Molecular and Functional Characterization of the *Arabidopsis thaliana* Response Regulator ARR18

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Dedicated to My Beloved Father

O. Veerabahu

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Abbreviations

2S1	Seed storage albumin 1
2S2	Seed storage albumin 2
35S	Cauliflower Mosaic Virus 35S promoter
ABA	Abscisic acid
AHK	*Arabidopsis* histidine kinase
AHP	*Arabidopsis* histidine-phosphotransfer protein
ARR	*Arabidopsis* response regulator
ASN1	Asparagine synthetase1
Asp	Aspartate
BiFC	Bimolecular fluorescence complementation
bZIP	basic leucine zipper
CHASE	cyclase/His kinase-associated sensing extracellular
CKI1	Cytokinin independent 1
CRF	Cytokinin response factor
CTR1	Constitutive triple response
EIN	Ethylene insensitive
Elf	EF-Tu
ERF	Ethylene response factor
ERS	Ethylene response sensor
FRET	Förster resonance energy transfer
FLIM	Fluorescence lifetime imaging
GFP	Green fluorescence Protein
H_2O_2	Hydrogen peroxide
HA	Hemagglutinin antibody
His	Histidine
HK	Histidine kinase
LSD1	Lesion stimulating disease 1
MIPS	Munich information center for protein sequences
MS	Murashige-skoog medium
NLS	Nuclear localization sequence
OD	Output domain
PEG	Polyethylene glycol
phyB	Phytochrome B

PR1	Pathogen related protein 1
proDH	Proline dehydrogenase
Q-RTPCR	Real time quantitative PCR
RD	Receiver domain
RD29B	Responsive to dessication 29B
RFP	Red fluorescence Protein
RR	Response Regulator
TCS	Two-component System
T-DNA	Transfer DNA
GUS/uidA	Beta-D-glucuronidase
Y2H	Yeast-two Hybrid-System

Zusammenfassung

Das Zweikomponentensystem hat sich zu einem wichtigen Signal-Antwort-Mechanismus in *Arabidopsis thaliana* entwickelt und besteht aus Hybrid-Histidinkinasen (AHKs), Histidin Phosphotransferproteinen (AHPs) und Responseregulatoren (ARRs), welche biochemisch über einen Histidin zu Aspartat Phosphorelay miteinander in Verbindung stehen. In der vorliegenden Arbeit wurde die funktion des Response-reulators ARR18 durch verschiedene funktionale und molekularbiologische Ansätze näher characterisiert. Es Konnte gezeigt werden, dass ARR18 ein aktiver Transkriptionsfaktor ist dessen Aktivität und Homo-dimerisierung hauptsächlich von seinem Phosphorylierungszustand abhängen. Weitere Untersuchungen zeigten, dass ARR18 auch Heterodimere mit bZIP63 bilden kann und dessen Transkriptionsaktivität negativ beeinflusst, was ebenfalls vom Phosphorylierungszustand von ARR18 abhängt. Des Weiteren wurde durch umfassende Untersuchungen gezeigt, dass ARR18 und bZIP63 antagonistische Regulatoren der osmotischen Stressantwort in *Arabidopsis thaliana* darstellen. Die Ergebnisse unserer Untersuchungen zeigen die Bedeutung des Phosphorylierungszustandes der ARR18 bei der Kontrolle jedes Signalmechanismus sowie die Bedeutung von Proteinen, die nicht zum Zweikomponentensystem gehören jedoch durch direkte Interaktion mit dessen Elementen diesem ein neues Ausmaß an Diversität und Komplexität verleihen.

1. Summary

The two-component signaling system evolved as an important sensing and responding mechanism in *Arabidopsis thaliana*, which consists of hybrid histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs) and response regulators (ARRs) biochemically linked by a histidine to aspartate phosphorelay. In our work, several functional and molecular biological approaches were performed to characterize and understand the function of the B-type response regulator ARR18. In our study, ARR18 has been found to be an active transcription factor whose activity and homodimerization mainly depend on its phosphorylation state. Further investigations revealed that ARR18 is also able to form heteromers with bZIP63 and negatively influences the transcriptional activity of bZIP63 in a process that again depends on the ARR18 phosphorylation state. Moreover, extensive studies showed that ARR18 and bZIP63 function as antagonistic regulators in osmotic stress signaling in *Arabidopsis thaliana*. The results of our study reveal the importance of the phosphorylation state of the ARRs in controlling each signaling mechanism and the importance of non-TCS related proteins which, by direct interaction with TCS elements, add a new degree of diversity and complexity to the TCS signaling network.

2. Introduction

2.1. Two-component signaling systems

Bacteria and higher plants are relatively less capable of escaping from changing environmental conditions, so they have to react quickly and efficiently on a broad spectrum of environmental changes to survive. To tackle this difficult task, both plants and bacteria relay on different signal perception and transduction systems; one of them is the two-component system (TCS). Although the TCS was first described in bacteria, there is clear evidence that such a signaling mechanism occurs also in *Archaeabacteria*, eukayotes like *Saccharomyces*, *Dictyostelium* and *Neurospora* and higher plants like *Arabidopsis*, rice and maize (Kennelly and Potts, 1996; Loomis et al., 1997; Pareek et al., 2006; Rudolph and Oesterhelt, 1995; Stock et al., 2000). The TCS is a His- to-Asp phosphorelay mechanism involving at least two different proteins, the histidine kinase (HK) and the response regulator (RR). Signaling is triggered by exogenous or endogenous stimulus that results in the autophosphorylation of the histidine kinase. The phosphate group is then transferred to the response regulator which mediates the cellular response.

2.1.1. Two-component system (TCS) in bacteria

The two-component signaling system in prokaryotes is involved in different processes like light perception, chemotaxis and osmotic sensing (Stock and Da Re, 2000). In bacteria there are two distinguishable TCS mechanisms. The first one refers to the classical model described above composed of two different protein types, the histidine kinases and the response regulators. This one-step mechanism seems to dominate the bacterial TCS system as, for example, in the osmoregulatory EnvZ/OmpR system of *Escherichia coli*. In this case, the phosphotransfer occurs in a single step from the histidine-containing transmitter domain of the histidine kinase to the aspartate present in the receiver domain of the cognate response regulator (His-Asp phosphorelay). Differing from the above described mechanism, bacteria also possess an unorthodox TCS, which is based on a multistep phosphorelay (His-Asp-His-Asp phosphorelay) and can involve two or more additional proteins (Appleby et al., 1996). For example, in the *Bacillus subtilis* sporulation control system, all signaling domains are separated from each other and belong to four independent proteins (Perego, 1998). Variations of this system have been also observed in *Saccharomyces cerevisiae, Pseudomonas putida,* and *Bordetella pertussis* (Perraud et al., 1999).

Another characteristic of the bacterial TCS is that its different elements can dimerize, for example, the HK EnvZ of *Escherichia coli* has been reported to form stable dimers for the recognition of the OmpR response regulator (Park et al., 1998). Also response regulators are able to form dimers in a process that is induced by phosphorylation. Phosphorylation-dependent dimerization has been reported for the response regulators of the OmpR, NarL, LytR, NtrC and PrrA families (Baikalov et al., 1996; Laguri et al., 2003; Lee et al., 2003; Martinez-Hackert and Stock, 1997; Nikolskaya and Galperin, 2002). Recently, phosphorylation-independent dimerization of the *Salmonella enterica* PhoP (Perron-Savard et al., 2005) and *Escherichia coli* PhoB response regulators (Mack et al., 2009) has also been reported.

2.1.2. Two-component signaling in *Arabidopsis thaliana*

The *Arabidopsis* two-component signaling system is established by a multistep phosphorelay mechanism that involves members of three protein families of canonical TCS elements. The *Arabidopsis* histidine kinases (AHKs), the *Arabidopsis* histidine-phosphotransfer proteins (AHPs) and the *Arabidopsis* response regulators (ARRs) (Hwang et al., 2002).

In the *Arabidopsis* TCS system, the AHKs are hybrid histidine kinases as they carry an additional receiver domain. In response to the specific stimulus, the cognate AHK gets autophosphorylated on its histidine-containing transmitter domain and the phosphorelay is initiated by the transfer of the phosphate residue to its aspartate containing receiver domain. Among the 11 AHKs present in *Arabidopsis thaliana*, five (ETR1, ETR2, EIN4, ERS1, ERS2) are ethylene receptors (Chang et al., 1993; Hua et al., 1998). The remaining six AHKs have been shown to be involved in cytokinin signaling (AHK2, AHK3, AHK4) (Higuchi et al., 2004; Inoue et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001), megagametophyte development (CKI1) (Pischke et al., 2002), stress signaling (AHK1) (Tran et al., 2007) and stomatal closure (AHK5) (Desikan et al., 2008).

There are 6 AHPs in *Arabidopsis* that mediate the phosphorelay between AHKs and ARRs. *AHP1* to *AHP5* genes encode proteins which contain the conserved histidine residue required for phosphorylation and are, therefore, called canonical AHPs (Suzuki et al., 1998). The protein encoded by *AHP6* is considered to be a pseudo-AHP, since it lacks the conserved hisitidine residue required for phosphorylation (Grefen and Harter, 2004; Suzuki et al., 1998).

The final elements of the *Arabidopsis* two-component signaling systems are the response regulators, which mediate the output activity of the TCS and are divided into 3 subgroups according to their protein properties. The A-type ARRs (ARR3, 4, 5, 6, 7, 8, 9, 15, 16, 17) are

small proteins that carry an Asp-containing receiver domain and a short C-terminal extension (Imamura et al., 1998). They have been reported to be mainly negative regulators of cytokinin signaling (Hwang and Sheen, 2001).

The B-type response regulators (ARR1, 2, 10, 11, 12, 13, 14, 18, 19, 20 and 21) are transcriptional regulators. They also comprise a receiver domain along with an extended C-terminal output domain, in which a GARP DNA-binding domain, a transactivation domain and at least one NLS is found (Grefen and Harter, 2004; Lohrmann and Harter, 2002; Sakai et al., 2000). Some of them, namely ARR1, ARR2 and ARR10, have been reported to bind the 5′-(A/G)GAT(T/C)-3′ nucleotide motif in a sequence-specific manner (Imamura et al., 2003). This motif is enriched in the promoter of primary cytokinin response genes such as *ARR4*, *ARR5*, *ARR6* and *ARR7* (Rashotte et al., 2003). Although all B-type response regulators are able to activate the transcription of the *ARR6* gene in *Arabidopsis* protoplasts (Hwang and Sheen, 2001), only in the case of ARR1, ARR2, ARR10 and ARR12, their ability to bind to a specific DNA sequence has directly been proven (Hwang and Sheen, 2001; Imamura et al., 2003; Mason et al., 2005; Sakai et al., 2000). While the involvement of B-type response regulators in cytokinin signaling has been studied in detail and they are considered to act as positive regulators of cytokinin signaling (Grefen and Harter, 2004; Hwang and Sheen, 2001), little is known about their role in other plant processes. One exception is ARR2 that functions downstream of ETR1 in ethylene signaling (Hass et al., 2004) and ethylene / H_2O_2-mediated stomatal closure (Desikan et al., 2006). Although some of the B-type ARRs have been well studied, for some their functions are still not known. One good example is ARR18, of which no information is available yet apart from its expression pattern and intracellular localization (Mason et al., 2004). The C-type ARRs (ARR22, ARR24) are structurally and functionally similar to the A-type ARRs but their expression is not induced by cytokinin. Recent studies suggest that they act phospho-histidine phosphatases (Gattolin et al., 2006; Horak et al., 2008; Kiba et al., 2004).

Similar to bacterial TCS members, *Arabidopsis* TCS elements have also been shown to form dimers. The transmitter domains of AHK2, 3 and 4 (Dortay et al., 2008; Dortay et al., 2006), full-length AHK3 and AHK4 (Caesar et al., 2011), and the ethylene receptors forms homo- and heterodimers *in vivo* (Gao et al., 2008; Grefen et al., 2008). The intermediate element AHPs (AHP1, 2, 3) also been reported to form homo and heterodimers (Dortay et al., 2008; Dortay et al., 2006; Punwani et al., 2010). However till now there is no information available regarding the dimerization of the *Arabidopsis* response regulators.

2.2. The role of the two component system in cytokinin signaling

Cytokinins are adenine derivatives and have been well recognized as an essential hormone involved in many aspects of plant growth, development and responses to the environment. These processes include cell division, root elongation (Cary et al., 1995), shoot development, stress tolerance, shoot meristem initiation, chloroplast biogenesis, photomorphogenesis, seed development, senescence, vascular patterning and apical dominance (Argueso et al., 2010; Kieber and Schaller, 2010; Muller, 2011; Muller and Sheen, 2007). The cytokinin receptor subfamily consists of three hybrid histidine kinases AHK2, AHK3 and AHK4 (CRE1/WOL) that exhibit structural similarity among each other (Heyl et al., 2011; Yamada et al., 2001). The N-terminus of these AHKs consists of two to three transmembrane domains followed by a CHASE (cyclase / histidine kinase associated sensory extracellular) domain, a histidine containing transmitter domain and a receiver domain with the phosphorylatable aspartate (Grefen and Harter, 2004). Notably all three cytokinin receptors carry an additional receiver-like domain between the transmitter and the receiver domain, for which the function is unknown (Grefen and Harter, 2004; Hwang and Sheen, 2001). The cytokinin is perceived by the CHASE domain (Hothorn et al., 2011) and initiates the autophosphorylation at the conserved histidine residue in the transmitter domain (Muller and Sheen, 2007; Yamada et al., 2001). During cytokinin signaling, the phosphate is then relayed firstly to the conserved aspartate residue in the receiver domain of the AHK, from there to an AHP (Dortay et al., 2006) and finally to an ARR. As described above, cytokinin activates B-type ARRs that act as transcription factors up-regulating *A-type ARRs* transcription (Hwang and Sheen, 2002). At the same time, A-type ARRs act as negative regulators of cytokinin signaling (Hwang and Sheen, 2001; To et al., 2004) establishing a negative feedback loop in the cytokinin signaling (Ferreira and Kieber, 2005; Lohrmann and Harter, 2002; Muller and Sheen, 2007).

AHK2, AHK3 and AHK4 have been shown to be able to bind to natural and synthetic cytokinins (Heyl et al., 2011; Hwang and Sheen, 2002; Inoue et al., 2001; Stolz et al., 2011; Suzuki et al., 2001), providing compelling evidence that these are true cytokinin receptors (Yamada et al., 2001). Although the receptors show structural similarities and functional overlap (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), it is clear that they differ in the number of transmembrane domains and their preference towards the different active derivatives of cytokinin (Romanov et al., 2006). Regarding their function, the different cytokinin receptors display specific but overlapping functions. For example, AHK4 is primarily involved in the development of primary root vascular tissue (Mahonen et al., 2000)

which also includes a role in regulating the sensitivity to cytokinin in root elongation (Inoue et al., 2001; Riefler et al., 2006). AHK3 plays a role in leaf senescence (Kim et al., 2006) and cytokinin control of the meristematic cell differentiation rate in primary roots (Raffaele Dello Ioio et al., 2007).

In cytokinin signaling, the AHP proteins shuttles from the cytoplasm to the nucleus in order to connect the membrane bound cytokinin receptors with the nuclear localized B-type response regulators (Punwani et al., 2010). Among the six AHP proteins in *Arabidopsis*, AHP1 to AHP5 acts as positive regulators in cytokinin signaling (Hutchison et al., 2006). Although their function seems to be quite redundant, 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 an increased seed size similar to that observed in the *ahk2/ahk3/ahk4* triple mutant (Hutchison et al., 2006; Riefler et al., 2006). The pseudo-AHP AHP6 has been shown to impair the phosphotransfer between AHKs and the canonical AHPs, most likely by directly competing with functional AHPs for interaction with the AHKs (Mahonen et al., 2006).

When the phosphate group finally reaches the nuclear localized B-type ARRs, cytokinin signaling target genes such as A-type *ARRs* and the cytokinin response factors CRF1 to 6 are activated (Rashotte et al., 2006). Among the B-type ARRs functional overlap seems to be the rule. Thus, only higher order mutant such as the *arr1/arr10/arr12* triple mutant reveal a decreased sensitivity to cytokinin (Ishida et al., 2008; Mason et al., 2005; Yokoyama et al., 2007). Apart from their function in cytokinin signaling, B-type ARRs, namely ARR1 and ARR2, are also involved in processes not related to cytokinin signaling such as ethylene signal transduction (Hass et al., 2004), ethylene / H_2O_2-mediated stomatal closure (Desikan et al., 2006), regulation of female gametophyte development and vegetative growth (Deng et al., 2010). Similarly, apart from the role as negative regulators of cytokinin signaling, the A-type response regulators also have specific roles in processes not always directly triggered by cytokinin. For instance, ARR5 mediates lateral root formation (Lohar et al., 2004), ARR7 is a negative regulator of cold stress (Jeon et al., 2010) and ARR16 is involved in leaf senescence (Ren et al., 2009).

Cross-talk between cytokinin signaling and other signaling pathways takes place during the entire life of the plant. A good example is the cross-talk between red light and cytokinin signaling mediated by the A-type response regulator ARR4 (Mira-Rodado et al., 2007; Sweere et al., 2001). ARR4 gets activated by phosphorylation in a process triggered by the cytokinin receptors (Ferreira and Kieber, 2005; Mira-Rodado et al., 2007). The active ARR4

is able to interact and stabilize the active Pfr-form of the red light photoreceptor phytochrome B (phyB) (Mira-Rodado et al., 2007; Sweere et al., 2001). Thus, in *Arabidopsis*, ARR4 overexpression results in modified phyB-mediated processes such as enhanced hypocotyl elongation and early flowering (Mira-Rodado et al., 2007). In contrast, *arr4* mutants display a reduced phyB response in view of the fact that no ARR4 protein is present to stabilize the photoreceptor (Mira-Rodado et al., 2007). Cytokinin is also involved in relaying the nitrogen nutritional status from roots to leaves (Sakakibara, 2006) and regulating sodium accumulation in the shoots by controlling the AtHKT1 gene in the roots *via* ARR1 and ARR12 (Mason et al., 2010). The cross-talk between cytokinin and auxin balances cell division and differentiation through ARR1 (Taniguchi et al., 2007). Cytokinin-activated ARR2 has also been shown to promote plant immunity *via* salicylic acid signaling in *Arabidopsis* (Choi et al., 2010).

2.3. The role of the two-component system in osmotic signaling

Drought and high-salinity conditions are considered to be among the solid threads to plant productivity (Hasegawa et al., 2000; Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). In order to combat such an adverse environment, plants have developed sensitive protection systems enabling them to respond and adapt to drought and other stresses. In *Arabidopsis*, one member of the two-component system (TCS) family, namely the *Arabidopsis* histidine kinase 1 (AHK1) has been reported to act as an osmosensor (Urao et al., 1999). The initial hint of AHK1 being an osmosensor came from a report showing that AHK1 was able to complement the yeast osmosensing deletion mutant *sln1* (Urao et al., 1999). Later on, AHK2, 3 and 4 were also reported to be involved in stress responses. While microarray analysis of an *ahk1* mutant indicated that, by the down-regulation of many stress inducible genes such as *RD29B*, AHK1 positively regulated stress responses (Tran et al., 2007), *ahk2, ahk3 and ahk4* mutants were strongly tolerant to stress by up-regulating many stress-regulated genes (Tran et al., 2007). Increased transcript levels of ABA biosynthetic genes during osmotic stress, which depend on the presence of AHK1, support the idea of signaling pathways that involve both ABA biosynthesis and AHK1. Other hormones such as cytokinin were also reported to be necessary for the AHK4 to function as a negative regulator (Wohlbach et al., 2008). The comparison of whole-genome expression levels in the *ahk1* mutant *versus* wild type plants under osmotic stress revealed that AHK1 is co-transcriptionally regulated with several A-type *Arabidopsis* response regulators including *ARR4*, *ARR5*, *ARR6*, *ARR8* and *ARR9* (Wohlbach et al., 2008) revealing a complex system in which ARR3, 4 and ARR8, 9 play antagonistic roles. AHK1 is

also involved in the regulation of desiccation processes during seed formation, since *ahk1* mutants shows reduced expression levels of seed storage proteins like *2S1*, *2S2* and *2S4* (Wohlbach et al., 2008).

According to the current model (Wohlbach et al., 2008), AHK1 signal transduction is initiated by the sensing of water stress at the plasma membrane; the signal is then passed through the response regulators ARR3/4 or ARR8/9. In the ABA-independent pathway, stress-responsive gene expression is then activated which in turn results in vegetative stress tolerance. In the ABA-dependant pathway, activation of AHK1 signal transduction induces ABA biosynthesis that consecutively stimulates *AHK1* expression to rapidly propagate the signal. Like in the ABA-independent pathway, this results in the induction of stress responsive gene expression and, thus, leading to vegetative stress tolerance. The AHK1-induced ABA signal can also function in regulating seed storage protein synthesis that results in seed desiccation tolerance.

2.4. *Arabidopsis* basic leucine zipper motif (bZIP) proteins

In *Arabidopsis,* the basic leucine zipper motif (bZIP) proteins act as transcription factors and regulate processes like stress and light signaling, pathogen defense, flower development and seed maturation (Jakoby et al., 2002). The *Arabidopsis* bZIP family can be subdivided into ten groups according to their basic regions and the presence of additional conserved motifs (Jakoby et al., 2002). The bZIP proteins have a leucine zipper motif and a basic region that comprises around 16 amino acid residues and contains a nuclear localization signal (Hurst, 1994). In order to bind DNA, a coiled-coil structure called the zipper has to be created in the bZIP protein. This is achieved by bringing the hydrophobic sides of the helices in the basic and the leucine zipper domains together (Hurst, 1994). Plant bZIP proteins can bind to the A-box (TACGTA), C-box (GACGTC) or G-box (CACGTG) (Hurst, 1995; Izawa et al., 1993) and also have been shown to bind non-palindromic binding sites (Choi et al., 2000; Fukazawa et al., 2000) in the promoters of their target genes.

The function in plant growth and development for most groups in the bZIP family is yet to be elucidated. Nevertheless, group-A bZIP transcription factors have been shown to be involved in ABA-mediated seed germination and seed development (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2001). ABA also regulates this group of bZIPs at transcriptional and post-translational levels modifying, for example, the phosphorylation status of the bZIP proteins (Finkelstein and Lynch, 2000). The two group-H members (AtbZIP56/HY5 and AtbZIP64/HYH) are known to be involved in light signaling and photomorphogenesis in *Arabidopsis* (Ang et al., 1998; Hardtke et al., 2000). Group-I bZIP protein members play a

role in the regulation of cell elongation by activating the genes involved in the gibberellin biosynthetic pathway and vascular development. The members of the group-D bZIPs family participate in development and pathogen defense (Miao et al., 1994; Xiang et al., 1997). Salicylic acid induces the expression of pathogenesis-related (PR) genes in response to pathogen attack (Zhou et al., 2000). Reports suggest that the group-D bZIP AtbZIP57/TGA4 interacts with the *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), which then binds the ethylene response element present in many PR gene promoters (Buttner and Singh, 1997). Thus, in response to pathogen attack, group-D bZIP proteins integrate systemic salicylic acid and ethylene signaling. Recently, the group-D AtbZIP22/TGA3 have been shown to interact specifically with the *Arabidopsis* response regulator ARR2 and by recruiting ARR2 to the *PR1* promoter, to activate expression of pathogen-related genes (Choi et al., 2010). While salicylic acid enhances the binding of both proteins to the *PR1* promoter, cytokinin induces ARR2 activation through its phosphorylation (Choi et al., 2010), suggesting that cytokinins modulate salicylic acid signaling to increase resistance against pathogens (Choi et al., 2010). The group-C bZIP transcription factors comprises bZIP9, 19, 25 and 63 and structural predictions revealed that they contain an extended leucine zipper with up to nine heptad repeats and a conserved phosphorylation site, which is proposed to regulate their nuclear translocation and DNA binding capacity (Ciceri et al., 1997). In monocot plants, this group of bZIP proteins regulate storage protein expression and the plant´s responses to environmental or pathogen challenge (Droge-Laser et al., 1997; Onodera et al., 2001; Vicente-Carbajosa et al., 1997). Group-S is the largest bZIP group in *Arabidopsis*. One of its members, AtBZIP11/ATB2, controls processes associated with metabolite transport or utilization and sucrose signaling (Rook et al., 1998). Moreover, homologues of *Arabidopsis* group-S bZIPs in other plant genera (tobacco, maize, *Antirrhinum*) are transcriptionally activated by different stress treatments like cold, drought and wounding and specifically expressed only in flowers (Kusano et al., 1995; Martinez-Garcia et al., 1998; Strathmann et al., 2001).

In order to activate the transcription of their target genes, bZIP transcription factors must form homo- or heterodimers. For example, all members of the C-group of bZIP transcription factors form specific heteromers with five members of the S1-group to control gene expression of the *proline dehydrogenase* gene (*ProDH*) during hypo-osmotic and oxidation stress (Weltmeier et al., 2009). Recently the heterodimerization of C/S1 bZIP factors has also been shown to regulate *Arabidopsis* seed maturation *via* the transcriptional regulation of seed storage protein genes *2S1* and *2S2* (Alonso et al., 2009; Lara et al., 2003) as well as to

reprogram amino acid metabolism at low energy stress by regulating *asparagine synthetase 1* (*ASN1*) and *proline dehydrogenase* (*ProDH*) gene transcription (Dietrich et al., 2011).

3. Aim of this work

This PhD thesis focused on the molecular and functional characterization of the *Arabidopsis* response regulator ARR18 as an output element of TCS signaling. The specific aims have been:

1. To characterize the molecular, cell biological and functional properties of the B-type response regulator 18 and identify its position within the TCS signaling network of *Arabidopsis* (see chapter 1).
2. To determine the functional role of the newly identified interaction between ARR18 and the C-group of bZIP transcription factors, namely bZIP63, in osmotic stress signaling in *Arabidopsis* (see chapter 2).

4. Outline of the thesis

Living organisms sense and respond to their environment using a wide range of signal transduction systems. In plants the two-component signaling (TCS) system is evolved as an important sensing and responding mechanism. In *Arabidopsis thaliana* the TCS is represented by histidine kinases (AHKs), histidine phosphotransfer proteins (AHPs) and response regulators (ARRs) and connected by a histidine to aspartate phosphorelay. Within recent years TCS systems have been identified in cytokinin, ethylene, stress, light, stomatal closure and circadian signaling but, the functional role of specific TCS elements in those mechanisms still needs to be elucidated. A good example of an unwell understood TCS component is the response regulator ARR18. We performed various functional and molecular biological approaches to characterize and understand the function of the *Arabidopsis thaliana* TCS element ARR18.

Chapter 1

The formation of homomers is necessary for the activity of the *Arabidopsis* B-Type Response Regulator 18 (ARR18)

The *Arabidopsis* response regulator 18 (ARR18) is one of the poorly characterized type-B response regulators in *Arabidopsis*. Our experimental data proves that ARR18 is nuclear localized and capable of transcriptionally activate the type-A response regulator ARR5. The ARR18 also able form homomeric dimers like the bacterial response regulators. Phosphorelay confirmed to play a very significant role in the ARR18 homodimerization ability, moreover our experiments with dominant active and dominant negative version of ARR18 confirmed that ARR18 could exist in active and inactive dimer forms. The transcriptional activation of ARR18 completely depends on its phosphorylated state, furthermore all the mutated ARR18 was localized in the nuclei like the native ARR18. This confirmed that the difference in transcriptional activity among the ARR18 mutants was not due to the miss-localization of the protein. ARR18 specifically interacts with AHP1, AHP2, AHP3 and AHP5 in yeast and in living plant cells. The ARR18 insertion mutant and ectopic overexpressors confirmed the involvement of ARR18 in cytokinin signalling.

Chapter 2

The type-B response regulator ARR18 mediates osmotic stress responses through direct interaction with bZIP transcription factors

ARR18 is reported to be one among the differentially expressed type-B response regulators upon stress and analysis of seed developmental stages identified ARR18 as an early seed specific gene. ARR18 insertion line (*arr18-1*) exhibited hypersensitivity to seed germination on mannitol. Osmosensor AHK1 mutant (*ahk1*) showed differential regulation of many genes with enriched ACGT core and these sites are directly recognized by the bZIP transcription factors. Surprisingly ARR18 interacted with three (bZIP9, 10 and 63) out of four C-group bZIP transcription factors which are known to be regulating seed storage and hypo-osmotic stress. The C-group bZIP factors have been reported to form heterodimers with S-group bZIP factors and synergistically activate the 2S1 promoter. When the influence of ARR18 on the transcriptional activity of C/S-group bZIP factors was tested, ARR18 significantly down regulated the promoter activity only in combination with bZIP63. Physical interaction of ARR18 with bZIP63 was investigated by different experiments including BiFc, recruitment assay and FRET assay, moreover this interaction was observed to be phosphorelay dependant. Dominant active ARR18 (D70E) lead to a severe down regulation of the 2S1 promoter but not the dominant negative ARR18 (D70N), also addition of cytokinin to the ARR18 leads to a down regulation. Interestingly down regulation was not observed when bZIP63 was tested with ARR18 on the ARR5 promoter. Analysis of bZIP63 insertion and overexpressing lines on mannitol showed hypo and hypersensitivity respectively which places bZIP63 as a negative regulator of seed germination upon osmotic stress. Thus bZIP63 appeared to be opposite to the effect exerted by ARR18.

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CHAPTER 1

The formation of homomers is necessary for the activity of the *Arabidopsis* B-Type Response Regulator 18 (ARR18).

Abstract

In higher plants, the two-component system (TCS) is a signaling mechanism based on a His-to-Asp phosphorelay. The *Arabidopsis* TCS involves three different types of proteins, namely the histidine kinases (AHKs), the histidine phosphotransfer proteins (AHPs) and the response regulators (ARRs). Among the response regulators, the B-type ARRs act as DNA-binding transcription factors. Although some of the B-type family members have been extensively studied, very limited information is available for others such as ARR18. In this study, we characterize in detail the molecular and functional properties of ARR18. ARR18 acts as a transcriptional regulator in plant cells and forms homodimers *in planta* as shown by FRET-FLIM studies. As demonstrated by mutational analysis, the aspartate at position 70 (D70) in the receiver domain of ARR18 acts as crucial phosphorylation site. The modification of D70 affects the response regulator´s ability to homodimerize and to activate its target genes but not its capacity to bind its cognate 5′-(A/G)GAT(T/C)-3 nucleotide motif. Furthermore, physiological investigations of *Arabidopsis* lines ectopically expressing ARR18 introduce ARR18 as a new member within the cytokinin-regulated response pathway regulating root elongation.

Introduction

Bacteria and higher eukaryotes like yeasts and plants relay on signaling mechanisms such as the two-component systems (TCS) to sense and react to a broad spectrum of environmental and endogenous stimuli. In general, the transduction and amplification of a signal within TCS is achieved through a histidine (His) to aspartate (Asp) phosphorelay mechanism. In bacteria, the conserved basic phosphotransfer happens predominantly between a histidine protein kinase (HK) and a response regulator (RR) protein (Stock et al., 1989). Upon stimulus, the bacterial HK autophosphorylates and regulates the phosphorylation status of the RR. The bacterial RRs are fundamental control elements functioning as a phosphorylation-triggered switch that mediates the cellular response. Most of the bacterial RRs are transcription factors that consist of a conserved N-terminal receiver domain and a C-terminal DNA-binding domain. The phosphorylated RRs modulate the transcriptional activity of their target genes (West and Stock, 2001). Activation of RR transcription factors in bacteria typically involves dimerization or higher-order oligomerization (Fiedler and Weiss, 1995; Galperin, 2006; Jeon et al., 2001; Stock et al., 2000). The recently solved structure of the *Escherichia coli* RR

PhoB indicates that the protein monomers associate to two different structural dimers depending on the phosphorylation state and exhibit a differential DNA binding capacity and ability of transcriptional regulation (Mack et al., 2009).

Higher plants such as *Arabidopsis thaliana* have developed a TCS phosphorelay which include hybrid histidine kinases (AHK), response regulators (ARR) and an intermediate element, the histidine phosphotransfer proteins (AHP) (Lohrmann and Harter, 2002; Schaller et al., 2002; Stock and Da Re, 2000). *Arabidopsis* has been reported to have 11 AHKs. Among them, three AHKs are shown to be cytokinin receptors, namely AHK2, AHK3 and AHK4/CRE1/WOL (Nishimura et al., 2004; Ueguchi et al., 2001; Yamada et al., 2001). Other AHKs are involved in ethylene signaling (Cho and Yoo, 2007; Grefen et al., 2008; Hua et al., 1998), megagametophyte development (Pischke et al., 2002), stress signaling (Tran et al., 2007) and H_2O_2-regulated stomatal closure (Desikan et al., 2008). The five canonical AHP family members AHP1 through AHP5 encode proteins which contain the conserved His residue that is required for the phosphorelay (Hutchison et al., 2006), while the *AHP6* gene encodes a non-phosphorylatable AHP and is therefore considered a pseudo AHP (Suzuki et al., 1998). Analysis of the complete genome of *Arabidopsis* reveals the existence of 23 ARRs. According to the domain composition and molecular function, the canonical ARRs can be classified as either type A, B or C (Grefen and Harter, 2004; Kiba et al., 2004). ARR18 is one of the 11 members of the type-B family. The type-B ARRs are transcriptional regulators, containing at least one nuclear localization signal (NLS), a transactivation domain and a DNA-binding GARP domain (Grefen and Harter, 2004). The DNA binding domain of some B-type ARRs, namely ARR1, ARR2 and ARR10, has been shown to bind to the nucleotide motif 5′-(A/G)GAT(T/C)-3′ *in vitro* in a sequence-specific manner (Imamura et al., 2003; Lohrmann, 1999; Sakai et al., 2000). These sequence motifs are enriched in the promoters of primary cytokinin response genes like those of the A-type response regulators *ARR4*, *ARR5*, *ARR6* and *ARR7* (Rashotte et al., 2003). Furthermore, these type-B response regulators are able to activate the transcription of an *ARR6-promoter-uidA* reporter gene in *Arabidopsis* protoplasts (Hwang and Sheen, 2001). Among the 11 type-B ARRs in the case of ARR1, ARR2, ARR10 and ARR12, their role as transcriptional regulators has directly been proven (Hwang and Sheen, 2001; Imamura et al., 2003; Mason et al., 2005; Sakai et al., 2000).

The ARRs are involved in multistep phosphorelay and receive the phosphate group at a conserved aspartate (Grefen and Harter, 2004). By mutation of this aspartate (D) to either a glutamate (E) or asparagine (N) two ARR18 protein versions can be engineered that either mimic a constitutive active state (D-to-E mutation) or a constitutive inactive state (D-to-N

mutation) of the protein (Hass et al., 2004; Hwang and Sheen, 2001; Mira-Rodado et al., 2007). Similar mutants were essential, for example, to understand how phosphorylation of ARR2 is required for the control of leaf longevity (Kim et al., 2006), and a dominant active ARR2 version was shown to regulate the transactivation ability of ARR2 on the ERF1 and ARR6 promoters (Hass et al., 2004; Kim et al., 2006).

Several members of the *Arabidopsis* TCS elements are reported to form dimers similar to the members of bacterial TCS. The cytoplasmic transmitter domains of AHK2, 3 and 4 (Dortay et al., 2008; Dortay et al., 2006) and the full-length ethylene receptors form homo- and heterodimers (Gao et al., 2008; Grefen et al., 2008). Three AHPs, namely AHP1, AHP2 and AHP3 have equally been reported to form homo- and heterodimers (Dortay et al., 2008; Dortay et al., 2006; Punwani et al., 2010). However, although RRs are hypothesized to undergo conformational changes after receiving the phosphate group from the AHPs that would allow the formation of functional dimers (Kim, 2008), no dimerization related proof is yet available for plant ARRs including ARR18.

In this paper we show that the ARR18 acts as a transcriptional regulator and forms homomers *in planta*. Moreover, we show that the aspartate in position 70 (D70) in the receiver domain of ARR18 functions as crucial phosphor-accepting site. The modification of D70 in ARR18 interferes with its homomerization capacity and transcriptional activity but not with its ability to bind DNA. The results of our physiological investigations introduce ARR18 as a new member of the cytokinin-regulated root elongation response.

Materials and Methods

Cloning and site-directed mutagenesis

All the clones used in our experiments were constructed using Gateway™ technology (Invitrogen). The entry clones were either obtained *via* BP-reaction in pDONR207 or through TOPO-reaction using the pENTR/D-TOPO vector. cDNA preparations derived from *Arabidopsis* flowers were used as template to clone ARR18 (AT5G58080). For the generation of truncated ARR18 versions, the ARR18 receiver domain ($ARR18^{RD}$) and the output domain ($ARR18^{OD}$) were amplified by PCR using the ARR18 entry clone. Site-directed mutagenesis of ARR18 was carried out on the ARR18 entry clones using QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The primer pairs used for cloning the D70E, D70N, D75E and D75N mutants were described in supplementary table 1. The binary constructs for GFP and RFP fusion protein expression under the control of the 35S promoter were obtained *via* LR-

reaction using the corresponding ARR18, ARR18^{D70E} and ARR18^{D70N} entry clones and destination vectors pH7WGF2.0 and pB7WGR2.0 (Karimi et al., 2002).

FRET-FLIM interaction studies

ARR18, LSD1 and different ARR18 mutant entry clones (ARR18^{D70E}, ARR18^{D70N}, ARR18^{D75E} and ARR18^{D75N}) were recombined *via* LR reaction into estradiol inducible pABindFRET, pABindGFP and pABindmCherry expression vectors (Bisson et al., 2009) for the FRET analysis. All binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 and infiltrated into the tobacco leaves (Schutze et al., 2009). The p19 protein from tomato bushy stunt virus cloned in pBIN61 (Voinnet et al., 2000) was used to suppress gene silencing in all the infiltration experiments. Transgene expression from the estradiol inducible promoter was induced 2–3 days after infiltration by brushing the leaves with 20µMß -estradiol supplemented with 0.1 % tween 20. The florescence lifetime was determined after 4hrs of induction using a custom build confocal stage scanning microscope (CSSM) (Elgass et al., 2009).

Arabidopsis protoplast transient expression assay

The constructs for the protoplast transformation assay were obtained by LR-reaction with the entry clones ARR18, ARR18^{D70E} and ARR18^{D70N} and destination vectors P_{UBI10} (Grefen et al., 2010). The reporter construct *P_{ARR5}:uidA* and the internal control construct *P_{35S}:NAN* previously described were used in the experiments (Brandstatter and Kieber, 1998; Kirby and Kavanagh, 2002). *Arabidopsis* cell culture protoplasts were isolated and transfected according to (Schutze et al., 2009). The amount of each transformed plasmid was constant through the entire experimental set. To keep the total DNA quantity at the same level in each sample, unrelated DNA was added when required. Transfected protoplasts were incubated in darkness overnight at 23°C. The samples were incubated for further 4h with or without 100 nM t-Zeatin. The *P_{35S}:NAN* construct was used as an internal control to normalize the variations of each transfection due to transformation efficiency and cell viability. GUS and NAN enzyme assays were performed as described (Ehlert et al., 2006). The results are shown as the means of relative GUS/NAN activities. All transient experiments were repeated at least four times.

Root growth inhibition assay

Seedlings were surface sterilized using 70% ethanol with 0.01% Triton X 100 for 10 minutes followed by 10 minutes incubation in 95% ethanol. Stratification for 24h in darkness at 4^0C was performed to break the seed dormancy and initiate synchronized germination. Seedlings were grown vertically under constant light on ½ strength MS medium without sucrose and supplemented with appropriate concentrations of kinetin for the root elongation assay. Root lengths were measured after eight days and seedlings that had not germinated within two days of culture were excluded from the analysis. Means and standard errors were calculated from at least 25 seedlings per line. Values were calculated relative to the root length in medium without kinetin from at least three independent replicates.

Protein-protein interaction studies in yeast

Yeast two-hybrid experiments were performed using the vectors from Matchmaker™ system (Clontech). The BD-ARR18RD clone was constructed by LR-reaction with the corresponding entry clone and the destination vector pGBKT7-DEST. The AD-AHPs were constructed as previously described (Horak et al., 2008). Yeast strain PJ69-4A was transformed using the lithium acetate/SS-DNA/PEG method (Grefen et al., 2009) and the transformed yeast was then used in growth complementation experiments. CSM-L$^-$, W$^-$ selective medium was used to test the presence of the BD and AD vectors and CSM-L$^-$, W$^-$, Ade$^-$ to test the interaction between the AD and BD hybrid proteins. In addition, yeast cultures were harvested and analyzed by western blot using α-HA and α-myc antibodies to determine the correct expression of the fusion proteins (Horak et al., 2008). The total protein amount of the lysate was quantified by Bradford protein assay and the β-galactosidase activity was then measured (Grefen et al., 2007) using three independent yeast clones *per* transformation.

BiFC interaction studies

The ARR18 and ARR18RD were recombined via LR-reaction into pSPYCE-35S were used for the BiFC experiments together with the AHPs in pSPYNE-35S (Horak et al., 2008). All the binary vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 and infiltrated to the tobacco leaves as described (Schutze et al., 2009). The p19 protein from tomato bushy stunt virus cloned in pBIN61 (Voinnet et al., 2000) was used to suppress gene silencing in all the infiltration experiments. BiFC analysis were done by examining abaxial

epidermis of infiltrated tobacco leaves for fluorescence by CLSM 2–3 days post infiltration. The expression of the BiFC fusions was determined by western blot of the transfected leaf tissues using α-HA and α-myc accordingly (Walter et al., 2004).

Construction of transgenic *Arabidopsis* lines and characterization of the *ARR18* T-DNA insertion lines

The construct containing ARR18 cDNA fused to GFP and driven by the *UBQ10* promoter (P_{UBI10}::ARR18-GFP) was produced using the ARR18 entry clone and destination vector P_{UBI10} (Grefen et al., 2010). The construct was then transformed into *Arabidopsis* ecotype Ws-2 *via* floral dip method (Zhang et al., 2006). Homozygous lines were selected with kanamycin and the protein expression of the transgene was confirmed by Western blot using α-GFP antibody and confocal laser scanning microscopy (CLSM) (Figure S4 a, b). To test if the T-DNA insertion in the *arr18-1* (Mason et al., 2005) results in an impaired transcription of the gene, semi-quantitative RT-PCR studies were carried out. RNA was isolated from flowers using the RNeasy mini kit (Invitrogen). 5 µg of total RNA were reverse transcribed using oligo-dT primer with RevertAid^{TM} H minus M-MuLV reverse transcriptase (Fermentas) and resulting cDNA was used as template for PCR with different number of cycles. ARR18 transcript levels were then compared to those amplified with CAB3 primers (Figure S4, c). The primer pair used to amplify ARR18 were as follow: (5´- GCAAGTATCATGT GACAAAGACAATGG -3´) and (5´- TGGTAAGGAACTCGGTGGGAATCTG-3´). Primer pair for CAB3: (5´-CGTCTAGATCAATGGCCGCCTCAACAATGG-3´) and (5´-CGAATTCGCTCAC TTTCCGGGAACAAAGTT-3´).

Results and Discussion

ARR18 is a transcription factor

Sequence analyses indicate that ARR18 is a member of the type-B ARR subfamily. Its N-terminal receiver domain is followed by a long C-terminal region where a potential nuclear localization signal, DNA-binding domain and trans-activation domain are located (Figure 1A). As this architecture is found in other B-type ARRs (Hass et al., 2004; Mason et al., 2004; Sakai et al., 2000), ARR18 might also function as a nuclear transcriptional regulator. Firstly, we tested whether the predicted NLS in the C-terminal domain of ARR18 protein is functional. Therefore, the subcellular localization of the GFP fusions of full length ARR18 as

well as its receiver and output domains ($ARR18^{RD}$ and $ARR18^{OD}$) was examined in transiently transformed tobacco (*Nicotiana benthamiana*) epidermal leaf cells by confocal laser scanning microscopy (CLSM). ARR2-RFP served as nuclear marker (Hass et al., 2004). This analysis revealed a predominantly nuclear localization of full length ARR18-GFP and $ARR18^{OD}$-GFP, whereas $ARR18^{RD}$-GFP was exclusively observed in the cytoplasm (Figure 1B). These data show that the output domain of ARR18 is essential for its nuclear localization and suggest that the single predicted NLS is responsible for ARR18 nuclear localization.

We next addressed the question, whether ARR18 is able to bind DNA and to activate known target genes of type-B ARRs. We tested the transcription activation capacity of ARR18 on the *ARR5* promoter, that contains the sequence motif 5′-(A/G)GAT(T/C)-3′ (Rashotte et al., 2003), using an activator / reporter gene assay in *Arabidopsis* protoplasts (Walter et al., 2004). Therefore, the *ARR5* promoter sequence was fused to the *uidA* reporter gene (P_{ARR5}*:uidA*) (Brandstatter and Kieber, 1998) and its activity determined relative to that of the NAN transformation efficiency control (Kirby and Kavanagh, 2002) in the presence of ARR18, ARR2 (positive control) (Hwang and Sheen, 2001) and the *Arabidopsis* G-/C-box binding bZIP transcription factor 63 (AtbZIP63) as negative control, (Kirchler et al., 2010). As shown in figure 1C, we observed an increased activity of the P_{ARR5}*:uidA* reporter gene when ARR18 and ARR2 were used as effectors. In contrast, bZIP63 failed to activate the reporter gene (Figure 1C). These results indicate that, similar to the other tested type-B ARRs, ARR18 recognizes the *ARR5* promoter *in vivo* and functions as a transcriptional regulator.

ARR18 forms homomers *in planta*

The dimerization of bacterial response regulators is been known for a long time (Fiedler and Weiss, 1995). The members of OmpR/PhoB from *Escherichia coli* (Bachhawat et al., 2005) as well as RcpA and RcpB from cyanobacteria (Benda et al., 2004) were also reported to form dimers. In such cases, the receiver domain mediates the formation of dimers in a phosphorylation-dependent manner (Chen et al., 2003; Mack et al., 2009; Toro-Roman et al., 2005). In contrast, in *Arabidopsis* or other higher plants, there are no reports about the capacity of B-type response regulators to form dimers. This failure might be mainly due to the strong transactivation capacity of the type-B ARRs in the yeast two-hybrid system.

To analyse whether ARR18 is able to form homodimers, we conducted *in planta* Förster resonance energy transfer - fluorescence lifetime imaging microscopy (FRET-FLIM) analysis in nuclei of transiently transformed tobacco epidermal leaf cells as described previously by

Caesar and colleagues (Caesar et al., 2011). Hence, the ARR18 protein was fused to GFP (ARR18-GFP) providing the donor fusion and to mCherry (ARR18-mCherry) providing the acceptor fusion for FRET-FLIM. The unrelated NLS-fused Lesion Simulating Disease 1 (LSD1-mCherry) acceptor fusion (Dietrich et al., 1997; Kaminaka et al., 2006) and ARR18-GFP alone served as negative controls. To determine the maximal FRET-FLIM efficiency, a GFP-mCherry fusion was attached to ARR18 resulting in ARR18-FRET (Bisson et al., 2009). The expression of all protein fusions was under the control of the estradiol-inducible promoter to ensure similar levels of protein accumulation (Bisson et al., 2009). Determined by CLSM, the fusion proteins accumulated to very similar levels in the nuclei 4 h after application of estradiol (Figure 2A). As expected, a strongly reduced donor GFP fluorescence lifetime (FLT) was observed for the ARR18-FRET fusion. When the FLT of ARR18-GFP was recorded in the presence ARR18-mCherry a significantly shorter lifetime was observed compared to that of ARR18-GFP alone and the ARR18-GFP / LSD1-mCherry control protein pair (Figure 2B). These FRET-FLIM data led to the conclusion that ARR18-GFP and ARR18-mCherry are located very closely to each other indicating their homodimerisation or homomerisation inside the nucleus. Thus, the homomerization of response regulators is not only restricted to bacteria but also occurs in plants.

Homomerization of ARR18 is phosphorylation-dependent

We analysed next, whether aspartate phosphorylation in the receiver domain of ARR18 is required for its homomerization in plant cells. The amino acid sequence alignment of the 11 B-type ARR receiver domains revealed the putative phosphorylation site of ARR18 in position 70 (Figure S2a). By site-directed mutagenesis, we substituted this aspartate to either a glutamate (D70E) or asparagine (D70N) and engineered ARR18 proteins that either mimics a constitutive phosphorylated state ($ARR18^{D70E}$, gain-of-function version) or a constitutive non-phosphorylated state ($ARR18D^{70N}$, loss-of-function version) (Hass et al., 2004; Hwang and Sheen, 2001; Mira-Rodado et al., 2007). Then, donor (GFP) and acceptor (mCherry) fusion constructs of these *ARR18* mutant versions were generated, and the encoded fusion proteins tested by FRET-FLIM for their association in the nuclei of transiently transformed tobacco cells. Again, on the basis of CLSM images, the fusion constructs were expressed to very similar levels (Figure S2b). As shown in figure 3A, we observed a significantly reduced FLT for the ARR18-GFP / $ARR18^{D70E}$-mCherry protein pair. When the loss-of-function version of ARR18 ($ARR18^{D70N}$) was used as acceptor, the FLT of ARR18-GFP was not

changed (Figure 3B). This implies that wild type ARR18 can form homomers with gain-of-function ARR18^{D70E} but not with the loss-of-function version of ARR18 (ARR18^{D70N}). In order to further confirm the function of the putative phosphorylation site and, thus, the importance of aspartate 70 for ARR18 homomerisation, we also generated mutations on the next conserved aspartate in position 75 (D75) in ARR18 to either glutamate (ARR18^{D75E}) or asparagine (ARR18^{D75N}) and used these constructs for FRET-FLIM experiments. We observed a significant reduction of the FLT of GFP, when both the ARR18^{D75N} and ARR18^{D75E} mutant versions were tested against wild type ARR18 (Figure 3B). This provides a clear hint that a gain- or loss-of-function mutation of other Asp in the receiver domain besides the Asp 70 (D70) has no influence on the homomerisation ability of ARR18. We also conclude that Asp 70 is the target amino acid for ARR18 phosphorylation and that the modification status of Asp 70 may regulate the homomerization of ARR18. The gain-of function ARR18^{D70E} homomerized with wild type ARR18, ARR18^{D70E} but not with ARR18^{D70N} (Figure 3C). However, when the loss-of-function ARR18^{D70N} version was tested for its homomerization ability, it associated only with ARR18^{D70N} and not with wild type ARR18 or ARR18^{D70E} (Figure 3D). This implies that ARR18 can form homodimers only when the ARR18 monomers are in the same modification state.

The transcriptional activity of ARR18 is phosphorylation-dependent

As shown above, the ability of ARR18 to form homomers depends on its phosphorylation state. Therefore, the question arises whether Asp 70 phosphorylation regulates the transcriptional activity of ARR18. This was again tested in *Arabidopsis* leaf protoplasts using P_{ARR5}*:uidA* as reporter gene. Wild type ARR18 and the different mutant versions of ARR18, namely ARR18^{D70E} and ARR18^{D70N} were co-transformed with P_{ARR5}*:uidA*, P_{35S}*:NAN* and relative GUS/NAN activity was determined. As shown above (Figure 1C), wild type ARR18 activated P_{ARR5}*:uidA* even in the absence of exogenous cytokinin (Figure 4A) indicating that the ectopic expression of ARR18 in protoplasts is sufficient to regulate the activity of the primary cytokinin responsive *ARR5* promoter. Gain-of-function ARR18^{D70E} activated the reporter gene even to a higher extend than wild type ARR18 (Figure 4A). In contrast, loss-of-function ARR18^{D70N} activated the promoter to less extend (Figure 4A). These results indicate that the transcriptional activity of ARR18 target genes is dependent on the Asp 70 phosphorylation state.

It has been reported that cytokinin further enhances the transcriptional activity of type-B response regulators ARR2 and ARR10 in *Arabidopsis* leaf protoplasts (Hwang and Sheen, 2001; Kim et al., 2006) and in a line overexpressing gain-of-function version of ARR2 (Hass et al., 2004). We, therefore, tested whether the cytokinin t-zeatin would have a similar effect on ARR18. Similar to ARR2, t-zeatin enhanced the effect of ARR18 on P_{ARR5}*:uidA* activity (Figure 4A). In the presence of cytokinin, wild type ARR18 upregulated the reporter gene activity to a level equivalent to that of the non-treated gain-of-function ARR18^{D70E} mutant. This suggests that in the absence of cytokinin, the ectopically expressed wild type ARR18 pool is not entirely phosphorylated in protoplasts. As expected, t-zeatin does not alter the activity of the P_{ARR5}*:uidA* reporter gene in neither ARR18^{D70E} nor ARR18^{D70N} versions. In the case of the gain-of-function ARR18^{D70E} version, the entire ARR18 pool mimics a phosphorylated and thus "active" form of the protein. Consequently, cytokinin addition does not affect this "active-ARR18" saturated system. Similarly, in the loss-of-function ARR18^{D70N} mutant, the entire ARR18 pool consists of an inactive protein that cannot be phosphorylated and thus activated in response to t-zeatin application (Figure 4A).

Dimerization or higher-order oligomerization is an essential component of transcriptional activation of bacterial RRs. For instance, the inactive dimer of the bacterial receiver domain of NtrC1 prevents oligomerization and, thus, transcriptional activation, while the formation of the active state dimer relieves this inhibition on transcriptional activation (Lee et al., 2003). The ability of ARR18^{D70N} to homomerize emphasizes that, similar to bacterial response regulators (Mack et al., 2009), non-phosphorylated, inactive *Arabidopsis* response regulator homomers may also have an inhibitory function on transcriptional activity in plants. It is important to know, whether the differential ability of the various ARR18 versions to regulate the *ARR5* promoter is caused by their differential subcellular localization. To address this, we constructed a set of different ARR18 mutant proteins fused to RFP (ARR18^{D70E}-RFP, ARR18^{D70N}-RFP) and independently co-expressed these fusions with ARR18-GFP in tobacco epidermal leaf cells. The co-localization study revealed that both mutant ARR18 fusions localized to the nucleus like wild type ARR18 (Figure 4B). This indicates that the phosphorylation state of ARR18 has no effect on its subcellular distribution.

ARR18 DNA–binding capacity is phosphorylation-independent

As reported above, activation of the P_{ARR5}*:uidA* reporter gene using the loss-of-function ARR18^{D70N} mutant is strongly impaired (Figure 4A). Therefore, the question of whether Asp

70 phosphorylation interferes with the DNA-binding activity of ARR18 arises. This was thus tested by competition binding assays in *Arabidopsis* leaf protoplasts by using P_{ARR5}*:uidA* as reporter gene. Wild type ARR18 and the different mutant versions of ARR18, namely ARR18^{D70E} and ARR18^{D70N} were co-transformed with P_{ARR5}*:uidA* and P_{35S}*:NAN* and relative GUS/NAN activity was determined. Competition was introduced by adding increasing amounts of ARR18^{D70E} and ARR18^{D70N} to the samples while keeping the amount of wild type ARR18 constant. Single transformations of ARR18, ARR18^{D70E} and ARR18^{D70N} with P_{ARR5}*:uidA* exhibited similar results to those observed above (see Figure 4A and 5). In the competition binding assay, equal amounts (1:1) of loss-of-function ARR18^{D70N} and wild type ARR18 do not alter the ARR18 reporter gene activity. In contrast, an increased ratio of the ARR18^{D70N} protein (1:3) results in an inhibition of the reporter gene activity (Figure 5). This clearly indicates that ARR18^{D70N} can bind to the 5′-(A/G)GAT(T/C)-3′ promoter region *in vivo* and, thus, competes with wild type ARR18. As expected, the addition of increasing amounts of the gain-of-function ARR18^{D70E} protein version (1:1 and 1:3) results in a correlated increase in the activity of the reporter gene (Figure 5). In conclusion, both ARR18^{D70N} and ARR18^{D70E} mutant proteins can compete with wild type ARR18 for the binding to the ARR5 promoter sequence *in vivo* indicating that the perturbation of Asp 70 does not interfere with the ARR18 DNA-binding capacity.

ARR18 is involved in root elongation in a cytokinin-dependent manner

The cytokinin-responsive subfamily-1 of B-type ARRs comprises seven proteins, namely ARR1, ARR2, ARR10, ARR11, ARR12, ARR14 and ARR18. Among them ARR1, ARR2, ARR10 and ARR12 have been previously implicated in cytokinin responses based on the phenotype of the corresponding loss-of-function mutants and overexpression studies in *Arabidopsis* plants or protoplasts (Hwang and Sheen, 2001; Imamura et al., 2003; Mason et al., 2005; Sakai et al., 2001). The majority of subfamily-1 *ARR* genes including *ARR18* are expressed particularly in tissues and organs where cytokinin plays a crucial regulatory role such as in the apical shoot meristem and developing leaves (Mason et al., 2004). Single mutants of the subfamily-1 *ARRs* exhibit a decreased sensitivity to cytokinin with respect to root elongation and shoot regeneration from calli (Ishida et al., 2008; Mason et al., 2005). The cytokinin insensitivity increased in higher order mutants such as the triple *arr1/arr10/arr12* mutant, which exhibited a root elongation phenotype comparable to that of an *ahk2/ahk3/ahk4* triple cytokinin receptor mutant (Ishida et al., 2008; Mason et al., 2005).

To get an insight of how ARR18 is integrated in the cytokinin-mediated response pathways of subfamily-1 ARRs, we tested two independent transgenic *Arabidopsis* lines ectopically expressing ARR18 (ARR18-GFP_{OX}1 and ARR18-GFP_{OX}2) (Figure S4 a, b) and an *ARR18* loss-of-function mutant (*arr18-1*) (Mason et al., 2005) (Figure S4 c) for their primary root growth response in the presence of exogenously applied cytokinin. Seedlings of these above described lines and their corresponding wild type (Ws-2) were germinated on medium containing different concentrations of kinetin and incubated vertically under constant light for eight days before measuring the primary root length. The root length of wild type seedlings was strongly reduced when treated with different concentrations of kinetin (Figure 6A, B). In contrast, *arr18-1* seedlings showed a slightly decreased sensitivity to cytokinin resulting in longer roots than those of wild type plants (Figure 6A, B). Both ARR18 overexpressors (ARR18-GFP_{OX}1 and ARR18-GFP_{OX}2) were more sensitive to cytokinin than wild type plants, resulting thus in a strongly reduced root length (Figure 6A, B). Although there is functional redundancy in the subfamily-1 of type-B ARRs, our data proof that ARR18 plays an additional role in the cytokinin-dependent root elongation response in *Arabidopsis*. Moreover we found that AHP 1, 2, 3 and 5 interacts with the ARR18 (Figure S3 a, b and c), which have been shown to function as positive regulators in cytokinin TCS signaling (Hutchison et al., 2006) and interact with the transmitter domain of the cytokinin receptors AHK2, AHK3 and AHK4 (Dortay et al., 2008; Geisler-Lee et al., 2007; Suzuki et al., 2001).

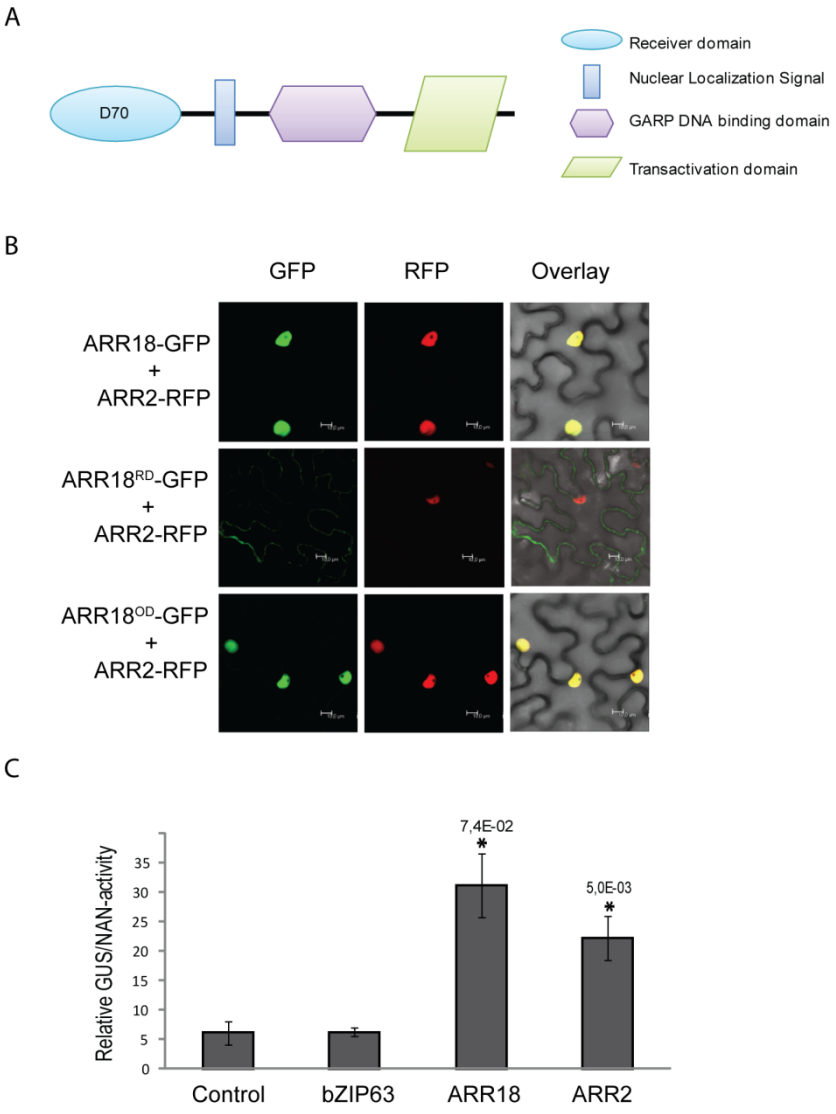

Figure 1: ARR18 localizes to the nucleus and activates the cytokinin-responsive ARR5:*uidA* reporter gene. (A) Scheme of the structural characteristics of the B-type response regulator ARR18. The conserved aspartate at position 70 (D70) is indicated. *Oval*; Receiver domain, *bar*; NLS, *rhomboid*; GARP DNA-binding domain; *parallelogram*; transactivation domain. (B) Confocal images of tobacco (*Nicotiana benthamiana*) epidermal leaf cells expressing the indicated GFP and RFP fusion proteins. Images from the emission channels for GFP (ARR18-GFP, ARR18RD-GFP, ARR18^{OD}-GFP) and RFP and overlays are shown. ARR2-RFP was used as nuclear marker. The bars represent 10µm. (C) Reporter gene activation assay. *Arabidopsis* protoplasts were co-transfected with the P_{ARR5}*:uidA* reporter construct (control), an effector plasmid expressing either ARR18, bZIP63 or ARR2 and the P_{35S}*:NAN* transformation efficiency standard. The relative GUS/NAN activity is shown on Y-axis. Mean values and standard deviations were calculated from three independent transfection experiments. * indicate statistically significant differences.

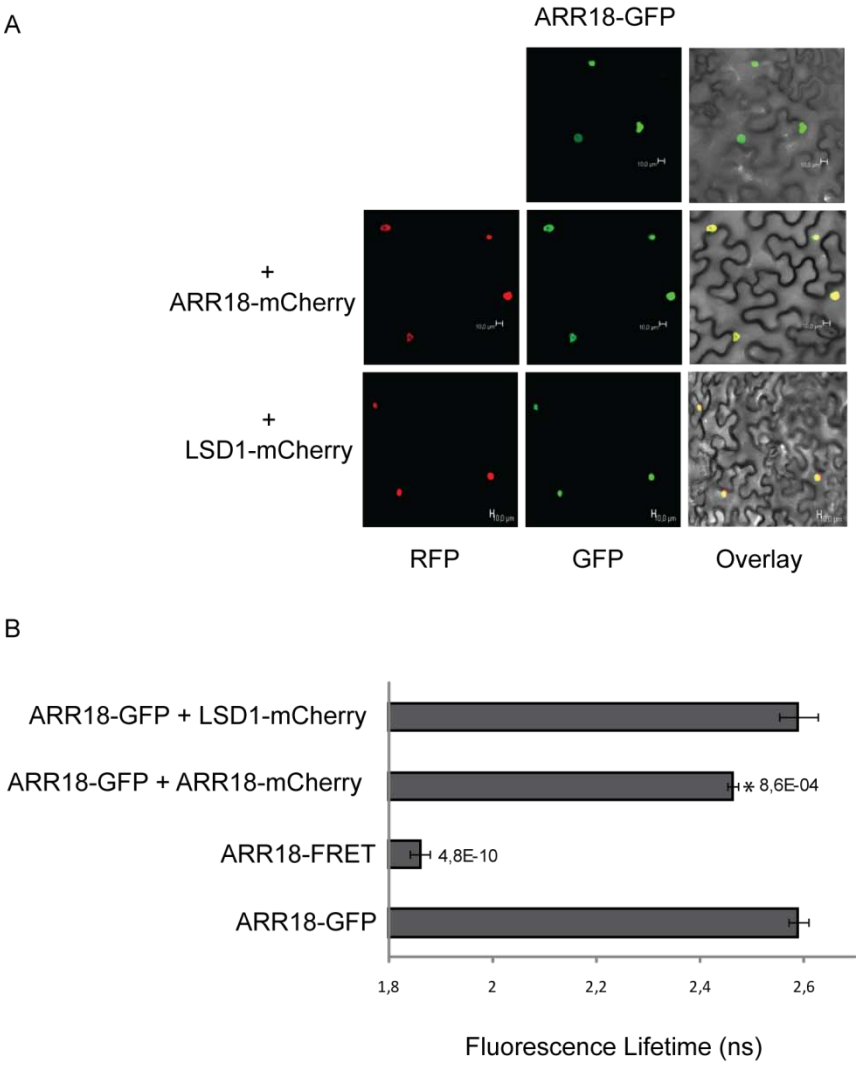

Figure 2: ARR18 forms homomers *in planta*. (A) Confocal images of tobacco epidermal leaf cells expressing the indicated GFP and mCherry fusion proteins. The emission channels for the GFP and RFP fluorescence and the overlays are shown. (B) FRET-FLIM interaction assay of ARR18 homomerisation. The indicated fusion proteins were expressed in tobacco cells and the fluorescence lifetime (FLT) of GFP was recorded. The individually transformed FRET fusion of ARR18 served as positive control. The single transformed GFP fusion of ARR18 was used to determine the FLT background value. Values are means and standard deviations of at least 15 independent measurements per experiment. * indicate significant FLT differences to the ARR18-GFP background control.

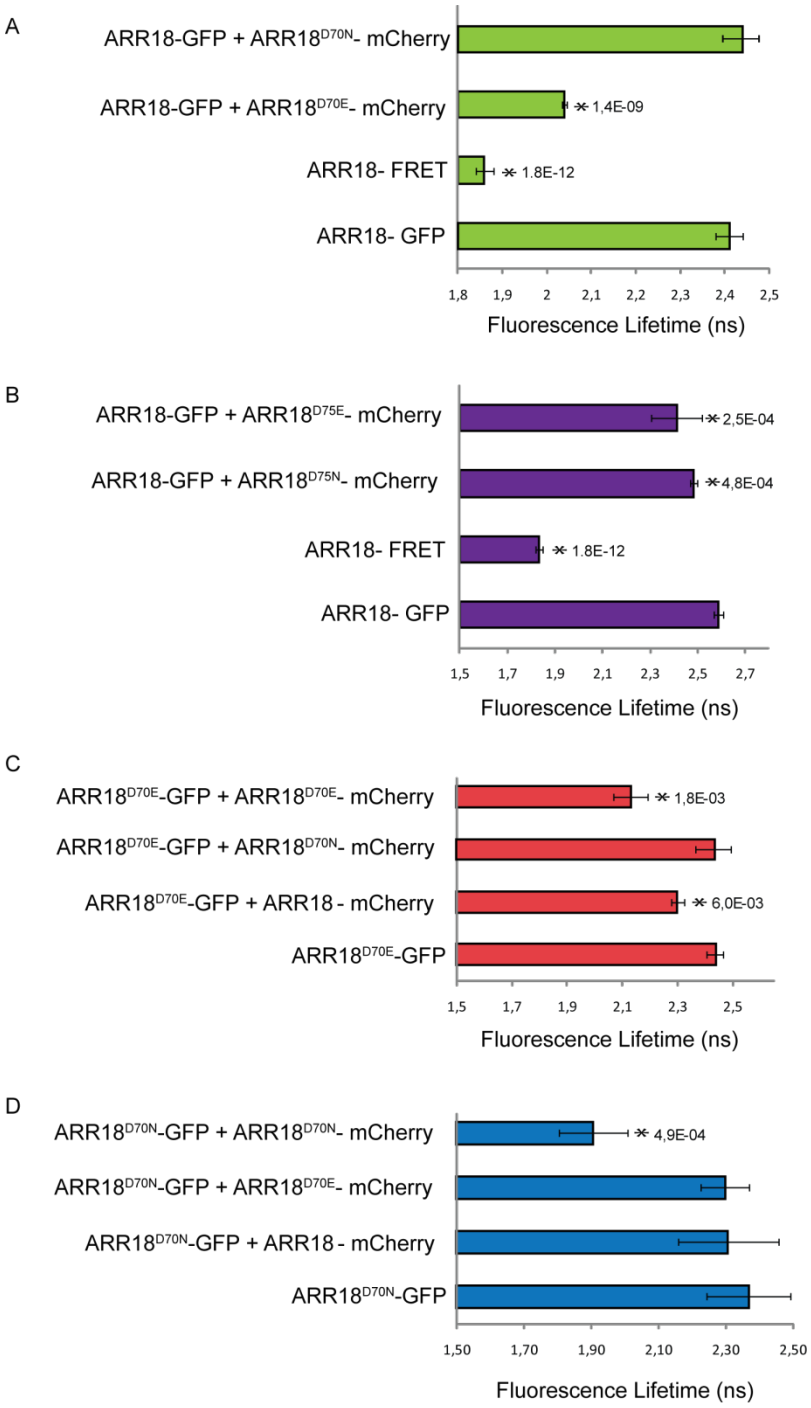

Figure 3: ARR18 homomerizes in a phosphorylation-dependent manner. FRET-FLIM studies of ARR18-GFP interaction with the phosphorylation mutants ARR18^{D70E}-mCherry, ARR18^{D70N}-mCherry (A), ARR18^{D75E} and ARR18^{D75N} (B). The interaction studies of ARR18^{D70E}-GFP (C) and ARR18^{D70N}-GFP (D) with the ARR18^{D70E}-mCherry, ARR18^{D70N}-mCherry and the ARR18-mCherry were also depicted. The fusion proteins were expressed in tobacco cells and the fluorescence lifetime (FLT) of GFP was recorded. The individually transformed FRET fusion of ARR18 served as positive control. The measurements of GFP-FLT in cells transformed with ARR18-GFP (A, B), ARR18^{D70E}-GFP (C) or ARR18^{D70N}-GFP (D) were used to determine the background FLT values. Values are presented as means and standard deviation of at least 15 independent measurements per experiment. * indicate significant FLT differences to the ARR18-GFP, ARR18^{D70E}-GFP or ARR18^{D70N}-GFP background controls.

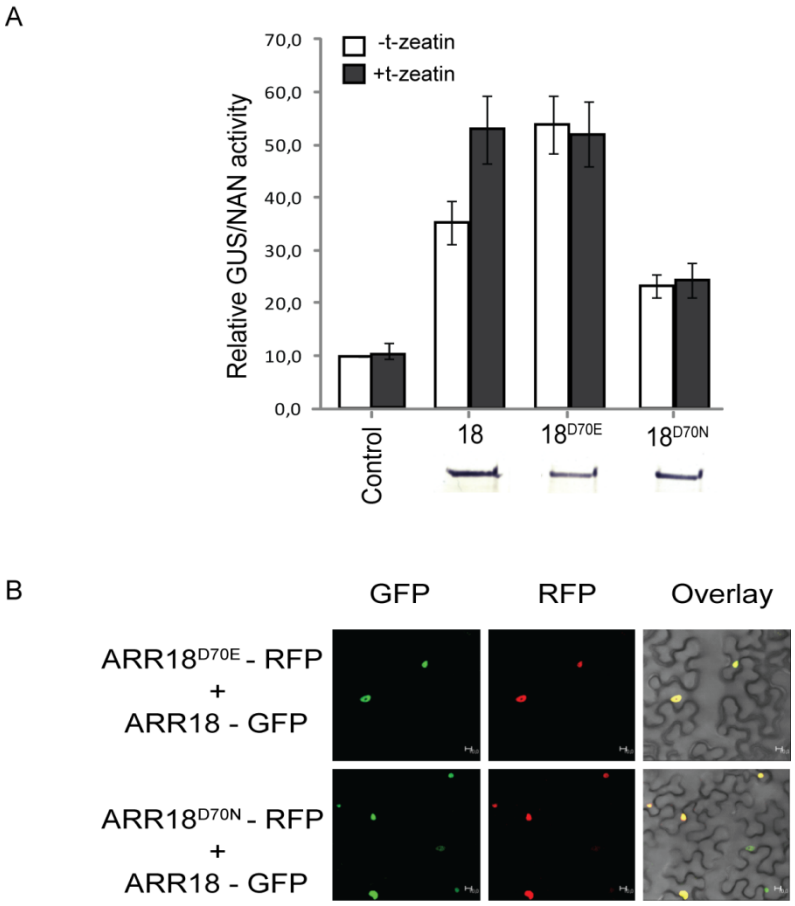

Figure 4: Asp70-to-Glu mutation enhances the transactivation capacity of ARR18 but not its intracellular distribution. (A) **Reporter gene activation assay.** *Arabidopsis* protoplasts were transfected with P_{ARR5}*:uidA* reporter gene alone (control) or in the combination with different effector plasmids expressing either ARR18 (18), ARR18^{D70E} (18^{D70E}) or ARR18^{D70N} (18^{D70N}) proteins. The co-transfection of the P_{35S}*:NAN* was used as transformation efficiency control. The transfected protoplasts were incubated with (grey) or without (white) 100nM *t*-zeatin. Mean values and standard deviations of GUS/NAN activity were calculated from four independent transfection experiments. Immunoblot with an anti-GFP antibody shows protein level of ARR18, ARR18^{D70}E, and ARR18^{D70N}. (B) Confocal images of tobacco epidermal leaf cells expressing the indicated RFP and GFP fusion proteins. The emission channels for the RFP, GFP and the overlays are shown. ARR18: GFP was used as nuclearmarker. The bars represent 10µm.

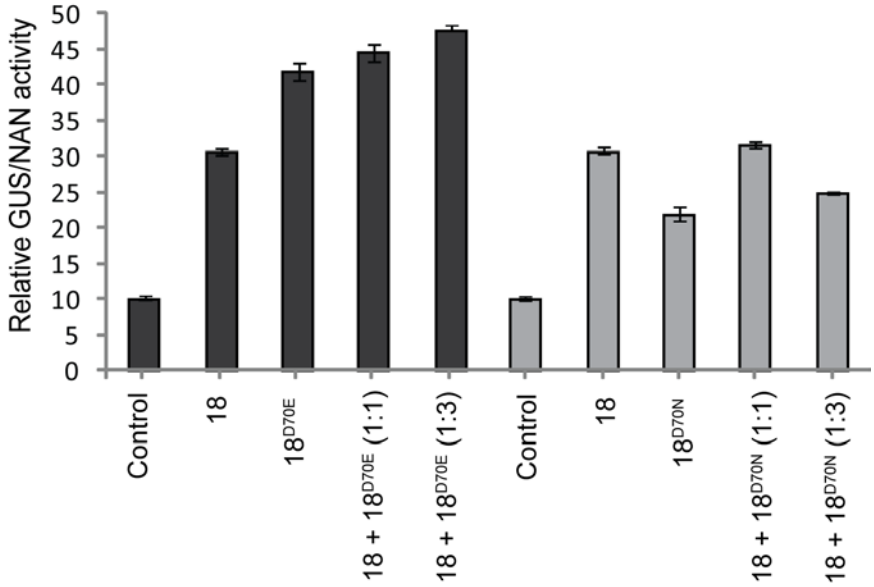

Figure 5: DNA binding activity of ARR18 is not affected by the Asp70 mutations. *Arabidopsis* protoplasts were transfected with P_{ARR5}*:uidA* reporter gene alone (control) or in combination with the different effector plasmids as indicated. The co-transfection of the P_{35S}*:NAN* was used as transformation efficiency control. DNA binding competition was performed by keeping the wild type ARR18 (18) at a constant level and increasing the ratio of ARR18^{D70E} (18^{D70E}) or ARR18^{D70N} (18^{D70N}) as indicated (1:1 and 1:3). Mean values and standard deviations of GUS/NAN activity were calculated from four independent transfection experiments.

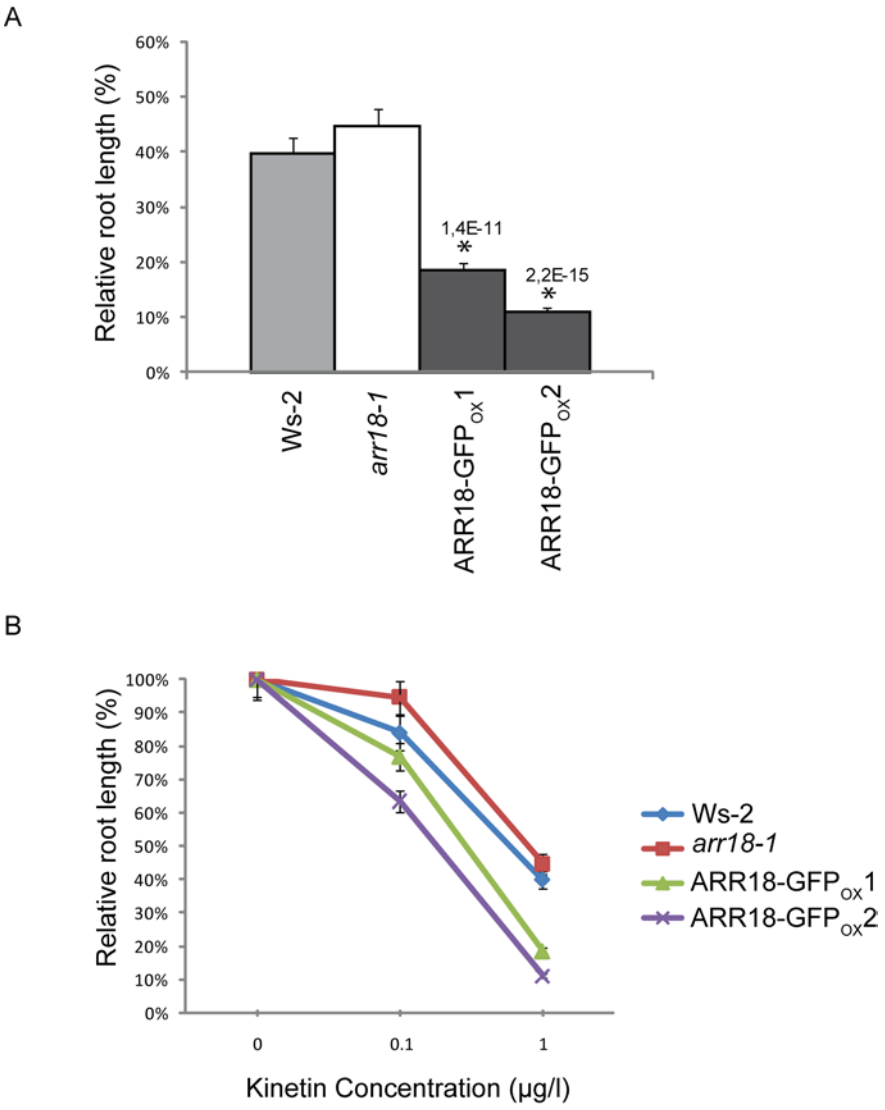

Figure 6: ARR18-overexpression lines and an *arr18* loss-of-function mutant shows altered root growth response in the presence of cytokinin. (A) *arr18* mutant (*arr18-1*) and two ectopic overexpressor (ARR18-GFP$_{OX}$1 and ARR18-GFP$_{OX}$2) seedlings on ½ MS plates supplemented with 1µg/L kinetin under constant light conditions at 23°C. The relative root length in the presence of kinetin is shown as percentage of the non-treated control. (B) Cytokinin dose response analysis of root growth of the indicated *Arabidopsis* lines (Ws-2, *arr18*-1, ARR18-GFP$_{OX}$1, ARR18-GFP$_{OX}$2). Seedlings were grown vertically on control plates and plates supplemented with the indicated concentrations of kinetin (0, 0.1 and 1µg/l). Root elongation was measured 8 days after sowing. Means and standard deviations of at least three independent replicates are presented (n > 25 per replicate). * indicate statistically significant differences compared to the wild type grown in the presence of cytokinin.

Name of the entry clone	Primers	Cloning strategy	Description
ARR18	5’CACCATGGAGTT TGAAGCACT 3’ 5’ AGGTGGAGGAAATGAATCAAAG 3‘	Topo Cloning into pENTRY	ARR18 full length
ARR18RD	5’GGGGACAAGTTTGTACAAAAAAGCA GGCTTAATGGAGTTTGGAAGCAC TGAAGA 3’ 5’GGGGACCACTTTGTACAAGAAAGCT GGGTCCTATTAGAGCAGTTTTGC GTAAGA 3’	BP Cloning into pDON207	ARR18 Receiver Domain
ARR18^{OD}	5' CACCATGCCACCTTCTGAATCTGAT 3' 5‘ AGGTGGAGGAAATGAATCAAAG 3’	Topo Cloning into pENTRY	ARR18 Output Domain
ARR18^{D70E}	5'TTTGATCTGGTGATAAGCGAAGTAGA GATGCCAGACACG 3' 5'CGTGTCTGGCATCTCTACTTCGCTTAT CACCAGATCAAA 3'	Site Directed Mutagenesis of pENTRY-ARR18	ARR18 containing the D70E mutation
ARR18^{D70N}	5'TTTGATCTGGTGATAAGCAATGTAGA GATGCCAGACACG 3' 5'CGTGTCTGGCATCTCTACATTGCTTA TCACCAGATCAAA 3'	Site Directed Mutagenesis of pENTRY-ARR18	ARR18 containing the D70N mutation
ARR18^{D75E}	5'AGCGATGTAGAGATGCCAGAGAAGC TTACGGATGGTTTT 3' 5'CAACTTAAAACCATCCGTCTCTGGCA TCTCTACATCGCT 3'	Site Directed Mutagenesis of pENTRY-ARR18	ARR18 containing the D75E mutation
ARR18^{D75N}	5'AGCGATGTAGAGATGCCAAACACGG ATGGTTTTAAGTTG 3' 5'CAACTTAAAACCATCCGTGTTTGGCA TCTCTACATCGCT 3'	Site Directed Mutagenesis of pENTRY-ARR18	ARR18 containing the D75N mutation

Table S1: Constructs and primers list used in the different cloning strategies. Table shows all the entry clones used in this study and the cloning method (Topo cloning, BP cloning and Site directed mutagenesis cloning). The forward and reverse primer used to amplify the desired genes is also listed.

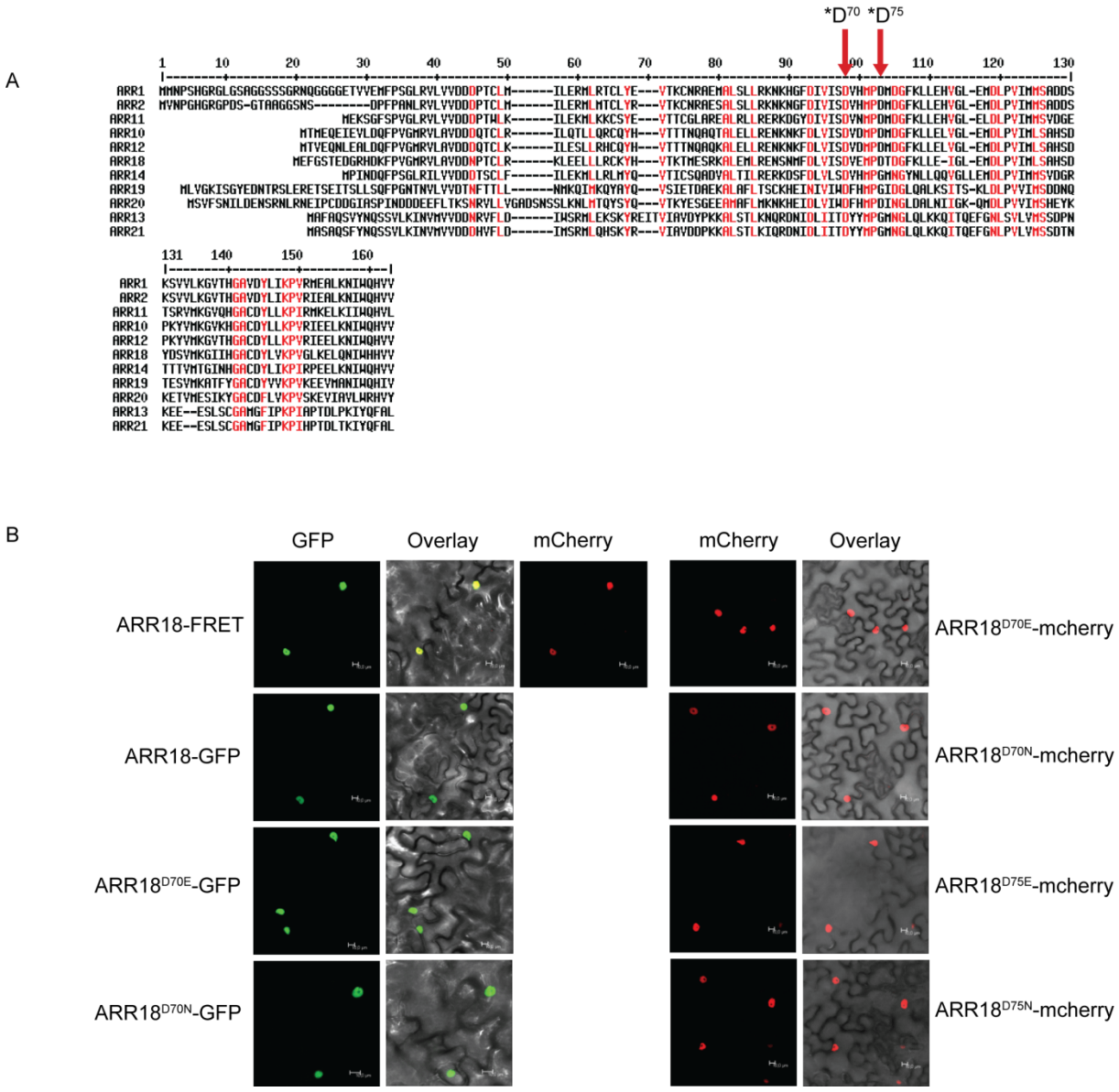

Figure S2: Alignment of the B-type ARRs and expression of the FRET constructs. (A) Amino acid sequences of receiver domains of the B-type response regulators were aligned. The highly conserved amino acids are highlighted in red. The two conserved aspartates are indicated as D^{70} and D^{75}. (B) Confocal images of *N.benthamiana* epidermal leaf cells expressing the indicated GFP and mCherry fusion proteins. The emission channels for the GFP, RFP fluorescence and the overlay are indicated above. The bars represent 10µm.

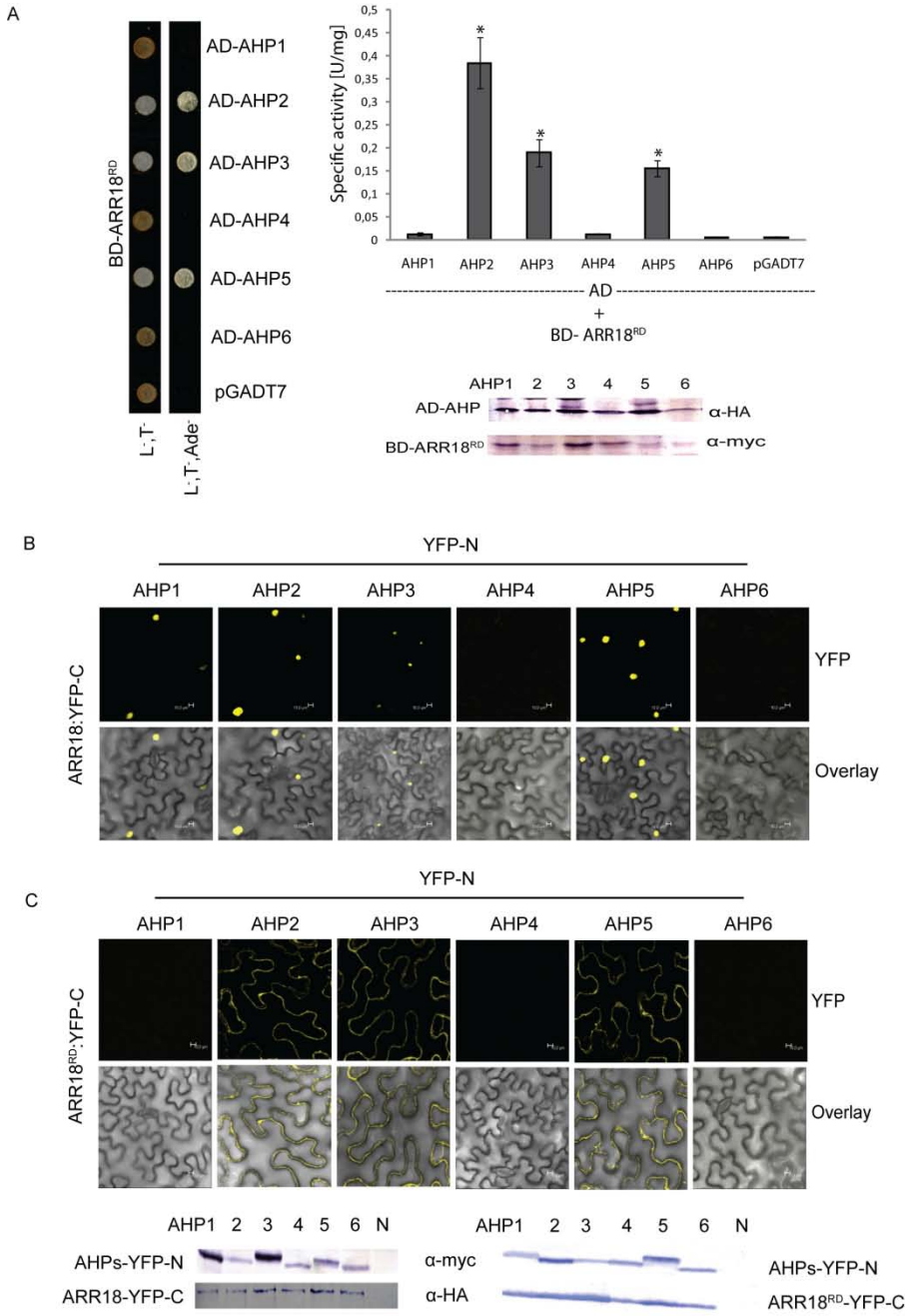

Figure S3: The interaction of ARR18 with AHP proteins. (A) Yeast clones expressing BD-ARR18RD and the indicated AD-AHP clones were cultivated for 4 days at 28°C on either vector selective media (L-, W-) or interaction selective media (L-, W-, Ade-). The pGADT7 vector expressing only the AD domain was used as a negative control. The specific β-galactosidase activity was measured in the extracts of three independent yeast clones expressing BD-ARR18RD and the indicated AD fusion proteins. In the western blot, the detection of the BD-ARR18RD was carried out with an antibody directed against the c-myc-tag (α-myc) and that of the AD fusion proteins with an antibody against the HA-tag (α-HA). CLSM images of tobacco leaf cells expressing the indicated YFP-C fusions of full-length ARR18 (B) or ARR18RD (C) and YFP-N fusions of AHP1 to AHP6. The upper panel shows the fluorescence images and the bottom panel the overlays. The bars represent 10 µm. Immunodetection of the YFP-N fusion proteins (AHPs) was carried out with an antibody against the c-myc-tag (α-myc) and of the YFP-C fusion protein (ARR18 or ARR18RD) with an antibody against the HA-tag (α-HA). N, extract from a non-transformed tobacco leaf.

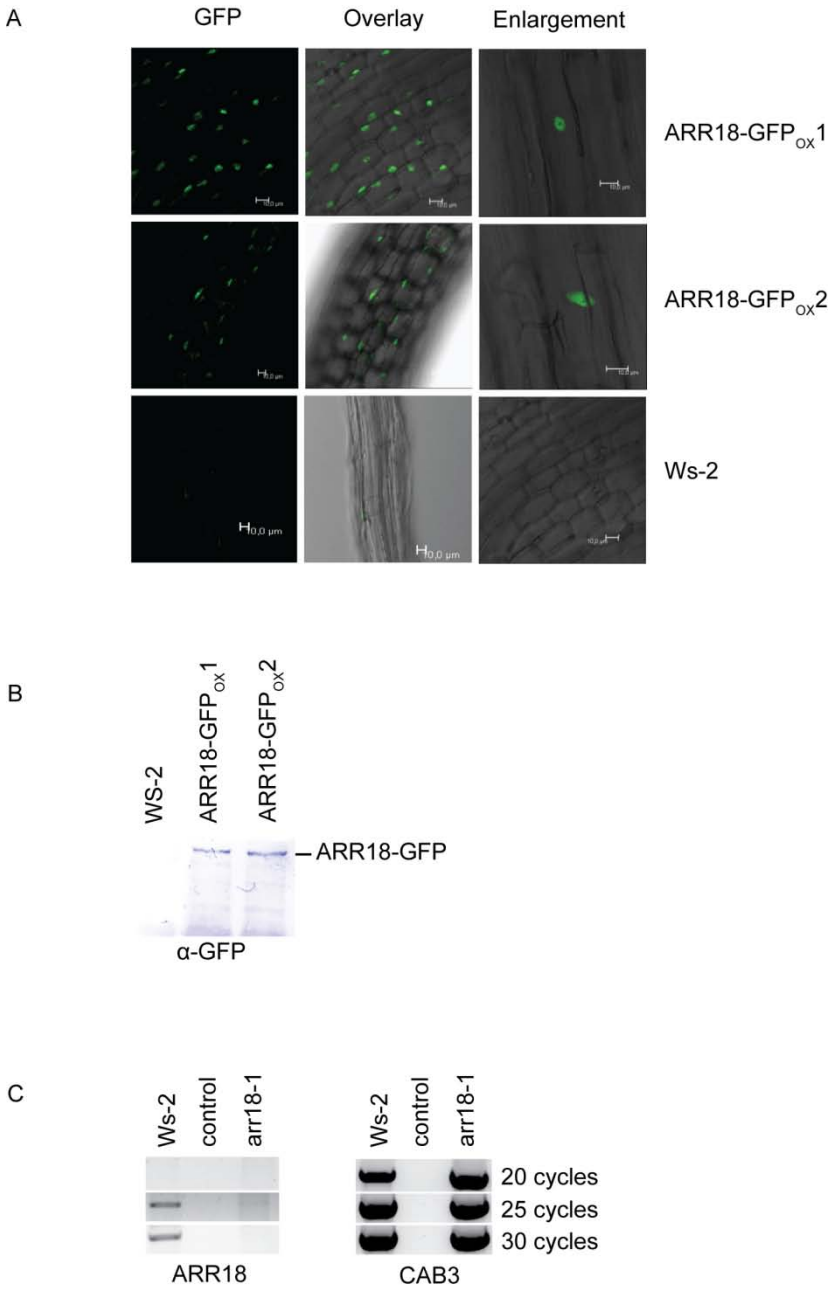

Figure S4: Characterization of the ARR18 knockout and ectopic-overexpressing lines. (A) Confocal images of wild type (Ws-2) and transgenic *Arabidopsis* (ARR18-GFP$_{OX}$1 and ARR18-GFP$_{OX}$2) root cells overexpressing ARR18-GFP. The bars represent 10 µm. (B) Western blot analysis of protein extracts from the ARR18 ectopic-overexpressors (ARR18-GFP$_{OX}$1, ARR18-GFP$_{OX}$2) analyzed for GFP fluorescence. Immuno detection of the ARR18-GFP fusion proteins was carried out with an antibody against the GFP-tag and wild type *Arabidopsis* (Ws-2) was used as a negative control. (C) Semi-Quantitative RT-PCR analysis of the steady-state expression level of *ARR18* transcript performed with the ARR18-specific primers and with *CAB3*-specific primers as a control. Three different cycle numbers were used (20, 25 and 30). To exclude any cross contamination the RT-PCR was performed also in the absence of cDNA (control).

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CHAPTER 2

The type-B response regulator ARR18 mediates osmotic stress responses through direct interaction with bZIP transcription factors.

Abstract

The receptor histidine kinase AHK1, a primary element of two-component system (TCS) in *Arabidopsis*, is proposed to function as osmo-sensing receptor. However, little information is available concerning the phosphorelay-mediated downstream signaling steps. The transcriptional regulation of the type-B response regulator *ARR18* suggests its involvement in plant water stress responses. Furthermore, the function of ARR18 as a positive regulator of plant responses to reduced water potential is demonstrated by a decreased germination of *arr18* mutant seeds under high osmolarity conditions. As a final element in the TCS phosphorelay, ARR18 is shown to affect the activity of promoters containing recognition sites for bZIP transcription factors. By several independent approaches we show that ARR18 physically interacts with bZIP63 *in vivo*. The ARR18-bZIP63 interaction depends on the ability of its receiver domain to accept the phosphate group. In contrast to wild type, a non-phosphorylatable ARR18 mutant does not affect the activity of a bZIP63-regulated seed storage promoter in protoplast-based gene reporter assays. Therefore, the ARR18 is among the first TCS components proposed to function as a positive regulator of osmotic stress responses probably downstream of AHK1. Furthermore, the phenotype of *bZIP63* transgenic lines under high osmolarity condition suggests its negative regulatory function in *Arabidopsis* osmotic stress responses both at the level of seed quality and stress marker gene expression. Our studies provide a new perspective in the understanding of the function of ARR18 and bZIP63 as antagonistic regulators in osmotic stress signaling in *Arabidopsis thaliana*.

Introduction

The discovery of the two-component signaling system in plant organisms, previously known as important regulatory circuit operating merely in prokaryotes, opened a new era in the understanding of plant signal-response pathways. Members of three distinct protein classes constitute the plant two-component system establishing a canonical multi-step phosphorelay, i.e. the transfer of the phosphate moiety from conserved histidine to aspartate. These three classes, which are encoded in *Arabidopsis* by approximately 40 genes, include the sensor histidine kinases, histidine phosphotransfer proteins, and response regulators (Grefen and Harter, 2004; Hwang and Sheen, 2001; Lohrmann and Harter, 2002).

Among the 11 *Arabidopsis* histidine kinases (AHKs) and histidine kinase-like proteins, AHK2, AHK3 and AHK4/CRE1 have been shown to function as cytokinin (CK) receptors

with the histidine-to-aspartate phosphorelay being reconstituted in the perception and transduction of the CK signal (Hejatko et al., 2009; Hwang and Sheen, 2001; Lohrmann and Harter, 2002; Muller, 2011). The ethylene (ET) receptor group consists of 5 genes namely ETR1, ETR2, ERS1, ERS2 and EIN4. Unlike in CK signalling, the phosphorelay does not play a major role in ET signaling. Still, there is evidence of a functional phosphorelay in ET signal transduction as well (Cho and Yoo, 2007; Hass et al., 2004; Scharein et al., 2008). Besides CK and ET signal transduction, the AHKs have been shown to be involved in the modulation of abscisic acid (ABA) responses, as well as in the plant responses to oxidative and drought stress (Desikan et al., 2008; Tran et al., 2010; Tran et al., 2007; Wohlbach et al., 2008).

Water deficit is one of the greatest challenges for plant growth and productivity. Water stress triggers a number of physiological and biochemical responses in plants, including alterations in hormone levels and gene expression. Although ABA is considered as a key player in stress responses (Hirayama and Shinozaki, 2007; Wilkinson and Davies, 2010), their precise control is achieved through the interaction of positive and negative regulators, which also include ABA-independent processes. Several reports show that the level of CK, which negatively regulates many ABA-mediated physiological processes, is reduced in stressed plants (Alvarez et al., 2008; Argueso et al., 2009). Similarly, other chemical signals, including pH, ethylene, gibberellins, malate, and amino acids, have been shown to be altered upon osmotic stress and have been discussed to be implicated in plant signaling and/or adaptation to changing environmental cues (Alvarez et al., 2008; Schachtman and Goodger, 2008; Wang et al., 2008). However, very little is known about the initial steps of water stress responses, including perception and signaling upstream of stress-induced ABA synthesis.

It has been shown that the *Arabidopsis* histidine kinase AHK1 suppresses lethality of the yeast *sln1Δsho1Δ* double mutant lacking its own osmosensor proteins under high salinity stress and activates the *HIGH OSMOLARITY GLYCEROL RESPONSE1* (HOG1) mitogen-activated protein kinase (Urao et al., 1999). The osmosensor function of AHK1 depends on its histidine kinase activity, since loss-of-function AHK1 variants generated by the substitution of the conserved His or Asp residues in the transmitter domain were not able to complement lethal phenotype of the yeast *sln1Δsho1Δ* mutant (Urao et al., 1999). Furthermore, AHK1 was shown to act as a positive regulator of drought and salt stress responses in *Arabidopsis*, functioning upstream of both ABA-mediated and ABA-independent signaling pathways (Tran et al., 2007; Wohlbach et al., 2008). Moreover, AHK1 has been not only suggested to directly

act as an osmosensor (Tran et al., 2007), but also to play a unique role in the regulation of desiccation processes during seed maturation (Wohlbach et al., 2008).

Induced by dehydration dramatic changes in metabolic processes, culminating in the adaptation of plants to changed environment, are coupled to the differences in the expression of number of genes. However, the molecular mechanism, which links the osmosensing AHK1 to downstream gene expression is still not understood. Several members of *Arabidopsis* type-A response regulators (ARRs), constituting the terminal components of AHK-triggered multi-step phosphorelay, namely the *ARR4*, *5*, *6*, *8*, and *9* have been identified as *AHK1* co-expressed genes, especially under water stress (Wohlbach et al., 2008). Higher order mutants of type-A ARRs are characterized by different sensitivity to osmotic stress. Whereas the *arr3,4,5,6* null mutant was found to be hypersensitive to osmotic stress during germination, the *arr5,6,8,9* null mutant was slightly insensitive as compared to wild type. The near wild type response of the *arr3,4,5,6,8,9* hextuple mutant led the authors to the conclusion that ARR3, 4 and ARR8, 9 might have opposite functions in the response to osmotic stress (Wohlbach et al., 2008). These results, however, do not elucidate the mechanism of the A-type ARR own regulation as well as the regulation of other genes, which are triggered by osmotic stress and are regulated in AHK1-dependent manner.

Apart from type-A ARRs, which consist merely of a receiver domain and, therefore, do probably not function as direct regulators of gene expression, a further subclass of putative transcription factors constitutes the so-called type-B ARRs. In addition to the receiver domain, they contain a large C-terminal region with a MYB-like GARP DNA-binding domain, at least one NLS and a transcription activation domain (Grefen and Harter, 2004; Hosoda et al., 2002). It has been shown for several type-B ARRs that they function as sequence-specific transcription factors (Lohrmann and Harter, 2002; Sakai et al., 2000) and directly trans-activate *type-A ARRs* genes (Hass et al., 2004; Hwang and Sheen, 2001; Sakai et al., 2001). Most of type-B ARRs have been characterized in detail uncovering their key function as positive regulators of CK signaling (Argyros et al., 2008; Hwang and Sheen, 2001; Lohrmann et al., 2001; Mason et al., 2004; Sakai et al., 2001; Tajima et al., 2004). More recent works point out that ARR2 also functions in shoot development (Che et al., 2008) and plant immunity (Choi et al., 2010), ARR1 and 12 in cell division and differentiation of the root meristem (Dello Ioio et al., 2008; Moubayidin et al., 2010). Despite this progress, their possible involvement in the plant´s water stress response has not yet been addressed. In part this might be due to the high level of functional redundancy, which makes it difficult to reveal a specific function of particular type-B ARR. Recently, it has been

demonstrated that ARR1 and 12, but not ARR2, 10 or 11, regulate sodium accumulation in *Arabidopsis* shoots, and enhance salt tolerance by controlling the expression of the high-affinity K+-transporter *AtHKT1;1* (Mason et al., 2010). However, it was not examined whether the *arr1,12* double mutant line display the alterations in the sensitivity to increased osmolarity, which accompanies high salt conditions. Nevertheless, taking into account the proposed osmo-sensing function of AHK1, it is worth to examine whether type-B ARRs are involved in phosphorelay-induced regulation of water stress-dependent gene expression.

Here, we report a positive function of ARR18, a member type-B ARRs, in seed germination under osmotic stress. By several independent approaches we show that ARR18 physically interacts with the *Arabidopsis* basic region leucine zipper transcription factor 63 (bZIP63) *in vivo*, and that this interaction depends on the ability of the conserved aspartate in the receiver domain of ARR18 to be phosphorylated. The regulation of stress marker gene expression as well as of seed germination rate in bZIP63 transgenic lines, that is opposite to that of ARR18, indicates the antagonistic function of ARR18 and bZIP63. Our findings suggest that the transcriptional regulation of osmotic stress-responsive genes is at least partially achieved *via* direct and specific interaction of type-B ARRs and bZIP transcription factors.

Materials and Methods

Plant material and growth conditions

Arabidopsis seeds of Col-0 and Ws ecotypes were used. For the hydroponics culture, seeds were surface sterilized and sown on 0.6% agar medium containing ½ -strength Hoagland salts (Jana Maresova et al., 2009), filled in 0.5ml reaction tubes and incubated in dark at 4^0C for 24 hrs. Then seeds were incubated in 12 h light/ 12 h dark conditions until the end of the experiment. After a week of germination, the bottom of the reaction tubes were cut off without disturbing the roots, and hanged in a box containing ¼-strength Hoagland solution. The plants were grown for 2-3 weeks and PEG treatment was performed by substitution of medium with fresh one of the same composition but containing different concentrations of PEG (0%, 10%, 15% and 20%). Plant leaves were collected at particular time interval (0h, 6h and 24h).

Germination experiments

Arabidopsis seeds were surface sterilized and sown on 0.8% agar media plates containing either only water or different concentrations of mannitol. After a 24h dark incubation at 4°C, plates were transferred to constant white light at 22°C. Germination tests were conducted using at least three replicates of 100 seeds each. Emergence of radical from seed coat was considered germination.

Cloning strategy

All clones were constructed using Gateway™ technology (Invitrogen). The Entry clones were generated either by Topo- or BP-reactions, combining the genes of interest into pENTR-TOPO or pDONR201 and 207 vectors respectively (Invitrogen). The ARR18 coding sequence was amplified using cDNA preparations from *Arabidopsis* inflorescences and all other genes described were amplified using cDNA preparations from the complete *Arabidopsis* plant. The different ARR18 mutant versions ($ARR18^{D70E}$, $ARR18^{D70N}$, $ARR18^{D75E}$, $ARR18^{D75N}$) were obtained via site directed mutagenesis using the full length ARR18 entry clone as template. The primer pairs are listed in Table S1.
For the BiFC assays, the corresponding cDNAs were recombined via LR- reaction into either pSPYNE-35S-GW or pSPYCE-35S-GW, which express N- and C-terminal YFP-fragments respectively (Schutze et al., 2009). To create GFP, RFP and GUS fusion proteins, the corresponding cDNAs were recombined via LR-reaction into pH7WGF2.0, pB7WGR2.0, pMDC107 and pMDC163 binary plant expression vectors respectively (Curtis and Grossniklaus, 2003; Karimi et al., 2002).

Transient transformation of *Nicotiana benthamiana* leaves

The corresponding constructs were transformed into the *Agrobacterium tumefaciens* strain GV3101 pMP90 and further infiltrated in *Nicotiana benthamiana* leaves according to Schutze et al. (2009). 2-3 days after infiltration, leaf (tobacco) epidermal cells were analyzed for fluorescence (YFP, GFP or RFP) by confocal laser scanning microscopy using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). Microscopy and western blot analyses were carried out as previously described (Schutze et al., 2009) using HA- and c-myc-specific antibodies (Roche).

FRET-FLIM interaction assays

For the FRET analysis, the ARR18, LSD1, NLS-LSD1, bZIP63 and different ARR18 mutant entry clones ($ARR18^{D70E}$, $ARR18^{D70N}$, $ARR18^{D75E}$ and $ARR18^{D75N}$) were recombined via LR reaction into estradiol inducible pABindFRET, pABindGFP and pABindmCherry expression vectors (Bisson et al., 2009). After transforming the corresponding vectors into *Agrobacterium tumefaciens* strain GV3101 pMP90 and infiltrating tobacco leaves as described above, plants were grown for 2-3 days. After this period, the estradiol inducible promoter was induced by brushing the leaves with 20µM ß-estradiol supplemented with 0.1 % Tween 20 to activate transgene expression. The GFP fluorescence lifetime was determined after 4 h of induction using a custom build confocal stage scanning microscope (CSSM) (Elgass et al., 2009).

Arabidopsis protoplast transient expression assay

bZIP63, bZIP53, ARR18 and the different ARR18 mutant versions ($ARR18^{D70E}$, $ARR18^{D70N}$, $ARR18^{D75E}$ and $ARR18^{D75N}$) were cloned by LR-reaction into the destination vector P_{UBI10} (Grefen et al., 2010). *Arabidopsis* cell culture protoplasts were isolated and transfected accordingly to Schutze et al. (2009). The amount of each transformed plasmid was constant through the entire experimental set. To keep the total DNA quantity at the same level in each sample, unrelated DNA was added when required. Transfected protoplasts were incubated in darkness overnight at 23°C. The *35S: NAN* construct was used as an internal control to normalize the variations of each transfection due to transformation efficiency and cell viability. GUS and NAN enzyme assays were performed as described in Ehlert et al. (2006). The results are shown as the means of relative GUS/NAN activities. All transient experiments were repeated at least three times.

Quantitative RT-PCR

The total RNA from corresponding plant material was isolated using RNeasy Plant Mini Kit (Qiagen) and a double DNA digestion was performed to ensure the absence of genomic DNA contamination. The cDNA was generated using either oligo-dT or random hexamer primers with M-MuLV Reverse Transcriptase (Fermentas). The optimal PCR conditions were verified by preparing dilution series. At least two PCR reactions (in triplicate repeats each) using two independent cDNA syntheses were performed for every treatment / gene. The amplification of

cDNA was performed with PerfeCta qPCR SuperMix or PerfeCta SYBR Green SuperMix (Quanta Biosciences, distributed by VWR) using, when necessary, the corresponding Probe (Universal ProbeLibrary Single Probes, Roche) accordingly to manufacturer. The PCR reactions were running in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad). The used primers are listed in the Table S2. Expression levels of each gene were normalized according to the expression of the following housekeeping genes: EF-1-alpha (AT5G60390), in case of SYBR reactions) and Ubi10 (AT4G05320), in case of using Universal ProbeLibrary Single Probes. The CFX Manager software (Version 1.1; Bio-Rad) has been used for the quantification of relative expression levels. The plant material has been collected from at least two independent experiments.

Generation of *Arabidopsis* transgenic lines

The bZIP63-GFPox *Arabidopsis* line that overexpresses driven by the 35S promoter bZIP63 fused to GFP was produced using bZIP63 cDNA cloned into the pH7WGF2.0 vector via LR-reaction. *Arabidopsis* Col-0 plants were transformed by floral dip method (Zhang et al., 2006) using the *Agrobacterium tumefaciens* strain GV3101 pMP90 and selected for hygromycin resistance.

Transient transformation of *Arabidopsis thaliana* seedlings

Arabidopsis thaliana seedlings were transiently transformed by vacuum infiltration (Marion et al., 2008)with *Agrobacterium tumefaciens* strain GV3101 pMP90 carrying the desired constructs, as described previously (Marion et al., 2008). To determine protein subcellular localization, cotyledon (*Arabidopsis*) epidermal cells were analyzed 2-3 days after infiltration for fluorescence (GFP or RFP) by 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).

Semi-quantitative RT-PCR

To test if the T-DNA insertion in the *ARR18* and *bZIP63* mutant results in an impaired transcription of the gene, semi-quantitative RT-PCR studies were carried out. Total RNA from *Arabidopsis* inflorescences and two weeks old *Arabidopsis* seedlings respectively was isolated using RNeasy Plant Mini Kit (Qiagen). RNA was reverse transcribed using oligo-dT

primer with M-MuLV Reverse Transcriptase (Fermentas) and the resulting cDNA was used as template for PCR with Taq polymerase. After 20-30 cycles for ARR18 and 33-35 cycles for bZIP63, PCR products were separated via agarose gel electrophoresis. The following primer sequences were used to amplify full length *bZIP63:* 5´ - CACCATGGAAAAAGTTTTCTCCGACGAAG-3´ and 5´-CTG ATCCCCAACGCTT CGAATACGTTTC -3´. To ensure the same cDNA amount in all tested samples, bZIP63 transcript levels were compared to those amplified with ACTIN2 primers: 5´- CTGCTCAATCT CATCTTCTTCC -3´ and 5´- GAGCTGCCTCATCATACTCG -3´. ARR18 RT-PCR was carried out using the primers described earlier (Mason et al., 2005) and compared with CAB3 primers 5'- CGTCTAGATCAATGGCCGCCTCAACAATGG-3' and 5'- CGAATTCGCTCACTTTCCGG GAACAAAGTT -3'.

DNA gel-blot analysis

For southern analysis, genomic DNA was isolated using Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences) accordingly to the manufacturer. 10-15 µg of isolated DNA was digested over night with 2 µl *BamHI* (Fermentas) in 30 µl end volume and separated on 1% agarose gel. The gel has been blotted over night onto positively charged nylon membrane (Roche) using 20x SSC buffer (0.3 M sodium citrate buffer, pH 7.0, 3 M NaCl). After short washing of the membrane in 5x SSC, the DNA has been cross-linked to the membrane by UV irradiation and pre-hybridized for approximately 6-8 h in hybridization buffer (5x SSC, 0,1% N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent (Roche), 50% formamide) at 37^{o}C. The T-DNA-specific probe was prepared by PCR using DIG-labeled dUTPs (Roche) and following primers: 5´- ATCATCCTGATCGACAAGACCGG -3' and 5'- ATGAT TGAACAAGATGGATTGCACG -3'. Denatured probe was added to hybridization buffer and the membrane was incubated over night at 42^{o}C followed by several washing steps of increased stringency. Following the last washing step (0.1x SSC, 0.1% SDS, 20 min at 68^{o}C), the membrane was blocked in 1% blocking reagent (Roche) in maleic acid buffer (pH 7.5) for 30 min and incubated for 30 min in 1:20,000 diluted in the blocking buffer anti-DIG-AP antibody (Roche). The detection of the signal was performed using CPD-Star (Roche) in accordance with manufacturer.

Results

ARR18 is an osmotic stress-responsive gene

In order to investigate whether and which type-B ARR genes respond to drought and osmotic stress at a transcriptional level, publicly available microarray data were analyzed using Genevestigator (https://www.genevestigator.com/gv/). Compared to type-A ARR genes, which are predominantly down-regulated under water stress in the majority of released experiments, the type-B ARR genes do not show a uniform regulation pattern (Figure S1A). The type-B ARRs genes, which are predominantly expressed at relatively higher level in all tissues and through all developmental stages, namely *ARR1*, *2*, *10*, *12* and *14* (Figure S1B,C), do not show any substantial treatment-dependent alteration in transcript levels (Figure S1A). By contrast, the type-B ARR genes, which are characterized by very low or tissue-specific expression, namely *ARR11*, *13/21*, *18* and *19* (Figure S1C); (Mason et al., 2004; Tajima et al., 2004), demonstrate high level of expression variability depending on the particular experiment (Figure S1A).

Such heterogeneity of the data might be due to the differences between the experimental settings. However it might also be caused by very low transcript levels and, thus, very low signal-to-noise ratios. To clarify this, we performed the Q-PCR analysis using total RNA from leaves of *Arabidopsis* plants under osmotic stress conditions. To induce osmotic stress, hydroponically grown plants were transferred to media containing different concentrations of polyethylene glycol (PEG 6000) for 6 h and 24 h. Whereas *ARR13*, *19* and *21* were so low expressed that their transcripts could not undoubtedly be detected in all the samples, the transcript level of most of type-B ARR genes did not substantially change in response to stress treatment (Figure 1A, B). The only exception was the *ARR18* transcript level which showed a significant increase when the plants have been transferred to 10 % or 15 % PEG-containing medium (Figure 1A, B). In contrast, most of the type-A ARR genes were rather repressed under our experimental conditions (Figure 1C, D). The failure to detect ARR13, 19 and 21 in vegetative tissue is in agreement with earlier reports considering their expression to be restricted to reproductive organs (Day et al., 2008; Mason et al., 2004; Tajima et al., 2004). Although *ARR18* showed high expression level in reproductive organs (Figure S1), it is also expressed in vegetative tissue (Figure S1). Taking into account the observed stress-induced expression of *ARR18*, and the absence of any functional characterization, we focused our work on this particular type-B ARR gene.

The germination of *arr18* seeds is impaired under high osmolarity conditions

To proceed with the functional characterisation of *ARR18*, we characterized two *Arabidopsis* lines, *arr18-1* and *arr18-2*, homozygous for T-DNA insertions in the *ARR18* gene in Ws-2 and Col-0 ecotypes respectively. Based on the position of the insertion, the mutants were predicted to be either null or hypomorphic (Mason et al., 2005). RT-PCR analysis of the mutant lines detected no *ARR18* transcripts in the *arr18-1* line (Figure S3A). In contrast, *ARR18* transcript was still detectable in the *arr18-2* line, carrying the T-DNA insertion in the 5'-untranslated region, although at comparatively lower level than in Col-0 plants (Figure S3A). Therefore, only the *arr18-1* line was used for further analyses.

Very detailed analyses of seed developmental stages identified *ARR18* as early seed specific gene (Day et al., 2008). We, therefore, investigated, whether the *arr18-1* mutation might cause alterations in seed physiology, especially with respect to water stress. When tested under control conditions, the seeds of the *arr18-1* line germinated to a similar rate as wild type (Ws-2; Figure 2A). In contrast, when seeds were incubated on mannitol-containing medium the germination rate of the *arr18-1* mutant line was significantly reduced compared to wild type (Figure 2B). Therefore, the absence of ARR18 results in an increased sensitivity of seeds to altered water potential. At the same time, we did not observe any substantial difference between wild type and mutant lines in the wilting rates when 3-week-old plants were transferred to PEG-containing medium. This would suggest that ARR18 acts as a positive regulator of the seed tolerance to water stress.

ARR18 interacts with the Opaque2 homologous bZIP transcriptional regulators *in planta*

The osmo-sensitivity of *arr18-1* germination resembles the phenotype observed in *ahk1* mutants. Osmotic stress caused numerous genes to be differentially regulated in the *ahk1* mutant as compared to wild type (Tran et al., 2007; Wohlbach et al., 2008). Since the function of AHK1 as potential osmosensor depends on the initiation of a phosphorelay (Wohlbach et al., 2008), a straight forward scenario would imply the HK-dependent activation of B-type ARR proteins. Similarly to other B-type ARRs, ARR18 contains a conserved GARP DNA-binding domain and functions as a transcriptional regulator (Veerabagu et al., unpublished). Therefore, ARR18 might function as the output element of a phosphotransfer cascade, directly linking an AHK1 phosphorelay circuit to differential gene expression. By searching

the upstream regions of AHK1-dependent genes, several significantly enriched *cis*-elements have been identified (Wohlbach et al., 2008). However, the (A/G)GAT(T/C) element, the binding site of type-B ARRs (Hosoda et al., 2002; Sakai et al., 2000) were not found. Instead, different variants of *cis*-elements containing the ACGT-core were among the most overrepresented. The ACGT core is known to be recognized by members of the basic region leucine zipper (bZIP) family of transcription factors, including ABA-dependent AREB/ABF factors (Hirayama and Shinozaki, 2007). In order to test, whether ARR18 could indirectly effect the expression of ACGT-core containing promoters, we initialized a bimolecular fluorescence complementation (BiFC)-based screening of bZIP factors for their interaction with ARR18. Because the main intention was to identify the components upstream of ABA signaling, the AREB/ABF factors were not considered. Instead, we focused on the *OPAQUE2 (O2)* homologues from *Arabidopsis*, so-called C-group bZIPs, namely *bZIP9, 10, 25* and *63* (Jakoby et al., 2002). Some of these bZIPs have been shown to be involved in the regulation of seed storage proteins (Alonso et al., 2009; Lara et al., 2003) and oxidation and hypoosmotic stress responses (Kaminaka et al., 2006; Weltmeier et al., 2006).

As shown in Figure 3A, BiFC fluorescence was clearly observed in the nuclei of transiently transformed tobacco (*Nicotiana benthamiana*) leaf cells, when ARR18 was co-expressed with bZIP9 and bZIP63, and to a weaker extent with bZIP10. No fluorescence was observed when ARR18 was co-expressed with bZIP25 or the non-bZIP protein Lesion Simulated Disease 1 (LSD1) (Dietrich et al., 1997). The expression of the split-YFP fusion proteins was confirmed by western-blot analysis using antibodies directed against the myc-tag (YFP-N fusions) or the HA-tag (YFP-C fusions; Figure 3B). These data suggest that ARR18 specifically interacts with bZIP9, 10 and 63 *in vivo*.

ARR18 represses the transcriptional activity of bZIP63/bZIP53 heterodimers

We next addressed the question, whether ARR18 could alter the transcriptional activity of the interacting bZIP factors. The members of C-group bZIP factors, including bZIP9 10 and 63, constitute transcriptionally highly active heterodimers with S1-group bZIPs (Ehlert et al., 2006). The cognate DNA elements recognized by, for instance, the S1-group member bZIP53 is known, and the synergistic activation of target promoters containing these elements by bZIP53 and C-group bZIPs has been demonstrated (Dietrich et al., 2011; Ehlert et al., 2006). Interestingly, some of the S1/C target genes appear to be not only differentially regulated in response to osmotic stress in wild type and *ahk1* plants, but are also de-regulated in the *ahk1*

seeds. Among others, these include the albumin genes *2S1* and *2S2*, the *PROLINE DEHYDROGENASE* (*ProDH*) gene and some *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) genes (Tran et al., 2007; Wohlbach et al., 2008). We, therefore, tested in transient protoplast assays, whether ARR18 is able to influence the transcriptional activity of bZIP53/C-group bZIP heterodimers using a *2S1 promoter:GUS* construct (*p2S1:GUS*) as reporter. When *p2S1-GUS* was co-transfected with either of the tested effector protein constructs alone, i.e. *bZIP* factors or *ARR18*, the GUS activity was similar to the control (Figure 3C; *p2S1:GUS* transformed alone). However, GUS activity increased significantly when the C-group *bZIPs* were co-transformed with *bZIP53*, confirming the reporter gene induction by the heterodimers of C- and S1-group bZIPs (Alonso et al., 2009; Ehlert et al., 2006). The co-transfection of *ARR18* together with *bZIP53* and C-group *bZIPs* did not lead to significant changes in the GUS activity for all but one protoplast set: In the presence of ARR18 the transcriptional activity of the bZIP53/bZIP63 heterodimer was significantly and specifically reduced (Figure 3C). Therefore, despite their *in planta* interaction, ARR18 had no effect on the transcriptional activation of *p2S1-GUS* mediated by the bZIP53/bZIP9 and bZIP53/bZIP10 heterodimers. These results indicate that the interaction of ARR18 with bZIP63 can cause the inhibition of the bZIP53/bZIP63-dependent gene transcription.

bZIP63 recruits ARR18 receiver domain to the nucleus

The regulatory receiver domain of RRs contains the phosphate-accepting aspartate which is directly involved in the phosphorelay downstream of HKs. We, therefore, examined whether the ARR18 receiver domain (RD) directly interacts with bZIP63. Using BiFC in transiently transformed tobacco leaf cells, an interaction of $ARR18^{RD}$ with bZIP63 was detected. Moreover, the BiFC fluorescence was only observed in the nuclei of the cells (Figure 4A), although $ARR18^{RD}$ usually showed a cytoplasmic localization in the absence of bZIP63 (Figure 4B). This indicates that bZIP63 recruits $ARR18^{RD}$ by protein-protein interaction to the nucleus.

Although BiFC is widely used in *in planta* protein-protein interaction studies (Schutze et al., 2009), false positive signals are observed when the fusion proteins accumulate to high levels. To exclude such possibility in our case, we designed and applied a nuclear recruitment approach: the cytoplasmic $ARR18^{RD}$-RFP fusion under the control of the strong 35S promoter was transiently co-expressed with bZIP63 fused either to GFP or GUS and the intracellular localization of $ARR18^{RD}$-RFP was monitored. When bZIP63:GFP was expressed under the

control of the strong *UBIQUITIN10* promoter (*pUBI10*) leading to its high nuclear accumulation, the virtually entire RFP signal was detected in the nucleus, indicating the nuclear recruitment of ARR18RD-RFP by bZIP63-GFP (Figure 4C). A significant nuclear recruitment of ARR18RD-RFP was also observed when bZIP63-GFP and bZIP63-GUS were expressed from the native *bZIP63* promoter and accumulated to lower nuclear levels. In these cases, cytoplasmic RFP signals were still detectable, most probably due to a higher amount of ARR18RD-RFP compared to *bZIP63* promoter-driven bZIP63-GFP/-GUS (Figure 4C). Similar results were revealed when 35S promoter-driven *ARR18RD-RFP* was expressed in *Arabidopsis* seedlings over-expressing bZIP63-GFP (Figure S2). Taken together, these data confirm the physical interaction between ARR18 and bZIP63 inside the nucleus.

The interaction of ARR18 with bZIP63 depends on aspartate phosphorylation

Next we determined, whether the interaction between ARR18 and bZIP63 depends on the phosphorylation status of the conserved aspartate (Asp70: Veerabagu et al., unpublished) in the response regulator´s receiver domain. In order to precisely examine this, we performed quantitative FRET-FLIM studies on nuclei of transiently transformed tobacco leaf cells (Harter et al., 2011). Therefore, we co-expressed ARR18 and bZIP63 in two C-terminal fusion variants: (i) ARR18 as donor molecule (ARR18-GFP) and bZIP63 as acceptor (bZIP63-mCherry) or (ii) bZIP63 as donor molecule (bZIP63-GFP) and ARR18 as acceptor molecule (ARR18-mCherry). As positive controls, the ARR18 or bZIP63 fused to GFP and mCherry in one molecule (FRET constructs) were used (Bisson et al., 2009). The individually transformed GFP fusions served as negative controls. The significant decrease in the fluorescence life time (FLT) of GFP indicated that ARR18 and bZIP63 associate inside the nucleus independent whether bZIP63-GFP or ARR18-GFP served as a donor (Figure 5A,B). In contrast, when ARR18-GFP was co-expressed with LSD1-mCherry no change in the FLT of GFP was observed (Figure 5A). The FRET-FLIM data substantiate the results of the BiFC and nuclear recruitment studies that ARR18 and bZIP63 specifically interact in the nuclei of plant cells.

We next examined whether the phosphorylation status of ARR18 influences its interaction with bZIP63. To this end, we generated mutant versions of ARR18 by substituting the phosphate-accepting Asp either with asparagine (Asn) (ARR18^{D70N}; non-phosphorylatable version that mimics a constitutive inactive form) or with glutamate (Glu) (ARR18^{D70E}; a constitutive active form mimicking a phosphorylated version) (Hass et al., 2004; Hwang and

Sheen, 2001; Mira-Rodado et al., 2007; Sweere et al., 2001). Both mutant versions were subjected as mCherry acceptor fusions to FRET-FLIM analysis using bZIP63-GFP as donor in transiently transformed tobacco leaf cells. In contrast to ARR18^{D70E}-mCherry, ARR18^{D70N}-mCherry did not associate with bZIP63-GFP (Figure 5C). To further test the specific role of Asp 70, we also mutagenized as a control the closely situated Asp75 to Asn or Glu. In this case, neither of the Asp75 substitutions influenced the interaction of ARR18-mCherry with bZIP63-GFP (Figure 5C). Furthermore, there was no interaction of bZIP63-GFP with LSD1-mCherry, which served as an additional control. These results indicate that wild type ARR18 and ARR18^{D70E} have a similar ability to interact with bZIP63 in the nuclei of living plant cells, whereas constitutive inactive ARR18^{D70N} is no longer capable to perform this interaction.

The phosphorylation status of ARR18 modulates bZIP53/bZIP63-dependent gene expression

As demonstrated above, the ARR18-bZIP63 association could be disrupted by a substitution of Asp70 to Asn. This substitution might also neglect the inhibitory effect of ARR18 on bZIP63-dependent gene expression. We, therefore, performed *p2S1-GUS* reporter gene assays with ARR18^{D70E} and ARR18^{D70N} (GFP fusions) as effector proteins in transiently transfected *Arabidopsis* protoplasts. Similarly to wild type *ARR18*, the transfection of the single *ARR18^{D70N}* or *ARR18^{D70E}* effector plasmids together with *p2S1-GUS* did not cause a substantial decrease in reporter gene activity (Figure 5D). Then, the mutant *ARR18* constructs were co-transfected together with the *p2S1-GUS* reporter as well as with *bZIP53* and *bZIP63*. The co-transfection of *ARR18^{D70N}* with both *bZIPs* did not have an effect on reporter gene activity (Figure 5D). In contrast, a significant inhibition of *p2S1-GUS* activity was observed when *ARR18^{D70E}* was co-transfected with *bZIP53* and *bZIP63* (Figure 5D). These data demonstrate that ARR18 is not only able to interact with bZIP63 but also to modulate the transcriptional activity of the bZIP53/bZIP63 heterodimer in an Asp70 phosphorylation-dependent manner.

We next tested the opposite scenario, i.e., whether bZIP63 might influence the transcriptional activity of ARR18. B-type ARRs have been shown to up-regulate the expression of type-A *ARR* genes via specific binding to *cis*-acting elements in their promoters. For instance, the promoter of the *ARR5* gene contains several copies of 5′-(A/G)GAT(T/C)-3′ element shown to be recognized by type-B ARRs including ARR1, 2 and 10 (Hwang and Sheen, 2001). By co-transfection of the *ARR18* effector construct with the *ARR5* promoter-driven GUS reporter

gene (*pARR5-GUS*) in *Arabidopsis* protoplasts a significant increase of GUS activity was observed compared to the no-effector control (Figure 5E). The additional co-transfection of bZIP63, however, had no effect on the *pARR5-GUS* activity (Figure 5E). In conclusion, we could show that the substitution of Asp70 to either Asn or Glu affects not only the interaction of ARR18 with bZIP63, but the expression of at least some bZIP53/bZIP63-dependent genes. Whereas phospho-mimicking Glu70 enhances the inhibitory effect of ARR18 on bZIP53/bZIP63 transactivity, $ARR18^{D70N}$ has no effect.

ARR18 and bZIP63 have opposite functions in seed germination and gene regulation

Our data derived from the protoplast assays suggest an opposite function of ARR18 and bZIP63, at least in the control of particular genes, namely *2S1*. This gene has also been found differently regulated in wild type and *ahk1* mutant seeds (Wohlbach et al., 2008). Since the germination assays have revealed hypersensitivity of *arr18* seeds to increased osmolarity, we analyzed the response of plants with altered levels of bZIP63 in the presence of similar stress stimuli. For this, we used a T-DNA insertion line in Ws background (T-DNA is inserted in the first exon). The RT-PCR analysis did not detect bZIP63 full-length transcript (Figure S3A), thus considering this line as a bZIP63 loss-of-function mutant (*bzip63*). Southern-blot analysis supported a single-copy genomic insertion (Figure S3B). As plants with increased bZIP63 level, three Col-0 lines stably transformed with bZIP63 either N-terminally HA-tagged (HA-$bZIP63_{ox}$), C-terminally GFP-tagged (bZIP63-GFP_{ox}), or non-tagged bZIP63 ($bZIP63_{ox}$) under the control of 35S promoter were used. The transcript levels of bZIP63 in these overexpressing lines are presented on Figure S3C.

The kinetics of seed germination of all the transgenic lines were similar to the corresponding wild type on control medium (Figure 6A, B), although bZIP63-GFP_{ox} demonstrated a slight retardation of germination during the first three days. However, on the medium containing 300 mM mannitol the seed germination of *bzip63* and the bZIP63 overexpressor lines was significantly different to the corresponding wild types. Whereas *bzip63* seeds started with testa disruption and radical production earlier than Ws seeds (Figure 6C), the seeds of all three overexpressors revealed retardation of germination compared to Col-0 (Figure 6D). Moreover, the kinetics of retardation correlated with the *bZIP63* transcript level in the corresponding lines (Figure S3C). Our results, therefore, indicate that, in contrast to ARR18, bZIP63 functions as a negative regulator of seed germination under osmotic stress conditions.

ARR18 and bZIP63 are functioning antagonistically in gene regulation

Taking into account the involvement of bZIP63 in water stress response, we tested whether changes in the osmolarity of the growth medium affect *bZIP63* expression. Upon low concentration of PEG (10%), no difference in *bZIP63* transcript level was observed in wild type plants as compared to untreated plants. However, the increase of the PEG concentration in growth medium progressively down-regulated *bZIP63* expression (Figure. 7A). Interestingly, the *bZIP63* mRNA level in *arr18-1* mutant plants was significantly lower already at 10% PEG compared to both Ws plants grown on 10% PEG and untreated *arr18-1* plants, and dropped even more at 15% PEG (Figure. 7A). Although the difference was not dramatic (approximately two-fold decrease relatively to Ws plants), it might be indicative of the alterations at gene expression level leading to the hypersensitivity of the *arr18-1* mutant to decreased water potential.

To further explore this observation, we analyzed the expression of the osmotic stress marker gene *RESPONSIVE TO DESSICATION 29B* (*RD29B*) in *arr18-1* plants exposed to low water potential for 6 h and 24 h. In accordance with numerous reports, *RD29B* transcript massively accumulated in PEG-treated wild type plants after onset of stress treatment in a concentration-dependent manner (Figure 7B-D). Comparable to the *bZIP63* transcript level, *arr18-1* was more sensitive than the wild type, accumulating increased amount of *RD29B* mRNA already after 6 h of 10% of PEG treatment. While the amount of *RD29B* mRNA in *arr18-1* mutant plants treated with 15% PEG for 6 h was still almost 3 times higher than in Ws-2 plants under the same treatment, practically no difference is found between *arr18-1* and Ws-2 plants at 20% PEG. A similar tendency is observed upon 24 h PEG treatment (Figure. 7B).

To further test whether *RD29B* is differently regulated in the *bzip63* mutant and the bZIP63 overexpressor lines, bZIP63-GFP_{ox} and *bzip63* plants together with the corresponding wild types were transferred to control medium or 20% PEG-containing medium for 6 h. Whereas the amount of *RD29B* mRNA in the mock-treated transgenic lines was comparable to that found in the wild type plants (Figure 7C, D), it significantly differed in plants subjected to low water potential. The bZIP63-GFP_{ox} line accumulated up to 100 times more *RD29B* transcript than Col-0 (Figure 7C). In contrast, the *RD29B* transcript level in *bzip63* was approximately 10 times lower than in Ws plants (Figure 7D), indicating that the observed phenotype was again opposite to this found for *arr18-1* mutant plants.

Discussion

The correlation between *arr18-1* and *ahk1* phenotypes suggests the role of ARR18 as downstream component of AHK1 signaling

Water deficit is among the major environmental constraints limiting plant growth and productivity. In order to properly adapt to water stress, the plants have had to develop a perception-transduction circuit sensing changes in water potential and activating coordinated multiple downstream responses. The histidine kinase AHK1 has been previously identified as an osmosensor that detects water stress in yeast and shown to mediate both ABA-dependent and -independent responses to water stress (Tran et al., 2007; Wohlbach et al., 2008). The ABA signaling pathway is considered to be a central response of plant cells to water stress and is initiated by the increase of its intracellular ABA levels. ABA is also important during the seed development, since the proper seed maturation and desiccation is vital for its quality and later germination ability. Consistent with its osmosensing properties, AHK1 has been shown to play a unique role in the regulation of desiccation during seed development (Wohlbach et al., 2008).

The main purpose of this study was to identify possible two-component signaling elements transferring the initial AHK1-derived signal to downstream targets and to get insight in the underlying molecular mechanism(s). The expression of *ARR18* during seed maturation and its up-regulation by mild osmotic stress has pointed towards its involvement in the induction of downstream responses. Indeed, our analysis of *arr18-1* mutant seeds has confirmed the necessity of ARR18 for the proper germination under low osmotic potential. This suggests that ARR18 is indispensable for the maturation of seeds. The loss of *AHK1* function resulted in the hypersensitivity of *ahk1* seeds to increased osmolarity (Tran et al., 2007; Wohlbach et al., 2008). A similar retardation of seed germination on hyperosmotic medium in *arr18-1* line clearly points to its role as downstream two-component signaling constituent. The increased transcript levels of ARR18 especially in the endosperm (Day et al., 2008) do not contradict its possible role as a positive regulator of the accumulation of seed storage components during seed development.

The ARR18 is not, however, the only B-type ARR preferentially expressed in the developing seeds. Two more B-type ARRs, namely ARR19 and ARR21, were identified as endosperm-preferred early seed specific genes (Day et al., 2008). It is therefore possible that they are functioning in part redundantly with ARR18. Otherwise, all three ARRs might amplify the AHK-generated signals through the regulation of different gene sets at particular seed

maturation stages. The analyses of single, double and triple mutants should help to answer this question.

B-type ARRs as modulators of bZIP transcriptional activity

We identified using different approaches bZIP63 as ARR18 interacting protein. With one exception (Choi et al., 2010), there is no report about the interaction of TCS- and bZIP-regulated processes. Through the interaction with the bZIP factor TGA3 ARR2 promotes the immunity of *Arabidopsis* plants to *Pseudomonas syringae*. Similarly to ARR18, the activation of ARR2 by phosphorylation was important for its effect of TGA3 on the transcriptional regulation of its target gene *PR1*. However, the interaction with TGA3 itself was not disrupted upon the substitution of conserved Asp80 to Asn in ARR2 (Choi et al., 2010). This is in the contrast to the interaction of ARR18 with bZIP63, where the substitution of phosphoaccepting Asp70 to Asn in the response regulator abolished the interaction with bZIP63 (see Figure 5). Furthermore, unlike ARR2, which activated the expression of *PR1* (Choi et al., 2010), ARR18 repressed the *p2S1*-driven *GUS* expression through its interaction with bZIP63. These results indicate significant differences between molecular mechanisms of B-type ARR action. Interestingly, the phosphorelay-dependent activation of ARR2 was triggered by cytokinin and required the functional cytokinin receptors AHK2 and AHK3. Unlike AHK1, the AHK2 and AHK3 have been shown to function as negative regulators in ABA, drought and high salinity stress signalling (Tran et al., 2010; Tran et al., 2007). The phenotype of *ahk2/ahk3* mutant under drought stress is opposite to this of *ahk1* and *arr18-1* mutants. Thus, CK receptors do not seem to function as specific upstream activators of ARR18 in seed germination during water stress. Therefore, not only different members of ARR and bZIP families are able to alter expression of particular genes through their interaction, but most probably different histidine kinases might trigger the formation of independent ARR-bZIP complexes upon receiving specific signal. Taken together, our findings suggest that physical interaction between B-type RRs and bZIP factors is not limited to a single signaling process but represents a regulatory mechanism functioning in various regulated processes in plants. Since prokaryotic organisms do not possess bZIP transcription factors, the link between two systems must have been established during plant-specific evolutionary processes.

Regulation of stress-responsive gene through ARR18-bZIP63 interaction

Consistent with the opposite function of ARR18 and bZIP63 on the activity of the *2S1* promoter, the loss-of-function *arr18-1* and *bzip63* lines demonstrated opposite sensitivity to hyperosmotic stress, both at the level of gene expression and seed germination. The impaired seed germination on the mannitol-containing medium was observed in *arr18-1* mutant line as well as in lines with increased bZIP63 expression; whereas the *bzip63* seeds were more tolerant to the decreased osmotic potential than wild type seeds (see Figures 2 and 6). At the same time, the induction of *RD29B* expression upon osmotic stress was increased in *arr18-1* and the bZIP63ox lines, but decreased in *bzip63*. Although *RD29B* is often used as a stress marker gene, the precise regulation of its expression is still not well understood. Its promoter contains several ABA Response Elements (ABREs, PyACGTGGC), which have been shown to be recognized by several ABRE-binding factors AREB/ABFs (Uno et al., 2000). The AREB/ABFs belong to the bZIP group-A factors and play a key role in ABA-dependent gene regulation (Hirayama and Shinozaki, 2007). Since bZIP63 can bind ACGT-containing sequences (Kirchler et al., 2010), the 100-fold increased *RD29B* expression observed in the bZIP63ox lines can therefore be caused by direct competition with AREB/ABFs for the binding to the ABRE sequence. In this case, however, it is difficult to understand why the absence of bZIP63 would lead to such a dramatic drop of *RD29B* transcript level upon water stress. Otherwise, it might be possible that the observed alteration in the sensitivity of *RD29B* promoter to osmotic stress in bZIP63ox and *bzip63* lines is indicative of the modulation in ABA levels or in the ABA-responsive mechanisms.

Compared to up to 100-fold alterations in *RD29B* transcript level in the bZIP63ox lines, it only increased about 10-fold at 10% PEG and 3-fold at 15% PEG in the *arr18-1* line. As *ARR18* is expressed in vegetative tissue at very low level (Mason et al., 2004; Tajima et al., 2004), it can probably bind only a small portion of the entire bZIP63 pool. Therefore, its absence would not cause the same strong difference in *RD29B* transcript level as it is observed between wild type and *bzip63*. Interestingly, the increase of *RD29B* transcript level in *arr18-1* is observed only at PEG concentrations below 15 %. This coincides with the strong down-regulation of *bZIP63* under high osmolarity conditions, and especially in *arr18-1* background (see Figure 7A), which reduces the bZIP63 for ARR18 regulation. These observations substantiate the proposed mechanism that ARR18 regulates bZIP63 activity through the direct protein-protein interaction.

Although the precise function of RD29B is yet unknown, it was recently reported that a *rd29b* loss-of-function line shows an increased resistance to salt stress-induced root growth inhibition (Msanne et al., 2011). Taking into account the increased osmolarity, which accompanies salt stress conditions, these data might be in agreement with our results: The hyper-accumulation of *RD29B* mRNA in bZIP63-GFP_{ox} and *arr18-1* coincides with an increased sensitivity of these lines to water stress, while *bzip63*, showing reduced *RD29B* level, was characterized by an increased tolerance. It would be interesting to know whether the disruption of *RD29B* gene activity can lead to increased seed germination rate under high osmolarity conditions; in the other words, whether the observed phenotype of the bZIP63 transgenic and *arr18-1* mutant lines directly depends on altered *RD29B* gene product level.

In conclusion it should be pointed out that, apart from bZIP63, ARR18 can also interact with other bZIP members of Opaque2 subfamily. Namely *bZIP10* and *bZIP25* are expressed in developing seeds and are known as important regulators of seed maturation, including accumulation of seed storage proteins (Alonso et al., 2009; Lara et al., 2003), which provide a source of nutrients for seedling development during germination and help embryo to withstand desiccation (Hundertmark and Hincha, 2008). Here, we concentrated primarily on bZIP63 as ARR18-interacting protein and showed the interaction-dependent down-regulation of the *2S1* promoter. It is however interesting to know, whether the interaction of ARR18 with bZIP9 or bZIP10 may also affect the expression of their target genes, and if yes, whether it would be the inhibitory or activating. This would further elucidate the complex regulatory network in the control of seed development and the response to water stress.

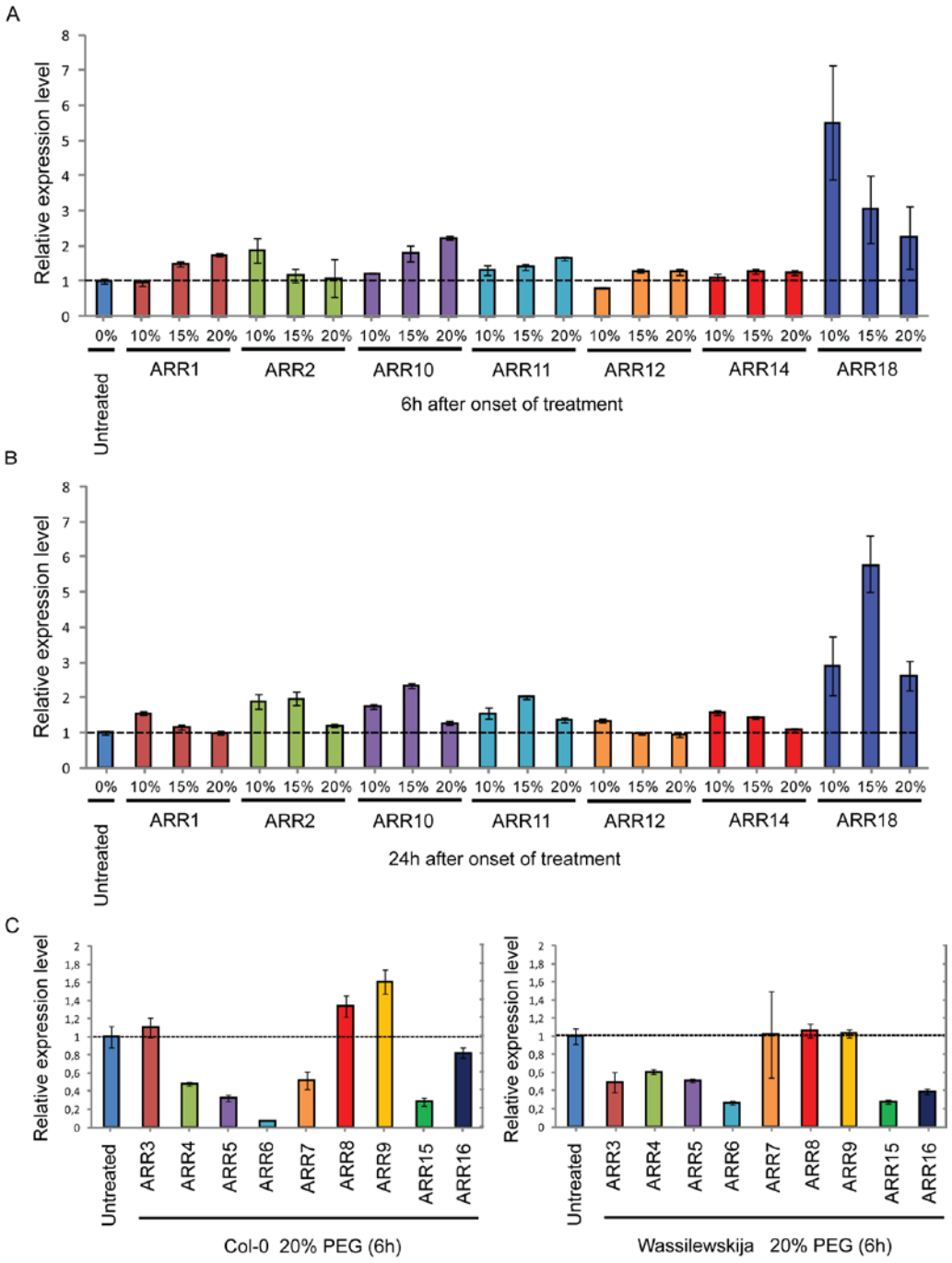

Figure 1: ARR18 is an osmotic stress-responsive gene. Relative expression levels of type-B *ARR*s in Wassilewskija ecotypes upon PEG treatments (0%, 10%, 15% and 20%) for 6 (A) and 24 h (B). Relative expression levels of type-A *ARR*s in Col-0 (C) and Wassilewskija (D) ecotypes treated for 6 h with 0% or 20% PEG 6000. A-D: For each gene, the values for control (0%) plants correspond to one, and those from treated plants are represented as the mean of relative fold changes compared to the control. The data pool consists of duplicates on two biological replicates; error bars represent SE.

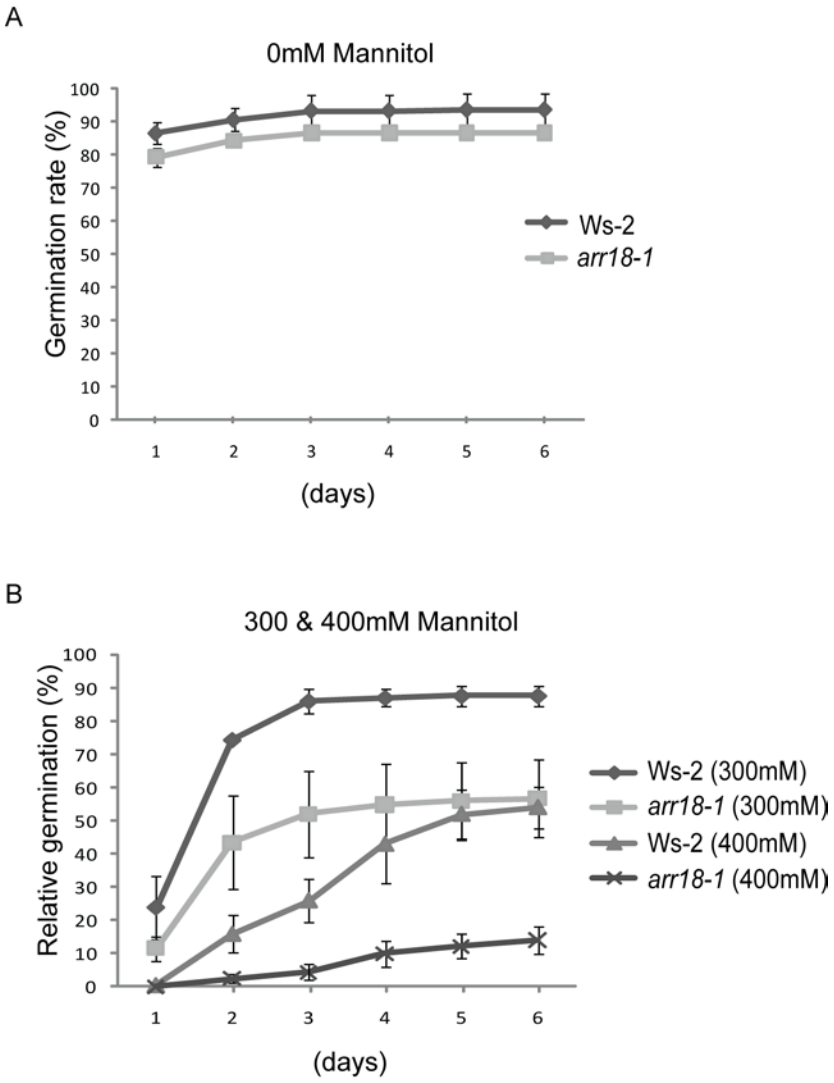

Figure 2: ARR18 acts as a positive regulator of seed germination under osmotic stress. Seeds of *arr18-1* and the wild type (WS-2) were sown on water agar medium either without mannitol (A) or with mannitol at the indicated concentrations (B). Plates were incubated at constant light at 22°C and germination rates measured at the indicated time points by counting the number of seeds with radical breaking through seed testa. The values represent the mean and standard deviation of three independent experiments (n > 100 per experiment).

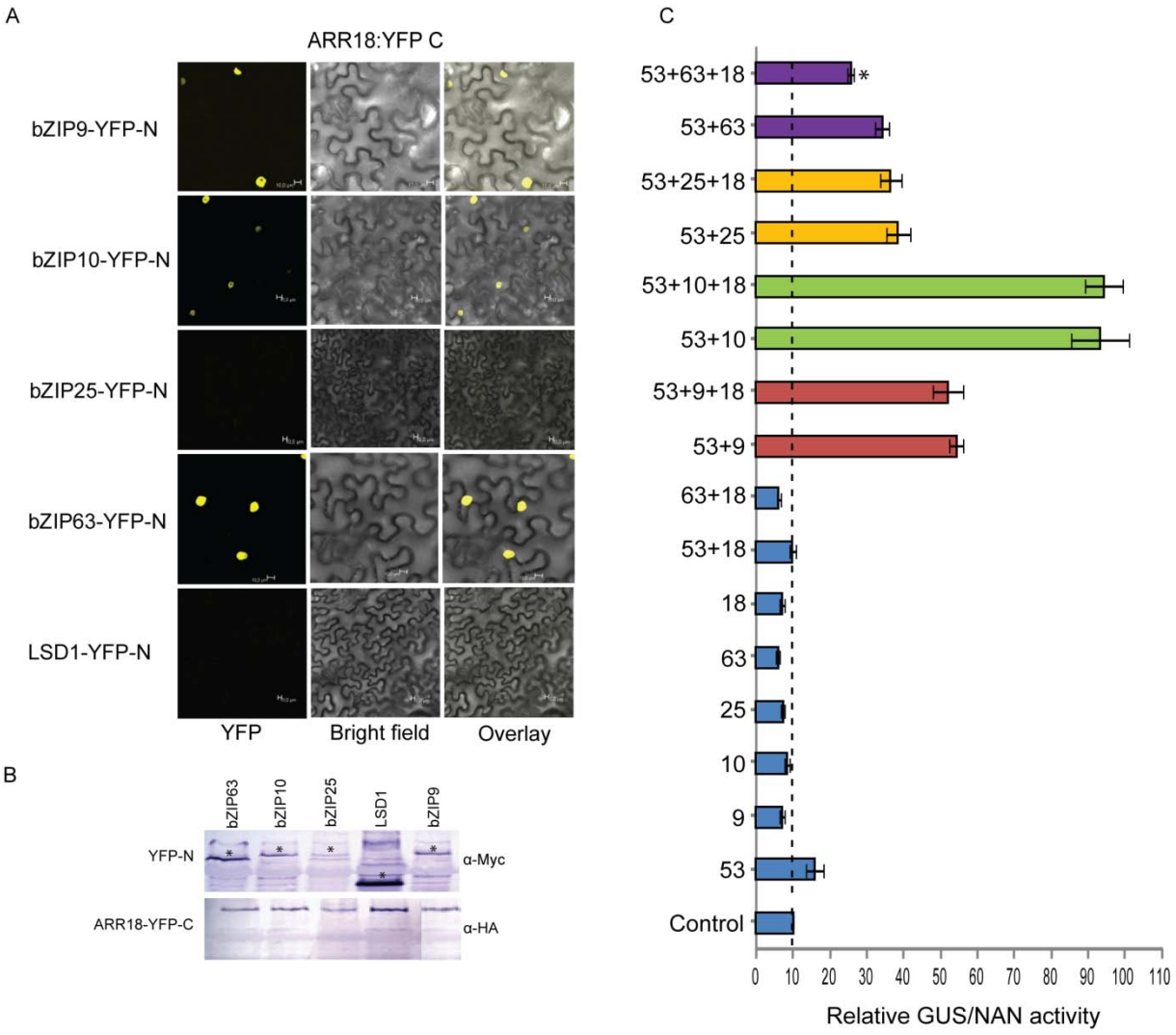

Figure 3: ARR18 interacts with group-C bZIPs in planta and represses the transcriptional activity of bZIP53/bZIP63 heterodimers. Confocal images of transiently transformed epidermal tobacco leaf cells co-expressing the indicated constructs. (A) BiFC assays of ARR18 (ARR18-YFP-C) and the different bZIP (bZIP-YFP-N) or LSD1 (LSD1-YFP-N) fusion proteins. Yellow fluorescence signal (left panel) and its corresponding overlay with bright field image (right panel) are shown. YFP-N: N-terminal fragment of the YFP protein; YFP-C: C-terminal fragment of the YFP protein. (B) Western blot analysis of protein extracts derived from the transformed tobacco leaves used for BiFC assays. The YFP-N (upper panel) and the YFP-C (lower panel) fusions were detected with c-myc- and HA-specific antibodies respectively. Bars represent 10µm. (C) *Arabidopsis* protoplasts were transfected with the *p2S1:uidA* reporter construct. The effector plasmids containing the coding sequences for *ARR18* and the bZIP factors were used in different combinations as indicated. Control: protoplasts are transfected only with reporter construct; 18: pUBI:ARR18-GFP; 53: pUBI:bZIP53-GFP; 9: pUBI:bZIP9-GFP; 10: pUBI:bZIP10-GFP; 25: pUBI:bZIP25-GFP; 63: pUBI:bZIP63-GFP. The 35S:NAN construct was used as transfection efficiency control. Mean values of GUS enzymatic activity and standard deviations were calculated from four independent transfections as relative to NAN activity.

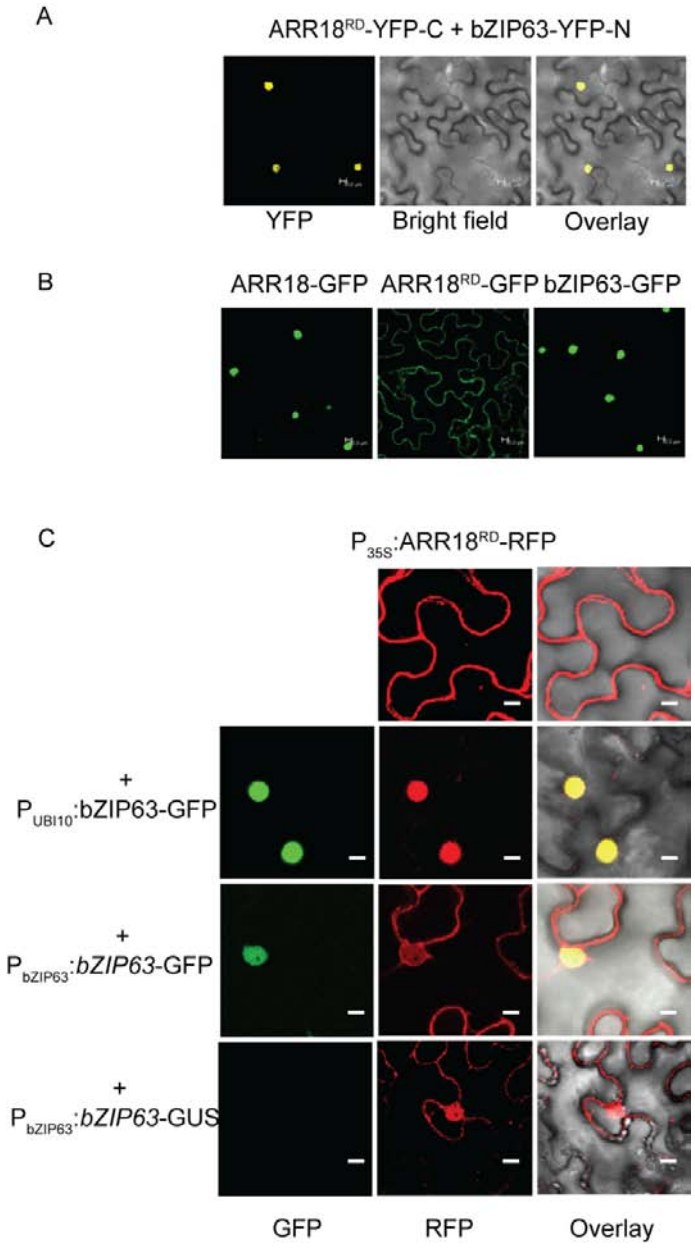

Figure 4: bZIP63 recruits ARR18RD to the nucleus. (A) Confocal images of infiltrated epidermal tobacco leaf cells co-expressing the ARR18 receiver domain fused to the C-terminal domain of the YFP protein (ARR18RD-YFP-C) and bZIP63 fused to the N-terminal domain of the YFP protein (bZIP63-YFP-N). The left panel shows the YFP fluorescence signal, the middle panel the corresponding bright field and the right panel the overlay. (B) Confocal images of abaxial epidermal leaf cells expressing the indicated GFP fusion proteins: ARR18 (35S:ARR18- GFP), ARR18RD (ARR18 receiver domain fused to GFP) and bZIP63 (pUBI: bZIP63-GFP). (C) Confocal images of tobacco leaf cells co-expressing the indicated bZIP63 fusion proteins together with ARR18RD-RFP fusion protein (recruitment assay). pUBI10:bZIP63-GFP: *bZIP63-GFP* cDNA fusion was driven by the *UBI10* promoter; pbZIP63:bZIP63-GFP: the *bZIP63* genomic fragment fused to *GFP*; pbZIP63:bZIP63-GUS: the *bZIP63* genomic fragment fused to *uid1;* and 35S:ARR18RD- RFP: the receiver domain of *ARR18* fused to RFP under the control of the *35S* promoter. The GFP fluorescence (left panels), the RFP fluorescence (middle panels) and the corresponding overlay (right panels) are shown.

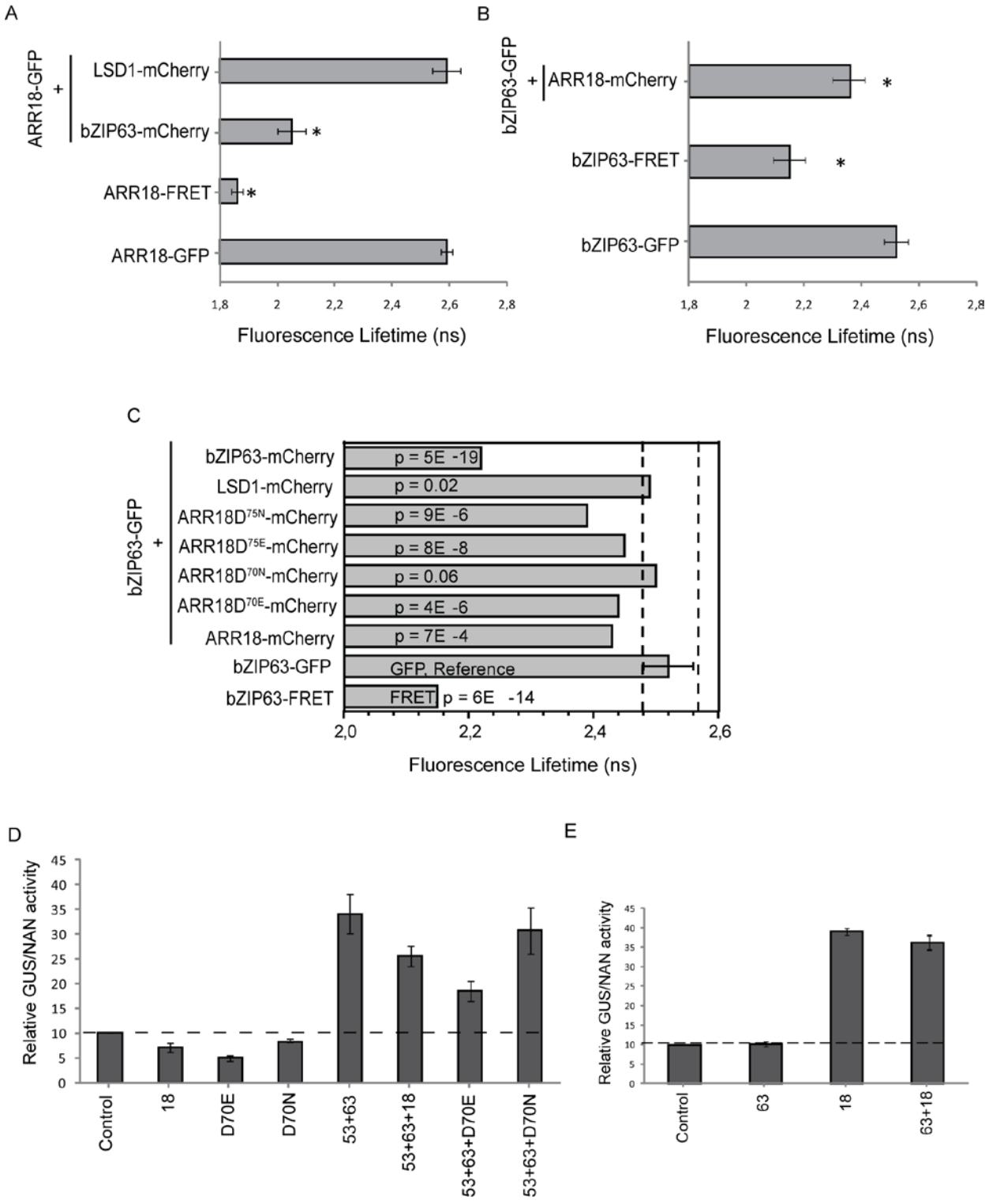

Figure 5: **ARR18 phosphorylation modulates bZIP53/bZIP63 dependent gene expression**. (A, B) FRET-FLIM results of *in planta* interaction between ARR18 and bZIP63, either with ARR18 acting as a donor molecule (ARR18-GFP) and bZIP63 as acceptor (bZIP63-mCherry) (A) or bZIP63 as donor (bZIP63-GFP) and ARR18 as acceptor (ARR18-mCherry) (B). * indicates significantly detected interaction. The lowest bar represents the GFP reference. (C) FRET-FLIM-verified *in planta* interaction between bZIP63 and various ARR18 mutants. The NLS-LSD1-mCherry together with the GFP fusion proteins transformed alone serve as negative controls, and both ARR18-FRET and

bZIP63-FRET serve as positive controls. The life time of GFP fluorescence in nanoseconds was determined and the data were analyzed by one-sided t-test (the corresponding p-values are shown). At least 15 independent measurements of each series were carried out for each experiment. Each experiment was reproduced at least once with similar results. (D) *Arabidopsis* protoplasts have been transfected with pARR5.*uidA* reporter constructs. The effector plasmids containing coding sequences of ARR18 and bZIP63 have been used in single and double combinations. Control: protoplasts are transfected only with reporter construct; 63: pUBI:bZIP63-GFP; 18: pUBI:ARR18-GFP. The 35S:NAN construct was used as internal control in all transformations. Mean values of GUS enzymatic activity and standard deviations were calculated from four independent transfections as relative to NAN activity. (E) Arabidopsis protoplasts were transfected with the *p2S1:uidA* reporter gene. Different effector plasmids containing coding sequences of ARR18, bZIP63, bZIP53 and different mutated forms of ARR18 (53: *pUBI:bZIP53-GFP*; 63: *pUBI:bZIP63-GFP*; 18: *pUBI:ARR18-GFP*; D70E: *pUBI:ARR18*^{D70E}*-GFP*; D70N: *pUBI:ARR18*^{D70N}*-GFP*.) were used either as single or in double and triple combinations as indicated. Control: protoplasts transfected with reporter construct alone (*p2S1-GUS*). The 35S: NAN construct was used as transformation efficiency control. Mean values of GUS enzymatic activity and standard deviations were calculated from four independent transfections as relative to NAN activity.

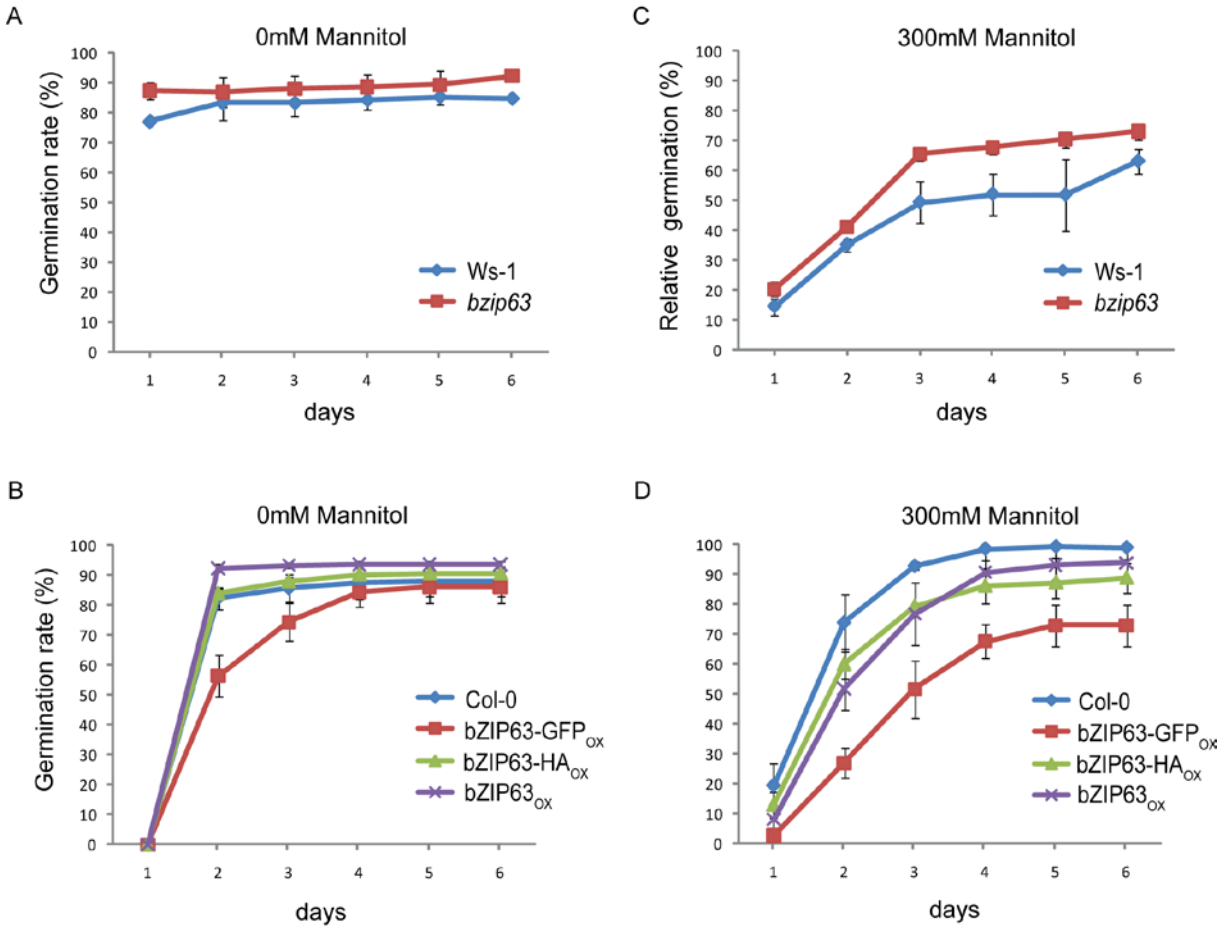

Figure 6: **bZIP63 functions as a negative regulator of seed germination under osmotic stress**. *bzip63* and overexpressors (bZIP63-GFP$_{ox}$,, HA-bZIP63$_{ox}$, bZIP63$_{ox}$) seeds together with their corresponding wild type (Ws, Col-0) were sown on water agar medium without mannitol (0 mM) (A, C) or 300 mM (B, D) mannitol. Experiments were carried out as in figure 2. Mean values and standard deviations were calculated from results of three independent experiments (n > 100 for each experiment).

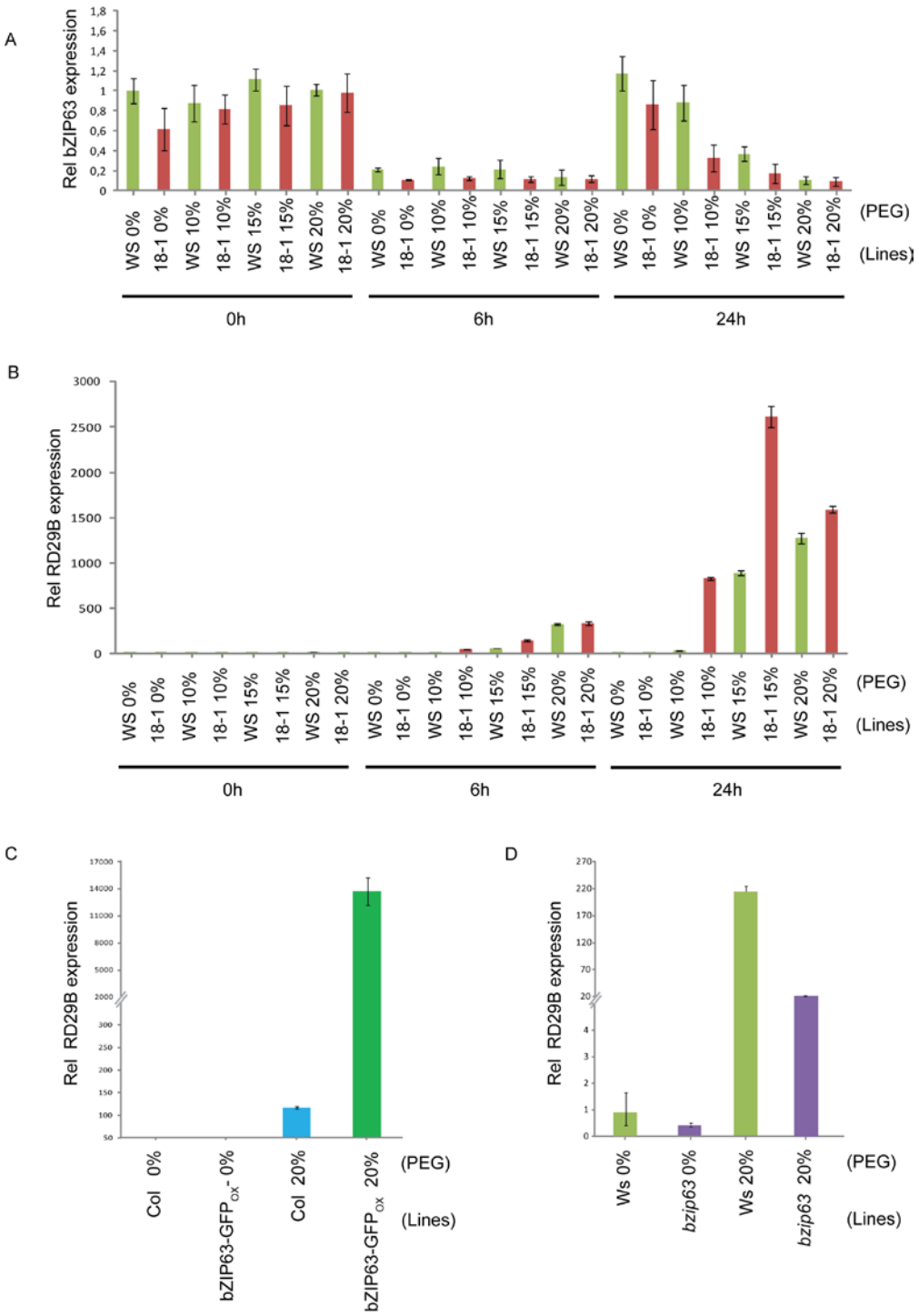

Figure 7: **Gene expression pattern in *bZIP63* and *ARR18* mutant plants:** Relative bZIP63 (A) and RD29B (B) expression levels in *arr18-1* and the corresponding wild type (Ws) treated for 6 and 24h with 0%, 10%, 15% or 20% PEG. (C, D) Relative *RD29B* gene expression levels in bZIP63-GFP$_{ox}$ (C) and *bzip63* (D) and the corresponding wild type plants upon 6 h treatment with 20% PEG. For every gene, the values for control (0%, or "untreated") plants correspond to one, and those from treated plants are represented as relative fold changes compared to the control.

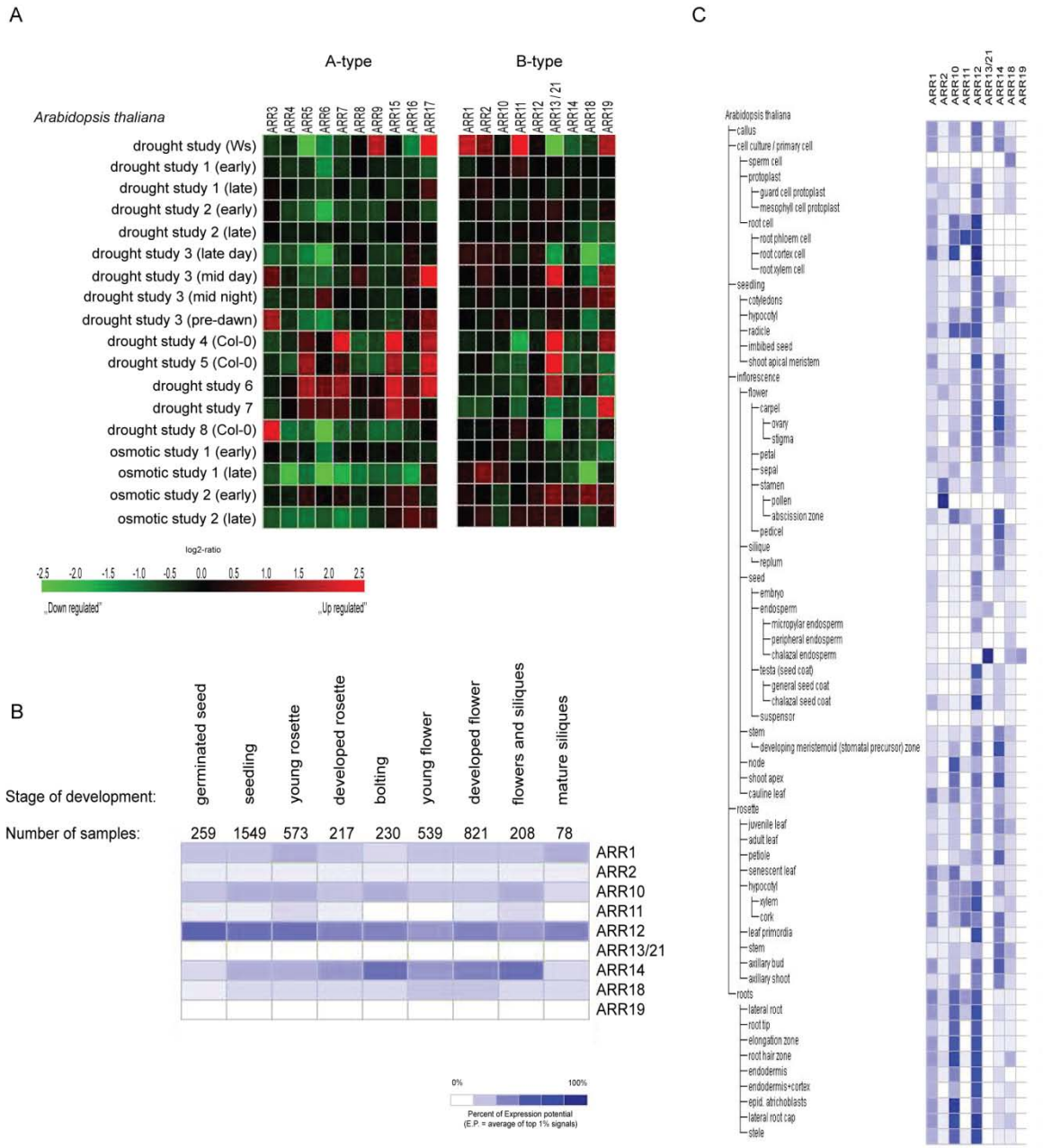

Figure S1: Expression of *Arabidopsis response regulators* (*ARR*s) genes upon water stress treatment. (A) Genevestigator-generated heat map of *ARR*s expression in plants subjected to various water stress treatments. The data of following *Arabidopsis* microarray experiments were used for the presentation: drought study (Zhang *et al.* 2008), drought study 1, 2 (Kilian *et al.* 2007), drought study 3 (Wilkins *et al.* 2010), drought study 4, 5 (Mizoguchi *et al.* 2010), drought study 6, 7 (Abdeen *et al.* 2010), drought study 8 (Giraud *et al.* 2008) and the osmotic studies 1, 2 (Kilian, Whitehead, Horak, Wanke, Weinl, Batistic, D'Angelo, Bornberg-Bauer, Kudla and Harter 2007). Increased expression levels relatively to untreated controls are shaded in red and decreased expression levels in shaded green. (B) The patterns of ARRs expression have been obtained from compiled *Arabidopsis* microarray experiments using Genevestigator analysis tools. Data are reported as absolute expression values shaded such that higher expression values are dark blue as indicated by the scale. The developmental stage and the number of samples taken are indicated. (C) ARRs expression data are obtained and reported as described in figure S1B. The considered *Arabidopsis* organs and the name of the ARRs are indicated.

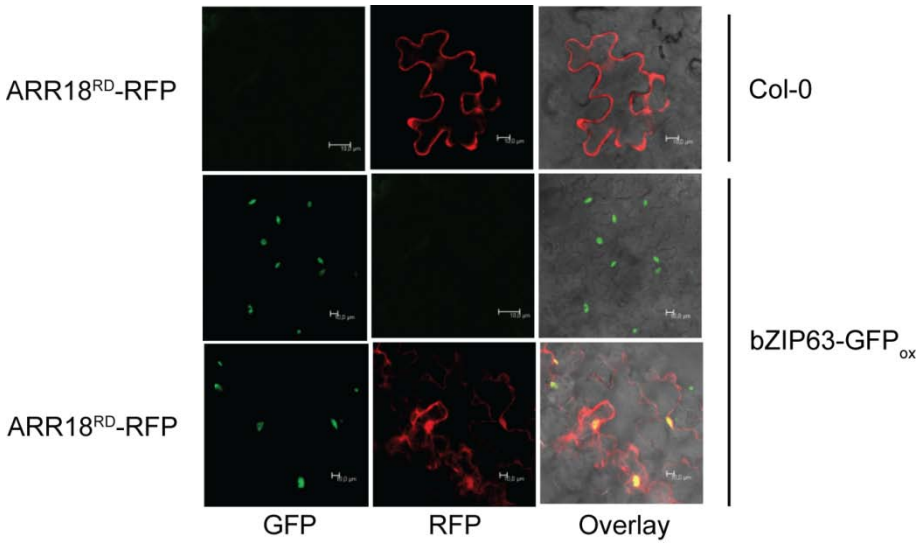

Figure S2: bZIP63 recruits ARR18RD to the nucleus. (A) CLSM images of either Col-0 or bZIP63-GFP$_{ox}$ *Arabidopsis* seedlings transiently expressing ARR18RD:RFP (recruitment assay). Left panel: GFP fluorescence image; middle panel: RFP fluorescence image; right panel: overlay of both fluorescence images. bZIP63-GFP$_{ox}$: *Arabidopsis* line expressing bZIP63 fused to GFP under the control of the *35S* promoter; ARR18RD: receiver domain of the ARR18 protein. Bars represent 10µM.

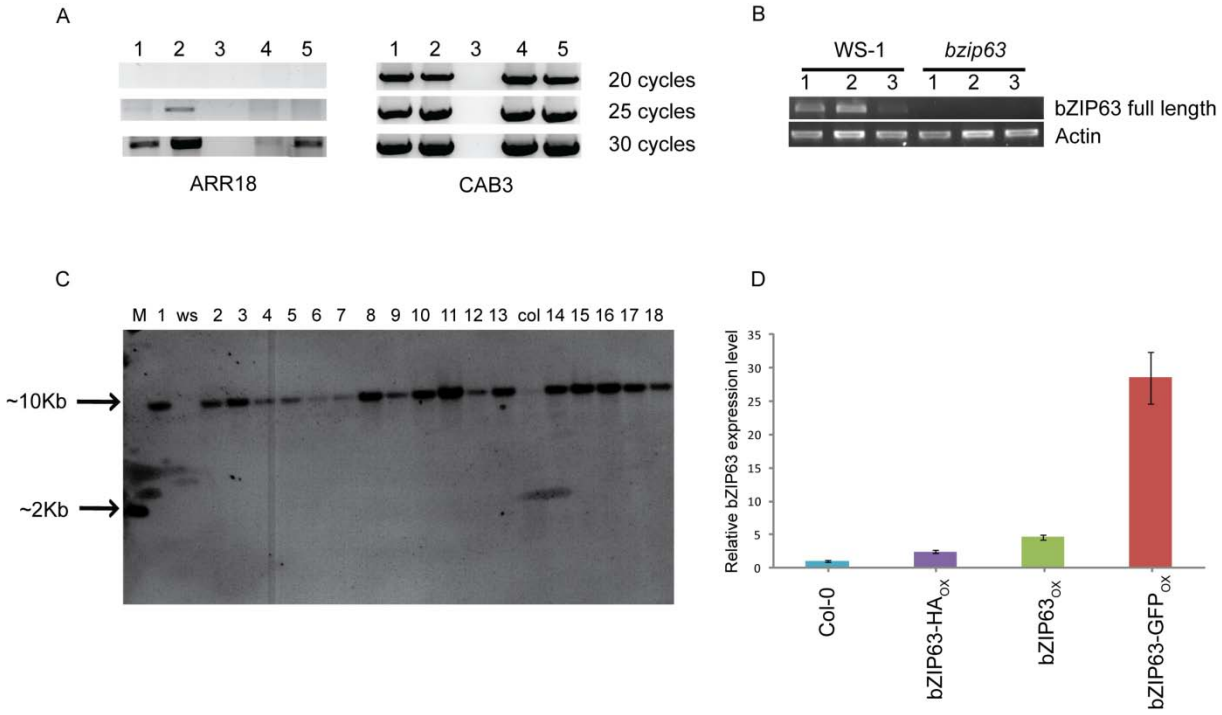

Figure S3: Molecular characterization of ARR18 knockout, bZIP63 knockout and overexpressing lines. (A) Semi-quantitative RT-PCR analysis on total RNA of inflorescences from *arr18-1* (lane 4), *arr18-2* (lane 5) and the corresponding wild type plants (Ws-2, lane 2; Col-0, lane 1) using *ARR18*-specific primers. CAB3 (At1G29910) expression served as control. The cycle numbers are indicated. (B) Semi-quantitative RT-PCR analysis of the steady-state level of bZIP63 transcripts. The cDNA was derived from total RNA extracted from 2 week old *bzip63ko* and wild type (Ws) seedlings. PCR was performed with bZIP63 specific primers (see Material and Methods). ACTIN2 specific primers were used as control for equal total RNA amount. (C) DNA gel-blot analysis using the total genomic DNA of wild type (Col-0 and Ws) and a number of *bzip63ko* plants (lanes 1-18). Isolated DNA was digested with *BamHI* and blotted onto positively charged nylon membrane. The detection of T-DNA insertion has been performed as described in Material and Methods. M, size marker. (D) The bZIP63 mRNA levels examined by quantitative RT-PCR in wild type (Col-0), bZIP63-HAox (homozygous line with *35S:HA:bZIP63* insertion), bZIP63ox (homozygous line with *35S:bZIP63* insertion) and bZIP63-GFPox (homozygous line with *35S:bZIP63:GFP* insertion) plants and indicated as relative expression levels. The relative level of *bZIP63* expression was normalized to the level of *Ubi10* and *EF-1* genes. Results are means from three independent experiments and error bars represent SD of expression changes.

	Construct	Primer Sequences
1	pENTR-TOPO-ARR18	5’-CACCATGGAGTTTGAAGCACT-3’ 5’-AGGTGGAGGAAATGAATCAAAG-3‘
2.	pDON207-ARR18RD	5’GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGTTTGGAAGCACTGAAGA-3’ 5’GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATTAGAGCAGTTTTGCGTAAGA-3’
3.	pENTR-TOPO-bZIP9	5’- CACCATGGATAATCACACAGCTAAAGAC -3’ 5’- AGGCCAGATGTCTGAGACGCAGCT-3’
4.	pENTR-TOPO-ARR18^{D70E}	5'TTTGATCTGGTGATAAGCGAAGTAGAGATGCCAGACACG 3' 5'CGTGTCTGGCATCTCTACTTCGCTTATCACCAGATCAAA 3'
5.	pENTR-TOPO-ARR18^{D70N}	5'TTTGATCTGGTGATAAGCAATGTAGAGATGCCAGACACG 3' 5'CGTGTCTGGCATCTCTACATTGCTTATCACCAGATCAAA 3'
6.	pENTR-TOPO-ARR18^{D75E}	5'AGCGATGTAGAGATGCCAGAGAAGCTTACGGATGGTTTT 3' 5'CAACTTAAAACCATCCGTCTCTGGCATCTCTACATCGCT 3'
7.	pENTR-TOPO-ARR18^{D75N}	5'AGCGATGTAGAGATGCCAAACACGGATGGTTTTAAGTTG 3' 5'CAACTTAAAACCATCCGTGTTTGGCATCTCTACATCGCT 3'
8.	pENTR-TOPO-bZIP10	5’-CACCATGAACAGTATCTTCTCCATTGACGA-3’ 5’ -GTCCACGCATTTTTTCGGCCATGC-3’
9.	pENTR-TOPO-bZIP25	5’-CACCATGCACATCGTCTTCTCTGTC-3’ 5’-ATGCTTGTGATTCCAATGGGGTAGG-3’
10.	pDONR201-bZIP63	5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAAAAGTTTTCTCCGACGAAG-3’ 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTGATCCCCAACGCTTCGAATAC-3’
11.	pENTR-TOPO-LSD1	5’- CACCATGCAGGACCAGCTGGTGTGTC-3’ 5’- CTTTTTGTCAGTTGTCACTC-3’
12.	pENTR-TOPO-NLS-LSD1	5’CACCATGAGCGAGCCTCCAAAAAAGAAGAGAAAGGTTGAACAGGACCAGCTGGTGTG -3’ 5’- CTTTTTGTCAGTcGTCACTCCAACAACAACATTG -3’
13.	pENTR-TOPO-bZIP63gen	5’-CACCTTGTAGGACAGTGATTTTTCCG-3’ 5’ –CTGATCCCCAACGCTTCGAATACGTTTC-3’

Table S1. Primer sequences used for cloning

	Gene	Primer Sequence	Product size (nt)	*Probe #
	B-type ARRs			
1	ARR1	TTGAAGAAACCGCGTGTCGTCT CCTTCTCAACGCCGAGCTGATTAA	83	
2.	ARR2	GGAAGAAGCGTAACGAGTGG TGTCACCGCCAGTATCTTCA	69	#65
3.	ARR10	TCAGAAATTCCGCGTTGCTCTGAA TGTGAGTCAATAGCCGCCCTGTTA	78	
4.	ARR11	AAGCTCCGTGTTGCACTCCCTTCA TAATGATGTCGGTGGACGGCGAAA	60	
5.	ARR12	TGATTAGCCACACCACTGATCCTC GGCCAGTCATCTTCAGAAATTCCG	60	
6.	ARR13	TTCAATCCGATGACAACAAGA TGTTCACGAAGGTCCAGTCA	60	#143
7.	ARR14	TCCTGGAAACTCGAAGAAGTCACG GAATCCGCTTTGGTACAGCTTTGT	110	
9.	ARR18	TTCATCGATAAATCTCCAAGGAA TTCTGGTGATGCCCCTGT	63	#65
10.	ARR19	CCGAAGAGCTTCACCAAAAA CTTGCAAGCATTCGACAAGT	81	#135
11.	ARR20	TCTTCTTGAGGCTCCTGAGGAGTG GCCAGTCATCTCCAGAAGTACCGT	68	
12.	ARR21	AGGTCCAGTCACCATCGTCTTCAT CAAGCATCAACAAGGAGAAGCGGA	98	
	A-type ARRs			
1.	ARR3	ACGAGAGACGTTAAAGTCGC CTAAGCTAATCCGGGACTCC	Hejátko et., 2009	
2.	ARR4	CCAGAGACGGTGGTGTTTCT GATTCAATTCCTCCGATACCAC	73	#136
3.	ARR5	TCAGAGAACATCTTGCCTCGT ATTTCACAGGCTTCAATAAGAAATC	76	#17
4.	ARR6	GAACATTTTGCCTCGTATTGATAG CGAGAGTTTTACCGGCTTCA	76	#41
5.	ARR7	TCCTGAAAGTCCTGGCATTGAGTA AAGTGACGACTGTAGAGAGTGGAA	135	
6.	ARR8	TACCAAGTTGAAACCTCATA GACCGAGGTTGTGATATCAT	Hejátko et., 2009	
7.	ARR9	TGGAGTCCCCACTGCAGTAG GACAGCGGTTGCGATACCGT	Hejátko et., 2009	
8.	ARR15	GAGAACATACAACCTCGTATAGAACAA GCTAATTTCACCGGTTTTAGCA	77	#68
9.	ARR16	ATCACCGATTACTGTATGCC GCTTCTGCAGTTCATGAGAT	Hejátko et., 2009	
10.	ARR17	ATTCCTACCCGCATCAACAA GATAGTTTCAGTGGCTTCTGCAT	71	#99
	Others			
1.	bZIP63	TCAGAACAAGCCTCTCTTGCT CACCAGAGAGCTCAGATCCA	82	#136
2.	RD29B	GAGGAGGATCGGATTATCTCAGTGGTGTAT AGTCTTCTTCGCGTCCTTGTCTTGATTTCT	Wohlbach et al., 2008	
3.	UBI10	TCACCGGAAAGACCATCACT CGGTGGGATACCCTCTTTG	92	#10; #39
4.	EF-1-alpha	GTAACAAGATGGATGCCACCACCCC TCCCTCGAATCCAGAGATTGGCACA	140	

*All probes are from Universal Probe Library Single Probes (Roche)
Table S2. Primer sequences used for qRT-PCR.

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7. Appendix

7.1. Curriculum Vitae

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1995 - 1998	B.Sc. Microbiology
1998 - 2000	M.Sc. Microbiology
2001 - 2002	Post M.Sc course in Plant Genetic Engineering
2002 - 2003	Worked as a Junior Research Fellow (JRF) in Spic Science Foundation, Chennai, India.
2004 - 2006	Worked as a Senior research Fellow (SRF) in Advance Centre for Plant Virology, Indian Agricultural Research Institute, New Delhi, India.
Since Oct 2006	PhD studies at the Center for Plant Molecular biology (ZMBP) University of Tübingen, Germany. Supported by the DAAD PhD scholarship and DFG.

Tübingen, 15.10.2011

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