Analysis of *AtPSKR1*, an LRR Receptor Protein Kinase, and other PSK-Signalling Components in Plant Defence Responses

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List of Abbreviations

ABA	Abscisic acid	LysM	Lysin motif
At	*Arabidopsis thaliana*	MAMPs	Microbe-associated molecular patterns
Avr	Avirulence factor	MAPKs	Mitogen-activated protein kinases
BAK1	BRI1-associated receptor kinase 1	NB-LRR	Nucleotide binding-site leucine-rich repeat
BRI1	brassinosteroid insensitive 1	PAMPs	Pathogen-associated molecular patterns
BRL1/2/3	BRI1-like 1/2/3	PCD	Programmed cell death
BRs	Brassinosteroids	PEPR1	Pep receptor 1
DAMPs	Damage-associated molecular patterns	PR	Pathogenesis-related
Dc	*Daucus carota*	PRR	Pattern-recognition receptor
DPI	Diphenylene iodonium	PSK	Phytosulfokine
EFR	EF-Tu receptor	PSKR	PSK receptor
EF-Tu	Elongation factor Tu from *Escherichia coli*	PTI	PAMP-triggered immunity
EMS	EXCESS MICROSPOROCYTES1	Pto	*Pseudomonas syringae* pv. *tomato*
ER	ERECTA	R protein	Resistance proteins
ERL1	ERECTA-like 1	RGFs	Root meristem growth factors
ET	Ethylene	RLKs	Receptor-like kinases
ETI	Effector-triggered immunity	ROS	Reactive oxygen species
ETS	Effector-triggered susceptibility	SA	Salicylic acid
Eix	Ethylene-inducing xylanase	SAR	Systemic acquired resistance
EXS	EXTRA SPOROGENOUS CELLS	SOT	Sulfotransferase
FLS2	Flagellin sensing 2	TE	Tracheary element
GUS	Beta-glucoronidase	TLRs	Toll-like receptors
HR	Hypersensitive response	TTSS	Type III secretion system
JA	Jasmonic acid	*Ws*	*Wassilewskija*
LPS	Lipopolysaccharides		

1 Introduction

1.1 Principles of plant immunity

For multicellular eukaryotic systems (including plants), the term immunity refers to the condition or ability of being immune, i.e. to have sufficient biological defences in order to avoid an infection, disease or other unwanted biological invasion (Nürnberger and Kemmerling 2009). Despite the remarkable similarities in the molecular organization of animal and plant systems for non-self recognition and anti-microbial defence, there are clear differences like the lack of an adaptive or acquired immunity in plants but not in animals (Nürnberger *et al.* 2004; Jones and Dangl 2006). Adaptive immunity is characterized by creation of antigen-specific receptors through somatic recombination in maturing lymphocytes (Medzhitov and Janeway 1997). Additionally, macrophages, neutrophils and dendritic cells, which form part of the circulatory blood system and are mobilized to the infection site in order to kill the invading organism or to limit its infection process, are absent in plants (Nürnberger *et al.* 2004). In plants, each cell possesses both a preformed and an inducible defence capacity, due to the plant lack of mobility, conversely to animals.

Plants must constantly defend themselves against microorganisms like bacteria, viruses, fungi, oomycetes, and invertebrates and even against other plants. In wild plant populations, plants are resistant to most microbes and rely on innate immune responses for their defence and on systemic signals arising from the infection sites; if disease occurs, it is usually restricted to a few plants and affects only a small amount of tissue. A successful infection, that is when disease occurs, rarely kills a plant.

Plant-microbe associations can result positive or detrimental for the plant host. For example, mycorrhizae form a mutualistic relationship with the roots of most plant species (Smith and Read 1997; Harrison 1999); but when the microbial colonization represents a disadvantage to the host, then such microbes are referred to as pathogens.

Plant pathogens have evolved different life strategies in order to be successful. Pathogenic bacteria thrive in the apoplast after entering via gas or water pores or via existing wounds (Jones and Dangl 2006). Nematodes and aphids feed by inserting a stylet, a small needlelike appendage, directly into a plant cell (Hussey 1989; Ng and Perry 2004). On the other hand, fungi can directly enter plant epidermal cells or extend hyphae, long and branching filamentous cells, on top of, between or through plant cells. Pathogenic and symbiotic fungi and oomycetes can invaginate feeding structures, named haustoria, into the host cell plasma membrane. The interface formed between the haustorial plasma membrane and the host

plasma membrane, called extrahaustorial matrix, is crucial for the outcome of the plant-microbe interaction. These pathogens deliver effector molecules (virulence factors) into the plant cell to heighten microbial fitness (Jones and Dangl 2006) by entirely different mechanisms than the type III secretion system (TTSS) delivery of bacterial effectors (Nishimura and Dangl 2010).

In parallel to the assorted pathogenic invading tools, plants have developed inducible defence responses that originally were thought to be triggered by two levels of microbial recognition, represented by the zigzag model (Jones and Dangl 2006) (Figure 1.1.1). The first recognition level implies conserved microbial molecules, known as pathogen- (or microbe-) associated molecular patterns (PAMPs/MAMPs) making use of pattern-recognition receptors (PRRs). PRRs refer to receptors that directly recognize molecules that are conserved among whole classes of microbes and symbionts but are absent from the host. This primary plant immune response is referred to as PAMP-triggered immunity (PTI) and is essential to plant innate immunity (Nürnberger *et al.* 2004; Zipfel and Felix 2005; Jones and Dangl 2006). PRRs can also recognize signals derived from the plant itself that were caused by microbes, like breakdown products of the cell wall (plant cell wall-derived oligogalacturonide fragments, cellulose fragments or cutin monomers); these signals are called damage-associated molecular patterns (DAMPs) (Lotze *et al.* 2007) or endogenous elicitors, as previously known. Plants with impaired PRRs are more susceptible to microbial infections, and pathogens that fail to avoid or suppress PTI are unable to cause disease (Zipfel *et al.* 2004; Zipfel *et al.* 2006). Successful bacterial pathogens manage to suppress PTI by secreting and delivering effectors via the TTSS directly into the cytoplasm of host cells, where their action results in effector-triggered susceptibility (ETS). Overcoming PTI constitutes an essential strategy of successful pathogens (Alfano and Collmer 2004; Chisholm *et al.* 2006; Jones and Dangl 2006). Successful pathogens among fungi and oomycetes appear to use a similar approach (Ellis *et al.* 2007; Kamoun 2007; Birch *et al.* 2008). In the course of evolution, some individual plants have developed resistance (R) proteins in order to recognize particular effectors directly or indirectly. Most of these R proteins are classified as nucleotide binding-site leucine-rich repeat proteins (NB-LRR proteins), which seem to act as guards of important host proteins, and the alteration or loss of the guardee is translated as a signal to induce effector-triggered immunity (ETI) (Jones and Dangl 2006). An extension of the 'guard hypothesis' has recently been presented as the 'decoy model', where a host protein that mimics the real virulence target of an effector can act as a decoy to trap the pathogen into a recognition event (Van der Hoorn and Kamoun 2008). ETI is an amplified PTI response and results in disease resistance and often in the hypersensitive cell death response (HR) at the infection site. This type of plant defence is known as ETI and constitutes the second level of microbial recognition (Chisholm *et al.* 2006; Jones and Dangl 2006).

Currently, an extension or variation to the zigzag model from Jones and Dangl 2006 has been proposed not only for describing the evolution of the oomycete-plant interactions (Hein *et al.* 2009), but also for the chitin signalling interaction between *C. fulvum* and tomato (Thomma *et al.* 2011). This variation contemplates the evolutionary contest on a molecular level between pathogen effectors and plant R proteins and virulence targets in the host beyond ETI, which result dictates whether ETI is activated or evaded, leading in the latter case to a second effector-triggered susceptibility (ETS2) and potentially to an additional ETI (ETI2) (Hein *et al.* 2009). This attack-and-response can conceptually occur iteratively with multiple rounds of ETS, followed by recognition, resulting in ETI (Nishimura and Dangl 2010). Yet another current interesting interpretation of activation of innate immunity in multicellular eukaryotic systems, is that the two forms of plants innate immunity, i.e. PTI and ETI, could be merged as one form only, since plants appear to perceive MAMPs, DAMPs and effectors as one and the same type of signal that indicates a situation of danger (Matzinger 2007; Boller and Felix 2009). Moreover, accumulative evidence indicates that separation between PAMPs and effectors, and between PRRs and R proteins, and so also between PTI and ETI, cannot be strictly maintained, leading to the idea that there is a continuum between PTI and ETI (Thomma *et al.* 2011).

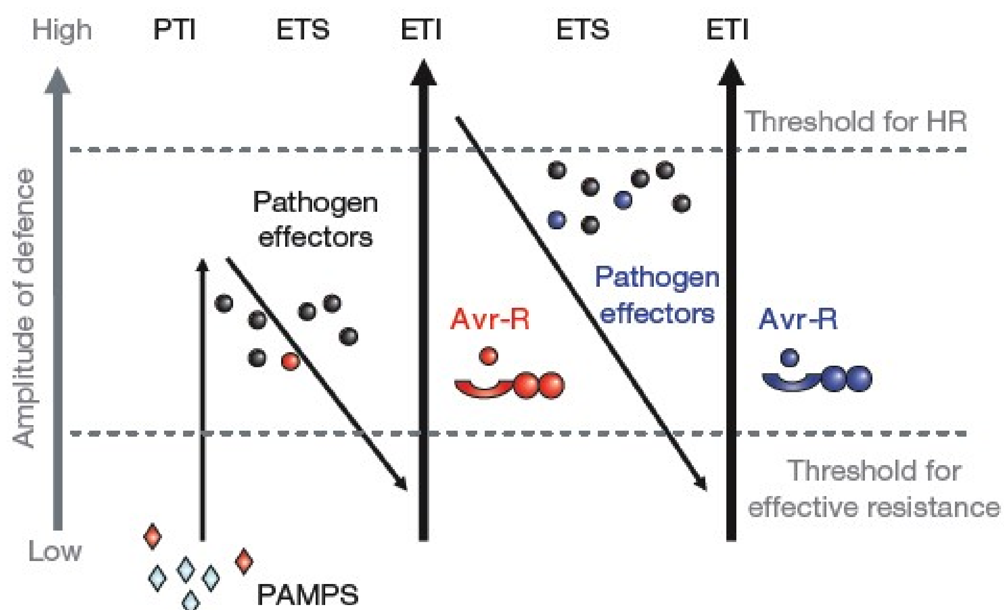

Figure 1.1 1: Zig-zag model representing the plant immune system

In phase 1, plants detect PAMPs via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI). In phase 4, new selected pathogen effectors (in blue) can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize on of the newly acquired effectors, resulting again in ETI. (Figure and explanatory text taken from Jones and Dangl 2006).

Defence responses can also be activated in the non-colonized organs of a plant, which is locally infected by a microbe/herbivore. Systemic acquired resistance (SAR) is an example of an inducible defence mechanism that is activated in the distal organs of a plant in response to a local infection of leaves with a pathogen (Vlot *et al.* 2008). SAR provides plants with enhanced resistance against subsequent attack by a wide array of pathogens. Systemic signals are responsible for the communication between the infected organ and the rest of the plant; methyl salicylate, jasmonates, azelaic acid among others account for these systemic signals (Shah 2009). Systemic expression of defence is also observed in plants colonized by beneficial microbes, like the case of root-inhabiting rhizobacteria that enhance resistance against foliar pathogens. This other form of systemic resistance is called induced systemic resistance (ISR) (van Loon 2007).

1.1.1 PAMPS and DAMPs

PAMPs are highly conserved structures typical of whole classes of pathogens (Medzhitov and Janeway 1997) that are essential for their survival and therefore are difficult to mutate or delete. These conserved structures are not exclusively characteristic to pathogenic microbes, thus the term MAMPs (microbe-associated molecular patterns) also applies. Among the known PAMPs that trigger immune responses in plants are lipopolysaccharides (LPS) (Meyer *et al.* 2001; Newman *et al.* 2002), flagellin (Felix *et al.* 1999), elongation factor EF-Tu (Kunze *et al.* 2004), Harpin (Wei *et al.* 1992; Lee *et al.* 2001) and cold shock protein (Felix and Boller 2003) from gram-negative bacteria; transglutaminase (Nürnberger *et al.* 1994; Brunner *et al.* 2002) and elicitins (Osman *et al.* 2001) from oomycetes; xylanase (Enkerli *et al.* 1999; Ron and Avni 2004), β-Glucans (Klarzynski *et al.* 2000; Yamaguchi *et al.* 2000) and chitin (Baureithel *et al.* 1994; Ito *et al.* 1997) from fungi. In general, plant cells have to deal with a variety of these signals when interacting with microorganisms in vivo. It is believed that defence responses triggered in plants arise from recognition of complex patterns that constitute the microbial surface, like in the case of phytopathogenic fungi that possess chitins, glycopeptides and ergosterol in their cell wall. Some PAMPs are even able to trigger HR (hypersensitive response), which is commonly involved with ETI responses, but in a species-specific manner. Moreover PAMPs are not only constitutive building blocks of microbial surface, since harpin proteins (like HrpZ, HrpN or PopA) are secreted only upon attempted microbial infection of plants (Alfano and Collmer 2004).

DAMPs or endogenous elicitors, resulting as breakdown products of the plant cell caused by pathogenic lytic enzymes, are also known to elicit plant immune responses (Vorwerk *et al.* 2004). In plants, cell wall-derived oligogalacturonide fragments, cellulose fragments or cutin monomers trigger plant immune responses that are indistinguishable from those caused by microbe-derived PAMPs (Fauth *et al.* 1998; Aziz *et al.* 2007). These plant-derived elicitors

bring to mind the animal tissue-derived 'danger' or 'alarm' signals. Moreover, in animals such signals are recognized by PRRs that also sense classical PAMPs (Gallucci and Matzinger 2001; Matzinger 2002). On the other hand, some peptides can be regarded as DAMPs, like systemin and *At*Pep1. Systemin is an 18-amino acid peptide, which resembles an endogenous elicitor. Because systemin is expected to be released only upon cell injury, it is believed that systemin acts as a DAMP for the neighboring cells in *Solanaceae* species (Ryan and Pearce 2003). *At*Pep1 is a 23-amino acid peptide from *Arabidopsis*, which is derived from PROPEP1 and induced in response to wounding. It associates with the innate immune responses through jasmonic acid (JA) and ethylene (ET) signalling pathways, thus resembling an endogenous signal for stress and wounding (Huffaker *et al.* 2006). The receptor of *At*Pep1 is PEPR1 which is a LRR-RK (LRR XI)(Yamaguchi *et al.* 2006).

1.1.2 PAMP perception by PRRs

In vertebrates PAMPs are perceived by a class of receptors that resemble the *Drosophila* Toll protein and are therefore called Toll-like receptors (TLRs) (Aderem and Ulevitch 2000; Girardin *et al.* 2002; Cook *et al.* 2004; Akira *et al.* 2006; Ferrandon *et al.* 2007; Medzhitov 2007). These receptor proteins are formed by extracytoplasmic leucine rich-repeat (LRR) domains, a transmembrane domain and a cytoplasmic TIR domain (*Drosophila* Toll and human interleukin-1 receptor) (Underhill and Ozinsky 2002; Cook *et al.* 2004) (Figure 1.1.2).

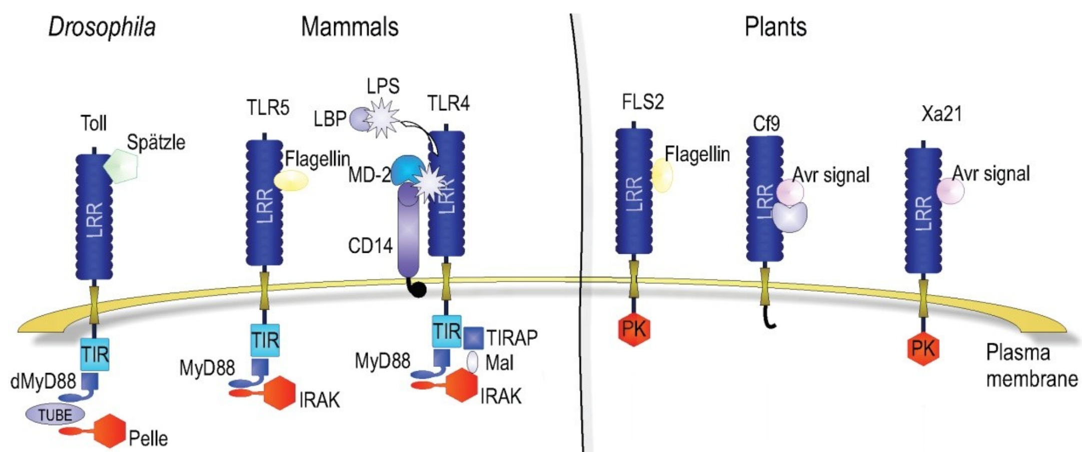

Figure 1.1.2: Conserved modular structures among PAMP receptor proteins, LRR-RLKs, in insects, mammals and plants.

Toll, Toll-like receptor 4 (TLR4), TLR5, FLS2 and the plant R genes Cf9 and Xa21 exemplify transmembrane receptors for the recognition of pathogen-associated molecular patterns (PAMPs) or Avr signals. (Figure modified from Nürnberger *et al.* 2004).

In *Arabidopsis*, the flagellin perception system appears to be highly similar to the systems in insects and animals for PAMP recognition. The *Arabidopsis* FLS2 (flagellin sensing 2) gene encodes a plasma membrane LRR-receptor kinase which recognizes bacterial flagellin through its extracytoplasmic LRR domain (Gomez-Gomez and Boller 2000; Chinchilla *et al.* 2006). Flagellin-induced immune responses restricted the growth of the virulent *P. syringae* pv. *tomato* strain DC3000 (*Pto* DC3000), whereas *fls2* mutants were more susceptible to this pathogen (Zipfel *et al.* 2004), emphasizing thus the importance of PTI in plant disease resistance. In *Arabidopsis*, flg22 (single stretch of 22 amino acid residues of the most conserved part in the N-terminus of flagellin (Felix *et al.* 1999)), also induces callose formation, accumulation of the defence protein PR1 and a strong inhibition of seedling growth (Gomez-Gomez *et al.* 1999). When structurally compared FLS2 and TLR5 (the human flagellin receptor (Hayashi *et al.* 2001)), both receptors appear to have a conserved modular structure, but nevertheless, they recognize different flagellin epitopes (Felix *et al.* 1999; Donnelly and Steiner 2002; Nürnberger *et al.* 2004). On the other hand, the *Arabidopsis* EFR (EF-Tu receptor) gene encodes a plasma membrane LRR-receptor kinase which recognizes elf18, a 18-amino acid fragment of *Escherichia coli* elongation factor Tu (EF-Tu) that constitutes a PAMP in *Arabidopsis* (Kunze *et al.* 2004). Interestingly, EFR resembles closely FLS2 (Gomez-Gomez and Boller 2000) and both PRRs belong to the same LRR family (LRR XII clade). Recently a third LRR-RLK, belonging also to the LRR XII clade, has been redefined as a PRR: XA21. In rice, XA21 (known previously as a resistance protein) confers resistance to the bacteria *Xanthomonas oryzae* pv. *oryzae* carrying the Avr gene AvrXA21 (Song *et al.* 1995). XA21's ligand is the type-I secreted protein Ax21, which first 17 amino-acids, named AxY^{S}22, are sufficient to trigger XA21-mediated resistance in rice (Lee *et al.* 2009). Because the sequence of Ax21 is conserved in all *Xanthomonas* species, as well as in the pathogenic bacteria *Xylella fastidiosa* and the human pathogen *Stenotrophomonas maltophilia (Han et al. 2011)*, this peptide is now recognized as a PAMP and its receptor redefined as a PRR (Lee *et al.* 2009; Segonzac and Zipfel 2011).

PRRs also recognize endogenous elicitors or DAMPs, like the peptide elicitor *At*Pep1 and its homologs (Pep2 to Pep6), which are endogenous amplifiers of innate immunity in Arabidopsis (Huffaker *et al.* 2006). These endogenous elicitors bind to PEPR1, a plasma membrane LRR receptor kinase belonging to the LRR XI clade, with and extracellular LRR domain and an intracellular protein kinase domain (Yamaguchi *et al.* 2006; Krol *et al.* 2010). Interestingly a close homolog of PEPR1, identified as PEPR2, was found to be a second receptor for the Pep1 and Pep2 peptides and to contribute to defence responses in Arabidopsis (Yamaguchi *et al.* 2010).

There are other plant PRRs with a different structure than the ones from FLS2 or EFR that recognize fungal chitin, fungal xylanase and oomycete glucans. In rice, perception of fungal chitin is mediated by both a plasma membrane LysM (lysin motif) receptor protein (LysM-P), named CEBiP (Kaku *et al.* 2006) and OsCERK1, a LysM-RLK, that associate in a ligand-dependent manner (Shimizu *et al.* 2010). In *Arabidopsis,* AtCERK1 has been implicated in chitin perception (Miya *et al.* 2007) and yet unidentified bacterial PAMP(s) (Gimenez-Ibanez

et al. 2009). Consequently, at least two types of LysM proteins in plants appear to act together (a chitin-binding protein and a transmembrane signalling partner) as a functional chitin receptor (Wan *et al.* 2008; Albert *et al.* 2010). In tomato, the Eix2 receptor of the fungal xylanase EIX (EIX, ethylene-inducing-xylanase) from *Trichoderma viride* is a receptor-like protein (RLP) (Ron and Avni 2004). This protein possesses an extracellular LRR domain, a transmembrane domain and a short cytoplasmic tail with a putative endocytosis signal but no kinase domain. It is believed that these LRR proteins (LRR-P) constitute another class of PRRs that are mechanistically similar to animal LRR-P-type PAMP receptors (Nürnberger and Kemmerling 2006; Bittel and Robatzek 2007). In soybean and French bean, the corresponding β-glucan-binding proteins (GBPs) have been identified (Mithöfer *et al.* 1996; Umemoto *et al.* 1997; Mithöfer *et al.* 1999; Mithöfer *et al.* 2000). Moreover, it is believed that the GBP associates with a PRR at the plasma membrane as part of a β-glucan elicitor receptor complex (Fliegmann *et al.* 2004; Zipfel and Felix 2005).

1.1.3 Signal transduction in PTI

When PAMPs activate PRRs, a subsequent host-signalling cascade occurs that terminates in the activation of pathogen immune responses. Among the earliest physiological responses to PAMPs in plant cell cultures are: alkalinization of the growth medium due to changes of ion fluxes (H^+, Ca^+) across the plasma membrane (Nürnberger *et al.* 2004); oxidative burst, recorded by H_2O_2-dependent luminescence of luminol (Chinchilla *et al.* 2007); activation of mitogen-activated protein kinases (MAPKs) which is accompanied by changes in protein phosphorylation (Nühse *et al.* 2000). Subsequently other PAMP responses occur, like the synthesis of the stress hormone ethylene (Spanu *et al.* 1994), receptor endocytosis (Robatzek *et al.* 2006) and gene activation (Zipfel *et al.* 2006; Libault *et al.* 2007). Among late responses after PAMP application are callose deposition (Gomez-Gomez *et al.* 1999) and seedling growth inhibition (Pearce *et al.* 2001; Navarro *et al.* 2006).

Protein kinase activity proved its crucial role in triggering rapid PAMP responses in plants such as changes in ion fluxes (Nürnberger *et al.* 1994; Felix and Boller 2003). In the case of FLS2, besides protein kinase activity, phosphorylation plays a crucial role in flagellin sensing/signalling (Robatzek *et al.* 2006). An important receptor kinase implicated in FLS2 and EFR function is BAK1 (BRI1-associated receptor kinase 1) (Chinchilla *et al.* 2007). BAK1, an LRR-RK, controls plant growth by heterodimerization with the brassinosteroid receptor BRI1 (Wang *et al.* 2001; Li *et al.* 2002). Beside its role as positive regulator of PTI, BAK1 appeared also to act as a negative regulator of plant cell death (Kemmerling *et al.* 2007). BAK1 constitutes an example of a plant LRR-RK with dual functions in plant development and immunity.

Receptor ligand-induced endocytosis has been proven to be a way to shut down PRR activity, like it is the case for FLS2 (Robatzek *et al.* 2006).

PAMP-triggered immunity requires a signal transduction from receptors to downstream components via the MAPK cascade. In plants, MAPK pathways are involved in the regulation of development, growth, programmed cell death and in responses to various environmental stimuli including cold, heat, reactive oxygen species, UV, drought and pathogen attack (Colcombet and Hirt 2008). Many of the known PAMPs were shown to activate MAP kinases, like flg22, which triggers a rapid and strong activation of MPK3, MPK4 and MPK6 (Pitzschke *et al.* 2009). MPK4 and MPK6 are also activated by harpin proteins, which are encoded by *hrp* (hypersensitive response and pathogenicity) genes in many plant pathogenic bacteria. After activation, induction of pathogenesis-related (PR) genes follows (Desikan *et al.* 2001), encoding for proteins with antimicrobial activities. PAMP-triggered MAPK pathways may exert both positive and negative regulation of PTI-associated responses in plants (Nürnberger and Kemmerling 2009). For example, flg22 has been shown to activate a MAPK cascade that suppresses various pathogen defence pathways, including callose deposition and *PR* gene expression (Ichimura *et al.* 2006).

1.1.4 Role of LRR-RLKs

Receptor-like kinases (RLKs) belong to the large RLK/Pelle gene family and only in *Arabidopsis thaliana* there are around 600 such kinases, which display key roles in plant growth, development and defence responses. Their biological function can be basically divided into two broad categories (Shiu and Bleecker 2001). The first category includes proteins that control plant growth and development, i.e. in *Arabidopsis* ERECTA is involved in determining organ shape (Torii *et al.* 1996), CLAVATA1 is involved in meristem maintenance (Clark *et al.* 1997), BRI1 is involved in cell growth regulation (Li and Chory 1997), maize CRINKLY4 is involved in controlling cell morphogenesis and differentiation (Becraft *et al.* 1996), in carrot PSKR is involved in cell proliferation control (Matsubayashi *et al.* 2002) and in *Arabidopsis* AtPSKR1 is involved in cellular longevity and potential for growth (Matsubayashi *et al.* 2006). The second category includes RLKs involved in plant-microbe interactions and defence responses. Here some proteins are associated to plant-pathogen interactions: rice Xa21 is involved in resistance to bacterial pathogens (Song *et al.* 1995); *Arabidopsis* FLS2 is the flagellin receptor (Gomez-Gomez and Boller 2000); *Arabidopsis* EFR is the receptor of EF-Tu (Zipfel *et al.* 2006), a bacterial elongation factor that triggers MAMP responses in Arabidopsis (Kunze *et al.* 2004); and tomato SR160 is involved in systemin signalling (Scheer and Ryan 2002). Among the rest of RLKs in this category, some are crucial for interaction with plant symbionts including NORK/SYMRK and

HAR1 in fungal and/or bacterial symbiosis (Stracke *et al.* 2002), and Lys-M RLKs that are involved in early stages of nodulation and Nod factor perception (Radutoiu *et al.* 2003).

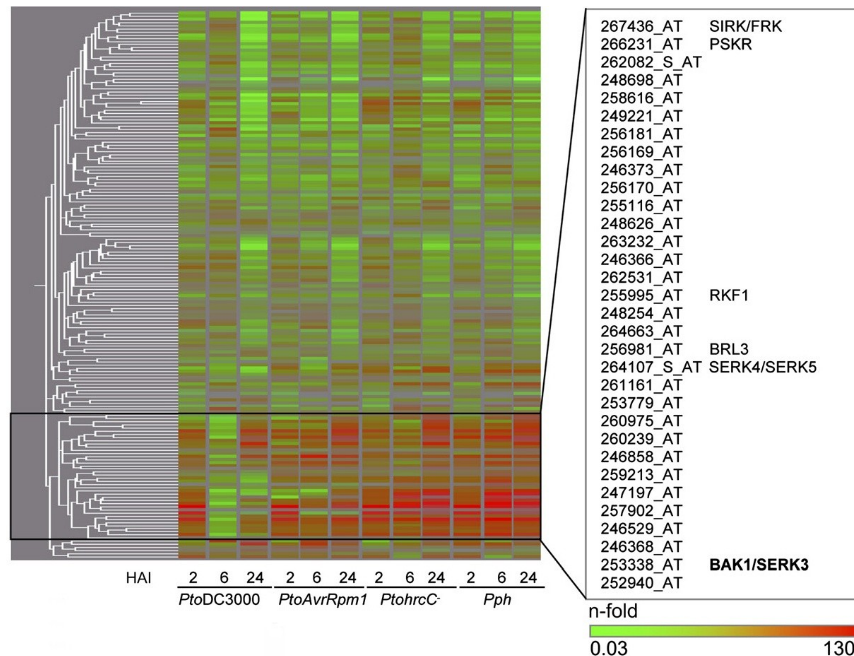

Figure 1.1.3: Microarray-based expression analysis of *Arabidopsis* LRR-RLK genes

Uncentered hierarchical cluster analysis of LRR-RLK gene expression in *Arabidopsis* plants infected for the indicated times (HAI) with bacterial strains *Pto* DC3000 (virulent), *Pto* AvrRpm1 (avirulent), *Pto* hrcC$^-$ (plant nonpathogenic), or *Pph* (nonpathogenic on *Arabidopsis*) with the Affymetrix ATH1 chip. Red and green indicate increased and decreased transcript accumulation, respectively, relative to control treatments (10mM $MgCl_2$). (Figure from Kemmerling *et al*. 2007)

Approximately a third of RLKs are constituted by the LRR-RLKs family (~ 235 in *Arabidopsis*) (Shiu *et al.* 2004), which is subdivided in 13 subfamilies based on kinase domain phylogeny (Shiu and Bleecker 2001). LRR-RLKs are anticipated to serve as a major class of surface pattern recognition receptors in plants (Nürnberger and Kemmerling 2006). This hypothesis is further supported by experiments of gene-expression profiling with *Arabidopsis* Col-0 plants infected with various *Pseudomonas syringae* strains, where gene-expression analysis revealed increased transcript accumulation for 32 genes, including BAK1, SERK4/SERK5 and PSKR1 (Figure 1.1.3) (Kemmerling *et al.* 2007); moreover, flagellin treatment yielded increased transcript levels of 28 LRR-RK-encoding genes (Navarro *et al.* 2004). Expression of flagellin-responsive LRR-RLK genes was also triggered by other PAMPs including bacterial LPS and fungal chitin (Zhang *et al.* 2002; Thilmony *et al.* 2006).

1.2 Bacteria, fungi and oomycetes as constant threat to plants

1.2.1 Plant pathogenic bacteria

Bacteria often live as epiphytes on the leaf surface without causing any obvious disease symptoms. However, under appropriate conditions of temperature and humidity, bacteria can enter the plant through natural openings such as stomata and hydathodes or via mechanical wounds (Hirano and Upper 2000). Once bacteria enter the intercellular spaces, the apoplast, they have to cope with preformed defence molecules in order to obtain nutrients and to multiply successfully inside of the host, which eventually would lead to the outcome of disease symptoms. Many gram-negative plant and animal pathogenic bacteria employ a type III secretion system (TTSS) to defeat and colonize their respective host organisms (Alfano and Collmer 2004; Cornelis 2006). TTSS are complex macromolecular machines that consist of the structural components of the apparatus itself, secreted proteins (including pore-forming translocators and effectors), chaperones and cytoplasmic regulators (Deane *et al.* 2010). TTSS facilitate the injection of bacterial effectors into eukaryotic cells to manipulate host physiology to the benefit of the pathogen (Büttner and Bonas 2006). These effectors interact with virulence targets inside of the plant cell in order to diminish or even abolish plant immune reactions. In plant pathogenic bacteria, TTSS are encoded by *hrp* genes (hypersensitive response and pathogenicity), whose products contribute to bacterial pathogenicity and elicit plant immune responses, including programmed cell death (hypersensitive response, HR), in non-host plants and in resistant cultivars of host plants (Alfano and Collmer 2004; Büttner and Bonas 2006). The hypersensitive response is a rapid local cell death at the infection site that restricts bacterial multiplication and is triggered by individual effector proteins in plants carrying a corresponding resistance gene (Dangl and Jones 2001). *hrp* genes were found in almost all major gram-negative bacterial plant pathogens, like *Pseudomonas syringae*, *Xanthomonas* spp., *Ralstonia solanacearum* and *Erwinia* spp., and in symbiotic Rhizobia (Fauvart and Michiels 2008). This suggests therefore a main role of the TTSS in mediating diverse plant-bacteria interactions (Alfano and Collmer 2004; He *et al.* 2004).

In order to deliver bacterial effector proteins from the cytoplasm of gram-negative bacteria to the plant cell interior, these effectors need to be transported across multiple physical barriers: the two bacterial membranes and the plasma membrane of the plant cell, that is surrounded by a thick cell wall (Figure 1.2.1, A). The effector transport is achieved through the TTSS-associated Hrp pilus, which is uniquely produced by phytopathogenic bacteria and is believed to have evolved to facilitate the establishment of infection structures across plant cell walls (Tampakaki *et al.* 2004). *P. syringae* pv. *tomato* (*Pto*) DC3000 HrpA1 is the major constituent of the Hrp pilus (Roine *et al.* 1997).

Pseudomonas syringae is a host-specific, hemibiotrophic bacterial plant pathogen that causes economically important diseases in a wide variety of plant species and is used as a model organism to understand the molecular basis of plant disease. The ability of *P. syringae* to enter and proliferate inside the plant depends on its ability to synthesize toxins, hormones and a type III secretion system that delivers effectors of diverse enzymatic activities, such as cysteine protease (Shao *et al.* 2002; Lopez-Solanilla *et al.* 2004; Kim *et al.* 2005), ubiquitin-like protease (Hotson *et al.* 2003; Roden *et al.* 2004), E3 ubiquitin ligase (Abramovitch *et al.* 2006; Janjusevic *et al.* 2006) and protein phosphatase activity (Bretz *et al.* 2003; Espinosa *et al.* 2003).

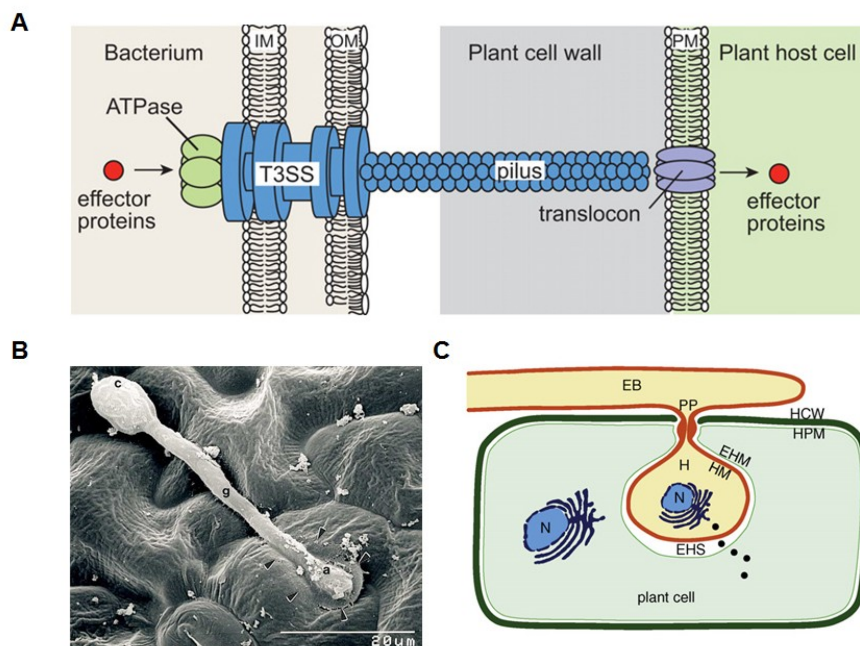

Figure 1.2.1: Effector delivery systems in plant pathogenic microbes

(A) Representation of the TTSS from plant pathogenic bacteria taken from Büttner and He 2009. (B) Germination and development of an appresorium on the surface of a tomato leaf taken from Prins *et al.* 2000. (C) Representation of the effector-exporting system in pathogenic fungi and oomycetes taken from Ellis *et al.* 2009. Penetrating peg (PP); external pathogen body (EB); host cell wall (HCW); extrahaustorial membrane (EHM); host plasma membrane (HPM); haustorial membrane (HM); extrahaustorial space (EHS); Haustoria (H); Nucleus (N).

1.2.2 Plant pathogenic fungi and oomycetes

The first step of pathogenic development for both necrotrophic - requiring dead host tissue- and biotrophic - requiring living host cells- fungal pathogens is the successful penetration of the plant surface. Penetration can occur directly via specialized infection structures, called appresoria, which promote the localized secretion of plant cell wall degrading enzymes or build up turgor and allow penetration through mechanical force (Howard *et al.* 1991; Bechinger *et al.* 1999). Another alternative is to use natural openings like stomata or wounds

for entry (Tucker and Talbot 2001). The infection strategy does not appear to be related to the subsequent lifestyle of the fungal pathogen (van Kan 2006). Some biotrophs like most rust fungi invade plant tissue via stomata, while other biotrophs like the smut fungi and the powdery mildew fungi form appressoria that allow direct entry into the plant epidermis (Klosterman *et al.* 2007). Necrotrophic pathogens kill the invaded cell by secretion of toxic compounds or induction of reactive oxygen species (ROS), and afterwards they feed on dead plant material.

In biotrophic interactions and during the initial stages of hemibiotrophic interactions (requiring living cells only at early infection stages) the infected plant cell stays alive. It is in these cases where the plant plasma membrane is invaginated and encases the infecting hyphae, thus forming a biotrophic interface. This interface, which can be established by intracellularly growing hyphae or by specialized structures (haustoria) (Figure 1.2.1, C), provides nutrients to the pathogen and facilitates exchange of signals maintaining the interaction (O'Connell and Panstruga 2006). It is now clear that haustoria-forming pathogens deliver numerous effector proteins into host cells across this interface. These effectors are secreted into the apoplast and must cross the extrahaustorial membrane before entering the plant cytoplasm, where they may target host proteins to manipulate host metabolism or can be recognized by host resistance proteins, resulting in triggering of the host defence response (Dodds *et al.* 2009). Recently, it has been shown that secreted proteins, from biotrophic fungi and oomycetes as well as from extracellular fungal pathogens, are subsequently delivered by a nonendomembrane pathway into the host cells by an yet unknown mechanism and that their recognition takes place inside plant cells (Dodds *et al.* 2009).

Alternaria brassicicola is a necrotrophic fungus that causes black spot disease and is an economically important seed-bourne fungal pathogen of *Brassicaceae* species. During host infection, *A. brassicicola* is exposed to high levels of defence compounds, such as phytoalexins and glucosinolate breakdown products, and the ability to overcome these antimicrobial metabolites is a key factor determining fungal virulence (Joubert *et al.* 2011). Camalexin, an indolic secondary metabolite, is the major phytoalexin synthesized by *Arabidopsis* and some other brassicaceous weeds (Glawischnig 2007), and it may cause damage to fungal membranes and activate a compensatory mechanism in fungal cells aimed at preserving membrane integrity (Sellam *et al.* 2007). Another necrotrophic fungus is *Botrytis cinerea,* which is capable of infecting more than 200 plant species (Elad *et al.* 2004). This pathogen not only attacks plants growing in the field or greenhouse but also the produce at post-harvest, usually beginning with a latent infection in the field and developing later during harvest, transport and storage (Williamson *et al.* 2007). Like a typical necrotroph, B. *cinerea* kills the cells surrounding the infection area and then obtains nutrients from the dead tissue (van Kan 2006; Williamson *et al.* 2007).

1.3 Plant development and plant immunity

1.3.1 Dual functions of ERECTA and BAK1

Some RLKs recognize more than one signal and have a dual function regulating different processes, e.g. development and response to biotic/abiotic stress. There are two well documented examples of plant LRR-RK with dual functions in plant development and immunity: ERECTA and BAK1. Dual roles for receptor proteins in development and immunity are also known from animal systems, exemplified by the *Drosophila* receptor TOLL that controls embryonic patterning and immunity against fungal infections in adult insects. (Lemaitre *et al.* 1996).

The ERECTA (*ER*) LRR-RK was initially identified as a developmental regulator in several ways, including inflorescence architecture, lateral organ shape, ovule development, stomatal patterning, and transpiration efficiency through its genetic interaction with two closely related paralogs (*ERL1* and *ERL2*) and the genes *TMM* and *EPF1* encoding, respectively, a receptor-like protein (RLP) and a peptide (Torii *et al.* 1996; Shpak *et al.* 2003; Shpak *et al.* 2004; Masle *et al.* 2005; Shpak *et al.* 2005; Hara *et al.* 2007; Pillitteri *et al.* 2007). Moreover, genetic interactions between *ER* and genes controlling different developmental and hormone-mediated pathways have been described (Fridborg *et al.* 2001; Mele *et al.* 2003). More recently, *ER* also emerged as a key regulator of basal resistance as *er* mutant alleles are more susceptible than wild-type plants to the soilborne bacterium *Ralstonia solanacearum* 14-25, the necrotrophic fungus *Plectosphaerella cucumerina* and the damping-off oomycete *Phythium irregulare* (Godiard *et al.* 2003; Llorente *et al.* 2005; Adie *et al.* 2007). *ER*-mediated developmental signalling pathway is well characterized in contrast to the *ER*-mediated immunity to necrotrophic and soilborne pathogens. Resistance to these types of pathogens is often genetically complex in *Arabidopsis* and depends on the precise regulation of the ethylene (ET), jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA) signalling pathways (Thomma *et al.* 1999; Adie *et al.* 2007). Recent genetic analyses also showed that variation in plant wall composition results in altered disease resistance responses in a number of plant-pathogen combinations, and it is speculated that ERECTA may be acting as a cell-wall integrity sensor responding to plant cell-wall fragments released by pathogens (Sánchez-Rodriguez *et al.* 2009).

BAK1 (BRI1-associated kinase 1) is an LRR-RLK which acts as a signalling partner of BRI1 (Brassinosteroid insensitive 1). Brassinosteroids (BRs), involved in various developmental processes and responses to biotic and abiotic stresses (Clouse and Sasse 1998), are perceived by the BRI1 receptor (Li and Chory 1997). Binding of BRs to preformed BRI1

homo-oligomers results in transphosphorylation and dimer stabilization (Wang *et al.* 2005), hetero-oligomerization with BAK1 (Wang *et al.* 2005) and activation of BR signalling. This BR-dependent association of BRI1 with BAK1 results in controlling developmental processes such as stem elongation, vascular differentiation, seed size, fertility, flowering time and senescence (Li *et al.* 2002; Nam and Li 2002; Wang *et al.* 2005). Nowadays the actual picture of BAK1 is as an adaptor and enhancer of BR signalling, probably in a tetrameric configuration with BRI1 homodimers; reciprocal and sequential phosphorylation of BAK1 and BRI1 results in stabilization of the complex and in full responsiveness to BR (Chinchilla *et al.* 2009). On the other hand, BAK1 displays other functions that are independent of BRs, such as negative regulation of plant cell death, since *bak1* mutants showed altered disease-resistance phenotypes to biotrophic and necrotrophic pathogens, which are likely the consequence of infection-induced deregulated cell death control (Kemmerling *et al.* 2007). BAK1 also appears to have a role as a positive regulator of PTI, since *bak1* mutants were partially insensitive to both flg22 and elf18. Flg22-dependent rapid heterodimerisation of FLS2 and BAK1 was demonstrated by co-immunoprecipitation experiments (Chinchilla *et al.* 2007). Hence, BAK1 has BR-independent immunity-associated functions. Later on, BAK1 was found to be target of bacterial effectors, considering that a key function of effectors is to modulate diverse host cellular activities and block defence responses (Block *et al.* 2008). AvrPto and AvrPtoB directly target BAK1 and interfere with the formation of FLS2/BAK1 and BRI1/BAK1 complexes (Shan *et al.* 2008). Moreover, recent studies with a candidate-based yeast two-hybrid approach revealed additional LRR-RLKs that have the potential to interact with BAK1. PEPR1 and its closest homolog PEPR2 appeared among the 4 proteins identified as BAK1 interactors in the previous studies (Postel *et al.* 2009). Currently, biochemical information has proven that BAK1 forms rapidly hetero-complexes of *de novo* phosphorylated BAK1 with FLS2 and also with other LRR-RKs, EFR and PEPR1, induced by flg22, EF-Tu and *At*Pep1, respectively (Schulze *et al.* 2010). Thus, rapid *de novo* phosphorylation of BAK1 appears to be a general activation mechanism to many LRR-receptor kinases (Schulze *et al.* 2010). Recently, another new function of BAK1 has emerged in that BAK1 might interact with LeEix1 (receptor of the ethylene-inducing xylanase, Eix, in tomato) in order to fine-tune defence responses. It is suggested that LeEix1, aided by BAK1, functions as a decoy for LeEix2, which is the only Eix receptor that mediates defence responses (Bar *et al.* 2010).

1.3.2 Peptide hormones in plant defence

Endogenous elicitors in plants are typically oligosaccharide fragments such as the ones derived from plant cell walls (DAMPs) by either carbohydrases produced from attacking pathogens or by endogenous enzymes of the plant that are synthesized in response to pathogen attacks (Di Matteo *et al.* 2006). Interestingly a new type of endogenous peptide

elicitor was isolated from Arabidopsis: *At*Pep1 (Huffaker *et al.* 2006). *At*Pep1 is a 23-amino acid peptide that was isolated as a substance which induces alkalinization in *Arabidopsis* cell cultures at subnanomolar concentrations (Huffaker *et al.* 2006). *At*Pep1 derives from a 93-amino-acid precursor protein, PROPEP1, whose gene is expressed in response to wounding, MeJA, ethylene and *At*Pep1 itself, and it is blocked in mutants with impaired JA and SA synthesis and by DPI (an inhibitor of reactive oxygen species) (Huffaker and Ryan 2007). Expression of a 35S::PROPEP1 gene in *Arabidopsis* resulted in a phenotype that constitutively expressed *PDF1.2* and exhibited an increased resistance to the oomycete *Pythium irregulare* (Huffaker *et al.* 2006). PROPEP1 belongs to a small gene family with six other annotated genes that encode precursors that contain sequences that are homologues to *At*Pep1 at their C-termini (Huffaker and Ryan 2007). PROPEP2 and PROPEP3 paralogs were the most highly expressed in response to pathogens and PAMPs, and were the only paralogs highly expressed in response to SA (Huffaker and Ryan 2007). *At*Pep1 together with its homologues appear to regulate expression of the defence protein PDF1.2 through the JA/Et defence signalling pathway. All six *At*Pep peptides induce PDF1.2 and PR1, besides of inducing the expression of its own precursor gene as well as PROPEP2 and PROPEP 3 (paralogs of PROPEP1). *At*Pep1 and its homologues are the first endogenous peptide defence signals found in *Arabidopsis*, and the existence of orthologues in other plants suggests that this gene family may have fundamental roles in amplifying plant defences associated with innate immunity (Ryan *et al.* 2007). When PAMPs induce expression of *At*PROPEP genes, *At*Pep peptides are produced that engage in a feedback loop to amplify defence signalling through both the MeJa/Et and SA pathways. Therefore, these peptides may be considered as PAMP amplifiers (Huffaker and Ryan 2007).

The receptor of *At*Pep1, called PEPR1, was identified by chemical crosslinking of the ligand and biochemical purification of the binding site, and this receptor was found to be a LRR-RLK, with a 26 extracellular LRRs, cysteine pairs flanking the LRR region, a transmembrane domain and an intracellular protein kinase domain (Yamaguchi *et al.* 2006). This receptor may be responsible for defence signalling by all six AtPep peptides, since all peptides derived from the sequences of the six paralogs competed for binding of radiolabeled *At*Pep1 (Yamaguchi *et al.* 2006). Another LRR-RLK with 72% similarity with PEPR1 was identified, and was named PEPR2. PEPR1 and PEPR2 belong to the LRR-RLK XI subfamily (Shiu and Bleecker 2003), which among others includes HAESA, CLV1, BAM1, BAM2 and BAM3, all of them corresponding to developmentally associated receptors. The *At*Pep1/PEPR1 ligand-receptor pair resembles flg22/FLS2 and elf18/EFR (Huffaker and Ryan 2007) since the overall response pattern of the plants to AtPep(s) is reminiscent to the response to MAMPs such as flg22 or elf18, since AtPep(s) increases cytosolic calcium and activates chloride channels followed by membrane depolarization in a strictly receptor-dependent manner together with growth inhibition and enhanced production of ROS and ethylene (Krol *et al.* 2010). Recently, it has been found that PEPR2 is a second receptor for Pep1, and that both

PEPR1 and PEPR2 are transcriptionally induced by wounding, treatment with MeJa, Pep peptides and PAMPs, and together they contribute to defence responses in *Arabidopsis* (Yamaguchi *et al.* 2010).

1.4 PSK-α, PSKR1 and other pathway components

1.4.1 PSK-α and its precursors

Callus formation and proliferation boost rapidly at high cell density but less significantly at low cell density. In order to promote cellular growth at low cell populations, researchers have used specialized culture techniques such as nurse cultures (Raveh *et al.* 1973), where target cells are grown close to but physically separated from high-density nurse cells, which led to the theory that cell-to-cell communication mediated by a chemical factor is involved in cell growth. This chemical factor is known as the ‘conditioning factor’, which was found in maize cell cultures to be highly hydrophilic and a neutral molecule (Birnberg *et al.* 1988) whereas in carrot it was as well hydrophilic and relatively heat stable (Bellincampi and Morpurgo 1987). Using a bioassay system, where asparagus mesophyll cells at low density were used to detect the mitogenic factor released by plant culture cells at a high density (Matsubayashi and Sakagami 1996), the active factor was found and identified as a sulfated peptide composed of five amino acids, and due to the presence of sulfate esters, the peptide was named phytosulfokine (PSK) (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH) (Matsubayashi and Sakagami 1996) (Figure 1.4.1). PSK, naturally and chemically synthesized, induces cellular dedifferentiation and proliferation of dispersed asparagus mesophyll cells. Further studies of PSK analogs identified the *N*-terminal tripeptide fragment as the active core of PSK (Matsubayashi *et al.* 1996). PSK is widely distributed among higher plants since it is present, with an identical structure, in conditioned medium derived from cell lines of several plants, such as asparagus (Matsubayashi and Sakagami 1996), rice (Matsubayashi *et al.* 1997), maize (Matsubayashi *et al.* 1997), *Zinnia* (Matsubayashi *et al.* 1999), carrot (Hanai *et al.* 2000) and *Arabidopsis* (Yang *et al.* 2001). Other additional functions of PSK include triggering of tracheary element (TE) differentiation of dispersed *Zinnia* mesophyll cells at nanomolar concentrations (Matsubayashi *et al.* 1999), promoting of various stages of plant growth such as somatic embryogenesis in carrot (Kobayashi *et al.* 1999; Hanai *et al.* 2000) and in *Cryptomeria japonica* (Igasaki *et al.* 2003), promoting effects on the formation of adventitious roots (Yamakawa *et al.* 1998) and a regulatory effect in pollen germination (Chen *et al.* 2000). Moreover, high concentrations of PSK showed a promotive effect on the growth and chlorophyll content of *Arabidopsis* seedlings when applied under high night-time temperature conditions, suggesting PSK may aid plants in their tolerance of heat stress (Yamakawa *et al.* 1999). Recently, a new function of the peptide PSK was proposed as a

promoter of root growth in *Arabidopsis* in a dose-dependent manner (Kutschmar *et al.* 2009) and that PSK signalling through its receptor promotes cell elongation via control of mature cell size (Kutschmar *et al.* 2009). Also lately it was suggested that PSK may be involved in the attenuation of stress response and healing of wound-activated cells during the early stage of TE differentiation (Motose *et al.* 2009).

Nowadays secreted peptides, like PSK, PSY1 or the newly identified RGFs (root meristem growth factors)(Matsuzaki *et al.* 2010), are recognized as important members of intercellular signals that coordinate and specify cellular functions in plants. Some of the secreted peptide hormones undergo complex posttranslational modifications that are mediated by specific enzymes that recognize particular sequences of multiple target peptides (Matsuzaki *et al.* 2010).

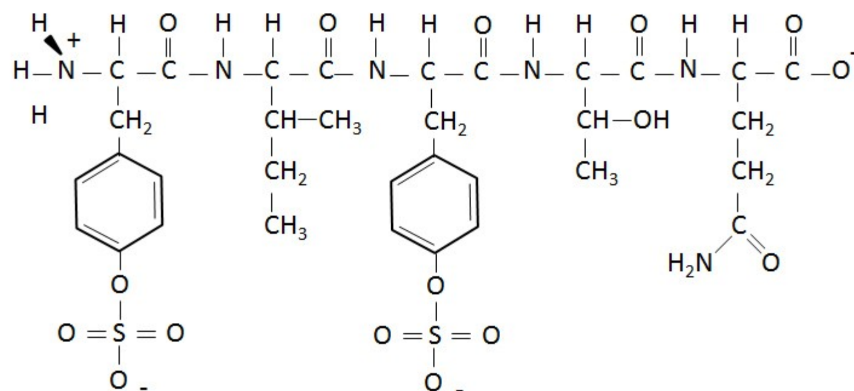

Figure 1.4.1: Chemical structure of PSK, (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH)

Linear representation of PSK-α, showing its amino acids and its hydrophobic nature with the two sulfated tyrosine residues.

The peptide PSK is produced via posttranslational sulfation of tyrosine residues and proteolytic processing of an ~80-amino acid precursor peptide that possesses a secretion signal at its N-terminus and a PSK sequence near the C-terminus flanked by dibasic amino acids residues, implying proteolytic processing similar to that of animal peptide hormones (Yang *et al.* 1999; 2001; Igasaki *et al.* 2003). Genes encoding PSK precursors are redundantly distributed throughout the genome (Yang *et al.* 2001) and are expressed in a variety of tissues in addition to calluses, including leaves, roots, stems (Matsubayashi *et al.* 2006). *Arabidopsis thaliana* possesses 5 paralogous genes which encode PSK precursors (Matsubayashi *et al.* 2006), where the only conserved amino acids within them is the five amino acid PSK domain. Certain residues between amino acids positions -25 and -1 are also highly conserved among the PSK precursors peptides, including multiple acidic residues, one cysteine pair, several hydrophobic residues, consecutive basic residues and one histidine residue.

1.4.2 PSK-α receptors

The PSK-binding protein was purified from microsomal fractions of carrot (*Daucus carota*, Dc) cells by detergent solubilization and ligand-based affinity chromatography using a PSK-Sepharose column. The identified protein was a 1021-amino acid LRR-RK, with an extracellular LRR domain, a single transmembrane domain and a cytoplasmic kinase domain. The overexpression of this receptor kinase in carrot cells caused enhanced callus growth in response to PSK and a substantial increase in the number of tritium-labeled PSK binding sites, suggesting that PSK and this receptor kinase act as a ligand-receptor pair (Matsubayashi *et al.* 2002). The PSK receptor protein was named PSKR1. The corresponding ligand-binding pocket was hereafter identified as the region of Glu^{503} –Lys^{517}, which deletion abolished completely the ligand binding activity of DcPSKR1. The binding region was found to be located at the island domain flanked by extracellular LRRs, indicating that the island domain of DcPSKR1 forms a ligand-binding pocket that directly interacts with PSK (Shinohara *et al.* 2006). The carrot PSK receptor exhibits high-percentage amino acid identity with one LRR-RLK found in the *Arabidopsis* genome. AtPSKR1 is the corresponding ortholog of DcPSKR1 and encodes a 1008-amino acid LRR-RK and shares a 60% amino acid sequence identity with its carrot counterpart. AtPSKR1 contains 21 tandem copies of LRRs, a 36-amino acid island domain between the 17th and the 18th LRR, a single transmembrane domain and a cytoplasmic kinase domain (Matsubayashi *et al.* 2006). The amino acid sequence in the island domain is highly conserved between DcPSKR1 and AtPSKR1. An island domain has also been found among the extracellular LRRs of the brassinosteroid receptor BRI1 and has been shown to be involved in ligand binding (Kinoshita *et al.* 2005). AtPSKR1 belongs to the LRR X subfamily (Shiu and Bleecker 2001), which also includes BRI1, BRL1, BRL2, BRL3 and EMS1/EXS (EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS), the latter required for specialization of tapetal cells (Canales *et al.* 2002). Northern blotting and promoter analysis (histochemical GUS staining of plants expressing *pAtPSKR1::GUS* fusions) revealed that AtPSKR1 is weakly but widely expressed in roots, leaves, stems, and flowers of 3-week old *Arabidopsis* plants as well as calluses (Matsubayashi *et al.* 2006). Loss-of-function mutants of AtPSKR1, named *pskr1-1*, germinated normally and developed normal cotyledons and hypocotyls phenotypically indistinguishable from wild-type plants. The root growth of mutant seedlings was slightly reduced compared to wild-type, whereas the so-called most impressive phenotype of the impaired *AtPSKR1* mutant plants was that individual cells gradually lost their potential to form calluses as the tissues matured. The *pskr1-1* calluses exhibited premature senescence with browning within 3 weeks of culture, resulting in formation of a smaller callus than wild type after 6 weeks; the senescence marker SEN1 (Oh *et al.* 1996) transcript was significantly increased in *pskr1-1* calluses. On the other hand,

pskr1-1 seedlings displayed a normal growth rate as wild-type seedlings, flowered normally and completed the normal life cycle, but their leaves exhibited premature senescence phenotypes 4 weeks after germination (Matsubayashi *et al.* 2006). Gain-of-function mutants, named *AtPSKR1ox*, were also examined. Their germination was also normal and indistinguishable from wild type; root growth of *AtPSKR1ox* was comparable to wild type; calluses of *AtPSKR1ox* proliferated vigorously and did not exhibit senescence even after 6 weeks of culture and displayed larger sizes; *AtPSKR1ox* seedlings also grew at almost the same rate as wild-type seedlings and completed normal life cycle, but they developed larger leaves than wild type and exhibited delayed senescence (Matsubayashi *et al.* 2006).

AtPSKR1 possesses two paralogous, PSKR2 and the PSY1 potential receptor, called here PSY1R; both paralogous genes encode LRR-RLKs that share 48.6% and 43.6% sequence identity with AtPSKR1, respectively (Amano *et al.* 2007). In the previous study, a 18-amino acid tyrosine-sulfated glycopeptides, PSY1, was identified in *Arabidopsis* cell suspension culture medium, whose perception depends on At1g72300, i.e. AtPSY1R. PSKR2 is also involved in PSK-α perception, therefore it is presented as an alternative PSK receptor, although less active than AtPSKR1. It is suggested that PSK and PSY1, two structurally distinct sulfated peptides, contribute redundantly to cellular proliferation, expansion and wound repair during plant growth and development (Amano *et al.* 2007).

1.4.3 PSK-α posttranslational modification

Little is known about the proteolytic processing of the PSK propeptide precursors, but the fact that PSK precursors have conserved di-basic residues 8-10 amino acids upstream from the mature peptide sequence (Matsubayashi and Sakagami 2006). Di-basic residues are characteristic of substrate sites for subtilases, subtilisin-like serine proteases (Barr 1991). The *Arabidopsis* genome contains 56 subtilase genes (Rautengarten *et al.* 2005) and recently it was found that PSK was proteolytically cleaved from one of its precursors, AtPSK4, by a specific plant subtilase, AtSBT1.1 (At1g01900), which is upregulated during the process of transferring root explants to tissue culture (Srivastava *et al.* 2008). Because AtSBT1.1 appears to be most specific for cleavage of PSK4, it is believed that other subtilases might be involved in the processing of other PSKs (Srivastava *et al.* 2008).

Protein tyrosine sulfation (or sulfatation) is a posttranslational modification restricted to proteins that transit the secretory pathway. The enzymes responsible for this reaction are tyrosylprotein sulfotransferases (TPSTs) and they catalyze the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl group of peptidyl tyrosine residues to form a tyrosine O^4-sulfate ester (Bettelheim 1954; Huttner 1982). The enzymes' subcellular localization in the *trans*-Golgi network and its widespread tissue and cellular

distribution have been well documented in animals, and several of them play important roles in inflammation, hemostasis, immunity and other processes (Moore 2003). In plants, this posttranslational modification is critical for the biological activities of the peptide hormones PSK and PSY1. Nevertheless, plant TPSTs have very limited sequence homology with those in the animal kingdom, suggesting that plants have evolved plant-specific TPSTs structurally different from their animal counterpart (Komori *et al.* 2009). Remarkably, an Arabidopsis TPST, At1g08030, was identified by affinity chromatography, whose recombinant version expressed in yeast catalyzed tyrosine sulfation of both PSK and PSY1 precursor polypeptides in vitro. This *At*TPST, a type I transmembrane protein localized in cis-Golgi, is expressed throughout the plant body and its highest levels of expression are in the root apical meristem (Komori *et al.* 2009). Most interestingly, this *At*TPST catalyzed tyrosine sulfation of the PSY1 precursor polypeptide more efficiently than it did the PSK precursor. Consequently, it is believed that there are yet uncharacterized tyrosine sulfated peptides or proteins involved in plant growth and development. Alternatively, *A. thaliana* contains 18 genes which conform the sulfotransferase family (SOT), that is known to catalyse the sulfate transfer from PAPS to hydroxyl groups of several classes of substrates (not exclusively to tyrosine residues) (Klein and Papenbrock 2004). They contain conserved amino acids motives which are involved in PAPS binding. Some of them have already a known function, like SOT5 with a marked preference for flavonols as substrate; or SOT10 with a strict specificity for brassinosteroids having 22R-, 23R-hydroxyl, and a 24S-methyl or ethyl group on the steroid side chain; or SOT14 which catalyses the sulphonation of 12-hydroxyjasmonate and thereby inactivate excess jasmonic acid in plants; SOT12 (*AtST1*) is inducible in response to pathogen infection and the pathogenesis-related signals, salicylic acid and methyl jasmonate and possess a stereo-specificity for 24-epibrassinosteroids.

1.5 Goal of this work

Plant receptors that reside at the cell surface or within the cytoplasm are usually in charge of surveilling pathogens that manage to overcome the natural plant cell barriers. Membrane bound plant PRRs include receptor-like kinases (RLKs) (Shiu and Bleecker 2003) that have an extracellular domain such as LRRs, lectin, lysine motif or wall associated kinases with a single transmembrane spanning region and a cytoplasmic kinase domain; receptor like proteins (RLPs) (Wang *et al.* 2008) that contain an extracellular LRR domain and a C-terminal membrane anchor but lack the cytoplasmic kinase domain; and polygalacturonase inhibiting proteins (PGIP) (Di Matteo *et al.* 2003) that have only an extracellular LRR domain. Remarkably, plants possess approximately 235 LRR-RLKs (Shiu *et al.* 2004) and a very significant number of them are assumed to act as PRRs in PAMP perception (Nürnberger and Kemmerling 2006). LRR-RLKs are known to regulate a wide variety of developmental and defence-related processes including cell proliferation, stem cell maintenance, hormone perception, host-specific as well as non-host-specific defence response, wounding response and symbiosis. Nevertheless yet, a small number of plant LRR-RLKs possess an annotated function in plant-pathogen interactions and even a smaller number are known to display double functions in plant defence and development. Within the scope of the AFGN (The Arabidopsis Functional Genomic Network) project and with the DAAD (German Academic Exchange Service) support, further functions for LRR-RLKs were investigated related to plant-pathogens interactions at the present work. Here, making use of reverse genetics, the potential involvement in plant defence of a plant hormone receptor, *At*PSKR1, previously known by regulating developmental processes, should be demonstrated.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals used in the laboratory have an analytic degree of purity, and if not mentioned, were provided by ICN (actually MP Biomedicals; Eschwege), Amersham Pharmacia Biotech (actually General Electrics, Freiburg), Biorad (München), Fluka (Buchs, CH), Merck (Darmstadt), Carl Roth (Karlsruhe), Serva (Heidelberg), Duchefa (Haarlem, NL) or Sigma (Steinheim). Organic solvents were ordered from Brenntag Chemiepartner GmbH NL (Plochingen), Fluka and Merck. The ingredients of growing medium were purchased from Difco Lab. (Detroit, USA), Invitrogen (Carlsbad, USA), Merck, Sigma and Duchefa (Haarlem, NL). Nucleic acids-modifying enzymes were purchased from Biomaster (Köln), Invitrogen, Stratagene (La Jolla, USA), New England Biolabs (Beverly, USA), Promega (Mannheim) and Amersham Pharmacia Biotech. Blotting membranes and radioactive chemicals were ordered from Amersham Pharmacia Biotech and Hartmann Analytic GmbH (Braunschweig). Oligonucleotides were delivered from MWG (Ebersberg). Phytosulfokine-α was purchased from NeoMPS (Strasbourg).

2.1.2 Growth medium

For selective media following antibiotics with the corresponding concentrations (Table 2.1.1) were added when medium reached a temperature of 55°C.

Table 2.1.1: Antibiotics and concentration for growth media

Antibiotic	Final concentration (µg/µl)
Ampicillin	50
Cycloheximide	50
Gentamicin	25
Kanamycin	50
Rifampicin	50 (from 25mg/ml in methanol)
Spectinomycin	100 or 200
Tetracycline	12,5 (from 12,5 mg/ml in ethanol)

For preparation of medium and buffers deionized and destillated water was used. Medium and buffers were sterilized for 20 min at 121°C, and solutions, whose ingredients were not heat-stable, were filter-sterilized.

An overview of often used medium is presented in Table 2.1.2. For solid medium 15 g/l of Bacto-Agar was added and for MS-Agar 8 g/l of Agar-Agar was used.

Table 2.1.2: Growth media

Medium	Ingredients
LB	10 g/l Bacto-Tryptone; 5 g/l Bacto-Yeast extract; 5 g/l NaCl
King's B	20 g/l Glycerol; 40 g/l Proteose-peptone 3; after autoclaving add 10 ml/l K_2HPO_4 and 10 ml/l $MgSO_4$ filter-sterilized.
$^1/_2$ MS	2,2 g/l MS-salt (Sigma, Duchefa); pH 5,7 adjusted with KOH
Sakai	34g/l of freshly grinded bean flour; 17 g/l oat bran with germ (Alnatura); 8,5 g/l sucrose; fill with H_2O until 1l; boil, let it soak for $^1/_2$ hour at 95°C, stir through a metal sieve, fill with H_2O until 1l and autoclave
PDB	12 g/l PD (Potato-dextrose, Duchefa)

2.2 Plant material and plant growth conditions

2.2.1 Plants

Wild type plants from the species *Arabidopsis thaliana var. Columbia 0* (Col-0) or *var. Wassilewskija 4* (Ws-4) were used during this work, together with the corresponding gene-deficient (knockout)-Lines, in which a specific gene was inactivated through a *T*-DNA insertion (Chapter 2.2.2). Seeds were sowed, like described in every particular experiment, either on previously vapour-sterilized GS90 soil (Gebr. Patzer) mixed with vermiculite or on ½ MS-Agar. Subsequently seeds were stored in the dark for 2 days at 4°C for stratification. Breeding of plants took place either in growing chambers with long-day conditions (16 h of light, 8 h of darkness) at 130 µmol/m^{2}s and 22°C or in Percival plant growth chambers (Percival, CLF) with short-day conditions (8 h of light, 16 h darkness) at 130 µmol/m^{2}s and 22°C with 40-60% humidity.

2.2.2 Gene inactivation through *T*-DNA insertion

The loss-of-function lines described in this work *pskr1-2*, *pskr1-4*, *pskr1-5*, *psy1r* and *pskr2* were acquired from the *Nottingham Arabidopsis Stock Centre* (NASC, http://nasc.nott.ac.uk). The line *pskr1-3* was acquired from the *Institut National de la Recherche Agronomique* (INRA, http://dbsgap.versailles.inra.fr). *pskr1-4* (N508585), *pskr1-5* (N506900), *psy1r* (N669833) and *pskr2* (N524464) arise from the Salk Institute (Baulcombe *et al.* 1986). *pskr1-2* (N829459) comes from the *Syngenta Arabidopsis Insertion Library* (SAIL) (McElver *et al.* 2001). *pskr1-3* comes from the *Arabidopsis thaliana* Resource Centre for Genomics (Bechtold *et al.* 1993) and Ws-4 is its corresponding background. The double mutants lines *pskr1-4*x*psy1r* (SALK_008585 x SALK_072802), *pskr2*x*pskr1-4* (SALK_024464 x SALK_008585) and *pskr2*x*psy1r* (SALK_024464 x SALK_072802) as well as the triple mutant line *3X* (SALK_008585 x SALK_072802 x SALK_024464) were created by Mike Wierzba from the Frans Tax laboratory, University of Arizona.

2.3 Phenotypic analyses

2.3.1 Root length experiments with PSK-α

In order to determine the root growth of *Arabidopsis thaliana var. Columbia 0* (Col-0) and *pskr1-4* after addition of PSK-α (NeoMPS), the respective seeds were sowed on $^1/_2$ MS-medium (Duchefa) with 0,8 % Agar-Selected (Sigma). PSK-α was added to medium after filter sterilization at a concentration of 0,1 µM and 1 µM. Seeds were surface-sterilized using a solution of 50% sodium hypochlorite and 0,1 % Triton X-100. Seeds were immersed in this solution for 10 min. Afterwards seeds were washed up to 8 times with sterilized water and first sowed in $^1/_2$ MS-medium without stressor. Seeds were stored in darkness at 4°C for 2 days (stratification). Then seedlings were allowed to grow in growing chambers under long-day conditions until root length reached approx. 1cm. These seedlings were then transferred to a $^1/_2$ MS-medium containing PSK-α. After 10 days the root length growth was assessed.

2.3.2 Phytopathogenic assays *in planta*

In order to find out whether the loss-of-function mutant lines used in this work compared to wild-type plants present either an increased resistance or increased susceptibility to various pathogens, 6-weeks old *Arabidopsis thaliana* plants, sowed on soil, were infected with bacteria and fungi (see next chapter). Bacteria used in this work are *Pseudomonas syringae*

pv. *tomato* DC3000 (*Pto* DC3000), *Pto* hrcC^{-}, *Pto* avrRpm1 and *Pseudomonas syringae* pv. *phaseolicola* (*Pph*). Additionally symptom development after application of necrotrophic fungi was investigated, using *Alternaria brassicicola* and *Botrytis cinerea*.

2.3.3 Bacterial growth analysis *in planta*

Arabidopsis plants were sowed on soil, and after a stratification period, plants were transferred to a plant growing chamber (Percival, CLF) under short-day conditions. When 6-week old, they were used for bacterial growth analysis. *Pseudomonas* strains (Chapter 2.3.2) were inoculated in a 50 ml over-night culture using King's B medium containing the corresponding antibiotics. Bacteria were incubated for 16 h at 28°C with 180 rpm. Bacterial cells were centrifuged for 10 min at 4°C with 3500 rpm and washed twice with 10mM $MgCl_2$. Concentration was adjusted to OD_{600nm} of 0,2, which corresponds approx. to 10^8 cfu/ml. For assays, where infection was performed manually, the final bacteria concentration was 10^4 cfu/ml in 10 mM $MgCl_2$. Corresponding bacterium was inoculated on the lower leaf surface, once on each side of the leaf axis using a syringe without a needle. For assays, where infection proceeded by spraying bacteria suspension on the leaf surface, the final bacteria concentration sprayed was 5 x 10^8 cfu/ml. The harvesting time points were 0, 1, 2 and 4 days post infection (dpi). Two pieces of each leaf were cut using a cork borer. Plant material was transferred to a reaction tube containing 100 µl of 10 mM $MgCl_2$ and then sheared with a grinder. From this suspension, various dilutions were made and plated on LB-Agar plates, and finally incubated for 48 hr at 28°C. LB-plates contained, besides the corresponding antibiotics, 50 µl/ml cycloheximide. Finally, the grown colonies were counted and the number of bacteria was determined. For statistical analysis, the average value was taken in account together with standard deviations. For each pathogen and harvesting time point, 2 leaves from 4 plants were inoculated (8 parallels). The number of independent repetitions is mentioned on each experiment.

2.3.4 Bacterial growth analysis in planta using PSK-α

Bacterial growth analyses were performed like described in Chapter 2.3.3 with the unique variation that plant material was homogenously pre-inoculated (24 hr before) with PSK-α or with H_2O (as control).

2.3.5 Analysis of susceptibility symptoms after infection with fungal pathogens

2.3.5.1 Infection with *Alternaria brassicicola*

Preparation of the *Alternaria brassicicola* spores was done like described by Thomma *et al*. 1999. The final concentration of spores suspension was adjusted with sterile water to $5x10^5$ spores/ml, where 5 µl of suspension were applied 6 times on the upper leaf surface (drop inoculation) and the infected plants were kept under 100% humidity conditions. The degree of infection was scored like described in Kemmerling *et al*. 2007.

2.3.5.2 Infection with *Alternaria brassicicola* using PSK-α

Assays were performed as described in Chapter 2.3.5.1 with the unique variation that the plant material was pre-inoculated (24 hr before) with the peptide PSK-α or with H_2O (as control).

2.3.5.3 Infection with *Botrytis cinerea*

The final spores concentration of the necrotroph *Botrytis cinerea* was adjusted with $^3/_4$ PDB medium till $5x10^5$ spores/ml. 2 leaves per plant were infected using 5 µl of spore suspension, which were deposited on the centre of the upper leaf surface. The infected plants were kept under 100% humidity conditions. The score of the degree infection was done like for *Alternaria brassicicola* (Chapter 2.3.5.1).

2.3.6 Staining of reactive oxygen species with 3,3'-Diaminobenzidine

Leaf material was collected and vacuum infiltrated with a solution of 1 mg/ml 3,3'-diaminobenzidine (DAB) (Sigma) for approx. 2 min. Following the staining solution was removed, and leaves were kept at 100% humidity conditions on darkness for approx. 5 hours at room temperature. The staining reaction was stopped by addition of 70% ethanol. Leaves were distain by exchanging the ethanol solution several times. A transmitted-light microscope was used for visualization of samples.

2.3.7 Staining of cell death with Trypan blue

After treating plant material with correspondent pathogens, leaves were dissected and submerged in a solution of Trypan blue (10 ml Lactic acid, 10 ml glycerol, 10 ml Aqua-phenol, 10 ml water, 300 mg Trypan blue stain, 80 ml ethanol). The staining proceeded on a boiling water bath for 30 s. Afterwards the staining solution was removed and a solution of 1

g/l chloralhydrate was added for distaining and plant material was incubated for several days with continuous exchange of distaining solution. A transmitted-light microscope was used for visualization of samples.

2.3.8 PCR determination of fungal biomass

DNA levels of the *A. brassicicola*-specific gene *cutinase* (*Abr*CUT) were determined relative to the DNA levels of the *Arabidopsis*-specific gene PR1. The amount of fungal biomass is measured as the qPCR signal from amplification of a fragment of fungal genomic DNA (AbrCUT) relative to the signal from amplification of a fragment of plant genomic DNA (PR1). Wild-type and *pskr1-4* mutant leaves were drop-inoculated with *A. brassicicola* suspension of $5x10^5$ spores/mL (Chapter 3.2.5.1), 2 x per leaf, on each side of the leaf. At indicated timepoints, leaf discs were punched out with a korkbohrer N. 2 around the infection site. Genomic DNA was extracted from samples (Chapter 2.5.1), and DNA amount was adjusted according to PR1 gene product.

2.3.9 Phytohormones content determination

Arabidopsis wild-type and *pskr1-4* mutant plants were infected with *Pto* DC3000 (Chapter 2.3.3) and *A. brassicicola* (Chapter 2.3.5.1.). At indicated timepoints, 400 mg of treated leaves were harvested and grinded using liquid nitrogen and transferred to 2 ml micro test tubes with screw-caps. The following procedures were performed in the ZMBP Analytics Department, Tübingen, by Karl Wurster.

Free analytes determination: Add 1,5 ml ethyl acetate with 0,530 mg 4-hydroxybenzoic acid/100ml as standard. Agitate samples for 2 hr at 25°C. Centrifuge 10 min at 14 000 U/min. Extract 1,2 ml of upper phase (organic phase) into a 1,5 ml micro test tube. Discard carefully remaining of organic phase. Evaporate ethyl acetate from samples in SpeedVac concentrator and dry further overnight with diminished vacuum together with phosphorus pentoxide (P_4O_{10}) as dehydrating agent. In the following day, dry further for some hours with full vacuum. Add to samples 70 µl MSTFA, agitate for 60 min at 40°C and then centrifuge. Transfer samples to GC-MS tubes for injection.

Conjugated analytes determination: Remove rest of ethyl acetate to remainings of previous extraction. Add 200 µl 3M HCl, vortex and agitate for 1 hr at RT. Neutralize with 3M ammonia solution. Add ethyl acetate and agitate samples 2 hr at 25°C and then centrifuge. Extract 700 µl from the supernatant and transfer to 1,5 ml micro test tube. Discard the rest. Evaporate ethyl acetate in SpeedVac concentrator and dry samples further overnight with phosphorus

pentoxide. Add to samples 70 µl MSTFA, agitate for 60 min at 40°C and then centrifuge. Transfer samples to GC-MS tubes for injection (volume injected = 1 µl).

2.3.10 Statistics

The data analysis was performed with the Software Microsoft Office Excel (© Microsoft Corporation).

2.3.10.1 Average and Standard deviations

For the analysis of gene expression, pathogenic growth, disease index or root length growth the arithmetic average values were determined together with the corresponding standard deviation.

2.3.10.2 Analysis of variance (ANOVA)

In order to determine a significant difference between values a statistical tool was employed: one-way Analysis of variance (ANOVA). p-Values < 0,05 were considered as significant.

2.4 General molecular-biological methods

2.4.1 Employed bacterial and fungal strains

On Table 2.4.1 the employed bacterial and fungal strains are listed together with their genotypes.

Table 2.4.1: Bacterial strains of the species *Pseudomonas syringae*

Strain	Genotype
Pseudomonas syringae pv. phaseolicola *(Pph)*	Rif^r
Pseudomonas syringae pv. tomato DC3000 (*Pto* DC3000)	Rif^r COR^+
Pseudomonas syringae pv. tomato DC3000 with a mutated type III secretion system hrcC (*Pto* hrcC-)	Rif^r Kan^r (nptII)
Pseudomonas syringae pv. tomato DC3000 carrying an effector avrRpm1 (*Pto* avrRpm1)	pVSP61-avrRpm1, Rif^r Tet^r

Further on, *Alternaria brassicicola* (MUCL 20297) and *Botrytis cinerea* (B05-10) were employed.

2.4.2 Cultivation of microorganisms

2.4.2.1 Cultivation of *Pseudomonas syringae* pv. *tomato*

Pseudomonas strains were incubated whether in King's B-Agar (for a longer storage time) or in LB-Agar (for bacterial growth assays) for approx. 48 hr at 28°C and with 180 rpm. Cultivation of bacteria in liquid medium was done in King's B-medium at 28°C with 180 rpm. Corresponding antibiotics were added according to the specific bacteria strains.

2.4.2.2 Cultivation of fungi

For A. *brassicicola and B. cinerea* spores, fungi were cultivated in Sakai Agar and 1x PD-Agar at RT alternating from white fluorescent light to long-day conditions for approx. 14 days. Spores could be harvested after 14 days, by covering the plates with 0,1% Tween 20 and by scrubbing the surface, with the use of a spatula Drigalski. The obtained material was filtered through Miracloth (Cal Biochem, LaJolla, USA) and was washed with sterile water twice. The number of spores was determined with a Fuchs-Rosenthal counter, then they were adjusted to a concentration of $4x10^7$ spores/ml. 1 vol. 50% glycerol was added and stored at -80°C until usage.

2.5 DNA analysis

2.5.1 Genomic plant DNA extraction

Two to three fresh plant leaves were transferred to a 1,5 ml micro test tube and were immediately frozen with liquid nitrogen. The frozen plant material was grinded using a cold plastic micropestle and 1 ml extraction buffer (100mM Tris/HCl pH 8,0; 50 mM EDTA pH 8,0; 500 mM NaCl; 1,5% SDS). After an incubation time of 10 min at 65°C, 300 µl of 5M potassium acetate buffer (60% 5M potassium acetate; 11,5 % acetic acid) were added and samples were incubated on ice for further 30 min. Hereafter, centrifuge samples for 10min at 20 800 x g at 4°C, and transfer the supernatant to a new 2 ml micro test tube. After adding 800 µl PCI (Phenol-Chloroform-Isoamyl alcohol, 25:24:1) mix carefully and centrifuge for 5min, 20 800x g, at 4°C, and again transfer supernatant to a 1,5 ml micro test tube. Add 500 µl of cold isopropanol for DNA precipitation and centrifuge for 10 min, 20 800 x g, at 4°C. Discard the supernatant and wash pellet once with 500 µl 70% ethanol (stored at -20°C),

centrifuge again and let the pellet air-dry. Resuspend pellet in 50 µl 10 mM Tris/HCl pH 8 and store at 4°C.

2.5.2 Nucleic acids quantification

Concentration of nucleic acids in solution was determined using a spectrophotometer (Ultrospec 2100 pro, Amersham) with a quartz cuvette exposed to ultraviolet light at 260nm wavelength. The following formulas allow the concentration calculation:

dsDNA: E_{260}= 1 ≡ 50 µl/ml

RNA: E_{260}= 1 ≡ 40 µg/ml

2.5.3 DNA restriction analysis

Incubation of a mix containing 1 to 5 µl of plasmid DNA, 2 µl 10x reaction buffer, 0,25 µl restriction enzyme and water (to have a final volume of 20 µl) was performed at 37°C for at least 4 hr or overnight .

2.5.4 DNA precipitation with ethanol

Before the actual DNA precipitation, an optional purification step could be done using the PCI method (for southern-hybridizations). Therefore, the volume was taken up to 150 µl, adding 150 µl PCI (phenol: chloroform: isoamyl alcohol, 25:24:1) and mixing. Centrifuge (5 min, 12 000 rpm, RT) then transfer supernatant to a new micro test tube. Add 0,1 vol 3 M sodium acetate and 2 vol cold 100% ethanol to the DNA solution. Incubate sample up to 12 hr at -20°C. Centrifuge sample (15 min, 20 000 x g, 4°C) and wash pellet with 70% ethanol. Air-dry pellet. Resuspend pellet in 15 µl H_2O.

2.5.5 Polymerase chain reaction – PCR

A list of the used primers together with its corresponding annealing temperature (T_{anneal}) is shown in the Appendix (see 7.1). For genotyping PCRs, a mix of <0,3 µmol genomic DNA, 0,5 µM 5'- and 3'-primers, 0,5 mM dNTP-mix, 1x *Taq* buffer and 0,05 U/µl *Taq* polymerase (Biomaster) was used, with the following PCR program: 94°C 2:00 min, 20-40x: 94°C 0:15 min, T_{anneal} 0:30 min, 72°C 1:00 min per 1 kbp; 72°C for 10 min, 10°C for unlimited time. The thermal cycler employed is from MJ Research (Waltham, USA).

Genotyping PCR for T-DNA insertion lines

For verifying the homozygocity of the inserted *T*-DNAs in the mutant plants used in this work, genomic DNA was isolated (Chapter 2.5.1) and PCRs were performed with the proper primer pairs combination. Primers for genotyping were at the 5' and 3' borders of the analysed insertion. In parallel, the *T*-DNA specific primer Lba1 in combination with the proper gene specific primer was used (Figure 2.5.1). The primers used for each experiment are mentioned further, respectively.

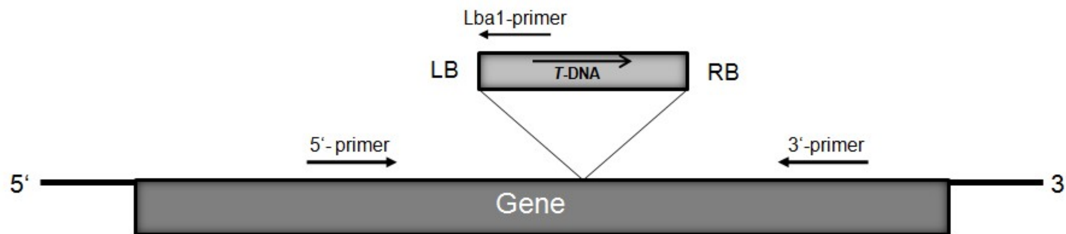

Figure 2.5.1: Positioning of *T*-DNA insertion and primers for genotyping

2.5.6 Agarose gel electrophoresis for DNA separation

The agarose gel electrophoresis allows the separation of DNA using an electric field applied to a gel matrix, where the DNA molecules negatively charged will move at different rates, determined by their mass when the charge to mass ratio (Z) of all species is uniform, toward the anode. Agarose is composed of long unbranched chains of uncharged carbohydrate without cross links resulting in a gel with large pores suitable for separation of macromolecules and macromolecular complexes. For DNA separation, agarose gels of 0,8 – 1,5 % were used in 1x TAE buffer (from 50x TAE buffer: 2M Tris-Acetate pH 8,0; 100 mM EDTA pH 8,0) with a voltage of 60 – 100 V. Fermentas 1 kb DNA ladder was used as a size marker. Visualization of DNA bands is achieved by addition of an ethidium bromide solution (10 µg/ml) which fluoresces under UV light (Infinity-3026 WL/26 MX, Peqlab) when intercalated into DNA, and bands are documented with help of a video system.

2.5.7 Purification of DNA fragments from agarose gels

DNA fragments, which were dyed with ethidium bromide, were extracted from the agarose gels aided by UV-light visualization. DNA-fragments purification is based on solubilization of agarose and selective absorption of DNA onto the silica-gels particles using QIAEX II *gel extraction kit* (Qiagen).

2.5.8 Radioactive labelling of nucleic acid fragments

The DNA probe for DNA hybridization was amplified from genomic DNA with specific primers (Appendix 7.2 and Table 7.1.1), then it was separated by agarose gel electrophoresis (Chapter 2.5.7) and then extracted from the corresponding gel (Chapter 2.5.8). By means of the *Megaprime DNA-labeling kits* (Amersham) the DNA fragment was labeled with radioactive 50 µCi [α-^{32}P]-dATP (Amersham) using the method of Feinberg and Vogelstein 1984. For removing unincorporated nucleotides, a gel filtration chromatography was performed with *Sephadex G-50 spin columns* (Amersham).

2.5.9 DNA hybridization (Southern hybridization)

Plant genomic DNA (Chapter 2.5.1) from wild-type plants and *pskr1-4* mutants was digested (Chapter 2.5.3) with 4 µl of the corresponding restriction enzyme, making a total reaction volume of 60 µl, for approx. 12 hr. A DNA purification followed by using the PCI method (Chapter 2.5.4). Samples were digested once again with the same restriction enzyme. DNA fragments were electrophoresed on an 1% agarose gel at 100 V for approx. 2,5 hr (Chapter 2.5.7). Using an alkaline transfer buffer (1M NaCl; 0,4 M NaOH), for denaturing double-stranded DNA, the DNA fragments were transfer to a nylon membrane by capillary action. The membrane was washed 3 times with 1M NaCl in 0,5 M Tris/HCl pH 7,2, and subsequently incubated at 80°C to attach permanently the transferred DNA to it. The membrane was blocked for at least 1hr at 42°C with Express hybridization buffer (ClonTech), then incubated overnight at 42°C in a rotation incubator with the pre-hybridization solution, which contained the radioactive labeled probe (Chapter 2.5.9). After hybridization, excess probe was washed 2 x 30 min from the membrane with washing solution I (2 x SSC; 0,1 % SDS) at RT, and then washed again 2 x 30 min between 42 till 65°C, depending on the desired stringency, with washing solution II (0,2 x SSC; 0,1 % SDS). The membrane was surrounded in Saran^{TM}-wrap and the pattern of hybridization was visualized on X-ray film by autoradiography, using a phosphoimager screen and reading software from FMBIO III Multi View (Hitachi).

2.5.10 DNA sequencing

A purified PCR-product (c = 20 ng/100bp, as air-dried pellet) was sent for sequencing to the MWG company.

2.5.11 DNA sequence analysis software

Sequencing data was analysed using the DNASTAR's Lasergene software as well as ClustalW2 (www.ebi.ac.uk/clustalw).

2.6 RNA analysis

2.6.1 Plant RNA extraction

In order to analyse specific gene expression based on certain stimulus, *Arabidopsis* plants (wild-type or mutants) were infected with the corresponding pathogens (Chapters 2.3.3 and 2.3.5.1) and samples (2 leaves per plant) were harvested at indicated timepoints and immediately pestled with liquid nitrogen. 1 ml of Trizol (Invitrogen) was added; samples were vigorously mix until thawn. Add 0,2 µl chloroform in order to extract the aqueous phase. Precipitate the nucleic acids (RNA) with 50 % isopropanol, then wash once pellet with 70 % ethanol and then air-dry pellet. Resuspend pellet in 35 µl DNAse- and RNAse-free water (Promega).

2.6.2 DNAse digestion of RNA

10 µg of RNA, extracted with Trizol (Chapter 2.6.1), were incubated at RT for 15 min with 1 x DNAse buffer and 1 U RNAse-free DNAse (Invitrogen). RNA was precipitated and extracted as described previously (Chapter 2.5.4).

2.6.3 Detection of transcript accumulation by semi-quantitative PCR

A RNA extraction (Chapter 2.6.1) precedes a DNAse digestion (Chapter 2.6.2) of the samples. By using M-MuLV reverse transcriptase enzyme (Fermentas) together with oligo dT-primers, synthesis of first strand cDNA arises from 5 µg RNA template. The following PCR product was performed with Taq-polymerase (Biomaster) as recommended by the manufacturer. Final concentration of used primers was 0,5 µM and the corresponding PCR conditions for them were adjusted according to the individual primers and the resulting fragment length (Chapter 2.5.5). For semi-quantitative assays, a fragment of the constitutively expressed elongation factor EF1-α was also amplified (as internal standard for templates).

3 Results

3.1 Selection of LRR-RLK candidates

The aim of the present work was to identify LRR-RLKs with an implication in pathogen perception and/or in pathogen defence in the model plant *Arabidopsis thaliana*, since Arabidopsis possesses many advantages for genome analysis, including a short generation time, small size, larger number of offspring and a relatively small nuclear genome. After the publication of the genomic sequence of Arabidopsis (2000 by the Arabidopsis Genome Initiative), further work has been done in the Receptor-like kinase proteins. The leucine-rich repeat (LRR) containing RLKs resulted to be the largest group of RLK in Arabidopsis with 216 genes (Shiu and Bleecker 2001), whereas a significant number of these kinases is predicted to serve as pattern recognition receptors (PRR) in PAMP perception (Nürnberger and Kemmerling 2006). The latter affirmation is based on the fact that transcript levels of multiple LRR-RK-encoding genes increased upon pathogen infection or PAMP treatment. In order to identify plant immunity-associated LRR-RLKs experiments of gene expression profiling with *Arabidopsis* Col-0 plants infected with various *Pseudomonas syringae* strains were conducted (Kemmerling et al. 2007). This analysis revealed that 32 genes manifested increased transcript accumulation; some of these genes were: BRL3, SERK4/SERK5, BAK1/SERK3 and *At*PSKR1, the phytosulfokine receptor 1(Kemmerling *et al.* 2007; Postel *et al.* 2009).

PSK, an endogenous sulfated pentapeptide secreted in plants, affects cellular potential for growth via specific binding to the LRR PSKR1 (At2g02220, in *Arabidopsis*). When the PSK receptor is not functional mutant plants present early senescence and formation of smaller calluses than wild-type plants (Matsubayashi *et al.* 2006). Now our aim is to find out if this LRR-RLK plays a role in pathogen defence in *Arabidopsis* or in regulation of plant defence mechanisms. Hereinafter it is described in detail what is the implication in pathogen defence for the *AtPSKR1* gene.

3.2 AtPSKR1

3.2.1 Expression of *AtPSKR1* after bacterial infection and elicitor treatment

As mentioned previously, *AtPSKR1* presents an interesting induction pattern after infection with various *Pseudomonas* strains (Figure 3.2.1, A). Here, it is observed how the endogenous transcript of *AtPSKR1* accumulates initially after initiated infection with different *Pseudomonas* strains, especially with the one strain defective in the TTSS, *Pto* hrcC$^-$, and the non-host strain, *Pph*. For verification of the Microarray data a semi-quantitative RT-PCR was performed (Figure 3.2.1, B).

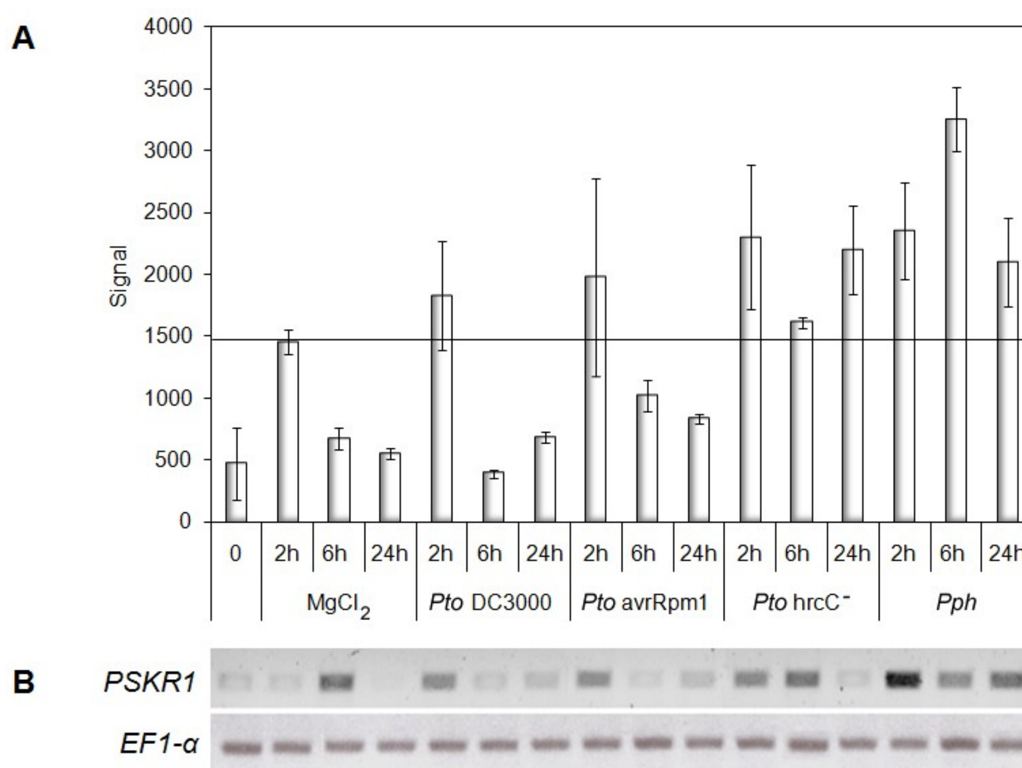

Figure 3.2.1: Microarray data and RT-PCR of WT plants after infiltration with various *Pseudomonas* strains

Plant material of Col-0 plants was harvested at 2, 6 and 24 h after inoculation with 10^8 cfu/ml *Pto* DC3000, *Pto* avrRpm1, *Pto* hrcC- and *Pph*, respectively, together with $MgCl_2$ (control) and RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Timepoint 0 represents untreated plants. (A) Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00106, AtGenExpress. Error bars represent the standard deviation of three replicates. The data were normalized by GCOS normalization. (B) For verification of the microarray data a semi-quantitative RT-PCR was performed using specific primers for *At*PSKR1 (Fragment size 549 bp; see Table 7.1.1 in Appendix for primer sequences). The total amount of cDNA was standardized with the elongation factor EF1α. (Fragment size 600 bp; see Table 7.1.1 in Appendix for primer sequences). The horizontal line marks the highest control value.

It is worth highlighting the characteristic induction pattern of *At*PSKR1 after infection with *Pto* DC3000. There is an initial accumulation of *At*PSKR1 transcript after infection with *Pto* DC3000, which decreases subsequently at 6h below the corresponding $MgCl_2$ control level and also slightly below the level of untreated plants. As the infection continues the transcript accumulation of *At*PSKR1 increases once more, but does not equal the level reached at 2h. The pattern observed with the avirulent strain *Pto* avrRpm1 resembles the control. On the contrary, with the non-pathogenic bacteria *Pto* hrcC$^-$ and Pph the *At*PSKR1 transcripts reach higher levels than the controls, reaching a maximum at 6h after infection with Pph. The RT-PCR (Figure 3.2.1, B) shows the same tendency as the Microarray data, i.e. initial endogenous transcript accumulation at 2 h after infection with the various used *Pseudomonas* strains, although with slight shifts in timepoints especially for the non-pathogenic bacteria. Here, it is important to mention the observed induction of *At*PSKR1 at 6 h after treatment with $MgCl_2$ presented in the RT-PCR: this transcript accumulation might be due to wounding, which might be as well the reason of the initial increased transcript accumulation of *At*PSKR1 at 2 h after treatment with $MgCl_2$ in the Microarray data.

PAMP or general elicitors perception by the host leads to rapid activation of defence mechanisms. Commonly, PAMP-induced early genes (within 1h) are functionally enriched for ones encoding enzymes for the synthesis of antimicrobial compounds and for proteins involved in signal perception and transduction, including receptor-like kinases, transcription regulatory factors, kinases and phosphatases (Navarro *et al.* 2004; Zipfel *et al.* 2004). Therefore, *At*PSKR1 induction after certain bacterial and oomycete elicitors was analysed (Figure 3.2.2). *At*PSKR1 transcript displays a clear accumulation of approx. 5-fold at early timepoints (1h and 4h) after treatment with bacterial HrpZ and Flg22 when compared to its corresponding control (H_2O). Conversely, when treated with bacterial LPS, *At*PSKR1 transcript is not significantly altered. Also noteworthy is the accumulation of AtPSKR1 transcript after treatment with oomycete elicitor NPP1 at early timepoints when compared to its corresponding control.

Thus *AtPSKR1* is initially downregulated after infection with the virulent bacterium *Pto* DC3000, whereas is visibly upregulated after infection with the non-pathogenic bacteria *Pto* hrcC$^-$ and Pph as well as after treatment with the bacterial elicitors HrpZ and flg22 and with the oomycete elicitor NPP1.

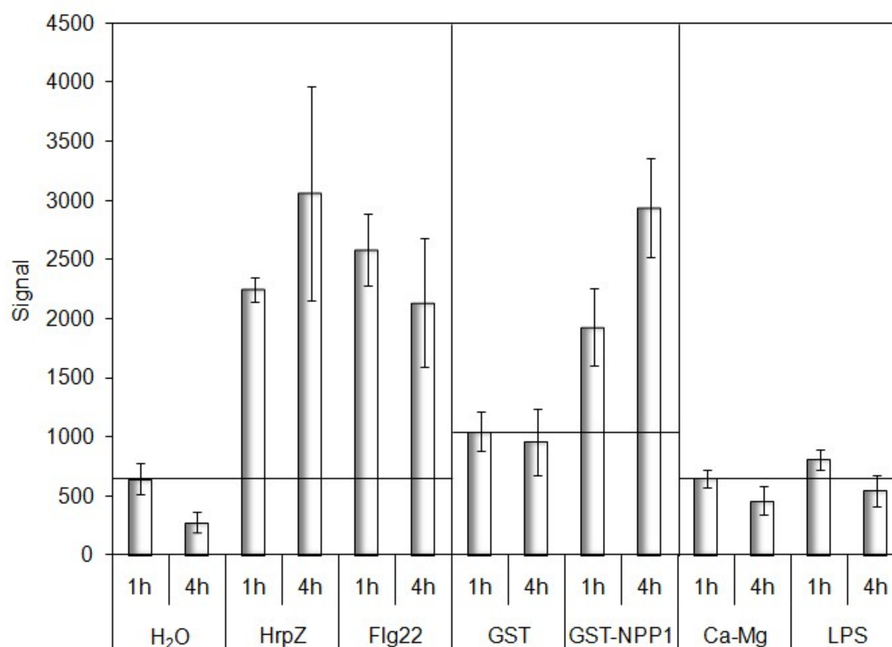

Figure 3.2.2: Microarray data of WT plants after treatment with bacterial and oomycete elicitors

Plant material of Col-0 plants was harvested at 1 and 4 h after infiltration with water (control for HrpZ and Flg22), 10 µM HrpZ, 1 µM Flg22, 1 µM GST (control for GST-NPP1), 1 µM GST-NPP1, 1mM $CaCl_2$ + 2.5 mM $MgCl_2$ (control for LPS), 100 µg/mL LPS. RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00107, AtGenExpress. Error bars represent the standard deviation of three replicates. The data were normalized by GCOS normalization. Horizontal lines mark the highest control values.

3.2.2 Expression of *AtPSKR1* after hormone and abiotic stress treatment

It is known that salicylic acid (SA)-mediated resistance is effective against biotrophs, whereas jasmonic acid (JA)-or ethylene-mediated responses are predominantly effective against necrotrophs and herbivorous insects (Glazebrook 2005). It has also been shown that auxin, a plant hormone that affects almost all aspects of plant growth and development, is involved in promoting pathogenesis (Chen *et al.* 2007) and auxin accumulation increases after *Pseudomonas* infection (Schmelz *et al.* 2003). Moreover it was shown recently that *P. syringae* infection dramatically induced the biosynthesis of ABA, and additionally, ABA inhibits the accumulation of SA and the expression of genes involved in basal resistance (de Torres-Zabala *et al.* 2007). Therefore, the role of phytohormones in the regulation of induced defences is crucial. Other plant hormones, like brassinosteroids, have also been implicated in plant defences (Nakashita *et al.* 2003). Based on this it is worth to investigate the effect of various hormones on the expression of *AtPSKR1*. Microarray data shows that addition of the auxin indole-3-acetic acid (IAA) and of 1-aminocyclopropan-1-carboxylic acid (ACC), precursor of ethylene biosynthesis, causes a slight increase of transcript accumulation of *AtPSKR1* (Figure 3.2.3); contrastingly, addition of ABA and methyl jasmonate (MJ), a

physiological active derivative of jasmonic acid (JA), represses the expression of *AtPSKR1* compared to the corresponding controls. Other hormones like Zeatin (a cytokinin), GA3 (gibelleric acid) and brassinolide (BL) do not affect significantly the transcript levels of *AtPSKR1* based on this data analysis (Appendix, Figure 7.4.1).

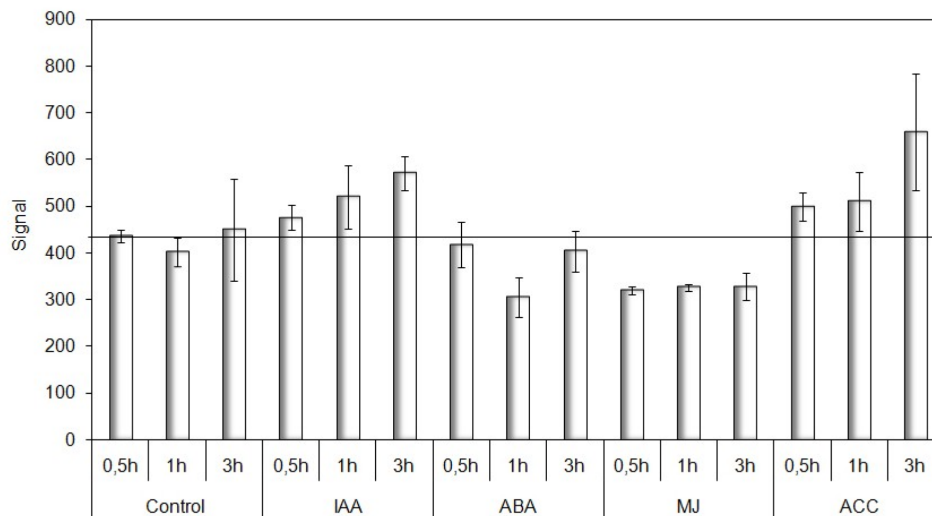

Figure 3.2.3: Microarray data of WT plants after treatment with various hormones

Plant material from 7 day old wild-type *Arabidopsis thaliana* seedlings of Col-0 was analysed. Plants were grown in liquid MS media under continuous light conditions at 23°C. After infiltration of 1µM IAA, 10 µM ABA, 10 µM MJ and 10 µM ACC plant material was collected and RNA was isolated and hybridized to the ATH1 GeneChip. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00110, AtGenExpress. Error bars represent the standard deviation of two replicates. The data were normalized by GCOS normalization. Horizontal line marks the initial control value.

Adaptation to abiotic stresses, such drought, salinity and low temperature, is one of the important roles of ABA among others in plant development (Xiong *et al.* 2002). But recently it has been shown that ABA also plays important roles in disease susceptibility, resistance to pathogen infection and interaction with other hormone-mediated biotic stress responses (Mauch-Mani and Mauch 2005; Melotto *et al.* 2006; de Torres-Zabala *et al.* 2007). On the other hand wounding stress on *Arabidopsis* leaves causes a powerful but transient protection against *Botrytis* infection (Lamotte *et al.* 2008). Therefore a revision of the effect of this abiotic stressors in *PSKR1* was performed (Figure 3.2.4). The expression levels of *PSKR1* on shoots were relatively low compared to the levels of *PSKR1* found on roots (approx. 0,5-fold less), hence no significant difference was observed. On the contrary, in roots it was noticeable that cold treatment led to a significant accumulation of *AtPSKR1* transcript levels after 12 hr, reaching a maximum level at 24h with a 3-fold increase compared to the control (Figure 3.2.4). A significant transcript accumulation after salt treatment starts even at earlier timepoints (at 1h), reaching an increase of almost 3-fold compared to the control at 3h (Figure 3.2.4). Drought and heat stress induce significant transcript accumulation solely at 1h and 3h after treatment, respectively, compared to the control on roots (Figure 3.2.4). Neither

osmotic, oxidative nor wound stress did cause a noticeable effect on *AtPSKR1* induction, either on shoots or on roots (Figure 7.4.2 in Appendix).

Thus *AtPSKR1* appears to be significantly upregulated especially upon stress with cold and salt, whereas drought and heat also induce its transcription but at rather earlier timepoints. Furthermore, *AtPSKR1* transcription does not appear to be significantly affected by phytohormones treatments.

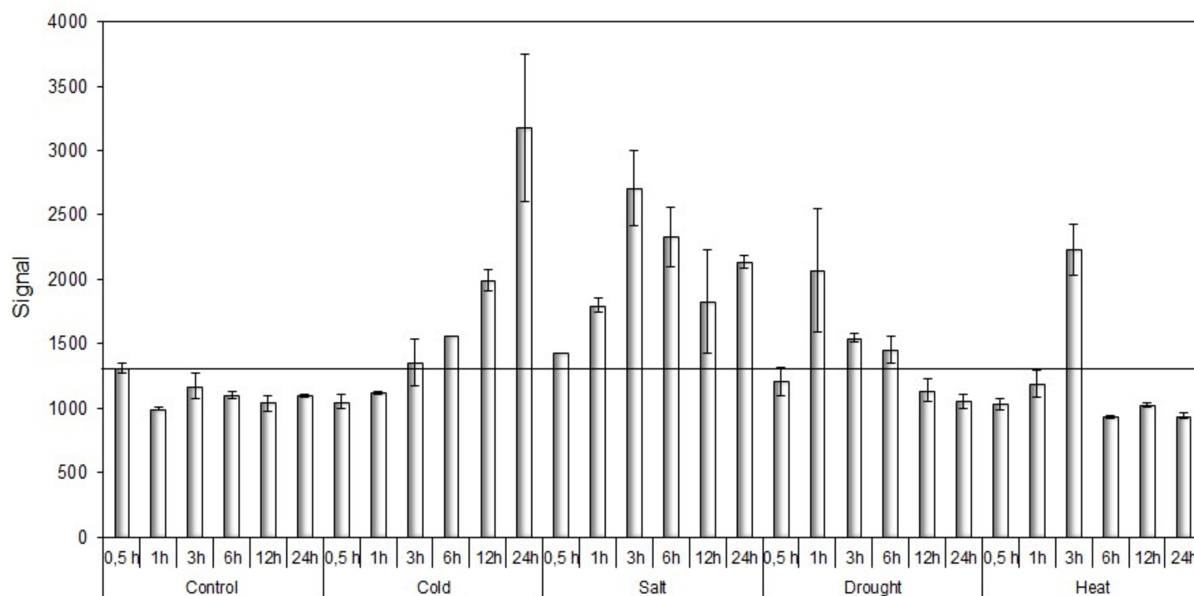

Figure 3.2.4: Microarray data of WT plant roots after treatment with abiotic stress

Plant material from 18 day old wild-type *Arabidopsis thaliana* roots of Col-0 was analysed. Seeds were sowed on rafts in Magenta boxes containing MS-Agar-media. After 2 days in the cold room (4°C, dark) the boxes were transferred to a long day chamber. Long day conditions were 16/8 hrs light/dark, 24°C, 50% humidity and 150 µEinstein/cm^2 sec light intensity. At day 11, the rafts were transferred in Magenta boxes containing MS-liquid-media. At day 16, stress treatment started at 3hr of light period: continous 4°C on crushed ice in cold chamber for cold stress; addition of 150 mM NaCl for salt stress; exposition to air stream for 15 min with loss of app. 10% fresh weight for drought stress; and 3 hours at 38°C followed by recovery at 25°C for heat stress. Samples were taken at 0.5, 1, 3, 6, 12 and 24 hr after treatment. RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00120, AtGenExpress. Error bars represent the standard deviation of two replicates. The data were normalized by GCOS normalization. Horizontal line marks the initial control value.

3.2.3 Analysis of T-DNA insertion lines

In order to analyse the potential role of *At*PSKR1 in pathogen defence *in vivo* we obtained various T-DNA insertion lines from the European *Arabidopsis* Stock Center (NASC), such as SALK lines (Alonso *et al.* 2003) and SAIL lines (Sessions *et al.* 2002), and from INRA

Versailles the FLAG line, which consist of loss-of-function mutants created by insertion of a *Agrobacterium T*-DNA so that the sequence of PSKR1 is disrupted (Figure 3.2.5)

Figure 3.2.5: Gene model and localization of T-DNA insertion lines of PSKR1

Each black triangle represents a T-DNA insertion, whereas the arrow represents the corresponding direction of the insertions. The white region corresponds to the promoter region of PSKR1. The exact positions of each T-DNA insertion are annotated with numbers, where 0 corresponds to the start codon.

Table 3.2.1: Description of *T*-DNA insertions used for in vivo analyses of *PSKR1*

Name	Stock name	NASC number	Background ecotype	Polymorphism site
pskr1-2	SAIL_673_H07	N829459	Col-0	Exon
pskr1-3	SALK_008585	N508585	Col-0	Exon
pskr1-4	FLAG_407_D02		Ws-4	Exon
pskr1-5	SALK_006900	N506900	Col-0	Promoter

Mutant lines impaired in *At*PSKR1 were named here: *pskr1-2* – *pskr1-5*, as shown above in Table 3.2.1. *pskr1-1*, not used here, corresponds to a *Ds* (Dissociation transposable element) insertion mutant used and cited by Matsubayashi *et al.* 2006. Here we worked predominantly with *pskr1-2* (cited and used by Amano *et al.* 2007) and *pskr1-3*, because of its parent ecotype, Col-0. In less extent, *pskr1-4* mutants were used, due to its less common parent ecotype, Ws-4 (Wassilewskija). *pskr1-5* mutants were not further used in this work due to the location of its *T*-DNA insertion in the promoter region. With aid of the previously mentioned mutants the effect of a non-functional PSK receptor protein in *Arabidopsis* should be investigated when confronted with diverse pathogens and stress circumstances. Therefore the proper genotyping of each mutant line was performed, followed by the control of remaining endogenous transcript and eventually with the checking of secondary T-DNA insertions present in the mutants.

3.2.3.1 Genotyping

In order to guarantee the homozygocity of the mutant lines, the corresponding genotyping via PCR was performed, taking 8 plants of each mutant line together with gene specific primer

pairs (Table 7.1.1). Corresponding *T*-DNA specific fragments were amplified only from the *pskr1-2*, *pskr1-3* and *pskr1-4* mutants, indicating that they all carry homozygous gene defects (Figure 3.2.6).

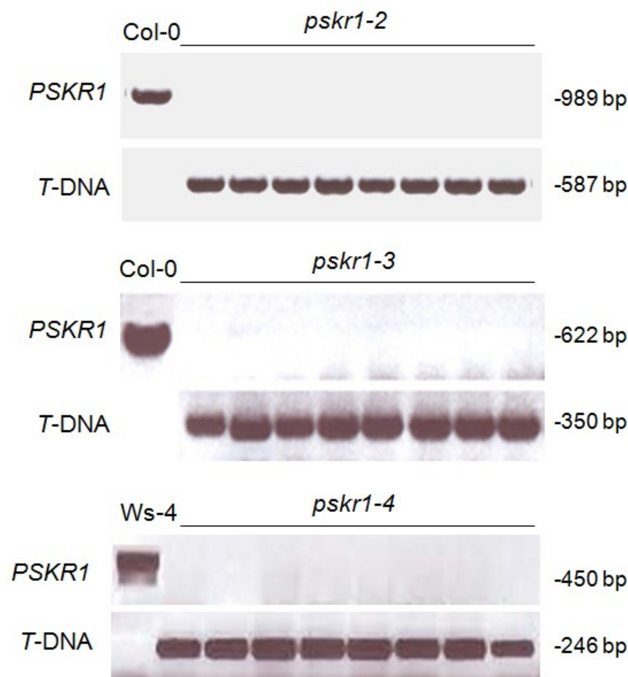

Figure 3.2.6: Genotyping PCRs of the various PSKR1 mutant plants

Plant material of 6-week old *Arabidopsis* plants was collected and genomic DNA was extracted, using the PCI method. The *PSKR1* specific fragments were amplified using the following primer pairs: for *pskr1-2* N506900/N533210 and 407D02-RP; for *pskr1-3* b-N508584/N50858 and N508584/; for *pskr1-4* 85407D02-LP and 407D02-RP. For *T*-DNA insertion specific fragments the following primers were used: for: *pskr1-2* Sail_LB and 407D02-RP; for *pskr1-3* b-Lba1 and N508584/85; for *pskr1-4* 407D02-LP and flag_LB4 (see Table 7.1.1 in Appendix for primer sequences).

3.2.3.2 Control of remaining endogenous transcript levels

Based on Microarray Data and on the verifying semi-quantitative RT-PCR it could be observed that the *PSKR1* transcript accumulated clearly at 2h after infection with *Pto* DC3000. Therefore, Col-0 and Ws-4 (controls) plants together with the corresponding mutant plants were infected via inoculation with *Pto* DC3000 and plant material was harvested 2h after infection. For *pskr1-2*, the absence of mRNA was previously verified by RT-PCR using gene-specific primers by Amano *et al.* 2007. Therefore, here we verified remaining transcripts for *pskr1-3* and *pskr1-4* mutant plants. For *pskr1-3*, the gene specific primer pairs were chosen in a way that the amplified fragment is localized 3’ from the insertion site. For *pskr1-4,* the gene specific primer pairs were localized 5’ and 3’ from the insertion site, due to the insertion direction. For ruling out variations in the amount of cDNA of each probe, a internal standard was used: the constitutively expressed elongation factor *EF1α.* Since *PSKR1* does not contain introns, the right positioning of the PRC products in the exon was guaranteed.

Transcript accumulation of *PSKR1* can be clearly observed in wild-type plants 2h after infection but not in *pskr1-3* and *pskr1-4* mutant plants, indicating a complete loss of PSKR1 transcript (Figure 3.2.7).

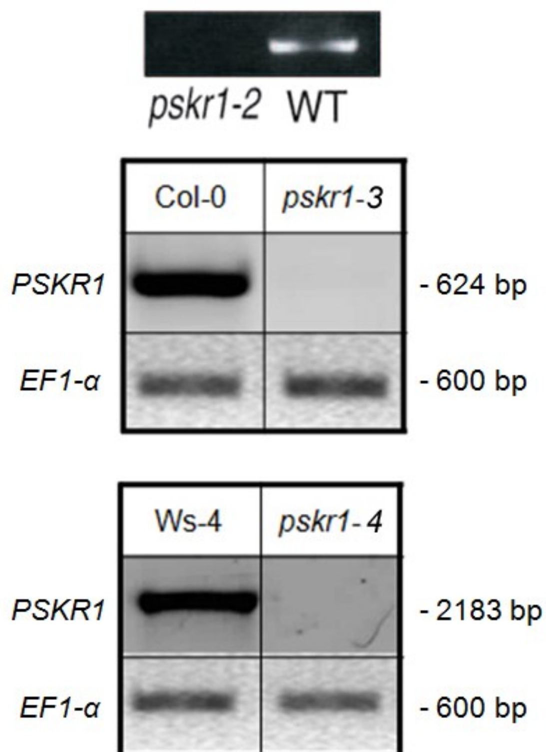

Figure 3.2.7: RT-PCR for controlling the PSKR1 endogenous levels

Upper picture was taken from Amano *et al.* 2007 and corresponds to remaining transcript verification in *pskr1-2* mutants. For the following pictures, 5 week old plants were infected with 10^8cfu/ml *Pto* DC3000, then 2hr later the plant material was collected and RNA was isolated. After cDNA synthesis, transcript levels were quantified via PCR. For amplification of the *PSKR1* transcript in *pskr1-3* primers 407D02_LP and N508584/85 were used; in *pskr1-4* primers b-N508584/N50858 and N508585/85 were used (see Table 7.1.1 in Appendix for primer sequences). For determination of the EF1α transcript, primers EF1α-s and EF1α-as were used.

3.2.3.3 Determination of T-DNA insertion number on mutant plants

In order to guarantee that potential phenotypes observed in the mutant plants correspond effectively to the effect of a non-functional gene, it is required to verify that the observed phenotype is present in different independent alleles of the gene under study, or to find out how many *T*-DNA insertions the corresponding mutants possess. Therefore, the number of *T*-DNA insertions was investigated for *pskr1-3*, which constitutes the main *T*-DNA insertion mutant line used in this work (Figure 3.2.8). For this purpose, genomic DNA was prepared, then fragmented using an appropriate restriction enzyme, *Eco*RI (Chapter 2.5.3) and together with a SALK *T*-DNA specific probe, which binds to the insertion on the left-border region (Appendix 7.2), Southern-Hybridization was performed (Chapter 2.5.9). Besides the 1126 bp expected band, there are other signals present, indicating the presence of multiple insertions in this specific mutant line. These multiple insertions could potentially diminish with backcrossings, but here we opt for performing the corresponding phenotypic analyses in parallel with the other independent mutant lines and verify and compare the resulting phenotypes.

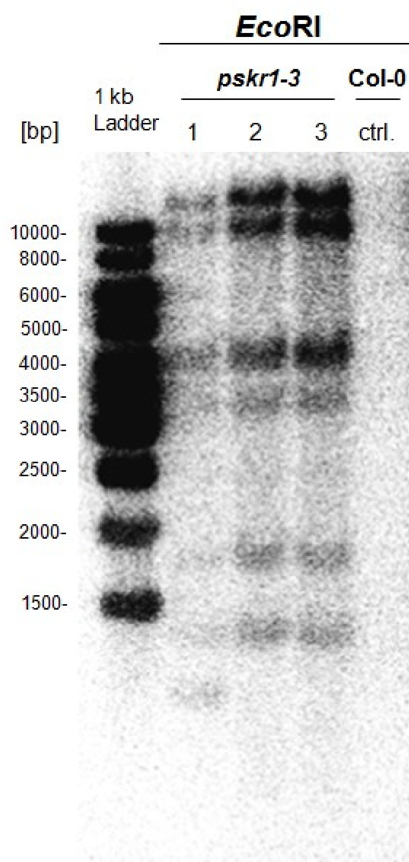

Figure 3.2.8: Southern-Hybridization with a *T*-DNA specific probe

Genomic DNA was extracted from plant material of 6-week old *pskr1-3* and Col-0 in parallel using the PCI method (Chapter 2.5.1). For identification of the *T*-DNA insertions number, Col-0 and *pskr1-3* DNA was fragmented with the enzyme *Eco*RI. Then a Southern-Hybridization was performed (Chapter 2.5.9) with a SALK *T*-DNA specific probe (Appendix 7.2). The expected fragment has a size of 1126 bp. The *pskr1-3* templates represent triplicates.

3.2.4 Phenotypic characterization of *PSKR1* impaired *T*-DNA insertion lines

It has been previously reported that *pskr1-1* seedlings, a Ds transposon insertion mutant, exhibit a normal growth and develop rosette leaves phenotypically indistinguishable from wild type for the first 3 weeks after germination. But afterwards a premature senescence phenotype is observed in leaves of *pskr1-1* plants at the late bolting stage (Matsubayashi *et al.* 2006). For the mutants available in this project the same growth and development phenotype was observed under the previously described growth conditions (Chapter 2.2.1). The *pskr1*-2, *pskr1-3* and *pskr1-4* seedlings grow at almost the same rate as wild-type seedlings, flower normally and complete the normal life cycle, as reported for the *pskr1-1* mutant (Matsubayashi *et al.* 2006) and present an earlier senescence phenotype compared to wild-type plants.

3.2.4.1 Analysis of bacterial pathogen growth in *pskr1* plants

Based on Microarray data and on previous semiquantitative RT-PCR (Chapter 3.2.1) it is to deduce that *PSKR1* is a gene induced by bacterial pathogens. In order to get to know the role of the receptor protein in pathogen defence, different strains of *Pseudomonas syringae* pv. *tomato* (*Pto*) with different pathogenicity were used. After infection of wild-type plants

together with *pskr1* mutants the bacterial growth was followed after 4 days. The various bacteria used here are: *Pto* DC3000 which is a virulent pathogen; *Pto* avrRpm1 which is an avirulent pathogen expressing the *avrRpm1* avirulent gene; and *Pto* hrcC- which is defective in the TTSS (Figure 3.2.9).

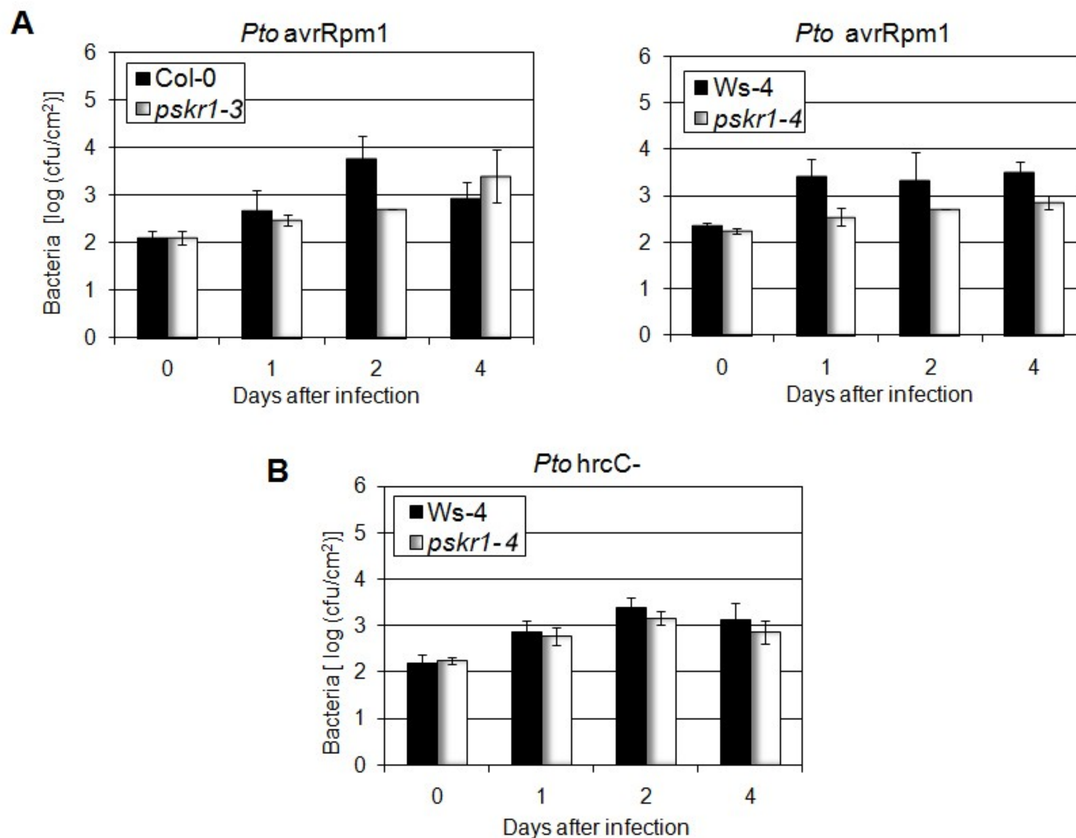

Figure 3.2.9: Bacterial growth after inoculation with different *Pseudomonas* strains

5 weeks-old plants from Col-0, Ws-4, *pskr1-3* and *pskr1-4*, respectively, were infected (A) with 10^5 cfu/ml *Pto* avrRpm1; plants from Ws-4 and *pskr1-4* were infected (B) with 10^5 cfu/ml *Pto* hrcC-. Bacterial growth was calculated (Chapter 2.3.3). Error bars represent the standard deviation of at least 6 replicates. The shown graphics represent two independent experiments for *pskr1-4* in (A); one experiment for *pskr1-3* in (A); two independent experiments for *pskr1-4* in (B).

When observing bacterial growth of the avirulent *Pto* avrRpm1 (*Pto* expressing AvrRpm1) and of the TTSS-deficient strain *Pto* hrcC- in wild-type plants, it comes out that there is an initial minimal increase in growth noticeable at day 1 and day 2 after initiated infection; afterwards the growth stops, which is interpreted as a non-successful growth of these bacteria in *Arabidopsis thaliana*. On the other hand, bacterial growth for *Pto* avrRpm1 is slightly reduced in *pskr1-3* and *pskr1-4* mutants when compared to wild-type plants on day 1 or day 2 after initiated infection. Nevertheless, growth for *Pto* hrcC- was only followed with *pskr1-4* mutants and showed no difference between wild-type plants and mutants along the followed infection time (Figure 3.2.9, B).

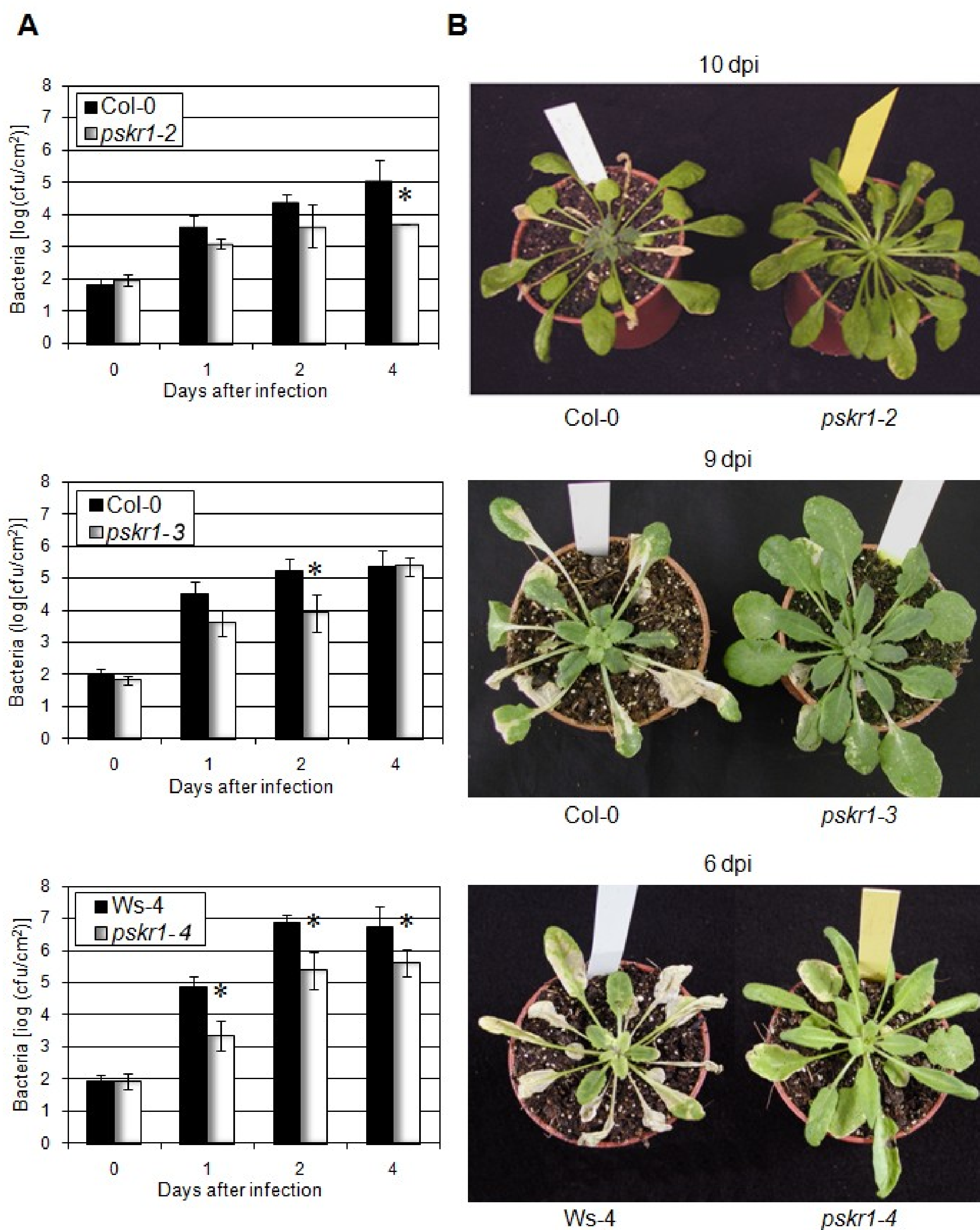

Figure 3.2.10: Bacterial growth and disease symptoms after infection with *Pto* DC3000

(A) 5 week-old plants were used to determine the number of grown bacteria at timepoints 0, 1, 2 and 4 days post infection (dpi). Wild type and the corresponding mutants were inoculated with 10^4 cfu/ml *Pto* DC3000 suspension (Chapter 2.3.3). The error bars represent the standard deviation of al least 6 replicates. These graphics represent the tendency observed in 2, 7 and 4 independent experiments for *pskr1-2*, *pskr1-3* and *pskr1-4*, respectively. (B) 5 week-old plants from wild type and the corresponding mutants plants were sprayed with 1_{OD} *Pto* DC3000 suspension and the macroscopic symptom development was followed at the above mentioned timepoints.

When observing bacterial growth of the virulent *Pto* DC3000 in wild-type plants, a clear successful growth is present already after 1 day of initiated infection, represented by a bacterial number increase of around 2 logs, indicating that *Pto* DC3000 manage to colonize the plant tissue satisfactorily. Interestingly, when bacterial growth of wild-type plants and *pskr1* mutants is compared, a significant decrease in bacteria numbers can be observed in *PSKR1*-impaired mutants at day 1 and 2 after infection, whereas at day 4 the bacterial growth in mutants equals the one in wild-type or is slightly diminished (Figure 3.2.10, A). The variations of bacterial amount seems to depend on the ecotype, for in Col-0 wild type the bacterial growth is less than in Ws-4. This increased bacterial resistance displayed by *pskr1* mutants is supported when spraying *Pto* DC3000 at a concentration of OD $_{600nm}$ = 1 (Figure 3.2.10, B), where mutant plants appeared more resistant than wild-type plants, showing less chlorotic lesions on leaves. These fitness symptoms are already visible at day 3 after spraying for the *pskr1-4* mutants, whereas for *pskr1-2* and *pskr1-3* mutants, symptoms appeared observable from day 5 on. It is also worth mentioning that after spraying *Pto* DC3000, bacterial numbers displayed the same tendency as for inoculation, that is, a decreased bacterial growth in *pskr1* mutants, but here the difference in growth was less significant between wild type and mutant plants (data not shown). The above bacterial growth experiments showed that plants impaired in PSKR1 displayed an enhanced resistance phenotype against *Pto* DC3000 infection.

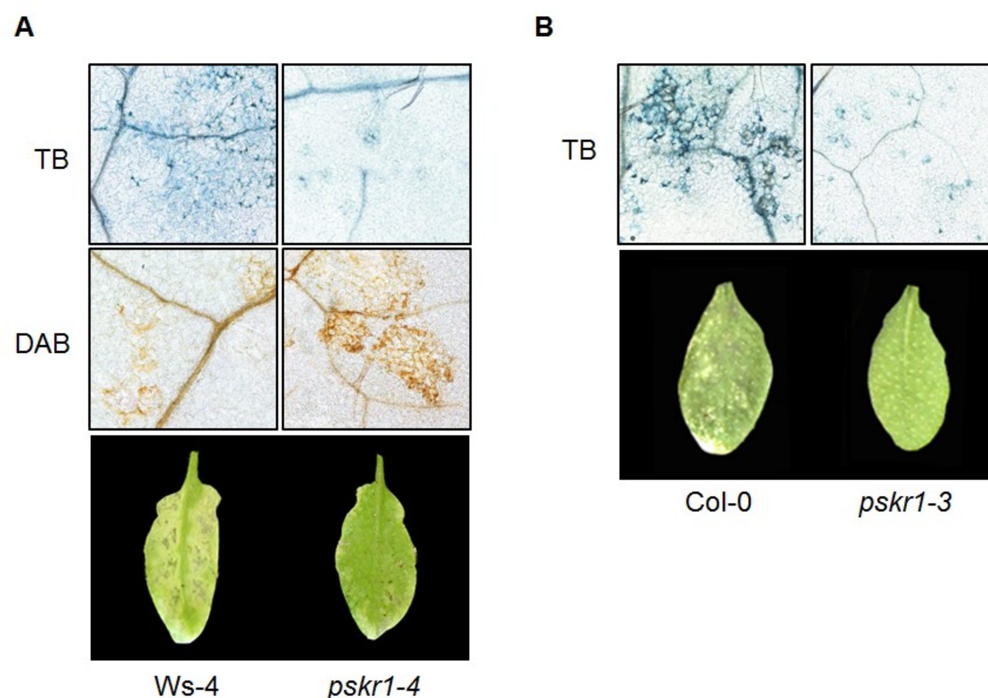

Figure 3.2.11: Disease symptoms after infection with *Pto* DC3000

5-week old plants were sprayed with $5x10^8$ cfu/ml *Pto* DC3000. Leaves were harvested (A) 3 dpi and were stained with Trypan blue (TB, Chapter 2.3.7) for visualization of cell death and with 3,3'-Diaminobenzidine (DAB, Chapter 2.3.6) for visualization of reactive oxygen species (ROS). (B) 7 dpi leaves were stained with trypan blue staining. Staining assays with *Pto* DC3000 were performed once.

When microscopic lesions were observed, *pskr1-3* and *pskr1-4* mutants displayed less cell death accumulation (micro-HRs) than the corresponding wild-type plants after infection with *Pto* DC3000 (Figures 3.2.11, A and B). Additionally *pskr1-4* showed increased accumulation of H_2O_2 when compared to wild-type plants (Figure 3.2.11, A); although here it's worth mentioning, that ROI detection is a very early response to PAMPs, so that the observed H_2O_2 accumulation, 72hr after infection, might not be part anymore of the *pskr1* mutant responses against *Pto* DC3000. Nevertheless, the healthier appearance of *pskr1* mutant plants infected with *Pto* DC3000 correlates with the less micro-HR accumulation when compared to wild-type plants.

3.2.4.2 Analysis of disease symptoms caused by necrotrophic fungi in *pskr1* plants

Previously it was shown that the mutation of *PSKR1* affects the growth of hemibiotrophic bacteria (Chapter 3.2.4.1). In order to investigate if this mutation affects the response to necrotrophic fungi, *A. thaliana* plants were challenged with the necrotrophic fungus *Alternaria brassicicola*. After application of *A. brassiccicola* spores on Col-0 or Ws-4 plants (controls) (Chapter 2.3.5), not-spreading lesions appear which are typical of an incompatible interaction. Symptom development was followed until 10 dpi and even then wild-type plants did not surrender to the fungal infection. Interestingly, when developing symptoms were compared with the ones from *pskr1* mutant plants, a more sensitive phenotype appeared in the mutants lacking a functional *PSKR1* gene. (Figure 3.2.12, A and B). After following the disease symptoms caused by *Alternaria brassicicola* (based on a ranking system with a score for each symptom; Chapter 2.3.5), *pskr1* mutant plants display more severe disease symptoms than wild-type plants, represented by darker lesion sites, broader chlorotic areas surrounding the infection site, expanded dead tissue and even the fungus was able to sporulate when symptoms were followed for more than 10 days (latest affirmation not shown) (Figure 3.2.12, B). It is worth mentioning that the difference in symptom severity after A. brassicicola infection seemed to be also ecotype-dependent, with Col-0 plants showing more pronounced symptoms than Ws-4 plants.

Further, when observed microscopically, it came out that *pskr1* mutant plants present an increased cell death accumulation on the infection sites caused by *A. brassicicola* observable even at early stages (Figure 3.2.13, A).

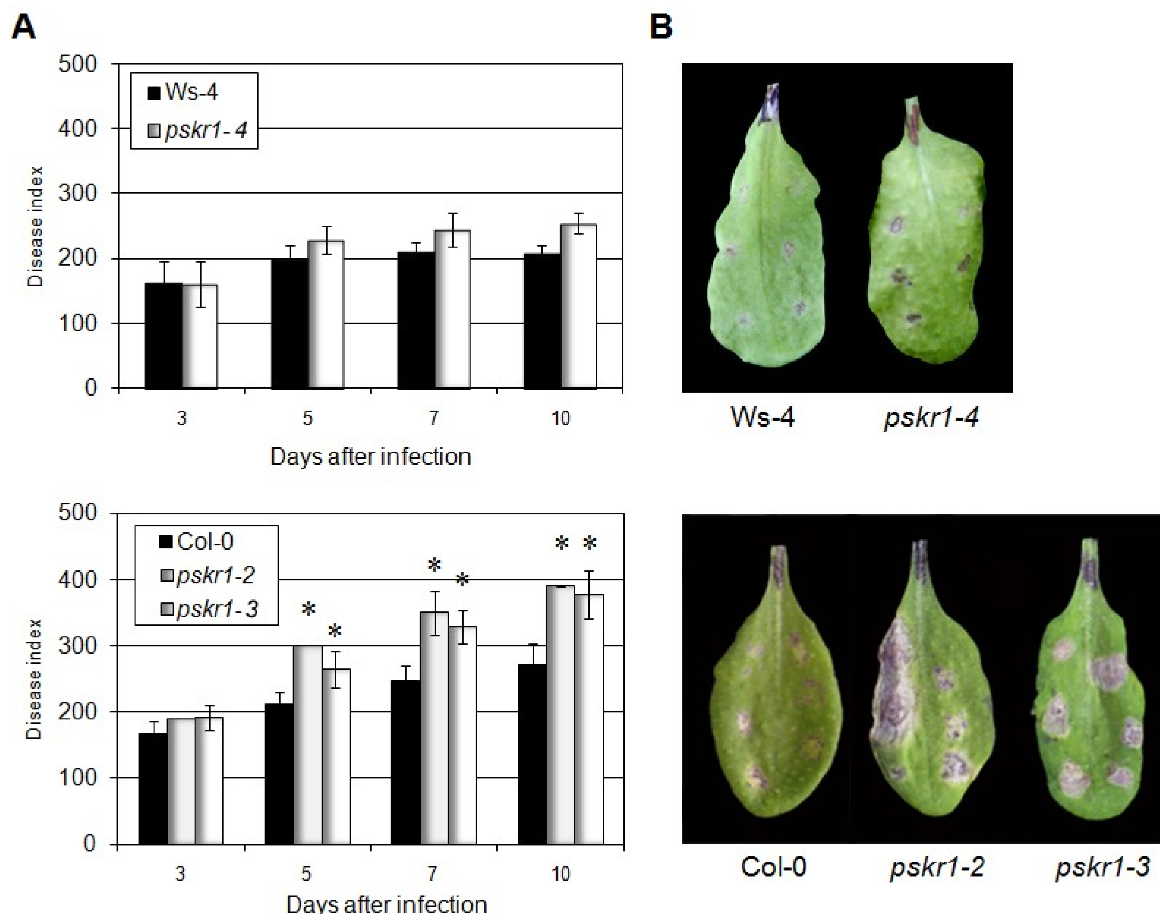

Figure 3.2.12: Disease development on Arabidopsis pskr1 mutants inoculated with *Alternaria brassicicola*

5-week-old plants were inoculated with a suspension of $5x10^5$ spores/ml of *Alternaria brassicicola*. (Chapter 2.3.5) (A) Symptom development was followed based on a score system until 10 dpi. These graphics represent the tendency observed in 2, 2 and 10 independent experiments for *pskr1-4*, *pskr1-2* and *pskr1-3*, respectively. (B) Pictures of detached leaves of *PSKR1* mutant plants infected with *Alternaria brassicicola* compared to the corresponding control plants. Pictures taken 7 dpi.

In order to asses the *Alternaria* growth in mutant platns, the fungal biomass was determined by semi-quantitative PCR (Chapter 2.3.8). *Alternaria* biomass is certainly higher in *pskr1* mutants compared to wild-type plants in the first 3 dpi (Figure 3.2.13, B), which might indicate that the fungus *A. brassicicola* thrives in *pskr1* mutant plants and when doing so, the fungus causes spreading lesions and manages to sporulate after aprox. 10 days (latest affirmation about sporulation observed repeatedly).

The response of *pskr1* mutant plants to the necrotrophic fungus *B. cinerea* (Chapter 2.3.5) was also tested. Different concentrations of the fungus spores together with disease symptomps assessments at different days rendered indistiguishable responses between the *pskr1* mutants and wild-type plants, like in the pictures shown below (Figure 3.2.14).

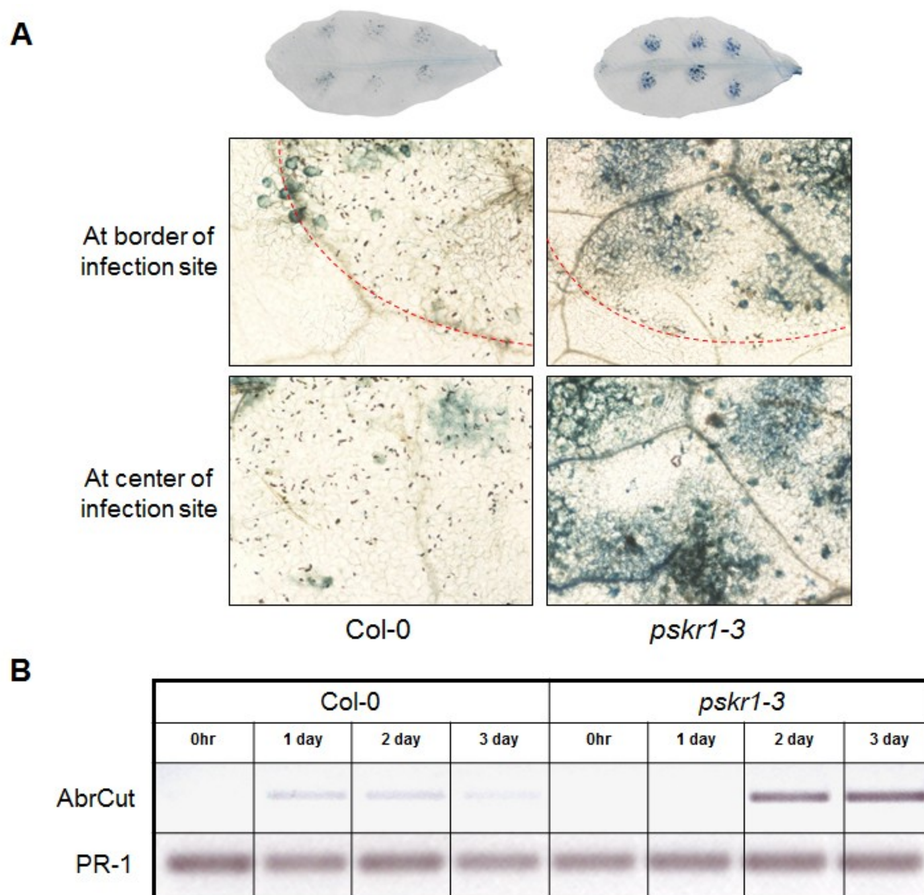

Figure 3.2.13: Microscopy pictures of disease development and fungal growth in *pskr1* mutants

5-week-old plants were inoculated with a $5x10^5$ spores/ml *Alternaria* suspension. (A) 4 dpi infected leaves were harvested and stained with trypan blue (Chapter 2.3.7) showing symptoms at the border of the infection sites (two upper square pictures) and at the center of infection site (two lower square pictures). Red dashed lines represent approximately the border of the infection site. Accumulation of cell death was also visible at macroscopic level (see pictures of whole leaves). Experiments were performed twice independently. (B) Growth of *A. brassicicola* in *planta* based on semi-quantitative PCR (qPCR) with *A. brassicicola*- and *Arabidopsis*-specific primers (Chapter 2.3.8; See Table 7.1.1 in Appendix for primer sequences). PCR performed once.

Figure 3.2.14: Disease development on Arabidopsis *pskr1* mutants drop-inoculated with *Botrytis cinerea*

5-week-old plants were inoculated with a suspension of $2,5x10^5$ spores/ml of *Botrytis cinerea*. (Chapter 2.3.5) Pictures were taken at 2 dpi. This picture represents the tendency observed in 3 independent experiments.

3.2.4.3 Induction of pathogen-inducible defence responses in pskr1 mutants

To investigate whether the mutation in *AtPSKR1* affects pathogen-inducible defence responses, the induction of a number of defence-related genes in *pskr1* mutant plants was characterized after infection with *A. brassicicola* and with *Pto*. DC3000.

JA-mediated defence plays an important role in regulating the expression of plant defence genes and resistance to necrotrophic pathogens. To determine whether the altered responses of the *pskr1* mutants to *A. brassicicola* is related to altered JA-mediated defence mechanisms, the induction of some JA-regulated defence genes after *A. brassicicola* infection was examined. *PDF1.2* is a marker for the jasmonate/ethylene-dependent defence responses (Penninckx *et al.* 1996; Penninckx *et al.* 1998; Thomma *et al.* 1998). *PDF1.2* was induced earlier in wild type plants in response to *A. brassicicola* infection compared to the *pskr1* mutant plants, where *PDF1.2* induction was delayed for 24 hr. Another JA/ET-dependent gene is PR4, whose transcription is enhanced in the *Arabidopsis*-*A. brassicicola* interaction (Trusov *et al.* 2008; Mukherjee *et al.* 2009). The transcript accumulation of PR4 appears reduced for the first day after infection but increased for the second day, resembling also a delayed induction in the *A. brassicicola* response (Figure 3.2.15).

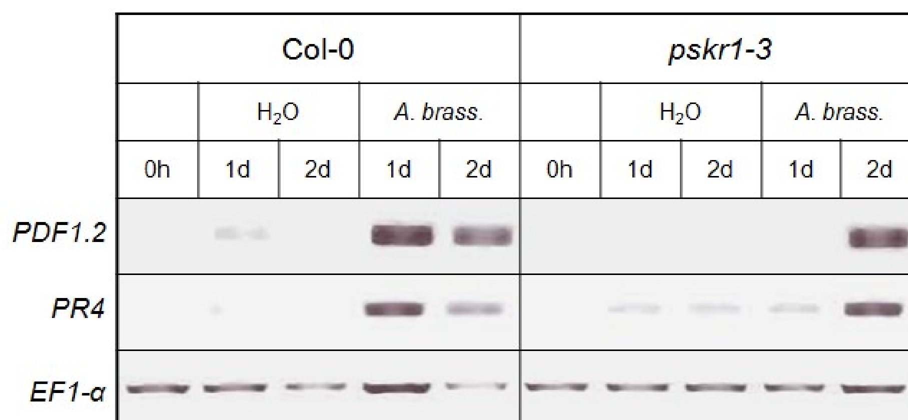

Figure 3.2.15: Pathogen-inducible defence responses in *pskr1* mutant plants after infection with *A. brassicicola*

5-week-old plants were inoculated with a $5x10^5$ spores/ml *A. brassicicola* suspension and H_2O (control). At indicated time points plant material was harvested and semiquantitative RT-PCR was performed using specific primers for PDF1.2 and PR4, JA-induced pathogenesis-related genes (see Table 7.1.1 in Appendix for primer sequences). The total amount of cDNA was standardised with the elongation factor EF1α. (Fragment size 600 bp). Pattern shown here represents the tendency of three independent experiments.

SA-mediated defence plays a critical role in plant defence against the bacterial pathogen *P. syringae*. SA-mediated defence mechanisms are associated with the expression of pathogenesis-related (PR) genes, including the *PR1* gene, which is often used as a reliable

molecular marker for SA-dependent SAR. Since *pskr1* mutant plants are more resistant to *Pto* DC3000 bacterium, the expression of *PR1* was examined in *pskr1* mutant plants after spray-infection with *Pto* DC3000 (Chapter 2.3.3). *PR1* is induced at high levels in wild type plants after bacterial infection. Whereas in *pskr1* mutant plants the induction of *PR1* appears to be activated earlier than for wild type, although more repetitions would be needed to confirm this (Figure 3.2.16).

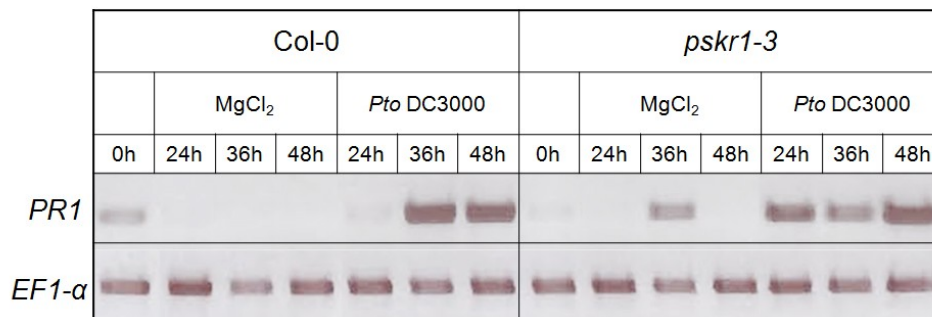

Figure 3.2.16: *PR1* response in *pskr1* mutant plants after infection with *Pto* DC3000

5-week-old plants were spray-infected with a 10^8 cfu/ml *Pto* DC3000 suspension. At indicated timepoints plant material was harvested and semiquantitative RT-PCR was performed using specific primers for PR1 (see Table 7.1.1 in Appendix for primer sequences). The total amount of cDNA was standardised with the elongation factor EF1α. (Fragment size 600 bp). Experiment performed once.

3.2.4.4 Analysis of pathogen-related hormone levels in *pskr1* mutant plants

Biotrophic pathogens, like *Pto* DC3000, are generally sensitive to defence responses that are regulated by SA, whereas pathogens with a necrotrophic lifestyle are commonly deterred by defences that are controlled by JAs and ET (Thomma *et al.* 2001; Glazebrook 2005).

The potential earlier induction of *PR1* in *pskr1* mutant plants, previously shown in Chapter 3.2.4.3, might indicate that production and/or perception of SA in *pskr1* plants is affected. Therefore, the total SA content was measured at 0 and 24hr post initiated infection with *Pto* DC3000. At basal levels (0hr), SA content in *pskr1-3* plants appeared to be slightly higher than in wild-type plants (Figure 3.2.17, A). After infection with virulent *Pto* DC3000, SA content increased in wild-type as well as in *pskr1-3* mutant plants, with *pskr1-3* mutants appearing to reach a slightly higher level of SA than wild-type plants at 24hr post infection (Figure 3.2.17, A). When plants were infected with *A. brassicicola*, SA content remained almost constant for wild-type plants but was significantly higher for *pskr1-3* mutant plants (Figure 3.2.17, B).

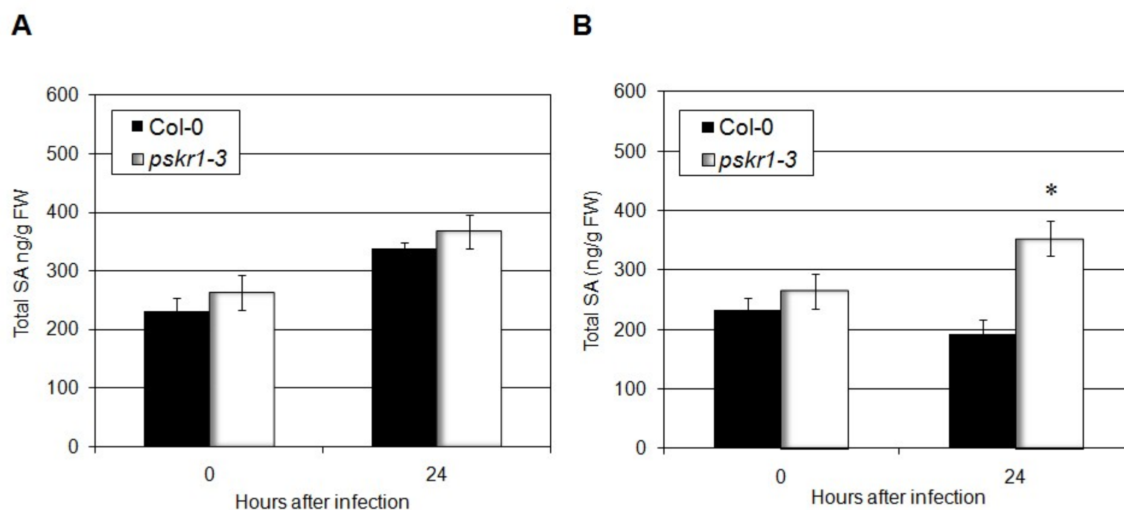

Figure 3.2.17: SA content in *pskr1-3* mutant plants at basal level and after pathogen infection

5-week old plants were used for measuring SA content. (A) Total SA content after spray-infection with a virulent 10^8 cfu/ml *Pto* DC3000 bacterial suspension. (B) Total SA content after spray-infection with a $5x10^5$ spores/ml *A. brassicicola* suspension. Error bars arise from 6 replicates for each indicated timepoint. Graphics above represent the tendency found in two independent experiments.

Auxin is an essential plant hormone in regulating plant growth and development and it is also involved in promoting plant disease susceptibility to *P. syringae* (Chen *et al.* 2007), a process that can be counteracted by SA (Wang *et al.* 2007). Free IAA (indolic acetic acid) content was measured at 0 and 24hr after initiated infection in wild-type and *pskr1-3* mutant plants. Basal levels of auxin in *pskr1-4* mutants appeared enhanced compared to wild-type plants (Figure 3.2.18). Upon infection with *Pto* DC3000 total auxin content in wild-type plants increased slightly after 24hr. Total auxin content in *pskr1-3* mutants remained similar than wild-type plants (Figure 3.2.18, A). Upon infection with the necrotroph *A. brassicicola,* total auxin levels in wild-type plants increased significantly at 24hr, whereas in *pskr1-3* mutant plants the IAA content remained basically constant throughout the experiment upon infection with the necrotrophic fungus (Figure 3.2.18, B).

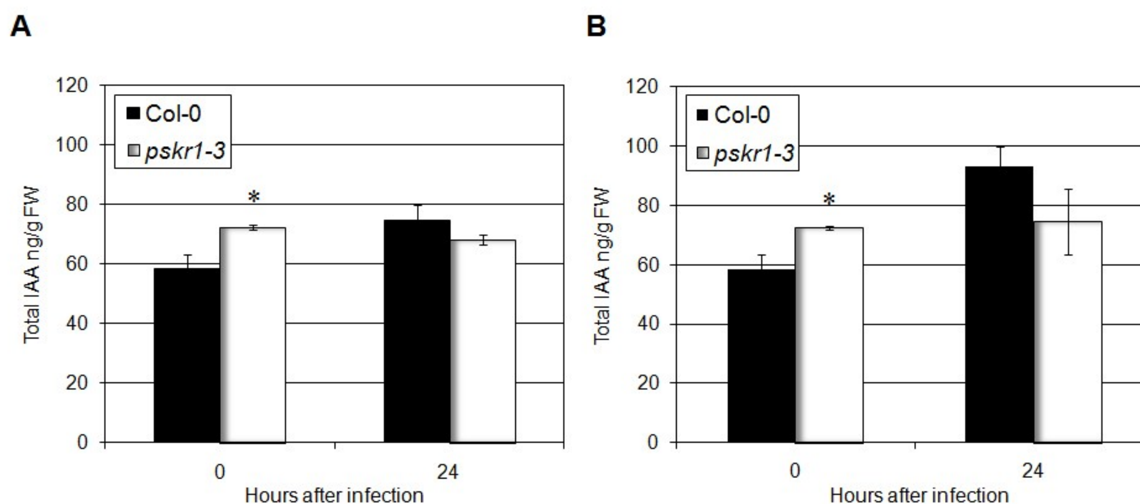

Figure 3.2.18: Total IAA content in *pskr1-3* mutant plants at basal level and after pathogen infection

5-week old plants were used for measuring IAA content. (A) IAA content after spray-infection with a virulent 10^8 cfu/ml *Pto* DC3000 bacterial suspension. (B) IAA content after spray-infection with a $5x10^5$ spores/ml *A. brasscicola* suspension. Error bars arise from 6 replicates for each indicated timepoint. Graphics above represent tendency found in two independent experiments.

3.2.5 Molecular properties of AtPSKR1

Based on overall amino acid similarity to carrot PSKR1(DcPSKR1), the PSK-α receptor in carrot (Matsubayashi *et al.* 2002), the correspondent receptor in *Arabidopsis thaliana* was identified (Matsubayashi *et al.* 2006). AtPSKR1 encodes a 1008-amino acid LRR-RK that shares a 60% amino acid sequence identity to DcPSKR1. The 150-kD AtPSKR1 protein contains a signal peptide, 21 tandem copies of extracellular LRR, a 36-amino acid island domain between the 17th and 18th LRR, a single transmembrane domain and a cytoplasmic kinase domain (Figure 3.2.19). AtPSKR1 is plasma membrane-localized (Matsubayashi *et al.* 2006). AtPSKR1 belongs to the LRR-X subfamily (Shiu and Bleecker 2001), which also contains other known kinases like BRI1, BRL1, BRL2 and BRL3 (Caño-Delgado *et al.* 2004)

Figure 3.2.19: Schematic representation of the protein At2g02220 obtained from Matsubayashi *et. al.* 2006

SP represents the signal peptide at the N-terminus (orange block), LRR is the Leucine-reach repeats domain (yellow blocks), Island is the island domain (green block between the LRR domain), TM represents the single transmembrane region (blue block) and finally the kinase domain at the C-terminus (red block).

3.2.6 LRR-domain in AtPSKR1

The LRR domain of AtPSKR1 consist of 21 LRR motifs, flanked by cysteine-rich domains. The C-terminal flanking domain contains 2 cysteine residues whereas the N-terminal flanking domain contains 2 cysteine residues (Figure 3.2.20).

Cysteine residues are of particular interest because it has been proposed that they can be involved in the formation of intramolecular and/or intermolecular disulfide bridges (Dievart and Clark 2003). The island domain in DcPSKR1 was found to be the ligand binding pocket that interacts directly with PSK-α by photoaffinity labeling (Shinohara *et al.* 2006). The amino acid sequences in the island domain are highly conserved between DcPSKR1 and At2g02220 (Matsubayashi *et al.* 2006), suggesting that in AtPSKR1 the island domain may be responsible for PSK binding.

```
63-               CNWTGITCN
 -SNNTGRVIRLELGNKKLSGKLSES     1
 -LGKLDEIRVLNLSRNFIKDSIPLS     2
 -IFNLKNLQTLDLSSNDLSGGIPTS     3
 -IN-LPALQSFDLSSNKFNGSLPSH     4
 ICHNSTQIRVVKLAVNYFAGNFTSG     5
 -FGKCVLLEHLCLGMNDLTGNIPED     6
 -LFHLKRLNLLGIQENRLSGSLSRE     7
 -IRNLSSLVRLDVSWNLFSGEIPDV     8
 -FDELPQLKFFLGQTNGFIGGIPKS     9
 -LANSPSLNLLNLRNNSLSGRLMLN     10
 -CTAMIALNSLDLGTNRFNGRLPEN     11
 -LPDCKRLKNVNLARNTFHGQVPES     12
 -FKNFESLSYFSLSNSSLANISSAL     13
GILQHCKNLTTLVLTLNFHGEALPDDS   14
 -SLHFEKLKVLVVANCRLTGSMPRW     15
 -LSSSNELQLLDLSWNRLTGAIPSW     16
 -IGDFKALFYLDLSNNSFTGEIPKS     17
 -LTKLESLTSRNISVNEPSPDFPFFMKRNESARALQYNQIFGFPPT
           IELGHNNLSGPIWEE     18
 -FGNLKKLHVFDLKWNALSGSIPSS     19
 -LSGMTSLEALDLSNNRLSGSIPVS     20
 -LQQLSFLSKFSVAYNNLSGVIPSG     21
  GQFQTFPNSSFESNHLCGEHRFPC
                      -638
 -LxxLxxLxxLxLxxNxLxGxIPxx
```

Figure 3.2.20: LRR domain of AtPSKR1

This analysis was performed between the amino acids 63 and 638 from *At*PSKR1; gray-highlighted characters represent the conserved residues in the LRR motif; flanking regions are represented by black-highlighted cysteines. Below is the amino acid consensus sequence motif for extracytoplasmic LRRs according to Torii 2004. The numbers on the right correspond to the LRRs present in AtPSKR1. The island domain is represented by green characters, between the 17th and 18th LRR motif.

3.2.7 Promoter analysis of AtPSKR1

Using the PLACE signal scan Programm (Higo *et al.* 1999) for analysing the promoter region of *AtPSKR1*, consisting of 1541 bp upstream of the initiation codon, a number of developmental- and abiotic-induced promoter *cis*-elements were found (Table 3.2.2).

Table 3.2.2: List of promoter *cis*-elements of *AtPSKR1* known in *Arabidopsis*

Cis-element (sequence)	Function	Number of apparitions
ARR1AT (NGATT)	ARR1 binding element in Arabidopsis; ARR1 is a type-B response regulator, implicated in the cytokinin signalling pathway (Argyros *et al.* 2008); N=G/A/C/T	19
CCAATBOX1 (CCAAT)	Acts cooperatively with HSEs (heat shock elements) to increase the hs (heat shock) promoter activity (Haralampidis *et al.* 2002; Wenkel *et al.* 2006)	2
POLASIG1 (AATAAA)	PolyA signal near upstream elements in *Arabidopsis* (Loke et al. 2005)	7
ACGTATERD1 (ACGT)	ACGT sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis (Simpson *et al.* 2003)	3
DRECRTCOREAT (RCCGAC)	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element found in many genes in Arabidopsis and in rice (Susuki *et al.* 2005); R=G/A	1
CCA1ATLHCB1 (AAMAATCT)	Binding site of the CCA1 protein (myb-related transcription factor) that interacts with the light harvesting chlorophyll protein Lhcb1*3 of *Arabidopsis thaliana*; related to regulation by phytochrome (Wang *et al.* 1997)	2
GATA-box (GATA)	Light-dependent *cis*-regulatory element (Teakle *et al.* 2002; Reyes *et al.* 2004)	10
HDZIP2ATATHB2 (TAATMATTA)	Binding site of the homeobox gene AtHB-2 that is regulated by light signals and functions as a negative autoregulator of its own gene (Ohgishi *et al.* 2001); M=C/A	1
T-box (ACTTTG)	"Tbox" found in the *Arabidopsis thaliana* GAPB gene promoter; mutations in the "Tbox" resulted in reductions of light-activated gene transcription; GAPB encodes the B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase(GADPH) of *Arabidopsis (Chan et al. 2001)*	2
LTRECOREATCOR15 (CCGAC)	Core of C/DRE sequence, which is essential for transcriptional activation in response to cold, drought and/or high salt treatments (Yamaguchi-Shinozaki and Shinozaki 1994)	1
MYC and MYB recognition sites	*cis*-elements in the drought-induced expression of the rd22 gene in *Arabidopsis* (Abe et al. 1997)	3x each
RAV1AAT (CAACA)	Binding consensus sequence of *Arabidopsis* transcription factor, RAV1 (Related to ABI3/VP1) (Kagaya *et al.* 1999) whose expression is stimulated by various external or environmental cues (low temperature, darkness, wounding, drought, salt stress and pathogen attack) (Fowler and Thomashow 2002; Lee *et al.* 2005; Sohn *et al.* 2006; Kagaya and Hattori 2009)	6
W-box core (TTGAC)	Essential for function and binding of WRKY transcription factors proteins (Eulgem *et al.* 2000)	7

The promoter region of *AtPSKR1* appears to be greatly composed by Arabidopsis *cis*-elements related to the signalling pathway of cytokinins, which are classic phytohormones that play important roles in the regulation of plant growth and development (Haberer and Kieber 2002; Howell *et al.* 2003). Moreover, various environmentally regulated elements are present in the *AtPSKR1* promoter region, like dehydration-, light-, drought, salt-regulated elements. Also noteworthy is the presence of a binding site for RAV1, a transcription factor implicated in the adaptation to a variety of environmental stimuli, including pathogen attack (Sohn *et al.* 2006; Kagaya and Hattori 2009). Interestingly, 7 copies of the W-Box core (TGAC) appeared in the analysed promoter region. Cis-acting W-boxes, (T)(T)TGAC(C/T), are present in numerous co-regulated *Arabidopsis* defence gene promoters (Maleck *et al.* 2001), and are recognized by several plant WRKY transcription factors (Eulgem *et al.* 2000). Various WRKY transcription factors act as negative regulators of plant defence whereas others positively modulate this response (Eulgem and Somssich 2007).

3.2.8 Kinase function of AtPSKR1

An analysis of the kinase domain of AtPSKR1 using the KinG Database (Krupa *et al.* 2004) classified this kinase into the group of RD kinases (Dardick and Ronald 2006), since it contains a conserved arginine (R) immediately before the the invariant aspartate (D) in subdomain VIb. Therefore, AtPSKR1 kinase may be regulated by activation loop phosphorylation. The same pattern is present in BRI1 but not in FLS2 or ERF, which are categorized like non-RD kinases. AtPSKR1 contains a glycine loop motif completely conserved (in subdomain I). Another worthy feature is the lack of a complete APE-motif in subdomain VIII. Consequently, the AtPSKR1 catalytic domain resembles remarkably BRI1 rather than FLS2 or EFR kinases (Figure 3.2.21).

```
                          I                 II               III
                       GxGxxGxV            VAxK               E
AtPSKR1         FDQANIIGCGGFGMVYKATLP--DGKKVAIKKLSGDCGQIEREFEAEVETLSRAQHPNLV
BRI1            FHNDSLIGSGGFGDVYKAILK--DGSAVAIKKLIHVSGQGDREFMAEMETIGKIKHRNLV
EFR             FSSTNLIGSGNFGNVFKGLLGP-ENKLVAVKVLNLLKHGATKSFMAECETFKGIRHRNLV
FLS2            FNSANIIGSSSLSTVYKGQLEDGTVIAVKVLNLKEFSAESDKWFYTEAKTLSQLKHRNLV
                * . .:**...:. *:*. *       * :  *        : * :* :*:   :* ***

                              IV                     V

AtPSKR1         LLRGFCFYKND-----RLLIYSYMENGSLDYWLHER-----NDGPALLKWKTRLRIAQGA
BRI1            PLLGYCKVGDE-----RLLVYEFMKYGSLEDVLHDP-----KKAGVKLNWSTRRKIAIGS
EFR             KLITVCSSLDSEGNDFRALVYEFMPKGSLDMWLQLEDLERVNDHSRSLTPAEKLNIAIDV
FLS2            KILGFAWESGK----TKALVLPFMENGNLEDTIHGS-------AAPIGSLLEKIDLCVHI
                 :   .   ..     : *:  :*  *.*:  ::              .   :  :.

                                  VIb               VII
                                HRDxKxxN            DFG
AtPSKR1         AKGLLYLHEGCDPHILHRDIKSSNILLDENFNSHLADFGLARLMSPYET-----HVST-D
BRI1            ARGLAFLHHNCSPHIIHRDMKSSNVLLDENLEARVSDFGMARLMSAMDT-----HLSVST
EFR             ASALEYLHVHCHDPVAHCDIKPSNILLDDDLTAHVSDFGLAQLLYKYDRESFLNQFSSAG
FLS2            ASGIDYLHSGYGFPIVHCDLKPANILLDSDRVAHVSDFGTARILGFREDGS--TTASTSA
                * .: :**      : * *:*.:*:***.:  ::::*** *:::   :        *
```

```
                        VIII            IX
                        APE
AtPSKR1         LVGTLGYIPPEYGQASVATYKGDVYSFGVVLLELLTDKRPVDMCKPKGCRDLISWVVKMK
BRI1            LAGTPGYVPPEYYQSFRCSTKGDVYSYGVVLLELLTGKRPTDS--PDFGDNNLVGWVKQH
EFR             VRGTIGYAAPEYGMGGQPSIQGDVYSFGILLLEMFSGKKPTDES--FAGDYNLHSYTKSI
FLS2            FEGTIGYLAPEFAYMRKVTTKADVFSFGIIMMELMTKQRPTSLNDEDSQDMTLRQLVEKS
                . ** ** .**:      : :.**:*:*::::*::: ::*..          :   .:

                    X                                        XI

AtPSKR1         HESRASEVFDPLIYSK-----E--NDKEMFRVLEIACLCLSENPKQRPTTQQLVSWL
BRI1            AKLRISDVFDPELMKE-----DPALEIELLQHLKVAVACLDDRAWRRPTMVQVMA--
EFR             LSGCTSSGGS------------NAIDEGLRLVLQVGIKCSEEYPRDRMRTDEAVREL
FLS2            IGNGRKGMVRVLDMELGDSIVSLKQEEAIEDFLKLCLFCTSSRPEDRPDMNEILTHL
                     .                   :  :   *::   * .. .  *    : :
```

Figure 3.2.21: Sequence alignment of the AtPSKR1 kinase domain with other receptor proteins

Sequences of AtPSKR1, BRI1, EFR and FLS2 were compared using ClustalW (http://align.genome.jp). Conserved sequences were highlighted in gray. Roman numerals above the sequences indicate the kinase subdomains as defined by (Hanks et al. 1988). Red letters represent the RD-motif preceding the catalytic loop in subdomain VIb.

3.3 *AtPSKR1* homologs

AtPSKR1 possesses two close homologs in the *Arabidopsis thaliana* genome (Amano *et al.* 2007), *AtPSKR2* (At5g53890) and *PSY1R* (At1g72300). Both genes encode LRR-RLKs that share 48,6% and 43,6% sequence identity with *At*PSKR1, respectively (Figure 3.3.1, A, B). These genes share various characteristics with AtPSKR1: they are intronless; they are genes of similar size (1036 amino acids for AtPSKR2 and 1095 amino acids for PSY1R against 1008 amino acids for AtPSKR1); they possess 21 LRR motifs; they possess an island domain of 36, 39 and 38 amino acids for AtPSKR1, AtPSKR2 and PSY1R, respectively, but with little similarity between them (Figure 3.3.1, C); they are RD kinases. (See Figure 7.3.4 in Appendix for sequence comparison).

*At*PSKR2 is involved in PSK perception and does interact with PSK, therefore it is an alternative PSK receptor but less active than *At*PSKR1 (Amano *et al.* 2007). Alternatively, PSY1R is the receptor of a tyrosine-sulfated glycopeptide, denominated PSY1, and it is not involved in PSK perception. Both sulfated peptides, PSK-α and PSY1, contribute redundantly to cellular proliferation, expansion and wound repair during plant growth and development (Amano *et al.* 2007). Hence, it seemed of utmost importance to verify the involvement of *AtPSKR1* homologes in the response of *pskr1* mutants against pathogens

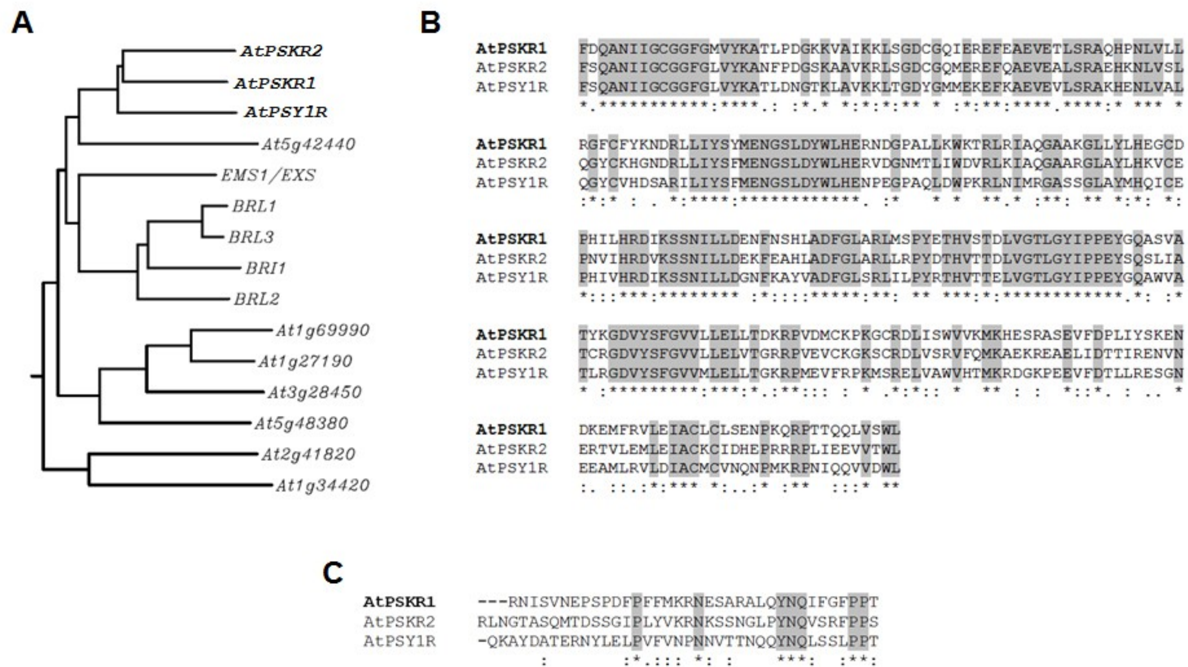

Figure 3.3.1: *AtPSKR1* and its closest homologes

(A) Phylogenetic tree of *Arabidopsis* LRR X subfamily. Alignment based on kinase domain amino acids using ClustalW (http://align.genome.jp); proteins in bold correspond to the PSK receptors and their closest homologs, AtPSKR2 and AtPSY1R. (B) Sequence alignment of the kinase domains of AtPSKR1, AtPSKR2 and AtPSY1R; residues highlighted in gray are conserved among these proteins. (C) Sequence alignment of the island domains from AtPSKR1, AtPSKR2 and AtPSY1R; residues highlighted in gray are conserved.

3.3.1 Expression of AtPSKR1 homologs after biotic and abiotic stress

Based on Microarray data (Zimmermann *et al.* 2004) *AtPSKR2* transcript accumulation remains *grosso modo* unaltered from its basal level after challenge with different *Pseudomonas* strains (Figure 3.3.2). Similarly, *AtPSY1R* shows slight variations from its basal transcript level in response to same *Pseudomonas* strains. Hence, it is noteworthy that both *AtPSKR2* and *AtPSY1R* transcriptional regulation is not altered significantly upon bacterial attack, especially when compared to the higher induction levels of *AtPSKR1* after treatment with TTSS-deficient and non-host *Pseudomonas* strains. Any of *AtPSKR1* homologs resemble the expression drop to basal levels at 6h after infection with the virulent *Pto* DC3000. (Figure 3.3.2)

When expression of *AtPSKR1* homologs after bacterial- and oomycete-elicitors treatment was checked (Genevestigator Microarray data), it results in a clear transcript accumulation of *AtPSKR1* after treatment with bacterial HrpZ and flg22, but not after bacterial LPS; also significant is the accumulation of *AtPSKR1* transcript after oomycete-derived NPP1.

Nevertheless, expression of the corresponding homologs, AtPSKR2 and AtPSY1R, does not undergo significant changes. (Figure 3.3.3)

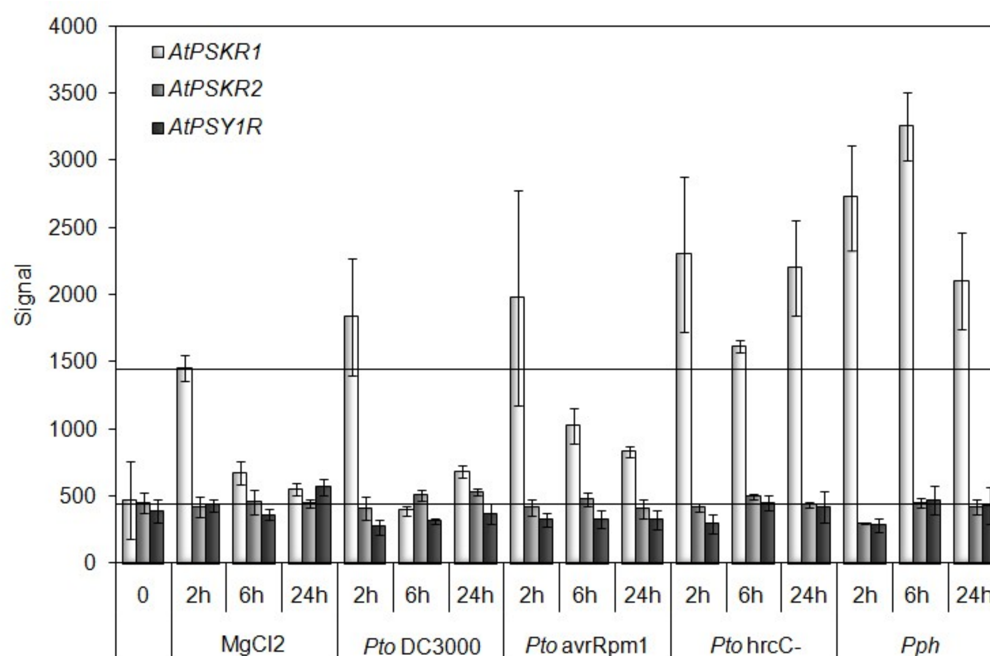

Figure 3.3.2: Microarray data after infiltration of wild type plants with various strains of *Pseudomonas*

Plant material of Col-0 plants was harvested at 2, 6 and 24h after inoculation with 10^8 cfu/ml *Pto* DC3000, *Pto* avrRpm1, *Pto* hrcC- and *Pph* together with $MgCl_2$ (control) and RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Timepoint 0 represents untreated plants. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00106, AtGenExpress. Error bars represent the standard deviation of three replicates. Horizontal black lines represent control levels of *AtPSKR1* (top line) and of *AtPSKR2* and *AtPSY1R* (bottom line).

The influence of abiotic stressors, like IAA, ABA, MJ and ACC, in the PSKR1 homologues was also investigated based on Microarray Data. AtPSKR2 resembles roughly the PSKR1 induction after treatment with ABA and MJ, i.e., its transcript accumulation is diminished when compared to corresponding controls after application of these two hormones. Application of IAA leads to a subtle increase of AtPSKR2 transcript after 1h. Interestingly, PSY1R displays a clearly different induction pattern after treatment with same hormones, where it appears downregulated by IAA, ABA, MJ and ACC, reaching a maximum of transcriptional downregulation after application of ABA at 3h (Figure 3.3.4; Table 7.4.1 in Appendix with relative values of induction after hormone treatment).

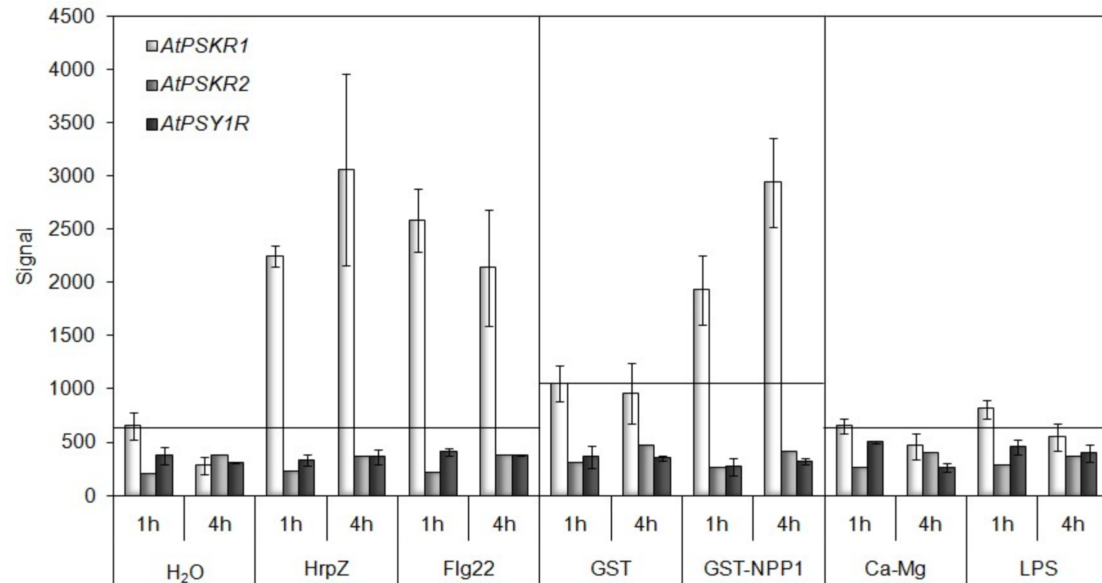

Figure 3.3.3: Microarray data after elicitors treatment of wild type plants

Plant material of 5 week old Col-0 plants was harvested at 1h and 4h after infiltration with bacterial-derived elicitor HrpZ (10 µM), Flg22 (1 µM) and LPS (100 µg/mL); and with oomycete-derived elicitor GST-tagged NPP1 (1 µM). RNA was isolated as described in (Chapter 2.6.1). Microarray data from AtGenExpress Initiative. The error bars represent the standard deviation of the corresponding values. Horizontal black lines represent control levels of *AtPSKR1.*

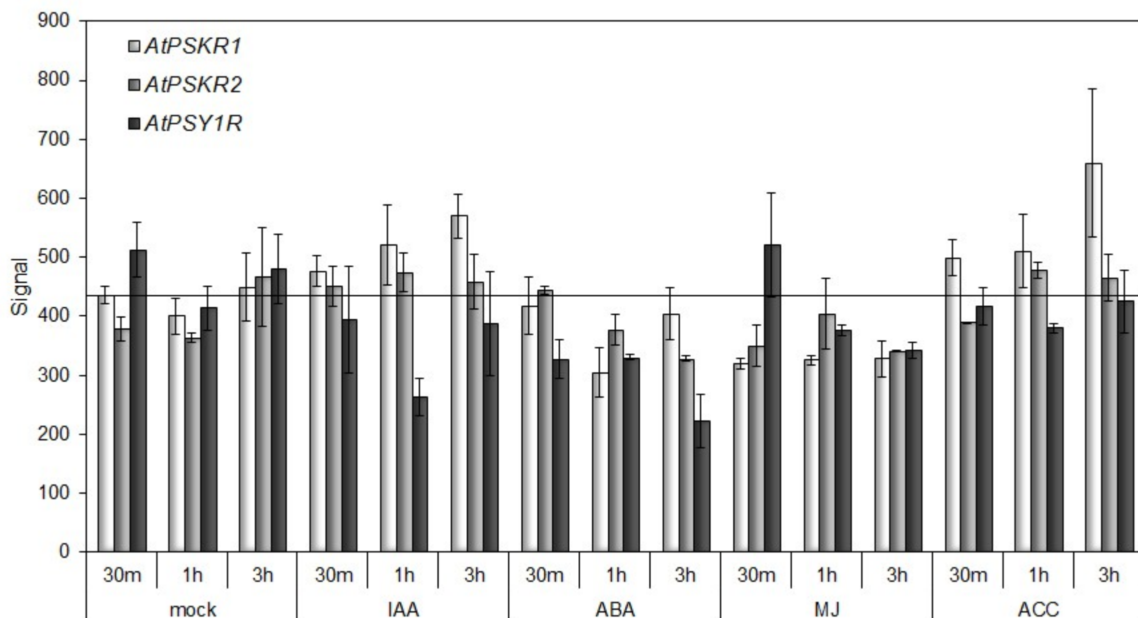

Figure 3.3.4: Microarray data after hormones treatment of wild type plants

Plant material of 7 week old Col-0 plants was harvested at 30min, 1h and 3 h after treatment with various hormones: IAA (1 µM); ABA (10 µM); MJ (10 µM); and ACC (10 µM). RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Microarray data from AtGenExpress Initiative. The error bars represent the standard deviation of the corresponding values.

3.3.2 Analysis of T-DNA insertion lines

A T-DNA insertion line from the European Arabidopsis Stock Center (NASC), SALK lines (Alonso *et al.* 2003), was obtained in order to verify the role of AtPSKR2 in plant defence. This line consists of a loss-of-function mutant created by insertion of a Agrobacterium *T*-DNA so that the sequence of the corresponding gene is disrupted. (Figure 3.3.5 and Table 3.3.1).

In order to verify the homozygous character of mutant plants, genotyping was performed, followed by control of remaining endogenous transcript.

Figure 3.3.5: Gene models and localization of *T*-DNA insertion lines of *AtPSKR2*

Black triangle represent a T-DNA insertion, whereas the arrow represents the corresponding direction of the insertion. The white region corresponds to the promoter region. The exact positions of *T*-DNA insertion is annotated with numbers, where 0 corresponds to the start codon.

Table 3.3.1: Description of the T-DNA insertion used for in vivo analyses of AtPSKR2

Name	Stock name	NACS number	Background ecotype	Polymorphism site
pskr2	SALK_024464	N524464	Col-0	Exon

3.3.2.1 Genotyping

In order to guarantee the homozygocity of the mutant lines, the corresponding genotyping via PCR was performed, taking 8 plants of each mutant line together with gene specific primer pairs (Table 7.1.1). Corresponding *T*-DNA specific fragments were amplified only from the *pskr2* mutants, indicating they carry homozygous gene defects (Figure 3.3.6). The control of remaining endogenous transcript levels of this exact mutant line was published in 2007 resulting to be a null allele (Amano *et al.* 2007).

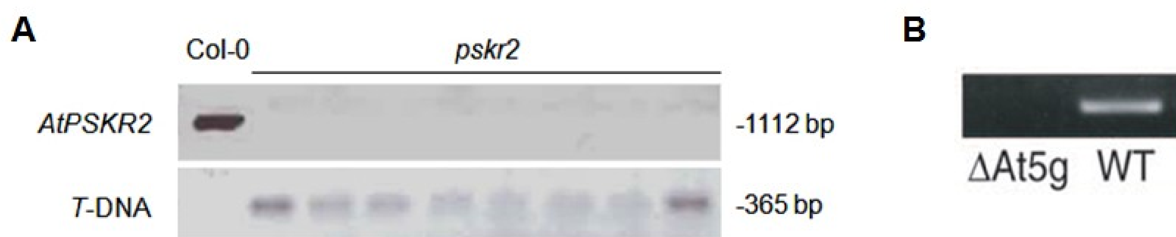

Figure 3.3.6: Genotyping PCRs of *AtPSKR2* and RT-PCR for controlling PSKR2 endogenous levels (picture modified from Amano *et al.* 2007)

(A) Plant material of 6-week old *Arabidopsis* plants was collected and genomic DNA was extracted, using the PCI method. The *PSKR2* specific fragments were amplified using the following primer pairs: for *pskr2* N524464-LP and N524464-RP. For *T*-DNA insertion specific fragments the following primers were used: for *pskr2* Lba1 and N524464-RP. (Table 7.1.1). (B) ΔAt5g corresponds to *AtPSKR2*. The absence of corresponding mRNA for loss-of-function mutant was verified by RT-PCR using gene-specific primers (Amano *et al.* 2007).

3.3.3 Phenotypic characterization of AtPSKR2 loss-of-function mutant

pskr2 seedlings exhibited a normal growth, under the previously described growth conditions (Chapter 2.2.1), and develop rosette leaves phenotypically indistinguishable from wild type plants, observations that were also reported by Amano (Amano *et al.* 2007). These mutant plants did not present the premature senescence phenotype of *pskr1-3* plants.

3.3.3.1 Analysis of bacterial pathogen growth in *pskr2* plants

Based on observations from Microarray data, *AtPSKR2* and *AtPSY1R* seem not to be recruited by *Arabidopsis* when challenged with biotic stressors. Because *pskr1-2*, *pskr1-3* and *pskr1-4* display a convincingly increased resistance to virulent *Pto* DC3000, *pskr2* was also tested with the same *Pseudomonas* strain.

The corresponding bacterial growth was followed until day 4. Virulent *Pto* DC3000 colonizes wild type plants satisfactorily, which is translated as an increased bacterial growth, observable even after 1 dpi (Figure 3.3.7). Bacterial growth in *pskr2* mutants resembles the one from wild type plants at 1, 2 and 4 dpi.

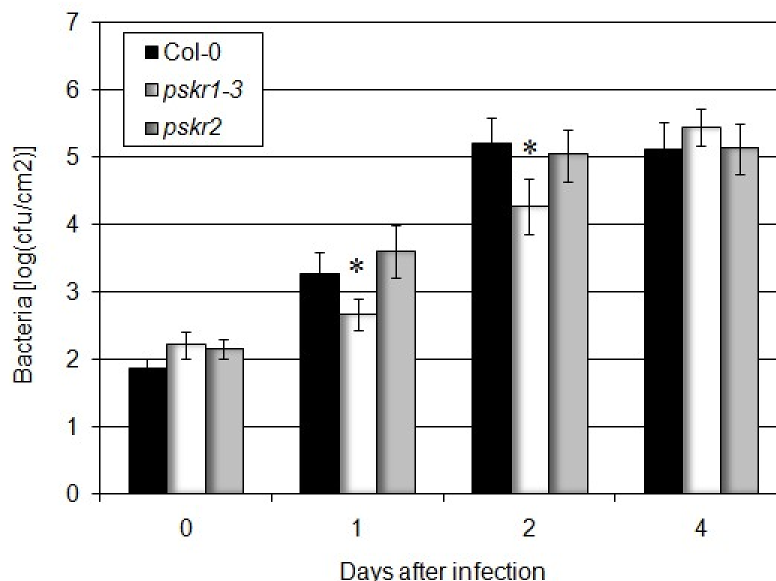

Figure 3.3.7: Bacterial growth curves after *Pto* DC3000 inoculation

5 week-old plants were used to determine the number of grown bacteria at timepoints 0, 1, 2 and 4 days post infection. Wild type and the corresponding mutants were inoculated with 10^4 cfu/ml *Pto* DC3000 suspension (Chapter 2.3.3). Error bars represent the standard deviation of at least 6 replicates. These graphics represent the tendency observed in two independent experiments.

3.3.4 Promoter analysis of *PSKR2* and *PSY1R* compared to *PSKR1*

Using the PLACE signal scan Programm (Higo *et al.* 1999), the promoter region of *AtPSKR2* and *AtPSY1R* was compared to the one from *AtPSKR1* (Chapter 3.2.7). The homologs sequences consisted of 1534 bp each upstream of the initiation codon. Following, the common *Arabidopsis* cis-promoter elements from these three genes are shown (Table 3.3.2). Unique *Arabidopsis* promoter elements were also searched in each gene and are presented in the following Tables 3.3.2, 3.3.3, 3.3.4 and 3.3.5.

There are only 3 *Arabidopsis cis*-elements that are unique in the *AtPSKR1* promoter compared with its two other homologs. These unique elements are related to various stress stimuli (dehydration, cold and drought) (Table 3.3.3). Alternatively, *AtPSKR2* contains in its promoter 6 unique elements. Some of these are related to developmental functions, and interestingly it contains two elements found in auxin-response genes in Arabidopsis (Table 3.3.4). *AtPSY1R* possesses 6 unique *Arabidopsis* cis-elements, as well, but related rather to light induction or to water stress (Table 3.3.5). Besides its differences, there are also several common elements shared by these three homologous genes (Table 3.3.2), like ARR1AT that is the binding site of a regulator implicated in the cytokinin signalling pathway (Argyros *et al.* 2008) and whose number constitutes the highest repeated Arabidopsis element in these three genes. Another repeatedly common element is the GATA-box, which is a light –

dependent cis-regualtory element (Teakle *et al.* 2002; Reyes *et al.* 2004). Interestingly, whereas all three gene promoters contain W-boxes, required elements for binding of WRKY transcription factor proteins (Eulgem *et al.* 2000), the AtPSY1R W-box number is less than the half of the other two genes. In general terms, it could be drawn that, regarding the number of repetitions or appearances of its common promoter elements, the promoter region of *AtPSKR1* is highly comparable to the one from *AtPSKR2*, with the *AtPSY1R* promoter rather differing from the other two promoter sequences.

Table 3.3.2: List of common *Arabidopsis* promoter *cis*-elements among *PSKR1*, *PSKR2* and *PSY1R*

Common *cis*-elements in PSKR1, PSKR2 and PSY1R	Function	Rep. in PSKR1	Rep. in PSKR2	Rep. in PSY1R
ACGTATERD1 (ACGT)	ACGT sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis (Simpson *et al.* 2003)	6	4	2
ARR1AT (NGATT)	ARR1 binding element in Arabidopsis; ARR1 is a type-B response regulator, implicated in the cytokinin signalling pathway (Argyros *et al.* 2008); N=G/A/C/T	19	20	17
CCAATBOX1 (CCAAT)	Acts cooperatively with HSEs (heat shock elements) to increase the hs (heat shock) promoter activity (Haralampidis *et al.* 2002; Wenkel *et al.* 2006)	2	6	4
GATA-box (GATA)	Light-dependent *cis*-regulatory element (Teakle *et al.* 2002; Reyes *et al.* 2004)	11	11	17
MYBCORE (CNGTTR)	Binding site for all animal MYB and at least two plant MYB proteins ATMYB1 and ATMYB2; ATMYB2 is involved in regulation of genes that are responsive to water stress in *Arabidopsis*	4	3	2
MYC recognition site (CANNTG)	MYC recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in *Arabidopsis*; binding site of ATMYC2 (previously known as rd22BP1); N=A/T/G/C; (Chinnusamy et al., 2004);)	6	12	10
POLASIG1 (AATAAA)	PolyA signal near upstream elements in *Arabidopsis* (Loke et al. 2005)	7	6	12
RAV1AAT (CAACA)	Binding consensus sequence of *Arabidopsis* transcription factor, RAV1 (Related to ABI3/VP1) (Kagaya *et al.* 1999) whose expression is stimulated by various external or environmental cues (low temperature, darkness, wounding, drought, salt stress and pathogen attack) (Fowler and Thomashow 2002; Lee *et al.* 2005; Sohn *et al.* 2006; Kagaya and Hattori 2009)	6	4	1

T-box (ACTTTG)	"Tbox" found in the *Arabidopsis thaliana* GAPB gene promoter; mutations in the "Tbox" resulted in reductions of light-activated gene transcription; GAPB encodes the B subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase(GADPH) of *Arabidopsis (Chan et al. 2001)*	2	3	1
WBOXATNPR1 (TTGAC)	"W-box" found in promoter of Arabidopsis thaliana NPR1 gene; They were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins (Eulgem *et al.* 2000; Chen and Chen 2002; Xu *et al.* 2006)	7	7	3

Table 3.3.3: List of *Arabidopsis* promoter *cis*-elements found only in *AtPSKR1*

Cis-element only in PSKR1 (Sequence)	Function	Repetitions
DRECRTCOREAT (RCCGAC)	Core motif of DRE/CRT (dehydration-responsive element/C-repeat) *cis*-acting element found in many genes in *Arabidopsis* and in rice (Susuki *et al.* 2005); R=G/A	1
CCA1ATLHCB1 (AAMAATCT)	Binding site of the CCA1 protein (myb-related transcription factor) that interacts with the light harvesting chlorophyll protein Lhcb1*3 of *Arabidopsis thaliana*; related to regulation by phytochrome (Wang *et al.* 1997)	2
LTRECOREATCOR15 (CCGAC)	Core of C/DRE sequence, which is essential for transcriptional activation in response to cold, drought and/or high salt treatments (Yamaguchi-Shinozaki and Shinozaki 1994)	1

Table 3.3.4: List of *Arabidopsis* promoter cis-elements found only in *PSKR2*

Cis-elements only in PSKR2 (sequence)	Function	Repetitions
ARFAT (TGTCTC)	ARF (auxin response factor) binding site found in the promoters of primary/early auxin response genes of *Arabidopsis* (Ulmasov *et al.* 1999; Nag *et al.* 2005)	3
SURECOREATSULTR11 (GAGAC)	Core of SURE (sulfur-responsive element) found in the promoter of SULTR1;1, a high-affinity sulfate transporter gene in *Arabidopsis*; SURE contains auxin response factor (ARF) binding sequence; involved in sulphur deficiency response (Maruyama-Nakashita *et al.* 2005)	4
L1BOXATPDF1 (TAAATGYA)	"L1 box" found in promoter of *Arabidopsis* PROTODERMAL FACTOR1 (PDF1) gene; involved in L1 layer-specific expression (Abe *et al.* 2001); Y=C/T	1

LEAFYATAG (CCAATGT)	Target sequence of LEAFY (transcription factor expressed throughout the flower) in the intron of AGAMOUS gene (with roles in specifying organ fate and in limiting stell cell proliferation) in *Arabidopsis* flowers (Lohmann *et al.* 2001)	1
PREATPRODH (ACTCAT)	Core sequence, named PRE (Pro- or hypoosmolarity-responsive element), necessary for efficient expression of *Pro*DH (Proline dehydrogenase) in response to L-Pro and hypoosmolarity (Satoh *et al.* 2002); similar to GCN4 motif; ATB2-binding site (Satoh *et al.* 2004)	1
ZDNAFORMINGATCAB1 (ATACGTGT)	"Z-DNA-forming sequence" found in the Arabidopsis chlorophyll a/b binding protein gene (cab1) promoter; involved in light-dependent developmental expression of the gene (Ha and An 1988); know also as "Z-box"; promoters containing the Z-box can respond to a broad spectrum of light (Yadav *et al.* 2002)	1

Table 3.3.5: List of A*rabidopsis* promoter *cis*-elements found only in *AtPSY1R*

Cis-elements only in PSY1R (sequence)	Function	Repetitions
ACGTTBOX (AACGTT)	"T-box" , type of ACGT cis-elements that are present in plant genes regulated by diverse environmental and physiological cues (Foster *et al.* 1994)	2
MYB2AT (TAACTG)	Binding site for ATMYB2, an Arabidopsis MYB homolog; ATMYB2 binds oligonucleotides that contained a consensus MYB recognition sequence (TAACTG); ATMYB2 is involved in regulation of genes that are responsive to water stress in *Arabidopsis* (Urao *et al.* 1993)	2
SBOXATRBCS (**CACCTCCA)**	"S-box" conserved in several rbcS (ribulose-1,5-bisphosphate carboxylase small subunit) promoters in *Arabidopsis*; ABI4 (Abscisic acid insensitive-4) binding site; important for the sugar and ABA responsiveness of CMA5 (Conserved modular arrangement 5; the shortest native light-responsive element of a photosynthetic gene promoter) (Acevedo-Hernandez *et al.* 2005)	1
SORLIP1AT (GCCAC)	One of the SORLIPs (Sequences Over-Represented in Light-Induced Promoters) in *Arabidopsis*; over-represented in light-induced cotyledon and root common genes and root-specific genes (Jiao *et al.* 2005)	2
SORLREP2AT (ATAAAACGT)	One of SORLREPs (Sequences Over-Represented in Light-Repressed Promoters) in *Arabidopsis* (Hudson and Quail 2003)	1
SREATMSD (TTATCC)	SRE (Sugar-repressive element) found in 272 of the 1592 down-regulated genes after main stem decapitation in *Arabidopsis* (Tatematsu *et al.* 2005)	1

3.3.5 Triple mutant of *AtPSKR1* and its homologs

In collaboration with the Franz Tax research group, from Arizona University, triple loss-of-function mutants of *PSKR1* with *PSKR2* and *PSY1R* were provided in order to clarify the homologs' implication in pathogen response, and to investigate the existence of a possible redundance in response to pathogen attack, since single *pskr2* mutants did not give a clear insight into the *pskr1* pathogenic phenotype. Below is a list of the obtained multiple mutants (Table 3.3.6).

Table 3.3.6: Description of multiple loss-of-function mutants of *PSKR1*, *PSKR2* and *PSY1R* obtained from the Tax Research group

Name	AGI	Stock Name
3X	At2g02220 X At1g72300 X At5g53890	SALK_008585 X SALK_072802 X SALK_024464

Triple loss-of-function mutants were genotyped by amplification of gene specific fragments using specific primer pairs (Figure 3.3.8). Plants, which did not display gene specific fragments for these genes, were propagated for further experiments. Triple mutant plants exhibited a reduced growth and a premature senescence (observations also described by Amano *et al.* 2007) compared to *pskr1* mutants, that is to say, triple mutant plants were clearly distinguishable from *pskr1* and wild-type plants because they remained smaller throughout their whole life cycle. Cross-complementation tests proved that AtPSKR1 and AtPSY1R mediate a signalling pathway by two distinct ligands, which redundantly contribute to cellular proliferation and plant growth (Amano *et al.* 2007).

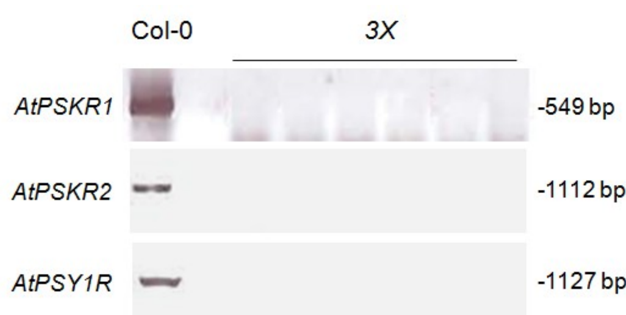

Figure 3.3.8: Genotyping PCRs of triple loss-of-function mutant of PSKR1, PSKR2 and PSY1R

Plant material of 5-week old Arabidopsis plants was collected and genomic DNA was isolated, using the PCI method (Chapter 2.5.1). The *PSKR1* gene specific fragments were amplified using the following primer pairs: At2g02220-F and At02220-R; for *PSKR2*: N524464-LP and N524464-RP; for *PSY1R*: N669833-LP and N669833-RP (see Table 7.1.1in Appendix for primer sequences).

3.3.5.1 Analysis of bacterial pathogen growth in triple mutants of *AtPSKR1*, *AtPSKR2* and *AtPSY1R*

Here the aim is to give some insight in the contribution of PSKR2 and PSY1R in the *pskr1-3* bacterial phenotype upon *Pto* DC3000 infection. Plants lacking all three functional receptors might cope differently with the virulent bacteria than plants lacking only one functional receptor. Here, using triple mutant plants, functional redundancy between *PSKR1*, *PSKR2* and *PSY1R* would be bypassed.

Wild type and mutant plants were grown and treated under the previously described conditions (Chapter 2.2.1). Bacterial growth of virulent *Pto* DC3000 was followed until 4 dpi.

From previous results (Chapter 3.3.3.1), mutant plants of the closest related receptor from *PSKR1*, *PSKR2*, did resemble wild-type bacterial growth. However here (Figure 3.3.9), triple loss-of-function mutants showed an enhanced resistance to *Pto* DC3000 when compared to wild-type plants; but when compared to *pskr1* mutants, bacterial growth in triple mutants was similar at 1 and 2 dpi, but interestingly, the growth remained reduced even until 4 dpi, implying perhaps the need of the other receptors for a longer termed effect, since *pskr1* mutant plants returned mostly to wild-type levels of resistance at 4 dpi. Thus, it would appear that *PSKR1* together with *PSKR2* and PSY1R might regulate negatively the defence process against *Pto* DC3000.

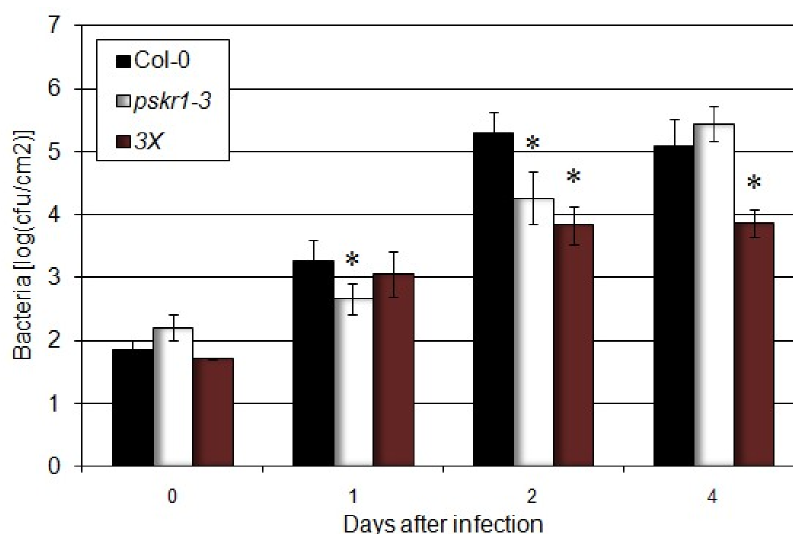

Figure 3.3.9: Bacterial growth curves after *Pto* DC3000 inoculation

5 week-old plants were used to determine the number of grown bacteria at time points 0, 1 and 2 days post infection. Wild type and the corresponding mutants were inoculated with 10^4 cfu/ml *Pto* DC3000 suspension (Chapter 2.3.3). Error bars represent the standard deviation of at least 6 replicates. This graphic represents the tendency observed in 4 independent experiments.

3.3.5.2 Analysis of disease symptoms caused by the necrotroph *A. brassicicola* triple mutants of *AtPSKR1*, *AtPSKR2* and *AtPSY1R*

Here the aim is to observe the combined effects of the impaired receptors *AtPSKR1*, *AtPSKR2* and *AtPSY1R* when challenged with the necrotroph *A. brassicicola* in order to figure out if the pathogenic phenotype of *pskr1* mutants is due uniquely to *AtPSKR1* or is rather a result of a combined work with its highly identical homologs.

Fungal assays were performed as described in Chapter 2.3.5. Disease symptoms were followed until day 10 post infection. Wild type plants did not surrender to the fungal infection, as expected. The triple mutant plants appeared to have more severe disease symptoms than *pskr1* plants (Figure 3.2.10).

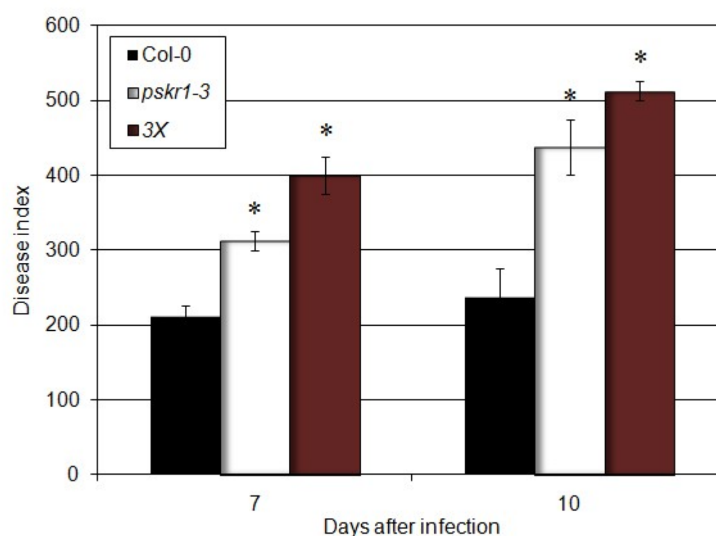

Figure 3.3.10: Disease development on *Arabidopsis* triple mutants of *PSKR1* and its closest homologs inoculated with *A. brassicicola*

5-week-old plants were drop-inoculated with a suspension of $5x10^5$ spores/ml of *Alternaria brassicicola*. The symptom development was followed based on a score system until 10 dpi. This graphic represents the tendency observed in three independent experiments.

The Table 3.3.7 summarizes the previous results and gives a general overview of the behavior from the single and multiple mutants when treated with the virulent *Pto* DC3000 and the necrotroph *A. brassicicola*.

Table 3.3.7: Phenotypic summary from single and multiple loss-of-function homologes of *PSKR1*

	Infection with *Pto* DC3000	Infection with *A. brassicicola*
pskr1	More resistant than wild type	More susceptible than wild type
pskr2	Wild type phenotype	(not tested)
3X	Similar to *pskr1* phenotype, with enhanced and longer lasting resistance	Similar to *pskr1* phenotype, with a more severe susceptibility

3.4 PSK-α precursors (prepro-phytosulfokines)

PSK-α is produced by enzymatic processing of an ~80-amino acid precursor peptide that has a secretion signal at its N-terminus (Yang *et al.* 1999). PSK precursor genes are redundantly distributed throughout the genome (Yang *et al.* 2001). The only conserved amino acids within PSK precursors are the five amino acid PSK domain, YIYTQ, and several conserved residues immediately upstream of the PSK domain (including dibasic amino acid residues).

There are 5 paralogous PSK precursor genes in Arabidopsis, *AtPSK1*, *AtPSK2*, *AtPSK3*, *AtPSK4* and *AtPSK5* (Yang *et al.* 2001) (Matsubayashi *et al.* 2006); they are expressed in a variety of tissue (roots, leaves, stems, flowers, siliques and calluses). *AtPSK1* is only expressed in roots. *AtPSK2* and *AtPSK4* have the strongest expression in leaves, and *AtPSK2*, *AtPSK4* and *AtPSK5* are more strongly expressed in lower mature leaves. *AtPSK4* is highly upregulated upon mechanical wounding (Matsubayashi *et al.* 2006). The fact the PSK precursors are expressed in almost all plant tissues but each with a different pattern might indicate also different uses for PSK production depending on specific factors at specific timepoints. Therefore, microarray data from each PSK precursor was analysed and pathogenic assays were performed in order to find a pathogenic phenotype that might relate to the *pskr1* mutants phenotype, thereby indicating an activation of the PSK receptor caused by one or more specific PSK precursors.

3.4.1 Expression of PSK precursors after bacterial infection and elicitor treatment

Microarray data from Genevestigator (Zimmermann *et al.* 2004) was analysed for each PSK precursor in *Arabidopsis*. *PSK2* and *PSK4* are the strongest induced genes after bacterial stress when compared with its respective control. *PSK3* and *PSK5* transcript accumulation is basically not altered after bacterial treatment when compared to the control (Figure 3.4.1). *PSK1* basal expression in leaves is extremely low, which correlates with the fact that *PSK1* is mainly expressed in roots. *PSK2* is upregulated after bacterial infection and interestingly it shows also a reduction of transcript accumulation at 6h post infection and subsequently a rise at 24h, resembling the induction pattern of *AtPSKR1* after *Pto* DC3000 infection. *PSK2* is also upregulated after infection with the avirulent *Pto* avrRpm1 and after infection with the deficient TTSS *Pto* hrcC$^-$ specially 24h post infection. More significant is the transcript accumulation of *PSK2* after infection with the non-host Pph, especially at 24h post infeciton. *PSK4* is the highest upregulated gene after bacterial stress from the PSK precursors, reaching a maximum induction always at 24 h post infection, with a maximum value of twelvefold increase after infection with the non-host Pph. However it is worth mentioning that a high upregulation of *PSK4* is also achieved by $MgCl_2$ (control) for the first two timepoints, indicating perhaps an upregulation due to wounding (Figure 3.4.1). The corresponding semiquantitative RT-PCRs represent the amount of transcript accumulation of each precursor gene, with the PSK1 transcript being the weakest and with PSK4 transcript being the strongest, which correlates with the Microarray data. Overall, a different induction pattern can be observed of each precursor gene after bacterial infection, suggesting perhaps independent regulatory roles in pathogen defence responses.

When upregulation of PSK precursors after elicitor treatment was analysed, *PSK4* was the highest upregulated gene, followed by *PSK2* and *PSK1*, with *PSK3* and *PSK5* hardly altering its transcript accumulation (Figure 3.4.2). *PSK2* and *PSK4* are also the strongest upregulated genes after infection with the oomycete *P. infestans* and the necrotroph fungus *B. cinerea* (Appendix, Figure 7.5.1).

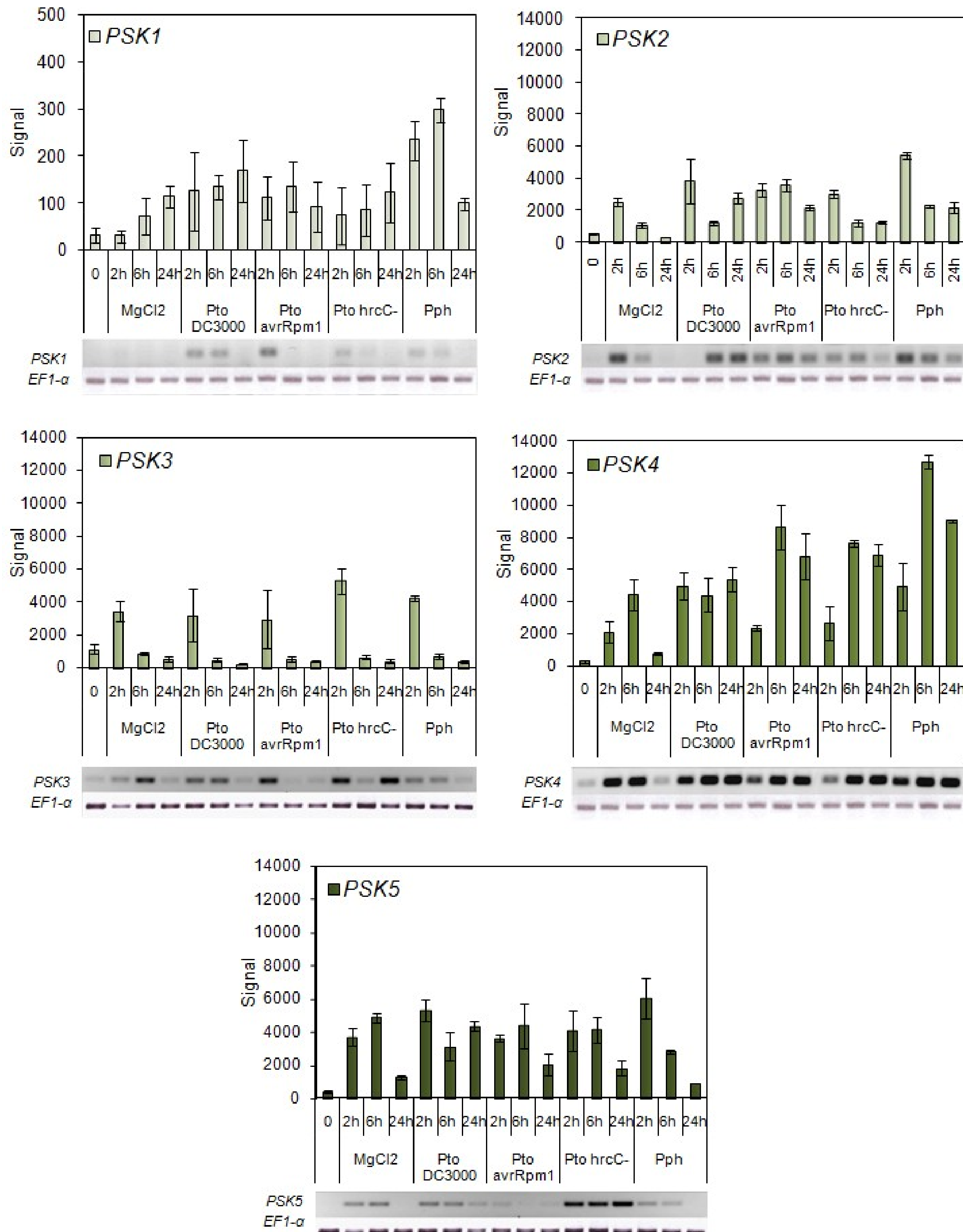

Figure 3.4.1: Microarray data of wild type plants after infiltration with various strains of *Pseudomonas*

Col-0 plant material was harvested at 2, 6 and 24h after inoculation with 10^8 cfu/ml *Pto* DC 3000, *Pto* avrRpm1, *Pto* hrcC- and *Pph* together with $MgCl_2$ (control); correspondent RNA was isolated (Chapter materials). 0h represents untreated plants. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00106, AtGenExpress. Error bars represent standard deviation three replicates. A semi-quantitative RT-PCR (below each gene induction graphic) was performed using specific primers for corresponding PSK precursor genes (see Table 7.1.1 in Appendix for primer sequences). The total amount of cDNA was standardized with the elongation factor EF1α. (Fragment size 600 bp).

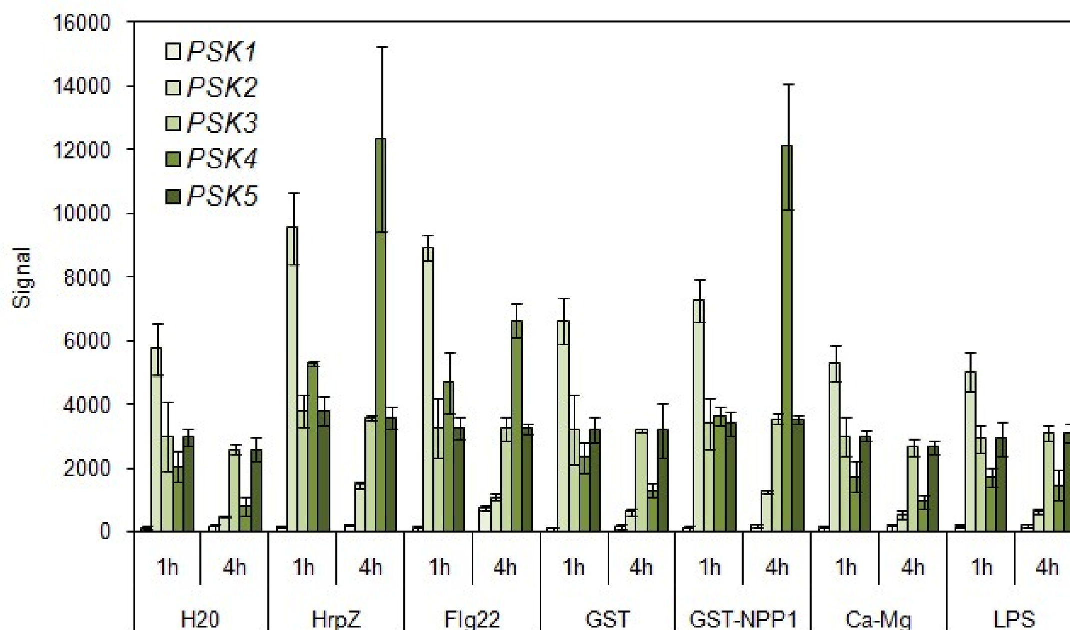

Figure 3.4.2: Expression of PSK-α precursors after various biotic treatment

Plant material of Col-0 plants was harvested at 1 and 4 h after infiltration with water (control for HrpZ and Flg22), 10 µM HrpZ, 1 µM Flg22, 1 µM GST (control for GST-NPP1), 1 µM GST-NPP1, 1mM $CaCl_2$ + 2.5 mM $MgCl_2$ (control for LPS), 100 ug/mL LPS. RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004); Experiment AT-00107, AtGenExpress. Error bars represent the standard deviation of three replicates. The data were normalized by GCOS normalization.

3.4.2 Analysis of *T*-DNA insertion lines from PSK-α precursors

In order to find out which of the phytosulfokine precursors is involved in pathogen defence responses that may act together with phytosulfokine receptor, we obtained various *T*-DNA insertion lines from the European *Arabidopsis* Stock Center (NASC) for three PSK precursors, such as SALK lines (Alonso *et al.* 2003) and SAIL lines (Sessions *et al.* 2002), which consist of loss-of-function mutants created by insertion of a *Agrobacterium T*-DNA so that the sequence of corresponding gene is disrupted (Figure 3.4.3). Mutant lines corresponding to only *PSK1*, *PSK3* and *PSK5* were available during the realization of this work.

Mutant lines impaired in *PSK1*, *PSK3* and *PSK5* were named here *psk1*, *psk3* and *psk5,* correspondingly (Table 3.4.1). With aid of the previously mentioned mutants the effect of a non-functional PSK precursor gene in *Arabidopsis* should be investigated when confronted with diverse pathogens and stress circumstances. Therefore the proper genotyping of each mutant line was performed.

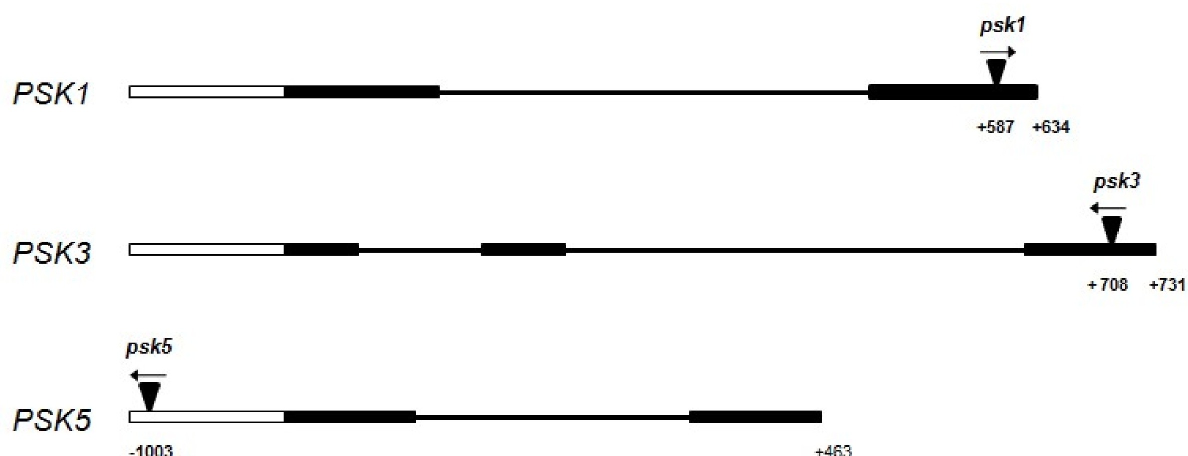

Figure 3.4.3: Gene model and localization of *T*-DNA insertion lines of *PSK1*, *PSK3* and *PSK5*

The white region corresponds to the promoter region of each gene. The black blocks represent exons and the black lines represent introns. Each black triangle represents a *T*-DNA insertion, whereas the arrows represent the corresponding direction of the insertions. The exact positions of each *T*-DNA insertion are annotated with numbers, where 0 corresponds to the start codon.

Table 3.4.1: Description of *T*-DNA insertions used for *in vivo* analyses of *PSK1, PSK2 and PSK5*

Name	Stock name	NACS number	Background ecotype	Polymorphism site
psk1	SALK_036304	N536304	Col-0	Exon
psk3	SAIL_378_F03	N817441	Col-0	Exon
psk5	SALK_043834	N543834	Col-0	Promoter

3.4.2.1 Genotyping

In order to guarantee the homozygocity of the mutant lines, the corresponding genotyping via PCR was performed, taking 8 plants of each mutant line together with gene specific primer pairs (Table 7.1.1). Corresponding *T*-DNA specific fragments were amplified only from the *psk1*, *psk3* and *psk5* mutants, indicating that they all carry homozygous gene defects (Figure 3.4.4).

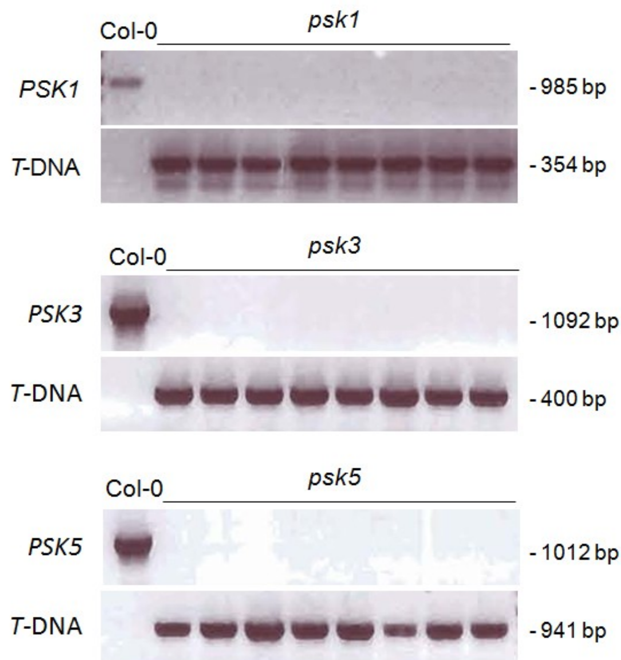

Figure 3.4.4: Genotyping PCRs of the various PSK precursors mutant plants

Plant material of 6-week old *Arabidopsis* plants was collected and genomic DNA was extracted, using the PCI method (Chapter 2.5.1). The corresponding gene specific fragments were amplified using the following primer pairs: for *psk1* N536304-LP and N536304-RP; for *psk3* N817441-LP and N817441-RP; for *psk5* N543834-LP and N543834-RP. For *T*-DNA insertion specific fragments the following primers were used: for: *psk1* b-Lba1 and N536304-RP; for *psk3* Sail_LB and N817441-RP; for *psk5* b-Lba1 and N543834-LP (Appendix, Table 7.1.1).

3.4.3 Phenotypic characterization of *PSK1*, *PSK2* and *PSK3* impaired T-DNA lines

psk1, *psk3* and *psk5* exhibit a normal growth and develop rosette leaves phenotypically indistinguishable from wild type under the previously described growth conditions (Chapter 2.2.1). The *psk1*, *psk3* and *psk5* seedlings grow at almost the same rate as wild-type seedlings, flower normally and complete the normal life cycle, and did not present an earlier senescence phenotype compared to wild-type plants.

3.4.3.1 Analysis of bacterial pathogen growth in *psk1*, *psk2* and *psk3* plants

Here, virulent bacterial growth was analized using the strain *Pto* DC3000 in the correspondent *psk1*, *psk2* and *psk3* mutant plants. The aim was to find out if any of the above mentioned precursors might have a similar response to bacteria like the main PSK receptor gene (Chapter 3.2.4.1) and thus, be possibly related to it in this bacterial defence response.

Mutant plants impaired in PSK1, PSK2 or PSK3 showed the same susceptibility to *Pto* DC3000 infection than wild-type plants, i.e. no difference in bacterial growth among this lines and the control line was observed when bacteria was inoculated on leaves (Figure 3.4.5, A) or when bacteria was sprayed (Figure 3.4.5, B).

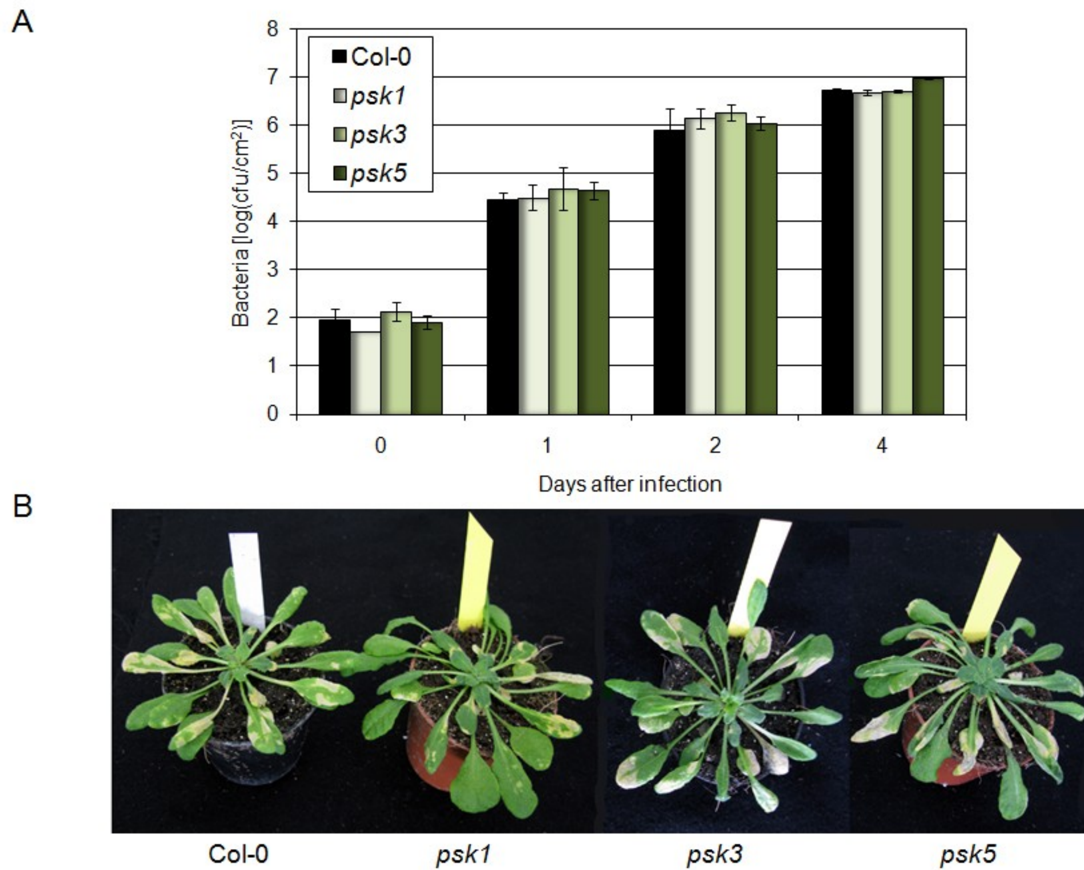

Figure 3.4.5: Bacterial growth after inoculation with *Pto* DC3000

(A) 5 week-old plants were used to determine the number of grown bacteria at timepoints 0, 1, 2 and 4 days post infection. Wild type and the corresponding mutants were inoculated with 10^4 cfu/ml *Pto* DC3000 suspension (Chapter 2.3.3). The error bars represent the standard deviation of at least 6 replicates. These graphics represent the tendency observed in one independent experiment. (B) 5 week-old plants from wild type and the corresponding mutants plants were sprayed with $1_{OD=600\ nm}$ *Pto* DC3000 suspension and the macroscopic symptom development shown above correspond to 10 dpi.

3.4.3.2 Analysis of disease symptoms caused by necrotrophic fungi in *psk1*, *psk3* and *psk5* plants

In order to investigate if these mutations affect the response to the necrotrophic fungus *A. brassicicola* and *Botrytis cinerea* in the same or similar way than in *pskr1* mutant plants, the aboved mentioned mutants together with wild-type plants were challenged and the disease symptoms were analysed (Chapter 2.3.5). After application of *A. brassiccicola* spores on Col-0 plants (controls) not-spreading lesions appear which are typical of an incompatible interaction. The same phyenotype as in wild-type plants was observed in all three PSK precursor mutants (Figure 4.3.6, A and B). In the case of *B. cinerea* spores application on control plants or mutants, the same response was observed in all tested plants, i.e. expanding necrotic lesions surrounding the application site, with a chlorosis zone around the

developing lesion, with disease symptoms from the PSK precursor mutants being indistinguishable from wild-type plants (Figure 3.4.6, C).

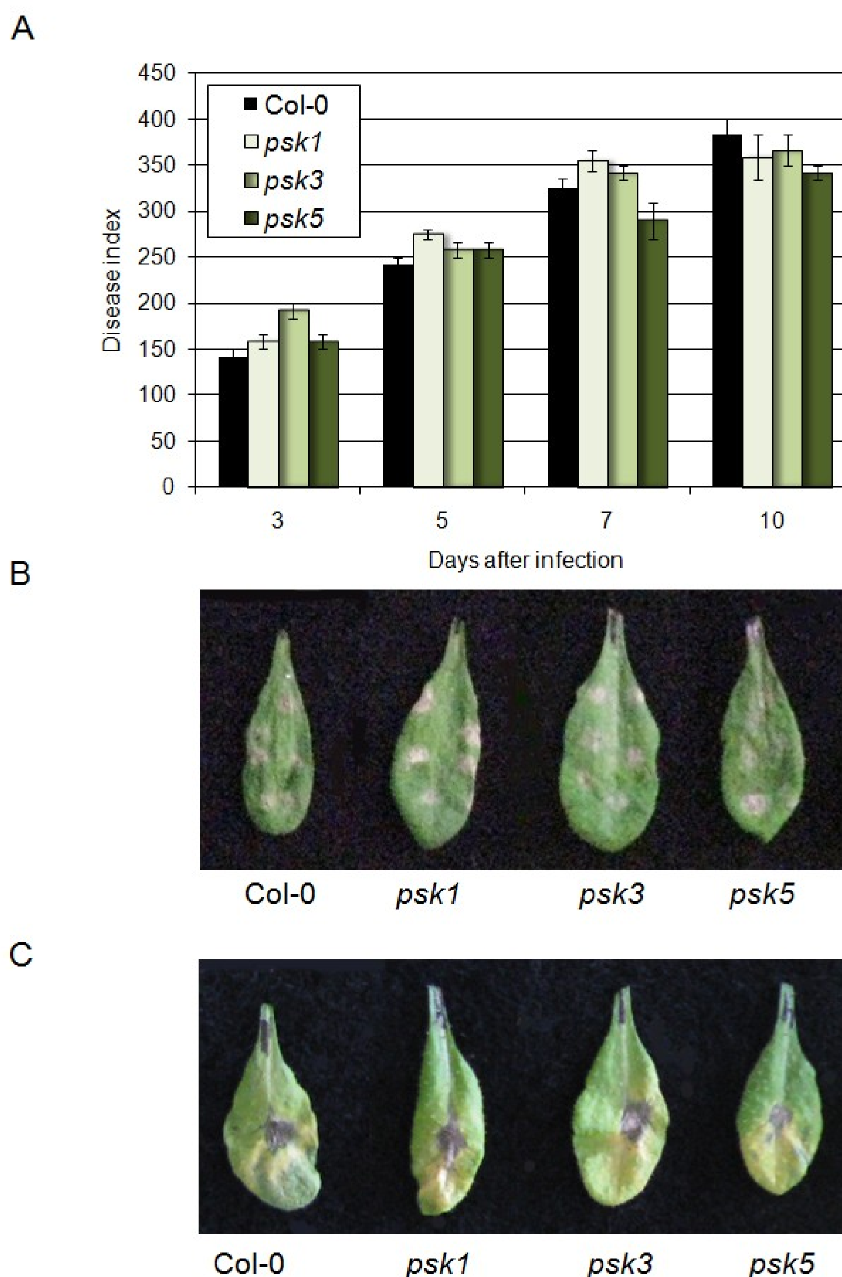

Figure 3.4.6: Disease development on *Arabidopsis psk1*, *psk3* and *psk5* mutants inoculated with necrotrophic fungi

5-week-old plants were inoculated with a suspension of $5x10^5$ spores/ml of *Alternaria brassicicola* (Chapter 2.3.5) and $2,5x10^5$ spores/ml of *Botrytis cinerea* (Chapter 2.3.5) (A) Symptom development was followed based on a score system until 10 dpi. Graphic represents tendency observed in two independent experiments. (B) Pictures of detached leaves of *psk1*, *psk3* and *psk5* mutant plants infected with *Alternaria brassicicola* compared to the corresponding control plants. Pictures were taken at 10 dpi. (C) Pictures of detached leaves of *psk1*, *psk3* and *psk5* mutant plants infected with *B. cinerea* compared to corresponding control plants. Pictures taken at 4 dpi.

3.4.3.3 Upregulation of PSK precursor genes after *A. brassicicola* infection

In order to clarify which PSK precursor gene or genes may regulate positively the defence pathway against *A. brassicicola* together with the *PSKR1* receptor, RT-PCRs were performed from wild-type plants previously infected with *A. brassciciola* (drop inoculation) (Figure 3.4.7). *PSK1* and *PSK3* genes were not induced in leaf tissue neither at 0hr nor after *A. brassicicola* infection, therefore are not shown. *PSKR1* is induced at early stages of infection with *A. brassicicola*, and *PSK2* resembles its induction in wild type plants. *PSK4* is lesser induced after infection but stronger after initial H_2O, indicating perhaps a wound stress response. *PSK5* is vaguely induced therefore not significant. This might indicate that when wild type plants are infected with the necrotroph *A. brasscicola*, the receptor *PSKR1* and mainly *PSK2* and perhaps *PSK4* (wound-induced upregulated) are activated, indicating their participation in plant defence responses: perhaps more PSK-α is secreted, by one or two precursors, and that in turn activates the receptor that might be involved in a defence pathway in *Arabidopsis*.

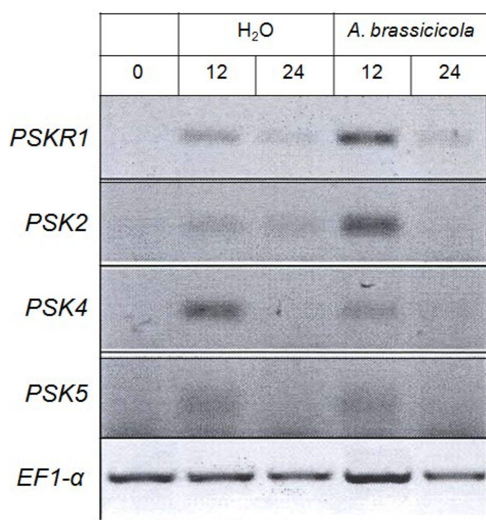

Figure 3.4.7: Upregulation of PSK precursor genes after infection with the necrotroph fungus *A. brassicicola*

5 week-old Col-0 plants were inoculated with a $5x10^5$ spores/ml *A. brassicicola* suspension. At mentioned timepoints (hr) plant material was harvested and semiquantitative RT-PCR was performed using specific primers for PSKR1, PSK1 (At1g13590-5' and AT1g13590-3'), PSK2 (At2g22860-for and At2g22860-rev), PSK3 (At3g44735-for and At3g44735-rev), PSK4 (At3g49780-5' and At3g49780-3') and PSK5 (At5g65870-5' and At5g65870-3') (See Table 7.1.1 in Appendix for primer sequences). Total amount of cDNA was standardised with the elongation factor EF1α.

3.4.4 Promoter analysis of PSK precursor genes

Using the PLACE signal scan Programm (Higo *et al.* 1999) for analysing the promoter region of *PSK1, PSK2, PSK3, PSK4 and PSK5*, consisting of 1125 bp each upstream of the initiation codon, a number of developmental- and abiotic-induced promoter *cis*-elements were found (Table 3.4.2).

Table 3.4.2: List of common *Arabidopsis* promoter *cis*-elements found in *PSK1*, *PSK2*, *PSK3*, *PSK4* and *PSK5*

Cis-element (Sequence)	Function	*PSK1*	*PSK2*	*PSK3*	*PSK4*	*PSK5*
ACGTATERD1 (ACGT)	ACGT sequence required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis (Simpson *et al.* 2003)	4	8	4	4	6
ARR1AT (NGATT)	ARR1 binding element in Arabidopsis; ARR1 is a type-B response regulator, implicated in the cytokinin signalling pathway (Argyros *et al.* 2008); N=G/A/C/T	15	15	10	10	9
CARGCW8GAT (CWWWWWWWWG)	A variant of CArG motif, with a longer A7T rich core; Binding site for AGL15 (AGAMOUS-like 15) (Tang and Perry 2003), a MADS domain protein that preferentially accumulates in developing plant embryos (Perry *et al.* 1996)	2	2	2	2	2
GATABOX (GATA)	Light-dependent *cis*-regulatory element (Teakle *et al.* 2002; Reyes *et al.* 2004)	7	12	13	13	13
GT1CONSENSUS (GRWAAW)	Consensus GT-1 binding site in many light-regulated genes; R=A/G; W=A/T; Intearction between a GT-1 cis-element and a GT-1-like transcription factor plays a role in pathogen- and salt-induced SCaM-4 gene expression in both soybean and Arabidopsis (Park *et al.* 2004).	11	9	16	14	15
IBOXCORE (GATAA)	"I box" conserved sequence upstream of light-regulated genes (Terzaghi and Cashmore 1995)	2	5	7	4	5
MYB1AT (WAACCA)	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes in Arabidopsis; W=A/T (Abe *et al.* 2003)	2	3	1	2	3
MYB and MYC recognition sites	*cis*-elements in the drought-induced expression of the rd22 gene in *Arabidopsis* (Abe et al. 1997)	4	10	15	17	7
POLASIG1 (AATAAA)	PolyA signal near upstream elements in *Arabidopsis* (Loke et al. 2005)	5	6	4	9	5
RAV1AAT (CAACA)	Binding consensus sequence of *Arabidopsis* transcription factor, RAV1 (Related to ABI3/VP1) (Kagaya *et al.* 1999) whose expression is stimulated by various external or environmental cues (low temperature, darkness, wounding, drought, salt stress and pathogen attack) (Fowler and Thomashow 2002; Lee *et al.* 2005; Sohn *et al.* 2006; Kagaya and Hattori 2009)	3	4	1	3	2
WBOXATNPR1 (TTGAC)	W-box found in promoter of ATNPR1; recognized specifically by SA-induced WRKY DNA binding proteins (Eulgem *et al.* 2000; Chen and Chen 2002)	5	6	4	2	4

The promoter region of PSK precursor genes resembles greatly the promoter regions of the PSK-α receptor proteins, AtPSKR1 and ATPSKR2, and the receptor of the sulfated peptide PSY1 (Chapter 3.3.4) in that they all appear greatly composed by Arabidopsis *cis*-elements related to the signalling pathway of cytokinins. Moreover, various environmentally regulated elements are also present in the precursor gene promoters, like dehydration-, light-, drought-regulated elements. Binding sites for RAV1, a transcription factor implicated in the adaptation to a variety of environmental stimuli, including pathogen attack (Sohn *et al.* 2006; Kagaya

and Hattori 2009), are higher in PSK2 than in the other precursors. AtPSKR1 also contains the most binding sites for this transcription factor (Chapter 3.3.4). Interestingly, PSK2 contains also the highest number of W-box copies, followed by PSK1. Cis-acting W-boxes, are present in numerous co-regulated *Arabidopsis* defence gene promoters (Maleck *et al.* 2001), and are recognized by several plant WRKY transcription factors (Eulgem *et al.* 2000). Various WRKY transcription factors act as negative regulators of plant defence whereas others positively modulate this response (Eulgem and Somssich 2007).

3.5 Tyrosylprotein sulfotransferases (TPSTs)

Another component of the PSK-α signalling pathway is the sulfation of the PSK-α precursor. Tyrosine sulfation is a common posttranslational modification found in peptides and proteins synthesized through the secretory pathway of most eukaryotes, including higher plants. This modification is thought to be mediated by an enzyme, tyrosylprotein sulfotransferase (TPST), which catalyzes the transfer of sulphate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the phenolic group of tyrosine. In plants there are only two tyrosine sulfated peptide hormones, PSK and PSY. As with tyrosine-sulfated peptide hormones in animals, tyrosine-sulfation of PSK and PSY is critical for their function (Matsubayashi *et al.* 1996). Recently, an *At*TPST has been identified that is able to catalyze tyrosine sulfation on both PSK and PSY precursor polypeptides in vitro; and showed in general a broad substrate sequence specificity (Komori *et al.* 2009). Therefore it is assumed that there are yet uncharacterized tyrosine-sulfated proteins involved in plant growth and development.

Here a microarray analysis of the sulfotransferase (SOT) family in *Arabidopsis* was performed in order to identify possible candidates responsible for PSK sulfation that are involved in pathogen defence.

3.5.1 Expression of SOTs after bacterial infection

For this analysis, sulfotransferases were divided into two groups: the first group showed signal control levels between 0 and 50; the second group showed levels from 50 upwards. For our purposes the first group was not taken into account for its basal levels were minimal (data not shown). The second group, with a signal higher than 50, was considered as more representative for these microarray data (Table 7.5.1). Table below presents relative values of each gene to its corresponding control.

Interestingly four SOTs appeared significantly upregulated after bacterial infection, whose values are highlighted in color. SOT15 appears highly upregulated after infection of virulent , avirulent or TTSS-deficient bacteria; in a lesser extent SOT15 is upregulated by the non-host bacteria Psph. SOT12 appeared to be as well upregulated specially by the virulent Pto DC3000. SOT16 and SOT17 showed some vague upregulation after infection with the non-host Psph.

Table 3.5 1: Relative values of microarray data from the *Arabidopsis* sulfotransferase family

	Pst DC3000			*Pst* avrRpm1			*Pst* hrcC-			Psph		
	2h	6h	24h	2h	6h	24h	2h	6h	24h	2h	6h	24h
SOT11	0,5	0,7	0,2	0,8	0,5	0,4	0,8	0,9	0,4	0,5	0,5	0,3
SOT12	0,9	1,5	6,3	1,2	2,2	1,9	0,5	0,5	0,9	1,0	0,6	0,9
SOT14	1,5	1,0	1,4	1,3	1,3	1,0	1,0	1,2	1,0	1,2	0,9	0,6
SOT15	2,1	1,3	29,3	1,9	1,4	17,4	1,9	1,2	11,8	2,2	1,0	3,9
SOT16	1,3	1,3	1,1	1,1	1,9	0,7	1,1	1,5	0,9	1,4	2,0	0,9
SOT17	0,7	0,8	0,7	0,6	1,5	1,1	0,4	1,2	1,1	0,8	2,3	1,0
SOT18	0,6	1,0	0,3	0,6	0,5	0,9	0,5	0,8	0,8	0,6	0,5	0,6

3.6 Effect of PSK-α in *pskr1* mutants pathogenic phenotype

PSK-α is present, with an identical structure, in conditioned medium derived from cell lines of many plants, including asparagus (Matsubayashi and Sakagami 1996), rice (Matsubayashi *et al.* 1997), maize (Matsubayashi *et al.* 1997), *Zinnia* (Matsubayashi *et al.* 1999), carrot (Hanai *et al.* 2000) and *Arabidopsis* (Yang *et al.* 2001), indicating its wide distributions among plants. PSK-α also promotes root growth (Amano *et al.* 2007). Lately, it was found that PSK-α signalling through AtPSKR1 affects root elongation in a dose-dependent manner primarily via control of mature cell size (Kutschmar *et al.* 2009). However, no data of the potential effects of PSK-α, as a sulfated peptide hormone, in bacteria or fungi was reported. Therefore, it resulted interesting to know whether exogenously applied PSK-α would alter the pathogenic phenotype observed in *pskr1* plants or not.

3.6.1 Activity verification of PSK-α

PSK-α was purchased from NEeoMPS Polypeptide Laboratories, Strasbourg, France. In order to test its activity, root length assays were performed following previously indicated protocol (Chapter 2.3.1). A low (0,1µM) and a relatively high concentration (1µM) of PSK- α was administered to the growth medium, and both concentrations proved to be suitable for

observing the increased root length of wild type plants treated with PSK- α compared to non-treated plants (control) (Figure 3.6.1).

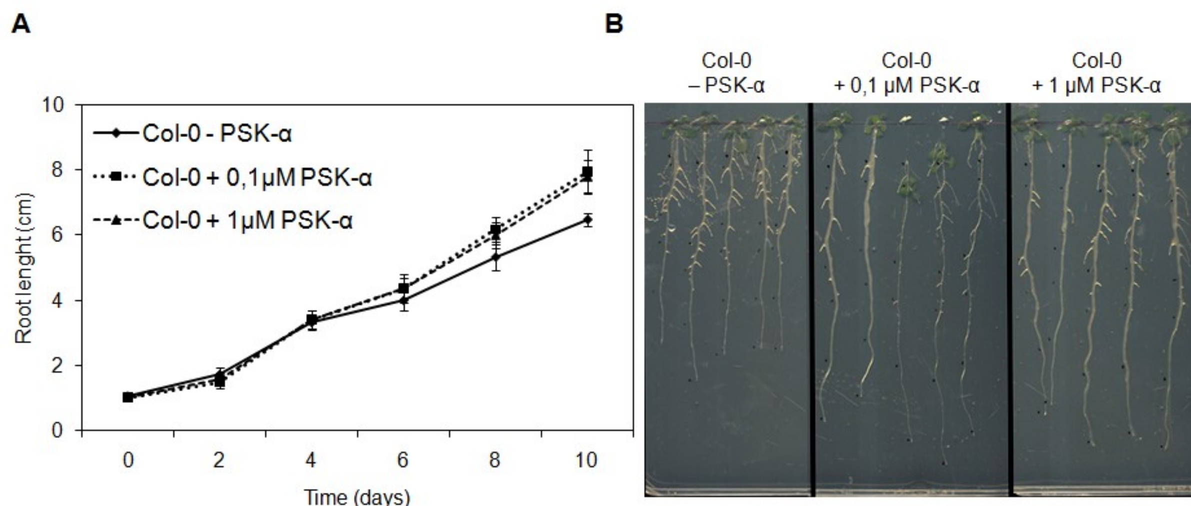

Figure 3.6.1: Activity of PSK-α in root growth assays

Col-0 seeds were surface-sterilized, then stratified and plated on MS medium until seedlings reached 1cm length approx. (Chapter 2.3.1). (A) Root growth measured in cm in MS medium containing 0 µM (control), 0,1 µM and 1 µM PSK-α. Error bars represent standard deviation of at least 5 replicates. (B) Picture of final length reached after 10 days with above-mentioned PSK-α concentrations. Experiment performed once.

3.6.2 Effect of PSK-α in bacterial growth and disease index after fungal attack

In order to asses a direct effect of the peptide in bacteria growth, *Pto* DC3000 was incubated for 2 days at 4°C in $MgCl_2$ with three different PSK-α concentrations (Chapter 2.3.4). Bacterial growth was measured at 0, 1 and 2 dpi. Bacteria incubated only in $MgCl_2$ showed a reduction in bacteria numbers already after the first day; reduction continued at the second day, as expected, since bacteria did not have a rich nutrient medium and suffered most likely from starvation. Interestingly when PSK-α was applied, the decrease in bacterial numbers was higher and in a dose-dependent manner, i.e., a higher concentration of PSK-α led to an increased reduction of bacterial colonies compared to a lower concentration of PSK-α. (Figure 3.6.2, A). Thus, a toxic effect or antimicrobial activity of PSK-α, as a disulfated peptide, in bacteria could be suspected.

To test whether direct application of PSK-α *in planta* leads as well to an increased plant resistance, the growth of *Pto* DC3000 was addressed after pre-treatment of leaves with PSK-α. Wild-type plants and *pskr1-3* and *pskr2* mutants (as controls) were pretreated with PSK-α 5 µM 24hr before challenge with bacteria, since a previous assay showed no variation in bacterial growth when wild type plants were co-inoculated with *Pto*DC3000 and PSK-α simultaneously (data not shown). Pre-treatment of plants 24 hr prior to inoculation was done

in plants for testing the flg22 effect in plant resistance (Zipfel *et al.* 2004). A decreased bacterial growth was observed in PSK-α pretreated wild-type plants compared to non-treated plants (Figure 3.6.2, B). Bacterial growth was also reduced in the controls, but most importantly, the tendency observed in non-treated plants was maintained, i.e., *pskr1-3* presented a higher bacterial resistance, whereas *pskr2* plants displayed a similar level of resistance than wild-type plants. Thus, the only observable effect of exogenously applied PSK-α, under these conditions, on bacterial growth might be antimicrobial (toxic) toward *Pto* DC3000.

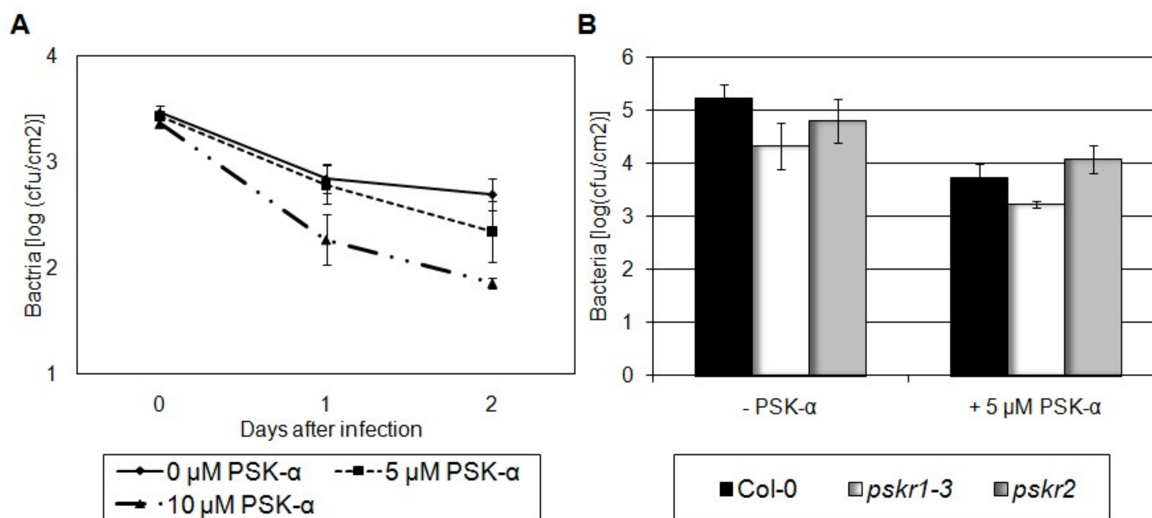

Figure 3.6.2: Effect of PSK-α in bacterial growth of *Pto* DC3000

(A) Virulent bacterial *Pto* DC3000 growth in $MgCl_2$ with and without PSK-α. Bacterial growth was followed until 2 dpi. Bacteria grew in three different mediums: 0 µM PSK- α (black diamonds), 5 µM (black squares) and 10 µM PSK-α (black triangles). This experiment was performed once. (B) Bacterial *Pto* DC3000 growth in pretreated plants with 5 µM PSK-α. Growth corresponds to day 2 after infection with *Pto* DC3000. These experiments were performed once.

The effect of pretreating plants with PSK-α prior to *A. brassicicola* infection was analysed by measuring the symptom development at 10 dpi. Wild type plants were firstly pre-treated with various concentrations of PSK-α (Figure 3.6.3, A). Here it came out that wild-type plants slightly increased their susceptibility toward *A. brassicicola* in a dosage-dependent manner. Afterwards to asses the effect of PSK-α addition in mutant plants lacking the functional receptor of this peptide, wild-type plants together with *pskr1-3* mutants were infected with 5 µM PSK-α, a higher concentration than the one used in planta assays of 1 µM PSK-α (Amano *et al.* 2007; Kutschmar *et al.* 2009). Here the difference in symptom development between plants pretreated whether with H_2O (control) or PSK-α was not significant for wild-type plants and *pskr1-3* mutants (Figure 3.6.3, B), in both cases *pskr1-3* plants displayed an enhanced susceptibility towards *A. brassicicola* than wild-type plants.

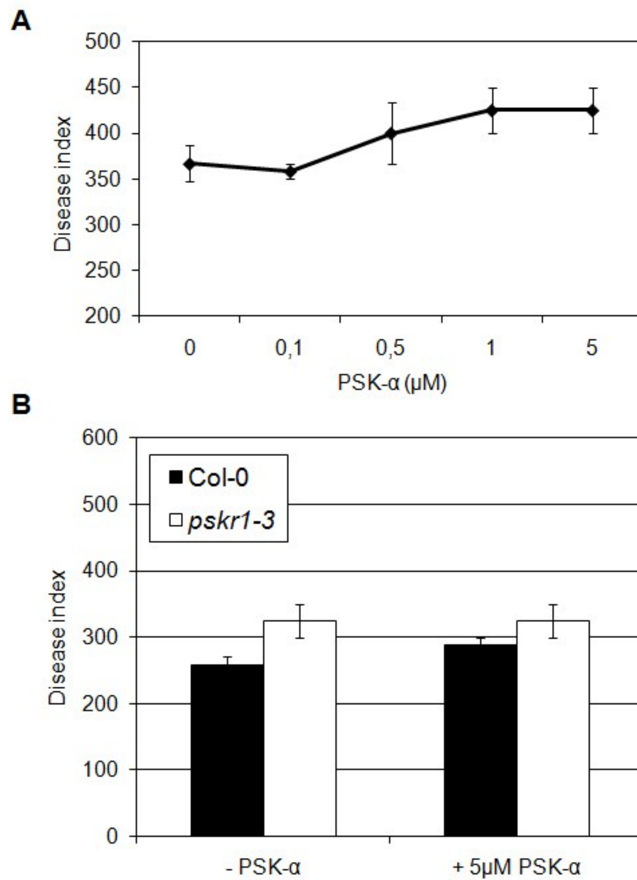

Figure 3.6.3: Effect of PSK-α in planta after infection with *A. brassicicola*

(A) 5 week-old plants were pretreated with various PSK-α concentrations: 0, 0,1, 0,5, 1 and 5µM. 24h later plants were inoculated with a suspension of $5x10^5$ spores/ml of *A. brassicicola.* The symptom development was followed based on a score system unti 10 dpi (B) 5 week-old plants were pretreated with H_2O (control) and 5 µM PSK-α. Procedure was exactly as described above. Graphics above corresponds to disease index of plants at 10 dpi. Error bars represent the standard deviation of 4 replicates. These experiments were performed once.

4 Discussion

Multicellular organisms such as plants and animals use cell surface receptors to sense and transduce chemical signals for cell-to-cell communications. One of the most important groups of cell surface receptors is the receptor-like protein kinase group (RLKs). Within this group and based on the structure of the extracellular domain, the leucine-rich repeat receptor kinase (LRR-RLK) subfamily arises and stands out playing critical roles in both animal and plant signalling pathways regulating growth, development, differentiation, cell death and pathogenic defence responses. Solely in *Arabidopsis thaliana*, there are at least 223 LRR-RLKs, representing thus one of the largest protein families. Nevertheless, only a handful of functional roles have been found for them. For instance, ERECTA (ER) regulates organ shape and inflorescence arquitecture (Torii *et al.* 1996); CLAVATA1 (CVL1) determines the balance between undifferentiated and differentiated shoot and floral meristem cells (Clark *et al.* 1997); BRI1 and BAK1 are a pair of RLKs involved in brassinosteroid (BR) signalling (Li and Chory 1997; Li *et al.* 2002; Nam and Li 2002); BAK1 possesses further BR-independent immunity-associated functions (Chinchilla *et al.* 2007; Kemmerling *et al.* 2007); FLAGELLIN-SENSITIVE 2 (FLS2) contributes to plant defence/pathogen-recognition (Gomez-Gomez and Boller 2000). Based on the Arabidopsis Functional Genomics Network (AFGN) initiative, further investigation of LRR-RLKs structure, activity and gene expression, among others, is pursued within the plant-pathogen interaction area. With this purpose, experiments of gene expression profiling with *Arabidopsis* Col-0 plants infected with various *Pseudomonas syringae* strains were conducted (Kemmerling et al. 2007). This analysis revealed that 32 genes manifested increased transcript accumulation and among these genes, *At*PSKR1, the PSK-α receptor 1, was found. Here, the novel involvement of this receptor protein in plant defence is supported with a potential cooperation of its closest homologs. Moreover, the potential participation in defence regulation of other components of the PSK signalling pathway, like PSK precursors, particularly PSK2 and TPSTs, is proposed.

4.1 *Arabidopsis* PSK-α receptor, *AtPSKR1*

4.1.1 Regulation of *AtPSKR1* gene expression

A noteworthy feature of *AtPSKR1* is its induction pattern after infection with various strains of *Pseudomonas syringae* bacteria, which points out the transcript accumulation of *AtPSKR1* especially upon infection with the non-pathogenic *Pto* hrcC^{-} and *Pph* (Chapter 3.2.1, Figure 3.2.1). Thus, the expression of the PSK receptor seems to be activated in non-compatible interactions. Conversely, its transcript accumulation is reduced upon infection with the

virulent *Pto* DC3000 at early timepoints. Previous and independent studies by Truman *et al.* revealed a number of significantly differentially expressed genes by bacterial effectors, among which *AtPSKR1* arose (Suplemental data from Truman *et al.* 2006). They also proved that certain PAMP-responsive genes display transcript suppression by bacterial effectors and are additionally induced by non-compatible interactions. Interestingly, this gene cluster was over-represented of encoding receptor kinases transcripts (Truman *et al.* 2006). Thus, based on our Microarray data analysis (Chapter 3.2.1) and the work from Truman and colleages, it could be hypothesized that *AtPSKR1* might be a component of the basal host response to bacterial infection, so that its relatively early and transient repression is necessary for the pathogen to attenuate further signalling pathways in compatible plant-pathogen interactions. Moreover, the early upregulation of *AtPSKR1* by bacterial elicitors, like flg22 and HrpZ, might highlight its potential importance in signal perception and transduction.

Based on our Microarray data analysis, *AtPSKR1* appears not to be significantly upregulated by hormones treatment, including ABA (Chapter 3.2.2). Conversely, *AtPSKR1* seems to be indeed upregulated by abiotic stressors like cold, high-salinity and drought (Chapter 3.2.2). A closer examination of the *AtPSKR1* promoter region reveals the presence of an essential *cis*-element, C/DRE, for transcriptional activation in response to cold, drought and/or high salt treatments (Chapter 3.2.7) that acts in a ABA-independent manner (Yamaguchi-Shinozaki and Shinozaki 1994). Interestingly, the presence of the ABRE-like *cis*-elements (ACGT) and the MYC and MYB recognition sites in the *AtPSKR1* promoter might be indicative of a regulatory system for ABA-responsive gene expression (Abe *et al.* 1997; Yamaguchi-Shinozaki and Shinozaki 2006). Hence, it could be hypothesized that *AtPSKR1* might undergo more than one type of stress-response regulation and that the presence of different promoter cis-acting elements in *AtPSKR1* might result in cross talk at the promoter level between ABA-independent and ABA-dependent pathways.

4.1.2 Phenotypic analysis for determining the *AtPSKR1* function in pathogen defence

In order to ultimately prove the involvement of *AtPSKR1* in defence against pathogens or pathogen perception, a reverse genetics approach was performed in which mutant lines impaired in *AtPKSR1* were anaylised with respect to its response to a variety of virulent and avirulent pathogens of bacterial or fungal origin, together with other abiotic factors. For this purpose, various homozygous and allele-independent mutant lines (*pskr1* mutants) were selected (Chapter 3.2.3.1) where no endogenous remaining transcript was present (Chapter 3.2.3.2). The use of different independent mutant lines was essential for verifying that further observed phenotypes correspond indeed to the lack of a functional AtPSKR1, since the mostly employed mutant line in this work (*pskr1-3*) did present at least two other *T*-DNA insertions (Chapter 3.2.3.3).

4.1.2.1 Effect of AtPSKR1-defective plants in pathogen defence

AtPSKR1 transcription is induced upon different pathogens and upon bacterial and oomycete elicitors (Chapter 3.2.1). Therefore, pathogen growth and symptom development after infection were anaylized in mutant lines impaired in *AtPSKR1*. Here, bacterial (virulent, avirulent and non-pathogenic strains) and fungal pathogens (necrotrophic) were tested (Chapter 3.2.4.1 and 3.2.4.2). Infection assays with the avirulent strain *Pto* avrRpm1 hinted an apparently slight enhanced resistance in the *pskr1* mutants compared to wild-type plants, whereas infection with the TTSS-defective strain Pto hrcC$^-$ pointed out no difference between mutants and wild-type plants. All this being indicative of an insignificant effect or influence of the impaired *AtPSKR1* against bacterial growth from both strains, despite the notable influence of these pathogens in *AtPSKR1* trascription observed in microarray data. Nevertheless, infection of *pskr1* mutant plants with the virulent strain *Pto* DC300 resulted in a clear and reproducible phenotype: the *pskr1* mutants enhanced resistance against virulent *Pto* DC3000 at days 1 and 2 after infection in comparison to wild-type plants. This phenotype was observable in different independent mutant lines, i.e. *pskr1-2*, *pskr1-3* and *pskr1-4*, with also different parent backgrounds (Col-0 and Ws-4) (Chapter 3.2.4.1). Moreover, when a bacterial suspension was sprayed, *pskr1* mutants remained healthier compared to wilted and chlorotic wild-type leaves. It is known that SA-dependent defence responses clearly play an important role in limiting *Pseudomonas syringae* growth, since mutants defective in SA signalling display enhanced susceptibility to virulent strains (Glazebrook 2005), like *eds1* (enhanced disease susceptibility 1), *pad4* (phytoalexin deficient 4), *eds5* (enhanced disease susceptibility 5), *sid2* (SA induction-deficient 2) among others. On the contrary, mutants associated with elevated levels of SA and increased expression of SA-regulated genes display an enhanced resistance against bacterial pathogens, like in the case of *pmr4-1* mutants (Flors *et al.* 2008), which are defective in a glucan callose synthase that is responsible for the production of pathogen-inducible callose, that display elevated levels of SA-dependent resistance and display constitutively expression of *PR-1* (Jacobs *et al.* 2003; Nishimura *et al.* 2003). Arabidopsis MPK4 is known as a regulator of PAMP-induced defence responses and loss-of-function *mpk4* mutants have elevated SA levels, accumulate pathogenesis-related transcripts and have increased resistance towards biotrophic pathogens, like *Pseudomonas syringae* and *Hyaloperonospora parasitica*, and moreover display an increased susceptibility towards the necrotroph *Alternaria brassicicola* (Petersen *et al.* 2000; Brodersen *et al.* 2006). Another example of compromised resistance against *P. syringae* is depicted by *At*eIF5A, a eukaryotic translation initiation factor that is involved in pathogen-induced cell death and development of disease symptoms in *Arabidopsis* (Hopkins *et al.* 2008). The antisense suppression of AteIF5A-2 inhibited the colonization of *Pto* DC3000 and curtailed the development of disease symptoms (infected leaves of the

transgenic plants did not become chlorotic). Nevertheless, *At*eIF5A appears to be involved in rather potentiating programmed cell death accompanying compatible host-pathogen interactions (Hopkins *et al.* 2008). Lastly, the *SIZ1* gene was shown to be required for SA and *PAD4*-mediated R gene signalling which in turn confers innate immunity in *Arabidopsis*. The *SIZ1* gene encodes an *Arabidopsis* SUMO (small ubiquitin-like modifier protein) E3 ligase and siz1 plants exhibit constitutive SAR accompanied by elevated accumulation of SA, increased expression of PR genes and increased resistance to bacterial *Pto* DC3000 (Lee *et al.* 2006). Thus, the enhanced resistance against *Pto* DC3000, the slightly higher SA content in *pskr1* mutant plant at basal levels (Chapter 3.2.4.4) and the potential earlier induction of the pathogenesis-related PR-1 gene upon *Pto* DC3000 infection in *pskr1* plants (Chapter 3.2.4.3), together with the previously mentioned supporting evidence, might indicate an implication of *AtPSKR1* in negative regulation of the SA-dependent defence response in *Arabidopsis* against the virulent *Pto* DC3000. Moreover, the presence of several W-boxes in the promoter of *AtPSKR1* (Chapter 3.2.7) might be supportive of a defence regulation function of the PSK-α receptor, whether negative or not, since WRKY proteins bind to W-box sequences in the promoters of pathogen-induced genes (Rushton and Somssich 1998; Eulgem *et al.* 2000). Direct transcriptional targets have been suggested for several WRKY factors and among potential targets receptor-like protein kinases have been found, with *At*SIRK (*Arabidopsis thaliana* senescence-induced receptor-like kinase) exemplifying nicely this theory since its transcriptional activation was found to be dependent on WRKY6 most likely through direct W-box interactions (Robatzek and Somssich 2002).

pskr1 mutants not only display a more resistant phenotype against the biotroph *Pto* DC3000, but also display an enhanced susceptibility towards the necrotroph *A. brassicicola.* The interaction among *Arabidopsis thaliana* and *A. brassicicola* is described as incompatible, since the necrotic tissue surrounding the infection site initially spreads, but then is restricted (Thomma *et al.* 1998; Thomma *et al.* 1999; van Wees *et al.* 2003). Subsequently there is a progressive yellowing of the leaf surrounding the infected area, which is reflected in a loss of average chlorophyll content as the diameter of the chlorotic area increases (Mukherjee *et al.* 2009). Interestingly, *pskr1* mutants display more severe disease symptoms (darker lesion sites, broader chlorotic areas surrounding the infection site, increased cell death accumulation) and surrender to the pathogen expansion which is depicted by a higher fungal mass accumulation (Chapter 3.2.4.2). Moreover, in *A. brassicicola* infected *pskr1* mutants JA-regulated defence genes induction was delayed (Chapter 3.2.4.3), which might indicate an impairment of the JA-signalling defence pathway. Defence-related signal crosstalk between SA and JA is well known with SA being a potent inhibitor of JA-dependent defence against necrotrophs (Spoel *et al.* 2007; Pieterse *et al.* 2009). It is known that resistance to the necrotrophs *A. brassicicola* and to *B. cinerea* depends on JA signalling and camalexin production and that mutations that block the JA signalling cause enhanced susceptibility (Glazebrook 2005), like in the case of *coi1* (*co*ronatine-*i*nsensitive1) mutants in *Arabidopsis*

that display a robust resistance to several *P. syringae* isolates, including the virulent *Pto* DC3000, which correlates with hyperactivation of PR-1 expression and accumulation of elevated SA levels (Kloek *et al.* 2001) and exhibit susceptibility to *A. brassicicola* (Thomma *et al.* 1998; van Wees *et al.* 2003). Recently, the essential role of COI1 was further corroborated since COI1 was found to be a JA receptor that binds directly to JA-isoleucine (JA-Ile) (Yan *et al.* 2009) and assembles the SCF^{COI1} complex (Xu *et al.* 2002; Wang *et al.* 2005), which targets proteins that act as repressors of JA-induced transcriptional responses for degradation and thus constitutes a central component of the JA-signalling (Staswick 2008). Thus, it is likely that *pskr1* mutant phenotypes towards *A. brassicicola* might arise as a result of an antagonistic signalling between JA and SA, since *pskr1* mutants do display higher levels of free SA after *A. brassicicola* infection compared to wild-type plants, indicating perhaps an abolished repression of SA accumulation. Thus, the inability in restricting SA levels of infected *pskr1* mutants might lead to an enhanced susceptibility toward the necrotrophic fungus *A. brassicicola* as an antagonistic effect to JA. The antagonistic cross-talk between SA and JA response pathways has been extensively studied (Gupta *et al.* 2000; Spoel *et al.* 2003; Brooks *et al.* 2004; Bostock 2005), although synergistic interactions have been described as well (Schenk *et al.* 2000; van Wees *et al.* 2000; Mur *et al.* 2006). Among the known molecular players in SA/JA cross talk are: NPR1, required for transduction of the SA signal (Dong 2004) and whose proposed function in wild-type plants is to regulate negatively SA production during hervibore attack and so suppress the SA/JA cross-talk to allow induction of JA-mediated defences against herbivores (Koornneef and Pieterse 2008); some transcription factors, like WRKY70, act as a positive regulator of SA-mediated defences while repressing the JA response (Li *et al.* 2004) or WRKY11 and WRKY17 that have been proposed to act as negative regulators of basal resistance in *Arabidopsis thaliana* (Journot-Catalino *et al.* 2006) and finally WRKY62 with a suggested repressive effect on the JA response (Mao *et al.* 2007); glutaredoxin GRX480, which catalyzes thiol disulfide reductions, is induced by SA-activated NPR1 and in turn it reacts with TGA transcription factors to suppress JA-responsive gene induction (Ndamukong *et al.* 2007); and finally MPK4 that was identified as a negative regulator for SA signalling and a positive regulator of JA signalling in Arabidopsis (Petersen *et al.* 2000). *mpk4* mutants displayed elevated SA levels and constitutive expression of SA-responsive *PR* genes, suppression of JA-responsive genes and enhanced susceptibility to *A. brassicicola* (Petersen *et al.* 2000), thus resembling partially the *pskr1* pathogenic phenotypes. So perhaps PSKR1 interacts directly or indirectly with at least one of the above mentioned SA/JA cross-talk molecular players, like with NPR1 before its nuclear relocalization or with MPK4 at the cytoplasm, since PSKR1 is membrane localized. It would result interesting to verify if the delay in JA-responsive genes induction and the enhanced susceptibility to *A. brassicicola* is indeed caused by an antagonistic effect of SA accumulation in *pskr1* mutants. Therefore, transgenic *pskr1*/NahG plants with low SA levels, since the transgene NahG encodes a salicylate hydroxylase that degrades SA to cathecol as it is formed (Delaney *et al.* 1994), could be treated with the necrotroph *A.*

brassicicola and various PR genes induction could be assessed again and even JA levels could be measured by using an improved method than the one used in this work.

The fact that *pskr1* plants do not show a difference in resistance agains *B. cinerea* infection but do show a enhanced susceptibility against *A. brassicicola*, a necrotroph as well, might be explained by a not affected Ethylene (ET) signalling pathway in *pskr1* plants. It is known that resistance to *B. cinerea*, unlike resistance to *A. brassicicola*, requires ET signalling (Ferrari *et al.* 2003; Glazebrook 2005), and that SA does not appear to contribute to resistance against B. *cinerea* (Ferrari *et al.* 2003). Thus, the affected resistance in *pskr1* mutants against *A. brassicicola* might be due to a defective ET-independent JA-signalling pathway problaby generated by a SA antagonistic effect.

4.2 *AtPSKR1* homologes: *AtPSKR2* and *AtPSY1R*

AtPSKR1 features two paralogous LRR-RLKs, *AtPSKR2* – an alternative but less active PSK receptor - and *AtPSY1R* – the receptor of a 18-aa tyrosine-sulfated glycopeptide called PSY1 (Amano *et al.* 2007). Besides sharing a relatively high percentage of sequence identity, these three LRR-RLKs have overlapping functions whose ligands, PSK and PSY1, are believed to contribute redundantly to cellular proliferation, expansion and wound repair during plant growth and development (Amano *et al.* 2007). Neverthless, a redundant or synergistic function of the receptor proteins in basal plant resistance is not known. Initially, after comparing transcript accumulation patterns (Chapter 3.3.1), it appeared that only *AtPSKR1* was involved in basal response to bacterial infection, since any of the homologes were neither up- nor down-regulated by virulent, avirulent or non-pathogenic bacteria, differing thus from *AtPSKR1*. Nevertheless, the fact that virulent bacterial growth at 1 and 2 dpi was unaltered in the single mutant plants *pskr2* (Chapter 3.3.3.1) and that triple mutants of the three closest homologs (*3X*) did show an enhanced resistance to the virulent *Pto* DC3000 and even mantained it after 4 dpi, showing longer resistant effect than the single mutants *pskr1* (Chapter 3.3.5.1), was a hint of potential functional redundancy between *PSKR2* and and perhaps *PSY1R* in negative regulation of basal resistance against *Pto* DC3000, with possibly a weaker contribution from *PSKR2* and *PSY1R*, since an impaired PSKR1 appeared to be necessary and sufficient for diminishing virulent bacterial growth for the first two days after initiated infection. Possibly the combined function in negative regulation of bacterial growth of PSKR1 and PSKR2 and perhaps PSY1R becomes truly relevant for later timepoints, since *pskr1* mutants displayed wild-type bacterial growth at 4 dpi whereas the triple mutants did halt the bacterial growth (Chapter 3.3.5.1).

PSKR2 and PSY1R seemed to contribute to the PSKR1 response against the non-host necrotroph *A. brassicicola,* since triple mutant plants did show an increase in disease symptoms compared to the previous single mutant *pskr1* (Chapter 3.3.5.2).

The similar and wide expression of AtPSKR1 and AtPSY1R in roots, leaves, stems, flowers, siliques and calluses (Matsubayashi *et al.* 2006; Amano *et al.* 2007) together with their wound-inducible nature (Amano *et al.* 2007) and the similarity in their promoter regions containing among others several binding consensus sequences of *Arabidopsis* transcription factor, RAV1 (Related to ABI3/VP1) (Kagaya *et al.* 1999) whose expression is stimulated by various external or environmental cues (low temperature, darkness, wounding, drought, salt stress and pathogen attack) (Fowler and Thomashow 2002; Lee *et al.* 2005; Sohn *et al.* 2006; Kagaya and Hattori 2009) and also W-boxes that are recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins (Eulgem *et al.* 2000; Chen and Chen 2002; Xu *et al.* 2006) indicating a similar biotic gene regulation (Chapter 3.3.4) may further support their redundant role in plant defence against the necrotroph *A. brassicicola* in *Arabidopsis thaliana*. It would result interesting to analyse the differences in induction of these genes in response to bacterial or fungal infection on their homologs background, i.e. to test if the induction of PSKR2 varies in *pskr1* or in *psy1r* mutant plants compared to wild-type plants, to verify the potential redundant function of these genes in plant defence.

In *Arabidopsis*, there are other receptor families related to plant growth and development as well as to plant immune responses, like is the case of the wall-associated kinases (WAK) family, which are likely the receptors of oligogalacturonides (OGs) and which comprises 5 tightly clustered genes (WAK1-WAK5) (Verica *et al.* 2003). WAK1 and WAK2 bind in vitro to OGs (Decreux *et al.* 2006; Kohorn *et al.* 2009). Conversly to PSKR1 and its homologs, silencing of WAK1 and WAK2 does not cause any phenotypic alterations likely due to functional redundancy (Wagner and Kohorn 2001), whereas reduction of all WAK proteins results in loss of cell expansion and a dwarf phenotype (Wagner and Kohorn 2001), the latter case resembling the triple mutant of PSKR1, PSKR2 and PSY1R. Recently, it has been shown that WAK1 is capable to sense OGs in vivo, by using chimeric receptors of EFR and WAK1, and to trigger a defence response that resembles the one normally activated by OGs effective against fungal and bacterial pathogens (Brutus *et al.* 2010). Another example of functional redundancy in Arabidopsis LRR-RLKs is BAK1 and its paralogs AtSERK4 (also known as BKK1 derived from BAK1-LIKE1, or BAK7) and AtSERK5, where AtSERK4/BKK1/BAK7 functions redundantly with BAK1 in positively regulating BR-dependent plant growth pathway and negatively regulating a BR-independent cell-death pathway (He *et al.* 2007). Lately, a direct interaction between AtSERK4/BKK1/BAK7 and BRI1 was found by FRET analysis (Fluorescence Resonance Energy Transfer) and overexpression of BAK7 can compensate for BAK1 in BR-mediated processes, specially in the absence of BAK1 (Jeong *et al.* 2010). Recently, other homologous genes with redundant

functions have been found in PEPR1 and PEPR2, with PEPR1 being an LRR-RLK and the receptor of the wound- indunced plant peptide *At*Pep1 (Yamaguchi *et al.* 2006) and PEPR2 being its homolog with 72% similarity (Ryan *et al.* 2007). *At*Pep1 triggers innate immune responses and enhanced resistance to *Pytium irregular*e infection (Huffaker *et al.* 2006; Huffaker and Ryan 2007). PEPR1 and PEPR2 are transcriptionally induced by wounding, treatment with methyl jasmonate, Pep peptides and PAMPs (Yamaguchi *et al.* 2010). Moreover, PEPR1 is a receptor for Pep1-Pep6 and PEPR2 is a receptor for Pep1 and Pep2, although with different binding affinities. Thus, PEPR1 and PEPR2, through perception of Peps, contribute to defence responses in Arabidopsis (Yamaguchi *et al.* 2010).

Another important point would be to test the induction of *At*PSKR1 and *At*PSKR2 upon perception of its own ligand, i.e. PSK-α, since other receptor proteins like WAK1 (Brutus *et al.* 2010), FLS2 (Zipfel *et al.* 2006), EFR (Zipfel *et al.* 2006) and PEPR1 (Huffaker and Ryan 2007) appear to be up-regulated upon perception of its corresponding ligands in order to establish a positive feedback to amplify the plant immune response (Huffaker and Ryan 2007; Boller and Felix 2009).

4.3 PSK-α precursors and their posttranslational modifications in plant defence

PSK precursors are redundantly distributed throughout the genome (Yang *et al.* 1999) and are found in a variety of angiosperm and gymnosperm plant species (Lorbiecke and Sauter 2002). *Arabidopsis thaliana* possesses 5 paralogous genes, *AtPSK1*, *AtPSK2*, *AtPSK3*, *AtPSK4* and *AtPSK5*, which encode PSK precursors (Matsubayashi *et al.* 2006), and that are expressed differently in a variety of tissue like roots, leaves, stems, flowers, siliques and calluses. After analyzing the diverse induction pattern of each precursor, it became clearer that *AtPSK2* and *AtPSK4* are the strongest up-regulated genes after bacterial stress (Chapter 3.5.1) and that the expression of the *AtPSK2* seems to be especially activated in non-compatible interactions, like in the case of the *AtPSKR1* gene. Additionally, *AtPSK2* features a down-regulation pattern at 6h post infection, resembling so the main PSK receptor *AtPSKR1* expression upon bacterial stress. In the case of *AtPSK4*, the notoriously high transcript accumulation might not be due exclusively to pathogenic stress, since it was found previously to be highly up-regulated by wounding (Matsubayashi *et al.* 2006). Another perhaps relevant precursor protein is *AtPSK5* which was also expressed in leaves, as well as *AtPSK2* and *AtPSK4* (Matsubayashi *et al.* 2006). Nevertheless, the transcript accumulation of *AtPSK5* did not seem to alter upon bacterial stress (Chapter 3.5.1). *AtPSK2* and *AtPSK4* precursors were the highest upregulated genes after treatment with elicitors or with the oomycete *P. infestans* and the necrotroph fungus *B. cinerea* according to our Microarray

Data analysis, suggesting perhaps their activation in plant defence responses (Chapter 3.5.1). However, pathogenic assays were not performed in planta with the all the corresponding PSK precursors mutant plants. *Pto* DC3000 bacterial growth in *psk1*, *psk3* and *psk5* mutant plants was comparable to the wild-type growth (Chapter, 3.4.3.1), indicating perhaps a lack of participation of these genes in basal defence against the virulent *Pto* DC3000 or a potential redundancy among these genes. The symptom development in these precursor mutants was also tested with two necrotrophs, *A. brassicicola* and *B. cinerea*, and they displayed a wild type response. Interestingly, it could be shown here that when *Arabidopsis* is attacked by the necrotroph *A. brassiciola*, transcripts from *AtPSKR1* and *AtPSK2* accumulate in a similar manner, which could be a hint of a joined participation of a PSK precursor together with the receptor of this peptide in a defence response against the necrotroph *A. brassicicola*. *AtPSK4* transcript also accumulates but differently, being induced mainly in the first 12 hr after initiated infection and not in a synchronized manner with *AtPSKR1*. Thus, PSK signalling might be involved in an Arabidopsis defence response against *A. brassicicola* through the participation of the PSK receptor *AtPSKR1* and mostly the *AtPSK2* precursor protein and perhaps *AtPSK4*. The remaining precursors do not seem to be involved in a defence response against *A. brasscicola* or *B. cinerea*, based on the preliminary pathogenic assays performed in this work. Moreover they are mainly expressed in other tissues, like *AtPSK1* expressed uniquely in roots, or *AtPSK3* and *AtPSK5* being weakly expressed in leaves (Matsubayashi *et al.* 2006). These results could be further confirmed by recent studies by Loivamäki *et al.* claiming that fungal infection induces the expression of *AtPSKR1* together with *AtPSK2* (and *AtPSK5* in a lesser extent) by studying the promoter activities using Promoter::GUS lines (Loivamäki *et al.* 2010). From our *cis*-element analysis of the promoter regions of each precursor gene, it could be observed that higher copies of the W-box binding site of NPR1 were present in the PSK2 promoter, followed by the PSK1 promoter, suggesting a more likely regulatory role in defence. Consequently, a phenotypic analysis of PSK2 loss-of-function mutants may give some insight in PSK precursor genes involvement in plant defence. On the other hand, a functional redundancy among these precursors should not be ignored, based on the broad similarity of its promoter regions and the broad presence of the different precursors in plant tissues.

Another important aspect of PSK signalling and of other peptides derived from larger precursors, is their post-translational proteolysis. To date, there is little information about the proteolytic processing of the PSK precursor proteins, which includes the peptide cleavage and the subsequent removal of residual N-terminal extensions and C-terminal tails to produce mature PSK (Matsubayashi and Sakagami 2006). Recently, a plant subtilase AtSBT1.1, which is upregulated during the process of transferring root explants to tissue culture, was found to cleave preferentially PSK4 from its precursor protein (Srivastava *et al.* 2008). Considering the preference of this subtilase for PSK4 and the fact that the Arabidopsis genome encodes a total of 56 subtilases with uncertain functions for the majority

of them (Rautengarten *et al.* 2005), it is likely that other subtilases are responsible for processing other PSKs. Thus, it remains still unclear which subtilase is involved in PSK2 processing with a potential relevant role in the complex regulation of PSK signalling in plant defence.

Another very decisive aspect of the PSK signalling regulation is its final post-translational modification: tyrosine sulfation. As with tyrosine-sulfated peptide hormones in animals, tyrosine-sulfation of PSK is critical for its function (Matsubayashi *et al.* 1996). Tyrosylprotein sulfotransferases (TPST) are the enzymes that catalyze the transfer of sulfate from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the hydroxyl group of peptidyl tyrosine residues to form a tyrosine O^4-sulfate ester (Bettelheim 1954; Huttner 1982). To date, only one TPST in Arabidopsis, called *At*TPST, have been found to catalyze the tyrosine sulfation of PSY1 more efficiently than of PSK *in vitro* (Komori *et al.* 2009). AtTPST is expressed throughout the plant body and the highest levels of expression are in the root apical meristem (Komori *et al.* 2009). Nevertheless, the facts that *At*TPST catalyzes preferentially the sulfate transfer to PSY1 rather than PSK and the existence of only one known TPST gene in Arabidopsis rather than two genes like in animal genomes (Moore 2009), might indicate the existence of another related tyrosine-sulfotransferase in plants. Here, we chosed to analyse the sulfotransferase (SOTs) family in Arabidopsis as potential sulfate transfer catalysts for PSK sulfation, since it was suggested previously that one of the 18 SOTs in Arabidopsis, enzymes that catalyze sulfate transfer from PAPS to hydroxyl groups of several classes of substrates (not exclusively to tyrosine residues), might be responsible for PSK sulfation processing (Klein and Papenbrock 2004). After the corresponding Microarray data analysis upon various bacterial (Chapter 3.5.1) and elicitor stresses (see Appendix) of the 18 genes encoding sulfotransferases in Arabidopsis, only SOT12 appears upregulated after virulent bacteria, SOT15 appears upregulated by virulent, avirulent and non-host bacteria, and lastly SOT16 and SOT17 appear upregulated uniquely by non-host Pph bacteria, therefore originally thought as potential candidates for PSK sulfation in pathogenic stress responses. In the case of SOT12, known as well as AtST1, it encodes a sulfotransferase with preference to 24-epicathasterone (a brassinosteroid) as a substrate (Marsolais *et al.* 2007); recently SOT12 was found to sulfate SA and *sot*12 mutant plants, upon *Pseudomonas syringae* challenge, accumulated less SA and were more susceptible to pathogen infection, consistently with the changes in SA levels (Baek *et al.* 2010). Therefore, SOT12 regulation in bacterial responses is most likely due to interaction with SA rather than to participation in the defence PSK signalling pathway. In the case if SOT15, known also as AtST2a, it is known to enconde a sulfotransferase that acts specifically on 11- and 12-hydroxyjasmonic acid (12-OHJA) (Gidda *et al.* 2003), with the latter displaying tuber-inducing properties (Yoshihara *et al.* 1989). Upon 12-OHJA application, SOT15 appeared upregulated indicating the presence of a feed-forward mechanism that controls the *in vivo* concentration of the free acid by sulfation or sulfonation (Gidda *et al.* 2003). Moreover, it was lately proprosed that 12-

OHJA forms in a JA-dependent manner and that JA signalling within the wound response can be switched off for a subset of wound-responsinve genes by hydroxylation of JA, i.e. its convertion to 12-OHJA (Miersch *et al.* 2008). Thus, SOT15 appears to be related perhaps to defence responses regarding its regulation properties toward JA, therefore being not likely to sulfate PSK in the potential PSK defence signalling. For the last two upregulated sulfotransferases upon non-host bacteria, SOT16 and SOT17, they are known to catalyse the last step of core glucosinolate biosynthesis (Piotrowski *et al.* 2004). Glucosinolates form a group of over 130 nitrogen-containing and sulfur-containing natural products found in vegetative and reproductive tissues of 16 plant families, but are most well known as major secondary metabolites in the Brassicaceae (Mithen 2001; Mikkelsen *et al.* 2002). SOT16 and SOT17 were found to be cytoplasmic with different substrate specificities and gene expression patterns (Klein *et al.* 2005). Glucosinolates are known to be involved in the plant defence response against microbial pathogen attack, resulting in reduced bacterial colonization and growth (Tierens *et al.* 2001; Brader *et al.* 2006), which might relate to the upregulation of the sulfotransferases SOT16 and SOT17 when non-host bacteria is applied on plants. Thus, all the upregulated sulfotransferases upon bacterial infection seem to relate to plant defence because of their substrate specificities to either SA, or a derivative of JA (12-OHJA) or to desulfoglucosinolates, but not likely due to an interaction with the peptide hormone PSK or its signal components.

4.4 PSK-α and its participation in plant defence

In the last years, several peptides and glycopeptides in plants have been found as a new class of plant signalling molecules during various aspects of plant growth and development and adaptation to abiotic and biotic stresses. These peptides are secreted and act as local signals recognized by membrane-localized receptor kinases, the largest family of receptor-like molecules in plants, and mediate cell-to-cell communication (Matsubayashi and Sakagami 2006). Among these peptide hormones is systemin, a 18-aa plant peptide hormone, that is involved in resistance of tomato against herbivore attack (Torii 2004). PSY1 and PSK-α are sulfated plant peptides that regulate plant growth (Amano *et al.* 2007). Active PSK-α contributes not only to cell proliferation in vitro but also regulates root growth in *Arabidopsis* via control of mature cell size (Matsubayashi *et al.* 2006; Kutschmar *et al.* 2009). Recently, it has been suggested that PSK is involved in attenuation of the stress response and healing of wound-activated cells during the early stages of TE (tracheary elements) differentiation in *Zinnia elegans* mesophyll cells, since administration of PSK significantly reduces the accumulation of transcripts for stress-related genes (Motose *et al.* 2009). A large majority of down-regualted genes in Zinnia upon PSK application are similar to various families of stress-related proteins, including chitinases, phenylpropanoid biosynthesis

enzymes, 1-aminocyclopropane-1-carboxylic acid synthase and receptor-like protein kinases (Motose *et al.* 2009), considering thus PSK as a negative regulator of the wound response in Zinnia. Here, after preliminary testing the activity of the synthesized PSK-α (Chapter 3.6.1), the effect of the peptide PSK was tested directly in bacteria growth. The fact that bacterial numbers did reduce in a dose-dependent manner in a low-nutrient medium and the fact that exogenous application of the peptide in plants also did result in a reduction on bacterial growth in all used lines including wild-type (Chapter 3.6.2), might suggest a possible toxic effect of the peptide PSK itself - at a concentration higher than the normally present in plants - against the virulent *Pto* DC3000 (perhaps by diminishing its fitness and so reducing its growth in planta).

Ultimately, PSK signalling is likely to be involved in not only plant development related processes like controlling cell proliferation in low-density cell cultures (Matsubayashi and Sakagami 1996) and regulating root growth by controlling cell size through *AtPSKR1* (Kutschmar *et al.* 2009), but also PSK signalling is likely to be involved in plant defence against the necrotroph *A. brassicicola*. Thus, AtPSKR1 may appear as a positive regulator of plant defence against *A. brassicicola* aided perhaps by its two homologs, *PSKR2* and *PSY1R*, with *PSKR1* being most likely the main component in this signalling pathway. *AtPSKR1* appears to be involved in the regulation of the SA-signalling pathway by controlling perhaps the SA levels in infected plants, since in the case of a necrotroph attack, a rise in SA might lead to an antagonistic decrease of JA, which would result detrimental for the plant since JA is a key factor in defence against necrotrophs. A tight regulation of the PSK signalling through *AtPSKR1* appears to be essential for *Arabidopsis* depending on the attacking pathogen, since *AtPSKR1* might act also as a negative regulator of plant defence against *Pto* DC3000. *AtPSKR1* may halt SA levels also during a bacterial attack, which assuredly would render a more susceptible plant. Accorlingly, the complex regulation of PSK-signalling through mainly *AtPSKR1* is evident and is also supported by the presence and diversitiy of the other components of this pathway, like the PSK precursors and the PSK posttranslational modifications that the mature PSK peptide has to undergo before being recognized by its receptor. In the specific case of PSK-signalling during *A. brassicicola* infection, the *PSK2* precursor gene seems to be one required for PSK production. The *PSK4* precursor gene cannot be yet excluded from the defence signalling pathway of PSK, although it appears mostly to be upregulated by wounding. It was suggested that PSK signalling might play a role in the wound response in Zinnia (Motose *et al.* 2009). Lately it has been confirmed that in Arabidopsis *PSK3* and *PSK5* precursor genes are the ones involved with AtPSKR1 in mechanical wounding responses (Loivamäki *et al.* 2010). Thus, the existence of five diverse PSK precursors is an indication of the importance of the fine-tuning of PSK-signalling in plant development and defence. Yet many questions are still

unanswered regarding the PSK-signalling. For instance, if *AtPSKR1* is supposedly involved in lowering the plant SA content during fungal and bacterial attack, the plant would need to suppress the *AtPSKR1* function of lowering SA in order to overcome a bacterial attack and succeed. This assumption could be supported by the observed early downregulation of *AtPSKR1* in infected leaves with *Pto*DC3000 at 6 hr post infection in the Microarray Data presented previoulsy. Thus, an early and transient repression of *AtPSKR1* is necessary for overcoming *Pto*DC3000 infection. Therefore, a potential repressor of *AtPSKR1* might exist. Another unidentified element of the PSK-signalling would be the subtilase that cleaves PSK from PSK2 in plant defence responses, i.e. upon fungal or bacterial attack. At present only the subtilase that cleaves PSK from PSK4 is known during the process of transferring root explants to tissue culture (Srivastava *et al.* 2008). If the same subtilase cleaves PSK2 in plant defence responses or another subtilase from the 56-members subtilase family in Arabidopsis is in charge, is still to be determined. Similarly, another posttranslational modification component of the PSK-signalling pathway not yet identified is perhaps the tyrosine sulfotransferase that catalyses the sulfation of PSK peptide during developmental and/or defence processes. In this work, although based on initial assays, it could be also suggested that the sulfated peptide PSK might possess a toxic effect toward bacteria and plants at the employed concentration of 5 µM. Once the receptors of PSK are saturated, the remaining non-recognized PSK appears to lower the fitness of the plant and even the fitness of the bacteria *Pto* DC3000 when reproduced in low-nutrient medium. An interesting approach to determine if the sulfated hydrophilic PSK peptide, due to its chemical composition, is responsible for this potential toxicity at a high concentration, would be to test a synthesized PSK without sulfated tyrosines in similar assays with diverse concentrations as the ones performed in this work.

A tentative model of the PSK-signalling pathway in plant defence responses is shown bellow, based on our own findings and on recently published data.

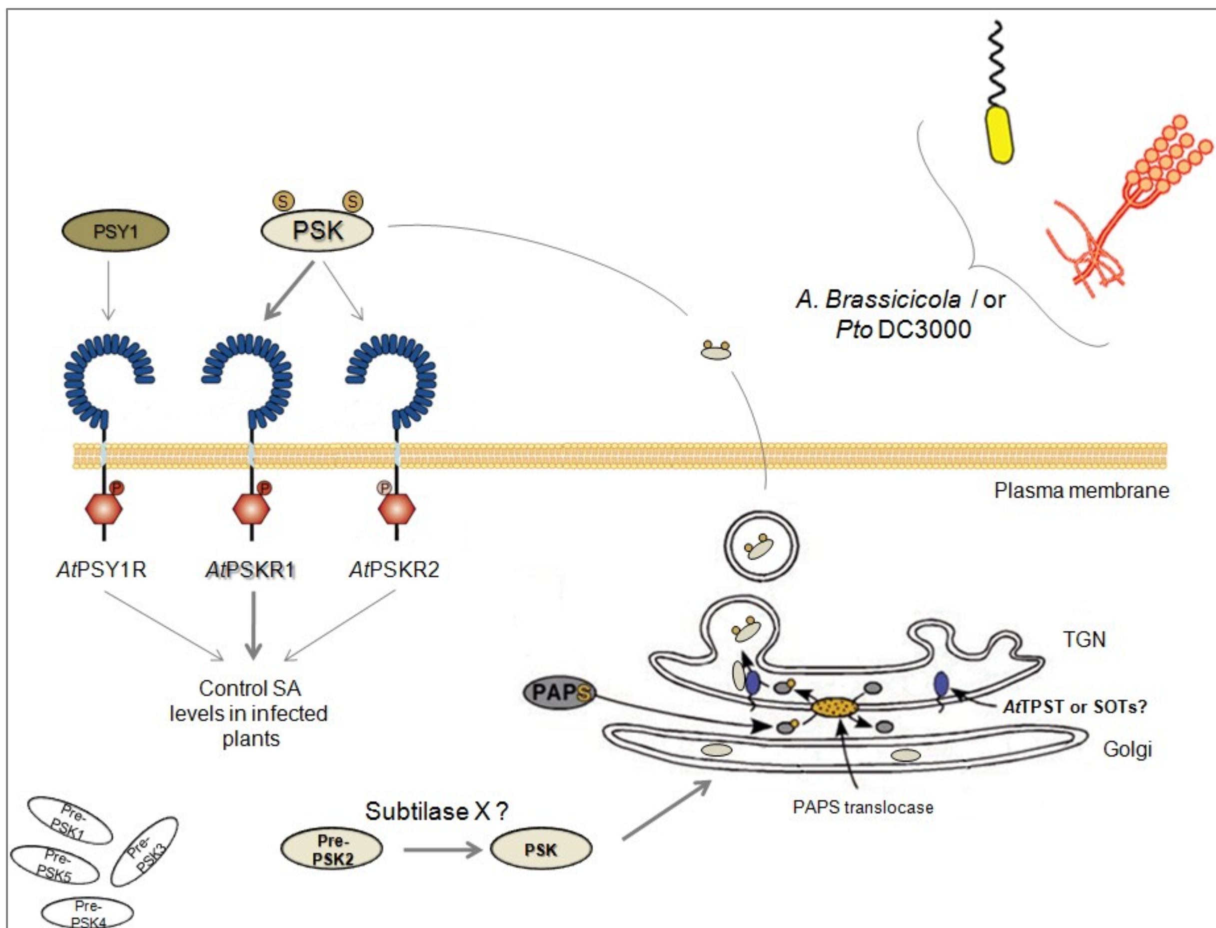

Figure 4.1: Scheme of the proposed PSK-signalling during A. *brassicicola* attack

5 Summary

The leucine-rich repeat (LRR) containing RLKs family constitutes the largest group of RLK in Arabidopsis with 216 genes (Shiu and Bleecker 2001), whereas a significant number of these kinases is predicted to serve as pattern recognition receptors (PRR) in PAMP perception, since transcript levels of multiple LRR-RK-encoding genes increased upon pathogen infection or PAMP treatment (Nürnberger and Kemmerling 2006). Gene expression profiling analysis with *Arabidopsis* Col-0 plants infected with various *Pseudomonas syringae* strains were conducted (Kemmerling et al. 2007) revealing that 32 LRR-RLK genes manifested increased transcript accumulation; some of these genes were: BRL3, SERK4/SERK5, BAK1/SERK3 and *At*PSKR1, the phytosulfokine receptor 1(Kemmerling *et al.* 2007; Postel *et al.* 2009). PSK, an endogenous sulfated pentapeptide secreted in plants, affects cellular potential for growth via specific binding to the LRR *At*PSKR1 (At2g02220, in *Arabidopsis*)(Matsubayashi *et al.* 2002; Matsubayashi *et al.* 2006). In this work, the potential role of *PSKR1* in regulation of basal plant defence in *Arabidopsis thaliana* was investigated. Here it was shown that PSK signalling through its receptor is involved in defence responses against the necrotroph *A. brassicicola* and the virulent bacteria *Pto*DC3000 with opposite regulatory roles, which could be initially explained by a potential participation of the PSK signalling in SA-JA crosstalk during pathogen attack in *Arabidopsis*. Therefore, a tight regulation of the PSK signalling through *AtPSKR1* appears to be essential for *Arabidopsis* depending on the attacking pathogen, since *AtPSKR1* might act as a positive regulator of plant defence against *A. brassicicola* and as a negative regulator of plant defence against *Pto* DC3000. Here it is also proposed that other PSK-signalling components are also involved in plant defence regulation, like PSKR2 and PSY1R (closest homologs of PSKR1), which appear to act redundantly with PSKR1 toward *A. brassicicola* and *Pto* DC3000 infection. Another component of the PSK signalling, like the *PSK2* precursor that seems to be required for PSK production during *A. brassicicola* infection, might also involved in plant defence. Although originally proposed to be upregulated by wounding (Matsubayashi *et al.* 2006), the PSK4 precursor cannot be yet excluded from the defence signalling pathway. Nevertheless, there are still unidentified elements of the PSK-signalling like the specific subtilase that cleaves PSK from PSK2 upon fungal or bacterial attack, or the tyrosine sulfotransferase that catalyses the sulfation of PSK peptide during developmental and/or defence processes. Because of the presence of multiple components with diferent roles in PSK signalling, and because of its apparent complex regulation, it seems likely that PSK signalling might be involved in dual roles in plant development and defence.

6 Literature

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7 Appendix

7.1 Primers

Table 7.1.1: Primers used in this work

Name	Sequence	Employed T_{anneal}
At2g02220-F	CGACCGGCTTTTAATCTACTCG	57 °C
At2g02220-R	GCAATCTCGAGAACCCGAAACATC	57 °C
EF-1α s	TCACATCAACATTGTGGTCATTGG	57 °C
EF-1α as	TTGATCTGGTCAAGAGCCTCAAG	57 °C
N506900/N533210	AGCTTCTTATTTCTTCTTCTTC	56 °C
407D02-RP	TCCTCAGGGATGTTACCAG	56 °C
407D02-LP	GACCGGAATCACCTGCAAT	56 °C
b-N508584/N50858	GGTTCGAT CCCGGTTTCT CTG	55 °C
N508584/85.	GAACAAGATTTGGATGCTGTGCTC	55 °C
Sail_LB	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	56 °C
flag_LB4	CGTGTGCCAGGTGCCCACGGAATAGT	51 °C
b-Lba1	TGGTTCACGTAGTGGGCCATCG	54-60 °C
PR1-s	TCGTCTTTGTAGCTCTTGTAGGTG	57 °C
PR1-as	TAGATTCTCGTAATCTCAGCTCT	57 °C
AbrCUT-5‘	CACTGCGCCCAATGATGAAC	57 °C
AbrCUT-3‘	GTAGCCGAACAACACGACACC	57 °C
PDF1.2-s	AATGAGCTCTCATGGCTAAGTTTGCTTCC	53 °C
PDF1.2-as	AATCCATGGAATACACACGATTTAGCAC	53 °C
PR3-for	ATGAGCGCTGCAAAGTCCTTC	58 °C
PR3-rev	GTGCTGTAGCCCATCCACCTG	58 °C
ATOSM-for	AAAAATGGCAAACCTCTTGGTC	53 °C
ATOSM-rev	GTTGTTGAATTGGTTCAAAGCG	53 °C
At2g22860-for	ATCGCAAACGTCTCCGCTTTGCTC	60 °C
At2g22860-rev	TCAAGGATGCTTCTTCTTCTGGGTA	60 °C
At3g49780-5'	CTCTCTTCTCAGGCTCCCATTATC	58 °C
At3g49780-3'	CAGAAACTTAGGGCTTGTGATTC	58 °C
At5g65870-5'	CTGCATCATCGCTCTTCTTCTC	52 °C
At5g65870-3'	GTGTAGATGTAATCAGTGTGAGC	52 °C

N536304-LP	CTCCTGGTGATAGTGCCTCTG	57 °C
N536304-RP	AAGTGGTATGCGATCCATCAC	57 °C
N817441-LP	ATGAGGACACTTCACTCGTGG	60 °C
N817441-RP	TGCATACCAATTAACATTTTCAAAG	60 °C
N543834-LP	TTGAGAAATATCAATAAAGATGAAGAGG	57 °C
N543834-RP	TAATGGGCCAAACCCTAAATC	57 °C

7.2 Probe for DNA-hybridization

7.2.1 SALK-southern probe

```
        T for L ──►
   1 CTGATGGGCT GCCTGTATCG AGTGGTGATT TTGTGCCGAG CTGCCGGTCG GGGAGCTGTT
  61 GGCTGGCTGG TGGCAGGATA TATTGTGGTG TAAACAAATT GACGCTTAGA CAACTTAATA
 121 ACACATTGCG GACGTTTTTA ATGTACTGGG GTGGTTTTTC TTTTCACCAG TGAGACGGGC
 181 AACAGCTGAT TGCCCTTCAC CGCCTGGCCC TGAGAGAGTT GCAGCAAGCG GTCCACGCTG
 241 GTTTGCCCCA GCAGGCGAAA ATCCTGTTTG ATGGTGGTTC CGAAATCGGC AAAATCCCTT
 301 ATAAATCAAA AGAATAGCCC GAGATAGGGT TGAGTGTTGT TCCAGTTTGG AACAAGAGTC
 361 CACTATTAAA GAACGTGGAC TCCAACGTCA AAGGGCGAAA AACCGTCTAT CAGGGCGATG
 421 GCCCACTACG TGAACCATCA CCCAAATCAA GTTTTTTGGG GTCGAGGTGC CGTAAAGCAC
 481 TAAATCGGAA CCCTAAAGGG AGCCCCCGAT TTAGAGCTTG ACGGGGAAAG CCGGCGAACG
 541 TGGCGAGAAA GGAAGGGAAG AAAGCGAAAG GAGCGGGCGC CATTCAGGCT GCGCAACTGT
 601 TGGGAAGGGC GATCGGTGCG GGCCTCTTCG CTATTACGCC AGCTGGCGAA AGGGGGATGT
 661 GCTGCAAGGC GATTAAGTTG GGTAACGCCA GGGTTTTCCC AGTCACGACG TTGTAAAACG
 721 ACGGCCAGTG AATTCCCGAT CTAGTAACAT AGATGACACC GCGCGCGATA ATTTATCCTA
 781 GTTTGCGCGC TATATTTTGT TTTCTATCGC GTATTAAATG TATAATTGCG GGACTCTAAT
 841 CATAAAAACC CATCTCATAA ATAACGTCAT GCATTACATG TTAATTATTA CATGCTTAAC
 901 GTAATTCAAC AGAAATTATA TGATAATCAT CGCAAGACCG GCAACAGGAT TCAATCTTAA
 961 GAAACTTTAT TGCCAAATGT TTGAACGATC GGGGAAATTC GAGCTCGGTA CCCGGGGATC
1001 CTCTAGAGTC CCCCGTGTTC TCTCCAAATG AAATGAACTT CCTTATATAG AGGAAGGGTC
             ◄──── GC248
1051 TTGCGAAGGA TAGTGGGATT GTGCGTC
```

Figure 7.2.1: SALK Southern probe
Underline bp correspond to used primers for probe amplification.

7.3 Sequences

7.3.1 AtPSKR1

7.3.1.1 *AtPSKR1*-promoter

```
                                                                   ARR1
-1541 CATCCTAAATTTGTCGTTTTTAAAAGAAAAAAGTATTAACGACAAAAAATAATCCTTTAG

-1481 CAATGAAAATTGGTGATAAAAAAACTGAAGAAAAAAGCAATGAAATTTATGTTCCTATAC
      LTRE-box
-1421 CGACTATTTTTTAGTTTCTTTTATATAAAATATAAAATAGTTTATATATAACCGTTCTTT
                     GATA-Box           ABRE-like
-1361 TATTTTTTTATCGTCTACAACTCACATACGTTACACTTTTATAGTTATACTTCTAAATTA
```

```
              W-Box              GATA-Box                                 MYC
-1301  GAAGTCAAGTTATTGTATAGTGATATTATAAACTTCAAGTTATACAAGTCCGGTCATATG
                            ARR1          ABRE-like ARR1
-1241  AAATTAATATTAGTAATTTGATTGGAATTATAACGTATTGATTTGGACATGTAAATAGCT
                                ARR1      MYB1
-1181  AAAAAAAAATAGCTCCTATATTAGATTCTTTTAACCAACAfAATACAACAATTAAGTTGA
      GATA-Box                        ARR1                    ARR1
-1121  TAACCTTCCTCTTTGAAGTATTTCAAGATTTTATGGAAAAAACTCAAGATTTTTTTCCGA
           GATA-Box         GATA-Box                  GATA-Box
-1061  AGCTTTTATCGGAAACTAAAGAATATCTAGTAGGTCTATTTTCATTCATTATCAACTTGC
                                 W-Box
-1001  GAAAAAAGAGAAACAATGTCAATAATTAATGTAAATAGAAAAAAAAAAAAAATTGTAGTTG

                                  W-Box/ABRE-like    W-Box GATA-Box
-941   AGAAGAGAGAGAGATGCAAAGGTGTCAACGTCCGTGCTTTGACTTCTATCTTTTCTCTTG
                     ARR1
-881   TCCTCATCTTAAATCCTCAAAGCTGACCTAACCGGTCACTCCCTTCTTCTCCGGTCATCT
        ATHB2-box                 ARR1                   MYB1 / MYC
-821   TTAATTATTATTCAATTAACCCATAATCAAATTTTAATATAAAAATTAACCATTTGCAAA
           ARR1
-761   ATAAAATCAATATAATTAATAAAAGCTACTCCTTTACCTTTTTATTGCATAGAAACTACT
                ARR1     MYC         ARR1        MYB1
-701   AAAATATAAATCTACCATTTGCAGAATAATCGGCAAAAAACCATAAAGAAACATTTATAG

-641   TCACATAATTTTGCATATATTTTTCTAAATTCGATCACTTTCATCATTCATTATTCATTA
       ARR1                     GATA-Box                         ARR1
-581   ATCGTTCCAAATTTTCTTCTGCCTATCCGTTGAACGACAAACTATTCAAAGTAAAAGATT
                                                     W-Box        ARR1
-521   TAGACAAAAAAAATAAAAAAAAATATTTAGAGAAAGACTTTCCTAAGTCAATGTTATTGATT
                                                W-Box            ARR1
-461   AAGACTTCATATATGACTAAACACACTCAATTATAATTGACAAAACACGGAAAAGATTTG
             GARE
-401   TCTATAACAAATTTTCCAAAAACACAAATTAATTCACAAAATATTTGGAAAAATAATATT
                                                 ARR1
-341   TATTCAAAGCTTCTTATTTCTTCTTCTTCTTTATTTAAAGATTTTTACTGTTTGCTCTGT
       GATA-Box                               GATA-Box
-281   ATCAGAAAAAAAGACCAAACACAACTCTTCCACTTTCTATCTCTCTCTAGAATTTTGCTTG
                                                   W-Box
-221   CATTAAAACAACACCCACTACTTGCTGTTGCGTGTTGAAGTCAAAAGTCGCCATTTTTCT
                                                         ARR1
-161   CTCTCTTGCTTCTTCATCTTCATTGATCATCATCTAAAATGGCAATAATCAGAAAACCCG
                         ARR1                     ARR1
-101   TTTCGTATACTCTCAGATTTCTCAAGGGTTTTTGTTGAATCTTAATTCTTCTCTCAAAGT

-41    TTCTTCCTTTATATTCTTCTTCTTCTTCCTCTGTTCTTGAAATG
```

Figure 7.3.1: *AtPSKR1* promoter sequence

All cis-elements are underlined and named. W-boxes are written in black; cytokinin signalling elements are written in red; a low-temperature responsive element is written in orange; light-regulated elements are written in light blue; dehydration responsive elements are written in green.

7.3.1.2 *AtPSKR1* gene sequence

```
                                         ← SALK_006900    N506900/533210 →
-380 acacaaatta attcacaaaa tatttggaaaaataatatttattcaaϒagct tcttatttct
-320 tcttcttctt tatttaaaga tttttactgt ttgctctgta tcagaaaaaa gaccaaacac
-260 aactcttcca ctttctatct ctctctagaa ttttgcttgc attaaaacaa cacccactac
-200 ttgctgttgc gtgttgaagt caaaagtcgc catttttctc tctcttgctt cttcatcttc
-140 attgatcatc atctaaaatg gcaataatca gaaaacccgt ttcgtatact ctcagatttc
```

```
 -80  tcaagggttt ttgttgaatc ttaattcttc tctcaaagtt tcttccttta tattcttctt
 -20  cttcttcctc tgttcttgaa ATGCGTGTTC ATCGTTTTTG TGTGATCGTC ATCTTCCTCA
  41  CAGAGTTACT ATGTTTCTTC TATTCCTCGG AATCTCAGAC CACCTCCAGG TGCCATCCAC
 101  ATGACCTCGA AGCCTTACGT GACTTCATAG CACATCTCGA ACCAAAACCA GATGGTTGGA
                                                 407D02-LP →
 161  TCAATTCTTC TTCTTCTACA GACTGCTGCA ACTGGACCGG AATCACCTGC AATTCAAACA
 221  ACACCGGAAG AGTTATTAGA TTGGAGCTTG GGAACAAAAA GCTGTCGGGG AAGTTGTCTG
 281  AATCTCTCGG GAAGCTAGAT GAGATTAGGG TTCTTAATCT CTCTCGAAAC TTCATCAAAG
 341  ATTCGATCCC TCTTTCGATT TTCAACTTGA AGAATCTACA AACTCTTGAT TTGAGCTCTA

                                         ← FLAG_407D02
 401  ATGATCTCTC CGGCGGAATCCCAACAAGTATAAATCTC♈CC AGCTCTGCAA AGTTTTGATC
 461  TTTCTTCAAA TAAATTCAAT GGGTCGCTTC CGTCTCATAT CTGCCATAAC TCTACTCAAA
 521  TTAGGGTTGT GAAACTTGCG GTGAACTACT TCGCCGGAAA CTTCACTTCC GGGTTTGGGA
                                                             ← 407D02-RP
 581  AATGTGTCTT GCTTGAGCAT CTCTGTCTTG GTATGAACGA TCTTACTGGT AACATCCCTG
 641  AGGATTTGTT TCATCTCAAA AGATTGAATC TTTTAGGGAT TCAAGAGAAT CGTCTCTCTG
 701  GTTCGTTGAG TCGTGAGATT AGGAATCTCT CAAGTCTTGT TCGTCTTGAT GTTTCTTGGA
 761  ATTTGTTTTC CGGTGAAATC CCTGATGTGT TCGACGAATT GCCTCAGTTA AAGTTTTTCT
 821  TAGGTCAGAC CAATGGATTC ATTGGAGGAA TACCTAAATC GTTGGCGAAT TCACCGAGTT
 881  TGAATCTGCT TAACTTGAGG AACAATTCTT TATCGGGTCG TTTGATGTTG AATTGTACGG
 941  CGATGATTGC TTTGAACTCT CTTGATTTAG GTACCAATAG ATTCAATGGG AGGTTACCTG
1001  AGAATCTACC GGATTGCAAG CGGTTAAAGA ACGTTAACCT CGCGAGGAAC ACCTTCCATG
1061  GACAAGTACC AGAGAGTTTC AAGAACTTCG AGAGCTTATC TTACTTCTCG TTATCGAATT
1121  CGAGTTTGGC TAATATCTCT TCAGCGCTTG GGATACTTCA GCATTGCAAG AACTTGACGA
1181  CTTTGGTTCT TACATTGAAT TTCCATGGAG AGGCTTTACC CGATGATTCA AGTCTTCATT
1241  TCGAGAAGCT TAAGGTGCTT GTAGTGGCGA ATTGTAGGCT TACTGGTTCG ATGCCGAGGT
1301  GGTTAAGCTC GAGTAATGAA CTTCAGTTGT TGGATCTTTC TTGGAACCGT TTAACCGGCG
1361  CTATCCCGAG CTGGATTGGT GACTTCAAGG CTCTGTTCTA CTTGGATTTA TCTAACAACT
1421  CGTTTACAGG AGAGATCCCT AAGAGCTTAA CTAAGTTAGA GAGTCTCACT AGCCGTAATA
1481  TCTCAGTCAA TGAGCCATCT CCTGATTTCC CGTTCTTTAT GAAAAGAAAC GAGAGCGCGA
1541  GAGCGTTGCA ATACAATCAG ATTTTCGGGT TCCCGCCAAC GATTGAGCTT GGTCATAACA
1601  ATCTCTCTGG ACCTATTTGG GAGGAGTTTG GTAATCTGAA GAAGCTTCAT GTGTTTGATT
1661  TGAAATGGAA TGCATTATCT GGATCAATAC CTAGCTCGCT TTCTGGTATG ACGAGCTTGG
                                            b-N508584/N50858 →
1721  AAGCTCTTGA TCTCTCTAAT AACCGTCTTT CGGGTTCGAT CCCGGTTTCT CTGCAACAGC
1781  TCTCGTTTCT GTCGAAGTTC AGTGTTGCTT ATAACAATCT CTCGGGAGTA ATACCTTCCG
1841  GTGGTCAGTT TCAGACGTTT CCAAACTCGA GCTTTGAGAG TAACCATCTC TGCGGGGAAC
1901  ACAGATTCCC CTGTTCTGAA GGTACTGAGA GTGCATTGAT CAAACGGTCA AGAAGAAGCA
1961  GAGGAGGTGA CATTGGAATG GCGATTGGGA TAGCGTTTGG TTCGGTTTTT CTTTTGACTC
2021  TTCTCTCGTT GATTGTGTTG CGTGCTCGTA GACGGTCAGG AGAAGTTGAT CCGGAGATAG
2081  AAGAATCCGA GAGCATGAAT CGTAAAGAAC TCGGAGAGAT TGGATCTAAG CTTGTGGTTT
2141  TGTTTCAGAG CAATGATAAA GAGCTCTCTT ATGATGACCT TTTGGACTCA ACAAATAGTT
2201  TTGATCAAGC TAACATCATT GGCTGTGGCG GGTTTGGTAT GGTTTACAAA GCAACGTTAC
2261  CAGACGGTAA GAAAGTTGCG ATCAAGAAGT TATCCGGTGA TTGCGGTCAA ATCGAAAGAG
```

```
                SALK_008585 →                  ← N508584/85
2321 AATTCGAAGC AGAAGTTGϒAAACACTCTCAA GAGCACAGCA TCCAAATCTT GTTCTTCTCC
                                   At2g02220-F →
2381 GAGGATTCTG TTTCTACAAA AACGACCGGC TTTTAATCTA CTCGTATATG GAAAACGGAA
2441 GCTTAGACTA TTGGCTACAC GAGCGTAACG ACGGTCCAGC GTTGTTGAAG TGGAAAACAC
2501 GTCTTAGAAT CGCTCAAGGT GCTGCAAAAG GGTTACTTTA CTTGCATGAA GGGTGTGATC
2561 CTCATATCTT ACACCGCGAT ATTAAATCGA GTAATATTCT TCTCGACGAG AATTTCAACT
2621 CTCATTTAGC GGATTTCGGA CTCGCAAGGC TGATGAGTCC TTACGAGACG CATGTAAGTA
2681 CTGATTTGGT TGGAACTTTA GGTTACATTC CTCCGGAATA CGGGCAAGCT TCGGTTGCTA
2741 CTTACAAAGG CGATGTGTAT AGTTTCGGAG TTGTGCTTCT CGAGCTTTTA ACCGATAAAA
2801 GACCGGTGGA TATGTGTAAA CCGAAAGGGT GTAGGGATCT GATCTCGTGG GTCGTCAAGA
2861 TGAAGCATGA GAGTCGAGCA AGCGAGGTTT TCGATCCGTT AATATACAGT AAAGAGAATG
                ← At2g02220R
2921 ATAAAGAGAT GTTTCGGGTT CTCGAGATTG CTTGTTTATG TTTAAGCGAA AACCCGAAAC
2981 AGAGGCCAAC GACTCAACAG TTAGTCTCTT GGCTTGATGA
```

Figure 7.3.2: *AtPSKR1* gene sequence

The coding sequence is writen in capital letters, the non-coding sequence in small letters. The corresponding primers (Table 7.1.1) are underlined. The T-DNA position is indicated by 'ϒ'. Arrows indicate the primers and the T-DNA insertion orientation.

7.3.1.3 AtPSKR1 protein sequence

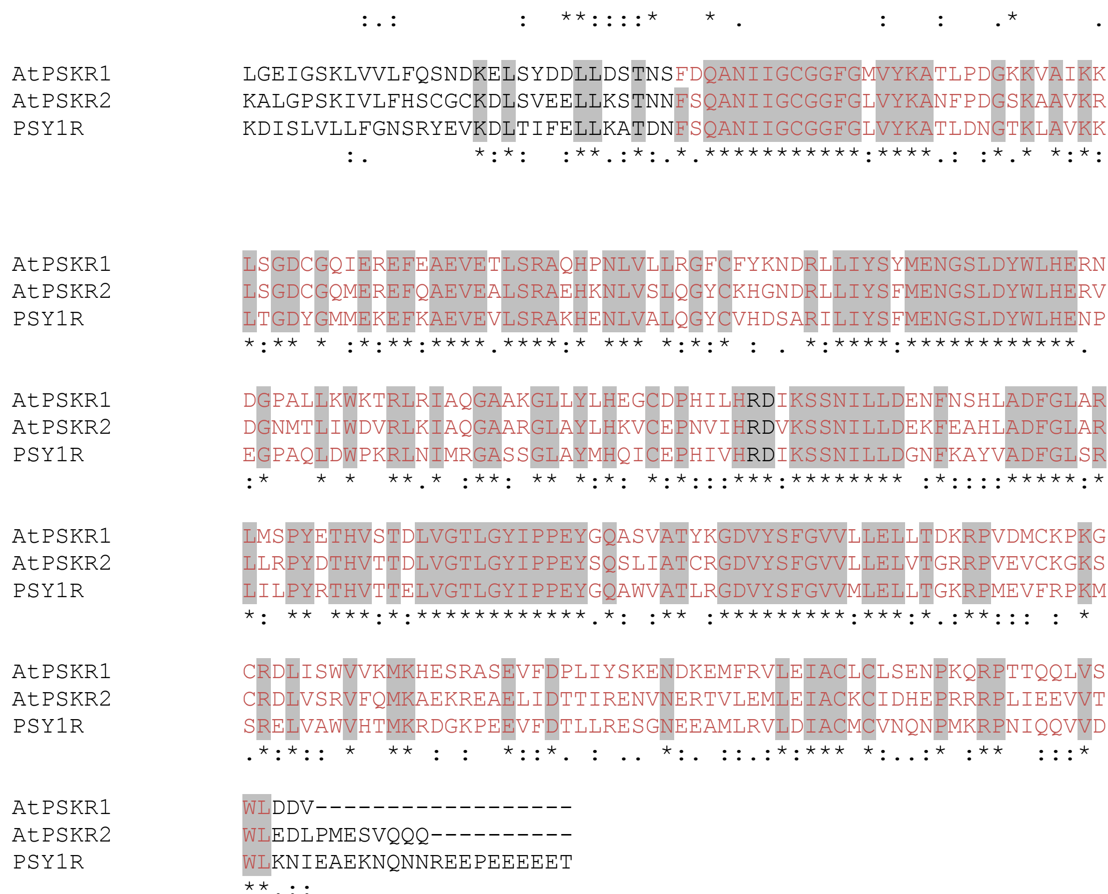
```
  1 MRVHRFCVIV IFLTELLCFF YSSESQTTSR CHPHDLEALR DFIAHLEPKP DGWINSSSST
 61 DCCNWTGITC NSNNTGRVIR LELGNKKLSG KLSESLGKLD EIRVLNLSRN FIKDSIPLSI
121 FNLKNLQTLD LSSNDLSGGI PTSINLPALQ SFDLSSNKFN GSLPSHICHN STQIRVVKLA
181 VNYFAGNFTS GFGKCVLLEH LCLGMNDLTG NIPEDLFHLK RLNLLGIQEN RLSGSLSREI
241 RNLSSLVRLD VSWNLFSGEI PDVFDELPQL KFFLGQTNGF IGGIPKSLAN SPSLNLLNLR
301 NNSLSGRLML NCTAMIALNS LDLGTNRFNG RLPENLPDCK RLKNVNLARN TFHGQVPESF
361 KNFESLSYFS LSNSSLANIS SALGILQHCK NLTTLVLTLN FHGEALPDDS SLHFEKLKVL
421 VVANCRLTGS MPRWLSSSNE LQLLDLSWNR LTGAIPSWIG DFKALFYLDL SNNSFTGEIP
481 KSLTKLESLT SRNISVNEPS PDFPFFMKRN ESARALQYNQ IFGFPPTIEL GHNNLSGPIW
541 EEFGNLKKLH VFDLKWNALS GSIPSSLSGM TSLEALDLSN NRLSGSIPVS LQQLSFLSKF
601 SVAYNNLSGV IPSGGQFQTF PNSSFESNHL CGEHRFPCSE GTESALIKRS RRSRGGDIGM
661 AIGIAFGSVF LLTLLSLIVL RARRRSGEVD PEIEESESMN RKELGEIGSK LVVLFQSNDK
721 ELSYDDLLDS TNSFDQANII GCGGFGMVYK ATLPDGKKVA IKKLSGDCGQ IEREFEAEVE
781 TLSRAQHPNL VLLRGFCFYK NDRLLIYSYM ENGSLDYWLH ERNDGPALLK WKTRLRIAQG
841 AAKGLLYLHE GCDPHILHRD IKSSNILLDE NFNSHLADFG LARLMSPYET HVSTDLVGTL
961 SEVFDPLIYS KENDKEMFRV LEIACLCLSE NPKQRPTTQQ LVSWLDDV
```

Figure 7.3.3: AtPSKR1 protein sequence with predigted domain structure

The protein sequence contains the LRR domain (underlined), the transmembrane domain (gray) and the kinase domain (bold).

7.3.1.4 Protein sequence of *At*PSKR1 and its closest homologs

```
AtPSKR1         -----------------MRVHRFCVIVIFLTELLCFFYSSESQTTSRCHPHDLEALRDFI
AtPSKR2         -----------------------------MVIILLLVFFVGSSVSQPCHPNDLSALRELA
PSY1R           MIDEKMRSKSIGPFVRQVKPLSPHMVLFVLLYVLSISVFFLTVSEAVCNLQDRDSLLWFS
                                             :  :* :     :     *: :* .:*  :

                                     CxWxGaSC
AtPSKR1         AHLEPK--PDGWINSSSSTDCCNWTGITCN-SNNTGRVIRLELGNKKLSGKLSESLGKLD
AtPSKR2         GALKNKSVTESWLNGSR---CCEWDGVFCEGSDVSGRVTKLVLPEKGLEGVISKSLGELT
PSY1R           GNVSSPVSPLHWN---SSIDCCSWEGISCDKSPEN-RVTSIILSSRGLSGNLPSSVLDLQ
                . :.    .  *        **.* *: *: *  . **  : * .: *.* :..*: .*

AtPSKR1         EIRVLNLSRNFIKDSIP-LSIFNLKNLQTLDLSSNDLSGGIPTSIN--------------
AtPSKR2         ELRVLDLSRNQLKGEVP-AEISKLEQLQVLDLSHNLLSGSVLGVVSGLKLIQ--------
PSY1R           RLSRLDLSHNRLSGPLPPGFLSALDQLLVLDLSYNSFKGELPLQQSFGNGSNGIFPIQTV
                .:  *:**:* :.. :*   :  *.:* .**** * :.* :    .

AtPSKR1         --------------------LPALQSFDLSSNKFNGSLPSHICHNSTQIRVVKLAVNYFA
AtPSKR2         --SLNISSNSLSGKLSDVGVFPGLVMLNVSNNLFEGEIHPELCSSSGGIQVLDLSMNRLV
PSY1R           DLSSNLLEGEILSSSVFLQGAFNLTSFNVSNNSFTGSIPSFMCTASPQLTKLDFSYNDFS
                                       *  :::*.* * *.: . :*  *  :  :.:: * :

AtPSKR1         GNFTSGFGKCVLLEHLCLGMNDLTGNIPEDLFHLKRLNLLGIQENRLSGSLSREIRNLSS
AtPSKR2         GNLDGLYNCSKSIQQLHIDSNRLTGQLPDYLYSIRELEQLSLSGNYLSGELSKNLSNLSG
PSY1R           GDLSQELSRCSRLSVLRAGFNNLSGEIPKEIYNLPELEQLFLPVNRLSGKIDNGITRLTK
                *::    . .  :. *  . * *:*::*. :: : .*: * :  * ***.:.. : .*:

AtPSKR1         LVRLDVSWNLFSGEIPDVFDELPQLKFFLGQTNGFIGGIPKSLANSPSLNLLNLRNNSLS
AtPSKR2         LKSLLISENRFSDVIPDVFGNLTQLEHLDVSSNKFSGRFPPSLSQCSKLRVLDLRNNSLS
PSY1R           LTLLELYSNHIEGEIPKDIGKLSKLSSLQLHVNNLMGSIPVSLANCTKLVKLNLRVNQLG
                *  * :  * :.. **. :.:*.:*. :    * : * :* **::...*  *:** *.*.

AtPSKR1         G-RLMLNCTAMIALNSLDLGTNRFNGRLPENLPDCKRLKNVNLARNTFHGQVPESFKNFE
AtPSKR2         G-SINLNFTGFTDLCVLDLASNHFSGPLPDSLGHCPKMKILSLAKNEFRGKIPDTFKNLQ
PSY1R           GTLSAIDFSRFQSLSILDLGNNSFTGEFPSTVYSCKMMTAMRFAGNKLTGQISPQVLELE
                *    :: : :  *  ***..* *.* :*..:  *  :. : :* * : *::.  . :::

AtPSKR1         SLSYFSLSNSSLANISSALGILQHCKNLTTLVLTLNFHGEALPDDS----SLHFEKLKVL
AtPSKR2         SLLFLSLSNNSFVDFSETMNVLQHCRNLSTLILSKNFIGEEIPNN-----VTGFDNLAIL
PSY1R           SLSFFTFSDNKMTNLTGALSILQGCKKLSTLIMAKNFYDETVPSNKDFLRSDGFPSLQIF
                ** ::::*:..:.::: ::.:** *::*:**::: ** .* :*.:        * .* ::

AtPSKR1         VVANCRLTGSMPRWLSSSNELQLLDLSWNRLTGAIPSWIGDFKALFYLDLSNNSFTGEIP
AtPSKR2         ALGNCGLRGQIPSWLLNCKKLEVLDLSWNHFYGTIPHWIGKMESLFYIDFSNNTLTGAIP
PSY1R           GIGACRLTGEIPAWLIKLQRVEVMDLSMNRFVGTIPGWLGTLPDLFYLDLSDNFLTGELP
                 :. * * *.:* ** . :.::::*** *:: *:** *:* :  ***:*:*:* :** :*

AtPSKR1         KSLTKLESLTSRN--ISVNEPSPDFPFFMKRNESARALQYNQIFGFPPTIELGHNNLSGP
AtPSKR2         VAITELKNLIRLNGTASQMTDSSGIPLYVKRNKSSNGLPYNQVSRFPPSIYLNNNRLNGT
PSY1R           KELFQLRALMSQKAYDATERNYLELPVFVNPNNVTTNQQYNQLSSLPPTIYIKRNNLTGT
                  : :*. *   :   :       :*.::: *: :    ***:  :**:* : .*.*.*.

AtPSKR1         IWEEFGNLKKLHVFDLKWNALSGSIPSSLSGMTSLEALDLSNNRLSGSIPVSLQQLSFLS
AtPSKR2         ILPEIGRLKELHMLDLSRNNFTGTIPDSISGLDNLEVLDLSYNHLYGSIPLSFQSLTFLS
PSY1R           IPVEVGQLKVLHILELLGNNFSGSIPDELSNLTNLERLDLSNNNLSGRIPWSLTGLHFLS
                *  *.*.** **:::*  * ::*:**..:*.: .** **** *.* * ** *:  * ***

                                                 CGxxxxxC
AtPSKR1         KFSVAYNNLSGVIPSGGQFQTFPNSSFESNH-LCGEHRFPCSEGTESALIKRSRRSRGGD
AtPSKR2         RFSVAYNRLTGAIPSGGQFYSFPHSSFEGNLGLCRAIDSPCDVLMSNMLNPKGSSRRNNN
PSY1R           YFNVANNTLSGPIPTGTQFDTFPKANFEGNPLLCGGVLLTSCDPTQHSTTKMGKGKVNRT
                 *.** * *:* **:* ** :**::.**.*  **     ..    .      .    .

AtPSKR1         IG---------MAIGIAFGSVFLLTLLSLIVLRARRS---------GEVDPEIEESESMNRKE
AtPSKR2         GGKFGRSSIVVLTISLAIGITLLLSVILLRISRKD--------VDDRINDVDEETISGVS
PSY1R           LVLG----LVLGLFFGVSLILVLLALLVLSKRRVNPGDSENAELEINSNGSYSEVPPGSD
```



```
                      :.:          :   **::::*    * .          :   :    .*      .

AtPSKR1         LGEIGSKLVVLFQSNDKELSYDDLLDSTNSFDQANIIGCGGFGMVYKATLPDGKKVAIKK
AtPSKR2         KALGPSKIVLFHSCGCKDLSVEELLKSTNNFSQANIIGCGGFGLVYKANFPDGSKAAVKR
PSY1R           KDISLVLLFGNSRYEVKDLTIFELLKATDNFSQANIIGCGGFGLVYKATLDNGTKLAVKK
                       :.        *:*:   :**.:*:.*.***********:****.: :*.* *:*:


AtPSKR1         LSGDCGQIEREFEAEVETLSRAQHPNLVLLRGFCFYKNDRLLIYSYMENGSLDYWLHERN
AtPSKR2         LSGDCGQMEREFQAEVEALSRAEHKNLVSLQGYCKHGNDRLLIYSFMENGSLDYWLHERV
PSY1R           LTGDYGMMEKEFKAEVEVLSRAKHENLVALQGYCVHDSARILIYSFMENGSLDYWLHENP
                *:** *  :*:**:****.****:* *** *:*:*  :  . *:****:************.

AtPSKR1         DGPALLKWKTRLRIAQGAAKGLLYLHEGCDPHILHRDIKSSNILLDENFNSHLADFGLAR
AtPSKR2         DGNMTLIWDVRLKIAQGAARGLAYLHKVCEPNVIHRDVKSSNILLDEKFEAHLADFGLAR
PSY1R           EGPAQLDWPKRLNIMRGASSGLAYMHQICEPHIVHRDIKSSNILLDGNFKAYVADFGLSR
                :*    * *  **.* :**: ** *:*: *:*::***:********  :*::::*****:*

AtPSKR1         LMSPYETHVSTDLVGTLGYIPPEYGQASVATYKGDVYSFGVVLLELLTDKRPVDMCKPKG
AtPSKR2         LLRPYDTHVTTDLVGTLGYIPPEYSQSLIATCRGDVYSFGVVLLELVTGRRPVEVCKGKS
PSY1R           LILPYRTHVTTELVGTLGYIPPEYGQAWVATLRGDVYSFGVVMLELLTGKRPMEVFRPKM
                *: ** ***:*:************.*: :** :*********:***:*.:**::: : *

AtPSKR1         CRDLISWVVKMKHESRASEVFDPLIYSKENDKEMFRVLEIACLCLSENPKQRPTTQQLVS
AtPSKR2         CRDLVSRVFQMKAEKREAELIDTTIRENVNERTVLEMLEIACKCIDHEPRRRPLIEEVVT
PSY1R           SRELVAWVHTMKRDGKPEEVFDTLLRESGNEEAMLRVLDIACMCVNQNPMKRPNIQQVVD
                .*:*:: *  ** : :  *::*. : .. *:. ::.:*:*** *:..:* :**   :::*

AtPSKR1         WLDDV------------------
AtPSKR2         WLEDLPMESVQQQ----------
PSY1R           WLKNIEAEKNQNNREEPEEEEET
                **.::
```

Figure 7.3.4: *At*PSKR1 protein sequence compared to its closes homologs.
Sequence alignment obtained using ClustalW (http://align.genome.jp). Amino acids highlighted in gray are conserved among these proteins. Characters in orange correspond to the island domains. Flanking conserved Cysteines are indicated with a consensus sequence above the alingment before and after the LRR domain. Characters in red indicate the catalytic kinase domain, in which the conserved RD residues appear in bold.

7.4 *AtPSKR1* and homologes expression

7.4.1 *AtPSKR1* expression after abiotic treatment

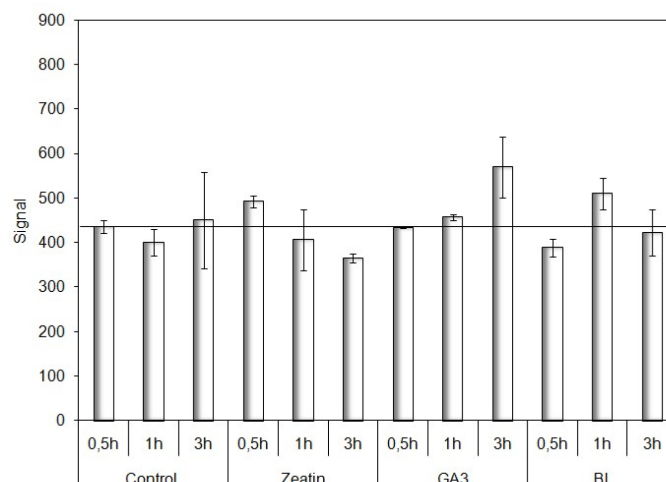


Figure 7.4.1: Microarray data of WT plants roots after treatment with various hormones

Plant material from 7 day old wild-type *Arabidopsis thaliana* seedlings of Col-0 was analysed. Plants were grown in liquid MS media under continuous light conditions at 23°C. After infiltration of 1µM Zeatin, 1µM GA3 and 10 nM BL, plant material was collected and RNA was isolated and hybridized to the ATH1 GeneChip. All measurements were taken in duplicates, whose average is shown. The data were normalized by GCOS normalization. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004).

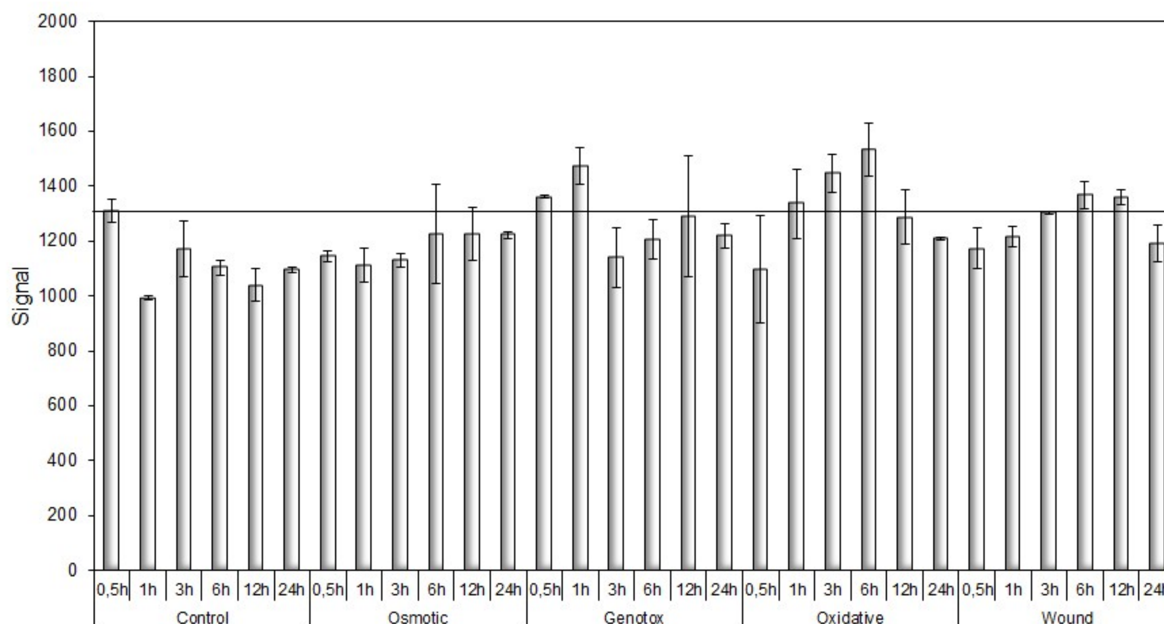


Figure 7.4.2: Microarray data of WT plants roots after treatment with abiotic stress

Plant material from 18 day old wild-type *Arabidopsis thaliana* of Col-0 was analysed. Seeds were sowed of rafts in Magenta boxes containing MS-Agar-media. After 2 days in the cold room (4°C, dark) the boxes were transferred to a long day chamber. At day 11, the rafts were transferred in Magenta boxes containing MS-liquid-media. Plants were grown under long day conditions with 16/8 hrs light/dark, 24°C, 50% humidity and 150 µEinstein/cm^2 sec light intensity. For osmotic stress: mannitol was added to the MS medium to a final concentration of 300 mM. For drought stress: rafts were exposed to a stream of air in a clean bench for 15 min; during this time period the plants lost 10% of their fresh weight. For wound stress: plants were wounded only by punctuation of the leaves with a custom made pin-tool consisting of 16 needles (about 2 needles/cm^2). Samples were taken at 30 min, 1h, 3h, 6h, 12h and 24h after treatment; control samples include timepoint 0. All measurements were taken in duplicates, the average of which is shown. RNA was isolated and hybridized to the ATH1 GeneChip. Data were normalized by GCOS normalization. This study is part of the AtGenExpress project, funded by the DFG (Kilian *et al.* 2007). The red line is intended to make evident the basal level of *AtPSKR1* transcripts.

7.4.2 *AtPSKR1, AtPSKR2 and AtPSY1R* after abiotic treatment

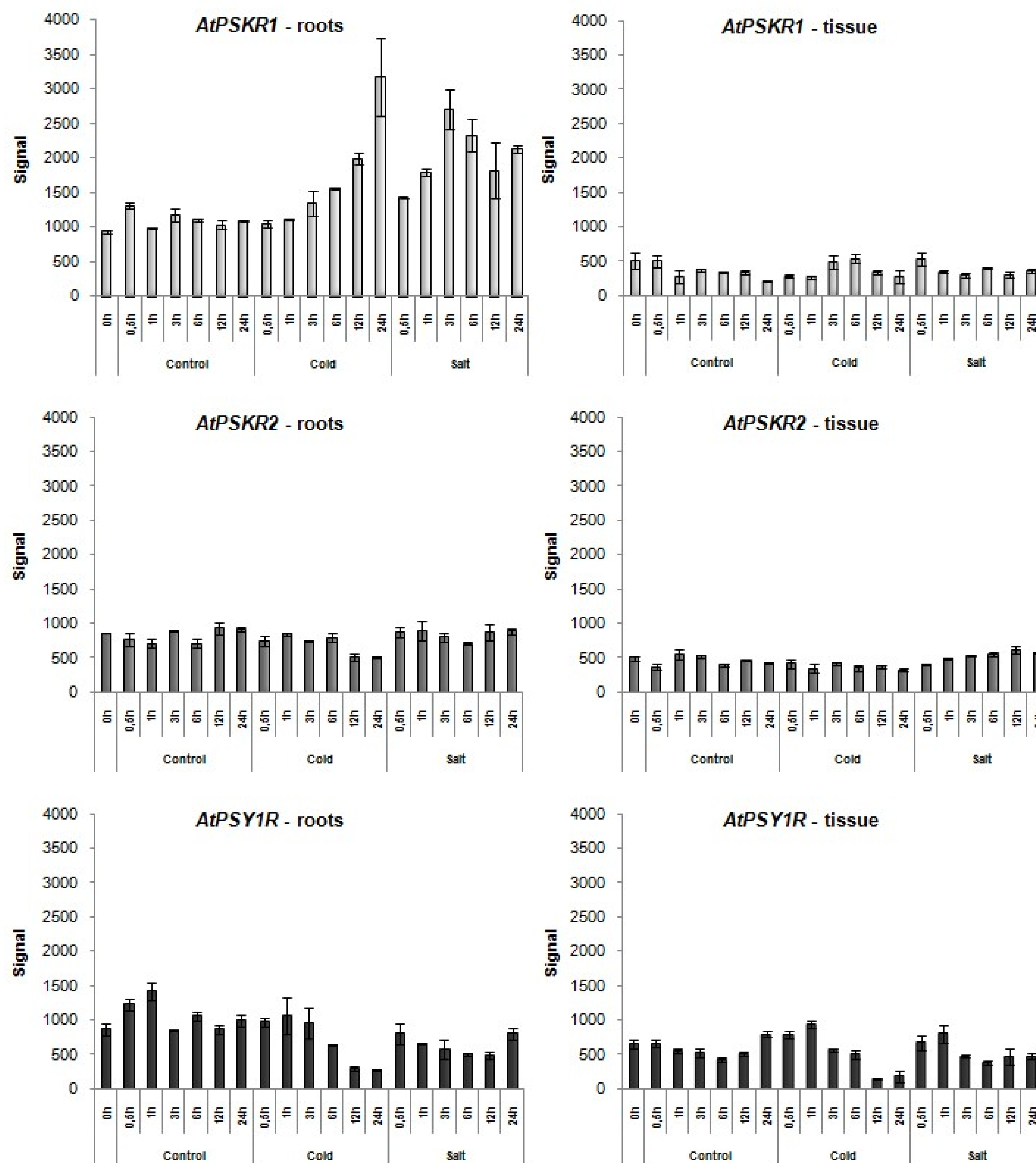


Figure 7.4.5: Expression of *AtPSKR1* and its homologs after abiotic treatment

Plant material from 18 day old wild-type *Arabidopsis thaliana* of Col-0 was analysed. Seeds were sowed of rafts in Magenta boxes containing MS-Agar-media. After 2 days in the cold room (4°C, dark) the boxes were transferred to a long day chamber. At day 11, the rafts were transferred in Magenta boxes containing MS-liquid-media. Plants were grown under long day conditions with 16/8 hrs light/dark, 24°C, 50% humidity and 150 µEinstein/cm^2 sec light intensity. For cold stress: continuous 4°C on crushed ice in cold chamber. For salt stress: 150 mN NaCl was used. Samples were taken at 30 min, 1h, 3h, 6h, 12h and 24h after treatment; control samples include timepoint 0. All measurements were taken in duplicates, the average of which is shown. RNA was isolated and hybridized to the ATH1 GeneChip. Data were normalized by GCOS normalization. This study is part of the AtGenExpress project, funded by the DFG (Kilian *et al.* 2007). The red line is intended to make evident the basal level of AtPSKR1 transcripts.

Table 7.4.1: Relative values of transcript accumulation from *AtPSKR1* and its homologs after hormone treatment

	IAA			ABA			MJ			ACC		
	30 min	1h	3h	30 min	1h	3h	30 min	1h	3h	30 min	1h	3h
AtPSKR1	1,09	1,30	1,27	0,96	0,76	0,90	0,73	0,81	0,73	1,14	1,27	1,47
AtPSKR2	1,19	1,31	0,98	1,17	1,04	0,70	0,93	1,11	0,73	1,03	1,31	1,00
AtPSY1R	0,77	0,63	0,81	0,64	0,80	0,46	1,02	0,91	0,71	0,81	0,92	0,89

7.5 PSK-precursors

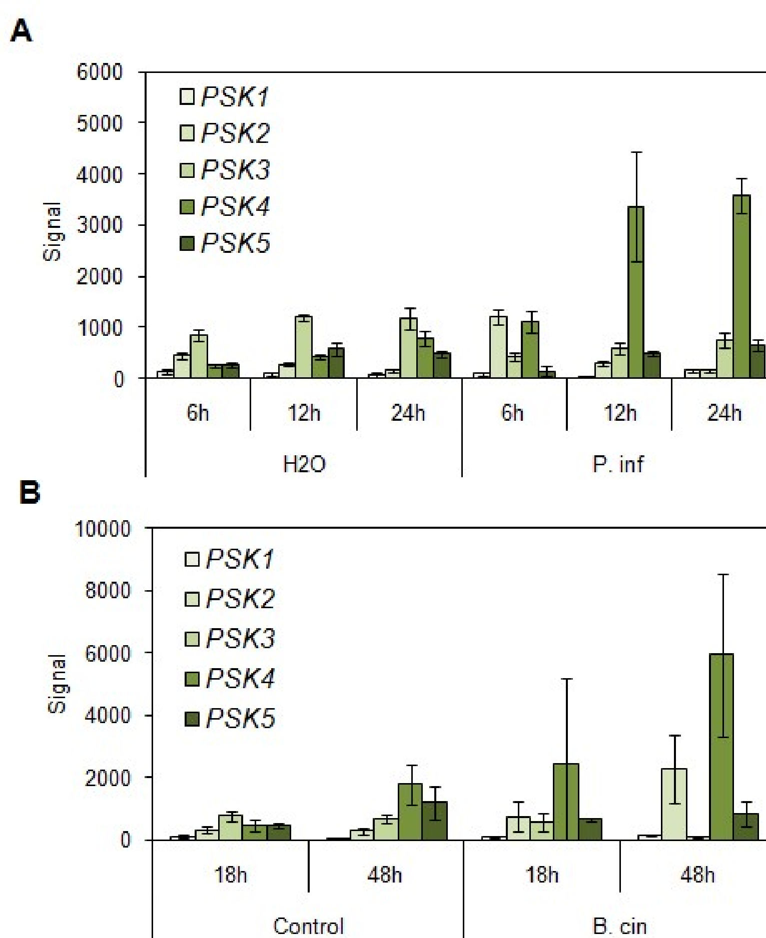


Figure 7.5.1: Microarray data of wild type plants after infiltration with an oomycete and with a necrotrophic fungus

(A) 5-week-old Col-0 plants were drop inoculated with as many as possible 10ul-drops per leaf of a suspension of 10^6 *Phytophthora infestans* spores/ml. Plants were grown under 8/16 hour light/dark conditions. RNA of samples harvested at the indicated time points was isolated. (B) 4-week-old Col-0 rosette leaves were drop inoculated with 5 µl of sterile potato broth, as control, or with conidiospores of *Botrytis cinerea* (collected with sterile water from 2-week-old plates, pelleted and resuspended in 24g L-1 sterile potato dextrose broth). Conidiospores were diluted to $5x10^5$ spores/ml and pre-germinated at RT for 3 hours. Plants were watered right before the experiment, leaving 500 ml of

water on the bottom, then inoculated, covered with a clear plastic lid. Leaves were harvested at the indicated timepoints. Error bars represent standard deviation of corresponding values.

7.6 Sulfotransferases

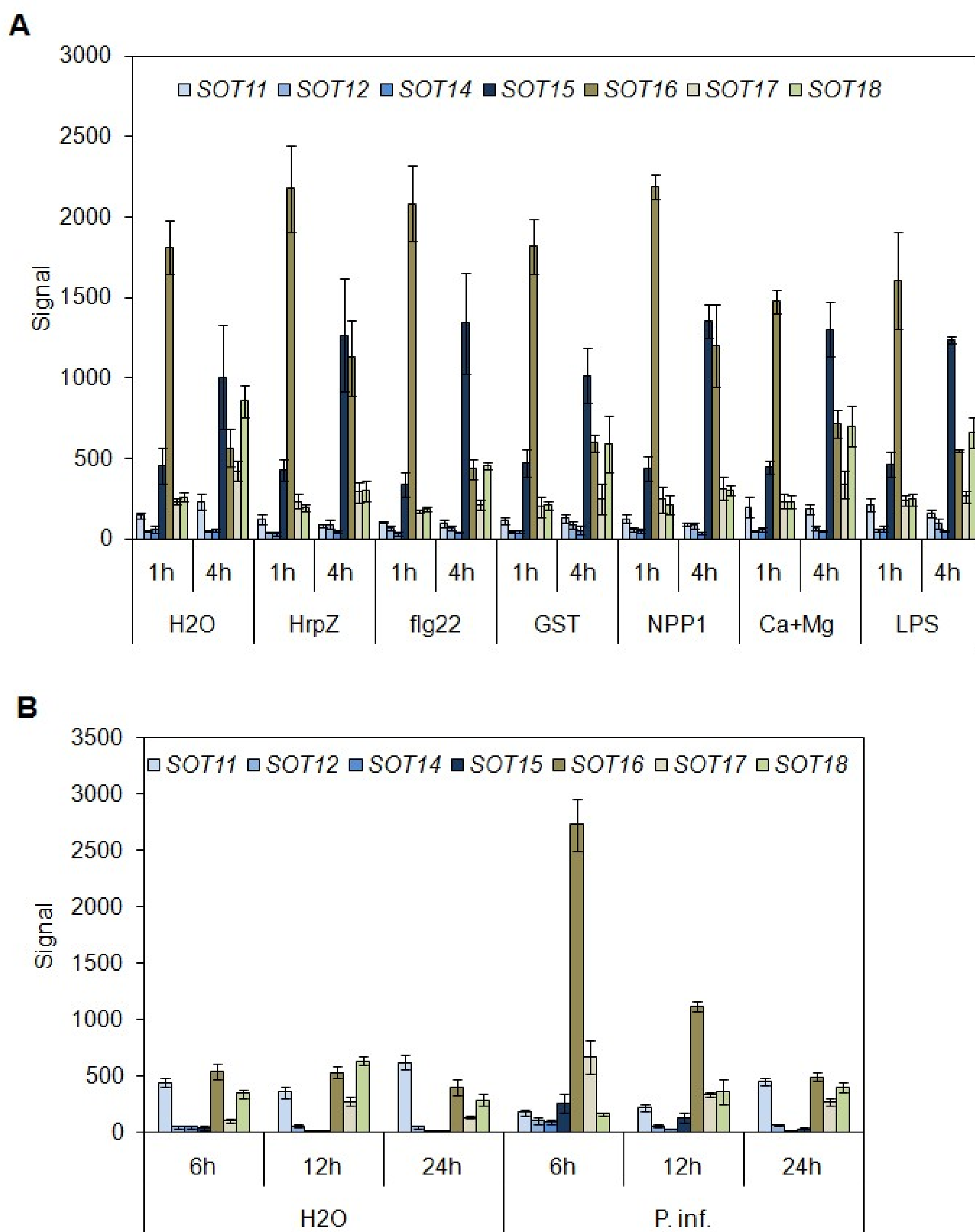


Figure 7.8.1: Expression of AtSOTs after various biotic treatments

(A) Plant material of Col-0 plants was harvested at 1 and 4 h after infiltration with water (control for HrpZ and Flg22), 10 µM HrpZ, 1 µM Flg22, 1 µM GST (control for GST-NPP1), 1 µM GST-NPP1, 1mM $CaCl_2$ + 2.5 mM $MgCl_2$ (control for LPS), 100 µg/mL LPS. RNA was isolated (Chapter 2.6.1) and hybridized to the ATH1 GeneChip. All measurements were taken in duplicates, whose average is shown. The data were normalized by GCOS normalization. Microarray data from GENEVESTIGATOR: Arabidopsis Microarray Database and Analysis Toolbox (Zimmermann *et al.* 2004). (B) 5 week-old Col-0 plants were treated with a suspension of 10^6 Phytophtora infestans spores/mL in water. Samples were taken at 6, 12 and 24hr post infection.

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