Novel roles of *ARR2* in pathogen response and flowering time

**Dissertation**

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Summary

Two-component system (TCS) and MAPK signalling cascades play essential roles in plant signal transduction. The *Arabidopsis* Response Regulator 2 (ARR2), a member of B-type response regulators in the two-component circuit, serves as a molecular hub integrating several incoming signals. A comparable hub function is also known for the MAPK signalling cascade. Based on preliminary experiments we have started to address the hypothesis that there exists a link between ARR2-dependent TCS and MAPK signalling cascade, i.e. a crosstalk of those two evolutionary divergent signal transduction systems.

Potential protein-protein interactions between ARR2 and MAPK cascade members (MKKKs, MKKs, and MPKs respectively), were tested. The results revealed that ARR2 as well as other tested B-type ARRs, but not the A-type ARRs, strongly interacted with MKKs and some MPKs where none of them interacted with MKKKs. Interestingly, despite a high sequence similarity with ARR2, ARR1 did not interact with the all those MAPK members ARR2 is interacting with. ARR2 was found to interact preferentially with MKK4 and MKK5, which play an important role in the biotic stress defence specially including responses to pathogen attacks. Pathogenesis assays applied on ARR2 and ARR1 mutants lines, using the necrotrophic, semi biotrophic and biotrophic pathogens differing in evolution and distinct defence strategies in plants, revealed us that ARR2 mutants showed difference only in necrotrophic stress responses but not in biotrophic stresses.

The detailed analysis of *arr1* and *arr2* single loss-of-function mutants as well as an *arr1 arr2* double mutant revealed an early flowering phenotype of these plants especially visible under short day conditions (SDs) with the double mutant having an additive effect compared to the single parents. Despite very similar sequence homology of the *ARR1* and *ARR2* genes, the loss of *ARR2* effects the flowering time was quite stronger than *ARR1* but they still function in the same signalling pathway. From the mechanistic point of view the lack of *ARR1/ARR2* activity causes down-regulation of specific floral repressor genes but not of genes that act as floral activators and this miss-regulation of expression is not due developmental defects but directly linked to floral induction. ARR2 expressed either in the shoot apical meristem (SAM) or in phloem companion cells recues the early flowering phenotype of the *arr2* single and the *arr1 arr2* double mutants. The crossing of *ARR1* and *ARR2* mutants with flowering time-related mutants revealed that *ARR1* and *ARR2* function predominantly independent of *Flowering Locus C* (*FLC*). However, this approach revealed that *Flowering Locus M* (*FLM*) acts epistatic to *ARR1* and *ARR2*.

These findings are revealing and describing special features of the TCS elements and their crosstalks with other signalling pathways and contributing to a better understanding of their physiological role.
Zusammenfassung


Protein-Protein-Interaktionen zwischen ARR2 und Proteinen der MAPK Signalkaskade (MKKKs, MKKs und MPK) wurden untersucht. Wir konnten zeigen, dass ARR2 und andere ARRs aus der Gruppe der B-Typ Response Regulatoren, aber nicht der A-Typ ARRs, stark mit MKKs und einigen MPKs interagieren. Dabei gab es keine Interaktion mit den MKKKs. Trotz einer hohen Sequenzähnlichkeit zwischen ARR2 und ARR1, interagiert ARR1 nicht mit allen Komponenten der MAPK Signalkaskade mit denen ARR2 wechselwirkt. Wir konnten zeigen, dass ARR2 mit MKK4 und MKK5 interagiert. Beide haben eine wichtige Rolle bei der Stressantwort der Pflanze, vor allem bei der Pathogenabwehr. Versuche an ARR2- und ARR1-Knockoutlinien mit nekrotrophen, hemibiotrophen und biotrophen Pathogenen, die jeweils evolutionär unterschiedliche Abwehrstrategien in Pflanzen hervorrufen, haben gezeigt, dass ARR2-Mutanten keine Unterschiede bei biotrophen, dafür aber bei nekrotrophen Stressantworten hervorrufen.


Diese Daten beschreiben spezielle Elemente des TCS und deren Interaktion mit anderen Signaltransduktionswegen, welche zu einem besseren Verständnis der physiologischen Rolle führt.
1 General Introduction

1.1 Two-component systems

Two-component transduction systems (TCS or TSCT) pathways represent principal means for coordinating responses to environmental changes in bacteria and also in plants, some fungi, archaea and protozoa, but it is absent in higher eukaryotes and metazoans (Koretke et al. 2000; Hwang et al. 2002; Capra and Laub 2012). In prokaryotes these systems are mostly composed of a receptor sensor histidine kinase (HK or SK) and its cognate response regulator (Koretke et al. 2000; Capra and Laub 2012). Histidine kinases sense a specific signal and generally form functional homodimers allowing kinases to autophosphorylate at an internal histidine (H, His) by the γ-phosphoryl group of ATP (Koretke et al. 2000). The high-energy phosphoryl group is further transferred to an aspartate (D, Asp) residue of the two-component receiver domain in the response regulator protein. Response regulators (RRs) typically contain a two-component receiver domain and an effector domain allowing the protein to modify and regulate cellular behaviour in a TCS-dependent manner (Koretke et al. 2000; Hwang et al. 2002). The name “two-component” actually originates from this fact: in its simplest case only two elements are involved in TCS signal transduction, however there are exceptions which are elaborated on below. HKs share very high homology their ATP-binding domains with Hsp90, type II topoisomerases and MutL (mismatch repair protein) (Dutta et al. 1999; Koretke et al. 2000; Capra and Laub 2012). These proteins are all members of GHKL superfamily and it is suggested that HKs originated from one of these ATPases and had further evolved by series of duplications, lineage-specific expansion and divergence (Capra and Laub 2012). Generally, HKs are bifunctional, i.e. they can also act as phosphatases of their response regulators (Capra and Laub 2012). In order to make dimers, autophosphorylate and then successfully transfer the γ-phosphate group, histidine kinases must contain the dimerization and histidine phosphotransfer domain (DHp) and the catalytic and ATP binding (CA) domain (Koretke et al. 2000). Other domains most frequent in HKs are the sensory binding domains Per Arnt Sim (PAS) and cGMP-specific phosphodiesterases Adenyl cyclases and FhlA (GAF), a conserved “linker” domain Histidine kinase Adenyl cyclases Methyl accepting proteins and Phophatases (HAMP) (Galperin et al. 2001). These periplasmic sensory domains are responsible for direct signal (PAS and GAF) recognition and relaying this information to the DHp and CA domains; consequently these domains more variable than the other ones as reflected by the large diversity of detectable signalling molecules (Moglich et al. 2009; Parkinson 2010). Besides these domains, HKs usually have transmembrane domains and other domains which specialise and define their function. As mentioned, HK autophosphorylation appears to be dependent on forming homodimers (Ashenberg et al. 2011). HAMP domains are likely to be involved in dimerization in a sensory dependent manner (Parkinson 2010). Formation of functional homodimers is common for almost all HKs as there is only a single observation of a physiologically functional heterodimer in cyanobacteria Pseudomonas aeruginosa (Goodman et al. 2009). Some HKs have an additional
receiver domain fused to their C-terminus; these are called hybrid histidine kinases (also abbreviated HKs) and almost 25% of all bacterial HKs belong to this group (Cock and Whitworth 2007). Hybrid HKs might have originated by fusion of upstream encoded HKs and RRs through the mutation of stop codons in operons (Qian et al. 2008). After a signal is perceived and autophosphorylation occurs in hybrid HKs, the high energy phosphate group is delivered from the DHp histidine to the cis internal receiver domain. This phosphate is then shuttled by other elements of the TCS in a typically a four-step relay via hybrid HK to HPt (a free DHp domain protein called a histidine phosphotransfer protein) to a classical RR protein, thus the ~P group moves from His to Asp to His to Asp. This multistep phospho-transfer is commonly referred to as a phosphorelay. In eukaryotes, hybrid HKs are found in the majority of systems (Koretke et al. 2000) although the real reason for such selective pressure is not known. It has been proposed that the spatial arrangement within the hybrid HK enforces the specificity of phosphotransfer avoiding crosstalk with other TCS pathways (Wegener-Feldbrugge and Sogaard-Andersen 2009). In support to this claim, it has been proven that HKs missing their receiver domains can phosphorylate non-cognate RRs even better than their own internal response regulator domain (Biondi et al. 2006; Wegener-Feldbrugge and Sogaard-Andersen 2009). Next crucial element of two-component system signalling is RRs, as up until now, HPt proteins have only been shown to be ~P shuttles and add little specificity to TCSs with the exception of YPD1, which has been shown to stabilize the phosphoryl~RR form of SSK1 (Janiak-Spens et al. 2000) and the Arabidopsis protein AHP6 which cannot receive a TCS phosphate. AHP6 has been shown regulate developmental and hormonal processes, presumably at the level of protein-protein interaction within the TCS network (Mahonen et al. 2006; Moreira et al. 2013). All RRs have an evolutionary well conserved receiver domain which pulls the phosphate group onto its Asp residue thereby causing conformal changes to the protein which activate or even inhibit it initiating an output response (Gao et al. 2007; Capra and Laub 2012). Numerous RRs possess DNA-binding output domains (Galperin 2006) whereby phosphorylation of the conserved Asp promotes also dimerization of receiver domains favouring the DNA binding and direct control of target gene transcription (Capra and Laub 2012). Other domains frequent within RRs are diguanylate cyclases and methyltransferases domains (Koretke et al. 2000). In bacteria, RRs are signal integrators as these organisms usually have, if not an equal number, more HKs than RRs (Koretke et al. 2000; Capra and Laub 2012). Free RRs are therefore the last direct step of TCS signalling pathways and they are the final factor responsible ascribed for causing TCS-dependent physiological changes (Capra and Laub 2012). The diversity of in bacterial RRs is a direct consequence of gene duplications, lateral gene transfer events and point mutations (Rabin and Stewart 1993). Divergent evolution of the RRs has enabled them to specifically recognize promoters and control different genes (Price et al. 2008). Fifty percent of all known RRs form homodimers upon phosphorylation (Gao and Stock 2010) and there are subsets of interfacial residues that enforce homo-dimerisation and prevent hetero-dimerisation which lie within the β4-α4-β5-α5 region of the conserved RR domain (Weigt et al. 2009; Szurmant and Hoch 2010; Capra and Laub 2012).
In both Archea and eukaryotes, TCS independently originated from bacteria by lateral gene transfer (Korete et al. 2000; Kim and Forst 2001). In plants, TCS plays important roles in developmental processes (Ren et al. 2009; El-Showk et al. 2013) and most likely they obtained their TCS from chloroplasts after integration of chloroplast genes into their nuclear genome (Martin et al. 2002). As mentioned, higher eukaryotes and metazoans do not possess TCS (Hwang et al. 2002; Schaller et al. 2011) and instead, TCS (histidine/aspartyl phosphorylation) has been replaced by serine/threonine/tyrosine phosphorylation perhaps due to the intrinsic liability of phosphoryl groups on Asp compared to its stability on serine, threonine or tyrosine (Capra and Laub 2012). Thus the idea is that eukaryotes as “compartmented” organisms need more stable and longer lived outputs for signal conduction from the cell membrane to the nucleus. Supporting this claim would be the direct modulation of activity MAPK kinase signalling pathway in Saccharomyces cerevisiae by the SLN1-YDP1-SSK1 TCS pathway (Posas et al. 1996). On the other hand this is not entirely the case in plants. Here TCS is directly controlling expression of many target genes as in classical TCSs (Hwang et al. 2012; El-Showk et al. 2013). One of the most described examples in plants is cytokinin signalling where the TCS plays a crucial role, and B-Type RRs directly modulate gene activity of A-Type RRs and other genes (Shi and Rashotte 2012; El-Showk et al. 2013). The shuttling of the phosphoryl group from hybrid HKs in the plasma membrane and endoplasmic reticulum by HPts to RRs in the nucleus (Shi and Rashotte 2012) could have been selected for in plants because it is strongly believed that the histidyl~P residue is more stable than an aspartyl~P residue (Korete et al. 2000; Capra and Laub 2012).
1.2 References for General Introduction


2 Aim of this work

Besides its functions in ethylene signal transduction, cytokinin and H$_2$O$_2$ signalling etc., the two-component-signalling system seems to play very significant roles in crosstalk and fine-tuning between distinct signalling pathways. These additional functions of the TCS might be essential for the plant’s fitness within the natural environment and also for their general survival. Based on these observations, this thesis intends to contribute to a better understanding of these special features of TCS and describe them. The specific aims of this thesis are:

- To investigate and determine the functional (physiological) role of ARR2 interactions with biotic stress-related members of MAPK cascade, obtaining new data by expanding the comprehensive ARRs/MAPKs module interaction map and determine of the roles of ARR1 and ARR2 in response to pathogen attack and different elements that cause biotic stress in plants (see Chapter 1).

- Characterisation of a novel flowering time phenotype in the ARR1 and ARR2 lacking mutants under non-inductive short day conditions discovered during this dissertation by resolving which floral pathway(s), their mechanism and nature of interaction, these two TCS elements are involved in (see Chapter 2).
3 Materials and Methods

3.1 Chemicals

All chemicals were ordered from Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvents were delivered by Brenntag Chemiepartner GmbH NL (Plochingen) and Merck. BASTA pesticide was ordered from Bayer Crop Science. The ingredients used for culture media were ordered from Invitrogen (Carlsbad, USA), Merck, Sigma and Duchefa (Haarlem, NL). Restriction enzymes, ligase and DNA modification enzymes used for nucleic acids studies were ordered from Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA), Promega (Mannheim) and Amersharm Pharmacia Biotech. Oligonucleotides were received from Eurofins MWG Operon (Ebersberg) and antibodies from the companies Sigma-Aldrich (Taufkirchen) and New England Biolabs (Beverly, USA). The synthetically generated fg22, elf18 peptides, PEN and Xac extracts were a kind gift from the Laboratory of Georg Felix.

3.1.1 Media

The different media used are presented in the following table. For media preparation deionized water was used and the media was sterilized by autoclaving for 20 minutes at 121°C. Bacto-agar 15g/L was used for solid media preparation, (BD) or 8g/L Select-Agar for MS plates (Sigma-Aldrich). Where it was needed, filter sterilized antibiotics were added to the sterilized medium at appropriate final concentrations as listed in Table 3.1

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ingredients per 1 liter</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Kings's B</td>
<td>20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) MgSO₄</td>
<td>Pseudomonas syringae</td>
</tr>
<tr>
<td></td>
<td>andKH₂PO₄</td>
<td></td>
</tr>
<tr>
<td>½ MS</td>
<td>2.2 g MS (Duchefa), pH 5.7 (KOH)</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>YPD</td>
<td>20g Bactopeptone (BD #211677), 20g Glucose (monohydrate), 10g Yeast extract (BD #212750),</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td></td>
<td>1000ml ddH₂O</td>
<td></td>
</tr>
<tr>
<td>CSM</td>
<td>20g Glucose (monohydrate), 6.6g Yeast nitrogen base (BD #291940), 0.64g CSM-(L-W, or L-W-A, Qbiogene #4520-012), 1000ml ddH₂O</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Z buffer</td>
<td>10,68g Na₂HPO₄, 5,5g NaH₂PO₄, 0,75g KCl, 246mg MgSO₄, 1000ml ddH₂O, adjust PH to 7,0</td>
<td>Saccharomyces cerevisiae</td>
</tr>
</tbody>
</table>
3.1.2 Antibiotics

Media were supplemented when required with antibiotics at the final concentrations listed in the following table:

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration (μg/μl)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbenicillin</td>
<td>100</td>
<td>Water</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>Water</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
<td>Methanol</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100</td>
<td>Water</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>50</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>

3.2 Vectors and Primers

All the vectors used for this thesis are listed directly within each method text section. Primers were synthesized by Eurofins MWG Operon. Primers stocks were kept at a 100 μM concentration diluted in nuclease-free water and stored at -20°C.

<table>
<thead>
<tr>
<th>Name of the mutant line</th>
<th>Sequence (5’→ 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>arr2-4</td>
<td>GAACGGGAAGGCTCAGAG</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>arr1-4</td>
<td>GAAGAACAACATGGATTCGATATAGTA</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>tDNA(SALK)</td>
<td>TGTTTCACGTAGTGCCGATCG</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>co-9</td>
<td>CAACCTCTACTCCCCGTAGC</td>
<td>Balasubramanian et al., PloS Gen. 2006</td>
</tr>
<tr>
<td>soc1-2</td>
<td>GGATCACATGGTGGGAAAACCTC</td>
<td>Yoo et al., Plant Phys 2005</td>
</tr>
<tr>
<td>flm-3</td>
<td>GATCGGTTTTTGGTTTAATG</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of the mutant line</th>
<th>Sequence (5’→ 3’)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARR21-145</td>
<td>ATGGTAAATCCGGTACCGAAG</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>ARR21-145</td>
<td>TCTTCTGTAATGGTATTGATC</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>ARR21-145</td>
<td>CCACTCGTACCTCGATTTC</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td>ARR21-145</td>
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3.3 Bacterial strains

3.3.1 Escherichia coli strain DH5α
The E. coli strain DH5α [F- (Φ80lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK- mK+) phoA supE44 λ– thi-1 gyrA96 relA1] was used for cloning of the different constructs. The E. coli strain DB3.1 [F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(rB-, mB-) ara14 galK2 lacY1 proA2 rpsL20(SmR) xyl5 Δleu mtl1] was used for the cloning and propagation of Gateway™ vectors carrying the ccdb gene.

3.3.2 Agrobacterium strain
The Agrobacterium tumefaciens strain GV3101 (T-DNA–vir{+} rif{R}) with helper plasmid pMP90 (gen{R}) or pMP90RK (gen{R}, kan{R}) was used.

3.4 Organisms

3.4.1 Organisms used for pathogen assays
Two biotrophs Pseudomonas syringae pv. tomato Pto DC3000 (marked with Rif{R} resistance) and the fungus Peronospora parasitica were used. Two necrothrophic fungi Alternaria brassicola and Botrytis cinerea were used.

3.4.2 Plant lines
For all experiments Columbia-0 (Col-0) ecotype of Arabidopsis thaliana was used, and all transgenic lines were generated in this ecotype. For some experiments Nicotiana benthamiana was also used. The T-DNA insertion mutant lines used in this work include arr1-4 (SALK 042196), arr2-4 (SALK 016143), flm-3 (SALK 141971), co (SAIL_24_H04), soc1-2 (Lee et al. 2000; Yoo et al. 2005) and the deletion mutant flc-3 (Michaels and Amasino 1999). The double mutant arr1-4 arr2-4 was crossed and isolated by Dr. Katharina Caesar. Other mutants listed in the work were made by crossing; the lines obtained are: flm-3 arr2-4, co arr2-4, soc1-2 arr2-4, flm-3 arr1-4 arr2-4, flc-3 arr1-4 arr2-4. All of the crossed lines were genotyped by PCR for known insertion markers and characterised by checking mRNA expression. Single mutants flc-3, flm-3, soc1-2 and co were kindly donated by Dr. Markus Schmid and Dr. Rebecca Schwab (Max-Planck Institute for Developmental Biology, Tübingen). ARR2 and ARR2D80N loss-of-function genes were (both in pDONR207 Gateway vector; previously cloned in the laboratory of Prof. Dr. Klaus Harter) and pJL-Blue Gateway entry plasmid (kindly donated by Dr. Rebecca Schwab) were recombined into the respective pGREEN-IIS Gateway vectors pFK-101 (BAR_FD_rfA+) and pHW-059 (BAR_SUC2_rfA+) described by (Mathieu et al. 2007). These constructs were transformed into Col-0 wild-type, arr1-4, arr2-4 or arr1-4 arr2-4 mutants. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000 (§3.5.6.1).
3.5 Cultivation

3.5.1 Growth of *Escherichia coli*

*E. coli* strains were cultivated overnight at 37°C either on LB-plates or in liquid LB medium at 230 rpm. Antibiotics were added into the media according to the resistance cassettes the strains were harboring.

3.5.2 Growth of *Pseudomonas syringae*

*P. syringae* Pto pv. DC3000 strains were grown for 24 to 48 hours at 28°C either on King’s B plates or in liquid King’s B medium at 180 rpm. For the determination of bacterial growth in infection assays the *Pseudomonas* strain was re-isolated from plant material and plated on LB plates containing cycloheximide in addition to rifamycin.

3.5.3 Growth of *Agrobacterium tumefaciens*

*A. tumefaciens* strains were cultivated for 48 hours at 28°C on LB-plates or liquid LB medium at 230 rpm. Additional antibiotics were added into the media according to the plasmid-DNA the strains were carrying.

3.5.4 Growth of *Alternaria brassicicola*

The cultivation of *A. brassicicola* and the preparation of the spores for the infection assays were personally performed in the Laboratory of Dr. Birigit Kemmerling exactly as previously described (Kemmerling et al. 2007).

3.5.5 Growth of *Peronospora parasitica* and *Botrytis cinerea*

Assays with *Peronospora parasitica* and *Botrytis cinerea* were both performed by our collaboration partners. The *Peronospora parasitica* assay was performed by the research group of Professor Dr. Volker Lipka from University of Göttingen and the *Botrytis cinerea* assay was done in the laboratory of Professor Dr. Jean-Pierre Métraux at the University of Fribourg, Switzerland. *Peronospora parasitica* was grown as previously described by (Jacobs et al. 2003) and *Botrytis cinerea* as described by (Ferrari et al. 2003).

3.5.6 Growth of *Arabidopsis thaliana* and *Nicotiana benthamiana*

3.5.6.1 Growth conditions for flowering time analysis

For flowering time analysis, plants were first stratified by being kept at 4°C for 2 to 4 days and then transferred to 22°C; this was defined as day 0. *Arabidopsis thaliana* plants were grown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) and for pathogen experiments soil was mixed with Vermiculite. Plants were grown in growth chambers on soil at 22°C under long days (LD) 16 hours light / 8 hours dark or short days (SD) 8 hours light / 16 hours dark (110 mEm⁻² s⁻¹, 50-60% humidity). Plants used for pathogen experiments were grown only under SDs. Plants complemented under tissue specific promoters were used in T2 generation and therefore they were pre-selected with 1:1000 dilution of BASTA (dilution from the stock, 183 g/L glufosinate; 200 g/L ammonium salt, Bayer CropScience). The soil was directly soaked in the BASTA solution.
3.5.6.2 Growth of *Nicotiana benthamiana*

*Nicotiana benthamiana* plants were cultivated in a mixture of soil and sand containing 0.1% (v/v) Confidor by the ZMBP Greenhouse (13 h light, 11 h darkness).

3.6 Standard molecular biology methods

General protocols were used for PCR, side directed mutagenesis, agarose gel electrophoresis, western blot, restriction digestion, ligation, transformation of bacteria and plasmid isolation (Sambrook and Russell 2001). Techniques done differently are explained directly in the text. Restriction enzymes were used according the manufacturer’s protocols (Fermentas and NEB). For the generation of PCR fragments either the *Taq* DNA-Polymerase or the *Pfu* DNA polymerase (cloning purposes; Fermentas) were used. GeneRuler DNA Ladder Mix (Fermentas) was used as size marker for the agarose gel electrophoresis. DNA fragments were extracted out of agarose gels or purified out of PCR reactions by using the Qiagen Gel Extraction Kit and Qiagen PCR Purification Kit (Qiagen).

3.6.1 Yeast-two-hybrid

Yeast two-hybrid experiments were performed using the Matchmaker™System (Clontech). Plasmids were constructed by LR-reaction of corresponding Entry clones and destination vectors pGBKTK7-DEST or pGADTK7-DEST (Horak et al. 2008). Primers for clones produced during this thesis project are listed in the general primer list (§3.2), the rest of the clones were made by Achim Hahn and can be found in the plasmid repositories of Prof. Dr. Klaus Harter. Clones with *ARR18* were kindly provided by Manikandan Veerabagu and clones with *ARR4* are from the lab of Dr. Virtudes Mira-Rodado. Yeast strain PJ69-4A (James et al. 1996) was transformed using lithium acetate/SS-DNA/PEG method (James et al. 1996; Horak et al. 2008). After 3 days of growth on vector selective media (CSM, -L, -W), 6 independent clones were picked, resuspended in ddH2O and 10μl were dropped on vector-selective media. Subsequently, 10μl of culture were dropped on vector- and interaction-selective media (CSM, -L, -W, -A) and incubated at 28°C. At day 3 the growth of the clones was monitored. In addition, yeasts from selective media were inoculated in selective media (CSM,-L,-W) harvested and analysed by western-blot using α-HA and α-myc antibodies to determine the correct expression of the fusion proteins (Horak et al. 2008).

3.6.2 Transient expression in tobacco leaves

Expression of proteins expressed transiently in tobacco leaves was performed as described previously (Marion et al. 2008). A single colony of *Agrobacterium tumefaciens* strain GV3101 pMP90 transformed with the desired constructs and was inoculated in 5ml of YEB-Medium (0.5% beef extract, 0.5% sucrose, 0.1% yeast extract, 0.05% MgSO4·7H2O) containing Rif/Gent/and vector-specific antibiotic at 28°C overnight. In the morning, 1 ml of the pre-culture was taken and re-inoculated into 5 ml of the same Medium. The same was done for *Agrobacterium* strain carrying the p19 RNAi-suppressor protein from tomato bushy stunt virus (Voinnet et al. 2003). Each culture was collected in a 15 ml Falcon Tube and centrifuged at 4000 rpm for 20 min. Bacteria pellets were then resuspended in AS-Medium (10 mM MgCl2, 10 mM MES [pH 5.6], 150 μM acetosyringone) to an optical density at 600 nm of
about 0.7-0.8. The resuspended bacteria (two potential interaction partners and p19 strain) were mixed 600 ml each, a 1:1:1 ratio, in a 2ml Eppendorf tube and incubated for 0.5 to 1 hours at 4°C.

The bacterial solution was injected into the entire leaf area through the abaxial sides using a 1 ml syringe; two leaves per plant were inoculated. After inoculation, the plants were kept in a tray with a hood at 25°C. Two days after the bacterial inoculation, the β-estradiol responsive promoters in the pABind vectors (Zimmermann and Nentwig 1989) and the N-terminal mRFP vector pB7WGR2.0 (Plant Systems Biology, Gent) were induced by application of β-estradiol by brushing a 20 μM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements and localization studies were performed 24 to 48 hours after β-estradiol application. Localization studies were performed with confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). Microscopy was carried out as previously described (Horak et al. 2008). Images were taken with HCX APO LW 20×/0.5 or the HCX PL APO 63×/1.2 W water-immersion objective.

3.6.3 Ethylene accumulation measurements
For ethylene measurements, leaf material of 4 week-old plants were cut into 1 mm thick strips and floated overnight in water. Afterward three leaf stripes (20 mg) were transferred in 6 ml glass vials containing 0.5 ml of an aqueous solution of the elicitor to be tested. The tubes were closed with a rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 3h incubation at 170 rpm at room temperature. Following PAMPs and their working concentrations were used: PEN from Penicilium schizogonium 1 μL/500 μL, Xanthomonas axonopodis citri 3 μL/500 μL, flg22 (0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM), elf18(0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM).

3.6.4 ROS (Reactive Oxygen Species) measurements
Leaf discs were excised from 6 week-old Arabidopsis plants and incubated in water overnight. The following day, the discs were transferred to a solution of 20 μM luminol L-012 (Wako Pure Chemical Industries) and 10 μg/mL (w/v) horseradish peroxidase in a 96-well-plate and treated with 100 nM flg22, 100 nM elf18, 1 μM chitin octamers or 1 mg/mL chitin (crab). The plates were analysed for a period of at least 30 min using a multiplate reader Centro LB 900 (Berthold Technologies). For each data point at least 9 replicates were measured.

3.6.5 Statistical analysis
Statistical analysis was performed using Microsoft Office Excel or JMP (SAS). The data represent the average of replicates with plus or minus standard error of the mean (SE). The significance of the differences was calculated using Student’s t-test, ANOVA or Fisher’s Least Significant Difference (LSD) test; which test was used is explicitly mentioned in the text, figures and tables.

3.6.6 ELM software for in silico predictions
ELM software (http://elm.eu.org) was used for MKK docking site identification in ARR2.
3.6.7 Cloning and site-directed mutagenesis

All the clones used in our experiments were constructed using Gateway™ technology (Invitrogen™ | Life Technologies). The Entry clones were obtained via BP-reaction in pDONR207. cDNA preparations derived from *Arabidopsis* leaves were used as template to clone *ARR2* (AT4G16110). For the generation of truncated *ARR2* versions, *ARR2*<sup>1-300</sup>, *ARR2*<sup>300-664</sup>, *ARR2*<sup>1-165</sup>, *ARR2*<sup>145-664</sup>, *ARR2*<sup>165-664</sup> and *ARR2*<sup>1-145</sup>, primers were made corresponding to the *ARR2* cDNA and the fragments were amplified by PCR using the *ARR2* Entry clone (see primer table §3.2). Site-directed mutagenesis of *ARR2* was carried out on the *ARR2* Entry clones using QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and the *D80E, D80N* *ARR2* mutants were already present at the Laboratory of Klaus Harter at the time when the experiments were performed.

The binary constructs for FRET-FLIM measurements (*pABind* vectors) under the control of the β-estradiol inducible promoter and for stable *Arabidopsis* transformations under the control of the *FD* and *SUC2* promoters were obtained via LR-reactions. LR-reactions were also done for yeast-two hybrid system destination vectors pGADT7 and pGBK7.

3.6.8 Quantitative RT-PCR (RT-qPCR)

Total RNA from corresponding material (leaves, shoot apical meristem or whole plant) was isolated using the "Universal RNA Purification Kit" (Roboklon, Germany) including a DNA digestion step to ensure the absence of genomic DNA contamination/presence. The cDNA was synthetized using oligo-dT or random hexamer primers with H-Minus Reverse Transcriptase (Fermentas). qPCR primer were tested for doubling time by diluting 1:2 and ensuring that there was exactly a loss of 1 CT; primer efficiency was thereafter assumed to be 100%. At least two PCR reactions (in triplicate repeats each) using two independent cDNA synthesis were performed for every treatment. The amplification of cDNA was performed with PerfeCta qPCR SuperMix or PerfeCta SYBR Green Super Mix (Quanta Biosciences, distributed by VWR) using, when necessary, the corresponding Probe (Universal ProbeLibrary Single Probes, Roche) accordingly to manufacturer. The PCR reactions were ran in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad). The used primers are listed in the §3.2. Expression levels of each gene were normalized according to the expression of the following housekeeping genes: *EF-1-α* (AT5G60390). The CFX Manager software (Version 1.1; Bio-Rad) has been used for the quantification of relative expression levels except for §4.2.3.4 for which the CT values were exported and ΔΔCT was manually processed in Excel to ensure proper normalisation.

3.6.9 ONPG assay

Before the Day One, 3 to 4 transformed yeast colonies were pooled and inoculated and incubated overnight at 28°C while shaking in 0.5ml of CSM-L/W liquid medium at 180 rpm.

**Day 1**: the tubes were taken from the shaker and 3 ml of YPD liquid medium was added in each one and put back to the shaker for an additional 3 hours. After that, the optical density at OD<sub>600</sub> was measured. The tubes were then centrifuged for one minute at 11000 rpm and
the pellet was re-suspended in 1 ml of Z Buffer (the same procedure was repeated three times). The tubes were frozen in liquid nitrogen and stored at -80°C.

**Day 2**: Frozen tubes were thawed in a 37°C water bath and then returned to liquid nitrogen; this cycle freezing/thawing was repeated at least 4 times. Blank control (with all following solutions) was set and OD_{600} was measured. Immediately after adding of 500 µl of Z buffer-ME (38.6mM β-Mercaptoethanol) and 160 µl of Z buffer/ONPG (13.3mM β-Mercaptoethanol) the timer was started and the tubes were incubated at 30°C until yellow colouring appeared. The reaction was stopped by the addition of 400 µl 1M Na_{2}CO_{3}. The tubes were centrifuged afterwards and the supernatant was transferred to another tube. The optical density was measured at OD_{420}. Galactosidase units were calculated as follows:

\[
\text{Galactosidase units} = 1000 \times \frac{OD_{420}}{t} \times V \times \frac{OD_{600}}{}
\]

with t being elapsed time in min, V is the volume of the yeast culture used for the assay, OD_{420} and OD_{600} the optical density of the yeast cultures used for the assay.

### 3.6.10 MPK assay using anti-phospho antibodies

Anti-phospho antibodies p44/42 (Cell Signaling Technology, NEB #9101) were used as they are known to specifically detect phosphorylated MPK3, MPK4 and MPK6 in *Arabidopsis thaliana*. 5 week-old plants grown under short day conditions were used. Total protein amount was extracted from 50-100 mg of leaf material. For protein extraction 50mM Tris/HCl [pH 7.5], 5mM EDTA pH 8 and 2mM DTT was used. Protease inhibitor cocktail tablets (Roche) were used. Protein concentration was determined with Bradford Reagent (Biorad-System) where 10µl of protein extract was added to 990µl Bradford-Solution (Bradford Reagent diluted 1: 5 in H_{2}O), exposed for 5 min at RT; and then the optical density (OD) was measured at 595 nm. To estimate protein concentration the following formula was used:

\[
\text{Protein concentration} \left[ \frac{mg}{ml} \right] = \frac{OD_{595}}{(0.0283 \times vol).protein extract used}
\]

### 3.6.11 Stomata measurement assays

Leaves from 5 week-old *Arabidopsis thaliana* plants were floated for 2 h under continuous illumination (120–150 µE m^{-2} s^{-1}) in MES/KCl buffer (5mM KCl/10mM MES/50µM CaCl_{2}, pH 6.15) as previously described by (Mira-Rodado et al. 2012). Once the stomata were fully open, leaves were treated with either 1µM t-zeatin or 10µM ABA for further 2 h. The leaves were subsequently homogenized individually in a Waring blender for 30 sec and the epidermal fragments collected on a 100 µm nylon mesh (SpectraMesh, BDH-Merck, UK) and placed on a microscope slide and covered with a coverslip. Stomata apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (Leica QWin software, Leica, UK). Lens 20X-0.75 magnification was used plus. Leaves were collected from plants also treated with *Pseudomonas syringae* DC3000 3 days after infection and directly homogenized without being exposed to additional light or being put in water. To
calculate stomata aperture, relative pixel distance values were measured in ImageJ software. For statistical analyses ANOVA was used.

3.6.12 Day length shifting experiments
This method was used for the synchronized flowering experiments. Plants were first grown at 8h light/16h dark (short days) at 23°C for 30 days and then shifted to 16h light/8h dark conditions (long days), (Maizel and Weigel 2004; Wahl et al. 2013). Apical centres (meristems) were harvested after removal of all visible leaves at day 0 and 5 (counting from the shifting day).

3.6.13 Generation of stable Arabidopsis thaliana transgenic lines
Stable transgenic plants were generated by Agrobacterium tumefaciens-mediated transformation using the floral-dip method (Clough and Bent 1998). For all transformations Agrobacterium GV3101::pMP90 or GV3101::pMP90RK strain were used. The RK strain possesses the RK2 replicase and trf gene required for the replication of RK plasmids. The Agrobacterium RK strain was co-transformed with pSoup vector which provides replication functions in trans for pGREEN-IIS destination vectors into which the different promoters used in this study (FD, SUC2) which had been cloned in front of a modified Gateway recombination cassette. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000.

3.6.14 Tissue fixation, embedding and sectioning of Arabidopsis thaliana apical meristem

3.6.14.1 Embedding
Day 1: Material was directly harvested into fixative FAA (Formaldehyde: Acetic Acid) in a little glass beaker. Fixative: FAA (Formaldehyde: Acetic Acid):

- 50% Ethanol
- 5% Glacial Acetic Acid
- 3.7% Formaldehyde
- 41.3% Water

Vacuum was applied and released (soft up and down with the vacuum) for 1 to 4 hours until the material became translucent and sank to the bottom. The FAA was afterwards changed and left in a cold-room overnight. FAA was replaced with 70% Ethanol; this step was repeated once to be sure all FAA is gone, after that material can be stored in falcon tubes for a long time.

Day 2: These steps were done at 4°C in a cold-room. 70% Ethanol was replaced with 85% Ethanol for 60 min. After one hour previous solution was replaced with 96% ethanol with Eosin and it was left overnight until a light pink colour emerged and tissue could be visualized.

Day 3: These steps were done at room temperature. The previous solution was replaced by 96% ethanol with Eosin for one hour, and then two times replaced by the same solution for one hour each time. After that the tissue was treated with 25% Histoclear with 75% Ethanol for 30 minutes and after each thirty minutes the percentage of Histoclear was increased by
25% where the percentage was decreased at the same amount until the percentage of Histoclear reached 100%. The tissue was further treated with the 100% Histoclear twice for 1 hour each time. After that paraplast chips were added 25% (v/v) of the solution and so left overnight at room temperature.

**Day4**: The tubes were placed at 42°C until the chips completely melted and one more ¼ volume of paraplast chips was constantly added until they completely melted. The tubes were afterwards moved to 60°C for several hours and parallel in another tube wax was melted overnight at 60°C to prepare for the next day.

**Day5**: Two wax changes approximately ¼ volume separated by several hours, were performed. The same procedure is for days 6 and 7.

**Day8**: The tissue was placed in sectioning moulds and stored at 4°C. The tissue was then ready for sectioning.

### 3.6.14.2 Sectioning
The sections made were 8µm thick. Slides were ProbeOne Plus from Fisher Biotechnology; they were pre-cleaned and charged. Moulds were trimmed in order to get as close to the tissue as possible. The ribbons were placed in 40°C water bath to remove compressions, and then they were fished out with a slide. They slides were kept on a heating plate at 42°C overnight. Thereafter, slides can be stored in a box at 4°C for a long time. Sections were scanned for clear visibility of two big air vacuoles; always the same plane was used for comparative measurements and statistics. Images were captured using a Leica DM-IRB microscope with a 20x lens.

### 3.6.15 Pathogen Assays with *Pseudomonas syringae* DC3000
Bacteria were inoculated from an LB plate into 50 ml LB medium with the corresponding antibiotics (rifampicin) and incubated over night at 28°C and shaken at 180 rpm in a shaker. The cells were harvested in the next morning by centrifugation in 50-ml falcon tubes at 3500 rpm at 4°C for 10 min. The pellet was resuspended in 10 mM MgCl₂ and the OD<sub>600</sub> was set to OD<sub>600</sub>=0.2 which refers to approximate concentration of 10⁸ cfu/ml. The cells were afterwards diluted up to density 1x10⁴ cfu/ml and used likewise for the assays. Bacteria were inoculated on two leaves per 5-week-old plant by infiltration with a needless syringe in the middle of a leaf apoplast half (two leaves per plant) ensuring that an area was covered that was always bigger than the cork borer used for extracting leaf discs. The infected leaves were marked for easy identification.

The leaves were harvested at the time points 0 h and 1, 2 and 4 days past infection. For each time point, five plants and two leaves from each plant per line were used. One disc was made from each leaf with the cork borer. The leaf discs were surface was sterilised by washing them in 70% (v/v) ethanol first for about 1 minute and then 1 minute in water. Afterwards the leaf discs were placed into Eppendorf tubes containing 100µl 10mM MgCl₂. Immediately after placing the leaf in the Eppendorf tube, it was homogenized with the sterile pistil and
additional 100µl 10mM MgCl₂ was added on the pistil in order to wash out the remains of the leaf discs. The homogenised material was plated on LB-plates with rifampicin and 50µg/ml cycloheximide (it prevents growth of eukaryotes like fungi). Extracts were serially diluted 5 times at 10-fold per dilution and dilutions anticipated to yield evaluable data were scored by plating out 10 µl (see Table 3.3). At least two technical replicates per sample were evaluated.

For those experiments involving a cytokinin treatment, trans-zeatin in a concentration of 1µM was used. Cytokinin was given directly to the 1x10⁴ cfu/ml bacterial dilution before infiltration. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

<table>
<thead>
<tr>
<th>Days/Dilutions</th>
<th>Undiluted</th>
<th>1:10³</th>
<th>1:10²</th>
<th>1:10¹</th>
<th>1:10⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>*</td>
<td>*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st day</td>
<td>*</td>
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<tr>
<td>2nd day</td>
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</tr>
<tr>
<td>4th day</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>

### 3.6.16 Infection with Alternaria brassicicola

*Alternaria brassicicola* spores used for infection assays were obtained as previously published (Thomma et al. 1999). Leaves of 5 week-old *Arabidopsis* plants were drop-inoculated with two 5 µl droplets of spore solution (5*10⁵ Spores/ml). Two leaves per plant and a minimum of 20 plants per line were infected. Plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal infection was scored using the following table (Table 3.4). The Disease Index was defined as previously published (see below and (Kemmerling et al. 2007)). For experiments with cytokinin, *trans*-zeatin was used at concentration of 1µM. Shortly before drop-inoculation, cytokinin was given to the 5*10⁵ Spores/ml bacterial dilution. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

### 3.6.16.1 Disease indexes assigned with their description

Scoring system was adopted from the laboratory of Dr. Birgit Kemmerling at the department of Biochemistry at ZMBP.

<table>
<thead>
<tr>
<th>RANK</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No symptoms</td>
</tr>
<tr>
<td>2</td>
<td>Light brown spots at infection site</td>
</tr>
<tr>
<td>3</td>
<td>Dark spots brown spots at infection site</td>
</tr>
<tr>
<td>4</td>
<td>Spreading necrosis</td>
</tr>
<tr>
<td>5</td>
<td>Leaf maceration</td>
</tr>
<tr>
<td>6</td>
<td>Sporulation</td>
</tr>
</tbody>
</table>

**Figure 3.1 Scoring scale for determination of disease index for plants treated with Alternaria brassicicola (A) Description of different disease indexes (B) Photograph of treated Arabidopsis leaves with spores where is the phenotype related with appropriate disease indexes (image provided by courtesy of Thierry Halter, Dr. Birgit Kemmerling’s group)**
3.6.17 Infection with *Peronospora parasitica*

Treatment with the virulent *Peronospora parasitica* isolate was done on 4 to 6 week-old soil-grown plants. Spore suspensions of *Peronospora parasitica* were prepared as described (Shah et al. 2001). Plants were sprayed with a suspension of conidiospores diluted in water (1x10^6 spores ml^-1). Inoculated plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation as previously published (Delaney et al. 1995).

3.6.18 Infection with *Botrytis cinerea*

*Botrytis cinerea* strain BMM was used (Zimmerli et al. 2000). The strain was grown on Difco potato dextrose agar (Becton Dickinson). Spores were harvested in water and then filtered and diluted in quarter-strength Difco potato dextrose broth (PDB) for inoculation as previously described (La Camera et al. 2011). For disease assays, 6 μL of spore solution (5 × 10^4 spores mL^-1) were deposited on three leaves (detached leaf assay) of 5 weeks old plants. Lesion diameters were measured after 3 days. Fungal growth was measured as previously described (Gachon and Saindrenan 2004). Plants were drop-inoculated and leafs discs were harvested at the indicated time points (0h, 12h, 24h, 36h, 48h post infection). The inoculated plants were kept under high humidity in a tray closed with a water-sprayed transparent lid.

3.6.19 FRET-FLIM and microscopy

Two days after the infection of Nb abaxial leaf surface with Agrobacteria carrying the desired binary plamids pABind vectors (Zimmermann and Nentwig 1989) were induced by application of β-estradiol by brushing a 20 μM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements were performed 24 to 48 hours after β-estradiol application as described in (Berendzen et al. 2012). The FRET-FLIM measurements were performed with a custom-built CSSM (confocal stage scanning microscope), based on a Zeiss Axiovert 135 TV, and equipped with a pulsed supercontinuum laser–source (SuperK™, NKT Photonics) as excitation light source operating at 471 nm and a repetition rate of 40 MHz. A microscope objective with high numerical aperture (Plan-Neofluar, 100×/1.30 oil, Zeiss) was used to focus the excitation light as well as to collect the fluorescence emission. The setup was equipped with a 500 nm dichroic mirror (FF500-Di01-25×36, Semrock) to block back-scattered excitation light and with a 527 nm bandpass filter (Semrock BrightLine BL527/20) to detect GFP-fluorescence. An avalanche photo diode (PDM series, Micro Photon Devices (MPD), Italy) served as a spectrally integrating detector to record fluorescence intensity. Lifetime decays were recorded using a time-correlated single photon counting board for data acquisition (PicoHarp 300, Picoquant, Software: SymPhoTime, Picoquant) and the MPD as a detector (Wanke et al. 2011).
3.7 References for Materials and Methods


4 Chapter 1
Interaction of Type-B TCS components with MAPK kinase cascade members and a putative crosstalk function in pathogen response

4.1 Introduction

4.1.1 Two-component system (TCS) in Arabidopsis thaliana
Like in all plants, the TCS in Arabidopsis thaliana is also a multi-step phosphorelay system (Hwang et al. 2002; Heyl and Schmulling 2003; El-Showk et al. 2013). TCS mediates physiological response to hormones (cytokinin and ethylene, cross-talk with auxin), nutrients, light and osmotic stress. Its components have important roles in the regulation of several developmental processes such as control of stem cell number in SAM, female gametophyte development, differentiation of root vascular tissue, just to name a few (Hwang et al. 2012; El-Showk et al. 2013). This involves members of three protein families: Arabidopsis histidine kinases (AHKs), Arabidopsis histidine phosphotransferase proteins (AHPs) and Arabidopsis response regulators (ARRs). Arabidopsis possess 11 AHKs and they are hybrid kinases, with an exception of ETHYLENE RESPONSE SENSOR 1 (ERS1) kinase (Guo and Ecker 2004). Based on their functional and structural characteristics the AHKs can be grouped into three subfamilies (Grefen and Harter 2004). Five AHKs have roles as ethylene receptors (Grefen and Harter 2004): ERS1, ERS2, ETHYLENE RESISTANT 1 (ETR1), ETR2 and ETHYLENE INSENSITIVE 4 (EIN4); ERS1 and ETR1 belong to the class I subfamily of AHKs (Grefen and Harter 2004) and are localised to the endoplasmic reticulum (Guo and Ecker 2004). ETR2, ERS2 and EIN4 belong to the class II of ethylene receptors (Grefen and Harter 2004; Guo and Ecker 2004) and have degenerated transmitter domains, i.e. they do not have histidine kinase activity as they lack at least one crucial amino acid to be functional (Guo and Ecker 2004). Kinase AHK1 is playing role in stress signalling as membrane-bound osmosensor (Urao et al. 1999) and CYTOKININ INSENSITIVE I (CKI1) is enrolled in female gametophyte development (Pischke et al. 2002; Hejatko et al. 2003). AHK5, or also known as CKI2, is missing a transmembrane domain (Urao et al. 1999; Pischke et al. 2002) and therefore some authors classify it as a separate group. The AHK5/CKI2 is playing a role in stomata closure signalling (Desikan et al. 2008) and it is predicted to be a cytoplasmic hybrid histidine kinase (Grefen and Harter 2004; Hass et al. 2004).

Members of the final subfamily respond to cytokinin: AHK2, AHK3 and AHK4/WOODEN LEG (WOL)/ CYTOKININ RESPONSE 1 (CRE1) (Hwang et al. 2002; Grefen and Harter 2004; El-
showk et al. 2013). briefly, these AHKs contain a ligand-binding CYCLASE HISTIDINE KINASE ASSOCIATED SENSORY EXTRACELLULAR (CHASE) domain at their N-terminus which is flanked by two transmembrane domains and followed on the cytoplasmic side by the C-terminus with a histidine kinase domain and output domain (suzuki et al. 2001; ueguchi et al. 2001; hwang et al. 2002). this sensor domain is like other bacterial histidine kinases PAS domains and their mechanism of function (Hothorn et al. 2011). the cytokinin receptors are localised not only on plasma membrane but also at the endoplasmic reticulum membrane highlighting that compartmentalization is important for cytokinin signal transduction (Caesar et al. 2011). These receptors show different sensitivity to different types of cytokinin hormones and also are expressed and functional in different tissues (Stolz et al. 2011; Lomin et al. 2012). AHK2 and AHK4/CRE1 have roughly the same high affinity to isopentenyladenine (iP) and t-zeatin but a lower affinity to dihydrozeatin whereas AHK3 has high affinity to dihydorzeatin compared to isopentenyladenine (Lomin et al. 2012). 

P ARR S: GUS experiments in ahk2, ahk3 and cre1 mutant backgrounds demonstrated that loss of cre1 affected promoter responses in the root while loss of ahk2, ahk3 crippled shoot responses (Stolz et al. 2011). Interestingly, AHK4 is known to be prevalent in the roots and AHK3 in the shoots (Lomin et al. 2012). If correctly interpreted, the receptors are expressed and receptive to the corresponding cytokinins that are transported from the opposing tissue, i.e. t-zeatin from root to shoot and iP's from shoot to root (Lomin et al. 2012).

There are 6 AHPs in Arabidopsis that mediate phosphorelay and shuttle through the nucleus between AHKs and ARR s. AHP1 to AHP5 are positive regulators in cytokinin signalling (Hutchison et al. 2006) and are also known as “canonical” AHPs because they contain the conserved histidine HPt residue thus they can carry the TCS-phosphate group on further to ARR s (suzuki et al. 1998). Despite their partial redundancy, different subsets of AHPs have been shown to trigger cytokinin responses in specific tissues and organs. For example the ahp2/ahp3/ahp5 triple loss-of-function mutant showed increased seed size similar to that observed in the ahk2/ahk3/ahk4 triple mutant (Hutchison et al. 2006; Riefler et al. 2006). Different combinations of ahp1-5 mutants do not show the same responses to exogenous cytokinin and its effects on chlorophyll content, lateral root formation or hypocotyl elongation (Hutchison et al. 2006). Generally speaking however, the loss of AHPs seems to be additive, as expected due to redundancy. AHP6 is called a “pseudo AHP” because it lacks the conserved histidine residue required for TCS-dependent phosphorylation: nevertheless, it is an active cytokinin signalling inhibitor (suzuki et al. 1998; Grefen and Harter 2004; Mahonen et al. 2006) (Moreira et al. 2013). Recently it has been reported that AHP2, AHP3, and AHP5, also work redundantly to control responses to drought stress in a negative and redundant manner as loss of these three AHPs resulted in a strong drought-tolerant phenotype that was associated with the stimulation of protective mechanisms (Nishiyama et al. 2013).

There are 23 functional ARR s in Arabidopsis and based on their protein properties and phylogeny they are divided into 3 subgroups: the type-A, type-B and pseudo-response regulators. The Type-A subfamily of ARR s are single-domain response regulators that
contains the members: ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16 and ARR17. Members of this subfamily only have a functional receiver domain (with functional Asp residue) and a short C-terminal tail (Grefen and Harter 2004). Members of the type-A subfamily generally act as negative regulators of cytokinin signalling based on genetic and transgenic studies (Lee et al. 2008; Hwang et al. 2012; El-Showk et al. 2013). So far only ARR4 has been described with a function outside of the TCS. ARR4 positively interacts with phytochrome B and stabilizes the active PhyB-Pfr form under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007).

The type-B response regulators have additional effector domains on their extended C-termini and are therefore able to activate the transcription in a TCS-dependent manner (Sakai et al. 2001). Their expression is not regulated by any stimulus tested so far (Grefen and Harter 2004). This subfamily consists of 11 members: ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20 and ARR21. The output domain of the B-Type members usually is composed of the GARP (named after Golden2 found in maize) DNA-binding domain, at least one NLS and C-terminal transactivation domain (Lohrmann and Harter 2002). B-Type response regulators work as transcription factors binding to target promoters that contain 5’-W/GAT/W-3’ motif (W represents either A or T) (Lohrmann and Harter 2002). Last group of response regulators are C-Type ARRs (Horak et al. 2008). Both type-A and type-B ARRs are now know to be regulated by the 26S proteasome (Ren et al. 2009; Kim et al. 2013; Kurepa et al. 2013).

Two genes (ARR22 and ARR24) encode single-response domain response regulators and are structurally very similar to type-A response regulators but their expression is not regulated by cytokinin. It has been recently suggested that they function as phosphatases for phosphates on histidine moiety of AHPs, but their expression domain is restricted to reproductive organs (Kiba et al. 2003; Gattolin et al. 2006; Horak et al. 2008).

Beside these three groups of the ARRs there is one more group, composed out of 9 members, called pseudo-response regulators (pseudo-RRs). These are response regulator proteins that have a mutation in the conserved response regulator aspartate (Asp) residue (Hwang et al. 2002). It has already been proven that pseudo-RRs regulate flowering time in Arabidopsis thaliana and that TOC1/APRR1, an Arabidopsis pseudo response regulator, is part of the plant circadian clock (Farre and Liu 2013). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate, they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Grefen and Harter 2004). The pseudo-response regulators can be split into two subfamilies (Matsushika et al. 2000): The APRR2 family which more similar to a classical response regulator structure with an N-terminal receiver domain followed by a DNA binding domain (also Myb-like GARP) and C-terminal effector extension; the spacing between the RR domain and the GARP domain is however larger than that of the type-B ARRs (Makino et al. 2000; Matsushika et al. 2000). The APRR1 family has an “inverted” response regulator structure:
there is an N-terminal RR domain, a DNA-binding CCT domain at the C-terminal end, and, presumptuously, the effector domain in-between these two domains (Makino et al. 2000). PRR9, PRR7, and PRR5 function as transcriptional repressors of CCA1 and LHY (Nakamichi et al. 2010) recently shown to bind to DNA via their C-terminal CCT domains (Gendron et al. 2012). The pseudo-response regulators are also targeted by the 26S proteasome (Baudry et al. 2010).

4.1.2 Crosstalk of TCS-related pathways in plants

Although most type-B ARRs have been described to only be involved in cytokinin signalling, an exception is ARR2. Recently it was discovered that cytokinin-activated ARR2 promotes plant immunity in *Arabidopsis* via salicylic acid signalling whereby ARR2 directly interacts with salicylic acid response factor TGA3 and increases pathogen resistance (Choi et al. 2010). ARR2 is also known to be involved in ethylene signalling by working downstream of *ETR1* (Hass et al. 2004). Furthermore, ARR2 is involved in ethylene and H2O2-mediated stomatal closure (Desikan et al. 2006). The crosstalk of TCSs with other plants signalling pathways became more evident after a comparative microarray analysis was done on 30-day-old *arr2* mutant and control Landsberg erecta (Ler) plants where the results showed altered expression of about 600 genes mostly related to biotic and abiotic stresses, ethylene and auxin signalling (Hass et al. 2004). The expression of dominant-active, non-phosphorylatable, *ARR2°D80E* (aspartate-to-glutamate mutation) caused even more dramatic changes in more than 16000 genes, and most of the disturbed genes are involved in hormone homoeostasis and its signal transduction, biotic and abiotic stress, photomorphogenesis, and others (Hass et al. 2004).

4.1.3 Mitogen-activated protein kinase cascade

Eukaryotic mitogen-activated protein kinase (MAPK) cascades transduce environmental and developmental signals into adaptive and programmed responses. In plants, MAPK cascades have evolved to regulate innate immunity, hormonal response, stress and developmental processes (Colcombet and Hirt 2008; Rodriguez et al. 2010). MAPK signalling cascade(s) are evolutionary conserved within eukaryotes (Schwartz and Madhani 2004) and have roles in abiotic stress, response to pathogens and pathogen-derived elicitors, plant hormones (ethylene, auxin), cell cycle and developmental processes (Tena et al. 2011; Rasmussen et al. 2012; Pathak et al. 2013; Smekalova et al. 2013). MAPK cascades transduce and amplify their signal by three different types of kinase members: *MAP kinase kinase kinase* (MKKK), *MAP kinase kinase* (MKK) and *MAP kinase* (MPK) (Rodriguez et al. 2010). In general, they function as a three tiered kinase cascade with each upstream member required to active the lower level eventually influencing genes expression (Rodriguez et al. 2010). Initially, a ligand activates plasma membrane receptors and these stimulated receptors activate MKKKs that are serine or threonine kinases that in turn phosphorylate MKKs at a conserved S/T-X3-5-S/T motif (X can be any amino acid) (Chang and Karin 2001; Rodriguez et al. 2010). The MKKs afterwards phosphorylate MPKs on threonine and tyrosine residues at a conserved T-X-Y repeat (Chang and Karin 2001; Rodriguez et al. 2010). Activity of MAPKs can be regulated/deactivated by serine/threonine or tyrosine phosphatases (Luan 2003). Different cascades can share kinase components but still maintain their signalling specificity by various
mechanisms like protein-protein interactions, scaffolding, cross-inhibition and feedback control (Whitmarsh and Davis 1998; Bardwell et al. 2001; Takekawa et al. 2005). MAPK cascades are present in all eukaryotes and is evolutionarily highly conserved which confirms its essential function in these organisms (Kim et al. 2003).

4.1.4 Mitogen-activated protein kinase cascade and its signalling in Arabidopsis thaliana

Around 60 MKKKs, 10 MKKs and 20 MPKs are found in the genome of Arabidopsis thaliana (Group 2002). Besides having the largest number of members, MKKKs also have the greatest variety in domain composition and primary structures among all the MAPK cascade members (Group 2002). Their nomenclature is still not totally defined as some authors have different opinions about this topic. Based on amino acid sequence of the kinase catalytic domains the Arabidopsis MKKKs are grouped into 2 main classes which are further divided into different subclasses (Group 2002; Rodriguez et al. 2010): MEKKs and the Raf-like kinases. The MEKK MKKKs family comprises members who kinase domains share significant similarity to previously described MKKKs, for example MEKK1 in mammals or to yeast STE1 (Group 2002). Members of this family are generally active in response to abiotic stress, especially drought, touch and high salinity (Mizoguchi et al. 1996). The Raf-like kinase class is named after RAF1 mammalian kinase. Interestingly, most members of this class have a PAS domain which is the most frequent sensor domain present among TCS histidine kinases and other pathways (Zhulin et al. 1997; Zwerger and Hirt 2001). The most studied members of this MKKK class are CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and ENHANCED DISEASE RESISTANCE 1 (EDR1) which are both involved in ethylene-mediated signalling and defence responses (Kieber et al. 1993; Huang et al. 2003). Among the Raf-like class there is also the ZIK sister clade for which no phosphorylation activity has been shown on MKKs (Figure 4.1) (Rodriguez et al. 2010).

The small number of putative MKKs in Arabidopsis suggests that crosstalk between various signal-transduction pathways might be concentrated at this level of the MAPK signalling cascade. It is known that one MKK can activate various different MPKs (Popescu et al. 2009). Considering their small number, the MKKs are very well described in Arabidopsis and rice and both are classified into 4 groups (A-D) (Group 2002; Hamel et al. 2006; Rodriguez et al. 2010). Members of the group A are MKK1, MKK2 and MKK6. Kinases MKK1 and MKK2 are involved in abiotic (cold, salinity) and biotic (especially innate immune response) stresses (Teige et al. 2004; Meszaros et al. 2006; Rodriguez et al. 2010), both acting upstream of MPK4 (Ichimura et al. 1998). Group B includes only one member: MKK3 which is distinguishing from the other kinases by the presence of a nuclear transport factor 2 (NTF2) domain (Kiegerl et al. 2000; Group 2002). Group C includes MKK4 and MKK5 (Group 2002) and they play very important roles in biotic stresses by providing resistance to bacterial and fungal pathogens controlling converging signals initiated by diverse pathogens (Asai et al. 2002; Group 2002; Rodriguez et al. 2010). In addition, both MKK4 and MKK5 are in the pathway downstream of YODA (MAPK kinase kinase) regulating cell fate specification in stomata development (Wang et al. 2007) and the ethylene synthesis pathway (Babula et al. 2006). Members of group D are MKK7, MKK8, MKK9 and MPK10 whose physiological roles are not yet precisely described. It is known that the MKK9-MPK3/MPK6 cascade promotes ETHYLENE-INSENSITIVE 3 (EIN3)-
mediated transcription in response to ethylene signalling (Yoo et al. 2008; Hahn and Harter 2009) and that M KK7 repressed expression causes deficiency or enhancement in auxin transport (Dai et al. 2006).

![Diagram](image)

**Figure 4.1 Relatedness of Arabidopsis MKKs based on their protein sequence**

The unrooted tree was made by aligning complete protein sequences with Clustal W (default settings) available on TAIR (http://www.arabidopsis.org/) of the (A) AtMKKs, (B) AtMKxs and (C) AtMPKs. Subfamilies are marked according to the classifications proposed in (Group 2002). The trees were draw with TreeView X.

The last members of the MAPK signalling cascade are MPKs. They are also grouped into 4 groups (A-D) based on their sequence homology (Group 2002; Rodriguez et al. 2010). In plants, MPKs contain either the TDY (present also in all mammals) or TEY phosphorylation motif which is phosphorylated by M KKs and causing MPK activation (Group 2002; Rodriguez et al. 2010). Members of groups A, B and C families contain TEY whereas only group D has the TDY phosphorylation motif. The most studied group of MPKs is Group A. Generally, members of this group are involved in environmental and hormonal responses (Group 2002). MPK3 and MPK6 are members of this group and they have been the most studied group.
among all MAP kinases. MPK3/MPK6 play roles in ethylene signalling downstream of MKK9 (Hahn and Harter 2009) whereas MPK6 is additionally required for interaction with ETHYLENE RESPONSE FACTOR 104 (ERF104) in ethylene responses regulated by flg22 (Bethke et al. 2009). Findings for the group B are mostly based on physiological studies of Arabidopsis MPK4 involved in pathogen defence and abiotic stresses (Widmann et al. 1999; Yuasa et al. 2001; Qiu et al. 2008). MPK4 is known to play important role in plant immunity, it functions as a negative regulator of pathogen defence and also interferes with stress signalling pathways at several distinct steps in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) as well as in effector-triggered immunity (ETI) (Berriri et al. 2012; Colcombet et al. 2012). Furthermore, it is also known that the Pseudomonas syringae HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses (Rasmussen et al. 2012). Data on C group are very limited and based on MPK7 whose expression is regulated by the circadian rhythm (Group 2002). Group D also has a C-terminal MKK5 docking domain (Rodriguez et al. 2010). It was found that some members of this group are induced by fungi and wounding in rice and alfalfa (He et al. 1999; Schoenbeck et al. 1999). One described member is MPK17. Generally not much is known about MPK17 but it is thought to be important, like whole group D, in plant sugar signal transduction (Sheen et al. 1999).

4.1.5 Plant pathogens and mechanisms of plant defence

4.1.5.1 Immunity

Plants are often attacked by a huge variety of microbial pathogens and herbivore insects. In order to respond to these threats they have developed numerous defence mechanisms and many of them are directly induced by pathogen attack (Glazebrook 2005). Generally, defence often begins with gene-for-gene pathogen recognition where the production of pathogen virulence effectors leads to their recognition by plants that carry correspondent resistance (R) genes (Glazebrook 2005). This kind of resistance is accompanied by fast reactive oxygen species (ROS) production also known as oxidative burst which is needed for a further process known as hypersensitive cell death (HR). Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism, removed by means of antioxidants and antioxidative enzymes (Kawano 2003) and was not suspected to provide meaningful information for the plant.

On one side, R gene-driven resistance activates salicylic acid (SA)-dependent signalling pathways which in turn lead to the activation of pathogenesis-related proteins (PR) that also contribute to resistance. This rapid activation of defence results in the limitation of pathogen growth. Innate immunity is generally initiated with recognition of conserved pathogen/microbial-associated molecular patterns (PAMPs/MAMPs) (Nishimura and Dangl 2010). Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of microbes (Boller and He 2009) and they are typically essential components of whole classes of pathogens, such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010). PAMPs are
perceived by pattern recognition receptors (PRRs), leading to activation of a series of immune responses, including the expression of defence genes, Reactive Oxygen Species (ROS) production, nitric oxide, ethylene, jasmonic acid (JA), and salicylic acid (SA) and activation of MAP kinase cascades (Jones and Dangl 2006; Tena et al. 2011; Yamaguchi et al. 2013). Activation of PRRs leads to PAMP-triggered immunity (PTI); PTI is often the first inducible response of a plant to PAMPs (Jones and Dangl 2006; Nishimura and Dangl 2010). The largest group within the *R* genes is binding site-leucine rich repeats (NBS-LRR) (Belkhadir et al. 2004), now mostly called NLR-dependent effector-triggered immunity (ETI) (Dangl et al. 2013). ETI is a second class of perception called effector-triggered immunity. This way of perception involves recognition by molecules called effectors which present intracellular receptors of pathogen virulence presenting, in contrast to PTI, co-evolutionary dynamics between the plant and pathogen. Generally, PTI and ETI give rise to similar responses. PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI, which is stronger and faster than PTI, is mostly active against adapted pathogens (Dodds and Rathjen 2010). Plants also respond to endogenous molecules which are released by pathogen invasion, cell wall or cuticular fragments, called danger-associated molecular patterns (DAMPs) (Dodds and Rathjen 2010).

Beside defence response controlled by SA pathways, defence responses can also be controlled by ethylene and/or jasmonic acid (JA) and this responses are mostly overlapping with response to wounding (Glazebrook 2005). Nevertheless, SA, JA and ethylene interacting between each other, SA and JA are reported to mutually inhibit the expression of many target genes whereas JA and ethylene are sometimes both acquired for expression of some genes but there are also some cases of negative interaction between their signalling.

**4.1.5.2 Biotrophic and necrotrophic pathogens**

According to their lifestyle pathogens are divided into biotrophs and necrotrophs. Biotrophs are pathogens that live and feed on living tissue; necrotrophs are defined as organisms that live and feed on dead tissue (Mcdowell and Dangl 2000; Thaler et al. 2004). Besides their different life style more important is the fact that Necrotrophic and biotrophic pathogens have evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004). SA is linked to resistance to biotrophic pathogens and is important to trigger the HR, a programmed cell death (PCD) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading (Glazebrook 2005; Colcombet and Hirt 2008) and regulate resistance against necrotrophic pathogens (Colcombet and Hirt 2008). However, some pathogens cannot be assigned as biotrophs or necrotrophs. There are also hemibiotrophic pathogens: hemibiotrophic pathogens incorporate aspects of both biotrophic and necrotrophic infection strategies. Often this involves an initial biotrophic infection phase during which the pathogen spreads in host tissue, followed by a necrotrophic phase during which host cell death is induced (Dodds and Rathjen 2010). However, the interactions between SA-, JA-, and ethylene-dependent pathways do not appear to be simple.
Complications could arise from different roles of these pathways in different stages of plant-pathogen interactions (Katagiri et al. 2002).

In order SA to accumulate two genes encoding enzymes similar to triacyl-glycerol lipases are required PHYTOALEXIN DEFICIENT 4 PAD4 and ENHANCED DISEASE SUSCEPTIBILITY 1 EDS1 (Zhou et al. 1998; Falk et al. 1999), raised SA levels in turn are activating various defense effector genes including PR-1 (Glazebrook 2005). The PR-1 effector gene for plant defense is activated by NPR1 and TGA-type transcription factors. NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) has function in regulation of systemic acquired resistance (SAR) in Arabidopsis (Rochon et al. 2006) and NPR1, as recently reported, plays the role of an SA receptor in vivo, in the signalling cascade leading to PR-1 activation (Wu et al. 2012). Systemic acquired resistance (SAR) means a mechanism of induced defence and implies long lasting protection against a broad spectrum of microorganisms. Salicylic acid is absolutely required by SAR as signalling molecule (Durrant and Dong 2004). Based on their physical interaction TGA transcription factors are considered as regulators of pathogenesis-related (PR) genes because of their physical interaction with the known positive regulator NPR1 (Kesarwani et al. 2007). Using reverse genetics approach it has been characterized 10 TGA factors in Arabidopsis so far and they all belong to the bZIP transcription factor family (Jakoby et al. 2002). When the level of SA are low, NPR1 exists as oligomeric form in cytoplasm, the monomers are held together by disulfide linkages which are getting reduced after the level of NPR1 increases and NPR1 is now present in monomeric form which as such can enter the nucleus and interact with the nuclear TGA$s$ and induce expression of PR-1 (Zhang et al. 2003).

As response to pathogen attack the levels of JA can also increase and as a consequence to that defence effector genes are increasing as well, especially VSP1 (Otani et al. 1998) and PDF1.2. The VEGETATIVE STORAGE PROTEIN 1 (VSP1) gene of Arabidopsis thaliana encodes a storage protein that accumulates in vegetative organs and it is jasmonate-responsive element (Guerineau et al. 2003). The PLANT DEFENSIN PDF1.2 gene in Arabidopsis encodes a plant defensin and it is commonly used as a marker for characterization of the jasmonate-dependent defence responses (Brown et al. 2003). Expression of the PDF1.2 requires both JA and ethylene (Glazebrook 2005). For known signalling activities of JA in Arabidopsis CORONATINE INSENSITIVE 1 (COI1) is required. COI1 gene encodes protein from F-box group and it is believed to act in proteolysis (Xie et al. 1998) but it can also bind histone deacetylases (Devoto et al. 2002). Beside the essential COI1, JA also requires members of MAPK cascade for its activity, the MPK4. MPK4 knock-out mutant failed to express the PD1.2. gene (Petersen et al. 2000). For ethylene connection to pathogen defence EIN2 is involved but that is not the only ethylene-related gene involved in pathogen defence (Lorenzo et al. 2003).

Pseudomonas syringae is a gram-negative, rod-shaped bacterium with polar flagella and it infects a wide variety of plants (Katagiri et al. 2002). Pseudomonas mostly acts as biotrophic pathogen (Zhao et al. 2003; Tsai et al. 2011). Peronospora parasitica is an oomyceteous
fungus from the Peronosporaceae family. In contrast to Pst DC3000, *Peronospora* is an obligate biotroph that is controlled by the salicylic acid-dependent SAR (systemic acquired resistance) pathway (Nawrath and Metraux 1999; Jambunathan et al. 2001). Defence responses under SA control are critical for resistance to both *Pst* DC3000 and *Peronospora parasitica* (Glazebrook 2001; Van Wees et al. 2003) (Nawrath and Metraux 1999; Jambunathan et al. 2001). *Alternaria brassicicola* belongs to phylum Ascomycota (family Pleosporaceae) and it known to be a very common plant pathogen. Black spot disease caused by *Alternaria brassicicola* is an important fungal disease affecting cruciferous crops (*Brassicaceae*). The interaction between *Arabidopsis thaliana* and *A. brassicicola* is a representative model system and objective estimation of disease progression is indispensable for accurate functional analyses (Su'udi et al. 2013). Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling, respectively (Van Wees et al. 2003). *Botrytis cinerea* is necrotrophic fungus belonging to the phylum Ascomycota (family Sclerotianiaecae) and it is a natural pathogen of *Arabidopsis thaliana* (P et al. 2007). *Botrytis* belongs in top 10 fungal pathogens in molecular plant pathology because it has an impact because of its broad host range causing severe damage: *Botrytis* causes bunch rot in viticulture or grey mould in horticulture (Dean et al. 2012). The fungus is considered as a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve infection (Amselem et al. 2011; Dean et al. 2012).

4.1.6 TCS type-B response regulator and MAPK cascade members are involved in pathogen signalling

MAPK cascade members found to be involved in pathogen signalling are *MPK3, MPK4* and *MPK6* (Colcombet and Hirt 2008). These kinases in *Arabidopsis* are activated by PAMPs, mostly by flg22 (Droillard et al. 2004). Very soon other MAPK cascade members, working upstream of MPK3/4/6 and downstream of the FLS2-BAK1, were found (Colcombet and Hirt 2008). These are MKKK1 and two MKKs, MKK4 and MKK5 (Colcombet and Hirt 2008). Nevertheless, in *mekk1* knock-out plants, flg22 was still able to activate MPK3 and MPK6 suggesting redundant effects at the MKK level (Ichimura et al. 2006; Suarez-Rodriguez et al. 2007). Interestingly, biosynthesis of ethylene is also triggered by flg22 via MPK6 which in turn activates *ACS6* (1-amino-cyclopropane-1-carboxylic acid) synthase 6 a crucial element in ethylene synthesis (Liu and Zhang 2004; Joo et al. 2008). Pst DC3000 induces MKK3 to positively regulate expression of *PRI*. On the other hand *mkk3-1* knock-out plants showed high susceptibility to *Pst* DC3000 and this effect was the opposite when the *mpk3-1* mutant was complemented by *MKK3* wild-type form or constitutive active *MKK3-EE* form (S235E and S411E, MKKK phosphorylation residues) (Doczi et al. 2007), that is, the downstream MPK targets are needed for a functional readout of MKK activity. Downstream elements for MKK3 thus appear to be Group C MPKs based on yeast-two-hybrid interaction studies; physiological roles were only confirmed with MPK7 (Zhang et al. 2007). Recently was found that ARR2 also contributes resistance against *Pst* DC3000. ARR2 cannot directly do this, but interacts with salicylic acid response factor TGA3 after it is picks up a TCS phosphate at D80, thereafter interacting with the TGA3/NPR1 dimer making ARR2/TGA3/NPR1 trimer complex.
that can bind to the PR1 promoter, activate it, and increase pathogen resistance. arr2-4 mutant plants in that study showed susceptibility to Pseudomonas (compared to the wild-type Col-0 plants) and also retarded induction of PR1 in response to SA (Choi et al. 2010). ARR2 was shown to pull down PR1, PR2, and ARR6 promoter DNA via ChIP assays. The association to DNA at PR1 however was mediated by TGA3 and not ARR2 (Choi et al. 2010), is suggests that a type of scaffolding role for ARR2 was discovered. Possibly other such roles for ARR2 and or type-B RRs independent of direct DNA binding could be discovered.

4.1.7 Cell-specificity effect of the MAPK cascade

Despite the fact that only couple members of the MAPK cascade control huge range of physiological responses, these signalling cascades are quite specific because they show cell specificity (Colcombet and Hirt 2008). For example, activity of MPK4 is increased in response to osmotic shock in Arabidopsis but only in suspension cells not in plantlets (Droillard et al. 2004). Subcellular-dependent function can be very well observed on ethylene-related roles of MPK6. The cascade MKK4-MPK6-ACS6 is involved in ethylene production whereas MKK9-MPK6-EIN3 cascade plays role in ethylene signalling. The MKK9–MPK3/MPK6 cascade promotes ETHYLENE-INSENSITIVE 3 (EIN3)-mediated transcription in ethylene signalling (Yoo et al. 2008; Hahn and Harter 2009). Ethylene as a ligand binds and inactivates the negative regulator CTR1 (MKKK family member) to activate the positive MKK9–MPK3/6 cascade (Yoo et al. 2008). This signalling pathway breaks a linear model and common MAPK signalling, and opens many questions, for example, both the real connection between CTR1 and MKK9 or how CTR1 is actually inhibiting the autophosphorylation of MKK9 through direct protein-protein interaction or in some other way (Hahn and Harter 2009). Supporting cell-specificity idea, scaffolding proteins have extremely important functions because they help MPK kinases to build physiologically functional cascades. Such an example is present in yeast where the C-terminal of the SSK1 RR binds to the N-terminus of the MKKK SSK2 inducing a conformational change which induces its autophosphorylation and activation of the kinase cascade (Posas and Saito 1998). Therefore it is very important to identify and describe these proteins in their crosstalk points and this work contributes to this issue.
4.2 Results

4.2.1 Background
The TCS and MAPK signal transduction pathways are two differently evolved signalling pathways both crucial for mediating control of physiological responses and development in plants (Grefen and Harter 2004; Hass et al. 2004; Mira-Rodado et al. 2007; Pathak et al. 2013; Smekalova et al. 2013). It has been known that ARR2 also functions in ethylene signalling as an arr2 Ler-0 knock-out shows an hyposensitive phenotype (Hass et al. 2004). Transcriptomic data of arr2 Ler-0 knock-out and ARR2 overexpression lines revealed drastic mis-regulation of various defence-related genes some of which are well known to be controlled/activated by MAPK cascade signalling (Hass et al. 2004). Preliminary data suggested that ARR2 co-localises and interacts with several MAP kinase kinases in Y2H and in vivo BIFC (Achim Hahn, personal correspondence). These findings indicated that there might be a functional connection of ARR2-dependent TCS signalling and MAPK cascades which were further explored in this thesis.

4.2.2 Interaction of ARR2 with MAPK cascade members

4.2.2.1 Response regulators Type A or B do not interact with MKKKs
MKKKs were tested for protein-protein interaction against ARR2 using the yeast-two-hybrid system as an in vivo heterologous approach. The large number of MKKK proteins made it impossible to test all of them, but nevertheless, three representative members were chosen for this experiment: Raf24, Raf43 and Raf41 due to their distinct sequence and functions (Jouannic et al. 1999; Group 2002). Besides ARR2, two other members of type-B family were chosen, ARR1 and ARR18, and a well-known type-B family member, ARR4. ARR1 has very high sequence homology with ARR2 and it is proposed to be redundant to ARR2 in many physiological functions (Sakai et al. 2000). ARR18 on the other hand is different from its group members ARR1 and ARR2 in sequence, expression pattern and presumably function (Mason et al. 2004; Veerabagu et al. 2012) even though these three type-B response regulators are all members of the subgroup I. ARR4 was taken as a typical member of type-A group of response regulators to distinguish B-type and A-type characteristics, if there are any.

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A (James et al. 1996) was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MKKKs were cloned with the GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. None of tested MKKK showed transactivation in yeast (only shown for Raf41) indicating that all three MKKKs could be evaluated in yeast. Nevertheless, when the colonies were plated on interaction-selective media (~W, L, A), none of these MKKKs interacted with any of tested ARRs (Figure 4.2). This experiment was repeated three times using all interaction elements and each time the same result was obtained. Based on this experiment, if there is intercommunication between this two signaling pathways, then it is downstream of MKKKs or requires other MKKK elements.
**Figure 4.2** *Arabidopsis* response regulators do not interact with selected MKKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (Raf24, Raf43, Raf41). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only Raf41 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

### 4.2.2.2 B-Type response regulators interact with MKK members

MKKs represent a point of signal convergence within MAPK signalling cascade (Mordret 1993). After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM-W, L medium. MKK were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. As before ARR1, 2, 4 and 18 were tested against MKKs: MKK1, MKK2, MKK4, MKK5. MKK3 is transitive in yeast and cannot be analyzed as a full-length protein and therefore was not tested (not shown). None of other tested MKK constructs showed transactivation in yeast (Figure 4.3) indicating that all three MKKs could be evaluated in yeast. Interaction was observed for ARR1, 2 and 18 with MKK1, MKK2, MKK4, MKK5, whereas no interaction was observed for ARR4 with any MKK tested (Figure 4.3).
4.2.2.3 ARR2 response regulator interacts also with MPKs

As the last members of MAPK signalling cascades, MPKs were also tested against ARR2. For this experiment MPK4 and MPK17 were chosen as they both have been described in the literature and have distinct evolutionally histories (Group 2002).

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MPKs were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. ARR1, 2, 4 and 18 were tested against MPK4 and MPK17 (Figure 4.4). Neither MPK showed transactivation in yeast indicating that each MPKs could be evaluated in yeast (data for MPK17 shown).

Figure 4.4 ARR2 interacted with MPKs in yeast-two-hybrid assays.

Proteins were expressed either as GAL4-AD-fusions (ARR2) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK17 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.
Both MPK4 and MPK17 showed interaction with ARR2. It is known that MKKs and MPKs interactions can be observed in yeast. Therefore, the MKK1/2/4/5 were tested against the two MPKs, MPK4 and MPK17. Interestingly, only MPK17 interacted with MKK5. MPK4 interacted with all four MKKs but a stronger interaction was observed with MKK4 and MKK5, especially with a preference for with MKK5 (Figure 4.5).

![Image](image.png)

**Figure 4.5 MPK4 and MPK17 interact with biotic stress-related MKKs in yeast-two-hybrid assays.**
Proteins were expressed either as GAL4-AD-fusions (MKK1, MKK2, MKK4 and MKK5) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK4 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

**4.2.2.4 MKK4 and MKK5 interacted with truncated versions of ARR2 containing only receiver or output domains**
In order to get a closer look at which regions of ARR2 are essential for ARR2-MKK interactions, ARR2 was truncated (Figure 4.6, C). Protein truncation ARR21–300 contains receiver domain, part of the DNA-binding GARP domain and two out of three nuclear localisation signals (NLSs) present in ARR2. ARR2300–664 carries the other half of the DNA-binding GARP domain and the third NLS motif. ARR21–145 contains a receiver domain only. ARR2145–664 contains the output domain with all three NLSs. ARR21–165 contains the receiver domain and one of three NLSs while the part. ARR2165–664 contains other two NLSs and the output domain. This information is summarized in Figure 4.6C as a graphic representation of the ARR2 protein domains, the location of MKK docking sites and the truncation positions.
Figure 4.6 MKK docking motifs are present on both receiver and output domains in ARR2. (A, B) Proteins were expressed either as GAL4-AD-fusions (ARR2^{1-145}, ARR2^{145-664}, ARR2^{1-165}, ARR2^{165-664}, ARR2^{1-300}, ARR2^{300-664}, ARR2^{51-81} and ARR2^{215-260}) or as GAL4-BD-fusions (MKK1, MKK2, MKK3, MKK4 and MKK5). (C) Cartoon presentation of the location of the truncated parts of ARR2 with MKK binding sites indicated in yellow. Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls shown in A. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown.

The various ARR2 truncations were tested against MKK1, MKK2, MKK4 and MKK5 for interaction using yeast-two-hybrid system (Figure 4.6, A,B). The receiver domain-containing truncations ARR2^{1-145}, ARR2^{1-165} and ARR2^{1-300} extended variant showed positive interaction only with MKK5; for all other tested kinase members no interaction was observed. On the other hand, the truncation containing the output domain ARR2^{145-664}, ARR2^{165-664} and the ARR2^{300-664} truncation showed very strong interaction with MKK4 and MKK5, but not with MKK1 or MKK2. These results suggested that there are a couple of different MKK docking sites within ARR2 that are widely dispersed along the protein. It is therefore possible that more than one, or maybe all, binding sites are needed. In summary, the type-B receiver domain and output domain can interact with MKKs.
4.2.2.5 MKK docking motifs are present on ARR2 in both receiver and output domains

MKK4 and MKK5 play an important role in the plant’s response to biotic stress, such as pathogen attack (Asai et al. 2002) and stomata development (Wang et al. 2007). The interaction of ARR2 with MKK4 and MKK suggested it would be useful to find out more about the number and position of MKK docking sites in ARR2 by applying an in silico approach. For this purpose ELM software (http://elm.eu.org) was used. ELM is a computational biology resource for investigating candidate functional sites in eukaryotic proteins reported to have high significance value (Dinkel et al. 2012). The docking interaction in the MAP kinase cascades is achieved through specific conserved regions on MKKs (docking groove) and MAPK-interacting molecules (the MKK docking motif). After evaluation of the raw output from ELM (Supplemental figure 1), docking motifs for MAPK cascade interacting molecules (e.g. MKKs, substrates, phosphatases) were predicted. There are at least two different MKK docking types: the classical docking motif and short peptide containing the sequence FxF (Sharrocks et al. 2000; Bardwell et al. 2001; Galanis et al. 2001). The classical docking motif is characterized by a cluster of at least two positively charged amino acids followed by a spacer of 2-to-6 residues from a hydrophobic-X-hydrophobic sequence, where the hydrophobic residues are long-chain aliphatics (usually Leu or Ile).

In the spacer and in the sequence immediately C-terminal to the hydrophobic-X-hydrophobic element, there is a high propensity for the presence of Pro, Asn, and/or Gly, which are residues that are both turn-forming and helix-breaking (Bardwell et al. 2001). The classic motif approximates \((R/K)X_{2-6}\PsiX\Psi\) where \(\Psi\) is a hydrophobic residue and X any residue. Four such docking motifs were found in ARR2 (Supplemental figure 1, figure 4.6). Interestingly, two of them were found on the receiver domain of the ARR2 at amino acid positions 56-66 (KCNRAESALSL) and 68-78 (RKNKNGFDIVI) amino acid positions. Two other potential docking domains were located in the output domain of ARR2 at amino acid positions 215-225 (KKPRVVWSVEL) and 244-252 (KKILEMMNV) (Figure 4.6, C). In order to check if these motifs are really docking sites for MKKs two truncation peptides of ARR2 to were made: ARR2\(^{51-81}\) and ARR2\(^{215-260}\). Only two fragments were made because of the fact that between the potential receiver domain docking sites only a very small physical distance lies between them and such small peptides would most likely be degraded in yeast. Surprisingly, both peptides ARR2\(^{51-81}\) and ARR2\(^{215-260}\) interacted only with MKK5 and that very strongly; No interactions were obtained with the other MKKs (Figure 4.6, B). This suggests that for all of the other MKKs other docking sites must be present which do not fit into the typical pattern for MKK docking domains, or, an alternative type of interaction domain exists. These experiments confirmed the previous observations in yeast, where truncated versions of ARR2 containing only receiver domain sequence were used, that receiver domain may also play role in ARR2-MKKs interactions and not just the output domain only. Thus it can be concluded that the inability of the ARR4 type-A RR to interact with MKKs is not due to an absence of output domain but rather due to difference in sequence of receiver domains between type-A and the types-B ARRs.
4.2.2.6 ARR2 does not need to be phosphorylated by TCS elements in order to interact with MKKs in Y2H

The ARR2 can obtain a phosphoryl group from the AHP TCS elements on its aspartate at the 80th position (Grefen and Harter 2004). Mutation of D80 to E (glutamate, Glu) increases the transactivation activity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects if expressed in plants (Hass et al. 2004). Mutation of D80 to N (asparagine, Asn) however, prevents TCS dependent phosphorylation (Kim et al. 2006) and impairs basal reporter-gene activation capacity. To check if the phosphorylation state of ARR2 at the D80 position influences MPK(K)-ARR2 protein interaction loss-of-function ARR2D80N mutant and gain of function ARRD80E protein variants were tested in the yeast-two-hybrid system.

![Figure 4.7 ARR2 interacts with the MKKs independent of TCS-mediated phosphorylation.](image)

Proteins were expressed either as GAL4-AD-fusions (ARR2D80E and ARR2D80N) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

MKK1-5 were used for this experiment. Both mutant versions of ARR2 showed exactly the same pattern like the wild-type protein (Figure 4.7). This suggests that ARR2 does not need to be phosphorylated by TCS elements for successful interaction with MKKs and that ARR2 might serve as scaffold protein in signal transduction between MAPK cascade elements and the TCS or yet other unknown, undescribed proteins.

4.2.2.7 ARR2 shows very strong interaction with biotic-stress-related MKKs in yeast

MKK1 and MKK2 are known to be involved in response to abiotic stress (e.g. osmotic stress, temperature change, high salinity) (Teige et al. 2004) whereas MKK4 and MKK5 are known for their role in biotic stress (e.g. pathogen interactions) (Asai et al. 2002). In the previous yeast experiments some interactions appeared stronger as judged by both the growth intensity of the colonies and the number of colonies that displayed positive interactions. In particular, MKK4 and MKK5 repeatedly displayed stronger interactions with ARR2, its variants and other B-types. Therefore, the interaction strength of the MKK interactions with ARR2 was quantified in yeast using serial dilutions and quantification of reporter-gene strength by the O-NPG assay.
**Figure 4.8** ARR2 shows strong interaction with biotic-stress-related MKKs in yeast.  
(A) Dilution series of fusion proteins co-expressed from the yeast-two-hybrid expression vectors: GAL4AD-ARR2 and MKK1, MKK2, MKK4 and MKK5 fused to N-terminal GAL4BD. (B) The β-galactosidase assay of the same constructs used for dilution series. The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Serial dilutions (1, 1:10, 1:10², 1:10³ and 1:10⁴) of the corresponding yeast culture was grown on selective medium and transformed PJ64-4A yeast cells with corresponding vectors were used for the O-NPG assay (β-galactosidase assay). Five colonies containing ARR2 and MKK1/2/4/5 were tested. As suspected, ARR2 interacted much stronger with biotic-related MKKs (MKK4 and MKK5) than the abiotic-related ones (MKK1 and MKK2) by the growth assay (Figure 4.8).

While the serial dilution method is informative, the more quantitative and less error-prone method is measuring β-galactosidase reporter-gene activity via the O-NPG assay. In this case, the O-NPG assay was extended to include ARR1 and ARR18. As before, ARR2 interacted stronger with MKK4/5 (~17 and ~20 O-NPG units for MKK4 and MKK5 respectively) than MKK1/2 (~5 and ~7 O-NPG units for MKK1 and MKK2 respectively). The same tendency was observed for ARR1 and ARR18 (Supplemental figure 2). It can be therefore concluded that, in general, MKK4 and MKK5 have a stronger affinity for B-type subclass I response regulators.
4.2.2.8  **ARR2 shows very strong interaction with biotic-stress-related M KKs in planta**

In order to see if this strong and specific interaction between ARR2 and MKK4 and MKK5 obtained in yeast is also the case in plant cells, FRET-FLIM (Förster Resonance Energy Transfer-Fluorescence Life Time Imaging) microscopy analysis was performed in planta. For this experiment the fluorescence lifetime of a Donor (i.e. GFP) is measured. A reduction in the lifetime is observed when an Acceptor (i.e. mCherry or mRFP) is within a distance of 10 Å or less; this distance is what is expected of protein-protein interactions (Caesar et al. 2011).

Since MKK3 is also known to be involved in biotic stress in a JA dependent manner (Doczi et al. 2007) and was previously not characterized in yeast due to its strong transactivation effect, MKK3 was also tested against ARR2 in planta using the FRET-FLIM approach.

ARR2 was cloned in-frame with GFP to make an ARR2::GFP fusion. The MKKs were therefore cloned in-frame with mCherry. A positive readout control was created by fusing ARR2 to a tandem GFP::mCherry coding frame via a short linker and subsequently called ARR2::FRET.

After cloning, the constructs were transformed into *Agrobacteria tumefaciens* and transiently expressed in tobacco (*Nicothiana bethamiana*) epidermal cells as a heterologous plant system. The negative control was ARR2::GFP transformed alone.

All three tested MKKs interacted with ARR2 (Figure 4.9). Both MKK4 and MKK5 interacted with ARR2 stronger than MKK3, with MKK5 having the strongest interaction with ARR2 (Figure 4.9, B). These in planta data confirmed the previous data obtained in the yeast-two hybrid system and lead to more stable and concrete evidence that these interactions, especially ones with MKK4 and MKK5, might be have physiologically functional roles in *Arabidopsis* to biotic stresses.
Figure 4.9 ARR2 interacts very strongly with biotic-stress-related MKKs in planta.  
(A) Cartoon representation of ARR2 and MKKs proteins fused for fluorescent proteins for FRET-FLIM measurements. (B) FRET-FLIM results of ARR2-GFP interaction with MKK3, MMK4 and MKK5 fused to mCherry. (C) CLSM photos of tobacco leaves showing localization of expressed ARR2-GFP fusion protein with indicated MKKs fused to mCherry fluorescent protein. The fusion proteins were transiently expressed in tobacco cells and the results are given as function of Chi² test (measurements and evaluation courtesy of Dr. Kirstin Elgass). ARR2-GFP and ARR2-GFP-mCherry were used as negative and positive controls respectively. This experiment was repeated two times with similar results.
4.2.3 Roles of ARR2 in pathogen-related phenomenon

As previously shown, FRET-FLIM in planta measurements and experiments using the yeast-two-hybrid system connect TCS type-B RR s to MKKs via physical interaction. ARR2 especially interacted strongly with MKK3, MKK4 and MKK5. Additionally, ARR1 and ARR2 interacted with MPK4 and MPK17 as well. MKKs play an important role in the plant’s response to biotic stress, such as pathogen attack (Asai et al. 2002). And interestingly, this is also the case for all three in planta tested MKKs: MKK4 and MKK5 (Nakagami et al. 2005; Popescu et al. 2009) as well as MKK3 (Doczi et al. 2007) clearly implicating the involvement of ARR1 and ARR2 in pathogen defence.

4.2.3.1 Reactive Oxygen Species (ROS) and ethylene measurements in ARR1 and ARR2 mutants after treatment with Pathogen-Associated Molecular Patterns (PAMPs)

As a first look into pathogen-related responses in ARR2, wild-type and mutants arr1-4, arr2-4 and arr1-4 arr2-4 lines were treated with PAMPs. As output, ROS and ethylene production were measured.

4.2.3.1.1 The arr1-4 arr2-4 double mutant showed differences in ROS production after treatment with flg22 and elf18

For ROS experiments, leaves from 5 to 6 week-old plants were used. Leaves were cut in discs from the same leaf surface and incubated in water overnight.

Figure 4.10 arr1-4 arr2-4 double mutant shows differences in ROS production after treatment with PAMPs. Arabidopsis leaves from different mutants were treated with elf18 (left) and flg22 (right). Mutants efr and fts22 were used as negative response controls. Letters a, b and c stand for biological experimental repetitions whereas in the c repetition only the arr1-4 arr2-4 double mutant was used along with control plants. Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level separately for the time points 13, 17 and 21 minutes.
The following day, the discs were transferred to a reaction solution and the ROS production was triggered by treatment with 100nM of flg22 or 100nM elf18. In addition to the type-B mutant lines, fls2 (Dunning et al. 2007; Krol et al. 2010) and efr mutants lacking leucine rich repeat-receptor-like kinases FLS2 (Flagellin-Sensitive 2) and EFR (Elongation Factor-Tu receptor), respectively (Krol et al. 2010). FLS2 recognizes bacterial flagellum (active epitope flg22) and EFR recognizes elongation factor Tu (active epitopes elf 18, elf13 and elf26).

Both times the same tendency was observed: arr1-4 arr2-4 double mutant significantly produced more ROS than the wild-type (p≤0.05 at time-points 13, 17, 21 min post treatment) in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments; the double mutant consistently however showed more ROS than the wild-type (Figure 4.10, experiment a versus b). Therefore, a third experiment was conducted using more replicates to confirm the previous two experiments. It could be concluded that the arr1-4 arr2-4 double mutant significantly produces more ROS in response to applied PAMPs, flg22 (p≤0.05 at time points 13, 17, 21 min post treatment) and elf18 (p≤0.05 at time-points 13, 17, 21 min post treatment) but it is unclear what the effect of the single mutants have on ROS production (Figure 4.10).

4.2.3.1.2 The arr1-4 arr2-4 double mutant did not show any difference in ethylene production after treatment with different PAMPs

*Figure 4.11 arr1-4 arr2-4 double mutant shows no difference in ethylene production after treatment with different PAMPs*

Arabidopsis leaves from arr1-4 arr2-4 double mutant and Col-0 wild-type were treated with (A) elf18 and flg22 using different concentrations: 0nM, 1nM, 10nM, 100nM and 1000nM (B) Fungal extracts used were 1µL of PEN and 3µL of Xac in a final volume of 500µL. Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level. This experiment was performed as two biological replicates each containing three technical replicates.

Ethylene production was measured as a response to different PAMPs flg22 and elf18 or crude extracts PEN from *Penicillum schizogonium* and Xac from *Xanthomonas axonopodis citri*. A dose response curve was obtained for Flg22 and Elf18 (0.1nM, 1nM, 10nM, 100nM, 1µM) but only single concentrations 1µL/500µL PEN and Xac 3µL/500µL for the crude extracts was
conducted. Based on results from obtained the previous ROS measurements and due to lack of growth space, only Col-0 wild-type and arr1-4 arr2-4 double mutant lines were used. The double mutant did not show any statistically significant difference in ethylene production to any of the tested PAMPs compared to the wild-type (Figure 4.11, A, B). Based on this experiment, it can be concluded that either there is no significant difference in ethylene production. Alternatively, perhaps the analytical system was not sensitive enough to detect subtler changes in ethylene production compared to that of the ROS system.

4.2.3.1.3 The arr1-4 arr2-4 double mutant did not show any difference in activation pattern of MPK3, MPK4 and MPK6 after treatment with flg22

The MPK kinases MPK3, MPK4 and MPK6 are known to be activated by phosphorylation in a flg22 dependent manner (Droillard et al. 2004). Activation of these MPKs can be analysed by using the phospho-p44/p42 MPK antibody (Brock et al. 2010; Montillet et al. 2013). As previously observed, flg22 was able to elicit a higher ROS response in the arr1-4 arr2-4 mutant background (§4.2.3.1.1). Thus by conclusion, it is possible that these MPK kinases are differentially activated in the arr1-4 arr2-4 mutant background compared to the wild-type. For this experiment 4 week-old plants were used and their leaves were infected by dropping of 1µM solution of flg22 or mock onto a leaf surface. Samples were collected at three time points: immediately after infection, 15 minutes and 30 minutes after treatment (Figure 4.12).

Before flg22 treatment only weak signals were obtained for MPK6 and MPK3 showing that these two kinases are phosphorylated at some basal level. After 15 minutes of flg22 treatment, the signals for MPK3/6 became much stronger and a third band representing MPK4 appeared (Figure 4.12). Half an hour after infection the phosphorylation pattern stayed
the same as the 15 minute time point, indicating that the peak flg22-dependent MPK activation level was already reached at the 15th minute or before. Mock treated leaves at 15 and 30 minutes after treatment maintained the same basal MPK3/6 pattering as the zero time point. In conclusion, no dramatic differences were observed between the wild-type and the double mutant in activation pattern or intensity with any of these three kinases.

4.2.3.2 Pathogen assays using biotrophs and necrotrophs
A broad spectrum of different pathogens was used in this work to cover both necrotrophic and biotrophic types of infection strategies. These pathogens were challenged on Col-0, arr1-4, arr2-4 and arr1-4 arr2-4 plant lines. It was very important to obtain various measurements of a given interaction with high spatial and temporal resolution. For necrotrophs, Alternaria brassicicola and Botrytis cinerea were tested. For biotrophs, the obligate biotrophic fungus Peronospora parasitica was tested as well as the biotrophic/hemibiotrophic bacterium Pseudomonas syringae pv. tomato DC3000 (Pto DC3000).

4.2.3.2.1 Pathogen assays using biotrophs
Pathogen assays with biotrophs were always performed using one bacterial and one fungal pathogen: the bacterium Pto DC3000 and fungus Peronospora parasitica.

4.2.3.2.1.1 There is no difference in response of ARR1 and ARR2 mutants to biotrophic Pst DC3000
This pathogenic assay was done using five-week old plants and treating them with Pto DC3000 bacterial strain. Bacterial cells were infiltrated into two leaves per plant. Bacterial cells at an approximate concentration of $10^4$ cfu/ml were infiltrated with a needless syringe into the middle of the leaf apoplastic space (two leaves per plant) to an area was always bigger than the cork borer used for cutting the leaf discs with which the experiment was further performed. Afterwards the leaves were harvested, at the time point 0h and 1st, 2nd and 4th days past infection, material was plated on LB-plates with antibiotics and number of colonies per plate/mutant formed was counted.

Based on number of grown colonies per plate the difference in resistance between the mutants was determined. Although this experiment was repeated six times none of them consistently showed any pattern that could be attributed to the loss of ARR1 or ARR2 or both. That is, any differences that could be observed in the second and fourth day were never reproduced in any other experiment (all repetitions are not shown but compare 4.13A with the 4.13B mock for an example.

Knowing that ARR2 has been implemented in Pto DC3000 pathogen resistance and that this response required both cytokinin and SA (Choi et al. 2010), a cytokinin treatment was added to the experimental design. t-zeatin was used at a working concentration of 1µM. Before infiltration cytokinin was given to the 1x10^4 cfu/ml bacterial dilution. The plants were also treated with cytokinin 3 days before the experiment by adding it to the water used for watering. No clear trend could be observed that could be ascribed to a combined effect of exogenous cytokinin and loss of ARR1 or ARR2 could be found. In fact, in this experiment, the cytokinin treatment resulted in reduced bacterial growth in all plant lines which was highly
statistically significant by day 4 (Supplement figure 3). This effect of cytokinin on Pto DC3000 growth has been observed before (Choi et al. 2010; Choi et al. 2011).

Figure 4.13 ARR1 and ARR2 mutant plants do not show reproducible differences in response to biotrophic Pseudomonas syringae pv. tomato DC3000. Leaves of Arabidopsis ARR1 and ARR2 single and double mutants and corresponding wild-type (Col-0) were treated with Pseudomonas. The evaluation of infection was done at day 0, 1, and 4. (A) Plant lines treated only with Pseudomonas syringae pv. tomato DC3000. (B) Plant lines treated either with Pseudomonas syringae pv. tomato DC3000 only (Mock) and also with 1µM t-zeatin (cytokinin). Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level (Supplemental figure 3). These experiments were repeated at least three times each with similar results. CFU stands for Colony Forming Units. Data are represented as box-and-whisker plots: the central horizontal line is the median (2nd quartile) and the boxed region extends from the beginning of the 1st quartile to the end of the 3rd quartile. Lines extending outside of the boxed region are 1.5 x (the respective interquartile range). Data points outside this region are shown as dots and are called outliers.
4.2.3.2.1.2 There is no difference in response of ARR1 and ARR2 mutants to Peronospora parasitica

Next arr1-4 arr2-4 plants were challenged with *Peronospora parasitica*. This experiment was performed in collaboration with the research group of Prof. Dr. Volker Lipka from University of Göttingen. Four to six-week old plants were inoculated by spraying the leaves with a *Peronospora parasitica* conidial suspension and the infected plants were observed after seven days.

![Image of plant leaves](image)

Figure 4.14 ARR1 and ARR2 mutant plants do not show differences in response to biotroph *Peronospora parasitica*.

Leaves of *Arabidopsis* wild-type (Col-0), ARR1 and ARR2 single and double mutants were sprayed with *Peronospora*. After seven days there were no visible differences among the mutant plants and the wild-type. This experiment was repeated once with similar results. Data were produced by the research group of Prof. Dr. Volker Lipka.

If there is any difference in resistance to *Peronospora*, a clear phenotypic difference would have been visible. However, since there was no visual difference obtained between the wild-type and the mutant plants there was not any need for counting of sporangiophores on the leaves (pers. communication V. Lipka). It was concluded that the mutant plants do not show any difference in resistance to *P. parasitica* compared to the wild-type (Figure 4.14).

4.2.3.2.1.3 Lack of differential response to biotrophs *Pst DC 3000* and *Peronospora parasitica* is not due to perturbation of stomata aperture regulation

It was proposed that a larger stomata aperture would make it easier for bacteria to enter into the plant, infect cells and form a stable microfilm in vascular bundles allowing it to further multiply and invade the plant. It is also known that cytokinin can cause stomata to open (Tanaka *et al.* 2006) and abscisic acid (ABA) causes them to close (Desikan *et al.* 2006). Initially, *Pst DC3000* also causes stomata to close (Zeng *et al.* 2010; Desclos-Theveniau *et al.* 2012), however after 3 hrs, the bacteria produce diffusible phytotoxin COR that reopens closed stomata to increase the number of sites for bacterial invasion (Melotto *et al.* 2008). Therefore an experiment was designed to test this hypothesis.
Figure 4.15 Regulation of stomata aperture in *ARR1* and *ARR2* lacking mutants in response to hormonal treatment with ABA and *t*-zeatin and *Pst DC 3000* *Pseudomonas* strain

For treatments with hormones at least five plants per line were used and from those 2 leaves per plant were treated with the hormones. Experiments with *Pst DC 3000* were done only for *arr1-4 arr2-4* double mutant and Col-0 whereby five plants per line were used and the stomata aperture size was measured 3 days after infection. This experiment was repeated once with similar results. The mean and SE is shown. Leaves were floated on water or water containing 10µM ABA or 1µM *t*-zeatin (see §3.6.11 for details). For statistical results see Supplemental Figure 4.

Leaves were treated with water (Control), ABA, *t*-zeatin for 2 hours or three days after *Pst DC3000* infection. The results are displayed as bar graphs (Figure 4.15) and the significance tests results are given in Supplemental Figure 4. Importantly for this work, it is known that ABA-dependent stomata closure is not dependent on *ARR2*, as the *arr2-4* responds like wild-type (Desikan et al. 2006). This response was replicated; that is, the stomata apertures were significantly closed in response to ABA treatment. On the contrary, treatment with *t*-zeatin resulted in a significant increase in stomata aperture for all plant lines. Plants sprayed with *Pst DC3000* also had stomata apertures wider than the non-treated controls (data only for Col-0 and *arr1-4 arr2-4* were obtained). The data showed that there was no difference in stomata aperture response in the mutant backgrounds compared to the wild-type. Furthermore, all plant lines responded the same way after treatment with different hormones and after application of *Pst DC3000* by spraying (Figure 4.15).

In conclusion, based on the preceding pathogen experiments and this stomata aperture measurement, there is no difference in the resistance of *arr1-4, arr2-4* or *arr1-4 arr2-4* plants to *Pseudomonas syringae Pst DC3000* under my experimental conditions.
4.2.3.2.2 Pathogen assays using necrotrophs

Two necrotrophic fungi *Alternaria brassicola* and *Botrytis cinerea* were challenged with Col-0 wild-type and the *arr1/2* mutants. Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling pathways, respectively (Van Wees *et al.* 2003). *Botrytis cinerea* is a natural necrotrophic pathogen of *Arabidopsis thaliana* (P *et al.* 2007) and is considered as a typical necrotroph that co-opts programmed cell death pathways in the host to achieve infection (Amselem *et al.* 2011; Dean *et al.* 2012).

4.2.3.2.2.1 *arr2*-4 single and *arr1*-4 *arr2*-4 double mutants are more susceptible to *Alternaria brassicola*

*Alternaria brassicola* was applied as a sporal suspension dropped (5µl droplets of spore solution of 5*10^5 Spores/ml) onto *Arabidopsis* leaves (two leaves per plant) and scored on the 7th and 10th day after infection using a disease progression index as in (Kemmerling *et al.* 2007) and summarized in Methods (§3.6.16). Statistically significant differences were obtained between *arr2*-4 single, and *arr1*-4 *arr2*-4 double mutants compared to the WT (Col-0) were computed using the mean. Single *arr2*-4 (p≤0.001) and the double *arr1*-4 *arr2*-4 mutants (p≤0.001) showed statistically significant susceptibility compared to the wild-type where the difference in *arr1*-4 single mutant (p≤0.01) was not statistically significant from wild-type (Figure 4.16, A).

The same experiment was therefore repeated in presence of 1 µM exogenous cytokinin, *t*-zeatin. Similar to the previous experiment with *Pst* DC3000, before the assay was performed, cytokinin was added to the fungal dilution. The plants were also treated with cytokinin by mixing it with water used for watering 3 days before the experiment was performed. Cytokinin induced some kind of resistance in all of the plants (Figure 4.16, B) and this resistance was statistically significant (see Supplementary Figures 5 and 6, Figure 4.16B). At the day 7 after treatment the disease index of cytokinin treated plants decreased from 270.8 by mock treated plants to 257.5 (p≤0.0029, LSD (Fisher’s Least Squared Difference Test). The same tendency was observed 10th day after treatment where the disease index of treated plants decreased from 306.0 to 294.4 (p≤0.0066 LSD (Fishers Least Squared Difference Test).

The *arr2*-4 and the double mutant are more sensitive irrespective of the cytokinin treatment to *Alternaria* which was statistically confirmed (see Supplemental Figure 5 and 6). In contrast, wild-type plants and the *arr1*-4 single mutant did not show differences in their disease indices. This means that *arr2*-4 is responsible for susceptibility phenotype observed in the double mutant (Figure 4.16, B).
Figure 4.16 *Arabidopsis thaliana* arr2-4 single and arr1-4 arr2-4 double mutants are susceptible to necrotroph *Alternaria brassicicola*

Leaves of arr1-4, arr2-4, arr1-4 arr2-4 and Col-0 were treated with *Alternaria* spores. The infection was evaluated at day 7 and 10. The data are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table) (A) Plant lines only treated with *Alternaria brassicicola* spores. Image insert to the right shows a representative leaf composition at Day 7 (B) Plant lines treated either with *Alternaria brassicicola* spores only (Mock) and also with 1µM t-zeatin. To the right, the pooled mean and ANOVA 95% confidence interval for all samples either treated or not treated with cytokinin is shown for Day 7 (ANOVA p ≤ 0.0029) and Day 10 (ANOVA p ≤ 0.0066); the cytokinin treated samples showed less disease symptoms. LSD (Fisher’s Least Squared Difference Test) was also used to compute significance levels at the α=0.05 level based on the mean (Supplemental Figures 5 and 6). For this experiment at least 20 plants per line were used and two leaves per plant were infected with *Alternaria* spores. This experiment was repeated at least three times with similar results.
4.2.3.2.2  arr1-4 arr2-4 double mutant is resistant to Botrytis cinerea

Botrytis cinerea is especially interesting as it is known to be a natural pathogen of Arabidopsis thaliana. Botrytis cinerea was applied to the single mutants, double mutant and wild-type by dropping a fungal suspension on the leaves. Susceptibility to B. cinerea was determined using a detached leaf assay and a visually identified by a lesion disease index. Lesion size has been used as an indicator of susceptibility to B. cinerea (Ferrari et al. 2003; Denby et al. 2004) and this has been shown to correlate with whole plant susceptibility and pathogen growth within the plant (Govrin and Levine 2000; Denby et al. 2004).

This experiment was performed in collaboration with Floriane L’Haridon from research group of Prof. Dr. Jean-Pierre Métraux from University of Fribourg, Switzerland. Hyphae and conidiophores were visually identified throughout the lesions and the infection sites were categorized according to their lesion sizes which are divided into three groups: Group 1 are lesions less than or equal to 2mm, group 2 lesions between 2 mm and 6 mm and the group 3 are lesions bigger than 6 mm. To reflect different severities of infection (Figure 4.17, A) the outgrowing lesion size was also determined (Figure 4.17, B) as well as the average lesion size (Figure 4.17, C). The lesions were measured three days after application of the pathogen.

As the data are categorical, they are presented in Figure 4.17 A and B as mosaic plots; alternative plots and statistics can be found in Supplemental Figure 7. The categorized lesion size is shown in Figure 4.17A and average lesion size is shown as a bar graph of the mean (Figure 4.17, C). As can be seen in Figure 4.17 A and B the severity of in infection was reduced in all mutant lines compared to Col-0. Col-0 had the majority of its lesions in Category 3 and its overall outgrowth index at 75% or more (87.5% of all lesions). In contrast, each ARR mutant had more lesions in Category 1 and 2, less in 3 (Figure 4.17, A). In fact looking only at Category 3 results, Col-0 was infected better with over 58.3% its lesions bigger than 6 mm. In contrast, the double mutant had only 31.3% of its lesions bigger than 6 mm. The single mutants showed in infection levels in-between wild-type and the double mutant: arr2-4 (40.6% ≥6 mm) and arr1-4 single mutant (43.8% ≥6 mm). This difference in infection efficiency is also evident from inspection of the outgrowth index, whereby only 58.3% of arr1-4, 54.2% of arr2-4 and 50.0% of arr1-4 arr2-4 leaves had an outgrowth index at 75% or more.

The average lesion size (Figure 4.17, C) again showed a clear picture of mutant resistance. Average lesion size for the wild-type was about 5.77 mm whereas the single mutants this value decreased to 4.39 mm for arr1-4 and 3.99 mm for arr2-4. In the double mutant the average lesion size was 3.52 mm only. Leaves treated with inoculation media alone did not develop any lesions or lesion-like symptoms (data not shown).

All of these parameters very clearly show that arr1-4 arr2-4 double mutant is more resistant to Botrytis compared to Col-0 wild-type. Interestingly the same tendency was observed in the single mutants as well, but only the difference by the double mutant was statistically significant when ascertained for average lesion size. These results clearly show the redundancy (additive effect) of the ARR1 and ARR2 in respect to defense against Botrytis.
Figure 4.17 *Arabidopsis thaliana* arr1-4 arr2-4 double mutants is susceptible to necrotroph *Botrytis cinerea*

Leaves of *Arabidopsis* ARR1 and ARR2 single and double mutants and corresponding wild-type (Col-0) were treated with *Botrytis cinerea*. The evaluation of infection was done 48hrs after the treatment. Data for (A,B) are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table). Data in C is the mean and SE. (A) Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by lesion disease index (Category 1: ≤ 2mm, Category 2: 2-6mm, Category 3: >6mm). (B) Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by outgrowth index in % (C) Average lesion size of the tested plant lines in mm. LSD (Fishers Least Squared Difference Test) was used to compute significance levels at the α ≤ 0.05 level using the mean; asterisk shows a significance difference compared to Col-0 only. For this experiment at least 20 plants per line were used. This experiment was repeated two times with similar results.
4.2.3.3 Response of common marker genes PR-1 and PDF1.2 with respect to Botrytis cinerea

Knowing that the double mutant was more resistant to Botrytis compared to the wild-type, leaf material of treated and not treated plants was harvested and the transcript levels of PR-1 and PDF 1.2 were quantified by RT-qPCR. PR-1 and PDF1.2 are induced in response to a variety of pathogens (Penninckx et al. 1996; Schenk et al. 2000). The PR-1 gene has been used to elucidate transcriptional control mechanisms regulating SAR (Pape et al. 2010) and expression of PR-1 is salicylic-acid responsive (Metraux et al. 1990; Zhang et al. 2013).

**Figure 4.18 Response of common marker genes PR-1 and PDF1.2 after treatment with Botrytis cinerea**

Relative expression levels of PR-1 and PDF1.2 genes in arr1-4, arr2-4, arr1-4 arr2-4 and Col-0. Gene expression was measured after 0, 12, 24, 36 and 48 hrs after treatment with Botrytis. For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. This mRNA extraction and RT-qPCR was repeated two times with similar results. For each gene, time point 0 for Col-0 was used as reference and EF-1-α was used as the internal control to calculate ΔΔCT values.

PDF1.2 belongs to the plant defensin (PDF) family encodes an ethylene- and jasmonate-responsive plant defensin protein (Penninckx et al. 1998; Ferrari et al. 2003). PDF1.2 mRNA levels are not responsive to salicylic acid treatments (Manners et al. 1998) although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene (Mur et al. 2006; Koornneef and Pieterse 2008).

For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. Infected leaves from both treated and non-treated plants were harvested after 0 hrs, 12 hrs, 24 hrs, 36 hrs and 48 hrs post inoculation.
and the mRNA expression levels for PR-1 and PDF1.2 were quantified. In non-treated plants PR-1 was, as expected, very low expressed in all harvested time points. The first significant up-regulation of the PR-1 was after 24 h where the mRNA level was higher in all mutants compared to the wild-type. This trend in rising expression continued until 36 hrs after inoculation and the levels of PR-1 were still higher in the mutants. However, there was no difference in expression among the mutants themselves (Figure 4.18). Two days after inoculation (48 hrs) the expression of PR-1 in all mutants maintained the same level as seen at 36 hrs while expression in the wild-type was increased. Thus, although the basal levels of PR-1 was higher in the mutant plants, the pattern of induction with respect to Botrytis was not altered in any way.

Expression level of PDF1.2 was very low in non-treated plants independently of the time point and the first increase of expression among the inoculated plants was visible 24 hrs after inoculation. The same tendency in all mutants and the wild-type plants was observed as an up-regulation at 24 hrs and 36 hrs post inoculation. Up until the 36 hrs time point, no difference was observed between either single mutants or the wild-type. However a difference was obtained by the double mutant with the level of PDF1.2 down-regulated. After 48 hrs, the expression level of PDF1.2 in the wild-type strongly went up compared to any of the mutants.

Based on these observations it must be concluded that even though regulation of PR-1 and PDF1.2 is slightly perturbed in arr1/2 plants, it does not appear reflect the resistance of arr1-4 arr2-4 to Botrytis. Thus even though PR-1 and PDF 1.2 are commonly used marker genes for these kind of studies, in this case, the genes themselves do not correlate directly to the resistance phenotype and are more a reflection of the current molecular status in the plants after infection.
4.3 Discussion

4.3.1 ARR2 interacts with MKK members

For the first time a connection has been shown between MAPK signalling cascades and the two-component signalling system in plants. These findings supported earlier presumptions and previous data (Hass et al. 2004) that ARR2 might play role in response in biotic stresses, especially in response to pathogens. This crosstalk was observed strictly downstream of MKKKs, at the level of MKKS and MPKs. This is because none of the tested response regulators (A- and B-type) interacted with any of tested MKKKs. In comparison, only type-B RRs interacted with MKKS and MPKs.

Nevertheless, although only type-B interacted with MKKS, they did not interact with all of them. All MAPK kinase kinase members, except MKK3, were tested against: ARR1, ARR2, ARR10, ARR14, ARR18 and ARR4 (type-A) using yeast-two-hybrid method (studies with MKK6-10, and with ARR10 and ARR14 were done in our laboratory in collaboration with Niklas Wallmeroth (Wallmeroth, Diplom, Tübingen). Besides MKK3, MKK9 showed also transactivation in yeast, but its growth on control medium still allowed for evaluation of protein-protein interactions. ARR10 interacted only with MKK4, MKK5 and MKK9 while ARR14 interacted only with MKK5. ARR1, ARR2, and ARR18 interacted with more MKKS whereas the A-type ARR ARR4 did not interact with any. This information has been summarized below (Table 4.1).

Table 4.1 Summary table of protein-protein interactions between MKKs and RRs

<table>
<thead>
<tr>
<th>MKK</th>
<th>ARR1</th>
<th>ARR2</th>
<th>ARR10</th>
<th>ARR14</th>
<th>ARR18</th>
<th>ARR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKK1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>MKK2</td>
<td>+</td>
<td>+</td>
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Despite very high sequence homology between ARR1 and ARR2, they did not interact with the same MKK members suggesting that the interactions could be rather specific. Interestingly, ARR2 interacted with the largest number of MKKS. On the converse side, MKK4, MKK5 and MKK9 interacted with the largest number of type-B ARR.
MKK7 and MKK9 both are class D MKKs and also play important roles in biotic stress responses. Only ARR2 and ARR18 interacted with MKK7. MKK7 is very well known as a negative regulator of polar auxin transport, defence response to bacterium and salicylic acid mediated signalling pathways (Dai et al. 2006). ARR1 is known to control polar auxin transport by directly controlling SHY2/IAA3, an Aux/IAA transcriptional repressor, expression, which in turn controls expression of PIN1, PIN3 and PIN7 in the vascular tissue (Taniguchi et al. 2007). As ARR1 did not interact with MKK7 in the yeast-two-hybrid, it is unclear if this rules out a role for MAPK cascades in planta. The high homology between ARR2 and ARR1, plus the fact that ARR12, the closest homolog to ARR10, also has been shown to have a role in regulating SHY2 (Moubayidin et al. 2010) leaves this possibility still open. ARR1, ARR2, ARR10, and ARR12 all complement the root cytokinin response phenotype of the arr1 arr12 mutant and therefore can functionally replace each other in root elongation assays (Hill et al. 2013). Taken together, it is worth speculating that ARR2 also has a role in polar auxin transport by controlling SHY2 expression and integrating signal information from MKK7. This hypothesis of course must be tested experimentally.

MKK9 is known to be enrolled in phosphorylation between MPK3 and MPK6 and it is independently involved in ethylene and caldalexin biosynthesis, response to salt stress and wounding (Xu et al. 2008). In addition, both MKK7 and MKK9 are thought to be involved in the innate immunity cell death response (Popescu et al. 2009). ARR1, ARR2, ARR10 and ARR18 all interacted with MKK9. That ARR2 has been described to also function downstream of ethylene signalling (Popescu et al. 2009) this implies that these B-types could mediate allosteric functions. Expression of constitutive active version MKK9EE lead to enhanced cell death in N. benthamiana transfection assays (Popescu et al. 2009). Whether or not type-B ARRs could be part of this process is not yet known.

As mentioned, ARR2 interacted with the most MKKs and of those most of them are hormonal/stress-related MKKs (MKK3, MKK4, MKK5, MKK7 and MKK9). Moreover, ARR2 strongly interacted with MKK4 and MKK5 compared to the abiotic-stress related MKKs MKK1 and MKK2. This difference of interaction strength was conferred by O-NPG assay and by making serial dilution of transformed yeast cells. Therefore, MKK4, MKK5, MKK3 were tested in planta by quantifying protein-protein interaction strength using FRET-FLIM. The FRET-FLIM results revealed strong interaction between ARR2 and MKK4, MKK5, MKK3 in planta. Thus, there is little doubt that these two protein classes do interact in living plant cells.

Two MPKs were tested against ARR2 and MKKs, the biotic stress-related MPKs MPK4 and MPK17 (Group 2002). Both MPK4 and MPK17 interacted with ARR2, ARR18 and ARR1. MPK4 is especially known to play very important roles in pathogen response (Rasmussen et al. 2012). It has been known that mpk4 knockout mutant plants show constitutive activation of SA-mediated defences (Petersen et al. 2000). Furthermore, MPK4 activity inhibits basal defences to virulent Pst DC3000 and pathogen-induced SA accumulation, MPK4 also plays role in ETI as compromising it and its activity negatively regulate ROS production induced by PAMPs (Berriri et al. 2012). The interaction of MPK4 with both biotic stress-related MKKs
MKK4 and MKK5 could be confirmed in this thesis. MPK17, whose physiological function(s) are not described yet, only interacted with MKK5. Recall however that MKK5 interacted with all of the type-B ARRs. MKK9 also interacted with the majority of type-B ARRs, albeit over its yeast-two-hybrid transactivation capacity. However, only MKKs 1 to 5 were tested against MPK17 in this thesis. Nevertheless, it is reported that MPK17 interacts with MKK9 and MKK10 in yeast-two-hybrid assays (Lee et al. 2008). Thus, although the interaction of MPK17 with MKK9 was not confirmed in this thesis, it suggests that a bona fide interaction network has been documented. Furthermore, expounding on the knowledge of roles for MKK5 and MKK9, the type-B ARRs are somehow involved in physiological responses related to biotic stress and/or hormonal regulation. With regards to MKK9 which is known to be involved in ethylene signalling downstream of CTR1 (Yoo et al. 2008) as a MKK9–MPK3/6 module. Haß et al. 2004 from our laboratory showed that ARR2 also responds to ethylene in an ETR1–TCS dependent manner. In addition, MKK4/5 have also been placed in a MKK4/5/9–MPK6 ethylene biosynthesis model (Hahn and Harter 2009; Zhao and Guo 2011). Interestingly, the most type-B ARR interactive MKKs MKK4/5/7/9 were shown to activate signalling cascades controlling cell death pathways in plants (Popescu et al. 2009). How these two signalling pathways converge on ethylene signalling is still a mystery, yet it suggests that part of the signal integration can occur at a protein-protein interaction level.

The FRET-FLIM analysis revealed that MKK3 and ARR2 interact in living plant cells. MKK3 is an activator of group C MPKs (MPK1, MPK2, MPK7, and MPK14) of which MKK3 also activates MPK7 in response to plant pathogen Pst DC3000 and ROS (Doczi et al. 2007). MKK3 is interesting as it is shown to play a physiological role in response to biotic stresses as a MKK3-MPK6 cascade important for JA signal transduction in Arabidopsis (Takahashi et al. 2007) and the same cascade plays crucial role in resistance to Salmonella infections in Arabidopsis (Schikora et al. 2008). Additionally, ARR2 showed very strong interaction with MKK4 and MKK5 compared to MKK1 and MKK2 and this interaction was also confirmed in planta. The MKK1 and MKK2 are mostly involved in abiotic stresses (Asai et al. 2002). For example MKK2 is specially activated by cold and salt stress and by stress-induced MKK1 (Teige et al. 2004). They have also been described with a role in biotic stress as a part of MKK1/2-MPK4 cascade functioning as negative regulator of SA-dependent systemic required resistance (Petersen et al. 2000) and positive regulator of ET- and JA-mediated defences (Brodersen et al. 2006; Qiu et al. 2008). Here, it is also possible the MKK1/2-MPK4 module is allosterically influenced by ARR2 and ARR1.

The presumptuously stronger interaction of MKK4 and MKK5 over the other MKKs with B-types was confirmed in yeast-serial dilution and quantitative O-NPG assays. MKK4/5 are known to be active in response to different biotic stresses but especially active and described in pathogen defence (Asai et al. 2002) where MKK4 is strongly induced when plants treated with Pseudomonas syringae or together with MKK9 against Phytophthora infestans (Menges et al. 2008). Even newly characterised homologue of MKK5 in cotton (Gossypium hirsutum) GhMKK5 affects pathogen resistance to the bacterial pathogen Ralstonia solanacearum by
elevating the expression of pathogen resistance (PR) genes (PR1a, PR2, PR4, PR5, and NPR1) (Zhang et al. 2012). Interestingly, the interaction capability of ARR2 was not due to the presence of the ARR2 type-B output domain, since a truncated version of ARR2 containing only its receiver domain was able to interact with MKK4 and MKK5. Furthermore, using an in silico approach, it was shown that MKK docking domains are dispersed throughout ARR2 and not restricted to its output domain. In total, four different MKK binding domains were predicted to be present in ARR2. All of these regions seem to be functional when tested by yeast-two-hybrid system based on the larger protein truncations. When tested as peptides, the receiver domain-based and output domain-based MKK docking motifs showed interaction only with MKK4 and MKK5. This observation was taken to confirm previous observations that these two MKKs interact much stronger with ARR2 than the others. Furthermore, the interaction of ARR2 with MKKs does not require phosphorylation by TCS elements on its D80 position in order to interact with any of tested MPK(K) cascade elements. This suggests that ARR2 might interact with MAPK cascade members independent of cytokinin or ethylene, i.e. TCS signalling. In fact, this would put ARR2 in novel signalling pathways which may or may not require its transcription factor roles. This potential function of ARR2 might make signalling pathways and protein-protein interaction more specific. One way to unravel TCS dependent signalling and the physiological role of MAPK cascades would be possible with constitutive expression of non-phosphorylatable ARR2<sup>D80N</sup> (Hwang and Sheen 2001; Choi et al. 2010). This was not performed at this time, but similar experiments with the constitutive TCS active form ARR2<sup>D80E</sup> were attempted. Readout however was not possible as mutant lines complemented with ARR2<sup>D80E</sup> driven under a constitutively active promoter were lethal in embryonic stage (Hass et al. 2004). In this work, attempts to bypass constitutive expression of ARR2<sup>D80E</sup> using a β-estradiol inducible promoter only for a few days after treatment still showed huge developmental defects, early necrosis and most likely embryonic lethality (see Supplemental Figure 10).

Taken together, the protein-protein interaction data suggest involvement of ARR2 in a biotic stress direction concerning its association with biotic stress-related MKKs and MPKs, and in particular pathways mediated by MKK4/5/7/9. This crosstalk of ARR2 with MKKs and MPKs could be a confirmation of the early presumptions that TCS and B-type response regulators per se play an important role in fine-tuning of different signals and in mediating crosstalk between different signalling pathways in aim of improving the fitness of plants for better adaptation within their natural ecosystems. Therefore, a connection to biotic stress and ARR1 and/or ARR2 was pursued in this thesis to provide a physiological basis for which a functional output could be eventually be assigned to. Provided that this is successful, cross-talk relationships between ARR1/2 and MKKs could be pursued.
4.3.2 *arr2-4* can be shown to be involved in pathogen responses to necrotrophs

Innate immunity is generally initiated with recognition of conserved pathogen-associated molecular patterns (PAMPs). As a first look into pathogen-related responses in *ARR2*, wild-type and *arr1-4, arr2-4* and *arr1-4 arr2-4* mutant lines were treated with PAMPs and as an output, ROS and ethylene production was measured.

Indeed, ROS measurement triggered by *flg22* and *elf18* showed that *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type or single mutants in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments but the double mutant however consistently showed more ROS than the wild-type. This was first hint that *ARR1* and *ARR2* are enrolled in response to pathogen defence. Since the double mutant showed the strongest effect in ROS production only the *arr1-4 arr2-4* double mutant and Col-0 wild-type were taken and tested against PAMPs with fungal origin (PEN, Xac) for ethylene production.

Double mutant here did not show any statistically significant difference in ethylene production to any of tested PAMPs compared to the wild-type. This approach only showed that there is no significant difference in ethylene production between the mutant and the wild-type but it doesn’t necessarily mean that the signalling pathways controlled by ethylene are not affected in the double mutant since differences in ROS production were observed. It could be that the differences in ethylene production between the double mutant and the wild-type are below the detection range of the machine used for ethylene measurements. Similarly, it could be that the ROS measurements were much more sensitive than those of ethylene.

Changes in ROS have been correlated with MKK and MPK activity (Pitzschke and Hirt 2009). Unfortunately, the lack of *ARR1* and *ARR2* did not cause any dramatic differences between the wild-type and the double mutant in MPK phosphorylation status in response to *flg22* of pathogen-response related MPK4, MPK3 and MPK6. This does not exclude that actually *ARR1* and *ARR2* may serve here as MKK scaffold proteins which redirect and specify pathogen-related signalling pathways, but it does suggest the function of *ARR1* and *ARR2* does not regulate MKK kinase activity.

The data up to this point suggested that *ARR2* (and other B-types) interact with MKKs and MPKs. The interaction with *ARR2* in yeast-two-hybrid and *in planta* was preferentially with MKK4 and MKK5, which are known to be involved in biotic stress. Furthermore, changes in ROS production with respect to *flg22* were also observed although this could not be corroborated with ethylene production or MPK phosphorylation status. Recent publications about *ARR2* and cytokinin have indicated a growing connection to pathogen stress (Choi et al. 2010). Therefore, pathogen assays were performed on *ARR1* and *ARR2* single and double mutants. For these assays necrotrophic and biotrophic pathogens were taken since each has evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004).
No any statistically significant differences between wild-type (Col-0) and the mutant lines were obtained using Pst DC3000 when applying it by leaf infiltration at 3 x 10^6 CFU. The same mutant lines were also twice challenged with Pst DC3000 in presence of 1μM exogenous cytokinin (t-zeatin). No biologically significant differences were found either. These results are in contrast to data who published a weak arr2-4 phenotype for resistance to Pst DC3000 at one log difference (Choi et al. 2010). The majority of their assays worked with overexpressor lines to show the effects of cytokinin and ARR2 variants however. Considering that our data are of very high quality as they were reproduced at least 5 times, we conclude that arr1-4, arr2-4 and arr1- arr2-4 do not confer any significant resistance to Pst DC3000 infection.

Peronospora is known to activate defence pathways via salicylic acid (SA) accumulation, which is a general characteristic of biotrophic pathogens (Glazebrook 2005), and independently of SA via RPPs (RECOGNITION OF PERONOSPORA PARASITICA) genes, especially via RPP7. RPPs represent R (resistance) genes, genes responsible for gene-dependent defence activation in Arabidopsis (Mcdowell et al. 2000). Here seems that ARR1 and ARR2 are not involved in these signalling pathways or that the other B-types work in a redundant fashion no evident from the yeast-two-hybrid data.

It could be postulated that the defence response could depend on stomata developmental/response defects. This is because ARR2 has been described to be involved in mediating stomatal closure in an H_2O_2-depenent manner (Desikan et al. 2006; Mira-Rodado et al. 2012) and that MKK4 and MKK5 are known to be involved in stomatal patterning (Lampard et al. 2009). Such same effects should be seen by spraying the bacteria onto plants. Stomata aperture was measured in mutant backgrounds and the wild-type treated with hormones (ABA and cytokinin) and after Pst DC3000 application. Although the wild-type and mutants closed in response to ABA as previously described (Desikan et al. 2006), wild-type and mutants also responded equally to cytokinin application or Pst DC3000 challenge. In fact, stomata in all the plant lines treated with Pst DC3000 were more open than non-treated control consistent with the release of diffusible phytotoxin COR by the bacteria which reopen closed stomata to increase the number of bacterial invasion sites (Melotto et al. 2008). Stomata aperture measurements were done 3 days after pathogen application which was more than enough to cause reopening of stomata. Here was shown that ARR1 and ARR2 are not any how involved in mechanism for recognition of phytotoxin COR and in this case they are not part of apparatus for regulation of stomata aperture. Therefore under our conditions and experimental approach we could not confirm the previously published observation that cytokinin modulates SA signalling by augmenting resistance against Pst DC3000, a process in which the SA/cytokinín-dependent interaction TGA3 and ARR2 is important (Choi et al. 2010). This discrepancy, despite the performed experiment where stomata aperture was measured and also no difference obtained, may still be due to differences in infection conditions.

Interestingly, cytokinin caused the stomata to open in the wild-type and the arr1/2 mutant lines. This means, that although there is a lack of two cytokinin responsive (Hill et al. 2013), type-B they are not crucial for stomatal aperture responses. This indicates that this response
is more than likely redundantly controlled by other B-types. On the other hand, the strong effects of previously reported for arr2-4 on H₂O₂-dependent closure point towards explaining MKK4/5/9 cross-talk by the fact that ARR2 interacts with more MKKs than does all the other type-B ARRs.

Generally speaking, JA-dependent signalling pathways play a crucial role in defence against necrotrophic pathogens (Glazebrook 2005). Therefore assays done with necrotrophic pathogens were performed in order to see if there could be a defect in regulation of jasmonic acid (JA)-dependent signalling pathways. The arr2-4 single mutant and arr1-4 arr2-4 double mutant but not arr1-4 single mutant, showed enhanced susceptibility to the necrotrophic fungus Alternaria brassicicola compared to the wild-type. This experiment strongly suggested that, despite their high homology, ARR2 but not ARR1, is the only one of the two involved in signalling pathways responsible for defence against Alternaria. It is known that COI1 (CORONATINE INSENSITIVE 1), COI1-dependent genes and camalexin biosynthesis-dependent genes are primarily responsible for its defence against Alternaria brassicicola (Van Wees et al. 2003). In almost all Arabidopsis mutants that show susceptibility to Alternaria, a perfect correlation has been observed between camalexin deficiency after A. brassicicola infection and susceptibility (Van Wees et al. 2003). However, it is known that even though infection by A. brassicicola and infection by P. syringae are quite different stimuli, genes under COI1 control are common for plant resistance against both pathogens but this is not the case with camalexin biosynthesis-dependent genes (Van Wees et al. 2003; Jones and Dangl 2006). Based on the data obtained with arr2-4 mutants, ARR2 is more likely involved in camalexin biosynthesis pathways where it might positively regulate its synthesis. Furthermore, ARR2 interacted with MKK3 further strenghtening a role of ARR2 in the MKK3-MPK6 JA signal transduction. How these two are intertwined is yet unclear at this point.

The effects of the necrotrophic fungus Botrytis cinerea was challenged on arr1/2. This pathogen is important for two reasons: Botrytis cinerea is known to activate distinct signalling pathways from Alternaria and more importantly, Arabidopsis thaliana is natural host of Botrytis cinerea. Based on measured disease index parameters, the arr1-4 arr2-4 double mutant clearly showed resistance to Botrytis compared to the wild-type and an additive effect compared to the single mutant parents. That is, the double mutant twice as less lesions (~30%) than the wild-type (~60%) lesions greater for equal to 6 mm (≥6 mm). Both single mutants showed the same resistance tendency and were statistically different from the wild-type. It was also observed that arr2-4 single mutant had a slight, non-significant tendency to be more resistant than the arr1-4 single mutant: it had 40% of lesions ≥6 mm whereas the arr1-4 had only 45%. Again this favours the role of ARR2 compared to ARR1 with respect to resistance against necrotrophic pathogens.

The common marker genes PR-1 and PDF1.2 were tested from the pooled material from the Botrytis cinerea challenged Arabidopsis plants. Despite the fact that PR-1 is rather SA marker and not suitable for infection with necrotrophs, expression of PR-1 was induced 36 hrs post infection, 12 hrs earlier than the wild-type. This expression was maintained at the same level
in the mutants one day later whereas PR-1 expression in the wild-type was strictly upregulated in the wild-type at 48 hrs. This would imply that the arr1/2 caused early induction of PR-1 in response to Botrytis infection. Based on the current models, the expression level of PDF1.2 should be increased in the mutant plants compared to the wild-type knowing the role of PDF1.2 as JA marker and is upregulated in response to necrotroph infection (Seo et al. 2001; Qi et al. 2012; Lu et al. 2013). Although PDF1.2 was induced in response to Botrytis infection as expected in all samples, it was not as highly induced in the mutants compared to the wild-type. Thus, the expression of this gene cannot be correlated with the mutant resistance phenotype. These results present a conundrum as to the mechanism of resistance to Botrytis. On one hand, we expected that PDF1.2 expression to be increased in the mutant plants, however dampened expression kinetics were observed. On the other hand, PR-1 was upregulated at an earlier time in the mutant plants but did not reach the same maximum as the wild-type at 48 hrs. Therefore, it is unclear if these two markers really can be correlated with the resistance phenotype of arr1/2. It is known that despite the wide use of PR-1 and PDF1.2 as markers for the induction SA- and JA-dependent pathogen pathways, the levels of both PR-1 and PDF1.2 expression do not clearly correlate with the level of susceptibility to Botrytis cinerea (Ferrari et al. 2003). So the observed differences in expression of these genes in the arr1/2 mutants are more a reflection of the current molecular status in the plants after infection, that is, it definitely showed that the current status was of plants under pathogen attack.

The dramatic difference in resistance between Botrytis cinerea and Alternaria brassicicola could be explained by the very wide, naturally variable resistance to necrotrophic plant pathogens which appears to be quantitative and polygenic (Micic et al. 2004; Rowe and Kliebenstein 2008). This tendency is exactly visible in the arr1-4 arr2-4 double mutant when the same mutants tested against two different necrotrophic pathogens showed completely opposite phenotypes. Furthermore, all of the parameters very clearly showed the redundancy (additive effect) of the ARR1 and ARR2 in respect to defence against Botrytis but not Alternaria, of which arr2-4 played the major role. In addition, the nature and extent of isolate-specific interaction between plants and necrotrophic pathogens is relatively unknown, and no qualitative naturally variable resistance genes effective against necrotrophic pathogens have been yet described (Jones and Dangl 2006; Rowe and Kliebenstein 2008). Provocatively, infection by the fungal pathogen Phytophthora infestans led to the rapid transcriptional induction of MKKK19, MKK9 and MKK4, while Botrytis cinerea infection led to the rapid transcriptional induction of completely different genes MKKK18, 19 and 20, Raf43, ZIK2, 8 (Menges et al. 2008). Although no direct genetic or transgenic connection has been shown between ARR1/2 and MKKs, a role for ARR1/2 is more than likely evaluable in necrotrophic but not biotrophic pathogen resistance as neither Pst DC3000 nor Peronospora parasitica differentially infected arr1/2 compared to their wild-type control. There are also reports that have shown signalling responses to bacterial and fungal pathogen attack are distinct, i.e. different genes are induced, especially with regard to MAPK cascades. It is still open if differences in resistance of arr1/2 mutants when tested against distinct pathogens is
correlated with MKK/type-B ARR crosstalk. If valid, the role of TCS-dependent phosphorylation, at least for protein-protein interaction is not required (so far judged by yeast-two-hybrid assays). In addition, arr1/2 mutants did not change the phosphorylation level of MPK3/4/6 in response to flg22. Therefore, regulation of MKK activity also does not appear to be mediated by type-B ARRs, although it was not tested if modulating the TCS-dependent phosphorylation state of ARR2 could influence this. It is therefore proposed that the proposed protein-protein crosstalk mediates protein target networks dynamics, i.e. their interaction affects binding/signalling kinetics of the two systems without actually controlling the physical TCS or MAPK cascade phosphorylation mechanism. As both the TCS and MAPK cascades target gene expression, the interaction of the two networks would modify their own information flow leading to changes in output gene expression similar to what has been observed for a TCS and MAPK cascade in yeast (Xu and West 1999).
4.4 References for Chapter 1


5 Chapter 2

ARR1 and ARR2 are involved in flowering time regulation of Arabidopsis thaliana under short days

5.1 Introduction

Flowering is a crucial step in plant development and it is characterised as a transition from vegetative to reproductive phase (Koornneef et al. 1998). Due to its importance of ensuring reproductive success, flowering is controlled by complex networks of genetic regulatory pathways (Coupland 1995; Srikanth and Schmid 2011; Poethig 2013). In the end, successful reproduction not only includes seed formation but also dispersion, germination and growth and complex regulatory mechanisms need to integrate both environmental and endogenous signals to ensure the next generation can also reproduce (Bernier et al. 1993; Koornneef et al. 1998; Srikanth and Schmid 2011). However, it is clear today that all these signalling pathways that control flowering are not strictly separated but that they work together, i.e. crosstalk in the regulation of flowering (Genoud and Metraux 1999; Franklin 2009). Surprisingly the number of common targets regulated by huge number of signalling pathways is quite small and they present central floral pathway integrator or “integrator genes” (Li et al. 2008; Srikanth and Schmid 2011). Even in self-fertile plants, flowering time is still strictly controlled by environmental and endogenous factors (Srikanth and Schmid 2011). Exogenous factors that strongly influence the flowering are day length, light quality and temperature (Coupland 1995; Reeves and Coupland 2000; Chen et al. 2004). On the other side, common endogenous factors that are regulating flowering are hormones (hormonal status of the plant), sugars and plant age (Blazquez et al. 1998; Wahl et al. 2013). Five genetic pathways have been identified that play role in control of flowering: the vernalization (originally jarovization) pathway, the photoperiod pathway (related to the day length and quality of perceived light), the gibberellin (GA) pathway, the autonomous pathway (endogenously controlled independent of photoperiod or gibberellin pathways), and plant age (also an endogenous pathway) (Srikanth and Schmid 2011).

5.1.1 Photoperiod-dependent flowering control

Photoperiod pathways present a cascade of events in charge for measurement day length and consequently initiating flowering (Valverde et al. 2004; Sawa et al. 2007). The organ for photoperiod perception is located exclusively in the leaves. Light perception in leaves is enabled by the presence of three main classes of photoreceptors specialized for different wavelengths: phototropins (blue light), phytochromes (red/far red light) and cryptochromes (blue light) (Franklin et al. 2005; Lariguet and Dunand 2005). Once a particular type of light-signal is perceived (varies between species) the flower-triggered substance called “florigen” is
produced and afterwards transmitted to the shoot apex to induce flowering (Colasanti and Sundaresan 2000). Even before the era of molecular biology had begun, it was very well known that plants measure and respond to the day length (Srikanth and Schmid 2011). This is due to the presence of the internal oscillators, genes that are regulated by the circadian clock (Harmer et al. 2000). The circadian clock is explained as a timekeeping mechanism in photoperiodism (Mizoguchi et al. 2005).

5.1.2 Photoperiod-dependent flowering control in Arabidopsis thaliana

In Arabidopsis thaliana, a circadian clock controlled flowering pathway includes the following gene members GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) that promote flowering specifically under long days (Fowler et al. 1999; Mizoguchi et al. 2005). Briefly, the circadian clock in Arabidopsis thaliana is composed and works as follows: The central oscillator generates a self-sustaining rhythm driven by two interacting feedback loops that are active at different times of day. The first one so called “morning loop” consists of CCA1 (CIRCADIAN AND CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), which encode closely related MYB transcription factors that regulate circadian rhythms in Arabidopsis, as well as PRR 7 and 9 (Pseudo-Response Regulators). The second “evening loop” consists of GI (GIGANTEA) and ELF4 (EARLY FLOWERING 4), both are involved in regulation of flowering time genes (Fowler et al. 1999; Mizoguchi et al. 2005; Kolmos and Davis 2007).

Classification of plants due to their photoperiodic responses is usually based on flowering. The two main photoperiodic response categories are short-day and long day-plants (Garner 1933). Short-day plants (SDPs) flower in short days (qualitative SDPs) or their flowering is accelerated by short days (quantitative SDPs). Long-day plants (LDPs) flower only in long days (qualitative LDPs) or their flowering is accelerated by long days (quantitative LDPs). The essential distinction between long-day and short-day plants is that flowering in LDP is promoted only when the day length exceeds a certain duration. This time course is called critical day length. To flower SDPs require a day length less than a critical day length (Yano et al. 2001; Kojima et al. 2002). Plants that flower under any photoperiodic condition are referred to as day-neutral plants (DNPs) (Wu et al. 2004; Lifschitz et al. 2006); examples are most of the desert annuals that flower quickly whenever the water is available. Arabidopsis thaliana is a facultative LDP, i.e. quantitative LDP means it flowers also under SDs but its flowering is accelerated by inductive long days (Hicks et al. 1996). Important to photoperiodic flowering is the so-called phenomenon “coincidence model” proposed in the mid-1930s by the German botanist Professor Erwin Bünning which brings together endogenous and exogenous signals. The circadian oscillator controls the timing of light-sensitive and light-insensitive phases only when the light signal is coincident with the appropriate phase of the circadian rhythm thereby allowing flowering induction under both non-inductive and inductive days (Srikanth and Schmid 2011). The first mutant discovered in Arabidopsis thaliana incapable of a photoperiodic flowering response was co mutant: a mutant lacking in CO (CONSTANS) which encodes a zinc finger transcription factor family protein which is controlling expression of other floral regulators (Putterill et al. 1995; Tran et al. 2007). The co
mutant has a very strong late flowering phenotype under inductive LD conditions only whereas at non-inductive SD, co flowers identical like the wild-type plant suggesting the CO to be a flower activator (Putterill et al. 1995). It was later discovered that the expression CO is regulated by the circadian clock with pick expression of CO around dusk (Suarez-Lopez et al. 2001). Expression pattern of CO represents an example of “coincidence model”. The CO proteins levels are also controlled however and CO only accumulates enough to promote flowering under LD when the light phase overlaps/coinides with the maximal pick of the CO mRNA (Valverde et al. 2004; Bohlenius et al. 2006). Research has shown that CO is posttranscriptional regulated and that during the dark phase the protein is tagged for degradation by ubiquitin and afterwards degraded in 26s proteasome and part of this is controlled by different photoreceptors (Valverde et al. 2004). It has been shown that PhyB signalling in the morning negatively regulates CO whereas PhyA and cryptochromes positively regulate the accumulation of CO protein in the evening (Lin 2000; Endo et al. 2013). This entire regulation of CO happens only in leaves (An et al. 2004; Ayre and Turgeon 2004), i.e. it does not transfer signal from the leaves to the shoots. Further downstream CO activates expression of other flowering-related genes which are transferring the signal information to the apical meristem and initiating flowering. One of them is FT (FLOWERING LOCUS T) gene (Kardailsky et al. 1999; Hisamatsu and King 2008). FT belongs to a small group of proteins that show structural similarities to mammalian phosphatidylethanolamine-binding protein (Ahn et al. 2006; Kim et al. 2013). Five more members belong to this family: TSF (TWIN SISTER OF FT), MFT (MOTHER OF FT AND TFL1), BFT (BROTHER OF FT AND TFL1), ATC (ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUES) and their major role is the regulation of photoperiodic flowering (Wigge 2011; Pin and Nilsson 2012; Kim et al. 2013). In the shoot apical meristem of Arabidopsis thaliana it seems that the FT protein, but not FT mRNA (Notaguchi et al. 2008), is needed to induce flowering, although there are some indications that FT mRNA could also be contributing flower induction but only together with its protein form (Li et al. 2009). In the shoot apex FT makes a complex with FD (Abe et al. 2005; Wigge et al. 2005). FD belongs to the bZIP transcription factor family and it is preferentially expressed in the shoot apex and required there for FT to promote flowering (Abe et al. 2005). This complex afterwards activates downstream targets such as SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) (Searle et al. 2006), APETELA 1 (API) (Wigge et al. 2005) and indirectly LFY (LEAFY) (Abe et al. 2005; Smith et al. 2011). SOC1 is floral activator required for CO to promote flowering, it acts downstream of FT (Yoo et al. 2005), API and LFY are meristem identity genes which generally promote the transition to flowering by further induction of floral homeotic genes (Yu et al. 2004).

5.1.3 Photoperiod-dependent flowering control in rice and other plants

The coincidence model is also present within neutral and short day plants like in tomato (Solanum lycopersicum) or rice (Oryza sativa), respectively. In tomato, the orthologue of FT is SFT (SINGLE-FLOWER TRUSS) and it is a floral activator just like in Arabidopsis (Lifschitz et al. 2006). Besides its commercial value and, in contrast to Arabidopsis, Oryza sativa belongs to the group of short day plants (Yano et al. 2001; Kojima et al. 2002). The rice genes Hd1
(Heading-date 1) and Hd3a (Heading-date 3a) encode homologs to Arabidopsis CO and FT respectively. Like CO in Arabidopsis, Hd1 has an identical expression pattern in rice (Kojima et al. 2002) with only difference between rice and Arabidopsis is that under short day conditions Hd1 acts as repressor of Hd3a expression. That is the reason why is the flowering in rice, in contrast to Arabidopsis, actually inhibited by coincidence of light and the Hd1 expression; when the light overlaps the maximum expression pick of Hd1 mRNA it blocks afterwards expression of Hd3a and inhibits the flowering. These three examples showed that CO-FT regulatory module is conserved among plants pointing its importance and differences in flowering time are most likely mediated through different strategies for regulating this module (Srikanth and Schmid 2011). Summed together, photoperiodism is a very complex mechanism and it is highly controlled and fine-tuned by different regulatory elements which impend on key, evolutionarily conserved genetic networks (Hayama and Coupland 2004; Franklin et al. 2005; Imaizumi and Kay 2006).

5.1.4 Connection between two-component system and flowering regulation in rice and Arabidopsis

Interestingly, in rice, FT-like gene expression can be controlled independently of Hd1 by the so-called Ehd1 (Early heading Date 1) gene encoding a B-type response regulator, part of two-component signal (TCS) transduction systems (Doi et al. 2004) suggesting an important role of TCS in regulating flowering. Ehd1 functions as a floral inducer and it induces FT-like and MADS box genes (e.g. the AP1 ortholog in Arabidopsis) (Doi et al. 2004).

TCS systems typically transduce a receptor-mediated signal into a phosphorelay from a histidine-kinase onto a conserved aspartate residue of a response regulator (RR) protein. In Arabidopsis, there are response regulator proteins that have a mutation in this conserved aspartate (Asp) residue: these proteins are called pseudo response regulators (pseudo-RRs) (Doi et al. 2004). It has been already proven that pseudo-RRs regulate flowering time in Arabidopsis thaliana and that TOC1/APRR1, an Arabidopsis pseudo response regulator, is part of the plant circadian clock. TOC1/APRR1 belongs to a small gene family in Arabidopsis (Matsushika et al. 2000) and it influences flowering time via the phase setting of CO expression (Yanovsky and Kay 2002). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate as they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Doi et al. 2004).

In Arabidopsis thaliana, ARR4 (an A-type response regulator) plays a very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007). PhyB, as above mentioned, is actively involved in expression control of CO in Arabidopsis thaliana and also Hd3a in Oryza sativa.

On the other hand it has been proven in various ways that B-Type response regulators are directly, positively regulating expression of the A-Type response regulators in Arabidopsis
(Grefen and Harter 2004; Brenner et al. 2012; Veerabagu et al. 2012). In addition to this, it was recently shown that floral regulator SVP (SHORT VEGETATIVE PHASE) is directly regulating A-type response regulators as well as CRFs (CYTOKININ RESPONSE FACTORS) (Gregis et al. 2013) was well as directly binding to STIP (STYMPY) newly reported component of cytokinin signalling pathway mediating cytokinin signalling during shoot meristem establishment in Arabidopsis thaliana (Skylar et al. 2010). Furthermore, it was also recently reported that cytokinin is influencing flowering time in Arabidopsis, especially under non-inductive short days (Bernier 2011; D’aloia et al. 2011). B-type response regulators are being discovered to be involved in fine-tuning and crosstalk of a multitude of signalling pathways in higher plants (Veerabagu et al. 2012; Zwack and Rashotte 2013). For example, ARR2, a member of B-type response regulators, is part of TCS signalling important for signal transmission of cytokinin and ethylene (detail about TCS signalling and members can be found in the introduction of the first chapter) (Rashotte et al. 2003; Grefen and Harter 2004; Hass et al. 2004; Mason et al. 2005; Zwack and Rashotte 2013). Despite the great sequence similarity of the B-types within the same subgroup they still show very high specificity among each other (Hill et al. 2013). The arr2 loss-of-function mutant plant in Landsberg displayed retarded growth and development including early flowering (Hass et al. 2004). ARR2 was also reported to function in ethylene signalling (Hass et al. 2004) and in response to pathogen attack (Choi et al. 2010) further demonstrating that B-types can be involved in multiple signalling pathways.

It was discovered that, when applied in hydroponic culture from the roots, in SDs, cytokinin bypasses FT and activates transcription of TSF in leaves to induce flowering (D’aloia et al. 2011). Furthermore, TSF interacts with FD and regulates expression of SOC1 which also seems to be controlled by cytokinin (D’aloia et al. 2011). On the other hand TSF and SOC1 also work independently of FD suggesting that FD and its paralogue FDP (FD PARALOG) (Abe et al. 2005) might share this function of SOC1 control (Bernier 2011; D’aloia et al. 2011). Cytokinin itself cannot induce flowering (Bernier 2011) but the fact that when cytokinin was added to roots of hydroponic-grown Arabidopsis plant was sufficient to induce flowering in SDs and that the cytokinin contents of leaves, phloem sap, and SAM (SHOOT APICAL MERISTEM) increased in response to LDs induction in Arabidopsis (Bernier 2011; D’aloia et al. 2011). In Sinapis cytokinin level is also increased in response to LDs induction (Corbesier et al., 2003) and it was reported co-dependent transportation of SaFT and cytokinin to the meristem suggests that cytokinin may play a part to regulatory effects attributed to “florigen” (Bernier 2011). However, biological functions of Arabidopsis B-type response regulators with respect to flowering have not yet been identified.

5.1.5 Temperature as floral regulator

Another factor, beside photoperiod, that is important in the regulation of flowering is temperature (Blazquez et al. 2003). Flowering can be controlled by temperature in two manners: by the process called vernalisation and also by ambient temperatures under which plants exposed to during their vegetative phase (Alonso-Blanco and Koornneef 2000; Koornneef et al. 2004).
5.1.5.1 Vernalisation

Vernalization is a process by which flowering is promoted when plants sense exposure to the prolonged cold temperatures (≤7°C). This is an adaptive trait that helps prevent flowering before winter is over thus permitting flowering in the favourable conditions of spring (Kim et al. 2009). In winter annual biennial and perennial plants, vernalization is an obligate process, whereas for summer annuals vernalization is facultative. For vernalization to work the temperature has to be a in range between 1 and 7°C for a time of 1 to 3 months depending on the plant species (Srikanth and Schmid 2011). In Arabidopsis, vernalization involves the recruitment of chromatin-modifying complexes to a clade of flowering repressors that are silenced epigenetically via histone modifications (Kim et al. 2009). This effect is known as “memory of winter” and it does not pass to the progeny but is reset during meiosis (Amasino 2004; Heo and Sung 2011). In Arabidopsis and cereals, vernalization results in the suppression of genes that repress flowering (Kim et al. 2009). Interestingly, in Arabidopsis, the most commonly used laboratory strains do not need to be vernalized, but some do flower very late if they are not vernalized first (Reeves et al. 2007; Srikanth and Schmid 2011). There are two dominant genes for vernalization in Arabidopsis thaliana: FRI (FRIGIDA) and FLC (FLOWERING LOCUS C) (Lee and Amasino 1995; Song et al. 2012) whereby FRI up-regulates the expression of FLC (Geraldo et al. 2009). FRI encodes a nuclear protein found only in plants (Johanson et al. 2000; Bari and Jones 2009) and FLC encodes a MADS-box DNA binding protein that functions as a repressor of flowering, i.e. flowering-time related genes (Deng et al. 2011). FLC represses expression of FT, FD, and SOC1 floral activators by direct interaction with their promoters (Hepworth et al. 2002; Helliwell et al. 2006). It has been proven that FLC also directly interacts with another MAD-box member, SVP which is also floral repressor that functions within the thermosensory pathway (Li et al. 2008). This interaction makes physiological and biological sense because it partially supresses FLC-mediated delay in flowering (Li et al. 2008; Bari and Jones 2009; Yoshida et al. 2009). Briefly, FLC is silenced in response to vernalization, after FLC been silenced VIN3 (VERNALIZATION INSENSITIVE 3) initially repressing FLC during the cold exposure by remodelling the chromation, i.e. by methylating lysine residues of histone H3 (Sung and Amasino 2004; Bond et al. 2009) than at least two crucial genes VRN1 and VRN2 (VERNALIZATION 1 and 2) maintain the epigenetic state of FLC (Bastow et al. 2004). Vernalization as a phenomenon is probably result of convergent evolution as outside of Brassicaceae no clear orthologs of FLC has been identified, making this a complicated research field (Srikanth and Schmid 2011).

5.1.5.1.1 Ambient temperature

Another factor that controls flowering is ambient temperature during its vegetative stage. Higher temperatures (27°C) accelerate flowering under non-inductive SDs for different Arabidopsis accessions serving as a substitute to inductive LDs (Balasubramanian et al. 2006). Ambient temperature effects on flowering are very diverse among different species, and even between different accessions of Arabidopsis thaliana. Many flowering time mutants also show temperature dependence, i.e. when exposed to higher temperatures they flowered earlier (e.g. photoreceptor mutants phyB, cry2 and most Arabidopsis fri/flc natural accessions)
FLM is like FLC a MADS-box protein and shares strong sequence similarity with FLC but despite this their physiological roles seem to be different (Scorteci et al. 2003). Microarray data showed that genes associated with alternative splice site selection are affected by thermal induction and it has been also proven that FLM has temperature-dependent alternative splicing and this splicing is an important regulator of flowering. (Werner et al. 2005; Srikanth and Schmid 2011) In the Waissilewskija ecotype FLM gene has four splice variants (α, β, γ and δ) whereas in Columbia (Col-0) accession FLM has two splice variants (β and δ) and they are both translated (Lee et al. 2013; Pose et al. 2013). FLM-β is the prevalent splice variant at lower temperatures (16°C) whereas FLM-δ dominates at higher temperatures (27°C) (Lee et al. 2013; Pose et al. 2013). Both splice variants are interact and make heterodimers with SVP independent of the transcriptional level which does not change with variation in temperature. Interestingly, they can also interact among each other making FLM-β x FLM-δ heterodimers but only FLM-β x FLM-β homodimers are possible (Lee et al. 2013; Pose et al. 2013). Proposed model of activity says that dependent of which splice variant β or δ is making heterodimers with SVP determinates later the activity of SVP-FLM heterocomplex (Lee et al. 2013). At low ambient temperatures formation of SVP-SVP and SVP-FLM-β complexes is favoured and they both repressing flowering. As the temperature is rising the FLM-β splice form is down-regulated and therefore SVP-FLM-δ complex formation is increased and it now accelerates flowering. Based on this model the role of temperature-dependent mRNA splicing in adaptation to climate change is observed and explained (Pose et al. 2013). Other known genes which are involved in regulating flowering in response to ambient temperatures are HSP70 (HEAT SHOCK PROTEIN 70) found to be highly correlated with an increase in temperature (Balasubramanian et al. 2006; Kumar and Wigge 2010), and also ARP6 (ACTIN RELATED PROTEIN 6) which is known to be a nuclear protein that represses flowering as maintaining the expression of FLC (Choi et al. 2005; Deal et al. 2005).

5.1.6 The autonomous pathway
Beside the endogenous hormonal factors that are influencing flowering it is important to mention that autonomous pathways are defined as those that delay flowering irrespective of day length (Mouradov et al. 2002). Genes involved in autonomous pathway can be grouped into two broad functional categories of genes that act by repressing FLC; these are: general remodelling and maintenance factors of FLC, and proteins that effect RNA processing (He et al. 2003; Srikanth and Schmid 2011).

5.1.7 Gibberellic acid pathway as a regulator of flowering
One of most potent endogenous control of flowering is hormonal control regulated by the gibberellic acid (GA) pathway (Blazquez et al. 1998). The effect of gibberellic acid on plant
growth and development has been known since the beginning of the last century when the rice plants infected with fungus *Gibberella fujikuroi* were growing so fast that they tipped over (Tudzynski 1999; Hsuan *et al.* 2011). Other developmental roles of GA, like its influence on flowering time, became familiar much later. Numerous GAs were discovered in plants but they are not all of them are biologically active; the active ones are GA1, GA3, GA4 and GA7 named according the order of their discovery (Hedden and Phillips 2000). After huge mutagenesis screenings for mutants that affect GA biosynthesis were detected. These mutants showed, beside various developmental defects, delay of flowering by *Arabidopsis* mostly under the non-inductive SDs (Wilson *et al.* 1992). The *ga1-3* mutant, lacking the gene for synthesis of ent-kaurene needed for GA1 formation (Sun *et al.* 1992) showed under inductive LDs almost normal flowering pattern whereas it was not able to flower at all at SDs, even when previously treated with exogenous GAs. Nevertheless, this flowering effect of GA is not strictly limited to SDs as demonstrated by the triple *gid1* mutant (lacking in all three *GID-GIBBERELLIC INSENSITIVE DWARF* receptors for GA). The *gid1* mutant flowers extremely late or not even at all under inductive LDs (Griffiths *et al.* 2006; Willige *et al.* 2007). Interestingly, GA works in parallel (i.e. independently) of CO as the *ga1-3 co* double mutant showed an additive late-flowering phenotype. of *co* under LD but on the other hand the levels of *FT* mRNA expression were increased after addition of exogenous GAs suggesting that GA are on some other way independently controlling expression of *FT* (Hisamatsu and King 2008). It was later shown that *FT* expression is controlled by GA through interaction of the GID1 receptor with members of the DELLA protein family, totally independent of CO (Sun 2010). DELLA proteins belong to the GRAS family of transcriptional regulators and work as repressors of plant growth and development (Hirsch and Oldroyd 2009).

**5.1.8 Other factors affecting flowering**

Other important endogenous factors that are promoting flowering are sugars (Bernier *et al.* 1993). It is known that sucrose can promote flowering in some plant species but this effect might be rather species-specific (Srikanth and Schmid 2011). Trehalose is another sugar that promotes flowering (Schluepmann *et al.* 2003). Furthermore, T6P (trehalose-6-phosphate) was proven to be a proxy for carbohydrate status in plants and that it is absolutely essential for expression of *FT* and *TSF* in the phloem companion cells (Wahl *et al.* 2013). The age of the plant was recently reported to play a role in the regulation in flowering time independent of photoperiod, vernalization or GA pathways, and instead works via the *miR156* microRNA level which decreases with increasing age of the plant (Wang *et al.* 2009). In this work an early flowering phenotype of *ARR1* and *ARR2* lacking mutants (*arr1-4, arr2-4* single and *arr1-4 arr2-4* double mutants) under SD was further investigated. This is the first time that B-type response regulators in *Arabidopsis* (LD facultative plant) have been shown to regulate flowering. TCS and probably cytokinin were suggested to play roles as regulators of flowering. Further *ARR1* and *ARR2* are acting independent of *FLC* floral repressor but therefore *FLM* floral repressor seems to be epistatic to both *ARR1* and *ARR2* response regulators.
5.2 Results

5.2.1 ARR2 mutants show early flowering phenotype under short day (SD) conditions

Previous work on ARR2 had been conducted with the arr2-1 to arr2-4 mutant allele in the Ler-0 ecotype (Hass et al. 2004). Work on this mutant showed connections between the two-component signalling pathway, genes related to defence and abiotic stress signalling and adaptation and ethylene signalling (Hass et al. 2004). In order to investigate further roles of ARR2 with its closest homolog ARR1, a mutant of ARR2 was obtained in the Col-0 background. This mutant, and a mutant of ARR1, arr1-4, also in the Col-0 background, were shown to be full knock-outs. Interestingly, arr1-4, arr2-4 single mutants and the arr1-4 arr2-4 double mutant displayed precocious flowering under non-inductive short day (SD) conditions, with the double mutant having an additive effect (Figure 5.1).

Despite the high sequence similarity and reported functional redundancy of ARR1 and ARR2 in regulating expression of certain common targets in response to cytokinin signal (Mason et al. 2005), the loss of ARR1 and ARR2 effects flowering time to a different degree.

The single arr1-4 and arr2-4 mutants flower one and two weeks earlier than wild-type control plants, respectively (Figure 5.1, B). In contrast, the arr1-4 arr2-4 double mutant flowers three weeks before the Col-0 control (Figure 5.1, B). The early flowering phenotype can be also observed as a reduction of rosette leaf number. The arr1-4 mutant induces flowering by more than 4 leaves, arr2-4 mutant by more than 7 leaves, while arr1-4 arr2-4 produces 15 leaves less than Col-0 (Figure 5.1, B). A trend towards early flowering was also observed when the arr1/2 mutants were grown under inductive long day (LD) conditions. However, only the arr1-4 arr2-4 double mutant flowered significantly earlier than wild-type (p = 0.0052; α=0.05) and produced 3 leaves less than the wild-type (Figure 5.1, C). In conclusion, the loss of ARR1, ARR2, or both genes simultaneously, resulted in early flowering under SD conditions. As ARR1 and ARR2 are known B-Type transcription factors, some of known flowering time-related genes (CO, SOC1, AP1, FT, FLC, FLM, TSF, TFL, LFY and FD) were profiled for alterations in expression patterns in the various mutants under short day and long day conditions.
Figure 5.1 Novel early flowering phenotype of arr1/2 mutants.
Photographs illustrating the phenotypes of wild-type (Col-0) and arr1-4, arr2-4 single mutants and arr1-4 arr2-4 double mutant grown under LDs (left panel) and SDs (right panel). Differences in Days Until Flowering (DUF) and number of Rosette Leaf Number (RLN). ‡Significance differences (α=0.05) with the wild-type were determined by LSD (Fischer’s Least Significant Differences) after the data passed one-way ANOVA (α=0.05). Each day length experiment was repeated twice with similar results.

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<tr>
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<th>DUF (Range)</th>
<th>RLN (Range)</th>
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| **A**
| Col-0 (wild type) | 31.2 (26-36) | 13.4 (12-16) | 20 |
| arr1-4 | 29.0 (24-36) | 13.4 (12-16) | 20 |
| arr2-4 | 24.7 (20-31) | 13.5 (20-31) | 20 |
| arr1-4 arr2-4 | 22.5 (20-31) | 12.5 (20-31) | 20 |

| **B**
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<th>RLN (Range)</th>
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<th>RLN range</th>
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<tr>
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<td>±11.8</td>
<td>±1.4</td>
<td>26-36</td>
<td>12-16</td>
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<td>48.0 (39-56)</td>
<td>±10.3</td>
<td>±1.6</td>
<td>95-119</td>
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</tr>
<tr>
<td>arr2-4</td>
<td>103.8 (85-116)</td>
<td>46.2 (36-54)</td>
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<td>±1.7</td>
<td>85-116</td>
<td>36-54</td>
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</tr>
<tr>
<td>arr1-4 arr2-4</td>
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<td>33.7 (20-45)</td>
<td>±12.4</td>
<td>±1.1</td>
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| **C**
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<th>RLN SD</th>
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<th>RLN range</th>
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5.2.2 Lack of the activity of ARR1/ARR2 genes causes down-regulation of specific floral repressors

In order to identify which flowering time pathways are disturbed in the arr1-4, arr2-4 and arr1-4 arr2-4 mutant lines, key flowering loci were tested for changes in expression with respect to Col-0. Gene expression data quantified by RT-qPCR was firstly obtained from leaves of 70-days-old SD-grown plants shortly before the arr1-4 arr2-4 double mutant, which displays the strongest acceleration in flowering, entered the reproductive phase. In agreement with the early flowering, expression of the floral repressors FLC and FLM were 2-fold and 4-fold reduced in leaves, respectively (Figure 5.2, A). In contrast, expression of the known floral repressor SVP was not affected. Similarly, expression if the floral activators CO, SOC1 and FT were also not affected with only FD showing a slight up-regulation (Figure 5.2,
B). Based on these observations the *arr1-4 arr2-4* double mutant causes a depression of floral repressors in leaf and the key LD inductive-pathway genes are not perturbed. Genes that are predominantly expressed at the meristem are also known to have very important roles in flowering initiation, especially in early flowering under short days (Koornneef *et al.* 1998). Therefore, some key meristem-localized genes were examined with the aim of profiling the state of the SAM from 70-day-old SD-grown plants.

![Figure 5.2](image)

**Figure 5.2** The transcript levels of the flowering time pathway-specific marker genes in the wild-type and *arr1-4 arr2-4* double mutant at DUF 70.  
(A) Expression of floral repressor genes *FLM*, *FLC* and *SVP* in the wild-type and double mutant plants. The expression of *FLM* and *FLC* in double mutant plants is down-regulated compared to the wild-type which is not the case with *SVP*.  
(B) Expression of floral activators *CO*, *SOC1*, *FD* and *FT*. *FT* levels are very low under the short days and it was taken as a control (i.e. levels are qPCR noise).  
(C and D) The transcript levels of the flowering time pathway-specific marker genes *FDP*, *TFL*, *LFY*, *AP1* and *TSF*.  
(E) Expression of *GA4* and *GA5*, marker genes for gibberellic acid pathway. RNA was isolated from SD plants short before flowering of the double mutants, tissue from leaves and/or shoot apical meristem was taken. For each line 15 plants were taken and pooled together, RT-qPCR was done using three biological replicates were used with three technical repetitions each and normalized by *ELF-1α*. Error bars indicate SD of the mean. Leaf material was used for (A, B) and for (C-E) material was taken from SAM.

In the SAM, the expression of the floral repressor *TERMINAL FLOWER 1* (*TFL1*) was reduced 1.7-fold, whereas expression of the floral activator *FD PARALOG* (*FDP*) was not significantly changed (Figure 5.2, C). In contrast, the meristem identity genes *LEAFY* (*LFY*) was 2-fold increased (Figure 5.2, B) and *APETALA* (*AP1*) and *TWIN SISTER OF FT* (*TSF*) were up-regulated
in the double mutant, 3-fold and 6-fold respectively. The meristem identity genes like AP1 and LFY play an important role in early flower initiation (Ferrandiz et al. 2000) and their higher expression confirms the early flowering state of the arr1-4 arr2-4 double mutant compared to Col-0.

Flower initiation in Arabidopsis thaliana under non-inductive short day conditions (Eriksson et al. 2006) is dependent on the biosynthesis of the plant hormone gibberellin (GA) and cross-talk between cytokinin and GA has been described (Greenboim-Wainberg et al. 2005). This dependency can be explained, at least partly, by GA regulation of the flower meristem identity gene LFY and the flowering time gene SOC1 (Eriksson et al. 2006). Gibberellic acid flowering pathway is mostly typical for non-inductive conditions, based on the observations of ga1 mutant (Wilson et al. 1992). GA4 and GA5 (gibberellin 3 β-hydroxylase 1 and 2) are involved in later steps of the gibberellic acid biosynthetic pathway (Hedden and Phillips 2000) and their mRNA levels were shown to somewhat parallel active GA levels in vivo (Achard et al. 2008). Therefore, the mRNA levels of GA4 and GA5 were examined in the SAM at DUF 70. Both GA4 and GA5 were unaffected in the double mutant in the SAM (Figure 5.2, E), presumptively indicating that ARR1 and ARR2 are not involved in the gibberellic acid flowering pathway.

In conclusion, the expression data obtained by RT-qPCR suggests that ARR1 and ARR2 are mostly involved in short-day, non-inductive flowering pathways. This is supported by the fact that the expression of floral repressors FLC and FLM was lower in the double mutant, whereas known floral activators were not dramatically altered in leaf. The SAM at DUF70 in the double mutant was clearly committed to flowering compared to the wild-type as evident by the high expression levels of API, LFY, and TSF. Double mutant was clearly, based on API expression, in different developmental stage than the wild-type. In order to investigate the previous observations experiments with different approach were further done.

5.2.3 Change in expression of floral repressors is strictly due to early flowering and not developmental effects

To ensure that the data reported above reflect true expression differences rather than differences in developmental stage, arr1-4 arr2-4 plants were grown for 30 days under non-inductive SD conditions and then shifted to the inductive photoperiodic long days. It has previously been shown that plants grown for 30 days under SDs are large and developed enough to rapidly enter the reproductive phase after a shift to LDs (Schmid et al. 2003). For Arabidopsis, plants are irreversibly committed to flowering after 3 days in LDs as indicated by the expression of the floral homeotic gene API (Schmid et al. 2003). Therefore flowering was induced in arr1-4 arr2-4 and wild-type plants at 30 days after germination by shifting them LD and plant material was harvested 0 (control) and 5 days after the shift.
After shifting, the expression of the floral repressors FLM, FLC and SVP was reduced in Col-0 while the expression of floral activator CO and floral pathway integrator SOC1 increased (Figure 5.3) indicating that SAM to the shift in photoperiod. AP1 expression (Figure 5.3, F) is suggests that plants were still in vegetative state before the shift and first after the shift under the inductive long days flowering was induced (the experiment as such was working).

Thus a vegetative phase was replaced by a reproductive phase. The expression level of the floral repressor FLC in arr1-4, arr2-4 and arr1-4 arr2-4 is decreased (about 2-fold) before the shift relative to Col-0 and stays reduced after the shift (Figure 5.3, A). FLM also follows this pattern, in that it is already reduced in expression before the shift and maintains this level after the shift (Figure 5.3, B). In contrast, the expression of floral repressor SVP decreased after the shift from SDs to LDs independently of the loss of ARR1 or ARR2 (Figure 5.3, C). Similarly to SVP, the expression of SOC1 is unchanged in the mutant backgrounds and SOC1 reached induction levels like that in Col-0 (Figure 5.3, D). CO also follows a similar pattern as SOC1, in that after the flowering shift induction, the expression of CO is increased in response to the shift to LD (Figure 5.3, E).

Taken together, these results suggest that the reduction of FLC and FLM expression might be causal for the early flowering observed in arr1-4 and arr2-4 single mutants and the arr1-4 arr2-4 double mutant.
5.2.4 Changes in expression of floral repressors are not due to an increase in size of the apical meristem

ARR1 and ARR2 are known to have roles in cytokinin signalling (Grefen and Harter 2004; Zwack and Rashotte 2013) and it is known that cytokinin has a positive role in regulating SAM size and activity (Tucker and Laux 2007; Werner and Schmulling 2009; Gupta and Rashotte 2012). Cytokinin is also required in the SAM for maintenance of cell division and prevention of cell differentiation (Jasinski et al. 2005; Gupta and Rashotte 2012). Therefore, the size of shoot apical meristem SAM was measured in mutant plants and compared to its wild-type in order to see if the early flowering phenotype could be ascribed to a difference in size or in morphology. For this experiment SAM of 30-day-old plants grown under SDs were examined (Figure 5.4).

![Figure 5.4 Size of shoot apical meristem of Col-0 arr1-4 and arr2-4 single mutants and arr1-4 arr2-4 double mutant.](image)

(A) Examples of the tissue taken and how measurements were made. Sections were scanned for clear visibility of the two large air vacuoles (arrows, upper image). Once found then the SAM width was measured (solid line, lower image). (B) Representative sections of shoot apical meristems of Col-0, arr1-4, arr2-4 and arr1-4 arr2-4 respectively. (C) Size of shoot apical meristems in single mutants and the double mutants from 30 day-old plants grown under SDs. At least 10 plants per line were used for statistical evaluation. Significance classes were computed using the Fisher’s Least Significant Difference (LSD, α=0.05) test after passing one-way ANOVA (α=0.05, p ≥ 0.0339). Classes not connected by a letter are significantly different. All of the mutants have means that are smaller than Col-0, but only arr2-4 and arr1-4 arr2-4 are statistically smaller than the wild-type. Error bars indicate SD of the mean. Scale bars, 100µm.

SAMs of Col-0 and arr1-4 single mutant did not show a statistical significant difference in their size (LSD, α=0.05) even though the mean of the arr1-4 similar to the arr2-4 and arr1-4 arr2-4 and are not statistically different (Figure 4, C). On the other hand, the SAMs of arr2-4 single mutant and the arr1-4 arr2-4 double mutant are statistically significantly smaller than in the wild-type (LSD, α=0.05). The SAM of the arr1-4 arr2-4 double mutant is not significantly different in size from arr2-4 or arr1-4 single mutants however (Figure 5.4, C).
suggests that the difference in SAM size is only due to lack of ARR2 and not ARR1, however one should keep in mind that the arr1-4 SAM was never observed to be in the same range as that of Col-0 (Figure 5.4, C) and was not statistically different from arr2-4 or the double mutant. It is possible that with a larger sample size the arr1-4 would have also been statistically smaller. For now, it is evident that the SAMs of arr1-4, arr2-4 and arr1-4 arr2-4 are not larger than Col-0. Thus, despite of even smaller size of SAM in the mutant plants they manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed (Figure 5.4, A, B).

5.2.5 ARR2 expressed either in the SAM or from phloem companion cells recues the early flowering phenotype of arr2-4 and arr1-4 arr2-4

The RT-qPCR results from the previous experiments indicated that there were changes in floral regulator expression in both leaf and the SAM. Investigation of the SAM indicated the early flowering phenotype could not be attributed to an increase in meristem size. Therefore as the action of ARR2 with respect to flowering time was unclear, the double mutant was complemented with two different tissue specific promoters, pSUC2 and pFD. pSUC2 is a vasculature-specific promoter expressed only in phloem (companion cells) of all green tissues of Arabidopsis such as rosette leaves, stems, and sepals. SUC2 encodes a plasma-membrane sucrose-H+ symporter (Truernit and Sauer 1995; Wippel and Sauer 2012). pFD is a meristem-specific promoter expressed in the shoot apex (Abe et al. 2005). FD is bZIP protein required for positive regulation of flowering (Abe et al. 2005; Wigge et al. 2005). Furthermore, it is known that ARR2 can be activated by phosphorylation of an aspartate (D80) in a Two-Component System (TCS) dependent manner (Grefen and Harter 2004). Mutation of D80 to E (Glutamate) mimics the phosphorylated state, increases the transactivation capacity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects (Hass et al. 2004). Mutation of D80 to N (Asparagine), however, prevents TCS dependent phosphorylation (Kim et al. 2006) and impairs basal gene activation capacity, yet D80N still appears to respond to cytokinin according to reporter gene assays (Hwang and Sheen 2001; Kim et al. 2006). Therefore, the arr2-4 and arr1-4 arr2-4 mutants were complemented using wild-type ARR2 and ARR2D80N cDNA versions under the control of the pSUC2 and pFD promoters (Tables 5.1 to5. 4). Due to the stronger flowering time effect observed by the loss of ARR2, arr1-4 plants were included as controls only and not complemented with the ARR2 at this time, thus this complementation assay focuses on the effect of the ARR2 gene.

All plants used for this experiment were in examined in the T2 generation and therefore preselected with BASTA. For each construct two or three independent lines were chosen and a minimum of 15 plants were used for the experiment. To rule out any secondary effects from the BASTA application and ensure that the vector backbones did not disturb the early flowering phenotype, all the mutant lines as well as the wild-type plants were transformed with empty vectors. This was done by using the “pJL blue” gateway compatible vector which only has a MCS (multiple cloning site (MCS) sequence between the gateway cassette. The results will be presented in the same order as they are given in Tables 5.1 to 5.4.
Table 5.1 shows the complementation results for ARR2 and ARR2D80N driven under the pSUC2 promoter in LDs. Two to three transgenic lines were chosen for each construct and genotype pair. The table presents the data for each line analysed; statistical analyses were performed by pooling the results from independent lines for each transgene. With regards to the two parameters DUF and rosette leaf number, DUF is more predictive and has a higher resolution as the number of rosette leaves was nearly invariant under our LD condition (total range 2 leaves). Therefore, only the results with respect to DUF will be presented for LDs. The control vector construct, here called “pJL-blue”, did not complement the flowering time phenotype. The wild-type flowered the latest (mean: 29 DUF), the double mutant the earliest (mean: 24 DUF) and the two single mutants in-between (means: 26 DUF for arr1-4 and 25 DUF for arr2-4). Thus even though the early flowering phenotype is comparably weak in LDs, the flowering time trend previously observed was not disturbed by the vector backbone nor the BASTA treatment. ARR2 driven by pSUC2 was able to complement the flowering defect to near wild-type levels in the arr2-4 single mutant (mean: 28 DUF same significance class as Col-0). Most remarkably, ARR2 D80N loss-off-function version driven under the pSUC2 was also able to partially complement (mean: 27 DUF) the early flowering phenotype of the single mutant under LDs. Neither construct was able to complement the double mutant however.

The complementation results for ARR2 and ARR2 D80N driven under the pFD promoter in LDs are given in Table 5.2. Like the pSUC2 experiment in LDs, the DUF parameter is more informative as the rosette number again is nearly invariant and has a small range (2 leaves). The control pJL-blue constructs lead to the same tendency with the wild-type flowering the latest (mean: 29 DUF), the double mutant earliest (mean: 25 DUF) and the single mutants in-between as previously observed. This again demonstrated that neither the BASTA application nor vector backbone disturbed the flowering phenotype of the respective genotypes. Expression of ARR2 from the pFD promoter was able to complement the single and double mutant (means: 28.5 and 29 DUF, respectively). A partial complementation was also observed with ARR2 D80N in the arr2-4 mutant but not the arr1-4 arr2-4 double mutant. Table 5.3 summarises the results for complementation of the various mutant lines for ARR2 and ARR2 D80N driven under the pSUC2 promoter in SDs. The control pJL-blue constructs once again did not disturb the flowering time with the wild-type flowering the latest (mean: 101 DUF with 53 leaves), the double mutant earliest (mean: 81 DUF with 41 leaves) and the single mutants in-between as previously observed. All the complemented lines used for these experiments showed within the same complementation same tendency (supplemental figures 8 and 9). The rosette leaf range is larger this time (range of 13 leaves) and overall reflects the DUF parameter but not only for the double mutant. The best complementation was observed in the double mutant (mean: 90 DUF with 48 leaves) with ARR2 D80N also partially, but significantly, complementing the mutant (mean: 86 DUF with 45 leaves). In contrast to the pSUC2 under LD conditions, neither ARR2 nor ARR2 D80N was able to even partially complement arr2-4 based on the significance classes. Nevertheless, this complementation of ARR2 did have a mean DUF of 89, nearly identical to that of the partially complemented double mutant.
### Table 5.1 Flowering time of transgenic lines driven under tissue specific SUC2 promoter (pSUC2) under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

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<th>Genotype</th>
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Table 5.2 Flowering time of transgenic lines driven under tissue specific FD promoter under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer’s Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes not connected by a letter are significantly different.
Finally, the complementation results for \textit{ARR2} and \textit{ARR2 D80N} driven under the \textit{pFD} promoter under SDs are in Table 5.4. As observed in the other three experiments, the control pJL-blue constructs did not disturb the flowering time: wild-type flowered the latest (mean: 108 DUF with 54 leaves), \textit{arr1-4} (mean: 88 DUF with 50 leaves), \textit{arr2-4} (mean: 84 DUF with 48 leaves) and the double mutant the earliest (mean: 81 DUF with 41 leaves). In contrast to the previous experiments \textit{ARR2} driven by the \textit{pFD} promoter was rescued both the \textit{arr2-4} (mean: 97 DUF with 50 leaves) and the \textit{arr1-4 arr2-4} (mean: 95 DUF with 50 leaves) mutants almost completely. Most remarkably, the \textit{ARR2D80N} was also able to, partially, and significantly, complement both the single \textit{arr2-4} (mean: 89 DUF with 47 leaves) and the double mutant (mean: 86.5 DUF with 45.5 leaves).

Taken together, even though full complementation was only observed under LDs, the complementation of the early flowering of \textit{arr2-4} under SDs was also partial, yet statistically significant, suppressed. This suggests that lack of \textit{ARR2} is indeed causal for the observed early flowering phenotype. \textit{ARR2} driven under \textit{pFD} showed the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the \textit{arr2-4} and \textit{arr1-4 arr2-4} mutation effects strongest. The rescue is slightly weaker when \textit{ARR2} is driven under \textit{pSUC2} but still significant in the double mutant. In LD conditions, \textit{ARR2} expressed from either promoter was able to complement the \textit{arr2-4} mutation, but only \textit{ARR2} expressed under the \textit{pFD} promoter was able to partially rescue the \textit{arr1-4 arr2-4} mutant. Mutant plants were also slightly complemented with loss-of-function \textit{ARR2 D80N} gene in the single and double mutants in SDs when driven under the \textit{pFD} promoter or by \textit{pSUC2} in \textit{arr1-4 arr2-4}. This effect of \textit{ARR2 D80N} was also observed in LDs but only in the \textit{arr2-4} mutant background. The \textit{ARR2D80N}-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type \textit{ARR2}-cDNA (Hwang and Sheen 2001; Choi et al. 2010; Veerabagu et al. 2012). The reason for this is most likely due to an incomplete inactivation of the \textit{ARR2} by the D80N mutation. Considering this fact, it can be still concluded that \textit{ARR2} needs to be phosphorylated by the TCS (Two-Component System) in order to best rescue the early flowering phenotype as only partial complementation was observed for the \textit{ARR2D80N} loss-of-function transgene. This strongly suggests that \textit{ARR2} may be a point of integration for the TCS and other flowering time pathways under SDs.
Table 5.3 Flowering time of transgenic lines driven under tissue specific SUC2 promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA (α=0.05) by using Fischer’s Least Significance Difference (LSD) with alpha levels α=0.01 for DUF and α=0.05 or RLN. Classes not connected by a letter are significantly different.

<table>
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<th>Genotype</th>
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<th>RLN</th>
<th>DUF SD</th>
<th>RLN SD</th>
<th>DUF range</th>
<th>RLN range</th>
<th>n</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>±6.3</td>
<td>±2.3</td>
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<td>46-52</td>
<td>15</td>
</tr>
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</tr>
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<td>47-50</td>
<td>15</td>
</tr>
<tr>
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Table 5.4 Flowering time of transgenic lines driven under tissue specific FD promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

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<th>Mean</th>
<th>Genotype</th>
<th>LSD Rosette</th>
<th>Mean</th>
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<td>B</td>
<td>50,3</td>
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<td>arr2-4 (Col-0) pFD::ARR2D80N</td>
<td>C</td>
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<td>C</td>
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<table>
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<th>DUF range</th>
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<td>49,4</td>
<td>±4,1</td>
<td>±5,7</td>
<td>78-93</td>
<td>40-57</td>
<td>15</td>
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<tr>
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<td>±2,3</td>
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<td>46,2</td>
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<td>±3,1</td>
<td>82-94</td>
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<td>49,8</td>
<td>±3,7</td>
<td>±3,0</td>
<td>90-100</td>
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<td>48,3</td>
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<td>51,0</td>
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<td>±3,1</td>
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<td>15</td>
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<td>39-49</td>
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</table>
5.2.6 ARR1 and ARR2 work mostly independent of FLC

FLOWERING LOCUS C (FLC) is a transcription factor that functions as a repressor of the floral transition. It is the main target of the vernalization pathway in Arabidopsis thaliana and is epigenetically silenced by the prolonged exposure of plants to cold. FLC was shown to bind to and directly block the transcriptional activation of SOC1 and FT (Helliwell et al. 2006; Srikanth and Schmid 2011).

Based on RT-qPCR data obtained from ARR1 and ARR2 knockouts, the arr1-4, arr2-4 and arr1-4 arr2-4 double mutants showed reduced levels of FLC compared to wild-type. In order to find out more about the nature of this interaction the arr1-4 arr2-4 double mutant was crossed with a deletion mutant for FLC, flc-3 (Michaels and Amasino 1999). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 11).

The flc-3 arr1-4 arr2-4 (mean: 28 DUF) triple mutant under inductive LD conditions behaved like the flc-3 single mutant (mean: 28 DUF), flowering about 6 days earlier (mean: 34 DUF) and producing 2 leaves less than the Col-0. The arr1-4 arr2-4 double mutant displayed an intermediate phenotype (mean: 32 DUF) (Figure 5.5, A (left panel), B). Under LDs, FLC appears to be epistatic to ARR1 and ARR2. Under non-photoperiodic SD conditions the flc-3 arr1-4 arr2-4 triple mutant (mean: 78 DUF) very clearly shows an additive effect. It flowers earlier than both parents, 8 days earlier than the flc-3 (mean: 87 DUF) about 2 weeks before arr1-4 arr2-4 (mean: 92 DUF) and more than 3 weeks before the Col-0 (mean: 111 DUF). The same flowering time was observed when this experiment was repeated under SD conditions but under a different light quality. These results confirm the consistency of an additive early flowering phenotype of the flc-3 arr1-4 arr2-4 triple mutant in SDs (Figure 5.5, A (right panel), D).

The flc-3 arr1-4 arr2-4 triple mutant repeatedly showed a clear tendency towards early flowering when compared to the flc-3 single mutant. However, the differences were not statistically significant. Therefore, the same experiment was repeated again under LDs but at 16°C temperature, which results in a mild delay in flowering. Interestingly, under these conditions, flc-3 arr1-4 arr2-4 flowered earlier than flc-3. The triple mutant flc-3 arr1-4 arr2-4 flowered over a week earlier that the wild-type (mean: 34 DUF) which was almost five days before arr1-4 arr2-4 double mutant (mean: 39 DUF) and two days earlier than flc-3 single mutant (mean: 36 DUF) (Figure 5.5, C), suggesting an additive effect on of FLC and ARR1 and ARR2 under inductive LD conditions.

Based on these experiments, the flc-3 arr1-4 arr2-4 triple mutant shows very consistent additive effect in early flowering under SDs and LDs (statistically significant only at 16°C). Based on this result ARR2 seems to work mainly independent of FLC. If there is any intercommunication between these two signalling pathways then it is rather small.
Figure 5.5 Flowering phenotype of arr1-4 arr2-4 flc-3 triple mutant.

(A) Photographs showing early flowering phenotype of arr1-4 arr2-4 double mutant, flc-3 single mutant and arr1-4 arr2-4 flc-3 triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. The arrow indicates the position of floral bud of Col-0. (B) Flowering times of mutant plants grown under long day conditions. (C) Flowering times of mutant plants grown at 16°C and 23°C under LD conditions. (D) Flowering times of mutant plants under SD conditions. Additive effect on flowering time by the triple mutant is very visible and consistent even under different light conditions (i.e. Chamber 1 and Chamber 2).

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.
5.2.7 ARR1 and ARR2 work in the same pathway upstream of FLM

FLOWERING LOCUS M (FLM) is a MADS-domain gene that acts as an inhibitor of flowering in Arabidopsis. Although the sequence of FLM is similar to that of FLC, FLM and FLC interact with different flowering pathways (Scortecchi et al. 2003). The FLM levels were also strongly down-regulated in both ARR1 and ARR2 knockouts and in the double mutant. Therefore, to get more insight into the potential interaction with FLM in respect to flowering time, the arr1-4 arr2-4 double mutant was crossed with flm-3 single knock-out mutant for FLM (Balasubramanian et al. 2006). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 12).

A

B

Table 5.6 Flowering phenotype of flm-3 arr1-4 arr2-4 triple mutant.

Flowering time was first determined for the flm-3 arr1-4 arr2-4 triple mutant under LD conditions. The triple mutant showed very early flowering (compared to the Col-0) and was indistinguishable from the flm-3 single mutant. The triple mutant flm-3 arr1-4 arr2-4 flowered in average 6 days earlier (mean: 24 DUF) which was the time same as flm-3 (mean: 101).
24 DUF), and both developed 3 rosette leaves less than the Col-0 (mean: 30 DUF). The double mutant arr1-4 arr2-4 (mean: 27 DUF) flowered somewhat later than the flm-3 mutants and produced one leaf less than Col-0 (Figure 5.6, A left panel, B). Clearly FLM is epistatic to ARR1 and ARR2.

Figure 5.6 Flowering phenotype of flm-3 arr2-4 double mutant.
(A) Photographs showing early flowering phenotype of arr2-4 and flm-3 single mutants and flm-3 arr2-4 double mutant under LDs (left panel) and SDs (right panel) compared to wild-type. (B) Flowering time of mutant plants grown under long day conditions. (C) Flowering time of mutant plants grown under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.

The flm-3 arr1-4 arr2-4 triple mutant and the flm-3 single mutant grown under non-inductive SD condition showed epistasis to flm-3 single mutant as well (Figure 5.6, A(right panel), C). The flm-3 and flm-3 arr1-4 arr2-4 double mutant mutants flowered approximately at the same time, i.e. more than 30 days earlier (mean: 77 DUF) than the wild-type (mean: 110 DUF) and over 10 days earlier than the arr1-4 arr2-4 double mutant (mean: 87 DUF). Both flm-3 containing mutants developed 20 rosette leaves less (mean: RLN 29) than Col-0 (mean: RLN 50). In contrast the arr1-4 arr2-4 double mutant made 10 leaves less than Col-0 (mean: RLN 40). Based on these results FLM is epistatic to ARR1 and ARR2 in LDs and SDs, and therefore ARR1 and ARR2 appear to be genetically involved within the same flowering pathway as FLM. ARR1 and ARR2 are hypostatic to FLM, i.e. they both probably act upstream of FLM). Epistasis of ARR2 to FLM was one more time confirmed on flm-3 arr2-4 double mutant. Under long
day conditions flm-3 arr2-4 (mean: 87 DUF) double mutant flowers at the same time like the flm (mean: 77 DUF) parent which is almost a week before the wild-type and 5 days before the arr2-4 single mutant. Single mutant flm-3 and the flm-3 arr2-4 double mutant also have the same number of rosette leaves (Figure 5.7, A (left panel) and B).

5.2.8 Initial experiments with crossings between arr2-4 with soc1-2 and co reveal unanticipated interactions

During this work it was possible to generate and analyse double mutants with co and soc1-2 lines for placing ARR2 within other known flowering time pathways. Recall that arr2-4 had the stronger flowering time phenotype compared to arr1-4. Floral activators SOC1 and CO, especially SOC1, are floral integrators converting most of the flowering pathways. CONSTANS (CO) promotes flowering of Arabidopsis in response to day length. Under LD conditions CO activates transcription of FLOWERING LOCUS T (FT) in the vascular tissue of leaves and initiates flowering (Corbesier et al. 2007). SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) is required for CO to promote flowering and integrates the long-day and vernalization/autonomous pathways (Samach et al. 2000). On the other hand SOC1 also integrates signalling from the GA-dependent pathway, a major flowering pathway under non-inductive short days (Moon et al. 2003). The SOC1 and FT genes are also regulated by a different flowering-time pathway that acts independently of CO and which is active also at the non-inductive SD conditions (Samach et al. 2000). As previously described, beside the similar role of CO and SOC1 as floral activators, they can also act independently of each other. Therefore, both arr2-4 soc1 and arr2-4 co double mutants were tested for flowering time under LD and SD conditions.

Under inductive LD conditions both double mutants were indistinguishable from their soc1 and co single mutant parents. The arr2-4 soc1 double mutant flowered 5 days later than the Col-0 and more than a week after its arr2-4 parent developing 7 rosette leaves more than Col-0 and arr2-4. Late flowering phenotype is especially remarkable by arr2-4 co (mean: 45 DUF) double mutant where flowering was delayed for more than two weeks compared to Col-0 (mean: 30 DUF) and more than 17 days than by arr2-4 (mean: 28 DUF), arr2-4 co double mutant developed 13 rosette leaves more than Col-0 and arr2-4 (Figure 5.8, A and B).

Under non-inducible SD conditions CO is unable to activate the FT flowering-pathway. When grown under SD conditions co single mutant and arr2-4 co double mutant show very similar flowering phenotype. They flower around 10 days (arr2-4 co double mutant) and 15 days (arr2-4 single mutant) (mean: 100 DUF) before the Col-0 (mean: 110 DUF) but make even 15 rosette leaves less than the wild-type). On the other hand, co and arr2-4 co double mutant flower only a couple of days later than the arr2-4 (mean: 96 DUF although they both made 10 leaves less than arr2-4 single parent (Figure 5.8, C).
Figure 5.8 Flowering phenotype of soc1 arr2-4 and co arr2-4 double mutants.

(A) Photographs showing late flowering phenotype of soc1 arr2-4 (middle panel) and co arr2-4 (left and right panel) double mutants under LDs compared to wild-type. (B) ARR2 is hypostatic to CO and SOC1.

(C) Flowering phenotype of soc1 arr2-4 and co arr2-4 under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.

Double mutant arr2-4 soc1 shows intermediate flowering phenotype compared to its parents. The arr2-4 soc1 (mean: 107 DUF) double mutant flowers around 9 days earlier than soc1 (mean: 116 DUF) single mutant and makes 5 rosette leaves less, the same double mutant flowers around 12 days later than arr2-4 single mutant and makes around 5 rosette leaves more than the arr2-4 single mutant (Figure 5.8, C).

Taken together, under inductive long days ARR2 seems to be hypostatic or even works independently of CO and SOC1. Under non-inductive short days CO-related-flowering pathways are not very active and arr2-4 co double mutant shows very similar phenotype like arr2-4 parent showing again indications that ARR2 might be hypostatic or independent to CO under SDs as well. Another arr2-4 soc1 double mutant shows similar phenotype like the wild-type which is in between of both single parents suggesting that under SD conditions ARR2 works partially independent and partially dependent of SOC1-flowering pathways.
5.3 Discussion

During this work, a novel early flowering phenotype was observed and confirmed for single mutants arr1-4 and arr2-4 and the double mutant arr1-4 arr2-4 with respect to flowering time. The early flowering phenotype is especially visible under non-inductive SD conditions for all three mutant lines. The arr1-4 arr2-4 double mutant flowers even earlier than either single mutant or the wild-type and this tendency was statistically significant between wild-type and the both single mutants. In contrast, under LD conditions, only the arr2-4 as single mutant showed slightly statistically significant differences as early flowering on the other hand was also observed for the arr1-4 single mutant but this tendency could not be statistically confirmed in LD. Nevertheless, these differences under inductive long days are from a physiological/functional point of view very subtle and were only revealed when plants were exposed to cool temperatures (16°C). In this case, the double mutant clearly flowered earlier than wild-type. Thus, although arr2-4, and presumably arr1-4, also affect flowering in LDs, the loss of these two genes clearly points much stronger roles of ARR2 as a flowering repressor under SD conditions.

Flowering of arr1-4 mutant in SDs is approximately one week earlier, arr2-4 flowers two weeks earlier, the arr1-4 arr2-4 double mutant flowers three weeks before the Col-0. This effect on days until flowering (DUF) was also reflected in rosette leaf number. It has been already proven that generally B-types from the subgroup I (Kim et al. 2012) can complement the arr10 arr12 cytokinin response-deficient mutant (Hill et al. 2013). This could be also the case with early flowering phenotype. Yet interestingly, despite redundancy and very similar sequence homology of ARR1 and ARR2 genes, we managed to prove that under the loss of ARR2 effects flowering more strongly than ARR1 suggesting specificity and affinity (dominance) of ARR2 over ARR1 with respect to flowering time despite their high sequence homology. Interestingly, overexpression of ARR2 was shown to have higher transactivation potential and response with and without the addition of cytokinin on the ARR6 promoter compared to ARR1 (Hwang and Sheen 2001). In addition, even though ARR2 contributes to primary root-elongation responses to exogenous cytokinin, ARR1 is the predominate cytokinin information carrier in this tissue (Mason et al. 2005). Thus, even though these two homologues can have different roles in different tissues, ARR1 and ARR2 showed an additive effect on flowering time, which is visible in the arr1-4 arr2-4 double mutant under both inductive- and non-inductive conditions. One can therefore conclude that both genes are involved in the same flowering time pathway and their role is especially crucial in the signalling pathways which are dominantly active under non-inductive SD conditions.

To check which genes are miss-regulated in the double mutant RT-qPCR analysis of familiar and well described floral regulators was performed. One scenario that would explain the early flowering phenotype is that ARR1 and ARR2 genes/pathway (cytokinin) positively regulates the expression of certain floral repressors (such as FLM, FLC, SVP). Alternatively, early flowering could be induced by activation of positive regulators of flowering (TSF, FD, CO) or floral integrators (SOC1). The chosen markers were applied in order to profile the state of
SAM at this chosen time point. For the first hints, leaf material and material from shoot apical meristem (SAM) from the wild-type and the double mutant was taken depending on where the tested gene is best expressed. The material was taken from plants grown under SDs and it harvested shortly before the double mutant entered in the reproductive phase (based on previous results).

Gene expression of floral activators CO, SOC1, FT and FDP did not show any statistically significant difference in the double mutant compared to the wild-type. FT is known to be expressed in LDs conditions and acts as a long-range signal in Arabidopsis (Valverde et al. 2004; Jaeger and Wigge 2007; Sawa and Kay 2011) and here this marker gene was used as a "control" to make sure that the plants were strictly grown under SD conditions and this is confirmed by its very low expression. Floral activators FD and TSF showed slight up-regulation, although only TSF was statistically significantly different compared to the wild-type. Interestingly, it was shown that under non inductive SDs exogenous cytokinin promotes early flowering via activation of the TSF (D’aloia et al. 2011). The exact mechanism of TSF activation is not known and it could be that ARR1 and ARR2 play role in its regulation knowing that both ARR1 and ARR2 are part of TCS and react to cytokinin treatment (Brenner et al. 2012). It might be that non phosphorylated form of ARR1 and ARR2 by TCS are somehow disturbing transcription of TSF and control flowering on that way. Unfortunately we have failed to produce the tsf-1 arr1-4 arr2-4 triple knock out mutant and make a closer look how these three genes are interacting on genetic level. In general this data clearly indicated that the expression profile of floral activators is not significantly changed in the double mutant and that these genes are mainly not responsible for the early flowering phenotype in the double mutant.

On the other hand, tested floral repressors showed quite a different expression pattern in the double mutant plants. The floral repressors FLC, FLM, SVP and TFL1 cover most of the crucial signalling pathways responsible for the flowering regulation in Arabidopsis (Coupland 1995; Koornneef et al. 1998; Song et al. 2013). Interestingly, three of tested floral repressors showed significant down-regulation of their expression in double mutant: FLC and FLM were quite reduced whereas TFL1 showed only slight reduction. These data show the importance of FLC and FLM among the rest floral repressors with respect to early flowering in arr1-4 arr2-4. On the other hand, the SVP floral repressor seems not to be miss-regulated by the lack of ARR1 or ARR2. This observation is of paramount importance and will be address later.

GA promotes flowering transition most strongly under environmental conditions such as SDs, especially when other regulatory pathways that promote flowering in Arabidopsis thaliana are not active (Moon et al. 2003; Porri et al. 2012). In an attempt to address this, showed that GA4 and GA5 reflected the state of active GA signalling in healthy plants (Achard et al. 2008). Therefore the expression of GA4 and GA5 were also monitored. Based on these two genes, we conclude that the GA flowering pathway is not disturbed in the double mutant as both GA4 and GA5 marker genes for this pathway are unaffected in the SAM in the double mutant.
This presumptively indicates that ARR1 and ARR2 are not involved in the GA flowering pathway but in other flowering signalling pathways active under non-inductive conditions.

Since meristem identity genes and some other genes predominately expressed in meristem play very important role in the initiation of the early flowering they were applied in order to profile the state of SAM at the chosen time point. For this purpose especially AP1 but also and LFY expression was tested. Expression of LFY and AP1 was 2-fold and 3-fold increased with respect to the wild-type. These markers confirmed the early flowering state of the arr1-4 arr2-4 double mutant compared to Col-0 at the time point where this process could not be visually obtained. Although the low expression of FT suggested that the plants were monitored before they were flowering, it could still have been possible that the shift to inflorescence meristem had already occurred. Therefore, to eliminate the possibility of unseen developmental effects, the flowering transition induced with 30-day old plants grown under non-inductive conditions and then shifted to inductive LDs. The material collected from SAM before and after the shift guaranteed that differences in expression profiles of floral regulators among the mutant plants were due to the miss-regulation of the flowering time only. Once more, it was demonstrated that the early flowering phenotype in both single parents and the wild-type comes from the miss-regulation, i.e. down-regulation of specific floral repressors, primarily FLC and FLM, as that their level was low even under non-inductive conditions and it remained unchanged after the shifting. Expression pattern of SVP floral regulator remained unaffected in any of mutant plants before the shift and responded as expected after the shift (was down-regulated) confirming the previous results and suggesting that ARR1 and ARR2 are specifically regulating only certain floral repressors under non-inductive SD conditions. Consistently, all of the tested floral activators showed their regular expression profile like wild-type, they were up-regulated after the shift from vegetative into reproductive state and showing that neither ARR1 nor ARR2 are not playing any role in transcriptional regulators of these floral activators at the not expression level. In conclusion, the early flowering phenotype was most probably a consequence of down-regulation of floral repressors but not of miss-regulation of floral activators.

Considering roles of ARR1 and ARR2 in cytokinin signalling and knowing the fact that cytokinin has a positive-correlative role in regulating SAM size (Werner and Schmulling 2009; Gupta and Rashotte 2012) and that SAM size has been correlated with flowering time (Jeong and Clark 2005), the SAM size of arr1-4, arr2-4, and arr1-4 arr2-4 warranted examination. Such information could be used to deduce if changes in expression of floral repressors were due to an increase in size of the apical meristem in the mutant plants. Therefore, plants were grown under the same conditions used in the shifting experiment. Both the arr2-4 single and arr1-4 arr2-4 double mutants had surprisingly statistically significant smaller SAMs than the wild-type and although the measurement population also showed a smaller size for arr1-4, its SAM did not pass the significance threshold (α=0.05). Thus, the smaller SAM fits more with a loss of cytokinin signal, which would be expected knowing that both ARR1 and ARR2 are active in cytokinin singling and despite the smaller SAM size in the mutant plants they
manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed either. In conclusion, the changes in expression of floral repressors were not due to an increase in size of the apical meristem.

Although the arr2-4 and arr1-4 mutants have been previously described (Mason et al. 2005) and presumed free of any other background mutations, we wanted to be sure that the early-flowering phenotype was due to the lack of ARR1 and ARR2. In order to check this presumption and to determine the origin/location of acting of the ARR2 with respect to early flowering phenotype the arr2-4 and arr1-4 arr2-4 mutants which had the strongest phenotype were complemented with ARR2-cDNA and an ARR2D80N-cDNA mutant. The ARR2D80N-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type ARR2-cDNA (Hwang and Sheen 2001; Choi et al. 2010; Veerabagu et al. 2012). Thus although it is not completely inert, ARR2D80N can be used to see if the wild-type protein, and therefore TCS phosphorylation-dependency, is necessary to rescue the early flowering phenotype.

The cDNA variants were driven under tissue-specific promoters pFD and pSUC2. For this purpose following vectors with promoter expressed in specific tissues were used: pSUC2 vasculature-specific promoter expressed in phloem (companion cells) only (Truemnitt and Sauer 1995; Wippel and Sauer 2012) and pFD-meristem-specific promoter expressed in shoot apical meristem only (Mathieu et al. 2007). Based on their expression pattern one can determine the most likely location where ARR2 protein activity is needed to complement the flowering time phenotype. Only T2 plants were able to be analysed, therefore a pre-selection was needed to remove any non-transgenic plants using BASTA; secondary effects were controlled by using an empty-vector backbone transformed into all of the mutant lines as well as the wild-type.

ARR2 driven under pFD shows the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the arr2-4 and arr1-4 arr2-4 mutation effects were most strongly observed. This effect in SDs is slightly weaker (partial) when ARR2 is driven under pSUC2 but still significant in the double mutant. In LDs conditions, ARR2 expressed from either promoter was able to complement the arr2-4 mutation, but only ARR2 expressed under the pFD promoter was able to partially rescue the arr1-4 arr2-4 mutant. Considered that both tissue-specific promoters are equally strong and having in mind that pSUC2 expression is on whole plant level one can conclude that ARR2 is most potently working in the SAM with respect to flowering time.

Some remarks are warranted: first, both promoters can lead to complementation (even if partial) meaning that function of ARR2 is deliverable (or needed) at the meristem but also throughout the plant. Taken together, the observed early flowering phenotype is truly a consequence of the lack of ARR2 as it can be rescued when it is expressed directly in the SAM by the pFD promoter or when it is expressed in phloem companion cells by pSUC2. Although not directly shown in the work if the lack of ARR2 and ARR1 means that cytokinin signalling
per-se is the culprit for the early-flowering phenotype, the idea is still passed around, albeit lightly, that cytokinin(s) in some form or fashion also could work in a “florigen” type function (Bernier 2011). These results are based on work in Sinapis alba (Sa) where connections to a simultaneous, but not necessarily, co-dependent transportation of SaFT and cytokinin to the meristem. Bernier proposed that some aspect of cytokinin, possibly on the organizing centre, contributes to flowering, but does not in itself cause flowering in Sinapis (Bernier 2011). This idea is interesting in lieu of the results here where a stronger effect is seen when ARR2 is expressed in the meristem, yet ARR2 still expressed in the phloem (distally) can also lead to complementation. Conflicting with these ideas is the observation that Arabidopsis can be induced to flower in SD with an 8 hour hydroponic treatment with BAP (D’aloia et al. 2011). This effect requires TSF and not FT (D’aloia et al. 2011). Recall that expression of TSF was altered in arr1-4 arr2-4.

FLC is known to interact with directly interacts with another MAD-box member SVP, SVP which is also floral repressor that functions within the thermosensory pathway (Li et al. 2008). SVP and FLC also target CYTOKININ RESPONSE 1 (CRE1) and other genes in cytokinin signalling (Gregis et al. 2013). SVP has been further connected to cytokinin via SVP regulation of STIMPY (STIP), but this occurs independently and downstream of FT and TSF (Gregis et al. 2013). STIP works downstream of cytokinin in establishing the SAM (Liu et al. 2009). Interestingly, FLC has been shown to be active in the SAM and in the vascular tissue directly regulating SOC1 and FT (reviewed in Andres and Coupland 2012). TSF was shown not to be bound by SVP (Gregis et al. 2013). In this work, arr1-4 and arr2-4 were also crossed to tsf-1 (Yamaguchi et al. 2005), but triple mutants were not identified, therefore it was not possible to determine the genetic interaction of arr1-4 or arr2-4 with tsf-1.

Knowing that the loss of ARR1 and ARR2 impair cytokinin signalling, it is tempting to speculate that this would explain the early flowering phenotype. In this regard, SVP has recently been uncovered to be involved in regulation of some cytokinin genes (Gregis et al. 2013). Considering that the expression data and mutant analysis (discussed below) suggest that ARR1 and ARR2 are not in the SVP-FLC pathway, it is still an open question if the early flowering phenotype can only be attributed to a loss of cytokinin single transmission. Second, other possible reasons for partial complementation could be that the plants used for this experiment were preselected in the T2 generation with most of them in the hemizygous state and thus not enough ARR2 was produced. Alternatively, the gene was not driven under its native promoter and perhaps ARR2 is also needed in other tissues. For example, it is known that ARR1 is active in controlling auxin flux by regulation of the auxin-response repressor protein SHY2 (SHORT HYPOCOTYL2) (Taniguchi et al. 2007; Chapman and Estelle 2009) and the functional characterisation of ARR1 and ARR2 have mostly been done with regards to cytokinin responses in the root (Mason et al. 2005; Argyros et al. 2008; Hill et al. 2013).

As said before, the connection to cytokinin, is compelling but not conclusive. In rice, it is known that a cytokinin-dependent TCS pathway exists that requires a B-type (EHD1) for aviation of FT homologues HD3A and RICE FLOWERING LOCUS T1 (RFT1) under SDs (Andres
and Coupland 2012). Provided that more evidence is mounted that connects more TCS components to this flowering time phenotype, then (although highly speculative at this point) it could be proposed that a TCS network also works in Arabidopsis to modulate flowering which may be analogous to that of rice (as it is not likely it is the same considering no orthologues can be found) or more indicative to the observations made in Sinapis. Nevertheless, this regulation mechanism in rice only supports our observation that TCS, or at least its B-Type response regulators ARR1 and ARR2, might also play role in flowering regulation of Arabidopsis thaliana under short day conditions as mutant plants lacking in ARR1 and ARR2 genes showed the early flowering phenotype under non-inductive short days in Arabidopsis. In support to this claim is a fact that ARR4 (A-type response regulator) in Arabidopsis thaliana plays very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007) and PhyB, as above mentioned, actively involved in expression control of CO in Arabidopsis thaliana and Hd3a in Oryza sativa (Ishikawa et al. 2011).

That said, is there any evidence that TCS signalling is required for the early-flowering time phenotype? Examination of the mutant plants showed that they were also slightly complemented with loss-of-function ARR2D80N in the single and double mutants under SDs when driven under the pFD promoter or by pSUC2 (albeit only in the arr1-4 arr2-4). This slight effect of ARR2D80N was also observed in LDs but only in the arr2-4 mutant background. As mentioned earlier, the reason for this is most likely due to an incomplete inactivation of the ARR2 by the D80N mutation which was proven by other groups (Hwang and Sheen 2001). ARR2 has a strong basal transactivation capacity unlike the other B-types without the presence of exogenous cytokinin; yet, it still can strongly activate promoter-reporters in a cytokinin-dependent manner (Hwang and Sheen 2001; Veerabagu et al. 2012). The ARR2-D80N mutation impairs, sometimes strongly, this basal transactivation capacity without destroying the cytokinin-dependent response, which is highly enhanced compared to mock controls (Hwang and Sheen 2001). Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS in order to best rescue the early flowering phenotype as only weak partial complementation were observed for the ARR2D80N loss-of-function gene. Type-B response regulators mediate most, if not all, of the immediate-early changes of gene expression induced by cytokinin. This is also the case with ARR1 and ARR2 member of the largest sub-class of response regulators expressed in almost all tissues which consists of seven members (ARR1, ARR2, ARR10, ARR12, ARR11, ARR14, and ARR18) (Brenner et al. 2012). This additionally supports the claim that the early flowering phenotype in the ARR1 and ARR2 lacking plants is originating due to the impaired signalling of cytokinin. Furthermore, the expression pattern of the ARR1 and ARR2 in almost all tissues is showing the importance of cytokinin in every tissue of the plant, and also explains the observation that the early flowering phenotype was rescued also when ARR2 expressed from phloem. On the other hand mutant complementation with ARR2D80E gain-of-function gene under constitutive active promoter is not possible while the plants showed embryo lethality (Hass et
Based on this fact the complementation with \textit{pSUC2} and \textit{pFD} tissue specific promoters could not possible while due to their high potency. Therefore \textit{arr1-4 arr2-4} was complemented with \textit{ARR2D80E} cloned into estradiol inducible \textit{pABind::GFP} vector. As expected the effect of the gain-of-function \textit{ARR2D80E} was very strong and lethal even for plants in T2 generation (supplemental figure 10) and was clearly visible 3 days after the hormonal treatment. Plants were getting various necrotic spots and dying very fast, whereas some of them managed to flower showing early flowering phenotype caused by stress. The formed siliques showed great variation in size and the seed yield was generally more than double lower compared with the wild-type containing certain number of aborted and sterile seed as well. This strongly suggests that \textit{ARR2} may be a “crosstalk point” between the TCS and some flowering time pathways under the short day conditions. Expression analysis, via RT-qPCR, revealed which genes were miss-regulated in the single and double mutants compared to the wild-type and after conducting the flowering time shift, it could be confirmed that the repressors FLC and FLM are down-regulated in \textit{arr1-4 arr2-4}. In order to determine which floral pathways \textit{ARR1} and \textit{ARR2} are involved in, \textit{arr1-4 arr2-4} or \textit{arr2-4} mutants were crossed to mutants in key floral regulatory pathways: \textit{flc-3}, \textit{flm-3}, \textit{tsf-1}, \textit{co}, and \textit{soc1}. Triple mutants were obtained and evaluated with \textit{flc-3} and \textit{flm-3}, whereas \textit{arr2-4} double mutants with \textit{co} and \textit{soc1} were also obtained and evaluated. Crosses with \textit{tsf} unfortunately were not evaluable. For these experiments \textit{arr2-4} single and \textit{arr1-4 arr2-4} double mutants were crossed against \textit{flc-3}, \textit{flm-3} single mutants lacking in these floral repressors as they showed the highest miss-regulation pattern in \textit{ARR1} and \textit{ARR2} lacking mutants. On the other hand, despite the fact that all tested floral activators did not show any expression miss-regulation in \textit{arr1-4 arr2-4}, \textit{co} and \textit{soc1} single mutants were crossed in order to see how and if these genes are interacting on genetic level.

When grown under inductive LD conditions at standard 22/23°C temperature the \textit{flc-3 arr1-4 arr2-4} triple mutant behaved like its \textit{flc-3} parent suggesting that FLC and the \textit{ARR1/2} are involved in the same pathway that controls flowering, \ie FLC is epistatic to both \textit{ARR1} and \textit{ARR2}. Nevertheless, early flowering phenotype is under LDs is by all the mutants quite weak and the temporal resolution of flowering was very low. In addition to this \textit{flc-3 arr1-4 arr2-4} triple mutant after each repeat under the LDs showed clear tendency to flower even earlier than the \textit{flc-3} single parent but none of the time this tendency could be statistically proven. Therefore, the same experiment was repeated again under LDs but at 16°C temperature. Lower temperature should generally slow down the flowering time and the discrete differences in flowering time between the different mutant lines should be observed in higher resolution. This was indeed the case at 16°C, plants generally flowered later than at 23°C. Interestingly, this time \textit{flc-3 arr1-4 arr2-4} triple mutant flowered even earlier that \textit{flc-3} parent suggesting the additive effect of FLC under \textit{ARR1} and \textit{ARR2} under inductive LD conditions. Surprisingly, \textit{flc-3} deletion mutant was in all this conditions showing the same ratio of flowering compared to the wild-type suggesting that FLC is probably active at the same level on both temperatures with respect to flowering time regulation, \ie it is active at 16°C as a repressor. It have been published that that there are some natural accessions that are
unresponsive to thermal induction despite having non-functional fri/flc alleles (Srikanth and Schmid 2011). Recently was also reported that flc-3 single mutant restores its temperature sensitivity first below 16°C and not at higher temperatures (Lee et al. 2013). Under non-inductive SD conditions the flc-3 arr1-4 arr2-4 triple mutant very clearly shows consistent additive effect in early flowering. The same pattern was observed also when this experiment was repeated under SD conditions in other growth chamber with different light intensity.

In conclusion, FLC appears to work partially independent of ARR1 and ARR2 which lack of expression has the same outcome with respect to early flowering phenotype making the triple mutant to flower even earlier then its parents in additive manner. This additive effect is especially good visible under SD conditions due to the enhanced activity of ARR1 and ARR2 under non-inductive short days with respect to early flowering. Under LD conditions at normal ambient temperature of 23°C additive effect could not be statistically proven, but there was each time the tendency present, due to very low involvement of ARR1 and ARR2 with respect to flowering time and therefore weak flowering time phenotype with the mutants. When plants grown under inductive LD conditions but at 16°C temperature additive effect could be observed and statistically proven. On the other hand, in flm-3 arr2-4 double and flm-3 arr1-4 arr2-4 triple mutants obtained early flowering phenotype was very clear. When grown on both inductive LD and non-inductive SD conditions both flm-3 arr2-4 double and flm-3 arr1-4 arr2-4 triple mutants showed identical phenotype like flm-3 single parent supporting previous expression data obtained by RT-qPCR. Single flm-3 mutant has very strong early flowering effect on both LD and SD conditions so it was very easy to proof and describe the obtained phenotype. Based on obtained results both ARR1 and ARR2 seem to work in the same pathway like FLM with respect to early flowering. Genetically observed FLM is epistatic to ARR1 and ARR2.

Despite the fact that all tested floral activators and signal integrators did not show any miss-regulation of their expression in tested ARR1 and ARR2 knock outs, CO and SOC1 lacking mutants were crossed with arr2-4 single mutant in order to examine their genetic interaction/hierarchy and see if there is some difference. When tested under inductive LDs co arr2-4 flowered more than two weeks after the wild-type identical to its co single mutant parent. This may suggest that under LDs CO may be epistatic to ARR2 or event that they work independent and because the weak phenotype of arr2-4 under LDs this difference could not be observed. When co arr2-4 double mutant was grown under non-inductive SD conditions it flowered earlier than the wild-type showing the identical phenotype like arr2-4 single parent. This is because co mutants are only delayed in long days, and CO mRNA is more abundant in long than in short days (Blazquez et al. 1997) and CO dependent pathways are not active under non-inductive SD conditions, only under LDs where the CO protein is stabilized by light and this leads to induction of the floral activators FT and SOC1 (Eriksson et al. 2006). The effect observed in the co arr2-4 double mutant is more due to the lack of ARR2 only.

Another tested mutant with SOC1 lacking floral signal integrator, soc1-2 arr2-4 when grown under inductive LD conditions showed the late flowering phenotype which was statistically
not different than soc1-2 single parent suggesting epistasis of SOC1 under ARR2. Nevertheless, soc1 arr2-4 each time showed tendency to flower slight earlier than the soc1-2 single mutant but because of the very weak flowering phenotype of arr2-4 under inductive conditions it could not be statistically proven but still independent working ARR2 and SOC1 with respect to flowering time cannot be excluded. This claim was supported by fact that when the soc1-2 arr2-4 double mutant was grown under non-inductive SD conditions it showed an intermediate flowering phenotype. The double mutant plant flowered approximately one week after arr2-4 single parent and also one week before the soc1-2 single parent. This all suggested that under non-inductive SD conditions, and most probably under inductive LD conditions, the ARR2 and SOC1 are working independent of each other with respect to flowering time which was before proven on expression level as well; ARR2 works here as negative regulator of flowering (alternatively positive regulator of floral suppressors) and SOC1 is known to be a positive regulator of flowering.
5.4 References for Chapter 2


### 6 Appendix

#### 6.1 Supplemental Figures

**Supplemental 1 Supplement to Figure 4.6. In silico predictions of MKK docking motifs on ARR2 based on ELM software**

The figure shows different predicted functional domains of ARR2; four docking motifs for the MKKs are marked (yellow). ELM - (the database of eukaryotic linear motifs, http://elm.eu.org). The docking motif for MKKs is called DOC_MAPK_1.
Supplemental 2 Supplement to Figure 4.8. oNPG data for interaction strength between ARR1 and ARR18 with biotic-stress-related MKKs in yeast.

The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Supplemental 3 Supplement to Figure 4.13B. Results of Fisher’s Least Significant Difference (LSD) test for pathogen assay with *Pseudomonas syringae Pst DC3000*

(A) LSD results of plants treated only with *Pst DC3000*. (B) LSD results of plants treated with PstDC3000 in addition of cytokinin (1 µM t-Zeatin). WT stands for Col-0. Data are presented for days: 0, 1, 2 and 4 post treatment. LSD tests were conducted at the 0.05 α level.
Supplemental 4 Supplement to Figure 4.15. Results of Fisher’s Least Significant Difference (LSD) test for measurements of stomata aperture

Wt stands for Col-0, 1-4 for arr1-4, 2-4 for arr2-4 and 1-4x2-4 for arr1-4 arr2-4. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.

<table>
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<tr>
<td>wt zeatin</td>
<td>A 5.2988914</td>
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<tr>
<td>2-4 zeatin</td>
<td>B 5.1199891</td>
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<tr>
<td>1-4x2-4 zeatin</td>
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<tr>
<td>1-4x2-4DC3000</td>
<td>C 4.7440645</td>
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<tr>
<td>1-4 zeatin</td>
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<tr>
<td>1-4ABA</td>
<td>G 3.1161346</td>
</tr>
</tbody>
</table>

Levels not connected by same letter are significantly different.

Supplemental 5 Supplement to Figure 4.16 Results of Fisher’s Least Significant Difference (LSD) test for pathogen assay with Alternaria brassicicola

(A) LSD results for plants treated with Alternaria only after 7 days (top) and after 10 days (bottom). (B) LSD results for plants treated with Alternaria in the presence and absence of cytokinin (1µM t-Zeatin). WT stands for Col-0. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.
Supplemental 6 Supplement to Figure 4.16B. Plants treated with cytokinin for pathogen assays with *Alternaria brassicicola*

Cytokinin application was monitored by detecting expression of *ARR4*, which is a known cytokinin responsive gene. Plants treated with 1 μM t-Zeatin showed increased expression of *ARR4* indirectly proving that cytokinin treatment was successful.
Supplemental 7 Supplement to Figure 4.17. Plants treated with cytokinin for pathogen assays with *Botrytis cinerea*.

(A) Left: Outgrowth Index data shown as mean and the 95% confidence interval. Right: LSD results for the Outgrowth Index data about the mean at the 0.1 $\alpha$ level. All of the mutants are significantly smaller than the wild-type at the 0.1 alpha level and those with arr2-4 at the 0.05 $\alpha$ level. (B) Left: Lesion Size data shown as mean and the 95% confidence interval. Right: LSD results for the Lesion Size about the mean at the 0.06 $\alpha$ level. All of the mutants are significantly smaller than the wild-type at the 0.06 $\alpha$ level.
Supplemental 8 to table 5.3. Flowering time of transgenic lines driven under tissue specific pSUC2 promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where pSUC2::ARR2 was complemented in arr2-4 mutant background. (B) Experiments where pSUC2::ARR2 was complemented in arr1-4 arr2-4 mutant background. All independent lines show the same tendency. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.

Supplemental 9 to table 5.4. Flowering time of transgenic lines driven under tissue specific FD promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where pFD::ARR2 was complemented in arr2-4 mutant background. (B) Experiments where pFD::ARR2 was complemented in arr1-4 arr2-4 mutant background. All independent lines show the same tendency and stronger complementation than under SUC2 promoter. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.
Supplemental 10 Phenotypic differences of arr1-4 arr2-4 transgenic lines transformed with pABind::ARR2D80E::GFP estradiol inducible vector when treated with estradiol or Mock control

(A) arr1-4 arr2-4 transformed with pABind::ARR2D80E::GFP estradiol inducible vector in T2 generation. Left photograph shows phenotypic differences of transgenic plant not induced (-E) and induced (+E) with β-estradiol. In addition photographs of disproportional silique size, reduced seed number and leaf changes, respectively, of transgenic line treated with β-estradiol are shown. (B) Difference in seed number and types of seeds between non-induced (left) and induced (right) transgenic line. For this experiment at least 10 plants per transgenic line were taken, total 3 independent lines were used, and 5 siliques per plant were used for statistical evaluation.
Supplemental 11 Transcriptional levels of ARR1, ARR2 and FLC in the triple flc-3 arr1-4 arr2-4 mutant knock out.

(A) Expression of ARR1 in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. (B) Expression of ARR2 in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. (C) Expression of FLC in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate with three technical repetitions each and normalized to ELF-1-α. Error bars indicate SD of the mean.
Supplemental 12 Transcriptional levels of *ARR1*, *ARR2* and *FLM* in the *triple flm-3 arr1-4 arr2-4* mutant knock out.

(A) Expression of *ARR1* in Col-0, *flm-3*, *arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. (B) Expression of *ARR2* in Col-0, *flm-3*, *arr2-4*, *arr1-4 arr2-4* and *flm-3 arr1-4 arr2-4* mutants. (C) Expression of *FLM* in Col-0, *flm-3*, *arr1-4 arr2-4*, and *flm-3 arr1-4 arr2-4* mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate was used with three technical repetitions and normalized to ELF-1-α. Error bars indicate SD of the mean.
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8 Acknowledgements

Oh, I thought I will never come to this point of my PhD and therefore I did not even think about what I would write in here...

First person coming on my mind is Klaus (and that not because of the fact that he is one who makes important decisions about this dissertation). When I go 6 years back in the past I remember myself, young and ambitious student of molecular biology just finished 3rd year of his studies, sitting in front of computer (exactly like now) and sending an Email to some Professor from Germany whose group is working on very interesting topic and me willing to spend whole my summer holiday in his lab. The “shock” occurred only one hour after that when Klaus wrote me back (using his direct, kind and polite style) and said that he would be very happy having me in his lab for a practical course. No one was happier than me in that moment! Ever since then Klaus has always been there to give me help, support and his never-ending optimism. Klaus you were/are a real DoktorVATER to me and thank you for that!

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Непроцењиву заслугу у целокупном мом животном развоју има, наравно, и мој комшилук из Церове (комшије Снежа и Горан) и касније Новог Сада (моји Снежа и Иван, Срчче-Прасе, Романа, дивни Мира и Влада). Хвала Вам на искреној и безусловној подршци, ручковима и вечерама, трпљења мојих провокација и разноразних испада, силним покушајима да ме „уразумите” и „доведете у ред” (мада ми је драго да се само делимично у томе успели!).

За крај, велико ХВАЛА моjoj породици - родитељима и брату, посебно моjoj mами, за сав труд и пожртвовање које су уложили да мог брата и мене изведу на „прави” пут.

ХВАЛА Вам Наталиja, Милице, Милане, Дивна, Светиславе где год да стe!

Овај рад посвећујем моjoj баки Нади и моjoj малим venturi-јима: Наталиji, Иви и Сергеjу!

Ваш Марко
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Summary

Two-component system (TCS) and MAPK signalling cascades play essential roles in plant signal transduction. The *Arabidopsis* Response Regulator 2 (ARR2), a member of B-type response regulators in the two-component circuit, serves as a molecular hub integrating several incoming signals. A comparable hub function is also known for the MAPK signalling cascade. Based on preliminary experiments we have started to address the hypothesis that there exists a link between ARR2-dependent TCS and MAPK signalling cascade, i.e., a crosstalk of those two evolutionary divergent signal transduction systems.

Potential protein-protein interactions between ARR2 and MAPK cascade members (MKKKs, MKKs, and MPKs respectively), were tested. The results revealed that ARR2 as well as other tested B-type ARRs, but not the A-type ARRs, strongly interacted with MKKs and some MPKs where none of them interacted with MKKKs. Interestingly, despite a high sequence similarity with ARR2, ARR1 did not interact with all those MAPK members ARR2 is interacting with. ARR2 was found to interact preferentially with MKK4 and MKK5, which play an important role in the biotic stress defence specially including responses to pathogen attacks. Pathogenesis assays applied on *ARR2* and *ARR1* mutants lines, using the necrothrophic, semi biotrophic and biotrophic pathogens differing in evolution and distinct defence strategies in plants, revealed us that ARR2 mutants showed difference only in necrotrophic stress responses but not in biotrophic stresses.

The detailed analysis of *arr1* and *arr2* single loss-of-function mutants as well as an *arr1 arr2* double mutant revealed an early flowering phenotype of these plants especially visible under short day conditions (SDs) with the double mutant having an additive effect compared to the single parents. Despite very similar sequence homology of the *ARR1* and *ARR2* genes, the loss of *ARR2* effects the flowering time was quite stronger than *ARR1* but they still function in the same signalling pathway. From the mechanistic point of view the lack of *ARR1/ARR2* activity causes down-regulation of specific floral repressor genes but not of genes that act as floral activators and this miss-regulation of expression is not due developmental defects but directly linked to floral induction. ARR2 expressed either in the shoot apical meristem (SAM) or in phloem companion cells recues the early flowering phenotype of the *arr2* single and the *arr1 arr2* double mutants. The crossing of *ARR1* and *ARR2* mutants with flowering time-related mutants revealed that *ARR1* and *ARR2* function predominantly independent of *Flowering Locus C* (*FLC*). However, this approach revealed that *Flowering Locus M* (*FLM*) acts epistatic to *ARR1* and *ARR2*.

These findings are revealing and describing special features of the TCS elements and their crosstalks with other signalling pathways and contributing to a better understanding of their physiological role.
Zusammenfassung


Protein-Protein-Interaktionen zwischen ARR2 und Proteinen der MAPK Signalkaskade (MKKKs, MKKs und MPK) wurden untersucht. Wir konnten zeigen, dass ARR2 und auch andere ARRs aus der Gruppe der B-Typ Response Regulatoren, aber nicht der A-Typ ARRs, stark mit MKKs und einigen MPKs interagieren. Dabei gab es keine Interaktion mit den MKKKs. Trotz einer hohen Sequenzänlichkeit zwischen ARR2 und ARR1, interagiert ARR1 nicht mit allen Komponenten der MAPK Signalkaskade mit denen ARR2 wechselwirkt. Wir konnten zeigen, dass ARR2 mit MKK4 und MKK5 interagiert. Beide haben eine wichtige Rolle bei der Stressantwort der Pflanze, vor allem bei der Pathogenabwehr. Versuche an ARR2- und ARR1-Knockoutlinien mit nekrotrophen, hemibiotrophen und biotrophen Pathogenen, die jeweils evolutionär unterschiedliche Abwehrstrategien in Pflanzen hervorrufen, haben gezeigt, dass die ARR2-Mutanten keine Unterschiede bei biotrophen, dafür aber bei nekrotrophen Stressantworten hervorrufen.


Diese Daten beschreiben spezielle Elemente des TCS und deren Interaktion mit anderen Signaltransduktionswegen, welche zu einem besseren Verständnis der physiologischen Rolle führt.
1 General Introduction

1.1 Two-component systems

Two-component transduction systems (TCS or TSCT) pathways represent principal means for coordinating responses to environmental changes in bacteria and also in plants, some fungi, archaea and protozoa, but it is absent in higher eukaryotes and metazoans (Koretke et al. 2000; Hwang et al. 2002; Capra and Laub 2012). In prokaryotes these systems are mostly composed of a receptor sensor histidine kinase (HK or SK) and its cognate response regulator (Koretke et al. 2000; Capra and Laub 2012). Histidine kinases sense a specific signal and generally form functional homodimers allowing kinases to autophosphorylate at an internal histidine (H, His) by the γ-phosphoryl group of ATP (Koretke et al. 2000). The high-energy phosphoryl group is further transferred to an aspartate (D, Asp) residue of the two-component receiver domain in the response regulator protein. Response regulators (RRs) typically contain a two-component receiver domain and an effector domain allowing the protein to modify and regulate cellular behaviour in a TCS-dependent manner (Koretke et al. 2000; Hwang et al. 2002). The name "two-component" actually originates from this fact: in its simplest case only two elements are involved in TCS signal transduction, however there are exceptions which are elaborated on below. HKs share very high homology their ATP-binding domains with Hsp90, type II topoisomerases and MutL (mismatch repair protein) (Dutta et al. 1999; Koretke et al. 2000; Capra and Laub 2012). These proteins are all members of GHKL superfamily and it is suggested that HKs originated from one of these ATPases and had further evolved by series of duplications, lineage-specific expansion and divergence (Capra and Laub 2012). Generally, HKs are bifunctional, i.e. they can also act as phosphatases of their response regulators (Capra and Laub 2012). In order to make dimers, autophosphorylate and then successfully transfer the γ-phosphate group, histidine kinases must contain the dimerization and histidine phosphotransfer domain (DHP) and the catalytic and ATP binding (CA) domain (Koretke et al. 2000). Other domains most frequent in HKs are the sensory binding domains Per Arnt Sim (PAS) and cGMP-specific phosphodiesterases Adenyl cyclases and FhIA (GAF), a conserved "linker" domain Histidine kinase Adenyl cyclases Methyl accepting proteins and Phophatases (HAMP) (Galperin et al. 2001). These periplasmic sensory domains are responsible for direct signal (PAS and GAF) recognition and relaying this information to the DHP and CA domains; consequently these domains more variable than the other ones as reflected by the large diversity of detectable signalling molecules (Moglich et al. 2009; Parkinson 2010). Besides these domains, HKs usually have transmembrane domains and other domains which specialise and define their function. As mentioned, HK autophosphorylation appears to be dependent on forming homodimers (Ashenberg et al. 2011). HAMP domains are likely to be involved in dimerization in a sensory dependent manner (Parkinson 2010). Formation of functional homodimers is common for almost all HKs as there is only a single observation of a physiologically functional heterodimer in cyanobacteria Pseudomonas aeruginosa (Goodman et al. 2009). Some HKs have an additional
receiver domain fused to their C-terminus; these are called hybrid histidine kinases (also abbreviated HKs) and almost 25% of all bacterial HKs belong to this group (Cock and Whitworth 2007). Hybrid HKs might have originated by fusion of upstream encoded HKs and RRs through the mutation of stop codons in operons (Qian et al. 2008). After a signal is perceived and autophosphorylation occurs in hybrid HKs, the high energy phosphate group is delivered from the DHp histidine to the cis internal receiver domain. This phosphate is then shuttled by other elements of the TCS in a typically a four-step relay via hybrid HK to HPt (a free DHp domain protein called a histidine phosphotransfer protein) to a classical RR protein, thus the ~P group moves from His to Asp to His to Asp. This multistep phospho-transfer is commonly referred to as a phosphorelay. In eukaryotes, hybrid HKs are found in the majority of systems (Koretke et al. 2000) although the real reason for such selective pressure is not known. It has been proposed that the spatial arrangement within the hybrid HK enforces the specificity of phosphotransfer avoiding crosstalk with other TCS pathways (Wegener-Feldbrugge and Sogaard-Andersen 2009). In support to this claim, it has been proven that HKs missing their receiver domains can phosphorylate non-cognate RRs even better than their own internal response regulator domain (Biondi et al. 2006; Wegener-Feldbrugge and Sogaard-Andersen 2009). Next crucial element of two-component system signalling is RRs, as up until now, HPt proteins have only been shown to be ~P shuttles and add little specificity to TCSs with the exception of YPD1, which has been shown to stabilize the phosphoryl~RR form of SSK1 (Janiak-Spens et al. 2000) and the Arabidopsis protein AHP6 which cannot receive a TCS phosphate. AHP6 has been shown regulate developmental and hormonal processes, presumably at the level of protein-protein interaction within the TCS network (Mahonen et al. 2006; Moreira et al. 2013). All RRs have an evolutionary well conserved receiver domain which pulls the phosphate group onto its Asp residue thereby causing conformational changes to the protein which activate or even inhibit it initiating an output response (Gao et al. 2007; Capra and Laub 2012). Numerous RRs possess DNA-binding output domains (Galperin 2006) whereby phosphorylation of the conserved Asp promotes also dimerization of receiver domains favouring the DNA binding and direct control of target gene transcription (Capra and Laub 2012). Other domains frequent within RRs are diguanylate cyclases and methyltransferases domains (Koretke et al. 2000). In bacteria, RRs are signal integrators as these organisms usually have, if not an equal number, more HKs than RRs (Koretke et al. 2000; Capra and Laub 2012). Free RRs are therefore the last direct step of TCS signalling pathways and they are the final factor responsible ascribed for causing TCS-dependent physiological changes (Capra and Laub 2012). The diversity of in bacterial RRs is a direct consequence of gene duplications, lateral gene transfer events and point mutations (Rabin and Stewart 1993). Divergent evolution of the RRs has enabled them to specifically recognize promoters and control different genes (Price et al. 2008). Fifty percent of all known RRs form homodimers upon phosphorylation (Gao and Stock 2010) and there are subsets of interfacial residues that enforce homo-dimerisation and prevent hetero-dimerisation which lie within the β4-α4-β5-α5 region of the conserved RR domain (Weigt et al. 2009; Szurmant and Hoch 2010; Capra and Laub 2012).
In both Archea and eukaryotes, TCS independently originated from bacteria by lateral gene transfer (Koretke et al. 2000; Kim and Forst 2001). In plants, TCS plays important roles in developmental processes (Ren et al. 2009; El-Showk et al. 2013) and most likely they obtained their TCS from chloroplasts after integration of chloroplast genes into their nuclear genome (Martin et al. 2002). As mentioned, higher eukaryotes and metazoans do not possess TCS (Hwang et al. 2002; Schaller et al. 2011) and instead, TCS (histidine/aspartal phosphorylation) has been replaced by serine/threonine/tyrosine phosphorylation perhaps due to the intrinsic liability of phosphoryl groups on Asp compared to its stability on serine, threonine or tyrosine (Capra and Laub 2012). Thus the idea is that eukaryotes as “compartmented” organisms need more stable and longer lived outputs for signal conduction from the cell membrane to the nucleus. Supporting this claim would be the direct modulation of activity MAPK kinase signalling pathway in Saccharomyces cerevisiae by the SLN1-YDP1-SSK1 TCS pathway (Posas et al. 1996). On the other hand this is not entirely the case in plants. Here TCS is directly controlling expression of many target genes as in classical TCSs (Hwang et al. 2012; El-Showk et al. 2013). One of the most described examples in plants is cytokinin signalling where the TCS plays a crucial role, and B-Type RRs directly modulate gene activity of A-Type RRs and other genes (Shi and Rashotte 2012; El-Showk et al. 2013). The shuttling of the phosphoryl group from hybrid HKs in the plasma membrane and endoplasmic reticulum by HPts to RRs in the nucleus (Shi and Rashotte 2012) could have been selected for in plants because it is strongly believed that the histidyl~P residue is more stable than an aspartyl~P residue (Koretke et al. 2000; Capra and Laub 2012).
1.2 References for General Introduction


2 Aim of this work

Besides its functions in ethylene signal transduction, cytokinin and H$_2$O$_2$ signalling etc., the two-component-signalling system seems to play very significant roles in crosstalk and fine-tuning between distinct signalling pathways. These additional functions of the TCS might be essential for the plant's fitness within the natural environment and also for their general survival. Based on these observations, this thesis intends to contribute to a better understanding of these special features of TCS and describe them. The specific aims of this thesis are:

- To investigate and determine the functional (physiological) role of ARR2 interactions with biotic stress-related members of MAPK cascade, obtaining new data by expanding the comprehensive ARRs/MAPKs module interaction map and determine of the roles of $ARR1$ and $ARR2$ in response to pathogen attack and different elements that cause biotic stress in plants (see Chapter 1).

- Characterisation of a novel flowering time phenotype in the $ARR1$ and $ARR2$ lacking mutants under non-inductive short day conditions discovered during this dissertation by resolving which floral pathway(s), their mechanism and nature of interaction, these two TCS elements are involved in (see Chapter 2).
3 Materials and Methods

3.1 Chemicals

All chemicals were ordered from Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvents were delivered by Brenntag Chemiepartner GmbH NL (Plochingen) and Merck. BASTA pesticide was ordered from Bayer Crop Science. The ingredients used for culture media were ordered from Invitrogen (Carlsbad, USA), Merck, Sigma and Duchefa (Haarlem, NL). Restriction enzymes, ligase and DNA modification enzymes used for nucleic acids studies were ordered from Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA), Promega (Mannheim) and Amersham Pharmacia Biotech. Oligonucleotides were received from Eurofins MWG Operon (Ebersberg) and antibodies from the companies Sigma-Aldrich (Taufkirchen) and New England Biolabs (Beverly, USA). The synthetically generated fg22, elf18 peptides, PEN and Xac extracts were a kind gift from the Laboratory of Georg Felix.

3.1.1 Media

The different media used are presented in the following table. For media preparation deionized water was used and the media was sterilized by autoclaving for 20 minutes at 121°C. Bacto-agar 15g/L was used for solid media preparation, (BD) or 8g/L Select-Agar for MS plates (Sigma-Aldrich). Where it was needed, filter sterilized antibiotics were added to the sterilized medium at appropriate final concentrations as listed in Table 3.1

<table>
<thead>
<tr>
<th>Medium</th>
<th>Ingredients per 1 liter</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td>10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Kings's B</td>
<td>20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) MgSO₄ and KH₂PO₄</td>
<td>Pseudomonas syringae</td>
</tr>
<tr>
<td>½ MS</td>
<td>2.2 g MS (Duchefa), pH 5.7 (KOH)</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>YPD</td>
<td>20g Bactopeptone (BD #211677), 20g Glucose (monohydrate), 10g Yeast extract (BD #212750), 1000ml ddH₂O</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>CSM</td>
<td>20g Glucose (monohydrate), 6,6g Yeast nitrogen base (BD #291940), 0,64g CSM-(L-W, or L-W-A, Qbiogene #4520-012), 1000ml ddH₂O</td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>Z buffer</td>
<td>10,68g Na₂HPO₄, 5,5g NaH₂PO₄, 0,75g KCl, 246mg MgSO₄, 1000ml ddH₂O, adjust PH to 7,0</td>
<td>Saccharomyces cerevisiae</td>
</tr>
</tbody>
</table>
3.1.2 Antibiotics

Media were supplemented when required with antibiotics at the final concentrations listed in the following table:

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration (μg/μl)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbenicillin</td>
<td>100</td>
<td>Water</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>Water</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>50</td>
<td>Methanol</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100</td>
<td>Water</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>50</td>
<td>Ethanol</td>
</tr>
</tbody>
</table>

3.2 Vectors and Primers

All the vectors used for this thesis are listed directly within each method text section. Primers were synthesized by Eurofins MWG Operon. Primers stocks were kept at a 100 μM concentration diluted in nuclease-free water and stored at -20°C.

Table 3.3 List of primers (primer sequences) used for genotyping and cloning

<table>
<thead>
<tr>
<th>Name of the mutant line</th>
<th>Sequence (5´-&gt; 3´)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CGA CGA GAA GAG CGA CGG ATG</td>
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<tr>
<td>arr2-4</td>
<td>GAACGGGAGGAGCTGAG</td>
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</tr>
<tr>
<td></td>
<td>GACCTGGATATTATCGGTGAGTATCC</td>
<td></td>
</tr>
<tr>
<td>arr1-4</td>
<td>GAAGAACAAACATGGATTTCCCATGTA</td>
<td>Laboratory Harter, ZMBP, MV</td>
</tr>
<tr>
<td></td>
<td>CCGTCAATAACCGAGTTGTTAAGATTG</td>
<td></td>
</tr>
<tr>
<td>tDNA(SALK)</td>
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<td>co-9</td>
<td>CAACCTCTACTCCCCCGTACG</td>
<td>Balasubramanian et al., PloS Gen. 2006</td>
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<td>GATGTCACCTGCACTTGCC</td>
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<td>soc1-2</td>
<td>GGATCATCGTGGAGGGGCAAACCT</td>
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<td>CTGAAAACATCTGACAAAAAGCTG</td>
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<td></td>
<td>TGGGTTCACGTAGTTGGGCCATCG</td>
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</tr>
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<td>flm-3</td>
<td>GATGCGGTTTTTGGTTTATG</td>
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<td>GCCATGAAATGTGCCCTTTATCG</td>
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</table>

Primers used for genotyping

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<tr>
<th>Name of the mutant line</th>
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<td>ARR2215-260</td>
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Table 3.4 List of primers (primer sequences) used for RTq-PCR

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</table>
3.3 Bacterial strains

3.3.1 *Escherichia coli* strain DH5α
The *E. coli* strain DH5α [F- (λφ80 lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ– thi-1 gyrA96 relA1] was used for cloning of the different constructs. The *E. coli* strain DB3.1 [F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS2O(rB-, mB-) ara14 galK2 lacY1 proA2 rpsL2O(Sm r) xyl2 Δleu mtI] was used for the cloning and propagation of Gateway™ vectors carrying the *ccdb* gene.

3.3.2 *Agrobacterium* strain
The *Agrobacterium tumefaciens* strain GV3101 (T-DNA - vir+ rifr) with helper plasmid pMP90 (genr) or pMP90RK (genr, kanr) was used.

3.4 Organisms

3.4.1 Organisms used for pathogen assays
Two biotrophs *Pseudomonas syringae* pv. tomato Pto DC3000 (marked with Rifr resistance) and the fungus *Peronospora parasitica* were used. Two necrothrophic fungi *Alternaria brasisicola* and *Botrytis cinerea* were used.

3.4.2 Plant lines
For all experiments Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used, and all transgenic lines were generated in this ecotype. For some experiments *Nicotiana benthamiana* was also used. The T-DNA insertion mutant lines used in this work include *arr1-4* (SALK 042196), *arr2-4* (SALK 016143), *flm-3* (SALK 141971), *co* (SAIL_24_H04), *soc1-2* (Lee et al. 2000; Yoo et al. 2005) and the deletion mutant *fic-3* (Michaels and Amasino 1999). The double mutant *arr1-4 arr2-4* was crossed and isolated by Dr. Katharina Caesar. Other mutants listed in the work were made by crossing; the lines obtained are: *flm-3 arr2-4*, *co arr2-4*, *soc1-2 arr2-4*, *flm-3 arr1-4 arr2-4*, *fic-3 arr1-4 arr2-4*. All of the crossed lines were genotyped by PCR for known insertion markers and characterised by checking mRNA expression. Single mutants *fic-3*, *flm-3*, *soc1-2* and *co* were kindly donated by Dr. Markus Schmid and Dr. Rebecca Schwab (Max-Planck Institute for Developmental Biology, Tübingen). *ARR2* and *ARR2D80N* loss-of-function genes were (both in pDONR207 Gateway vector; previously cloned in the laboratory of Prof. Dr. Klaus Harter) and pJL-Blue Gateway entry plasmid (kindly donated by Dr. Rebecca Schwab) were recombined into the respective pGREEN-IIS Gateway vectors pFK-101 (BAR_FD_rfA*) and pHW-059 (BAR_SUC2_rfA+) described by (Mathieu et al. 2007). These constructs were transformed into Col-0 wild-type, *arr1-4*, *arr2-4* or *arr1-4 arr2-4* mutants. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000 (§3.5.6.1).
3.5 Cultivation

3.5.1 Growth of *Escherichia coli*

*E. coli* strains were cultivated overnight at 37°C either on LB-plates or in liquid LB medium at 230 rpm. Antibiotics were added into the media according to the resistance cassettes the strains were harboring.

3.5.2 Growth of *Pseudomonas syringae*

*P. syringae Pto* pv. DC3000 strains were grown for 24 to 48 hours at 28°C either on King’s B plates or in liquid King’s B medium at 180 rpm. For the determination of bacterial growth in infection assays the *Pseudomonas* strain was re-isolated from plant material and plated on LB plates containing cycloheximide in addition to rifamycin.

3.5.3 Growth of *Agrobacterium tumefaciens*

*A. tumefaciens* strains were cultivated for 48 hours at 28°C on LB-plates or liquid LB medium at 230 rpm. Additional antibiotics were added into the media according to the plasmid-DNA the strains were carrying.

3.5.4 Growth of *Alternaria brassicicola*

The cultivation of *A. brassicicola* and the preparation of the spores for the infection assays were personally performed in the Laboratory of Dr. Birigit Kemmerling exactly as previously described (Kemmerling et al. 2007).

3.5.5 Growth of *Peronospora parasitica* and *Botrytis cinerea*

Assays with *Peronospora parasitica* and *Botrytis cinerea* were both performed by our collaboration partners. The *Peronospora parasitica* assay was performed by the research group of Professor Dr. Volker Lipka from University of Göttingen and the *Botrytis cinerea* assay was done in the laboratory of Professor Dr. Jean-Pierre Métraux at the University of Fribourg, Switzerland. *Peronospora parasitica* was grown as previously described by (Jacobs et al. 2003) and *Botrytis cinerea* as described by (Ferrari et al. 2003).

3.5.6 Growth of *Arabidopsis thaliana* and *Nicotiana benthamiana*

3.5.6.1 Growth conditions for flowering time analysis

For flowering time analysis, plants were first stratified by being kept at 4°C for 2 to 4 days and then transferred to 22°C; this was defined as day 0. *Arabidopsis thaliana* plants were grown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) and for pathogen experiments soil was mixed with Vermiculite. Plants were grown in growth chambers on soil at 22°C under long days (LD) 16 hours light / 8 hours dark or short days (SD) 8 hours light / 16 hours dark (110 mEm⁻² s⁻¹, 50-60% humidity). Plants used for pathogen experiments were grown only under SDs. Plants complemented under tissue specific promoters were used in T2 generation and therefore they were pre-selected with 1:1000 dilution of BASTA (dilution from the stock, 183 g/L glufosinate; 200 g/L ammonium salt, Bayer CropScience). The soil was directly soaked in the BASTA solution.
3.5.6.2 Growth of Nicotiana benthamiana

*Nicotiana benthamiana* plants were cultivated in a mixture of soil and sand containing 0.1 % (v/v) Confidor by the ZMBP Greenhouse (13 h light, 11 h darkness).

3.6 Standard molecular biology methods

General protocols were used for PCR, side directed mutagenesis, agarose gel electrophoresis, western blot, restriction digestion, ligation, transformation of bacteria and plasmid isolation (Sambrook and Russell 2001). Techniques done differently are explained directly in the text. Restriction enzymes were used according the manufacturer’s protocols (Fermentas and NEB). For the generation of PCR fragments either the *Taq* DNA-Polymerase or the *Pfu* DNA polymerase (cloning purposes; Fermentas) were used. GeneRuler DNA Ladder Mix (Fermentas) was used as size marker for the agarose gel electrophoresis. DNA fragments were extracted out of agarose gels or purified out of PCR reactions by using the Qiagen Gel Extraction Kit and Qiagen PCR Purification Kit (Qiagen).

3.6.1 Yeast-two-hybrid

Yeast two-hybrid experiments were performed using the Matchmaker™System (Clontech). Plasmids were constructed by LR-reaction of corresponding Entry clones and destination vectors pGBK7-DEST or pGADT7-DEST (Horak et al. 2008). Primers for clones produced during this thesis project are listed in the general primer list (§3.2), the rest of the clones were made by Achim Hahn and can be found in the plasmid repositories of Prof. Dr. Klaus Harter. Clones with *ARR18* were kindly provided by Manikandan Veerabagu and clones with *ARR4* are from the lab of Dr. Virtudes Mira-Rodado. Yeast strain PJ69-4A (James et al. 1996) was transformed using lithium acetate/SS-DNA/PEG method (James et al. 1996; Horak et al. 2008). After 3 days of growth on vector selective media (CSM, -L, -W), 6 independent clones were picked, resuspended in ddH₂O and 10μl were dropped on vector-selective media. Subsequently, 10μl of culture were dropped on vector- and interaction-selective media (CSM, -L, -W, -A) and incubated at 28°C. At day 3 the growth of the clones was monitored. In addition, yeasts from selective media were inoculated in selective media (CSM,-L,-W) harvested and analysed by western-blot using α-HA and α-myc antibodies to determine the correct expression of the fusion proteins (Horak et al. 2008).

3.6.2 Transient expression in tobacco leaves

Expression of proteins expressed transiently in tobacco leaves was performed as described previously (Marion et al. 2008). A single colony of *Agrobacterium tumefaciens* strain GV3101 pMP90 transformed with the desired constructs and was inoculated in 5ml of YEB-Medium (0.5% beef extract, 0.5% sucrose, 0.1% yeast extract, 0.05% MgSO₄·7H₂O) containing Rif/Gent/ and vector-specific antibiotic at 28°C overnight. In the morning, 1 ml of the pre-culture was taken and re-inoculated into 5 ml of the same Medium. The same was done for *Agrobacterium* strain carrying the p19 RNAi-suppressor protein from tomato bushy stunt virus (Voinnet et al. 2003). Each culture was collected in a 15 ml Falcon Tube and centrifuged at 4000 rpm for 20 min. Bacteria pellets were then resuspended in AS-Medium (10 mM MgCl₂, 10 mM MES [pH 5.6], 150 μM acetosyringone) to an optical density at 600 nm of
about 0.7-0.8. The resuspended bacteria (two potential interaction partners and p19 strain) were mixed 600 ml each, a 1:1 ratio, in a 2ml Eppendorf tube and incubated for 0.5 to 1 hours at 4°C.

The bacterial solution was injected into the entire leaf area through the abaxial sides using a 1 ml syringe; two leaves per plant were inoculated. After inoculation, the plants were kept in a tray with a hood at 25°C. Two days after the bacterial inoculation, the β-estradiol responsive promoters in the pABind vectors (Zimmermann and Nentwig 1989) and the N-terminal mRFP vector pB7WGR2.0 (Plant Systems Biology, Gent) were induced by application of β-estradiol by brushing a 20 μM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements and localization studies were performed 24 to 48 hours after β-estradiol application.Localization studies were performed with confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). Microscopy was carried out as previously described (Horak et al. 2008). Images were taken with HCX APO LW 20×/0.5 or the HCX PL APO 63×/1.2 W water-immersion objective.

### 3.6.3 Ethylene accumulation measurements

For ethylene measurements, leaf material of 4 week-old plants were cut into 1 mm thick strips and floated overnight in water. Afterward three leaf stripes (20 mg) were transferred in 6 ml glass vials containing 0.5 ml of an aqueous solution of the elicitor to be tested. The tubes were closed with a rubber septa and ethylene accumulating in the free air space was measured by gas chromatography (GC-14A Shimadzu) after 3h incubation at 170 rpm at room temperature. Following PAMPs and their working concentrations were used: PEN from *Penicillium schizogonium* 1 μL/500 μL, *Xanthomonas axonopodis citri* 3 μL/500 μL, flg22 (0,1 nM, 1 nM, 10 nM, 100 nM, 1 μM), elf 18(0,1 nM, 1 nM, 10 nM, 100 nM, 1 μM).

### 3.6.4 ROS (Reactive Oxygen Species) measurements

Leaf discs were excised from 6 week-old *Arabidopsis* plants and incubated in water overnight. The following day, the discs were transferred to a solution of 20 μM luminol L-012 (Wako Pure Chemical Industries) and 10 μg/mL (w/v) horseradish peroxidase in a 96-well-plate and treated with 100 nM flg22, 100 nM elf18, 1 μM chitin octamers or 1 mg/mL chitin (crab). The plates were analysed for a period of at least 30 min using a multiplate reader Centro LB 900 (Berthold Technologies). For each data point at least 9 replicates were measured.

### 3.6.5 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel or JMP (SAS). The data represent the average of replicates with plus or minus standard error of the mean (SE). The significance of the differences was calculated using Student’s t-test, ANOVA or Fisher’s Least Significant Difference (LSD) test; which test was used is explicitly mentioned in the text, figures and tables.

### 3.6.6 ELM software for *in silico* predictions

ELM software (http://elm.eu.org) was used for MKK docking site identification in ARR2.
3.6.7 Cloning and site-directed mutagenesis

All the clones used in our experiments were constructed using Gateway™ technology (Invitrogen™ | Life Technologies). The Entry clones were obtained via BP-reaction in pDONR207. cDNA preparations derived from Arabidopsis leaves were used as template to clone ARR2 (AT4G16110). For the generation of truncated ARR2 versions, ARR2\(^{1-300}\), ARR2\(^{300-664}\), ARR2\(^{1-165}\), ARR2\(^{145-664}\), ARR2\(^{165-664}\) and ARR2\(^{1-145}\), primers were made corresponding to the ARR2 cDNA and the fragments were amplified by PCR using the ARR2 Entry clone (see primer table §3.2). Site-directed mutagenesis of ARR2 was carried out on the ARR2 Entry clones using QuikChange® Site-Directed Mutagenesis Kit (Stratagene) and the D80E, D80N ARR2 mutants were already present at the Laboratory of Klaus Harter at the time when the experiments were performed. The binary constructs for FRET-FLIM measurements (pABind vectors) under the control of the β-estradiol inducible promoter and for stable Arabidopsis transformations under the control of the FD and SUC2 promoters were obtained via LR-reactions. LR-reactions were also done for yeast-two hybrid system destination vectors pGADT7 and pGBKT7.

3.6.8 Quantitative RT-PCR (RT-qPCR)

Total RNA from corresponding material (leaves, shoot apical meristem or whole plant) was isolated using the “Universal RNA Purification Kit” (Roboklon, Germany) including a DNA digestion step to ensure the absence of genomic DNA contamination/presence. The cDNA was synthetized using oligo-dT or random hexamer primers with H-Minus Reverse Transcriptase (Fermentas). qPCR primer were tested for doubling time by diluting 1:2 and ensuring that there was exactly a loss of 1 CT; primer efficiency was thereafter assumed to be 100%. At least two PCR reactions (in triplicate repeats each) using two independent cDNA synthesis were performed for every treatment. The amplification of cDNA was performed with PerfeCta qPCR SuperMix or PerfeCta SYBR Green Super Mix (Quanta Biosciences, distributed by VWR) using, when necessary, the corresponding Probe (Universal ProbeLibrary Single Probes, Roche) accordingly to manufacturer. The PCR reactions were ran in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad). The used primers are listed in the §3.2. Expression levels of each gene were normalized according to the expression of the following housekeeping genes: EF-1-α (AT5G60390). The CFX Manager software (Version 1.1; Bio-Rad) has been used for the quantification of relative expression levels except for §4.2.3.4 for which the CT values were exported and \(\Delta\Delta CT\) was manually processed in Excel to ensure proper normalisation.

3.6.9 ONPG assay

Before the Day One, 3 to 4 transformed yeast colonies were pooled and inoculated and incubated overnight at 28°C while shaking in 0.5ml of CSM-L/W liquid medium at 180 rpm.

Day 1: the tubes were taken from the shaker and 3 ml of YPD liquid medium was added in each one and put back to the shaker for an additional 3 hours. After that, the optical density at \(OD_{600}\) was measured. The tubes were then centrifuged for one minute at 11000 rpm and
the pellet was re-suspended in 1 ml of Z Buffer (the same procedure was repeated three times). The tubes were frozen in liquid nitrogen and stored at -80°C.

**Day 2:** Frozen tubes were thawed in a 37°C water bath and then returned to liquid nitrogen; this cycle freezing/thawing was repeated at least 4 times. Blank control (with all following solutions) was set and OD_{600} was measured. Immediately after adding of 500 µl of Z buffer-ME (38.6mM β-Mercaptoethanol) and 160 µl of Z buffer/ONPG (13.3mM β-Mercaptoethanol) the timer was started and the tubes were incubated at 30°C until yellow colouring appeared. The reaction was stopped by the addition of 400 µl 1M Na_{2}CO_{3}. The tubes were centrifuged afterwards and the supernatant was transferred to another tube. The optical density was measured at OD_{420}. Galactosidase units were calculated as follows:

\[
\text{Galactosidase units} = \frac{1000 \times OD_{420}}{t \times V \times OD_{600}}
\]

with t being elapsed time in min, V is the volume of the yeast culture used for the assay, OD_{420} and OD_{600} the optical density of the yeast cultures used for the assay.

### 3.6.10 MPK assay using anti-phospho antibodies

Anti-phospho antibodies p44/42 (Cell Signaling Technology, NEB #9101) were used as they are known to specifically detect phosphorylated MPK3, MPK4 and MPK6 in *Arabidopsis thaliana*. 5 week-old plants grown under short day conditions were used. Total protein amount was extracted from 50-100 mg of leaf material. For protein extraction 50mM Tris/HCl [pH 7.5], 5mM EDTA pH 8 and 2mM DTT was used. Protease inhibitor cocktail tablets (Roche) were used. Protein concentration was determined with Bradford Reagent (Biorad-System) where 10µl of protein extract was added to 990µl Bradford-Solution (Bradford Reagent diluted 1: 5 in H_{2}O), exposed for 5 min at RT; and then the optical density (OD) was measured at 595 nm. To estimate protein concentration the following formula was used:

\[
\text{Protein concentration} \left[\frac{\text{mg}}{\text{ml}}\right] = \frac{OD_{595}}{(0.0283 \times \text{vol. protein extract used})}
\]

### 3.6.11 Stomata measurement assays

Leaves from 5 week-old *Arabidopsis thaliana* plants were floated for 2 h under continuous illumination (120–150 µE m^{-2} s^{-1}) in MES/KCl buffer (5mM KCl/10mM MES/50µM CaCl_{2}, pH 6.15) as previously described by (Mira-Rodado et al. 2012). Once the stomata were fully open, leaves were treated with either 1µM t-zeatin or 10µM ABA for further 2 h. The leaves were subsequently homogenized individually in a Waring blender for 30 sec and the epidermal fragments collected on a 100 µm nylon mesh (SpectraMesh, BDH-Merck, UK) and placed on a microscope slide and covered with a coverslip. Stomata apertures from epidermal fragments were then measured using a calibrated light microscope attached to an imaging system (Leica QWin software, Leica, UK). Lens 20X-0.75 magnification was used plus. Leaves were collected from plants also treated with *Pseudomonas syringae* DC3000 3 days after infection and directly homogenized without being exposed to additional light or being put in water. To
calculate stomata aperture, relative pixel distance values were measured in ImageJ software. For statistical analyses ANOVA was used.

3.6.12 Day length shifting experiments
This method was used for the synchronized flowering experiments. Plants were first grown at 8h light/16h dark (short days) at 23°C for 30 days and then shifted to 16h light/8h dark conditions (long days), (Maizel and Weigel 2004; Wahl et al. 2013). Apical centres (meristems) were harvested after removal of all visible leaves at day 0 and 5 (counting from the shifting day).

3.6.13 Generation of stable Arabidopsis thaliana transgenic lines
Stable transgenic plants were generated by Agrobacterium tumefaciens-mediated transformation using the floral-dip method (Clough and Bent 1998). For all transformations Agrobacteria GV3101::pMP90 or GV3101::pMP90RK strain were used. The RK strain possesses the RK2 replicase and trf gene required for the replication of RK plasmids. The Agrobacterium RK strain was co-transformed with pSoup vector which provides replication functions in trans for pGREEN-IIS destination vectors into which the different promoters used in this study (FD, SUC2) which had been cloned in front of a modified Gateway recombination cassette. Transgenic plants were selected with BASTA (Bayer) at a dilution of 1:1000.

3.6.14 Tissue fixation, embedding and sectioning of Arabidopsis thaliana apical meristem
3.6.14.1 Embedding
Day 1: Material was directly harvested into fixative FAA (Formaldehyde: Acetic Acid) in a little glass beaker. Fixative: FAA (Formaldehyde: Acetic Acid):

- 50% Ethanol
- 5% Glacial Acetic Acid
- 3.7% Formaldehyde
- 41.3% Water

Vacuum was applied and released (soft up and down with the vacuum) for 1 to 4 hours until the material became translucent and sank to the bottom. The FAA was afterwards changed and left in a cold-room overnight. FAA was replaced with 70% Ethanol; this step was repeated once to be sure all FAA is gone, after that material can be stored in falcon tubes for a long time.

Day 2: These steps were done at 4°C in a cold-room. 70% Ethanol was replaced with 85% Ethanol for 60 min. After one hour previous solution was replaced with 96% ethanol with Eosin and it was left overnight until a light pink colour emerged and tissue could be visualized.

Day 3: These steps were done at room temperature. The previous solution was replaced by 96% ethanol with Eosin for one hour, and then two times replaced by the same solution for one hour each time. After that the tissue was treated with 25% Histoclear with 75% Ethanol for 30 minutes and after each thirty minutes the percentage of Histoclear was increased by
25% where the percentage was decreased at the same amount until the percentage of Histoclear reached 100%. The tissue was further treated with the 100% Histoclear twice for 1 hour each time. After that paraplast chips were added 25% (v/v) of the solution and so left overnight at room temperature.

**Day 4:** The tubes were placed at 42°C until the chips completely melted and one more ¼ volume of paraplast chips was constantly added until they completely melted. The tubes were afterwards moved to 60°C for several hours and parallel in another tube wax was melted overnight at 60°C to prepare for the next day.

**Day 5:** Two wax changes approximately ¼ volume separated by several hours, were performed. The same procedure is for days 6 and 7.

**Day 8:** The tissue was placed in sectioning moulds and stored at 4°C. The tissue was then ready for sectioning.

### 3.6.14.2 Sectioning
The sections made were 8µm thick. Slides were ProbeOne Plus from Fisher Biotechnology; they were pre-cleaned and charged. Moulds were trimmed in order to get as close to the tissue as possible. The ribbons were placed in 40°C water bath to remove compressions, and then they were fished out with a slide. They slides were kept on a heating plate at 42°C overnight. Thereafter, slides can be stored in a box at 4°C for a long time. Sections were scanned for clear visibility of two big air vacuoles; always the same plane was used for comparative measurements and statistics. Images were captured using a Leica DM-IRB microscope with a 20x lens.

### 3.6.15 Pathogen Assays with *Pseudomonas syringae* DC3000
Bacteria were inoculated from an LB plate into 50 ml LB medium with the corresponding antibiotics (rifampicin) and incubated over night at 28°C and shaken at 180 rpm in a shaker. The cells were harvested in the next morning by centrifugation in 50-ml falcon tubes at 3500 rpm at 4°C for 10 min. The pellet was resuspended in 10 mM MgCl₂ and the OD₆₀₀ was set to OD₆₀₀=0.2 which refers to approximate concentration of 10⁸ cfu/ml. The cells were afterwards diluted up to density 1x10⁴ cfu/ml and used likewise for the assays. Bacteria were inoculated on two leaves per 5-week-old plant by infiltration with a needless syringe in the middle of a leaf apoplast half (two leaves per plant) ensuring that an area was covered that was always bigger than the cork borer used for extracting leaf discs. The infected leaves were marked for easy identification.

The leaves were harvested at the time points 0 h and 1, 2 and 4 days past infection. For each time point, five plants and two leaves from each plant per line were used. One disc was made from each leaf with the cork borer. The leaf discs were surface was sterilised by washing them in 70% (v/v) ethanol first for about 1 minute and then 1 minute in water. Afterwards the leaf discs were placed into Eppendorf tubes containing 100μl 10mM MgCl₂. Immediately after placing the leaf in the Eppendorf tube, it was homogenized with the sterile pistil and
additional 100μl 10mM MgCl2 was added on the pistil in order to wash out the remains of the leaf discs. The homogenised material was plated on LB-plates with rifampicin and 50μg/ml cycloheximide (it prevents growth of eukaryotes like fungi). Extracts were serially diluted 5 times at 10-fold per dilution and dilutions anticipated to yield evaluable data were scored by plating out 10 µl (see Table 3.3). At least two technical replicates per sample were evaluated.

For those experiments involving a cytokinin treatment, trans-zeatin in a concentration of 1µM was used. Cytokinin was given directly to the 1x10⁴ cfu/ml bacterial dilution before infiltration. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

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</table>

3.6.16 Infection with *Alternaria brassicicola*

*Alternaria brassicicola* spores used for infection assays were obtained as previously published (Thomma et al. 1999). Leaves of 5 week-old *Arabidopsis* plants were drop-inoculated with two 5 µl droplets of spore solution (5*10⁵ Spores/ml). Two leaves per plant and a minimum of 20 plants per line were infected. Plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal infection was scored using the following table (Table 3.4). The Disease Index was defined as previously published (see below and (Kemmerling et al. 2007)). For experiments with cytokinin, trans-zeatin was used at concentration of 1µM. Shortly before drop-inoculation, cytokinin was given to the 5*10⁵ Spores/ml bacterial dilution. Three days before the experiment was performed plants were also treated with cytokinin by mixing it with water to the working concentration and applied by watering.

3.6.16.1 Disease indexes assigned with their description

Scoring system was adopted from the laboratory of Dr. Birgit Kemmerling at the department of Biochemistry at ZMBP.

![Scoring scale for determination of disease index for plants treated with *Alternaria brassicicola* (A) Description of different disease indexes (B) Photograph of treated *Arabidopsis* leaves with spores where is the phenotype related with appropriate disease indexes (image provided by courtesy of Thierry Halter, Dr. Birgit Kemmerling’s group)
3.6.17 Infection with *Peronospora parasitica*

Treatment with the virulent *Peronospora parasitica* isolate was done on 4 to 6 week-old soil-grown plants. Spore suspensions of *Peronospora parasitica* were prepared as described (Shah et al. 2001). Plants were sprayed with a suspension of conidiospores diluted in water (1x10⁶ spores ml⁻¹). Inoculated plants were kept under humid conditions at 18°C for 1 week and then scored for fungal sporulation as previously published (Delaney et al. 1995).

3.6.18 Infection with *Botrytis cinerea*

*Botrytis cinerea* strain BMM was used (Zimmerli et al. 2000). The strain was grown on Difco potato dextrose agar (Becton Dickinson). Spores were harvested in water and then filtered and diluted in quarter-strength Difco potato dextrose broth (PDB) for inoculation as previously described (La Camera et al. 2011). For disease assays, 6 µL of spore solution (5 x 10⁴ spores mL⁻¹) were deposited on three leaves (detached leaf assay) of 5 weeks old plants. Lesion diameters were measured after 3 days. Fungal growth was measured as previously described (Gachon and Saindrenan 2004). Plants were drop-inoculated and leaf discs were harvested at the indicated time points (0h, 12h, 24h, 36h, 48h post infection). The inoculated plants were kept under high humidity in a tray closed with a water-sprayed transparent lid.

3.6.19 FRET-FLIM and microscopy

Two days after the infection of Nb abaxial leaf surface with Agrobacteria carrying the desired binary plamids pABind vectors (Zimmermann and Nentwig 1989) were induced by application of β-estradiol by brushing a 20 µM β-estradiol (in 0.1% Tween-20) solution onto the abaxial leaf surface. FRET measurements were performed 24 to 48 hours after β-estradiol application as described in (Berendzen et al. 2012). The FRET-FLIM measurements were performed with a custom-built CSSM (confocal stage scanning microscope), based on a Zeiss Axiovert 135 TV, and equipped with a pulsed supercontinuum laser–source (*SuperK™*, NKT Photonics) as excitation light source operating at 471 nm and a repetition rate of 40 MHz. A microscope objective with high numerical aperture (Plan-Neofluar, 100×/1.30 oil, Zeiss) was used to focus the excitation light as well as to collect the fluorescence emission. The setup was equipped with a 500 nm dichroic mirror (FF500-Di01-25×36, Semrock) to block back-scattered excitation light and with a 527 nm bandpass filter (Semrock BrightLine BL527/20) to detect GFP-fluorescence. An avalanche photo diode (PDM series, MicroPhoton Devices (MPD), Italy) served as a spectrally integrating detector to record fluorescence intensity. Lifetime decays were recorded using a time-correlated single photon counting board for data acquisition (PicoHarp 300, Picoquant, Software: SymPhoTime, Picoquant) and the MPD as a detector (Wanke et al. 2011).
3.7 References for Materials and Methods


4 Chapter 1
Interaction of Type-B TCS components with MAPK kinase cascade members and a putative crosstalk function in pathogen response

4.1 Introduction

4.1.1 Two-component system (TCS) in Arabidopsis thaliana
Like in all plants, the TCS in Arabidopsis thaliana is also a multi-step phosphorelay system (Hwang et al. 2002; Heyl and Schmulling 2003; El-Showk et al. 2013). TCS mediates physiological response to hormones (cytokinin and ethylene, cross-talk with auxin), nutrients, light and osmotic stress. Its components have important roles in the regulation of several developmental processes such as control of stem cell number in SAM, female gametophyte development, differentiation of root vascular tissue, just to name a few (Hwang et al. 2012; El-Showk et al. 2013). This involves members of three protein families: Arabidopsis histidine kinases (AHKs), Arabidopsis histidine phosphotransferase proteins (AHPs) and Arabidopsis response regulators (ARRs). Arabidopsis possess 11 AHKs and they are hybrid kinases, with an exception of ETHYLENE RESPONSE SENSOR 1 (ERS1) kinase (Guo and Ecker 2004). Based on their functional and structural characteristics the AHKs can be grouped into three subfamilies (Grefen and Harter 2004). Five AHKs have roles as ethylene receptors (Grefen and Harter 2004): ERS1, ERS2, ETHYLENE RESISTANT 1 (ETR1), ETR2 and ETHYLENE INSENSITIVE 4 (EIN4); ERS1 and ETR1 belong to the class I subfamily of AHKs (Grefen and Harter 2004) and are localised to the endoplasmic reticulum (Guo and Ecker 2004). ETR2, ERS2 and EIN4 belong to the class II of ethylene receptors (Grefen and Harter 2004; Guo and Ecker 2004) and have degenerated transmitter domains, i.e. they do not have histidine kinase activity as they lack at least one crucial amino acid to be functional (Guo and Ecker 2004). Kinase AHK1 is playing role in stress signalling as membrane-bound osmosensor (Urao et al. 1999) and CYTKININ INSENSITIVE I (CKI1) is enrolled in female gametophyte development (Pischke et al. 2002; Hejatko et al. 2003). AHK5, or also known as CKI2, is missing a transmembrane domain (Urao et al. 1999; Pischke et al. 2002) and therefore some authors classify it as a separate group. The AHK5/CKI2 is playing a role in stomata closure signalling (Desikan et al. 2008) and it is predicted to be a cytoplasmic hybrid histidine kinase (Grefen and Harter 2004; Hass et al. 2004).

Members of the final subfamily respond to cytokinin: AHK2, AHK3 and AHK4/WOODEN LEG (WOL)/ CYTKININ RESPONSE 1 (CRE1) (Hwang et al. 2002; Grefen and Harter 2004; El-
Showk et al. 2013). Briefly, these AHKs contain a ligand-binding CYCLASE HISTIDINE KINASE ASSOCIATED SENSORY EXTRACELLULAR (CHASE) domain at their N-terminus which is flanked by two transmembrane domains and followed on the cytoplasmic side by the C-terminus with a histidine kinase domain and output domain (Suzuki et al. 2001; Ueguchi et al. 2001; Hwang et al. 2002). This sensor domain is like other bacterial histidine kinases PAS domains and their mechanism of function (Hothorn et al. 2011). The cytokinin receptors are localised not only on plasma membrane but also at the endoplasmic reticulum membrane highlighting that compartmentalization is important for cytokinin signal transduction (Caesar et al. 2011). These receptors show different sensitivity to different types of cytokinin hormones and also are expressed and functional in different tissues (Stolz et al. 2011; Lomin et al. 2012). AHK2 and AHK4/CRE1 have roughly the same high affinity to isopentenyladenine (iP) and t-zeatin but a lower affinity to dihydrozeatin whereas AHK3 has high affinity to dihydrozeatin compared to isopentenyladenine (Lomin et al. 2012). PARR5::GUS experiments in ahk2, ahk3 and cre1 mutant backgrounds demonstrated that loss of cre1 affected promoter responses in the root while loss of ahk2, ahk3 crippled shoot responses (Stolz et al. 2011). Interestingly, AHK4 is known to be prevalent in the roots and AHK3 in the shoots (Lomin et al. 2012). If correctly interpreted, the receptors are expressed and receptive to the corresponding cytokinins that are transported from the opposing tissue, i.e. t-zeatin from root to shoot and iP from shoot to root (Lomin et al. 2012).

There are 6 AHPs in Arabidopsis that mediate phosphorelay and shuttle through the nucleus between AHKs and ARRs. AHP1 to AHP5 are positive regulators in cytokinin signalling (Hutchison et al. 2006) and are also known as “canonical” AHPs because they contain the conserved histidine HPt residue thus they can carry the TCS-phosphate group on further to ARRs (Suzuki et al. 1998). Despite their partial redundancy, different subsets of AHPs have been shown to trigger cytokinin responses in specific tissues and organs. For example the ahp2/ahp3/ahp5 triple loss-of-function mutant showed increased seed size similar to that observed in the ahk2/ahk3/ahk4 triple mutant (Hutchison et al. 2006; Riefler et al. 2006). Different combinations of ahp1-5 mutants do not show the same responses to exogenous cytokinin and its effects on chlorophyll content, lateral root formation or hypocotyl elongation (Hutchison et al. 2006). Generally speaking however, the loss of AHPs seems to be additive, as expected due to redundancy. AHP6 is called a “pseudo AHP” because it lacks the conserved histidine residue required for TCS-dependent phosphorylation: nevertheless, it is an active cytokinin signalling inhibitor (Suzuki et al. 1998; Grefen and Harter 2004; Mahonen et al. 2006) (Moreira et al. 2013). Recently it has been reported that AHP2, AHP3, and AHP5, also work redundantly to control responses to drought stress in a negative and redundant manner as loss of these three AHPs resulted in a strong drought-tolerant phenotype that was associated with the stimulation of protective mechanisms (Nishiyama et al. 2013).

There are 23 functional ARRs in Arabidopsis and based on their protein properties and phylogeny they are divided into 3 subgroups: the type-A, type-B and pseudo-response regulators. The Type-A subfamily of ARRs are single-domain response regulators that
contains the members: ARR3, ARR4, ARR5, ARR6, ARR7, ARR8, ARR9, ARR15, ARR16 and ARR17. Members of this subfamily only have a functional receiver domain (with functional Asp residue) and a short C-terminal tail (Grefen and Harter 2004). Members of the type-A subfamily generally act as negative regulators of cytokinin signalling based on genetic and transgenic studies (Lee et al. 2008; Hwang et al. 2012; El-Showk et al. 2013). So far only ARR4 has been described with a function outside of the TCS. ARR4 positively interacts with phytochrome B and stabilizes the active PhyB-Pfr form under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007).

The type-B response regulators have additional effector domains on their extended C-termini and are therefore able to activate the transcription in a TCS-dependent manner (Sakai et al. 2001). Their expression is not regulated by any stimulus tested so far (Grefen and Harter 2004). This subfamily consists of 11 members: ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14, ARR18, ARR19, ARR20 and ARR21. The output domain of the B-Type members usually is composed of the GARP (named after Golden2 found in maize) DNA-binding domain, at least one NLS and C-terminal transactivation domain (Lohrmann and Harter 2002). B-Type response regulators work as transcription factors binding to target promoters that contain 5´-W/GAT/W-3´ motif (W represents either A or T) (Lohrmann and Harter 2002). Last group of response regulators are C-Type ARRs (Horak et al. 2008). Both type-A and type-B ARRs are now know to be regulated by the 26S proteasome (Ren et al. 2009; Kim et al. 2013; Kurepa et al. 2013).

Two genes (ARR22 and ARR24) encode single-response domain response regulators and are structurally very similar to type-A response regulators but their expression is not regulated by cytokinin. It has been recently suggested that they function as phosphatases for phosphates on histidine moiety of AHPs, but their expression domain is restricted to reproductive organs (Kiba et al. 2003; Gattolin et al. 2006; Horak et al. 2008).

Beside these three groups of the ARRs there is one more group, composed out of 9 members, called pseudo-response regulators (pseudo-RRs). These are response regulator proteins that have a mutation in the conserved response regulator aspartate (Asp) residue (Hwang et al. 2002). It has already been proven that pseudo-RRs regulate flowering time in Arabidopsis thaliana and that TOC1/APRR1, an Arabidopsis pseudo response regulator, is part of the plant circadian clock (Farre and Liu 2013). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate, they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Grefen and Harter 2004). The pseudo-response regulators can be split into two subfamilies (Matsushika et al. 2000): The APRR2 family which more similar to a classical response regulator structure with an N-terminal receiver domain followed by a DNA binding domain (also Myb-like GARP) and C-terminal effector extension; the spacing between the RR domain and the GARP domain is however larger than that of the type-B ARRs (Makino et al. 2000; Matsushika et al. 2000). The APRR1 family has an “inverted” response regulator structure:
there is an N-terminal RR domain, a DNA-binding CCT domain at the C-terminal end, and, presumptuously, the effector domain in-between these two domains (Makino et al. 2000). PRR9, PRR7, and PRR5 function as transcriptional repressors of CCA1 and LHY (Nakamichi et al. 2010) recently shown to bind to DNA via their C-terminal CCT domains (Gendron et al. 2012). The pseudo-response regulators are also targeted by the 26S proteasome (Baudry et al. 2010).

4.1.2 Crosstalk of TCS-related pathways in plants

Although most type-B ARRs have been described to only be involved in cytokinin signalling, an exception is ARR2. Recently it was discovered that cytokinin-activated ARR2 promotes plant immunity in *Arabidopsis* via salicylic acid signalling whereby ARR2 directly interacts with salicylic acid response factor TGA3 and increases pathogen resistance (Choi et al. 2010). *ARR2* is also known to be involved in ethylene signalling by working downstream of *ETR1* (Hass et al. 2004). Furthermore, *ARR2* is involved in ethylene and H$_2$O$_2$-mediated stomatal closure (Desikan et al. 2006). The crosstalk of TCSs with other plants signalling pathways became more evident after a comparative microarray analysis was done on 30-day-old *arr2* mutant and control Landsberg erecta (Ler) plants where the results showed altered expression of about 600 genes mostly related to biotic and abiotic stresses, ethylene and auxin signalling (Hass et al. 2004). The expression of dominant-active, non-phosphorylatable, *ARR2*$_{D80E}$ (aspartate-to-glutamate mutation) caused even more dramatic changes in more than 16000 genes, and most of the disturbed genes are involved in hormone homoeostasis and its signal transduction, biotic and abiotic stress, photomorphogenesis, and others (Hass et al. 2004).

4.1.3 Mitogen-activated protein kinase cascade

Eukaryotic mitogen-activated protein kinase (MAPK) cascades transduce environmental and developmental signals into adaptive and programmed responses. In plants, MAPK cascades have evolved to regulate innate immunity, hormonal response, stress and developmental processes (Colcombet and Hirt 2008; Rodriguez et al. 2010). MAPK signalling cascade(s) are evolutionary conserved within eukaryotes (Schwartz and Madhani 2004) and have roles in abiotic stress, response to pathogens and pathogen-derived elicitors, plant hormones (ethylene, auxin), cell cycle and developmental processes (Tena et al. 2011; Rasmussen et al. 2012; Pathak et al. 2013; Smekalova et al. 2013). MAPK cascades transduce and amplify their signal by three different types of kinase members: *MAP kinase kinase kinase* (MKKK), *MAP kinase kinase* (MKK) and *MAP kinase* (MPK) (Rodriguez et al. 2010). In general, they function as a three tiered kinase cascade with each upstream member required to active the lower level eventually influencing genes expression (Rodriguez et al. 2010). Initially, a ligand activates plasma membrane receptors and these stimulated receptors activate MKKKs that are serine or threonine kinases that in turn phosphorylate MKks at a conserved S/T-$X_3$-S/T motif (X can be any amino acid) (Chang and Karin 2001; Rodriguez et al. 2010). The MKKs afterwards phosphorylate MPks on threonine and tyrosine residues at a conserved T-X-Y repeat (Chang and Karin 2001; Rodriguez et al. 2010). Activity of MAPks can be regulated/deactivated by serine/threonine or tyrosine phosphatases (Luan 2003). Different cascades can share kinase components but still maintain their signalling specificity by various
mechanisms like protein-protein interactions, scaffolding, cross-inhibition and feedback control (Whitmarsh and Davis 1998; Bardwell et al. 2001; Takekawa et al. 2005). MAPK cascades are present in all eukaryotes and is evolutionarily highly conserved which confirms its essential function in these organisms (Kim et al. 2003).

4.1.4 Mitogen-activated protein kinase cascade and its signalling in Arabidopsis thaliana

Around 60 MKKKs, 10 MKKs and 20 MPKs are found in the genome of Arabidopsis thaliana (Group 2002). Besides having the largest number of members, MKKKs also have the greatest variety in domain composition and primary structures among all the MAPK cascade members (Group 2002). Their nomenclature is still not totally defined as some authors have different opinions about this topic. Based on amino acid sequence of the kinase catalytic domains the Arabidopsis MKKKs are grouped into 2 main classes which are further divided into different subclasses (Group 2002; Rodriguez et al. 2010): MEKKs and the Raf-like kinases. The MEKK MKKKs family comprises members who kinase domains share significant similarity to previously described MKKKs, for example MEKK1 in mammals or to yeast STE1 (Group 2002). Members of this family are generally active in response to abiotic stress, especially drought, touch and high salinity (Mizoguchi et al. 1996). The Raf-like kinase class is named after RAF1 mammalian kinase. Interestingly, most members of this class have a PAS domain which is the most frequent sensor domain present among TCS histidine kinases and other pathways (Zhulin et al. 1997; Zwerger and Hirt 2001). The most studied members of this MKKK class are CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) and ENHANCED DISEASE RESISTANCE 1 (EDR1) which are both involved in ethylene-mediated signalling and defence responses (Kieber et al. 1993; Huang et al. 2003). Among the Raf-like class there is also the ZIK sister clade for which no phosphorylation activity has been shown on MKKs (Figure 4.1) (Rodriguez et al. 2010).

The small number of putative MKKs in Arabidopsis suggests that crosstalk between various signal-transduction pathways might be concentrated at this level of the MAPK signalling cascade. It is known that one MKK can activate various different MPKs (Popescu et al. 2009). Considering their small number, the MKKs are very well described in Arabidopsis and rice and both are classified into 4 groups (A-D) (Group 2002; Hamel et al. 2006; Rodriguez et al. 2010). Members of the group A are MKK1, MKK2 and MKK6. Kinases MKK1 and MKK2 are involved in abiotic (cold, salinity) and biotic (especially innate immune response) stresses (Teige et al. 2004; Meszaros et al. 2006; Rodriguez et al. 2010), both acting upstream of MPK4 (Ichimura et al. 1998). Group B includes only one member: MKK3 which is distinguishing from the other kinases by the presence of a nuclear transport factor 2 (NTF2) domain (Kiegerl et al. 2000; Group 2002). Group C includes MKK4 and MKK5 (Group 2002) and they play very important roles in biotic stresses by providing resistance to bacterial and fungal pathogens controlling converging signals initiated by diverse pathogens (Asai et al. 2002; Group 2002; Rodriguez et al. 2010). In addition, both MKK4 and MKK5 are in the pathway downstream of YODA (MAPK kinase kinase) regulating cell fate specification in stomata development (Wang et al. 2007) and the ethylene synthesis pathway (Babula et al. 2006). Members of group D are MKK7, MKK8, MKK9 and MPK10 whose physiological roles are not yet precisely described. It is known that the MKK9–MPK3/MPK6 cascade promotes ETHYLENE-INSENSITIVE 3 (EIN3)-
mediated transcription in response to ethylene signalling (Yoo et al. 2008; Hahn and Harter 2009) and that M KK7 repressed expression causes deficiency or enhancement in auxin transport (Dai et al. 2006).

Figure 4.1 Relatedness of Arabidopsis MKKks based on their protein sequence
The unrooted tree was made by aligning complete protein sequences with Clustal W (default settings) available on TAIR (http://www.arabidopsis.org/) of the (A) AtMKKKs, (B) AtMKKs and (C) AtMPKs. Subfamilies are marked according to the classifications proposed in (Group 2002). The trees were draw with TreeView X.

The last members of the MAPK signalling cascade are MPKs. They are also grouped into 4 groups (A-D) based on their sequence homology (Group 2002; Rodriguez et al. 2010). In plants, MPKs contain either the TDY (present also in all mammals) or TEY phosphorylation motif which is phosphorylated by MKKs and causing MPK activation (Group 2002; Rodriguez et al. 2010). Members of groups A, B and C families contain TEY whereas only group D has the TDY phosphorylation motif. The most studied group of MPKs is Group A. Generally, members of this group are involved in environmental and hormonal responses (Group 2002). MPK3 and MPK6 are members of this group and they have been the most studied group
among all MAP kinases. MPK3/MPK6 play roles in ethylene signalling downstream of MKK9 (Hahn and Harter 2009) whereas MPK6 is additionally required for interaction with ETHYLENE RESPONSE FACTOR 104 (ERF104) in ethylene responses regulated by flg22 (Bethke et al. 2009). Findings for the group B are mostly based on physiological studies of Arabidopsis MPK4 involved in pathogen defence and abiotic stresses (Widmann et al. 1999; Yuasa et al. 2001; Qiu et al. 2008). MPK4 is known to play important role in plant immunity, it functions as a negative regulator of pathogen defense and also interferes with stress signalling pathways at several distinct steps in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) as well as in effector-triggered immunity (ETI) (Berriri et al. 2012; Colcombet et al. 2012). Furthermore, it is also known that the Pseudomonas syringae HopAI1 effector irreversibly inactivates MPK4 to prevent immune responses (Rasmussen et al. 2012). Data on C group are very limited and based on MPK7 whose expression is regulated by the circadian rhythm (Group 2002). Group D also has a C-terminal MKKs docking domain (Rodriguez et al. 2010). It was found that some members of this group are induced by fungi and wounding in rice and alfalfa (He et al. 1999; Schoenbeck et al. 1999). One described member is MPK17. Generally not much is known about MPK17 but it is thought to be important, like whole group D, in plant sugar signal transduction (Sheen et al. 1999).

4.1.5 Plant pathogens and mechanisms of plant defence

4.1.5.1 Immunity

Plants are often attacked by a huge variety of microbial pathogens and herbivore insects. In order to response to these threats they have developed numerous defence mechanisms and many of them are directly induced by pathogen attack (Glazebrook 2005). Generally, defence often begins with gene-for-gene pathogen recognition where the production of pathogen virulence effectors leads to their recognition by plants that carry correspondent resistance (R) genes (Glazebrook 2005). This kind of resistance is accompanied by fast reactive oxygen species (ROS) production also known as oxidative burst which is needed for a further process known as hypersensitive cell death (HR). Reactive oxygen species (ROS) were initially recognized as toxic by-products of aerobic metabolism, removed by means of antioxidants and antioxidative enzymes (Kawano 2003) and was not suspected to provide meaningful information for the plant.

On one side, R gene-driven resistance activates salicylic acid (SA)-dependent signalling pathways which in turn lead to the activation of pathogenesis-related proteins (PR) that also contribute to resistance. This rapid activation of defence results in the limitation of pathogen growth. Innate immunity is generally initiated with recognition of conserved pathogen/microbial-associated molecular patterns (PAMPs/MAMPs) (Nishimura and Dangl 2010). Pathogen-associated molecular patterns, or PAMPs, are molecules associated with groups of pathogens that are recognized by cells of the innate immune system. These molecules can be referred to as small molecular motifs conserved within a class of microbes (Boller and He 2009) and they are typically essential components of whole classes of pathogens, such as bacterial flagellin or fungal chitin (Dodds and Rathjen 2010). PAMPs are
perceived by pattern recognition receptors (PRRs), leading to activation of a series of immune responses, including the expression of defence genes, Reactive Oxygen Species (ROS) production, nitric oxide, ethylene, jasmonic acid (JA), and salicylic acid (SA) and activation of MAP kinase cascades (Jones and Dangl 2006; Tena et al. 2011; Yamaguchi et al. 2013). Activation of PRRs leads to PAMP-triggered immunity (PTI); PTI is often the first inducible response of a plant to PAMPs (Jones and Dangl 2006; Nishimura and Dangl 2010). The largest group within the R genes is binding site-leucine rich repeats (NBS-LRR) (Belkhadir et al. 2004), now mostly called NLR-dependent effector-triggered immunity (ETI) (Dangl et al. 2013). ETI is a second class of perception called effector-triggered immunity. This way of perception involves recognition by molecules called effectors which present intracellular receptors of pathogen virulence presenting, in contrast to PTI, co-evolutionary dynamics between the plant and pathogen. Generally, PTI and ETI give rise to similar responses. PTI is generally effective against non-adapted pathogens in a phenomenon called non-host resistance, whereas ETI, which is stronger and faster than PTI, is mostly active against adapted pathogens (Dodds and Rathjen 2010). Plants also respond to endogenous molecules which are released by pathogen invasion, cell wall or cuticular fragments, called danger-associated molecular patterns (DAMPs) (Dodds and Rathjen 2010).

Beside defence response controlled by SA pathways, defence responses can also be controlled by ethylene and/or jasmonic acid (JA) and this responses are mostly overlapping with response to wounding (Glazebrook 2005). Nevertheless, SA, JA and ethylene interacting between each other, SA and JA are reported to mutually inhibit the expression of many target genes whereas JA and ethylene are sometimes both acquired for expression of some genes but there are also some cases of negative interaction between their signalling.

4.1.5.2 Biotrophic and necrotrophic pathogens
According to their lifestyle pathogens are divided into biotrophs and necrotrophs. Biotrophs are pathogens that live and feed on living tissue; necrotrophs are defined as organisms that live and feed on dead tissue (Mcdowell and Dangl 2000; Thaler et al. 2004). Besides their different life style more important is the fact that Necrotrophic and biotrophic pathogens have evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004). SA is linked to resistance to biotrophic pathogens and is important to trigger the HR, a programmed cell death (PCD) to locally counteract pathogen attack and progression. ET and JA play a role in the control of PCD spreading (Glazebrook 2005; Colcombet and Hirt 2008) and regulate resistance against necrotrophic pathogens (Colcombet and Hirt 2008). However, some pathogens cannot be assigned as biotrophs or necrotrophs. There are also hemibiotrophic pathogens: hemibiotrophic pathogens incorporate aspects of both biotrophic and necrotrophic infection strategies. Often this involves an initial biotrophic infection phase during which the pathogen spreads in host tissue, followed by a necrotrophic phase during which host cell death is induced (Dodds and Rathjen 2010). However, the interactions between SA-, JA-, and ethylene-dependent pathways do not appear to be simple.
Complications could arise from different roles of these pathways in different stages of plant-pathogen interactions (Katagiri et al. 2002).

In order SA to accumulate two genes encoding enzymes similar to triacyl-glycerol lipases are required PHYTOALEXIN DEFICIENT 4 PAD4 and ENHANCED DISEASE SUSCEPTIBILITY 1 EDS1 (Zhou et al. 1998; Falk et al. 1999), raised SA levels in turn are activating various defense effector genes including PR-1 (Glazebrook 2005). The PR-1 effector gene for plant defense is activated by NPR1 and TGA-type transcription factors. NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) has function in regulation of systemic acquired resistance (SAR) in Arabidopsis (Rochon et al. 2006) and NPR1, as recently reported, plays the role of an SA receptor in vivo, in the signalling cascade leading to PR-1 activation (Wu et al. 2012). Systemic acquired resistance (SAR) means a mechanism of induced defence and implies long lasting protection against a broad spectrum of microorganisms. Salicylic acid is absolutely required by SAR as signalling molecule (Durrant and Dong 2004). Based on their physical interaction TGA transcription factors are considered as regulators of pathogenesis-related (PR) genes because of their physical interaction with the known positive regulator NPR1 (Kesarwani et al. 2007). Using reverse genetics approach it has been characterized 10 TGA factors in Arabidopsis so far and they all belong to the bZIP transcription factor family (Jakoby et al. 2002). When the level of SA are low, NPR1 exists as oligomeric form in cytoplasm, the monomers are held together by disulfide linkages which are getting reduced after the level of NPR1 increases and NPR1 is now present in monomeric form which as such can enter the nucleus and interact with the nuclear TGAs and induce expression of PR-1 (Zhang et al. 2003).

As response to pathogen attack the levels of JA can also increase and as a consequence to that defence effector genes are increasing as well, especially VSP1 (Otani et al. 1998) and PDF1.2. The VEGETATIVE STORAGE PROTEIN 1 (VSP1) gene of Arabidopsis thaliana encodes a storage protein that accumulates in vegetative organs and it is jasmonate-responsive element (Guerineau et al. 2003). The PLANT DEFENSIN PDF1.2 gene in Arabidopsis encodes a plant defensin and it is commonly used as a marker for characterization of the jasmonate-dependent defence responses (Brown et al. 2003). Expression of the PDF1.2 requires both JA and ethylene (Glazebrook 2005). For known signalling activities of JA in Arabidopsis CORONATINE INSENSITIVE 1 (COI1) is required. COI1 gene encodes protein from F-box group and it is believed to act in proteolysis (Xie et al. 1998) but it can also bind histone deacetylases (Devoto et al. 2002). Beside the essential COI1, JA also requires members of MAPK cascade for its activity, the MPK4. MPK4 knock-out mutant failed to express the PD1.2 gene (Petersen et al. 2000). For ethylene connection to pathogen defence EIN2 is involved but that is not the only ethylene-related gene involved in pathogen defence (Lorenzo et al. 2003).

Pseudomonas syringae is a gram-negative, rod-shaped bacterium with polar flagella and it infects a wide variety of plants (Katagiri et al. 2002). Pseudomonas mostly acts as biotrophic pathogen (Zhao et al. 2003; Tsai et al. 2011). Peronospora parasitica is an oomyceteous
fungus from the Peronosporaceae family. In contrast to Pst DC3000, Peronospora is an obligate biotroph that is controlled by the salicylic acid-dependent SAR (systemic acquired resistance) pathway (Nawrath and Metraux 1999; Jambunathan et al. 2001). Defence responses under SA control are critical for resistance to both Pst DC3000 and Peronospora parasitica (Glazebrook 2001; Van Wees et al. 2003) (Nawrath and Metraux 1999; Jambunathan et al. 2001). Alternaria brassicicola belongs to phylum Ascomycota (family Pleosporaceae) and it known to be a very common plant pathogen. Black spot disease caused by Alternaria brassicicola is an important fungal disease affecting cruciferous crops (Brassicaceae). The interaction between Arabidopsis thaliana and A. brassicicola is a representative model system and objective estimation of disease progression is indispensable for accurate functional analyses (Su'udi et al. 2013). Resistance against Alternaria requires the Arabidopsis phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling, respectively (Van Wees et al. 2003). Botrytis cinerea is necrotrophic fungus belonging to the phylum Ascomycota (family Sclerotianiacae) and it is a natural pathogen of Arabidopsis thaliana (P et al. 2007). Botrytis belongs in top 10 fungal pathogens in molecular plant pathology because it has an impact because of its broad host range causing severe damage: Botrytis causes bunch rot in viticulture or grey mould in horticulture (Dean et al. 2012). The fungus is considered as a typical necrotroph, which co-opts programmed cell death pathways in the host to achieve infection (Amselem et al. 2011; Dean et al. 2012).

4.1.6 TCS type-B response regulator and MAPK cascade members are involved in pathogen signalling

MAPK cascade members found to be involved in pathogen signalling are MPK3, MPK4 and MPK6 (Colcombet and Hirt 2008). These kinases in Arabidopsis are activated by PAMPs, mostly by flg22 (Droillard et al. 2004). Very soon other MAPK cascade members, working upstream of MPK3/4/6 and downstream of the FLS2-BAK1, were found (Colcombet and Hirt 2008). These are M KK K1 and two MKKs, MKK4 and MKK5 (Colcombet and Hirt 2008). Nevertheless, in mkk1 knock-out plants, flg22 was still able to activate MPK3 and MPK6 suggesting redundant effects at the M KK K level (Ichimura et al. 2006; Suarez-Rodriguez et al. 2007). Interestingly, biosynthesis of ethylene is also triggered by flg22 via MPK6 which in turn activates ACS6 (1-amino-cyclopropane-1-carboxylic acid) synthase 6 a crucial element in ethylene synthesis (Liu and Zhang 2004; Joo et al. 2008). Pst DC3000 induces M KK K to positively regulate expression of PRI. On the other hand mkk3-1 knock-out plants showed high susceptibility to Pst DC3000 and this effect was the opposite when the mpk3-1 mutant was complemented by M KK K wild-type form or constitutive active M KK K-EE form (S235E and S411E, M KK K phosphorylation residues) (Doczi et al. 2007), that is, the downstream MPK targets are needed for a functional readout of M KK K activity. Downstream elements for M KK K thus appear to be Group C MPKs based on yeast-two-hybrid interaction studies; physiological roles were only confirmed with MPK7 (Zhang et al. 2007). Recently was found that ARR2 also contributes resistance against Pst DC3000. ARR2 cannot directly do this, but interacts with salicylic acid response factor TGA3 after it is picks up a TCS phosphate at D80, thereafter interacting with the TGA3/NPR1 dimer making ARR2/TGA3/NPR1 trimer complex
that can bind to the *PR1* promoter, activate it, and increase pathogen resistance. *arr2-4* mutant plants in that study showed susceptibility to *Pseudomonas* (compared to the wild-type Col-0 plants) and also retarded induction of *PR1* in response to SA (Choi et al. 2010). ARR2 was shown to pull down *PR1*, *PR2*, and *ARR6* promoter DNA via ChIP assays. The association to DNA at *PR1* however was mediated by TGA3 and not ARR2 (Choi et al. 2010), is suggests that a type of scaffolding role for ARR2 was discovered. Possibly other such roles for ARR2 and or type-B RRs independent of direct DNA binding could be discovered.

4.1.7 Cell-specificity effect of the MAPK cascade

Despite the fact that only couple members of the MAPK cascade control huge range of physiological responses, these signalling cascades are quite specific because they show cell specificity (Colcombet and Hirt 2008). For example, activity of *MPK4* is increased in response to osmotic shock in *Arabidopsis* but only in suspension cells not in plantlets (Droillard et al. 2004). Subcellular-dependent function can be very well observed on ethylene-related roles of *MPK6*. The cascade MKK4-MPK6-ACS6 is involved in ethylene production whereas MKK9-MPK6-EIN3 cascade plays role in ethylene signalling. The MKK9–MPK3/MPK6 cascade promotes *ETHYLENE-INSENSITIVE 3 (EIN3)*-mediated transcription in ethylene signalling (Yoo et al. 2008; Hahn and Harter 2009). Ethylene as a ligand binds and inactivates the negative regulator CTR1 (MKKK family member) to activate the positive MKK9–MPK3/6 cascade (Yoo et al. 2008). This signalling pathway breaks a linear model and common MAPK signalling, and opens many questions, for example, both the real connection between CTR1 and MKK9 or how CTR1 is actually inhibiting the autophosphorylation of MKK9 through direct protein-protein interaction or in some other way (Hahn and Harter 2009). Supporting cell-specificity idea, scaffolding proteins have extremely important functions because they help MPK kinases to build physiologically functional cascades. Such an example is present in yeast where the C-terminal of the SSK1 RR binds to the N-terminus of the MKKK SSK2 inducing a conformational change which induces its autophosphorylation and activation of the kinase cascade (Posas and Saito 1998). Therefore it is very important to identify and describe these proteins in their crosstalk points and this work contributes to this issue.
4.2 Results

4.2.1 Background

The TCS and MAPK signal transduction pathways are two differently evolved signalling pathways both crucial for mediating control of physiological responses and development in plants (Grefen and Harter 2004; Hass et al. 2004; Mira-Rodado et al. 2007; Pathak et al. 2013; Smekalova et al. 2013). It has been known that ARR2 also functions in ethylene signalling as an arr2 Ler-0 knock-out shows an hyposensitive phenotype (Hass et al. 2004). Transcriptomic data of arr2 Ler-0 knock-out and ARR2 overexpression lines revealed drastic mis-regulation of various defence-related genes some of which are well known to be controlled/activated by MAPK cascade signalling (Hass et al. 2004). Preliminary data suggested that ARR2 co-localises and interacts with several MAP kinase kinases in Y2H and in vivo BIFC (Achim Hahn, personal correspondence). These findings indicated that there might be a functional connection of ARR2-dependent TCS signalling and MAPK cascades which were further explored in this thesis.

4.2.2 Interaction of ARR2 with MAPK cascade members

4.2.2.1 Response regulators Type A or B do not interact with MKKKs

MKKKs were tested for protein-protein interaction against ARR2 using the yeast-two-hybrid system as an in vivo heterologous approach. The large number of MKKK proteins made it impossible to test all of them, but nevertheless, three representative members were chosen for this experiment: Raf24, Raf43 and Raf41 due to their distinct sequence and functions (Jouannic et al. 1999; Group 2002). Besides ARR2, two other members of type-B family were chosen, ARR1 and ARR18, and a well-known type-B family member, ARR4. ARR1 has very high sequence homology with ARR2 and it is proposed to be redundant to ARR2 in many physiological functions (Sakai et al. 2000). ARR18 on the other hand is different from its group members ARR1 and ARR2 in sequence, expression pattern and presumably function (Mason et al. 2004; Veerabagu et al. 2012) even though these three type-B response regulators are all members of the subgroup I. ARR4 was taken as a typical member of type-A group of response regulators to distinguish B-type and A-type characteristics, if there are any.

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A (James et al. 1996) was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MKKKs were cloned with the GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. None of tested MKKK showed transactivation in yeast (only shown for Raf41) indicating that all three MKKKs could be evaluated in yeast. Nevertheless, when the colonies were plated on interaction-selective media (-W, L, A), none of these MKKKs interacted with any of tested ARRs (Figure 4.2). This experiment was repeated three times using all interaction elements and each time the same result was obtained. Based on this experiment, if there is intercommunication between this two signaling pathways, then it is downstream of MKKKs or requires other MKKK elements.
Figure 4.2 *Arabidopsis* response regulators do not interact with selected MKKKs in yeast-two-hybrid assays.
Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (Raf24, Raf43, Raf41). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only Raf41 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

4.2.2.2 **B-Type response regulators interact with MKK members**
MKKs represent a point of signal convergence within MAPK signalling cascade (Mordret 1993). After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM-W, L medium. MKK were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. As before ARR1, 2, 4 and 18 were tested against MKKs: MKK1, MKK2, MKK4, MKK5. MKK3 is transitive in yeast and cannot be analyzed as a full-length protein and therefore was not tested (not shown). None of other tested MKK constructs showed transactivation in yeast (Figure 4.3) indicating that all three MKKs could be evaluated in yeast. Interaction was observed for ARR1, 2 and 18 with MKK1, MKK2, MKK4, MKK5, whereas no interaction was observed for ARR4 with any MKK tested (Figure 4.3).
4.3 Type-B *Arabidopsis* response regulators interacted with MKKs in yeast-two-hybrid assays.
Proteins were expressed either as GAL4-AD-fusions (ARR1, ARR2, ARR4, ARR18) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown for simplification.

### 4.2.2.3 ARR2 response regulator interacts also with MPKs
As the last members of MAPK signalling cascades, MPKs were also tested against ARR2. For this experiment MPK4 and MPK17 were chosen as they both have been described in the literature and have distinct evolutionally histories (Group 2002).

After cloning the ORFs into the yeast-two-hybrid expression vectors, yeast strain PJ69-4A was transformed and colonies carrying the auxotrophic markers were selected on CSM –W-L media. MPKs were cloned with a GAL4-BD fusion and ARRs were cloned as GAL4-AD fusions. ARR1, 2, 4 and 18 were tested against MPK4 and MPK17 (Figure 4.4). Neither MPK showed transactivation in yeast indicating that each MPKs could be evaluated in yeast (data for MPK17 shown).

### 4.4 ARR2 interacted with MPKs in yeast-two-hybrid assays.
Proteins were expressed either as GAL4-AD-fusions (ARR2) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK17 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.
Both MPK4 and MPK17 showed interaction with ARR2. It is known that MKKs and MPKs interactions can be observed in yeast. Therefore, the MKK1/2/4/5 were tested against the two MPKs, MPK4 and MPK17. Interestingly, only MPK17 interacted with MKK5. MPK4 interacted with all four MKKs but a stronger interaction was observed with MKK4 and MKK5, especially with a preference for with MKK5 (Figure 4.5).

![Figure 4.5 MPK4 and MPK17 interact with biotic stress-related MKKs in yeast-two-hybrid assays.](image)

Proteins were expressed either as GAL4-AD-fusions (MKK1, MKK2, MKK4 and MKK5) or as GAL4-BD-fusions (MPK4 and MPK17). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls; only MPK4 is shown. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

### 4.2.2.4 MKK4 and MKK5 interacted with truncated versions of ARR2 containing only receiver or output domains

In order to get a closer look at which regions of ARR2 are essential for ARR2-MKK interactions, ARR2 was truncated (Figure 4.6, C). Protein truncation ARR2$^{1-300}$ contains receiver domain, part of the DNA-binding GARP domain and two out of three nuclear localisation signals (NLSs) present in ARR2. ARR2$^{300-664}$ carries the other half of the DNA-binding GARP domain and the third NLS motif. ARR2$^{1-145}$ contains a receiver domain only. ARR2$^{145-664}$ contains the output domain with all three NLSs. ARR2$^{1-165}$ contains the receiver domain and one of three NLSs while the part. ARR2$^{165-664}$ contains other two NLSs and the output domain. This information is summarized in Figure 4.6C as a graphic representation of the ARR2 protein domains, the location of MKK docking sites and the truncation positions.
Figure 4.6 MKK docking motifs are present on both receiver and output domains in ARR2. (A, B) Proteins were expressed either as GAL4-AD-fusions (ARR2^{1-145}, ARR2^{145-664}, ARR2^{1-165}, ARR2^{165-664}, ARR2^{1-300}, ARR2^{300-664}, ARR2^{51-81}, and ARR2^{215-260}) or as GAL4-BD-fusions (MKK1, MKK2, MKK3, MKK4 and MKK5). (C) Cartoon presentation of the location of the truncated parts of ARR2 with MKK binding sites indicated in yellow. Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls shown in A. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results; representative colonies are shown.

The various ARR2 truncations were tested against MKK1, MKK2, MKK4 and MKK5 for interaction using yeast-two-hybrid system (Figure 4.6, A,B). The receiver domain-containing truncations ARR2^{1-145}, ARR2^{1-165} and ARR2^{1-300} extended variant showed positive interaction only with MKK5; for all other tested kinase members no interaction was observed. On the other hand, the truncation containing the output domain ARR2^{145-664}, ARR2^{165-664} and the ARR2^{300-664} truncation showed very strong interaction with MKK4 and MKK5, but not with MKK1 or MKK2. These results suggested that there are a couple of different MKK docking sites within ARR2 that are widely dispersed along the protein. It is therefore possible that more than one, or maybe all, binding sites are needed. In summary, the type-B receiver domain and output domain can interact with MKKs.
MKK4 and MKK5 play an important role in the plant’s response to biotic stress, such as pathogen attack (Asai et al. 2002) and stomata development (Wang et al. 2007). The interaction of ARR2 with MKK4 and MKK suggested it would be useful to find out more about the number and position of MKK docking sites in ARR2 by applying an *in silico* approach. For this purpose ELM software (http://elm.eu.org) was used. ELM is a computational biology resource for investigating candidate functional sites in eukaryotic proteins reported to have high significance value (Dinkel et al. 2012). The docking interaction in the MAP kinase cascades is achieved through specific conserved regions on MKKs (docking groove) and MAPK-interacting molecules (the MKK docking motif). After evaluation of the raw output from ELM (Supplemental figure 1), docking motifs for MAPK cascade interacting molecules (e.g. MKKs, substrates, phosphatases) were predicted. There are at least two different MKK docking types: the classical docking motif and short peptide containing the sequence FxF (Sharrocks et al. 2000; Bardwell et al. 2001; Galanis et al. 2001). The classical docking motif is characterized by a cluster of at least two positively charged amino acids followed by a spacer of 2-to-6 residues from a hydrophobic-X-hydrophobic sequence, where the hydrophobic residues are long-chain aliphatics (usually Leu or Ile).

In the spacer and in the sequence immediately C-terminal to the hydrophobic-X-hydrophobic element, there is a high propensity for the presence of Pro, Asn, and/or Gly, which are residues that are both turn-forming and helix-breaking (Bardwell et al. 2001). The classic motif approximates \((R/K)X_{2-6}\Psi X\Psi\) where \(\Psi\) is a hydrophobic residue and \(X\) any residue. Four such docking motifs were found in ARR2 (Supplemental figure 1, figure 4.6). Interestingly, two of them were found on the receiver domain of the ARR2 at 56-66 (KCNRAESALSL) and 68-78 (RKNKNGFDIVI) amino acid positions. Two other potential docking domains were located in the output domain of ARR2 at amino acid positions 215-225 (KKPRVVWSVEL) and 244-252 (KKILEMMNV) (Figure 4.6, C). In order to check if these motifs are really docking sites for MKKs two truncation peptides of ARR2 to were made: ARR2\(^{51-81}\) and ARR2\(^{215-260}\). Only two fragments were made because of the fact that between the potential receiver domain docking sites only a very small physical distance lies between them and such small peptides would most likely be degraded in yeast. Surprisingly, both peptides ARR2\(^{51-81}\) and ARR2\(^{215-260}\) interacted only with MKK5 and that very strongly; No interactions were obtained with the other MKKs (Figure 4.6, B). This suggests that for all of the other MKKs other docking sites must be present which do not fit into the typical pattern for MKK docking domains, or, an alternative type of interaction domain exists. These experiments confirmed the previous observations in yeast, where truncated versions of ARR2 containing only receiver domain sequence were used, that receiver domain may also play role in ARR2-MKKs interactions and not just the output domain only. Thus it can be concluded that the inability of the ARR4 type-A RR to interact with MKKs is not due to an absence of output domain but rather due to difference in sequence of receiver domains between type-A and the types-B ARRs.
### 4.2.2.6 ARR2 does not need to be phosphorylated by TCS elements in order to interact with MKKs in Y2H

The ARR2 can obtain a phosphoryl group from the AHP TCS elements on its aspartate at the 80\textsuperscript{th} position (Grefen and Harter 2004). Mutation of D80 to E (glutamate, Glu) increases the transactivation activity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects if expressed in plants (Hass et al. 2004). Mutation of D80 to N (asparagine, Asn) however, prevents TCS dependent phosphorylation (Kim et al. 2006) and impairs basal reporter-gene activation capacity. To check if the phosphorylation state of ARR2 at the D80 position influences MPK(K)-ARR2 protein interaction loss-of-function ARR2D80N mutant and gain of function ARRD80E protein variants were tested in the yeast-two-hybrid system.

![Figure 4.7](image_url)

**Figure 4.7 ARR2 interacts with the MKKs independent of TCS-mediated phosphorylation.**

Proteins were expressed either as GAL4-AD-fusions (ARR2D80E and ARR2D80N) or as GAL4-BD-fusions (MKK1, MKK2, MKK4 and MKK5). Yeast cells were incubated for 4 days at 28°C on either vector selective (CSM-L, W) or interaction selective (CSM-L, W, A) media. The free AD domain was expressed along with BD-fusions for transactivation controls. For all interactions PJ96-4A was used. This experiment was repeated at least three times with similar results.

MKK1-5 were used for this experiment. Both mutant versions of ARR2 showed exactly the same pattern like the wild-type protein (Figure 4.7). This suggests that ARR2 does not need to be phosphorylated by TCS elements for successful interaction with MKKs and that ARR2 might serve as scaffold protein in signal transduction between MAPK cascade elements and the TCS or yet other unknown, undescribed proteins.

### 4.2.2.7 ARR2 shows very strong interaction with biotic-stress-related MKKs in yeast

M KK1 and MKK2 are known to be involved in response to abiotic stress (e.g. osmotic stress, temperature change, high salinity) (Teige et al. 2004) whereas MKK4 and MKK5 are known for their role in biotic stress (e.g. pathogen interactions) (Asai et al. 2002). In the previous yeast experiments some interactions appeared stronger as judged by both the growth intensity of the colonies and the number of colonies that displayed positive interactions. In particular, MKK4 and MKK5 repeatedly displayed stronger interactions with ARR2, its variants and other B-types. Therefore, the interaction strength of the MKK interactions with ARR2 was quantified in yeast using serial dilutions and quantification of reporter-gene strength by the O-NPG assay.
Figure 4.8 ARR2 shows strong interaction with biotic-stress-related MKKs in yeast. (A) Dilution series of fusion proteins co-expressed from the yeast-two-hybrid expression vectors: GAL4AD-ARR2 and MKK1, MKK2, MKK4 and MKK5 fused to N-terminal GAL4BD. (B) The β-galactosidase assay of the same constructs used for dilution series. The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Serial dilutions (1, 1:10^1, 1:10^2, 1:10^3 and 1:10^4) of the corresponding yeast culture was grown on selective medium and transformed PJ64-4A yeast cells with corresponding vectors were used for the O-NPG assay (β-galactosidase assay). Five colonies containing ARR2 and MKK1/2/4/5 were tested. As suspected, ARR2 interacted much stronger with biotic-related MKKs (MKK4 and MKK5) than the abiotic-related ones (MKK1 and MKK2) by the growth assay (Figure 4.8).

While the serial dilution method is informative, the more quantitative and less error-prone method is measuring β-galactosidase reporter-gene activity via the O-NPG assay. In this case, the O-NPG assay was extended to include ARR1 and ARR18. As before, ARR2 interacted stronger with MKK4/5 (~17 and ~20 O-NPG units for MKK4 and MKK5 respectively) than MKK1/2 (~5 and ~7 O-NPG units for MKK1 and MKK2 respectively). The same tendency was observed for ARR1 and ARR18 (Supplemental figure 2). It can be therefore concluded that, in general, MKK4 and MKK5 have a stronger affinity for B-type subclass I response regulators.
4.2.2.8 ARR2 shows very strong interaction with biotic-stress-related MKKs in planta

In order to see if this strong and specific interaction between ARR2 and MKK4 and MKK5 obtained in yeast is also the case in plant cells, FRET-FLIM (Förster Resonance Energy Transfer-Fluorescence Life Time Imaging) microscopy analysis was performed in planta. For this experiment the fluorescence lifetime of a Donor (i.e. GFP) is measured. A reduction in the lifetime is observed when an Acceptor (i.e. mCherry or mRFP) is within a distance of 10 Å or less; this distance is what is expected of protein-protein interactions (Caesar et al. 2011).

Since MKK3 is also known to be involved in biotic stress in a JA dependent manner (Doczi et al. 2007) and was previously not characterized in yeast due to its strong transactivation effect, MKK3 was also tested against ARR2 in planta using the FRET-FLIM approach.

ARR2 was cloned in-frame with GFP to make an ARR2::GFP fusion. The MKKs were therefore cloned in-frame with mCherry. A positive readout control was created by fusing ARR2 to a tandem GFP::mCherry coding frame via a short linker and subsequently called ARR2::FRET. After cloning, the constructs were transformed into Agrobacteria tumefaciens and transiently expressed in tobacco (Nicothiana bethamiana) epidermal cells as a heterologous plant system. The negative control was ARR2::GFP transformed alone.

All three tested MKKs interacted with ARR2 (Figure 4.9). Both MKK4 and MKK5 interacted with ARR2 stronger than MKK3, with MKK5 having the strongest interaction with ARR2 (Figure 4.9, B). These in planta data confirmed the previous data obtained in the yeast-two hybrid system and lead to more stable and concrete evidence that these interactions, especially ones with MKK4 and MKK5, might be have physiologically functional roles in Arabidopsis to biotic stresses.
Figure 4.9 ARR2 interacts very strongly with biotic-stress-related MKKs in planta.
(A) Cartoon representation of ARR2 and MKKs proteins fused for fluorescent proteins for FRET-FLIM measurements. (B) FRET-FLIM results of ARR2-GFP interaction with MKK3, MMK4 and MKK5 fused to mCherry. (C) CLSM photos of tobacco leaves showing localization of expressed ARR2-GFP fusion protein with indicated MKKs fused to mCherry fluorescent protein. The fusion proteins were transiently expressed in tobacco cells and the results are given as function of Chi² test (measurements and evaluation courtesy of Dr. Kirstin Elgass). ARR2-GFP and ARR2-GFP-mCherry were used as negative and positive controls respectively. This experiment was repeated two times with similar results.
4.2.3 Roles of ARR2 in pathogen-related phenomenon
As previously shown, FRET-FLIM in planta measurements and experiments using the yeast-two-hybrid system connect TCS type-B RRs to MKKs via physical interaction. ARR2 especially interacted strongly with MKK3, MKK4 and MKK5. Additionally, ARR1 and ARR2 interacted with MPK4 and MPK17 as well. MKKs play an important role in the plant’s response to biotic stress, such as pathogen attack (Asai et al. 2002). And interestingly, this is also the case for all three in planta tested MKKs: MKK4 and MKK5 (Nakagami et al. 2005; Popescu et al. 2009) as well as MKK3 (Doczi et al. 2007) clearly implicating the involvement of ARR1 and ARR2 in pathogen defence.

4.2.3.1 Reactive Oxygen Species (ROS) and ethylene measurements in ARR1 and ARR2 mutants after treatment with Pathogen-Associated Molecular Patterns (PAMPs)
As a first look into pathogen-related responses in ARR2, wild-type and mutants arr1-4, arr2-4 and arr1-4 arr2-4 lines were treated with PAMPs. As output, ROS and ethylene production were measured.

4.2.3.1.1 The arr1-4 arr2-4 double mutant showed differences in ROS production after treatment with flg22 and elf18
For ROS experiments, leaves from 5 to 6 week-old plants were used. Leaves were cut in discs from the same leaf surface and incubated in water overnight.

![Figure 4.10 arr1-4 arr2-4 double mutant shows differences in ROS production after treatment with PAMPs.](image)

Arabidopsis leaves from different mutants were treated with elf18 (left) and flg22 (right). Mutants efr and fts22 were used as negative response controls. Letters a, b and c stand for biological experimental repetitions whereas in the c repetition only the arr1-4 arr2-4 double mutant was used along with control plants. Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level separately for the time points 13, 17 and 21 minutes.
The following day, the discs were transferred to a reaction solution and the ROS production was triggered by treatment with 100nM of flg22 or 100nM elf18. In addition to the type-B mutant lines, *fls2* (Dunning *et al.* 2007; Krol *et al.* 2010) and *efr* mutants lacking leucine rich repeat-receptor-like kinases *FLS2* (*Flagellin-Sensitive 2*) and *EFR* (*Elongation Factor-Tu receptor*), respectively (Krol *et al.* 2010). *FLS2* recognizes bacterial flagellum (active epitope flg22) and *EFR* recognizes elongation factor Tu (active epitopes elf 18, elf13 and elf26).

Both times the same tendency was observed: *arr1-4 arr2-4* double mutant significantly produced more ROS than the wild-type (*p*≤0.05 at time-points 13, 17, 21 min post treatment) in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments; the double mutant consistently however showed more ROS than the wild-type (Figure4.10, experiment a verses b). Therefore, a third experiment was conducted using more replicates to confirm the previous two experiments. It could be concluded that the *arr1-4 arr2-4* double mutant significantly produces more ROS in response to applied PAMPs, flg22 (*p*≤0.05 at time points 13, 17, 21 min post treatment) and elf18 (*p*≤0.05 at time-points 13, 17, 21 min post treatment) but it is unclear what the effect of the single mutants have on ROS production (Figure 4.10).

4.2.3.1.2 The *arr1-4 arr2-4* double mutant did not show any difference in ethylene production after treatment with different PAMPs

![Ethylene production response to different PAMPs](image)

**Figure 4.11** *arr1-4 arr2-4* double mutant shows no difference in ethylene production after treatment with PAMPs

*Arabidopsis* leaves from *arr1-4 arr2-4* double mutant and Col-0 wild-type were treated with (A) elf18 and flg22 using different concentrations: 0nM, 1nM, 10nM, 100nM and 1000nM (B) Fungal extracts used were 1µl of PEN and 3µl of Xac in a final volume of 500µl. Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level. This experiment was performed as two biological replicates each containing three technical replicates.

Ethylene production was measured as a response to different PAMPs flg22 and elf18 or crude extracts PEN from *Penicillium schizogonium* and Xac from *Xanthomonas axonopodis citri*. A dose response curve was obtained for Flg22 and Elf18 (0.1nM, 1nM, 10nM, 100nM, 1µM) but only single concentrations 1µL/500µL PEN and Xac 3µL/500µL for the crude extracts was
conducted. Based on results from obtained the previous ROS measurements and due to lack of growth space, only Col-0 wild-type and arr1-4 arr2-4 double mutant lines were used. The double mutant did not show any statistically significant difference in ethylene production to any of the tested PAMPs compared to the wild-type (Figure 4.11, A, B). Based on this experiment, it can be concluded that either there is no significant difference in ethylene production. Alternatively, perhaps the analytical system was not sensitive enough to detect subtler changes in ethylene production compared to that of the ROS system.

4.2.3.1.3 The arr1-4 arr2-4 double mutant did not show any difference in activation pattern of MPK3, MPK4 and MPK6 after treatment with flg22

The MPK kinases MPK3, MPK4 and MPK6 are known to be activated by phosphorylation in a flg22 dependent manner (Droillard et al. 2004). Activation of these MPKs can be analysed by using the phospho-p44/p42 MPK antibody (Brock et al. 2010; Montillet et al. 2013). As previously observed, flg22 was able to elicit a higher ROS response in the arr1-4 arr2-4 mutant background (§4.2.3.1.1). Thus by conclusion, it is possible that these MPK kinases are differentially activated in the arr1-4 arr2-4 mutant background compared to the wild-type. For this experiment 4 week-old plants were used and their leaves were infected by dropping of 1µM solution of flg22 or mock onto a leaf surface. Samples were collected at three time points: immediately after infection, 15 minutes and 30 minutes after treatment (Figure 4.12).

Before flg22 treatment only weak signals were obtained for MPK6 and MPK3 showing that these two kinases are phosphorylated at some basal level. After 15 minutes of flg22 treatment, the signals for MPK3/6 became much stronger and a third band representing MPK4 appeared (Figure 4.12). Half an hour after infection the phosphorylation pattern stayed

![Figure 4.12](image-url)
the same as the 15 minute time point, indicating that the peak flg22-dependent MPK activation level was already reached at the 15th minute or before. Mock treated leaves at 15 and 30 minutes after treatment maintained the same basal MPK3/6 patterning as the zero time point. In conclusion, no dramatic differences were observed between the wild-type and the double mutant in activation pattern or intensity with any of these three kinases.

4.2.3.2 Pathogen assays using biotrophs and necrotrophs

A broad spectrum of different pathogens was used in this work to cover both necrotrophic and biotrophic types of infection strategies. These pathogens were challenged on Col-0, arr1-4, arr2-4 and arr1-4 arr2-4 plant lines. It was very important to obtain various measurements of a given interaction with high spatial and temporal resolution. For necrotrophs, *Alternaria brassicola* and *Botrytis cinerea* were tested. For biotrophs, the obligate biotrophic fungus *Peronospora parasitica* was tested as well as the biotrophic/hemibiotrophic bacterium *Pseudomonas syringae pv. tomato* DC3000 (*Pto* DC3000).

4.2.3.2.1 Pathogen assays using biotrophs

Pathogen assays with biotrophs were always performed using one bacterial and one fungal pathogen: the bacterium *Pto* DC3000 and fungus *Peronospora parasitica*.

4.2.3.2.1.1 There is no difference in response of ARR1 and ARR2 mutants to biotrophic Pst DC3000

This pathogenic assay was done using five-week old plants and treating them with *Pto* DC3000 bacterial strain. Bacterial cells were infiltrated into two leaves per plant. Bacterial cells at an approximate concentration of 10^4 cfu/ml were infiltrated with a needless syringe into the middle of the leaf apoplastic space (two leaves per plant) to an area was always bigger than the cork borer used for cutting the leaf discs with which the experiment was further performed. Afterwards the leaves were harvested, at the time point 0h and 1st, 2nd and 4th days past infection, material was plated on LB-plates with antibiotics and number of colonies per plate/mutant formed was counted.

Based on number of grown colonies per plate the difference in resistance between the mutants was determined. Although this experiment was repeated six times none of them consistently showed any pattern that could be attributed to the loss of *ARR1* or *ARR2* or both. That is, any differences that could be observed in the second and fourth day were never reproduced in any other experiment (all repetitions are not shown but compare 4.13A with the 4.13B mock for an example.

Knowing that ARR2 has been implemented in *Pto* DC3000 pathogen resistance and that this response required both cytokinin and SA (Choi et al. 2010), a cytokinin treatment was added to the experimental design. *t*-zeatin was used at a working concentration of 1µM. Before infiltration cytokinin was given to the 1x10^4 cfu/ml bacterial dilution. The plants were also treated with cytokinin 3 days before the experiment by adding it to the water used for watering. No clear trend could be observed that could be ascribed to a combined effect of exogenous cytokinin and loss of *ARR1* or *ARR2* could be found. In fact, in this experiment, the cytokinin treatment resulted in reduced bacterial growth in all plant lines which was highly
statistically significant by day 4 (Supplement figure 3). This effect of cytokinin on Pto DC3000 growth has been observed before (Choi et al. 2010; Choi et al. 2011).

Figure 4.13 ARR1 and ARR2 mutant plants do not show reproducible differences in response to biotrophic Pseudomonas syringae pv. tomato DC3000.

Leaves of Arabidopsis ARR1 and ARR2 single and double mutants and corresponding wild-type (Col-0) were treated with Pseudomonas. The evaluation of infection was done at day 0, 1, and 4. (A) Plant lines treated only with Pseudomonas syringae pv. tomato DC3000. (B) Plant lines treated either with Pseudomonas syringae pv. tomato DC3000 only (Mock) and also with 1µM t-zeatin (cytokinin). Fisher’s Least Significant Difference (LSD) test was used to compute significance levels at the 0.05 α level (Supplemental figure 3). These experiments were repeated at least three times each with similar results. CFU stands for Colony Forming Units. Data are represented as box-and-whisker plots: the central horizontal line is the median (2nd quartile) and the boxed region extends from the beginning of the 1st quartile to the end of the 3rd quartile. Lines extending outside of the boxed region are 1.5 x (the respective interquartile range). Data points outside this region are shown as dots and are called outliers.
4.2.3.2.1.2 There is no difference in response of ARR1 and ARR2 mutants to *Peronospora parasitica*

Next arr1-4 arr2-4 plants were challenged with *Peronospora parasitica*. This experiment was performed in collaboration with the research group of Prof. Dr. Volker Lipka from University of Göttingen. Four to six-week old plants were inoculated by spraying the leaves with a *Peronospora parasitica* conidial suspension and the infected plants were observed after seven days.

![Image showing wild-type and mutant plants](image)

**Figure 4.14 ARR1 and ARR2 mutant plants do not show differences in response to biotroph *Peronospora parasitica***.

Leaves of *Arabidopsis* wild-type (Col-0), ARR1 and ARR2 single and double mutants were sprayed with *Peronospora*. After seven days there were no visible differences among the mutant plants and the wild-type. This experiment was repeated once with similar results. Data were produced by the research group of Prof. Dr. Volker Lipka.

If there is any difference in resistance to *Peronospora*, a clear phenotypic difference would have been visible. However, since there was no visual difference obtained between the wild-type and the mutant plants there was not any need for counting of sporangiophores on the leaves (pers. communication V. Lipka). It was concluded that the mutant plants do not show any difference in resistance to *P. parasitica* compared to the wild-type (Figure 4.14).

4.2.3.2.1.3 Lack of differential response to biotrophs *Pst DC 3000* and *Peronospora parasitica* is not due to perturbation of stomata aperture regulation

It was proposed that a larger stomata aperture would make it easier for bacteria to enter into the plant, infect cells and form a stable microfilm in vascular bundles allowing it to further multiply and invade the plant. It is also known that cytokinin can cause stomata to open (Tanaka *et al.* 2006) and abscisic acid (ABA) causes them to close (Desikan *et al.* 2006). Initially, *Pst DC3000* also causes stomata to close (Zeng *et al.* 2010; Desclos-Theveniau *et al.* 2012), however after 3 hrs, the bacteria produce diffusible phytotoxin COR that reopens closed stomata to increase the number of sites for bacterial invasion (Melotto *et al.* 2008). Therefore an experiment was designed to test this hypothesis.
Figure 4.15 Regulation of stomata aperture in *ARR1* and *ARR2* lacking mutants in response to hormonal treatment with ABA and *t*-zeatin and *Pst DC 3000* *Pseudomonas* strain

For treatments with hormones at least five plants per line were used and from those 2 leaves per plant were treated with the hormones. Experiments with *Pst DC 3000* were done only for *arr1-4 arr2-4* double mutant and Col-0 whereby five plants per line were used and the stomata aperture size was measured 3 days after infection. This experiment was repeated once with similar results. The mean and SE is shown. Leaves were floated on water or water containing 10µM ABA or 1µM *t*-zeatin (see §3.6.11 for details). For statistical results see Supplemental Figure 4.

Leaves were treated with water (Control), ABA, *t*-zeatin for 2 hours or three days after *Pst DC3000* infection. The results are displayed as bar graphs (Figure 4.15) and the significance tests results are given in Supplemental Figure 4. Importantly for this work, it is known that ABA-dependent stomata closure is not dependent on *ARR2*, as the *arr2-4* responds like wild-type (Desikan *et al.* 2006). This response was replicated; that is, the stomata apertures were significantly closed in response to ABA treatment. On the contrary, treatment with *t*-zeatin resulted in a significant increase in stomata aperture for all plant lines. Plants sprayed with *Pst DC3000* also had stomata apertures wider than the non-treated controls (data only for Col-0 and *arr1-4 arr2-4* were obtained). The data showed that there was no difference in stomata aperture response in the mutant backgrounds compared to the wild-type. Furthermore, all plant lines responded the same way after treatment with different hormones and after application of *Pst DC3000* by spraying (Figure 4.15).

In conclusion, based on the preceding pathogen experiments and this stomata aperture measurement, there is no difference in the resistance of *arr1-4, arr2-4* or *arr1-4 arr2-4* plants to *Pseudomonas syringae Pst DC3000* under my experimental conditions.
4.2.3.2.2  Pathogen assays using necrotrophs

Two necrotrophic fungi *Alternaria brassicola* and *Botrytis cinerea* were challenged with Col-0 wild-type and the *arr1/2* mutants. Resistance against *Alternaria* requires the *Arabidopsis* phytoalexin, camalexin and jasmonic acid (JA)-dependent signalling pathways, respectively (Van Wees et al. 2003). *Botrytis cinerea* is a natural necrotrophic pathogen of *Arabidopsis thaliana* (P et al. 2007) and is considered as a typical necroph that co-opts programmed cell death pathways in the host to achieve infection (Amselem et al. 2011; Dean et al. 2012).

4.2.3.2.2.1  *arr2-4* single and *arr1-4 arr2-4* double mutants are more susceptible to *Alternaria brassicicola*

*Alternaria brassicicola* was applied as a sporal suspension dropped (5µl droplets of spore solution of 5*10^5* Spores/ml) onto *Arabidopsis* leaves (two leaves per plant) and scored on the 7th and 10th day after infection using a disease progression index as in (Kemmerling et al. 2007) and summarized in Methods (§3.6.16). Statistically significant differences were obtained between *arr2-4* single, and *arr1-4 arr2-4* double mutants compared to the WT (Col-0) were computed using the mean. Single *arr2-4* (*p*≤0.001) and the double *arr1-4 arr2-4* mutants (*p*≤0.001) showed statistically significant susceptibility compared to the wild-type where the difference in *arr1-4* single mutant (*p*≤0.01) was not statistically significant from wild-type (Figure 4.16, A).

The same experiment was therefore repeated in presence of 1 µM exogenous cytokinin, *t*-zeatin. Similar to the previous experiment with *Pst DC3000*, before the assay was performed, cytokinin was added to the fungal dilution. The plants were also treated with cytokinin by mixing it with water used for watering 3 days before the experiment was performed. Cytokinin induced some kind of resistance in all of the plants (Figure 4.16, B) and this resistance was statistically significant (see Supplementary Figures 5 and 6, Figure 4.16B). At the day 7 after treatment the disease index of cytokinin treated plants decreased from 270.8 by mock treated plants to 257.5 (*p*≤0.0029, LSD (Fisher’s Least Squared Difference Test). The same tendency was observed 10th day after treatment where the disease index of treated plants decreased from 306.0 to 294.4 (*p*≤0.0066 LSD (Fishers Least Squared Difference Test).

The *arr2-4* and the double mutant are more sensitive irrespective of the cytokinin treatment to *Alternaria* which was statistically confirmed (see Supplemental Figure 5 and 6). In contrast, wild-type plants and the *arr1-4* single mutant did not show differences in their disease indices. This means that *arr2-4* is responsible for susceptibility phenotype observed in the double mutant (Figure 4.16, B).
Figure 4.16 Arabidopsis thaliana arr2-4 single and arr1-4 arr2-4 double mutants are susceptible to necrotroph Alternaria brassicicola

Leaves of arr1-4, arr2-4, arr1-4 arr2-4 and Col-0 were treated with Alternaria spores. The infection was evaluated at day 7 and 10. The data are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table) (A) Plant lines only treated with Alternaria brassicicola spores. Image insert to the right shows a representative leaf composition at Day 7 (B) Plant lines treated either with Alternaria brassicicola spores only (Mock) and also with 1µM t-zeatin. To the right, the pooled mean and ANOVA 95% confidence interval for all samples either treated or not treated with cytokinin is shown for Day 7 (ANOVA p ≤ 0.0029) and Day 10 (ANOVA p ≤ 0.0066); the cytokinin treated samples showed less disease symptoms. LSD (Fisher’s Least Squared Difference Test) was also used to compute significance levels at the α=0.05 level based on the mean (Supplemental Figures 5 and 6). For this experiment at least 20 plants per line were used and two leaves per plant were infected with Alternaria spores. This experiment was repeated at least three times with similar results.
4.2.3.2.2  *arr1-4 arr2-4 double mutant is resistant to Botrytis cinerea*

*Botrytis cinerea* is especially interesting as it is known to be a natural pathogen of *Arabidopsis thaliana*. *Botrytis cinerea* was applied to the single mutants, double mutant and wild-type by dropping a fungal suspension on the leaves. Susceptibility to *B. cinerea* was determined using a detached leaf assay and a visually identified by a lesion disease index. Lesion size has been used as an indicator of susceptibility to *B. cinerea* (Ferrari *et al.* 2003; Denby *et al.* 2004) and this has been shown to correlate with whole plant susceptibility and pathogen growth within the plant (Govrin and Levine 2000; Denby *et al.* 2004).

This experiment was performed in collaboration with Floriane L’Haridon from research group of Prof. Dr. Jean-Pierre Métraux from University of Fribourg, Switzerland. Hyphae and conidiophores were visually identified throughout the lesions and the infection sites were categorized according to their lesion sizes which are divided into three groups: Group 1 are lesions less than or equal to 2 mm, group 2 lesions between 2 mm and 6 mm and the group 3 are lesions bigger than 6 mm. To reflect different severities of infection (Figure 4.17, A) the outgrowing lesion size was also determined (Figure 4.17, B) as well as the average lesion size (Figure 4.17, C). The lesions were measured three days after application of the pathogen.

As the data are categorical, they are presented in Figure 4.17 A and B as mosaic plots; alternative plots and statistics can be found in Supplemental Figure 7. The categorized lesion size is shown in Figure 4.17A and average lesion size is shown as a bar graph of the mean (Figure 4.17, C). As can be seen in Figure 4.17 A and B the severity of in infection was reduced in all mutant lines compared to Col-0. Col-0 had the majority of its lesions in Category 3 and its overall outgrowth index at 75% or more (87.5% of all lesions). In contrast, each *ARR* mutant had more lesions in Category 1 and 2, less in 3 (Figure 4.17, A). In fact looking only at Category 3 results, Col-0 was infected better with over 58.3% its lesions bigger than 6 mm. In contrast, the double mutant had only 31.3% of its lesions bigger than 6 mm. The single mutants showed in infection levels in-between wild-type and the double mutant: *arr2-4* (40.6% ≥6 mm) and *arr1-4* single mutant (43.8% ≥6 mm). This difference in infection efficiency is also evident from inspection of the outgrowth index, whereby only 58.3% of *arr1-4*, 54.2% of *arr2-4* and 50.0% of *arr1-4 arr2-4* leaves had an outgrowth index at 75% or more.

The average lesion size (Figure 4.17, C) again showed a clear picture of mutant resistance. Average lesion size for the wild-type was about 5.77 mm whereas the single mutants this value decreased to 4.39 mm for *arr1-4* and 3.99 mm for *arr2-4*. In the double mutant the average lesion size was 3.52 mm only. Leaves treated with inoculation media alone did not develop any lesions or lesion-like symptoms (data not shown).

All of these parameters very clearly show that *arr1-4 arr2-4* double mutant is more resistant to *Botrytis* compared to Col-0 wild-type. Interestingly the same tendency was observed in the single mutants as well, but only the difference by the double mutant was statistically significant when ascertainment for average lesion size. These results clearly show the redundancy (additive effect) of the *ARR1* and *ARR2* in respect to defense against *Botrytis*. 57
Figure 4.17 *Arabidopsis thaliana* arr1-4 arr2-4 double mutants is susceptible to necrotroph *Botrytis cinerea*

Leaves of *Arabidopsis* ARR1 and ARR2 single and double mutants and corresponding wild-type (Col-0) were treated with *Botrytis cinerea*. The evaluation of infection was done 48hrs after the treatment. Data for (A,B) are shown as mosaic plots which are a graphical representation of an automatically computed two-way frequency table (also called a Contingency Table). Data in C is the mean and SE. (A) Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by lesion disease index (Category 1: \(\leq 2\)mm, Category 2: 2-6mm, Category 3: >6mm). (B) Susceptibility of plant lines to *Botrytis cinerea* are visually presented in a mosaic plot by outgrowth index in %. (C) Average lesion size of the tested plant lines in mm. LSD (Fishers Least Squared Difference Test) was used to compute significance levels at the \(\alpha \leq 0.05\) level using the mean; asterisk shows a significance difference compared to Col-0 only. For this experiment at least 20 plants per line were used. This experiment was repeated two times with similar results.
4.2.3.3 Response of common marker genes PR-1 and PDF1.2 with respect to Botrytis cinerea

Knowing that the double mutant was more resistant to Botrytis compared to the wild-type, leaf material of treated and not treated plants was harvested and the transcript levels of PR-1 and PDF 1.2 were quantified by RT-qPCR. PR-1 and PDF1.2 are induced in response to a variety of pathogens (Penninckx et al. 1996; Schenk et al. 2000). The PR-1 gene has been used to elucidate transcriptional control mechanisms regulating SAR (Pape et al. 2010) and expression of PR-1 is salicylic-acid responsive (Metraux et al. 1990; Zhang et al. 2013).

Figure 4.18 Response of common marker genes PR-1 and PDF1.2 after treatment with Botrytis cinerea

Relative expression levels of PR-1 and PDF1.2 genes in arr1-4, arr2-4, arr1-4 arr2-4 and Col-0. Gene expression was measured after 0, 12, 24, 36 and 48 hrs after treatment with Botrytis. For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. This mRNA extraction and RT-qPCR was repeated two times with similar results. For each gene, time point 0 for Col-0 was used as reference and EF-1-α was used as the internal control to calculate ΔΔCT values.

PDF1.2 belongs to the plant defensin (PDF) family encodes an ethylene- and jasmonate-responsive plant defensin protein (Penninckx et al. 1998; Ferrari et al. 2003). PDF1.2 mRNA levels are not responsive to salicylic acid treatments (Manners et al. 1998) although jasmonate and salicylic acid can act synergistically to enhance the expression of this gene (Mur et al. 2006; Koornneef and Pieterse 2008).

For this experiment all of the treated material for each plant line from both biological experiments (§4.2.3.3) was pooled together. Infected leaves from both treated and non-treated plants were harvested after 0 hrs, 12 hrs, 24 hrs, 36 hrs and 48 hrs post inoculation.
and the mRNA expression levels for \textit{PR-1} and \textit{PDF1.2} were quantified. In non-treated plants \textit{PR-1} was, as expected, very low expressed in all harvested time points. The first significant up-regulation of the \textit{PR-1} was after 24 h where the mRNA level was higher in all mutants compared to the wild-type. This trend in rising expression continued until 36 hrs after inoculation and the levels of \textit{PR-1} were still higher in the mutants. However, there was no difference in expression among the mutants themselves (Figure 4.18). Two days after inoculation (48 hrs) the expression of \textit{PR-1} in all mutants maintained the same level as seen at 36 hrs while expression in the wild-type was increased. Thus, although the basal levels of \textit{PR-1} was higher in the mutant plants, the pattern of induction with respect to \textit{Botrytis} was not altered in any way.

Expression level of \textit{PDF1.2} was very low in non-treated plants independently of the time point and the first increase of expression among the inoculated plants was visible 24 hrs after inoculation. The same tendency in all mutants and the wild-type plants was observed as an up-regulation at 24 hrs and 36 hrs post inoculation. Up until the 36 hrs time point, no difference was observed between either single mutants or the wild-type. However a difference was obtained by the double mutant with the level of \textit{PDF1.2} down-regulated. After 48 hrs, the expression level of \textit{PDF1.2} in the wild-type strongly went up compared to any of the mutants.

Based on these observations it must be concluded that even though regulation of \textit{PR-1} and \textit{PDF1.2} is slightly perturbed in \textit{arr1/2} plants, it does not appear reflect the resistance of \textit{arr1-4 arr2-4} to \textit{Botrytis}. Thus even though \textit{PR-1} and \textit{PDF 1.2} are commonly used marker genes for these kind of studies, in this case, the genes themselves do not correlate directly to the resistance phenotype and are more a reflection of the current molecular status in the plants after infection.
4.3 Discussion

4.3.1 ARR2 interacts with MKK members

For the first time a connection has been shown between MAPK signalling cascades and the two-component signalling system in plants. These findings supported earlier presumptions and previous data (Hass et al. 2004) that ARR2 might play role in response in biotic stresses, especially in response to pathogens. This crosstalk was observed strictly downstream of MKKKs, at the level of MKKs and MPKs. This is because none of the tested response regulators (A- and B-type) interacted with any of tested MKKKs. In comparison, only type-B RRs interacted with MKKs and MPKs.

Nevertheless, although only type-B interacted with MKKs, they did not interact with all of them. All MAPK kinase kinase members, except MKK3, were tested against: ARR1, ARR2, ARR10, ARR14, ARR18 and ARR4 (type-A) using yeast-two-hybrid method (studies with MKK6-10, and with ARR10 and ARR14 were done in our laboratory in collaboration with Niklas Wallmeroth (Wallmeroth, Diplom, Tübingen). Besides MKK3, MKK9 showed also transactivation in yeast, but its growth on control medium still allowed for evaluation of protein-protein interactions. ARR10 interacted only with MKK4, MKK5 and MKK9 while ARR14 interacted only with MKK5. ARR1, ARR2, and ARR18 interacted with more MKKs whereas the A-type ARR ARR4 did not interact with any. This information has been summarized below (Table 4.1).

Table 4.1 Summary table of protein-protein interactions between MKKs and RRs

+ means positive scored interaction, - stands for negative scored interaction, (+) stands for slightly transactive protein but a scorable interaction could still be observed, and blank means interaction was not tested. All interactions, except the one with MKK3 (grey labelled), were observed based on yeast two-hybrid system. Fields in blue represent my own results and the rest of data were produced in our laboratory by Niklas Wallmeroth during his Diploma thesis.

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Despite very high sequence homology between ARR1 and ARR2, they did not interact with the same MKK members suggesting that the interactions could be rather specific. Interestingly, ARR2 interacted with the largest number of MKKs. On the converse side, MKK4, MKK5 and MKK9 interacted with the largest number of type-B ARRs.
MKK7 and MKK9 both are class D MKKs and also play important roles in biotic stress responses. Only ARR2 and ARR18 interacted with MKK7. MKK7 is very well known as a negative regulator of polar auxin transport, defence response to bacterium and salicylic acid mediated signalling pathways (Dai et al. 2006). ARR1 is known to control polar auxin transport by directly controlling SHY2/IAA3, an Aux/IAA transcriptional repressor, expression, which in turn controls expression of PIN1, PIN3 and PIN7 in the vascular tissue (Taniguchi et al. 2007). As ARR1 did not interact with MKK7 in the yeast-two-hybrid, it is unclear if this rules out a role for MAPK cascades in planta. The high homology between ARR2 and ARR1, plus the fact that ARR12, the closest homolog to ARR10, also has been shown to have a role in regulating SHY2 (Moubayidin et al. 2010) leaves this possibility still open. ARR1, ARR2, ARR10, and ARR12 all complement the root cytokinin response phenotype of the arr1 arr12 mutant and therefore can functionally replace each other in root elongation assays (Hill et al. 2013). Taken together, it is worth speculating that ARR2 also has a role in polar auxin transport by controlling SHY2 expression and integrating signal information from MKK7. This hypothesis of course must be tested experimentally.

MKK9 is known to be enrolled in phosphorylation between MPK3 and MPK6 and it is independently involved in ethylene and calmalexin biosynthesis, response to salt stress and wounding (Xu et al. 2008). In addition, both MKK7 and MKK9 are thought to be involved in the innate immunity cell death response (Popescu et al. 2009). ARR1, ARR2, ARR10 and ARR18 all interacted with MKK9. That ARR2 has been described to also function downstream of ethylene signalling (Popescu et al. 2009) this implies that these B-types could mediate allosteric functions. Expression of constitutive active version MKK9EE lead to enhanced cell death in N. benthamiana transfection assays (Popescu et al. 2009). Whether or not type-B ARRs could be part of this process is not yet known.

As mentioned, ARR2 interacted with the most MKKs and of those most of them are hormonal/stress-related MKKs (MKK3, MKK4, MKK5, MKK7 and MKK9). Moreover, ARR2 strongly interacted with MKK4 and MKK5 compared to the abiotic-stress related MKKs MKK1 and MKK2. This difference of interaction strength was conferred by O-NPG assay and by making serial dilution of transformed yeast cells. Therefore, MKK4, MKK5, MKK3 were tested in planta by quantifying protein-protein interaction strength using FRET-FLIM. The FRET-FLIM results revealed strong interaction between ARR2 and MKK4, MKK5, MKK3 in planta. Thus, there is little doubt that these two protein classes do interact in living plant cells.

Two MPKs were tested against ARR2 and MKKs, the biotic stress-related MPKs MPK4 and MPK17 (Group 2002). Both MPK4 and MPK17 interacted with ARR2, ARR18 and ARR1. MPK4 is especially known to play very important roles in pathogen response (Rasmussen et al. 2012). It has been known that mpk4 knockout mutant plants show constitutive activation of SA-mediated defences (Petersen et al. 2000). Furthermore, MPK4 activity inhibits basal defences to virulent Pst DC3000 and pathogen-induced SA accumulation, MPK4 also plays role in ETI as compromising it and its activity negatively regulate ROS production induced by PAMPs (Berriri et al. 2012). The interaction of MPK4 with both biotic stress-related MKKs
MKK4 and MKK5 could be confirmed in this thesis. MPK17, whose physiological function(s) are not described yet, only interacted with MKK5. Recall however that MKK5 interacted with all of the type-B ARRs. MKK9 also interacted with the majority of type-B ARRs, albeit over its yeast-two-hybrid transactivation capacity. However, only MKKs 1 to 5 were tested against MPK17 in this thesis. Nevertheless, it is reported that MPK17 interacts with MKK9 and MKK10 in yeast-two-hybrid assays (Lee et al. 2008). Thus, although the interaction of MPK17 with MKK9 was not confirmed in this thesis, it suggests that a bona fide interaction network has been documented. Furthermore, expounding on the knowledge of roles for MKK5 and MKK9, the type-B ARRs are somehow involved in physiological responses related to biotic stress and/or hormonal regulation. With regards to MKK9 which is known to be involved in ethylene signalling downstream of CTR1 (Yoo et al. 2008) as a MKK9–MPK3/6 module. Haß et al. 2004 from our laboratory showed that ARR2 also responds to ethylene in an ETR1-TCS dependent manner. In addition, MKK4/5 have also been placed in a MKK4/5/9-MPK6 ethylene biosynthesis model (Hahn and Harter 2009; Zhao and Guo 2011). Interestingly, the most type-B ARR interactive MKKs MKK4/5/7/9 were shown to activate signalling cascades controlling cell death pathways in plants (Popescu et al. 2009). How these two signalling pathways converge on ethylene signalling is still a mystery, yet it suggests that part of the signal integration can occur at a protein-protein interaction level.

The FRET-FLIM analysis revealed that MKK3 and ARR2 interact in living plant cells. MKK3 is an activator of group C MPKs (MPK1, MPK2, MPK7, and MPK14) of which MKK3 also activates MPK7 in response to plant pathogen Pst DC3000 and ROS (Doczi et al. 2007). MKK3 is interesting as it is shown to play a physiological role in response to biotic stresses as a MKK3-MPK6 cascade important for JA signal transduction in Arabidopsis (Takahashi et al. 2007) and the same cascade plays crucial role in resistance to Salmonella infections in Arabidopsis (Schikora et al. 2008). Additionally, ARR2 showed very strong interaction with MKK4 and MKK5 compared to MKK1 and MKK2 and this interaction was also confirmed in planta. The MKK1 and MKK2 are mostly involved in abiotic stresses (Asai et al. 2002). For example MKK2 is specially activated by cold and salt stress and by stress-induced MKK1 (Teige et al. 2004). They have also been described with a role in biotic stress as a part of MKK1/2-MPK4 cascade functioning as negative regulator of SA-dependent systemic required resistance (Petersen et al. 2000) and positive regulator of ET- and JA-mediated defences (Brodersen et al. 2006; Qiu et al. 2008). Here, it is also possible the MKK1/2-MPK4 module is allosterically influenced by ARR2 and ARR1.

The presumptuously stronger interaction of MKK4 and MKK5 over the other MKKs with B-types was confirmed in yeast-serial dilution and quantitative O-NPG assays. MKK4/5 are known to be active in response to different biotic stresses but especially active and described in pathogen defence (Asai et al. 2002) where MKK4 is strongly induced when plants treated with Pseudomonas syringae or together with MKK9 against Phytophthora infestans (Menges et al. 2008). Even newly characterised homologue of MKK5 in cotton (Gossypium hirsutum) GhMKK5 affects pathogen resistance to the bacterial pathogen Ralstonia solanacearum by
elevating the expression of pathogen resistance (PR) genes (PR1a, PR2, PR4, PR5, and NPR1) (Zhang et al. 2012). Interestingly, the interaction capability of ARR2 was not due to the presence of the ARR2 type-B output domain, since a truncated version of ARR2 containing only its receiver domain was able to interact with MKK4 and MKK5. Furthermore, using an in silico approach, it was shown that MKK docking domains are dispersed throughout ARR2 and not restricted to its output domain. In total, four different MKK binding domains were predicted to be present in ARR2. All of these regions seem to be functional when tested by yeast-two-hybrid system based on the larger protein truncations. When tested as peptides, the receiver domain-based and output domain-based MKK docking motifs showed interaction only with MKK4 and MKK5. This observation was taken to confirm previous observations that these two MKKs interact much stronger with ARR2 than the others. Furthermore, the interaction of ARR2 with MKKs does not require phosphorylation by TCS elements on its D80 position in order to interact with any of tested MPK(K) cascade elements. This suggests that ARR2 might interact with MAPK cascade members independent of cytokinin or ethylene, i.e. TCS signalling. In fact, this would put ARR2 in novel signalling pathways which may or may not require its transcription factor roles. This potential function of ARR2 might make signalling pathways and protein-protein interaction more specific. One way to unravel TCS dependent signalling and the physiological role of MAPK cascades would be possible with constitutive expression of non-phosphorylatable ARR2D80N (Hwang and Sheen 2001; Choi et al. 2010). This was not performed at this time, but similar experiments with the constitutive TCS active form ARR2D80E were attempted. Readout however was not possible as mutant lines complemented with ARR2D80E driven under a constitutively active promoter were lethal in embryonic stage (Hass et al. 2004). In this work, attempts to bypass constitutive expression of ARR2D80E using a β-estradiol inducible promoter only for a few days after treatment still showed huge developmental defects, early necrosis and most likely embryonic lethality (see Supplemental Figure 10).

Taken together, the protein-protein interaction data suggest involvement of ARR2 in a biotic stress direction concerning its association with biotic stress-related MKKs and MPKs, and in particular pathways mediated by MKK4/5/7/9. This crosstalk of ARR2 with MKKs and MPKs could be a conformation of the early presumptions that TCS and B-type response regulators per se play an important role in fine-tuning of different signals and in mediating crosstalk between different signalling pathways in aim of improving the fitness of plants for better adaptation within their natural ecosystems. Therefore, a connection to biotic stress and ARR1 and/or ARR2 was pursued in this thesis to provide a physiological basis for which a functional output could be eventually be assigned to. Provided that this is successful, cross-talk relationships between ARR1/2 and MKKs could be pursued.
Innate immunity is generally initiated with recognition of conserved pathogen-associated molecular patterns (PAMPs). As a first look into pathogen-related responses in \textit{ARR2}, wild-type and \textit{arr1-4}, \textit{arr2-4} and \textit{arr1-4 arr2-4} mutant lines were treated with PAMPs and as output, ROS and ethylene production was measured.

Indeed, ROS measurement triggered by flg22 and elf18 showed that \textit{arr1-4 arr2-4} double mutant significantly produced more ROS than the wild-type or single mutants in response to both applied PAMPs. Although there were differences between the single mutants, these observations were not consistent between experiments but the double mutant however consistently showed more ROS than the wild-type. This was first hint that \textit{ARR1} and \textit{ARR2} are enrolled in response to pathogen defence. Since the double mutant showed the strongest effect in ROS production only the \textit{arr1-4 arr2-4} double mutant and Col-0 wild-type were taken and tested against PAMPs with fungal origin (PEN, Xac) for ethylene production.

Double mutant here did not show any statistically significant difference in ethylene production to any of tested PAMPs compared to the wild-type. This approach only showed that there is no significant difference in ethylene production between the mutant and the wild-type but it doesn’t necessarily mean that the signalling pathways controlled by ethylene are not affected in the double mutant since differences in ROS production were observed. It could be that the differences in ethylene production between the double mutant and the wild-type are below the detection range of the machine used for ethylene measurements. Similarly, it could be that the ROS measurements were much more sensitive than those of ethylene.

Changes in ROS have been correlated with MKK and MPK activity (Pitzschke and Hirt 2009). Unfortunately, the lack of \textit{ARR1} and \textit{ARR2} did not cause any dramatic differences between the wild-type and the double mutant in MPK phosphorylation status in response to flg22 of pathogen-response related MPK4, MPK3 and MPK6. This does not exclude that actually \textit{ARR1} and \textit{ARR2} may serve here as MKK scaffold proteins which redirect and specify pathogen-related signalling pathways, but it does suggest the function of \textit{ARR1} and \textit{ARR2} does not regulate MKK kinase activity.

The data up to this point suggested that \textit{ARR2} (and other B-types) interact with MKKs and MPKs. The interaction with \textit{ARR2} in yeast-two-hybrid and \textit{in planta} was preferentially with MKK4 and MKK5, which are known to be involved in biotic stress. Furthermore, changes in ROS production with respect to flg22 were also observed although this could not be corroborated with ethylene production or MPK phosphorylation status. Recent publications about \textit{ARR2} and cytokinin have indicated a growing connection to pathogen stress (Choi \textit{et al.} 2010). Therefore, pathogen assays were performed on \textit{ARR1} and \textit{ARR2} single and double mutants. For these assays necrotrophic and biotrophic pathogens were taken since each has evolved differently leading to distinct defence strategies in plants and thus activation of different genes required for pathogenicity (Idnurm and Howlett 2001; Oliver and Ipcho 2004).
No any statistically significant differences between wild-type (Col-0) and the mutant lines were obtained using *Pst* DC3000 *when* applying it by leaf infiltration at $3 \times 10^6$ CFU. The same mutant lines were also twice challenged with *Pst* DC3000 in presence of $1\mu$M exogenous cytokinin (*t-zeatin*). No biologically significant differences were found either. These results are in contrast to data who published a weak *arr2-4* phenotype for resistance to *Pst* DC3000 at one log difference (Choi *et al.* 2010). The majority of their assays worked with overexpressor lines to show the effects of cytokinin and *ARR2* variants however. Considering that our data are of very high quality as they were reproduced at least 5 times, we conclude that *arr1-4*, *arr2-4* and *arr1- arr2-4* do not confer any significant resistance to *Pst* DC3000 infection.

*Peronospora* is known to activate defence pathways via salicylic acid (SA) accumulation, which is a general characteristic of biotrophic pathogens (Glazebrook 2005), and independently of SA via *RPPs* (*RECOGNITION OF PERONOSPORA PARASITICA*) genes, especially via *RPP7*. *RPPs* represent R (resistance) genes, genes responsible for gene-dependent defence activation in *Arabidopsis* (Mcdowell *et al.* 2000). Here seems that *ARR1* and *ARR2* are not involved in these signalling pathways or that the other B-types work in a redundant fashion no evident from the yeast-two-hybrid data.

It could be postulated that the defence response could depend on stomata developmental/response defects. This is because *ARR2* has been described to be involved in mediating stomatal closure in an H$_2$O$_2$-dependent manner (Desikan *et al.* 2006; Mira-Rodado *et al.* 2012) and that *MKK4* and *MKK5* are known to be involved in stomatal patterning (Lampard *et al.* 2009). Such same effects should be seen by spraying the bacteria onto plants. Stomata aperture was measured in mutant backgrounds and the wild-type treated with hormones (ABA and cytokinin) and after *Pst* DC3000 application. Although the wild-type and mutants closed in response to ABA as previously described (Desikan *et al.* 2006), wild-type and mutants also responded equally to cytokinin application or *Pst* DC3000 challenge. In fact, stomata in all the plant lines treated with *Pst* DC3000 were more open than non-treated control consistent with the release of diffusible phytotoxin COR by the bacteria which reopen closed stomata to increase the number of bacterial invasion sites (Melotto *et al.* 2008). Stomata aperture measurements were done 3 days after pathogen application which was more than enough to cause reopening of stomata. Here was shown that *ARR1* and *ARR2* are not any how involved in mechanism for recognition of phytotoxin COR and in this case they are not part of apparatus for regulation of stomata aperture. Therefore under our conditions and experimental approach we could not confirm the previously published observation that cytokinin modulates SA signalling by augmenting resistance against *Pst* DC3000, a process in which the SA/cytokinin-dependent interaction TGA3 and *ARR2* is important (Choi *et al.* 2010). This discrepancy, despite the performed experiment where stomata aperture was measured and also no difference obtained, may still be due to differences in infection conditions.

Interestingly, cytokinin caused the stomata to open in the wild-type and the *arr1/2* mutant lines. This means, that although there is a lack of two cytokinin responsive (Hill *et al.* 2013), type-B they are not crucial for stomatal aperture responses. This indicates that this response
is more than likely redundantly controlled by other B-types. On the other hand, the strong effects of previously reported for \textit{arr2-4} on H$_2$O$_2$-dependent closure point towards explaining MKK4/5/9 cross-talk by the fact that ARR2 interacts with more MKKs than does all the other type-B ARRs.

Generally speaking, JA-dependent signalling pathways play a crucial role in defence against necrotrophic pathogens (Glazebrook 2005). Therefore assays done with nectrotrophic pathogens were performed in order to see if there could be a defect in regulation of jasmonic acid (JA)-dependent signalling pathways. The \textit{arr2-4} single mutant and \textit{arr1-4 arr2-4} double mutant but not \textit{arr1-4} single mutant, showed enhanced susceptibility to the necrotrophic fungus \textit{Alternaria brassicicola} compared to the wild-type. This experiment strongly suggested that, despite their high homology, \textit{ARR2} but not \textit{ARR1}, is the only one of the two involved in signalling pathways responsible for defence against \textit{Alternaria}. It is known that COI1 (CORONATINE INSENSITIVE 1), COI1-dependent genes and camalexin biosynthesis-dependent genes are primarily responsible for its defence against \textit{Alternaria brassicicola} (Van Wees \textit{et al}. 2003). In almost all \textit{Arabidopsis} mutants that show susceptibility to \textit{Alternaria}, a perfect correlation has been observed between camalexin deficiency after \textit{A. brassicicola} infection and susceptibility (Van Wees \textit{et al}. 2003). However, it is known that even though infection by \textit{A. brassicicola} and infection by \textit{P. syringae} are quite different stimuli, genes under COI1 control are common for plant resistance against both pathogens but this is not the case with camalexin biosynthesis-dependent genes (Van Wees \textit{et al}. 2003; Jones and Dangl 2006). Based on the data obtained with \textit{arr2-4} mutants, \textit{ARR2} is more likely involved in camalexin biosynthesis pathways where it might positively regulate its synthesis. Furthermore, \textit{ARR2} interacted with MKK3 further strengthening a role of \textit{ARR2} in the MKK3-MPK6 JA signal transduction. How these two are intertwined is yet unclear at this point.

The effects of the necrotrophic fungus \textit{Botrytis cinerea} was challenged on \textit{arr1/2}. This pathogen is important for two reasons: \textit{Botrytis cinerea} is known to activate distinct signalling pathways from \textit{Alternaria} and more importantly, \textit{Arabidopsis thaliana} is natural host of \textit{Botrytis cinerea}. Based on measured disease index parameters, the \textit{arr1-4 arr2-4} double mutant clearly showed resistance to \textit{Botrytis} compared to the wild-type and an additive effect compared to the single mutant parents. That is, the double mutant twice as less lesions (~30%) than the wild-type (~60%) lesions greater for equal to 6 mm (≥6 mm). Both single mutants showed the same resistance tendency and were statistically different from the wild-type. It was also observed that \textit{arr2-4} single mutant had a slight, non-significant tendency to be more resistant than the \textit{arr1-4} single mutant: it had 40% of lesions ≥6 mm whereas the \textit{arr1-4} had only 45%. Again this favours the role of \textit{ARR2} compared to \textit{ARR1} with respect to resistance against necrotrophic pathogens.

The common marker genes \textit{PR-1} and \textit{PDF1.2} were tested from the pooled material from the \textit{Botrytis cinerea} challenged \textit{Arabidopsis} plants. Despite the fact that \textit{PR-1} is rather SA marker and not suitable for infection with necrotrophs, expression of \textit{PR-1} was induced 36 hrs post infection, 12 hrs earlier than the wild-type. This expression was maintained at the same level
in the mutants one day later whereas PR-1 expression in the wild-type was strictly upregulated in the wild-type at 48 hrs. This would imply that the arr1/2 caused early induction of PR-1 in response to Botrytis infection. Based on the current models, the expression level of PDF1.2 should be increased in the mutant plants compared to the wild-type knowing the role of PDF1.2 as JA marker and is upregulated in response to necrotroph infection (Seo et al. 2001; Qi et al. 2012; Lu et al. 2013). Although PDF1.2 was induced in response to Botrytis infection as expected in all samples, it was not as highly induced in the mutants compared to the wild-type. Thus, the expression of this gene cannot be correlated with the mutant resistance phenotype. These results present a conundrum as to the mechanism of resistance to Botrytis. On one hand, we expected that PDF1.2 expression to be increased in the mutant plants, however dampened expression kinetics were observed. On the other hand, PR-1 was upregulated at an earlier time in the mutant plants but did not reach the same maximum as the wild-type at 48 hrs. Therefore, it is unclear if these two markers really can be correlated with the resistance phenotype of arr1/2. It is known that despite the wide use of PR-1 and PDF1.2 as markers for the induction SA- and JA-dependent pathogen pathways, the levels of both PR-1 and PDF1.2 expression do not clearly correlate with the level of susceptibility to Botrytis cinerea (Ferrari et al. 2003). So the observed differences in expression of these genes in the arr1/2 mutants are more a reflection of the current molecular status in the plants after infection, that is, it definitely showed that the current status was of plants under pathogen attack.

The dramatic difference in resistance between Botrytis cinerea and Alternaria brassicicola could be explained by the very wide, naturally variable resistance to necrotrophic plant pathogens which appears to be quantitative and polygenic (Micic et al. 2004; Rowe and Kliebenstein 2008). This tendency is exactly visible in the arr1-4 arr2-4 double mutant when the same mutants tested against two different necrotrophic pathogens showed completely opposite phenotypes. Furthermore, all of the parameters very clearly showed the redundancy (additive effect) of the ARR1 and ARR2 in respect to defence against Botrytis but not Alternaria, of which arr2-4 played the major role. In addition, the nature and extent of isolate-specific interaction between plants and necrotrophic pathogens is relatively unknown, and no qualitative naturally variable resistance genes effective against necrotrophic pathogens have been yet described (Jones and Dangl 2006; Rowe and Kliebenstein 2008). Provocatively, infection by the fungal pathogen Phytophthora infestans led to the rapid transcriptional induction of MKKK19, MKK9 and MKK4, while Botrytis cinerea infection led to the rapid transcriptional induction of completely different genes MKKK18, 19 and 20, Raf43, ZIK2, 8 (Menges et al. 2008). Although no direct genetic or transgenic connection has been shown between ARR1/2 and MKKs, a role for ARR1/2 is more than likely evaluable in necrotrophic but not biotrophic pathogen resistance as neither Pst DC3000 nor Peronospora parasitica differentially infected arr1/2 compared to their wild-type control. There are also reports that have shown signalling responses to bacterial and fungal pathogen attack are distinct, i.e. different genes are induced, especially with regard to MAPK cascades. It is still open if differences in resistance of arr1/2 mutants when tested against distinct pathogens is
correlated with MKK/type-B ARR crosstalk. If valid, the role of TCS-dependent phosphorylation, at least for protein-protein interaction is not required (so far judged by yeast-two-hybrid assays). In addition, arr1/2 mutants did not change the phosphorylation level of MPK3/4/6 in response to flg22. Therefore, regulation of MKK activity also does not appear to be mediated by type-B ARRs, although it was not tested if modulating the TCS-dependent phosphorylation state of ARR2 could influence this. It is therefore proposed that the proposed protein-protein crosstalk mediates protein target networks dynamics, i.e. their interaction affects binding/signalling kinetics of the two systems without actually controlling the physical TCS or MAPK cascade phosphorylation mechanism. As both the TCS and MAPK cascades target gene expression, the interaction of the two networks would modify their own information flow leading to changes in output gene expression similar to what has been observed for a TCS and MAPK cascade in yeast (Xu and West 1999).
4.4 References for Chapter 1


5 Chapter 2

**ARR1** and **ARR2** are involved in flowering time regulation of *Arabidopsis thaliana* under short days

### 5.1 Introduction
Flowering is a crucial step in plant development and it is characterised as a transition from vegetative to reproductive phase (Koornneef *et al.* 1998). Due to its importance of ensuring reproductive success, flowering is controlled by complex networks of genetic regulatory pathways (Coupland 1995; Srikanth and Schmid 2011; Poethig 2013). In the end, successful reproduction not only includes seed formation but also dispersion, germination and growth and complex regulatory mechanisms need to integrate both environmental and endogenous signals to ensure the next generation can also reproduce (Bernier *et al.* 1993; Koornneef *et al.* 1998; Srikanth and Schmid 2011). However, it is clear today that all these signalling pathways that control flowering are not strictly separated but that they work together, *i.e.* crosstalk in the regulation of flowering (Genoud and Metraux 1999; Franklin 2009). Surprisingly the number of common targets regulated by huge number of signalling pathways is quite small and they present central floral pathway integrator or “integrator genes” (Li *et al.* 2008; Srikanth and Schmid 2011). Even in self-fertile plants, flowering time is still strictly controlled by environmental and endogenous factors (Srikanth and Schmid 2011). Exogenous factors that strongly influence the flowering are day length, light quality and temperature (Coupland 1995; Reeves and Coupland 2000; Chen *et al.* 2004). On the other side, common endogenous factors that are regulating flowering are hormones (hormonal status of the plant), sugars and plant age (Blazquez *et al.* 1998; Wahl *et al.* 2013). Five genetic pathways have been identified that play role in control of flowering: the vernalization (originally jarovization) pathway, the photoperiod pathway (related to the day length and quality of perceived light), the gibberellin (GA) pathway, the autonomous pathway (endogenously controlled independent of photoperiod or gibberellin pathways), and plant age (also an endogenous pathway) (Srikanth and Schmid 2011).

#### 5.1.1 Photoperiod-dependent flowering control
Photoperiod pathways present a cascade of events in charge for measurement day length and consequently initiating flowering (Valverde *et al.* 2004; Sawa *et al.* 2007). The organ for photoperiod perception is located exclusively in the leaves. Light perception in leaves is enabled by the presence of three main classes of photoreceptors specialized for different wavelengths: phototropins (blue light), phytochromes (red/far red light) and cryptochromes (blue light) (Franklin *et al.* 2005; Lariguet and Dunand 2005). Once a particular type of light-signal is perceived (varies between species) the flower-triggered substance called “florigen” is
produced and afterwards transmitted to the shoot apex to induce flowering (Colasanti and Sundaresan 2000). Even before the era of molecular biology had begun, it was very well known that plants measure and respond to the day length (Srikanth and Schmid 2011). This is due to the presence of the internal oscillators, genes that are regulated by the circadian clock (Harmer et al. 2000). The circadian clock is explained as a timekeeping mechanism in photoperiodism (Mizoguchi et al. 2005).

5.1.2 Photoperiod-dependent flowering control in *Arabidopsis thaliana*

In *Arabidopsis thaliana*, a circadian clock controlled flowering pathway includes the following gene members GIGANTEA (GI), CONSTANS (CO) and FLOWERING LOCUS T (FT) that promote flowering specifically under long days (Fowler et al. 1999; Mizoguchi et al. 2005). Briefly, the circadian clock in *Arabidopsis thaliana* is composed and works as follows: The central oscillator generates a self-sustaining rhythm driven by two interacting feedback loops that are active at different times of day. The first one so called “morning loop” consists of CCA1 (CIRCADIAN AND CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), which encode closely related MYB transcription factors that regulate circadian rhythms in *Arabidopsis*, as well as PRR 7 and 9 (Pseudo-Response Regulators). The second “evening loop” consists of GI (GIGANTEA) and ELF4 (EARLY FLOWERING 4), both are involved in regulation of flowering time genes (Fowler et al. 1999; Mizoguchi et al. 2005; Kolmos and Davis 2007).

Classification of plants due to their photoperiodic responses is usually based on flowering. The two main photoperiodic response categories are short-day and long day-plants (Garner 1933). Short-day plants (SDPs) flower in short days (qualitative SDPs) or their flowering is accelerated by short days (quantitative SDPs). Long-day plants (LDPs) flower only in long days (qualitative LDPs) or their flowering is accelerated by long days (quantitative LDPs). The essential distinction between long-day and short-day plants is that flowering in LDP is promoted only when the day length exceeds a certain duration. This time course is called critical day length. To flower SDPs require a day length less than a critical day length (Yano et al. 2001; Kojima et al. 2002). Plants that flower under any photoperiodic condition are referred to as day-neutral plants (DNPs) (Wu et al. 2004; Lifschitz et al. 2006); examples are most of the desert annuals that flower quickly whenever the water is available. *Arabidopsis thaliana* is a facultative LDP, i.e. quantitative LDP means it flowers also under SDs but its flowering is accelerated by inductive long days (Hicks et al. 1996). Important to photoperiodic flowering is the so-called phenomenon “coincidence model” proposed in the mid-1930s by the German botanist Professor Erwin Bünning which brings together endogenous and exogenous signals. The circadian oscillator controls the timing of light-sensitive and light-insensitive phases only when the light signal is coincident with the appropriate phase of the circadian rhythm thereby allowing flowering induction under both non-inductive and inductive days (Srikanth and Schmid 2011). The first mutant discovered in *Arabidopsis thaliana* incapable of a photoperiodic flowering response was co mutant: a mutant lacking in CO (CONSTANS) which encodes a zinc finger transcription factor family protein which is controlling expression of other floral regulators (Putterill et al. 1995; Tran et al. 2007). The co
mutant has a very strong late flowering phenotype under inductive LD conditions only whereas at non-inductive SD, co flowers identical like the wild-type plant suggesting the CO to be a flower activator (Putterill et al. 1995). It was later discovered that the expression CO is regulated by the circadian clock with pick expression of CO around dusk (Suarez-Lopez et al. 2001). Expression pattern of CO represents an example of "coincidence model". The CO proteins levels are also controlled however and CO only accumulates enough to promote flowering under LD when the light phase overlaps/coincides with the maximal pick of the CO mRNA (Valverde et al. 2004; Bohlenius et al. 2006). Research has shown that CO is posttranscriptional regulated and that during the dark phase the protein is tagged for degradation by ubiquitin and afterwards degraded in 26s proteasome and part of this is controlled by different photoreceptors (Valverde et al. 2004). It has been shown that PhyB signalling in the morning negatively regulates CO whereas PhyA and cryptochromes positively regulate the accumulation of CO protein in the evening (Lin 2000; Endo et al. 2013). This entire regulation of CO happens only in leaves (An et al. 2004; Ayre and Turgeon 2004), i.e. it does not transfer signal from the leaves to the shoots. Further downstream CO activates expression of other flowering-related genes which are transferring the signal information to the apical meristem and initiating flowering. One of them is FT (FLOWERING LOCUS T) gene (Kardailsky et al. 1999; Hisamatsu and King 2008). FT belongs to a small group of proteins that show structural similarities to mammalian phosphatidylethanolamine-binding protein (Ahn et al. 2006; Kim et al. 2013). Five more members belong to this family: TSF (TWIN SISTER OF FT), MFT (MOTHER OF FT AND TFL1), BFT (BROTHER OF FT AND TFL1), ATC (ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUES) and their major role is the regulation of photoperiodic flowering (Wigge 2011; Pin and Nilsson 2012; Kim et al. 2013). In the shoot apical meristem of Arabidopsis thaliana it seems that the FT protein, but not FT mRNA (Notaguchi et al. 2008), is needed to induce flowering, although there are some indications that FT mRNA could also be contributing flower induction but only together with its protein form (Li et al. 2009). In the shoot apex FT makes a complex with FD (Abe et al. 2005; Wigge et al. 2005). FD belongs to the bZIP transcription factor family and it is preferentially expressed in the shoot apex and required there for FT to promote flowering (Abe et al. 2005). This complex afterwards activates downstream targets such as SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) (Searle et al. 2006), APETELA 1 (AP1) (Wigge et al. 2005) and indirectly LFY (LEAFY) (Abe et al. 2005; Smith et al. 2011). SOC1 is floral activator required for CO to promote flowering, it acts downstream of FT (Yoo et al. 2005), AP1 and LFY are meristem identity genes which generally promote the transition to flowering by further induction of floral homeotic genes (Yu et al. 2004).

5.1.3 Photoperiod-dependent flowering control in rice and other plants

The coincidence model is also present within neutral and short day plants like in tomato (Solanum lycopersicum) or rice (Oryza sativa), respectively. In tomato, the orthologue of FT is SFT (SINGLE-FLOWER TRUSS) and it is a floral activator just like in Arabidopsis (Lifschitz et al. 2006). Besides its commercial value and, in contrast to Arabidopsis, Oryza sativa belongs to the group of short day plants (Yano et al. 2001; Kojima et al. 2002). The rice genes Hd1
(Heading-date 1) and Hd3a (Heading-date 3a) encode homologs to Arabidopsis CO and FT respectively. Like CO in Arabidopsis, Hd1 has an identical expression pattern in rice (Kojima et al. 2002) with only difference between rice and Arabidopsis is that under short day conditions Hd1 acts as repressor of Hd3a expression. That is the reason why is the flowering in rice, in contrast to Arabidopsis, actually inhibited by coincidence of light and the Hd1 expression; when the light overlaps the maximum expression pick of Hd1 mRNA it blocks afterwards expression of Hd3a and inhibits the flowering. These three examples showed that CO-FT regulatory module is conserved among plants pointing its importance and differences in flowering time are most likely mediated through different strategies for regulating this module (Srikanth and Schmid 2011). Summed together, photoperiodism is a very complex mechanism and it is highly controlled and fine-tuned by different regulatory elements which impend on key, evolutionarily conserved genetic networks (Hayama and Coupland 2004; Franklin et al. 2005; Imaizumi and Kay 2006).

5.1.4 Connection between two-component system and flowering regulation in rice and Arabidopsis

Interestingly, in rice, FT-like gene expression can be controlled independently of Hd1 by the so-called Ehd1 (Early heading Date 1) gene encoding a B-type response regulator, part of two-component signal (TCS) transduction systems (Doi et al. 2004) suggesting an important role of TCS in regulating flowering. Ehd1 functions as a floral inducer and it induces FT-like and MADS box genes (e.g. the AP1 ortholog in Arabidopsis) (Doi et al. 2004).

TCS systems typically transduce a receptor-mediated signal into a phosphorelay from a histidine-kinase onto a conserved aspartate residue of a response regulator (RR) protein. In Arabidopsis, there are response regulator proteins that have a mutation in this conserved aspartate (Asp) residue: these proteins are called pseudo response regulators (pseudo-RRs) (Doi et al. 2004). It has been already proven that pseudo-RRs regulate flowering time in Arabidopsis thaliana and that TOC1/APRR1, an Arabidopsis pseudo response regulator, is part of the plant circadian clock. TOC1/APRR1 belongs to a small gene family in Arabidopsis (Matsushika et al. 2000) and it influences flowering time via the phase setting of CO expression (Yanovsky and Kay 2002). Even though pseudo-RRs have lost their ability to accept the phosphorelay phosphate as they are constitutively active because the highly conserved Asp is mutated to a glutamate naturally mimicking a phosphorylated Asp (Doi et al. 2004).

In Arabidopsis thaliana, ARR4 (an A-type response regulator) plays a very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007). PhyB, as above mentioned, is actively involved in expression control of CO in Arabidopsis thaliana and also Hd3a in Oryza sativa.

On the other hand it has been proven in various ways that B-Type response regulators are directly, positively regulating expression of the A-Type response regulators in Arabidopsis
In addition to this, it was recently shown that floral regulator SVP (SHORT VEGETATIVE PHASE) is directly regulating A-type response regulators as well as CRFs (CYTOKININ RESPONSE FACTORS) (Gregis et al. 2013) was well as directly binding to STIP (STYMPY) newly reported component of cytokinin signalling pathway mediating cytokinin signalling during shoot meristem establishment in Arabidopsis thaliana (Skylar et al. 2010). Furthermore, it was also recently reported that cytokinin is influencing flowering time in Arabidopsis, especially under non inductive short days (Bernier 2011; D’aloia et al. 2011). B-type response regulators are being discovered to be involved in fine-tuning and crosstalk of a multitude of signalling pathways in higher plants (Veerabagu et al. 2012; Zwick and Rashotte 2013). For example, ARR2, a member of B-type response regulators, is part of TCS signalling important for signal transmission of cytokinin and ethylene (detail about TCS signalling and members can be found in the introduction of the first chapter) (Rashotte et al. 2003; Grefen and Harter 2004; Hass et al. 2004; Mason et al. 2005; Zwick and Rashotte 2013). Despite the great sequence similarity of the B-types within the same subgroup they still show very high specificity among each other (Hill et al. 2013). The arr2 loss-of-function mutant plant in Landsberg displayed retarded growth and development including early flowering (Hass et al. 2004). ARR2 was also reported to function in ethylene signalling (Hass et al. 2004) and in response to pathogen attack (Choi et al. 2010) further demonstrating that B-types can be involved in multiple signalling pathways.

It was discovered that, when applied in hydroponic culture from the roots, in SDs, cytokinin bypasses FT and activates transcription of TSF in leaves to induce flowering (D’aloia et al. 2011). Furthermore, TSF interacts with FD and regulates expression of SOC1 which also seems to be controlled by cytokinin (D’aloia et al. 2011). On the other hand TSF and SOC1 also work independently of FD suggesting that FD and its paralogue FDP (FD PARALOG) (Abe et al. 2005) might share this function of SOC1 control (Bernier 2011; D’aloia et al. 2011). Cytokinin itself cannot induce flowering (Bernier 2011) but the fact that when cytokinin was added to roots of hydroponic-grown Arabidopsis plant was sufficient to induce flowering in SDs and that the cytokinin contents of leaves, phloem sap, and SAM (SHOOT APICAL MERISTEM) increased in response to LDs induction in Arabidopsis (Bernier 2011; D’aloia et al. 2011). In Sinapis cytokinin level is also increased in response to LDs induction (Corbesier et al., 2003) and it was reported co-dependent transportation of SaFT and cytokinin to the meristem suggests that cytokinin may play a part to regulatory effects attributed to “florigen” (Bernier 2011). However, biological functions of Arabidopsis B-type response regulators with respect to flowering have not yet been identified.

5.1.5 Temperature as floral regulator

Another factor, beside photoperiod, that is important in the regulation of flowering is temperature (Blazquez et al. 2003). Flowering can be controlled by temperature in two manners: by the process called vernalisation and also by ambient temperatures under which plants exposed to during their vegetative phase (Alono-Blanco and Koornneef 2000; Koornneef et al. 2004).
5.1.5.1 Vernalisation

Vernalization is a process by which flowering is promoted when plants sense exposure to the prolonged cold temperatures (≤7°C). This is an adaptive trait that helps prevent flowering before winter is over thus permitting flowering in the favourable conditions of spring (Kim et al. 2009). In winter annual biennial and perennial plants, vernalization is an obligate process, whereas for summer annuals vernalization is facultative. For vernalization to work the temperature has to be in range between 1 and 7°C for a time of 1 to 3 months depending on the plant species (Srikanth and Schmid 2011). In Arabidopsis, vernalization involves the recruitment of chromatin-modifying complexes to a clade of flowering repressors that are silenced epigenetically via histone modifications (Kim et al. 2009). This effect is known as “memory of winter” and it does not pass to the progeny but is reset during meiosis (Amasino 2004; Heo and Sung 2011). In Arabidopsis and cereals, vernalization results in the suppression of genes that repress flowering (Kim et al. 2009). Interestingly, in Arabidopsis, the most commonly used laboratory strains do not need to be vernalized, but some do flower very late if they are not vernalized first (Reeves et al. 2007; Srikanth and Schmid 2011). There are two dominant genes for vernalization in Arabidopsis thaliana: FRI (FRIGIDA) and FLC (FLOWERING LOCUS C) (Lee and Amasino 1995; Song et al. 2012) whereby FRI up-regulates the expression of FLC (Geraldo et al. 2009). FRI encodes a nuclear protein found only in plants (Johanson et al. 2000; Bari and Jones 2009) and FLC encodes a MADS-box DNA binding protein that functions as a repressor of flowering, i.e. flowering-time related genes (Deng et al. 2011). FLC represses expression of FT, FD, and SOC1 floral activators by direct interaction with their promoters (Hepworth et al. 2002; Helliwell et al. 2006). It has been proven that FLC also directly interacts with another MAD-box member, SVP which is also floral repressor that functions within the thermosensory pathway (Li et al. 2008). This interaction makes physiological and biological sense because it partially suppresses FLC-mediated delay in flowering (Li et al. 2008; Bari and Jones 2009; Yoshida et al. 2009). Briefly, FLC is silenced in response to vernalization, after FLC been silenced VIN3 (VERNALIZATION INSENSITIVE 3) initially repressing FLC during the cold exposure by remodelling the chromation, i.e. by methylating lysine residues of histone H3 (Sung and Amasino 2004; Bond et al. 2009) than at least two crucial genes VRN1 and VRN2 (VERNALIZATION 1 and 2) maintain the epigenetic state of FLC (Bastow et al. 2004). Vernalization as a phenomenon is probably result of convergent evolution as outside of Brassicaceae no clear orthologs of FLC has been identified, making this a complicated research field (Srikanth and Schmid 2011).

5.1.5.1.1 Ambient temperature

Another factor that controls flowering is ambient temperature during its vegetative stage. Higher temperatures (27°C) accelerate flowering under non-inductive SDs for different Arabidopsis accessions serving as a substitute to inductive LDs (Balasubramanian et al. 2006). Ambient temperature effects on flowering are very diverse among different species, and even between different accessions of Arabidopsis thaliana. Many flowering time mutants also show temperature dependence, i.e. when exposed to higher temperatures they flowered earlier (e.g. photoreceptor mutants phyB, cry2 and most Arabidopsis fri/flc natural accessions)
(Blazquez et al. 2003). Nevertheless, this is not the case when Nd-1 or svp were tested. Nd-1 accession mutant showed masked effect when grown at 27°C (Balasubramanian et al. 2006) as this mutant was shown to have a deletion of the floral repressor FLM (FLOWERING LOCUS/MADS AFFECTING FLOWERING1) as a major cause for early flowering of Nd-1 in short days at 23 °C (Werner et al. 2005; Balasubramanian et al. 2006).

FLM is like FLC a MADS-box protein and shares strong sequence similarity with FLC but despite this their physiological roles seem to be different (Scortecci et al. 2003). Microarray data showed that genes associated with alternative splice site selection are affected by thermal induction and it has been also proven that FLM has temperature-dependent alternative splicing and this splicing is an important regulator of flowering. (Werner et al. 2005; Srikanth and Schmid 2011) In the Waissilewskija ecotype FLM gene has four splice variants (α, β, γ and δ) whereas in Columbia (Col-0) accession FLM has two splice variants (β and δ) and they are both translated (Lee et al. 2013; Pose et al. 2013). FLM-β is the prevalent splice variant at lower temperatures (16°C) whereas FLM-δ dominates at higher temperatures (27°C) (Lee et al. 2013; Pose et al. 2013). Both splice variants are interact and make heterodimers with SVP independent of the transcriptional level which does not change with variation in temperature. Interestingly, they can also interact among each other making FLM-β x FLM-δ heterodimers but only FLM-β x FLM-β homodimers are possible (Lee et al. 2013; Pose et al. 2013). Proposed model of activity says that dependent of which splice variant β or δ is making heterodimers with SVP determinates later the activity of SVP-FLM heterocomplex (Lee et al. 2013). At low ambient temperatures formation of SVP-SVP and SVP-FLM-β complexes is favoured and they both repressing flowering. As the temperature is rising the FLM-β splice form is down-regulated and therefore SVP-FLM-δ complex formation is increased and it now accelerates flowering. Based on this model the role of temperature-dependent mRNA splicing in adaptation to climate change is observed and explained (Pose et al. 2013). Other known genes which are involved in regulating flowering in response to ambient temperatures are HSP70 (HEAT SHOCK PROTEIN 70) found to be highly correlated with an increase in temperature (Balasubramanian et al. 2006; Kumar and Wigge 2010), and also ARP6 (ACTIN RELATED PROTEIN 6) which is known to be a nuclear protein that represses flowering as maintaining the expression of FLC (Choi et al. 2005; Deal et al. 2005).

5.1.6 The autonomous pathway
Beside the endogenous hormonal factors that are influencing flowering it is important to mention that autonomous pathways are defined as those that delay flowering irrespective of day length (Mouradov et al. 2002). Genes involved in autonomous pathway can be grouped into two broad functional categories of genes that act by repressing FLC; these are: general remodelling and maintenance factors of FLC, and proteins that effect RNA processing (He et al. 2003; Srikanth and Schmid 2011).

5.1.7 Gibberellic acid pathway as a regulator of flowering
One of most potent endogenous control of flowering is hormonal control regulated by the gibberellic acid (GA) pathway (Blazquez et al. 1998). The effect of gibberellic acid on plant
growth and development has been known since the beginning of the last century when the rice plants infected with fungus *Gibberella fujikuroi* were growing so fast that they tipped over (Tudzynski 1999; Hsuan *et al.* 2011). Other developmental roles of GA, like its influence on flowering time, became familiar much later. Numerous GAs were discovered in plants but they are not all of them are biologically active; the active ones are GA1, GA3, GA4 and GA7 named according the order of their discovery (Hedden and Phillips 2000). After huge mutagenesis screenings for mutants that affect GA biosynthesis were detected. These mutants showed, beside various developmental defects, delay of flowering by *Arabidopsis* mostly under the non-inductive SDs (Wilson *et al.* 1992). The ga1-3 mutant, lacking the gene for synthesis of ent-kaurene needed for GA1 formation (Sun *et al.* 1992) showed under inductive LDs almost normal flowering pattern whereas it was not able to flower at all at SDs, even when previously treated with exogenous GAs. Nevertheless, this flowering effect of GA is not strictly limited to SDs as demonstrated by the triple gid1 mutant (lacking in all three *GID-GIBBERELLIC INSENSITIVE DWARF* receptors for GA). The gid1 mutant flowers extremely late or not even at all under inductive LDs (Griffiths *et al.* 2006; Willige *et al.* 2007). Interestingly, GA works in parallel (i.e. independently) of CO as the ga1-3 co double mutant showed an additive late-flowering phenotype. of co under LD but on the other hand the levels of *FT* mRNA expression were increased after addition of exogenous GAs suggesting that GA are on some other way independently controlling expression of *FT* (Hisamatsu and King 2008). It was later shown that *FT* expression is controlled by GA through interaction of the GID1 receptor with members of the DELLA protein family, totally independent of CO (Sun 2010). DELLA proteins belong to the GRAS family of transcriptional regulators and work as repressors of plant growth and development (Hirsch and Oldroyd 2009).

### 5.1.8 Other factors affecting flowering

Other important endogenous factors that are promoting flowering are sugars (Bernier *et al.* 1993). It is known that sucrose can promote flowering in some plant species but this effect might be rather species-specific (Srikanth and Schmid 2011). Trehalose is another sugar that promotes flowering (Schuepmann *et al.* 2003). Furthermore, T6P (trehalose-6-phosphate) was proven to be a proxy for carbohydrate status in plants and that it is absolutely essential for expression of *FT* and *TSF* in the phloem companion cells (Wahl *et al.* 2013). The age of the plant was recently reported to play a role in the regulation in flowering time independent of photoperiod, vernalization or GA pathways, and instead works via the *miR156* microRNA level which decreases with increasing age of the plant (Wang *et al.* 2009). In this work an early flowering phenotype of *ARR1* and *ARR2* lacking mutants (*arr1-4, arr2-4* single and *arr1-4 arr2-4* double mutants) under SD was further investigated. This is the first time that B-type response regulators in *Arabidopsis* (LD facultative plant) have been shown to regulate flowering. TCS and probably cytokinin were suggested to play roles as regulators of flowering. Further *ARR1* and *ARR2* are acting independent of *FLC* floral repressor but therefore *FLM* floral repressor seems to be epistatic to both *ARR1* and *ARR2* response regulators.
5.2 Results

5.2.1 ARR2 mutants show early flowering phenotype under short day (SD) conditions

Previous work on ARR2 had been conducted with the arr2-1 to arr2-4 mutant allele in the Ler-0 ecotype (Hass et al. 2004). Work on this mutant showed connections between the two-component signalling pathway, genes related to defence and abiotic stress signalling and adaptation and ethylene signalling (Hass et al. 2004). In order to investigate further roles of ARR2 with its closest homolog ARR1, a mutant of ARR2 was obtained in the Col-0 background. This mutant, and a mutant of ARR1, arr1-4, also in the Col-0 background, were shown to be full knock-outs. Interestingly, arr1-4, arr2-4 single mutants and the arr1-4 arr2-4 double mutant displayed precocious flowering under non-inductive short day (SD) conditions, with the double mutant having an additive effect (Figure 5.1).

Despite the high sequence similarity and reported functional redundancy of ARR1 and ARR2 in regulating expression of certain common targets in response to cytokinin signal (Mason et al. 2005), the loss of ARR1 and ARR2 affects flowering time to a different degree.

The single arr1-4 and arr2-4 mutants flower one and two weeks earlier than wild-type control plants, respectively (Figure 5.1, B). In contrast, the arr1-4 arr2-4 double mutant flowers three weeks before the Col-0 control (Figure 5.1, B). The early flowering phenotype can be also observed as a reduction of rosette leaf number. The arr1-4 mutant induces flowering by more than 4 leaves, arr2-4 mutant by more than 7 leaves, while arr1-4 arr2-4 produces 15 leaves less than Col-0 (Figure 5.1, B). A trend towards early flowering was also observed when the arr1/2 mutants were grown under inductive long day (LD) conditions. However, only the arr1-4 arr2-4 double mutant flowered significantly earlier than wild-type (p= 0.0052; α=0.05) and produced 3 leaves less than the wild-type (Figure 5.1, C). In conclusion, the loss of ARR1, ARR2, or both genes simultaneously, resulted in early flowering under SD conditions. As ARR1 and ARR2 are known B-Type transcription factors, some of known flowering time-related genes (CO, SOC1, AP1, FT, FLC, FLM, TSF, TFL, LFY and FD) were profiled for alterations in expression patterns in the various mutants under short day and long day conditions.
Figure 5.1 Novel early flowering phenotype of arr1/2 mutants.
Photographs illustrating the phenotypes of wild-type (Col-0) and arr1-4, arr2-4 single mutants and arr1-4 arr2-4 double mutant grown under LDs (left panel) and SDs (right panel). Differences in Days Until Flowering (DUF) and number of Rosette Leaf Number (RLN). ‡Significance differences (α=0.05) with the wild-type were determined by LSD (Fischer’s Least Significant Differences) after the data passed one-way ANOVA (α=0.05). Each day length experiment was repeated twice with similar results. Flowering time data of the mutants grown under SDs (B) and LDs (C). DUF, days until flowering; RLN, rosette leaf number; n, number of individuals.

5.2.2 Lack of the activity of ARR1/ARR2 genes causes down-regulation of specific floral repressors

In order to identify which flowering time pathways are disturbed in the arr1-4, arr2-4 and arr1-4 arr2-4 mutant lines, key flowering loci were tested for changes in expression with respect to Col-0. Gene expression data quantified by RT-qPCR was firstly obtained from leaves of 70-days-old SD-grown plants shortly before the arr1-4 arr2-4 double mutant, which displays the strongest acceleration in flowering, entered the reproductive phase. In agreement with the early flowering, expression of the floral repressors FLC and FLM were 2-fold and 4-fold reduced in leaves, respectively (Figure 5.2, A). In contrast, expression of the known floral repressor SVP was not affected. Similarly, expression if the floral activators CO, SOC1 and FT were also not affected with only FD showing a slight up-regulation (Figure 5.2, B).
B). Based on these observations the \textit{arr1-4 arr2-4} double mutant causes a depression of floral repressors in leaf and the key LD inductive-pathway genes are not perturbed. Genes that are predominantly expressed at the meristem are also known to have very important roles in flowering initiation, especially in early flowering under short days (Koornneef et al. 1998). Therefore, some key meristem-localized genes were examined with the aim of profiling the state of the SAM from 70-day-old SD-grown plants.

Figure 5.2 The transcript levels of the flowering time pathway-specific marker genes in the wild-type and \textit{arr1-4 arr2-4} double mutant at DUF 70.

(A) Expression of floral repressor genes \textit{FLM}, \textit{FLC} and \textit{SVP} in the wild-type and double mutant plants. The expression of \textit{FLM} and \textit{FLC} in double mutant plants is down-regulated compared to the wild-type which is not the case with \textit{SVP}. (B) Expression of floral activators \textit{CO}, \textit{SOC1}, \textit{FD} and \textit{FT}. \textit{FT} levels are very low under the short days and it was taken as a control (i.e. levels are qPCR noise). (C and D) The transcript levels of the flowering time pathway-specific marker genes \textit{FDP}, \textit{TFL}, \textit{LFY}, \textit{AP1} and \textit{TSF}. (E) Expression of \textit{GA4} and \textit{GA5}, marker genes for gibberellic acid pathway. RNA was isolated from SD plants short before flowering of the double mutants, tissue from leaves and/or shoot apical meristem was taken. For each line 15 plants were taken and pooled together, RT-qPCR was done using three biological replicates were used with three technical repetitions each and normalized by \textit{ELF-1-α}. Error bars indicate SD of the mean. Leaf material was used for (A, B) and for (C-E) material was taken from SAM.

In the SAM, the expression of the floral repressor \textit{TERMINAL FLOWER 1} (\textit{TFL1}) was reduced 1.7-fold, whereas expression of the floral activator \textit{FD PARALOG} (\textit{FDP}) was not significantly changed (Figure 5.2, C). In contrast, the meristem identity genes \textit{LEAFY} (\textit{LFY}) was 2-fold increased (Figure 5.2, B) and \textit{APETALA} (\textit{AP1}) and \textit{TWIN SISTER OF FT} (\textit{TSF}) were up-regulated.
in the double mutant, 3-fold and 6-fold respectively. The meristem identity genes like AP1 and LFY play an important role in early flower initiation (Ferrandiz et al. 2000) and their higher expression confirms the early flowering state of the arr1-4 arr2-4 double mutant compared to Col-0.

Flower initiation in *Arabidopsis thaliana* under non-inductive short day conditions (Eriksson et al. 2006) is dependent on the biosynthesis of the plant hormone gibberellin (GA) and cross-talk between cytokinin and GA has been described (Greenboim-Wainberg et al. 2005). This dependency can be explained, at least partly, by GA regulation of the flower meristem identity gene LFY and the flowering time gene SOC1 (Eriksson et al. 2006). Gibberellic acid flowering pathway is mostly typical for non-inductive conditions, based on the observations of ga1 mutant (Wilson et al. 1992). GA4 and GA5 (gibberellin 3 ß-hydroxylase 1 and 2) are involved in later steps of the gibberellic acid biosynthetic pathway (Hedden and Phillips 2000) and their mRNA levels were shown to somewhat parallel active GA levels in vivo (Achard et al. 2008). Therefore, the mRNA levels of GA4 and GA5 were examined in the SAM at DUF 70. Both GA4 and GA5 were unaffected in the double mutant in the SAM (Figure 5.2, E), presumptively indicating that ARR1 and ARR2 are not involved in the gibberellic acid flowering pathway.

In conclusion, the expression data obtained by RT-qPCR suggests that ARR1 and ARR2 are mostly involved in short-day, non-inductive flowering pathways. This is supported by the fact that the expression of floral repressors FLC and FLM was lower in the double mutant, whereas known floral activators were not dramatically altered in leaf. The SAM at DUF70 in the double mutant was clearly committed to flowering compared to the wild-type as evident by the high expression levels of API, LFY, and TSF. Double mutant was clearly, based on API expression, in different developmental stage than the wild-type. In order to investigate the previous observations experiments with different approach were further done.

### 5.2.3 Change in expression of floral repressors is strictly due to early flowering and not developmental effects

To ensure that the data reported above reflect true expression differences rather than differences in developmental stage, arr1-4 arr2-4 plants were grown for 30 days under non-inductive SD conditions and then shifted to the inductive photoperiodic long days. It has previously been shown that plants grown for 30 days under SDs are large and developed enough to rapidly enter the reproductive phase after a shift to LDs (Schmid et al. 2003). For *Arabidopsis*, plants are irreversibly committed to flowering after 3 days in LDs as indicated by the expression of the floral homeotic gene API (Schmid et al. 2003). Therefore flowering was induced in arr1-4 arr2-4 and wild-type plants at 30 days after germination by shifting them LD and plant material was harvested 0 (control) and 5 days after the shift.
Figure 5.3 Expression of floral regulators in SAM before and after photoperiodic induction.

Transcription levels of genes known floral repressors (A-C), activators/integrators (D,E) or meristem identity genes (F) in wild-type Col-0, arr1-4, arr2-4 and arr1-4 arr2-4. RNA was isolated from the shoot apical meristems (SAM) of 30-day-old plants grown in SD at DUF 30 (Day 0) and after being shifted to LD (Day 5). For each line at least 15 plants were taken and pooled together, RT-qPCR was performed using two biological replicates and three technical replicates each and normalized to ELF-1-α. Error bars indicate SD of the mean.

After shifting, the expression of the floral repressors FLM, FLC and SVP was reduced in Col-0 while the expression of floral activator CO and floral pathway integrator SOC1 increased (Figure 5.3) indicating that SAM to the shift in photoperiod. AP1 expression (Figure 5.3, F) is suggests that plants were still in vegetative state before the shift and first after the shift under the inductive long days flowering was induced (the experiment as such was working).

Thus a vegetative phase was replaced by a reproductive phase. The expression level of the floral repressor FLC in arr1-4, arr2-4 and arr1-4 arr2-4 is decreased (about 2-fold) before the shift relative to Col-0 and stays reduced after the shift (Figure 5.3, A). FLM also follows this pattern, in that it is already reduced in expression before the shift and maintains this level after the shift (Figure 5.3, B). In contrast, the expression of floral repressor SVP decreased after the shift from SDs to LDs independently of the loss of ARR1 or ARR2 (Figure 5.3, C). Similarly to SVP, the expression of SOC1 is unchanged in the mutant backgrounds and SOC1 reached induction levels like that in Col-0 (Figure 5.3, D). CO also follows a similar pattern as SOC1, in that after the flowering shift induction, the expression of CO is increased in response to the shift to LD (Figure 5.3, E).

Taken together, these results suggest that the reduction of FLC and FLM expression might be causal for the early flowering observed in arr1-4 and arr2-4 single mutants and the arr1-4 arr2-4 double mutant.
5.2.4 Changes in expression of floral repressors are not due to an increase in size of the apical meristem

ARR1 and ARR2 are known to have roles in cytokinin signalling (Grefen and Harter 2004; Zwack and Rashotte 2013) and it is known that cytokinin has a positive role in regulating SAM size and activity (Tucker and Laux 2007; Werner and Schmulling 2009; Gupta and Rashotte 2012). Cytokinin is also required in the SAM for maintenance of cell division and prevention of cell differentiation (Jasinski et al. 2005; Gupta and Rashotte 2012). Therefore, the size of shoot apical meristem SAM was measured in mutant plants and compared to its wild-type in order to see if the early flowering phenotype could be ascribed to a difference in size or in morphology. For this experiment SAM of 30-day-old plants grown under SDs were examined (Figure 5.4).

**Figure 5.4** Size of shoot apical meristem of Col-0 arr1-4 and arr2-4 single mutants and arr1-4 arr2-4 double mutant.

(A) Examples of the tissue taken and how measurements were made. Sections were scanned for clear visibility of the two large air vacuoles (arrows, upper image). Once found then the SAM width was measured (solid line, lower image).

(B) Representative sections of shoot apical meristems of Col-0, arr1-4, arr2-4 and arr1-4 arr2-4 respectively. (C) Size of shoot apical meristems in single mutants and the double mutants from 30-day-old plants grown under SDs. At least 10 plants per line were used for statistical evaluation. Significance classes were computed using the Fisher’s Least Significant Difference (LSD, $\alpha=0.05$) test after passing one-way ANOVA ($\alpha=0.05$, $p \geq 0.0339$). Classes not connected by a letter are significantly different. All of the mutants have means that are smaller than Col-0, but only arr2-4 and arr1-4 arr2-4 are statistically smaller than the wild-type. Error bars indicate SD of the mean. Scale bars, 100$\mu$m

SAMs of Col-0 and arr1-4 single mutant did not show a statistical significant difference in their size (LSD, $\alpha=0.05$) even though the mean of the arr1-4 similar to the arr2-4 and arr1-4 arr2-4 and are not statistically different (Figure 4, C). On the other hand, the SAMs of arr2-4 single mutant and the arr1-4 arr2-4 double mutant are statistically significantly smaller than in the wild-type (LSD, $\alpha=0.05$). The SAM of the arr1-4 arr2-4 double mutant is not significantly different in size from arr2-4 or arr1-4 single mutants however (Figure 5.4, C).
suggests that the difference in SAM size is only due to lack of ARR2 and not ARR1, however one should keep in mind that the arr1-4 SAM was never observed to be in the same range as that of Col-0 (Figure 5.4, C) and was not statistically different from arr2-4 or the double mutant. It is possible that with a larger sample size the arr1-4 would have also been statistically smaller. For now, it is evident that the SAMs of arr1-4, arr2-4 and arr1-4 arr2-4 are not larger than Col-0. Thus, despite of even smaller size of SAM in the mutant plants they manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed (Figure 5.4, A, B).

5.2.5  **ARR2 expressed either in the SAM or from phloem companion cells recues the early flowering phenotype of arr2-4 and arr1-4 arr2-4**

The RT-qPCR results from the previous experiments indicated that there were changes in floral regulator expression in both leaf and the SAM. Investigation of the SAM indicated the early flowering phenotype could not be attributed to an increase in meristem size. Therefore as the action of ARR2 with respect to flowering time was unclear, the double mutant was complemented with two different tissue specific promoters, pSUC2 and pFD. pSUC2 is a vasculature-specific promoter expressed only in phloem (companion cells) of all green tissues of *Arabidopsis* such as rosette leaves, stems, and sepals. SUC2 encodes a plasma-membrane sucrose-H+ symporter (Truernit and Sauer 1995; Wippel and Sauer 2012). pFD is a meristem-specific promoter expressed in the shoot apex (Abe *et al.* 2005). FD is bZIP protein required for positive regulation of flowering (Abe *et al.* 2005; Wigge *et al.* 2005). Furthermore, it is know that ARR2 can be activated by phosphorylation of an aspartate (D80) in a Two-Component System (TCS) dependent manner (Grefen and Harter 2004). Mutation of D80 to E (Glutamate) mimics the phosphorylated state, increases the transactivation capacity of ARR2 (Hwang and Sheen 2001) and leads to severe developmental defects (Hass *et al.* 2004). Mutation of D80 to N (Asparagine), however, prevents TCS dependent phosphorylation (Kim *et al.* 2006) and impairs basal gene activation capacity, yet D80N still appears to respond to cytokinin according to reporter gene assays (Hwang and Sheen 2001; Kim *et al.* 2006). Therefore, the arr2-4 and arr1-4 arr2-4 mutants were complemented using wild-type ARR2 and ARR2D80N cDNA versions under the control of the pSUC2 and pFD promoters (Tables 5.1 to 5.4). Due to the stronger flowering time effect observed by the loss of ARR2, arr1-4 plants were included as controls only and not complemented with the ARR2 at this time, thus this complementation assay focuses on the effect of the ARR2 gene.

All plants used for this experiment were in examined in the T2 generation and therefore preselected with BASTA. For each construct two or three independent lines were chosen and a minimum of 15 plants were used for the experiment. To rule out any secondary effects from the BASTA application and ensure that the vector backbones did not disturb the early flowering phenotype, all the mutant lines as well as the wild-type plants were transformed with empty vectors. This was done by using the "pJL blue" gateway compatible vector which only has a MCS (multiple cloning site (MCS) sequence between the gateway cassette. The results will be presented in the same order as they are given in Tables 5.1 to 5.4.

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Table 5.1 shows the complementation results for *ARR2* and *ARR2D80N* driven under the *pSUC2* promoter in LDs. Two to three transgenic lines were chosen for each construct and genotype pair. The table presents the data for each line analysed; statistical analyses were performed by pooling the results from independent lines for each transgene. With regards to the two parameters DUF and rosette leaf number, DUF is more predictive and has a higher resolution as the number of rosette leaves was nearly invariant under our LD condition (total range 2 leaves). Therefore, only the results with respect to DUF will be presented for LDs. The control vector construct, here called “pJL-blue”, did not complement the flowering time phenotype. The wild-type flowered the latest (mean: 29 DUF), the double mutant the earliest (mean: 24 DUF) and the two single mutants in-between (means: 26 DUF for *arr1-4* and 25 DUF for *arr2-4*). Thus even though the early flowering phenotype is comparably weak in LDs, the flowering time trend previously observed was not disturbed by the vector backbone nor the BASTA treatment. *ARR2* driven by *pSUC2* was able to complement the flowering defect to near wild-type levels in the *arr2-4* single mutant (mean: DUF 28 same significance class as Col-0). Most remarkably, *ARR2 D80N* loss-off-function version driven under the *pSUC2* was also able to partially complement (mean: 27 DUF) the early flowering phenotype of the single mutant under LDs. Neither construct was able to complement the double mutant however.

The complementation results for *ARR2* and *ARR2 D80N* driven under the *pFD* promoter in LDs are given in Table 5.2. Like the *pSUC2* experiment in LDs, the DUF parameter is more informative as the rosette number again is nearly invariant and has a small range (2 leaves). The control pJL-blue constructs lead to the same tendency with the wild-type flowering the latest (mean: 29 DUF), the double mutant earliest (mean: 25 DUF) and the single mutants in-between as previously observed. This again demonstrated that neither the BASTA application nor vector backbone disturbed the flowering phenotype of the respective genotypes. Expression of *ARR2* from the *pFD* promoter was able to complement the single and double mutant (means: 28.5 and 29 DUF, respectively). A partial complementation was also observed with *ARR2 D80N* in the *arr2-4* mutant but not the *arr1-4 arr2-4* double mutant. Table 5.3 summarises the results for complementation of the various mutant lines for *ARR2* and *ARR2 D80N* driven under the *pSUC2* promoter in SDs. The control pJL-blue constructs once again did not disturb the flowering time with the wild-type flowering the latest (mean: 101 DUF with 53 leaves), the double mutant earliest (mean: 81 DUF with 41 leaves) and the single mutants in-between as previously observed. All the complemented lines used for these experiments showed within the same complementation same tendency (supplemental figures 8 and 9). The rosette leaf range is larger this time (range of 13 leaves) and overall reflects the DUF parameter but not only for the double mutant. The best complementation was observed in the double mutant (mean: 90 DUF with 48 leaves) with *ARR2 D80N* also partially, but significantly, complementing the mutant (mean: 86 DUF with 45 leaves). In contrast to the *pSUC2* under LD conditions, neither *ARR2* nor *ARR2 D80N* was able to even partially complement *arr2-4* based on the significance classes. Nevertheless, this complementation of *ARR2* did have a mean DUF of 89, nearly identical to that of the partially complemented double mutant.
Table 5.1 Flowering time of transgenic lines driven under tissue specific SUC2 promoter (pSUC2) under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

DUF     RLN     DUF SD    RLN SD    DUF range RLN range n
---      ---      ---        ---        ---        ---        ---

**Long Day**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DUF</th>
<th>RLN</th>
<th>DUF SD</th>
<th>RLN SD</th>
<th>DUF range</th>
<th>RLN range</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Col-0) pSUC2::Jlblue</td>
<td>29.0</td>
<td>11.7</td>
<td>±1.6</td>
<td>±1.4</td>
<td>27-32</td>
<td>10-13</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 pSUC2::Jlblue #1</td>
<td>25.6</td>
<td>9.6</td>
<td>±1.3</td>
<td>±0.9</td>
<td>23-26</td>
<td>9-12</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 pSUC2::Jlblue #2</td>
<td>25.3</td>
<td>10.1</td>
<td>±1.7</td>
<td>±0.8</td>
<td>23-27</td>
<td>8-10</td>
<td>15</td>
</tr>
<tr>
<td>arr2-4 pSUC2::Jlblue</td>
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<td>±2.8</td>
<td>±1.1</td>
<td>23-31</td>
<td>8-12</td>
<td>15</td>
</tr>
<tr>
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<td>9.4</td>
<td>±0.8</td>
<td>±1.3</td>
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<td>7-12</td>
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</tr>
<tr>
<td>arr1-4 arr2-4 pSUC2::ARR2 #1</td>
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<td>10.9</td>
<td>±1.8</td>
<td>±1.0</td>
<td>25-30</td>
<td>10-12</td>
<td>15</td>
</tr>
<tr>
<td>arr2-4 pSUC2::ARR2 #2</td>
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<td>10.7</td>
<td>±1.8</td>
<td>±1.0</td>
<td>25-31</td>
<td>9-12</td>
<td>15</td>
</tr>
<tr>
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<td>11.3</td>
<td>±3.2</td>
<td>±2.3</td>
<td>24-33</td>
<td>8-14</td>
<td>20</td>
</tr>
<tr>
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<td>10.3</td>
<td>±1.5</td>
<td>±0.5</td>
<td>23-28</td>
<td>9-14</td>
<td>15</td>
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<td>10.8</td>
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<td>±1.0</td>
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<td>15</td>
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<td>10.7</td>
<td>±1.5</td>
<td>±1.1</td>
<td>23-28</td>
<td>9-12</td>
<td>15</td>
</tr>
<tr>
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<td>10.6</td>
<td>±1.4</td>
<td>±1.4</td>
<td>23-27</td>
<td>9-14</td>
<td>15</td>
</tr>
</tbody>
</table>

Genotype                          | LSD DUF | Mean | Genotype                          | LSD Rosette | Mean |
---                                | --------|------|-----------------------------------|-------------|------|
| WT (Col-0) pSUC2::Jlblue          | 29.0     | A    | WT (Col-0) pSUC2::Jlblue          | 11.0        | A    |
| arr2-4 (Col-0) pSUC2::ARR2        | 28.1     | B    | arr1-4 Xarr2-4 (Col-0) pSUC2::ARR2D80N | 10.8        | A    |
| arr2-4 (Col-0) pSUC2::ARR2D80N    | 26.8     | B    | arr2-4 (Col-0) pSUC2::ARR2        | 10.8        | A    |
| arr1-4 (Col-0) pSUC2::Jlblue      | 26.1     | C    | arr1-4 Xarr2-4 (Col-0) pSUC2::Jlblue | 10.5        | A    |
| arr2-4 (Col-0) pSUC2::Jlblue      | 25.7     | C    | arr2-4 (Col-0) pSUC2::ARR2        | 10.3        | B    |
| arr1-4 Xarr2-4 (Col-0) pSUC2::Jlblue | 25.3   | C    | arr2-4 (Col-0) pSUC2::Jlblue      | 9.9         | B    |
| arr1-4 Xarr2-4 (Col-0) pSUC2::ARR2 | 24.0     | D    | arr1-4 Xarr2-4 (Col-0) pSUC2::Jlblue | 9.5         | C    |
Table 5.2 Flowering time of transgenic lines driven under tissue specific FD promoter under long day inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

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<th>Mean</th>
<th>Genotype</th>
<th>LSD Rosette</th>
<th>Mean</th>
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<td>11,9</td>
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<td>28,9</td>
<td>arr2-4 (Col-0) pFD::ARR2</td>
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<td>11,4</td>
</tr>
<tr>
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<td>26,8</td>
<td>arr2-4 (Col-0) pFD::ARR2</td>
<td>A</td>
<td>11,3</td>
</tr>
<tr>
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<td>A</td>
<td>28,4</td>
<td>WT (Col-0) pFD::Jlblue</td>
<td>B</td>
<td>10,8</td>
</tr>
<tr>
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<td>B C</td>
<td>26,4</td>
<td>arr1-4Xarr2-4 (Col-0) pFD::ARR2</td>
<td>A</td>
<td>10,2</td>
</tr>
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<td>C D</td>
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<td>C D</td>
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<td>C D</td>
<td>10,0</td>
</tr>
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<td>24,8</td>
<td>arr1-4Xarr2-4 (Col-0) pFD::Jlblue</td>
<td>D</td>
<td>9,6</td>
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</table>

<table>
<thead>
<tr>
<th>Long Day</th>
<th>DUF</th>
<th>RLN</th>
<th>DUF SD</th>
<th>RLN SD</th>
<th>DUF range</th>
<th>RLN range</th>
<th>n</th>
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<tbody>
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<td>11,5</td>
<td>±1,7</td>
<td>±1,7</td>
<td>27-32</td>
<td>8-13</td>
<td>15</td>
</tr>
<tr>
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<td>±1,7</td>
<td>27-32</td>
<td>8-13</td>
<td>15</td>
</tr>
<tr>
<td>pFD:pJL-blue #5</td>
<td>29,3</td>
<td>11,4</td>
<td>±1,7</td>
<td>±1,4</td>
<td>27-32</td>
<td>8-13</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 pFD:pJL-blue #1</td>
<td>26,8</td>
<td>10,0</td>
<td>±1,6</td>
<td>±1,9</td>
<td>24-29</td>
<td>7-12</td>
<td>15</td>
</tr>
<tr>
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<td>10,5</td>
<td>±1,5</td>
<td>±2,1</td>
<td>24-28</td>
<td>7-14</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 pFD:pJL-blue #3</td>
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<td>10,2</td>
<td>±1,2</td>
<td>±1,4</td>
<td>25-28</td>
<td>7-12</td>
<td>15</td>
</tr>
<tr>
<td>arr2-4 pFD:pJL-blue #1</td>
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<td>9,6</td>
<td>±1,0</td>
<td>±2,2</td>
<td>23-26</td>
<td>7-11</td>
<td>15</td>
</tr>
<tr>
<td>arr2-4 pFD:pJL-blue #5</td>
<td>24,8</td>
<td>10,1</td>
<td>±1,2</td>
<td>±1,2</td>
<td>23-26</td>
<td>8-11</td>
<td>15</td>
</tr>
<tr>
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<td>10,3</td>
<td>±1,2</td>
<td>±1,5</td>
<td>23-26</td>
<td>8-13</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 arr2-4 pFD:pJL-blue #1</td>
<td>24,6</td>
<td>9,4</td>
<td>±1,3</td>
<td>±1,5</td>
<td>23-27</td>
<td>7-12</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 arr2-4 pFD:pJL-blue #3</td>
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<td>9,6</td>
<td>±1,1</td>
<td>±1,7</td>
<td>24-27</td>
<td>7-13</td>
<td>15</td>
</tr>
<tr>
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<td>9,6</td>
<td>±1,4</td>
<td>±1,5</td>
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<td>7-11</td>
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<td>10,5</td>
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<td>±1,1</td>
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<td>11,8</td>
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<td>±1,2</td>
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<td>10-13</td>
<td>15</td>
</tr>
<tr>
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<td>11,4</td>
<td>±2,0</td>
<td>±1,4</td>
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<td>9-14</td>
<td>10</td>
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<td>arr2-4 pFD:ARR2D80N #2</td>
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<td>11,4</td>
<td>±2,0</td>
<td>±1,4</td>
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<td>9-13</td>
<td>15</td>
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<td>9-15</td>
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<tr>
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<td>11,6</td>
<td>±1,6</td>
<td>±1,3</td>
<td>26-31</td>
<td>10-13</td>
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<td>±2,0</td>
<td>±1,7</td>
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<td>10,9</td>
<td>±1,6</td>
<td>±1,7</td>
<td>24-29</td>
<td>7-12</td>
<td>15</td>
</tr>
<tr>
<td>arr1-4 arr2-4 pFD:ARR2D80N #2</td>
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<td>10,7</td>
<td>±1,5</td>
<td>±1,5</td>
<td>24-29</td>
<td>8-12</td>
<td>15</td>
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</tbody>
</table>
Finally, the complementation results for \textit{ARR2} and \textit{ARR2 D80N} driven under the \textit{pFD} promoter under SDs are in Table 5.4. As observed in the other three experiments, the control pJL-blue constructs did not disturb the flowering time: wild-type flowered the latest (mean: 108 DUF with 54 leaves), \textit{arr1-4} (mean: 88 DUF with 50 leaves), \textit{arr2-4} (mean: 84 DUF with 48 leaves) and the double mutant the earliest (mean: 81 DUF with 41 leaves). In contrast to the previous experiments \textit{ARR2} driven by the \textit{pFD} promoter was rescued both the \textit{arr2-4} (mean: 97 DUF with 50 leaves) and the \textit{arr1-4 arr2-4} (mean: 95 DUF with 50 leaves) mutants almost completely. Most remarkably, the \textit{ARR2^{D80N}} was also able to, partially, and significantly, complement both the single \textit{arr2-4} (mean: 89 DUF with 47 leaves) and the double mutant (mean: 86.5 DUF with 45.5 leaves).

Taken together, even though full complementation was only observed under LDs, the complementation of the early flowering of \textit{arr2-4} under SDs was also partial, yet statistically significant, suppressed. This suggests that lack of \textit{ARR2} is indeed causal for the observed early flowering phenotype. \textit{ARR2} driven under \textit{pFD} showed the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the \textit{arr2-4} and \textit{arr1-4 arr2-4} mutation effects strongest. The rescue is slightly weaker when \textit{ARR2} is driven under \textit{pSUC2} but still significant in the double mutant. In LD conditions, \textit{ARR2} expressed from either promoter was able to complement the \textit{arr2-4} mutation, but only \textit{ARR2} expressed under the \textit{pFD} promoter was able to partially rescue the \textit{arr1-4 arr2-4} mutant. Mutant plants were also slightly complemented with loss-of-function \textit{ARR2 D80N} gene in the single and double mutants in SDs when driven under the \textit{pFD} promoter or by \textit{pSUC2} in \textit{arr1-4 arr2-4}. This effect of \textit{ARR2 D80N} was also observed in LDs but only in the \textit{arr2-4} mutant background. The \textit{ARR2D80N-cDNA} mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type \textit{ARR2-cDNA} (Hwang and Sheen 2001; Choi et al. 2010; Veerabagu et al. 2012). The reason for this is most likely due to an incomplete inactivation of the \textit{ARR2} by the D80N mutation. Considering this fact, it can be still concluded that \textit{ARR2} needs to be phosphorylated by the TCS (Two-Component System) in order to best rescue the early flowering phenotype as only partial complementation was observed for the \textit{ARR2D80N} loss-of-function transgene. This strongly suggests that \textit{ARR2} may be a point of integration for the TCS and other flowering time pathways under SDs.
Table 5.3 Flowering time of transgenic lines driven under tissue specific SUC2 promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>DUF</th>
<th>RLN</th>
<th>DUF SD</th>
<th>RLN SD</th>
<th>DUF range</th>
<th>RLN range</th>
<th>n</th>
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<td>±4,4</td>
<td>90-109</td>
<td>47-60</td>
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</tr>
<tr>
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<td>48-57</td>
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<td>±2,9</td>
<td>94-115</td>
<td>50-58</td>
<td>15</td>
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<td>±2,0</td>
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<td>±4,4</td>
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<tr>
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<td>45,3</td>
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<td>±8,8</td>
<td>±1,8</td>
<td>75-100</td>
<td>42-47</td>
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</tr>
</tbody>
</table>

Genotype | LSD DUF | Mean | Genotype | LSD Rosette | Mean |
---------|---------|------|----------|-------------|------|
WT (Col-0) pSUC2::Jlblue | A | 100,8 | WT (Col-0) pSUC2::Jlblue | A | 53,1 |
arr1-4 arr2-4 (Col-0) pSUC2::ARR2 | B | 90,0 | arr1-4 (Col-0) pSUC2::Jlblue | B | 49,1 |
arr2-4 (Col-0) pSUC2::ARR2 | B | 89,1 | arr2-4 (Col-0) pSUC2::ARR2 | B | 47,8 |
arr1-4 (Col-0) pSUC2::Jlblue | C | 88,9 | arr2-4 (Col-0) pSUC2::Jlblue | B | 47,4 |
arr2-4 (Col-0) pSUC2::Jlblue | B | 86,6 | arr1-4 arr2-4 (Col-0) pSUC2::ARR2 | B | 47,4 |
arr1-4 arr2-4 (Col-0) pSUC2::ARR2D80N | C | 86,0 | arr1-4 arr2-4 (Col-0) pSUC2::ARR2D80N | C | 46,0 |
arr2-4 (Col-0) pSUC2::ARR2D80N | D | 85,0 | arr1-4 arr2-4 (Col-0) pSUC2::ARR2D80N | D | 44,9 |
arr1-4 arr2-4 (Col-0) pSUC2::Jlblue | D | 81,1 | arr1-4 arr2-4 (Col-0) pSUC2::Jlblue | E | 41,3 |
Table 5.4 Flowering time of transgenic lines driven under tissue specific FD promoter under short day non-inductive conditions.

DUF, days until flowering; RLN, rosette leaf number; n, number of individuals; #, identifier for individual transgenic line. All transgenic lines used for this experiment were in T2 generation and preselected with BASTA. Significance levels were computed after passing ANOVA ($\alpha=0.05$) by using Fischer’s Least Significance Difference (LSD) with alpha levels $\alpha=0.01$ for DUF and $\alpha=0.05$ or RLN. Classes not connected by a letter are significantly different.

<table>
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<th>LSD DUF</th>
<th>Mean</th>
<th>Genotype</th>
<th>LSD Rosette</th>
<th>Mean</th>
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<td>WT (Col-0) pFD::Jlblue</td>
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<td>arr1-4 (Col-0) pFD::Jlblue</td>
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<td>arr1-4Xarr2-4 (Col-0) pFD::ARR2</td>
<td>B</td>
<td>95,4</td>
<td>arr1-4Xarr2-4 (Col-0) pFD::Jlblue</td>
<td>B C</td>
<td>49,7</td>
</tr>
<tr>
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<td>arr2-4 (Col-0) pFD::ARR2</td>
<td>B C</td>
<td>49,6</td>
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<tr>
<td>arr1-4 (Col-0) pFD::Jlblue</td>
<td>C</td>
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<td>arr2-4 (Col-0) pFD::Jlblue</td>
<td>C D</td>
<td>47,9</td>
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<td>F</td>
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<th>Short Day</th>
<th>DUF</th>
<th>RLN</th>
<th>DUF SD</th>
<th>RLN SD</th>
<th>DUF range</th>
<th>RLN range</th>
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<tbody>
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<td>45-56</td>
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<tr>
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<td>49.4</td>
<td>±4.1</td>
<td>±5.7</td>
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<td>40-57</td>
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</tr>
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<td>arr1-4 pFD:pJL-blue #3</td>
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<td>±4.0</td>
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<td>47-55</td>
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<td>±4.8</td>
<td>74-89</td>
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<tr>
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<td>48.3</td>
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<td>±4.3</td>
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<td>41.8</td>
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<td>±3.8</td>
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<td>79-91</td>
<td>39-49</td>
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5.2.6  **ARR1 and ARR2 work mostly independent of FLC**

FLOWERING LOCUS C (FLC) is a transcription factor that functions as a repressor of the floral transition. It is the main target of the vernalization pathway in *Arabidopsis thaliana* and is epigenetically silenced by the prolonged exposure of plants to cold. FLC was shown to bind to and directly block the transcriptional activation of SOC1 and FT (Helliwell *et al.* 2006; Srikanth and Schmid 2011).

Based on RT-qPCR data obtained from *ARR1* and *ARR2* knockouts, the *arr1-4, arr2-4* and *arr1-4 arr2-4* double mutants showed reduced levels of FLC compared to wild-type. In order to find out more about the nature of this interaction the *arr1-4 arr2-4* double mutant was crossed with a deletion mutant for FLC, *flc-3* (Michaels and Amasino 1999). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 11).

The *flc-3 arr1-4 arr2-4* (mean: 28 DUF) triple mutant under inductive LD conditions behaved like the *flc-3* single mutant (mean: 28 DUF), flowering about 6 days earlier (mean: 34 DUF) and producing 2 leaves less than the Col-0. The *arr1-4 arr2-4* double mutant displayed an intermediate phenotype (mean: 32 DUF) (Figure 5.5, A (left panel), B). Under LDs, FLC appears to be epistatic to *ARR1* and *ARR2*. Under non-photoperiodic SD conditions the *flc-3 arr1-4 arr2-4* triple mutant (mean: 78 DUF) very clearly shows an additive effect. It flowers earlier than both parents, 8 days earlier than the *flc-3* (mean: 87 DUF) about 2 weeks before *arr1-4 arr2-4* (mean: 92 DUF) and more than 3 weeks before the Col-0 (mean: 111 DUF). The same flowering time was observed when this experiment was repeated under SD conditions but under a different light quality. These results confirm the consistency of an additive early flowering phenotype of the *flc-3 arr1-4 arr2-4* triple mutant in SDs (Figure 5.5, A (right panel), D).

The *flc-3 arr1-4 arr2-4* triple mutant repeatedly showed a clear tendency towards early flowering when compared to the *flc-3* single mutant. However, the differences were not statistically significant. Therefore, the same experiment was repeated again under LDs but at 16°C temperature, which results in a mild delay in flowering. Interestingly, under these conditions, *flc-3 arr1-4 arr2-4* flowered earlier than *flc-3*. The triple mutant *flc-3 arr1-4 arr2-4* flowered over a week earlier that the wild-type (mean: 34 DUF) which was almost five days before *arr1-4 arr2-4* double mutant (mean: 39 DUF) and two days earlier than *flc-3* single mutant (mean: 36 DUF) (Figure 5.5, C), suggesting an additive effect on of FLC and *ARR1* and *ARR2* under inductive LD conditions.

Based on these experiments, the *flc-3 arr1-4 arr2-4* triple mutant shows very consistent additive effect in early flowering under SDs and LDs (statistically significant only at 16°C). Based on this result *ARR2* seems to work mainly independent of FLC. If there is any intercommunication between these two signalling pathways then it is rather small.
Figure 5.5 Flowering phenotype of arr1-4 arr2-4 flc-3 triple mutant.

(A) Photographs showing early flowering phenotype of arr1-4 arr2-4 double mutant, flc-3 single mutant and arr1-4 arr2-4 flc-3 triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. The arrow indicates the position of floral bud of Col-0. (B) Flowering times of mutant plants grown under long day conditions. (B) Flowering times of mutant plants grown at 16°C and 23°C under LD conditions. (C) Flowering times of mutant plants under SD conditions. Additive effect on flowering time by the triple mutant is very visible and consistent even under different light conditions (i.e. Chamber 1 and Chamber 2).

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<th>RLN SD</th>
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<td>12-15</td>
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<td>±0.4</td>
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<td>10-12</td>
<td>15</td>
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<td>±1.9</td>
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<td>±4.2</td>
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<td>71-83</td>
<td>27-32</td>
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5.2.7  ARR1 and ARR2 work in the same pathway upstream of FLM

FLOWERING LOCUS M (FLM) is a MADS-domain gene that acts as an inhibitor of flowering in Arabidopsis. Although the sequence of FLM is similar to that of FLC, FLM and FLC interact with different flowering pathways (Scortecci et al. 2003). The FLM levels were also strongly down-regulated in both ARR1 and ARR2 knockouts and in the double mutant. Therefore, to get more insight into the potential interaction with FLM in respect to flowering time, the arr1-4 arr2-4 double mutant was crossed with flm-3 single knock-out mutant for FLM (Balasubramanian et al. 2006). The triple mutant was identified by PCR (see Methods) and confirmed at the expression level (supplemental figure 12).

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Figure 5.6 Flowering phenotype of flm-3 arr1-4 arr2-4 triple mutant.

(A) Photographs showing early flowering phenotype of arr1-4 arr2-4 double mutant, flm-3 single mutant and flm-3 arr1-4 arr2-4 triple mutant under LDs (left panel) and SDs (right panel) compared to wild-type. Epistatic effect of FLM on ARR1 and ARR2 on early flowering is very good visible in both long-and short days.

(B) Flowering time of mutant plants grown under long day conditions. (C) Flowering time of mutant plants grown under short day conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.

The flowering time was first determined for the flm-3 arr1-4 arr2-4 triple mutant under LD conditions. The triple mutant showed very early flowering (compared to the Col-0) and was indistinguishable from the flm-3 single mutant. The triple mutant flm-3 arr1-4 arr2-4 flowered in average 6 days earlier (mean: 24 DUF) which was the time same as flm-3 (mean: 30 DUF)
24 DUF), and both developed 3 rosette leaves less than the Col-0 (mean: 30 DUF). The double mutant arr1-4 arr2-4 (mean: 27 DUF) flowered somewhat later than the flm-3 mutants and produced one leaf less than Col-0 (Figure 5.6, A left panel, B). Clearly FLM is epistatic to ARR1 and ARR2.

![Figure 5.6](image)

**Figure 5.6** Flowering phenotype of **flm-3 arr2-4** double mutant.
(A) Photographs showing early flowering phenotype of **arr2-4** and **flm-3** single mutants and **flm-3 arr2-4** double mutant under LDs (left panel) and SDs (right panel) compared to wild-type. (B) Flowering time of mutant plants grown under long day conditions. (C) Flowering time of mutant plants grown under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.

The **flm-3 arr1-4 arr2-4** triple mutant and the **flm-3 single** mutant grown under non-inductive SD condition showed epistasis to **flm-3** single mutant as well (Figure 5.6, A(right panel), C). The **flm-3** and **flm-3 arr1-4 arr2-4** triple mutant mutants flowered approximately at the same time, i.e. more than 30 days earlier (mean: 77 DUF) than the wild-type (mean: 110 DUF) and over 10 days earlier than the **arr1-4 arr2-4** double mutant (mean: 87 DUF). Both **flm-3** containing mutants developed 20 rosette leaves less (mean: RLN 29) than Col-0 (mean: RLN 50). In contrast the **arr1-4 arr2-4** double mutant made 10 leaves less than Col-0 (mean: RLN 40). Based on these results FLM is epistatic to **ARR1** and **ARR2** in LDs and SDs, and therefore **ARR1** and **ARR2** appear to be genetically involved within the same flowering pathway as FLM. **ARR1** and **ARR2** are hypostatic to **FLM**, i.e. they both probably act upstream of **FLM**. Epistasis of **ARR2** to **FLM** was one more time confirmed on **flm-3 arr2-4** double mutant. Under long
day conditions flm-3 arr2-4 (mean: 87 DUF) double mutant flowers at the same time like the flm (mean: 77 DUF) parent which is almost a week before the wild-type and 5 days before the arr2-4 single mutant. Single mutant flm-3 and the flm-3 arr2-4 double mutant also have the same number of rosette leaves (Figure 5.7, A (left panel) and B).

5.2.8 Initial experiments with crossings between arr2-4 with soc1-2 and co reveal unanticipated interactions

During this work it was possible to generate and analyse double mutants with co and soc1-2 lines for placing ARR2 within other known flowering time pathways. Recall that arr2-4 had the stronger flowering time phenotype compared to arr1-4. Floral activators SOC1 and CO, especially SOC1, are floral integrators converting most of the flowering pathways. CONSTANS (CO) promotes flowering of Arabidopsis in response to day length. Under LD conditions CO activates transcription of FLOWERING LOCUS T (FT) in the vascular tissue of leaves and initiates flowering (Corbesier et al. 2007). SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1) is required for CO to promote flowering and integrates the long-day and vernalization/autonomous pathways (Samach et al. 2000). On the other hand SOC1 also integrates signalling from the GA-dependent pathway, a major flowering pathway under non-inductive short days (Moon et al. 2003). The SOC1 and FT genes are also regulated by a different flowering-time pathway that acts independently of CO and which is active also at the non-inductive SD conditions (Samach et al. 2000). As previously described, beside the similar role of CO and SOC1 as floral activators, they can also act independently of each other. Therefore, both arr2-4 soc1 and arr2-4 co double mutants were tested for flowering time under LD and SD conditions.

Under inductive LD conditions both double mutants were indistinguishable from their soc1 and co single mutant parents. The arr2-4 soc1 double mutant flowered 5 days later than the Col-0 and more than a week after its arr2-4 parent developing 7 rosette leaves more than Col-0 and arr2-4. Late flowering phenotype is especially remarkable by arr2-4 co (mean: 45 DUF) double mutant where flowering was delayed for more than two weeks compared to Col-0 (mean: 30 DUF) and more than 17 days than by arr2-4 (mean: 28 DUF), arr2-4 co double mutant developed 13 rosette leaves more than Col-0 and arr2-4 (Figure 5.8, A and B).

Under non-inducible SD conditions CO is unable to activate the FT flowering-pathway. When grown under SD conditions co single mutant and arr2-4 co double mutant show very similar flowering phenotype. They flower around 10 days (arr2-4 co double mutant) and 15 days (arr2-4 single mutant) (mean: 100 DUF) before the Col-0 (mean: 110 DUF) but make even 15 rosette leaves less than the wild-type. On the other hand, co and arr2-4 co double mutant flower only a couple of days later than the arr2-4 (mean: 96 DUF although they both made 10 leaves less than arr2-4 single parent (Figure 5.8, C).
Figure 5.8 Flowering phenotype of *soc1 arr2-4* and *co arr2-4* double mutants.

(A) Photographs showing late flowering phenotype of *soc1 arr2-4* (middle panel) and *co arr2-4* (left and right panel) double mutants under LDs compared to wild-type. (B) *ARR2* is hypostatic to *CO* and *SOC1*. (C) Flowering phenotype of *soc1 arr2-4* and *co arr2-4* under SD conditions. DUF, days until flowering; RLN, rosette leaf number; n, number of individuals. Significance tests with LSD (α=0.05) were performed on DUF after passing one-way ANOVA (α=0.05). LSD classes are given as letters in superscript. Classes not connected by a letter are significantly different.

Double mutant *arr2-4 soc1* shows intermediate flowering phenotype compared to its parents. The *arr2-4 soc1* (mean: 107 DUF) double mutant flowers around 9 days earlier than *soc1* (mean: 116 DUF) single mutant and makes 5 rosette leaves less, the same double mutant flowers around 12 days later than *arr2-4* single mutant and makes around 5 rosette leaves more than the *arr2-4* single mutant (Figure 5.8, C).

Taken together, under inductive long days *ARR2* seems to be hypostatic or even works independently of *CO* and *SOC1*. Under non-inductive short days *CO*-related-flowering pathways are not very active and *arr2-4 co* double mutant shows very similar phenotype like *arr2-4* parent showing again indications that *ARR2* might be hypostatic or independent to *CO* under SDs as well. Another *arr2-4 soc1* double mutant shows similar phenotype like the wild-type which is in between of both single parents suggesting that under SD conditions *ARR2* works partially independent and partially dependent of *SOC1*-flowering pathways.
5.3 Discussion

During this work, a novel early flowering phenotype was observed and confirmed for single mutants arr1-4 and arr2-4 and the double mutant arr1-4 arr2-4 with respect to flowering time. The early flowering phenotype is especially visible under non-inductive SD conditions for all three mutant lines. The arr1-4 arr2-4 double mutant flowers even earlier than either single mutant or the wild-type and this tendency was statistically significant between wild-type and the both single mutants. In contrast, under LD conditions, only the arr2-4 as single mutant showed slightly statistically significant differences as early flowering on the other hand was also observed for the arr1-4 single mutant but this tendency could not be statistically confirmed in LD. Nevertheless, these differences under inductive long days are from a physiological functional point of view very subtle and were only revealed when plants were exposed to cool temperatures (16°C). In this case, the double mutant clearly flowered earlier than wild-type. Thus, although arr2-4, and presumably arr1-4, also affect flowering in LDs, the loss of these two genes clearly points much stronger roles of ARR2 as a flowering repressor under SD conditions.

Flowering of arr1-4 mutant in SDs is approximately one week earlier, arr2-4 flowers two weeks earlier, the arr1-4 arr2-4 double mutant flowers three weeks before the Col-0. This effect on days until flowering (DUF) was also reflected in rosette leaf number. It has been already proven that generally B-types from the subgroup I (Kim et al. 2012) can complement the arr10 arr12 cytokinin response-deficient mutant (Hill et al. 2013). This could be also the case with early flowering phenotype. Yet interestingly, despite redundancy and very similar sequence homology of ARR1 and ARR2 genes, we managed to prove that under the loss of ARR2 effects flowering more strongly than ARR1 suggesting specificity and affinity (dominance) of ARR2 over ARR1 with respect to flowering time despite their high sequence homology. Interestingly, overexpression of ARR2 was shown to have higher transactivation potential and response with and without the addition of cytokinin on the ARR6 promoter compared to ARR1 (Hwang and Sheen 2001). In addition, even though ARR2 contributes to primary root-elongation responses to exogenous cytokinin, ARR1 is the predominate cytokinin information carrier in this tissue (Mason et al. 2005). Thus, even though these two homologues can have different roles in different tissues, ARR1 and ARR2 showed an additive effect on flowering time, which is visible in the arr1-4 arr2-4 double mutant under both inductive- and non-inductive conditions. One can therefore conclude that both genes are involved in the same flowering time pathway and their role is especially crucial in the signalling pathways which are dominantly active under non-inductive SD conditions.

To check which genes are miss-regulated in the double mutant RT-qPCR analysis of familiar and well described floral regulators was performed. One scenario that would explain the early flowering phenotype is that ARR1 and ARR2 genes/pathway (cytokinin) positively regulates the expression of certain floral repressors (such as FLM, FLC, SVP). Alternatively, early flowering could be induced by activation of positive regulators of flowering (TSF, FD, CO) or floral integrators (SOC1). The chosen markers were applied in order to profile the state of
SAM at this chosen time point. For the first hints, leaf material and material from shoot apical meristem (SAM) from the wild-type and the double mutant was taken depending on where the tested gene is best expressed. The material was taken from plants grown under SDs and it harvested shortly before the double mutant entered in the reproductive phase (based on previous results).

Gene expression of floral activators $CO$, $SOC1$, $FT$ and $FDP$ did not show any statistically significant difference in the double mutant compared to the wild-type. $FT$ is known to be expressed in LDs conditions and acts as a long-range signal in Arabidopsis (Valverde et al. 2004; Jaeger and Wigge 2007; Sawa and Kay 2011) and here this marker gene was used as a “control” to make sure that the plants were strictly grown under SD conditions and this is confirmed by its very low expression. Floral activators $FD$ and $TSF$ showed slight up-regulation, although only $TSF$ was statistically significantly different compared to the wild-type. Interestingly, it was shown that under non inductive SDs exogenous cytokinin promotes early flowering via activation of the $TSF$ (D’aloia et al. 2011). The exact mechanism of $TSF$ activation is not known and it could be that $ARR1$ and $ARR2$ play role in its regulation knowing that both $ARR1$ and $ARR2$ are part of TCS and react to cytokinin treatment (Brenner et al. 2012). It might be that non phosphorylated form of $ARR1$ and $ARR2$ by TCS are somehow disturbing transcription of $TSF$ and control flowering on that way. Unfortunately we have failed to produce the $tsf-1$ $arr1-4$ $arr2-4$ triple knock out mutant and make a closer look how these three genes are interacting on genetic level. In general this data clearly indicated that the expression profile of floral activators is not significantly changed in the double mutant and that these genes are mainly not responsible for the early flowering phenotype in the double mutant.

On the other hand, tested floral repressors showed quite a different expression pattern in the double mutant plants. The floral repressors $FLC$, $FLM$, $SVP$ and $TFL1$ cover most of the crucial signalling pathways responsible for the flowering regulation in Arabidopsis (Coupland 1995; Koornneef et al. 1998; Song et al. 2013). Interestingly, three of tested floral repressors showed significant down-regulation of their expression in double mutant: $FLC$ and $FLM$ were quite reduced whereas $TFL1$ showed only slight reduction. These data show the importance of $FLC$ and $FLM$ among the rest floral repressors with respect to early flowering in $arr1-4$ $arr2-4$. On the other hand, the $SVP$ floral repressor seems not to be miss-regulated by the lack of $ARR1$ or $ARR2$. This observation is of paramount importance and will be address later.

GA promotes flowering transition most strongly under environmental conditions such as SDs, especially when other regulatory pathways that promote flowering in Arabidopsis thaliana are not active (Moon et al. 2003; Porri et al. 2012). In an attempt to address this, showed that $GA4$ and $GA5$ reflected the state of active GA signalling in healthy plants (Achard et al. 2008). Therefore the expression of $GA4$ and $GA5$ were also monitored. Based on these two genes, we conclude that the GA flowering pathway is not disturbed in the double mutant as both $GA4$ and $GA5$ marker genes for this pathway are unaffected in the SAM in the double mutant.
This presumptively indicates that $ARR1$ and $ARR2$ are not involved in the GA flowering pathway but in other flowering signalling pathways active under non-inductive conditions.

Since meristem identity genes and some other genes predominately expressed in meristem play very important role in the initiation of the early flowering they were applied in order to profile the state of SAM at the chosen time point. For this purpose especially $AP1$ but also and $LFY$ expression was tested. Expression of $LFY$ and $AP1$ was 2-fold and 3-fold increased with respect to the wild-type. These markers confirmed the early flowering state of the $arr1-4$ $arr2-4$ double mutant compared to Col-0 at the time point where this process could not be visually obtained. Although the low expression of FT suggested that the plants were monitored before they were flowering, it could still have been possible that the shift to inflorescence meristem had already occurred. Therefore, to eliminate the possibility of unseen developmental effects, the flowering transition induced with 30-day old plants grown under non-inductive conditions and then shifted to inductive LDs. The material collected from SAM before and after the shift guaranteed that differences in expression profiles of floral regulators among the mutant plants were due to the miss-regulation of the flowering time only. Once more, it was demonstrated that the early flowering phenotype in both single parents and the wild-type comes from the miss-regulation, i.e. down-regulation of specific floral repressors, primarily $FLC$ and $FLM$, as that their level was low even under non-inductive conditions and it remained unchanged after the shifting. Expression pattern of $SVP$ floral regulator remained unaffected in any of mutant plants before the shift and responded as expected after the shift (was down-regulated) confirming the previous results and suggesting that $ARR1$ and $ARR2$ are specifically regulating only certain floral repressors under non-inductive SD conditions. Consistently, all of the tested floral activators showed their regular expression profile like wild-type,: they were up-regulated after the shift from vegetative into reproductive state and showing that neither $ARR1$ nor $ARR2$ are not playing any role in transcriptional regulators of these floral activators at the not expression level. In conclusion, the early flowering phenotype was most probably a consequence of down-regulation of floral repressors but not of miss-regulation of floral activators.

Considering roles of $ARR1$ and $ARR2$ in cytokinin signalling and knowing the fact that cytokinin has a positive-correlative role in regulating SAM size (Werner and Schmulling 2009; Gupta and Rashotte 2012) and that SAM size has been correlated with flowering time (Jeong and Clark 2005), the SAM size of $arr1-4$, $arr2-4$, and $arr1-4$ $arr2-4$ warranted examination. Such information could be used to deduce if changes in expression of floral repressors were due to an increase in size of the apical meristem in the mutant plants. Therefore, plants were grown under the same conditions used in the shifting experiment. Both the $arr2-4$ single and $arr1-4$ $arr2-4$ double mutants had surprisingly statistically significant smaller SAMs than the wild-type and although the measurement population also showed a smaller size for $arr1-4$, its SAM did not pass the significance threshold ($\alpha=0.05$). Thus, the smaller SAM fits more with a loss of cytokinin signal, which would be expected knowing that both $ARR1$ and $ARR2$ are active in cytokinin singling and despite the smaller SAM size in the mutant plants they
manage to flower earlier than the wild-type. Differences in anatomy or morphology of SAMs in mutant plants and the wild-type were not observed either. In conclusion, the changes in expression of floral repressors were not due to an increase in size of the apical meristem.

Although the arr2-4 and arr1-4 mutants have been previously described (Mason et al. 2005) and presumed free of any other background mutations, we wanted to be sure that the early-flowering phenotype was due to the lack of ARR1 and ARR2. In order to check this presumption and to determine the origin/location of acting of the ARR2 with respect to early flowering phenotype the arr2-4 and arr1-4 arr2-4 mutants which had the strongest phenotype were complemented with ARR2-cDNA and an ARR2D80N-cDNA mutant. The ARR2D80N-cDNA mutant version cannot be phosphorylated by the TCS anymore, and is less responsive to cytokinin than the wild-type ARR2-cDNA (Hwang and Sheen 2001; Choi et al. 2010; Veerabagu et al. 2012). Thus although it is not completely inert, ARR2D80N can be used to see if the wild-type protein, and therefore TCS phosphorylation-dependency, is necessary to rescue the early flowering phenotype.

The cDNA variants were driven under tissue-specific promoters pFD and pSUC2. For this purpose following vectors with promoter expressed in specific tissues were used: pSUC2 vasculature-specific promoter expressed in phloem (companion cells) only (Truernit and Sauer 1995; Wippel and Sauer 2012) and pFD-meristem-specific promoter expressed in shoot apical meristem only (Mathieu et al. 2007). Based on their expression pattern one can determine the most likely location where ARR2 protein activity is needed to complement the flowering time phenotype. Only T2 plants were able to be analysed, therefore a pre-selection was needed to remove any non-transgenic plants using BASTA; secondary effects were controlled by using an empty-vector backbone transformed into all of the mutant lines as well as the wild-type.

ARR2 driven under pFD shows the strongest effect and can almost completely complement the flowering phenotype of both the single and the double mutant in SDs where the arr2-4 and arr1-4 arr2-4 mutation effects were most strongly observed. This effect in SDs is slightly weaker (partial) when ARR2 is driven under pSUC2 but still significant in the double mutant. In LDs conditions, ARR2 expressed from either promoter was able to complement the arr2-4 mutation, but only ARR2 expressed under the pFD promoter was able to partially rescue the arr1-4 arr2-4 mutant. Considered that both tissue-specific promoters are equally strong and having in mind that pSUC2 expression is on whole plant level one can conclude that ARR2 is most potently working in the SAM with respect to flowering time.

Some remarks are warranted: first, both promoters can lead to complementation (even if partial) meaning that function of ARR2 is deliverable (or needed) at the meristem but also throughout the plant. Taken together; the observed early flowering phenotype is truly a consequence of the lack of ARR2 as it can be rescued when it is expressed directly in the SAM by the pFD promoter or when it is expressed in phloem companion cells by pSUC2. Although not directly shown in the work if the lack of ARR2 and ARR1 means that cytokinin signalling...
per-se is the culprit for the early-flowering phenotype, the idea is still passed around, albeit lightly, that cytokinin(s) in some form or fashion also could work in a “florigen” type function (Bernier 2011). These results are based on work in *Sinapis alba* (*Sa*) where connections to a simultaneous, but not necessarily, co-dependent transportation of *SaFT* and cytokinin to the meristem. Bernier proposed that some aspect of cytokinin, possibly on the organizing centre, contributes to flowering, but does not in itself cause flowering in *Sinapis* (Bernier 2011). This idea is interesting in lieu of the results here where a stronger effect is seen when *ARR2* is expressed in the meristem, yet *ARR2* still expressed in the phloem (distally) can also lead to complementation. Conflicting with these ideas is the observation that *Arabidopsis* can be induced to flower in SD with an 8 hour hydroponic treatment with BAP (D’aloia et al. 2011). This effect requires *TSF* and not *FT* (D’aloia et al. 2011). Recall that expression of *TSF* was altered in *arr1-4 arr2-4*.

FLC is known to interact with directly interacts with another MAD-box member SVP, SVP which is also floral repressor that functions within the thermosensory pathway (Li et al. 2008). SVP and FLC also target CYTOKININ RESPONSE 1 (CRE1) and other genes in cytokinin signalling (Gregis et al. 2013). SVP has been further connected to cytokinin via SVP regulation of STIMPY (STIP), but this occurs independently and downstream of *FT* and *TSF* (Gregis et al. 2013). *STIP* works downstream of cytokinin in establishing the SAM (Liu et al. 2009). Interestingly, *FLC* has been shown to be active in the SAM and in the vascular tissue directly regulating *SOC1* and *FT* (reviewed in Andres and Coupland 2012). *TSF* was shown not to be bound by SVP (Gregis et al. 2013). In this work, *arr1-4* and *arr2-4* were also crossed to *tsf-1* (Yamaguchi et al. 2005), but triple mutants were not identified, therefore it was not possible to determine the genetic interaction of *arr1-4* or *arr2-4* with *tsf-1*.

Knowing that the loss of *ARR1* and *ARR2* impair cytokinin signalling, it is tempting to speculate that this would explain the early flowering phenotype. In this regard, SVP has recently been uncovered to be involved in regulation of some cytokinin genes (Gregis et al. 2013). Considering that the expression data and mutant analysis (discussed below) suggest that *ARR1* and *ARR2* are not in the *SVP-FLC* pathway, it is still an open question if the early flowering phenotype can only be attributed to a loss of cytokinin single transmission. Second, other possible reasons for partial complementation could be that the plants used for this experiment were preselected in the T2 generation with most of them in the hemizygous state and thus not enough *ARR2* was produced. Alternatively, the gene was not driven under its native promoter and perhaps *ARR2* is also needed in other tissues. For example, it is known that *ARR1* is active in controlling auxin flux by regulation of the auxin-response repressor protein SHY2 (SHORT HYPOCOTYL2) (Taniguchi et al. 2007; Chapman and Estelle 2009) and the functional characterisation of *ARR1* and *ARR2* have mostly been done with regards to cytokinin responses in the root (Mason et al. 2005; Argyros et al. 2008; Hill et al. 2013).

As said before, the connection to cytokinin, is compelling but not conclusive. In rice, it is known that a cytokinin-dependent TCS pathway exists that requires a B-type (*EHD1*) for aviation of *FT* homologues *HD3A* and *RICE FLOWERING LOCUS T1* (*RFT1*) under SDs (Andres...
and Coupland 2012). Provided that more evidence is mounted that connects more TCS components to this flowering time phenotype, then (although highly speculative at this point) it could be proposed that a TCS network also works in Arabidopsis to modulate flowering which may be analogous to that of rice (as it is not likely it is the same considering no orthologues can be found) or more indicative to the observations made in Sinapis. Nevertheless, this regulation mechanism in rice only supports our observation that TCS, or at least its B-Type response regulators ARR1 and ARR2, might also play role in flowering regulation of Arabidopsis thaliana under short day conditions as mutant plants lacking in ARR1 and ARR2 genes showed the early flowering phenotype under non-inductive short days in Arabidopsis. In support to this claim is a fact that ARR4 (A-type response regulator) in Arabidopsis thaliana plays very important role in stabilization of active PhyB-Pfr under extended red light conditions and thus functions as modulator of photomorphogenesis (Sweere et al. 2001; Mira-Rodado et al. 2007) and PhyB, as above mentioned, actively involved in expression control of CO in Arabidopsis thaliana and Hd3a in Oryza sativa (Ishikawa et al. 2011).

That said, is there any evidence that TCS signalling is required for the early-flowering time phenotype? Examination of the mutant plants showed that they were also slightly complemented with loss-of-function ARR2D80N in the single and double mutants under SDs when driven under the pFD promoter or by pSUC2 (albeit only in the arr1-4 arr2-4). This slight effect of ARR2D80N was also observed in LDs but only in the arr2-4 mutant background. As mentioned earlier, the reason for this is most likely due to an incomplete inactivation of the ARR2 by the D80N mutation which was proven by other groups (Hwang and Sheen 2001). ARR2 has a strong basal transactivation capacity unlike the other B-types without the presence of exogenous cytokinin; yet, it still can strongly activate promoter-reporters in a cytokinin-dependent manner (Hwang and Sheen 2001; Veerabagu et al. 2012). The ARR2-D80N mutation impairs, sometimes strongly, this basal transactivation capacity without destroying the cytokinin-dependent response, which is highly enhanced compared to mock controls (Hwang and Sheen 2001). Considering this fact, it can be still concluded that ARR2 needs to be phosphorylated by the TCS in order to best rescue the early flowering phenotype as only weak partial complementation were observed for the ARR2D80N loss-of-function gene. Type-B response regulators mediate most, if not all, of the immediate–early changes of gene expression induced by cytokinin. This is also the case with ARR1 and ARR2 member of the largest sub-class of response regulators expressed in almost all tissues which consists of seven members (ARR1, ARR2, ARR10, ARR12, ARR11, ARR14, and ARR18) (Brenner et al. 2012). This additionally supports the claim that the early flowering phenotype in the ARR1 and ARR2 lacking plants is originating due to the impaired signalling of cytokinin. Furthermore, the expression pattern of the ARR1 and ARR2 in almost all tissues is showing the importance of cytokinin in every tissue of the plant, and also explains the observation that the early flowering phenotype was rescued also when ARR2 expressed from phloem. On the other hand mutant complementation with ARR2D80E gain-of-function gene under constitutive active promoter is not possible while the plants showed embryo lethality (Hass et
al. 2004). Based on this fact the complementation with pSUC2 and pFD tissue specific promoters could not possible while due to their high potency. Therefore arr1-4 arr2-4 was complemented with ARR2D80E cloned into estradiol inducible pABind::GFP vector. As expected the effect of the gain-of-function ARR2D80E was very strong and lethal even for plants in T2 generation (supplemental figure 10) and was clearly visible 3 days after the hormonal treatment. Plants were getting various necrotic spots and dying very fast, whereas some of them managed to flower showing early flowering phenotype caused by stress. The formed siliques showed great variation in size and the seed yield was generally more than double lower compared with the wild-type containing certain number of aborted and sterile seed as well. This strongly suggests that ARR2 may be a “crosstalk point” between the TCS and some flowering time pathways under the short day conditions. Expression analysis, via RT-qPCR, revealed which genes were miss-regulated in the single and double mutants compared to the wild-type and after conducting the flowering time shift, it could be confirmed that the repressors FLC and FLM are down-regulated in arr1-4 arr2-4. In order to determine which floral pathways ARR1 and ARR2 are involved in, arr1-4 arr2-4 or arr2-4 mutants were crossed to mutants in key floral regulatory pathways: flc-3, flm-3, tsf-1, co, and soc1. Triple mutants were obtained and evaluated with flc-3 and flm-3, whereas arr2-4 double mutants with co and soc1 were also obtained and evaluated. Crosses with tsf unfortunately were not evaluable. For these experiments arr2-4 single and arr1-4 arr2-4 double mutants were crossed against flc-3, flm-3 single mutants lacking in these floral repressors as they showed the highest miss-regulation pattern in ARR1 and ARR2 lacking mutants. On the other hand, despite the fact that all tested floral activators did not show any expression miss-regulation in arr1-4 arr2-4, co and soc1 single mutants were crossed in order to see how and if these genes are interacting on genetic level.

When grown under inductive LD conditions at standard 22/23°C temperature the flc-3 arr1-4 arr2-4 triple mutant behaved like its flc-3 parent suggesting that FLC and the ARR1/2 are involved in the same pathway that controls flowering, i.e. FLC is epistatic to both ARR1 and ARR2. Nevertheless, early flowering phenotype is under LDs is by all the mutants quite weak and the temporal resolution of flowering was very low. In addition to this flc-3 arr1-4 arr2-4 triple mutant after each repeat under the LDs showed clear tendency to flower even earlier than the flc-3 single parent but none of the time this tendency could be statistically proven. Therefore, the same experiment was repeated again under LDs but at 16°C temperature. Lower temperature should generally slow down the flowering time and the discrete differences in flowering time between the different mutant lines should be observed in higher resolution. This was indeed the case at 16°C, plants generally flowered later than at 23°C. Interestingly, this time flc-3 arr1-4 arr2-4 triple mutant flowered even earlier that flc-3 parent suggesting the additive effect of FLC under ARR1 and ARR2 under inductive LD conditions. Surprisingly, flc-3 deletion mutant was in all this conditions showing the same ratio of flowering compared to the wild-type suggesting that FLC is probably active at the same level on both temperatures with respect to flowering time regulation, i.e. it is active at 16°C as a repressor. It have been published that that there are some natural accessions that are
unresponsive to thermal induction despite having non-functional fri/flc alleles (Srikanth and Schmid 2011). Recently was also reported that flc-3 single mutant restores its temperature sensitivity fist below 16°C and not at higher temperatures (Lee et al. 2013). Under non-inductive SD conditions the flc-3 arr1-4 arr2-4 triple mutant very clearly shows consistent additive effect in early flowering. The same pattern was observed also when this experiment was repeated under SD conditions in other growth chamber with different light intensity.

In conclusion, FLC appears to work partially independent of ARR1 and ARR2 which lack of expression has the same outcome with respect to early flowering phenotype making the triple mutant to flower even earlier then its parents in additive manner. This additive effect is especially good visible under SD conditions due to the enhanced activity of ARR1 and ARR2 under non-inductive short days with respect to early flowering. Under LD conditions at normal ambient temperature of 23°C additive effect could not be statistically proven, but there was each time the tendency present, due to very low involvement of ARR1 and ARR2 with respect to flowering time and therefore weak flowering time phenotype with the mutants. When plants grown under inductive LD conditions but at 16°C temperature additive effect could be observed and statistically proven. On the other hand, in flm-3 arr2-4 double and flm-3 arr1-4 arr2-4 triple mutants obtained early flowering phenotype was very clear. When grown on both inductive LD and non-inductive SD conditions both flm-3 arr2-4 double and flm-3 arr1-4 arr2-4 triple mutants showed identical phenotype like flm-3 single parent supporting previous expression data obtained by RT-qPCR. Single flm-3 mutant has very strong early flowering effect on both LD and SD conditions so it was very easy to proof and describe the obtained phenotype. Based on obtained results both ARR1 and ARR2 seem to work in the same pathway like FLM with respect to early flowering. Genetically observed FLM is epistatic to ARR1 and ARR2.

Despite the fact that all tested floral activators and signal integrators did not show any miss-regulation of their expression in tested ARR1 and ARR2 knock outs, CO and SOC1 lacking mutants were crossed with arr2-4 single mutant in order to examine their genetic interaction/hierarchy and see if there is some difference. When tested under inductive LDs co arr2-4 flowered more than two weeks after the wild-type identical to its co single mutant parent. This may suggest that under LDs CO may be epistatic to ARR2 or event that they work independent and because the weak phenotype of arr2-4 under LDs this difference could not be observed. When co arr2-4 double mutant was grown under non-inductive SD conditions it flowered earlier than the wild-type showing the identical phenotype like arr2-4 single parent. This is because co mutants are only delayed in long days, and CO mRNA is more abundant in long than in short days (Blazquez et al. 1997) and CO dependent pathways are not active under non-inductive SD conditions, only under LDs where the CO protein is stabilized by light and this leads to induction of the floral activators FT and SOC1 (Eriksson et al. 2006). The effect observed in the co arr2-4 double mutant is more due to the lack of ARR2 only.

Another tested mutant with SOC1 lacking floral signal integrator, soc1-2 arr2-4 when grown under inductive LD conditions showed the late flowering phenotype which was statistically
not different than soc1-2 single parent suggesting epistasis of SOC1 under ARR2. Nevertheless, soc1 arr2-4 each time showed tendency to flower slight earlier than the soc1-2 single mutant but because of the very weak flowering phenotype of arr2-4 under inductive conditions it could not be statistically proven but still independent working ARR2 and SOC1 with respect to flowering time cannot be excluded. This claim was supported by fact that when the soc1-2 arr2-4 double mutant was grown under non-inductive SD conditions it showed an intermediate flowering phenotype. The double mutant plant flowered approximately one week after arr2-4 single parent and also one week before the soc1-2 single parent. This all suggested that under non-inductive SD conditions, and most probably under inductive LD conditions, the ARR2 and SOC1 are working independent of each other with respect to flowering time which was before proven on expression level as well; ARR2 works here as negative regulator of flowering (alternatively positive regulator of floral suppressors) and SOC1 is known to be a positive regulator of flowering.
5.4 References for Chapter 2


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6 Appendix

6.1 Supplemental Figures

Supplemental 1 Supplement to Figure 4.6. *In silico* predictions of MKK docking motifs on ARR2 based on ELM software.

The figure shows different predicted functional domains of ARR2; four docking motifs for the MKKs are marked (yellow). ELM - (the database of eukaryotic linear motifs, http://elm.eu.org). The docking motif for MKKs is called DOC_MAPK_1.
Supplemental 2 Supplement to Figure 4.8. oNPG data for interaction strength between ARR1 and ARR18 with biotic-stress-related MKKs in yeast.
The β-galactosidase activity was measured in the extracts of three independent yeast clones. Diluted yeast colonies were incubated for 2 days at 28°C on interaction selective (CSM-L, W, A) media. The experiment was repeated at least two times.

Supplemental 3 Supplement to Figure 4.13B. Results of Fisher’s Least Significant Difference (LSD) test for pathogen assay with Pseudomonas syringae Pst DC3000
(A) LSD results of plants treated only with Pst DC3000. (B) LSD results of plants treated with PstDC3000 in addition of cytokinin (1 µM t-Zeatin). WT stands for Col-0. Data are presented for days: 0, 1, 2 and 4 post treatment. LSD tests were conducted at the 0.05 α level.

Levels not connected by same letter are significantly different.
Supplemental 4 Supplement to Figure 4.15. Results of Fisher’s Least Significant Difference (LSD) test for measurements of stomata aperture

Wt stands for Col-0, 1-4 for arr1-4, 2-4 for arr2-4 and 1-4x2-4 for arr1-4 arr2-4. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.

Supplemental 5 Supplement to Figure 4.16. Results of Fisher’s Least Significant Difference (LSD) test for pathogen assay with Alternaria brassicicola

(A) LSD results for plants treated with Alternaria only after 7 days (top) and after 10 days (bottom). (B) LSD results for plants treated with Alternaria in the presence and absence of cytokinin (1µM t-Zeatin). WT stands for Col-0. Control represents MOCK treatment. LSD tests were conducted at the 0.05 α level.
Supplemental 6 Supplement to Figure 4.16B. Plants treated with cytokinin for pathogen assays with *Alternaria brassicicola*

Cytokinin application was monitored by detecting expression of *ARR4*, which is a known cytokinin responsive gene. Plants treated with 1 µM t-Zeatin showed increased expression of *ARR4* indirectly proving that cytokinin treatment was successful.
Supplemental 7 Supplement to Figure 4.17. Plants treated with cytokinin for pathogen assays with *Botrytis cinerea*.

(A) Left: Outgrowth Index data shown as mean and the 95% confidence interval. Right: LSD results for the Outgrowth Index data about the mean at the 0.1 $\alpha$ level. All of the mutants are significantly smaller than the wild-type at the 0.1 alpha level and those with arr2-4 at the 0.05 $\alpha$ level. (B) Left: Lesion Size data shown as mean and the 95% confidence interval. Right: LSD results for the Lesion Size about the mean at the 0.06 $\alpha$ level. All of the mutants are significantly smaller than the wild-type at the 0.06 $\alpha$ level.
Supplemental 8 to table 5.3. Flowering time of transgenic lines driven under tissue specific pSUC2 promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where pSUC2::ARR2 was complemented in arr2-4 mutant background. (B) Experiments where pSUC2::ARR2 was complemented in arr1-4 arr2-4 mutant background. All independent lines show the same tendency. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.

Supplemental 9 to table 5.4. Flowering time of transgenic lines driven under tissue specific FD promoter under short day non-inductive conditions (alternative representation).
(A) Experiments where pFD::ARR2 was complemented in arr2-4 mutant background. (B) Experiments where pFD::ARR2 was complemented in arr1-4 arr2-4 mutant background. All independent lines show the same tendency and stronger complementation than under SUC2 promoter. Lines used for this experiment were in T2 generation and preselected with BASTA. Mean values and standard deviations are shown.
Supplemental 10 Phenotypic differences of arr1-4 arr2-4 transgenic lines transformed with pABind::ARR2D80E::GFP estradiol inducible vector when treated with estradiol or Mock control

(A) arr1-4 arr2-4 transformed with pABind::ARR2D80E::GFP estradiol inducible vector in T2 generation. Left photograph shows phenotypic differences of transgenic plant not induced (-E) and induced (+E) with β-estradiol. In addition photographs of disproportional silique size, reduced seed number and leaf changes, respectively, of transgenic line treated with β-estradiol are shown. (B) Difference in seed number and types of seeds between non-induced (left) and induced (right) transgenic line. For this experiment at least 10 plants per transgenic line were taken, total 3 independent lines were used, and 5 siliques per plant were used for statistical evaluation.
Supplemental 11 Transcriptional levels of ARR1, ARR2 and FLC in the triple flc-3 arr1-4 arr2-4 mutant knock out.

(A) Expression of ARR1 in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. (B) Expression of ARR2 in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. (C) Expression of FLC in flc-3, arr2-4, arr1-4 arr2-4 and flc-3 arr1-4 arr2-4 mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate with three technical repetitions each and normalized to ELF-1-α. Error bars indicate SD of the mean.
Supplemental 12 Transcriptional levels of ARR1, ARR2 and FLM in the triple flm-3 arr1-4 arr2-4 mutant knock out.

(A) Expression of ARR1 in Col-0, flm-3, arr1-4 arr2-4 and flm-3 arr1-4 arr2-4 mutants. (B) Expression of ARR2 in Col-0, flm-3, arr2-4, arr1-4 arr2-4 and flm-3 arr1-4 arr2-4 mutants. (C) Expression of FLM in Col-0, flm-3, arr2-4, arr1-4 arr2-4 and flm-3 arr1-4 arr2-4 mutants. For RNA isolation leaves were taken from 3-week-old plants grown under SD. RT-qPCR was done using one biological replicate was used with three technical repetitions and normalized to ELF-1-α. Error bars indicate SD of the mean.
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7 Curriculum Vitae

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Непроцењиву заслугу у целокупном мом животном развоју има, наравно, и мој комшилук из Церове (комшије Снежа и Горан) и касније Новог Сада (моји Снежа и Иван, Срче-Прасе, Романа, дивни Мира и Влада). Хвала Вам на искреној и безусловној подршци, ручковима и вечерама, трпљења мојих провокацији и разноразних испада, силним покушајима да ме „уразумите“ и „доведете у ред“ (мада ми је драго да се само делимично у томе успели!).

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