Tomato Receptors of the Bacterial Cold Shock Protein and the Plant Peptide Signal Systemin

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1 Summary

Plants have large families of plasma membrane localized receptors that can sense extracellular signals and initiate appropriate cytoplasmic responses. The csp22 peptide derived from bacterial cold shock protein (CSP) has been known as an immune elicitor for more than a decade, but the corresponding receptor has not been identified. Discovered more than a quarter-century ago as the first plant peptide hormone, systemin was shown to be critical for systemic wound response and anti-herbivore defense in tomato. The receptor for this peptide hormone also remains elusive, since the previously proposed receptor SR160 is a tomato homolog of the brassinosteroid receptor BRI1 and its role as systemin receptor could not be corroborated in later work.

Our work started with the observation that the wild tomato *Solanum pennellii*, in contrast to the cultivated tomato *Solanum lycopersicum*, lacks responsiveness to csp22 and systemin, indicating natural variation in perception of both peptides. By making use of well-defined introgression lines of these two species, we mapped the genes responsible for csp22 and systemin sensing to a common genomic region on chromosome 3. This region contains around two dozen genes encoding potential cell surface receptors. Functional analysis of these individual receptor candidate genes expressed in leaves of young *N. benthamiana* plants revealed one leucine-rich repeat-receptor kinase (LRR-RK) that strongly enhanced responsiveness to csp22, which we named cold shock protein receptor (CORE), and two other closely related LRR-RKs that conferred sensitivity to systemin, which we named systemin receptor 1 and 2 (SYR1, SYR2). CORE and SYR1 showed high affinity and specificity for their respective ligands in receptor binding assays. They also proved functional when heterologously expressed in *Arabidopsis* cells. Furthermore, ectopic expression of CORE can confer *Arabidopsis* increased resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Presence of SYR1, while not decisive for local and systemic wound responses as previously reported, is important for defense against insect herbivory in tomato plants.

2 ZUSAMMENFASSUNG

Pflanzen besitzen große Familien von membranständigen Rezeptoren, die extrazelluläre Signale erkennen und spezifische cytoplasmatische Zellantworten einleiten. Ein schon seit Jahrzehnten bekanntes Molekül, das eine Immunantwort in Pflanzen auslöst ist das bakterielle „cold-shock Protein“, bzw. dessen Peptidepitop csp22. Der zugehörige csp22-erkennende Rezeptor war bislang unbekannt. Das erste Peptidhormon „Systemin“ wurde bereits vor über 25 Jahren in Tomatenpflanzen entdeckt und ist an der systemischen Wundreaktion und bei der Verteidigung gegen Herbivoren beteiligt. Der zuerst identifizierte Systeminrezeptor SR160, ein Brassinosteroid Rezeptor Homolog (BRI1) der Tomatenpflanze, stellte sich jedoch als falsch heraus; es konnte nicht gezeigt werden, dass dieser Systemin spezifisch erkennt.

Die hier beschriebene Arbeit startete mit der Beobachtung, dass *Solanum pennellii*, im Gegensatz zur kultivierten Tomate *Solanum lycopersicum*, nicht auf Behandlungen mit csp22 oder Systemin reagiert, was auf eine natürliche Variation deren Perzeption hinweist. Mit Hilfe eines genetischen Screens von definierten Introgressionslinien der beiden Tomatenspezies gelang es uns, einen Abschnitt auf Chromosom 3 der Tomate zu identifizieren, auf dem um die 20 mögliche Kandidatengene für Rezeptoren lokalisiert sind. Nach Klonierung und heterologer Expression dieser Rezeptorkandidatengene in jungen Blättern von *Nicotiana benthamiana*, wurden Blattproben mit den Peptiden behandelt und hinsichtlich der Produktion des Phytohormons Ethylen getestet. So konnten eine Leucin-reiche Rezeptorkinase (LRR-RK) für csp22 („cold-shock protein receptor“, CORE), sowie zwei LRR-RKs als Rezeptoren für Systemin (Systeminrezeptor, SYR1 und SYR2) identifiziert werden. CORE und SYR1 sind beide hochaffine und spezifische Rezeptoren für ihre jeweiligen Peptidliganden csp22 bzw. Systemin. Beide Rezeptoren funktionieren auch in anderen pflanzlichen Systemen wie z.B. in der Modellpflanze *Arabidopsis*. CORE erhöht nach Expression in *A. thaliana* die Resistenz gegenüber dem bakteriellen Pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Für SYR1 konnte eine Beteiligung an der Verteidigung gegen Herbivoren in Tomatenpflanzen bestätigt werden, während die Funktion von SYR1 an der lokalen und systemischen Wundantwort nicht ausschlaggebend ist.

3 Introduction

3.1 General introduction

As multicellular and sessile organisms, higher plants must respond to different internal and external signals to orchestrate growth, development and interaction with the ever-changing environment. Some of these signals are sensed by histidine kinases[1]. Many other signals are sensed by plasma membrane-localized receptor kinases (RKs, also called receptor like kinases) [1,2]. Higher plants have evolved large arrays of RKs to fulfil various cellular functions. For example, the dicot plants *Arabidopsis* and tomato both have at least 600 RKs[3,4], while the monocot plant rice has over a thousand RKs[5]. All of these RKs comprise an extracellular domain, a single pass transmembrane domain and an intracellular kinase domain (Figure 3.1). A group of similar proteins, termed receptor like proteins (RLPs), are also involved in signal perception. Unlike RKs, RLPs lack the kinase domain (Figure 3.1)[2]. So far, relatively few of these cell surface receptors have been linked to specific biological functions, and an ever smaller number of them have been paired with their cognate ligands.

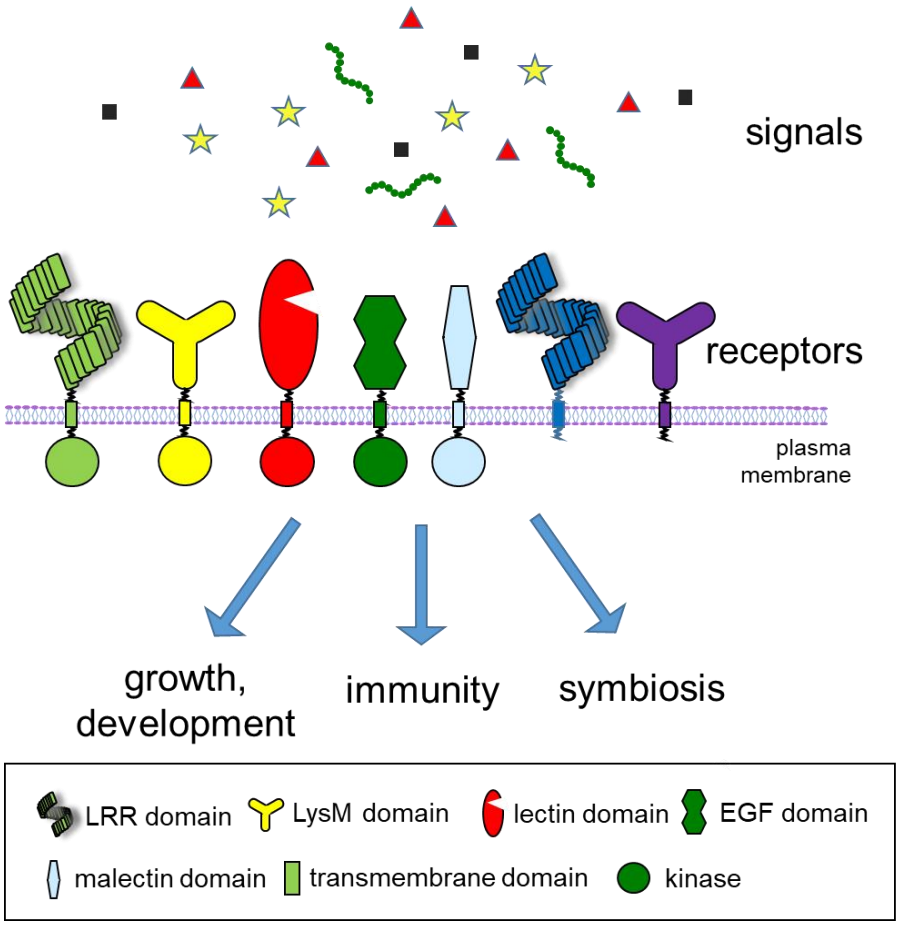

Figure 3.1 Schematic model of signal perception by plant cell surface receptors. Extracellular domains determine the ligand binding specificity whereas kinase domain is important for triggering intracellular signal output.

3.2 Biological functions of cell surface receptors

A number of RKs and RLPs play crucial roles in plant growth and development. Brassinosteroid insensitive 1 (BRI1), a leucine-rich-repeat receptor kinases (LRR-RK), perceives the phytohormone brassinolide (BL) and influences multiple developmental processes, such as cell elongation[6-8]. BL signaling involves two other LRR-RKs, Somatic Embryogenesis Receptor Kinase1 (SERK1) and BRI1-associated receptor kinase 1 (BAK1), two members of the SERK family, both of which act as co-receptors[9-13]. Some RKs and RLPs shape development through mediating plant peptide signaling[14,15]. The Phytosulfokine Receptor1/2 (PSKR1/2)[16,17], Plant peptide containing Sulfated tYrosine 1 (PSY1) Receptor (PSY1R)[18] and FERONIA[19] are also involved in regulating cell expansion, in a way through peptide signal sensing. A group of proteins including CLAVATA1 (CLV1), CLAVATA2 (CLV2), CORYNE, Receptor-like Protein Kinase 2 (RPK2), Arabidopsis CRinkly 4 (ACR4), Barely Any Meristem 1-3 (BAM1-3) and Root Meristem Growth Factor Receptor (RGFR) regulate meristem maintenance in shoots and roots by sensing the peptide CLAVATA3 (CLV3), other CLAVATA3/Embryo surrounding region-related (CLE) Like peptides or Root meristem Growth Factor(RGF) peptides[20-28]. In addition, root vascular development[29,30], Casparian strip formation[31,32], floral abscission[33,34], stomatal patterning[35,36] and plant fertilization[37-39] are also in part regulated by cell surface receptors.

Cell surface receptors also involved in the recognition of microbes or other organisms. Many RKs and RLPs can perceive conserved microbe-associated molecule patterns (MAMPs, also called pathogen-associated molecular patterns, PAMPs), host-derived damage-associated molecular patterns (DAMPs) or molecular patterns derived from other invaders. Perception of such molecular patterns at the cell surfaces triggers so called pattern triggered immunity (PTI), and receptors involved in recognition of these patterns are collectively called pattern recognition receptors (PRRs).[40]. Despite differences in kinetics and amplitude, activated PRRs induce stereotypical responses that are shared among this type of receptors. The events associated with ligand dependent activation of PRRs include formation of receptor complexes, activation of mitogen-activated protein kinases (MAPKs), Ca^{2+} influx, alteration of other ion fluxes across the plasma membrane, burst of reactive oxygen species (ROS), biosynthesis of ethylene and expression of numerous defense related genes (Figure 3.2)[40]. Associated with these responses, activation of PRRs leads to reinforcement of barriers

against invaders through remodeling of the cytoskeletal[41], callose deposition[42], closure of stomata[43] and closure of plasmodesmata[44]. Production of jasmonic acid, salicylic acid and phytoalexins is also induced to fend off further invasions[45-47].

Many plants have an array of PRRs to recognize different components of pathogenic microorganisms and trigger PTI. For example, the model plant *Arabidopsis* has evolved various PRRs to detect bacterial pathogens through recognition of flagellin[48-50], elongation factor Tu (EF-Tu)[51,52], peptidoglycans (PGNs)[53,54] and lipopolysaccharides (LPS)[40,55]. Some plants even produce multiple PRRs to sense the same protein via different epitopes, exemplified by the two tomato LRR-RKs flagellin sensing 2 (FLS2) and FLS3 which detect two distinct epitopes of bacterial flagellin[56,57].

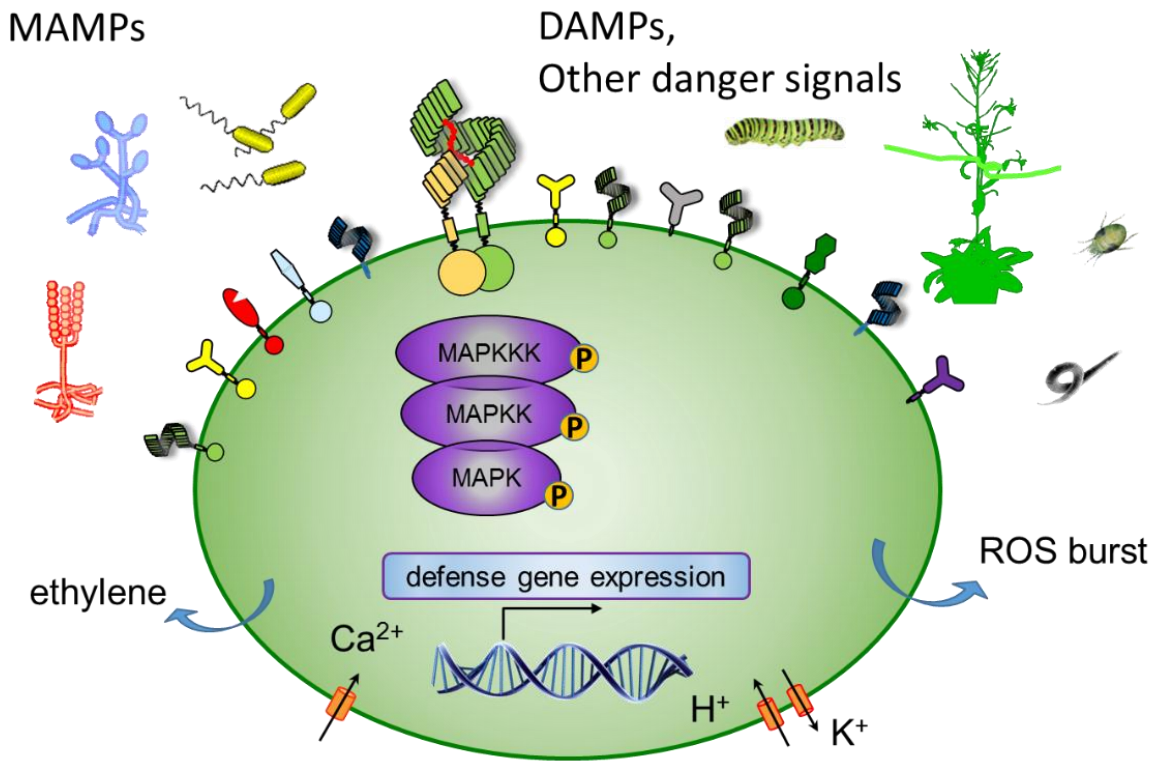

Figure 3.2 Early immune responses upon pattern recognition. Plant PRRs can sense MAMPs, DAMPs and danger signals derived from non-microbial enemies and trigger immunity. Some of the immune responses appear fast after pattern recognition, varying from a few seconds to some hours.

Recognition of *N*-acetyl-D-glucosamine (NAG) containing signals by Lysin-motif (LysM) containing receptors allows plants to detect fungal pathogens via chitin fragments, but this type of receptor is also used to establish connections with symbiotic microbes. In *Lotus japonicus*, NAG containing nodulation (Nod) factors are sensed by a receptor

complex containing the LysM-RKs Nodulation Factor Receptor 1/5 (NFR1/5)[58-61]. In *Medicago truncatula*, this task is performed by the LysM-RK complex containing Nod Factor Perception (NFP) and LysM domain-containing receptor-like Kinase 3 (LYK3)[62,63]. Additionally, NFR1/5 and NFP/LYK3 are also involved in sensing of mycorrhizal lipochito- oligosaccharides, which are important for plant-arbuscular mycorrhizal fungi interaction.

3.3 Known ligand-cell surface receptor pairs

The extracellular domains of cell surface receptors differ drastically in architecture and size. This domain is crucial for ligand binding and, consequently, for ligand specificity of the receptor. In complex with their respective ligands, these domains also determine the interaction with the corresponding co-receptors[2], leading to different downstream signaling events and, finally, different physiological responses. Among all the cell surface receptors, LRR type of receptors form the biggest group and have been implicated with a wide range of functions.

LRR-RKs/RLPs are well-known for recognizing proteinaceous ligands[2], many of which are endogenous peptides that are synthesized and secreted by the plant cells. Based on structure, these peptides can be divided into two groups, posttranslationally modified small peptides and cysteine-rich peptides[14].

The binding specificity and affinity of posttranslationally modified small peptides are altered by tyrosine sulfation, proline hydroxylation and hydroxyproline arabinosylation[14]. CLV3, the ligand of the LRR-RK CLV1, is a hydroxyproline O-arabinosylated 13-amino-acid (aa) glycopeptide[64]. The Tracheary element Differentiation Inhibitory Factor (TDIF) peptide, the ligand for TDIF Receptor (TDR)/Phloem intercalated with XYlem (PXY), also has hydroxylated proline residues[29,30]. Inflorescence Deficient in Abscission (IDA), another peptide with hydroxylated proline, is recognized by two LRR-RKs HAESA and HAESA-Like 2[33,34]. *Arabidopsis* root derived C-terminally Encoded Peptide 1 (CEP1), one more peptide with proline hydroxylation, is perceived by the shoot localized LRR-RKs CEPR1/2 to mediate nitrogen demand signaling[65]. Phytosulfokine (PSK), a 5-aa sulfated peptide, is recognized by two LRR-RKs PSKR1/2[16,17]. PSY1, an 18-aa tyrosine-sulfated glycopeptide, is recognized by the LRR-RK PSY1R. Two other groups of tyrosine-sulfated glycopeptides, RGFs and Casparian Strip Integrity Factors (CIFs) are

recognized by the LRR-RKs RGFRs[66-69] and SCHENGEN3 (SGN3)/ GASSHO1/2(GSO1/2)[31,32], respectively.

Cysteine-rich peptides can be recognized by LRR-RKs as well as receptors with other ectodomains. The Epidermal Patterning Factor (EPF) peptides are recognized by two LRR-RKs ERECTA (ER) and ERECTA-Like 1 (ERL1) to determine stomatal patterning[35,36]. The LURE peptides[70] are sensed by a receptor complex including 3 LRR-RKs Male DIScoverer1 (MDIS1), MDIS1-Interacting receptor like Kinase1 (MIK1) and MIK2[39]. Whereas the Rapid ALkalinization Factors (RALFs) are ligands for receptors of the *Catharanthus roseus* RLK1-Like (CrRLK1L) family which have malectin containing ectodomains[19,37,38,71]. RALF1 and RALF23, are the ligands for FERONIA[19,71]. RALF4 and RALF19, are recognized by ANXUR1/2 (ANX1/2), paralogs of FERONIA[37,38]. Additionally, RALF4 and RALF19 can be sensed by two other CrRLK1Ls, Buddha's Paper Seal 1 (BUPS1) and BUPS2[37]. Intriguingly, the spatially differentially expressed BUPS1/2 and ANX1/2 bind RALF4/19 with similar affinity, demonstrating the complexity of peptide signaling in plant life, and in this case, plant fertilization[37,38].

LRR-RKs/RLPs are also well characterized for perceiving proteinaceous molecular patterns. One model peptide ligand-receptor pair is the flg22 peptide (epitope of bacterial flagellin) and its corresponding receptor FLS2[72,73]. Flg22 represents the most conserved N-terminal part of bacterial flagellin and acts as potent elicitor in vast range of seed plants, including *Arabidopsis*, tomato, tobacco and grapes[48,74]. Recognition of flg22 by FLS2 leads to rapid heterodimerization with BAK1, with flg22 acting as a molecular glue between the LRR ectodomains of FLS2 and BAK1[73,75]. This flg22 dependent complex formation is crucial for the immune signaling cascade[75]. Other peptide-PRR pairs include the elf18 peptide (epitope of bacterial EF-Tu) recognized by the LRR-RK EF-Tu Receptor (EFR)[52], the flgII-28 peptide (2^{nd} epitope of bacterial flagellin) recognized by receptor FLS3[57], the xup25 peptide recognized by the LRR-RK XPS1[76], and the peptide nlp20 (epitope of the microbial Necrosis and ethylene-inducing peptide 1-Like Proteins, NLPs) recognized by the LRR-RLP RLP23[77]. The small Plant elicitor peptides (Peps) and PAMP-Induced secreted Peptides (PIPs) are also recognized by receptors with LRR domains, namely Peps recognized by Pep Receptor 1/2 (PEPR1/2) [78-80] and PIP1/2 recognized by RLK7[81]. A few small proteins have been reported to be ligands for LRR containing receptors. In tomato, the LRR-

RLP Eix2 can bind the ethylene-inducing xylanase (EIX) from *Trichoderma* and transmit EIX triggered signaling[82]. In *Arabidopsis*, Responsiveness to Botrytis Polygalacturonases1 (RBPG1), a LRR-RLP, can recognize fungal endopolygalacturonases[83].

LysM-RKs/RLPs can recognize NAG containing symbiotic signals, as exemplified by the receptor-ligand pair NFR1/5-Nod Factor and ExoPolysaccharide Receptor 3 (EPR3)- exopolysaccharide[60,84]. In addition, they can also sense NAG containing pathogenic signals, including chitin[85-88] and PGN[53,54]. The first chitin receptor, named chitin-elicitor binding protein (CEBiP), was identified in rice. CEBiP is a LysM-RLP with high binding affinity to chitin oligosaccharides[85]. Later, the LysM-RK Chitin Elicitor Receptor Kinase 1 (CERK1), which has no chitin binding activity, was also shown indispensable for full chitin elicited signaling in rice[86-88]. In Arabidopsis, *At*CERK1 was first proposed to be the only chitin receptor, and its homo-dimerization upon chitin binding was demonstrated to be crucial for chitin-induced immunity[89,90]. Later, AtLYK5, another LysM-RK, was found to be another chitin receptor with higher chitin binding affinity[91]. Interestingly, *At*LYK5 associates with *At*CERK1 in presence of chitin[91]. PGN was recognized by two LYM1 and LYM3, two LysM-RLPs in Arabidopsis[54] and OsLYP4 and OsLYP6, two LysM-RLPs in rice[92].

Receptors with other ectodomains have also been implicated in ligand perception. The L-lectin receptor kinase named Does not Respond to Nucleotides 1 (DORN1) can bind extracellular ATP (eATP) and mediate eATP-dependent response. The Wall-Associated Kinase 1(WAK1) which has an Epidermal Growth Factor (EGF) ectodomain was shown to recognize oligogalacturonides[93].

3.4 Orphan ligands with unknown receptors

Despite the exciting discovery of some new ligand-receptor pairs, many more receptors remain elusive regarding to their function and cognate ligands. On the other hand, an number of specific molecules have been identified that actively trigger physiological responses at very low concentrations[40,94], indicating they act as ligands for yet-unknown receptors.

Sytemin, an 18-aa peptide purified from tomato, was the first plant peptide reported with a signaling function[95]. Derived from the C-terminus of its precursor prosystemin[96], systemin was shown to be important for systemic wound signaling and resistance

against insect herbivory[95,97]. Similar to MAMP perception, perception of systemin at subnanomolar concentration induces rapid changes in ion fluxes across the plasma membrane and ethylene biosynthesis [98]. The long search for its receptor culminated in the identification of Systemin Receptor 160 (SR160), which subsequently turned out to be a tomato ortholog of the BL receptor BRI1[99,100]. The role of SR160 as systemin receptor was called into question because *cu3*, a tomato *bri1/sr160* mutant, can still respond to systemin in the same way as the wild type tomato[101,102]. Although the discovery of systemin has inspired the identification of many other plant peptide signals and their cognate receptors, systemin still remains an orphan ligand.

In contrast to systemin, many other plant peptide signals identified later are secreted peptides. Hydroxyproline-rich systemins (HypSys), a glycopeptide discovered in tobacco, tomato and sweet potato plants, has also been implicated with an anti-herbivore function[103-105]. Its receptor remains to be identified as well. Some members of the CLE family peptides have been shown to regulate different aspect of plant development[14], yet identification of the corresponding receptors is missing, which is also the case for most members of the RALF family peptides[106,107]. A few more other cysteine-rich peptides including Plant DeFensins (PDFs) are reported to mediate immunity against microbes or development through yet-unknown receptors[107]. Furthermore, as more than a thousand signaling peptides are predicted to be encoded by *Arabidopsis* genome[14], it is foreseeable that many more peptides will be found and their receptors will need to be identified to understand their function.

In addition to plant peptides, many signals derived from microbes or other organisms also lack their corresponding plant receptors[94]. Among them, a few have even been identified for more than two decades. Gp8c, one of the most active glycopeptides derived from yeast invertase, can induce ethylene biosynthesis in tomato[108]. Pep-13, a 13-aa peptide identified from a fungal glycoprotein, can bind parsley plasma membrane with high affinity and trigger multiple responses[109]. Ergosterol, the main sterol of most higher fungi, can induce extracellular alkalinization of tomato cells with an EC50 of 10 pM[110]. Recently, some new molecules have been shown to have MAMP activity, like the Cellulose-Binding Elicitor Lectin (CBEL) from Oomycetes[111], CD 2-1 from bacterial flagellin[112] and EFa50 from bacterial EF-Tu[113]. None of these have yet been matched with a receptor. Bacterial cold shock proteins (CSPs) are also capable of triggering physiological response in tomato[114]. Functioning as RNA chaperons and transcription anti-terminators, CSPs can make up more than 10% total protein in

bacteria[115]. Csp22/csp15, derived from the highly conserved nucleic acid binding motif RNP-1 at the N-terminal of CSPs, can induce immunity at subnanomolar concentrations[114]. Despite the fact that CSPs were identified as MAMPs more than a decade ago, the receptor remained elusive before the start of this work.

Herbivore-associated molecular patterns (HAMPs), a term analogous to MAMP, has been adopted to describe herbivore-derived elicitors found in oral secretions, saliva and oviposition fluid[116,117]. HAMPs like inceptins, caeliferins and volictins can induce synthesis of stress hormones and emission of defense related volatiles[118-120]. Although there have been a few reports demonstrating the involvement of RKs in HAMP-mediated immunity[121,122], how HAMPs are sensed by plants remains poorly understood.

3.5 Strategies to identify cell surface receptors

Numerous efforts have been made to identify the cognate plant receptors for known ligands. Taking PRRs as examples, forward genetics and reverse genetics approaches have been used in most of the successful cases. Biochemical purification has also led to the successful discovery of a few receptors[94].

3.5.1 Forward Genetics

A forward genetics approach involves the search of a mutant or variant that differs in the recognition of a given ligand. Usually, the non-responsiveness of a given mutant or variant is associated with the absence of the receptor, but not with components in the downstream signaling pathways, as controlled by presence of response triggered by other molecular patterns. By analysis of the offspring (usually the F2 generation) from genetic crosses of responsive plants with non-responsive plants, a locus or loci responsible for pattern sensing can be determined either by map-based cloning or next generation sequencing.

The Arabidopsis FLS2 was identified using an ethyl methanesulfonate (EMS)-mutagenized population of the ecotype *Landsberg erecta* for seedlings that show no growth inhibition in the presence of flg22[72]. Screening mutagenized seedlings from the *Arabidopsis* Col-0 for non-responsiveness led to the identification of DORN1 as the receptor for eATP [123] and LORE as the LPS receptor[124].

Natural variants or populations generated via breeding programs are another important source for such screenings. Recently, Cuscuta Receptor 1 (CuRe1) has been identified

as the receptor for Cuscuta factor by making use of natural variation between *S. pennellii* and *S. lycopersium* and a collection of well-defined introgression lines between these two species[125]. Another tomato PRR FLS3 was discovered exploiting the natural variation of flagellin flgII-28 epitope sensing among tomato heirloom varieties[57]. The rice XA21 receptor was identified via map-based cloning[126]. In Arabidopsis, several LRR-RLPs, including RLP1, RLP30 and RLP42, were also found to be PRRs by ecotype screening with their respective ligands[83,127,128] .

3.5.2 Reverse genetics

In Arabidopsis, several collections of RK or RLP T-DNA insertion lines have been generated, with the hope of deciphering the physiological function of these genes in immunity as well as other aspects of plant life. Screening a collection of flg22-induced LRR-RK mutant lines led to the identification of EFR[52]. Similarly, the PIPs receptor RLK7 was discovered by testing 6 XI LRR-RK mutant lines of PAMP- or infection-upregulated genes[81]. XPS1 was identified from a screen of 187 LRR-RK mutant lines[76], and the NLP receptor RLP23 was found by screening LRR-RLP T-DNA insertion lines[77].

LysM receptor mutants in Arabidopsis and rice have been evaluated for their roles in chitin and PGN sensing. This led to the findings that *At*CERK1, *At*LYK5 and *At*LYM2 are required for chitin perception[44,89,91] and that *At*LYM1 and *At*LYM3 are PGN receptors in Arabidopsis[54].

Unlike the model plant Arabidopsis, many other plants have a rather limited mutant library. This severely hinders the identification of some family- or species-specific PRRs. The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) mediated gene editing technology opens up new possibilities for plant genome engineering[129]. Its applicability has been proved in several plants[130-133]. Recently, a collection of XII LRR-RK tomato mutants has been generated using the CRISPR/Cas9-mediated mutagenesis[134], providing a new tool for uncovering PRRs in crops.

3.5.3 Biochemical approach

Biochemical purification has only been successful for identifying a few bona fide PRRs. Photoaffinity labeling of *At*Pep1 led to the purification of its binding protein PEPR1, and accompanying genetic evidence could verify PEPR1 as the *At*Pep1 receptor[79].

Similarly, *Os*CEBiP was found as a chitin binding protein by affinity labeling[135], and later proved to be important for chitin induced immune signaling in rice[85].

Recently, an interesting strategy has been proposed to use a co-receptor as molecular bait to fish out PRRs based on the ligand-dependent interaction of PRRs with the co-receptor BAK1. As proof of concept, a *N. benthamiana* LRR-RLP CSPR, was identified as the receptor for csp22[136]. However, the role of *Nb*CSPR in csp22 sensing could not be confirmed[137]. Thus the legitimacy of this approach still needs to be evaluated.

3.6 Aim of the thesis

In the past few years, significant progress has been made elucidating the identity of plant cell surface receptors and their cognate ligands. However, given the large number, redundancy, and functional complexity of these receptors, our knowledge remains very limited and more receptors are waiting to be matched with functions and/or ligands.

In tomato, bacterial cold shock protein[114], systemin[95], fungal glycopeptide (gp8c)[108], ergosterol[110], flgII-28[138] and the fungal preparation Pen[139] can all induce immunity via yet unknown receptors. By making use of natural variations of ligand sensing among different tomato species, we aim to identify the receptors for some of these ligands listed above. In addition, we try to determine the binding affinity of putative receptors with their ligands, and make clear how perception of these ligands contribute to plant immunity.

4 Results and Discussion

4.1 Natural variation of molecular pattern sensing in tomato

In the cultivated tomato *S. lycopersicum*, immunity can be triggered by elicitors including csp22[114], systemin[95], fungal glycopeptide (gp8c)[108], ergosterol[110], flgII-28[138] and the fungal preparation Pen[139]. Before the start of this work, all of these had yet to be matched with corresponding receptors. In order to identify novel immune receptors, we tested whether there is natural variation of pattern sensing in a collection of wild tomato species, using ethylene biosynthesis as a convenient output. Indeed, we observed an interesting variation among these tomato species. All the species tested responded to flgII-28, whereas they were insensitive to one or a few other elicitors used in the assay (Table 4.1.1). This indicates the none-responsiveness is due to the lack of the corresponding receptors, and not due to a generally impaired immune signaling pathway.

Among all the species tested, *S.lycopersicum* and *S.pennellii* were of particular interest. Firstly, *S. lycopersicum* was sensitive to csp22, systemin and glycopeptide, while *S. pennellii* responded to none of them. Secondly and more importantly, a collection of well-defined introgression lines (ILs) between these two species was available[140,141]. The primary collection of these introgression lines covers ~98% of *S. pennellii* genome, missing only a part of chromosome 6 which has a lethal effect[140,141]. These lines, which were instrumental for discovering the tomato CuRe1 as a receptor detecting the parasitic plant *Cuscuta*[125], offered a ready-to-use tool for mapping the locus or loci of potential receptors for csp22, systemin and gp8c.

We continued to screen this primary collection of ILs for induction of ethylene biosynthesis in response to csp22, systemin and gp8c. Individual ILs showed notable difference in ethylene induction. However, a few lines were completely insensitive to elicitor treatment, showing ethylene levels similar to the water treated controls. (Figure 4.1.1). By chance, the none-responsiveness to all three elicitors was found in the same ILs, IL3-2 and IL3-3 (Figure 4.1.1).

Tomato Species	Ethylene Biosynthesis					
	csp22	systemin	gp8c	ergosterol	flgII-28	Pen
Solanum lycopersicum	**++**	**++**	**++**	**++**	**++**	**++**
Solanum sitiens	**++**	**++**	**-**	**+**	**+**	**++**
Solanum peruvianum	**+**	**-**	**++**	**+**	**++**	**++**
Solanum cheesmaniae	**+**	**++**	**-**	**+**	**++**	**++**
Solanum habrochaites f. glabratum	**+**	**+**	**-**	**-**	**++**	**++**
Solanum lycopersicum var. cerasiforme	**+**	**++**	**++**	**-**	**++**	**++**
Solanum habrochaites	**+**	**+**	**++**	**+**	**++**	**++**
Solanum ochranthum	**-**	**+**	**-**	**-**	**++**	**+**
Solanum corneliomuelleri	**-**	**++**	**+**	**-**	**n.d.**	**-**
Solanum pennellii	**-**	**-**	**-**	**+**	**++**	**++**

Table 4.1.1 Induction of ethylene biosynthesis in response to 6 elicitors in 10 tomato species. Ethylene biosynthesis is shown as fold induction over water control, with "++" meaning ≥ 3 folds, "+" meaning < 3 fold and ≥ 1.5 fold and "-" meaning no induction. n.d., not determined. Ethylene biosynthesis in leaf pieces were measured 3h after treatment with with 100 nM csp22, 100 nM systemin, 10 µl/ml gp8c preparation, 10 µM ergosterol, 1µM flgII-28 or 90 µg/ml Pen. Crude screening was done once with 2 technical replicates. Data shown for *S. lycopersicum* and *S.pennellii* was representative of at least three independent experiments.

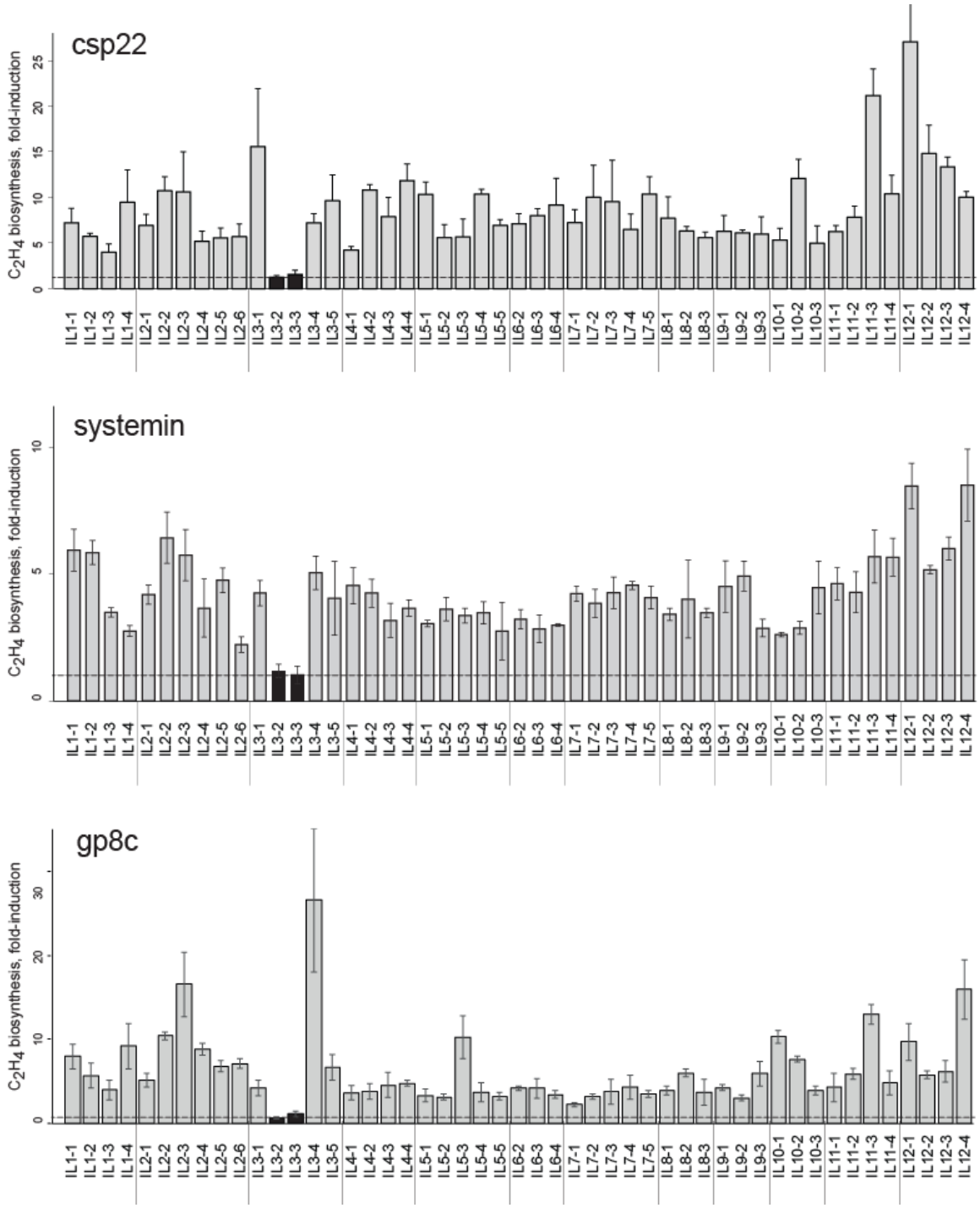

Figure 4.1.1 Induction of ethylene biosynthesis triggered by csp22, systemin or gp8c in introgression lines derived from a cross between cultivated (*S. lycopersicum*) and wild tomato (*S. pennellii*)[140,141]. Ethylene biosynthesis (fold-induction over H_2O controls) in leaf pieces was measured 3 h after treatment with 100 nM csp22, 100 nM systemin or 10 µl/ml gp8c preparation. Bars and error bars show fold-induction as mean ± S.D. of n = 3 replicates.

Both of these ILs are non-contiguous ILs, and each carries 2 individual introgression bins. The overlapping introgression bin of these two ILs is ~39 Mbp, containing ~1200 annotated genes (Figure 4.1.2), around 2 dozen of which encode putative receptors

(Table 4.1.2). Based on the knowledge that most of the identified PRRs with proteinaceous ligands contain a large extracellular LRR domain, we focused on those with more than 10 LRRs as our primary receptor candidates.

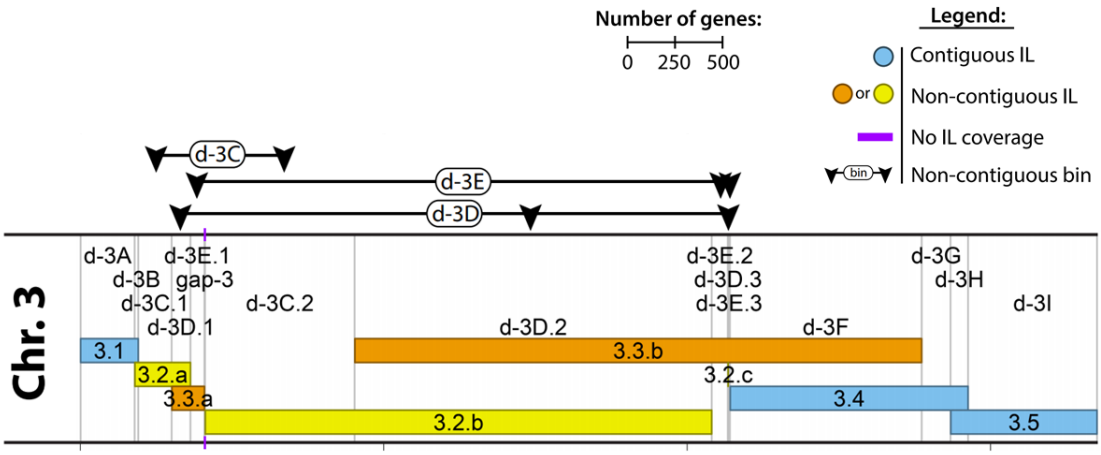

Figure 4.1.2 Map of *S.pennellii* introgression line chromosome 3. This figure is modified from Supplementary Figure 4 of Chitwood *et.al*., 2013[141].

In chapter 4.2, we describe in detail the identification of the tomato protein CORE as receptor for csp22 and its role in immunity against bacterial pathogens. In chapter 4.3, we describe in detail the identification of SYR1 as receptor for systemin and its role in wound signaling and resistance against insect herbivory in tomato plants. All of the receptor candidates mentioned in chapter 4.2 and 4.3 were also tested for response to gp8c, and none of them was shown to be responsible for gp8c sensing. Other candidates need to be cloned and analyzed to further identify the receptor for fungal glycopeptide (gp8c).

Gene	Protein type	Polypeptide length	Notes
Solyc03g059020	LRR-RK	711	
Solyc03g059490	LRR-RK	1125	Tested
Solyc03g062660	LRR-RK	873	Tested
Solyc03g063650	Lectin-RK	759	
Solyc03g064010	LRR-RK	628	
Solyc03g078360	Lectin-RK	792	
Solyc03g078370	Lectin-RK	789	
Solyc03g078520	LRR-RK	607	
Solyc03g080060	Lectin-RK	641	
Solyc03g082450 *SYR2*	LRR-RK	1115	Tested
Solyc03g082470 *SYR1*	LRR-RK	1115	Tested
Solyc03g082780	LRR-RLP	781	
Solyc03g083510	LRR-RLP	386	
Solyc03g093330	LRR-RK	982	Tested
Solyc03g093380	LRR-RK	865	Tested
Solyc03g095490	LRR-RK	635	
Solyc03g096190 *CORE*	LRR-RK	1042	Tested
Solyc03g098150	RK with unknown ectodomain	1165	
Solyc03g098400	LRR-RK	1032	

Table 4.1.2 List of receptor candidates from the overlapping region of IL3-2 and IL3-3.

4.2 The pattern recognition receptor CORE of Solanaceae detects bacterial cold shock protein

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This work aimed to identify the tomato receptor for bacterial cold shock protein and evaluate its role in bacterial resistance. I contributed to the experimental design and performed all the experiments with help from co-authors. I analyzed the data and wrote the manuscript together with Prof. Georg Felix with input from co-authors.

4.2.1 Abstract

Plants and animals recognize microbial invaders by detecting microbe-associated molecular patterns (MAMPs) by cell surface receptors. Many plant species of the *Solanaceae* family detect the highly conserved nucleic acid binding motif RNP-1 of bacterial cold shock proteins, represented by the peptide csp22, as a MAMP. Here, we exploited the natural variation in csp22 perception observed between cultivated tomato (*Solanum lycopersicum*) and *Solanum pennellii*, to map and identify the Leucine-Rich Repeat (LRR) receptor kinase CORE of tomato as the specific, high-affinity receptor site for csp22. Corroborating its function as genuine receptor, heterologous expression of CORE in *Arabidopsis thaliana* conferred full sensitivity to csp22 and, importantly, it also rendered these plants more resistant to infection by the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Our study also confirms the biotechnological potential of enhancing plant immunity by interspecies transfer of highly effective pattern recognition receptors like CORE to different plant families.

4.2.2 Introduction

Active defense against microbial pathogens depends on the detection of the infectious agents. As part of their innate immune system, plants use membrane-bound pattern recognition receptors (PRRs) to detect a variety of microbe-associated molecular patterns (MAMPs)[40]. Most of the plant PRRs identified so far belong to the multi-membered protein families of receptor-like proteins (RLPs) and receptor-like kinases (RLKs)[3,142-144]. Although the number of RLKs and RLPs with functional attributions is steadily increasing[145], the vast majority of these putative receptors remain orphan with respect to their biological functions and ligands. In turn, there is an increasing number of specific molecules that trigger responses in plant cells at nanomolar concentrations, indicating that they might act as ligands for as yet unidentified surface receptors. Matching such signals with their respective receptor proteins as ligand-receptor pairs remains a major task for plant research.

More than a decade ago we identified the highly conserved nucleic acid binding motif RNP-1 of bacterial cold shock proteins (CSPs) as a MAMP for the innate immune system of tomato (*S. lycopersicum*), tobacco (*N. tabacum*), potato (*S. tuberosum*), but not for plant species outside the *Solanales*[114] like *A. thaliana* or rice . CSPs were

named based on their characteristic hyper-accumulation in bacteria after rapid lowering of the incubation temperature by >10 °C (cold shock). However, proteins belonging to the CSP family are also constitutively expressed or are induced under stress other than cold shock[115]. Originally, it came as a surprise that plants have an immunodetection system directed against a protein which naturally resides in the cytoplasm of the bacteria. Nevertheless, the sensitivity and the specificity of the plant response to the membrane impermeable peptides csp15 and csp22, which represent the RNP-1 epitope with the MAMP activity, suggested that perception of this MAMP occurred via a specific PRR located at the surface of the plant cells.

The current study started with the observation that the wild tomato *Solanum pennellii*, in contrast to the cultivated tomato *Solanum lycopersicum*, showed no response to csp22, indicating that *S. pennellii* lacks an essential component of the pattern recognition system. *S. lycopersicum* and *S. pennellii* are closely related and crosses between these species have been used to produce a collection of recombinant inbred lines that were very comprehensively characterized for their genomic and transcriptomic properties[140,141,146]. This collection, together with the genomic information now available for *S. lycopersicum* and *S. pennellii* (*https://**solgenomics**.net/*), provided an excellent tool for mapping responsiveness to csp22 and helped subsequent identification of the receptor kinase CORE as the PRR for csp22. CORE acts as a genuine receptor for csp22 since it binds csp22 with high affinity and specificity and is sufficient to confer responsiveness to the csp22 MAMP when heterologously expressed in *A. thaliana*.

In the course of our studies we became aware of the work by Saur et al. who very recently published on the importance of the receptor-like protein *Nb*CSPR from *Nicotiana benthamiana* for perception of the csp22 peptide[136]. Since *Nb*CSPR is clearly distinct from the receptor kinase CORE described in our work we compared both types of receptors to evaluate their roles in csp22 perception.

4.2.3 Results

The wild tomato *S. pennellii* lacks responsiveness to csp22

Defense-related responses, including induction of ethylene biosynthesis and an oxidative burst, to treatment with the csp22 peptide or its shorter version csp15 have been reported for several solanaceous plants[114]. Here, we started out with the observation that leaf tissue of *Solanum pennellii*, in contrast to the closely related cultivated tomato *S. lycopersicum*, does not respond with production of ethylene when treated with csp22 (Fig. 4.2.1A). However, much like *S. lycopersicum, S. pennellii* responded to unrelated MAMPs like flg22[48], flgII-28[138] and the fungal preparation Pen1[139], indicating that *S. pennellii* only lacks the pattern recognition system specific for csp22.

In order to identify the gene(s) underlying the natural variation in csp22 perception we made use of a collection of well-defined recombinant inbred lines (ILs) from crosses between *S. lycopersicum* and *S. pennellii*[140,141,146]. We tested the 49 lines of the original Eshed-Zamir-collection, comprising introgressions covering ~98% of the *S. pennellii* genome, for responsiveness to csp22. The individual ILs exhibited considerable variation in fold-induction of ethylene biosynthesis but only the lines IL 3-2 and IL 3-3 proved insensitive to csp22 and ethylene biosynthesis remained at the level of untreated controls (Fig. 4.2.1B). The overlapping introgressions in IL 3-2 and IL 3-3[141] (Fig. 4.2.1C) cover parts on chromosome 3 that encompass ~1200 predicted genes (*https://**solgenomics**.net*), including ~30 genes coding for receptor-like kinases or receptor-like proteins. We set out to test these candidates for their possible function as csp22 receptors by transient expression in leaves of *N. benthamiana*. We observed that leaves of young *N. benthamiana* plants, at the stage suitable for Agrobacterium-mediated transient transformation[147], either showed no or only a marginal response to csp22, while leaves from older plants responded to csp22 with a clear increase in the production of ethylene (Fig. 4.2.2A). Focusing on genes predicted to code for receptors with signal peptides for export and large apoplastic domains, such as receptors with LRR-domains comprising >10 LRRs, we tested a first subset of candidate receptors by transient expression in young *N. benthamiana* plants. Out of these five candidate genes only Solyc03g096190 led to significant responsiveness to csp22 when assayed for induction of ethylene biosynthesis or the stimulation of an oxidative burst (Fig. 4.2.2B, C and D). Leaves expressing this receptor-like kinase, tentatively termed

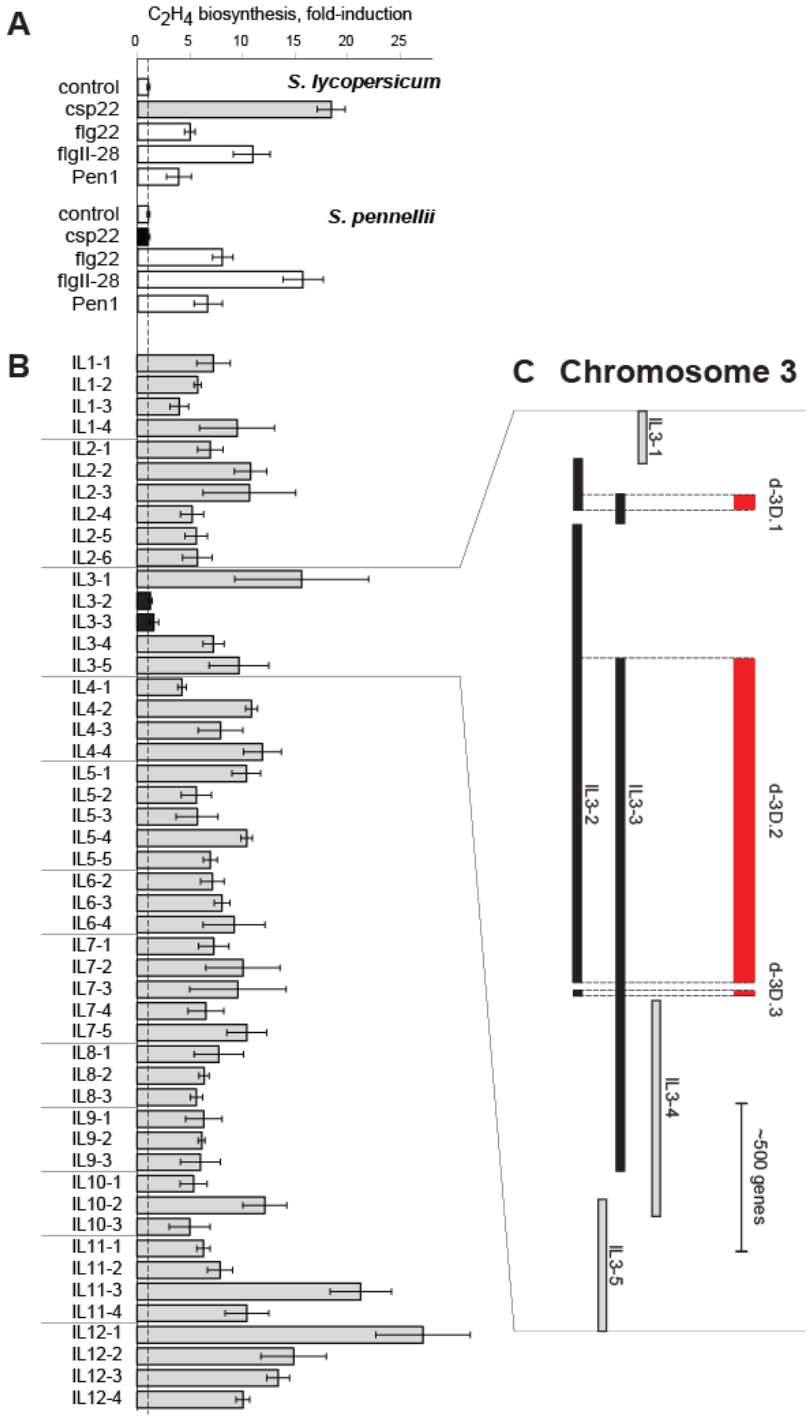


Figure 4.2.1. Csp22-dependent induction of ethylene biosynthesis in tomato (*S. lycopersicum*), *S. pennellii* and a collection of recombinant inbred lines. A) Ethylene biosynthesis in leaf slices of *S. lycopersicum* and *S. pennellii* treated for 3 h with H_2O (control), 1 µM csp22, 1 µM flg22, 1 µM flgII-28 or 90 µg/ml of the fungal preparation Pen1. B) Induction of ethylene biosynthesis (fold-induction over H_2O controls) in leaf slices of recombinant inbred lines between *S. lycopersicum* and *S. pennellii*[140] treated for 3 h with 100 nM csp22. Bars and error bars show fold-induction as mean ± S.D. of n = 3 replicates. Ethylene production in controls treated with H_2O ranged from 0.11 to 0.58 nmol C_2H_4 per g of fresh weight. C) Genetic map of the recombinant inbred lines with introgressions of the genomic parts of *S. pennellii* in chromosome 3 of *S. lycopersicum* (adapted from the Suppl. Figure 4 of Chitwood *et al.*, 2013[141]).

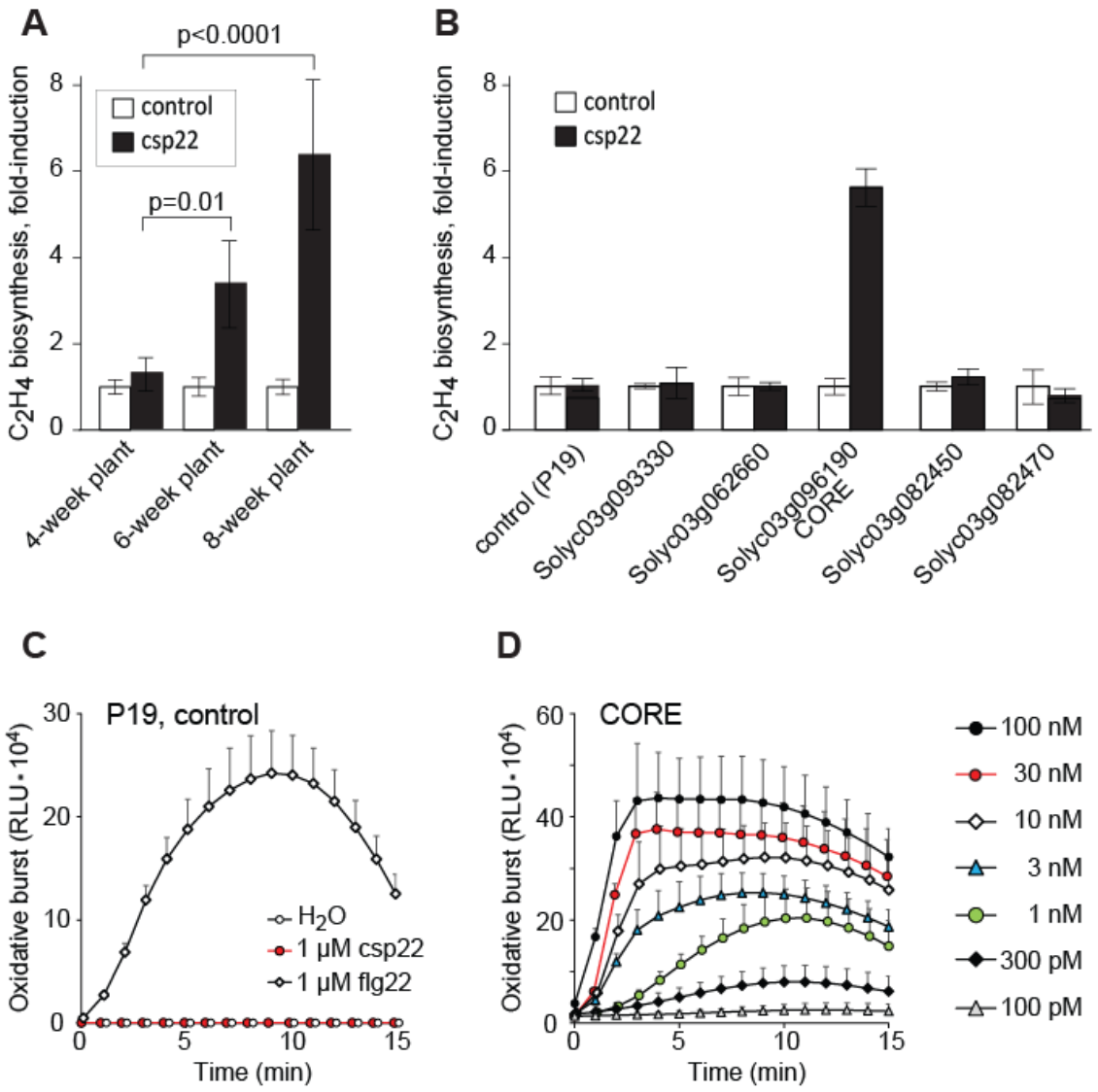


Figure 4.2.2. Csp22-dependent induction of MAMP responses in non-transformed *N. benthamiana* and in *N. benthamiana* expressing receptor candidate genes from tomato (*S. lycopersicum*). A) Response to csp22 in *N. benthamiana* depends on the plant age. Effect of 1 µM csp22 on ethylene production in leaves of non-transformed *N. benthamiana* plants of different age. B) Ethylene response to csp22 in 4-week old *N. benthamiana* plants after transient transformation with *Agrobacteria* carrying Ti plasmids with the P19 gene alone (control) or the P19 gene and a gene encoding a candidate receptor from tomato as indicated. Bars and error bars in A) and B) show fold-induction of ethylene as mean ± S.D. of n = 3 (triplicate control treatments and triplicate peptide treatments). Ethylene production in controls not treated with csp22 ranged from 0.12 to 0.28 nmol C_2H_4 per g of fresh weight. C) and D) Oxidative burst in 4-week old *N. benthamiana* leaves transformed with a plasmid coding for P19 (C) or a plasmid encoding P19 and Solyc03g096190 (CORE); D) in response to flg22 (1 µM) or csp22 at the concentration indicated. Values and error bars show means + S.D. of n = 6 replicates. Results shown are representative for n ≥ 3 independent repetitions of the experiments.

CORE for Cold shock protein Receptor, showed a significant response to csp22 down to concentrations of 0.3 nM csp22 (Fig. 4.2.2D), thus matching the sensitivity of csp22 perception in *N. tabacum* and tomato described before[114]. In complementation assays

with the *CORE* gene stably introduced into the non-responsive lines IL 3-2 and IL 3-3 we observed clear response to the csp22 peptide (Suppl. Fig. 4.2.1), indicating that loss-of-function in IL 3-2 and IL 3-3 is only due to lack of functional CORE.

CORE is a LRR-receptor kinase with structural resemblance to EFR and Xa21

CORE encodes a leucine-rich repeat receptor-like kinase with 22 LRRs, interrupted at LRR11 with a short island domain of 6 amino acids (Suppl. Fig. 4.2.2). With this architecture it strongly resembles EFR, the receptor for bacterial EF-Tu found in *Brassicaceae* like *A. thaliana*[52], and XA21, the receptor for the bacterial peptide RaxX21-sY from rice[126,148], respectively. As exemplified for the comparison of CORE with EFR (Suppl. Fig. 4.2.2), the sequence identity between these receptors is high only for the cytoplasmic kinase domain (≥50 %) and the amino acids at positions forming the solenoid CORE of the LRR stacks (≥60 %) but low for the amino acids predicted to form the surface of the apoplastic LRR domains (≤22 %). This observation predicts distinct surfaces of the LRR domains that might explain specificity of EFR for elf18, Xa21 for RaxX21-sY and CORE for csp22, respectively.

S. pennellii, although consistently lacking responsiveness to csp22, encodes a close homolog of CORE (97 % overall aa identity) on chromosome 3 as well (https://**solgenomics**.net). In comparison to *S. lycopersicum*, however, the promoter region of CORE in *S. pennellii* has a 23 bp deletion and a 3.2 kb insertion (Suppl. Fig. 4.2.3A) that might be responsible for the lack of *CORE* expression (Suppl. Fig. 4.2.3B). Indeed, when expressed in *N. benthamiana* leaves under the control of the 35S promoter *SpCORE* conferred responsiveness to csp22 in a manner similar to CORE from tomato (Suppl. Fig. 4.2.4A), suggesting that the lack of responsiveness in *S. pennellii* arises from a malfunction of the promoter.

Close homologs of CORE, with high conservation also at positions forming the surface of the LRR domain, can be found in several *Solanaceous* plants, including *S. pimpinellifolium*, potato (*S. tuberosum*), eggplant (*S. melongena*) and *N. tabacum* but not in the genomic sequences available from plants outside of this family.

Notably, the genome of *N. benthamiana*[149] also harbors a gene coding for a CORE homolog (79 % aa identity). When ectopically expressed under the control of the 35S promoter in leaves of young *N. benthamiana* plants this *NbCORE* also conferred sensitivity to csp22 with significant induction of ROS to ≥1 nM csp22 (Suppl. Fig. 4.2.4B).

This result is of particular interest with respect to the *Nb*CSPR protein that was very recently reported as an essential component of csp22 perception in *N. benthamiana*[136]. This protein, dubbed *Nb*CSPR for RECEPTOR-LIKE PROTEIN REQUIRED FOR CSP22 RESPONSIVENESS, is a receptor like protein that shares no obvious sequence relationship with CORE. However, in our hands, *NbCSPR* cloned under the 35S promoter and ectopically expressed in leaves of young *N. benthamiana* plants did not confer responsiveness to csp22, at least not in a manner comparable to that found after expression of the *CORE* genes from tomato or *N. benthamiana* (Suppl. Fig. 4.2.4).

In correlation with responsiveness to csp22 in young and older *N. benthamiana* plants, *NbCSPR* has been reported to show an age-dependent expression pattern with low expression in young and higher expression in older *N. benthamiana* plants, respectively[150]. We corroborated this pattern of expression for *NbCSPR* but we found clearly higher expression also for *NbCORE* in 6 weeks old plants compared to 4 weeks old plants (Suppl. Fig. 4.2.4E). Since ectopic expression of *NbCORE* but not *NbCSPR* conferred responsiveness to csp22, we conclude that expression of *NbCORE* is the limiting factor in these young *N. benthamiana* plants.

CORE interacts with BAK1 in a ligand-dependent manner

As part of a common molecular activation mechanism, ligand binding of LRR receptor kinases like BRI1, FLS2 and EFR triggers rapid complex formation with a second type of LRR receptor kinase such as BAK1/SERK3 or another member of the SERK family[151]. We tested whether CORE undergoes a similar complex formation by co-expressing CORE with a myc-tagged form of tomato SERK3a[152] in *N. benthamiana* leaves. SERK3a was only found to co-precipitate with CORE-GFP after treatment with csp22 or csp15 but not after treatment with the structurally unrelated peptide elf18 or the inactive peptide analog csp15-Ala10 (Fig. 4.2.3). Thus, CORE interacts with the co-receptor SERK3a in a ligand-dependent manner, providing further evidence for this protein to act as a genuine receptor of bacterial cold shock protein.

CORE binds csp22 with high affinity and specificity

CORE-GFP protein immunoadsorbed to anti-GFP beads was used in direct binding assays with labeled csp22. As a ligand label, we used a chemiluminescent acridinium ester that allows detection down to the femtomole or even attomole range of label[153]. Coupling of the acridinium ester to the N-terminus of csp22 did not affect the MAMP activity and acri-csp22 showed the same specific activity as csp22 when assayed for

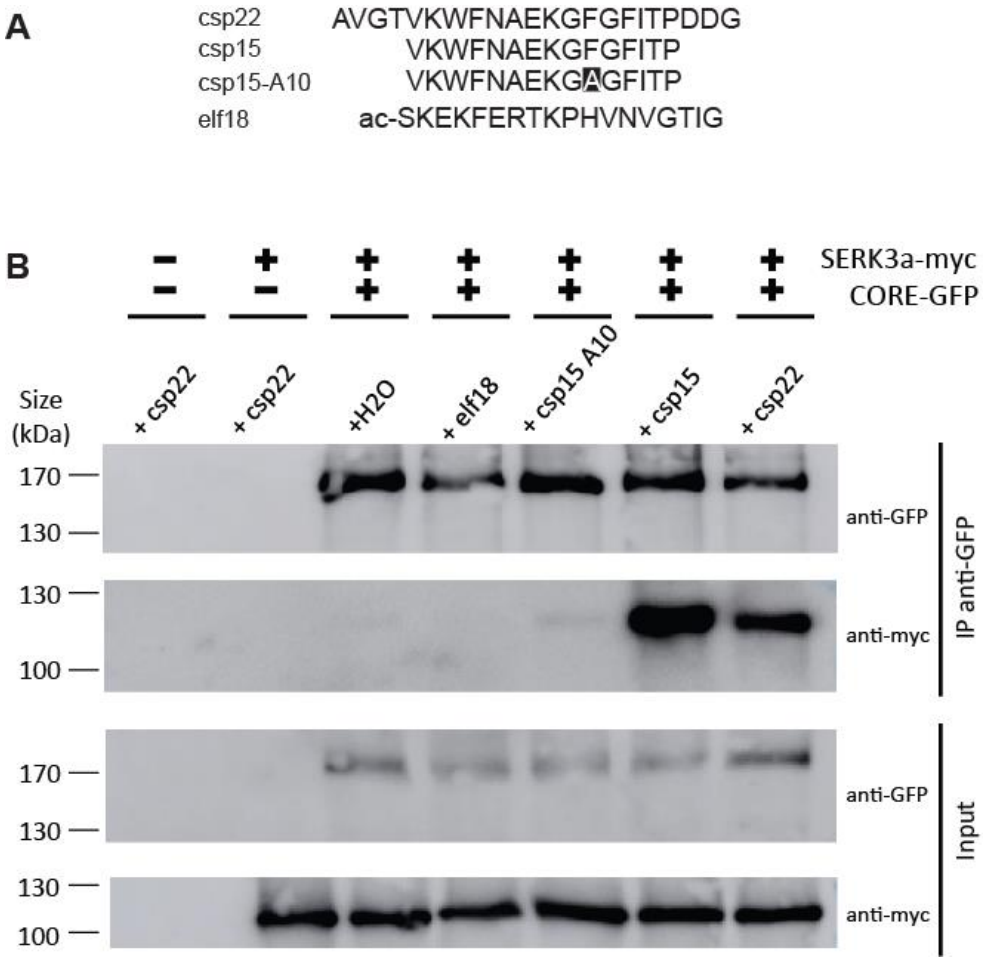


Figure 4.2.3. CORE forms a complex with SERK3 in response to treatment with csp22 or csp15. A) CSP and EF-Tu derived peptides used in the experiments. Csp22 and csp15 act as strong agonists, csp15-A10 as a weak antagonist in the csp-response of tomato and tobacco [114]. Elf18 is a potent agonist for EFR [51]. B) Leaves of *N. benthamiana* plants, transiently transformed with *p35S-SERK3a-myc* (SERK3a from tomato) and *p35S-CORE-GFP* (as indicated), were treated for 2 min by pressure infiltration of H_2O or solutions with 1 µM of the peptide indicated. Elf18 and csp15-A10 served as negative controls for perception by CORE. Detergent solubilized extracts, either before (input) or after immunoadsorption to anti-GFP beads (IP anti-GFP), were assayed on Western blots for presence of CORE-GFP and SERK3a-myc with the respective antibodies.

induction of an oxidative burst in tomato leaves (Suppl. Fig. 4.2.5A and 4.2.5B, respectively). For standard binding assays immunoprecipitates were incubated with 10 nM acri-csp22 to determine total binding or with 10 nM acri-csp22 and a 1000-fold excess of unlabeled csp22 to assay for non-specific binding, respectively (Suppl. Fig. 4.2.5C and D). Specific binding (total binding minus non-specific binding) for acri-csp22 was reproducibly detected with immunoprecipitates from plants expressing *CORE* homologs from tomato and *N. benthamiana* (*NbCORE*) but not from plants expressing *EFR* or no GFP-tagged receptor construct (control), respectively (Fig. 4.2.4A). Similarly, no specific binding could be found with immunoprecipitates of *Nb*CSPR that contained

amounts of GFP-tagged protein similar to the ones in the immunoprecipitates with CORE (Fig. 4.2.4A and B).

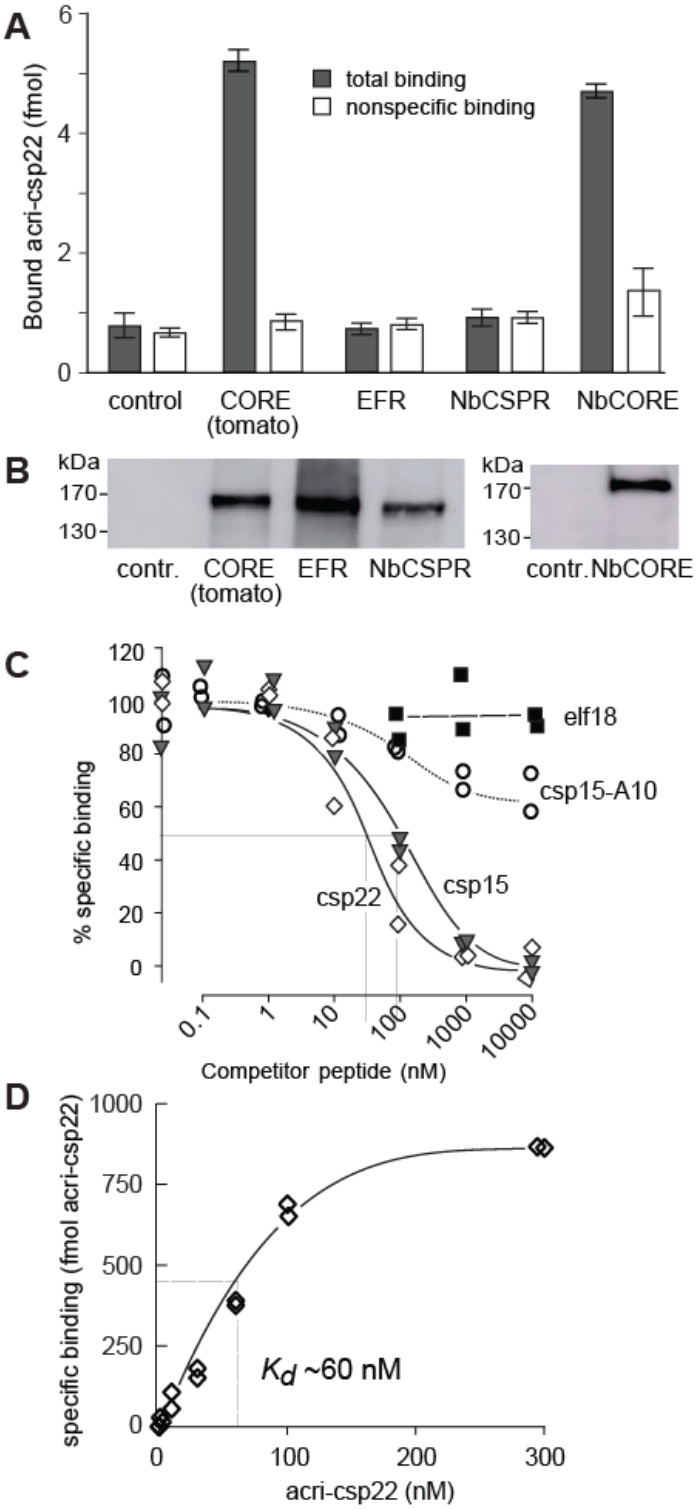


Figure 4.2.4. CORE binds csp22 and csp15 with high affinity and high specificity. A) Binding of acri-csp22 (1 nM) in the absence (total binding) or presence of 10 µM unlabeled csp22 (nonspecific binding) to immunoprecipitates from *N. benthamiana* leaves expressing P19 (control) alone or in combination with GFP-tagged versions of CORE (tomato), EFR, NbCSPR or NbCORE as indicated. Specific binding can be calculated by subtracting the nonspecific binding from the total binding. Bars and error bars show means ± S.D. of n = 3 replicates. B) Presence of CORE, NbCSPR, EFR and NbCORE in immunoprecipitates after detection on Western blots with anti-GFP antibodies. C) Specificity of binding to CORE from tomato in competition experiments with 0.3 nM of acri-csp22 and different amounts of the unlabeled peptides as indicated. D) Saturation of specific binding with increasing amounts of acri-csp22. Experiments in A) to D) are representative for n≥2 independent repetitions of the experiments.

In correlation with their activity as inducers of immune responses[114], csp22 and csp15 effectively competed with acri-csp22 for binding to CORE, with half maximal

competition reached at concentrations of ~50 nM for both peptides (Fig. 4.2.4C). In contrast, only a weak competition was observed with the inactive csp15-A10 analog and no competition was found with the peptide elf18 even at micromolar concentrations (Fig. 4.2.4C), demonstrating specificity of the receptor binding site. Saturation experiments with increasing concentrations of acri-csp22 showed simple saturation kinetics resulting in a dissociation constant, Kd of ~60 nM (Fig. 4.2.4D).

The receptor CORE is sufficient to confer csp22 perception to cells of A. thaliana

As a first assay to test whether CORE functions also in cells of non-*solanaceous* plants we used mesophyll protoplasts from *A. thaliana* leaves with a luciferase reporter under the control of the MAMP-responsive promoter of ´flg22-induced receptor-like kinase1´ (pFRK1) as a well-established and sensitive monitoring system[154,155]. Protoplasts from leaves of the double mutant *efr fls2* were transformed with *CORE* or *EFR* as a positive control (Fig. 4.2.5A and B). Protoplasts transformed with *EFR* gained responsiveness to the elf18 peptide but remained insensitive to csp22 (Fig. 4.2.5A). In turn, protoplasts expressing *CORE* showed clearly induced luciferase activity in response to csp22 or csp15 but not to treatment with the inactive peptide csp15-A10 or the structurally unrelated peptide elf18 (Fig. 4.2.5B). Similarly, the *Nb*CORE homolog was functional as csp22 receptor in Arabidopsis protoplasts as well (Suppl. Fig. 4.2.6). In contrast, we could not detect any responsiveness to csp22 in protoplasts transformed with *NbCSPR* (Fig. 4.2.5C). Protoplasts with CORE responded to concentrations of csp22 as low as 1 nM but not to micromolar concentrations of the inactive analog csp15-A10 (Fig. 4.2.5D). These results demonstrate that the CORE protein alone is sufficient to confer a specific and highly sensitive perception system for csp22 to *A. thaliana* cells.

Arabidopsis plants expressing CORE show enhanced resistance to *P. syringae* pv. *tomato* DC3000

Similar to the protoplasts, *A. thaliana* plants stably transformed with *CORE* showed high sensitivity for the csp22 peptide, illustrated by the induction of a clear oxidative burst to treatments with ≥1 nM of csp22 (Fig. 4.2.6A). We used these transgenic plants to test whether CORE as an additional PRR would affect resistance against plant pathogenic bacteria. First, we spray-inoculated plants with *Pseudomonas syringae* pv. *tomato* DC 3000 and we consistently observed weaker symptom development in plants expressing *CORE* in comparison to non-transformed wildtype plants (Fig. 4.2.6B). In further experiments with pressure-infiltration of the bacteria we reproducibly observed significantly lower titers of bacteria in leaves of plants transformed with CORE than in

wildtype plants (Fig. 4.2.6C). Our results show that CORE from tomato can be easily transferred to a heterologous plant where this additional PRR contributes to resistance against bacterial pathogens.

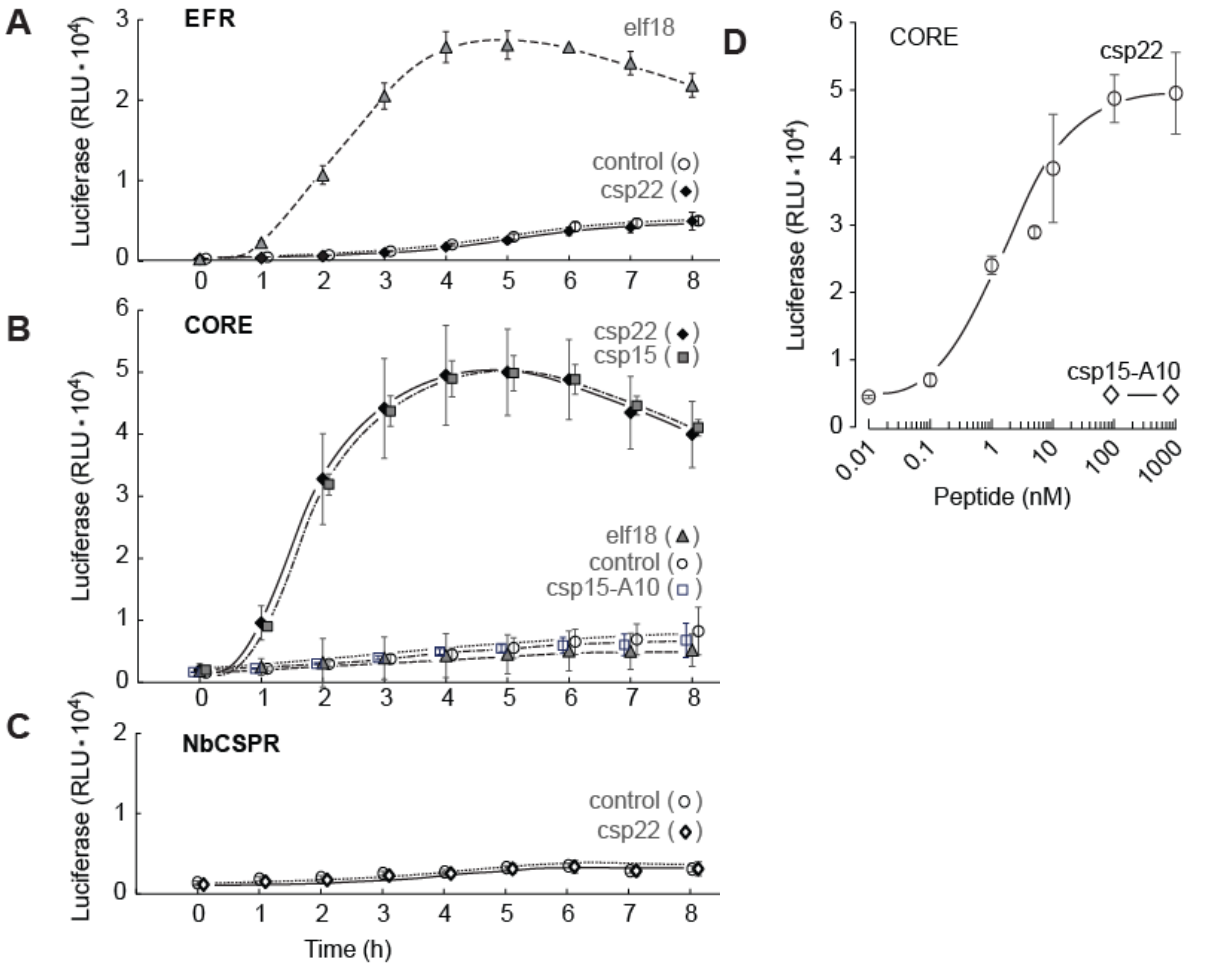


Figure 4.2.5. The receptor kinase CORE is sufficient to confer responsiveness to csp22 when expressed in cells of *A. thaliana*. Protoplasts from leaves of *Arabidopsis* plants lacking EFR were transformed with *pFRK1-Luc* in combination with either *p35S-EFR-GFP* (A), *p35S-CORE-GFP* (B) or *p35S-NbCSPR-GFP* (C), respectively. Results show luciferin-dependent light emission in response to treatment with H_2O (control) or 1 µM of the peptides indicated. D) Dose dependence of the *pRK1-Luc* induction in protoplasts expressing CORE. Values and error bars show means ± S.D. of n = 3 replicates. Results are representative for n≥3 independent repetitions of the experiments.

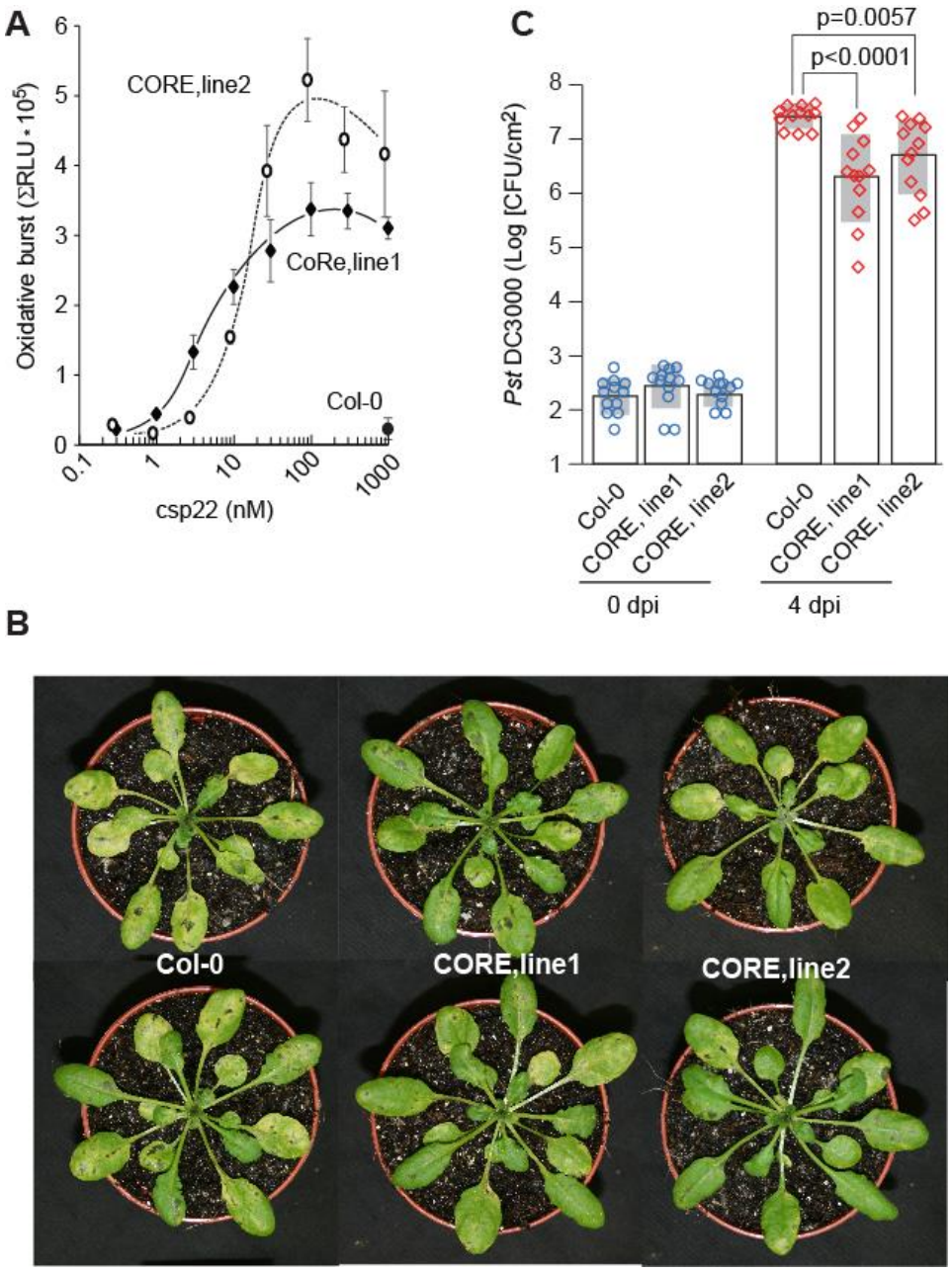


Figure 4.2.6. The receptor kinase CORE expressed in *A. thaliana* is fully functional and contributes to resistance against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000). A) Oxidative burst in 2 independent lines of Arabidopsis Col-0 plants stably transformed with *p35S-CORE-GFP* (homozygous T2 generation). Leaf pieces were treated with different concentrations of csp22 and oxidative burst was measured as luminol-dependent light emission integrated over 15 min. Values and error bars show means and ± S.D. of n = 6 replicates. B) Presence of CORE reduces development of disease symptoms after spray inoculation with *Pst* DC3000. Pictures show representative plants 4 days after inoculation. C) Presence of CORE restricts growth of *Pst* DC3000 after pressure infiltration. Bacteria were quantified in extracts of leaves by serial dilutions at day 0 and 4 after inoculation (dpi). Values are shown for n = 12 samples from 6 plants. Means are indicated by bars and standard variations by shaded areas, respectively. Pairwise comparison of the values at 4 dpi show significance levels of p = <1% for both transgenic lines according to Student´s T test. The experiments shown are representative for 3 independent repetitions.

4.2.4 Discussion

The novel receptor kinase CORE exhibits the features expected for a genuine receptor of the MAMP csp22, including high-affinity and specificity for the csp22 ligand and the capacity to confer responsiveness to csp22 to *A. thaliana* plants that have no endogenous perception system for this MAMP. Thus, csp22 and CORE add to the still rather small list of MAMP/PRR pairs for which interaction between ligand and receptor has been demonstrated.

CORE is but the second PRR for which a role in csp22 perception has been postulated. In a very recent publication the receptor-like protein *Nb*CSPR has been reported to have an essential function for perception of the csp22 peptide in *N. benthamiana*[136]. *Nb*CSPR homologs occur exclusively in some species of the *Solanaceae* family[136] but seem absent from *S. lycopersicum* where the most closely related proteins with ~51% amino acid identity (http://solgenomics.net/) are the RLPs EIX1 and EIX2 that function as receptors for fungal xylanase[82]. In turn, currently available genomic information predicts homologs for *CORE* for various *Solanaceae*, including species that belong to the genera of *Solanum* and *Nicotiana*. The *CORE* homolog of *N. benthamiana* encodes a functional receptor (Suppl. Fig. 4.2.4A) with affinity for the csp22 ligand (Fig. 4.2.4A). Like *NbCSPR*[136], the *NbCORE* gene showed age-dependent expression that could explain age-dependence of the csp22 responses in *N. benthamiana* (Suppl. Fig. 4.2.4E).

Presence of *NbCSPR* and *NbCORE* in *N. benthamiana* suggested that this species might have two PRRs for CSP. Evolution of two perception systems for MAMPs residing on a common bacterial protein has a precedence with tomato being able to recognize bacterial flagellin via the distinct peptide epitopes flg22 and flgII-28 by FLS2 and FLS3, respectively[56,57]. However, the recognition of an identical MAMP like csp22 by two distinct PRRs, *Nb*CSPR and *Nb*CORE in *N. benthamiana*, would be novel. In our attempts to directly compare the functions of CORE and *N*bCSPR we were not able to corroborate the relevance of *Nb*CSPR for csp22 perception. Rather, in our hands, ectopic or heterologous expression of *NbCSPR* did neither lead to responsiveness to csp22 in leaves of young *N. benthamiana* plants (Suppl. Fig. 4.2.4C) nor in cells of *A. thaliana* (Fig. 4.2.5C). Additionally, *Nb*CSPR exhibited no measurable affinity for the csp22 ligand (Fig. 4.2.4A). At present, we cannot explain our failure to reproduce the results reported in Saur et al.[136] but we would rather conclude that

*Nb*CSPR is neither sufficient nor necessary for csp22 perception. Our results do not explain other possible functions the *Nb*CSPR protein might have. They also do not explain the reduced csp22 response observed in plants silenced for *NbCSPR*[136], but silencing of this gene or an off-target might interfere with the csp22 perception in very indirect ways. Perception via the receptor kinase CORE instead of the RLP *Nb*CSPR, however, might explain why silencing of SOBIR1, a protein kinase that interacts with *Nb*CSPR[136] and is thought to be essential for the functioning of RLPs but not RLKs[156], had no apparent effect on the response[136].

The pattern recognition system of plants is redundant, with multiple PRRs contributing to the detection of a given type of pathogen. For example, the well-studied model host *A. thaliana* can detect the bacterial pathogen *Pst* DC3000 via PRRs with specificities for flagellin, EF-Tu, LPS and peptidoglycan[48,51,124,157]. This redundancy of perception systems may render the loss of a single PRR less critical and allow for the considerable natural variation observed between closely related species, accessions or even strains of plants with respect to the presence of individual PRRs. Such variations have been instrumental for the identification of most of the PRRs identified so far, including Xa21 from rice[126], EIX1/2, CuRe1 and FLS3 from tomato[57,82,125] and FLS2, RLP30 and ReMAX from Arabidopsis[49,128,158]. Non-functionality of CORE in *S. pennellii* is due to lack of expression caused by changes in its promoter region (Suppl. Fig. 4.2.3). Similarly, responsiveness of young *N. benthamiana* plants appears to be limited by low expression of *NbCORE* at this developmental stage (Suppl. Fig. 4.2.4E). Whether this age-dependent expression pattern reflects an adaptive function for the immune system of *N. benthamiana* or rather partial loss of this PRR will be an interesting area for further studies.

Apart from loss of function, natural variation also implies the more intriguing process leading to the evolution of novel recognition specificities. Indeed, an ongoing evolutionary arms race between pathogens and plant hosts appears to drive rapid diversification of patterns exposed by the pathogens and pattern recognition systems by the hosts[5,40,143]. Interestingly, whereas some PRRs, such as FLS2 detecting bacterial flagellin via flg22, occur in many species of angiosperms and even gymnosperms[159], most of the PRRs seem to be restricted to a rather limited range of species within individual plant families or genera.

Here, the appearance of CORE in species of the family of *Solanaceae* might serve as an interesting model case. The CORE protein shows striking structural resemblance to EFR from Arabidopsis and XA21 from rice, respectively. Apart from the kinase domain, this resemblance covers features of the apoplastic LRR domain with the same number of 22 LRRs and a 6 aa insert at the same position in LRR 11 (Suppl. Fig. 4.2.2). While clearly related at positions forming the interior of the solenoid of stacked LRRs, amino acids at the solvent-exposed surface of the LRR domain exhibit only very modest conservation. Overall, this suggests that these receptors have a common internal structure but a highly divergent surface in their LRR domains. This might explain the different ligand specificities of the receptors with Xa21 detecting RaxX21-sY[148], EFR detecting elf18[52] and CORE detecting csp15/csp22, respectively. Thereby, at least as far as tested for elf18 and csp22 in this report, these receptors are highly selective, showing binding and receptor activation only with their genuine ligand peptides (Figs. 4.2.3, 4.2.4 and 4.2.5). This opens interesting new questions as to how the LRR domains of such PRRs can evolve resulting in strong variation in half of the positions that form the surface while keeping the other half forming the structural scaffold essentially unchanged.

The perception of cytoplasmic bacterial proteins like CSP and EF-Tu by specific cell surface receptors of plants seems to defy common sense. However, EF-Tu and CSPs are highly abundant proteins and small amounts of them are found in secretomes of bacteria[160]. Whether this is due to a small fraction of dead and lysed bacteria or to a controlled release process, such as general permeability changes occurring as part of osmotic adaptation in bacteria[161], is currently unknown. Importantly though, the amounts of such MAMPs that get exposed to the highly sensitive PRRs appear to be sufficient for activation of plant immunity. Experimentally, the best evidence for this is the significant reduction of bacterial colonization, which depended on the presence of either EFR[52] or CORE (Fig. 4.2.6), respectively. These results also show that transfer of a highly effective PRR like CORE can enlarge and enhance the repertoire of immunodetectors and contribute to increased plant resistance in a recipient plant like *A. thaliana*. This provides further evidence that transfer of PRRs can function even between species from different taxonomic families and strengthens the option of biotechnological approaches to use the divergent repertoires of PRRs in different plants to enhance pathogen recognition and disease control in crop species beyond the boundaries of classic breeding.

4.2.5 Material and Methods

Plant Material

Arabidopsis thaliana plants were grown in phytochambers (8-h photoperiod, 22 °C, and 40%-65% relative humidity). Tomato plants and *Nicotiana benthamiana* plants were cultivated in the greenhouse with a 14-h photoperiod, day temperature of 25 °C, night temperature of 19 °C. Recombinant inbred lines between *S. lycopersicum* and *S. pennellii*[162] were obtained from the Tomato Genetics Resource Center (UC Davis; http://tgrc.ucdavis.edu/).

Materials

Peptides were synthesized by GenScript and the derivatization of csp22 with the acridinium ester was performed as described[153].

Bioassays with plant tissue or Arabidopsis protoplasts

Ethylene measurement and oxidative burst were performed as described[155]. For oxidative burst, leaf pieces were floated in 96-well plates (1 piece/well) containing 100 µl substrate solution with 20 µM L-012 (Waco) and 2 µg/ml horseradish peroxidase (Applichem) and light emission after treatment with control or peptide solutions was measured with a luminometer (Mithras LB 940, Berthold) in 1 min intervals.

Transient expression in leaf mesophyll protoplasts of *efr fls2* Arabidopsis mutant plants were performed as described[154,155]. Aliquots of 300'000 protoplasts were co-transformed with 30 µg DNA plasmid *pFRK1-luciferase* as a MAMP-inducible reporter[154] and 30 µg plasmid DNA encoding CORE, NbCSPR or EFR. Protoplasts were re-suspended in W5-solution with 200 µM luciferin (D-Luciferin, firefly, PJK) and distributed in a 96-well-plate (100 µl aliquots; 20'000 protoplasts per well). After ~14 h of incubation in the dark, the cells were treated with the peptides to be tested and luciferase activity was quantified *in vivo* as light emitted by the protoplasts (RLU; light units measured by a luminometer Mithras LB 940).

Receptor Cloning

Receptor candidates were amplified from tomato (M82) genomic DNA using Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific) and the primers indicated in Suppl. Table 4.2.1. PCR products were cloned into the pK7FWG2.0 expression vector (https://gateway.psb.ugent.be) between a CaMV35S promoter and a C-terminal GFP tag. All constructs were verified by sequencing.

Transient Expression in N. benthamiana

A. tumefaciens (strain GV3101) harboring the desired receptor constructs were grown in LB medium, collected by centrifugation, and resuspended in infiltration solution (10 mM $MgCl_2$, 150 µM acetosyringone) to an OD600 of 1.0. After further incubation at room temperature for 1 to 2 h, bacteria with receptor constructs were mixed 1 : 1 with bacteria carrying the silencing inhibitor P19[147], diluted 2 times with infiltration solution and pressure infiltrated into leaves of 4-week old *N. benthamiana* plants. Leaves were cut into pieces 24 h after infiltration, floated overnight on H_2O and used for ethylene-induction assays and oxidative burst measurements. For preparing immunoprecipitates leaves were harvested ~40-48 h after infiltration.

Immunoprecipitation

Membrane proteins of *N. benthamiana* leaves expressing the receptor constructs of interest were solubilized as described[73] and immuno-adsorbed via their GFP-tags on magnetic agarose GFP-Trap® beads (ChromoTek, IZB Martinsried, Germany). Western blots were developed with anti-GFP antibodies (Torrey Pines Biolabs) or anti-myc antibodies (Sigma-Aldrich), followed by staining with secondary antibodies coupled to alkaline phosphatase and CDP-Star (Roche) as substrate.

Binding assays

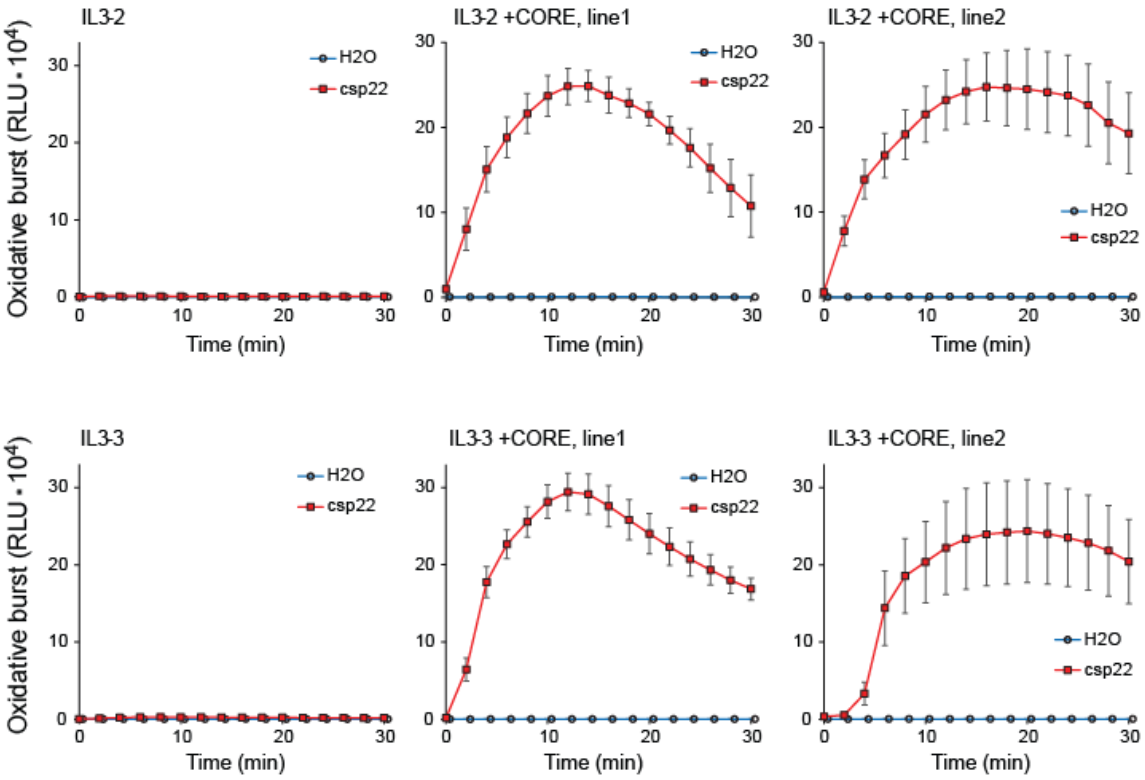
GFP-Trap beads with immunoadsorbed receptors were washed twice with solubilization buffer (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 % deoxycholic acid and 1% Nonidet P‑40), twice with 25 mM Tris-HCl, pH 8.0, 150 mM NaCl and twice with binding buffer (25 mM MES, pH 6.0, 10 mM NaCl, and 3 mM $MgCl_2$). Beads were re-suspended in binding buffer (10 µl beads per 100 µl of total volume) and supplied with acri-csp22 either alone or with different concentrations of unlabeled peptides as competitors. After 20 min incubation on ice, unbound ligands were removed by two rounds of rapid washing with 1 ml binding buffer. Beads were re-suspended in 10 µl citric acid (5 mM) and transferred to measuring tubes containing 90 µl citric acid, and acridinium ester was measured by integrating light emission of the sample for 10 s in a single tube luminometer (FB12; Berthold) after adding 150 µl reacting solution with 100 mM NaOH and 20 mM H_2O_2.

Bacterial Growth Assay

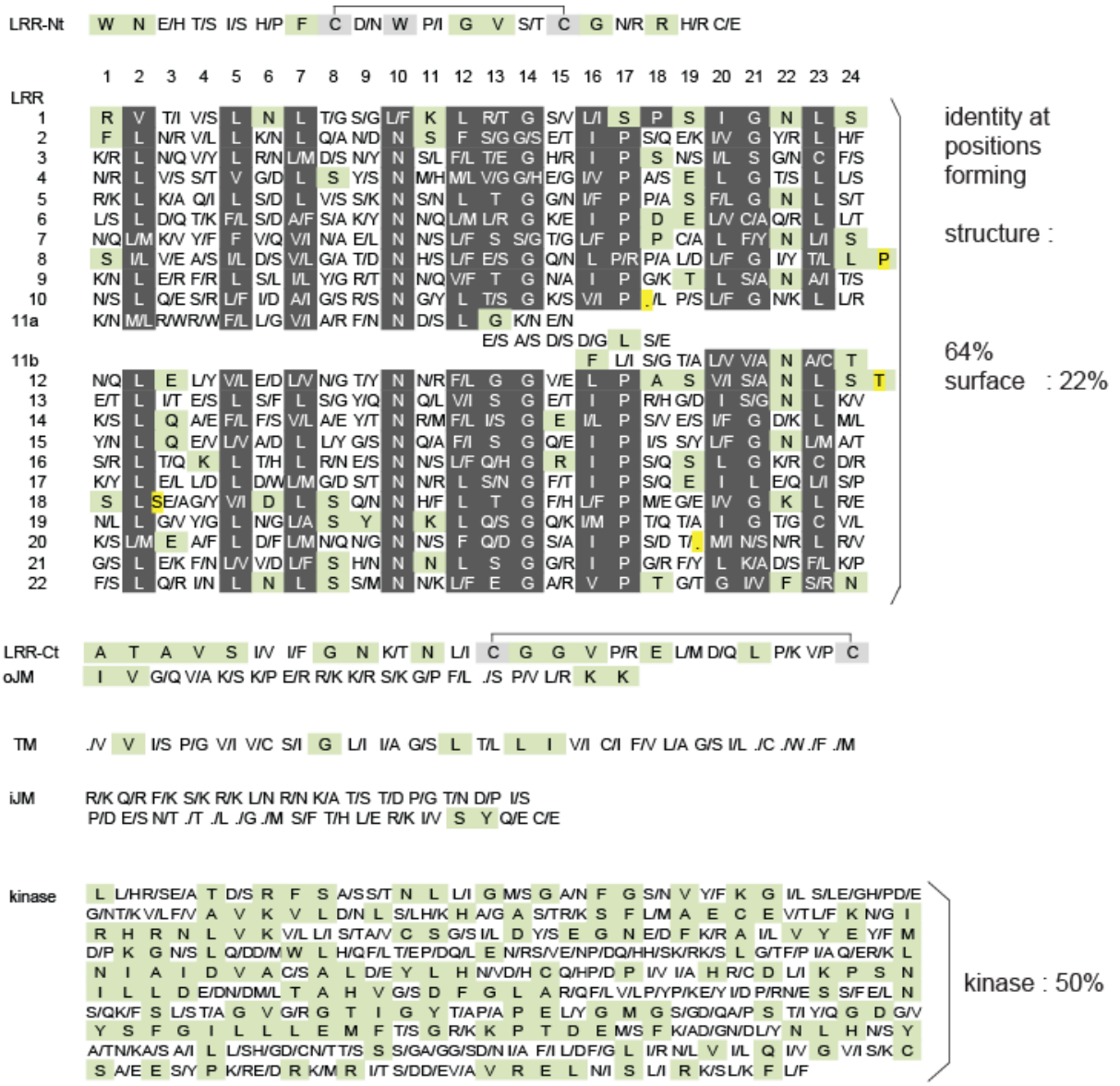
Pseudomonas syringae pv. *tomato* DC3000 was grown for ~16 h in LB medium at 28°C, and resuspended in 10 mM $MgCl_2$ at a concentration of 10^4 colony-forming units

(cfu) ml^{-1} for pressure infiltration assays or at $5*10^8$ cfu ml^{-1} for spray inoculation assays, respectively.

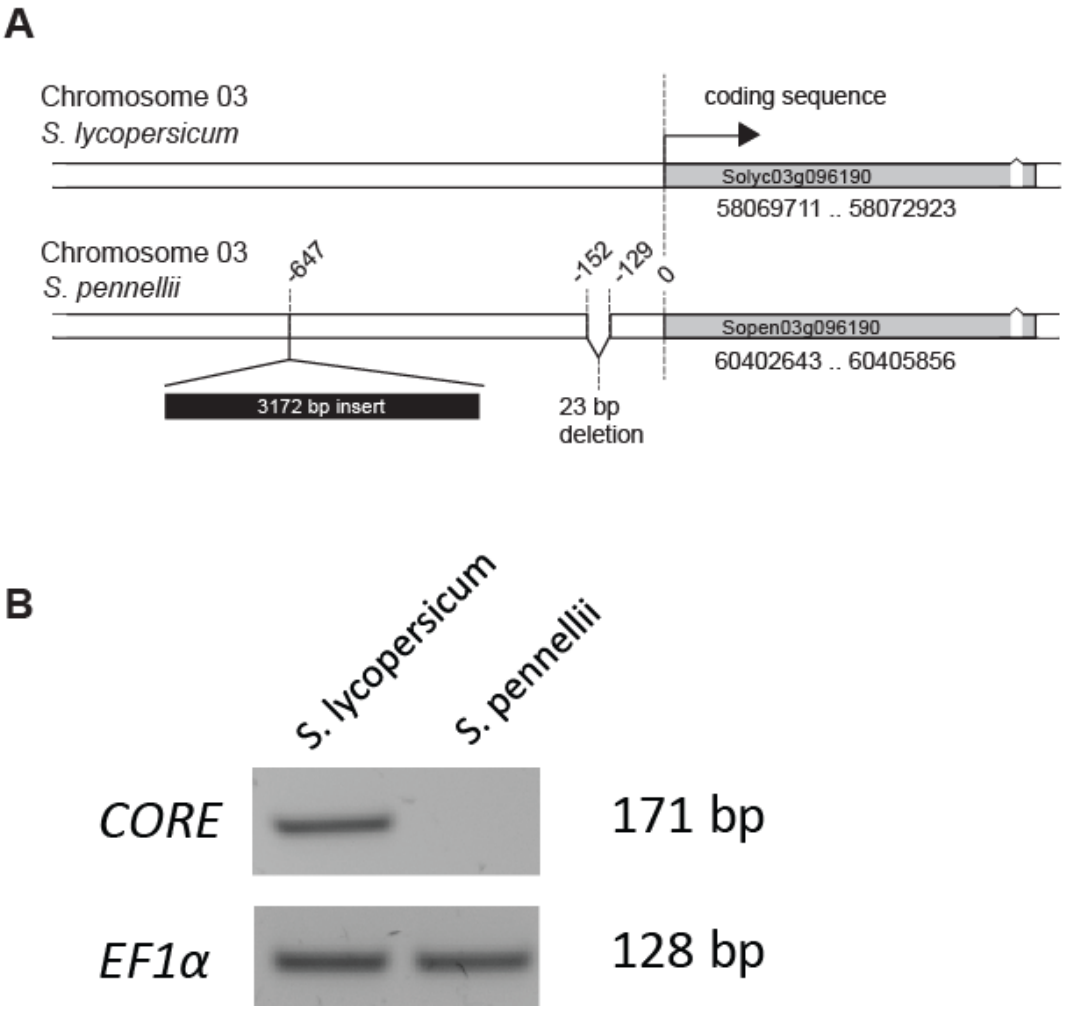
4.2.6 Supplementary Data



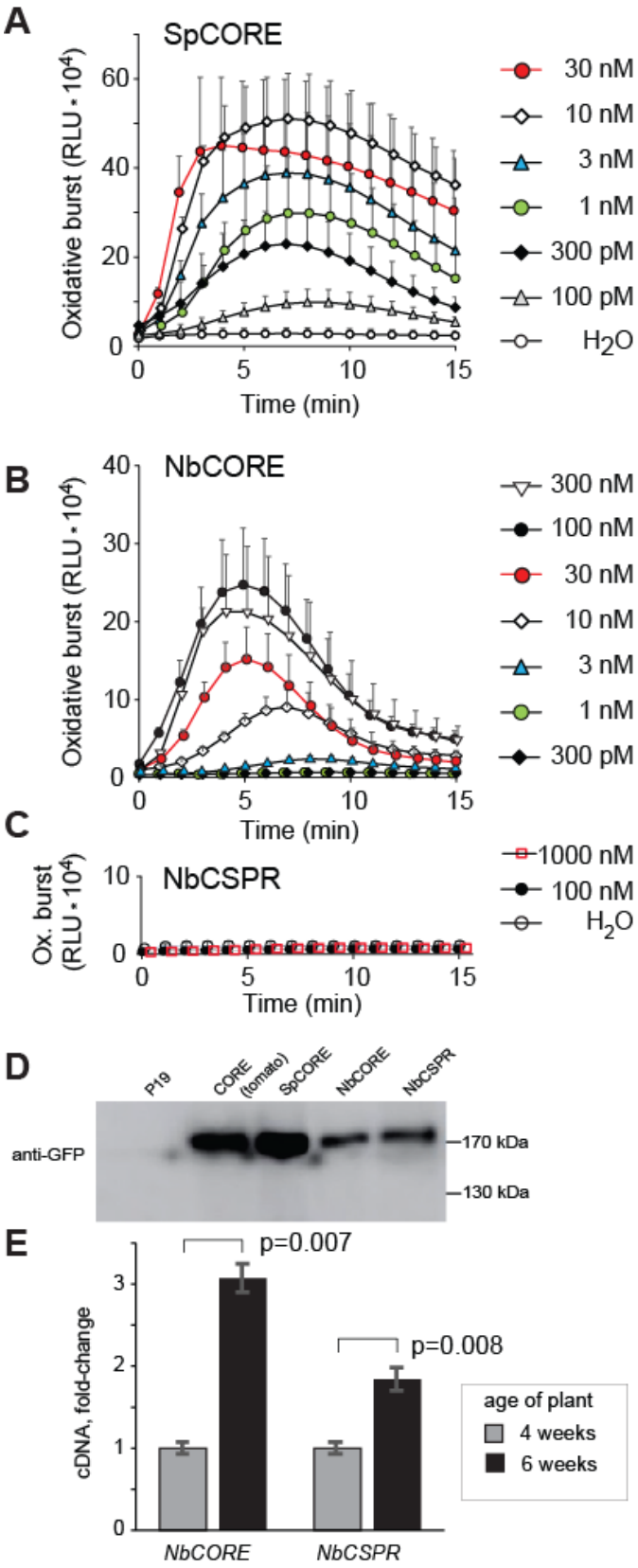
Supplementary Figure 4.2.1. The introgression lines IL3-2 and IL3-3 gain responsiveness to csp22 when complemented with *CORE*. Oxidative burst in response to treatment with 100 nM csp22 is shown for the untransformed lines IL3-2 and IL3-3, and for the introgression lines stably transformed with *p35S-CORE-GFP*. Two lines (T2 generation), representing independent transformation events, were tested. Values and error bars show means ± S.D. of n = 4 replicates.



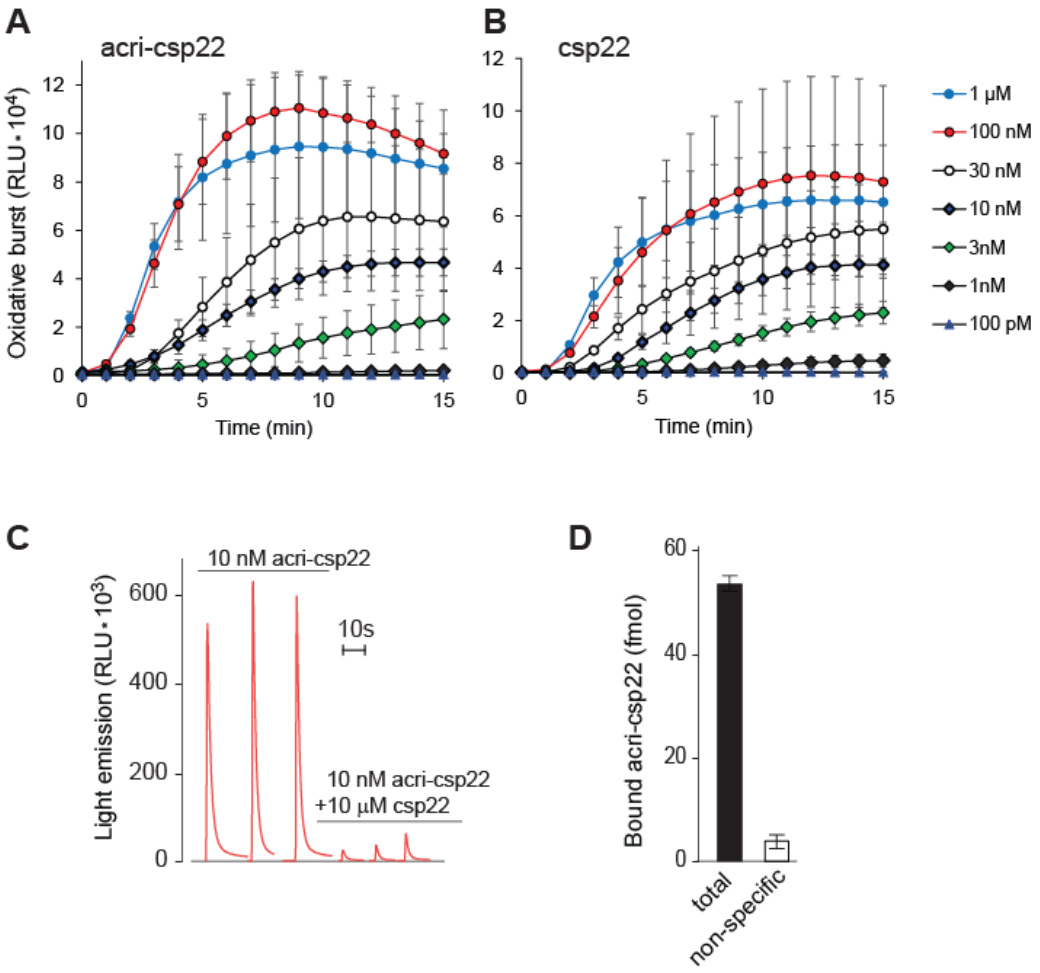
Supplementary Figure 4.2.2. Comparison of the primary structures of the LRR receptor kinases encoded by CORE (Solyc03g096190) from tomato and EFR (At5g20480) from Arabidopsis. Single letters indicate positions with identical amino acids (aa, green underlay) while two letters separated by "/" indicate divergent aa residues, respectively. Positions with deletions or insertions of single aa in the repeats are highlighted in yellow. Amino acids in the LRR domain predicted to form the CORE of the solenoid structure according to the 24 aa plant LRR consensus xLxxLxLxxNxLS/TGxIPxxLGxLx (with other non-hydrophobic aa occasionally substituting for L) are indicated with white letters on black underlay. Remarkably, the positions predicted to form the surface of the LRR domain (x in the consensus) show particularly low conservation (22 %), with several of the conserved sites belonging to potential N-glycosylation sites NxS/T. LRR-Nt and LRR-Ct, domains with conserved Cys-Cys bridges that form N- and C-terminal ends of the LRR domain; oJM, outer juxtamembrane domain; TM, transmembrane domain; iJM, inner juxtamembrane domain. Not shown are the N-terminal sequences with the signal peptides for export via ER and the unrelated C-terminal sequences of 9aa in CORE and 30 aa in EFR, respectively.



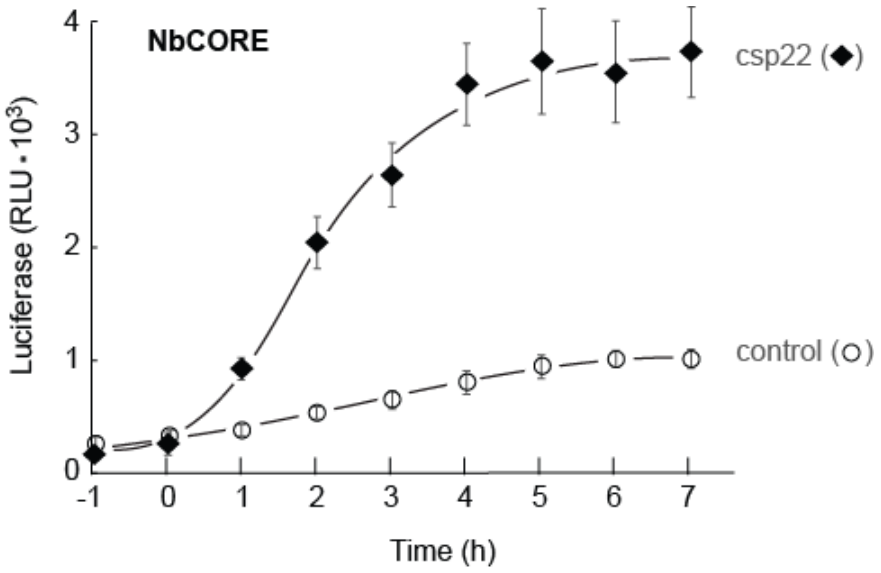
Supplementary Figure 4.2.3. A) Schematic representation of the *CORE* genes in the *S. lycopersicum* and *S. pennellii* genomes. In comparison to *S. lycopersicum*, the upstream promoter region in *S. pennellii* has a 23 bp deletion and a 3.2 kb insert of sequences of Solanum-specific elements related to >PRSiTERT00100080 and Copia-like retrotransposons TLC1.1 (Ty1). B) RT-PCR of *CORE* and *EF1α* (control) with mRNA from *S. lycopersicum* and *S. pennellii*.



Supplementary Figure 4.2.4. *S. pennellii* and *N. benthamiana* encode functional CORE homologs. Leaves of young *N. benthamiana* plants were transiently transformed with *SpCORE* (A), *NbCORE* (B) or *NbCSPR* (C) under the control of the 35S promoter and assayed for induction of an oxidative burst in response to different doses of csp22. Values and error bars show means + S.D. of n = 6 replicates. D) Western blot of crude leaf extracts with antibodies against the GFP-tags present on all three constructs. E) Results of quantitative real-time PCR assays for *NbCORE* and *NbCSPR* with cDNA from leaves of 4 and 6 week old *N. benthamiana* plants, respectively. Values (fold-increase) are normalized on the respective levels in 4-week plants and represent mean and standard deviations of n = 3 biological replicates. With respect to the expression level of *NbEF1α* used as an internal standard the expression of *NbCSPR* was four times higher than that of *NbCORE* in 4-week old plants.



Supplementary Figure 4.2.5. Acri-csp22 is a fully active MAMP and a suitable ligand in binding assays. A) and B) Acri-csp22 is as active as csp22 when tested in different concentrations for the induction of ROS production in tomato leaf pieces. Results are shown as means ± S.D. of n = 6 replicates. C) and D) Principles of measuring specific binding with acri-csp22. Immunoprecipitates of CORE on immunobeads were incubated with low concentrations of acri-csp22 (0.3 to 10 nM) alone ("total binding") or in presence of a high molar excess of unlabeled csp22 (1 to 10 µM)("nonspecific binding"). Beads were washed to remove unbound ligand and chemiluminescence of the acridinium esters remaining on the beads was measured by the addition of reaction solution containing 100 mM NaOH and 20 mM H_2O_2. C) Tracings show examples for the flash-type of chemiluminescence (as relative light units (RLU) in the luminometer). The Integrals of these flashes over the first 10 s were used for quantification. D) The amounts (fmol) of acri-csp22 were derived by comparison with a standard curve obtained with different doses of acri-csp22.



Supplementary Figure 4.2.6. NbCORE, the CORE homolog of *N. benthamiana*, is functional as csp22 receptor in *A. thaliana* cells. Protoplasts from leaves of Arabidopsis plants were transformed with *pFRK1-Luc* in combination with *p35S-NbCORE-GFP*. Results show luciferin-dependent light emission in response to treatment with H_2O (control) or 1 µM of csp22. Values and error bars show means ± S.D. of n = 3 replicates.

Gene name	Usage	Primer name	Primer sequence (5’ – 3’)
Solyc03g062660	cloning	660Fw	CACCATGACAGTACAGTGTATTG
		660R	AGAATGAGTATGGGTGGAAG
Solyc03g082450	cloning	450Fw	CACCATGTTCTTGTTTATGGTAA
		450R	CATCTGATTTAACTCGACAAG
Solyc03g082470	cloning	470Fw	CACCATGTTCTTGTTTGATGTTG
		470R	TAACTCGACAAGAGACCTTAC
Solyc03g093330	cloning	330Fw	CACCATGAAACACTTCTCTCCATTC
		330R	ACACTCCACATTAGCTTTTTGAG
Solyc03g096190 (*CORE*)	cloning	190Fw	CACCATGATTCTCCCAAAGAATTCTC
		190R	TAACTTTTTCTTCCGGTATGCTTG
SpCORE	cloning	SpCORE Fw	CACCATGATTCTCCCAAAGAATTCTC
		SpCORE R	ATGCAGGTTGAAGCTTATAACTTTTTC
NbCORE	cloning	NbCORE Fw	CACCATGATTTTTCCAAGTCATAC
		NbCORE R	CACCTGTTCTTTCCAGTATGC
NbCSPR	cloning	NbCSPR Fw	CACCATGAAAAGTGAGAGATTTTTAT
		NbCSPR R	ACTCCAGAGCACCTTCAATC
CORE	RT-PCR	CORE q Fw	GCGATCTGAAGCCAAGCAAT
		CORE q R	CCCATACCTAATTCTGGAGGTGT
SlEF1α	RT-PCR	SlEF1α q Fw	CTCCGTCTTCCACTTCAGG
		SlEF1α q R	TCAGTTGTCAAACCAGTAGGG
NbCORE	Real Time PCR	NbCORE q Fw	TGGCATTCGACAGTTTGGTG
		NbCORE q R	CAGACCCAAAACCACCCATG
NbCSPR	Real Time PCR	NbCSPR q Fw	CAAGTTCAGGAGGGGATGGT
		NbCSPR q R	GCTACACTGCACACCTTTCC
NbEF1α	Real Time PCR	NbEF1α q Fw	TGTGGAAGTTTGAGACCACCC
		NbEF1α q R	GCAAGCAATGCGTGCTCAC

Supplementary Table 4.2.1. Primers used in cloning, RT-PCR and Real-Time PCR.

4.3 The systemin receptor SYR1 enhances resistance of tomato against herbivorous insects

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This work aimed to identify the tomato receptor for the peptide hormone systemin and evaluate its role in resistance against herbivorous insects. I contributed to the experimental design and performed all the experiments with help from co-authors. I analyzed the data and wrote the manuscript together with Prof. Georg Felix with input from co-authors. The experiment shown in Figure 4.3.3c was done in collaboration with Dr. Axel Mithöfer from Max Planck Institute for Chemical Ecology.

4.3.1 Abstract

The discovery in tomato of systemin, the first plant peptide hormone[95,163], was a fundamental change for the concept of plant hormones. While before this report peptide hormones were assumed to be absent in plants, numerous other peptides have since been shown to play regulatory roles in many aspects of the plant life, including growth, development, fertilization and interactions with symbiotic organisms[14,15,107,164]. The "role model" peptide hormone systemin, an 18-amino acid peptide derived from a larger precursor protein[165], was proposed to act as the spreading signal that triggers systemic defense responses observed in plants after wounding or attack by herbivores[95,96,166]. An initial attempt to identify the systemin receptor culminated in the isolation of the leucine-rich repeat (LRR) receptor kinase SR160[100,167] which turned out to be a tomato homolog of Brassinosteroid Insensitive 1 (BRI1). BRI1 is one of the best studied plant receptors, and it mediates the regulation of growth and development in response to the steroid hormone brassinolide (BL)[7,8,168]. However, whereas the role of SR160/BRI1 as BL receptor was not disputed, its role as systemin receptor could not be corroborated by others[101,102,169]. Here, we demonstrate that perception of systemin depends on the two closely related LRR-receptor kinases SYR1 and SYR2 and not on SR160/BRI1. SYR1 acts as a genuine systemin receptor that binds systemin with high affinity and specificity. Further, we show that presence of SYR1, while not decisive for local and systemic wound responses, is important for defense against insect herbivory.

4.3.2 Results and discussion

Treatment of tomato (*Solanum lycopersicum*) and other *Solanum* species with systemin induces an array of defense-related responses including the accumulation of proteinase inhibitors (PINs), increase of ethylene biosynthesis and induction of an oxidative burst[95,98,170]. Using ethylene biosynthesis as a convenient output we observed that, in contrast to *S. lycopersicum*, the wild tomato species *S. pennellii* lacked responsiveness to systemin (Fig. 4.3.1a). Progeny from crosses between these closely related species have been used to establish collections of tomato introgression lines (ILs) with specific parts of their genome replaced by homologous parts of the *S. pennellii* genome[140,141]. We tested a collection of 49 precisely defined ILs[141] for response to systemin and found that lines IL3-2 and IL3-3, with replacements of overlapping regions in chromosome 3, lacked responsiveness to systemin (Supplementary Fig.4.3.1). By chance, the non-responsiveness to systemin was associated with the same two ILs that in previous work helped to map and identify the pattern recognition receptor CORE which specifically detects the csp22 peptide from bacterial cold shock protein[137]. We therefore tested the collection of candidate receptor genes established in this previous study for their potential role in systemin sensing when expressed in *Nicotiana benthamiana*, a species that has no endogenous perception system for systemin[167].

Two of these genes, Solyc03g082450 and Solyc03g082470, conferred clear induction of ethylene biosynthesis and production of reactive oxygen species (ROS) in response to treatment with systemin (Fig. 4.3.1b and c). These genes encode two closely related LRR-RLKs (89% identity, Supplementary Fig. 4.3.2) that we tentatively named systemin receptor 1 and 2 (SYR1 and SYR2), respectively. For comparison, we also expressed SR160/BRI1 that has previously been postulated as the systemin receptor in tomato[100,167]. However, while accumulating to similar levels as SYR2 and SYR1 (Supplementary Fig.4.3.3a), SR160/BRI1 in leaves of *N. benthamiana* did not confer responsiveness to micromolar concentrations of systemin (Fig. 4.3.1b-d). In contrast, leaves expressing SYR1 responded to subnanomolar concentrations of systemin, resulting in half-maximal stimulation (EC50) at ~0.03 nM systemin (Fig. 4.3.1d). Leaf pieces with SYR2 were less sensitive and responded with an EC50 of >30 nM systemin (Fig. 4.3.1d). A similar pattern of responsiveness to systemin was observed after heterologous expression of these receptors in Arabidopsis thaliana protoplasts (Fig.

4.3.1e). While no induction occurred in cells with SR160/BRI1, systemin-dependent induction of the reporter gene construct *pFRK1::Luciferase*[154] occurred with an EC50 of 0.1 nM with SYR1, and ~3 µM with SYR2, respectively.

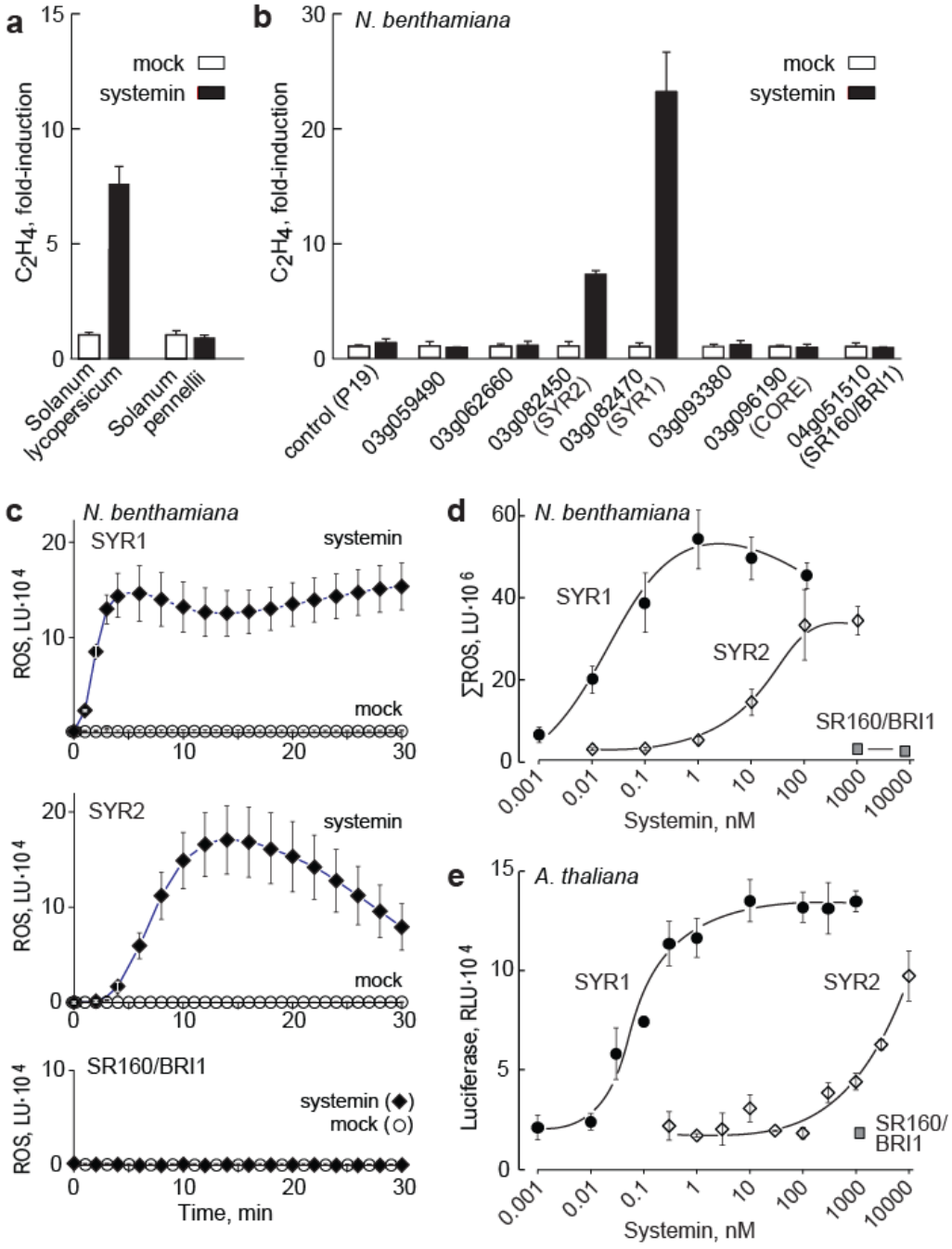


Figure 4.3.1. The tomato genes SYR1/Solyc03g082470 and SYR2/Solyc03g082450 provide responsiveness to systemin when heterologously expressed in N. benthamiana or A. thaliana. a) S. lycopersicum but not its wild relative S. pennellii responds with increased ethylene biosynthesis to treatment with 100 nM systemin. b) and c) Systemin-dependent induction of ethylene biosynthesis and reactive oxygen species (ROS) in N. benthamiana leaves transiently transformed with different receptor candidates. d) Production of ROS (integral over 30 min) for leaf pieces treated with different concentrations of systemin. e) Activity of the luciferase reporter in transformed A. thaliana protoplasts treated with different concentrations of systemin for 3 h. a) to e) values and bars indicate mean ± SD of n=3 replicates for of ethylene, n=6 for ROS, and n=3 for luciferase, respectively. Controls for expression of the transgenes are shown in Supplementary Fig.4.3.3. Data are representative for at least three independent experiments.

Leaves of *S. pennellii* and the tomato ILs IL3-2 and IL3-3 showed no responses to systemin, indicating lack of functional SYR1 and SYR2 in these plants (Fig. 4.3.1a, Supplementary Fig.4.3.1). Comparison of the corresponding genomic regions in *S. lycopersicum* and *S. pennellii*[146,171] shows a 56 base pair (bp) deletion leading to a premature translational stop in the *S. pennellii* SYR1 homolog (Supplementary Fig. 4.3.4a). The adjacent *S. pennellii* SYR2 gene contains rearrangements in the region immediately 5´ of the coding region, explaining the absence of this transcript in IL3-2 and IL3-3 as observed in RNAseq data[141], and in cDNA prepared from *S. pennellii* leaves (Supplementary Fig. 4.3.4b).

A BLAST search for SYR-type genes in current databases shows that tomato, potato, eggplant and pepper all have homologs of both, SYR1 and SYR2 (Supplementary Fig.4.3.5). Similar to the occurrence of prosystemin genes encoding the systemin signal described earlier[172], the occurrence of SYR1 and SYR2 receptors seem to be restricted to the *Solanoideae* subfamily. In contrast, only single SYR-like genes seem present in representative species of the sister subfamily *Nicotianoideae* and in other higher plants. Although forming distinct groups, the SYR and SYR-like genes are close relatives of the PEPRs, receptor kinases which also recognize endogenous peptides as danger signal[173,174].

Leaves of potato and pepper respond to systemin much like tomato leaves (Supplementary Fig.4.3.6a). We cloned the SYR1 and SYR2 homologs of potato and pepper for expression and functional assessment in leaves of *N. benthamiana*. As observed for SYR1 from tomato, the SYR1 homologs of potato and pepper conferred high sensitivity to systemin with a ROS response triggered with an EC_{50} of ~30 pM (Supplementary Fig. 4.3.6b and c). Similarly, the SYR2 homologs of potato and pepper resembled SYR2 from tomato and significant ROS induction was only observed with concentrations of >10 nM systemin. Conservation of SYR1 and SYR2 pairs in these plants might hint at a role of SYR2 as low-affinity receptor for fine-tuning of systemin responses or, alternatively, at a role in the perception of a different, perhaps systemin-related, ligand. Similarly, one might hypothesize that the SYR-like receptors occurring in many plants species might serve as receptors for endogenous signal peptides. Importantly, however, SYR1 has an unequivocal function as systemin receptor and SYR1 is sufficient to confer high sensitivity to systemin to cells of *N. benthamiana* and *A. thaliana* that contain no SYR1 or SYR2.

In order to test for direct interaction of systemin with the receptor candidates, systemin derivatives were labeled with an acridinium ester for sensitive detection via chemiluminescence[153] or with biotin for detection via streptavidin, respectively. While previous work showed that modification of the N-terminus leads to strong reduction of biological activity, modification at the C-terminal end had less severe effects[175,176]. Compared to systemin, the two C-terminally modified peptides systemin-acri and systemin-biotin both showed somewhat reduced biological activity on SYR1 and SYR2 (Supplementary Fig.4.3.7 and Table 4.3.1) but their specific binding to SYR1 could be readily detected (Fig. 4.3.2a). Systemin-acri shows binding to immunoprecipitates of SYR1 but not to immunoprecipitates of SYR2 or SR160/BRI1, respectively. The binding of the labeled systemin to SYR1 was competed in a concentration-dependent manner, reaching 50% inhibition (IC_{50}) at ~6 nM with systemin (Fig. 4.3.2b, Supplementary Table 4.3.1). No competition of binding was observed with the structurally unrelated peptide AtPep1. In good agreement with their respective biological activity as weaker agonists or competitive antagonists of the systemin response[175,176], the systemin derivatives systemin-Ala17, systemin-Ala13 and systemin1-14 competitively inhibited binding of systemin-acri (Fig. 4.3.2b, Supplementary Table 4.3.1).

To examine the interaction of the receptor proteins with the ligand, the systemin-biotin derivative was used in affinity-crosslinking experiments *in planta*. *N. benthamiana* leaves expressing the GFP-tagged receptors were first incubated with the systemin-biotin derivative, either alone or together with an excess of non-modified systemin, and, subsequently, with a chemical crosslinker. When analyzed for the presence of biotin, immunoprecipitates of SYR1 showed clear labeling which was absent in samples treated with an excess of non-modified systemin (Fig. 4.3.2c), indicating direct and specific interaction of systemin with SYR1. In contrast, specific crosslinking of systemin-biotin was not detectable under these conditions with either SYR2 or SR160/BRI1, respectively.

Overall, affinity of SYR2 appeared to be too low for detection in binding assays with the compromised C-terminally modified systemin as a ligand. On the contrary, binding with SYR1 clearly demonstrates that this protein acts as a specific, high-affinity receptor for systemin.

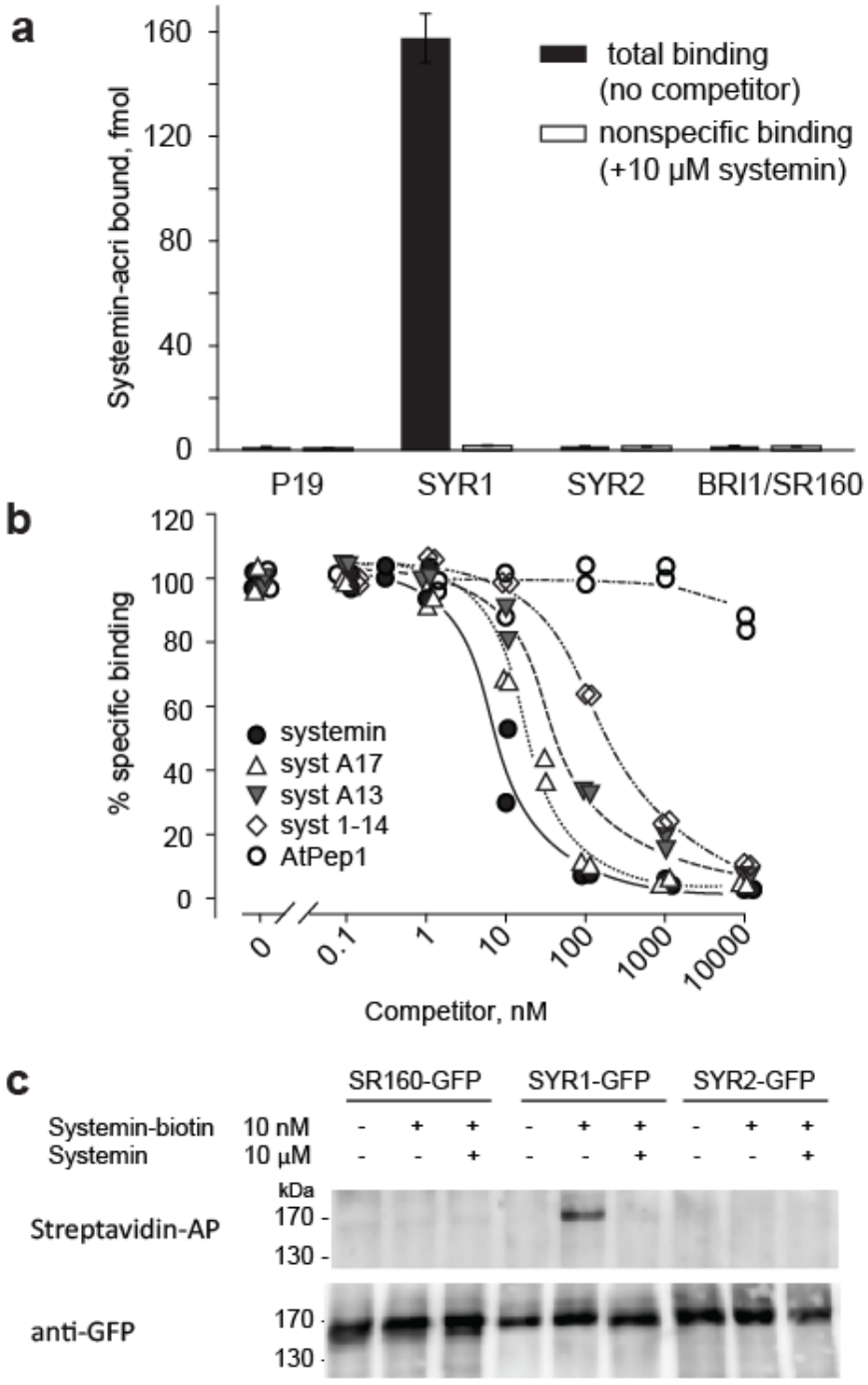


Figure 4.3.2. SYR1 binds systemin with high affinity and specificity. **a**) Competitive binding assays with receptor proteins adsorbed to immunobeads via their GFP-tags. Specific binding is the difference between binding of 1 nM systemin-acri in the absence (total binding) and presence of 10 µM non-modified systemin (nonspecific binding). Bars and error bars show mean ± SD of n = 3 replicates. **b**) Competitive binding assays with SYR1 on immunobeads and various concentrations of different peptides in duplicates as indicated. **c**) Affinity-crosslinking of systemin-biotin with receptor proteins *in planta*. Solubilized proteins were immunoprecipitated and analyzed for GFP-tagged (lower panel) and biotinylated proteins (upper panel). Data are representative for at least three independent experiments.

In order to study whether the high-affinity receptor SYR1 alone can restore systemin perception in tomato plants we produced lines of IL3-3 stably transformed with *SYR1*. Two such transgenic lines were tested and both responded to systemin like tomato wildtype plants, as exemplified by the induction of ethylene (Fig. 4.3.3a) and the induction of the proteinase inhibitor gene *PIN1* (Fig. 4.3.3b). Thus, IL3-3 and these transgenic lines, differing only in the transgene *SYR1*, provide a suitable experimental model to study and revisit the physiological function of systemin perception. Systemin and its perception in tomato plants was originally implicated in local and systemic wound responses. However, we observed that mechanical wounding caused local and systemic induction of the *PIN1* gene irrespective of the presence or absence of SYR1 (and SYR2) in the tomato and IL3-3 plants (Fig. 4.3.3c). These results are in line with reports which favor other long distance signals for systemic wound responses such as jasmonic acid, H_2O_2, hydraulic-changes or electric-waves[177-182].

Contribution to resistance against chewing insect larvae in tomato was another important function associated with the expression of the prosystemin gene in tomato[183]. We performed feeding assays, using larvae of the generalist herbivore *Spodoptera littoralis*. Larvae on IL3-3 plants gained significantly more mass compared with the ones that fed on tomato wildtype or IL3-3 plants complemented with *SYR1* (Fig. 4.3.3d), demonstrating that systemin perception contributes to resistance of tomato plants against insect herbivory.

In conclusion, systemin perception in species of the *Solanoideae* subfamily depends on the presence of the SYR1/SYR2 pair of receptors. Whether SYR2 is a low affinity receptor or has a paralogous function as receptor for a different ligand remains to be studied. SYR1, however, acts as a high-affinity, *bona fide* systemin receptor. Our results further show that presence of a functional SYR1, while not the decisive factor for the wound response, plays an important role in resistance to herbivorous insects such as the generalist *S. littoralis*. The systemin receptor at hand will now allow for approaches to elucidate the physiological roles, the evolutionary origin and the adaptive value of this highly sensitive and specific receptor-ligand pair.

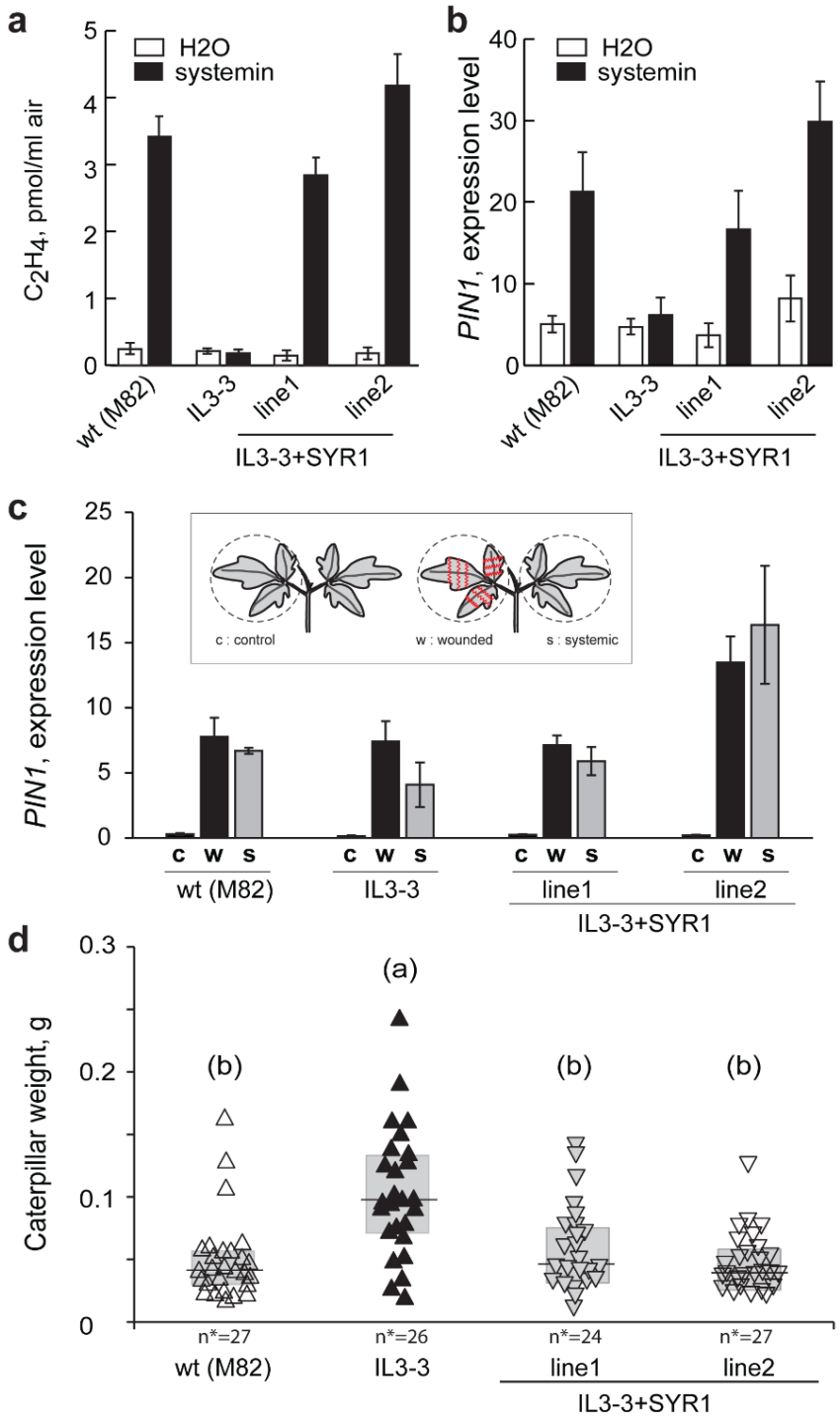


Figure 4.3.3. Systemin perception is not essential for wound responses but contributes significantly to resistance against herbivory by insect larvae of S. littoralis. a) and b) Expression of *SYR1* in IL3-3 restores induction of ethylene and expression of PIN1 to systemin. Expression levels are relative to the level of EF1α. Bars and error bars represent mean ±S.D. of n = 4 biological replicates. c) Local and systemic induction of *PIN1* mRNA after wounding also occurs in the absence of SYR1. Leaves from control plants (c) and leaves from treated plants, separated into wounded leaves (w) and systemic leaves (s), respectively, were assayed for expression of *PIN1* mRNA as in b). d) Weight of S. littoralis larvae after feeding for 7 days. Shaded boxes with horizontal lines indicate quartiles and medians, different letters statistical significance at the $p < 0.01$ level (T-test). Data from one representative experiment is shown; however, significant difference between IL3-3 and the lines with functional systemin perception was observed in 4 independent experiments with 10 plants per genotype and 3 caterpillars per plant. n* indicates the number of larvae recovered and weighed at 7 d. Data are representative for at least three independent experiments.

4.3.3 Methods

Plant material and growth conditions

Tomato (M82), potato (*Solanum tuberosum* L. cv. Désirée), hot pepper (*Capsicum annuum* cv CM334), and *Nicotiana benthamiana* plants were maintained in greenhouse with a 14-h photoperiod and a 25°C /19°C day/night program. Introgression lines obtained from crosses between *Solanum lycopersicum* cv M82 and *Solanum pennellii*[140,141] were provided by the Tomato Genetics Resource Center (UC Davis; http://tgrc.ucdavis.edu/). *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown at 22°C with an 8-hour photoperiod in growth chambers.

Peptides

Peptides were synthesized by standard FMOC technology or ordered from GenScript. Derivatization with acridinium ester or biotin was performed according to the method described before[153].

Bioassays with plant tissue or Arabidopsis protoplasts

Ethylene and ROS measurements were conducted as described[155], except for the substrate solution in the ROS burst assay, which contained 20 µM L-012 (Wako) and 2 µg/ml horseradish peroxidase (Applichem). Transformation of *Arabidopsis* mesophyll protoplast and monitoring of the *pFRK1::Luciferase* reporter[154] were done as described[137].

Treatment of tomato seedlings with peptides

Tomato seedlings (~18 days after sowing (DAS)) were cut at their base and fed with 100 nM systemin (or water as a control) via the transpiration stream for one hour. Plantlets were then transferred to water and leaves of 3 plants per replicate sample were analyzed after further incubation for 8 hours.

Wounding assays

Plantlets with two fully expanded leaves were used for experiments (~18 DAS). The three leaflets of one leaf were pinched with a hemostat twice across the midrib (time zero). Three hours later, the same leaflets were wounded once more. Wounded and systemic leaves (second leaf on the same seedling) and leaves from non-treated seedlings were analyzed for PIN1 mRNA 12 hours after the first wounding.

Cloning of receptor candidates

Receptor candidates were amplified from genomic DNA isolated from tomato (M82), pepper (CM334), or potato (Désirée), respectively, using Phusion Hot Start II DNA polymerase (Thermo Fisher Scientific) and gene specific primers indicated in Supplementary Table2. PCR products were first cloned into pENTR/D-TOPO (Invitrogen) and then integrated between the Cauliflower Mosaic Virus (CaMV) *35S* promoter and a C-terminal GFP tag into pK7FWG2.0 (transient expression) or pB7FWG2.0 expression vectors (https://gateway.psb.ugent.be). All constructs were verified by Sanger sequencing.

Plant Transformation

Agrobacteria-mediated transient expression of receptor candidates in *N. benthamiana* was performed as described [155]. Leaf pieces for ethylene and ROS assays were cut 1 day after agro-infiltration, floated on water and used the following day. Plant material for binding assays was harvested 2 days after agro-infiltration. Stable transformation of the introgression line IL3-3 was conducted according to[184]. In brief, cotyledon segments of 10-day-old seedlings were co-cultivated for 2 days with *A.tumefaciens* strain GV3101 containing *p35S::SYR1:GFP* in pB7FWG2.0 with BASTA resistance. R Resistant plants were regenerated from calli and tested for segregation of resistance and systemin response. Homozygous progeny (T2) of lines 1 and 2, representing two independent transformation events, were selected for further experiments.

Binding assays

Binding with the acridinium-labeled peptide ligand and receptor proteins immunoadsorbed to anti-GFP beads was done as previously described[137]. For affinity-crosslinking, *N. benthamiana* transiently expressing *SYR1-GFP*, *SYR2-GFP* or *SR160-GFP* under the 35S promoter were infiltrated with 10 nM systemin-biotin alone or 10 nM systemin-biotin together with 10 µM systemin for 2 min, followed by infiltration with 2 mM crosslinker EGS (ethylene glycol bis(succinimidyl succinate)) in 50 mM HEPES (pH 7.5). Leaf samples were harvested 30 min later and ground into fine power in liquid nitrogen. Solubilized proteins from 300 mg leaf material were immunoprecipitated using GFP-trap (Chromo Tek, IZB Martinsried, Germany) as described[137]. Protein blots were developed either with anti-GFP antibodies (Torrey Pines Biolabs) or a Streptavidin-alkaline phosphatase conjugate (Roche) for biotin detection.

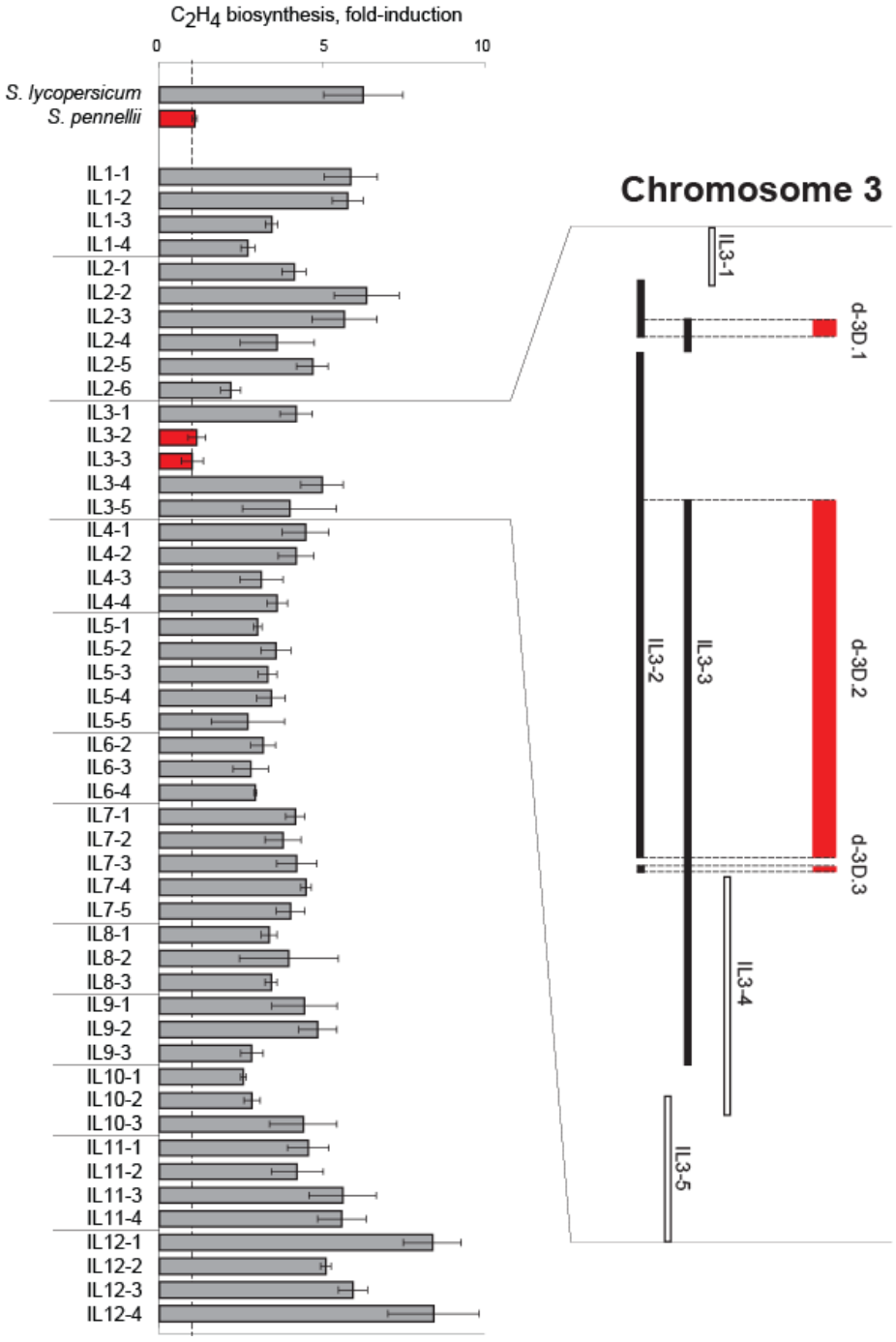
RNA extraction, RT-PCR and qRT-PCR

Total RNA extraction from tomato leaves was done with the RNeasy Plant mini Kit (Qiagen). After DNA digestion (DNase I, Thermo Fisher Scientific), total RNA was used as template to generate cDNA (First Strand cDNA Synthesis Kit, Thermo Fisher Scientific). Semi-quantitative reverse transcription (RT) PCR and real time qRT-PCR were performed with the primers listed in SupplementaryTable2 and levels of expression were expressed relative to *EF1α* used as an internal standard.

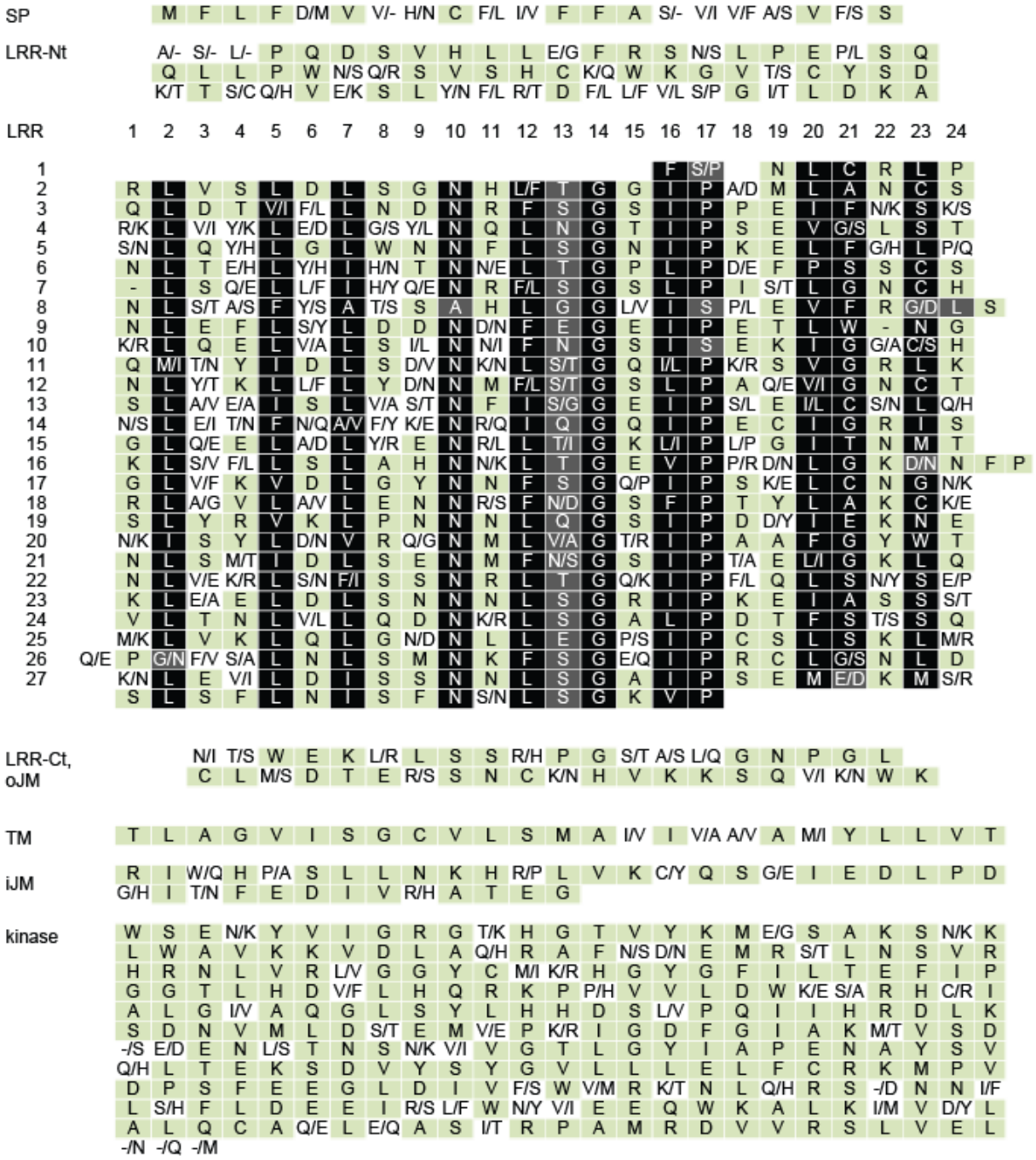
Insect feeding assay

First instar larvae of *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) were prepared as described[185]. Larvae were placed on 4 weeks old plants (3 larvae per plant, ~3 mg fresh weight each) and individual plants were contained within polyethylene bags. Larval weight was determined 7 days later.

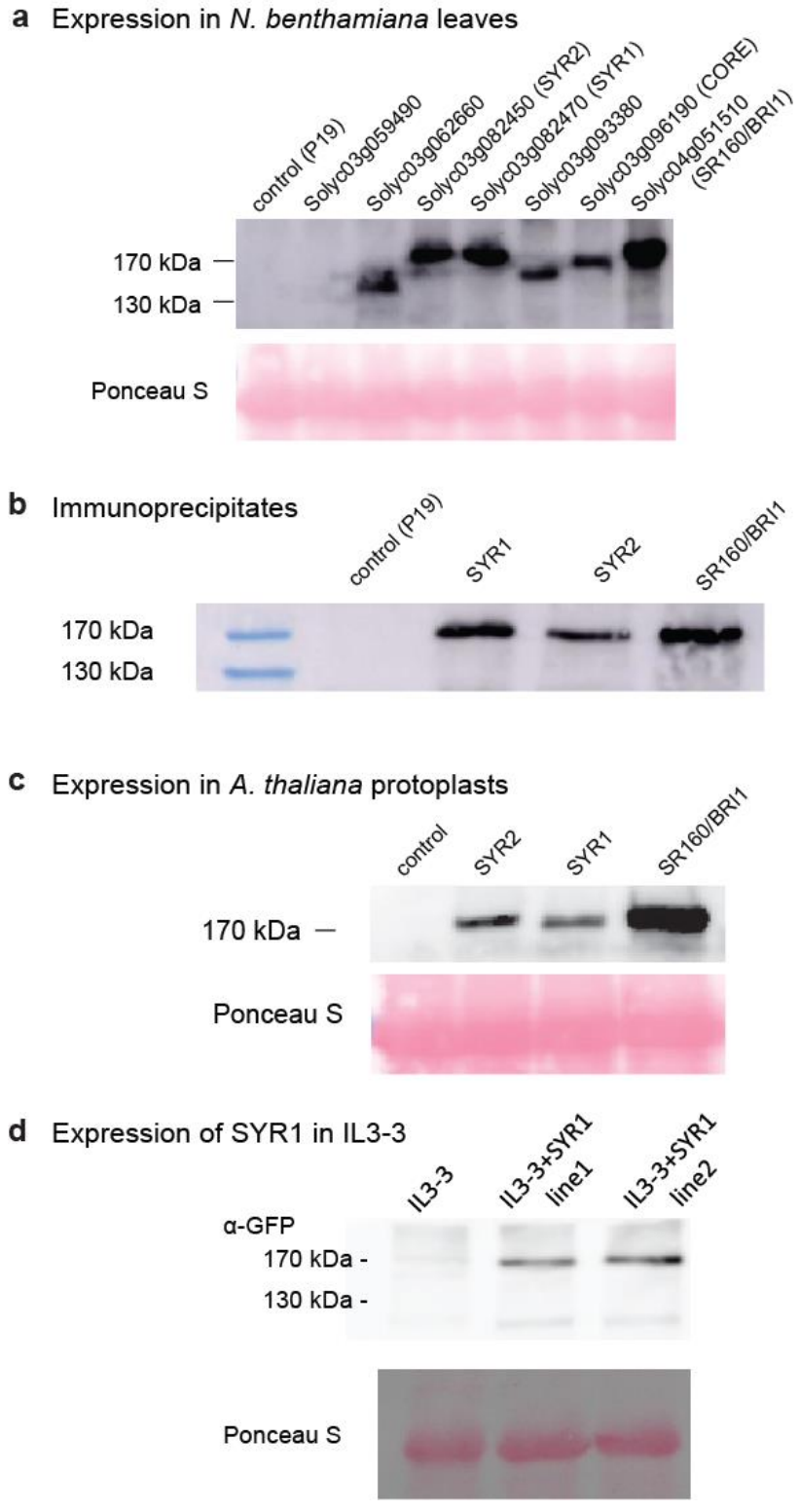
4.3.4 Supplementary Data



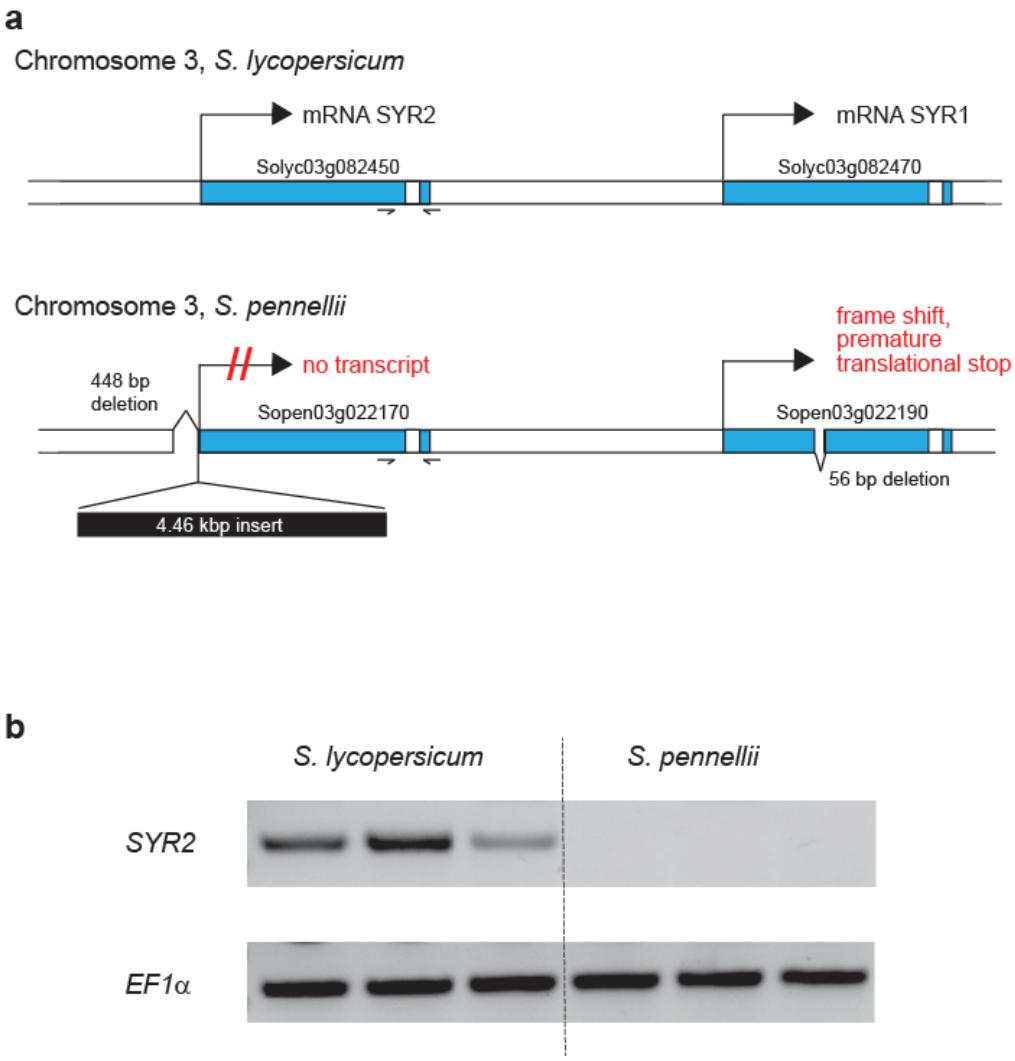
Supplementary Figure 4.3.1. Systemin-dependent induction of ethylene biosynthesis in introgression lines derived from a cross of cultivated (*S. lycopersicum*) and wild tomato (*S. pennellii*)[140,141]. Left panel: Ethylene biosynthesis (fold-induction over H_2O controls) in leaf slices treated for 3 h with 100 nM systemin. Bars and error bars show fold-induction as mean ± S.D. of n = 3 replicates. Ethylene production in controls treated with H_2O ranged from 0.11 to 0.6 nmol C_2H_4 per g of fresh weight. Right panel: Schematic representation of the introgressions of the genomic parts of *S. pennellii* in chromosome 3 of *S. lycopersicum* as described in the DRYAD repository (http://dx.doi.org/10.5061/dryad.rm5v5). The introgressions common to IL3-2 and and IL3-3 (d-3D, red bar) comprise ~1200 annotated genes.



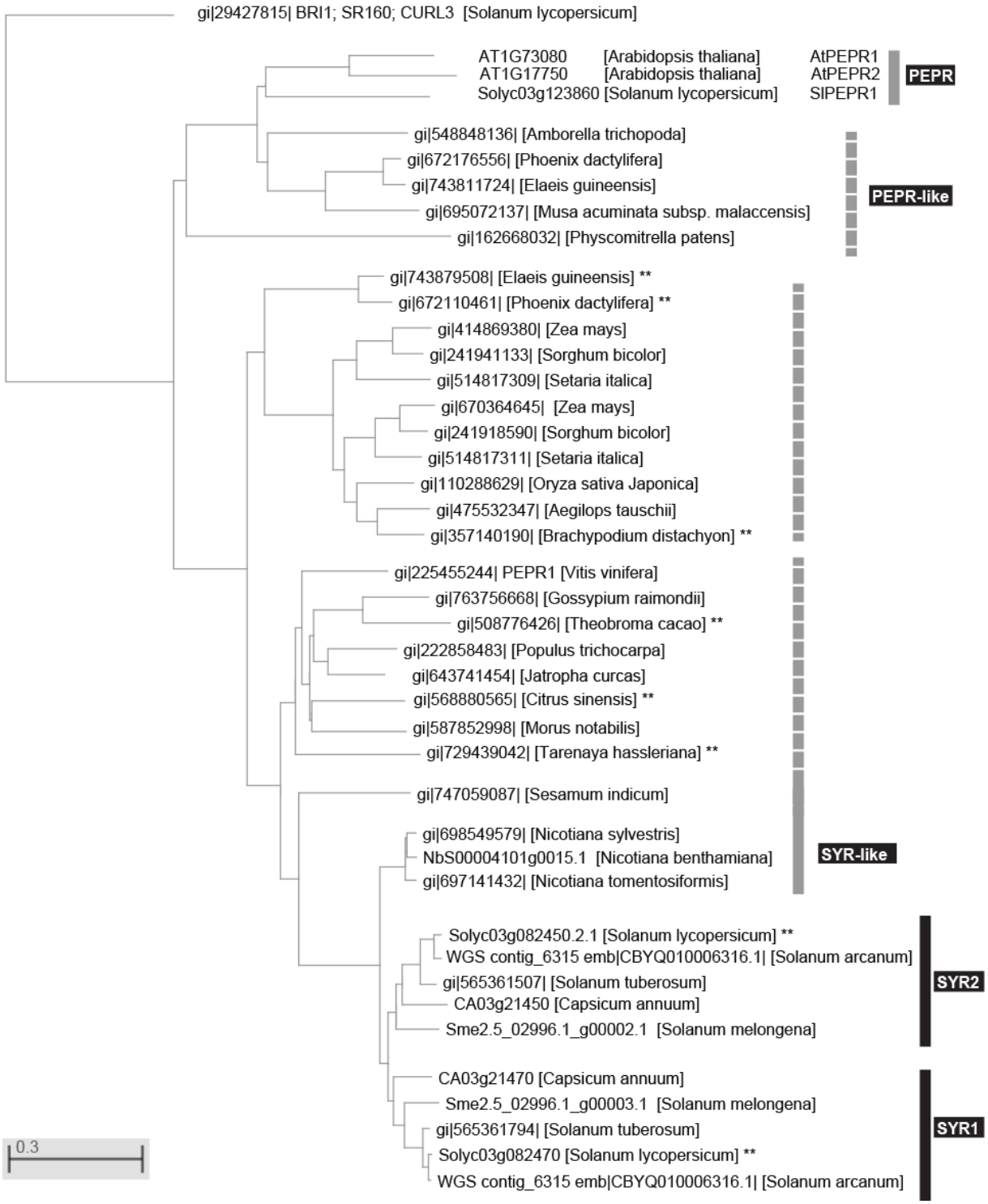
Supplementary Figure 4.3.2. Comparison of the primary structures of the LRR receptor kinases encoded by Solyc03g082450 (*SYR2*) and Solyc03g082470 (*SYR1*). Amino acids in the LRR domain predicted to form the solenoid structure according to the 24 aa plant LRR consensus xLxxLxLxxNxLS/TGxIPxxLGxLx (with other non-hydrophobic aa occasionally substituting for L) are indicated with white letters on black underlay. Single letters (green underlay) indicate positions with identical amino acids, two letters separated by "/" indicate divergent aa residues, respectively. SP, signal peptide for export via ER; LRR-Nt and LRR-Ct, domains with predicted C-C disulfide bridges that form N- and C-terminal ends of the LRR domain; oJM, outer juxtamembrane domain; TM, transmembrane domain; iJM, inner juxtamembrane domain.



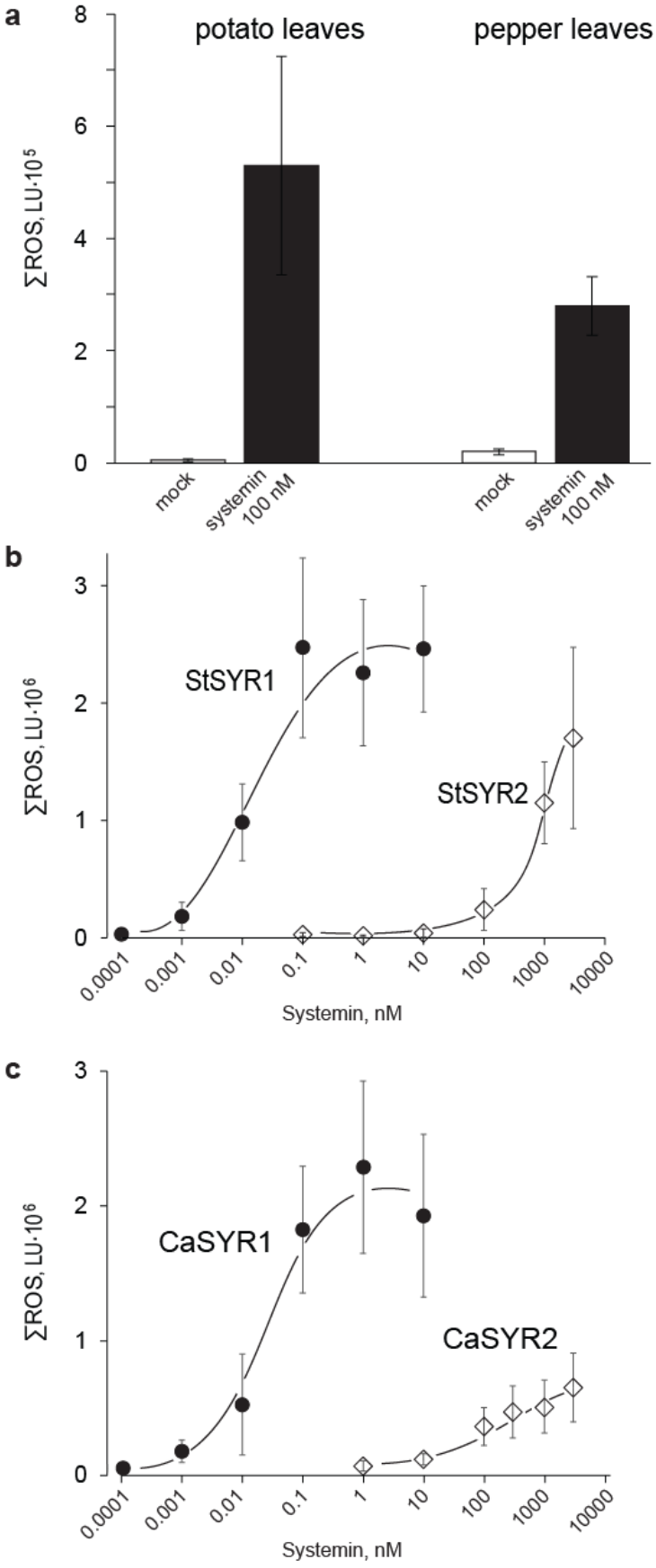
Supplementary Figure 4.3.3. Expression controls for receptor constructs expressed in *N. benthamiana* leaves (**a** and **b**), in *A. thaliana* protoplasts (**c**) or in stably transformed IL3-3 plants (**d**). Western blots were developed with antibodies against the GFP-tag present on the receptor constructs. Ponceau-S staining shows equal loading of proteins on blots with crude extracts (**a**, **c** and **d**).



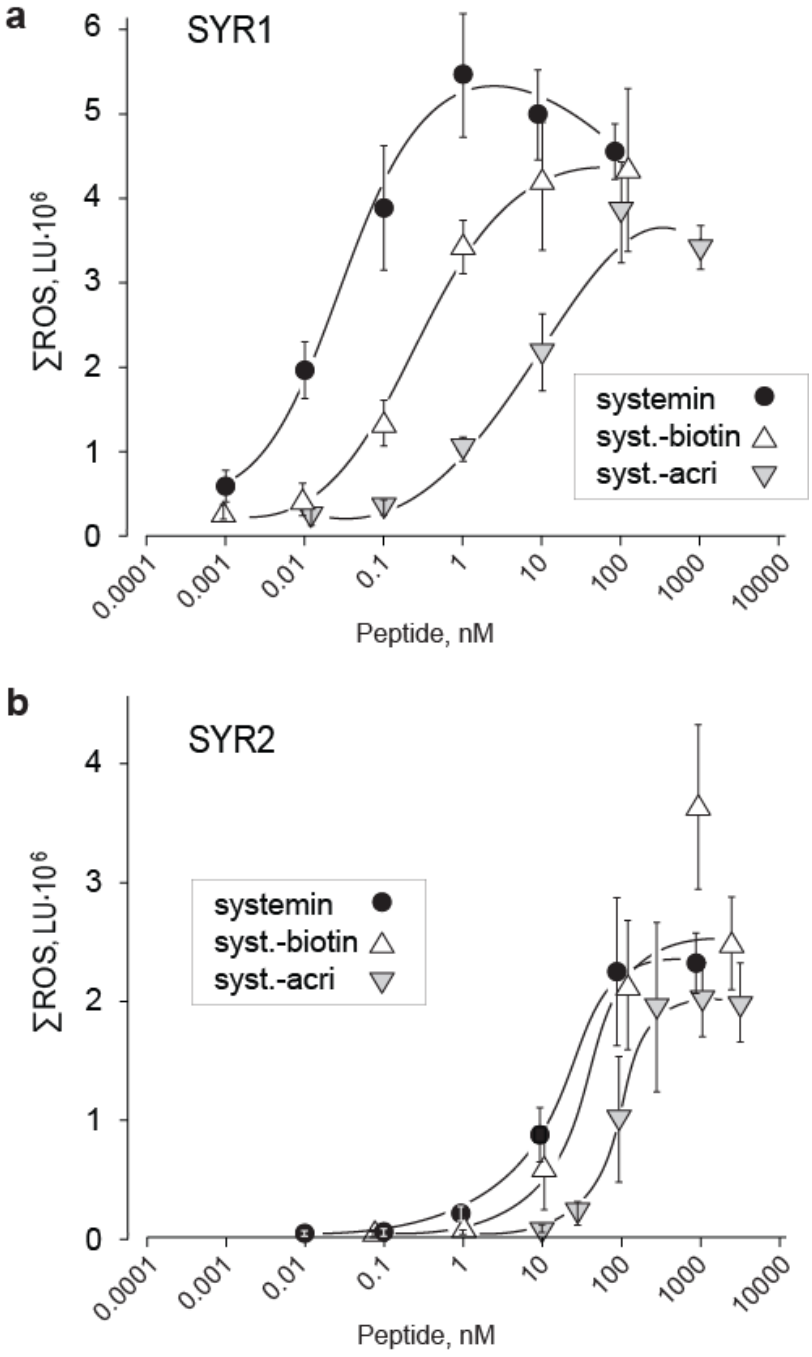
Supplementary Figure 4.3.4. *S. pennellii* lacks responsiveness to systemin, indicating a lack of the SYR1 and SYR2 receptors. **a**) Schematic representation of the region in chromosome 3 encoding SYR1 and SYR2 in *S. lycopersicum* and *S. pennellii*. In comparison to Solyc03g082470, the Sopen03g022190 gene has a 56 bp deletion that leads to a frameshift and premature stop of the translation of *SYR1*. In comparison to Solyc03g082450, the coding region of Sopen03g022170 shows little alteration but its 5´upstream promoter region exhibits major rearrangements. **b**) RT-PCR of *SYR2* using a primer pair (indicated by half arrows) amplifying 144 bp of the cDNA spanning the small intron in the 3´region of *SYR2* shows the presence of *SYR2* mRNA in *S. lycopersicum* but not in *S. pennellii*.



Supplementary Figure 4.3.5. Phylogenetic tree (Cobalt) established with sequences most closely related to SYR1 in different plant species. Sequences were obtained by BLAST searches with SYR1 in public databases (PubMed/NCBI, Solgenomics). ** sequences that are annotated as PEPR or PEPR-like in the databases that are better grouped as SYR and SYR-likes.



Supplemental Figure 4.3.6. Systemin perception in potato and pepper by the SYR1 and SYR2 homologs of potato (*S. tuberosum*) and pepper (*Capsicum annuum*). **a**) Non-transformed leaves of potato and pepper respond with ROS production to treatment with (tomato-)systemin. **b**) and **c**) Dose-dependent systemin-induced production of ROS as integral over 30 min in *N. benthamiana* leaf pieces expressing *StSYR1* or *StSYR2* and *CaSYR1 or CaSYR2*, respectively. Values and bars indicate mean ± S.D. of n = 6 replicates.



Supplementary Figure 4.3.7. C-terminal modifications of systemin affect their efficiency to stimulate responses via SYR1 and SYR2. **a**) and **b**) ROS inducing activity of systemin analogs tested with *N. benthamiana* transiently expressing *SYR1 or SYR2*, respectively. Total ROS is shown as integral over 30 min. Values and bars indicate mean ± S.D. of n = 6 replicates.

		SYR1		SYR2
		EC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)
systemin-acri	AVQSKPPSKRDPPKMQTD-NNKLK-acri	10	n.d.	200
systemin-biot	AVQSKPPSKRDPPKMQTD-NNKLK-biotin	0.2	n.d.	30
systemin	**AVQSKPPSKRDPPKMQTD**	**0.02**	**6**	**20**
syst.-A17	AVQSKPPSKRDPPKMQAD	antagonist	15	n.d.
syst.-A13	AVQSKPPSKRDPAKMQTD	1000	50	n.d.
syst.1-14	AVQSKPPSKRDPPK	antagonist	200	n.d.
AtPep1	ATKVKAKQRGKEKVSSGRPGQHN	>10´000	>10´000	>10´000

n.d. : not determined

Supplementary Table 4.3.1. Sequence and specific activities of peptides used in this study. EC_{50} values indicate concentrations required for induction of half maximal ROS production in *N. benthamiana* leaves expressing either SYR1 or SYR2, respectively. IC_{50} values indicate the concentrations of peptide required to reduce binding of systemin-acri to SYR1 by 50% as deduced from binding competition experiments shown in Fig. 2B.

Gene	Usage	Primer name	Primer sequence (5' – 3')
Solyc03g059490	cloning	059490 Fw	CACCATGGGTCGTTGTTGTTTTGTC
		059490 R	ACACGACGGGGGTTGAAGTTGC
Solyc03g062660	cloning	062660 Fw	CACCATGACAGTACAGTGTATTG
		062660 R	AGAATGAGTATGGGTGGAAG
Solyc03g082450 (*SYR2*)	cloning	082450 Fw	CACCATGTTCTTGTTTATGGTAA
		082450 R	CATCTGATTTAACTCGACAAG
Solyc03g082470 (*SYR1*)	cloning	082470 Fw	CACCATGTTCTTGTTTGATGTTG
		082470 R	TAACTCGACAAGAGACCTTAC
Solyc03g093380	cloning	093380 Fw	CACCATGGATTTCAAGTTACAA
		093380 R	AAATTTGTTTGAGAAATTGC
Solyc03g096190 (*CORE*)	cloning	096190 Fw	CACCATGATTCTCCCAAAGAATTCTC
		096190 R	TAACTTTTTCTTCCGGTATGCTTG
Solyc04g051510 (*SR160*)	cloning	SR160 Fw	CACCATGAAAGCTCACAAAACTG
		SR160 R	AAGGTGTTTGCTCAGCTCATTG
CA03g21450 (*CaSYR2*)	cloning	CaSYR2 Fw	CACCATGTTATTGTTTTTGGTTAATTG
		CaSYR2 R	AAGTTTCATTTCTCCTCTTTTACATATATG
CA03g21470 (*CaSYR1*)	cloning	CaSYR1 Fw	CACCATGTTCTTGATTCTGGTTAATTG
		CaSYR1 R	TTTATTCTCTTTACATCTTTTATTTAATTCGA
gi|565361794| (*StSYR1*)	cloning	StSYR1 Fw	CACCATGTTCTTGTTTGATCTTGTTC
		StSYR1 R	TAACTCGACAAGAGACCTTAC
gi|565361507| (*StSYR2*)	cloning	StSYR1 Fw	CACCATGTTCTTGTTTCTGGTTAATTG
		StSYR1 R	CATCTATGATTTAACTCGACAAGAG
SYR2	RT-PCR	SYR2 q Fw	AGTATCAGACTCTGATGAAAACTCG
		SYR2 q R	CAGAAGAGCTCCAGTAGAAGAAC
EF1α	RT-PCR /qRT-PCR	EF1α q Fw	CTCCGTCTTCCACTTCAGG
		EF1α q R	TCAGTTGTCAAACCAGTAGGG
PIN1	qRT-PCR	PIN1 q Fw	GCAACTTCCTTTGAAACTCTC
		PIN1 q R	GTACACCAATAAGTTCTGGCC

Supplementary Table 4.3.2. List of primers.

4.4 General discussion

Making use of natural variation of molecular pattern sensing in tomato species and a collection of tomato introgression lines, we identified two LRR-RKs as novel PRRs, namely CORE as the receptor for bacterial CSPs and SYR1 as the receptor for systemin. Extopic expression of *CORE* in plants insensitive to csp22 like *N.benthamiana*, *Arabidopsis* and the tomato IL3-2 and IL3-3 is sufficient to confer CSP sensitivity that matches the one observed in tomato (Fig. 4.2.2, Fig 4.2.5 and Supp. Fig.4.1.1). Similarly, expression of *SYR1* alone is sufficient to confer systemin sensitivity to the insensitive *N.benthamiana*, *Arabidopsis* and the tomato IL3-3 (Fig. 4.3.1 and Fig. 4.3.3). In addition, CORE and SYR1 could be demonstrated to bind their respective ligands with high specificity and affinity (Fig. 4.2.4 and Fig. 4.3.2), matching the sensitivity observed in tomato[98,114]. SYR2, a close homolog of SYR1, is also functional when expressed in insensitive plants, yet no obeservable sytemin binding activity can be detected. Thus, SYR2 is only taken as a putative systemin receptor.

Interestingly, both CORE and SYR1 are the second receptors reported for their respective ligands. *Nb*CSPR, a LRR-RLP shares no obvious similarity with CORE, was rescently reported to be required for CSP responsiveness in *N. benthamiana* and able to confer CSP responsiveness to *Arabidopsis*[136]. Similarly, SR160/*Sl*BRI1, a receptor protein distinct from SYR1, was previously implicated to recognize systemin and mediate systemin triggered immunity[100,167]. However, in our hands, no binding activity of *Nb*CSPR and SR160 with their respective ligands could be observed (Fig. 4.2.4 and Fig. 4.3.2). Futhermore, we found that *Nb*CSPR is neither sufficient nor essential for csp22 mediated signaling (Fig. 4.2.5 and Supp. Fig. 4.2.4). We also found that SR160 is not sufficient for systemin sensing (Fig. 4.3.1). Based on these observation, we would rather conclude that *Nb*CSPR is not CSP receptor and SR160 is not systemin receptor.

What defines a genuine receptor? By definition, a receptor is the site that specifically interacts and binds a signal and transduces this signal into a physiological response. However, in addition to receptors, co-receptors or other components involved in ligand processing can also associate with the ligands, as exemplified by TOO MANY MOUTHS, a co-receptor with binding affinity for the EPF peptide[35]. It is also possible that proteins associated with receptor complexes interact with a ligand indirectly. In

either case, binding affinity, specificity and the capability to transmit signals should be checked to determine the role of ligand binding proteins. EFR, one of the best studied receptor, may serve as a good example of genuine receptor. EFR binds its ligand elf18 with high specificity and affinity, with a Kd of 0.8 nM[52], matching the sensitivity obeserved in *Arabidopsis* plants[51]. Furthermore, heterologous expression of EFR alone in elf18 nonresponsive rice can confer full elf18 sensitivity[186]. Like EFR, CORE and SYR1 clearly fulfil these criteria and act as genuine receptors for their respective ligands.

Sensing the same ligand via two different receptors has been reported beofore. In Arabidopsis, CERK1 and LYK5, two distinct LysM-RKs, have both been reported to bind chitin albeit with clearly different affinities[89-91]. *At*CERK1 and *At*LYK5 are both invloved in chitin induced immunity. Arabidopsis *cerk1* mutant is completely insensitve to chitin[89,91], while *lyk5* mutant has a severly impaired chitin response[91]. Interestingly, LYK5 associates with CERK1 in a chitin-dependent manner[91]. It seems that chitin perception is regulated by a complex including CERK1 and LYK5. It is also possible that CSP perception is regulated by a receptor complex including CORE, BAK1(Figure 4.2.3) and other components. However, *Nb*CSPR is not likely one of them, since we did not find *Nb*CSPR in a protein complex pulled down via NbCORE. The role of SR160/BRI1 as BL receptor has been well examined[7,8,102,168]. Although we conclude it is not the systemin receptor, we do not exclude its possible function in immunity.

The observation that SYR2, a SYR1 homolog without clear systemin binding activity, can also confer systemin sensitivity (Fig 4.3.1 and Fig 4.3.2), again shows the complexity and redundancy of signal sensing by cell surface receptors. Similar cases have been obeserved before. CLV2, a LRR-RLP with no obvious CLV3 binding activity[187], can transmit CLV3 signal in parallell to CLV1[24]. *At*LYK4, in addition to AtCERK1, is invloved in chitin signaling, athough it does not bind chitin directly[91]. Some other receptors can function redundantly both in ligand binding and signal transduction, and they are very often close homologs. Both PEPR1 and PEPR2 can bind Pep1 and Pep2 to transmit signals[79,80], although PEPR1 alone is responsible for sensing Pep3-7[79]. Other plant peptides including PSK[15,16], CEPs[65] and IDA[33,34] are also perceived by mutiple receptors.

Identification of novel PRRs like CORE further contributes to the source of new disease resistance traits for crop breeding. Several studies have demonstrated that interfamily

transfer of a PRR can confer responsiveness to an otherwise inactive MAMP/DAMP[188]. Heterologous expression of *AtEFR* leads to increased resistance to *Ralstonia solanacearum* and *Xanthomonas perforans* in tomato[189], enhanced bacterial leaf blight and bacterial brown stripe resistance in rice and enhanced bacterial halo blight resistance in wheat[186,190,191]. In our hands, expression of tomato CORE can confer *Arabidopsis* increased resistance to *Pseudomonas syringae* pv. *tomato* DC 3000 (Fig. 4.2.6), demonstrating its biotechnological potential in generating bacterial disease resistant crops.

The identification of SYR1 answers the long-standing question of how systemin is perceived. With the *bona fide* receptor at hand, more research can now be conducted to explore the elements and physiological function of systemin perception. Many systemin triggered immune responses are same as those observed in PTI. Study of elements involved in systemin signaling pathways will also provide insights into understanding factors that determine the generality and specificity of plant innate immunity. Since the discovery of systemin, many more bioactive plant peptides have been characterized as signals, yet systemin still stays as an outlier, as it exists solely in the Solanoideae subfamily. Given the fact that many other bioactive peptides exist as multi-copy families, searching for systemin-like peptides and dissecting their mechanism of expression, processing, recognition and regulation will extend our knowledge about evolution of plant peptide signaling.

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6 Abbreviations

BAK1	Brassinosteroid insensitive 1-Associated receptor Kinase 1
BAM	Barely Any Meristem
BL	Brassinolide
BRI1	Brassinosteroid Insensitive 1
BUPS	Buddha’s Paper Seal
CBEL	Cellulose-Binding Elicitor Lectin
CEBiP	Chitin-Elicitor Binding Protein
CEP1	C-terminally Encoded Peptide 1
CEPR	C-terminally Encoded Peptide Receptor
CERK1	Chitin Elicitor Receptor Kinase 1
CIF	Casparian strip Integrity Factors
CLEL	CLAVATA3/Embryo surrounding region-related (CLE)-Like
CLV1	CLAVATA1
CLV2	CLAVATA2
CLV3	CLAVATA3
CORE	Cold shock protein Receptor
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CSP	Cold Shock Protein
CSPR	Receptor-like protein Required for CSP22 Responsiveness
CuRe1	Cuscuta Receptor 1
DAMP	Damage-Associated Molecular Pattern
DORN1	Does not Respond to Nucleotides 1
eATP	Extracellular ATP
EFR	EF-Tu Receptor
EF-Tu	ElongationFactor Thermo unstable
EGF	Epidermal Growth Factor
EIX	Ethylene-Inducing Xylanase
eMax	enigmatic MAMP of *Xanthomonas*

EMS	Ethyl methanesulfonate
EPF	Epidermal Patterning Factor
ER	ERECTA
ERL1	ERECTA-Like1
FLS2	Flagellin Sensing 2
FLS3	Flagellin-Sensing 3
GLV	GOLVEN
GSO	GASSHO
HAE	HAESA
HAMP	Herbivore-Associated Molecular Pattern
HSL2	HAESA-LIKE2
HypSys	Hydroxyproline-rich Systemins
IDA	Infloresence Deficient in Abscission
LPS	Lipooligosaccharide
LRR	Leucine-Rich-Repeat
LYK	LysM domain–containing receptor-like Kinases
LYM	Lysin Motif domain protein
LYP	Lysin motif-containing Protein
LysM	Lysin-Motif
MAMP	Microbe-Associated Molecule Pattern
MAPK	Mitogen-Activated Protein Kinase
MDIS 1	Male Discoverer1
MIK	MDIS1-Interacting Receptor Like Kinase
NAG	*N*-acetyl-D-glucosamine
NFP	Nod-factor Perception
NFR	Nod-factor Receptor
NLP	Necrosis and ethylene-inducing peptide 1-Like Protein
Nod	Nodulation
OG	Oligogalacturonide
PAMP	Pathogen-Associated Molecular Pattern

Pep	Plant elicitor peptide
PEPR	Plant Elicitor PeptideReceptor
PGN	Peptidoglycan
PIP	PAMP-Induced secreted Peptide
PRR	Pattern Recognition Receptor
PSK	Phytosulfokine
PSKR	Phytosulfokine Receptor
PSY1	Plant Peptide Containing Sulfated Tyrosine 1
PSY1R	Plant Peptide Containing Sulfated Tyrosine 1 Receptor
PTI	Pattern Triggered Immunity
PXY	Phloem intercalated with Xylem
RALF	Rapid Alkalinization Factor
RBPG1	Responsiveness to Botrytis Polygalacturonases1
ReMax	Receptor of eMax
RGF	Root meristem Growth Factor
RGFR	Root meristem Growth Factor Receptor
RK	Receptor Kinase
RLP	Receptor Like Protein
ROS	Reactive Oxygen Species
RPK2	Receptor-like Protein Kinase 2
SERK	Somatic Embryogenesis Receptor Kinase
SGN3	SCHENGEN3
SYR	Systemin Receptor
TDIF	Tracheary element Differentiation Inhibitory Factor
T-DNA	Transfer DNA
TDR	TDIF Receptor
WAK	Wall-Associated Kinase
Xoo	*Xanthomonas oryzae* pv. *oryzae*
XPS1	Xanthine/uracil Permease Sensing 1

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