The tyrosine-sulfated peptide receptors PSKR1 and PSY1R modulate *Arabidopsis* immune responses

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Abbreviations

amiRNA	Artificial microRNA
avr	Avirulence factor
BR	Brassinolide
CC	Coiled coil
cfu	Colony forming unit
Col-0	Columbia-0, *Arabidopsis* ecotype
DAMP	Danger-associated molecular pattern
DNA	Deoxyribonucleic acid
Ef-Tu	Elongation factor Tu
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
flg22	Active peptide from flagellin
JA	Jasmonic acid
KDa	KiloDalton
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LysM	Lysin motif
MAPK	Mitogen-activated protein kinase
OG	Oligogalacturonides
PAMP	Pathogen-associated molecular pattern
PTI	PAMP triggered immunity
Pto	*Pseudomonas syringae* pv. *tomato*
PSKα	Phytosulfokine
PSY1	Plant peptide containing sulfated tyrosine 1
pv.	Pathovar
RGF	Root meristem growth factor
ROS	Reactive oxygen species
RLK	Receptor-like kinase
RNA	Ribonucleic acid
R-protein	Resistance-protein
SA	Salicylic acid
SEM	Standard error of the mean
T3SS	Type three secretion system
TIR	Toll/interleukin-1 receptor
T-DNA	Transfer-DNA
Ws-4	Wassilewskija-4, *Arabidopsis* ecotype

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1 Introduction

1.1 Plant-pathogen interactions

In the lifetime of a plant, attack from pathogens can be expected from a variety of disease causing microorganisms such as bacteria, viruses and fungi. And yet, even though pathogens pose a continuous threat, plants defenses are seldom overcome largely due to physical barriers of the plant epidermis, antimicrobial effects of secondary compounds and by the activation of inducible defense-related responses (Hammond-Kosack and Jones, 1996). However, unlike mammals, plants lack mobile defense cells and instead rely on the innate immunity of the individual cell and signals originating from the site of infection for induction of defense programs (Jones and Dangl, 2006). These inducible defenses rely on the ability to differentiate self from non-self. Recognition of non-self is mediated by two layers of plant defense programs, the first being initiated by cell surface receptors called pattern recognition receptors that perceive pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) which are highly conserved molecules that are often essential for the fitness and/or survival of microbial organisms. This form of defense is termed PAMP-triggered immunity (PTI). Some of the earliest observable responses upon PAMP perception include the mobilization of ions across the plasma membrane, with an influx of Ca^{2+} and H^{+} as well as an efflux of K^{+} (Jabs et al., 1997; Pugin et al., 1997; Felix and Boller, 2003; Garcia-Brugger et al., 2006), the generation of reactive oxygen species (ROS) (Torres et al., 2006) and the activation of mitogen-activated protein kinases (MAPKs) (Colcombet and Hirt, 2008). Activation of MAPKs precedes the subsequent activation of transcription factors such as members of the WRKY family that ultimately drive the expression of defense-responsive genes such as *PR1* (Turck et al., 2004) and *FRK1* (Asai et al., 2002; Robatzek and Somssich, 2002; Journot-Catalino et al., 2006), camalexin (Qiu et al., 2008; Mao et al., 2011) and salicylic acid (SA) biosynthetic genes (van Verk et al., 2011) as well as RLK genes (Du and Chen, 2000). Later responses of PAMP perception includes plant cell wall fortification through the accumulation and deposition of callose, elevated levels of SA and inhibition of

seedling growth (Nicaise et al., 2009). The result of successful induction of PTI is restriction of pathogen growth and ultimately thwarted microbial attack (Boller and He, 2009). The second layer of plant defense relies on Resistance- (R-) protein-mediated detection of virulence promoting effectors utilized by pathogens in an attempt to overcome PTI and is termed effector-triggered immunity (ETI). The establishment of ETI leads to a stronger form of PTI that results in even higher levels of SA accumulation and often the onset of a type of programmed cell death known as the hypersensitive response (Jones and Dangl, 2006; Gassmann and Bhattacharjee, 2012).

1.2 Interactions between salicylate and jasmonate signaling pathways in defense responses

Upon pathogen detection, plant cells undergo reprogramming of their cellular homeostasis to prioritize defense over their normal developmental functions. The exact nature of this reprogramming has been shown to correlate with the various infection strategies employed by various pathogens. As biotrophic pathogens, such as *Hyaloperonospora parasitica*, *Pseudomonas syringae* and the turnip crinkle virus, rely on living host cells for nutrition, programmed cell death at the site of invasion is a common mechanism of plant-defense in the presence of this class of pathogens. Necrotrophs, such as *Alternaria brassicicola* and *Botrytis cinerea*, on the other hand, feed on dead tissue (Spoel and Dong, 2008). It is therefore of paramount importance that plants activate the appropriate defense response according to the type of invading pathogen; the SA-signaling pathway chiefly protects against biotrophic pathogens and viruses, whereas jasmonic acid (JA)-signaling predominantly combats necrotrophic pathogens (Vlot et al., 2009).

However, in nature, plants often are confronted with simultaneous or subsequent attack by multiple pathogens and therefore possess complex regulatory elements to balance resistance to biotrophic and necrotrophic invaders while minimizing the associated energy

costs associated with defense responses (Koornneef and Pieterse, 2008). One of the well-studied master regulators of SA signaling is Non-expressor of PR genes1 (NPR1). NPR1 localization changes from the cytoplasm to the nucleus upon SA-mediated cellular redox alterations. Once inside the nucleus, NPR1 interacts with TGA transcription factors, leading to the activation of SA-responsive genes and the deactivation of JA-inducible genes (Spoel et al., 2003). WRKY transcription factors have also been implicated in SA/JA crosstalk. WRKY70 was reported to act as a node of regulation between these two signaling pathways based on the finding that its overexpression leads to both the enhanced expression of *PR* genes and the suppression of JA-inducible *PDF1.2* (Li et al., 2004). Two additional transcription factors, WRKY11 and WRKY17, have been proposed to act as negative regulators of WRK70, adding an additional layer of control over SA/JA signaling (Journot-Catalino et al., 2006; Koornneef and Pieterse, 2008). Another player involved in the orchestration between these two signaling pathways is the MAP kinase MPK4. Petersen et al. (2000) found that *mpk4* mutants accumulated elevated SA levels, and show constitutive expression of SA-inducible genes, suppression of JA-inducible genes and enhanced susceptibility to the necrotrophic pathogen *A. brassicicola*. Furthermore, the MPK4 substrate MKS1 has been demonstrated to interact with both MPK4 and WRKY33, coupling the kinase activity of MPK4 to a defense-related transcription factor (Andreasson et al., 2005). The complex of MPK4 and WRKY33 is dependent on MKS1 and exists as a nuclear complex even in the absence of pathogens (Qiu et al., 2008). However, upon treatment with *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 or the flagellin derivative flg22, MPK4 phosphorylation of MKS1 leads to the dissociation of WRK33, allowing WRK33 to target the promoter of the antimicrobial camalexin biosynthesis gene *PAD3* (Qiu et al., 2008).

While several lines of evidence suggest that SA/JA crosstalk can fine tune plant defense responses against various pathogens, some pathogens have evolved mechanisms that manipulate these signaling pathways to promote virulence (Spoel and Dong, 2008). One well characterized example of this mode of induced susceptibility involves coronatine, a phytotoxin produced by several strains of *Pseudomonas syringae* (Mittal and Davis, 1995)

that is structurally analogous to the JA derivative JA isoleucine. It is thought that coronatine allows the biotrophic pathogen *Pseudomonas syringae* strains to alter host signaling, leading to enhanced virulence. Indeed, coronatine has been shown to promote the reopening of stomata, providing the bacteria with a route for entry into the plant. Upon the perception of PAMPs such as flg22 or lipopolysaccharide (LPS), stomatal closure is rapidly induced in an SA-dependent manner, thereby physically preventing microbial access to the apoplast for colonization (Melotto et al., 2006). However, compared to wild type, coronatine deficient *Pto* DC3000 mutants failed to reverse stomatal closure induced by PAMP perception. Furthermore, *coi1-20*, a mutant allele of the jasmonate receptor CORONATINE INSENSITIVE1 (COI1), failed to efficiently reopen stomata in response to wild type *Pto* DC3000. Together, this data demonstrates that hijacking the *Arabidopsis* JA signaling pathway via coronatine is indeed a virulence strategy employed by *Pto* DC3000 to overcome salicylate induced defense responses.

In a recent report, it was shown that the necrotrophic fungal pathogen *Cochliobolus victoriae*, the causal agent of victoria blight of oats, also exploits plant signaling to enhance virulence (Lorang et al., 2012). Pathogens typically utilize effectors to disable defense responses and experimental results show that the fungal effector victorin, a cyclized pentapeptide, physically interacts with the thioredoxin TRX-h5. Interestingly, TRX-h5 has previously been shown to be required for the redox control that regulates the conformation and activity of NPR1 (Tada et al., 2008). As an effector target, TRX-h5 is guarded by the R-protein LOV1 (Lorang et al., 2012). Binding of victorin to TRX-h5 activates LOV1 signaling and the induction of cell death, ultimately leading to susceptibility instead of resistance. The authors of this report suggest that this is likely due to the facilitation of *C. victoriae*'s necrotrophic exploitation of LOV1 induced cell death. The fact that *lov1* null mutants are resistant to *C. victoriae* corroborates these findings. It further suggests that victorin was not evolved to inhibit TRX-h5-mediated defense responses, but rather to exploit SA-associated defense responses for the promotion of necrotrophic disease susceptibility.

1.3 Receptor like kinases in plants

The best studied pattern recognition receptors belong to the *Arabidopsis* RLK/Pelle class of protein kinases which is composed of over 600 members (Shiu and Bleecker, 2003). Many of the proteins in this family are receptor-like kinases (RLKs) and approximately two thirds are predicted to have extracellular domains Shiu and Bleecker, 2003). These extracellular domains such as lysine motif (LysM) domains, epidermal growth factor-like repeats and leucine-rich repeats (LRRs) that are involved in ligand binding and influence a large number of plant processes including cell wall interactions mediated by WAK1, developmental control regulated by BRI1 and disease resistance responses associated with FLS2 activity (Shiu and Bleecker, 2003; Gish and Clark, 2011). The RLK gene family is one of the most diverse in *Arabidopsis* and the disparate nature of the extracellular domains among its members is thought to be the evolutionary result of pathogen pressure (Shiu and Bleecker, 2003; Smith et al., 2011). Despite the hyper-variability of the extracellular portions of RLKs, they typically contain a single transmembrane domain and an intracellular cytoplasmic protein kinase domain involved in the initiation of downstream signaling and responses.

1.3.1 RLKs involved in plant immunity

Plants recognize a wide range of PAMPs. PAMPs identified so far include proteinaceous molecules from bacteria such as flagellin (Felix et al., 1999), EF-Tu (Kunze et al., 2004), harpin (Lee et al., 2001a; Lee et al., 2001b) a 23 amino acid peptide corresponding to HpaG, a *Xanthomonas* harpin (Kim et al., 2004), and a 13 amino acid peptide (Pep13) conserved in transglutaminases found in oomycetes (Nürnberger et al., 1994). Non-proteinaceous PAMPs include cell wall components like fungal chitin (Felix et al., 1993), oomycete heptaglucan (Albersheim and Valent, 1978), bacterial LPS and peptidoglycans (Silipo et al., 2005; Gust et al., 2007). The mode of perception of many of these PAMPs is unknown but some have been shown to be perceived by receptors that are localized at the cell surface and are involved in the transduction of non-self-perception across the cell membrane.

Flagellin-Sensing 2 (FLS2) is one of the most prominently studied RLKs involved in plant defense-signaling (Gomez-Gomez and Boller, 2000) and was identified in a mutant screen due to its insensitivity to the PAMP flg22, a synthetic peptide derived from the most highly conserved 22 amino-acid portion of the N terminal region of bacterial flagellin (Felix et al., 1999). The molecular structure of FLS2 is a typical LRR-RLK. It consists of an N-terminal extracellular leucine rich domain with 28 repeats, a single-pass transmembrane motif, a cytoplasmic C-terminal protein kinase domain, and belongs to the LRR-RLK subfamily XII, which is populated by 10 members (Gomez-Gomez and Boller, 2000). While subsequent experiments have demonstrated that FLS2 indeed binds flg22 and the exact nature of this binding remains elusive, the LRR domains 9 to 15 have been shown to be important for FLS2 function (Dunning et al., 2007). Wild type plants pre-treated with flg22 trigger resistance responses to the bacterial pathogen *Pto* DC3000 (Gomez-Gomez and Boller, 2000; Zipfel et al., 2004). Additionally, *Arabidopsis* mutants lacking a functional FLS2 protein show susceptibility to *Pto* DC3000, indicating that PAMP perception has a salient impact on plant disease resistance (Zipfel et al., 2004).

Another well characterized PRR belonging to the LRR-RLK gene family is the receptor for bacterial EF-Tu and is referred to as EFR (EF-Tu Receptor) (Zipfel et al., 2006). Similarly to FLS2, EFR has a typical LRR-RLK structure containing 21 LRRs and has been shown to bind a minimal motif of 18 amino acids corresponding to the N-terminal portion of EF-Tu (elf18) with the binding site narrowed down to LRRs 19-21 (Albert et al., 2010). Along with FLS2, EFR belongs to the LRR-RLK subfamily XII (Shiu and Bleecker, 2003; Zipfel et al., 2006). *Arabidopsis efr* mutants show enhanced susceptibility to *Agrobacterium tumefaciens*, resulting in higher efficiency in T-DNA transformation, suggesting that EFR perception of EF-Tu reduces transformation efficiency mediated by this bacterium (Zipfel et al., 2006). Furthermore, transient expression of *EFR* in *Nicotiana benthamiana*, plants that lack an endogenous EF-Tu perception system, resulted in the conference of elf18 sensitivity, indicating that EFR is the receptor for EF-Tu (Zipfel et al., 2006).

Chitin, a structural component of fungal cell walls, as well as its partially deacetylated form chitosan, was one of the earliest described PAMPs that elicit plant immune responses (Felix et al., 1993). In *Arabidopsis*, chitin perception is mediated by CERK1 (Miya et al., 2007; Wan et al., 2008). CERK1, a plasma membrane protein with three extracellular LysM domains and a cytoplasmic kinase domain, has been shown to directly bind insoluble chitin (Iizasa et al., 2010; Petutschnig et al., 2010). *Arabidopsis* plants lacking CERK1 are more susceptible to the fungal pathogens *A. brassicicola* and *Erysiphe cichoracearum* (Miya et al., 2007; Wan et al., 2008). Furthermore, exogenous application of chitooligosaccharides enhances resistance to both fungal and bacterial pathogens (Wan et al., 2008), highlighting the broad level of resistance brought about by PAMP perception. These findings link the CERK1 receptor to the establishment of resistance through the perception of fungal derived chitin.

Bacterial peptidoglycan, a major bacterial cell wall component, also elicits plant defense responses (Willmann et al., 2011; Gust et al., 2012). Pretreatment with peptidoglycan confers resistance to bacterial infection in tomato (Nguyen et al., 2010). Recently, Willmann et al. (2011) described two LysM containing RLKs, LYM1 and LYM3 as receptors of peptidoglycan in *Arabidopsis*. Both LYM1 and LYM3 are membrane bound, contain extracellular LysM domains but lack an intracellular signaling domain (Gust et al., 2012). Both LYM1 and LMY3 physically bind peptidoglycan and both are required for full responsiveness to peptidoglycan (Willmann et al., 2011). *lym1* and *lym3* single mutants both exhibit increased susceptibility to infection with *Pto* DC3000, but this phenotype is not enhanced in the double mutant *lym1/lym3*, suggesting that these receptors do not function redundantly and may be part of the same recognition system (Willmann et al., 2011). Although chitin perception is not affected by *lym1* or *lym3* mutation, *cerk1* mutants phenotypically mimic the peptidoglycan insensitivity and the heightened susceptibility to *Pto* DC3000 observed in the *lym1* and *lym3* mutants; this suggests a scenario where peptidoglycan binding is mediated by the receptors LYM1 and LYM3, relying on the intracellular kinase activity of CERK1 (Willmann et al., 2011).

In addition to PAMP receptors, another set of receptors monitor host derived signals known as danger-associated molecular patterns (DAMPs) and were first described in animal systems (Matzinger, 1994; Lotze et al., 2007). In plants, these endogenous signaling molecules, such as cell wall fragments, cutin monomers and host encoded peptides typically accumulate in the apoplast in response to damage associated with pathogen attack (Boller and Felix, 2009). The endogenous peptide elicitor, *At*PEP1, is derived from the precursor protein PROPEP1, which is strongly induced by cell wall degradation, wounding, JA and PAMP perception (Huffaker et al., 2006). An additional five genes (*PROPEP2-PROPEP6*) related to *PROPEP1* have been identified in the *Arabidopsis* genome (Huffaker et al., 2006). The receptors for *At*PEPs, referred to as PEPR1 and PEPR2, were identified in *Arabidopsis* as LRR-RLKs with 26 and 25 extracellular LRR motifs, respectively, and belong to the LRR subfamily XI (Yamaguchi et al., 2006; Yamaguchi et al., 2010). All six *At*PEP peptides induce both *PDF1.2* and *PR-1* (Huffaker and Ryan, 2007) and *At*PEP1-*At*PEP6 pre-treatment significantly reduced the growth of *Pst* DC3000 (Yamaguchi et al., 2010). Interestingly, some of the *AtPEP* genes are induced by PAMP treatment, leading to the production of *At*PEP peptides that activate defense responses, supporting the supposition that the *At*PEP/PEPR ligand receptor system acts in a feedback loop to amplify plant defense-signaling via the salicylic acid (SA) and JA/ethylene (ET) pathways (Huffaker and Ryan, 2007; Ryan et al., 2007). Recently, Tintor et al. (2013) demonstrated that EFR signaling requires ET for the induction of *PROPEP2* but not *PROPEP3* activation, implying that EFR induces various *PROPEP* genes through additional signaling pathways. The authors of this report also show that EFR signaling defects in plants with a disabled ET-signaling pathway can be partially compensated by the activation of PEP receptors.

Another form of DAMP perception is mediated by the wall-associated kinase (WAK) family that is encoded in the *Arabidopsis* genome by five clustered genes WAK1-WAK5 (Verica et al., 2003). These receptors belong to the WAK-like subfamily of RLKs that is populated by 26 members (Shiu and Bleecker, 2003) which contain several extracellular epidermal growth factor-like repeats, and a cytoplasmic kinase domain (Verica et al., 2003) and are tightly

associated with pectin, a constituent of the cell wall (He et al., 1996). Inactivation of individual WAK gene expression does not cause any phenotypic alteration, most likely due to functional redundancy (Wagner and Kohorn, 2001). On the other hand, inducible silencing of all *WAK* genes leads to the loss of cell expansion and a dwarf phenotype (Lally et al., 2001; Wagner and Kohorn, 2001). WAK1 was recently shown to sense oligogalacturonides (OGs), molecules derived from the plant cell wall (Hematy et al., 2009), *in vivo* (Brutus et al., 2010). It has long been known that fungal polygalacturonases degrade pectin, releasing OGs, and these molecules function as danger signals and induce the expression of defense-related genes (Hahn et al., 1981; Hematy et al., 2009). Importantly, exogenous application of OGs induces resistance to *Botritis cinerea*, demonstrating that this host cell wall derived elicitor plays an important role in the regulation of plant defense responses (Ferrari et al., 2007).

From a reverse genetic approach aimed at identifying LRR-RLKs involved in defense responses, *bak1* mutants were shown to be defective in containing the spread of necrosis after infection with both bacterial and fungal pathogens in a brassinosteroid-independent manner (Kemmerling et al., 2007). BAK1 (BRI1-associated kinase 1) is an LRR-RLK that consist of 4 and a half extracellular LRR domains, a single transmembrane domain and an intracellular kinase domain (Hecht et al., 2001) and belongs to the LRR-RLK subfamily II (Shiu and Bleecker, 2003). Interestingly, double mutants affecting *BAK1* and *BKK1* (BAK1-LIKE1), which also belongs to the LRR/RLK subfamily II (Shiu and Bleecker, 2003), show enhanced spontaneous cell death and seedling lethality 10 days after germination that is associated with constitutive defense-gene expression (He et al., 2007). This suggests that BAK1 and BKK1 negatively regulate cell-death development (He et al., 2007).

Recent work has demonstrated that both BAK1 and BKK1 interact with FLS2 and EFR and are required for their signaling activity (Chinchilla et al., 2007; Roux et al., 2011). Plants that carry *BAK1* null mutations still show normal binding of flg22 but are affected in flg22-triggered responses, suggesting that BAK1 is not involved in ligand binding (Chinchilla et al., 2007). FLS2 and BAK1 interact in a ligand-dependent manner *in vivo* within minutes of flg22

stimulation (Chinchilla et al., 2007). Recently, a novel allele of BAK1, *bak1-5*, was identified that specifically blocks innate immune responses (Schwessinger et al., 2011). *bak1-5* plants carry a point mutation in its cytoplasmic kinase domain, accumulate similar levels of protein compared to wild type BAK1 and are not impaired in cell death control (Schwessinger et al., 2011). However, upon flg22 or elf18 treatment, *bak1-5* plants display a stark reduction in FLS2 and EFR-mediated responses (Schwessinger et al., 2011). These phenotypes were enhanced to greater levels in *bak1-5*/*bkk1-1* double mutant plants, demonstrating that the co-receptors BAK1 and BKK1 are required for activation of FLS2 and EFR PAMP signaling complexes (Roux et al., 2011).

1.3.2 RLKs involved in plant development

Perhaps one of the best characterized plant RLKs to date is the brassinosteroid (BR) receptor *Brassinosteroid Insensitive 1* (BRI1), which also interacts with the regulatory RLK BAK1 (Li et al., 2002; Nam and Li, 2002). BRs are signaling molecules involved in various developmental processes such as cell elongation, vascular differentiation, root growth and senescence (Kim and Wang, 2010) as well as responses to both biotic and abiotic stresses (Albrecht et al., 2012; Belkhadir et al., 2012). A forward genetic screen identified the *bri1* mutant as a dwarf plant insensitive to BR treatment (Clouse et al., 1996; Kauschmann et al., 1996). The cloning of the *BRI1* gene revealed that BRI1 is an LRR-RLK with 24 LRRs and an island domain between the twentieth and twenty-first LRRs (Li and Chory, 1997) and is a member of the LRR-RLK subfamily X (Shiu and Bleecker, 2003). It was later demonstrated that the island domain and the flanking LRR21 of BRI1 can physically bind BR (Wang et al., 2001; Kinoshita et al., 2005) and that this binding activates its cytoplasmic kinase domain, leading to autophosphorylation and allowing for signal transduction to other proteins (Wang et al., 2005; Kim and Wang, 2010). Recently, Hothorn et al. (2011) solved the structure of the extracellular portion of BRI1 and found that the LRR domains form a superhelix.

Furthermore, the authors of this report suggest that BR binding by BRI1 generates a docking platform for co-receptors required for BRI1 activation such as BAK1.

BAK1 was identified as a signaling partner of BRI1 using activation tagging and a yeast two-hybrid screen (Li et al., 2002; Nam and Li, 2002). Ligand dependent association of BAK1 and BRI has been shown to be required for the full potential of BR signaling (Wang et al., 2008). The interaction between BRI1 and BAK1 requires their kinase activities as kinase-dead BRI1 mutants do not interact with BAK1, and vice versa, upon BR treatment (Wang et al., 2008). Furthermore, it has been demonstrated that BAK1 is transphosphorylated by BRI1 and this results in enhanced BRI1-BAK1 association (Wang et al., 2008; Yun et al., 2009). BAK1 then transphosphorylates BRI1 resulting in the full activation of the BR signaling complex (Wang et al., 2008). BAK1 belongs to a small subfamily of RLKs referred to as Somatic Embryogenesis Receptor Kinases (SERKs) (Li, 2010) that also belong to the LRR-RLK subfamily II (Shiu and Bleecker, 2003; Chinchilla et al., 2009). The closest BAK1 paralog BKK1 has been shown to function redundantly with BAK1 in regulating BR signaling (He et al., 2007). Recently, Gou et al. (2012) demonstrated that SERKs are indispensable for brassinosteroid signaling by showing that BRI1 phosphorylation activity upon BR treatment is completely lost in *serk1*/*bak1*/*bkk1* triple mutants.

One of the first identified RLKs was CLAVATA1 (CLV1), an RLK with 21 LRRs that is involved in the maintenance of floral and shoot apical meristem size (Clark et al., 1997). Meristematic tissue undergoes constant organogenesis by maintaining a delicate balance of undifferentiated and differentiated cells. *Arabidopsis* plants lacking CLV1 result in the excess accumulation of undifferentiated cells in meristematic tissue, supporting its role in the maintenance of this developmentally important tissue (Clark et al., 1997). Recent work has demonstrated that CLV1 binding of CLAVATA3 (CLV3), a member of the secreted CLV3/embryo-surrounding region (CLE) family of arabinosylated peptides, regulates stem cell specification and inhibits cell division in the shoot apical meristem (Ogawa et al., 2008). CLAVATA2 (CLV2) is an LRR-receptor-like protein, which lacks an intracellular signaling

domain and is also required for maintenance of shoot apical meristems (Jeong et al., 1999; Fiers et al., 2005; Wang et al., 2011). The transmembrane kinase CORYNE (CRN) functions together with CLV2 in regulating the shoot apical meristem (Miwa et al., 2008; Muller et al., 2008), making preformed complexes in the ER which is required for both of these proteins to localize to the plasma membrane (Bleckmann et al., 2010). Confocal microscopy, luciferase complementation imaging and FRET analysis has suggested that CLV1, CLV2 and CRN form a signaling complex *in vivo* (Muller et al., 2008; Bleckmann et al., 2010; Meng and Feldman, 2010; Zhu et al., 2010) and this complex possesses an affinity for diverse set of CLE peptides (Guo et al., 2009).

HAESA is another RLK associated with plant developmental processes and also contains 21 LRRs (Horn and Walker, 1994). *Arabidopsis* plants with reduced *HAESA* expression result in delayed abscission of floral organs, implicating another RLK in coordinating plant developmental processes (Jinn et al., 2000). Interestingly, double mutants affecting *HAE* and *HAESA-LIKE2* (*HSL2*), another an RLK, retain their floral organs indefinitely (Cho et al., 2008). Butenko et al. (2003) identified the *Arabidopsis* mutant *inflorescence deficient in abscission* (*ida*) that is also involved in organ shedding. Mutations affecting *IDA*, in a fashion similar to *hae*/*hsl2* double mutant plants, indefinitely retain floral organs after the shedding of mature seeds, whereas the overexpression of *IDA* leads to earlier abscission events (Butenko et al., 2003; Stenvik et al., 2008). *IDA* proteins have a C-terminal domain referred to as EPIP, and treatment of plants with synthetic IDA EPIP peptides rescued the abscission deficient phenotype of *ida* but not *hae/hsl2*, suggesting that IDA is a ligand for the RLKs HAE and HSL2 (Stenvik et al., 2008).

PSKR1 and PSY1R are two additional RLKs that have been shown to be involved in controlling cell proliferation (Matsubayashi et al., 2002; Amano et al., 2007). These receptors bind the sulfated peptides PSKα and PSY1, respectively. The various works that have demonstrated that CLV1, CLV2, HAESA, PSKR1 and PSY1R all bind peptides has revealed

that LRR-RLK perception of endogenously encoded peptide ligands is a common mode of communicating signals associated with plant development.

1.4 Plant peptide signaling

Multicellular organisms rely on cell-to-cell signaling for proper growth, development and signaling. In higher plants, this signaling is largely mediated by small lipophilic hormones such as auxins, cytokinins, gibberellins, abscisic acid, ethylene, brassinosteroids and jasmonates, allowing for communication between cells in various developmental stages (Matsubayashi and Sakagami, 2006). However, due to the important roles played by these hormones, the relevance of secreted peptides in intercellular communication has been largely overlooked despite the significant contribution these molecules have been shown to play in animals (Amano et al., 2007). In recent years it has been demonstrated that, in plants, endogenous peptides play a role in a wide variety of plant responses. For instance, defense responses have been associated with tobacco systemins (Pearce et al., 2001), self-incompatibility is regulated by the SCR peptide binding to the receptor SRK (Schopfer et al., 1999; Tantikanjana et al., 2009), floral organ abscission is controlled by perception of IDA by HAESA (Butenko et al., 2003; Cho et al., 2008), maintenance of root stem cell niche is regulated by RGFs (Matsuzaki et al., 2010), stomatal patterning is coordinated through EPF1 (Hara et al., 2007) and cell proliferation and differentiation is promoted by phytosulfokine (PSKα) and PSY1 binding to the receptors PSKR1 and PSY1R, respectively (Matsubayashi and Sakagami, 1996; Matsubayashi et al., 2006; Amano et al., 2007).

1.4.1 Phytosulfokines and other tyrosine-sulfated plant peptides

Division of plant cells grown in culture has been documented to be directly related to the initial cell density of the culture; if cells are cultured below a critical minimum density, growth is arrested (Matsubayashi and Sakagami, 2006). However, stimulation of cellular proliferation

in low density cultures is induced by supplementation with conditioned medium from rapidly growing cell cultures (Stuart and Street, 1969). By means of chromatographic techniques, a heat stable and protease sensitive biologically active growth factor named phytosulfokine was purified from conditioned medium of asparagus and identified as a tyrosine sulfated pentapeptide with the sequence sYIsYTQ (Matsubayashi and Sakagami, 1996). Perfectly conserved PSKα has also been identified in conditioned culture medium obtained from maize, rice, carrot and zinnia, hinting at its universal existence in the plant kingdom (Yang et al., 2000).

Five paralogous PSKα precursor genes have been identified in the *Arabidopsis* genome (Yang et al., 2001; Matsubayashi et al., 2006). The predicted protein encoded by each gene contains a putative secretion signal at the N terminus and the PSKα sequence near to the C terminus (Matsubayashi et al., 2006). While the PSKα sequence is conserved, the five *PSK* genes have undergone high levels of diversification as indicated by their divergent sequences. Furthermore, diversification has also been reported for these genes in terms of their expression patterns (Matsubayashi et al., 2006). *PSK1* has been shown to be expressed specifically in root tissue, whereas *PSK2, PSK4* and *PSK5* are primarily expressed in lower mature leaves (Matsubayashi et al., 2006). Promoter-GUS transgenic lines revealed that *PSK2*, *PSK3*, *PSK4* and *PSK5* are expressed in cotyledons and leaves with the highest level of expression in vascular bundles (Matsubayashi et al., 2006). Upon treatment with the necrotrophic fungal pathogens *Sclerotinia slerotiorum* and *Alternaria brassicicola*, promoter-GUS analysis revealed that of the five PSK genes, only *PSK2* was induced transcriptionally (Loivamaki et al., 2010). Several publications have reported wound responsiveness for *PSK3, 4* and *5* (Matsubayashi et al., 2006; Kilian et al., 2007; Loivamaki et al., 2010).

PSKα has also been shown to be involved in various other physiological responses other than cellular proliferation. For instance, PSKα has promotive effects on chlorophyll synthesis and adventitious root formation and root elongation (Yamakawa et al., 1998; Amano et al.,

2007; Kutschmar et al., 2009). PSKα also promotes somatic embryogenesis (Hanai et al., 2000; Igasaki et al., 2003), tracheary element differentiation *in vitro* (Matsubayashi et al., 1999; Motose et al., 2009), adventitious bud formation (Yang et al., 1999) and pollen germination (Chen et al., 2000).

An expanded search for additional sulfated peptides from conditioned *Arabidopsis* suspension cell culture medium led to the identification of a peptide consisting of 18 amino acid residues referred to as *p*lant peptide containing *s*ulfated t*y*rosine 1 (PSY1) (Amano et al., 2007). The PSY1 sequence was shown to be derived from the C-terminal region of the predicted protein encoded by *At5g58650*, which contains a putative secretion signal at the N-terminus. Further analysis revealed that PSY1 is not only tyrosine sulfated but also glycosylated with three L-arabinose subunits on the 16th hydroxylated proline. The *Arabidopsis* genome contains two additional PSY1 precursor paralogs with a high level of similarity in the PSY1 domain. Unlike the PSK domains, however, the PSY1 domains are not perfectly conserved. PSY1 expression was determined to be highest in tissues such as leaves, stems and flowers but substantially lower in roots. However, histochemical staining of promoter-GUS lines revealed that PSY1 expression is particularly high in marginal regions of leaves, shoot apical meristem and the elongation zone of roots. Additionally, PSY1 was reported to be transcriptionally up-regulated by mechanical wounding (Amano et al., 2007).

To assess the physiological function of PSY1, Amano et al. (2007) analyzed 35S-*PSY1* lines and found that they, compared to wild type plants, developed longer roots and larger cotyledons, and that this was mainly due to larger cell size. Exogenous application of PSY1 to *Arabidopsis* seedlings resulted in the stimulation of root growth at the 100nM level. Furthermore, when added to the culture media of *Arabidopsis* suspension cells, PSY1 induced cellular proliferation in a dose-dependent manner. Interestingly, PSY1 was also able to promote proliferation of asparagus mesophyll cells, suggesting that this growth promoting peptide signaling pathway is conserved in a distantly related plant species (Amano et al., 2007).

1.4.2 Processing of PSK and PSY1 precursors proteins is required for an active peptide

In order to fully activate signaling cascades that lead to the induction of physiological responses associated with PSKα- and PSY1-signaling, precursor polypeptides must be posttranslationally modified to yield mature signaling compounds (Matsubayashi and Sakagami, 1996; Amano et al., 2007). Tyrosine sulfation is a common posttranslational modification of proteins in animals transported through the trans-golgi network and is important for the function of a diverse group of secreted proteins (Moore, 2003; Matsubayashi, 2012). In humans, tyrosine sulfation is mediated by two related transmembrane proteins, tyrosylprotein sulfotransferase -1 (TPST) and TPST-2, which localize to the *trans*-golgi network (Beisswanger et al., 1998; Ouyang et al., 1998). However, no candidate TPST encoding genes were identified in the *Arabidopsis* or other plant genomes based on sequence similarity, suggesting that TPST activity may have evolved in a structurally distinct form in plants (Komori et al., 2009). By means of affinity purification using microsomal fractions, Komori et al. (2009) successfully isolated an *Arabidopsis* TPST using a PSY1 oligopeptide as an affinity probe. Recombinant TPST expressed in yeast catalyzed tyrosine sulfation of PSY1 and PSK precursor polypeptides *in vitro*. Plants lacking a functional *TPST* display a semi-dwarf phenotype, early senescence and stunted root growth (Komori et al., 2009). The root meristem defects of *tpst* mutant plants can be restored by another sulfated peptide known as the root meristem growth factor RGF1, implicating peptide sulfation activity in maintaining root meristem cell niche (Matsuzaki et al., 2010). Interestingly, *TPST* expression was found to be transcriptionally induced by the growth regulating phytohormone auxin which is also critical for the specification and maintenance of root stem cell niche (Dinneny and Benfey, 2008). Furthermore, *tpst* mutation results in down-regulation of auxin biosynthetic genes and auxin transport genes, consequently affecting auxin distribution, suggesting that peptide sulfation and auxin signaling are tightly linked (Zhou et al., 2010).

As PSKs and PSY1 peptides are thought to be sulfated in the *trans*-golgi network, it is likely that this modification takes place prior to secretion in a similar fashion documented for sulfated proteins in animal cells (Baeuerle and Huttner, 1987). Srivastava et al. (2008) identified the subtilase AtSBT1.1 as being required for PSK4 proteolytic cleavage in response to callus and shoot induction as well as wounding. Based on YFP fusion proteins, it was determined that AtSBT1.1 and PSK4 accumulate in the extracellular matrix. Coupled with the fact that AtSBT1.1 has a slightly acidic pH optimum similar to that found in the apoplast, the authors of this work propose that this is the site of PSK proteolysis. Interestingly, of the 56 various subtilases encoded by *Arabidopsis* (Rautengarten et al., 2005), only null mutations in *AtSBT1.1* halted PSK4 cleavage, hinting that substrate recognition of AtSBT1.1 is highly specific (Srivastava et al., 2008). Indeed, processing of PSK2 and PSK5 preproproteins were much slower than for PSK4. Furthermore, products of AtSBT1.1 proteolysis with PSK1 and PSK3 as substrates were barely detectable, suggesting that other subtilases might be required to process other PSK preproproteins.

1.4.3 Perception of PSK and PSY1 is mediated by LRR-RLKs

It has been proposed that the production of mature PSKα is stimulated in response to auxin and cytokinin and that it functions as an autocrine growth factor that regulates plant developmental processes (Matsubayashi et al., 1999). First data supporting the mode of PSKα perception was reported from experiments using rice suspension cells. Differential centrifugation revealed that radio labeled PSKα binds to plasma membrane enriched fractions (Matsubayashi et al., 1997). Ligand based affinity chromatographic techniques using carrot microsomal fractions later identified a 120 KDa LRR-RLK trans-membrane protein that specifically interacts with PSKα (Matsubayashi et al., 2002). The sequence of this receptor revealed an N-terminal signal peptide, 21 LRRs with an island domain between repeats 17 and 18, a transmembrane domain and a cytoplasmic kinase domain. Overexpression of this phytosulfokine receptor (*Dc*PSKR1) resulted in enhanced PSKα

binding in membrane fractions as well as accelerated growth in response to PSKα treatment compared to control cells. On the other hand, silencing of the carrot PSKR1 receptor strongly inhibited callus growth after PSKα treatment compared to control cells.

Based on amino acid sequence similarity, the orthologous receptor in *Arabidopsis* was identified (Matsubayashi et al., 2006). The sequence of *Arabidopsis* PSKR1 was found to be 60% identical at the amino acid level compared to *Dc*PSKR1, similarly containing an N-terminal signal peptide, 21 LRRs with an island domain between repeats 17 and 18, a transmembrane domain and a cytoplasmic kinase domain. PSKR1 belongs to the LRR subfamily X (Shiu and Bleecker, 2003). This subfamily is populated by 16 members including the brassinosteroid receptors BRI1 (Li and Chory, 1997), BRI1-Like1 and BRI1-Like3 (Cano-Delgado et al., 2004), a negative regulator of plant defense responses known as BIR1 (Gao et al., 2009) and a regulator of anther cell patterning known as EMS1 (Zhao et al., 2002). Analysis of the PSKR1 receptor showed that its affinity to PSKα was similar to that of *Dc*PSKR1. Phenotypic characterization of receptor insertion mutants revealed that *pskr1* plants germinated normally, developing normal cotyledons and hypocotyls indistinguishable from wild type plants. Although root growth was moderately reduced in *pskr1* insertion mutants compared to wild type plants, no morphological differences were detected in the above ground portion of the plants (Matsubayashi et al., 2006). While the root growth of *PSKR1* overexpression lines was comparable to wild type, the rate of callus tissue formation from leaf discs was enhanced, whereas the *pskr1* insertion mutant senesced within 3 weeks of culture, leading to strongly reduced callus formation. Although wild type control tissue did gradually show senescence after three weeks of culture, *PSKR1* overexpression lines did not show any signs of senescence after 6 weeks of growth. Both *pskr1* insertion mutants and *PSKR1* overexpressor seedlings grew at the same rate as wild type plants and flowered normally. However, leaves of *pskr1* plants exhibited premature senescence by 4 weeks after germination and were fully senescent by 6 weeks after germination. On the other hand, leaves of *PSKR1* overexpressors grew larger than wild type and exhibited a delayed

senescence phenotype, suggesting that the PSKR1 receptor plays an important role in the regulation of senescence (Matsubayashi et al., 2006).

In an effort to uncover receptors paralogous to PSKR1, Amano et al. (2007) identified a second PSKR receptor in *Arabidiopsis* known as PSKR2. This receptor is predicted to encode an LRR-RLK with 23 LRR motifs and an island domain between LRR 18 and 19, a transmembrane domain and a cytoplasmic kinase domain. PSKR2 also belongs to the LRR subfamily X and has 48.6% sequence identity compared to PSKR1 at the amino acid level. Photoaffinity labeling experiments using BY-2 tobacco cells showed that the PSKR2 receptor indeed binds PSKα. *PSKR2* insertion mutants germinated normally, and were phenotypically indistinguishable from wild type plants. To test the impact of PSKR2 on PSKα perception, both *PSKR1* and *PSKR2* mutant plants were exogenously treated with PSKα. While PSKα-mediated root growth promotion was strongly inhibited in *PSKR1* mutants, *PSKR2* mutants were only moderately affected. Furthermore, a role for PSKα-signaling in hypocotyl cell elongation has been proposed by Stuhrwohldt et al. (2011). However, while hypocotyls of etiolated *pskr1* insertion mutants were found to be significantly shorter than those of wild type plants, *pskr2* insertion mutants were unaffected in this response. Taken together, the lack of altered responses observed in *pskr2* insertion mutants suggests a limited role for PSKR2 in PSKα perception and signaling.

In the search for PSKR1 paralogs, Amano et al. (2007) also identified a receptor required for the perception of PSY1 (PSY1R). This receptor is predicted to encode an LRR-RLK with 23 LRR motifs and an island domain between LRR 18 and 19, a transmembrane domain and a cytoplasmic kinase domain. PSY1R also belongs to the LRR subfamily X and has 43.6% sequence identity with PSKR1 at the amino acid level. T-DNA insertion mutant analysis revealed that *psy1r* plants germinate normally and the leaves of three week old *psy1r* plants were morphologically indistinguishable from wild type plants. When grown on media supplemented with PSKα, root growth promotion in *psy1r* insertion mutants was similar to wild type plants, indicating that PSY1R is not involved in the perception of PSKα. When

grown on media supplemented with PSY1, however, *psy1r* insertion mutants were significantly less sensitive to its root growth promoting properties than wild type plants, leading to the conclusion that PSY1R is involved in the perception of PSY1. Additionally, the roots of *pskr1* insertion mutants elongated to a similar level compared to wild type plants in the presence of PSY1, supporting the conclusion that PSKR1 and PSY1R perceive two structurally distinct ligands that contribute redundantly to cellular proliferation and root growth (Amano et al., 2007).

Highlighting these redundant growth promoting characteristics, the *pskr1/pskr2/psy1r* triple receptor mutant exhibit reduced root length and cotyledon size in seedlings. Microscopic analysis showed that the smaller root size resulted from decreased cell size and a reduced size of the shoot apical meristem. Mature *pskr1/pskr2/psy1r* triple receptor mutant plants had a dwarf phenotype due to both a decrease in cell number and cell size. Furthermore, they display decreased callus formation upon cutting leaf disks or wounding, suggesting a role for PSY1 and PSKα-signaling in not only growth and development, but also wound repair (Amano et al., 2007). Interestingly, *TPST* mutations result in similar phenotypes compared with *pskr1/pskr2/psy1r* plants indicating that both ligand and receptor are important for PSY1- and PSKα-signaling (Komori et al., 2009).

1.5 Aims of the thesis

Cell surface localized RLKs have been implicated in a number of plant processes including cell wall interactions, developmental control and disease resistance (Gish and Clark, 2011). Over 600 RLKs, two thirds of which possess extracellular domains, are encoded by the *Arabidopsis* genome (Smith et al., 2011). The presence of such an abundance of receptors underpins their importance in *Arabidopsis* signaling. To gain insights into receptor like kinases involved in plant defense responses, a reverse genetic approach was taken to identify candidate RLKs transcriptionally up-regulated by pathogen and/or PAMP treatment.

Screening of the expression patterns of all RLK genes gave a first hint for a role of the phytosulfokine receptor PSKR1 in plant immunity. Initial work revealed that PSKR1 has a strong impact on defense responses to the biotrophic pathogen *Pto* DC3000 and necrotrophic pathogen *Alternaria brassicicola*. Interestingly, PSKR1 was originally identified as the receptor for PSKα, a sulfated peptide that plays a role in plant cellular dedifferentiation and proliferation (Matsubayashi and Sakagami, 1996; Matsubayashi et al., 2002). The fact that PSKR1 has such a salient impact on plant immunity raised the question how PSKα-signaling modulates defense responses, whether the observed modulation of immunity is a direct result of PSKα perception, and whether additional peptide receptors redundantly modulate plant immunity.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals

All used standard chemicals were of standard purity and purchased from Sigma-Aldrich (Taufkirchen), Carl Roth (Karlsruhe), Merck (Darmstadt), Qiagen (Hilden), Invitrogen (Karlsruhe), Duchefa (Haarlem, the Netherlands), Molecular Probes (Leiden, the Netherlands), Fluka (Buchs, Switzerland) und BD (Sparks, USA), unless noted otherwise in the text. Restriction enzymes, ligase and DNA modification enzymes were purchased from Fermentas (St. Leon- Rot) and New England Biolabs (Beverly, USA). Oligonucleotides were received from Eurofins MWG Operon (Ebersberg) and antibodies from the companies Sigma-Aldrich (Taufkirchen), New England Biolabs (Beverly, USA) and Acris Antibodies GmbH (Herford). The synthetically generated Flg22 peptide was a kind gift from Georg Felix.

2.1.2 Media

Table 2-5 summarizes the media used in this work. All media were prepared using deionized water and sterilized by autoclaving for 20 minutes at 121°C. For solid media 15g/l Bacto-agar (BD) or 8g/l Select-Agar for MS plates (Sigma-Aldrich) was added to the medium. If necessary, antibiotics were added to the sterilized medium at appropriate final concentrations as listed in Table 2-6.

Table 2-5. Media used

Medium	Ingredients per 1 liter	species
LB	10 g Bacto-Tryptone, 5 g NaCl, 5 g Yeast extract (YE)	*Escherichia coli*
Kings's B	20 g glycerol, 40 g Proteose Pepton 3, after autoclaving addition of 0.1 % (v/v) MgSO4 and KH2PO4	*Pseudomonas syringae*
½ MS	2.2 g MS (Duchefa), pH 5.7 (KOH)	*Arabidopsis thaliana*

Table 2-6. Antibiotics used

Antibiotics	concentration (µg/µl)	solvent
Carbenicillin	100	Water
Kanamycin	50	Water
Rifampicin	50	Methanol
Spectinomycin	100	Water
Tetracyclin	50	Ethanol

2.1.3 Vectors

The vectors used for this thesis project are listed in Table 2-7.

Table 2-7. Vectors used

pDONR201	Ori Puc, rrnB, T2, rrnB,T1, attP1, attP2, ccdB,Cm^r, Kan^r	Invitrogen
pDONR207	Ori Puc, rrnB, T2, rrnB,T1, attP1, attP2, ccdB,Cm^r, $Gent^r$	Invitrogen
pCR8/GW/TOPO	Ori Puc, rrnB, T2, rrnB,T1, attP1, attP2, ccdB, Sm/Sp^r	Invitrogen
pDEST15	PT7, RBS, GST, attR1, attR2, ccdB, Cmr, PT7, bla Promotor, Amp^r, pBR322 origin, ROP orf	Invitrogen
pDEST17	PT7, RBS, His6-tag, attR1, attR2, ccdB, Cmr, PT7, bla, Promotor, Ampr, pBR322 origin, ROP, orf	Invitrogen
pH35GWG	attR1, attR2, ccdB, Hyg^r, Kan^r, GFP	Steve Clouse, 2010 project
pK2GW7	attR1, attR2, ccdB, Kan^r, Sm/Sp^r	karimi et al. 2005
pH2GW7	attR1, attR2, ccdB, Hygr, Sm/Spr	karimi et al. 2005
pB7YWG2	attR1, attR2, ccdB, Ba^r, Sm/Sp^r, eYFP	karimi et al. 2005
pH7CWG2	attR1, attR2, ccdB, Ba^r, Sm/Sp^r, eCFP	karimi et al. 2005
pUB-Dest-cYFP	attR1, attR2, ccdB, Bar, Sm/Sp^r, cYFP	Grefen et al. 2010
pUB-Dest-nYFP	attR1, attR2, ccdB, Bar, Sm/Sp^r, nYFP	Grefen et al. 2010

2.1.4 Primers

The primers used in this work are listed in the Appendix, Table 8-11.

2.2 Organisms

2.2.1 Bacteria and fungi

The bacterial strains used in this work are listed in table2-8.

Table 2-8. Bacterial strains

Species	Strain	Genotype
Escherichia coli	DH5α	supE44 ΔlacU169 (Φ80 lacZM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1
	TOP10	mcrA, delta (mrr-hsdRMS-mcrBC), phi, 80delta lac delta M15, delta lacX74, deoR, recA1, araD139 delta (ara, leu), 7697, galU, galK, lambda$^-$, rpsL, endA1, mupG
	BL21AI	F-ompT hsdSb(rb-mb-) gal dcm araB::T7RNAP-tetA
Pseudomonas syringae pv*. tomato*	*Pto* DC3000	Rif^r
Agrobacterium tumefaciens	GV3103::pMP90	T-DNA$^-$ vir$^+$ rif^r, pMP90 gen^r

2.2.2 Plant lines

All experiments were conducted using the *Arabidopsis thaliana* ecotypes Columbia-0 (Col-0) or Wassilewskija (Ws-4) or *Nicotiana benthamiana* and transgenic lines generated in these ecotypes. The T-DNA insertion mutant lines used in this work include *pskr1-3* (SALK_008585), *pskr1-5* (FLAG_407D02), *pskr2* (SALK_024464) and *psy1r* (SALK_072802C) and were obtained from the Nottingham Arabidopsis Stock Centre. The *tpst-1* (SALK_009847) line was kindly donated by the laboratory of Yoshikatsu Matsubayashi. *PSKR1-GFP* transgenic lines were kindly donated by the laboratory of Dr. Frans Tax and contain a 2.4-kb promoter region of *PSKR1* plus the coding region amplified from Col-0 genomic DNA, were cloned into pCR8-/GW-TOPO and recombined into pBIB-BASTA-GFP. Transgenic Arabidopsis lines harboring the constructs p35S-*PSK2*, p35S-

PSK4 (in pK2GW7) and p35S-*PSKR1* (in pH2GW7) were kindly donated by the laboratory of Dr. Herald Keller. Entire coding sequences amplified by PCR using sequence-specific primers (Table 8-11) and were recombined into the respective Gateway vectors described by Karimi et al. (2002). Constructs were transformed into wild type or *pskr1-3/pskr2/psy1r* mutants via Agrobacterium tumefaciens strain GV3101-mediated transformation.

2.3 Cultivation condition of the organisms

2.3.1 Growth of *Escherichia coli*

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

2.3.2 Growth of *Pseudomonas syringae*

P. syringae Pto pv. DC3000 strains were grown for 24-48 hours at 28°C either on King's B-plates or in liquid King's B medium at 180 rpm. For the determination of bacterial growth in infection assays the *Pseudomonas* strain was re-isolated from plant material (see 2.6.1) and plated on LB plates containing cycloheximide in addition to other antibiotics.

2.3.3 Growth of *Agrobacterium tumefaciens*

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

2.3.4 Growth of *Alternaria brassicicola*

The cultivation of *A.Brassicicola* and the preparation of the spores for the infection assays were performed as published previously (Kemmerling et al., 2007).

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

A. thaliana seeds were sown on steam-sterilized GS90-soil (Gebr. Patzer GmbH) mixed with vermiculite or after surface-sterilization with chlorine gas on sterile ½ MS plates. After stratification of the seeds for two days at 4°C and in the dark the plants were grown in environmental chambers either in long-day (16 h light, 8 h darkness) or short-day (8 h light, 16 h darkness) under standard conditions (150µmol/cm_2s light, 40-60 % humidity, 22°C). *N.benthamiana* plants were cultivated in a mixture of soil and sand containing 0.1 % (v/v) Confidor in the greenhouse (13 h light, 11 h darkness).

2.4 Methods

2.4.1 General molecular biology methods

Standard protocols were used for PCR, agarose gel electrophoresis, restriction digestion, ligation, transformation of bacteria and plasmid isolation (Sambrook and Russell, 2001). The transformation of TOP10 cells was performed according to the manufacturer's protocols (Invitrogen). The enzymes were used according the manufacturer's protocols (Fermentas and NEB). For the generation of PCR fragments either the *Taq* DNA-Polymerase or the *Pfu* DNApolymerase (cloning purposes; Fermentas) were used. GeneRuler DNA Ladder Mix (Fermentas) was used as size marker for the agarose gel electrophoresis. DNA fragments were extracted out of agarose gels or purified out of PCR reactions by using the Qiagen Gel Extraction Kit and Qiagen PCR Purification Kit (Qiagen).

2.4.2 Cloning

The constructs were generated by Gateway Technology (Invitrogen). Gateway-cloning was performed according to the manufacturer's recommendations (Invitrogen). To obtain Gateway-compatible inserts gene-specific adaptor primers were used in the first PCR. The essential recombination sites were then completed in a second PCR using the Gateway-primers attB1 and attB2 (see Table 8-11). The resulting inserts were then subcloned into pDONR201 or pDONR207 (Invitrogen) by using the BP clonase reaction and afterwards

inserted into the expression vectors by using the LR clonase reaction following the manufacturer's specifications (Invitrogen).

2.4.3 DNA isolation

Genomic DNA from plant tissue for genotyping purposes was isolated as outlined in Edwards et al. (1991). For sequencing purposes plasmid-DNA was isolated and column-purified using the QIAprep Spin MiniPrep Kit (Qiagen). Sequencing of the generated constructs was performed by the companies Eurofins MWG Operon (Ebersberg) and GATC Biotech AG (Konstanz). The sequence analysis was performed using the Lasergene DNA*STAR software.

2.4.4 RNA isolation

Total RNA from leaves or seedlings was isolated using the Trizol method according to the standard protocol (Chomczynski and Sacchi, 2006). For seedling samples the standard volumes were reduced to one-third. At the end of the isolation procedure the RNA pellet was re-suspended in ddH_2O (leaf RNA in 20-40 µL and seedling RNA in 10 µL) and stored at -20°C.

2.4.5 Quantitative Real-time PCR

2.5 µL DNase treated seedling RNA (amounts not adjusted) or 1 µg DNase treated leaf RNA was used for cDNA synthesis (in 20µl total reaction volume). Leaf cDNA was diluted 3 to 5 fold for qRT-PCR experiments, whereas seedling cDNA was used undiluted. RT-qPCR amplifications and measurements were performed with the iQ5 Multicolour Real Time PCR detection system from Bio-Rad. RT-qPCR amplifications were monitored using the ABsolute SYBR Green Fluorescein Mix (Thermo Scientific). The gene expression data was quantified using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The normalization of the expression levels was done using the CT values obtained for the *EF-1α* gene. All

quantifications were made in duplicate on RNA samples obtained from three independent experiments.

2.4.6 Stable transformation of *Arabidopsis thaliana*

Arabidopsis plants were stably transformed by the floral dip-method (Clough and Bent, 1998). 500 ml liquid LB medium containing appropriate antibiotics was inoculated with a preculture of selected agrobacteria and cultivated for further 18 – 24 hours. The cells were collected by centrifugation for 20 minutes at 4500 x g and resuspended in fresh 5 % (w/v) saccharose solution at a density of 0.8 (OD_{600nm}). After addition of 0.02 % (v/v) Silwet, young *Arabidopsis* flowers were dipped for one minute into the bacterial suspension. Afterward the plants were incubated at 100 % humidity for 24 hours. Seeds from floral-dipped plants were then screened for resistance against Basta or kanamycin.

2.4.7 Transient transformation of *Nicotiana benthamiana*

Agrobacterium tumefaciens-mediated transient transformation was used for transient expression of *Arabidopsis* genes in tobacco. The bacterial strain carrying the appropriate expression vector was cultured as described in 2.3.3. After harvesting the cells by centrifugation for 10 minutes at 2000 x g they were washed for two times with 10mM $MgCl_2$. The density of the culture was diluted to 5 x 10^4 cfu/ml and 150 µM acetosyringone was added. The bacterial suspension was then incubated shaking at RT for 3-6 hours. Afterwards the suspension was mixed 1:1 with a suspension of bacteria carrying an expression construct of p19 (Voinnet et al., 2003) and the mixture was then infiltrated into the leaves of 3 week-old tobacco plants. The leaf tissue was analyzed 2-4 days post infection for the presence of the protein.

2.4.8 Genotyping analysis of T-DNA insertion lines

The T-DNA lines used in this work were analyzed for their genotype. Since diploid plants contain two copies of each gene and are thus able to segregate it was necessary to confirm that the T-DNA insertion lines used for the experiments were homozygous. The discrimination between WT, heterozygous insertion and homozygous insertion lines was achieved by two sets of PCR reactions. In the WT-PCR, primers were used which bind a region flanking the T-DNA insertion (product amplified only in the WT plants, the large size of the T-DNA insertion inhibits the amplification in mutants). In the second PCR a T-DNA specific left border a primer (Lba primer, Table 8-11) was used in a combination with a gene-specific primer allowing an amplification product only in plants carrying a T-DNA insertion. Thus, homozygous plants should show a product only in the Lba-PCR.

2.4.9 Generation of knock-down lines

To investigate the role of phytosulfokine precursors in more detail, artificial microRNAs (amiRNAs) were utilized to silence *PSK* genes. amiRNAs were generated that are predicted to target *PSK2*, *PSK4*, *PSK1* and *5* or *PSK2* and *6* using the program Web MicroRNA Designer (WMD, http://wmd3.weigelworld.org) (Schwab et al., 2005). The coding region of amiRNAs were generating by PCR. After amplification and gel extraction, the four amiRNAs were cloned into the PCR8-TOPO vector, subcloned into the vector pB2GW7 and subsequently transformed into *Agrobacterium tumefaciens* and finally transformed into *Arabidopsis*.

2.5 Biochemical methods

2.5.1 Protein extraction from plant tissue

Total protein was extracted from plant tissue using a protein extraction buffer containing detergents for solubilization of membrane-bound proteins (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 % (v/v) Nonidet P40 and 1 protease inhibitor cocktail tablet/10 ml from Roche). The

plant tissue was first homogenized in liquid N_2 and after addition of the extraction buffer the sample was incubated for 1 hour at 4°C. Afterwards the soluble proteins were separated from the insoluble proteins and cell debris in a centrifugation step (20 minutes 20000 x g at 4°C) and used for further analysis.

2.5.2 Immunoprecipitation

For immunoprecipitation experiments, 200 mg of leaf tissue was crushed and extracted as detailed in 2.5.1. Antibodies were bound to Protein A Agarose (Roche), after washing with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, by coincubation with gentle rotation for 1 hour at 4°C. The Protein A Agarose coupled with an antibody was then incubated with protein extracts for 2 hours with gentle rotation at 4°C and subsequently washed two times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and one time with 50 mM Tris-HCl, pH 7.5, incubated at 95°C for 5 minutes and subjected to SDS-PAGE and western blot analysis.

2.5.3 SDS-PAGE

SDS polyacrylamide gel electrophoresis was performed as described (Sambrook, 2001) using the gel chamber system of BioRad. 10% SDS- polyacrylamide gels were used as separating gels (with 5 % stacking gels) for the discontinuous SDS-PAGE by the method of Laemmli (1970). The Prestained Protein Ladder Mix (Fermentas) was used as a protein marker.

2.5.4 Western blot analysis

For the western blot analysis the proteins were transferred after SDS-PAGE onto a PVDF membrane (GE Healthcare) using a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) for one hour at 100V. Nonspecific binding sites were blocked by incubation of the membrane for 1 hour at RT with 5 % (w/v) milk in 1x PBST (140 mM NaCl, 2.7 mM KCl, 10 mM

Na_2HPO_4, 1.8 mM KH_2PO_4, 0.1 % (v/v) Tween 20). Afterwards the membrane was incubated with a primary antibody overnight at 4°C. Then the membrane was washed 3 time for 5 minutes with 1 x PBST and incubated for 1.5 hours with a secondary antibody and then washed again 3 times for 5 minutes with 1 x PBST. The signal of peroxidase-coupled secondary antibody was detected using the Enhanced Chemiluminescence Kit (GE Healthcare) according to the manufacturer's instructions.

2.5.5 Coomassie blue stain

For non-specific staining of proteins after SDS-PAGE a Coomassie blue R-250 stain (0.125 % (w/v) Coomassie blue R-250, 50 % (v/v) MeOH, 10 % (v/v) acetic acid) was used. After incubation for 30 minutes at RT the excess stain was removed by washing 10 % (v/v) acetic acid.

2.5.6 Salicylate measurements

The analysis of SA content was performed in collaboration with the ZMBP analytics department at the University of Tübingen. 200 mg plant leaves were homogenized in liquid nitrogen and free analytes were extracted at 28°C for 60min with 1.5ml ethylacetate, containing 0.1% (v/v) formic acid and the internal standard 3-hydroxybenzoeic acid. After centrifugation (10,000g for 15min), 1.2ml supernatant was dried over phosphorus pentoxide (at 200mbar) overnight. For derivatization, a 1:1 mixture of methanol and Trimethylsilyldiazomethane (2M in Diethylether, Sigma) was used (70 µl, 25°C, 15 min). One µl was injected onto the GC column.

For hydrolysis of analyte derivatives, the supernatant of the last centrifugation step was removed and the pellet was incubated at 28°C for 60min with 200µl 3M hydrochloric acid. After neutralization with 3M ammonia, 1 ml ethylacetate, containing 0.1% (v/v) formic acid and the internal standards 3-hydroxybenzoeic acid was added to extract (at 28°C for 60min)

the analytes. After centrifugation (10,000g for 15min), 0.7 ml supernatant was dried over phosphorus pentoxide (at 200mbar) overnight. Derivatization was performed as described above. One µl was injected onto the GC column.

Analytes were determined by GC/MS (Agilent 6890 GC and Agilent 5973 single quad mass spectrometer), using the split injection mode and an SPB-50 column (30m, 0.25mm diameter, Supelco). The GC oven temperature was held at 70°C for 5min, then ramped at 5°C per min to 280°C and afterwards held for an additional 10min at 280°C. Helium was used as carrier gas with a flow rate of 1ml/min. Detection of the analytes was performed by electron impact ionization single quadrupole mass spectrometry operated in selected ion monitoring mode.

2.5.7 SDS PAGE and in-gel digestion for MS/MS analysis

To identify interaction partners of PSKR1, PSKR1-GFP was immunoprecipitated. Recovered proteins were processed in collaboration with the Proteome Center at the University of Tübingen. Complete protein eluates of the immunoprecipitation experiments were submitted to a gel run on a 1D SDS PAGE (NuPAGE 12% precast Bis/Tris gels, Invitrogen). The proteins were visualized by staining using the Novex Colloidal Blue Staining Kit (Invitrogen) according to the manufacturer's instructions, each lane was devided into 4 sections for in-gel digestion. Destaining was performed by washing three times with 10 mM ABC and acetonitrile (ACN) (1:1, v/v) and was followed by protein reduction with 10mM DTT in 20mM ABC for 45 min at 56°C, and alkylation with 55 mM iodoacetamide in 20 mM ABC for 30 min at room temperature in the dark. The gel pieces were then washed twice for 20 min in destaining solution followed by dehydration with ACN. The liquid was removed and gel pieces were swollen at room temperature by adding 13 ng/mL sequencing-grade trypsin (Promega) in 20 mM ABC. Digestion of proteins was performed at 37°C overnight. The resulting peptides were extracted in three subsequent incubation steps with 30% ACN/3% TFA; with 80% ACN/0.5% acetic acid; and with 100% ACN. Supernatants were combined,

ACN was evaporated in a vacuum centrifuge, and peptides were desalted using C_{18} StageTips.

2.5.8 NanoLC-MS/MS analysis

In collaboration with the Proteome Center at the University of Tübingen, all digested peptide mixtures were separated on a nanoLC (Easy-nLC , Thermo Fisher Scientific, formerly Proxeon Biosystems) coupled to a LTQ-Orbitrap-XL (Thermo Fisher Scientific) through a nano-LC-MS interface (Proxeon Biosystems). Binding and chromatographic separation of the peptides was performed on a 15-cm fused silica emitter of 75-mm inner diameter (New Objective), in-house packed with reversed-phase ReproSil-Pur C18-AQ 3-mm resin (Dr. Maisch GmbH). The peptide mixtures were injected onto the column in HPLC solvent A (0.5% acetic acid) at a flow rate of 500 nL/min and subsequently eluted with a 127-min segmented gradient of 5%–90% HPLC solvent B (80% ACN in 0.5% acetic acid) at a flow rate of 200 nL/min. MS data acquisition was conducted in the positive ion mode. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full-scan MS spectra were acquired in the mass range of m/z 300–2000 in the orbitrap mass analyzer at a resolution of 60,000. An accumulation target value of 10^6 charges was set and the lock mass option was used for internal calibration (Olsen et al., 2005). The 10 most intense ions were sequentially isolated and fragmented in the linear ion trap using collision-induced dissociation (CID) at the ion accumulation target value of 5000 and default CID settings. The ions already selected for MS/MS were dynamically excluded for 90 sec. The resulting peptide fragment ions were recorded in the linear ion trap.

2.5.9 Data processing and analysis of MS/MS data

In collaboration with the Proteome Center at the University of Tübingen, raw files were processed using the MaxQuant software (v.1.2.2.9) (Cox and Mann, 2008). Raw MS spectra were first processed by the Quant module to generate peak lists. To retrieve peptide sequences from the processed spectra, the integrated Andromeda peptide search engine (Cox et al., 2011) was utilized. The processed MS spectra were searched against a decoy Arabidopsis thaliana database (Uniprot organism 3702 reference proteome as of 12/25/2012) containing 33317 forward protein entries plus the sequences of 248 commonly observed contaminants. In the database search, carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and acetylation (protein N termini) were set as variable modifications. The mass tolerances for precursor and fragment ions were set to 6 ppm and 0.5 Da, respectively. A false discovery rate (FDR) of 1% was set at the peptide, protein and phosphorylation site level.

2.6 Bioassays

2.6.1 Infection with *Pseudomonas syringae*

For the bacterial infection assay *Pseudomonas syringae* pv. *tomato* DC3000 was diluted with 10mM $MgCl_2$ to a density of 1 x 10^4 cfu/ml and was then infiltrated with a 1ml-needleless syringe into the leaf apoplast. Two leaves per plant and 8 plants were infected per genotype. The growth of bacteria was determined 0, 1, 2 and 4 days post infection. For bacterial growth quantification, the infected leaves were harvested and washed for one minute with 70 % (v/v) EtOH followed by one minute with water. Afterwards 2 leaf discs (5 mm diameter) per leaf were homogenized in 200 µl 10 mM $MgCl_2$. 10 µl of each homogenate were then plated undiluted and in different dilutions onto LB agar plates and incubated at 28°C for 24-48 hours. The growth of bacteria was determined by colony counting and mean values and standard error were determined.

2.6.2 Infection with *Alternaria brassicicola*

Alternaria brassicicola spores used for the infection assays were obtained as published previously (Thomma et al., 1999). Leaves were drop-inoculated with 5µl droplets of aqueous spore suspensions at a concentration of $5x10^5$ spores/ml. Two leaves per plant and a minimum of 15 plants per line were infected. To avoid positional effects plants of different lines were randomly distributed in the tray and incubated at 100% relative humidity. Fungal growth was scored at 7, 10 and 13 days after inoculation by symptom classification: 1 (no symptoms), 2 (light necrotic lesions), 3 (severe necrotic lesions), 4 (spreading of lesions beyond infection site), 5 (whole leaf affected) and 6 (sporulation of the fungus). From this scoring, disease index (Kemmerling et al., 2007) was calculated.

2.6.3 Elicitation assays in leaves or seedlings

Leaves of 4-6 week old plants were infiltrated using a needle-less syringe with flg22 solutions and harvested after indicated time points. For the seedlings elicitations seedlings were first cultivated on sterile ½ MS agar plates for 5-6 days in long-day growth conditions followed by transfer to liquid MS medium supplemented with 1 % (w/v) saccharose (4-6 seedlings in 200µl medium/well, 24 well plate) and equilibrated overnight. After addition of flg22, the seedlings were harvested after four hours for gene expression analysis. For seedling growth inhibition assays, seedlings were treated with 0.1M NaCl and 0.1% BSA with and without flg22 and seedling mass was recorded after 5 days.

2.6.4 PSKα promotion of root elongation

To test the promotion of root growth by PSKα, Col-0 seeds were germinated on sterile ½ MS agar plates and were placed in a long day growth chamber. Plates were placed vertically in the growth chamber to prevent roots from entering the agar medium and allowed to grow for 7 days. Seedlings were then transferred to ½ MS agar plates containing either 100 nm PSKα or water as a control. After 6 days, root lengths were measured using the software ImageJ.

2.7 Microscopy and histochemistry

2.7.2 Aniline blue stain

The induction of callose deposits upon PAMP treatment was analyzed by aniline blue (water blue) staining (Gomez-Gomez et al., 1999). Leaves were infiltrated with the different PAMPs and incubated for 24 hours and subsequently incubated with a fixing solution (1% (v/v) glutaraldehyde; 5mM citric acid; 90mM Na_2HPO_4; pH7.4) for 24 hours at room temperature. After fixation the leaf tissue was bleached with 100% (v/v) EtOH for 1-2 days. The leaves were then transferred to 50% (v/v) EtOH and afterwards equilibrated in 67 mM K_2HPO_4 (pH 12.0) and finally stained for 1 hour at room temperature in 0.1% (w/v) aniline blue dissolved in 67 mM K_2HPO_4 (pH 12.0). The stained leaves were transferred to a microscope slide in 70% (v/v) glycerol and 30% (v/v) staining solution and examined by UV epifluorescence microscopy. Quantification of Analine blue staining was performed using the software ImageJ by averaging the mean signal detected by the software.

2.7.3 Trypan blue stain

Trypan blue staining was used to visualize dead cells and fungal structures after infection with *Alternaria brassicicola*. Infected leaves were treated for 1 minute with trypan blue stain (10 ml lactic acid, 10 ml 100% glycerol, 10ml Aqua-Phenol, 10 ml ddH2O, 80 ml EtOH and 300 mg Trypan blue) and afterwards bleached with a 1mg/ml chloral hydrate solution and examined by light microscopy.

2.7.4 Bimolecular fluorescence complementation

Bimolecular fluorescence complementation experiments were conducted by cloning PSKR1, PSY1R, BAK1 and FLS2 into the ubiquitin-10 promotor driven pUB-Dest vector system

(Grefen et al., 2010) followed by transiently co-expression in *Nicotiana benthamiana* by *Agrobacterium tumefaciens*-mediated transient transformation (see 2.4.7). After 48 hours, leaves were transferred to a microscope slide and mounted in water and examined by UV epifluorescence microscopy.

2.8 Statistical analysis

Statistical analysis was performed using Microsoft Office Excel or JMP. The data represent the average of replicates with plus or minus standard error of the mean (SE). The significance of the differences observed was calculated using either the t-test or ANOVA.

3 Results

LRR-RLKs often act as regulators of defense responses and the genes encoding these receptors are often transcriptionally up-regulated by pathogen treatment (Boller and Felix, 2009). Using a reverse genetics approach, Postel et al (2010) identified 49 pathogen-inducible LRR-RLK genes in *Arabidopsis* including the previously described PSKR1 receptor. PSKR1 perceives endogenous tyrosine-sulfated peptides referred to as phytosulfokines resulting in growth promotion and various developmental processes. PSKR1 was identified as being transcriptionally up-regulated by treatment with the type three secretion system deficient strain *Pto* DC3000 *hrcC*- and the non-host pathogen *Pseudomonas phaseolicola*. *PSKR1* expression was also up-regulated by treatment with the PAMPs flg22, hrpZ and NPP1. As such, mutants affecting *PSKR1* as well as the closely related LRR-RLKs *PSKR2* and *PSY1R* were functionally characterized to determine their role in defense responses.

3.1 The influence of sulfated peptide signaling on plant defense

3.1.1 Genetic characterization of T-DNA insertion lines

As the T-DNA insertion lines required for the functional analysis of *PSKR1*, *PSKR2*, *PSY1R* and *TSPT* were not all homozygous, genotyping by PCR was conducted for *pskr1-3*, *pskr2* and *psy1r* (Figure 1a). The double receptor mutants *pskr1-3/pskr2*, *pskr1-3/psy1r* and *pskr2/psy1r* (Figure 1b) and the triple receptor mutant *pskr1-3/pskr2/psy1r* (Figure 1c) as well as the tyrosylprotein sulfotransferase insertion line *tpst-1* (Figure 1d) were also selected for homozygous lines using genotyping PCR.

3.1.2 Influence of *PSKR1* on resistance responses to bacterial and fungal pathogens

Since the results from a former student researching the effect of phytosulfokine signaling were based on lines that were not all homozygous, several experiments regarding the effect

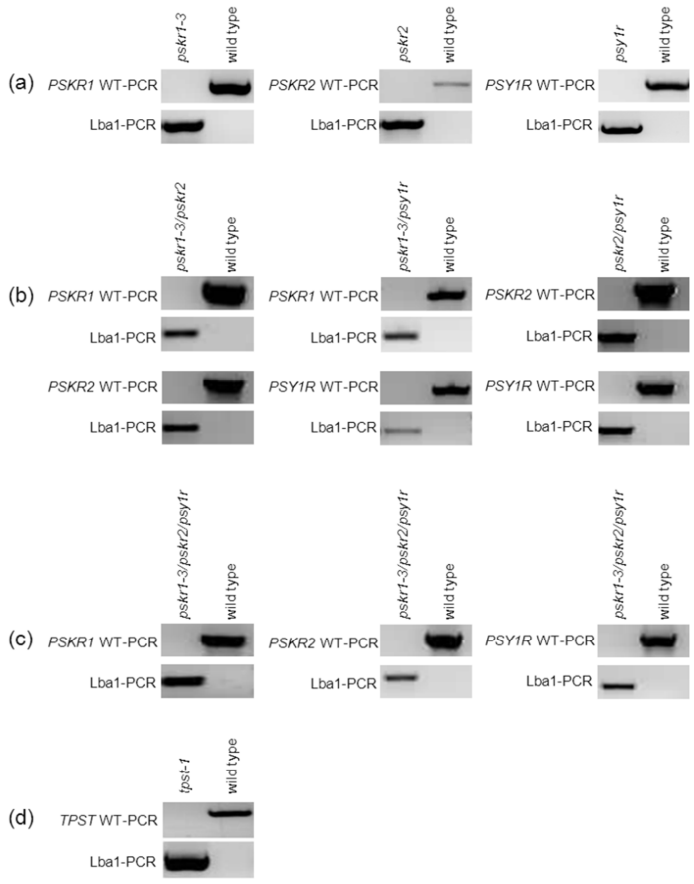

Figure 1. Genotyping T-DNA insertion mutants of *PSKR1*, *PSKR2*, *PSY1R* and *TPST* in single and multiple mutant lines.
Leaf genomic DNA was isolated from Col-0 wild type and mutant plants and PCR was performed for the purpose of genotyping using either gene specific wild type (WT) primers or T-DNA specific (Lba1) primers. Lines tested were the single receptor mutants *pskr1-3*, *pskr2*, and *psy1r* (a), the double mutants *pskr1-3/pskr2*, *pskr1-3/psy1r* and *pskr2/psy1r* (b), the triple receptor mutant *pskr1-3/pskr2/psy1r* (c) and the tyrosylprotein sulfotransferase *tpst-1* (d).

of sulfated peptide receptors on plant defense were necessarily repeated. Analysis of the loss-of-function allele *pskr1-3* revealed an enhanced resistance phenotype to infection with *Pto* DC3000 in bacterial growth assays (Figure 2). Bacterial growth was reduced by up to two orders of magnitude in the *pskr1* mutants. This shows that loss of *PSKR1* has a strong positive impact on resistance to biotrophic bacterial pathogens.

To further characterize the role of PSKR1 in defense responses to an additional plant pathogen, *pskr1* mutants were inoculated with the necrotrophic fungus *Alternaria brassicicola* that shows an incompatible interaction with Arabidopsis Col-0 plants (Thomma et al., 1999). This resulted in higher disease indices, greater lesion sizes and stronger disease symptoms

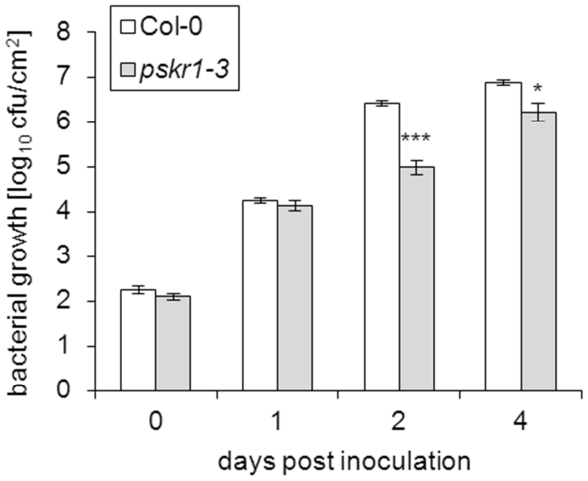

Figure 2. Mutants lacking *PSKR1* are more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000.
Bacterial growth was monitored at 0 to 4 days post inoculation with 10^4 cfu (colony forming units) ml^{-1} *Pto* DC3000 in five-week-old plants of the indicated genotype. Mean values of the number of bacteria are presented ± SEM of at least 6 biological replicates, asterisks represent significant differences from Col-0 (**p*<0.5, ****p*<0.001, Student's t-test). Representative results of three experiments are presented.

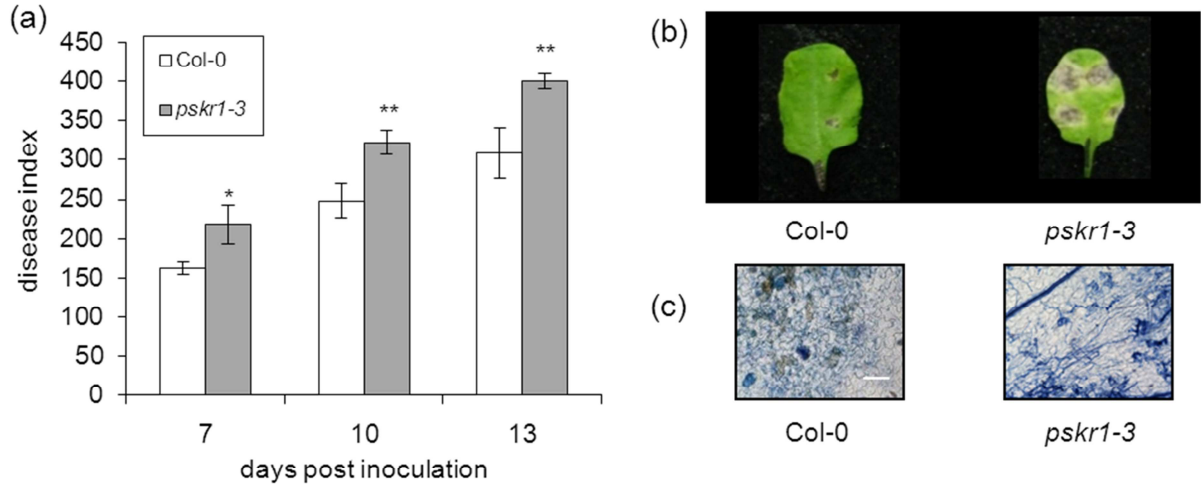

Figure 3. *PSKR1* mutants are more susceptible to the fungal pathogen *Alternaria brassicicola*.
Disease symptoms were monitored at 7, 10 and 13 days after inoculation of 5-week-old plants of the indicated genotype with 10^5 spores ml^{-1}. Mean values of disease indices are presented ± SEM of at least 15 replicates, asterisks represent significant differences from Col-0 (*P < 0.05, **P < 0.01, Student's t-test) (a). Pictures of representative leaves were taken 13 days after inoculation (b). Leaves were subjected to trypan blue staining for visualization of mycelial growth 10 days after inoculation (c). Scale bar represents 0.2 mm. Representative results of three experiments are presented.

in *pskr1-3* mutants compared with wild type plants (Figure 3a,b). At the microscopic level, trypan blue staining showed that *pskr1-3* mutant plants developed higher levels of cell death than wild type plants in response to fungal infection (Figure 3c). Furthermore, while fungal growth stopped at the stage of germinated spores at the zone of inoculation in wild type plants, this was not the case in *pskr1-3* plants, where the mycelium grew and spread outside this zone, suggesting that loss of *PSKR1* has a negative effect on fungal resistance.

3.1.3 Influence of *PSKR2* and *PSY1R* on resistance responses to bacterial and fungal pathogens

The small subfamily of LRR-RLKs including PSKR1, PSKR2 and PSY1R was reported to function in a partially redundant manner in PSKα- and PSY1-mediated cell proliferation (Amano et al., 2007). Since we found that PSKR1 has a strong impact on the resistance of plants to *Pto* DC3000, mutants in its close paralogs *pskr2* and *psy1r* were subjected to bacterial infection experiments to determine if they have additional functions in defense

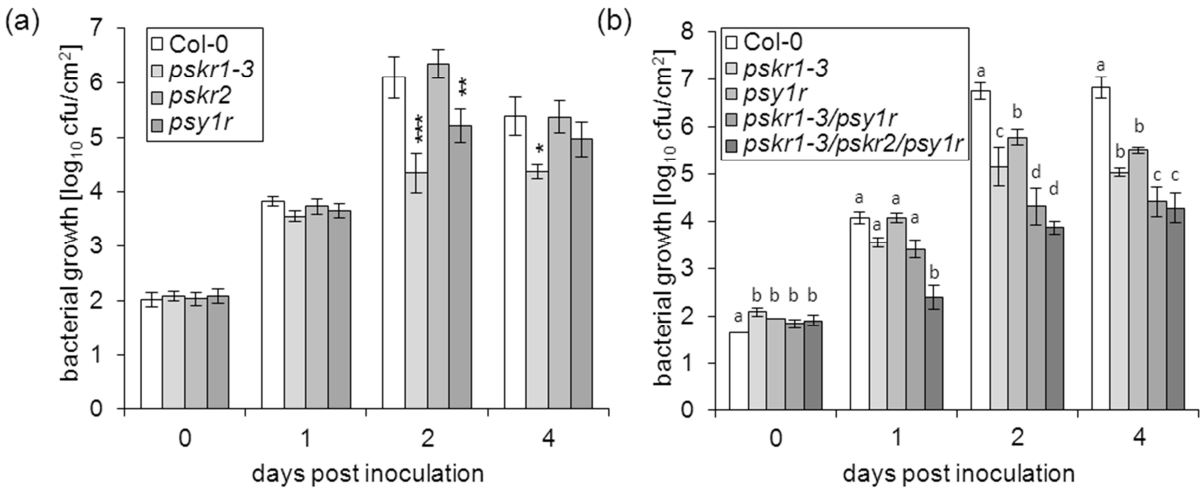

Figure 4. Loss of *PSKR1* and *PSY1R* but not *PSKR2* has an effect on resistance to the bacterial pathogen *Pto* DC3000.
Bacterial growth was monitored at 0 to 4 days post-inoculation with 10^4 cfu ml^{-1} *Pto* DC3000 in single mutants (a) as well as in double and triple mutants (b). Mean values are presented ± SEM of at least six biological replicates. Representative results of four experiments are presented. Asterisks represent significant differences from Col-0 (*P < 0.05, **P < 0.01, ***P < 0.001, Student's t-test). Bars with different letters are significantly different based on one-way ANOVA (P < 0.1, Student's t-test). Representative results of three experiments are presented.

responses. While bacterial growth on *pskr2* mutant plants was not significantly different than wild type on any of the days tested, loss of *PSY1R* lead to approximately 10-fold decreased growth of *Pto* DC3000 two days post inoculation (Figure 4a). Double and triple mutants of all three genes were tested in all combinations to assess the impact of each gene on bacterial resistance. Interestingly, *pskr1-3/psy1r* double mutant plants displayed an additive resistance phenotype to *Pto* DC3000, with just over 100-fold less growth than in wild type plants (Figure 4b). The triple mutant *pskr1-3/pskr2/psy1r* did not show any further differences from the *pskr1-3/psy1r* double mutant. Furthermore, the *pskr2* mutation in the *pskr1-3* and *psy1r* mutant background did not affect the bacterial resistance phenotypes observed in the single mutants, confirming a limited role for *PSKR2* in *Pto* DC3000 resistance (Figure 5).

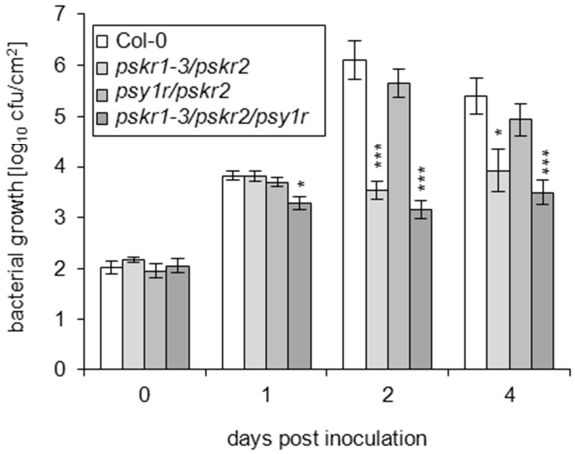

Figure 5. *pskr2* does not affect *pskr1* or *psy1r Pto* DC3000 resistance phenotypes in double and triple mutants.
Bacterial growth was monitored at 0 to 4 days post inoculation with 10^4 cfu ml^{-1} *Pto* DC3000. Mean values are presented ± SEM, asterisks represent significant differences from Col-0 (**p*<0.5, ***p*<0.01, ****p*<0.001, Student's t-test). Representative results of three experiments are presented.

In further characterizing this subfamily of LRR-RLKs, *pskr1*, *pskr2* and *psy1r* mutant plants were then subjected to fungal disease-rating experiments. It was observed that *pskr2*, like in bacterial growth assays, did not show any significant differences in defense responses compared to wild type plants (Figure 6). On the other hand, the loss of *PSY1R* resulted in increased symptom formation, disease index, lesion size and fungal growth after infection with *Alternaria brassicicola* (Figure 6). In combination, *pskr1-3/psy1r* double mutants cause a very high disease index, severe lesion formation and strong mycelial growth compared with

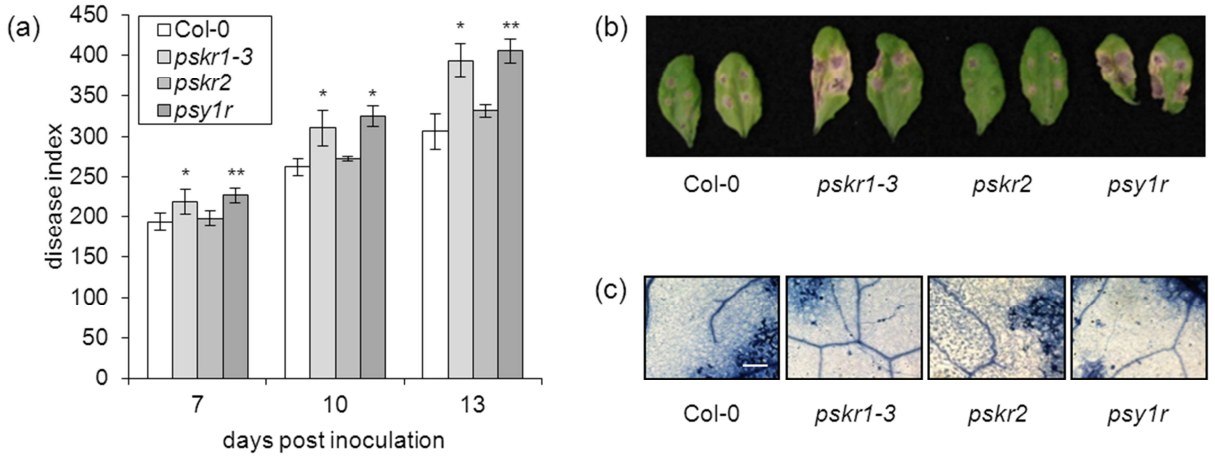

Figure 6. Mutants affecting *PSY1R* but not *PSKR2* are more susceptible to the fungal pathogen *Alternaria brassicicola*.
Disease symptoms were monitored at 7, 10 and 13 days after inoculation with 10^5 spores ml^{-1}. Mean values are presented ± SEM of at least 15 replicates, asterisks represent significant differences from Col-0 (*P < 0.05, **P < 0.01, Student's t-test) (a). Pictures of representative leaves were taken 13 days after inoculation (b). Leaves were subjected to trypan blue staining for visualization of mycelial growth 10 days after inoculation (c). Scale bar represents 0.2 mm. Representative results of four experiments are presented.

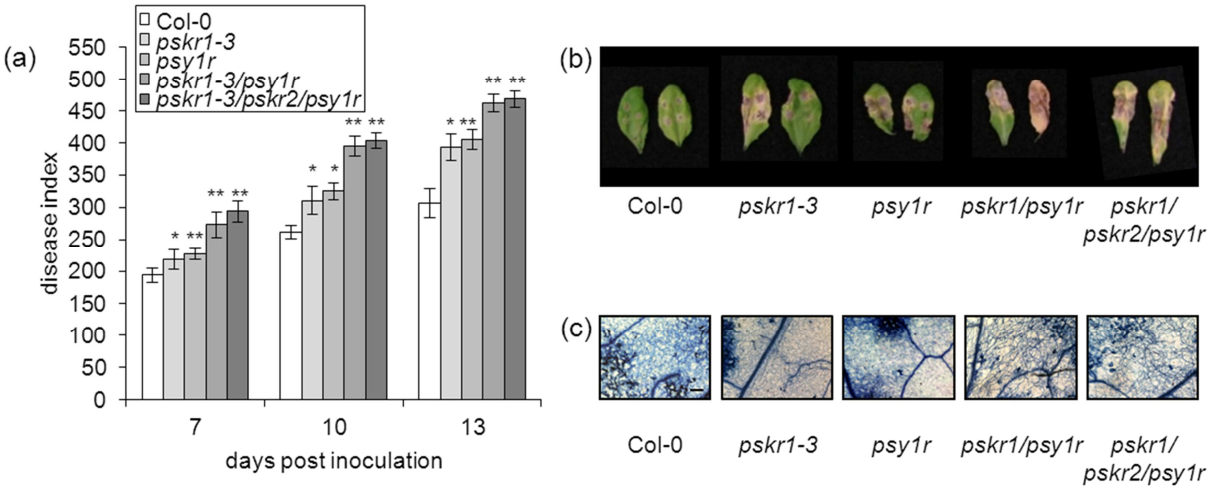

Figure 7. The *Alternaria brassicicola* susceptibility phenotype of *pskr1* and *psy1r* mutants is additive in double mutants.
Disease symptoms were monitored at 7, 10 and 13 days after inoculation with 10^5 spores ml^{-1}. Mean values are presented ± SEM of at least 15 replicates, asterisks represent significant differences from Col-0 (*P < 0.05, **P < 0.01, Student's t-test) (a). Pictures of representative leaves were taken 13 days after inoculation (b). Leaves were subjected to trypan blue staining for visualization of mycelial growth 10 days after inoculation (c). Scale bar represents 0.2 mm. Representative results of four experiments are presented.

wild type plants (Figure 7). The *pskr2* mutation had little effect on these *A. brassicicola* phenotypes in all mutant combinations tested (Figure 8). Since, in addition, the double mutant *pskr1-3/psy1r* displayed the same level of symptoms as the triple mutant *pskr1-3/pskr2/psy1r* (Figure 7), the role of *PSKR2* in defense against *Alternaria brassicicola* seems to be marginal.

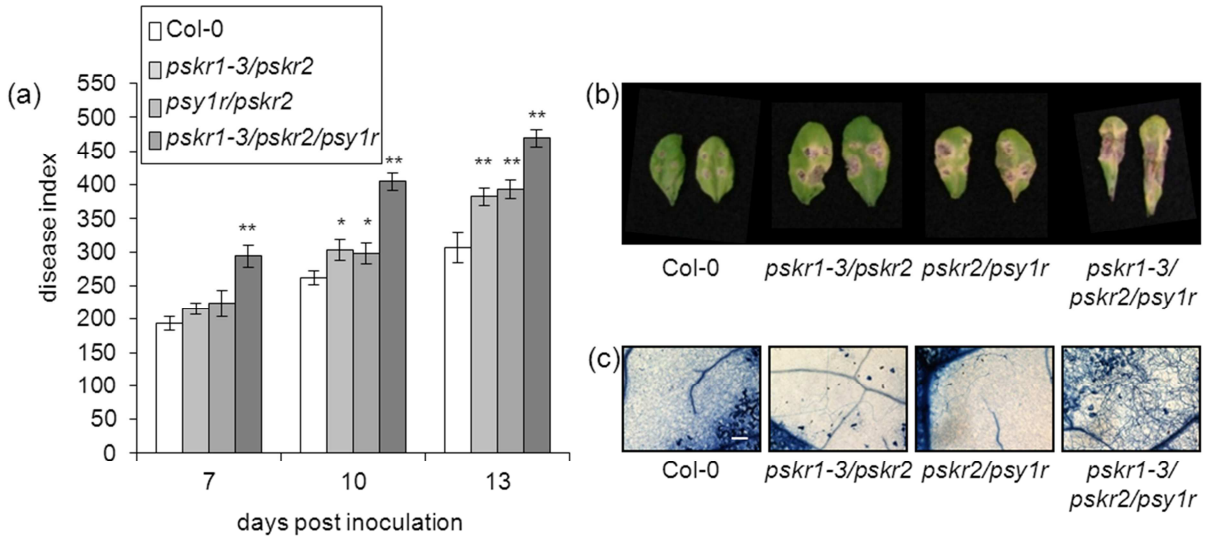

Figure 8. *pskr2* does not affect *pskr1* or *psy1r Alternaria brassicicola* susceptibility phenotypes in double and triple mutants.
Disease symptoms were monitored at 7, 10 and 13 days after inoculation with 10^5 spores ml^{-1}. Mean values are presented ± SEM of at least 15 replicates, asterisks represent significant differences from Col-0 (*p<0.5, **p<0.01, Student's t-test) (a). Pictures of representative leaves were taken 13 days after inoculation (b). Leaves were subjected to trypan blue staining for visualization of mycelial growth 10 days after inoculation (c). Scale bar represents 0.2 mm. Representative results of four experiments are presented.

3.1.4 Influence of PSKR1 and PSY1R on PAMP responses

Based on microarray data (Postel et al., 2010), *PSKR1* transcription is up-regulated by PAMPs. To address whether mutations in *PSKR1* and *PSY1R* alter PAMP responses, plants were treated with 100 nm flg22 and callose deposition was visualized by aniline blue staining (Figure 9a,b). Microscopically, it is obvious that the receptor mutants produce an increased amount of callose after PAMP treatment compared with wild type plants. Quantification reveals that *psy1r* plants have a very similar amount of increased callose staining as pskr1 mutants (both about 1.7-fold) compared with wild type plants indicating that both PSKR1 and PSY1R receptors suppress PAMP-induced callose formation. The triple mutant

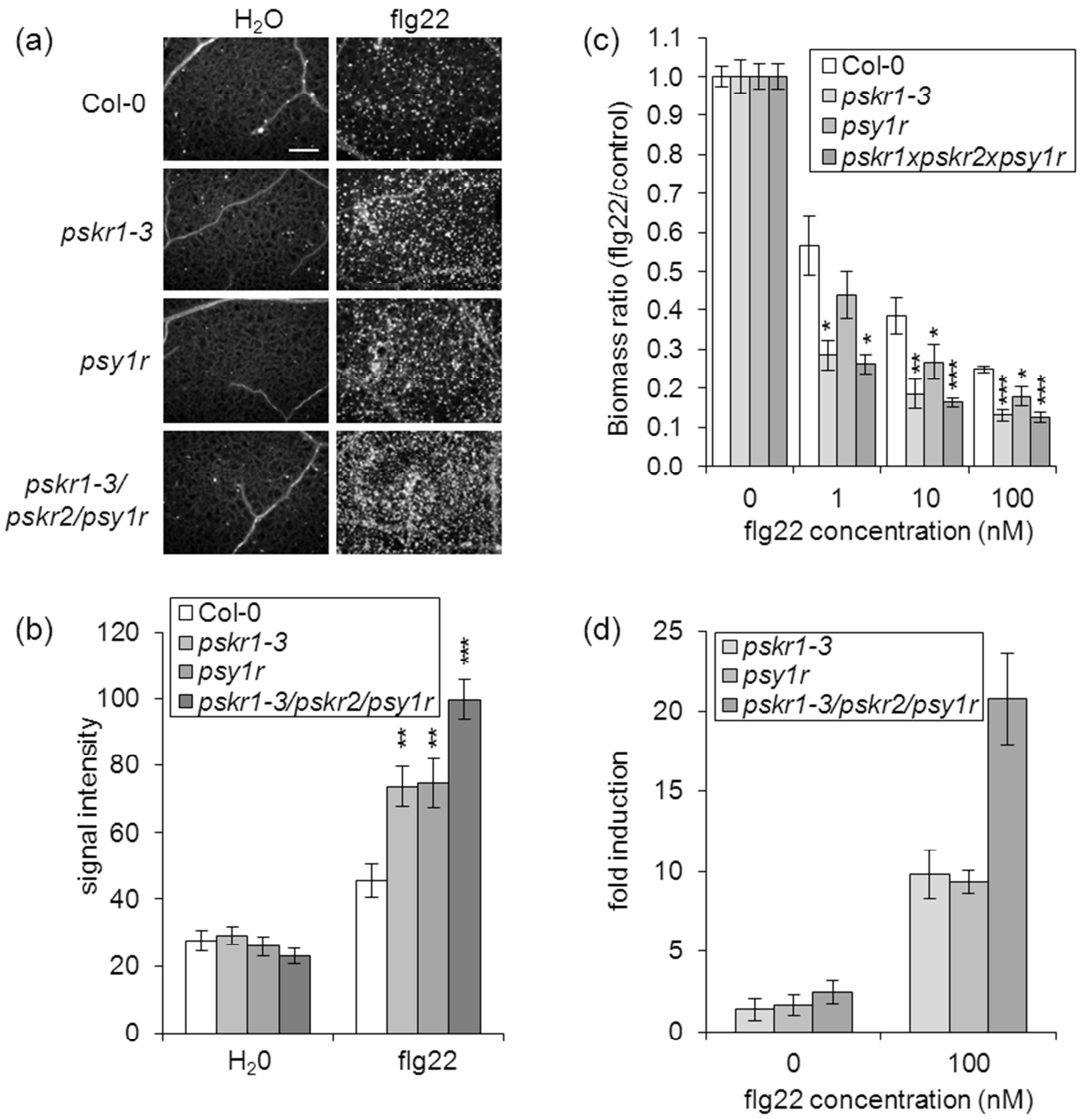

Figure 9. *PSKR1* and *PSY1R* mutants are hypersensitive to pathogen-associated molecular pattern treatment.
Adult plants were infiltrated with or without 100 nm flg22 for 24 hours. (a) Callose deposition visualized by aniline blue staining. Scale bar represents 0.2 mm. (b) Signal intensity plotted as mean values ± SEM from 10 replicates. (c) Biomass of flg22-induced inhibition of seedling growth presented as mean values ± SEM from six replicate samples containing eight seedlings each. (d) For *FRK1* gene expression analysis, quantitative RT-PCR experiments were performed on cDNA generated from three independent biological replicates. Expression values were normalized to *EF1α* and then expressed as a ratio to Col-0 and presented as fold induction. Bars represent mean ratios ± SEM. Representative results of three experiments are presented. Asterisks represent significant differences from Col-0 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test).

pskr1/*pskr2*/*psy1r* reflects the additive effects of both receptors with 2.4-fold more callose being detected. To further characterize alterations in PAMP responses, seedling growth inhibition, which usually accompanies PAMP responses and *FRK1* expression, was studied. After flg22 treatment, all three mutants showed a strongly enhanced seedling growth

inhibition compared with wild type at the 10 and 100 nm levels, with slightly less responsiveness in *psy1r* mutants reflected by insignificant changes at the 1 nm level (Figure 9c). Adult leaves were treated with 100 nm flg22 for 4 hours and the induction of *FRK1* was measured by quantitative RT-PCR. Mutants in *pskr1-3*, *psy1r* and *pskr1-3*/*pskr2*/*psy1r* all displayed an enhanced induction of *FRK1* compared with wild type upon treatment, suggesting that both PSKα and PSY1-signaling is required for down-regulating PAMP responses (Figure 9d). Cumulatively, these data show that PAMP responses are suppressed by PSKR1 and PSY1R signaling, suggesting that PSKα and probably PSY1 act in a negative regulatory loop to prevent over-responsiveness to PAMPs.

3.1.5 Characterization of defense phenotypes associated with *pskr1* and *psy1r* mutations at the molecular level

To further characterize the effect of *pskr1* and *pskr1*/*pskr2*/*psy1r* mutations on defense responses at the molecular level, plants were treated with *Pto* DC3000 and SA levels were analyzed. In *pskr1-3* mutant plants, SA levels are about 1.6-fold higher than in wild type plants at 12 and 24 h post-inoculation. In the triple mutant *pskr1-3**l**pskr2**l**psy1r* plants, SA accumulation was delayed, but by 24 h post-inoculation the plants contained about two times more SA than wild type plants (Figure 10a). Real-time PCR-based gene expression analysis was performed after *Pto* DC3000 treatment to assess whether *PR* gene expression was altered in the mutants. The single *pskr1-3* and the triple *pskr1-3**l**pskr2**l**psy1r* mutant exhibited significantly higher induction of *PR1* than wild type plants (three- and eight-fold, respectively) (Figure 10b). In the case of *PR2*, the induction was about three- and five-fold higher in *pskr1-3* and *pskr1-3**l**pskr2**l**psy1r*, respectively (Figure 10c). Expression levels of *FRK1**,*** a marker for PAMP-induced gene expression, peaked at 12 h post-inoculation with an increased induction of about seven- and twelve-fold in *pskr1-3* and *pskr1-3**l**pskr2**l**psy1r* mutants, respectively (Figure 10d). On the other hand, upon *Pto* DC3000 treatment, both

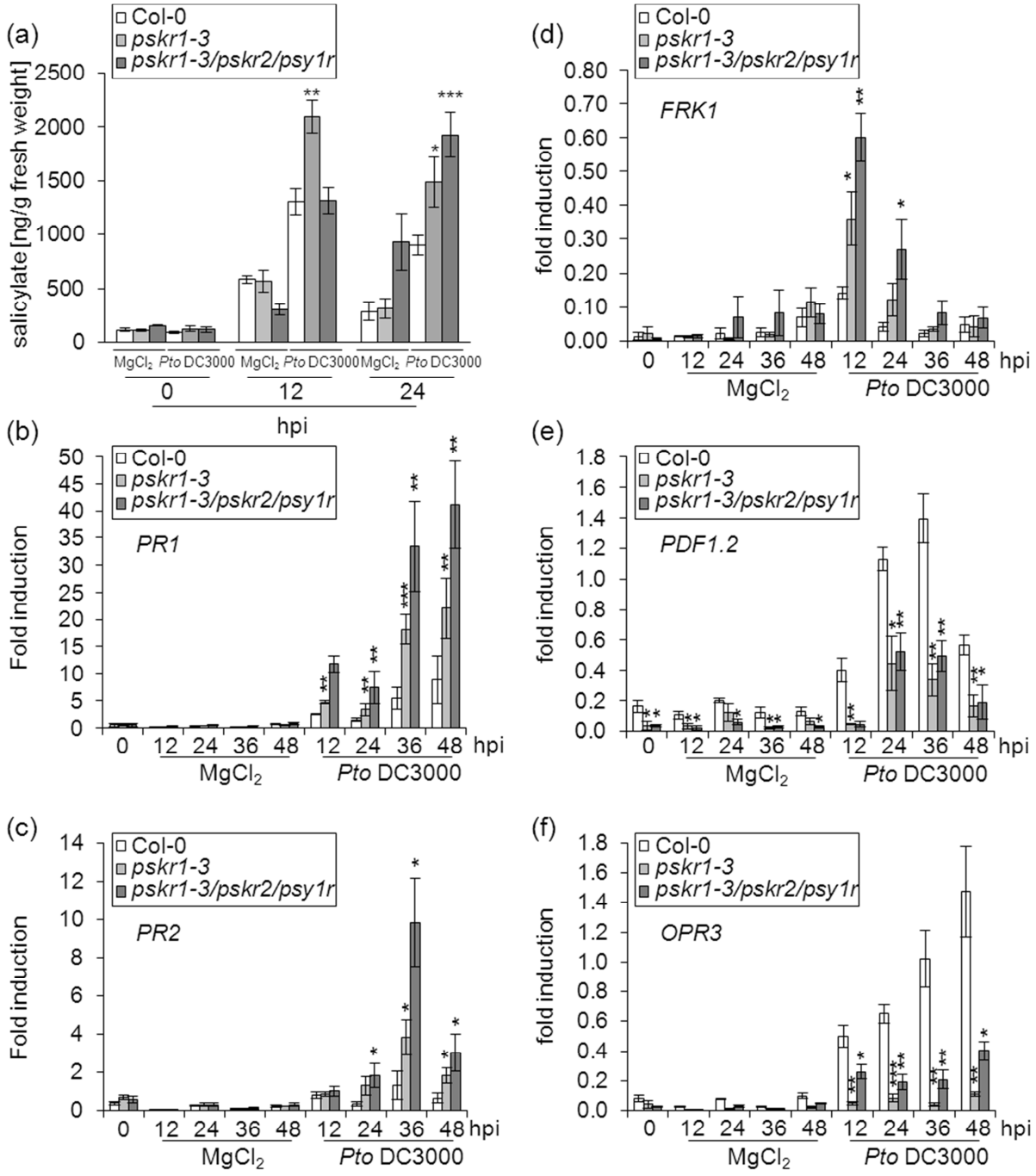

Figure 10. SA content and SA marker gene expression in *pskr1-3* and *pskr1lpskr2lpsy1r* triple mutants after *Pto* DC3000 infection are enhanced while JA marker gene expression is reduced.
After treatment of 5-week-old plants of the indicated genotypes with 10^8 cfu ml^{-1} *Pto* DC3000, SA content was determined by GC-MS 0, 12 and 24 h after infection. Mean values are presented ± SEM of six biological replicates (a). For marker gene expression analysis of *PR1*(b), *PR2* (c), *FRK1* (d), *PDF1.2* (e), and *OPR3* (f), quantitative RT-PCR experiments were performed on cDNA generated from three independent biological replicates. Expression values were calculated as a ratio to *EF1α* and presented as fold induction. Bars represent mean ratios ± SEM. Representative results of three experiments are presented. Asterisks represent significant differences from Col-0 (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, Student's *t*-test).

pskr1-3 and *pskr1-3**l**pskr2**l**psy1r* plants expressed lower levels of *PDF1.2**,*** a marker gene for JA-dependent defense responses***,*** as compared with wild type plants. In both mutant plants, expression of *PDF1.2* was lower in uninoculated mutant plants (about 30- and 100-fold, respectively) and remained lower during *Pto* DC3000 infection (Figure 10e). In the case of *OPR3*, untreated *pskr1-3* and *pskr1**l**pskr2**l**psy1r* plants also had fewer transcripts than the wild type. Induction of *OPR3* never reached wild type levels in either mutant plant (about ten- and two-fold less, respectively) (Figure 10f). Taken together, SA- and PAMP-responsive genes are transcriptionally up-regulated in *pskr1* and more strongly in *pskr1-3**l**pskr2**l**psy1r* mutants upon *Pto* DC3000 treatment, while JA-responsive genes are down-regulated. This observation may reflect a shift in the antagonistic hormone signaling pathways to the advantage of SA signaling.

3.1.6 Effect of tyrosine peptide sulfation on plant defense responses

In animals and plants, peptide tyrosine sulfation is mediated by a unique membrane localized TPST (Moore, 2003; Matsubayashi, 2012). Loss-of-function mutants in *TPST* mimic the triple receptor mutant phenotypes and have additional phenotypes comparable to plants lacking sulfated peptides or their corresponding receptors (Komori *et al*., 2009; Zhou *et al*., 2010; Stuhrwohldt *et al*., 2011). Mutants in TPST show a strong bacterial resistance phenotype after infection with *Pto* DC3000, phenocopying the effects observed in the triple receptor mutants (Figure 11a). Enhanced PAMP-induced callose formation is found in *tpst-1* mutants as well as seedling growth inhibition and *FRK1* gene expression (Figure 12). Furthermore, susceptibility to *A. brassicicola* was strongly enhanced and indistinguishable from that of the triple receptor mutant (Figure 11b–d). Taken together, these data show that peptide tyrosine sulfation is necessary for balancing bacterial susceptibility and fungal resistance, two antagonistically regulated defense pathways.

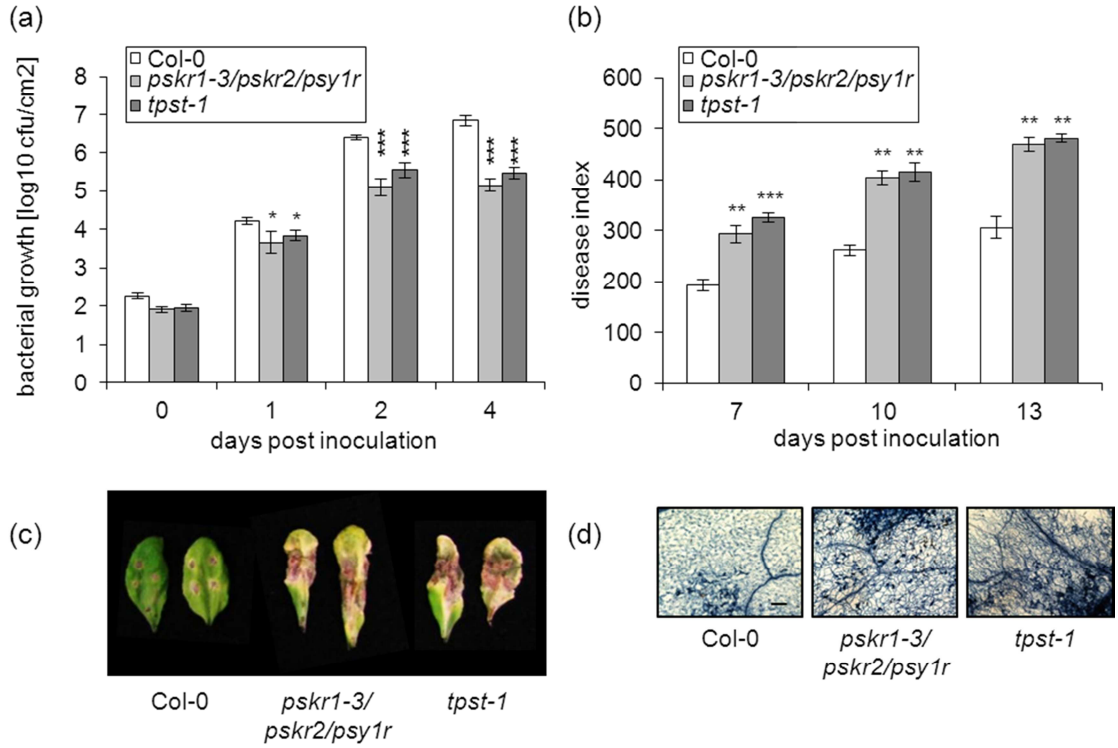

Figure 11. *tpst-1* mutant plants lacking sulfated peptides show increased resistance to *Pto* DC3000 and decreased resistance to *Alternaria brassicicola*.
(a) Bacterial growth was monitored at 0 to 4 days post-inoculation with 10^4 cfu ml^{-1} *Pto* DC3000 in 5-week-old plants. Mean values of bacterial numbers are presented ± SEM of at least six biological replicates. (b) Plants were inoculated with 10^5 spores ml^{-1} of *Alternaria brassicicola*. Mean values of disease scoring are presented ± SEM of at least 15 replicates. *Alternaria brassicicola* treated plants were photographed 13 days after inoculation (c) or subjected to trypan blue staining 10 days after inoculation (d). Scale bar represents 0.2 mm. Representative results of four experiments are presented. Asterisks represent significant differences from Col-0 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test).

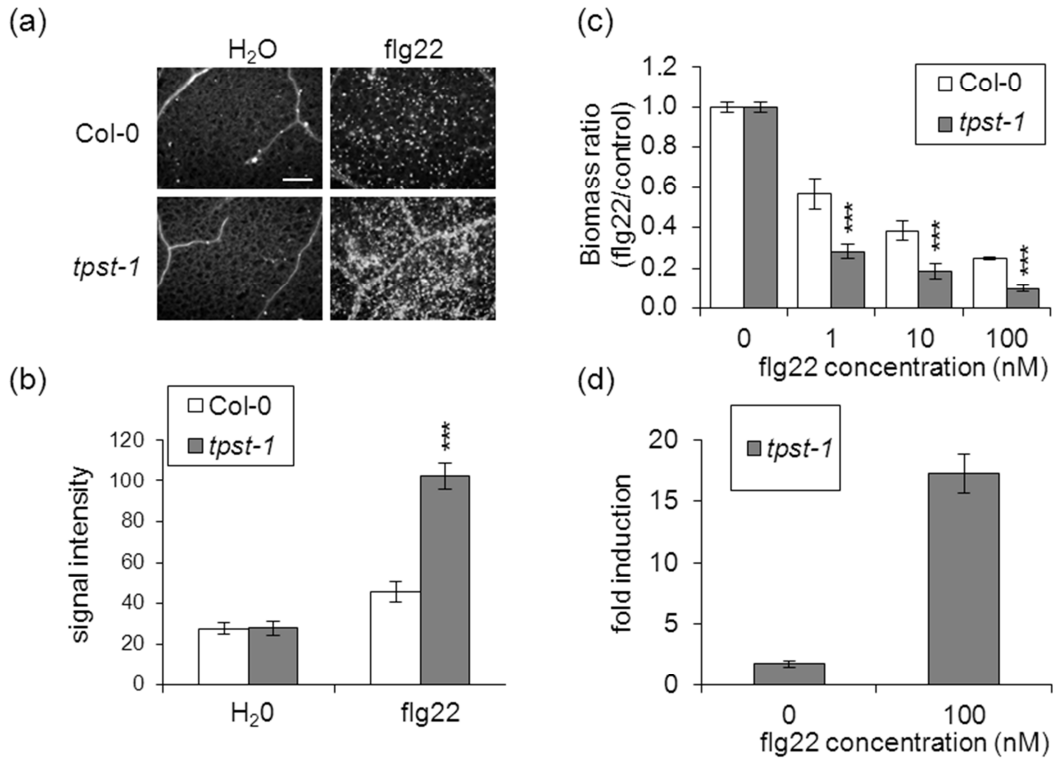

Figure 12. PAMP responses are altered in *tpst* mutants.
Adult plants were infiltrated with or without 100 nM flg22 for 24 hours and (a) callose deposition was visualized by aniline blue staining. Signal intensity was plotted as mean values ± SEM from 10 replicates. Asterisks represent significant differences from Col-0 (***p<0.001, Student's t-test) (b). Biomass of flg22 induced inhibition of seedling growth was presented as mean values ± SEM from 6 replicate samples containing 8 seedlings each. Asterisks represent significant differences from Col-0 (***p<0.001, Student's t-test) (c). For *FRK1* gene expression analysis, quantitative RT-PCR experiments were performed on cDNA generated from three independent biological replicates. Expression values were normalized to *EF1α* and then expressed as a ratio to Col-0 and presented as fold induction. Bars represent mean ratios ± SEM (d). Scale bar represents 0.2 mm. Representative results of three experiments are presented.

3.1.7 Ectopic expression of *PSK2*, *PSK4* and *PSKR1* affects *Arabidopsis* defense

The previous results indicate that PSKR1 and PSY1R receptors are necessary for balancing defense responses, as is peptide tyrosine sulfation. To prove that these phenomena are indeed due to PSK signaling, PSK2-, PSK4- and PSKR1-encoding regions were overexpressed in *Arabidopsis* (provided provided by Herald Keller) and these lines were tested in bacterial and fungal infection assays. Indeed, expression of all three proteins leads to opposite effects in infection assays compared to *pskr1* and *psy1r* mutants. Upon treatment with *Pto* DC3000, *PSK2*-, *PSK4*- and *PSKR1*-overexpressing plants display a susceptibility phenotype, allowing approximately 10-fold more bacterial growth than in wild

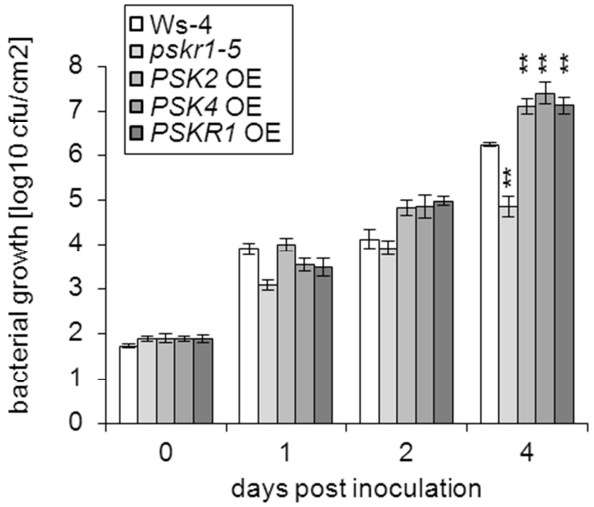

Figure 13. Overexpression of *PSK2*, *PSK4* and *PSKR1* enhances susceptibility to *Pto* DC3000. Bacterial growth was monitored at 0 to 4 days post-inoculation with 10^4 cfu ml^{-1} *Pto* DC3000 in 5-week-old plants. Mean values of bacterial numbers are presented ± SEM of at least six biological replicates Asterisks represent significant differences from Col-0 (**P < 0.01, Student's *t*-test). Representative results of three experiments are presented.

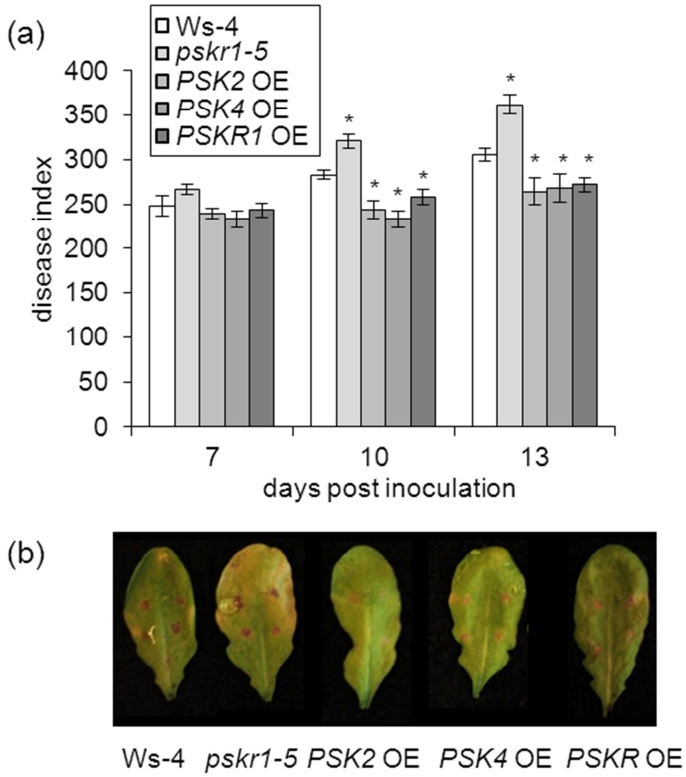

Figure 14. Overexpression of *PSK2*, *PSK4* and *PSKR1* increases resistance to *Alternaria brassicicola*. Plants were inoculated with 10^5 spores ml^{-1} *Alternaria brassicicola*. Mean values of disease scoring are presented ± SEM of at least 15 replicates (a). *Alternaria brassicicola* treated plants were photographed 13 days after inoculation (b). Asterisks represent significant differences from Col-0 (*P < 0.05, Student's *t*-test). Representative results of three experiments are presented.

type plants (Figure 13). On the other hand, treatment with *A. brassicicola* revealed an enhanced resistance phenotype in the overexpressing plants (Figure 14a,b). Taken together, these gain-of-function experiments support that PSKα-signaling has a strong effect on both defense responses to biotrophic and necrotrophic pathogens.

3.1.8 Complementation of *pskr1/pskr2/psy1r* triple receptor mutant with *PSKR1-GFP*

To show that the loss-of-function phenotypes in *pskr1* mutants are indeed due to the loss of *PSKR1*, the coding region of *PSKR1* fused to *GFP* under its native promoter was expressed in the *pskr1-3*/*pskr2*/*psy1r* mutant background (provided by Frans Tax). Expression of *PSKR1-GFP* in the triple mutant background leads to a partial restoration of both the bacterial resistance (Figure 15) and fungal susceptibility phenotypes (Figure 16a,b). The partial character of the complementation might be a result of the construct used, but may also support the impact of the second receptor PSY1R which is not restored in these plants. Taken together, these results show that PSKR1 and most likely also PSY1R play a partially redundant role in plant immunity with antagonistic effects on bacterial and fungal resistance.

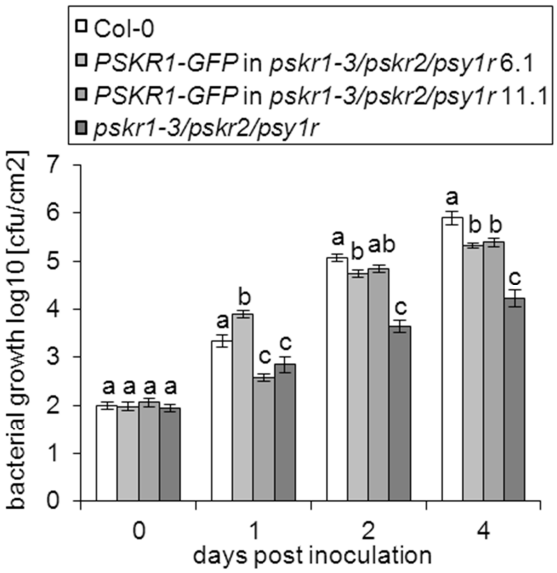

Figure 15. Expression of GFP-tagged *PSKR1* partially complements the *Pto* DC3000 resistance phenotype of *pskr1-3/pskr2/psy1r* plants.
Plants were inoculated with 10^4 cfu ml^{-1} *Pto* DC3000 and growth was monitored at 0 to 4 days after infection. Mean values of bacterial numbers are presented ± SEM of at least 6 biological replicates. Bars with different letters are significantly different based on one-way ANOVA ($p<0.05$, Student's t-test)

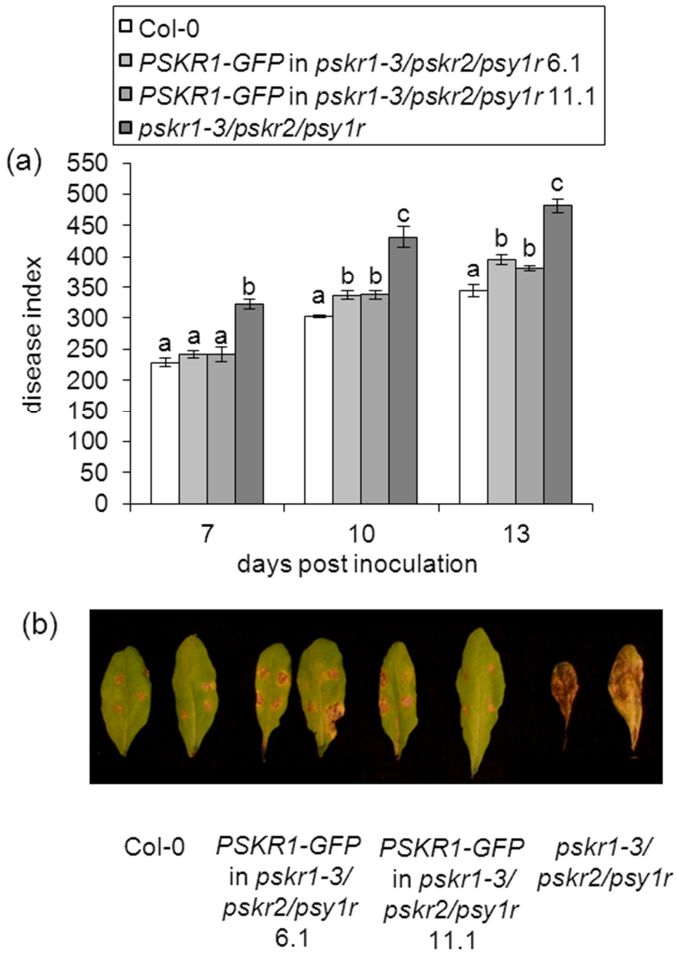

Figure 16. Expression of GFP-tagged *PSKR1* partially complements the *Alternaria brasicicola* susceptibility phenotype of *pskr1-3/pskr2/psy1r* plants.
Plants were treated with 10^5 spores ml^{-1} *Alternaria brassicicola.* Disease indices were rated at days 7, 10 and 13. Mean values are presented ± SEM of at least 15 replicates. Bars with different letters are significantly different based on one-way ANOVA ($p<0.05$, Student's t-test) (a). *Alternaria brassicicola* treated plants were photographed 13 days after inoculation (b). Representative results of three experiments are presented.

3.1.9 The effect of PSKα on plant immunity

To determine if secondary effects such as growth or morphological differences in the mutants might be the reason for the immunity-related phenotypes, we tested if exogenous PSKα application can be directly tied to defense responses. Application of PSKα to wild type plants slightly promotes root growth (Figure 17) but has only weak effects on bacterial resistance (Figure 18). In contrast, *tpst-1* plants were partially rescued from the bacterial resistance

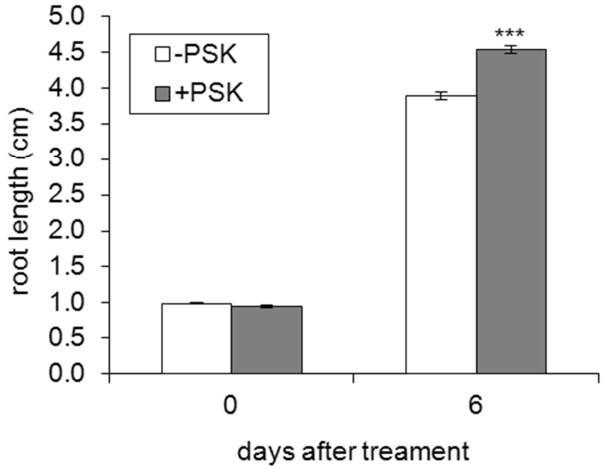

Figure 17. PSKα treatment leads to enhanced root growth in Col-0 seedlings.
To test that our preparation of PSKα is active, seedlings were grown on 1/2 MS media containing either 100nm PSKα or buffer only. Root length measurements were taken 6 days after transfer. Bars represent mean values of 30 replicate seedlings ± SEM. Asterisks represent significant differences from Col-0 (***p<0.001, Student's t-test).

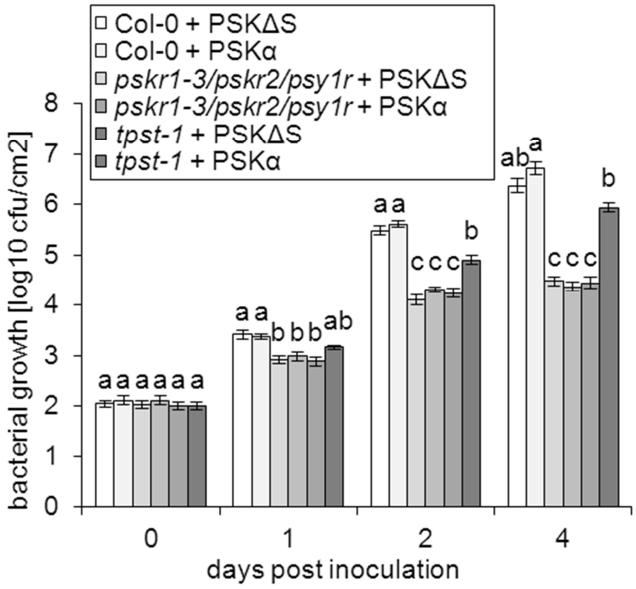

Figure 18. Pre-treatment of plants with PSKα partially complements the bacterial resistance phenotype of *tpst* mutants.
Plants were treated with 5×10^{-6} M PSKα or non-sulfated PSK (PSKΔS) 24h prior to infiltration with 10^4 cfu ml^{-1} *Pto* DC3000. Bacterial growth was monitored at 0 to 4 days post-inoculation. Mean values are presented ± SEM of at least six biological replicates. Bars with different letters are significantly different based on two-way anova (Tukey's honestly significant difference, P<0.05). Representative results of four experiments are presented.

phenotype by PSKα treatment (Figure 18). *tpst-1* plants treated with inactive non-sulfated PSK allowed about 25-fold less bacterial growth than control treated wild type plants. However, upon treatment with active PSKα, bacteria grew about 30-fold more in *tpst-1* plants and only nine-fold less compared with wild type plants. The *pskr1-3**l**pskr2**l**psy1r* triple mutants were completely insensitive to PSKα treatment, as expected. These data show that the defense-related phenotypes are due to direct effects of PSKα-signaling rather than due to secondary effects from developmental differences in the mutants.

3.1.10 Generation of silencing lines of *PSK* genes

As the overexpression of *PSK2* and *PSK4* has a salient impact on plant defense responses to *Pst* DC3000 and *A. brassicicola* (Figure 13), and T-DNA insertion lines for only *PSK1*, *PSK3*, *PSK5* and *PSK6* exist, amiRNA constructs for *PSK2* and *PSK4* were designed. Additionally, as certain sequences identified were predicted to simultaneously silence *PSK2* and *PSK6* as well as *PSK1* and *PSK5*, constructs aimed at targeting these genes were also generated. *Arabidopsis* Col-0 wild type plants as well as *psk1* and *psk5* T-DNA insertion mutants were transformed by *Agrobacterium tumefaciens* with the goal of silencing single and multiple *PSK* genes. Transformed plants were selected on the basis of Basta resistance conferred by the vector pB2GW7 and seeds from two separate transformation events were collected from the T_2 generation (Table 3-9). At this point in time, the effectiveness of the targeted gene silencing is pending characterization. These lines will be helpful tools for the analysis of responses downstream of PSKα perception as endogenous levels of PSKα are almost saturating and hinder the identification of specific PSKα-induced responses.

Table 3-9. Overview of ami*PSK* transformed plants

Genetic background	Transformed construct	*PSK* target
Col-0	pB2GW7-amiPSK2	*PSK2*
Col-0	pB2GW7-amiPSK4	*PSK4*
Col-0	pB2GW7-amiPSK1/5	*PSK1* and *PSK5*
Col-0	pB2GW7-amiPSK2/6	*PSK2* and *PSK6*
psk1	pB2GW7-amiPSK4	*PSK4*
psk1	pB2GW7-amiPSK2/6	*PSK2* and *PSK6*
psk5	pB2GW7-amiPSK4	*PSK4*
psk5	pB2GW7-amiPSK2/6	*PSK2* and *PSK6*

3.2 Identification of sulfated peptide receptor interacting partners

3.2.1 PSKR1 and PSY1R interact with BAK1

Since BAK1 is required for a diverse set of plant physiological responses (Mazzotta and Kemmerling, 2011), the possibility that BAK1 acts as a co-receptor of PSKR1 and PSY1R was explored. Results of bimolecular fluorescence complementation analysis using a transient expression system in *Nicotiana benthamiana* support an interaction between both PSKR1 and BAK1 as well as between PSY1R and BAK1, but not between PSKR1 or PSY1R and FLS2 (Figure 19a). These interactions were further corroborated by co-immunoprecipitation experiments utilizing full length PSKR1- and PSY1R-C-terminal GFP fusions and a BAK1 C-terminal Myc fusion in the transient expression system of *Nicotiana benthamiana* (Figure 19b). Both interactions between PSKR1 or PSY1R and BAK1 in bimolecular fluorescence complementation and co-immunoprecipitation were detectable in the absence of exogenously supplied PSKα or PSY1, presumably due to the presence of these peptides in *Nicotiana benthamiana* leaves (Yang et al., 2000). Due to this fact, it is not possible using this system to determine if the interaction between PSKR1 or PSY1R and BAK1 is ligand-induced.

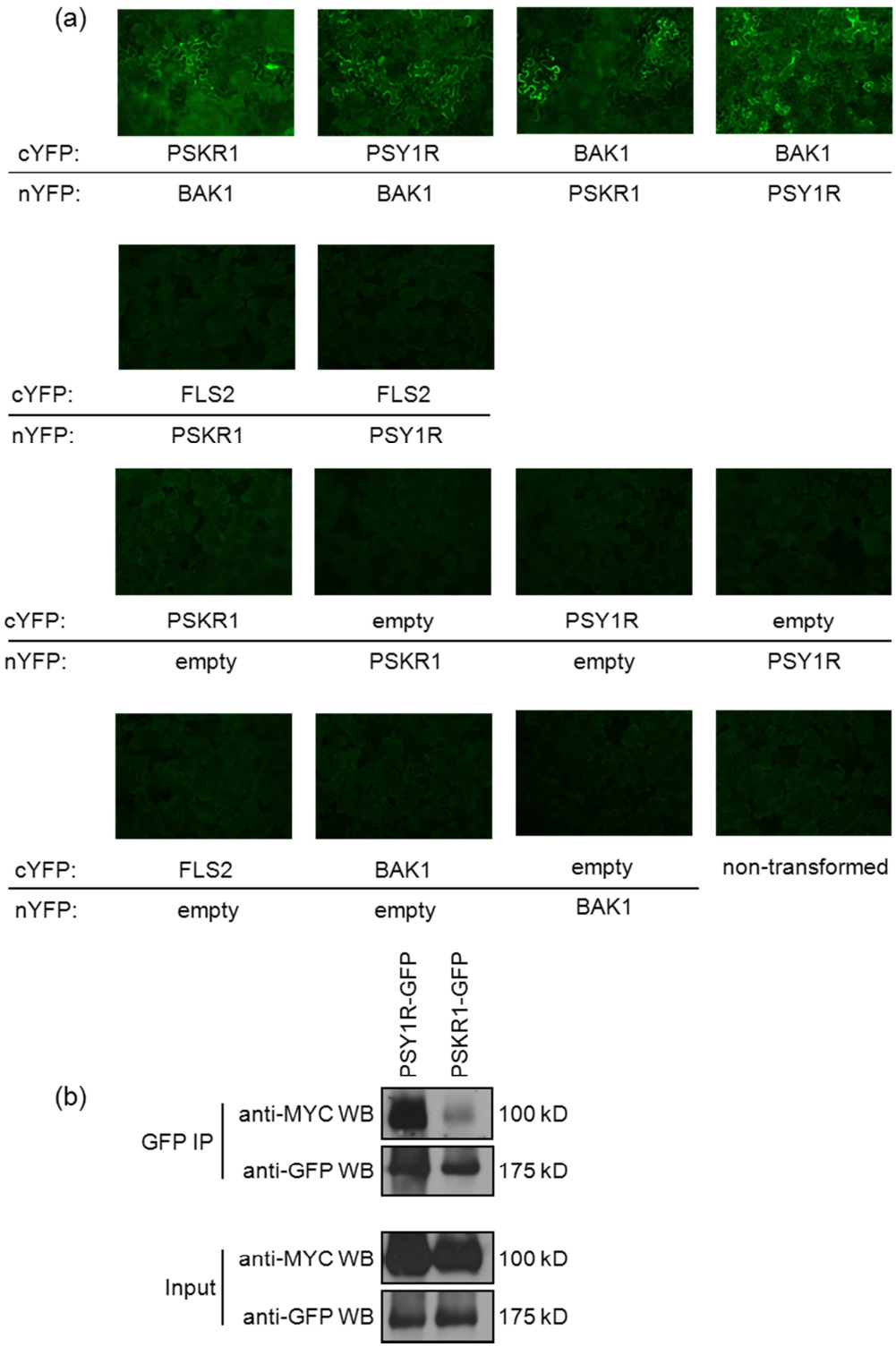

Figure 19. PSKR1 and PSY1R interact with BAK1.
PSKR1, *PSY1R*, *BAK1* and *FLS2* were cloned into ubiquitin-10 promotor containing vectors fused to the respective tags and expressed in *Nicotiana benthamiana* leaves by *Agrobacterium* infiltration. Bimolecular fluorescence complementation indicating interaction of the respective proteins was visualized by epifluorescence microscopy (a). *PSKR1* and *PSY1R* were cloned into 35S-promoter containing vectors with a GFP fusion tag and *BAK1* was cloned into a 35S-promoter containing vector with a MYC fusion tag and expressed in *Nicotiana benthamiana* leaves by *Agrobacterium* infiltration. Total protein (Input) preparations were subjected to anti-YFP immunoprecipitation followed by immunoblot analysis with anti-Myc antibodies to detect BAK1 and with anti-GFP antibodies to detect PSKR1 and PSY1R (b).

3.2.2 Identification of PSKR1-GFP interactors *in vivo*

To identify additional interaction partners of PSKR1, PSKR1-GFP proteins were expressed in *Arabidopsis* under its native promoter in the *pskr1-3/pskr2/psy1r* mutant background. Leaves of *PSKR1-GFP* expressing plants were treated with either PSKα or PSKΔS (a non-sulfated PSKα peptide) for 24 hours prior to tissue harvest. *pskr1-3/pskr2psy1r* mutant plants treated with PSKα served as a negative control. PSKR1-GFP proteins were immunoprecipitated and subsequently analyzed by mass spectrometry to identify co-immunoprecipitated proteins. Of the peptides identified, PSKR1 was determined to be the most abundant in the protein mixture analyzed (Table 3-10). Candidate PSKR1 interaction partners presented in Table 3-10 were selected based on low signal intensity in the negative control to eliminate proteins that were non-specifically co-immunoprecipitated and based on predicted localization to either the plasma membrane or cytosol. The candidate list includes the bacterial defense associated membrane protein HIR1 (Hypersensitive Induced Reaction 1), the cytosolic redox control proteins GSNOR1 (s-nitrosoglutathione reductase 1) and GLYR1 (Glyoxylate Reductase 1), the H(+)-ATPase 2 proteins AHA2 and AHA11, the aquaporins PIP1;4, PIP2;6 and TIP2;1, as well as the 14-3-3 proteins ω, λ, υ, κ and μ. Of all these proteins, only the 14-3-3 proteins λ and κ showed an interaction that was triggered by exogenous treatment with PSKα.

Table 3-10. Mass spectrometric analysis of PSKR1-GFP interaction partners from *Arabidopsis* leaves*

	PSKR1 - PSKα		PSKR1 - PSKΔS		pskr1-3/pskr2/psy1r - PSKα	
protein	sequence coverage (%)	signal intensity	sequence coverage (%)	signal intensity	sequence coverage (%)	signal intensity
PSKR1	11.2	33,920,000	13.4	65,707,000	0	0
HIR1	5.6	942,900	10.1	402,650	0	0
GSNOR1	5.6	815,930	3.1	86,096	0	0
GLY1R	11.8	1,886,300	8.3	884,000	4.5	232,600
AHA2	6	425,120	8.8	331,390	4.8	0
AHA11	5.9	339,490	4.9	245,860	2.7	0
PIP1;4	3.5	2,325,800	3.5	6,668,700	0	0
PIP2;6	4.2	324,050	4.2	469,600	0	0
TIP2;1	7.2	723,330	7.2	392,880	0	0
14-3-3 ω	16.2	360,240	13.5	551,560	3.9	0
14-3-3 λ	7.7	569,170	3.7	0	3.7	0
14-3-3 υ	18.7	353,340	22.4	1,642,300	3.7	0
14-3-3 κ	7.7	430,090	3.8	0	3.8	0
14-3-3 μ	10.9	2,278,900	11.2	1,385,800	3.6	0

* Leaf tissue of *pskr1-3/pskr2/psy1r* plants expressing *PSKR1-GFP* were treated with PSKα or PSKΔS 24 hours prior to tissue harvest and immunoprecipitation. *pskr1-3/pskr2/psy1r* plants treated with PSKα served as a negative control. Raw data was processed with a setting of 1 percent for the false discovery rate. The best interaction candidates based on low signal intensity in the negative control to eliminate proteins that were non-specifically co-immunoprecipitated and on predicted localization to the plasma membrane or cytosol are presented with their associated values of sequence coverage and signal intensity.

4 Discussion

Arabidopsis receptor-like kinases belong to the RLK/Pelle class of protein kinases which is populated by over 600 members (Shiu and Bleecker, 2003). Of these, two thirds are predicted to contain extracellular domains such as LRRs that are proposed to be involved in ligand binding (Shiu and Bleecker, 2003). LRR-RLKs are involved in perceiving signals that regulate various plant processes such as plant growth and development as well as plant defense responses (Gish and Clark, 2011). Although *Arabidopsis* encodes such a vast number of putative LRR-RLKs, only a few have been functionally characterized. Using a reverse genetics approach, Postel et al. (2010) identified 49 pathogen-inducible LRR-RLK genes. Among those identified was the previously described phytosulfokine receptor *PSKR1* (Postel et al., 2010). Here, the involvement of PSKR1 and a closely related LRR-RLK PSY1R are shown to be critical for regulating defense responses.

4.1 Pathogen defense responses and sulfated peptide receptors

As reported by Postel et al. (2010) elevated levels of *PSKR1* transcript accumulates upon treatment with *Pto* DC3000 hrcC$^-$, a mutant strain of *Pto* DC3000 lacking a type three secretion system, and the non-host pathogen *Pseudomonas phaseolicola*. Likewise, treatment with elicitors of PTI also induces *PSKR1* transcript accumulation. This finding led to the hypothesis that PSKR1 signaling might be involved in plant defense-signaling. To test this hypothesis, *pskr1* mutant plants were infected with the pathogen *Pto* DC3000 and experimental results indicate that the loss of this receptor in *Arabidopsis* leads to enhanced resistance compared to wild type plants. On the other hand, non-host resistance to the necrotrophic fungus *Alternaria brassicicola* is impaired in *pskr1* plants, indicating that the regulation of defense responses is differentially altered in these mutant plants.

Studies from the last ten years have revealed that extensive cross talk exists between plant hormone signaling pathways including SA and JA (Robert-Seilaniantz et al., 2007). In

general, SA-mediated responses predominantly combats biotrophic pathogens, resulting in enhanced *PR* gene expression and hypersensitive cell death, whereas JA signaling defends against necrotrophic pathogens and is accompanied by the induction of plant defensins such as *PDF1.2* (Kazan and Manners, 2008; Vlot et al., 2009). Although synergism between these two pathways has been reported (Mur et al., 2006; Spoel and Dong, 2008), a number of studies indicate that SA and JA signaling is mutually antagonistic (Glazebrook, 2005; Jones and Dangl, 2006; Koornneef and Pieterse, 2008; Spoel and Dong, 2008; Vlot et al., 2009). The antagonistic results obtained for biotrophic and necrotrophic infection on pskr1 mutants indicate that the balance between SA and JA is affected in these mutants.

In *pskr1* mutants, the balance between the hormone signaling pathways SA and JA is significantly shifted toward SA responses. Both *pskr1* and the triple mutant *pskr1/pskr2/psy1r* plants exhibit elevated levels of SA upon *Pto* DC3000 infection. Furthermore, SA-responsive *PR* gene induction is also enhanced after bacterial infection, providing further evidence that the SA-mediated signaling pathway is up-regulated in comparison to wild type plants. These data support a scenario in wild type plants where sulfated peptide receptor activation leads to the suppression of the SA-mediated signaling pathway. This is in line with the finding that in *Zinnia*, exogenous PSKα treatment represses defense-associated gene expression such as *PR1*, *chitinases* and several genes involved in SA biosynthesis (Motose et al., 2009). The authors of this report also conclude that PSKα-signaling attenuates stress responses via the suppression of SA signaling. The studies utilizing *Arabidopsis* presented here demonstrate that the altered SA responses in *pskr1* and *pskr1/pskr2/psy1r* plants are correlated with heightened resistance to a biotrophic pathogen and, antagonistically, loss of resistance to a necrotrophic pathogen and suggest that the PSKα perception leads to the negative regulation of SA-dependent defense responses.

In *pskr1* and *pskr1/pskr2/psy1r* plants, the enhanced levels of SA-mediated signaling are accompanied by repressed JA-mediated responses. Although the levels of JA content are not altered in either *pskr1* or *pskr1/pskr2/psy1r* plants upon infection with *Pto* DC3000, the

JA-responsive marker genes *PDF1.2* and *OPR3* are markedly reduced compared to wild type plants, supporting a scenario where PSKα perception in wild type plants leads to a shift in defense hormone homeostasis resulting in the down-regulation of SA-associated responses and an up-regulation of JA-associated responses. This finding is in line with previous reports that *PSKR1* and the PSKα precursor genes *PSK3*, *PSK4* and *PSK5* are transcriptionally up-regulated by wounding (Matsubayashi et al., 2006; Kilian et al., 2007; Loivamaki et al., 2010), a response that is regulated by JA signaling (Reymond et al., 2000). Furthermore, senescence responses, which are at least partially regulated by SA-mediated signaling including the up-regulation of *PR1* expression (Robatzek and Somssich, 2001, 2002; Buchanan-Wollaston et al., 2005; Spoel and Dong, 2008), were prematurely induced in *pskr1* mutants and delayed in *PSKR1*-overexpressing plants (Matsubayashi et al., 2006). This is in agreement with the findings reported here, where the loss of PSKα perception leads to a shift in SA-JA homeostasis. As over-amplification of the SA-mediated biotrophic defense pathway leaves plant vulnerable to necrotrophic pathogens, and vice versa (Spoel and Dong, 2008), the induction of sulfated peptide signaling by PAMPs might be necessary for maintaining balanced defenses against all pathogens following an initial boost of PTI by PAMPs.

4.2 PAMP responses and sulfated peptide receptors

The expression of *PSKR1* is induced by the non-host pathogen *Pseudomonas syringae* pv. *phaseolicola* and the non-pathogenic bacteria *Pto* DC3000 HrcC$^-$, which are unable to suppress PTI, as well as by direct exposition to treatment with the PAMPs flg22 (Felix et al., 1999), HrpZ (Lee et al., 2001b) and NPP1 (Fellbrich et al., 2002). In testing whether genes associated with sulfated peptide signaling play a role in the development of PTI, we found that *pskr1, psy1r*, *pskr1/pskr2/psy1r* and *tpst* mutant plants have enhanced flg22 responses such as callose deposition, *FRK1* expression and seedling growth inhibition compared to wild type plants. Similar results were recently published by Igarashi et al. (2012). However, the

authors of this study reported no alteration of *FRK1* induction by flg22 in *pskr1* mutant seedlings. Results presented here show a strong induction of *FRK1* upon flg22 treatment in the adult leaves of the mutants tested. This could indicate that the experimental differences are attributable to the developmental stage of the tissue in question. To confirm this, *FRK1* expression levels were analyzed in seedlings after treatment with flg22 using our experimental conditions, and indeed, no significant differences were found between *pskr1* and wild type plants (Figure 20), indicating that the differences in the observed experimental results are indeed due to the developmental stage of tissue being used. This finding correlates well with the observation that the PSKα precursor genes *PSK2*, *PSK4* and *PSK5* are predominantly expressed in mature leaf tissue and are induced during senescence (Matsubayashi and Sakagami, 2006). However, *pskr1* plants did show enhanced *FRK1* expression upon treatment with elf18 (Igarashi et al., 2012). This suggests that flg22- and elf18-induced defense responses might be differentially sensitive to the tissue or age specific PSKα/PSY1-regulated predisposition for altered PAMP responses.

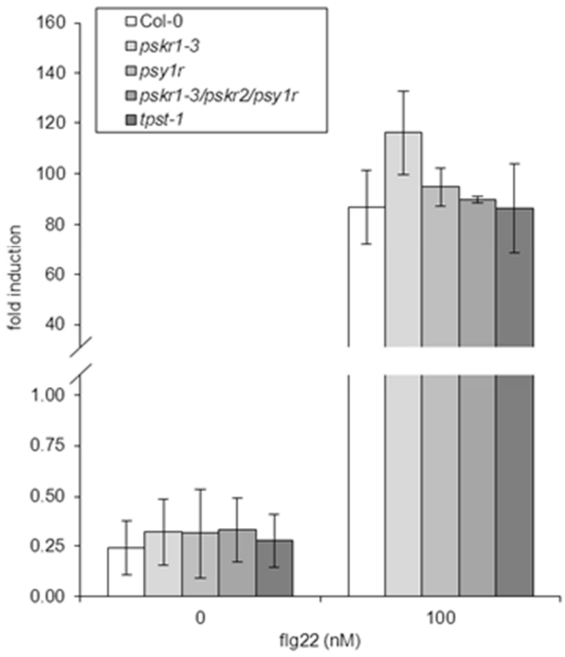

Figure 20. *FRK1* gene expression in seedlings treated with flg22**.**
After treatment of 10-day-old seedlings with 100 nM flg22 or water, quantitative RT-PCR experiments were performed on cDNA generated from three independent biological replicates to analyze *FRK1* gene expression. Expression values were calculated as a ratio to the housekeeping gene *EF1α* and presented as fold induction. Bars represent mean ratios ± SEM.

Perception of the bacterial PAMP EF-Tu by its cognate receptor EFR leads to the induction of a strongly overlapping set of responses compared to perception of flg22 by FLS2 (Zipfel et al., 2006). Plants mutated in the *EFR* gene exhibit a heightened level of susceptiblity to the virulent bacteria *Agrobacterium tumefaciens*, linking PAMP perception and resistance to this bacterial pathogen (Zipfel et al., 2006). Interestingly, exogenous application of PSKα causes greater than five-fold increase in the efficiency of *Agrobacterium*-mediated transformation of carrot hypocotyl tissue (Matsubayashi et al., 2004). Furthermore, *pskr1* mutant plants developed significantly less crown gall growth after *Agrobacterium* inoculation (Loivamaki et al., 2010). Both Matsubayashi et al. (2004) and Loivamaki et al. (2010) suggest that the enhanced level of *Agrobacterium* transformation is due to the elevated level of cellular proliferation associated with phytosulfokine signaling. Work presented here as well as by Igarashi et al. (2012) suggests that the increased virulence of *Agrobacterium* may actually be due to the suppressive effect of PSKα- and PSY1-mediated signaling on PTI.

4.3 Phytohormones and sulfated peptide signaling

Plant hormones play a pivotal role in integrating developmental and environmental signals that both influence plant architecture and stress responsiveness (Kazan and Manners, 2009). Several publications have demonstrated that, on the one hand, defense associated hormones SA and JA affect developmental processes such as growth repression and, on the other hand, plant hormones associated with growth regulation such as auxin affect plant defense and microbial pathogenesis (Robert-Seilaniantz et al., 2007; Robert-Seilaniantz et al., 2011). For instance, Wang et al. (2007) demonstrated that SA signaling leading to the restriction of pathogen growth is accompanied by the down-regulation of genes involved in auxin transport, perception and responses. The authors of this study also show that auxin insensitivity leads to increased resistance to *P. syringae*, suggesting that an intact auxin signaling pathway promotes susceptibility to biotrophic pathogens. On the other hand,

decreasing auxin signaling via auxin pathway mutants or by pharmacologic inhibition leads to attenuated defense responses against necrotrophic fungi such as *Plectosphaerella cucumerina*, *Botrytis cinerea* and *Alternaria brassicicola* (Llorente et al., 2008; Qi et al., 2012). These data suggest that auxin and JA synergistically promote resistance to necrotrophic pathogens.

A role for auxin has also been suggested for PSKα-induced cellular proliferation. Eun et al. (2003) reported that carrot cells treated with PSKα fail to proliferate in the absence of auxin. The authors of this work also demonstrate that the production of mature PSKα is stimulated by the addition of auxin to carrot cell culture medium. As discussed above, a role for PSKα-signaling in the development of *Agrobacterium* induced crown gall formation has been suggested (Loivamaki et al., 2010). Crown galls develop upon transfer of T-DNA from *Agrobacterium* into the plant host and integration into its genome (Magori and Citovsky, 2012). *Agrobacterium* T-DNA encodes, among others, several genes involved in auxin biosynthesis and their expression in plants cells lead to abnormal cellular proliferation (Magori and Citovsky, 2012). The finding that PSKα precursor genes and *PSKR1* are expressed in crown galls and that *pskr1* mutants significantly reduce crown gall size is in line with the interconnectedness of PSKα and auxin signaling. Furthermore, the expression of *TPST* has been reported to be transcriptionally induced by auxin (Zhou et al., 2010). Additionally, mutations affecting *TPST* leads to the down-regulation of auxin biosynthetic genes and auxin transport genes and subsequently an alteration in auxin distribution (Zhou et al., 2010).

Based on these associations, the link between PSKα and auxin can be used to explain the phenotypes observed in sulfated peptide signaling mutants. First, PSKα-mediated cellular proliferation requires auxin. Second, auxin has been shown to have a negative impact on senescence (Osborne, 1959; Lim et al., 2007; Kim et al., 2011) which has also been reported for the PSKα-mediated signaling pathway (Matsubayashi et al., 2006; Amano et al., 2007; Komori et al., 2009). Third, auxin has been reported to have a suppressive effect on SA-

inducible *PR* gene expression (Park et al., 2007; Wang et al., 2007; Ding et al., 2008) and to promote the expression of the JA-inducible defensin *PDF1.2* (Qi et al., 2012). This is perfectly in line with the findings presented here, where sulfated peptide signaling represses the SA pathway and promotes the JA pathway. Lastly, the increased resistance to the biotrophic pathogen *Pto* DC3000 and antagonistically, the increased susceptibility to the necrotrophic pathogen *Alternaria brassicicola* observed in sulfated peptide signaling mutants closely match the effects exerted by the auxin pathway on the establishment of defense responses (Tiryaki and Staswick, 2002; Wang et al., 2007; Llorente et al., 2008; Qi et al., 2012). Taken together, this set of experimental results suggests that sulfated peptide signaling, perhaps together with auxin, regulate the balance of defense responses for the promotion of senescence prevention and growth. However, Motose et al. (2009) have reported that, in *Zinnia*, PSKα suppresses the induction of stress-responsive genes in the absence of auxin and conclude that it is dispensable for PSKα-mediated stress responses. Detailed experiments aimed at quantifying hormone levels and their interplay in various signaling pathways is required to more fully understand the influence of PSKα on hormone homeostasis.

4.4 Sulfated peptide perception integrates growth and defense responses

PSKα was initially characterized as a secreted conditioning factor critical for the proliferation of *Asparagus* cells grown in culture (Matsubayashi and Sakagami, 1996). Several years later, the PSY1 peptide was identified as an additional secreted conditioning factor in *Arabidopsis* cell cultures (Amano et al., 2007). Both of these secreted peptides have been shown to promote *Arabidopsis* root growth by increasing both cell number and size (Amano et al., 2007). Application of both PSKα and PSY1 also leads to an increased production of callus tissue in *Arabidopsis* (Matsubayashi et al., 2006; Amano et al., 2007). Results presented here, however, show that sulfated peptide signaling additionally has a strong

effect on the establishment of plant defense responses. As plant growth and immunity consume high amounts of energy (Ali et al., 2007), it is essential that plants prioritize the allocation of limited resources between these two biological processes to maximize fitness. The high number of mutant plants exhibiting constitutive defense responses that result in severe dwarf phenotypes such as *cpr1* (Bowling et al., 1994), *cpr5* (Bowling et al., 1997), *cpr22* (Yoshioka et al., 2006), *ssi4* (Shirano et al., 2002), *bon1*/*cpn1* (Hua et al., 2001; Jambunathan et al., 2001) and *bir1* (Gao et al., 2009) highlight the negative consequences of overactive immune responses on plant growth. Based on these studies, it is clear that the negative regulation of defense is critical to plant development. Such a role was assigned to PSKα-signaling in *Zinnia*, where the induction of several stress-related genes encoding proteinase inhibitors and *PR* proteins were reduced upon PSKα application (Motose et al., 2009). Additionally, *PSK1*, *PSK2*, *PSK4*, *PSY1* and *PSKR1* gene expression is activated upon pathogen attack and PAMP treatment. Therefore, sulfated peptide signaling might be required for restricting energy intensive defense responses for the benefit of senescence prevention and growth.

4.5 Sulfated peptide receptor redundancy

The PSKα receptor PSKR1 has a closely related paralog, PSKR2 (Amano et al., 2007). While PSKR2 expressed in tobacco BY-2 cells has been shown to bind PSKα by photoaffinity labeling *in vitro* (Amano et al., 2007), its role in PSKα-signaling seems to be marginal. Mutants affecting the *PSKR2* gene still respond to PSKα-mediated promotion of root elongation, most likely due to the effects of *PSKR1*. Moreover, PSKα-mediated root growth promotion in *pskr1/psy1r* double mutants is only about a fifth of that observed in Col-0 wild type plants, suggesting that the role of PSKR2 in perceiving PSKα is limited (Amano et al., 2007). While hypocotyl elongation of *pskr1* mutants is significantly reduced compared to wild type plants, *pskr2* mutants showed no difference compared to wild type controls suggesting PSKR2 plays no role in this physiological response (Stuhrwohldt et al., 2011).

Data presented here and by Igarashi et al. (2012) show that PSKR2 also plays no role in regulating PAMP signaling or resistance responses to biotrophic or necrotrophic pathogens. While PSKR2 can still bind PSKα, the fact that PSKR2 signaling seems to be minor suggests that this receptor might not be functional. However, recent data has shown that when *PSKR2* is expressed at extremely high levels, root lengths are increased as is the promotion of hypocotyl elongation (Hartmann et al., 2013). This suggests that the limited role of PSKR2 in PSKα-signaling might be related to receptor abundance rather than loss of functionality. On the other hand, this finding could indicate that the activity of PSKR2 is greatly reduced compared to that of PSKR1. While gene duplication occurs at a high rate in eukaryotes, which is highlighted by the vast number of RLKs encoded by the *Arabidopsis* genome (Shiu and Bleecker, 2003), most gene duplicates are lost, from an evolutionary standpoint, within a short period of time (Lynch and Conery, 2000). However, the *PSKR2* gene has been retained in the *Arabidopsis* genome suggesting that selective pressure is driving its retention. While it cannot be ruled out that *PSKR2* will eventually be relegated to the status of a pseudogene, it is entirely possible that selective pressure has led to, or is leading to novel functionality of the PSKR2 receptor.

The PSY1 receptor PSY1R is also closely related to PSKR1 (Amano et al., 2007). PSY1 perception by PSY1R leads to an overlapping set of physiological responses with PSKR1 such as root growth promotion, the induction of callus tissue proliferation and wound repair (Amano et al., 2007). Amano et al. (2007) further state that PSY1 has no impact on PSKα perception and that PSY1R does not bind PSKα and suggest that both peptides redundantly contribute to similar physiological processes. In contrast to PSKR2, however, PSY1R has a strong impact on repressing responses that restrict biotrophic pathogenesis and PAMP signaling and, antagonistically, enhancing resistance to necrotrophic pathogens. Based on multiple mutant analysis, the overlapping effects of *pskr1* and *psy1r* seem to be additive in all cases tested. This could explain the partial character of *PSKR1* complementation of the triple receptor mutant and leaves room to speculate that an intact PSY1-signaling pathway is

required for full complementation. Taken together, these data suggest that PSKα and PSY1-signaling also act redundantly to modulate plant defense responses.

4.6 PSKα has a direct effect on defense against *Pseudomonas syringae*

The receptor mutants *pskr1* and *psy1r* have strong additive phenotypes which alter the outcome of plant-pathogen interactions. However, the possibility exists that these phenotypes could stem from developmental defects in the receptor mutants independent of sulfated peptide perception. To test this theory, *tpst* mutants, which are impaired in tyrosine sulfotransferase activity, were analyzed. The enhanced induction of PAMP signaling and the observed alteration in defense responses to *Pto* DC3000 and *Alternaria brassicicola* phenotypically mimicked the triple receptor mutant *pskr1/pskr2/psy1r*. As *TSPT* exists as a single copy gene in the *Arabidopsis* genome, this finding supports the conclusion that no additional tyrosine sulfated peptides are involved in modulating these defense responses. In another approach, *PSK2*, *PSK4* and *PSKR1* were separately overexpressed in the *Arabidopsis* wild type background. These plants, which presumably have heightened levels of PSKα-signaling, showed the same phenotype observed for *PSKR1* overexpressing plants, exhibiting heightened susceptibility to *Pto* DC3000 and increased resistance to *Alternaria brassicicola*. This shows that overrepresentation of receptor and ligand result in the same phenotypes further supporting that the observed phenotypes are indeed due to PSKα-signaling.

To directly show that the outcome of *Pto* DC3000 resistance responses is caused by PSKα-signaling, plants were exogenously treated with PSKα 24 hours prior to inoculation with *Pto* DC3000. Wild type plants showed only a moderate statistically insignificant increase in susceptibility after treatment with PSKα. This is in line with various reports that exogenous application of PSKα leads to only slight alterations of physiological outcomes (Motose et al., 2009; Stuhrwohldt et al., 2011; Igarashi et al., 2012). The lack of increased bacterial growth in wild type plants after PSKα treatment could be due to imperfect timing of peptide delivery

since overexpression of the preproprotein encoding genes *PSK2* and *PSK4* lead to a strong impact on bacterial and fungal resistance. However, *tpst* mutant plants, which are likely devoid of tyrosine sulfated peptides, show a clear but partial complementation of the bacterial resistance phenotype upon exogenous application of PSKα. The partial nature of this complementation allows for the speculation that the redundant function of the PSY1 peptide is required for full complementation of *tpst* mutant bacterial resistance phenotype. The triple receptor mutant is completely insensitive to PSKα treatment due to loss of both PSKR receptors and rules out any notion that PSKα has an effect on bacterial growth. Taken together, these data support the idea that sulfated peptide signaling has a direct effect on plant defense responses and are not attributable to morphological alterations associated with *tpst* or *pskr1*/*pskr2*/*psy1r* mutant phenotypes.

4.7 BAK1 as a signaling component of sulfated peptide receptors

Both PSKR1 and PSY1R belong to the LRR-RLK subfamily X (Shiu and Bleecker, 2003) and several members of this subfamily are characterized. Perhaps the best characterized RLK is BRI1, a receptor involved in the perception of brassinosteroids which are involved in various developmental processes such as cell elongation, vascular differentiation, root growth and senescence (Kim and Wang, 2010) as well as responses to both biotic and abiotic stresses (Albrecht et al., 2012; Belkhadir et al., 2012). The SERK family members BAK1 and BKK1, which belong to the LRR-RLK subfamily II (Shiu and Bleecker, 2003) have been shown to interact with BRI1 and are required for the full potential of BR signaling (He et al., 2007; Wang et al., 2008). Additional characterized members of the LRR-RLK subfamily X (Shiu and Bleecker, 2003) include the additional brassinosteroid receptors BRL1 and BRL3 (Cano-Delgado et al., 2004), a negative regulator of plant defense responses known as BIR1 (Gao et al., 2009) and a regulator of anther cell patterning known as EMS1 (Zhao et al., 2002). BAK1 has been shown to interact with BIR1 (Gao et al., 2009) and the SERK family members SERK1 and SERK2 have been shown to influence anther cell patterning,

suggesting that they might function together with EMS1 (Albrecht et al., 2005; Colcombet et al., 2005).

Based on the fact that SERK proteins have been shown to be important for the signaling activities of RLKs belonging to the LRR-RLK subfamily X, it was hypothesized that BAK1 might be required for PSKR1 and PSY1R signaling. Using BIFC and coimmunoprecipitation experiments, BAK1 was found to interact with both PSKR1 and PSY1R in transiently overexpressing tobacco tissue. The interaction was detectable even in the absence of exogenous application of PSKα or PSY1 peptides. Since the penptapeptide region corresponding to PSKα is perfectly conserved in preproPSKs from several plant species including both monocots and dicots (Yang et al., 2000) it is likely that PSKα is present in *Nicotiana benthamiana* leaves. However, at this time, no reports have indicated the presence of a PSY1 gene in plant species other than *Arabidopsis* (Amano et al., 2007). Therefore, it is not possible to determine whether or not the interaction between PSKR1 or PSY1R and BAK1 is ligand dependent in this experimental system. The evidence linking BAK1 to PSKR1 and PSY1R signaling is intriguing as it offers the first details of early molecular responses downstream of tyrosine-sulfated peptide perception.

Upon BR perception, transphosphorylation events occur between BRI1 and BAK1 (Wang et al., 2008). This raises the question of whether PSKR1 and PSY1R also initiate such transphosphorylation events upon ligand binding. Detailed experiments aimed at assessing the kinase activities of these RLKs will help shed light on this possibility. At this point in time, no downstream targets of tyrosine-sulfated peptide perception have been documented. Nevertheless, data presented here show that *pskr1* and *psy1r* mutants accumulate elevated levels of SA and *PR* gene expression and initiate heightened PTI. A recent report has demonstrated that PAMP treatment leads to the accumulation of SA in *Arabidopsis* leaves (Tsuda et al., 2008). In light of this and the newly identified interaction between two sulfated peptide receptors and BAK1, it is conceivable that PSKR1 and PSY1R repress biotrophic defense responses by tying up a portion of the available pool of BAK1 required for FLS2-

and/or EFR-mediated defense responses. However, recent evidence has suggested that negative regulation of PAMP responses by BR occurs independently of BAK1 (Albrecht et al., 2012). Therefore, it is entirely plausible that PSKR1 and PSY1R signaling, together with BAK1, activates an unknown pathway independent of PAMP receptors to modulate plant defense responses.

4.8 Identification of additional *PSKR1* interaction partners

To identify additional interaction partners of PSKR1, *PSKR1-GFP* expressed in *pskr1/pskr2/psy1r Arabidopsis* lines were immunoprecipitated and subjected to mass spectrometric analysis. Non-transformed *pskr1/pskr2/psy1r* plants were used as a control line. *PSKR1-GFP* expressing plants were treated with either PSKα or non-sulfated PSK peptide in an attempt to identify ligand induced complex formation. However, of the proteins that co-immunoprecipitated with PSKR1-GFP, there was none that were identified exclusively in samples prepared after PSKα treatment, suggesting either that these putative interaction partners may be found in preformed complexes with PSKR1 prior to ligand binding or that endogenous levels of PSKα in adult leaf tissue are high enough to stimulate complex formation. This finding is in line with the fact that exogenous application of PSKα has only marginal effect on wild type plants, unlike sulfotransferase deficient *tpst* mutant plants.

In this experiment, peptides from the Hypersensitive Induced Reaction 1 protein (HIR1) were detected in both PSKα and PSKΔS treatment, although the signal detected in PSKα pretreated plants was more than double that found in PSKΔS treatment. HIR1 is a protein that accumulates upon treatment with flg22 or *Pto* DC3000 hrcC$^-$ and its overexpression results in the reduction of *Pto* DC3000 growth in *Arabidopsis* plants (Qi et al., 2011). The HIR1 protein localizes to the plasma membrane, is enriched in detergent resistant microdomains, interacts with the R-protein RPS2 and contributes quantitatively to RPS2-mediated establishment of ETI upon recognition of the avirulence determinant AvrRpt2 (Qi et

al., 2011). Two additional proteins identified by this method include S-nitrosoglutathione reductase (GSNOR1) and glyoxylate reductase 1 (GLYR1). While these proteins were detected in control treatments of PSKΔS, the signal intensity detected for both GSNOR1 and GLYR1 was an order of magnitude greater in PSKα treatments. GSNOR1 and GLYR1 are both reductase enzymes involved in controlling the cellular redox state. GLYR1 is proposed to function in the detoxification of aldehydes during stress responses thereby contributing to the redox balance in *Arabidopsis* (Allan et al., 2009). GSNOR1 positively regulates thermotolerance (Lee et al., 2008), SA-mediated defense responses (Feechan et al., 2005) and the establishment of the hypersensitive response (Chen et al., 2009) by modulating cellular S-nitrosothiol levels.

Another class of putative PSKR1 interaction partners includes proteins involved in membrane transport such as the *Arabidopsis* H(+)-ATPases (AHA) AHA2 and AHA11, which are proton pumps. In *Arabidopsis*, there are 11 AHA proteins and their activation leads to the establishment of pH and membrane potential gradients across the plasma membrane (Palmgren, 2001). Furthermore, the activity of plant proton pumps has been associated with cell growth (Hager et al., 1991; Frías et al., 1996), blue-light membrane depolarization (Spalding and Cosgrove, 1992) and responses to the fungal elicitor fusicoccin (Marre, 1979). While a specific physiological role for AHA11 has not yet been reported, Liu et al. (2009) identified AHA2 as a RIN4 interacting protein in *Arabidopsis* guard cells. Guard cell opening is mediated by activation of proton pumps (Assmann and Schwartz, 1992) and RIN4 interaction with AHA2 has been proposed to regulate stomatal aperture to prevent the entry of bacterial pathogens into plant leaves during infection (Liu et al., 2009). In addition, three members of the aquaporin super family were also identified as putative PSKR1 interaction partners and include PIP1;4, PIP2;6 and TIP2;1. While aquaporins have been classically associated with membrane water permeability, recent data suggests that certain isoforms of the 35 aquaporin genes encoded by the *Arabidopsis* genome are involved in transporting a wide range of small neutral molecules such as formamide, glycerol, urea, CO_2 and H_2O_2 (Maurel, 2007).

Mass spectrometric analysis of co-immunoprecipitated PSKR1 also suggests that PSKR1 might interact with the 14-3-3 proteins ω, λ, υ, κ and μ. 14-3-3 proteins have been shown to bind phosphorylated consensus motifs of a wide variety of target proteins (Oecking and Jaspert, 2009). Interestingly, 14-3-3 proteins have been implicated in regulating hormone signaling pathways such as abscisic acid, gibberellin and BR signaling (Oecking and Jaspert, 2009). *Arabidopsis* encodes 15 isoforms of 14-3-3 proteins and their various structural properties have been suggested to determine their subcellular localization (Sehnke et al., 2002). Interestingly, Chang et al. (2009) identified an interaction between 14-3-3 Ω and BRI1 as well as BAK1. This, together with the identification of various 14-3-3 proteins that putatively interact with PSKR1 might suggest that 14-3-3 proteins are important for modulating RLK activity.

The identification of putative PSKR1 interaction partners from this experiment will be a valuable starting point for the elucidation of components involved in PSKα-signaling transduction.

4.9 Model of tyrosine-sulfated signaling in *Arabidopsis*

Based on work presented here and in various reports, a model of tyrosine-sulfated peptide signaling in *Arabidopsis* is proposed (Figure 21). Upon pathogen challenge or PAMP treatment, a subset of *PSK* and *PSY1* precursor genes as well as *PSKR1* is transcriptionally up-regulated. The preproproteins PSK and PSY1, designated pPSK or pPSY1, are tyrosine-sulfated by TPST which most likely takes place in the Golgi apparatus (Komori et al, 2009). The preproproteins are thought to be proteolytically processed in the apoplast at least in part by subtilisins (Srivasta et al, 2008). The fully processed PSKα and PSY1 peptides are then able to bind to their respective receptors, PSKR1 and PSY1R (Matsubayashi et al, 2002; Amano et al, 2007). Activation of these receptors, which may be dependent on the co-receptor BAK1, leads to the suppression of SA signaling and consequently suppressed resistance to biotrophic pathogens and senescence (Matsubayashi et al, 2006) as well as the

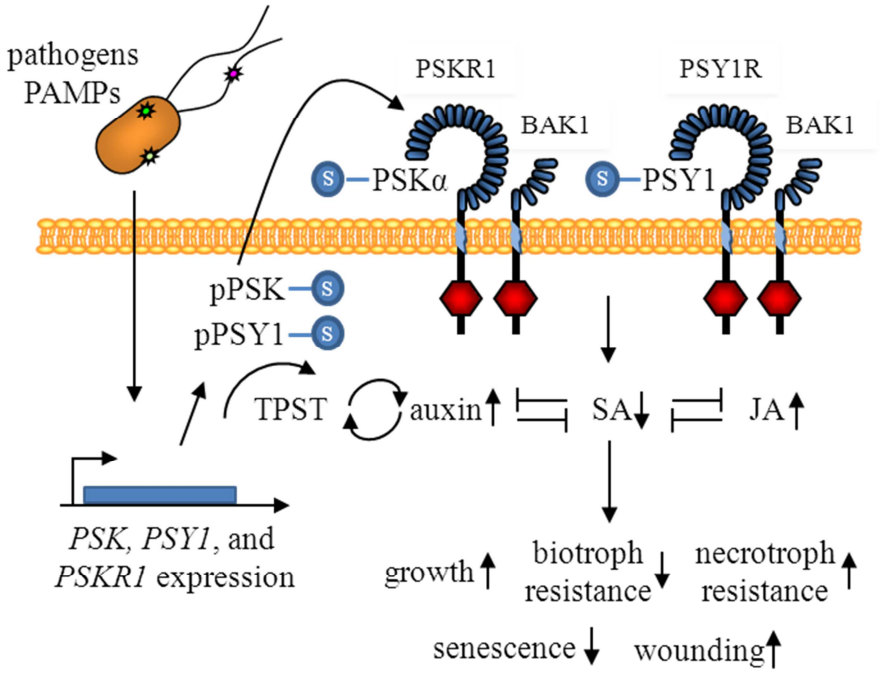

Figure 21. The involvement of tyrosine-sulfated peptide signaling in plant defense responses
PAMPs are depicted as a bacterial cell or star therein. The preproproteins PSK and PSY1 are referred to as pPSK and pPSY1, respectively. Sulfated preproprotein sulfation is depicted as an 'S' inside a blue circle. Receptor models are represented by blue extracellular leucine rich repeats, a single light blue transmembrane domain and a red cytosolic kinase domain. Arrows represent up-regulation (↑) or down-regulation (↓) of the specific hormone pathway or associated physiological process. Blocked arrows (⊢ or ⊣) represent mutually antagonistic pathways.

up-regulation of JA signaling and consequently increased resistance to necrotrophic pathogens and increased wounding responsiveness (Matsubayashi et al, 2006; Amano et al, 2007). PSKα and PSY1-signaling also promotes plant growth (Matsubayashi and Sakagami, 1996; Amano et al., 2007) and this growth is promoted by auxin (Eun et al., 2003). TPST expression is induced by auxin signaling and TPST activity promotes the up-regulation of auxin signaling (Zhou et al., 2010), suggesting that auxin and TPST pathways are tightly linked. As auxin has been reported to antagonize SA signaling and promote JA signaling (Kazan and Manners, 2009), it is possible that the regulation of defense responses by sulfated peptide signaling pathways is mediated via auxin.

5 Summary

Plants as sessile organisms cannot escape when confronted with harmful microbial pathogens. However, they are armed with sophisticated surveillance systems and inducible responses that provide effective defenses for self-protection. Innate immunity forms the basis for self-defense in higher organisms including mammals and invertebrates, as well as plants. During the basal immune response, conserved microbial signatures referred to as PAMPs are perceived by cell surface receptors and trigger a variety of defense responses leading to the protection of the plant ultimately thwarting the invasive pathogen. These cell surface receptors often come in the form of the LRR-RLK. In *Arabidopsis*, the RLK/Pelle class of protein kinases is composed of over 600 members (Shiu and Bleecker, 2003). Many of the proteins in this family are receptor-like kinases (RLKs) and approximately two thirds are predicted to have extracellular domains (Shiu and Bleecker, 2003). Based on a reverse genetics screen, Postel et al. (2010) identified 49 pathogen-inducible LRR-RLK genes including the previously described PSKR1 receptor. PSKR1 perceives endogenous tyrosine-sulfated peptides referred to as PSKα resulting in growth promotion and various developmental processes. PSY1R, an LRR-RLK closely related to PSKR1, perceives a structurally distinct sulfated peptide referred to as PSY1 that induces a strikingly similar set of physiological responses compared to PSKα. In this work, both PSKR1 and PSY1R, but not a second PSKα binding receptor PSKR2, were shown to suppress resistance to biotrophic bacteria and PAMP responses and antagonistically promote resistance to necrotrophic fungal infection. The antagonistic effect on biotrophic and necrotrophic pathogen resistance is reflected by an altered phytohormone balance with enhanced salicylate and reduced jasmonate responses in the receptor mutants, suggesting that PSKR1 and PSY1R suppress SA-dependent defense responses. Multiple mutant analyses revealed that *pskr1* and *psy1r* mutant phenotypes are additive, indicating that these receptors redundantly modulate plant defense responses. Mutant plants lacking TPST, a protein with tyrosylprotein sulfotransferase activity that can sulfate PSKα and PSY1 preproproteins *in vitro*,

phenotypically mimics mutant plants lacking both PSKR1 and PSY1R. PSKα feeding experiments utilizing *tpst* mutants partially restored the bacterial defense phenotype, revealing that PSKα has a direct effect on plant defense. These results, taken together, suggest a mechanism where the PSKR subfamily integrates both growth-promoting and defense signals mediated by sulfated peptides to coordinate developmental programs and the balance of resistance responses to biotrophic and necrotrophic pathogens.

6 Zusammenfassung

Pflanzen als sessile Organismen können Angriffen von mikrobiellen Pathogenen nicht entfliehen. Nichtsdestotrotz sind sie aber mit ausgeklügelten Überwachungssystemen und induzierbaren Abwehrmechanismen ausgestattet, die eine effektive Verteidigung ermöglichen. Die angeborene Immunität ist die Basis der Selbstverteidigung in höheren Organismen wie Säugetieren, Evertebraten und auch Pflanzen. Konservierte mikrobielle Signaturen, sogenannte PAMPs (*pathogen-associated molecular patterns*), werden von Oberflächenrezeptoren erkannt und lösen eine Reihe von verschiedenen Abwehrreaktionen aus, die schlussendlich zum Schutz der Pflanze und zur Abwehr der Pathogeninfektion führen. Diese Oberflächenrezeptoren gehören vorwiegend zur Gruppe der leuzinreichen Rezeptorkinasen (LRR-RLKs). In Arabidopsis weist die RLK/Pelle Familie, zu denen auch die LRR-RLKs gehören, über 600 Mitgliedern auf (Shiu and Bleecker, 2003) und für ungefähr zwei Drittel davon wurden extrazelluläre Domänen vorhergesagt (Shiu and Bleecker, 2003). Basierend auf einem revers-genetischen *screen* haben Postel et al. (2010) 49 pathogen-induzierbare LRR-RLK Gene identifiziert, einschließlich des kürzlich beschriebenen Phytosulfokin-Rezeptors PSKR1. Die Erkennung von endogenen Tyrosin-sulfatierten Peptiden, sogenannte Phytosulfokine (PSKα), durch PSKR1 führt zur Wachstumsförderung und hat Einfluss auf weitere inhärente Entwicklungsprozesse in Pflanzen. PSY1R, eine mit PSKR1 eng verwandte LRR-RLK, erkennt ein strukturell verschiedenartiges aber ebenfalls Tyrosin-sulfatiertes Peptid, welches als PSY1 bezeichnet wird und löst sehr ähnliche physiologische Reaktionen aus wie PSKα. In dieser Arbeit konnte gezeigt werden, dass sowohl PSKR1 als auch PSY1R, jedoch nicht PSKR2, ein weiterer PSKα-bindender Rezeptor, die Resistenzreaktionen von Pflanzen gegen biotrophe Bakterien und die Reaktionen auf PAMPs unterdrücken und im Gegenzug die Resistenz gegen nekrotrophe Pilze stärken. Der antagonistische Effekt auf die Resistenz gegen biotrophe und nekrotrophe Organsimen wird durch die veränderte Phytohormonbalance mit erhöhten Salizylat- (SA) und verringerten Jasmonat (JA)-Antworten in den Rezeptormutanten untermauert. Dies legt

nahe, dass PSKR1 und PSY1R SA-anhängige Abwehrprozesse unterdrücken. Die Analyse multipler Mutanten hat gezeigt, dass *pskr1* und *psy1r* Mutantenphänotypen additiv sind, was nahelegt, dass beide Rezeptoren redundant die pflanzliche Abwehr modulieren. Pflanzenmutanten, denen die Tyrosin-Protein-Sulfotransferase TPST fehlt, ein Protein, das PSKα und PSY1 *in vitro* sulfatieren kann, zeigen dieselben Phänotypen wie Mutanten, denen die beiden Rezeptoren PSKR1 und PSY1R fehlen. Desweiteren führt die exogene Applikation von PSKα in *tpst-* Mutanten zu einer partiellen Komplementation der Bakterienphänotypen. Diese Daten beweisen, dass PSKα einen direkten Effekt auf die pflanzliche Immunität hat. Eine Zusammenfassung aller Daten legt ein Model nahe, in dem die PSK-Rezeptor-Unterfamilie, basierend auf der Perzeption von endogenen sulfatierten Peptiden, wachstumsinduzierende und Abwehr-Signale integriert und die Balance zwischen den antagonistisch regulierten Abwehrreaktionen gegen biotrophe und nekrotrophe Pathogene aufrechterhält.

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8 Appendix

Table 8- 11. Primers used in this work

primer name	primer sequence
PR1-F	GTGGGTAGCGAGAAGGCTA
PR1-R	ACTTTGGCACATCCGAGTCT
PR2-F	ACGGCCAACATCCATCTAGACT
PR2-R	GAGTACCCTGGATCGTTATCAACA
FRK1-F	GATGGCGGACTTCGGGTTATC
FRK1-R	CGAATAGTACTCGGGGTCAAGGTAA
PDF1.2-F	AGGGGTTTGCGGAAACAGTAA
PDF1.2-R	CGTAACAGATACACTTGTGTGC
OPR3-F	GGACGCAACTGATTCTGACCCAC
OPR3-R	CGTAGGCGTGGTAGCGAGGTTG
EF1α-F	GAGGCAGACTGTTGCAGTCG
EF1α-R	TCACTTCGCACCCTTCTTGA
PSKR1-F	GAACAAGATTTGGATGCTGTGCTC
PSKR1-R	GGTTCGATCCCGGTTTCTCTG
PSKR2-F	GAGAACTTGTTGGAGCTCACG
PSKR2-R	TTTTGGGATGTGAGCGTTTAG
PSY1R-F	GAGAACCTTTAGCTGCCCAAC
PSY1R-R	ACCATGATTTCAGCGGTGATC
PSKR1 TF-F2	TTTTAGGAGTTTTAGAGACATACGGGAA
PSKR1 TF-R	GACATCATCAAGCCAAGAGACTAACTGT
35S PSKR1-F	AAAAAGCAGGCTGTTCTTGAAATGCGTGTTCATCG
35S-PSKR1-R	AGAAAGCTGGGTCTAGACATCATCAAGCCAAGAGAC
35S-PSK2-F	AAAAAGCAGGCTTCACCATGGCAAACGTCTCCGCTTTGC
35S-PSK2-R	AGAAAGCTGGGTGTCAAGGATGCTTCTTCTTCTGG
35S-PSK4-F	AAAAAGCAGGCTTCACCATGGGTAAGTTCACAACCATTT
35S-PSK4-R	AGAAAGCTGGGTGTCCACCTCCGGATCAGGGCTTGTGATTCTGAGTA
Salk-Lba	TGGTTCACGTAGTGGGCCATCG
attB1	GGGGACAACTTTGTACAAAAAAGCAGGCT
attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT
PSK2-I miR-s	GATTAATCAGTATGAGCGACCGATCTCTCTTTTGTATTCC
PSK2-II miR-a	GATCGGTCGCTCATACTGATTAATCAAAGAGAATCAATGA
PSK2-III miR*s	GATCAGTCGCTCATAGTGATTATTCACAGGTCGTGATATG
PSK2-IV miR*a	GAATAATCACTATGAGCGACTGATCTACATATATATTCCT
PSK1/5-I miR-s	GATGTAATCAGTGTGAGCGACTATCTCTCTTTTGTATTCC
PSK1/5-II miR-a	GATAGTCGCTCACACTGATTACATCAAAGAGAATCAATGA
PSK1/5-III miR*s	GATAATCGCTCACACAGATTACTTCACAGGTCGTGATATG
PSK1/5-IV miR*a	GAAAGTAATCTGTGTGAGCGATTATCTACATATATATTCCT
PSK2/6-I miR-s	GATGCAATCTTCGTCGTCCGCAATCTCTCTTTTGTATTCC
PSK2/6-II miR-a	GATTGCGGACGACGAAGATTGCATCAAAGAGAATCAATGA
PSK2/6-III miR*s	GATTACGGACGACGATGATTGCTTCACAGGTCGTGATATG
PSK2/6-IV miR*a	GAAGCAATCATCGTCGTCCGTAATCTACATATATATTCCT

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Lebenslauf

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