

STER1, a novel receptor-like kinase, functions in MAMP signalling in *Arabidopsis*

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Statement of Originality

The material in this thesis has not been previously submitted for a degree in any university, and to the best of my knowledge contains no material previously published or written by another person except where due acknowledgement is made in the thesis itself.

ABSTRACT

S. epidermidis has long been recognized as an important opportunistic pathogen accounting for the majority of nosocomial infections alongside *S. aureus*. However, in spite of this, our understanding of the *S. epidermidis* virulence mechanisms is still limited. Previous studies have emphasized various analogies in innate immunity against pathogens in plant, invertebrate and mammalian hosts. When compared to *in vivo* animal models, plant models are an attractive alternative and experiments using the *S.aureus-Arabidopsis* pathosystem have shown potential for this approach with *S. epidermidis*. In this study, an *Arabidopsis-S.epidermidis* system was established aiming to identify possible bacterial virulence traits. As *S. epidermidis* is not a true plant pathogen it fails to multiply *in planta*; however, most *S. epidermidis* strains tested generated a salicylic acid (SA)-dependent necrotic phenotype in *Arabidopsis* 5 days-post inoculation. Additionally, inoculation with boiled bacteria generated the same visual response as live cells, suggesting a pre-existent, heat stable molecule underlies the plant visual response. Taken together, this data suggests the necrotic response is a visual expression of MAMP perception. Subsequent exploitation of the *Arabidopsis* natural variation through QTL analysis, resulted in the isolation of the STER1 (*Staphylococcus* elicitor response 1) gene. This gene is essential for the visual response to *S. epidermidis* 18888 and encodes a membrane localized DUF26-containing receptor-like kinase. The *ster1-1* mutant remained asymptomatic following inoculation with Gram-positive *S. epidermidis* 18888 and Gram-negative *B. ambifaria*, suggesting STER1's likely role in the recognition of a common molecule. Peptidoglycan (PGN), an essential bacterial membrane component was considered a likely candidate and isolated from both species. Inoculation with *B. ambifaria* PGN generates a plant response that mirrors the one seen with bacterial suspensions of the same organism. By contrast, pure *S. epidermidis* 18888 PGN does not trigger a visual response in *Arabidopsis*. Instead, an *S. epidermidis* 18888 membrane fraction (MF), consisting of PGN, teichoic acid (TA) and an uncharacterized capsular polysaccharide (CPS), was found to generate a necrotic response similar to live cells. Treatment with *S. epidermidis* MF and *B. ambifaria* PGN triggered stereotypical defence responses, such as PR1 up-regulation and cell death in wild-type plants, but not in the *ster1-1* mutant. Additionally, pre-treatment with *S. epidermidis* MF and *B. ambifaria* PGN also restricted *Pst* DC3000 growth in wild-type plants only, thus emphasizing a likely

role for STER1 in basal resistance and PGN perception. In conclusion, the data obtained in this study implicate STER1 in PGN and possibly specialized CPS recognition, either as a receptor, co-receptor or essential signalling component.

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Table of abbreviations

Ba, *B. ambifaria*

CeBiP, chitin oligosaccharide elicitor-binding protein

CERK1, chitin elicitor receptor kinase 1

CPS, *S. epidermidis* capsular polysaccharide

CRK, cysteine rich kinase

DAMP, damage-associated molecular pattern

DAP, diaminopimelic acid

dpi, days post-inoculation

DUF26, domain of unknown function 26

Ef-Tu, elongation factor Tu

EIX, ethylene inducing xylanase

EPS, exopolysaccharide

ET, ethylene

ETI, effector triggered immunity

Flg, flagellin

FLS2, flagellin sensing 2

GBP, glucan binding protein

GFP, green fluorescent protein

hpi, hours post-inoculation

HR, hypersensitive response

JA, jasmonic acid

LPS, lipopolysaccharide

LRR, leucine rich repeat

LysM, lysine motif

MAMP, microbe-associated molecular pattern

MAPK, mitogen-activated protein kinase

MDP, muramyl peptide

MF, *S. epidermidis* membrane fraction

NIL, near-isogenic lines

NO, nitric oxide

NOD, nucleotide-binding oligomerisation domain

PGN, peptidoglycan

PGRP, peptidoglycan recognition protein

PIA, polysaccharide intracellular adhesion

PNAG, poly N-acetyl glucosamine

PR, pathogenesis related

PRR, pattern recognition receptor

Pst, *Pseudomonas syringae* pv. *tomato*

PTI, PAMP(MAMP) triggered immunity

QTL, quantitative trait loci

RIL, recombinant inbred lines

RLK, receptor-like kinase

RLP, receptor-like protein

ROS, reactive oxygen species

SA, salicylic acid

SAR, systemic acquire resistance

Se, *S. epidermidis*

SNP, single nucleotide polymorphism

STER 1, *Staphylococcus* elicitor response 1

TA, teichoic acid

TLR, Toll-like receptor

TTSS, type III secretion system

Xcc, *Xanthomonas campestris* pv. *campestris*

INTRODUCTION

A. The plant innate immune system

1. Overview

Plants are under constant attack above and below-ground by a variety of assailants, which range from microbes, such as bacteria, viruses and fungi, to nematodes and insects (McDowell and Simon, 2008). As a result, plants' survival depends on efficient recognition and fast defence responses.

At first glance, plants may appear to be at a disadvantage compared to animals when it comes to mounting resistance due to several constraints, such as being rooted into place coupled with their inability to produce mobile defender cells characteristic of an adaptive immune system (Jones and Dangl, 2006). In effect, plants rely on the ability of each plant cell to autonomously recognize the presence of diverse invaders and on systemic signals originating from the infection site (Nicaise et al, 2009); while this explains why pathogen-plant interactions occasionally fall in the pathogen's favour, resulting in epidemics such as fungal chestnut blight, this is the exception rather than the rule (McDowell and Simon, 2008). Throughout evolution, plants have developed multiple effective lines of defence and are resistant to most microbes and pathogens.

In the biomedical field, researchers had classically focused on adaptive immunity, however more than ten years ago innate immunity started getting increasing attention, with the discovery of Toll-like receptors (TLRs) and more specifically TLR4 (Medzhitov and Janeway, 1997). While the acquired immune system's strength resides in its ability to form an almost unlimited repertoire of recognition specificities for invading pathogens through developmental shuffling of immunoglobulin/ T-cell receptor genes, this elegant system cannot deploy in the absence of innate immunity (Akira et al, 2006). Unlike vertebrate systems, both plants as well as nonvertebrate animals (eg. *Drosophila melanogaster*) rely exclusively on this evolutionarily ancient first line of defence (Boller and Felix, 2009). The plant immune system has two branches, currently defined as PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Chisolm et al, 2006). This terminology and its associated four-phased "zigzag model" were proposed by Jones and Dangl in 2006 and met immediate acceptance, subsequently replacing the earlier notions of

horizontal (basal) and vertical (R-gene based) disease resistance. Once a microbe penetrates the initial physical/chemical barriers and gains access to the plant interior, inducible defences against it are activated owing to these interconnected surveillance systems. Firstly, membrane-anchored receptor-like proteins, termed pattern-recognition receptors (PRRs), recognize slowly evolving pathogen (microbe) associated molecular patterns (PAMPS/MAMPS), such as flagellin or lipopolysaccharide (LPS) and PTI ensues resulting in microbial growth prevention (Figure 1.1). However, successful Gram-negative pathogens have evolved systems aimed at evading and suppressing PTI, by evolving effectors which subvert plant defence responses following secretion through the type III secretion system (TTSS).

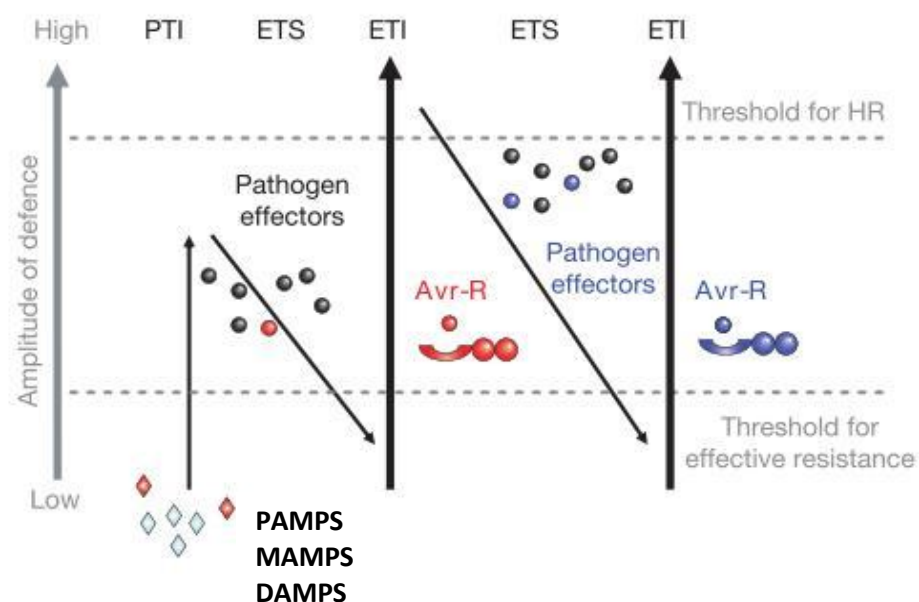


Figure 1.1. The Dangl-Jones zigzag model illustrating the output of the immune system.

In phase 1, plants detect PAMPs/MAMPs/DAMPs (red/grey diamonds) via PRRs and set off PAMP triggered immunity (PTI); In phase 2, successful pathogens evade this first line of defence and deploy effectors (red/black spheres), which results in effector-triggered susceptibility (ETS). In phase 3, the presence of the effector molecule is recognized by an NB-LRR protein in a specific manner (gene for gene Avr-R interaction, red), thus ensuing effector-triggered immunity (ETI) and ultimately a hypersensitive response (HR). In phase 4, pathogens that have acquired a new effector (blue spheres), suppress ETI and prompt ETS. Selection will favour plants with the ability to recognize this new threat and trigger ETI. (modified from Jones & Dangl, 2006)

Direct or indirect recognition of the presence of these effector molecules leads to ETI, a more specialized mechanism of microbial detection, which relies on polymorphic intracellular nucleotide-binding leucine rich repeat (NB-LRR) proteins encoded by *R* genes (Bent and Mackey, 2007). ETI, essentially an enhanced, faster PTI, ultimately culminates in the hypersensitive response (HR), which is a strong defence reaction characterized by apoptotic cell death and local necrosis. Finally, natural selection will favour on one hand pathogens that have diversified their effectors but on the other hand, plants able to prompt ETI in spite of this microbial advantage are also favoured (Jones and Dangl, 2006).

2. MAMP perception in plants (PTI)

Different pathogens employ different strategies to gain entry to the apoplast, where the water and nutrients available will facilitate their proliferation. Preformed barriers on the cell surface, such as the cuticle, cell wall and antimicrobial peptides are the first obstacles to pathogen ingress. In fact, several studies have successfully concentrated on expressing diverse thionins, a class of low-molecular weight antimicrobial peptides in plants in an attempt to provide protection from phytopathogenic bacteria and fungi (Castro and Fontes, 2005; Oard and Enright, 2006).

In order to circumvent these defensive structures, some organisms will operate by stealth, looking to minimize damage to the host, while others will release cell wall degrading enzymes in an attempt to lyse plant cells (Hematy et al, 2009). For example, bacteria will gain entrance through stomata, hydathodes or wounds. In contrast, fungi will either extend hyphae or introduce haustoria in order to feed, while nematodes and aphids will insert a stylet directly into the cells. When the structural barriers of the plant tissue are breached, the resulting products function as endogenous elicitors or damage-associated molecular patterns (DAMPs) (Nurnberger et al, 2004; Tor et al, 2009). A classic example of such molecules are cell wall fragments, such as oligogalacturonides and cutin monomers, released when fungal pathogens discharge degrading enzymes (d'Ovidio et al, 2003; Schweizer et al, 1996). Cytoplasmic peptides released upon injury can also function as danger signals, as shown with systemin in tomato plants and AtPEP1 in *Arabidopsis* (Lotze et al, 2007; Huffaker et al, 2006). The presence of these endogenous plant-derived structures leads to essentially the same salicylic acid (SA) or jasmonic acid (JA)-mediated systemic responses as in the case of MAMP immunity. Such responses include: release of reactive oxygen species (ROS),

changes in ion fluxes, production of ethylene (ET) and induction of pathogenesis-related (PR) proteins.

Perception of MAMPs/DAMPs by PRRs will trigger an active defence response of an appropriate intensity and duration, called basal resistance in plants, which is thought to keep non adapted (non-host) pathogens in check. This form of resistance, now termed PTI, is very similar to innate immunity in animals. However, should one of these initial defence responses fail, disease development will be initiated by the pathogen and an additional layer of resistance, ETI, will come into play (Boller and Felix, 2009). The discovery of plants with enhanced-disease susceptibility (*eds*) showed that even susceptible plants defend themselves against pathogens, but at a basal level which is insufficient to mount an effective response (Parker et al, 1996; Wiermer et al, 2005; Xing and Chen, 2006). While it is tempting to speculate that basal resistance is a result of the MAMP-PRR interaction, it is still unclear how these events converge into a stereotypical defence response. Genetically dissecting PTI is quite a challenge, as it is probably the result of multiple MAMP recognition events and the impairment of one pathway might not have a discernable impact on the overall plant-microbe interaction. Moreover, it still remains to be seen if microbial elicitors exhibit their defence-eliciting characteristics in natural encounters between plants and pathogens (Nurnberger et al, 2004; Gómez-Gómez and Boller, 2002).

2.1 Microbe-associated molecular patterns (MAMPs)

MAMPs are defined as molecular components that are highly conserved within a class of microbes and whose functions are essential towards fitness or survival (Medzhitov and Janeway, 1997). In spite of the increasing number of identified MAMPs recognized by plants, very few plant PRRs have been discovered to date (Table 1.1).

2.1.1 Proteinaceous bacterial MAMPs

Flagellin is the major structural protein of eubacterial flagella and is essential for bacterial motility and pathogenicity. Responsiveness to this protein is shared among most higher plants, indicating that detection of flagellin is evolutionarily ancient (Boller and Felix, 2009). The flagellar building block comprises three regions, namely two highly-conserved N- and C-terminal chains and one hypervariable central region (Ramos et al, 2004). Consistent with a MAMP definition, a 22-amino acid synthetic peptide (flg22), corresponding to a

highly conserved part of the N-terminus, was found to act as a potent elicitor at picomolar concentrations in plants (Felix et al, 1999). However, this minimal active epitope can vary between plants, as exemplified with the shortened version flg15 which is active in tomato, but also acts as an antagonist in *Arabidopsis* (Robatzek et al, 2007). Additionally, monocots, such as rice, show a weaker response to flg22 compared to full length flagellin (Takai et al, 2008). Once flagellin is perceived by the PRR FLS2, a whole array of transient immune responses is triggered including ROS production and activation of the mitogen-activated protein kinase (MAPK) signalling cascade. Downstream, later responses include callose deposition, commonly used as a marker to assess MAMP reactions, and seedling growth inhibition; the latter characteristic ultimately enabled FLS2 mapping (Gomez-Gomez et al, 1999). In addition, pretreatment with flg22 has also been shown to prime plant responses to subsequent pathogen challenges (Zipfel et al, 2004). Finally, although the main difference between PTI and ETI is generally thought to be HR, recent findings have revoked this dogma when several flagellins from *Pseudomonas syringae* pv. *tabaci* 6605 were shown to trigger cell death (Naito et al, 2008). Nevertheless, in some rare cases bacteria are able to mask recognition of flagellin and evade the immune response. Sometimes the glycosylation status of the protein is of significance, but in other instances, phytopathogenic bacteria exhibit mutated residues within the recognized epitope, thus impairing flagellin perception (Felix et al, 1999; Sun et al, 2006; Taguchi et al, 2009). This reflects an efficient strategy developed by successful pathogens alongside effector secretion.

Elongation factor Tu (EF-Tu), the most abundant bacterial protein, acts as a potent MAMP solely in *Arabidopsis* and other *Brassicaceae* (Kunze et al, 2004). Its inability to prompt defence responses in other plant families suggests EF-Tu perception is an innovation in *Brassicaceae* (Boller and Felix, 2009). This elicitor was discovered serendipitously while working with boiled extracts derived from bacteria lacking a flagellin gene (FliC-). Similar to flagellin, EF-Tu is highly conserved in all bacteria and its MAMP activity has been attributed to its acetylated N-terminus. Furthermore, while elicitor activity was restricted to a 18 amino acid peptide (elf18) which triggers equivalent responses at subnanomolar concentrations, a shorter peptide (elf12) was shown to act as a competitive antagonist for EF-Tu-related elicitors such as crude FliC- bacterial extracts. Although EF-Tu is involved in mRNA translation and is therefore intracellular, it is nonetheless perceived at the host plasma membrane by the PRR EFR (Zipfel et al, 2006). This apparent paradox can be explained by

taking into account bacterial lysis during plant colonization. As plants perceive very low concentrations of Ef-Tu, it is possible that dying bacteria release enough elicitor to set off an immune response. Moreover, EF-Tu is present in the secretome of several bacteria and it functions as an adhesion factor at the bacterial surface (Zipfel, 2008).

AvrXa21 is a conserved novel type I-secreted sulfated peptide whose production is controlled in *Xanthomonas oryzae* pv *oryzae* (Xoo) by a dual component system (Lee et al, 2006; Lee et al, 2008). As expected with a MAMP, AvrXa21 is conserved among all the *Xanthomonas* strains sequenced and appears to be essential for cell survival as suggested by the decreased fitness of *rax* mutants (Nicaise et al, 2009; Lee et al, 2006). However, as in the case of flagellin and glycosylation, in this case sulfation status seems to provide specificity to the system. AvrXa21 is recognized by the membrane protein Xa21 in rice (Song et al, 1995). Although initially designated as an R-gene, Xa21 is now regarded as a PRR. This change in opinion was prompted on one hand by Xa21's striking similarity to EFR and on the other hand by the fact that AvrXa21 does not encode one of the usual effectors (Lee et al, 2006). Instead, it would appear this conserved, secreted peptide acts as a quorum sensing signal, a class of molecules previously shown to act as MAMPs as demonstrated with acyl homoserine lactones in tomato (Schuhegger et al, 2006). This finding is not surprising considering bacteria's ability to switch from a free-living to a biofilm lifestyle towards plant colonization and it is likely that plants have in turn developed a system to "listen in" on these intercellular signals.

Several other proteinaceous MAMPs have been characterized at a biochemical level, but their receptors have yet to be identified. Bacterial cold shock proteins are potent elicitors in *Solanaceae*, and similarly to flg22 and elf 18, a synthetic peptide corresponding to the highly conserved RNP1 domain of these proteins triggers an immune response at subnanomolar concentrations (Felix and Boller, 2003). Tobacco also recognizes bacterial superoxide dismutase (SodM) from *Xanthomonas campestris* pv. *campestris* (Xcc) and *E. coli* (Watt et al., 2006), while both tobacco and parsley harbour a perception system for different bacterial harpins (Kim et al, 2004; Engelhardt et al, 2009). Finally, siderophores, a class of small iron-chelating molecules with peptide backbones, are similar in structure for numerous microbes and could also function as MAMPs in dicots, as demonstrated with pseudobactin (Wandersman and Delepelaire, 2004; Meziane et al, 2005).

2.1.2 Carbohydrate bacterial MAMPs

Lipopolysaccharide (LPS) is a ubiquitous component of the external membrane of Gram negative bacteria which acts as a MAMP in dicots and monocots (Newman et al, 2007). These heat-stable complex amphiphilic molecules have a tripartite structure consisting of a membrane-anchored lipid A moiety, a core oligosaccharide and an O-antigen polysaccharide; the presence or lack of the latter dictates whether the LPS is smooth type or rough type (LOS), respectively. The lipid A part of LPS is highly conserved among bacteria and is as effective as the whole molecule in inducing defence responses in *Arabidopsis* (Zeidler et al, 2004; Silipo et al, 2005). Moreover, the acylation and phosphorylation status of this lipid A moiety has an impact on the elicitation capabilities of LPS (Silipo et al, 2008). Interestingly, an antagonist of lipid A in mammals shows similar effects in plants when applied simultaneously with an active lipid A fraction (Erbs et al, 2008a). However, the endotoxic activity of LPS does not reside in lipid A alone, as the core oligosaccharide and synthetic oligorhamnans, which are common components of the otherwise highly variable O-chain, also lead to stereotypical defence responses (Silipo et al, 2005; Bedini et al, 2005). Whereas LPS and other exopolysaccharides activate early defence reactions such as nitric oxide (NO) production and induction of defence-related genes, they are also capable of suppressing responses such as calcium signalling and HR (Tellstrom et al, 2007; Aslam et al, 2008; Esposito et al, 2008). Furthermore, pretreatment with LPS alters the growth pattern of subsequently infiltrated plant pathogens (Newman et al, 2002). Although the identity of an LPS receptor remains elusive, endocytic uptake of Xcc LPS following membrane localization has been shown in tobacco, suggesting receptor mediated LPS internalization could be necessary for mounting a defence response (Gross et al., 2005).

Exopolysaccharides (EPS) from several bacteria, including xanthan from *Xanthomonas*, alginate from *Pseudomonas* and amylovoran from *Erwinia*, have long been associated with plant pathogenesis (Denny, 1995). Furthermore, capsule polymers such as alginate are important for full virulence in multiple model systems, including *Arabidopsis*, *C. elegans* and a mouse model (Yorgey et al, 2001). When colonizing the intercellular spaces of their hosts, many bacteria produce EPS which contribute to biofilm formation. Consistent with a MAMP definition, this bacterial coating is required for bacterial epiphytic survival and it is also largely conserved within species (Yu et al, 1999; Sutherland, 1994). In addition, alginate gene expression in *P. syringae* seems to be required irrespective of attempting to

colonise susceptible or resistant plant hosts (Keith et al, 2003). EPS can also act as a virulence factor by disrupting specific defence responses. More specifically, xanthan suppresses innate immunity by chelating apoplastic calcium and suppressing callose deposition (Aslam et al, 2008; Yun et al, 2006) resulting in recent reviews classifying EPS as an effector rather than a MAMP (Boller and Felix, 2009).

Peptidoglycan (PGN) is an essential and unique component of the bacterial envelope which provides structure and rigidity to the cell, thus enabling it to resist osmotic pressure. While PGN is the major constituent of the Gram positive cell wall, Gram negative bacteria harbour a relatively thin layer of PGN which is restricted to the periplasmic space. PGN is a polymer of alternating N-acetylglucosamine (GlcNAc) and N-acetyl-muramic acid (MurNAc) residues in beta-1-4 linkage which are cross-linked by short peptides (Gust et al, 2007). Whereas the glycan backbone is mostly invariant among bacteria, the peptide subunit and interpeptide bridge reveal species specific differences (Boneca, 2005). Recent research has shown that both Gram positive and Gram negative intact PGN can act as a MAMP (Gust et al, 2007; Erbs et al, 2008b). However, findings regarding the minimal structure which is required for recognition differ. It would appear perception of Gram positive PGN depends on the sugar backbone (Gust et al, 2007), while muropeptides derived from PGN are more active elicitors than intact PGN in the case of Gram negative bacteria (Erbs et al, 2008b). Taken together, these findings suggest plants may maintain a perception system similar to *Drosophila*, which displays the ability to distinguish between the two types of PGN (Leulier et al, 2003). Differences between the elicitation abilities of two different Gram negative pathogens also paralleled differences in their muropeptide structure, hence implying that pathogens may have also evolved PGN modifications to evade recognition (Erbs et al, 2008b). Just as LPS, PGNs are orphan MAMPs, with a receptor yet to be identified. The PGN disaccharide backbone resembles that of chitin, the major component of the fungal cell wall. However, PGN and chitin seem to engage different perception systems (Gust et al, 2007; Zipfel et al, 2009). LysM (lysine motif) domain proteins, such as CEBiP in rice and CERK1 in *Arabidopsis*, have been previously shown to be involved in chitin perception (Kaku et al, 2006; Miya et al, 2007). This domain was originally identified in peptidoglycan-degrading enzymes, so it is conceivable it may also be involved in plant PGN recognition (Buist et al, 2008). In addition, muropeptide detection might require internalization and subsequent intracellular recognition, as demonstrated with Nod factors in mammalian cells (Girardin et al, 2003).

Table 1.1. Selected microbe associated molecular patterns recognized by plants

Pathogens/Microbes	MAMP	Active epitope	Responsive plants	Receptor	References
Bacteria					
Gram-negative bacteria	Flagellin	flg22	Most plants	FLS2 (LRR-RLK)	Gomez-Gomez et al, 1999
	Elongation factor Tu (EF-Tu)	elf18	Brassicaceae	EFR (LRR-RLK)	Zipfel et al, 2006
	AvrXa21	Undefined	Rice	Xa21 (LRR-RLK)	Lee et al, 2008
	Cold shock protein	RNP1 motif (csp15 peptide)	Solanaceae, tobacco, tomato	Unknown	Felix and Boller, 2003
	Lipopolysaccharide (LPS)	Lipid A	Arabidopsis, tobacco, pepper	Unknown	Newman et al, 2002
	Exopolysaccharide (EPS)?	Undefined	Arabidopsis, legumes, Rosaceae	Unknown	Aslam et al, 2008
	Peptidoglycan (PGN)	PGN-derived muropeptides	Arabidopsis	Unknown	Erbs et al, 2008b
Gram-positive bacteria	Cold shock protein	RNP1 motif	Solanaceae, tobacco, tomato	Unknown	Felix and Boller, 2003
	Peptidoglycan (PGN)	Sugar backbone	Arabidopsis	Unknown	Gust et al, 2007
Oomycetes	β -glucans	Branched β -heptaglucoside (HG)	Fabaceae	GBP	Umemoto et al, 1997
	Transglutaminase	Pep13	Parsley, potato	Unknown	Hahlbrock et al, 1995
	Elicitins	Undefined	Tobacco	Unknown	Osman et al, 2001
	Necrosis-inducing proteins	Undefined	Legumes, tobacco, Arabidopsis	Unknown	Fellbrich et al, 2002
Fungi	Chitin	Chitin oligosaccharides	Tomato, Arabidopsis, rice, wheat	CERK1? (LySM-RLK)	Miya et al, 2007
	Xylanase	TKLGE pentapeptide	Tobacco, tomato	LeEIX2 (LRR-RLP)	Ron and Avni, 2004
	Ergosterol	Undefined	Tobacco, tomato	Unknown	Granado et al, 1995
	Invertase	N-manosylated peptide	Tomato	Unknown	Boller et al, 1995

Finally, a new class of surface active bacterial substances, namely biosurfactants, have gained MAMP status recently. To be more specific, rhamnolipids derived from *Pseudomonas aeruginosa* triggered immune recognition in grapevine (Varnier et al., 2009), while cyclic lipopeptides belonging to multiple strains of *Bacillus subtilis* stimulated defense responses in tobacco (Jourdan et al., 2009).

2.1.3 Oomycete and fungal MAMPs

Oomycetes and fungi constitute major classes of plant pathogens which infect a wide range of crops and grasses. As a result, a lot of studies have been aimed at researching both the molecules that facilitate pathogen invasion, as well as the corresponding plant defence mechanisms (Table 1.1).

Members of the *Fabaceae* plant family maintain a detection system for branched 1,3-1,6- β -glucans which are the main polysaccharide components of the oomycete cell wall. More specifically, soybean exploits the presence of a 1,3-1,6- β -heptaglucoside (HG), the smallest active elicitor compound from cell walls of the oomycete *Phytophthora sojae* (Shibuya and Minami, 2001). Similar to other systems (eg. LPS in mammals), a soluble glucan binding protein (GBP) has been purified (Umemoto et al, 1997). However, this extracellular membrane-bound glucanase was shown to be essential, but not sufficient for β -glucan elicitor-dependent disease resistance (Fliegmann et al, 2004). This finding is partially supported by the fact that while GBP proteins have been identified in other plant families, plants tested outside the *Fabaceae* group showed no responsiveness to HG. The current view on the role of GBP is that it releases oligoglucoside fragments that may act as ligands for an unknown high affinity binding site on the same protein (Fliegmann et al, 2004). It is also possible GBP associates with a PRR at the plasma membrane, but experimental evidence to that effect is currently lacking.

Plants recognize a number of other cell wall oomycete proteins and the abundant 42 kDa cell-wall glycoprotein of *Phytophthora megasperma* is a prime example of this. Experiments conducted with parsley cells pinpointed the surface exposed 13 amino acid peptide Pep13 as the minimal active epitope (Hahlbrock et al, 1995). This highly conserved oligopeptide is present within a transglutaminase enzymatic domain and has been shown to elicit a defence response in potato (Brunner et al, 2002). Consistent with the definition of a

MAMP, mutational analysis revealed that amino acids within Pep13 that are indispensable for transglutaminase activity are also required for the activation of plant defence responses (Brunner et al, 2002). So far, the existence of a high affinity binding site for Pep13 in parsley has been demonstrated, but its molecular characterization remains elusive (Nurnberger et al, 2004).

Additional proteinaceous oomycete-derived elicitors include the *Phytophthora* protein NPP1, the *Pythium aphanidermatum*-derived protein PaNie, various sterol binding proteins termed elicitins and Nep-1 like proteins. Necrosis inducing NPP1 is yet another cell-wall protein that evoked the same responses as Pep13 in parsley and a series of defence responses in *Arabidopsis* that culminate in a HR (Fellbrich et al, 2002). Defence reactions measured in carrot, tobacco and tomato were identical for a *P. aphanidermatum* crude elicitor preparation and the purified PaNie protein; however, these responses have not been replicated in oat and maize, suggesting monocotyledonous plants are unable to perceive this signal (Veit et al, 2001). Elicitins, such as the *Phytophthora* INF1 10kDa haloprotein, are potent inducers of the HR in tobacco (Osman et al, 2001); the intensity, specificity and consistency of the HR induction seems to directly relate to small sequence changes in elicitins (Huitema et al, 2005). *Fusarium oxysporum*-derived Nep-1 like secreted proteins trigger a comprehensive immune response and subsequent localized cell death in *Arabidopsis* and dicots only (Qutob et al, 2006).

Finally, arachidonic acid is a lipophilic oomycete MAMP that has not been explored in much depth up to date. This substance accumulates in membranes and appears to be perceived in a selective and specific manner in potato (Boller et al, 1995).

Chitin, a β -1,4-linked linear polymer of N-acetylglucosamine, is a major constituent of the cell walls of most higher fungi. N-acetylchitooligosaccharides derived from chitin are potent MAMPs and unlike other fungal elicitors, they are recognized by all of the higher plant species tested (Felix et al, 1993). It has been hypothesized that plants employ enzymes called chitinases towards decreasing the viability of the invading fungal pathogen and generating the aforementioned elicitor-active chitin fragments (Shibuya and Minami, 2001). Mounting evidence supporting this suggestion includes induction of chitinase genes by fungal infection, accumulation of chitinases at infection sites in planta and increased resistance of transgenic plants overexpressing chitinases (Jach et al, 2005; Wan et al, 2008). The cloned and purified rice chitin binding protein CEBiP is a receptor-like protein (RLP) with two

extracellular LysM domains, a transmembrane domain and a short cytoplasmic tail (Kaku et al., 2006). While silencing of CEBiP in rice leads to a reduction in chitin binding and responsiveness, the lack of an intracellular domain may imply that this protein is assisted in its PRR function by another receptor-like kinase (RLK). In the follow-up, the working assumption for pinpointing the *Arabidopsis* receptor for chitin was that LysM domains are likely involved in perception. Reverse genetics approaches lead to the identification of the *cerk1* mutant, which was completely unresponsive to chitin (Miya et al, 2007; Wan et al, 2008). Interestingly, RLKs like CERK1 that couple LysM motifs with kinase domains are present exclusively in plants (Zhang et al, 2007a). Nevertheless, CERK1 does not appear to be restricted to chitin perception, as *cerk1* mutants are also more susceptible to the adapted bacterium *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 alongside adapted fungi (Gimenez-Ibanez et al, 2009a). As other LysM binding molecules such as chitin and Nod factors can be excluded in the case of *Pst* DC3000, PGN was briefly regarded as the MAMP possibly engaging CERK1. However, this possibility was eventually excluded, leaving no clues as to the identity of the bacterial MAMP involved (Gimenez-Ibanez et al, 2009b). Accordingly, recent in vitro studies have shown that CERK1 directly binds chitin, but not PGN (Iizasa et al, 2010). Unexpectedly, the RLK's phosphorylation status remained unchanged upon chitin binding. As a result, it is tempting to speculate that *Arabidopsis* contains a homologue of CEBiP and that these two proteins act in concert as a functional receptor unit.

Similarly to oomycetes, fungi secrete a variety of proteins that are recognized as elicitors; these include xylanase and yeast invertase-derived glycopeptides (Basse et al, 1993; Boller et al, 1995). The fungal elicitor ethylene-inducing xylanase (EIX) can elicit HR and other plant defence responses in tobacco and tomato independently of its enzymatic products (Boller and Felix, 2009). The TKLGE pentapeptide, a surface-exposed epitope of EIX, has been shown to be essential for its elicitor activity (Rotblat et al., 2002). The tomato receptor for EIX, encoded by the LeEIX1/2 genes, is the only RLP involved in canonical MAMP perception (Ron and Avni, 2004). While both can bind EIX independently, only LeEIX2 is an activator of HR. Furthermore, mutating the endocytosis signal of LeEIX2 abolished subsequent HR, suggesting that, just as in the case of FLS2, receptor endocytosis is important for defence-related signal transduction (Ron and Avni, 2004; Robatzek et al, 2006).

Ergosterol, the main sterol of higher fungi is perceived by tomato cells and tobacco

plants at subnanomolar concentrations (Granado et al, 1995; Lochman et al, 2006). Perception of this lipophilic MAMP is highly sensitive and selective, with plants recognizing fungal ergosterol and its derivatives exclusively and not endogenous plant sterols. Such a perception system targeted to a non-self sterol coupled to the ergosterol-induced cell desensitization is reminiscent of the steroid perception and subsequent receptor adaptation systems in animals (Granado et al, 1995). However, in spite of these interesting findings, little progress has been made towards elucidating the perception mechanism for this fungal membrane component.

2.2. Pattern recognition receptors (PRRs)

Pattern recognition receptors (PRRs) mediate the perception of conserved microbial signatures in plants and animals. Upon MAMP detection, the PRRs activate signalling cascades leading to the subsequent activation of innate immune responses.

2.2.1. PRRs in mammalian systems

Research into innate immunity components was initially overshadowed by the discovery of antibodies and B and T cells, all elements of the more sophisticated adaptive immunity system. Interestingly, it is now apparent that the innate immune system is critical in clearing and moderating pathogen replication until the adaptive immune system can mount a more specific and robust response (West et al, 2006). Innate immunity is phylogenetically conserved in animal systems and initiates infection control via germline-encoded, nonclonal receptors (Medzhitov and Janeway, 1997). More specifically, the major players in extracytosolic and cytosolic MAMP perception in mammals are the Toll-like receptors (TLRs) and the nucleotide-binding oligomerisation (NOD) proteins.

TLRs are membrane-anchored proteins that consist of a leucine rich repeat (LRR) ectodomain, a short transmembrane domain and a carboxyterminal cytosolic Toll and IL-1 receptor homology (TIR) domain (Gay and Gangloff, 2007). Depending on ligand accessibility, these immune sensors are present either on cell surfaces or endosomal compartments. To date, 13 TLRs have been identified in mammals (10 in humans and 13 in mice, respectively); TLRs 10, 12 and 13 have yet to be characterized (Table 1.2).

Table 1.2. Mammalian TLRs and NODs

Receptor	Agonist	Species	Co-receptor	Localization
TLR1	Triacyl lipopeptides	Bacteria	TLR2	Plasma membrane
TLR2	Lipoteichoic acids	Gram positive bacteria	TLR1, TLR6	Plasma membrane
	Atypical LPS	Gram negative bacteria		
	PGN?	Bacteria		
	PNAG?	Gram positive bacteria		
	Lipoproteins	Mycobacteria		
	Zymosan	Fungi		
TLR3	Double stranded RNA	Viruses		Endosomes
TLR4	LPS	Gram-negative bacteria	CD14, MD-2	Plasma membrane
TLR5	Flagellin	Bacteria		Plasma membrane
TLR6	Diacyl lipopeptides	Mycobacteria		Plasma membrane
	Zymosan	Fungi		
TLR7	Single stranded RNA	Viruses		Endosomes
TLR8	Single stranded RNA	Viruses		Endosomes
TLR9	CpG DNA motifs	Viruses and bacteria		Endosomes
TLR10	?	?		?
TLR11	?	Uropathogenic bacteria		?
	Profilin-like protein	Protozoa		
TLR12	?	?		?
TLR13	?	?		?
NOD1	DAP-PGN	Gram-negative bacteria		Cytoplasm
NOD2	MDP-PGN	Bacteria		Cytoplasm

References: **TLR1**, Jin et al, 2007; **TLR2**, Strunk et al, 2010; **TLR3**, Alexopoulou et al, 2001; **TLR4**, Park et al, 2009; **TLR5**, Hayashi et al, 2001; **TLR6**, Takeuchi et al, 1999; **TLR7**, Hemmi et al, 2002; **TLR8**, Heil et al, 2004; **TLR9**, Hochrein et al, 2009; **TLR10,12 and 13**, Akira et al, 2006; **TLR11**, Zhang et al, 2004; **NODs**, Rosentiel et al, 2008.

Crystallographic analyses have revealed that the TLR extracellular domain exhibits a typical horseshoe-shaped solenoid structure which contributes towards receptor dimerization (Bacalla et al, 2009). The hydrophobic residues that form the core of this secondary structure are conserved, and ligand specificity is presumably conferred through the side chains of variable residues and/or cooperation between different TLRs. The engagement of TLRs by various microbial components leads to the activation of signalling cascades which culminate in the induction of host defence genes. Subsequent to ligand binding, TLRs undergo conformational changes and recruit adaptor molecules to their TIR domain; these are MyD88, TIR-associated protein (TIRAP)/MyD88adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN- β (TRIF)/TIR-domain-containing molecule (TICAM1) and TRIF-related adaptor molecule (TRAM) (Akira et al, 2006). In turn, MyD88 and TRIF lead to the

activation of distinct signaling pathways, resulting in the production of proinflammatory cytokines and interferons, respectively (Albiger et al, 2007). So far, the cell-membrane localized TLR4 is the most extensively studied TLR.

LPS, the most studied TLR ligand, activates the innate immune system via TLR4 leading to the production of numerous proinflammatory mediators (West et al, 2006). First, LPS is processed by the soluble LPS binding protein (LBP) and soluble CD14 (sCD14), an LRR-containing protein. Once LPS is cleaved from the bacterial membrane, an LPS-LBP-sCD14 complex forms which will ultimately deliver the glycolipid to the membrane glycosylphosphatidylinositol (GPI)-anchored CD14. LPS is subsequently transferred to MD-2, which associates with the extracellular portion of TLR4 prior to TLR4 oligomerization (Shimazu et al, 1999). The recent elucidation of the crystal structure of the MD2-TLR4-LPS complex has provided new insights into the molecular basis of this mechanism (Park et al, 2009). More specifically, an LPS with six lipid chains was shown to interface with a large hydrophobic pocket in MD-2 through five lipid chains and with TLR4 via the remaining chain. This interaction promoted the formation of an m-shaped receptor multimer composed of two copies of the TLR4–MD-2–LPS complex arranged symmetrically.

The intracellular NOD-LRR receptor family seems to play a pivotal role for the recognition of intracellular MAMPs. Proteins in this family possess a ligand sensing LRR domain, a nucleotide binding oligomerization domain (NOD) and a domain for the initiation of signalling, such as CARD (Rosenstiel et al, 2008). Although initial studies identified LPS as a NOD2 ligand, it is now well established that NOD1 and NOD2 recognize PGN derived peptides, namely γ -d-glutamyl-meso-diaminopimelic acid (DAP) and muramyl peptide (MDP), respectively (Table 1.2) (Girardin et al, 2003; Chamaillard et al, 2003). This recognition process is highly specific and can be abolished by either mutating critical residues within the LRR domain or minimal changes of the ligand (Girardin et al, 2005; Tanabe et al, 2004). Taken together, these findings suggest the LRR domain recognizes the PGN-derived peptides, although evidence of direct ligand-NOD protein binding is still lacking. Interestingly, NOD1 and NOD2 are structurally, but not functionally related to cytosolic plant R proteins. Such R proteins contrast with NODs in that they recognize distinct effector molecules from pathogenic bacteria instead of conserved bacterial structures. This difference partly explains why plant genomes encode more than 150 NB-LRR proteins as opposed to only 25 in humans (Chamaillard et al, 2003).

2.2.2. PRRs In *Drosophila*

Most of the genes involved in *Drosophila* host defence are highly similar to genes implicated in mammalian innate immune defences (Hoffmann, 2003). In contrast to mammals, the repertoire of bacterial motifs recognized by the immune system of the fruit fly seems to be very narrow and appears to be restricted to PGN and its derivatives (Charroux et al, 2009). Two major pathways control the expression of antimicrobial defences during infection. The activation of a Toll pathway accounts primarily for the response to infections by fungi and Gram-positive bacteria, while the Imd (immune deficiency) pathway underlies resistance to Gram-negative bacteria (Figure 1.2). Toll, the namesake of the TLR family, is a transmembrane protein with extracellular LRRs and an intracellular TIR domain, while Imd is a cytoplasmic protein. Once the insect immune system discerns between the various classes of microorganisms, a range of effector molecules is produced, among which antimicrobial peptides are prominent.

In the case of Gram positive bacteria, once the GNPB1 glucanase fragments the peptidoglycan and presents it to PGRP-SA and/or PGRP-SD (two PGN recognition proteins), a binary or ternary complex is formed. Toll activation during the immune response is dependent on the Spaetzle protein, which requires proteolytic cleavage for full biological activity (Mizuguchi et al, 1998). The protease cascade responsible for processing Spaetzle is triggered once deleterious bacterial or fungal presence is acknowledged through PGN-PGRP complex formation. Conversely, The Imd pathway is primarily targeted against Gram negative bacteria (Figure 1.2). Activation of this signalling mechanism relies on a putative transmembrane protein that is also a member of the PGRP family (PGRP-LC and/or PGRP-LE) (Royet et al, 2005). So far, the molecular basis of the Imd pathway activation via PGRP-LC remains elusive.

PGRPs are evolutionarily conserved proteins which share a conserved L-shaped PGN binding groove with one deep and one shallow end (Guan et al, 2004; Guan and Mariuzza, 2007). While mammals also express four PGRP proteins, these differ from insect proteins as far as localization and function goes. In addition, plants and nematodes do not appear to express the specific proteins (Charroux et al, 2009). According to a phylogenetic analysis of insect PGRPs, two categories emerge: PGRPs which hydrolyze PGN, and non-catalytic PGRPs, which activate proteolytic cascades or signaling pathways (Dziarski and Gupta,

2006). The PGN-PGRP interaction is one of the few receptor-ligand complexes characterized at the molecular level by X-ray crystallography. *Drosophila* distinguishes between the two types of PGN by exploiting the nature of the third residue in the peptide stem, namely lysine (Lys) for Gram-positive bacteria and DAP for Gram-negative bacteria.

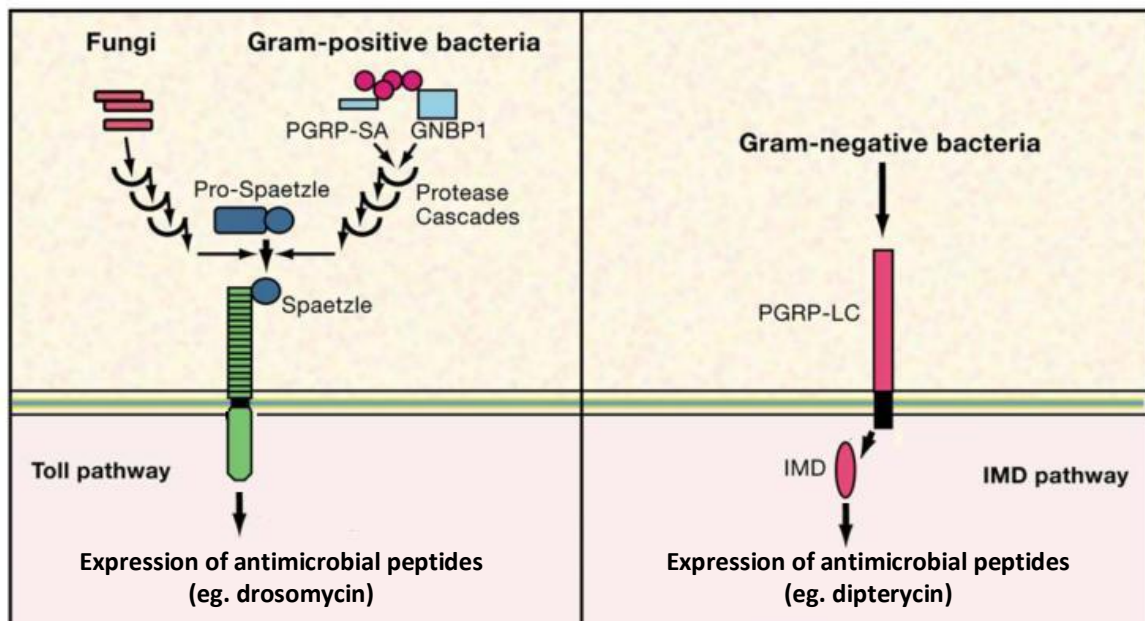


Figure 1.2. *Drosophila* innate immune recognition

In *Drosophila*, the Toll pathway is essentially triggered during infection by fungi and Gram-positive bacteria. Toll activation is mediated by binding of a cleaved form of the cytokine Spaetzle. This cytokine is cleaved in the blood by a circulating protease activated subsequently to Gram positive Lys type PGN recognition, which requires the presence of peptidoglycan-recognition protein PGRP-SA concomitantly with the Gram-negative bacteria binding protein GNBPs. During Gram-negative bacterial infection, microbial sensing occurs at the plasma membrane of immune-responsive cells by the transmembrane receptor PGRP-LC leading to the activation of the Imd pathway by an unknown mechanism (adapted from Akira et al, 2006).

Studies conducted using monomeric DAP-PGN and the recombinant PGRP-LE revealed a tendency towards receptor dimerization upon ligand binding (Lim et al, 2006). Nevertheless, the two PGRP molecules contribute differently to PGN binding and subsequent dimer formation. The PGN tetrapeptide stem lies in the PGN-binding cleft of one PGRP-LE molecule; the second PGRP molecule interacts solely with the disaccharide moiety of the PGN via a surface exposed α -helix (Lim et al, 2006). Finally, the identity of three key

residues in the PGN binding cleft underlies Gram-positive and/or Gram-negative PGN specific recognition (Swaminathan et al, 2006).

2.2.3. Plant PRRs

Pattern recognition receptors (PRRs) are key players in important plant processes such as growth and development, disease resistance, hormone perception and self-incompatibility. Almost all of the PRRs characterized to date are transmembrane proteins with or without an intracellular signalling domain (receptor-like kinases or receptor-like proteins, respectively). However, there is emerging evidence that some apoplastic and cytoplasmic proteins might function as PRRs as well.

2.2.3.1. Receptor like kinases (RLKs)

Signal perception through cell surface receptors, that are generally comprised of an extracellular ligand binding domain and an intracellular kinase domain, is a common feature in living organisms. The RLK gene family is one of the largest in *Arabidopsis*, accounting for almost 2.5% of the annotated protein-coding genes (Shiu and Bleecker, 2001a). RLK genes were established early in land plant evolution and the corresponding proteins play roles ranging from growth regulation to defence response (Lehti-Shiu et al, 2009). Interestingly, the subsequent dramatic expansion of this family has been restricted to subfamilies required towards withstanding fast-evolving pathogens (Afzal et al, 2008). On the basis of the presence or absence of the extracellular domain, members of the RLK serine-threonine kinase family are categorized as RLKs or RLCKs (receptor-like cytoplasmic kinases). Moreover, RLKs can be classified according to the structural characteristics of their extracellular domains (Figure 1.3).

2.2.3.1.1. LRR-RLKs

In *Arabidopsis*, the LRR domain represents the largest class of RLK extracellular motifs. The LRR-RLKs can be divided into several subfamilies, with each displaying characteristic numbers and locations of LRRs within the extracellular N-terminus (Figure 1.3). The LRR motif often participates in protein-protein interactions, hence suggesting

this extracellular domain binds proteinaceous ligands or protein-ligand complexes (Kobe and Kajava, 2001). So far, the majority of plant RLKs shown to be involved in developmental and/or defence responses belong to the LRR class of receptor kinases. Furthermore, the assumption that LRR-RLKs are the major players in pattern recognition has been supported by microarray data showing that various PAMP treatments resulted in LRR-RLK transcript upregulation (Nurnberger and Kemmerling, 2006; Zipfel et al, 2004).

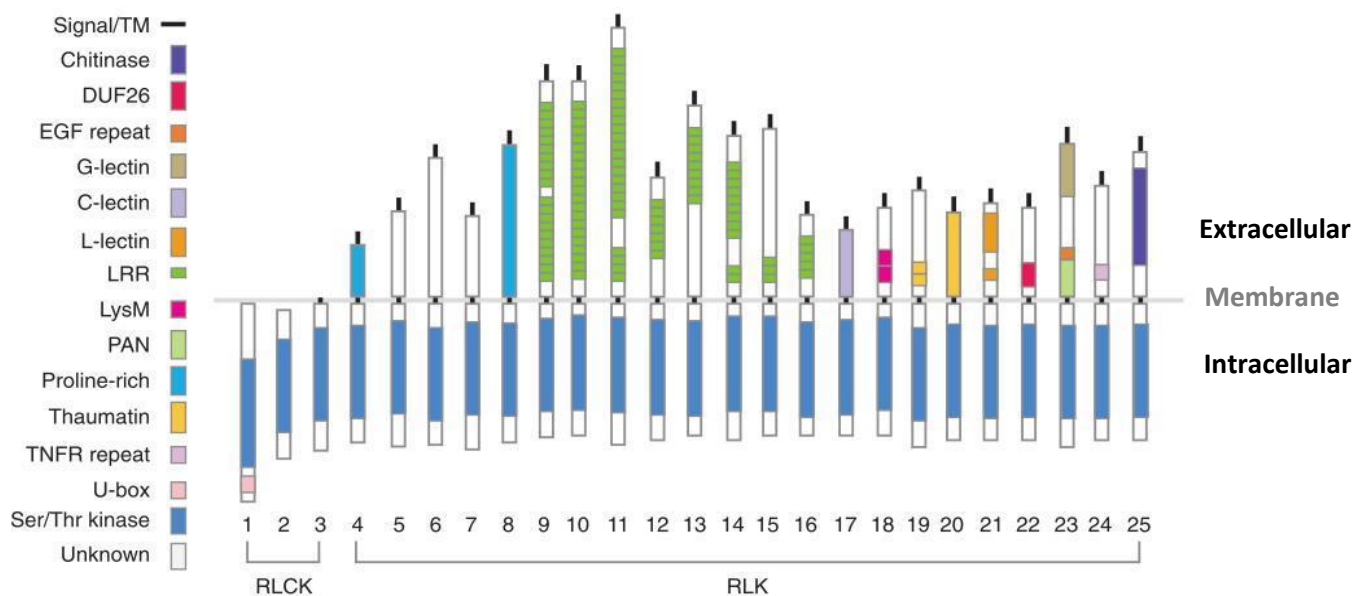


Figure 1.3. Representative members of the receptor-like kinase (RLK) family.

On the basis of the presence or absence of the extracellular domain, members of the RLK serine-threonine kinase family are categorized as RLKs or RLCKs (receptor-like cytoplasmic kinases). RLKs can be further classified according to the structural characteristics of their extracellular domains. TM, transmembrane region; DUF, domain of unknown function; EGF, epidermal growth factor; G-lectin, agglutinin; C-lectin, C-type lectin; L-lectin, legume lectin; LRR, leucine-rich repeat; LysM, lysin motif; PAN, plasminogen/apple/nematode protein domain; TNFR, tumor necrosis factor receptor; Ser/Thr, serine/threonine. (adapted from Shiu and Bleecker, 2001a)

FLS2, a member of the LRR XII RLK family which mediates the recognition of a highly conserved fragment of bacterial flagellin, is the first receptor protein identified in *Arabidopsis* (Gomez-Gomez and Boller, 2002; Chinchilla et al, 2006). While the extracellular domain of this receptor adopts a stereotypical horseshoe-like solenoid structure, the intracellular kinase region encompasses a non-canonical non-RD catalytic loop, viewed as a

hallmark of PRR kinases (Dardick and Ronald, 2006). Homologues of FLS2 with a high degree of conservation are present in the genomes of all higher plants studied, except for the moss *Physcomitrella patens* which appears to lack a flagellin perception system (Boller and Felix, 2009). Several studies have emphasized the role of FLS2 in bacterial resistance, as lack of flagellin perception in *Arabidopsis* and *N. benthamiana* leads to enhanced susceptibility to virulent, weakly virulent and non-adapted bacteria (Zipfel et al, 2004; Hann and Ratjen, 2007). Although FLS2 was shown to directly bind to flg peptides via LRRs 9-15, the exact binding site is still unknown (Dunning et al, 2007).

The EFR protein has a similar domain structure to FLS2 and belongs to the same RLK family. Although EF-Tu perception is restricted to *Brassicaceae*, several EFR putative orthologs have been detected in other plant genomes; it is presumed such proteins function as PRRs for other MAMPs, as demonstrated with the rice Xa21 receptor. EFR clearly contributes towards bacterial resistance as *Arabidopsis efr* mutants have been shown to be more susceptible to *Agrobacterium tumefaciens* and to hypovirulent strains of *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (Zipfel et al, 2006; Zipfel, 2009). In addition, transient expression of *Arabidopsis* EFR in *N. benthamiana* leaves restored EF-Tu/elf responsiveness, confirming downstream PRR signalling is conserved between the two plant species (Zipfel et al, 2006). Furthermore, expression of *Arabidopsis* EFR in *N. benthamiana* and tomato rendered these plant species more resistant to phytopathogenic bacteria (Lacombe et al, 2010). Recent studies based on a forward genetic screen for *Arabidopsis* elf18-insensitive (*elfin*) mutants has revealed several endoplasmic reticulum (ER) machinery components are required for PRR accumulation in innate immunity (Li et al, 2009). In effect, both the ER protein complex comprising stromal-derived factor-2 (SDF2) as well as the N-glycosylation status appear to be important for EFR biogenesis and function (Nekrasov et al, 2009; Haweker et al, 2010).

Although LRR-RLKs act to transduce an extracellular recognition event into an intracellular signalling cascade, it has become apparent that additional interactions with other LRR-RLKs are necessary to attain full function. Recent work has shown that FLS2 and EFR require the presence of SERK3/BAK1 towards integrating a perception event into downstream MAMP responses (Chinchilla et al, 2007; Heese et al, 2007) (Figure 1.4). BAK1, a member of the LRR-II subfamily, is an interacting partner of BRI1, a receptor like kinase which perceives brassinosteroids necessary for growth and development. Although dispensable for flg22 binding, the positive signalling regulator BAK1 is recruited into the

FLS2 receptor complex at a very early stage (Chinchilla et al, 2007). The current model for FLS2 activation implies that a conformational change triggered upon flagellin binding allows for FLS2-BAK1 receptor association through their LRR domains and subsequent intracellular signalling events (Chinchilla et al, 2009); this latter stage is reliant on the kinase activity of BAK1 as well as on the intracellular kinase BIK1 which ultimately transphosphorylates the BAK1-FLS2 complex (Schulze et al, 2010; Lu et al, 2010). Consistent with its role as an ubiquitous adaptor and/or signal amplifier, loss of BAK1 function in *Arabidopsis* and *N. benthamiana* leads to reduced responsiveness to flagellin and other MAMPs, such as LPS and PGN (Shan et al., 2008). Nevertheless, the weaker effect of the *bak1* mutation on elf18 responses coupled to the residual flg22 sensitivity in *bak1* null mutants suggests that the receptor complexes might involve additional proteins. BKK1/SERK4, the closest paralog of BAK1, and SERK1 were regarded as potential candidates due to their known interaction with BRI1 (Chinchilla et al, 2007). However, the possibility of a functional redundancy between BAK1 and other SERKs in MAMP signalling has been tested and *serk* single mutants (*serk1* to *serk5*) showed no defect in flg22 or elf18 perception (Chinchilla et al, 2007). Furthermore, a double *bak1bkk1* mutant dies at the seedling stage due to constitutive necrosis (He et al, 2007). While this phenomenon precludes further analyses, it also suggests BKK1 is part of a constitutive defence response; this conclusion is partly supported by recent studies which demonstrate both *bak1* and *bkk1* mutants are more susceptible to turnip crinkle virus (TCV) (Yang et al, 2010).

In addition to PAMPs, animal and plant cells can recognise molecules from damaged host cells that are normally not available for recognition, called DAMPs (Lotze et al, 2007). These endogenous elicitors differ according to the plant species investigated. For example, the small peptide systemin triggers a defence response in tomato plants. Although the tomato ortholog of BRI1, Cu3, was initially designated as the systemin receptor, subsequent work demonstrated an additional LRR-RLK is required for systemin recognition (Lanfermeijer et al, 2008). In *Arabidopsis*, the endogenous peptide elicitor AtPep1 is directly recognized by the LRR-RLK PEPR1 (Huffaker et al, 2006; Yamaguchi et al, 2006). In addition, recent bioinformatic studies have shed light on the signalling events associated with DAMP perception, by identifying BAK1 as an interactor of PEPR1 and 2 (Postel et al, 2010). This peptide molecule is derived from the PROPEP1 protein, whose constitutive expression leads to enhanced resistance to the fungal root pathogen *Pythium irregulare*.

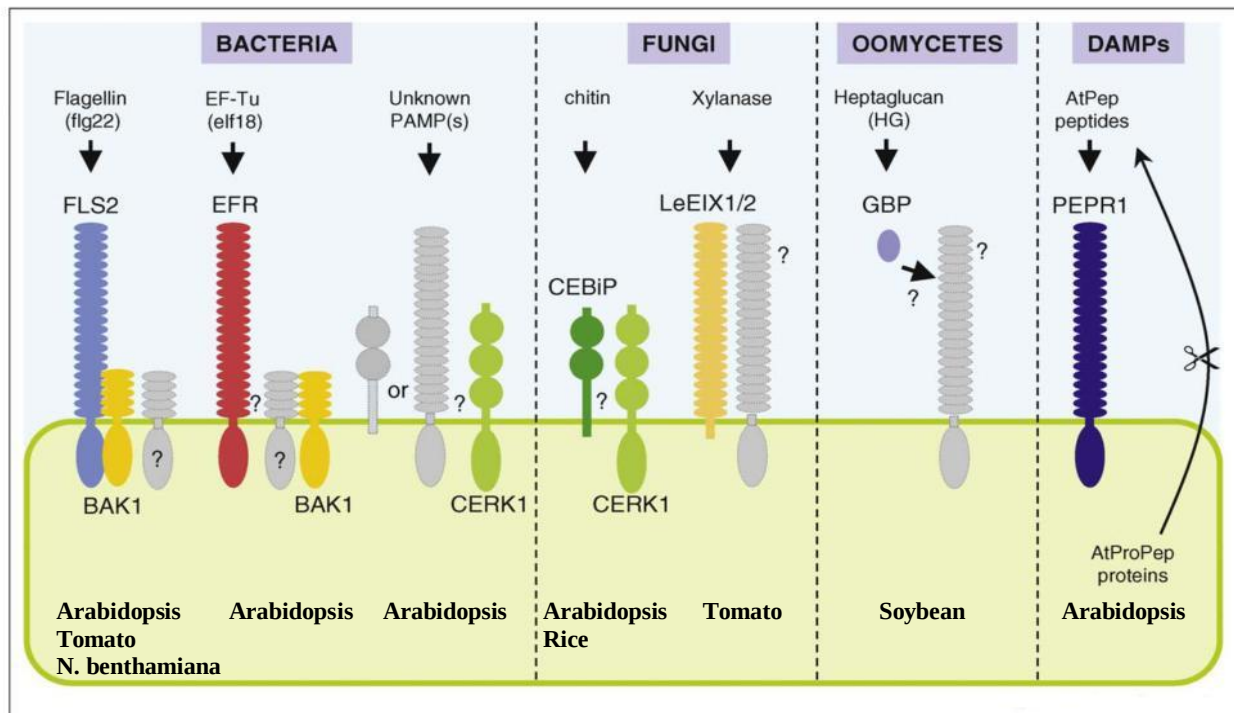


Figure 1.4. Plant RLKs/RLPs and their signalling adapters.

Bacterial flagellin (flg22) and EF-Tu (elf18) are recognised by the Arabidopsis LRR-RKs FLS2 and EFR, respectively. FLS2, and potentially EFR, oligomerise with BAK1 and maybe other SERK proteins in a ligand-dependent manner. The Arabidopsis LysM RK CERK1 mediates recognition of chitin and an unknown MAMP in anti-bacterial immunity. The chitin high-affinity-binding site in rice corresponds to the RLP CEBiP. In tomato, xylanase is recognised by the RLPs LeEIX1 and LeEIX2. In soybean, the soluble glucan-binding protein (GBP) directly binds oomycetal heptaglucon. The Arabidopsis LRR-RK PEPR1 recognise the endogenous AtPep peptides that act as DAMPs. The ProPep proteins are AtPep precursors which can be induced by their own peptides, thus ensuring a positive signal amplification loop (modified from Zipfel, 2009)

Interestingly, all PROPEP proteins can be induced by their respective AtPep peptides, MAMPs such as flg22 and elf18, salicylic acid, jasmonic acid or ethylene. Taken together, these data suggest AtPep1 acts as a positive signal amplification loop towards mounting a rapid and robust defence against invaders (Ryan et al, 2007) (Figure 1.4).

RLKs also play a major role in the perception of developmental cues and the clades of RLKs that are known to be involved in development contain similar numbers of genes in *Arabidopsis* and rice (Morillo and Tax, 2006). BRI1, a member of the LRR X subfamily involved in brassinolide perception and growth regulation, is one of the best characterized receptors in plants (Li and Chory, 1997). Other examples of LRR-RLK engaged in development include CLV1, which adjusts meristem size, and HAESA, which regulates the

abscission of floral organs (Clark et al, 1997; Stenvik et al, 2008). However, some RLKs can play a role in both defence responses and development and one such LRR-RLK is ERECTA. Although initially pinpointed as a developmental regulator for organ shape, subsequent studies have implicated this gene in *Arabidopsis* resistance to the bacterium *Ralstonia solanacearum* and the fungus *Plectosphaerella cucumerina* (Llorente et al, 2005).

2.2.3.1.2. LecRKs

The second largest class of extracellular motifs found in RLKs are various sugar binding motifs or lectins. The lectin receptor protein kinases (LecRKs) exhibit 3 types of extracellular motifs: legume lectin motifs (L-lectins), agglutinin motifs (G-lectins, formerly named B-lectins) and C-type lectin motifs (C-lectin) (Figure 1.3). Legume lectins have been extensively characterized in terms of their three-dimensional structure and ligand specificity (Hervé et al, 1996; DeHoff et al, 2009). Their binding to complex oligosaccharides and hormone ligands such as auxins is well documented, but little is known about their biological roles in plants (Barre et al, 2002). Nevertheless, some reports have implicated L-lectins in *Medicago truncatula* nodulation, abscisic acid (ABA) regulation and plant defence (Navarro-Gochicoa et al, 2003; Xin et al, 2009; Kanzaki et al., 2008). For example, a legume-like lectin receptor kinase from *N. benthamiana* was reported to interact intracellularly via its kinase domain with the *P. infestans* elicitor INF1 and play a role in the subsequent INF1-induced cell death (Kanzaki et al., 2008). This result led to the speculation that perhaps receptors use their extracellular lectin domains to anchor to a particular area of the plasma membrane prior to receptor endocytosis and subsequent signal amplification. The second type of lectins, G-lectins, specifically bind α -D-mannose (Hester et al, 1995). However, the most well studied member of this class is the S-locus receptor kinase (SRK) which functions in self-incompatibility in *Brassicaceae* (Stein et al, 1991). While this receptor detects the pollen ligand SCR (S-locus cysteine rich protein), it does not appear to employ the lectin domain for this recognition event (Shiba et al, 2001). The third class of lectins, C (calcium-dependent) type lectins, are ubiquitous in mammals, where they mediate innate immune responses through non-self sugar-moiety recognition. By contrast, *Arabidopsis* has a single gene encoding a protein with a C-type lectin motif, whose function is yet to be elucidated (Bouwmeester and Govers, 2009).

2.2.3.1.3. LysM-RLKs

In addition to the lectin motifs, other types of sugar-binding motifs are also present in RLKs, with LysM RLKs being the most studied so far (Figure 1.4). The LysM domain is an ancient and ubiquitous protein module that binds peptidoglycan and structurally related molecules, with GlcNAc being the most commonly bound sugar component (Zhang et al, 2007a). The *Arabidopsis* CERK1 RLK has three LysM domains. Mounting evidence suggests that while CERK1 is the receptor for fungal chitin and an unknown MAMP from *Pst DC3000*, it does not recognize PGN (Iizasa et al, 2010; Gimenez-Ibanez et al, 2009b). Nonetheless, this RLK is critical for elicitor signalling and resistance to pathogens. As previously shown for BAK1, the type III effector AvrPtoB directly targets CERK1 to disrupt defence responses elicited by this receptor (Shan et al, 2008; Gimenez-Ibanez et al, 2009a). LysM RLKs such as the *Lotus* NFR1 and NFR5 are involved in the perception of bacterial chitin-like molecules (Nod factors) during the nitrogen fixing legume–*Rhizobium* symbiosis (Radutoiu et al, 2003).

2.2.3.1.4. DUF26-RLKs

The third largest class of RLKs consists of CRKs (cysteine-rich kinases) which contain the DUF26 (domain of unknown function) extracellular motif (Figure 1.3). All DUF26 genes contain two DUF26 domains consisting of a conserved cysteine motif (C-X8-C-X2-C). Although this pattern has been identified in 45 membrane kinases and several secreted proteins involved in diverse plant processes, its function is still unknown (Strain and Muse, 2005). There are four classes of DUF26 domain containing proteins: proteins with a signal peptide for secretion, DUF26 RLPs, GPI anchored proteins and RLKs. The representatives of the first class were shown to localize to the apoplast (Thomas et al, 2008). In the case of RLPs, the *Arabidopsis* PDL1a is targeted to the plasmodesmata, where it modulates cell-to-cell trafficking (Thomas et al, 2008). By contrast, the rice OsRMC protein which mediates root development and JA signal transduction localizes to the plasma membrane (Jiang et al, 2007). Interestingly, DUF26 proteins were also secreted by rice cells in response to the rice blast fungus *Magnaporthe grisea*, suggesting that DUF26 might play an important role in both plant development and defence (Kim et al, 2009). Both the GPI-anchored and RLK proteins localize to the plasma membrane (Thomas et al, 2008). Almost

all *Arabidopsis* DUF26 RLKs are located on chromosome four, as opposed to the broad distribution of LRR-RLKs (Shiu and Bleecker, 2001b). A bioinformatic study aimed at identifying positively selected sites in the *Arabidopsis* RLK gene family found that 9 of the 12 groups of RLKs under positive selection had either DUF26 or LRR receptor domains (Strain and Muse, 2005). Such findings emphasize the apparent significance of DUF26 proteins. Members of the DUF26-RLK family have been shown to be induced by SA and pathogens, with CRK5 being implicated in regulating cell death (Czernic et al, 1999; Chen et al, 2004). In addition, overexpression of CRK13 in *Arabidopsis* was shown to confer resistance to *Pst* DC3000, by prompting increased SA accumulation (Acharya et al, 2007). Recently, the crystal structure of the DUF26-containing antifungal protein Gnk2 from *Ginkgo biloba* was reported (Miyakawa et al, 2009). This protein displays a positively charged surface and it was proposed its antifungal activity results from the interaction with negatively charged phospholipids and/or phosphomannan on the fungal-cell surface (Miyakawa et al, 2009).

2.2.3.2. Receptor like proteins (RLPs)

Receptor-like proteins (RLPs) are transmembrane proteins with extracellular LRRs, a short cytoplasmic tail, but lacking an intracellular signalling domain. The number of predicted RLPs varies according to the species studied, with 57 identified in *Arabidopsis* and over 90 in rice (Fritz-Laylin et al, 2005). These surface receptors are involved in growth, development as well as plant defences (Tor et al, 2009).

Many of the *Arabidopsis* and rice RLPs cluster into four distinct clades, three of which include RLPs known to be involved in plant defence (Fritz-Laylin et al, 2005). Functional characterization of AtRLP genes has proved difficult, as most homologous AtRLP genes reside at the same locus. Such genomic clustering may underline functional redundancy in single knockouts, thus making it difficult to uncover the role of these genes. In addition, while a study using an RNA interference approach in *Arabidopsis* confirmed previously characterized defence- associated phenotypes, it did not succeed in identifying new ones (Ellendorf et al, 2008). A number of known RLPs encode R proteins, as exemplified by the *Cf* (resistance to *Cladosporium fulvum*) and *Ve* (resistance to *Verticillium spp*) genes in tomato (Kawchuk et al, 2001; Kruijt et al, 2005). The first RLP identified was

the tomato *Cf9* gene that mediates resistance to *C. fulvum* strains carrying the *Avr9* avirulence gene. RLPs known to play a role in MAMP immunity are the LeEIX1/2 xylanase receptor of tomato and the rice chitin binding protein CeBiP (Ron and Avni, 2004; Kaku et al., 2006) (Figure 1.4). Studies performed to date have shown only LeEIX2 can transduce the signal when expressed transiently in tobacco. LeEIX proteins require endocytosis in order to interact with cytoplasmic host proteins before initiating defence-related intracellular signalling. A yeast two-hybrid screen pinpointed the cytoplasmic T-SUMO protein as an interacting partner mediating EIX-induced signalling (Hanania et al, 1999). Recently, additional RLPs have been linked to innate immunity. For example, while AtRLP30 and possibly AtRLP18 were shown to contribute to nonhost resistance against *Pseudomonas syringae* pv. *phaseolicola*, AtRLP52 was shown to confer resistance to the non-adapted fungal pathogen *Erysiphe cichoracearum* (Wang et al, 2008; Ramonell et al, 2005).

RLPs also play significant roles in plant development. For example, *Arabidopsis* CLAVATA2 (CLV2, AtRLP10) and Too Many Mounths (TMM, AtRLP17) are essential for meristem and stomatal development, respectively (Jeong et al, 1999; Nadeau and Sack, 2002). CLV2 was shown to be required for the stability and accumulation of the LRR-RLK CLV1 (Jeong et al, 1999). Consequently, it has been proposed a CLV1-CLV2 heterodimer may act as a receptor for the extracellular peptide ligand CLV3 (Ogawa et al, 2008). Additionally, while an interacting partner is yet to be isolated for TMM, a study has implicated this RLP in the negative regulation of some members of the ERECTA RLK family (Shpak et al, 2005). Taken together, the aforementioned findings have helped shape the current model for RLP-dependent signalling. The current assumption is that RLPs function in combination with RLK-type receptors, thus succeeding in relaying the message from the extracellular environment to the intracellular milieu.

2.3. Defence-related downstream responses, signalling and regulation

Time course studies conducted using MAMPs and DAMPs point to a stereotypical defence reaction, indicating multiple signals converge intracellularly to yield a common response. This defence reaction consists of early and late defence reactions.

2.3.1. Early defence responses

The first easily detectable physiological response to MAMPs is the alkalization of the growth medium within the first few minutes due to changes of ion fluxes across the plasma membrane. Although fluxes of H^+ , K^+ and Cl^- have been observed after MAMP treatments, Ca^{2+} influx has been found to be a critical step in plant innate immunity (Jabs et al, 1997; Blume et al, 2000). As in animal systems, Ca^{2+} is thought to act as a secondary messenger molecule in plants, functioning in numerous cell-signalling cascades (Ma and Berkowitz, 2007). Elevation of cytoplasmic Ca^{2+} levels, due to an influx from the apoplast, is perceived by numerous calcium binding proteins such as calmodulin (CaM) (Du et al, 2009). Some of these Ca^{2+} sensors have been implicated in the control of ROS and SA production (Garcia-Brugger et al, 2006; Wang et al, 2009). Further proof of Ca^{2+} involvement in PTI comes from studies demonstrating that Xcc employs exopolysaccharide (EPS) to chelate the cation as a virulence strategy (Aslam et al, 2008). In spite of the aforementioned studies documenting Ca^{2+} involvement in PTI, little is known about cation channels; the only Ca^{2+} channel identified by homology to animal systems was shown not to impact cytosolic Ca^{2+} homeostasis (Ranf et al, 2008).

Another early response to MAMPs is the rapid and transient production of ROS in an oxidative burst. Although ROS accumulation is mainly associated with pathogen recognition and activation of plant defences, these highly toxic reduced oxygen forms can also act in abiotic stresses as well as development (Apel and Hirt, 2004; Foreman et al, 2003). In the former case, the membrane-localized NADPH oxidases (respiratory burst oxidase homologs, Rboh) are considered the source of apoplastic reactive species such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Torres et al, 2005). *Arabidopsis* AtrbohD and AtrbohF are responsible for nearly all ROS produced in leaves in response to pathogens, with *atrbohD* mutants displaying impairment of ROS production after flg22 treatment (Zhang et al, 2007b). Several roles have been postulated for ROS in PTI such as direct antibiotic activity, establishment of cell-wall barriers by cross-linking glycoproteins and secondary stress signals (Torres, 2010). Nevertheless, ROS are also invariably linked with ETI and HR, albeit intracellular ROS species seem to be required in this case alongside apoplastic ones (Ashtamker et al, 2007). Successful pathogens have developed ways to block the oxidative burst; for example, the *Pseudomonas syringae* effectors AvrPtoB and HopAI1 suppress ROS production as part of their virulence strategy (Gimenez-Ibanez et al, 2009a; Zhang et al,

2007b). In addition to ROS, the reactive nitrogen species (RNS) nitric oxide (NO) has been shown to play a role in biotic stresses (Spadaro et al, 2010). This gaseous molecule is produced in response to bacterial and viral pathogens, orchestrating a wide range of responses ranging from defence gene regulation to defence hormone production and HR development (Asai et al, 2008). NO is thought to regulate target protein structure and function by promoting the oxidation of highly reactive cysteine residues, a process often resulting in S-nitrosylation (Spadaro et al, 2010). Nevertheless, due to the complexity of NO-related signalling networks, the identity of the enzymatic source of NO remains elusive (Leitner et al, 2009).

Finally, another early response to microbial challenge is the activation of the mitogen-activated protein kinase (MAPK) signalling cascade. Minimally composed of three elements (MAPKKK, MAPKK and MAPK), this phosphorelay links upstream receptors to downstream signalling targets in *Arabidopsis* (Pitzschke et al, 2009). The most studied MAPKs to date are MPK4, a negative regulator of biotic interactions, and MPK 3/6, which are positive mediators of defence responses. Apart from their role in flg22 and AtPep1-induced signalling, MPK 3/6 have also been shown to contribute to camalexin-based fungal resistance (Asai et al, 2002; Huffaker et al, 2006; Ren et al, 2008). Interestingly the same phosphatase, AP2C1, regulates both the MPK4 and MPK 3/6 antagonistic pathways (Schweighofer et al., 2007). Activation of the MAPK cascade is accompanied by changes in protein phosphorylation and WRKY transcription factors activity resulting in defence gene expression (Nuhse et al, 2007; Pandey and Somssich, 2009). For example, while WRKY22/29 act downstream of MPK 3/6 in flagellin perception, the MPK4 interactor WRKY33 is a positive regulator of resistance towards necrotrophic fungi (Asai et al, 2002; Zheng et al, 2006). The significance of the MAPK pathway is further emphasized as its components are prominent targets of bacterial effectors; both MPK3 and 6 are directly targeted for dephosphorylation by the *Pseudomonas* effector HopAI1 (Zhang et al, 2007b).

Although not as rapid as the aforementioned early responses, PRR endocytosis occurs within the first 30 minutes after MAMP treatment. Analysis of a functional FLS2-GFP fusion revealed FLS2 undergoes ligand-induced endocytosis following flg22 stimulation (Robatzek et al, 2006). Whilst this result parallels similar findings obtained for BRI1 and BAK1, it remains unclear whether internalization is required for MAMP signalling as such or simply for the removal and degradation of the activated receptor (Rusinova et al, 2004; Geldner et al, 2007).

As expected, infection perception is accompanied by a swift change in plant gene expression. Treatment of *Arabidopsis* plants with flg22 and elf26 resulted in the induction or downregulation of a series of genes within 30 minutes (Navarro et al, 2004; Zipfel et al, 2006). The pattern of gene regulation is very similar for both elicitors, indicating MAMP signalling through different PRRs converges at an early stage. Interestingly, RLKs feature prominently among the induced genes, suggesting the existence of a positive feedback loop aimed at enhancing PRR perception capabilities (Zipfel et al, 2004). Conversely, flg22 treatment results in a downregulation of auxin genes and responses which is mediated by RNA silencing (Navarro et al, 2006). The bacterial effector AvrPto has also been shown to target the microRNA machinery to achieve full virulence, thus emphasizing the role of gene silencing in plant immunity (Navarro et al, 2008).

2.3.2. Late defence responses

Subsequent to the early responses listed so far, a plethora of intermediate and long term responses ensue upon microbial perception. During plant-microbe interactions, bacteria attempt to get entry to the apoplast by using stomata as one of the entry points. Consequently, flg22 and LPS treatment, as well as *Pst* DC3000 infiltration were shown to induce stomatal closure in *Arabidopsis* within 2 hours (Melotto et al, 2006). In response, DC3000 secretes the JA mimic coronatine, a phytotoxin that reverts closure (Brooks et al, 2005; Melotto et al, 2006).

The accumulation of callose, a plant β -1,3-glucan polymer synthesized between the cell wall and plasma membrane is a classical marker of PTI responses. This late response can be recorded after treatment with PAMPs or non-infectious pathogens, with successful microbes preventing this type of plant cell wall reinforcement (Hauck et al, 2003). Moreover, the *Pto* DC3000 TTSS mutant *hrcC* exhibits 20-fold more growth in *Arabidopsis* plants deficient in the callose synthase PMR4 compared to wild-type plants (Kim et al, 2005a).

Pathogen infection has profound effects on hormonal pathways, with SA, JA and ET functioning as classical defence hormones (Bari and Jones, 2009). Production of ethylene is among the earliest MAMP responses (Felix et al, 1999). By contrast, SA contributes to longer-term plant defence strategies. A well established defence signal in dicots, SA has been implicated in both PTI, where it promotes callose deposition, and ETI, where it helps establish systemic acquired resistance (SAR) a few hours after HR development (DebRoy et

al, 2004; Shah, 2009). In addition, localized applications of non-adapted bacteria, flg22 or LPS were shown to trigger SA accumulation consistent with SAR initiation, which confers enhanced resistance against a broad spectrum of pathogens (Mishina and Zeier, 2007). The significance of SA-dependent basal resistance is further emphasized by the finding that *P. syringae* encodes suppressors aimed at preventing such plant defence strategies (DebRoy et al, 2004). Analyses of plants in which endogenous SA levels were altered have provided evidence supporting SA's role as a critical defence signal, as well as helped elucidate SA-mediated signalling. While the lipase-like protein EDS1 and its interacting partner PAD4 act upstream of SA in basal resistance and ETI, downstream signalling is largely dependent on NPR1 (Falk et al, 1999; Feys et al, 2001; Pieterse and VanLoon, 2004). The outcome of these events is transcriptional activation of SA-regulated genes, such as PR-1, via the TGA or WRKY transcription factors. Regarding the interaction with other hormonal pathways, SA generally antagonizes JA signalling, with jasmonates controlling resistance against pathogens with different infection strategies (biotroph and necrotroph, respectively) (Spoel and Dong, 2008). Additionally, other plant hormones such as abscisic acid and auxin have been implicated in plant defence, with the latter acting as a suppressor of PTI subsequent to *Pto* DC3000 infection (O'Donnell et al, 2003; Ghanashyam and Jain, 2009).

3. Effector triggered immunity (ETI)

By detecting generic conserved components of most microorganisms, plants can restrict pathogen growth and efficiently counteract infection. However, PTI is ineffective against adapted pathogens which use virulence factors or effectors to actively suppress components of the general defence response (Figure 1.1). As a result of an evolutionary arms race, plants have developed resistance (*R*) genes, whose products generally detect effector presence and activity by monitoring modifications of their targets. Based on the features of known virulence factors, effectors are small, secreted proteins, which are rich in cysteines and show no obvious homology to known proteins (Gohre and Robatzek, 2008; Stergiopoulos and de Wit, 2009). Gram-negative bacterial pathogens use type III secretion systems (TTSS) to deliver type III effector proteins into plant cells, in an effort to optimize conditions for pathogen growth while subverting plant defence responses (Grant et al, 2006). For example, the phytopathogen *P. syringae* secretes 20 to 30 effectors during infection, with

representatives such as AvrRpt2 and AvrRpm1 inhibiting flg22-induced defences in *Arabidopsis* and promoting the growth of TTSS-deficient bacteria (Chang et al, 2005; Kim et al, 2005a). Several studies have focused on the aforementioned effector proteins and their interaction with RIN4, a negative regulator of *R* protein-mediated defences in *Arabidopsis* (Mackey et al, 2002; Mackey et al, 2003). While AvrRpt2 cleaves and degrades RIN4, AvrRpm1 inhibits the signalling pathway by phosphorylating its target (Kim et al, 2005b; Mackey et al, 2002). Such effector-mediated changes to host proteins are monitored by the RPS2 and RPM1 *R* proteins respectively, enabling the indirect detection of effectors and the activation of plant resistance (“guard hypothesis”). Additional examples of *P. syringae* effector-mediated PTI suppression include AvrPto-related suppression of callose deposition and nonhost HR (Hauck et al, 2003; Hann and Ratjen, 2007), as well as inhibition of the MAPK cascade due to threonine modification by the protein phosphothreonine lyase HopAI1 (Zhang et al, 2007b).

Although it would appear indirect detection of effectors is the prevalent plant strategy, there have been some reports of direct interaction between some *R* proteins and effectors (Tang et al, 1996; Deslandes et al, 2003). *R* genes represent a flexible component of the plant immune system, with many being polymorphic (Bent and Mackey, 2007). Most *R* genes cloned to date give rise to an intracellular class of proteins exhibiting a nucleotide binding (NB) site and an LRR domain (Tammeling and Takken, 2008). Furthermore, the members of the NB-LRR protein family can be divided into coiled-coil (CC) NB-LRR and Toll-interleukin-1 receptor (TIR) NB-LRR according to their N-terminal domain which influences the requirement for specific downstream defence components (Feys and Parker, 2000). *R* proteins rapidly initiate ETI, a defence response including oxidative burst and defence-associated gene expression, which culminates in HR, a localized form of programmed cell death (PCD), and SAR. Nevertheless, this type of immunity is tightly regulated as such a response is costly for a plant and its uncontrolled proliferation could be fatal. As a result, NB-LRR are kept in check by several autoinhibitory mechanisms relying on intramolecular interactions between the various domains (Rairdan and Moffet, 2006). Additionally, as a counter strategy to *R* proteins, pathogens have evolved new sets of effectors that suppress HR-based immunity. While studies conducted with the *Pst* DC3000 effector AvrPtoB in *N. benthamiana* showed this protein has anti PCD activity, the *Pseudomonas syringae* pv. *phaseolicola* VirPphA was identified as the plasmid borne gene that enabled the bacteria to evade HR-based resistance (Abramovitch et al, 2003; Jackson et al, 1999).

Unlike for Gram negative bacteria, no TTSS has been discovered for fungal pathogens which deliver effectors to the plant apoplast via the haustorium. While enzyme activity has been demonstrated for a few of these proteins, their role in virulence and the manner by which they gain access to the plant cell remains unclear (Chisolm et al, 2006; Stergiopoulos and de Wit, 2009). For example, the two effector proteins Avr2 and Avr4 from *Cladosporium fulvum* have been characterized in depth; while Avr2 inhibits the tomato Rcr3 cysteine protease, Avr4 is believed to shield the fungal cell wall against plant chitinases (Rooney et al, 2005; van den Burg et al, 2003). In the case of oomycetes, effector are delivered via the appressorium (Kebdani et al, 2010). Effector inventories can be identified from the oomycete genome sequence by using the highly conserved RxLR motif as a starting point (Thines and Kamoun, 2010), but such predictions need to be coupled to functional analyses to confirm their role in virulence (Win et al, 2007). Since fungal and oomycete effectors seem to be recognized both directly and indirectly by their host, it remains unclear which form of identification is prevalent in their case (Jia et al, 2000; Rooney et al, 2005).

B. Using Arabidopsis to study *Staphylococcus* virulence mechanisms

1. *Arabidopsis thaliana*: a plant model for animal pathogenesis

Arabidopsis has long been adopted as a model organism and is by far the most thoroughly studied flowering plant (Koornneef and Meinke, 2010). Its short life cycle, small plant and genome size and prolific seed production through self-pollination make *Arabidopsis* a prime candidate for studying plant biology. However, the important breakthroughs that have established it as the organism of choice are its genetic tractability and the development of efficient transformation methods (Theologis et al, 2000; Clough and Bent, 1998). Additionally, the variation among natural accessions has been a valuable resource for analysing gene function and features of evolutionary ecology at the molecular level (Koornneef et al, 2004; Shindo et al, 2007). For example, the accessions Landsberg *erecta* (Ler) and Columbia (Col) exhibit contrasting reactions when infiltrated with *Xcc* which has allowed the identification of 3 loci contributing to plant resistance (Buell and Somerville, 1997).

Following the initial report of *Arabidopsis* infiltration with *P. syringae*, a lot of studies using this model were targeted at dissecting plant-agricultural pathogen interactions (Dong et al, 1991). The finding that the opportunistic human pathogen *P. aeruginosa* strain UCBPP-PA14 could infect both plants and animals suggested pathogens use similar virulence strategies on a range of hosts (Rahme et al, 1995). Accordingly, several *P. aeruginosa* virulence factors, such as *gacA* and phospholipase C were identified/confirmed by first screening mutants using a plant model, while other promising candidates such as alginate were discounted (Rahme et al, 1997; Yorgey et al, 2001). These seminal studies, coupled with the exemption from ethical considerations compared to animal models, has helped establish *Arabidopsis* as a heterologous host for several Gram positive and Gram negative mammalian pathogens. The Gram negative pathogens *Salmonella enterica* and enterohemorrhagic *E. coli* were shown to colonize *Arabidopsis*, while it was confirmed Gram positive *Staphylococcus aureus* and the opportunist *Enterococcus faecalis* employ the same virulence determinants in animal and plant systems (Cooley et al, 2003; Prithiviraj et al, 2005; Jha et al, 2005). However, identifying conserved bacterial virulence factors is not the only advantage of establishing this framework. As outlined previously, the plant and vertebrate innate immune systems share a multitude of elements and functions including the presence of PRRs, MAPK-based downstream signalling and ROS and defensin production. Furthermore, a comparative analysis of 289 human disease-related genes showed 60% have a homologue in *Arabidopsis*, implying that the molecular processes required for host defence might share similarities (Rubin et al, 2000). Identifying elements that contribute to innate immunity in a plant host could potentially have profound implications for elucidating resistance mechanisms in other systems, such as the genetically less-tractable human hosts. In addition, clarifying how a pathogen overcomes these defence components could help dissect virulence mechanisms in more detail. Various *Arabidopsis* mutants exhibit different features as far as pathogen infection, tolerance and susceptibility are concerned. Characterizing such mutants could reveal receptors targeted by microbial factors or metabolites that either interfere with virulence mechanisms or are directly toxic to the pathogen (van Baarlen et al, 2007).

2. *Staphylococcus spp.*

Staphylococci are common Gram positive bacterial colonizers of the skin and mucous membranes of humans and other mammals. Although initially regarded as an innocuous microorganism, *S. epidermidis* is currently acknowledged as an important opportunistic pathogen, ranking first alongside its relative *S. aureus* as the causative agent of nosocomial infections (Otto, 2009). As a ubiquitous colonizer of the human epithelia, *S. epidermidis* is a common source of infections on indwelling medical devices (Uckay et al, 2009). Although such infections are rarely life threatening, their frequency and the fact that they are difficult to treat renders them problematic. More specifically, *S. epidermidis* accidentally acquired during device insertion proceeds to become infectious by forming a biofilm with inherent antibiotic resistance (Hoiby et al, 2010).

Although usually in a benign relationship with its host, *S. epidermidis* does maintain a low virulence level aimed at enhancing persistence through evading the immune system rather than aggressively attacking the host. By contrast to *S. aureus*, this Gram positive bacterium's toxin arsenal is mostly limited to phenol soluble modulins (PSMs) (Vuong et al, 2004a). These short, amphipathic α -helical peptides display proinflammatory and occasional human neutrophil lysing functions (Mehlin et al, 1999; Wang et al, 2007). *S. epidermidis* also circumvents other components of the innate immune system such as cationic antimicrobial peptides by decreasing the anionic charge of the bacterial surface through several mechanisms (Peschel et al, 1999; Peschel et al, 2001). It is unclear why the specialized, acquired immune response is unable to clear long lasting *S. epidermidis* infections despite specific antibody production (Pourmand et al, 2006). Possible reasons for this occurrence include the presence of exopolymers that protect bacteria from antibody recognition, as well as the possibility that the immune system reacts less strongly to prevalent colonizing bacteria.

Persistence of staphylococcal infections is due to biofilm formation, a multicellular structure encased in a self-produced polymer matrix consisting of polysaccharide, protein and DNA (Hoiby et al, 2010). Biofilm development typically involves attachment to the host matrix, secretion of molecules that aid intracellular aggregation, maturation, structuring and detachment. Microarray and expression profiling studies have demonstrated *S. epidermidis* displays genome wide adaptation to the biofilm mode of growth (Yao et al, 2005). In this case, adhesion to matrix proteins is mediated by bacterial surface proteins, such as AtlE and covalently anchored proteins, such as the members of the Sdr family (Figure 1.5) (Heilmann et al, 1997; McCrea et al, 2005). After this initial step, subsequent intracellular aggregation is

mediated by the PNAG exopolysaccharide, Aap and Bap proteins, as well as teichoic acids (Figure 1.5).

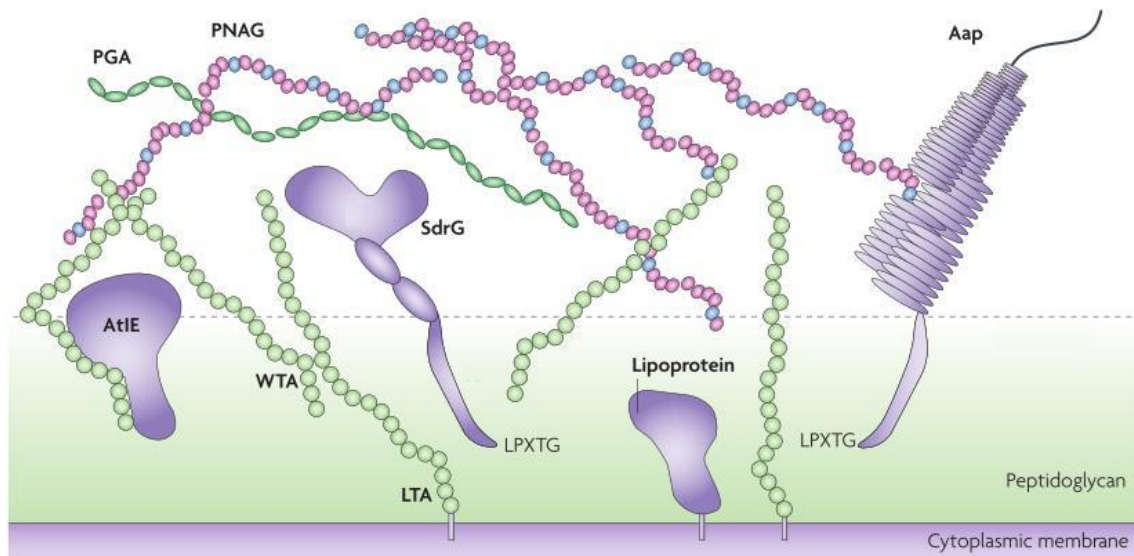


Figure 1.5. The *S. epidermidis* cell surface.

Proteins such as AtIE and SdrG assure matrix adhesion, while Aap is important for intracellular aggregation. SdrG and Aap are covalently linked to the peptidoglycan layer via the C-terminal LPXTG motif. Lipoproteins, wall teichoic acid (WTA) and lipoteichoic acid (LTA) are embedded in the peptidoglycan layer. The PNAG exopolymer is cationic (blue shading) and is thought to interact with negatively charged polymers (green shading) such as WTA, LTA and poly- γ glutamic acid (PGA). (modified from Otto, 2009).

Many *S. epidermidis* strains produce a poly N-acetylglucosamine (PNAG) homopolymer which differs from other PNAG polymers found in nature (eg. chitin) by its β 1-6 linkage (Mack et al, 1996). Also named PIA (polysaccharide intercellular adhesin), this capsular exopolysaccharide plays a major part in infection in several model systems (Rupp et al, 1999; Begun et al, 2007). Alongside poly- γ glutamic acid (PGA), the other exopolymer produced by *S. epidermidis*, PNAG protects the bacteria from innate host defences (Kocianova et al, 2005; Kristian et al, 2008). Moreover, PNAG, as well as *S. epidermidis*, were reported to stimulate TLR2, although this result needs to be confirmed using deletion mutants to rule out potential contaminants (Stevens et al, 2009; Strunk et al, 2010). Although most extracellular polysaccharides isolated from *S. epidermidis* were ultimately confirmed to be closely related or identical to PIA, a distinct sulfated 20 kDa polysaccharide has also been

described (Karamanos et al, 1997). The biosynthesis of PNAG is under complex regulation and depends on the gene products of the *ica* (intracellular adhesion) locus (O’Gara et al, 2007). Firstly, membrane localized IcaA and IcaD proteins produce a chain from activated N-acetylglucosamine (GlcNAc) monomers which is exported via the IcaC protein (Gerke et al, 1998). Subsequently, the cell surface located enzyme IcaB proceeds to partially de-acetylate the GlcNAc residues, thus introducing positive charges in the polymer that are important for PNAG-related biological functions (Vuong et al, 2004b).

Compared to *S. epidermidis*, the more versatile *S. aureus* employs a large array of virulence factors to cause disease on a multitude of hosts (Bost et al, 1999; Sifri et al, 2003). Although biofilm formation is also required for achieving full virulence, what sets *S. aureus* apart from its less virulent cousin is the prevalence of its secreted exotoxins (Dinges et al, 2000). These molecules have cytotoxic effects in humans, thus impairing the immune system. The production of these molecules and other virulence determinants is controlled by major global regulators such as *sarA* and *agr* (Chien et al, 1999; Beenken et al, 2010). In experiments mirroring the work done with *P. aeruginosa*, the human pathogen *S. aureus* was shown to elicit typical bacterial disease symptoms on *Arabidopsis* leaves and roots (Prithiviraj et al, 2005). Furthermore, mutants deficient in the *sarA* and *agr* regulators as well as an alpha-toxin mutant, all impaired in biofilm formation, showed reduced virulence in both plant and animal models of pathogenicity. Plant resistance to *S. aureus* correlated with high levels of SA, with subsequent experiments suggesting this phenolic metabolite not only triggers defence responses in the plant, but also acts directly on the pathogen to diminish its virulence by disrupting biofilm formation (Prithiviraj et al, 2005). Moreover, recent work making use of an *Arabidopsis* PR1:GUS reporter line has pinpointed the highly immunogenic *S. aureus* PGN as a MAMP (Gust et al, 2007).

3. Aim of this work

Primarily known for its commensal life style, *S. epidermidis* has recently emerged as an important opportunistic pathogen, mainly due to its ability to colonize polymer surfaces. Although a wide array of potential virulence factors has been investigated so far, it remains unclear why the host immune system has difficulty clearing *S. epidermidis* infections.

Previous studies have emphasized various analogies in innate immunity against pathogens in plant, invertebrate and mammalian hosts. By comparison, plant models are an attractive option and experiments using *S. aureus* and *A. thaliana* have shown potential for this approach with *S. epidermidis*.

So far, a lot of potential elicitors have been identified in plants, but only a few PRRs. By contrast, multiple receptors have been isolated in mammals, but the identity of their corresponding ligands remains elusive. Further studies are needed to identify how and which MAMPs are perceived and how their perception contributes to disease resistance. The aim of this project was to characterize the *S. epidermidis*-*A.thaliana* interaction. To this end, a dual approach was undertaken in this study. Firstly, natural variation of *Arabidopsis* accessions was exploited in order to map the plant loci underpinning plant responses to *S.epidermidis*. Secondly, the nature of the bacterial elicitors underlying the plant phenotypic reaction was researched.

The initial aim of this study was to establish the *Arabidopsis*- *S. epidermidis* pathosystem. Initial experiments focused on surveying the ability of *S. epidermidis* to infect *A. thaliana*. Towards this end, plant phenotypic responses, *in planta* bacterial growth and defence-associated markers were assessed following inoculation with *S. epidermidis*. Furthermore, an extensive panel of 46 accessions were tested for their response to *S. epidermidis* in order to investigate natural variation of the plant response.

Following the identification of accessions displaying contrasting reactions to this bacterium, QTL (quantitative trait loci) analysis was undertaken in order to determine the genetic basis of the plant phenotypic response to *S. epidermidis* infection. Investigation of F1 and F2 populations, coupled with a candidate gene approach allowed for the identification of the gene that forms the basis of the necrotic response to *S. epidermidis* 18888. Subsequent studies focused on mutant plant characterization, as well as on surveying the role of the identified gene in plant defence and more specifically, MAMP perception.

The remaining goal of this project was to identify the *S. epidermidis* microbial features underlying the plant phenotypic response. In view of this, potential MAMPS were purified and a series of defence parameters were assessed in *Arabidopsis* subsequent to MAMP exposure. The minimal structure required for elicitation was also evaluated.

Taken together, these approaches allowed for the identification of novel plant defence components involved in PTI, as well as for the isolation of microbial elicitors that trigger well-characterized defence responses.

2. MATERIALS AND METHODS

Plant material

All *Arabidopsis* accessions and mutants used in this study (Table 2.1 and 2.2) were grown at 21°C with 60% humidity under light (around 100 $\mu\text{mol m}^{-2}\text{s}^{-1}$) in an 10h light/14h dark cycle in environment-controlled chambers. *N. benthamiana* plants were grown in the same conditions, but with a 16h light/8 hour dark cycle.

Bacterial strains

Bacterial strains used in this study, their growth requirements and antibiotic selection are listed in table 2.3. The identity of the *Staphylococcus epidermidis* 18888 lab strain was confirmed by fatty acid profiling. Unless otherwise stated, this strain were used for all plant experiments.

Plant phenotypic studies

Bacterial cultures were spun down and resuspended in 10mM MgCl_2 at the required concentration. 5 week-old plants were pressure infiltrated on the abaxial side of the leaf using a needless 1 mL syringe. At least 3 leaves per plant and 3 plants per strain were used for each experiment. All *Staphylococcus* strains were infiltrated at a concentration of 10^8 cfu/mL (equivalent of $\text{OD}_{600}=0.2$). All Pst strains were infiltrated at a concentration of 5×10^6 cfu/mL (equivalent of $\text{OD}_{600}=0.002$). For Xcc experiments, plants were submerged for 30 seconds in a 10^7 cfu/mL (equivalent of $\text{OD}_{600}=0.05$) suspension supplemented with 0.02% v/v Silwet L-77 and kept covered for 2 days to maintain humidity. Experiments were repeated several times and representative results are shown.

Table 2.1. Individual accessions used in this project

accession	region	population	stock center
Cvi-0	Cape Verde Isl.	Cape Verde Isl.	CS22614
Lz-0	France	Puy-de-Dome	CS22615
Ei-2	Germany	Eifel	CS22616
Gu-0	Germany	Guckingen	CS22617
Ler-1	Poland	Landsberg	CS22618
Nd-1	Germany	Niederzenz	CS22619
C24	Portugal	Coimbra	CS22620
CS22491	Russia	Konchezero	CS22621
Wei-0	Switzerland	Weiningen	CS22622
Ws-0	Ukraine	(Wassilewskija)/Djnepr	CS22623
Yo-0	USA	Yosemite Nat. Park	CS22624
Col-0	USA (Germany?)	Columbia	CS22625
An-1	Belgium	Antwerpen	CS22626
Van-0	Canada	UBC, Vancouver	CS22627
Br-0	Czech Republic	Brno (Brunn)	CS22628
Est-1	Estland	Estland	CS22629
Ag-0	France	Argentat	CS22630
Gy-0	France	La Miniere	CS22631
Ra-0	France	Randan, Puy-de-Dome	CS22632
Bay-0	Germany	Bayreuth	CS22633
Ga-0	Germany	Gabelstein	CS22634
Mrk-0	Germany	Markt/Baden	CS22635
Mz-0	Germany	Merzhausen/Ts.	CS22636
Wt-5	Germany	Wietze	CS22637
Kas-2	India	Kashmir	CS22638
Ct-1	Italy	Catania	CS22639
Mr-0	Italy	Monte/Tosso	CS22640
Tsu-1	Japan	Tsushima	CS22641
Mt-0	Libya	Martubad/Cyrenaika	CS22642
Nok-3	Netherlands	Noordwijk	CS22643
Wa-1	Poland	Warsaw	CS22644
Fei-0	Portugal	St. Maria d. Feiria	CS22645
Se-0	Spain	San Eleno	CS22646
Ts-1	Spain	Tossa de Mar	CS22647
Ts-5	Spain	Tossa de Mar	CS22648
Pro-0	Spain	Proaza, Asturias	CS22649
LL-0	Spain	Llagostera	CS22650
Kondara	Tajikistan	Pamiro-Alay	CS22651
Shahdara	Tajikistan	Pamiro-Alay	CS22652
Sorbo	Tajikistan	Pamiro-Alay	CS22653
Kin-0	USA	Kindalville, MI	CS22654
Ms-0	Russia	Moscow	CS22655
Bur-0	Ireland	Burren	CS22656
Edi-0	Scotland	Edinburgh	CS22657
Oy-0	Norway	Oystese	CS22658
Ws-2	Ukraine	(Wassilewskija)/Djnepr	CS22659

Table 2.2. Plant lines used in this project

Plant lines	Accession	Source
<i>eds1-2</i>	Ler	B. Feys ^a
<i>pad4-2</i>	Ler	B. Feys ^a
<i>nahG</i>	Ler	B. Feys ^a
<i>etr1-1</i>	Col-0	NASC (N237)
<i>ein2-1</i>	Col-0	NASC (N3071)
<i>bak1-4</i>	Col-0	D. Chinchilla ^b
<i>bkk1-1</i>	Col-0	T. Hamann ^a
<i>cerk1-2</i>	Col-0	K. Shirasu ^c
<i>fls2</i>	Col-0	D. Chinchilla ^a
<i>efr</i>	Col-0	D. Chinchilla ^a
<i>fls2/efr</i>	Col-0	D. Chinchilla ^a

^aImperial College London

^bInstitute of Botany, University of Basel

^cRiken Plant Institute, Japan

Bacterial growth assays

Leaves of 5 week-old plants were syringe infiltrated with bacterial suspensions prepared in 10mM MgCl₂. 3 leaf discs per plant (approx. 0.5cm² each) were harvested using a cork borer at the designated time points post-infection. Leaf discs were ground in 500 µL of 10mM MgCl₂ using an eppendorf pestle. The resulting suspension was serially diluted and plated on media with appropriate selection (Table 2.3) in order to determine the cfu cm⁻² value.

All *Staphylococcus* strains were infiltrated at a concentration of 10⁷ cfu/mL (equivalent of OD₆₀₀=0.02). Pst strains were infiltrated at a concentration of 10⁵ cfu/mL (equivalent of OD₆₀₀=0.0002). For each strain, 3 leaves per plant and three plants per time point were used. After incubation, plates corresponding to a dilution yielding between 100-500 colonies were used to count colony forming units. Each data point represents average bacterial numbers of 3 replicates and error bars indicate standard error. Experiments were repeated at least three times and representative results are shown.

Table 2.3. Strains used in this project

Strain	Media	Antibiotic concentration	Temperature	Incubation time
<i>S. epidermidis</i> 18888	LB	Rifampicin 40µg/mL	37°C	24h
<i>S. epidermidis</i> ATCC12228	TSB	-	37°C	24h
<i>S. epidermidis</i> ATCC35983	TSB	-	37°C	24h
<i>S. epidermidis</i> RP62A	TSB	-	37°C	24h
<i>S. epidermidis</i> ATCC14990	TSB	-	37°C	24h
<i>S. epidermidis</i> SE-17	TSB	-	37°C	24h
<i>S. epidermidis</i> SE-20	TSB	-	37°C	24h
<i>S. epidermidis</i> SC-15	TSB	-	37°C	24h
<i>S. epidermidis</i> SE-11	TSB	-	37°C	24h
<i>S. epidermidis</i> 8400	TSB	-	37°C	24h
<i>S. epidermidis</i> 9142	TSB	-	37°C	24h
<i>S. epidermidis</i> 1457	TSB	-	37°C	24h
<i>S. epidermidis</i> 8400 M10 Δica	TSB	-	37°C	24h
<i>S. epidermidis</i> 9142 M10 Δica	TSB	-	37°C	24h
<i>S. epidermidis</i> 1457 M10 Δica	TSB	-	37°C	24h
<i>S. haemolyticus</i> ATCC 29970	TSB	-	37°C	24h
<i>S. saprophyticus</i> ATCC 15305	TSB	-	37°C	24h
<i>S. lugdunensis</i> ATCC43809	TSB	-	37°C	24h
<i>S. hominis</i> ATCC 27844	TSB	-	37°C	24h
<i>S. xylosus</i> ATCC 29971	TSB	-	37°C	24h
<i>Burkholderia ambifaria</i> 19182	LB	Gentamycin 10µg/mL	37°C	24h
<i>Pseudomonas syringae</i> pv tomato (Pst) DC3000	LB	Rifampicin 40µg/mL	28°C	48h
Pst DC3000 (avrRpm1)	LB	Rifampicin 40µg/mL	28°C	48h
Pst DC3000 (avrRpt2)	LB	Rifampicin 40µg/mL	28°C	48h
<i>Xanthomonas campestris</i> pv campestris (Xcc) 8004	NYG	Rifampicin 50µg/mL	25°C	48h
Xcc 8397 ΔEPS	NYG	Rifampicin 50µg/mL	25°C	48h
<i>Agrobacterium tumefaciens</i> GV3101	LB	Rifampicin 50µg/mL Kanamycin 50µg/mL	28°C	48h
<i>Agrobacterium tumefaciens</i> (pGWB2)	LB	Rifampicin 50µg/mL Hygromycin 50µg/mL	28°C	48h
<i>Agrobacterium tumefaciens</i> (pGWB2-ster1)	LB	Rifampicin 40µg/mL Hygromycin 50µg/mL	28°C	48h
<i>Agrobacterium tumefaciens</i> (pGWB5)	LB	Rifampicin 40µg/mL Hygromycin 50µg/mL	28°C	48h
<i>Agrobacterium tumefaciens</i> (pGWB5-ster1)	LB	Rifampicin 40µg/mL Hygromycin 50µg/mL	28°C	48h

LB, Luria Bertani; TSB, tryptic soy broth; NYG, nutrient yeast glycerol.

RIL and NIL mapping

A base set of 50 Cvi/Ler (Cape verde islands/Landsberg erecta) recombinant inbred lines (RILs) and a full set of Cvi/Ler near-isogenis lines (NILs) were obtained from NASC (stock no. N22477, N717143, N717144, N717145, N717146, N717147) (Alonso Blanco et al, 1998; Keurentjes et al, 2007).

For RIL mapping, 4 plants per line per experiment were grown in a randomized arrangement in order to avoid variations resulting from localized growth parameter variations. 4-week old plants were infiltrated with a 10^8 cfu/mL *Staphylococcus* suspension and symptoms were observed 5 days post-inoculation. Symptom severity of each RIL was scored by visual inspection as follows: 1= no symptoms, 2= patchy chlorosis, 3= strong chlorosis, 4= chlorosis with necrotic patches, 5= necrosis. Genotypic data for segregating molecular markers (20 evenly spread AFLP markers for each chromosome) was obtained from <http://www.dpw.wau.nl/natural/resources/populations/CVI/>. Data was analyzed using the Windows QTL Cartographer software v 1.17 (<http://statgen.ncsu.edu/qtlcart/>). Interval mapping (IM) and composite interval mapping (CIM) functions were used to map the QTL and a significance LOD threshold of 2.5 was assigned after 1000 permutations.

NIL mapping was used to confirm the QTL position obtained through RIL mapping. A core set of NILs providing full genome coverage were infiltrated and symptoms were observed 5 days post-inoculation (Keurentjes et al, 2007; Supplemental table 1).

Plant genomic DNA extraction

Leaf material from each plant was ground in extraction solution (100mM Tris HCl pH 8.0; 50mM EDTA pH 8.0; 100mM NaCl; 2% SDS; 0.1 mg/mL proteinase K) using an eppendorf pestle. Samples were shaken at 28°C for 30 minutes and 400µL of phenol chloroform pH 8.0 was added; after centrifuging at maximum speed, the upper aqueous layer was removed to a clean eppendorf. Subsequent to the addition of 500 µL isopropanol and 50µL 3M NaAc pH 5.2, samples were spun at maximum speed for 2 minutes and the resulting pellet was resuspended in 500 µL TE pH 8.0 (10mM Tris HCl pH 8.0; 1mM EDTA pH 8.0). Following precipitation with 500µL ethanol/ 50µL 3M NaAc pH 5.2 and centrifugation at maximum speed for 5 minutes, the DNA pellet was resuspended in 50µL TE pH 8.0. A dilution of 1/20 of this DNA stock was used for amplification by polymerase chain reaction (PCR).

Polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR)

PCR was performed in a 20 μ L reaction containing 1 μ mol of each primer by using a thermocycler programmed for 30 cycles of 94 °C 30 seconds; 55 °C, 30 seconds; 72 °C, specific extension time (1 minute per kb).

For RT-PCR, RNA was extracted from leaf material of 4 week plants using TRI REAGENT (Sigma). Briefly, samples were snap-frozen and homogenized with a mortar and pestle using liquid nitrogen. 100 mg of tissue was transferred to an eppendorf and 1 mL of TRI REAGENT was added. Samples were allowed to stand at room temperature for 5 minutes. After the addition of 200 μ L of chloroform, samples were vigorously shaken for 15 minutes, allowed to stand for 10 minutes and centrifuged at 12000g for 15 minutes at 4°C. The upper aqueous phase was transferred to a new tube and after the addition of 500 μ L of isopropanol, samples were allowed to stand for 10 minutes and centrifuged at 12000g for 10 minutes at 4°C. Subsequently, the resulting RNA pellet was washed with 75% ethanol, vortexed and centrifuged at 7500g for 5 minutes at 4°C. The RNA pellet was air-dried for 5 minutes and resuspended in RNase-free water. RNA was quantified using a spectrophotometer to read the OD₂₆₀ of a 1/100 dilution (1 OD₂₆₀= 40 μ g/mL RNA). 2 μ g of RNA and an oligodT primer (5'- TTTTTTTTTTTTTTTTTTTTTTTTTTV-3' V= mixture of A, C and G) were used for cDNA synthesis using the SuperScript III Reverse transcriptase kit (Invitrogen) as instructed. cDNA was used as template for amplification by PCR with gene specific primers. All semi-quantitative RT-PCR amplifications were performed with 25 cycles.

DNA sequencing

PCR products were subjected to agarose gel electrophoresis and gel purified using the QIAquick Gel Extraction Kit (QIAGEN). Purified DNA was quantified by comparison with the DNA ladder (GeneRuler 1kb DNA ladder, Fermentas) subsequent to gel electrophoresis. The sequencing reaction was performed in a 10 μ L volume containing 200ng of DNA and 300nM of primer by using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The assembled reactions were subjected to amplification as follows: an initial step of 96°C, 1 minute; 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes; final step of 4°C for 10 minutes. Subsequently, samples were sequenced using an AbiPrism3730 capillary sequencer (John Innes Genome Laboratory).

Marker analysis of Col/Cvi F2 population

The same Cvi and Ler stocks used to generate the Cvi/Ler RIL and NIL populations (NASC stocks N8580 and N8581) were used to generate F1 and F2 Cvi/Ler populations. The F1 and F2 were examined after *Staphylococcus* inoculation (10^8 cfu/mL) in order to determine dominance and segregation ratios.

For generating the F1 progeny, fine tweezers were used to emasculate an individual flower. Fresh pollen from three to four independent donor stamens was dabbed onto each single well-developed stigma. Mature siliques containing F1 seed were harvested and allowed to dry. F1 seeds were grown and allowed to self pollinate in order to generate the F2 seeds. The identity of the cross was confirmed using PCR and primers for CAPS marker N1 (table 2.4).

The AFLP (amplified fragment length polymorphism) marker map available for the Cvi/Ler NIL population was used to narrow down the genomic region of interest for subsequent analysis (chromosome 4: 11102901bp-12776081bp). CAPS (cleaved amplified polymorphic sequence) markers were developed for this region using the SNP database msqt.weigelworld.org/nordborg/index.html (table 2.4). An additional SSLP (simple sequence length polymorphism) marker (CIW7) was obtained from the database at www.arabidopsis.org.

DNA was extracted from 140 non-responsive Cvi x Col F2 plants and individuals showing a crossover event in the region of interest were selected for progeny testing. PCR was performed in a 20µL reaction using genomic DNA as template and marker specific primers (Table 2.4). For CAPS markers, resulting PCR products were digested with 0.5µl restriction enzymes (NEB) (Table 2.4) and subjected to agarose gel electrophoresis to reveal an ecotype specific polymorphism. For CIW7, PCR products were run on a 3% agarose gel to reveal an ecotype specific 23bp difference. As the mapping was done using Ler and the F2 lines have Columbia-0 (Col-0) as a parental, these ecotypes were crossed and the F1 and F2 generations were tested with *Staphylococcus* to confirm segregation.

Table 2.4. CAPS and SSLP markers used in this study

Marker name	Marker type	Position on chromosome 4 (bp)	Primers (forward and reverse) (5'-3')	Restriction enzyme	Fragment sizes (bp)
N2	CAPS	10876274	AAACCTCTGTTTTGTCCATTTACTG TGCTATTAGTCCACAGCTCATATTC	AluI	Ler=Col=400+100 Cvi=500
B1	CAPS	11325000	AACCGAAAGTCGCCAAGAAC AGCTGTGTCTGTGTTTTGCATT	XmnI	Ler=Col=500 Cvi=400+100
CIW7	SSLP	11524400	AATTTGGAGATTAGCTGGAAT CCATGTTGATGATAAGCACAA	-	Ler=123 Col=130 Cvi=100
B2	CAPS	12325000	TTTCTGCACAACAGAAAGAACG GATTTGTGTAGGCAGATGGTGA	AccI	Ler=Col=500 Cvi=300+200
N1	CAPS	12776081	TGCTAAGTCAAACTAACGAACCA GGGCTCTACATGCATCAAATC	DdeI	Ler=Col=1080+821 Cvi=919+821

In addition to the F2 plants, selected NIL lines were also mapped for the region in question using the markers in table 2.4.

ster1 mutant identification using T-DNA insertion knockout lines

A T-DNA insertion line (all in Col-0 background) corresponding to every gene in the RLK cluster (At4g23130 up to At4g23320) was identified using the T-DNA Express database at www.signal.salk.edu. All lines were obtained from NASC (table 2.5).

Plants were inoculated with a *Staphylococcus* suspension and symptoms were observed 5 days post-inoculation. If a homozygous line was not available, 24 plants of the segregating line were tested in order to detect differences in symptom development. The asymptomatic GABI Kat line GABI232B02 was renamed *ster1-1* (*Staphylococcus* *elicitor* *response* 1) and chosen for further analysis.

Table 2.5. T-DNA insertion lines for members of RLK cluster

Gene	Insertion line	NASC code	HM line
At4g23130	SALK 063519	N666247	√
At4g23140	SALK 020652	N520652	X
At4g23150	GABI 232B02	N422190	X
At4g23160	SALK 101066	N671790	√
At4g23170	SALK 080814	N580814	X
At4g23180	SALK 116653	N663753	√
At4g23190	SALK 055108	N555108	√
At4g23200	SALK 033837	N533837	X
At4g23210	SALK 085128	N666588	√
At4g23220	SAIL 893B09	N879807	X
At4g23230	SALK 067729	N666323	√
At4g23240	SALK 021280	N512280	X
At4g23250	SALK 114137	N614137	X
At4g23260	SALK 090966	N668642	√
At4g23270	SALK 004196	N504196	X
At4g23280	GT 5 109385	N180512	X
At4g23290	SALK 022512	N522512	X
At4g23300	SALK 019124	N665431	√
At4g23310	SALK 051873	N659172	√
At4g23320	SALK 101875	N601875	X

HM, homozygous; √, available; X, not available

SALK lines- Alonso et al, 2003; GABI Kat-Rosso et al, 2003;

SAIL lines- Sessions et al, 2001; GT, JIC gene trap lines

Analysis of ster1 T-DNA mutant lines

Several knockout lines for the At4g23150 gene were identified using the T-DNA Express database and all lines were obtained from NASC (Table 2.6). 24 plants from each line were inoculated with a 10^8 cfu/mL *Staphylococcus* suspension and symptoms were observed 5 days post-inoculation. DNA was extracted from each plant and PCR was performed with 2 pairs of primers (one for the wild-type gene, one specific to the T-DNA insertion) to assign genotype (Table 2.7). The PCR product obtained using primers specific for the T-DNA insertion was sequenced to verify insertion sites.

RNA was extracted from identified homozygous mutants (from heterozygous mutants in the case of SALK 151109 and WiscDsLox50A210) and RT-PCR was performed using full cDNA specific primers (forward primer 5'-AACCCCTTTTGTCTCTCTCTT-3'and

reverse primer 5'- GCGCTGGTAAAGCCATTGTA-3') in order to check transcript levels and confirm knockouts.

Table 2.6. *Ster1* T-DNA mutant lines used in this study

Insertion line	Name	NASC code	Confirmed insertion site
WiscDsLox361B10	<i>ster 1-6</i>	N852799	5' (368 bp upstream ATG)
WiscDsLox336D10	<i>ster 1-7</i>	N851666	5' (282 bp upstream ATG)
Salk151109	<i>ster 1-2</i>	N651109	5' (77 bp upstream ATG)
GK232B02	<i>ster 1-1</i>	N422190	2nd intron
WiscDsLox50A210	<i>ster 1-3</i>	N858983	5th exon
Salk018871	<i>ster 1-4</i>	N518871	7th exon (12bp upstream TGA)
WiscDsLox434B3	<i>ster 1-5</i>	N855622	3' (28bp downstream TGA)

WiscDsLox lines, Woody et al, 2007

Table 2.7. Primer pairs used to genotype *ster1* T-DNA mutant lines

Insertion line	WT gene primers (FW and RV) (5'-3')	T-DNA insert primers (FW and RV) (5'-3')
WiscDsLox361B10	ACAAAATTCCAGGTTTTGACG GGCTAGGAAACGAGGATCTTG	AACGTCCGCAATGTGTTATTAAGTTGTC GGCTAGGAAACGAGGATCTTG
WiscDsLox336D10	ACAAAATTCCAGGTTTTGACG GGCTAGGAAACGAGGATCTTG	AACGTCCGCAATGTGTTATTAAGTTGTC GGCTAGGAAACGAGGATCTTG
Salk151109	AACTTGCCAAAACGTCTTG TTGGGTTTTGCTCAACAAATG	AACTTGCCAAAACGTCTTG GCGTTCAAAGTCGCTAAG
GK232B02	CCTTCTTTGCAGGATCTGTG TTTCTACGGGCTTGTCAATG	GGGCTACACTGAATTGGTAGCTC TTTCTACGGGCTTGTCAATG
WiscDsLox50A210	TTTCAAATGGAACCGAAGTTG TGTTGGTCGTTGATTGATCTG	AACGTCCGCAATGTGTTATTAAGTTGTC TGTTGGTCGTTGATTGATCTG
Salk018871	GAGACAGATGACGCACAGGA TGCGATGTGTGAACAGAAGG	ATTTTGCCGATTTTCGGAAC TGCGATGTGTGAACAGAAGG
WiscDsLox434B3	GAGACAGATGACGCACAGGA TGCGATGTGTGAACAGAAGG	AACGTCCGCAATGTGTTATTAAGTTGTC TGCGATGTGTGAACAGAAGG

FW, forward; RV, reverse

All true homozygous mutants were backcrossed to wild-type Col-0 plants. The resulting F1 generation was inoculated with a *Staphylococcus* suspension (10^8 cfu/mL) and phenotypes were scored 5 days post-inoculation. The GK232B02 homozygous mutant was re-isolated

from the ♀Col-0 x ♂GK232B02 F2 population; also, 72 individuals from this F2 population were genotyped and phenotype was observed post *Staphylococcus* inoculation in an effort to establish dosage effects. The presence of truncated transcripts in *ster1-1* was assessed using the following pairs of primers: **(1)** 5'-CAAATCAAGGAACTCAAAGAAAA-3' and **(2)** 5'-ACGAGGATCTAAATCAGACATTGA-3' to amplify the whole *STER1* cDNA; primers **(1)** and **(3)** 5'-AACTTGGCCAAAAGTGTCTTG-3' to amplify the first exon; and primers **(4)** 5'-TTTCAAATGGAACCGAAGTTG-3' and **(2)** to amplify exons 5,6 and 7.

Heterozygous lines of SALK151109 and WiscDsLox50A210 lines were analyzed for embryo lethality. Briefly, siliques of 7 week-old plants were dissected longitudinally along the natural line of dehiscence using a forceps and the silique content was observed using a dissecting microscope. In the case of the absent embryos, only a small structure at the seed attachment point was visible. Aforementioned heterozygous lines were crossed to each other and siliques from F1 plants were analyzed for embryo lethality.

Generation of ster1-1 complemented lines

A full length At4g23150 open-reading frame cDNA clone in Gateway pENTR223 entry vector was obtained from the SALK institute genomic analysis laboratory (Kayoko et al, 2003). The *STER1* cDNA was transferred into the pGWB2 destination vector (GenBank: AB289765.1) by performing the LR reaction with The Gateway LR Clonase enzyme mix kit (Invitrogen) as instructed. For the generation of GFP constructs, *STER1* cDNA without the stop codon was amplified and cloned into the Gateway pENTR223 entry vector using the pENTR directional TOPO cloning kit (Invitrogen) as directed. The cDNA was further subcloned into the pGWB5 destination vector (GenBank: AB289768.2). All generated complementation constructs, as well as the empty destination vectors, were transferred into *Agrobacterium tumefaciens* GV3101 cells by electroporation. Cells were grown on plates containing antibiotics (Table 2.3) and confirmed by colony PCR (vector primers: forward 5'-GACCATGATTACGCCAAGCTT-3' and reverse 5'-ACGATCGGGGAAATTCGAGCTC-3'; gene specific primers: forward 5'-TTTCTACGGGCTTGTTCAATG-3' and reverse 5'-TGTTGGTCGTTGATTGATCTG-3'). The different *Agrobacterium* strains were further used for plant transformation by the floral dip method as previously described (Clough and Bent, 1998). Briefly, *A. tumefaciens* strain GV3101 containing the plasmids on interest was grown in LB media with appropriate antibiotics (Table 2.3) at 28°C overnight. Cultures were diluted 1/100 into fresh LB and

grown for another 24 hours. Pelleted cells were resuspended in 5% sucrose at an OD₆₀₀ of 0.8 and the suspension was supplemented with 0.05% Silwet L-77. Inflorescences of plants at the right developmental stage (just prior to flowering, with plants exhibiting a lot of unopened buds) were submerged in the *Agrobacterium* suspension for 1 minute. Plants were covered with a transparent lid overnight to maintain high humidity and allow plants to take up the suspension. Seeds from individual pots were pooled, harvested and selected on half strength Murashige Skoog (MS) medium. Firstly, seeds were sterilized by one wash with 70% ethanol for 5 minutes, 1 wash with 10% bleach/ 1% SDS for 5 minutes and 6 washes with water for 1 minute. Approximately 9000 seeds (~180 mg) were resuspended in 1% agarose and spread on half MS plates (15 cm diameter) containing hygromycin (5 µg/mL for Cvi transformants selection; 30 µg/mL for Col-0 and GK232B02 transformants). Primary transformants were transferred to soil and DNA was extracted to confirm presence of the complementation construct by PCR (gene specific primers: forward 5'-AACCCTTTTGTCTCTCTCTT-3' and reverse 5'- GCGCTGGTAAAGCCATTGTA-3'; vector primers (amplifying hygromycin gene): forward 5'-ATTTGTGTACGCCCCGACAGT-3' and reverse 5'-GATGTAGGAGGGCGTGGATA-3').

The asymptomatic accession Cvi and the non-responsive T-DNA mutant GK232B02 (*ster1-1*) were complemented with a copy of the gene, while a GFP tagged version of the same gene was introduced in the symptomatic accession Col-0 to generate a GFP-tagged line (table 2.8). Both the primary transformants and their progeny were infiltrated with a *Staphylococcus* suspension in order to confirm phenotype was successfully complemented.

Table 2.8. Transgenic lines generated in this study

Transgenic line	Vector used	Line type
Cvi::ster1	pGWB2-ster1	Complemented line
Cvi (EV)	pGWB2 empty vector	Complemented line control
Cvi::ster1-GFP	pGWB5-ster1	Complemented line
GK232B02::ster1	pGWB2-ster1	Complemented line
GK232B02 (EV)	pGWB2 empty vector	Complemented line control
Col(ster1-GFP)	pGWB5-ster1	GFP-tagged line
Col (EV)	pGWB5 empty vector	GFP-tagged line control

Bioinformatics

STER1 protein model and homolog sequences were obtained from the UniProtKB database (www.uniprot.org). Sequences were aligned using the ClustalW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Accession specific At4g23150 sequences were extracted from the re-sequencing data available at 1001genomes.org. Microarray data mining was performed using GeneVestigator v3 (Hruz et al, 2008; www.genevestigator.com).

Analysis of *ster1* defect in *Cvi*

RNA was extracted from accessions *Cvi*-0 and *Col*-0 and converted to cDNA using an oligodT primer. RT-PCR was performed using primers **(1)** 5'-CAAATCAAGGAACTCAAAGAAAA-3' and **(2)** 5'-ACGAGGATCTAAATCAGACATTGA-3' to amplify the whole *ster1* cDNA; primers **(1)** and **(3)** 5'-AACTTGGCCAAAACCTGTCTTG-3' to amplify the first exon; primers **(4)** and **(5)** 5'-TCTAGCAATCCCACCAATGA-3' to amplify exons 2,3 and 4; and primers **(6)** 5'-TTTCAAATGGAACCGAAGTTG-3' and **(2)** to amplify exons 5,6 and 7.

Cellular localization of *STER1* protein in *N. benthamiana* and *A. thaliana*

The GFP tagged *STER1* protein was transiently expressed in *N. benthamiana* using *Agrobacterium* infiltration. Briefly, *Agrobacterium tumefaciens* (pGWB5-*STER1*) GV3101 cells were grown in LB media with appropriate antibiotics (Table 2.1) overnight; cultures were diluted 1/10 into fresh LB and grown for another 24 hours. Cells were pelleted, washed once in infiltration media (10 mM MES pH 5.6, 10mM MgCl₂, 200μM acetosyringone) and resuspended to an OD₆₀₀ of 1.0. 6-week old *N. benthamiana* plants were inoculated by pressure infiltration using a 1mL syringe and the area of infiltration was outlined with a marker pen. Expression of the fluorescent protein and localization in the leaf cells was observed after 48 hours using a Leica confocal microscope and a 40x objective. A plasma membrane marker (pm-cb CD3-1002; Nelson et al, 2007) was used for comparison.

STER1 localization was also imaged in 2-week old *Arabidopsis Col* (*STER1*-GFP) plant lines using the same settings.

Boiled elicitor assays

Overnight cultures of *S. epidermidis* were resuspended in 10mM MgCl₂ and adjusted to a an OD₆₀₀ of 0.2. Suspensions were boiled (100°C for 15 minutes) and allowed to cool. 100 µL of the boiled suspension was plated on LB with no selection to ensure cells were killed by the treatment. Plants were inoculated with the heat-killed cells and phenotypes were observed 5 days post-inoculation.

To generate the soluble elicitor, overnight cultures of *S. epidermidis* were resuspended in 10mM MgCl₂ and adjusted to a an OD₆₀₀ of 2.0. Suspensions were boiled (100°C for 15 minutes), allowed to cool and centrifuged at 13000g for 10 minutes to remove cell debris. The supernatant was removed and passed through a sterile 0.2 µm filter. The resulting suspension was treated with 0.2 mg/mL proteinase K and incubated at 37°C for 6 hours. The removal of proteins was verified by SDS-PAGE and silver staining as previously described (Sambrook et al, 2001). The enzyme was inactivated by incubation at 70°C for 15 minutes. A negative control of MgCl₂ and proteinase K was also performed in parallel. Plants were inoculated with all the different fractions and phenotype was observed 5 days post-inoculation.

Peptidoglycan purification

Peptidoglycan (PGN) purification was performed as described by deJonge et al (1992) with some modifications. 10 litre cultures of each Gram-negative *B. ambifaria* 19182 and Gram-positive *S. epidermidis* were grown overnight at 37°C with shaking and pelleted by centrifugation for 30 minutes at 8000g (room temperature). Cells were freeze-dried for 48 hours and 5g of cells were used as starting material for PGN purification. Cells were resuspended in cold water and added in droplets to an equal volume of 8% boiling SDS solution. The resulting suspension was boiled at 100°C for 30 minutes in order to inactivate autolysins which hydrolyze PGN and samples were left at room temperature overnight. Insoluble polymeric PGN was recovered by centrifugation for 30 minutes at 8000g (room temperature) and cells were washed with water several times until no SDS was detected. SDS presence was assessed by the method of Hayashi (Hayashi, 1975). SDS treatment removes contaminating proteins, non-covalently bound lipoproteins and lipopolysaccharides. At this stage, samples were freeze dried and only the *Staphylococcus* fraction was passed through a french press and washed twice with water. Subsequently, both *Burkholderia* and *Staphylococcus* samples were treated with 100 µg/mL α-amylase (EC 3.2.2.1, Sigma) for 5

hours at room temperature to remove glycogen. Afterwards, 100 µg/mL of trypsin (EC 3.4.21.4, Sigma) was added in the presence of 50µM (NH₄)HCO₃ (final concentration) and samples were incubated with stirring at room temperature overnight to remove LPXTG proteins in Gram-positive bacteria and lipoproteins in Gram-negative bacteria. Enzymes were inactivated by boiling for 5 minutes in 1% SDS (final concentration). PGN was recovered by centrifugation at room temperature (8000g/30 minutes), samples were washed until no SDS was detected and subsequently freeze dried.

At this stage, *Staphylococcus* samples only were split in half. Only one half was treated with HF, thus generating a pre-HF and a post-HF PGN preparation (MF and pure PGN, respectively). 49% HF was added to the freeze-dried PGN sample (90mg PGN/1 mL 49% HF) and the suspension was incubated with stirring at 4°C for 48 hours. This mild acid hydrolysis allows for the removal of secondary polysaccharides covalently bound to PGN by phosphodiester bounds such as teichoic acids, capsules, poly (β 1-6 GlcNAc). PGN was recovered by centrifugation for 30 minutes at 9000g (room temperature) and washed several times with water until pH was neutral and the acid was removed.

The *Staphylococcus* pre-HF and post-HF PGN preparations and the *Burkholderia* PGN sample were washed twice with 8M LiCl, twice with 100mM EDTA to remove any polypeptidic contaminations and twice with acetone to remove lipoteichoic acid and traces of LPS. PGN was resuspended in the minimum amount of water, lyophilized and weighed to measure PGN amounts.

In order to determine sample composition and purity, all three PGN preparations underwent GC-MS analysis, which was performed with a SLB capillary column (0.18 mm³ 20 m, Supelco).

Muropeptide preparation

The isolated PGN (pure *Burkholderia* and *Staphylococcus* PGN fraction) were resuspended in 12.5 mM phosphate buffer pH 5.5 (20 mg PGN/ 1 mL buffer) and degraded with 500 units of mutanolysin (1U/µL) from *Streptomyces globisporus* ATCC21553 (Sigma-Aldrich) at 37°C overnight. The enzyme reaction was stopped by boiling for 5 minutes and insoluble contaminants were removed by centrifugation for 20 minutes at 9000g (room temperature). The generated muropeptides were dissolved in 0.5 M sodium borate buffer (pH 9.0), and solid sodium borohydride was added immediately. After incubation for 2 hours in the dark at room temperature, excess borohydride was destroyed with 2M HCl. Methanol was added to

both samples and the alcohol was evaporated using a rotary evaporator. Desalting of soluble mucopeptides was performed by reverse phase HPLC and peaks were collected, pooled and lyophilized. Peak identity was confirmed by NMR spectroscopy.

Teichoic acid purification

Teichoic acid (TA) was purified according to Peschel et al, 1999 (WTA isolation protocol).

Elicitor phenotypic assays

All elicitors (*B. ambifaria* and *S. epidermidis* PGN, *S. epidermidis* MF, *S. epidermidis* TA) were freshly prepared at a concentration of 50 µg/mL in 10mM MgCl₂. For all phenotypic elicitor assays, elicitor suspensions were infiltrated into leaves of 5 week-old *Arabidopsis* plants using a blunt end 1mL syringe and phenotypes were observed 5 days post-inoculation.

Fungal treatment

Botrytis cinerea was cultured on potato glucose agar (Fluka) supplemented with 500ug/ml spectinomycin and incubated at 18-20°C under UV lighting. Spores were harvested by flooding the plate with sterile distilled water and filtering through a layer of Miracloth. Spores were counted using a haemocytometer and adjusted to a final concentration of 2×10^5 spores in 1/8th strength potato dextrose broth (PDB, Fluka). Leaves were placed in transparent boxes lined with moist tissue to maintain humidity and inoculated with 10µl droplets of spore suspension on the abaxial side. Boxes were kept under light (10h light/14 hour dark light cycle). Symptom development was assessed 5 days post inoculation by visual inspection using the following scoring system: 0=no lesions, 0.5=small lesions within the droplet, 1=lesion within the droplet, 2=spreading lesion, 3=collapse, 4=collapse with sporulation.

Staining procedures

Trypan blue staining was used to visualize dead cells as described (Koch and Slusarenko, 1990). Suspensions were infiltrated into *Arabidopsis* leaves with a blunt end 1mL syringe and samples were collected at indicated time points. Leaves were covered in staining solution (25% v/v of each H₂O, lactic acid, phenol and glycerol plus 0.025% w/v trypan blue) and boiled for 5 minutes. After cooling, the staining solution was replaced with destaining solution (250% w/v chloral hydrate) and samples were shaken for 7 days until leaves were

cleared. Afterwards, leaves were mounted in 60% glycerol and observed using a Leica light microscope. Pst DC3000 (avrRpm1) was used as a positive control (10^7 cfu/mL), while 10mM MgCl₂ functioned as a negative control.

DAB staining was performed to visualize H₂O₂ as previously described (Thordal-Christensen et al, 1997). Briefly, leaves were infiltrated with the different suspensions and samples were collected at selected time points. Leaves were covered with a 0.1% DAB solution pH 3.8 (3,3'- diaminobenzidine, Fluka) and incubated in the dark overnight. Chlorophyll was cleared by incubation in 96% ethanol at 37°C overnight. Samples were washed twice with water and mounted in 60% glycerol for microscopic investigation using a Leica light microscope. Pst DC3000 (avrRpm1) was used as a positive control (10^7 cfu/mL), while 10mM MgCl₂ functioned as a negative control.

Aniline blue staining was performed to visualize callose deposition as previously described (Hauck et al, 2003). Briefly, leaves were infiltrated with the different suspensions and samples were collected 24 hours post-inoculation. Leaves were cleared of chlorophyll by incubation in 96% ethanol at 37°C overnight. After 2 washes with water, leaves were immersed in the aniline blue solution (1% w/v aniline blue in 150mM K₂HPO₄, pH 9.5/KOH) and incubated in the dark overnight. Samples were mounted in 60% glycerol and observed under UV fluorescence (filter excitation min 340nm max 380nm) with a 10x objective. 1 μ M flg22 was used as a positive control, while 10mM MgCl₂ functioned as a negative control. The number of callose deposits was determined using the ImageJ software (<http://rsbweb.nih.gov/ij>). Mean counts were made per three fields of view in three replicate leaves from *Arabidopsis* plants.

For all staining assays, *S. epidermidis* and *B. ambifaria* were infiltrated at a concentration of 10^8 cfu/mL and PGN fractions (*Staphylococcus* MF and PGN, *Burkholderia* PGN) were used at a concentration of 50 μ g/ mL. All experiments were performed at least 3 times, with 3 leaves per plant and 3 plants per treatment per experiment.

Calcium measurements

Cytosolic calcium concentrations were measured by calcium-induced aequorin luminescence using transgenic *Arabidopsis* Col plants expressing cytosolic apoaequorin under the control of the CaMV 35S promoter (Knight et al, 1996). Mature leaves of 4 week old plants were cut into 1-mm strips and floated on 100 μ L of water supplemented with 10 μ M coelenterazine (Biosynth) in a 12-well plate (10 strips/well). For aequorin reconstitution, plates were

incubated in the dark for at least 12 hours. Subsequently, 3 leaf strips were transferred to each well containing 400 μ L of water. MAMPs were applied by the addition of 400 μ L of a two-fold concentrated solution in water at a final concentration of 100 μ g/mL for the *Staphylococcus* MF fraction and *Burkholderia* PGN and 5 μ M flg22. Measurements were performed immediately using a luminometer consisting of a 9829A photomultiplier tube with a 1.5-kV potential from a FACT50 air-cooled thermoelectric housing and an AD2 amplifier/discriminator (all from Thorn EMI) and the data output was recorded continuously for 45 minutes. Controls were performed by addition of an equal volume of water. Aequorin imaging was performed using an intensified CCD camera (model EDC-02), with camera control unit (HRPCS-2) and image acquisition and processing software (IFS216), all from Photek (St Leonards-on-Sea, UK) as described previously (Knight and Knight, 2000). Relative luminescence values were calculated by quantifying luminescence for each sample and using water values as standard.

STER1 internalization assay

Detached leaves of 2 week-old *Arabidopsis* Col-0 plants expressing a STER1-GFP tagged protein under the control of the CaMV 35S promoter were floated on elicitor solutions (100 μ g/mL *Staphylococcus* MF and PGN, *Burkholderia* PGN; 10 μ M flg22; 10mM MgCl₂). After 15 minutes, leaves were observed using a Leica confocal microscope under a 40x objective.

Analysis of defense gene induction

PGN and derived fractions were dissolved in 10mM MgCl₂ at a concentration of 10 μ g/mL and infiltrated, by using a needleless syringe into leaves of 5-week old *A. thaliana* wild-type Col-0 and *ster1-1* mutant plants. An additional set of leaves was infiltrated with 10mM MgCl₂, *S. epidermidis* and *B. ambifaria* suspensions (10⁸ cfu/mL).

Table 2.9. Primers used to assess defence gene expression

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
PR1	GTAGGTGCTCTTGTTCTTCCC	CACATAATCCCACGAGGATC
PDF 1.2	TCATCATGGCTAAGTTTGCTTC	GTAAAATACACACGATTTAGCACCA
STER1	AACCCTTTTGTCCTCTCTCTCTT	GCGCTGGTAAAGCCATTGTA
ACTIN	ATGAAGATTAAGGTCGTGGCA	TCCGAGTTTGAAGAGGCTAC

Samples were collected 24 hours post-inoculation and RNA was extracted using TRI REAGENT (Sigma). RT-PCR was performed to assess PR1, PDF 1.2 and STER1 expression following the different treatments. Primer pairs used are listed in table 2.9. Band intensities were quantified using the ImageJ software (<http://rsbweb.nih.gov/ij>) and actin values were used as a standard.

Electrolyte leakage assay

Electrolyte leakage was measured in order to follow HR development as described (Kwon et al, 2003). Plants were inoculated with different suspensions as follows: 10^8 cfu/mL *Pst* DC3000 (avrRpm1), 10mM MgCl₂, 50 µg/mL of PGN-derived fractions. After inoculation (3 leaves per plant, 3 plants per treatment per experiment), plants were left to take up the inoculum for one hour. Afterwards, six 0.5 cm² leaf discs per plant (2 leaf discs per leaf) were harvested using a cork borer and transferred to a Petri dish with 25 mL of purified water. All plates were shaken for 2 hours to remove excess inoculum. Subsequently, leaf discs were transferred to fresh Petri dishes (50 mm diameter) containing 6 mL of purified water. Conductivity readings were performed for 96 hours at designated time points. The entire content of the Petri dishes (liquid and leaf discs) were transferred to individual tubes and boiled for 15 minutes. After cooling, the conductivity of each sample was recorded (100% electrolyte leakage). This value was used to determine the percentage of electrolyte leakage for each time point.

MAMP priming assay

Plants were inoculated with several solutions as follows: 1µM flg22, 10mM MgCl₂, 50µg/mL *Burkholderia* PGN, 5µg/mL *Staphylococcus* MF fraction, 50µg/mL *Staphylococcus* PGN. After 24 hours, plants were co-infiltrated with *Pst* DC3000 (10^6 cfu/mL) and leaf discs were collected at designated time points for growth curve assays. LB plates with serial dilutions were incubated at 28°C for 48 hours after which the colony forming units were counted.

Agrobacterium-mediated transient expression in N. benthamiana

The GFP tagged STER1 protein was transiently expressed in *N. benthamiana* using *Agrobacterium* infiltration. Briefly, *Agrobacterium tumefaciens* (pGWB5-STER1) GV3101 cells were grown in LB media with appropriate antibiotics (Table 2.3) overnight; cultures were diluted 1/10 into fresh LB and grown for another 24 hours. Cells were pelleted, washed

once in infiltration media (10 mM MES pH 5.6, 10mM MgCl₂, 200µM acetosyringone) and resuspended to an OD₆₀₀ of 1.0. 6-week old *N. benthamiana* plants were inoculated by pressure infiltration using a 1 mL syringe and the area of infiltration was outlined with a marker pen. After 48 hours, leaves were co-infiltrated with 100µg/mL *Burkholderia* PGN, 100µg/mL *Staphylococcus* MF and PGN fractions and 10⁸ cfu/ mL *S. epidermidis* and *B. ambifaria* suspensions. All MAMP solutions were prepared in infiltration media. Symptoms were observed for 7 days after co-infiltration.

3. RESULTS

3.1. A study of *S. epidermidis* virulence using *A. thaliana*

3.1.1. Introduction

A pathogenic model in which both the bacterium and its host are amenable to genetic manipulation can greatly facilitate the understanding of bacterial pathogenesis.

Arabidopsis is genetically tractable and has been used as an experimental model for identifying bacterial virulence factors (Rahme et al, 1995; Prithiviraj et al, 2005; Jha et al, 2005). This small plant has long been adopted as a model organism and is by far the most thoroughly studied flowering plant (Koornneef and Meinke, 2010). The variation among natural accessions, coupled to the availability of a plethora of mutants, make *Arabidopsis* an ideal candidate as a host system. Several genes relevant to mammalian pathogenesis have been confirmed by using plants as model hosts, including the *P. aeruginosa gacA* quorum sensing regulator and the *E. faecalis fsr* quorum-sensing system (Rahme et al, 1997; Jha et al, 2005).

S. epidermidis, a common Gram-positive bacterial colonizer of the skin and mucous membranes, is currently regarded as an important opportunistic pathogen (Otto, 2009). Although usually in a benign relationship with its host, *S. epidermidis* does maintain a low virulence level aimed at enhancing persistence through evading the immune system rather than aggressively attacking the host. Nevertheless, the specialized, acquired immune response fails to clear long lasting *S. epidermidis* infections despite specific antibody production (Pourmand et al, 2006). As a result, more studies aimed at elucidating *S. epidermidis* virulence mechanisms are required. Previous studies have emphasized various analogies in innate immunity against pathogens in plant, invertebrate and mammalian hosts. Despite *Staphylococcus* species being able to infect both plants and animals, so far mostly *in vivo* animal models have been used for research (Rupp et al, 1999; Prithiviraj et al, 2005). By comparison, plant models are an attractive option and experiments using the *S.aureus*-*Arabidopsis* pathosystem have shown potential for this approach with *S. epidermidis*.

Aim

The initial aim of this study was to establish the *Arabidopsis*- *S. epidermidis* pathosystem. Initial experiments focused on surveying the ability of *S. epidermidis* to infect *A. thaliana*.

The infection procedure was optimized using a few commonly used accessions initially. Plant phenotypic responses, as well as *in planta* bacterial growth were assessed following inoculation with *S. epidermidis*. Defence-associated markers such as cell death and callose deposition were followed to further characterize the plant-bacteria interaction. Following this initial analysis, an extensive panel of 46 accessions were tested for their response to *S. epidermidis* in order to investigate natural variation of the plant response. Additionally, well characterized MAMP and defence signalling mutants were tested for altered responses to *S. epidermidis*.

3.1.2. Growth of *S. epidermidis* on *A. thaliana*

The aim of this study was to assess the ability of *S. epidermidis* to infect *Arabidopsis* plants. All experiments were performed using a *Staphylococcus* lab strain designated *S. epidermidis* 18888.

Initially, commonly used *Arabidopsis* accessions Ler, Col-0 and Cvi-0 were surveyed following syringe-inoculation with *S. epidermidis* (Figure 3.1). While Ler and Col-0 reacted strongly to *S. epidermidis*, Cvi-0 showed no obvious symptoms (Figure 3.1 A and B). The disease-like symptoms consist of chlorosis that becomes visible by 3dpi and develops into necrosis by 5 dpi. The symptoms are restricted to the inoculated leaf area (Figure 3.1B). Leaves of different ages have been inoculated throughout the experiments with consistent phenotypic responses, indicating that the response is not dependent on leaf age.

In planta bacterial growth was measured at 0, 2 and 4 dpi for accessions Ler and Cvi-0 to assess whether phenotypic variation correlates with differences in bacterial growth. In all cases, *S. epidermidis* bacterial numbers decreased from the starting inoculum at day 0, resulting in eventual clearing of the bacteria by 3 dpi, coincident with the appearance of phenotypic symptoms (Figure 3.1C). As a control, Ler and Cvi-0 accessions were infiltrated with *Pseudomonas syringae* pathovar *tomato* (Pst) DC3000, a virulent bacterial pathogen of *Arabidopsis*. These accessions were shown to support exponential *in planta* growth of Pst DC3000, confirming that the ecotypes tested were capable of supporting a compatible pathogen under the conditions tested (Figure 3.1 D).

To test whether the infection conditions were optimal for *S. epidermidis* growth, several parameters were varied such as inoculum concentration, humidity and temperature.

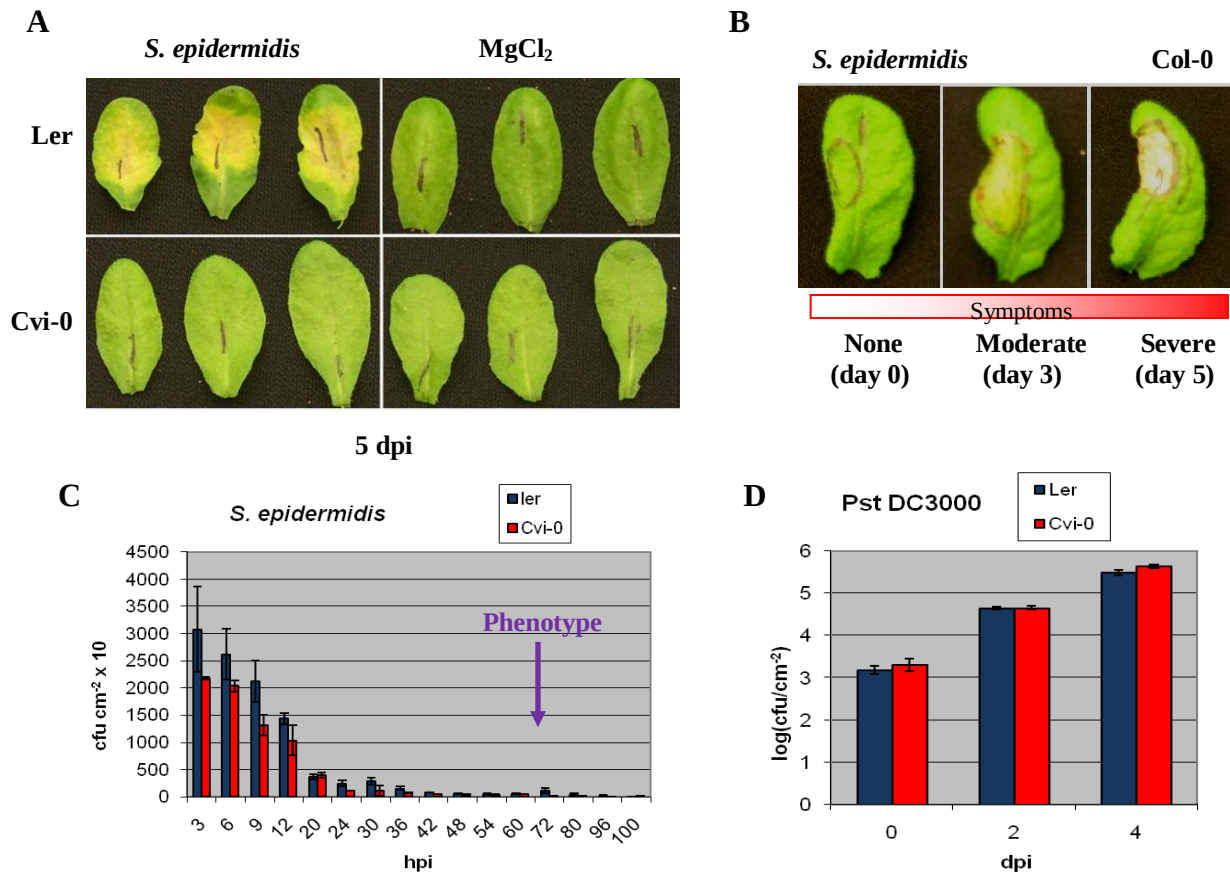


Figure 3.1. Infection of *A. thaliana* with *S. epidermidis* 18888. (A) Phenotype observed 5 dpi after *S. epidermidis* 18888 inoculation (10^8 cfu/mL) of accessions Ler and Cvi-0 ($MgCl_2$ used as negative control); (B) Symptom development in accession Col-0 after *S. epidermidis* 18888 infiltration; (C) *S. epidermidis* 18888 growth curve in Ler and Cvi-0; time point when phenotype becomes visible is indicated by arrow; (D) Pst DC3000 growth curve in Ler and Cvi-0;

Firstly, inoculum levels as high as 10^9 cfu/mL ($OD_{600}=2.0$) were used for infiltration and although this resulted in higher bacterial titres for each time point, the overall descending growth pattern remained unchanged. Secondly, in order to test temperature effects on *S.epidermidis* growth, plants were either incubated at $37^{\circ}C$ for defined time intervals immediately following inoculation or transferred to a growth cabinet at $30^{\circ}C$ for the duration of the experiment following inoculation. Both of these experimental variations had no discernible positive effect on *S.epidermidis* proliferation *in planta*. Increased humidity, achieved by covering the plants with a transparent lid for 24 hours, did not impact bacterial growth but seemed to accelerate phenotype development. Taken together, these data suggest *S. epidermidis* is unable to establish itself and grow in *Arabidopsis* leaves and that bacterial proliferation is not the underlying cause of the necrotic phenotypes observed in the accessions tested.

3.1.3. *S. epidermidis* elicits defence responses in *Arabidopsis*

In order to study plant defence responses elicited by *S. epidermidis* several histochemical analyses were performed following bacterial inoculation. Usually, PTI is associated with callose deposition, which is a form of cell-wall based extracellular defence, while ETI results in programmed cell death which correlates with reactive oxygen intermediates release.

Resistance-associated markers were monitored as follows: *S. epidermidis*-related cell death was investigated by lactophenol-trypan blue, in-situ peroxides were visualized by staining with DAB (3', 3' diaminobenzidine), while aniline blue allowed for the visualisation of cell wall callose deposits (Figure 3.2 and 3.4).

By comparison to *Pst* (*avrRpm1*), *S. epidermidis* induces cell death at a slower rate in Col-0 (Figure 3.2A). While at 12hpi some cell death is visible adjacent to the bacterial inoculation site only, by 24hpi the entire inoculated area displays a low level of staining. At 48hpi, trypan blue staining revealed extensive cell death comparable to that seen for *Pst* (*avrRpm1*). The amplitude of the cell death phenotype closely correlated with ROS release in Col-0 (Figure 3.2B). Low levels of DAB staining were discernible for *S. epidermidis* compared to *Pst* (*avrRpm1*). Additionally, this analysis was also performed with the asymptomatic accession Cvi-0, which displayed no cell death or peroxide accumulation across all time-points compared to Col-0 (Figure 3.2 C and D). Electrolyte leakage was also used to monitor cell death progression for 48 hours in both accessions (Figure 3.3). Electrolyte leakage values obtained for *S. epidermidis* were very low compared to measurements taken for *Pst* (*avrRpm1*). Nevertheless, electrolyte leakage values recorded following *S. epidermidis* treatment were higher in Col-0 than in Cvi-0. These data are in accordance with the results of the trypan blue staining.

S. epidermidis infiltration generated very few callose deposits at 24 or 48hpi in Col-0 (Figure 3.4). Callose deposition represents a localized response to incompatible pathogens and their MAMPs. Accordingly, inoculation with flg22 lead to abundant callose deposits (score of 732 and 900, respectively). By contrast, response to *S. epidermidis* was very weak (scores of 28 and 44, respectively).

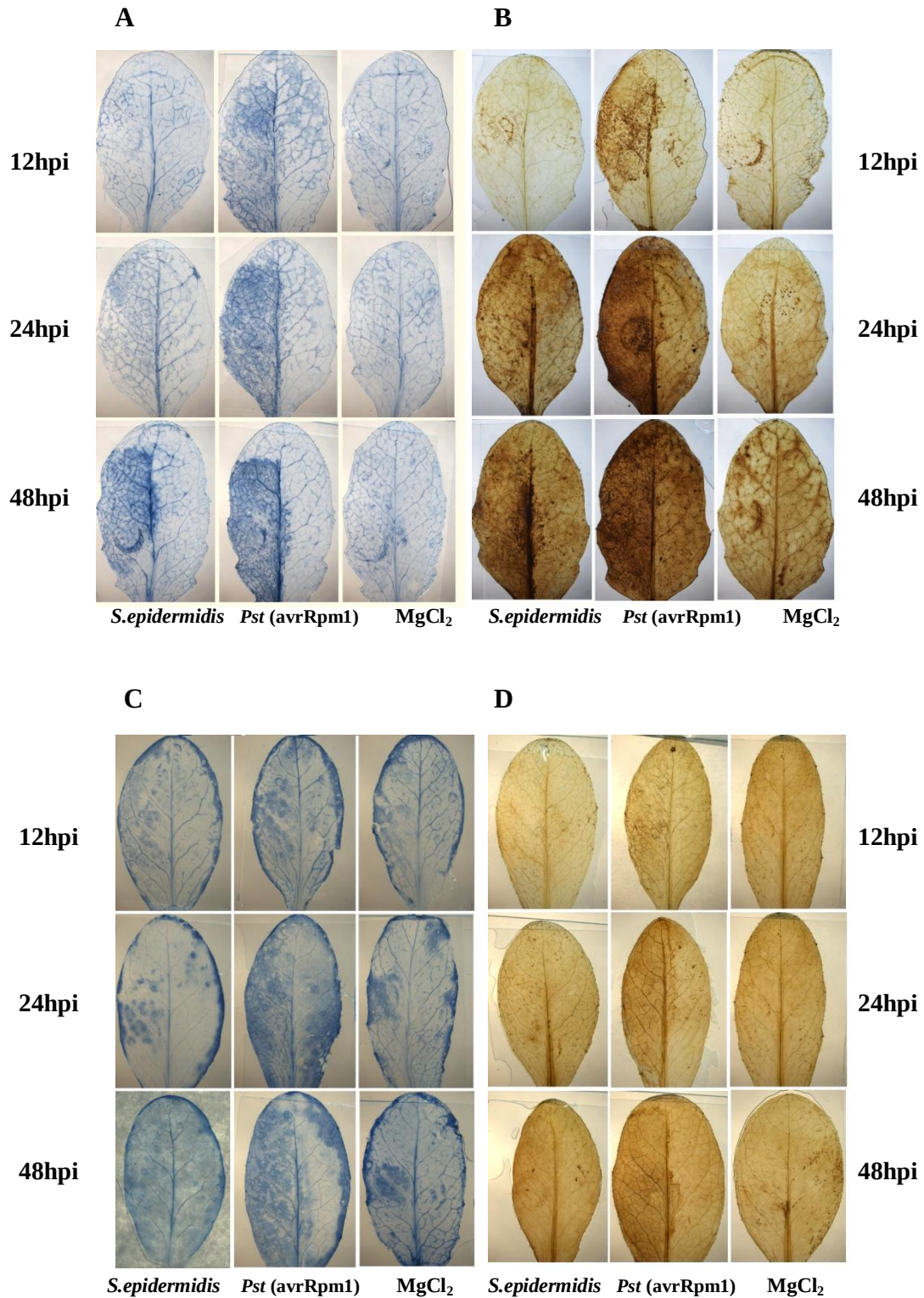


Figure 3.2. Cell death and ROS release following *S. epidermidis* inoculation.

(A),(C) Trypan-blue staining and (B),(D) DAB staining of Col-0 and Cvi-0 leaves, respectively; leaves were inoculated as indicated; The inoculum concentration used was 5×10^7 cfu/mL for *Pst* (avrRpm1) and 10^8 cfu/mL for *S. epidermidis* 18888; 10mM MgCl_2 was used as mock treatment. Only the left half of the leaf was infiltrated. Representative leaves for each timepoint are shown.

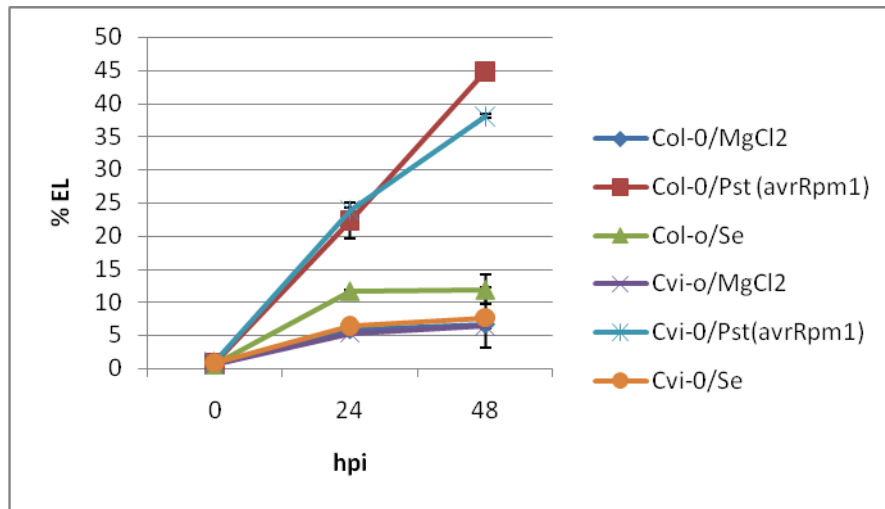


Figure 3.3. Electrolyte leakage following *S. epidermidis* inoculation. Electrolyte leakage was measured for Col-0 and Cvi-0 at specified time points following inoculation with *Pst* (avrRpm1) (5×10^7 cfu/mL) and *S. epidermidis* 18888 (Se) (10^8 cfu/mL). 10mM MgCl₂ was used as mock treatment. Measurements were expressed as % electrolyte leakage (EL).

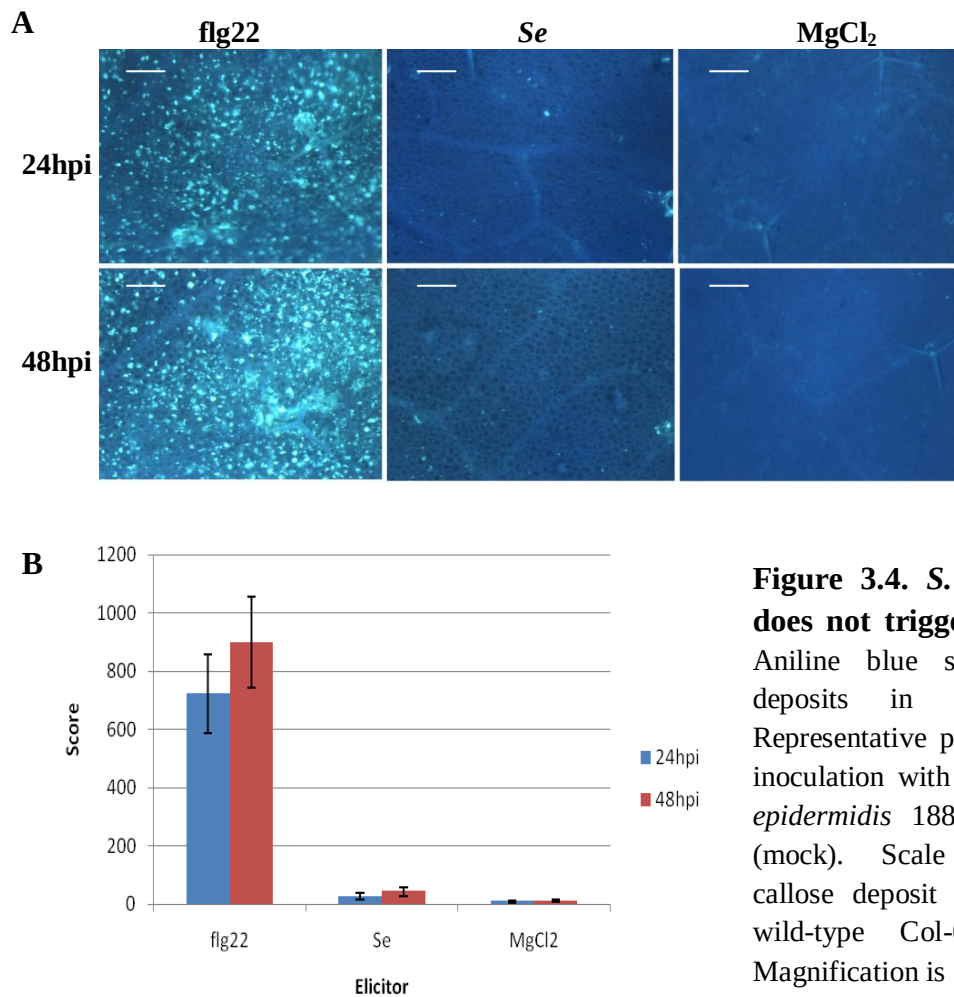


Figure 3.4. *S. epidermidis* inoculation does not trigger callose deposition (A) Aniline blue staining to detect callose deposits in Col-0 24 and 48hpi. Representative photos were taken following inoculation with 1 μ M flg22, 10^8 cfu/mL *S. epidermidis* 18888 (Se) or 10mM MgCl₂ (mock). Scale bar is 0.2mm; (B) Mean callose deposit counts made in leaves of wild-type Col-0 following inoculation. Magnification is 10x.

3.1.4. Natural variation of phenotypic responses to *S. epidermidis* among *Arabidopsis* accessions.

A collection of 46 *Arabidopsis* accessions (Figure 3.5) with a wide geographic distribution was tested for phenotypic responses and *in planta* bacterial growth. The phenotypic response was assessed 5 days post-inoculation (dpi) and scored from 1 to 5 according to symptom severity as follows: 1, asymptomatic; 2, mild chlorosis; 3, chlorosis; 4, enhanced chlorosis with necrotic patches; 5, necrosis. The majority of *Arabidopsis* accessions tested reacted strongly to *Staphylococcus* infection (eg. Ler, Col-0), while three accessions showed no obvious symptoms (Cvi-0, Tsu-1 and Ws-2) (Figure 3.5). *In planta* bacterial growth was measured at 0, 2 and 4 dpi for all accessions included in the panel to assess whether phenotypic variation correlates with differences in bacterial growth. In all cases, an eventual clearing of the pathogen was recorded, with growth patterns following the same trend as that seen for Ler and Cvi-0 (Figure 3.1C and data not shown).

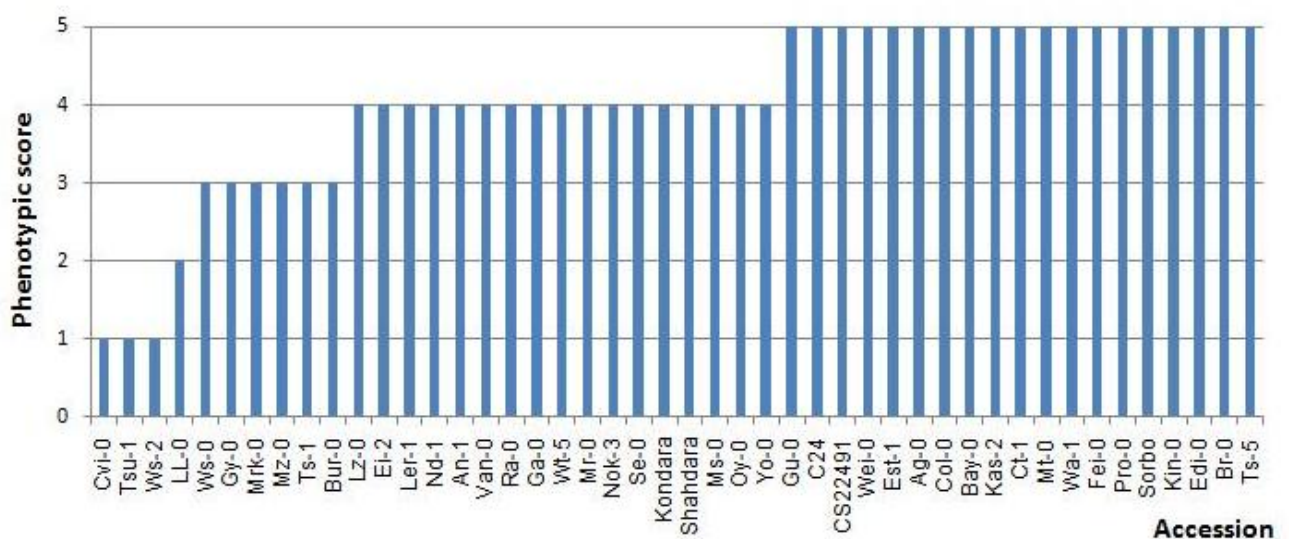


Figure 3.5. Natural variation of phenotypic responses to *S. epidermidis* 18888 among *Arabidopsis* accessions. Phenotypic scores were assigned as follows: 1, asymptomatic; 2, mild chlorosis; 3, chlorosis; 4, enhanced chlorosis with necrotic patches; 5, necrosis;

3.1.5. *Arabidopsis* responses to *Staphylococcus* spp.

A range of *Staphylococcus* species and *S. epidermidis* strains was infiltrated into *Arabidopsis* Col-0 leaves to test whether the phenotypic reaction observed is specific to *S. epidermidis* strain 18888.

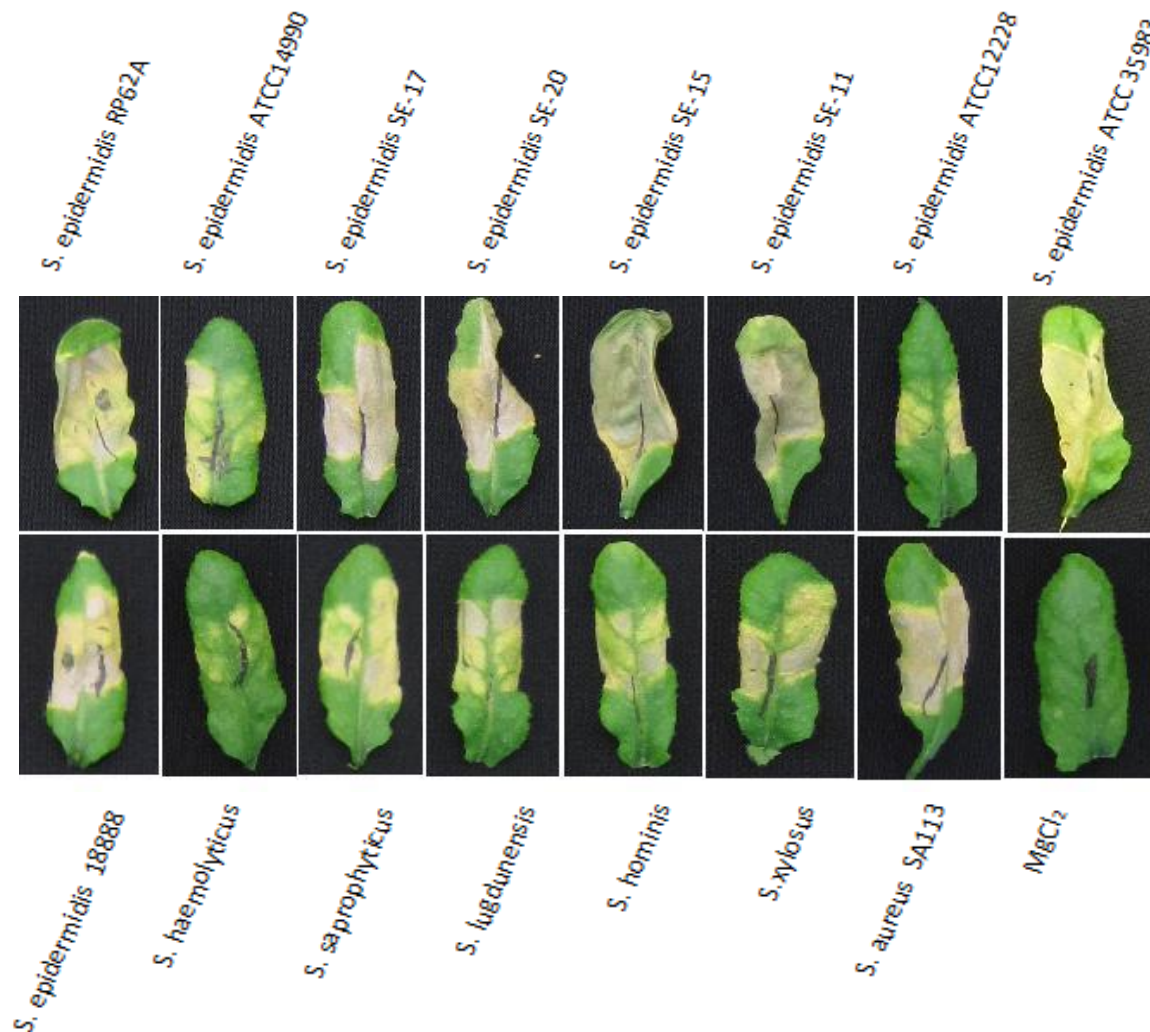


Figure 3.6. Infiltration of *A. thaliana* Col-0 with *Staphylococcus* spp.

Five-week old wild-type Col-0 plants were inoculated with several *Staphylococcus* strains as indicated (10^8 cfu/mL). Representative leaves were chosen and phenotypes were observed 5dpi. The *Staphylococcus* lab strain 18888 was used as a positive control and $MgCl_2$ functioned as a negative control.

Accession Col-0 was inoculated with several strains of *S. epidermidis*, including the sequenced biofilm-negative strain *S. epidermidis* ATCC12228 and biofilm-positive strain *S. epidermidis* RP62A, as well as *S. aureus* (Figure 3.6). Infiltration of all *S. epidermidis* strains tested resulted in a phenotype similar to the one produced by 18888, with the exception of *S. epidermidis* ATCC14990 and ATCC12228. Additional commensal *Staphylococcus* species were also tested. While inoculation with these *Staphylococcus* species did result in a phenotypic response, this was milder than phenotypes observed for *S. epidermidis* 18888, consisting mainly of chlorosis rather than necrosis. *S. haemolyticus* infiltration gave rise to the least pronounced phenotype (Figure 3.6). *S. aureus* SA113, a well-characterized human pathogen was also tested. Inoculation with *S. aureus* lead to a necrotic response which closely resembled the phenotypes seen for *S. epidermidis* 18888.

3.1.6. *S. epidermidis* triggers plant defence in an SA-dependent manner

In order to gain further insight into the *S. epidermidis* 18888 infection, well characterized MAMP and defence signalling mutants were tested for altered responses to *S. epidermidis*.

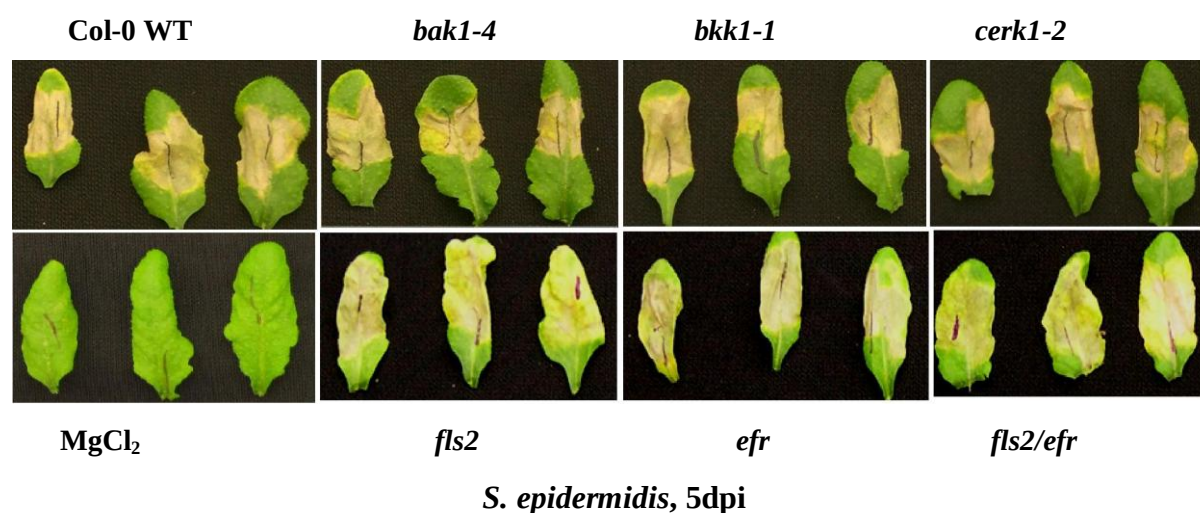


Figure 3.7. Role of well-studied PRRs and adaptor RLKs in *S. epidermidis* perception. Indicated mutants were inoculated with *S. epidermidis* (10^8 cfu/mL) and phenotypes were observed 5dpi. 10mM MgCl_2 was used as mock treatment.

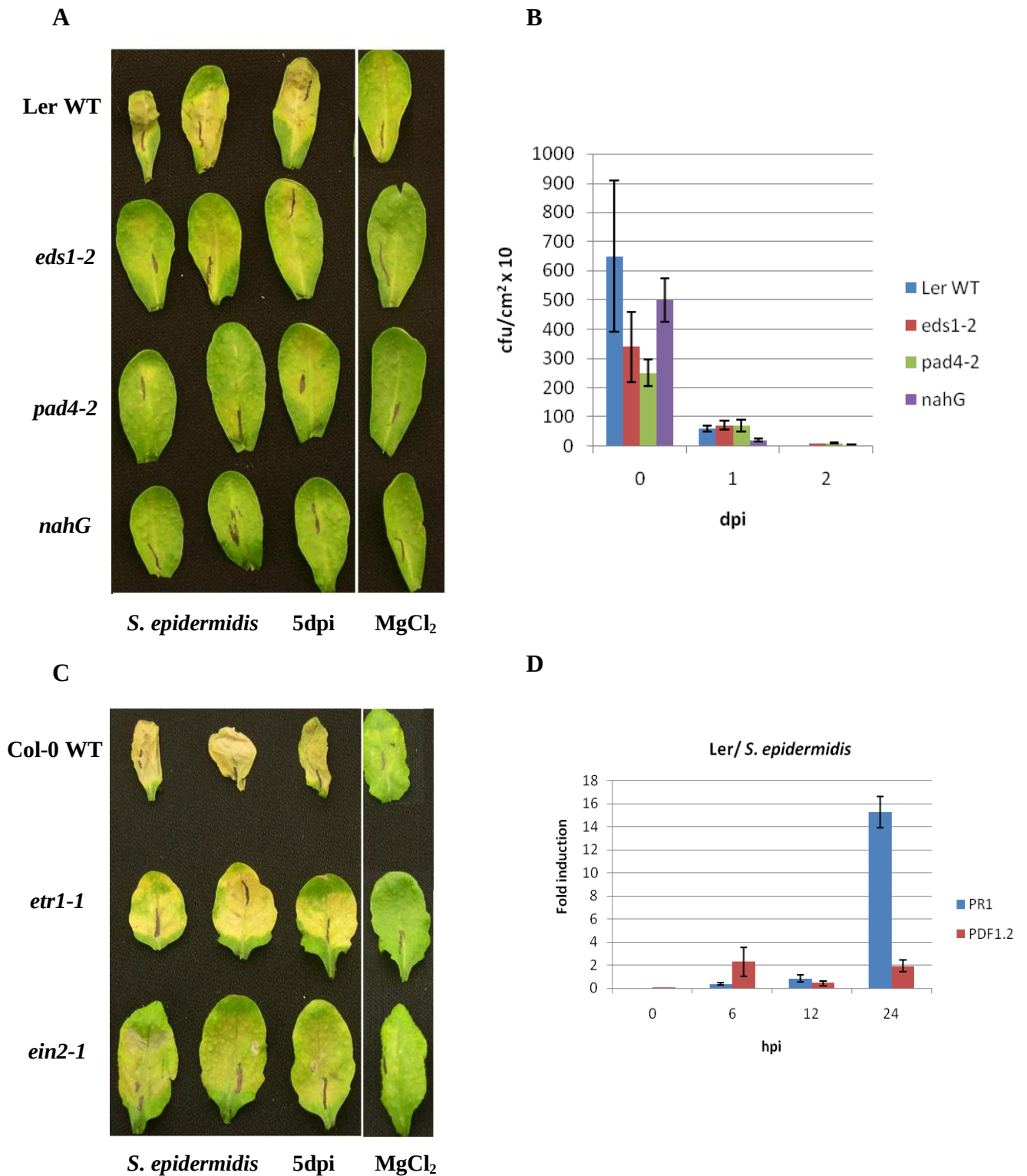


Figure 3.8. Involvement of SA and ET signalling pathways in *S. epidermidis* responses. (A) *eds1-2*, *pad4-2* mutants and the *nahG* transgenic line deficient in the SA-pathway remain asymptomatic following *S. epidermidis* inoculation; (B) *S. epidermidis* growth curve in SA pathway mutants; (C) *Arabidopsis* ET-mutants *etr1-1* and *ein2-1* show a reduced phenotype following *S. epidermidis* inoculation; (D) Induction of PR1 and PDF1.2 genes in *Arabidopsis* Ler following *S. epidermidis* treatment (10^8 cfu/mL); fold-induction values were obtained by comparison to MgCl_2 -treated tissue following normalization to actin values;

Several mutants impaired in MAMP responses were tested; these included *Arabidopsis* lines lacking well-known PRRs FLS2 and EFR, as well as mutants missing signalling adaptors like BAK1, BKK1 and CERK1 (Figure 3.7). As expected, no significant difference was observed in the FLS2 mutant after inoculation with non-flagellated *S. epidermidis* (Figure 3.7). Furthermore, all mutants displayed phenotypes similar to Col-0 following inoculation with *S. epidermidis*, suggesting these potential adaptor RLKs and PRRs do not contribute towards *S. epidermidis* responses.

After pathogen recognition and defence signal initiation, two main pathways ensure the establishment of systemic resistance (SAR): the SA-pathway and the JA-ET pathway. In order to investigate the contribution of these signalling pathways in *S. epidermidis* responses, mutants deficient in SA signalling, such as *eds1-2* and *pad4-2*, as well lines defective in ET perception, such as *ein2-1* and *etr1-1*, were challenged with *S. epidermidis*. Additionally, a *nahG* line encoding a salicylate hydroxylase that degrades SA was also included in the panel. In the mutants impaired in the SA-pathway, the macroscopic response to *S. epidermidis* was abolished (Figure 3.8A). Additionally, the *S. epidermidis* growth pattern remained unchanged in the *eds1-2*, *pad 4-2* mutants and the *nahG* transgenic line (Figure 3.8B). Mutants defective in the ET pathway also displayed attenuated phenotypes following *S. epidermidis* inoculation (Figure 3.8C). More specifically, *etr1-1* and *ein2-1* displayed less severe chlorotic symptoms 5 days-post inoculation as opposed to the necrotic phenotype displayed by the wild-type.

The ability of *S.epidermidis* to induce PR1 and PDF1.2 gene expression was also tested. While PR1 is a molecular marker for the induction of the SA-pathway and plant innate immune responses in general, PDF1.2 expression correlates with the JA-ET pathway induction. Only low levels of PR-1 transcript were detected prior to 24 hpi, when a 15-fold increase was observed. By contrast, PDF1.2 expression was not significantly up-regulated in response to *S. epidermidis* at any of the time points analyzed (Figure 3.8D). In conclusion, analysis of host gene expression pinpoints the SA-pathway, and not the JA-ET pathway, as the main participant in defence-associated signal transduction following *S.epidermidis* infection in Ler.

3.1.7. Discussion

Current understanding of *S. epidermidis* virulence is incomplete and the major assay presently being used for assessment of *S. epidermidis* pathogenicity is the animal infection models (eg. rat agar bead mortality model). The *Arabidopsis* infection model has previously been successfully used to identify *P. aeruginosa* virulence traits (Rahme et al, 1997). This study looks at the feasibility of using *A. thaliana* as an alternative for studying *S. epidermidis* pathogenicity.

Infiltration of the *Arabidopsis* reference accession Col-0 with *S. epidermidis* 18888 causes a strong necrotic reaction. A comparison of the *S. epidermidis* response of 46 naturally occurring *Arabidopsis* accessions showed that all but Cvi-0, Tsu-1 and Ws-2, showed a reaction upon bacterial inoculation. Nevertheless, the bacterium fails to multiply *in planta* in any of the accessions tested, suggesting *S. epidermidis* is not an adapted pathogen of *Arabidopsis*. By contrast, the bacterial titres of *S. aureus* (Prithiviraj et al, 2005) and the plant pathogen *Pst* DC3000 increase exponentially over time following inoculation in *Arabidopsis*; The phenotypic data, coupled to the inability of the pathogen to grow suggests that *Arabidopsis* is not susceptible to *S. epidermidis*, but rather that the contrasting reactions seen in different accessions are actually the expression of two distinct defence responses. To be more detailed, MAMP-triggered immunity limits pathogen growth after recognizing a microbial pattern and a successful pathogen suppresses this response through its type III effectors to allow for further colonization (Jones and Dangl, 2006). Since the study of the 46 accession panel did not yield an accession capable of supporting *S. epidermidis* growth, it could be inferred that the phenotypic responses observed in plants are a result of MAMP-based bacterial recognition rather than successful colonization. Nevertheless, defence responses to non-pathogenic bacteria are not usually accompanied by such strong visual symptoms in *Arabidopsis*. For example, the TTSS *Pst* DC3000 mutant *hrcC*, a strain that is unable to transport effectors and thus serves as an elicitor of the MAMP response, does not multiply or cause visible disease symptoms in *Arabidopsis* (Hauck et al, 2003). The necrotic response to *S. epidermidis* becomes visible 3dpi, although the bacteria is cleared within 36 hours. This could suggest *Arabidopsis* either recognizes a microbial feature that becomes more abundant or accessible after bacterial lysis, or that bacteria release a toxic compound during lysis.

Several analyses were performed in an effort to dissect plant host responses when challenged with this bacterium. Plant defence has two layers: PTI and ETI. In most cases, PTI is associated with callose deposition, which is a form of cell-wall based extracellular defence, and reactive oxygen intermediates release while ETI culminates in programmed cell death. *S. epidermidis* inoculation generated cell death and ROS accumulation in Col-0, while none of these defence-related responses was visible for Cvi-0. Additionally, inoculation of Col-0 resulted in higher electrolyte leakage values than those seen for Cvi-0 over a 48 hour period. Nevertheless, very low overall levels of electrolyte leakage were recorded compared to those seen for *Pst* (avrRpm1). This finding was unexpected, considering the necrotic, severe response seen for *S. epidermidis*. This result indicates that infiltration with an *S. epidermidis* bacterial suspension does not cause membrane damage resulting in electrolyte leakage. Although the inability of *S. epidermidis* to multiply *in planta* would suggest the phenotypes seen are the expression of a basal defence response, *S. epidermidis* does not trigger callose deposition. As this form of localized defence response is quite generalized and can be seen for most plant-bacteria interactions (Boller and Felix, 2009), it is conceivable that a *S. epidermidis* 18888 molecule prevents the formation of callose deposits. In fact, effectors and MAMPs such as xanthan have been shown to suppress cell wall based defences (Hauck et al, 2003; Yun et al, 2006).

Multiple studies support the role for each of the SA, JA and ET pathways in MAMP triggered immunity (Wang et al, 2009; Tsuda et al, 2009; Gomez-Gomez et al, 1999). For example, SA was produced in response to flg22 or challenge with *Pst* DC3000 *hrcC*, a strain that is unable to transport effectors and thus serves as an elicitor of the MAMP response (Tsuda et al, 2008). Similarly, ethylene was shown to accumulate upon flg22 treatment (Gomez-Gomez et al, 1999). In the *eds1-2*, *pad4-2* mutants and the *nahG* line which are defective in the SA-pathway, the macroscopic response to *S. epidermidis* was abolished. This finding, coupled to marker gene PR1 up-regulation 24hpi suggests the SA-pathway is required for the macroscopic plant defence response to *S. epidermidis*. Similarly, SA was shown to mediate *Arabidopsis* resistance to *S. aureus* (Prithiviraj et al, 2005). The transgenic line *nahG* (defective in SA accumulation) and the mutant *npr1-1* (non-expressor of PR protein) were highly susceptible to root inoculation with *S. aureus* NCTC8325, with SA pre-treatment reducing the ability of this bacteria to inflict *npr1-1* mortality.

In addition, ethylene mutants also displayed a reduction in the necrotic response that did not correlate with an increase in PDF1.2 expression. Recent research has indicated the

ethylene-insensitive mutants used in the present study (*etr1-1* and *ein 2-1*) display reduced FLS2 steady-state levels and are impaired in the oxidative burst triggered by flg22 (Mersmann et al, 2010). In a similar manner, responses to *S. epidermidis* might be reduced in ethylene mutants due to low ROS levels and/or a lack of PRR accumulation. These assumptions are being addressed currently by testing *RbohD/F* mutants.

Tests conducted with a larger *Staphylococcus* panel including several *S. epidermidis* strains, *S. aureus* and other commensal staphylococci confirmed that Col-0 is responsive to all *Staphylococcus* species tested so far. This finding prompted the suggestion *Arabidopsis* recognizes a conserved *Staphylococcus* microbial feature and that variations in phenotype could be traced back to specific structural modifications or lack of this feature. Inoculation of known *Arabidopsis* mutants impaired in MAMP perception, such as *efr* and *bak1-4*, with *S. epidermidis* 18888 resulted in necrotic phenotypes mirroring that of wild-type Col-0. This result suggests a hypothetical *S. epidermidis* MAMP would perhaps function through a distinct PRR and/or signalling pathway than those described up to date.

Leaf inoculation with *S. aureus* SA113 produced phenotypes similar to those reported for *S. aureus* NCTC8325 (Prithiviraj et al, 2005), while almost all *S. epidermidis* strains induced necrotic plant reactions. The biofilm-negative strain *S. epidermidis* ATCC12228 causes a reduced phenotype in *Arabidopsis*, also suggesting a molecule required for or involved in biofilm formation may be recognized by the plant in this case. Quorum sensing signals, such as acyl homoserine lactones (AHLs) and AvrXa21, required for biofilm establishment have been shown to act as MAMPs in tomato and rice, respectively (Schuhegger et al, 2006; Lee et al, 2006). More specifically, AHLs induce defence responses such as SA accumulation and PR-1 gene expression in tomato at micromolar levels, i.e., at the same level at which they function in bacteria (Schuhegger et al, 2006).

In conclusion, inoculation with *S. epidermidis* triggers an SA-dependent necrotic phenotype in *Arabidopsis*, which appears to be the expression of a basal defence response rather than the result of successful colonization. Additionally, the extent of natural variation of phenotypic responses to *S. epidermidis* among *Arabidopsis* accessions allows for the genetical dissection of the *Arabidopsis*-*S. epidermidis* interaction. Taken together, these results indicate that *Arabidopsis* can serve as a valuable model system to study *S. epidermidis* -host interaction.

3.2. Mapping of *STER1*, an *Arabidopsis* gene controlling the response to *S. epidermidis*

3.2.1. Introduction

Arabidopsis is a species which displays large intraspecific natural genetic variation for many traits, presumably reflecting adaptations (Koornneef et al, 2004). Most of the natural phenotypic variation existing among *Arabidopsis* accessions is of quantitative nature, with traits displaying quantitative variation being under multigenic control. Nevertheless, in a few cases, the variation among wild accessions is due to single gene differences with large discrete effects, such as the those determining the erecta phenotype of some accessions or some components of disease resistances, such as FLS2 (Torii et al, 1996; Gomez-Gomez et al, 1999).

The first step in the forward genetic analysis of natural variation corresponds to QTL analysis or mapping, namely the identification and localization of genomic regions containing the loci that affect a quantitative trait. Although QTL analyses are increasingly used to study complex traits in *Arabidopsis*, such as yield traits (Keurentjes et al, 2006), only a few studies have focused on the genetic basis of quantitative variation in resistance and susceptibility of pathogens (Denby et al, 2004; Perchevied et al, 2006). As an example, Perchevied et al (2006) investigated the genetic basis of partial resistance to *Pst* DC3000 by exploiting a phenotypic difference between the Bay-0 and Shahdara accessions. Quantitative evaluation of a recombinant inbred line (RIL) population derived from these accessions allowed for the identification of two major and two minor QTLs, which were further confirmed using near-isogenic lines (NILs).

Aim

The aim of this section of the project was the identification of the genes underpinning the phenotypic response to *S. epidermidis* 18888. This was initially investigated using QTL analysis. Accessions Ler and Cvi-0 display contrasting reactions to this bacterium, thus enabling the use of a RIL population. The Ler X Cvi RIL population has already been used to analyse more than 40 traits (Koornneef et al, 2004) and was employed in order to determine the genetic basis underlying the differential response to *S. epidermidis* infection.

Subsequently, a NIL population with genomic Cvi-0 regions introgressed into a Ler genetic background (Keurentjes et al, 2007) was used to confirm the QTLs obtained through QTL analysis and narrow down the genomic interval that forms the basis of the necrotic response to *S. epidermidis* 18888. Additional analysis included fine mapping using an F2 population and a candidate gene approach employing T-DNA mutants. This strategy as a whole allowed for the identification of the gene controlling the response to *S. epidermidis* 18888.

3.2.2. A single gene controls *Arabidopsis* responses to *S. epidermidis*

Inoculation of 10 individual Cvi-0 X Ler F1 plants with *S. epidermidis* 18888 resulted in symptom formation at 5 dpi, indicating the Ler allele is dominant (Table 3.1). Similarly, the Col-0 allele was shown to be dominant over the Cvi-0 allele, suggesting a loss of function in the Cvi-0 accession. Segregation of the response was measured in a Cvi-0 x Col-0 F2 population; out of 616 F2 plants, 469 showed a necrotic response, while 147 were non-responsive, consistent with a one-gene model of inheritance (3:1 ratio). At the same time, the inheritance of the response was assessed in Col-0 x Ler F1 and F2 populations. As both accessions are responsive to *S. epidermidis*, the F1 response was not informative, but all F2 plants tested were responsive, thus confirming the same gene (or two tightly linked genes) controls the response in both Col-0 and Ler.

Additional crosses were performed and analysed after bacterial inoculation (Table 3.1). Phenotypic assessment of F1 hybrids generated by crossing symptomatic accessions (Col-0, Ler) and/or asymptomatic accessions (Cvi-0, Ws2, Tsu1) revealed a single gene controls the necrotic reaction to *S. epidermidis* 18888 in all *Arabidopsis* accessions included in this study. As Ler and Col alleles are dominant, the expectation would be that if Ws2 and Tsu1 alleles were different from the Cvi-0 one, the corresponding F1 Cvi-0 x Ws2 and Cvi-0 X Tsu1 crosses would be responsive to *Staphylococcus* inoculation. As both F1 and one F2 generation of this crosses proved asymptomatic following inoculation, it would appear the same defect renders accessions Cvi-0, Ws2 and Tsu-1 non-responsive to *S. epidermidis*.

Taken together, this data suggests a single gene most likely controls the response to *S. epidermidis* in *Arabidopsis*. This gene was named *STER1* (Staphylococcus elicitor response 1).

Table 3.1. Inheritance of responsiveness to *S. epidermidis* 18888 in different crosses

Progeny	Responsive	Non-responsive	Conclusion
F1			
Cvi-0 x Ler	10	-	Ler allele is dominant
Cvi-0 x Col-0	12	-	Col-0 allele is dominant
Col-0 x Ler	16	-	Non-informative
Cvi-0 x Ws2	-	11	The same gene is at the basis of the lack in response in Cvi-0 and Ws2
Cvi-0x Tsu1	-	9	The same gene is at the basis of the lack in response in Cvi-0 and Tsu1
F2			
Cvi-0 x Col-0	469	147	3:1 ratio fits one gene model of inheritance ($X^2=0.365$, $p>0.5$)
Col-0 x Ler	72	-	The same gene (or two tightly linked genes) controls the response in Ler and Col-0
Cvi-0 x Ws2	-	70	The same gene is at the basis of the lack in response in Cvi-0 and Ws2

3.2.3. QTL mapping of *STER1* using Cvi-0 x Ler RIL and NIL populations

Natural variation is currently viewed as an important tool for biology as it represents a potential source for uncovering novel host factors underlying important biological processes. For instance, genetic variation found for resistance to biotic factors - such as bacteria- has been shown to be linked to certain variants in disease resistance genes (Koornneef et al., 2004).

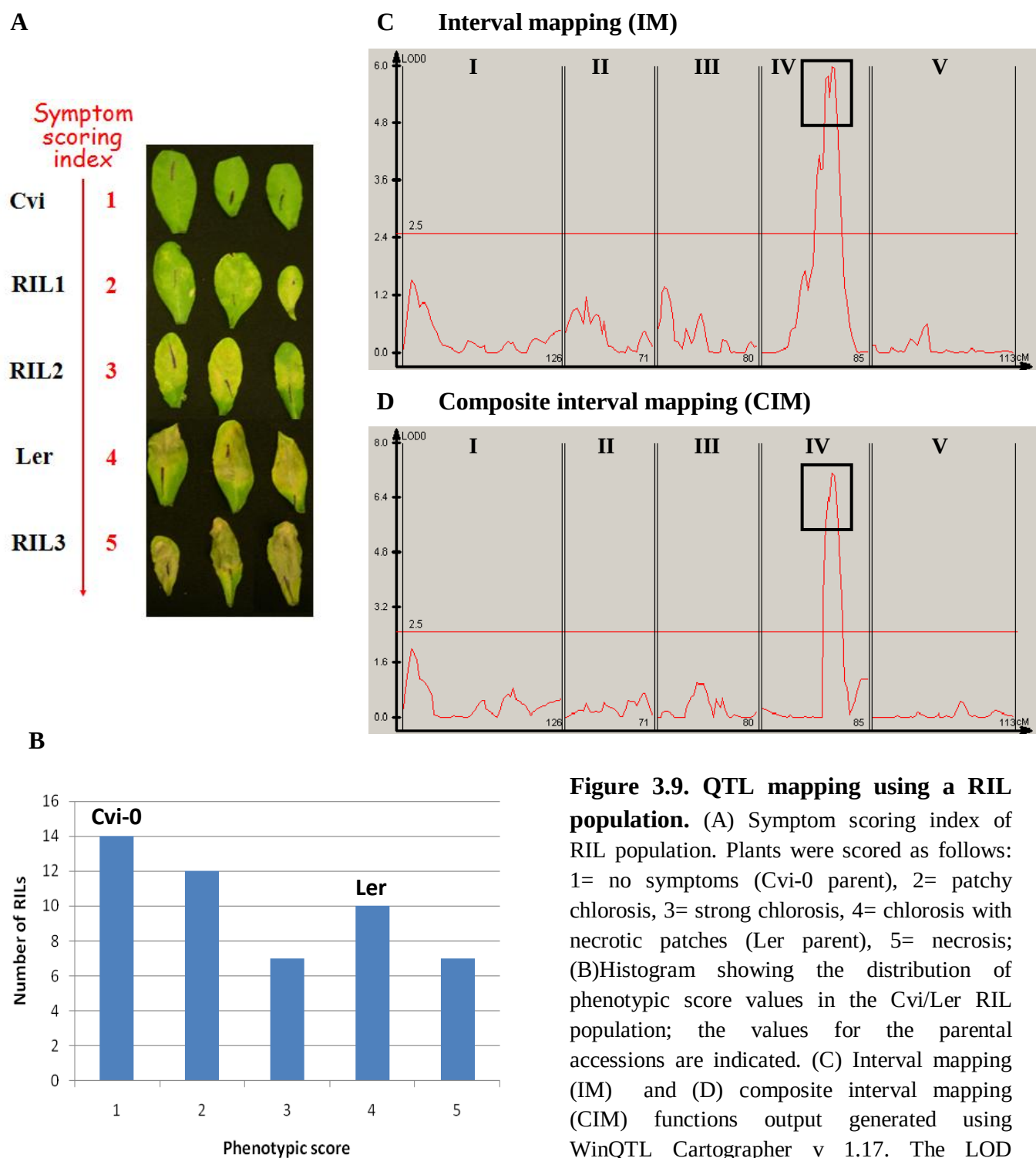


Figure 3.9. QTL mapping using a RIL population. (A) Symptom scoring index of RIL population. Plants were scored as follows: 1= no symptoms (Cvi-0 parent), 2= patchy chlorosis, 3= strong chlorosis, 4= chlorosis with necrotic patches (Ler parent), 5= necrosis; (B) Histogram showing the distribution of phenotypic score values in the Cvi/Ler RIL population; the values for the parental accessions are indicated. (C) Interval mapping (IM) and (D) composite interval mapping (CIM) functions output generated using WinQTL Cartographer v 1.17. The LOD threshold established after 1000 permutations is 2.5. The abscissa corresponds to the genetic map in cM, while the ordinate corresponds to the LOD scores. Output for all 5 *A. thaliana* chromosomes (I-V) is shown and the QTL on chromosome 4 is highlighted.

In order to investigate the genetic factors controlling the necrotic response of *Arabidopsis* to *S. epidermidis*, quantitative trait loci (QTL) analysis was performed using a recombinant inbred line (RIL) population derived from a cross between the asymptomatic accession Cvi-0 and the accession Ler that shows a strong necrotic reaction (Figure 3.1 A; Alonso-Blanco et al, 1998).

One of the requirements of QTL analysis is that the parents of the RIL population must display a strong, quantitative difference in the trait of interest; accessions Cvi-0 and Ler meet this condition, since the trait scored in this case is at the extremes of the phenotypic response curve to *S. epidermidis* 18888. The parents used for generating the RIL population were shown to exhibit *S. epidermidis* 18888 phenotypes indistinguishable from the Cvi-0 and Ler accessions that are part of the panel shown in Figure 3.1A. A base set of 50 RILs was used for the analysis and plants were assigned a score from 1 to 5 based on the severity of the phenotype seen 5 dpi, where a value of 1 corresponded to a lack of phenotype, as seen for Cvi-0, while a score of 4 was given for the Ler phenotype (Figure 3.9 A).

The RIL distribution of phenotypic scores appears slightly skewed towards lower values, however the overall trend is that of continuous variation suggesting a quantitative control of the response (Figure 3.9 B). Two types of analyses were performed using the compiled data: interval mapping (IM) and composite interval mapping (CIM) (Figure 3.9 C,D). While IM provides a systematic way to scan the whole genome for evidence of a QTL, CIM takes more variables into account, such as marker regression coefficients, and is performed in order to refine an IM result. Generally, a primary QTL is established if, in a certain region, the CIM test exceeds its threshold value and if the IM and CIM output closely resembles each other (Schiff et al, 2001). However, if only the CIM test surpasses its threshold, a secondary QTL is still considered valid as this function is considered superior to IM.

In this case, the threshold calculated for the data set after 1000 random permutations was 2.5. The output obtained (Figure 3.9 C,D) indicates that the necrotic response to *S. epidermidis* 18888 in *Arabidopsis* accession Ler is essentially controlled by one major QTL on chromosome 4. The QTL spans a 24 cM confidence interval (44cM- 68cM) and was assigned a LOD score of 7. Although the LOD score is quite low, this is to be expected as a visual trait was scored using a base set of only 50 RILs. The percentage of the phenotypic variance of the trait (R^2) explained by the QTL on chromosome 4 is 35%. Considering the

total phenotypic variance (TR^2) calculated using WinQTL Cartographer is 59%, this indicates that a chromosomal location with a high effect was identified in this study.

In order to confirm the QTL identified through RIL mapping, a panel of Cvi/Ler near-isogenic lines (NILs) covering the whole genome was employed (Keurentjes et al, 2007). The NILs were developed by introgressing genomic regions from the Cvi accession into the Ler genetic background.

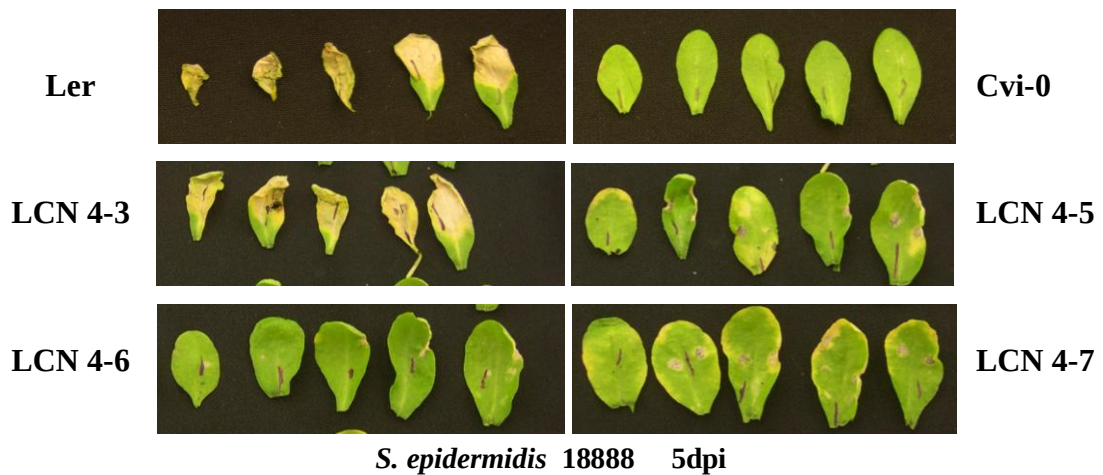
Table 3.2. Core set of NILs tested with *S. epidermidis* 18888

A core set of NILs introgressed Cvi segments covering all 5 chromosomes was infiltrated with *S. epidermidis* and symptoms were observed 5 dpi; the chromosome and position for each Cvi-0 introgression are indicated in the second and third column; the phenotypic reaction to *S. epidermidis* 18888 is indicated in the fourth column. √-presence of necrotic response; X-absence of necrotic response

NIL line (LCN)	Chromosome	Introgression position (start-end) (cM)*	Phenotypic response (5dpi)
1-3	1	0.0-15.1	√
1-8	1	10.9-126.0	√
1-10	1	21.7-31.4	√
1-13	1	29.9-45.1	√
1-16	1	40.7-65.5	√
1-22	1	59.4-94.5	√
1-26	1	87.0-126.0	√
2-2	2	0-22.2	√
2-4	2	16.0-36.0	√
2-7	2	34.9-64.4	√
2-20	2	63.2-71.3	√
3-3	3	0.7-40.5	√
3-9	3	22.3-62.9	√
3-14	3	43.2-81.1	√
3-15	3	69.1-81.1	√
4-3	4	27.9-43.6	√
4-5	4	42.5-57.7	X
4-6	4	57.7-67.8	X
4-7	4	71.1-85.3	X
5-1	5	0.3-7.3	√
5-6	5	14.2-43.3	√
5-8	5	38.1-78.6	√
5-13	5	62.2-107.5	√
5-15	5	83.7-106.8	√
5-19	5	106.8-112.1	√

*Distances are listed in accordance with the supplemental table of Keurentjes et al (2006) using the values corresponding to the closest Cvi marker left and right of the recombination point, respectively;

A



B

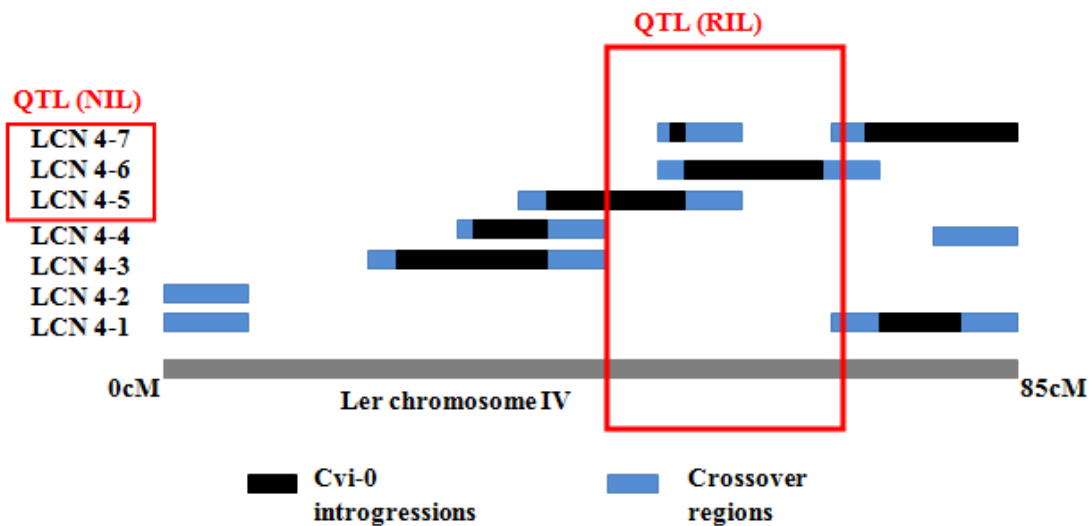


Figure 3.10. Confirmation of QTL position using NIL lines. (A) Phenotypic response of core chromosome 4 NILs to *S. epidermidis*18888 infiltration ; Ler and Cvi-0 parents were used as controls; NILs 4-5, 4-6 and 4-7 were asymptomatic following *S. epidermidis* 18888 inoculation; (B) Graphical representation of the Ler/Cvi NIL (LCN) lines for chromosome 4; Solid black bars represent the genetic position of Cvi-0 introgressions in individual NILs, blue bars represent crossover regions between markers used for the genotyping of the lines; Ler chromosome IV is represented as a solid grey bar; The region corresponding to the QTL mapped using RILs (spanning from 44cM-68cM) is highlighted by a red rectangle –QTL(RIL). Asymptomatic lines are indicated by the red rectangle on the left – QTL (NIL).

Although RIL mapping is widely used, analyses with this type of populations are limited because of the masking effects of major QTLs. By contrast, NIL populations also allow for the detection of smaller effect QTLs. A core set of NILs with introgressed Cvi segments covering all 5 chromosomes was infiltrated with *S. epidermidis* and symptoms were observed 5 dpi (Table 3.2). NILs 4-5, 4-6 and 4-7 which carry overlapping Cvi-0 introgressions on chromosome 4 consistent with the initial QTL position, failed to develop a necrotic reaction following infiltration (Figure 3.10 A,B). No additional QTLs were detected using this NIL-based mapping approach.

3.2.4. Fine mapping of *STER1* using a Cvi-0 x Col-0 F2 population

Phenotypic assessment of a Col-0 x Ler F2 population following inoculation confirmed a single gene, named *STER1*, controls the visual response to *S. epidermidis* 18888. This enabled the use of a Col-0 x Cvi-0 F2 population for fine mapping, as well as allowing for the candidate gene approach using T-DNA mutants which are generally in a Col-0 background.

Initially, the AFLP mapping data available for the Cvi/Ler NIL population was used as a starting point in order to narrow down the genomic region of interest (Figure 3.11). Cvi-0 introgressions in NILs 4-5, 4-6 and 4-7 share a common region on chromosome 4 between 54.5 and 62.5 cM, markers AD.115L (AFLP1) and CH.70L (AFLP4), respectively. This interval was narrowed down further to the region between 56.1 and 62.5 cM using NIL 3-18 which has an additional Cvi-0 introgression on chromosome 4 between 52.6 and 56.1 cM and is responsive to *S. epidermidis* 18888 (Figure 3.11).

NIL lines 4-5, 4-6 and 4-7 were further genotyped using CAPS (cleaved amplified polymorphic sequence) and SSLP (simple sequence length polymorphism) markers. While CAPS markers N2, B1, B2 and N1 were developed for this region using the Nordborg SNP database, an additional pre-existent SSLP marker (CIW7) was obtained from a marker database (see Materials and Methods). After this additional run of genotyping, the interval of interest was further narrowed down to the region between the B1 and B2 markers using NILs 4-5 and 4-7 which are asymptomatic and have a Cvi-0 introgression between N2 and B2.

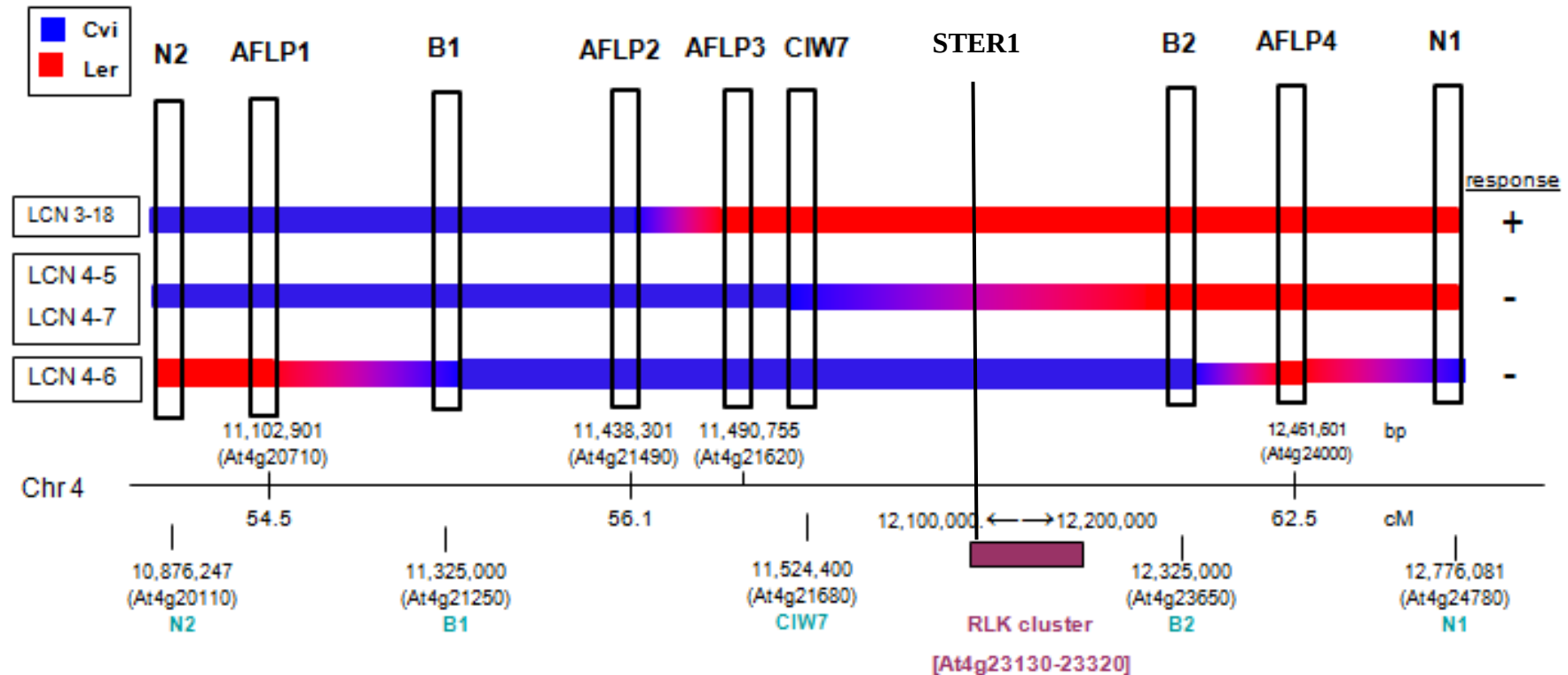


Figure 3.11. *STER1* genomic region on chromosome 4. The AFLP data available for the Cvi/Ler NIL population was used to narrow down the region towards fine-mapping of the locus of interest. NILs 4-5, 4-6 and 4-7 share a common region on chromosome 4 between 54.5 and 62.5 cM- markers AD.115L (AFLP1) and CH.70L (AFLP4). Intermediary markers FD167.L (AFLP2) and SNP3 (AFLP3) were used to map crossover regions. CAPS (N2,B1,B2,N1) and SSLP (CIW7) markers were also used to genotype selected NIL lines. Red solid bars represent regions mapping as Cvi-0, blue solid bars represent regions mapping as Ler and crossover regions between markers are represented by gradients. Marker positions are indicated in bp for all markers and in cM for AFLP markers. Marker identity at given positions (Cvi-0 or Ler) is highlighted by the black rectangles. An RLK cluster of 20 genes spanning ~100kbp, containing the *STER1* gene, is present between markers CIW7 and B2.

A Cvi-0 x Col-0 F2 population was used for further fine mapping. In a first phase, 200 F2 plants were inoculated with *S. epidermidis* 18888 and the resulting 44 non-responsive plants were selected for further analysis. Choosing plants that show the recessive phenotype simplifies the genetic analysis, as these plants will be homozygous for Cvi-0 at the *STER1* locus and this eliminates the need for F3 progeny testing. CAPS markers B1 and B2 and SSLP marker CIW7 were used for genotyping this first batch of non-responsive F2 plants.

Table 3.3. Genetic recombinants in B1-B2 genomic interval

F2 no.	Marker genotype				F3 progeny response	Confirmed <i>STER1</i> genotype
	B1	CIW7	<i>STER1</i>	B2		
3	H	H	Cvi	Cvi	23/23 asymptomatic	Cvi
4	H	H	Cvi	Cvi	21/21 asymptomatic	Cvi
7	H	H	Cvi	Cvi	24/24 asymptomatic	Cvi
9	H	H	Cvi	Cvi	23/23 asymptomatic	Cvi
42	Cvi	Cvi	Cvi	H	23/23 asymptomatic	Cvi

H, heterozygous

Only 5 plants out of the total 44 displayed crossovers between the chosen markers (Table 3.3). The F3 progeny were tested to confirm the accuracy of the initial phenotypic scoring process. As expected, all F3 progeny tested were asymptomatic following *S. epidermidis* 18888 infiltration. This first round of genotyping coupled with F3 progeny testing confirmed that *STER1*, the gene of interest resides between markers CIW7 and B2.

Another 96 non-responsive F2 plants were genotyped using the CIW7 and B2 markers (Table 3.4). The recombination frequencies obtained for both markers are smaller than 30%, thus conferring unequivocal evidence of linkage. The map distances calculated using the Kosambi mapping function suggested the gene of interest is closer to marker B2 than to marker CIW7 (Figure 3.12).

Table 3.4. Map distances between *STER1* and markers on chromosome 4 assigned using F2 genotyping data

Marker	Genotype			r (%)	D(cM)	Sr	Sd
	Cvi	H	Col				
CIW7	86	16	0	5.35	5.37	1.62	1.64
B2	96	7	0	2.50	2.50	1.12	1.12

H, heterozygous; r, recombination frequency; D, map distance in cM, calculated using the Kosambi mapping function; Sr/Sd standard error of recombination frequency/map distance

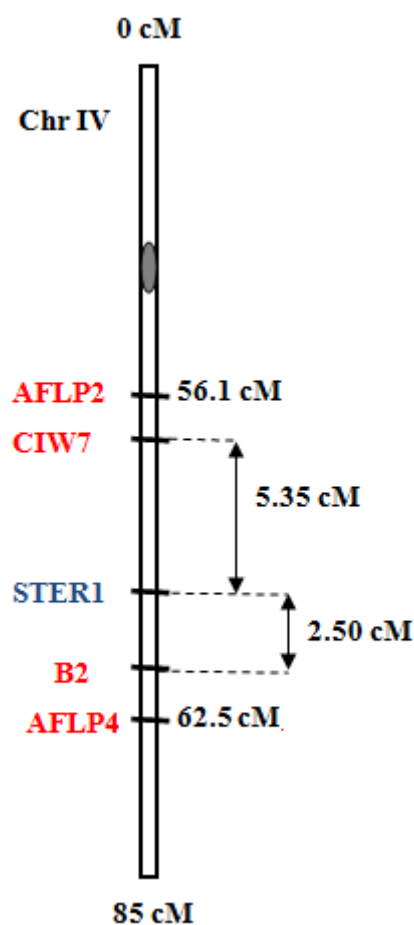


Figure 3.12. Graphical representation of linkage map. The *STER1* gene is flanked by SSLP marker CIW7 and CAPS marker B2. Known map positions for NIL genotyping markers AFLP2 and AFLP4 are indicated. Map distances were calculated using the Kosambi mapping function.

At this stage no additional markers inside the 800 kbp region between CIW7 and B2 were available. A scan of the genomic area between the two markers revealed an RLK cluster encompassing 20 genes, from At4g23130 to At4g23320 (Figure 3.11); these genes encode for

cysteine-rich RLKs that exhibit two extracellular DUF26 domains. This finding, coupled with the well-documented role of RLKs in *Arabidopsis* defence prompted a candidate gene approach.

T-DNA knockout lines in each gene in the cluster were isolated and examined after inoculation with *S. epidermidis* (Table 3.5.). The only asymptomatic mutant was a homozygous mutant in gene At4g23150 (CRK7), which was renamed *STER1* (*Staphylococcus elicitor response 1*). More specifically, a Col-0 GABI-Kat line with a T-DNA insertion in the second intron of the gene, named *ster1-1*, showed no symptoms following inoculation with *S. epidermidis*.

Table 3.5. CRK mutants phenotypic reaction to *S. epidermidis* 18888.

T-DNA knockout lines in each gene in the CRK cluster were tested with 10^8 cfu/mL *S.epidermidis* 18888 and observed 5dpi.

Gene	CRK no.	Phenotypic response (5dpi)
At4g23130	CRK5	√
At4g23140	CRK6	√
At4g23150	CRK7	X
At4g23160	CRK8	√
At4g23170	CRK9	√
At4g23180	CRK10	√
At4g23190	CRK11	√
At4g23200	CRK12	√
At4g23210	CRK13	√
At4g23220	CRK14	√
At4g23230	CRK15	√
At4g23240	CRK16	√
At4g23250	CRK17	√
At4g23260	CRK18	√
At4g23270	CRK19	√
At4g23280	CRK20	√
At4g23290	CRK21	√
At4g23300	CRK22	√
At4g23310	CRK23	√
At4g23320	CRK24	√

CRK, cysteine-rich RLK

√, responsive; X-non-responsive

3.2.5. Analysis of *ster1* mutants

All available T-DNA insertion mutants in gene At4g23150 (Figure 3.13A) were subsequently tested for their response to *S.epidermidis* inoculation. T-DNA insertion sites for all plant lines were confirmed (Table 3.6) and homozygous lines were isolated for all mutants, except *ster1-2*, *ster1-3* and *ster1-7*. RT-PCR was performed in order to confirm the isolated homozygous mutants were true knockouts (Figure 3.13B). *Ster1-2*^{+/-} and *ster1-3*^{+/-} heterozygous lines were also tested.

Table 3.6. T-DNA insertion mutants in *STER1*

Insertion line	Name	Confirmed insertion site
GK232B02	<i>ster1-1</i>	2nd intron
Salk151109	<i>ster1-2</i>	5' (77 bp upstream ATG)
WiscDsLox50A210	<i>ster1-3</i>	5th exon
Salk018871	<i>ster1-4</i>	7th exon (12bp upstream TGA)
WiscDsLox434B3	<i>ster1-5</i>	3' (28bp downstream TGA)
WiscDsLox361B10	<i>ster1-6</i>	5' (368 bp upstream ATG)
WiscDsLox336D10	<i>ster1-7</i>	5' (282 bp upstream ATG)

RT-PCR analysis using full *STER1* cDNA primers confirmed *ster1-1*, *ster1-4* and *ster1-5* are true knockouts, while showing that the *STER1* transcript is still present in the *ster1-6* homozygous mutant (Figure 3.13B). A *STER1* transcript was also detected in *ster1-2*^{+/-} and *ster1-3*^{+/-} heterozygous lines as expected, but at lower levels than those seen for the wild-type.

In order to assess whether *ster1-2*, *ster1-3* and *ster1-7* mutants could not be isolated due to a developmental defect, siliques of heterozygous *ster1-2*^{+/-}, *ster1-3*^{+/-} and *ster1-7*^{+/-} lines were opened and analyzed.

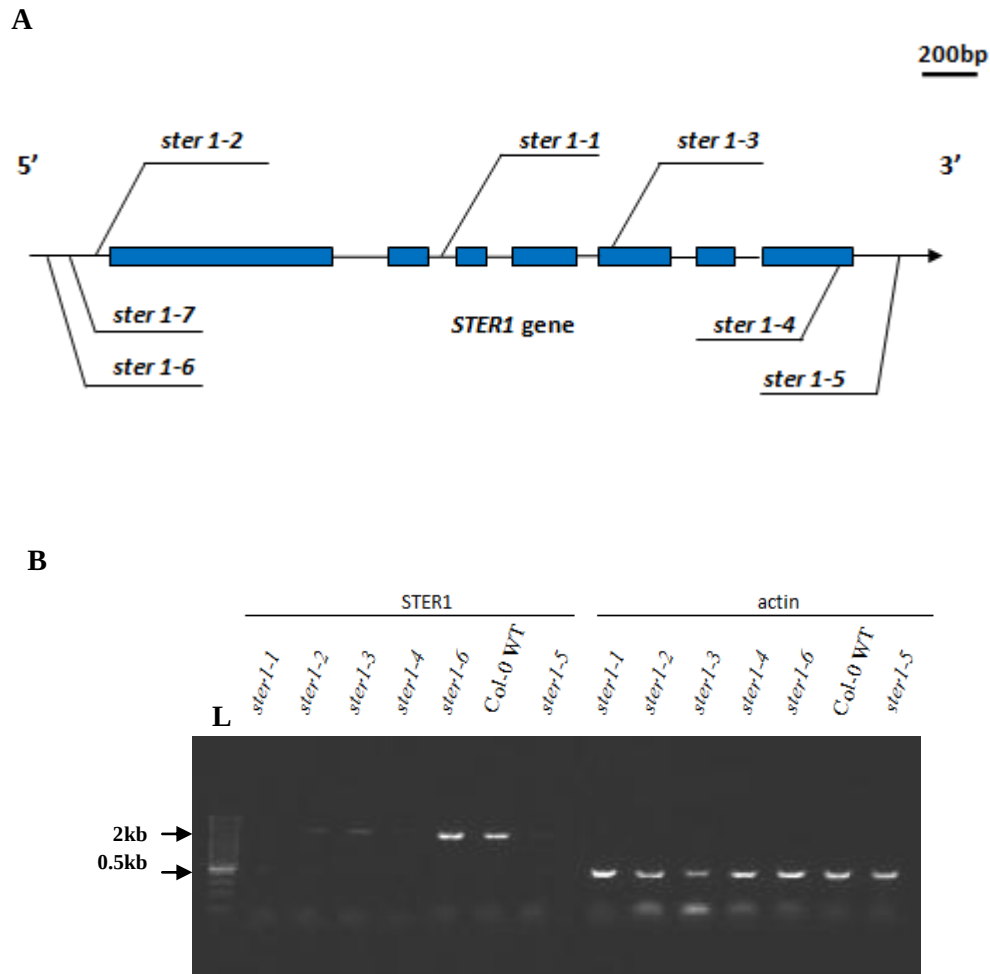


Figure 3.13. Analysis of *ster1* mutants. (A) Position of T-DNA insertions in the different *ster1* mutants; blue boxes represent exons and black lines represent introns; (B) RT-PCR results performed with cDNA derived from RNA extracted from mutants (*ster1-1*, *ster1-4* to *ster1-6*), heterozygous lines (*ster1-2*^{+/−} and *ster1-3*^{+/−}) and wild-type Col-0, as indicated; Primers amplifying the full *STER1* cDNA were used to assess if mutants are true knockouts; actin was used as a control; The GeneRuler 100bp Plus DNA ladder (Fermentas) (Ladder or L) was used to determine molecular weights in each case.

This analysis revealed 1 in 4 embryos was missing in *ster1-2*^{+/−} and *ster1-3*^{+/−} lines suggesting *STER1* also has a developmental function (Figure 3.14). By contrast, siliques of a *ster1-7*^{+/−} line appeared healthy. 20 plants were genotyped with several pairs of primers towards isolating a *ster1-7* homozygous mutant, but without success; it could be that this number was not sufficient for detecting a homozygous mutant or that the developmental defect in this case was at the germination stage rather than the seed stage; the latter possibility was not assessed during this study.

The possibility of *STER1*-related embryo lethality was assessed in some depth. Firstly, a large number of siliques from each homozygous mutant was analyzed along the Col-0 wild-type. A 3:1 ratio of present embryos versus missing embryos was observed for both the *ster1-2*^{+/-} and *ster1-3*^{+/-} lines, and not Col-0 (Table 3.7). These results were consistent with a potential role for *STER1* in development.

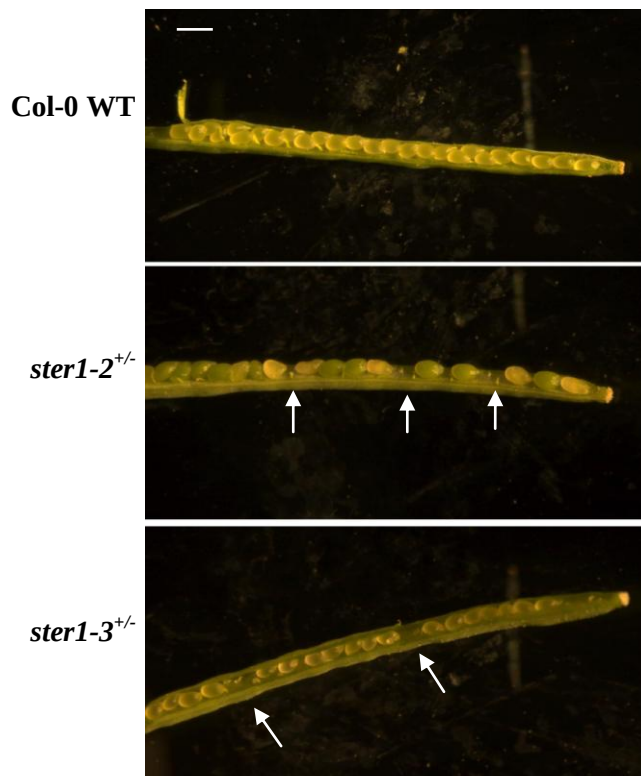


Figure 3.14. Phenotypic analysis of *ster1-2*^{+/-} and *ster1-3*^{+/-} heterozygous lines.

Embryo development phenotype of heterozygous *ster1-2*^{+/-} and *ster1-3*^{+/-} plants; arrows indicate missing embryos; siliques shown are representative of siliques observed for each genotype (scale bar is 0.8 mm).

Table 3.7. Summary of phenotypic data obtained for *ster1-2*^{+/-} and *ster1-3*^{+/-} mutants.

65 siliques (3 siliques/plant) were analyzed for each genotype as indicated.

n=65	Genotype	Present	Missing	X ²	Observations
AA	Col WT	1260	46	-	NS
Aa	<i>ster1-2</i> ^{+/-}	935	345	2.604	Fits 3:1 ratio
Ab	<i>ster1-3</i> ^{+/-}	969	342	0.826	Fits 3:1 ratio

NS, not significant

Subsequently, a cross between these 2 heterozygous mutants was performed and siliques were analyzed to confirm the phenotype is caused by these alleles and not an unrelated defect/ T-DNA insertion. The expectation was that 25% of the siliques will display a wild-type phenotype, with the rest exhibiting the missing/undeveloped embryo phenotype. Nevertheless, the phenotype did not segregate as expected in the cross between the two heterozygous mutants, as all siliques analyzed appeared healthy. This suggests *STER1* is not the underlying cause of this missing embryo phenotype (Table 3.8).

Table 3.8. Analysis of *ster1-2*^{+/-}/*ster1-3*^{+/-} crosses.

Mutants were crossed to each other and crosses were performed in both directions. Genotypes are described in a simplified manner as follows: AA-wild-type; Aa- *ster1-2*^{+/-}; Ab- *ster1-3*^{+/-}

Aa/Ab	(n=14)	Frequency(%)	Expected(%)	No. of siliques analyzed	Present	Missing	Observation
AA	1	7.14	25	6	111	9	NS
Aa	3	21.42	25	18	325	36	NS
Ab	4	28.57	25	24	524	6	NS
ab	6	42.57	25	36	729	26	NS
Ab/Aa	(n=12)						
AA	2	16.66	25	12	226	12	NS
Aa	4	33.33	25	24	518	10	NS
Ab	3	25	25	18	333	34	NS
ab	3	25	25	18	328	14	NS

NS, not significant

Ster1-1, the line identified as asymptomatic through the candidate gene approach, as well as *ster1-4* and *ster1-5* were asymptomatic following inoculation with *S. epidermidis* (Figure 3.15 B). These mutants displayed no obvious developmental defects. However, 4-week-old *ster1-1* mutants grown in short-day conditions displayed mild growth acceleration compared to wild-type Col-0, which became more distinct when plants reached 5 to 6 weeks old (Figure 3.15 A). F1 hybrids generated by back-crossing *ster1-4* and *ster1-5* to Col-0 were responsive to *S. epidermidis*, confirming a defect in *STER1* accounts for the lack of response in these lines, rather than an unrelated defect/ T-DNA insertion (Figure 3.15C). Although the F1 cross of *ster 1-1* and Col-0 developed patchy chlorosis in response to *S. epidermidis*, the magnitude of the response was considerably diminished compared to wild-type Col-0. As the identity of this cross has been confirmed by PCR, it was assumed the lack of response could be due to a dosage or an interference effect. Firstly, a *ster1-1* X Col-0 F2 population was

A

Col-0 WT *ster1-1*



B

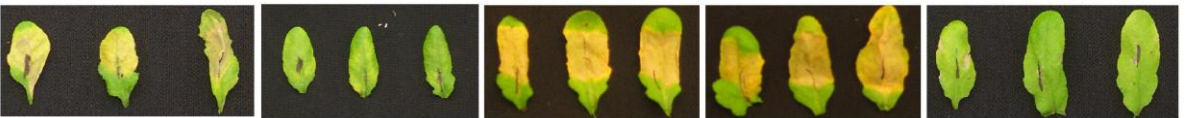
S.epidermidis 18888 $MgCl_2$



Col-0 WT *ster1-1* *ster 1-4* *ster 1-5* Col-0 WT

C

S.epidermidis 18888 $MgCl_2$



Col-0 WT Col-0 x *ster1-1* F1 Col-0 x *ster1-4* F1 Col-0 x *ster1-5* F1 Col-0 WT

100

47 *ster1-1* X Col-0 F2 plants were inoculated with *S. epidermidis* 18888 and their response was assigned as follows: WT, similar to Col-0 phenotype (necrosis); HZ, similar to *ster1-1*^{+/-} phenotype (patchy chlorosis); HM-similar to *ster1-1* phenotype (asymptomatic) (Table 3.9). The same plants were also genotyped and data was compared towards verifying the accuracy of the phenotypic scoring. Comparison of the two sets of data revealed that in most cases the phenotype matched the genotype, with the exception of 5 F2 plants that were scored as asymptomatic although they had a heterozygous genotype. Taken together, this data could suggest the reduced response of the F1 hybrid is the result of a dosage effect.

Table 3.9. Phenotypic analysis of a *ster1-1* X Col-0 F2 population

The response to *S. epidermidis* of 47 F2 plants was assessed and assigned as follows: WT, similar to Col-0 phenotype (necrosis); HZ, similar to *ster1-1*^{+/-} phenotype (patchy chlorosis); HM-similar to *ster1-1* phenotype (asymptomatic). The same plants were genotyped and data was compared.

	WT	HZ	HM
Genotype	14	22	11
Phenotype	14	17	16
Correctly assigned	14/14	17/22	11/11
Incorrectly assigned	0	5/22 HZ assigned as HM	0

WT, wild-type; HZ, heterozygous; HM, homozygous

Additionally, RNA was extracted from *ster1-1* and Col-0 leaves to assess whether any incomplete, truncated RNAs are being produced. Potential incomplete transcripts were detected in *ster1-1* although no full length transcript could be amplified (Figure 3.16). 5' and 3' RACE trials aimed at identifying the origin of these truncated RNA fragments were unsuccessful.

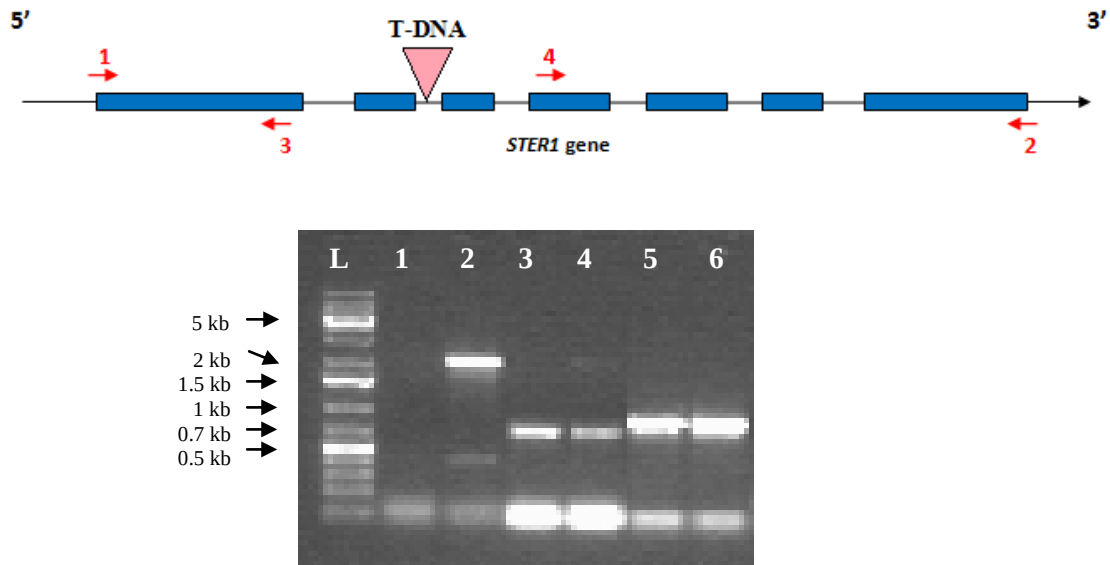


Figure 3.16. Analysis of *STER1* transcripts in *ster1-1*. Agarose gel electrophoresis of PCR reaction performed with primers as follows: 1+2, lanes 1,2; 1+3, lanes 3,4; 4+2, lanes 5,6; *ster1-1* cDNA was used as template for products in lanes 1,3,5; Col-0 cDNA was used as template for products in lanes 2,4,6; The GeneRuler 1kb DNA ladder (Fermentas) (Ladder or L) was used to determine molecular weights in each case. Position of primers along the *STER1* gene are indicated by red arrows; blue boxes represent exons and black lines are introns; the T-DNA insert in the second intron is represented by a pink triangle;

3.2.6. *STER1* complementation analysis

Following mutant analysis, complementation analysis was undertaken. A vector expressing *STER1* cDNA under a CaMV 35S promoter was introduced into accession Cvi-0 and *ster1-1* by *Agrobacterium*-mediated transformation. Three independent complemented Cvi-0 lines were recovered from the selection procedure and confirmed by PCR. Two of these lines were further analyzed, with segregation data for these two lines being consistent with a single T-DNA insertion. Both complemented Cvi-0 lines displayed the same necrotic phenotype as Col-0 subsequent to *S. epidermidis* 18888 inoculation (Figure 3.17).

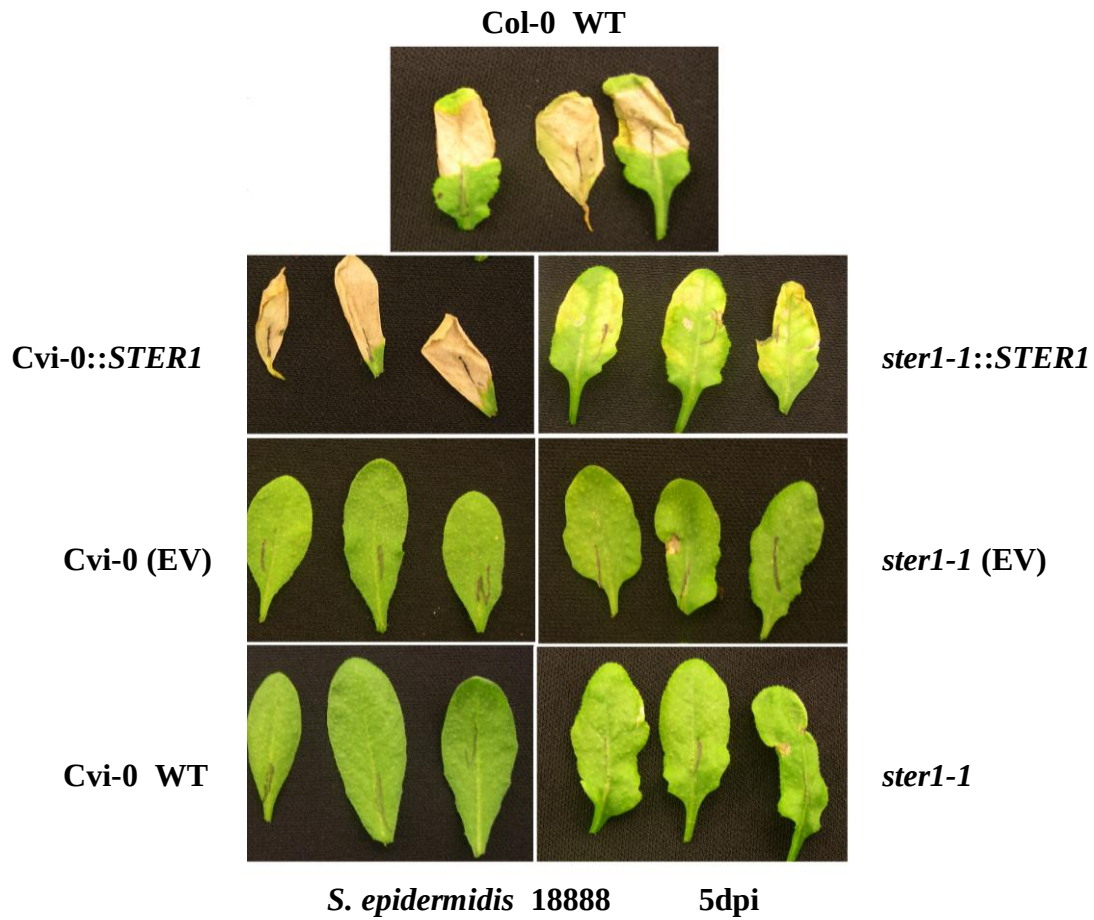


Figure 3.17. Complementation of Cvi-0 and *ster 1-1*. Cvi-0 and *ster1-1* were transformed with a 35S driven *STER1* cDNA construct. Control lines were transformed with the empty pGWB2 vector (EV). All plant lines were inoculated with *S. epidermidis* 18888 (10^8 cfu/mL) and phenotypes were observed 5dpi.

Similarly, three independent complemented *ster1-1* lines were recovered, with two showing a phenotypic reaction to *S. epidermidis* 18888 that was once again less pronounced than in Col-0 (Figure 3.17). This finding suggests the occurrence of an interference effect rather than a dosage effect, but further work is necessary to confirm this assumption. Plants complemented with the empty vector behaved similarly to untransformed plants as expected (Figure 3.17). Taken together, complementation analysis data confirmed *STER1* is the gene underlying the response to *S. epidermidis* 18888.

3.2.7. Discussion

The difference in responsiveness to *S. epidermidis* 18888 between Ler and Cvi-0 allowed for the use of a Cvi X Ler RIL population in an effort to dissect the basis of the necrotic phenotype. QTL analysis revealed the necrotic reaction to *S. epidermidis* 18888 is controlled by one major locus on chromosome IV; this finding was confirmed using NILs (Keurentjes et al, 2006). Although no minor QTLs were identified in this study, the major QTL did not account for the whole of the phenotypic variance suggesting there are additional QTL involved in this plant-bacterium interaction. The omission of these loci may result from the choice of a high significance threshold (2.5) or from masking effects of the major QTL. In addition, it is unlikely an analysis based on phenotypic scoring using just 50 RILs is suitable to detect negligible or very small contributions to a visual reaction. Evaluating a different parameter that allows for more detailed quantification might reveal additional QTLs. For example, Perchevied et al (2006) investigated the genetic basis of partial resistance to Pst DC3000 by exploiting the differential in bacterial growth observed between the Bay-0 and Shahdara accessions. However, bacterial titres are not informative in the case of *S. epidermidis* 18888; another option explored was chlorophyll content evaluation, but reproducibility between and within experiments was not optimal.

Presence of *STER1* is essential for the necrotic response to *S. epidermidis* 18888 to ensue. This gene encodes an RLK (CRK7) which is consistent with the assumption that the phenotypic response following inoculation is a form of MAMP perception. CRK7 is part of a cluster of 20 CRKs displaying DUF26 extracellular domains. A few of these CRKs are highly similar to CRK7 and could potentially function in absence of *STER1* to maintain the necrotic response to *S. epidermidis* 18888. However, tests conducted with T-DNA mutants confirmed there is no redundancy and a defect in this gene is sufficient for abolishing *S. epidermidis* 18888 responsiveness. As a further confirmation of a role of *STER1* in *S. epidermidis* 18888 responses, a recent study has confirmed *STER1*(CRK7) is expressed at low levels in *ein2-1* (Wraczek et al, 2010), a mutant which displayed attenuated responses to *S. epidermidis* 18888 compared to wild-type Col-0 (Figure 3.8).

Although all T-DNA insertion mutants available for *ster1* were tested, only three were suitable for further analysis. Unexpectedly, two T-DNA insertion lines displayed an embryo development phenotype, implying *STER1* is also involved in development. When grown alongside in short day conditions, a *ster 1-1* mutant displays slightly accelerated plant growth

compared to wild-type Col-0. Additionally, a recent study focusing on cocultivation of *Arabidopsis* with several bacterial and fungal isolates found *S. epidermidis* and *B. cepacia* promote plant growth (Vespermann et al, 2007). Since *STER1* is the gene underlying *S. epidermidis* 18888 recognition, it is possible this gene is also involved in promoting the plant growth observed by Vespermann et al. Similarly, previous studies have implicated the *ERECTA* RLK in both development and resistance to fungi (Llorente et al, 2005). Nevertheless, analysis of a *ster1-2^{+/-} /ster1-3^{+/-}* cross suggested a *STER1* defect does not underlie this phenotype. Nevertheless, additional experiments are necessary to confirm this finding. Over-expression of *STER1* in both *ster1-2^{+/-}* and *ster1-3^{+/-}* plants, as well as silique analysis of F1 and F2 plant derived from back-crossing with Col-0 must be performed before arriving to a definite conclusion.

Over-expression of *STER1* was sufficient to restore *S. epidermidis* responsiveness to asymptomatic accession Cvi-0. Nevertheless, a complemented *ster1-1* mutant reacted to *S. epidermidis* 18888 at a reduced level compared to wild-type Col-0. The phenotypic analysis of an *ster1-1*x Col-0 F2 population suggested a possible dosage effect. Nevertheless, over 20% of heterozygous plants were assigned incorrectly. Additionally, *ster1-2^{+/-}* and *ster1-3^{+/-}* lines that express *STER1* at reduced levels compared to the wild-type display a necrotic response to *S. epidermidis* 18888 that is reminiscent of wild-type Col-0. The presence of truncated transcripts was also detected in *ster1-1*. This asymptomatic line appears to produce a transcript corresponding to the first exon of the gene and the kinase domain. The 5' and 3' ends of these transcripts could not be sequenced in spite of several efforts. The presence of these truncated products, coupled with the reduced responsiveness of a *ster1-1* complemented mutant, could argue for the existence of an interference effect seen in the *ster1-1* mutant. In a similar fashion, expression of a truncated tomato polygalactouronase was shown to inhibit the expression of the endogenous gene in transgenic plants (Smith et al, 1990). Nevertheless, the RT-PCR findings in *ster1-1* first need to be confirmed by Northern blotting to eliminate the chance of false positives. Additionally, a Cvi-0 complementing *STER1* transgene should be crossed into *ster1-1* to confirm the existence of an interference effect, or alternatively Col-0 should be transformed with constructs expressing truncated proteins and tested with *S. epidermidis* 18888 to assess whether the wild-type phenotype is diminished. Taken together, current data indicates an interference effect rather than a dosage effect is responsible for the attenuated phenotypes seen in *ster1-1^{+/-}* and the complemented *ster1-1* line.

3.3. STER1 is required for the *Arabidopsis* response to bacterial membrane-derived elicitors

3.3.1. STER1 is a novel receptor-like kinase

3.3.1.1. Introduction

STER1 was isolated as the gene underlying the response to *S. epidermidis* 18888. This gene encodes for a cysteine-rich receptor-like kinase (CRK7) and is part of a cluster of 20 CRKs on chromosome four. CRKs make up the third largest class of RLKs and display extracellular DUF26 (domain of unknown function) domains consisting of a conserved cysteine motif (C-X8-C-X2-C) which are unique to plants. Although this structural pattern has been identified in several membrane kinases and secreted proteins involved in diverse plant processes, its function is still unknown (Strain and Muse, 2005). Members of this CRK cluster have been shown to be induced by SA and pathogens, with CRK5 being implicated in regulating cell death (Czernic et al, 1999; Chen et al, 2004) and overexpression of CRK13 in *Arabidopsis* conferring resistance to *Pst* DC3000 (Acharya et al, 2007). Additionally, the crystal structure of the DUF26-containing antifungal protein Gnk2 from *Ginkgo biloba* was recently reported (Miyakawa et al, 2009).

Aim

Following the isolation of *STER1*, the aim of this study was to further characterize the *STER1* gene. In view of this goal, several public databases were consulted towards identifying possible *STER1* defects in asymptomatic accessions and characteristic protein features, as well as assessing gene expression. Additionally, *STER1* localization was assessed both in a transient *N. benthamiana* system and in stable *Arabidopsis* transformants. Finally, the response of *ster1-1* to well-studied plant pathogens and known MAMPs has been studied in order to study the role of *STER1* in plant defence and MAMP perception.

3.3.1.2. STER1 is a cysteine-rich receptor-like kinase (CRK)

According to recent nomenclature, the CRK subfamily consists of 44 members, with a cluster of 20 CRKs on chromosome four encompassing CRK5 to CRK24. STER1 (or CRK7) is a transmembrane protein kinase encompassing two DUF26 motifs in its extracellular domain (Figure 3.18). The cytoplasmic Ser/Thr kinase domain is predicted to harbour a nucleotide (ATP) binding site and an active site containing an RD catalytic loop. In addition, several potential N-glycosylation sites are present along the first extracellular DUF26 domain.

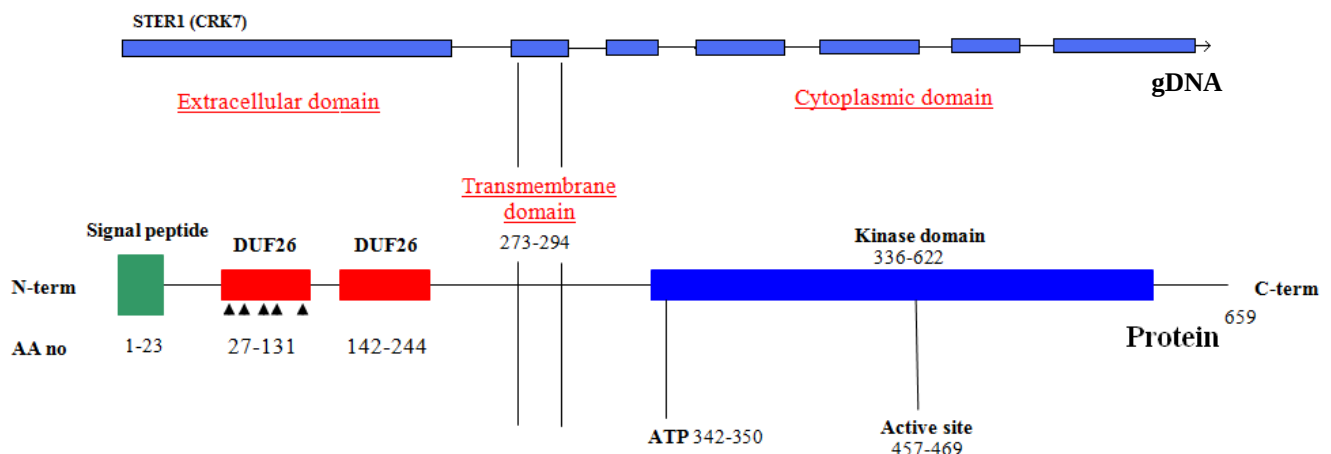


Figure 3.18. Predicted STER1 domain structure. STER1 domain structure was predicted using the information from the UniProtKB database. The *ster1* gene is represented by blue boxes (exons) and blue lines (introns). The first exon encodes for the extracellular domain containing a signal peptide (green block) and 2 DUF26 domains (red blocks). Potential N-glycosylation sites are indicated by black arrowheads. The second exon encodes for the transmembrane domain. The remaining exons encode for the cytoplasmic domain encompassing the serine-threonine kinase domain (blue box). The kinase domain contains a nucleotide (ATP) binding site (342-350) and the kinase active site (457-469). gDNA, genomic DNA, N-term, N-terminus, C-term, C-terminus, AA no., aminoacid number, DUF26, domain of unknown function 26, ATP, adenosine triphosphate.

Phylogenetic analysis of kinase domains of CRKs in the tandem arrays on chromosome IV led to the suggestion that tandem duplications and an internal chromosomal duplication may contribute to the expansion of the CRK gene family (Shiu and Bleecker, 2001b). In the present study, the phylogenetic relationship of the CRKs pertaining to the cluster was inferred using the full protein sequence, as both the extracellular receptor domains and the intracellular kinase domains may contribute to the difference in the

biological activity of these CRKs. All CRKs contained in the cluster display two extracellular DUF26 domains and a kinase domain, with the exception of CRK9, which lacks the transmembrane and the protein kinase domains, thus being considered a pseudogene, and CRK23, which displays an additional DUF26 domain. Phylogenetic analysis of the 19 CRKs revealed STER1 belongs to a subgroup which includes CRK 8, 9, 10 and 15 (Figure 3.19). In spite of this close structural relation, the T-DNA mutant lines corresponding to these genes responded to *S.epidermidis* in a similar fashion to wild-type Col-0 (see Chapter 3.2).

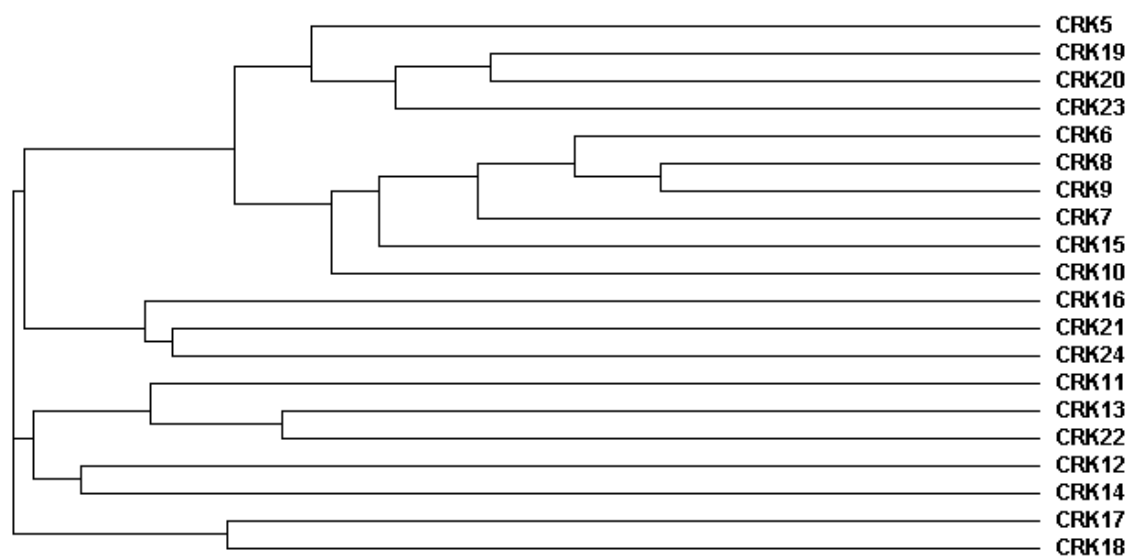


Figure 3.19. Phylogenetic analysis of the 20 CRKs forming a cluster on chromosome four.

Members of the CRK cluster were further analysed to research the extent of sequence homology and degree of conservation. Except for the conserved novel cysteine motifs (C-X8-C-X2-C) in the DUF26 domains, the CRKs share limited sequence homology with each other as far as the extracellular domain goes (Figure 3.20). By contrast, there is a high degree of conservation within the kinase region between the CRKs (35% sequence identity) (Figure 3.21). The kinase active site containing the RD catalytic loop also shows a high degree of conservation. In an effort to establish whether STER1 is an active kinase, its kinase domain was compared to kinase domains of other known plant functional kinases such as FLS2, EFR, BAK1 and MPKs (Figure 3.22). This analysis revealed the active site, as well as adjacent regions, are highly conserved between STER1 and the other kinases. Such a highly conserved architecture would suggest STER1 is a functional kinase.

C-X8-C-X2-C motif in DUF26 domain

CRK21	-----APTCIQRSDFKANGPYDINLRAMLSSLPSPRVKD--NEGFYK-TPFKPGPNIAHGLG	CSRGITTTQDCSDC	TSVSHLLHTCPNQAEADW--SGDSLCLVRYSNHLINGS	108
CRK24	-----QVCLERSGFFTPNSTYDLNRRVLLSSLPINLVIA--IDGFYT-TWIGHEPNRAYGLG	CVPGIDAHSCSDC	ILANARLLQNCNTQTEKIVWR--IDRTVCLVRYSNRFSYSS	107
CRK16	-----QKCG-KTGLFKPNDKYDINRHLSSLSASNVA--RGGFYN-ASIGQGPDRLYASGT	CIQSGSEPELCSAC	DSAFIRVVKCHNTEALDWSSFYEEYPCMIRYSNRSFFGL	108
CRK13	-----QTCIENRKYFTPNGTYSNRRLLSSLPNNTAS--RDGFYI-GSIGEEQDRVYALGN	CIPKSTPSPDCSNC	KGAAGNLQDCVQNTDAYYWA--LDPTLCLVRYSNISFSGS	107
CRK22	-----QTCIENRKYFTPNGTYSNRRLLSSLPNNTAS--QDGFYI-GSIGEEQDRVYALGN	CIPRSTPSPDCFNC	KGAAGNLQDCVQNTDAYYWA--LDPTLCLVRYSNISFSGS	107
CRK11	-----QTCITDKGIFRPNGTYSNRRLLSSLPNNTVD--QDGLYNGSIGQQPNRVYAIGN	CIPGSTSEDCSDC	KKESEFFLKNCPNQTEAYSWE--GEPTLCVRYSNISFSGS	108
CRK17	-----QTCIDNTAGSFKNSTYDNNRRLLLSTFASNVA--QNGYFN-GSFGLTDRVYAMGN	CAPGAEPDVCSCNC	KNTAEGLLQICLNQTDGFSWS--GEETLCLVRYSNKSFSGL	108
CRK18	-----QTCIDNTGTGFIIPNSPYDKNRRLILSTILASNVA--QEGYFI-GSIGIAPDQVFATGN	CAPGSEPDVCSLC	RSTSESLQSCLDQADAFW--GEETLCLVRYANRPFSGL	107
CRK14	-----QECG-KTGFVPQSRYETNRGLLLSSLPASNVA--RGGFYN-SSIGQGPDRVYALGN	CIEGAEPDVCSDC	EYASNLLDTCNQTGLEAF--EKRIILCMVRYSNSSFFGS	106
CRK12	-----LQTLPCINITYFIIPNSTYDNNRRLILSSLPNNTS--HFGFFN-GSIGQAPNRVYAVGN	CLPGTEEEESCIGC	LSASNTLLETCLTEENALNI--ANRTICMIRYSDTSFVGS	109
CRK8	-----QNPFFYLNDHCPNRTTYSSTYSTNLKTLSSSFASRNAS--YSTGFQN-IRAGQTPDRVTGLFI	CRGDSLSEVCSNC	AFSVNESLTRCPNQREAVFY--EECILRYSHKNILST	111
CRK9	-----QDPFFYLNYHCPNRTTYSSTYSTNLKTLSSSFASRNAS--YSTGFQN-VTVGQTPDLVTGLFI	CRGDSLSEVCSNC	AFSVNDEALTRCPNQREAVFY--EECILRYSDKNILST	111
CRK7	-----QDPFRFLAYCPNATTYSSNSTYLTNLKTLSSLSRNAS--YSTGFQN-ATVGQALDRVTGLFI	CRGDSVSEVCRNC	TFAVNNTFSRCPNQREAVFY--EECILRYSHKNILST	111
CRK6	-----QDPFFYLNYHCPNRTTYSSTYSTNLKTLSSLSRNAS--YSTGFQN-ATAGKAPDRVTGLFI	CRGDSVSEVCRNC	AFSVNQTNLNCPKQREAVFY--EQCLIRYSHKNILST	111
CRK15	-----VENVFYIKHICPNTTYSRNPSYLTNLKTLSSLSAPNAS--YSTGFQS-ARAGQAPDRVTGLFI	CRGDSVPAVCRNC	AFSINDTLVQCPSERSVFY--DECMLRYSDQNILST	112
CRK10	-----QDPYTVYHTQNTANYTSTYNNLKTLLASLSRNAS--YSTGFQN-ATVGQAPDRVTGLFI	CRGDSVTEVCRNC	SFAVNDTLTRCPNQREAVFY--DECVLRYSNQNILST	111
CRK23	-----VYVHVCPSWATFPASSTYMTNLITLLSSSPAS--YSTGFQN-ATAGKHPDRVTGLFI	CRGDSVSEVCRNC	SFAVNETSTRCPNREAVFY--DQCTLRYSNRNILST	107
CRK19	-----QNTFFYLHNCSTITTYSSNSTYSTNLKTLSSLSRNAS--YSTGFQT-ATAGKAPDRVTGLFI	CRGDSVSEVCRNC	TFAVNETSTRCPNKEGVFY--EQCLIRYSNRNIVAT	112
CRK20	-----QNPFFYLHNCSTITTYSSNSTYSTNLKTLSSLSRNAS--YSTGFQN-ATAGQAPDMVTGLFI	CRGNSVSEVCRNC	ALSVNESLRCNPREAVFY--EQCMRLYSNRNILST	111
CRK5	QLQDPTVYGVHCTN--RISRNSIYFSLQTLTLLSSNNAY-FSLGSHS-LTKGQNSDMVFLYL	CRGDLSPESCRECT	IFAAKDTRSRCPNKEGVFIQY--DECMLGYSDRNIAMD	111
	. * * : * : . * : . * * : * : . * * : * : .			
CRK21	LDEDIWAEYIEYKYNISFGQINLTFKSTQWALMDRVINKVDGS-----LYANSIQELGSPFRSIAIA	CNKDLTKLNCCKLQHLRIDNRSCCRGIQVGYIARTSCFM	215	
CRK24	LGMEILRSD-----NYTRDF-QANLTDLITWALMIRMIDQASYL-----YYAAGIRKLETSISR-IYGFV	CSRDLSLQNCNTKCLQNVVVEYRSCCRGRQGGIILRPSCFI	208	
CRK16	LEMTFFFKN-----YNATDFQVNLTEFYQKWEALMLGVIADAISPN-----PKFYGAGTGKIG--IQTVYAFV	CSKDLSPWNCSCRLGRNVMDNYKLSCSGKPRGHSFSPSCYM	211	
CRK13	AAFWEIEPQYL-VLNTATIA-SNLTEFKTIWEDLTSRTITAASAARSTPSS--SDNHYRVDVFANLT--KFQNIYALM	CTPDISSEDENCLQGVLEYSQCCGNNTGGVVMRPICF	219	
CRK22	AAFWEIEPQYL-VLNTATIA-SDLTDFKNIWEDLTSRTITAASAARSTPSS--SDNHYRVDVFANLT--KFQNIYALM	CTPDISSEDENCLQGVLEYSQCCGNNTGGVVMRPICF	219	
CRK11	A--DLNPRNW-LTNTGDL-SNLTEFTKIWEGLMGRMISAATAKSTPSS--SDNHYSADSAVLT--PLLNIYALM	CTPDLSSGDCENCLQSAIDYQSCCSQKRGVVMRPICF	217	
CRK17	LG--LEPSND-FFNVEIRKEDQKFDVDFELMFTQIGASSSVRNNSNLSLSGKYAKDVAP--VYGNISVVMQ	CTPDVSSKDMCLQRLSLDFYKKWYNGKRGTIILRPSCFI	222	
CRK18	LV--MDPLGA-IFNTGELN-TNQTVDIEWNLTSSMIAGITSSSGGNN--SSKYYSDIALVP--DFKNISALM	CTPDVSSDCNCLQNVVDYDNCRRGHQGGVMSRPNCF	216	
CRK14	LK--AEPHFY-IHNVDDIT-SNLTEFDQWEEELARMIASSTPSS--KRYKYAADVAALT--AFQIYALM	CTPDLSEDCHCLQGVSVGYETCCNGKQGGIVYRASCVF	211	
CRK12	FE--LEPHRE--FLSHGYKTNETFNTVWSRLTQRMVQEAASSTDATWS--GAKYYTADVAALP--DSQTYLVMQ	CTPDLSPAECNCLQTESVVMYQSCCLGRQGGISIVRLSCAF	218	
CRK8	-VTYEGELLMR-NPNNISSIQNRDQFIDLQVSNMNGAANEAAANS-----SRKFTSTIKTELT--SLQTYLGLV	CTPDLARQDCFCQLTSSINRM--MPLFRIGARQFWPSCNS	214	
CRK9	AITNEGEFILS-NTNTISPNQSQINQFIVLVQSNMNGAANEAAANS-----SRKFTSTIKTELT--ELQTYLGLV	CTPDLTSQDCCLQRLTSSINR--MPLSRIGARQFWPSCNS	214	
CRK7	AITNEGEFILR-NPNHISPIQNIQNFIVLVLSNMNGAIAEAAAN-----PRKFTSTIKTELT--ALQTYLGLV	CTPDLRSQNCMLQTLSSINR--MPLSRIGARQFWPSCNS	214	
CRK6	AITNEGEFILS-NTNTISPNQSQIDGFTSFVSSSTSEAAAGKAANS-----SRKLYTNWTELT--AYQNYLGLL	CTPDLTRADCLQSLQSSING--MALSRIGARLYWPSCNTA	214	
CRK15	-LAYDGAWIRM-NGN-ISIDQNMNRKDFVSSSTMNGAAGKAASS-----PRKFTYVKAATW--ALQTYLGLV	CTPDLTRQDCFCQLTSSINR--MPLYKTGGRTLYSSCNS	213	
CRK10	LITGGVILVN-TRNVTSNQ--DLLSDLVLPNLQAATVALNS-----SKKFGTRKNNFT--ALQSFYGLV	CTPDLTRQDCSRLQVLVINQ--IPTDRIGARINPSCTS	211	
CRK23	SNTNGGIILAN-SQNMITSNEQ--ARFKDLVLTTMNGAIAEAAANS-----SKRFDARSANFT--TLHSLYTLV	CTHDLTRQDCSRLQQLIINQ--LPTEKIGGQFIVPSCSS	207	
CRK19	LMTDGMFMQS-ARNPLSVKQ--DQFRDLVLTLMNLAAVEAARS-----FKKWAVRKIDLN--ASQSLYGMV	CTPDLREQDCDLQKIGINQ--VTYDKIGGRTLLPSCAS	212	
CRK20	LMTDGMFMQ--ARNPLSVKQ--DRFRDLVLTLMNLAAVEAARS-----IKRFAVKTFDLN--ALQSLYGMV	CTPDLTEQDCDLQQLIINQ--VTYDKIGGRTFLPSCTS	211	
CRK5	TVTTTTITWN-TQKVTDQS--DRFNDAVLSLMMKSAEEAANST-----SKKFAVKKSDFS--SSQSLYASV	CTPDLTSEDVCMQLQSSIKE--LYFNKVGGRFLVPSCNS	212	
	:			
CRK21	RWDLQPLGLFINGMLPTPPSELONGHS-----NTTKK-----	DGKNISTGS--	257	
CRK24	RWELYPFLGLFDN-IRPR-----	DGKSIST--	234	
CRK16	RWDLQYFYGFIEYRASPTLPRE--KGRI-----	SELSD--	251	
CRK13	RWQLFTFSKAFHNITLATTPPLSPPLQRPVVASQPPSADNDRKKRDN-----SSGKISMK--	275		
CRK22	RWQLFTFSKAFHNITLATPP--KPPMN--VPRPPSVGHGANITDN-----DSRGVSAGIV--	270		
CRK11	RWDLTYTSNAPDNLTVASPPP--EPPVT--VPQP--AGDQDNPTNN-----DSKGISAGVVV--	268		
CRK17	RWELYTFGAFDSINARHPP--PPRPLSPPLKTPSVTNITTKK-----NDSIRISGGT--	276		
CRK18	RWEYVYFSGAIDQNLKPS--PPS-VTSPSPIA-----NITK-----	NDSIRISGGK--	260	
CRK14	RWELFPFSEAFSRISLAPPQ--SPAFPTLPAVIN--TATKK-----	GSITIS--	255	
CRK12	RAELYPFGGAFVTMARPLSPPPSLIKKGEFFAKFMSNSQEPVKMIQNLYVVLIFY-----FLDSGKFSTET--	287		
CRK8	RYELYAFYNETAIGT-----PSPPLFPGSTPPLTSPS-----IPGKSGNSTV--	257		
CRK9	RYELYSFYNETTIGT-----PSP-----PPPSPS-----RAN--	242		
CRK7	RYELYDFYNETAIGT-----PPPP--LPPLASPS-----LSDKSGNSN--	250		
CRK6	RYELYPFYNESAIEPTPLPPPPPPPPRESLVSTPPISSSS-----LPKSGNSTV--	265		
CRK15	RYELFAFYNETTVRT-----QQAPPPLPSPSTPLVTSFS-----LPKGSWNNS--	256		
CRK10	RYEYIAYFYESAVP-----PPPP--PPSISTPP-----VSAP--P-----RSGKDGNSK--	251		
CRK23	RFELCLFYNSAVITPQPQNSAPPFP-----PTSIPTSPRGLNSRFLITCLSAVSFEKFDKLLKPGFICILIKIVSENTGKGNSS--	290		
CRK19	RYDNYAFYNESNVG-----TPQDSSPR-----	PGKGNSS--	242	
CRK20	RYDNYEFYNEFNVG-----TPQDSSPR-----	PGKGNSS--	241	
CRK5	RYEYVYFYKETIETG-----VLPPPVSAAPPLVSTPS-----FPPGKGNST--	255		
	* : :			

Figure 3.20. Alignment of the extracellular domains of the 20 members of the CRK cluster. Sequences were taken from UniProtKB and aligned with ClustalW2. C-X8-C-X2-C conserved motifs in DUF26 domains are enclosed in red boxes,


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FLS2      FNSANIIGSSSLSTVYKQLEDG-TVIIVKVLNLKEFSAESDKWIFYTEAKTLSQLKHRNL 59
EFR       FSSTNLIGSGNFGNVFKGLGPNKLVAVKVLNLLKHGATKS--FMAECETFKGIRHRNL 58
BAK1      FSNKNILGRGGFGKVYKGRADG-TLVAVKRL-KEERTQGELQFQTEVEMISMAVHRNL 58
BRI1      FHNDSLIGSGGFGDVYKAILKDG-SAVAIAKKL-IHVSQGQ-DREFMAEMETIGKIKHRNL 57
CRK7      FSENNKIGRGGFGDVYKGTFSNG-TEVAVKRL--SKTSEQGDTEFKNEVVVVANLRHKNL 57
MPK3      RPPIIPIGRGAYGIVCSVLDTETNELVAMKKIANAFDNHMDAKRTLREIKLLRHLDHENI 60
MPK6      KPPIMPIGKAYGIVCSAMNSETNESVAIKKIANAFDNKIDAKRTLREIKLLRHMDHENI 60
          :* . . * .          :*: :          * . . *:
          :

FLS2      VKILG----FAWESGKTKALVLPFMENGNLEDTIHGSAAPIG-----SLLEKIDLCVH 108
EFR       VKLIIVCSSLDSEGNDFRALVYEFMPKGSMDMWLQLEDLERVNDHSRSLTPAEKLNIAID 118
BAK1      LRLRG-----FCMTPTERLLVYPYMANGSVASCLRERPESQP-----PLDWPKRQRIALG 108
BRI1      VPLL-----YCKVGDERLLVYEFMKYGSLEDVLDHPKAGV-----KLNWSTRRKIAIG 107
CRK7      VRILG-----FSIEREERILVYEVENKSLDNFLFDPAKKGQ-----LYWTQRYHIIG 106
MPK3      IAIRD-----VVPPLRRQFSDVYISTELMDTDLHQIIRSNO-----SLSEEHCQYFLYQ 110
MPK6      VAIRD-----IIPPLRNAFNDVYIAYELMDTDLHQIIRSNO-----ALSEEHCQYFLYQ 110
          : :          . : : : : :          :

          Kinase active site

FLS2      IASGIDYLHSGYGFPVHCDLKPANILLSDRVAHVSDFGTARILG--FREDGSTTASTS 166
EFR       VASALEYLHVHCHDFVAHCDIKPSNILLDDLTAVHSDFGLAQLLYKYDRESFLNQFSSA 178
BAK1      SARGLAYLHDHCDPKIHRDVKAANILLDEEFAVVGDFGLAKLMD-----YKDVHTVT- 162
BRI1      SARGLAFLHHCSPHIIHRDMKSSNVLLDENLEARVSDFGMARLMS-----AMDTHLSVS 162
CRK7      IARGILYLHQDSRLTIHRDLKASNILLADMPNPKIADFGMARIFG-----MDQTQQNTS 161
MPK3      LLRGLKYIHSAN---IHRDLKPSNLLNANCDLKICDFGLARPTS-----ENDFMTE 160
MPK6      ILRGLKYIHSAN---VHRDLKPSNLLNANCDLKICDFGLARVTS-----ESDFMTE 160
          . : :*: : * :*: :*: : : : : : : : : : : : :

FLS2      AFEGTIGYLAPEFAYMRKVTTKADVFSFGIIMMELMTKQRPTSLNDEDSQD-MTLRQLVE 225
EFR       GVRGTIGYAAPEYGMGGQPSIQGDVYSFGILLLEMFSGKKPT--DESFAGD-YNLHSYTK 235
BAK1      AVRGTIGHIAPEYLSTGKSSEKTDVFGYGVMLLELITGQRAFDLARLANDDDVMLLDWVK 222
BRI1      TLAGTPGYVPPEYYQSFRCSTKGDVYSYGVVLELLTGKRPTDSPDFGDNN---LVGWVK 219
CRK7      RIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSFIETDDAQ--DLVTHAW 219
MPK3      YVVTWRWYRAPELLNSSDYTAADVWSVGCIFMELMN-RKPLFPKGDHVHQMRLLTLLG 219
MPK6      YVVTWRWYRAPELLNSSDYTAADVWSVGCIFMELMD-RKPLFPGRDHVHQLRLLMELIG 219
          . . . : * : * : : : : : : : : : : :

FLS2      KSIGNGRKGMVRVLDMELGDSIVSLKQEEAIEDFLKLCFCTSSRPEDRPMNEILTHLM 285
EFR       SILSG-----CTSSGGSNAIDGLRLVLQVGIKCSEYPRDRMRMTDEAVRELI 283
BAK1      GLLKEKK----LEALVDVDLQGNKYDEEVEQL--IQVALLCTQSSPMERPKMSEVVRMLE 276
BRI1      QHAKLR-----ISDVFDPELMKEDPALEIELLQHLKVAVACLDRAWRRPTMVQVMAMFK 274
CRK7      RLWRNG-----TALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAMSTISVMLT 273
MPK3      TPTESD-----LGFTHNEDAKRYIRQLPNFPRQPLAKLFSHVNPMAIDLVDRLMTFDP 272
MPK6      TPSEEE-----LEFLN-ENAKRYIRQLPPYPRQSITDKFPTVHPLAIDLIEKMLTFDP 271
          . : : : : :

FLS2      K----- 286
EFR       SIRSKEF----- 290
BAK1      GDGLAERWEEWQ--- 288
BRI1      EI----- 276
CRK7      SNTMALPAPQQPGF- 287
MPK3      NRRITVEQALNHQYL 287
MPK6      RRRITVLDALAHPYL 286

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Figure 3.22. Alignment of the protein kinase domains of STER1 and known plant functional kinases. Sequences of known plant functional Ser/Thr kinases (RLKs FLS2, EFR, BAK1, BRI1 and MPK3/6 intracellular kinases) were taken from UniProtKB and aligned with ClustalW2. The kinase active site is encased with a blue box.

In an effort to elucidate possible genomic differences in the *STER1* gene that result in significant changes at the *STER1* protein level, the recent sequencing data available from the 1001genomes.org database was used to detect sequence polymorphism between available accessions. Ecotypes falling into different phenotypic score ranges were chosen as follows: Cvi-0 and Tsu-1 (score of 1); Bur-0 (score of 3); Ler-1 (score of 4); Col-0, Est-1 and Bay-0 (score of 5). According to the data provided, several SNPs resulted in an amino acid change in the resulting protein sequence (Figure 3.23). The *STER1* protein variant corresponding to Tsu-1 displayed the most changes compared to the Col-0 reference sequence (22 substitutions). Fourteen of these substitutions were like-for-like amino acid changes, such as A285V (both amino acids with hydrophobic side chains) or E314D (both amino acids with negatively charged side chains). These type of changes are less likely to impact protein function. By contrast, 5 Tsu-1 *STER1* substitutions involve a change of charge, with an amino acid with a charged side chain being replaced by a an amino acid with a hydrophobic side chain (eg. S107R, N209D). These substitutions could adversely affect protein folding and/or function. Similarly, proline and glycine replacements (eg. P266S, G440V) can impact protein structure. Nevertheless, Tsu-1 also exhibits a premature STOP codon (position 650) leading to a predicted truncation of 9 aminoacids. It is likely that this change accounts for the lack of *STER1* function in Tsu-1. Est-1 and Bay-0 also display amino acid substitutions, but these are unlikely to be significant as these accessions react to *S. epidermidis* at the same extent as Col-0 (phenotypic score of 5) (eg. L45F, L144M, V224S and I226T for Est-1; L461H for Bay-0). According to the current sequencing data, the Cvi-0 *STER1* genomic sequence, as well as the 5' and 3' adjacent intergenic regions are the same as that of Col-0. Nevertheless, in spite of several efforts, the Cvi-0 *STER1* gene could not be amplified by PCR to confirm the sequencing data. Additionally, RT-PCR confirmed no full *STER1* transcript was present in Cvi-0 by comparison to Col-0 (Figure 3.24). The presence of possible truncated transcripts was also investigated using additional pairs of primers and a partial transcript corresponding to the first exon was detected in Cvi-0. The RT-PCR analysis suggests that either the sequencing results are incorrect or that additional elements required for *STER1* expression are lacking in Cvi-0.

Cvi-0	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTYL	TNLKTLSSLS	SSRN
Ler	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTYL	TNLKTLSSLS	SSRN
Col-0	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTYL	TNLKTLSSLS	SSRN
Bay-0	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTYL	TNLKTLSSLS	SSRN
Bur-0	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTY	TNLKTLSSLS	SSRN
Est-1	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPNATTYSSNSTY	TNLKTLSSLS	SSRN
Tsu-1	1	MSSLFPFIFLFLFSFLT	SFRASAQDPRLAYYCPN	TTYSSNSTY	TNLTLSSLS
Cvi-0	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Ler	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Col-0	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Bay-0	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Bur-0	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Est-1	61	ASYSTGFQ	NATVGQALDRVTGLF	LCRGDVSPEVCRNCVT	FAVNNTFSRCPNQREAVFY
Tsu-1	61	ASYSTGFQ	NATVGQALDR	ATGLF	LCRGDVSPEVCRNCVT
Cvi-0	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Ler	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Col-0	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Bay-0	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Bur-0	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Est-1	121	ECILRYSHKNIL	STAITNEGEFI	MRNP	NHISPIQNQINQ
Tsu-1	121	ECILRYSHKNIL	STAITNEGEFILRNP	NHISPIQNQINQ	TNLVLSNMNQIAIEAADNPR
Cvi-0	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPFSRIGARQFWPSCNSRYE
Ler	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPFSRIGARQFWPSCNSRYE
Col-0	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPFSRIGARQFWPSCNSRYE
Bay-0	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPFSRIGARQFWPSCNSRYE
Bur-0	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPFSRIGARQFWPSCNSRYE
Est-1	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPV
Tsu-1	181	KFSTIKTEL	TALQTFYGLVQCTP	DLSRQNCMCLTSS	INRMPPSRIGARQFWPSCNSRYE
Cvi-0	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Ler	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Col-0	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Bay-0	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Bur-0	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Est-1	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVAVLIFIAGYCFFAKR
Tsu-1	241	LYDFYNETA	IGTPPPPLPLASPS	LDKSGNSNVVVAVVVP	IIVVVLIFIAGYCFFAKR
Cvi-0	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Ler	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Col-0	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Bay-0	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Bur-0	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Est-1	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Tsu-1	301	AKKTYGTT	PALDEDDKTTIES	LQLDYRAIQ	AATNDFSENNKIGRGGFGDVYKGTFSNGTE
Cvi-0	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Ler	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Col-0	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Bay-0	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Bur-0	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Est-1	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Tsu-1	361	VAVKRLSKT	SEQGDETFKNEVVVVAN	LRHKNLVRILGFS	IEREERILVY EYVENKSLDNF
Cvi-0	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Ler	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Col-0	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Bay-0	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Bur-0	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Est-1	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP
Tsu-1	421	LFDPAKKG	QLYWTQRYHI	IIGGIARGILYLHQ	SRLTIIHRDLKASNILLDADMNP

Cvi-0	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Ler	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Col-0	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Bay-0	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Bur-0	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Est-1	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Tsu-1	481	GMARIFGMDQTQQNTSRIVGTYGYMSPEYAMRGQFSMKSDVYSFGVLVLEIISGRKNNSF	
Cvi-0	541	IETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Ler	541	IETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Col-0	541	IETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Bay-0	541	NETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Bur-0	541	NETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Est-1	541	NETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Tsu-1	541	NETDDAQDLVTHAWRLWRNGTALDLVDPFIADSCRKSEVVRCTHIGLLCVQEDPVKRPAM	
Cvi-0	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDKSMSDLDP	659
Ler	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDKSMSDLDP	659
Col-0	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDKSMSDLDP	659
Bay-0	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDKSMSDLDP	659
Bur-0	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDKSMSDLDP	659
Est-1	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDDSSVSDLDP	659
Tsu-1	601	STISVMLTSNTMALPAPQQPGFFVRSRPGTNRLDSDQSTTNKSVTVSIDD-----	650

Figure 3.23. Alignment of *Arabidopsis* accession-specific STER1 proteins. STER1 sequences were derived from the re-sequencing data available at 1001genomes.org and aligned with ClustalW2.

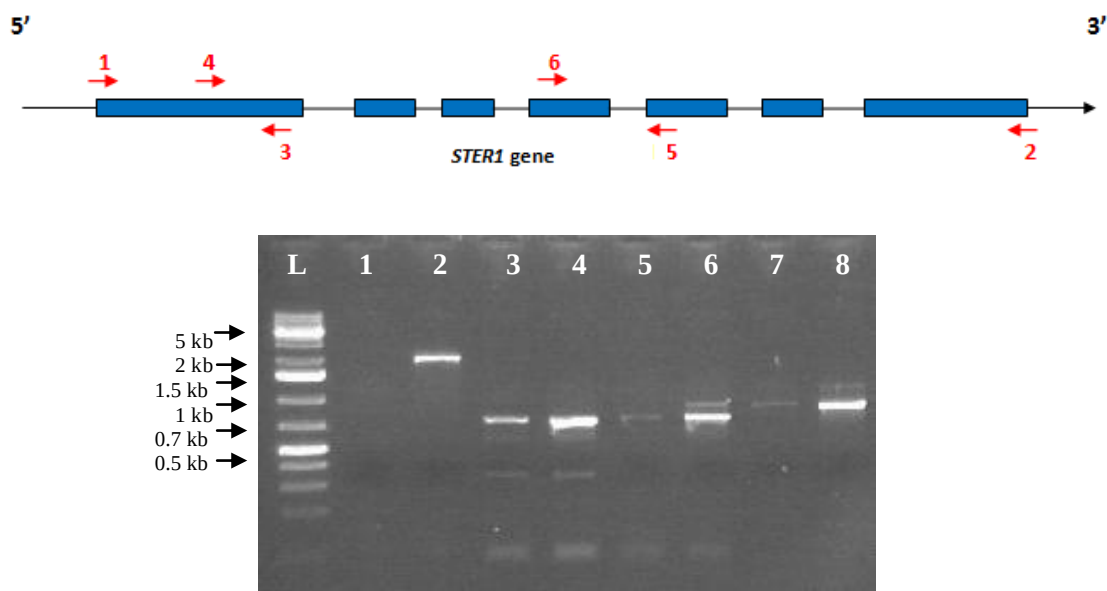


Figure 3.24. Analysis of *STER1* defect in *Cvi-0*. Position of primers along the *STER1* gene are indicated by red arrows; blue boxes represent exons and black lines are introns; Agarose gel electrophoresis of PCR reaction performed with primers as follows: 1+2, lanes 1,2; 1+3, lanes 3,4; 4+5, lanes 5,6; 6+2, lanes 7,8; *Cvi* cDNA was used as template for products in lanes 1,3,5,7; *Col* cDNA was used as template for products in lanes 2,4,6,8; The GeneRuler 1kb DNA ladder (Fermentas) (Ladder or L) was used to determine molecular weights.

3.3.1.3. Pattern of *STER1* expression

The *Arabidopsis* microarray database Genevestigator V3 (Hruz et al, 2008) was accessed to investigate *STER1* gene expression. *STER1* is highly expressed in rosette leaves, and the hypocotyls, with the senescent leaves displaying the highest expression (Figure 3.25A). This data was verified by RT-PCR, which revealed *STER1* mRNA was present in young rosette leaves and senescent leaves only; no *STER1* transcript was detected in shoot, flower or seedlings (Figure 3.25B).

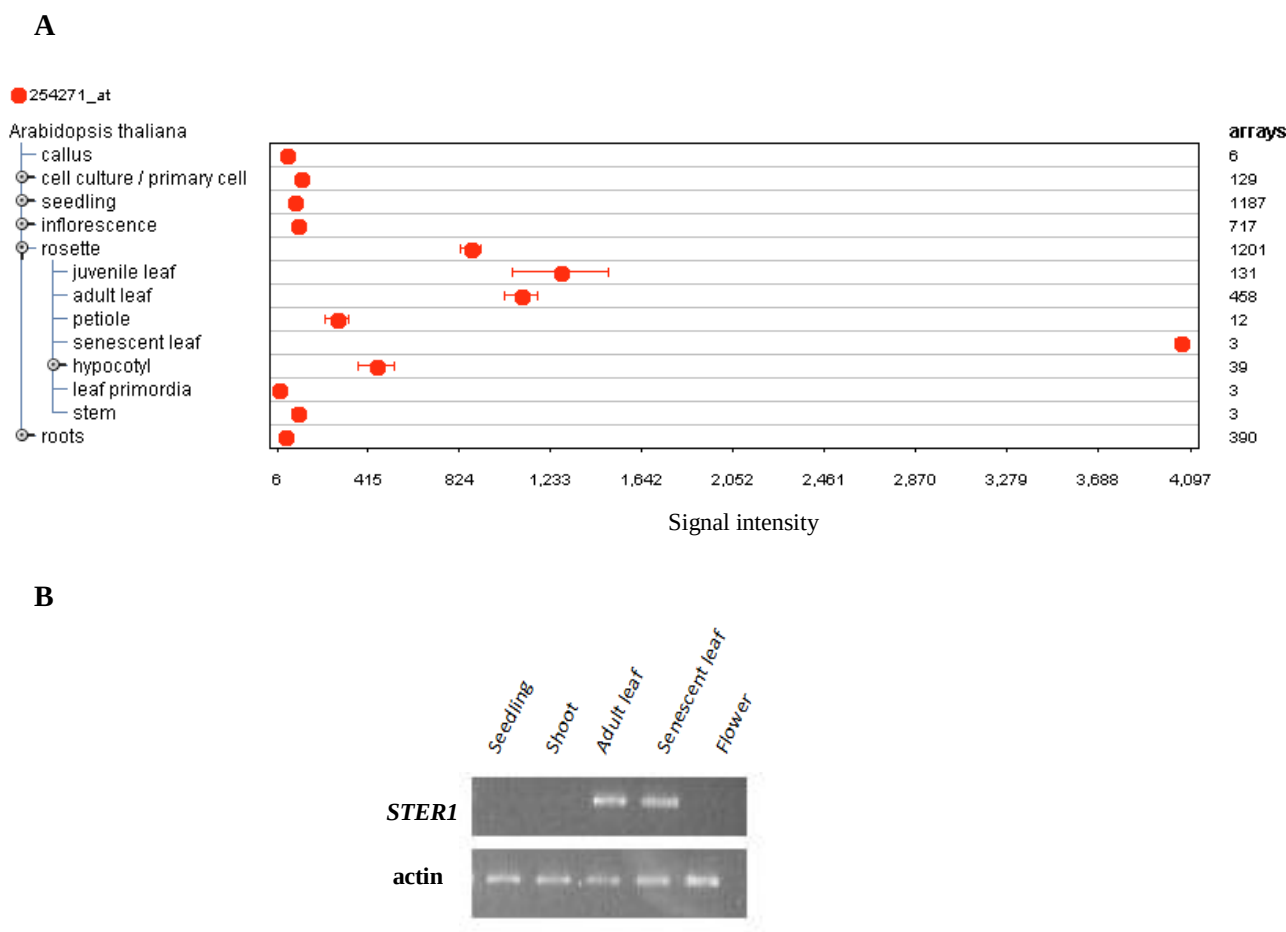


Figure 3.25. Tissue specific gene expression of *STER1*. *STER1* tissue specific gene expression according to (A) Genevestigator and (B) RT-PCR

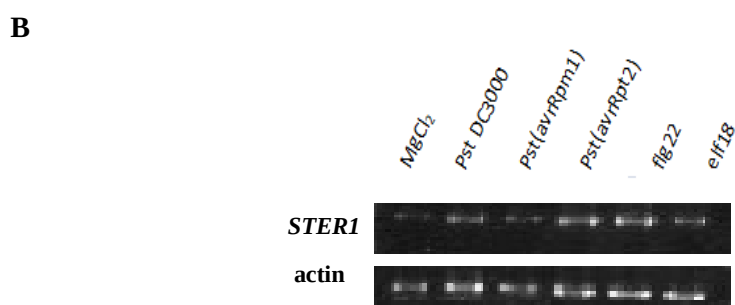
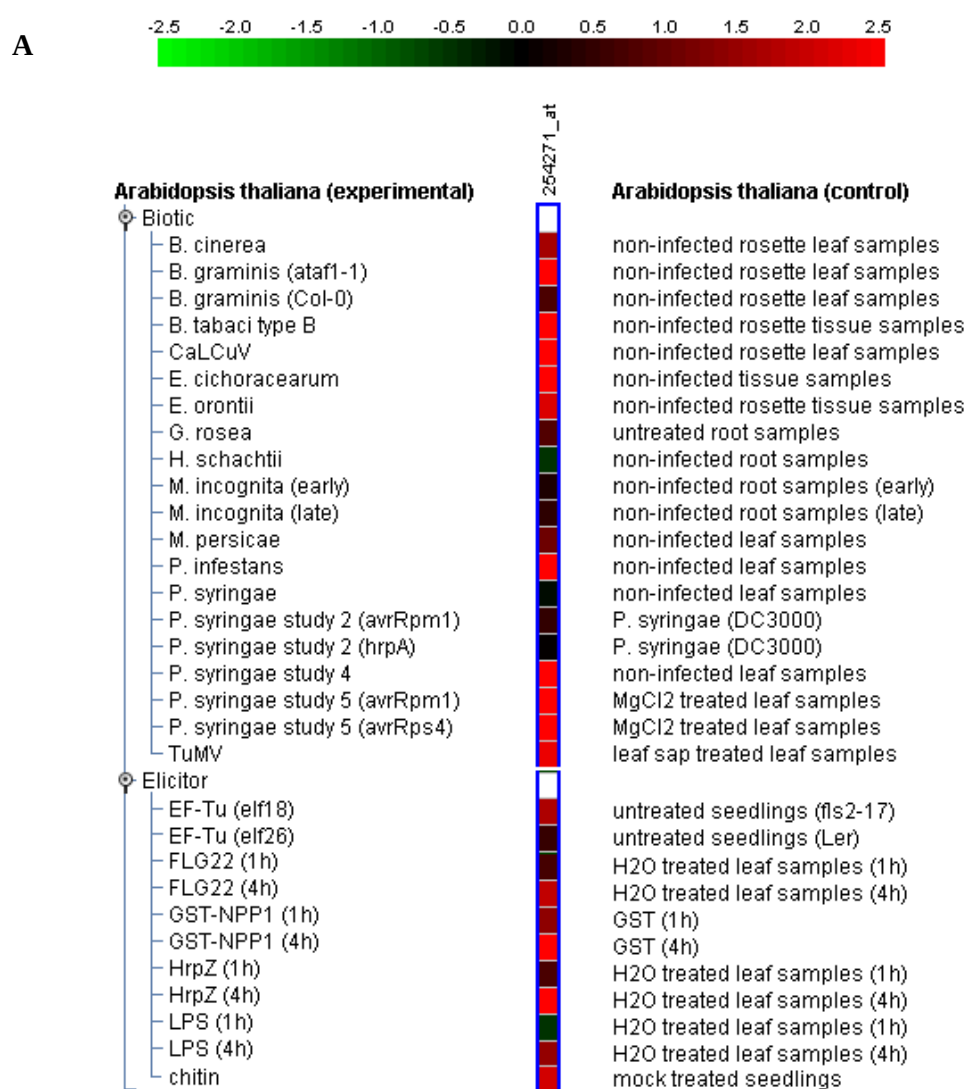


Figure 3.26. *STER1* expression pattern in response to different stimuli. Expression patterns according to (A) Genevestigator and (B) RT-PCR are presented; *STER1* is mainly up-regulated in response to biotic stimuli and known elicitors; a threshold of ± 1.5 was considered to denote a significant change in the expression pattern. For RT-PCR, Col-0 was inoculated with bacterial suspensions (10^8 cfu/mL) and active peptide solutions (1 μ M) and samples were collected 4hpi.

According to the microarray data, *STER1* is up-regulated in response to biotic stimuli, such as bacterial, fungal and viral pathogens, and known MAMPs, such as flagellin, chitin and LPS (Figure 3.26A). RT-PCR analysis corroborated some of this data, showing *STER1* is significantly up-regulated within 4 hours of treatment with bacterial pathogens *Pst* DC3000 and *Pst* DC3000 (avrRpt2) and the active peptide flg22 (Figure 3.26B). Additionally, an increase was also recorded following *Pst* (avrRpm1) and elf18 treatment, but at lower levels than those seen for the other stimuli.

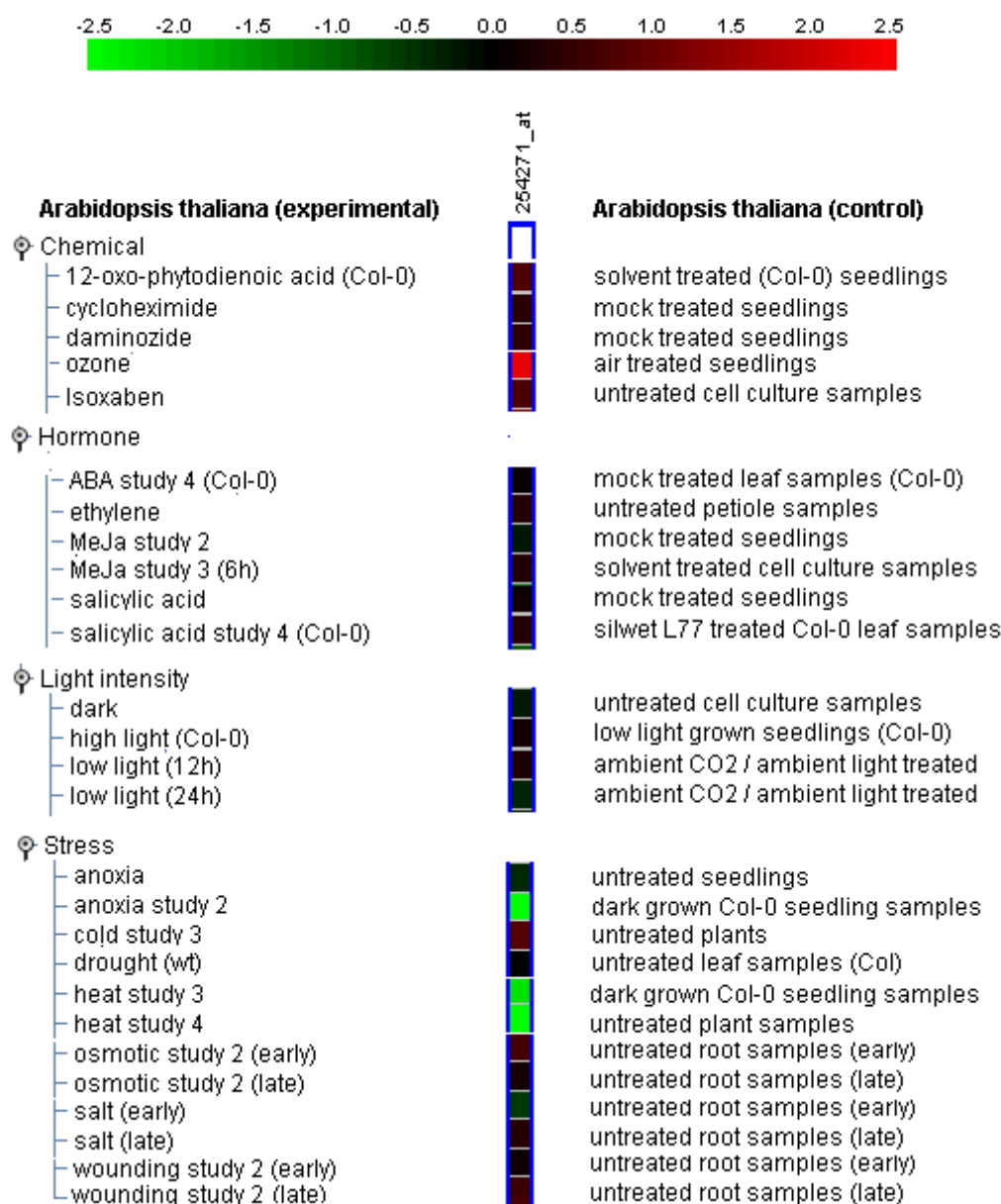


Figure 3.27. *STER1* expression pattern in response to abiotic stimuli obtained from Genevestigator. A threshold of ± 1.5 was considered to denote a significant change in the expression pattern.

According to Genevestigator, *STER1* expression remains mostly unchanged in response to abiotic treatments and stresses. However, there are some exceptions, with stresses such as apoxia and heat leading to a down-regulation of *STER1* expression, while exposure to ozone results in up-regulation of this gene (Figure 3.27).

Finally, time-dependent *STER1* expression in response to *S. epidermidis* 18888 was also assessed by RT-PCR. This analysis revealed *STER1* is significantly up-regulated at 24hpi (23-fold induction) (Figure 3.28). No significant change in *STER1* expression was recorded at earlier time-points.

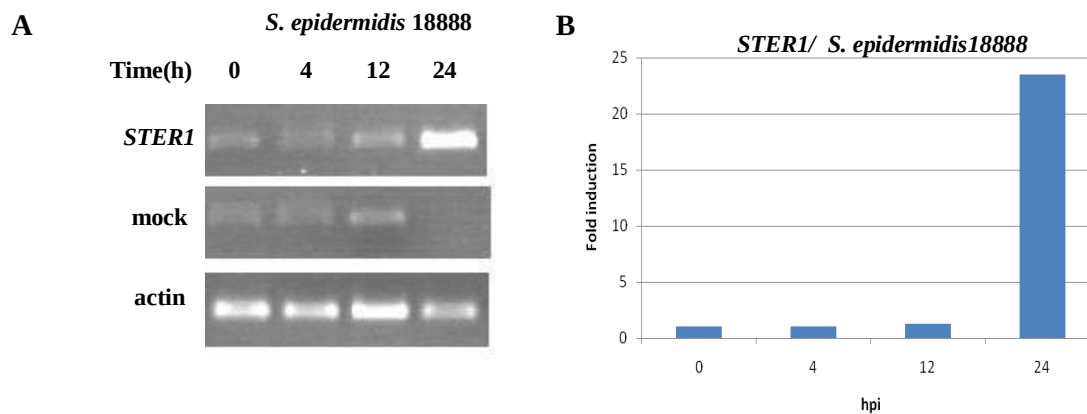


Figure 3.28. *STER1* time-dependent expression in response to *S. epidermidis*. (A) RT-PCR analysis of *STER1* expression following inoculation with *S. epidermidis* (10^8 cfu/mL). 10mM $MgCl_2$ was used as a mock treatment. (B) Quantification of RT-PCR data. Fold-induction values were obtained by comparison to $MgCl_2$ -treated tissue following normalization to actin values; this experiment was repeated twice with similar results;

3.3.1.4. Subcellular localization of *STER1*

In order to assess *STER1* subcellular localization in leaf tissue, a 35S driven construct encoding *STER1*-GFP carboxy-terminal fusion protein was generated.

Subsequently, two strategies were employed consisting of transient expression in *N. benthamiana* leaves and Agrobacterium-based transformation of Arabidopsis Cvi-0 and Col-0 ecotypes.

Confocal microscopy revealed that the *STER1*-GFP fusion protein localized to the plasma membrane in *N. benthamiana* cells (Figure 3.29 A).

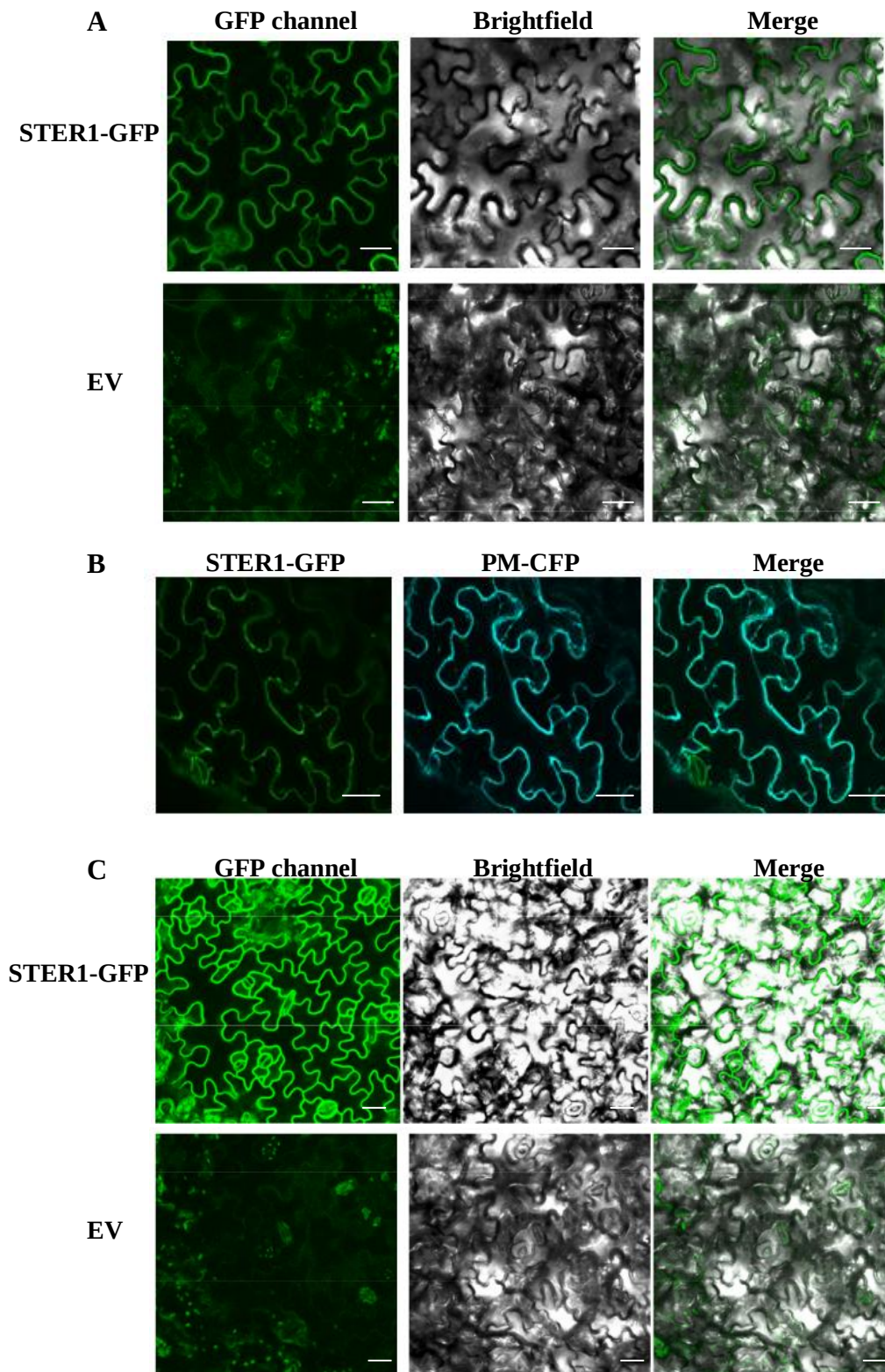


Figure 3.29. Subcellular localization of STER1. (A) Cellular localization using transient expression in *N. benthamiana* leaves (scale bar is 20 μm) (B) Confocal imaging of *N. benthamiana* leaves co-infiltrated with STER1-GFP and a plasma membrane marker fused to CFP (scale bar is 16 μm). (C) Visualization of STER1-GFP localization in *Arabidopsis* Col-0::STER1-GFP line. (scale bar is 40 μm). In all cases, the STER1-GFP fusion protein localized to the plasma membrane. GFP, green fluorescent protein, EV, empty vector, PM, plasma membrane marker, CFP, cyan fluorescent protein

STER1-GFP localization was compared with a plasma membrane marker fused to CFP (Figure 3.29 B), thus confirming STER1 membrane localization.

STER1 localization was also imaged in the leaves of three independent *Arabidopsis* transformant lines, with all lines displaying similar levels of GFP fluorescence. In this case, expression occurred in the plasma membrane of epidermal cells as well as in the guard cells lining the stomata, a possible entry site for invading bacteria (Figure 3.29 C). No specific subcellular localization was visible for the empty vector control (GFP only) in either plant systems.

Plasma membrane localization is consistent with STER1 being a surface receptor that recognizes an extracellular ligand, possibly released by or pertaining to the bacteria in the apoplast.

3.3.1.5. STER1 involvement in plant defence

To assess whether STER1 plays a role in disease resistance, *ster 1-1* mutants were challenged with different well-studied bacterial and fungal plant pathogens.

Phenotypic reactions seen after exposure to *Pst* DC3000 and *Pst* DC3000 (*avrRpm1*) suggest there is no apparent effect on basal resistance or hypersensitive response in the *ster1-1* mutant, respectively (Figure 3.30 A,B). In addition, no difference in the bacterial growth patterns was observed between the wild-type and the mutant plants (Figure 3.28C). Similar symptoms were observed for bacterial inoculation of *Pst* DC3000 regardless of inoculation method used (syringe infiltration or spraying) or inoculum concentration (Figure 3.30A). Trypan-blue staining and an electrolyte leakage assay were performed in order to examine in a quantitative way HR-related cell death induced after *Pst* (*avrRpm1*) inoculation. Since *Pst* (*avrRpm1*) triggers a very rapid HR, the response to a slower HR inducer, *Pst* (*avrRpt2*), was also tested. These assays revealed no significant differences between the *ster 1-1* mutant and the wild-type (Figure 3.30 D, E).

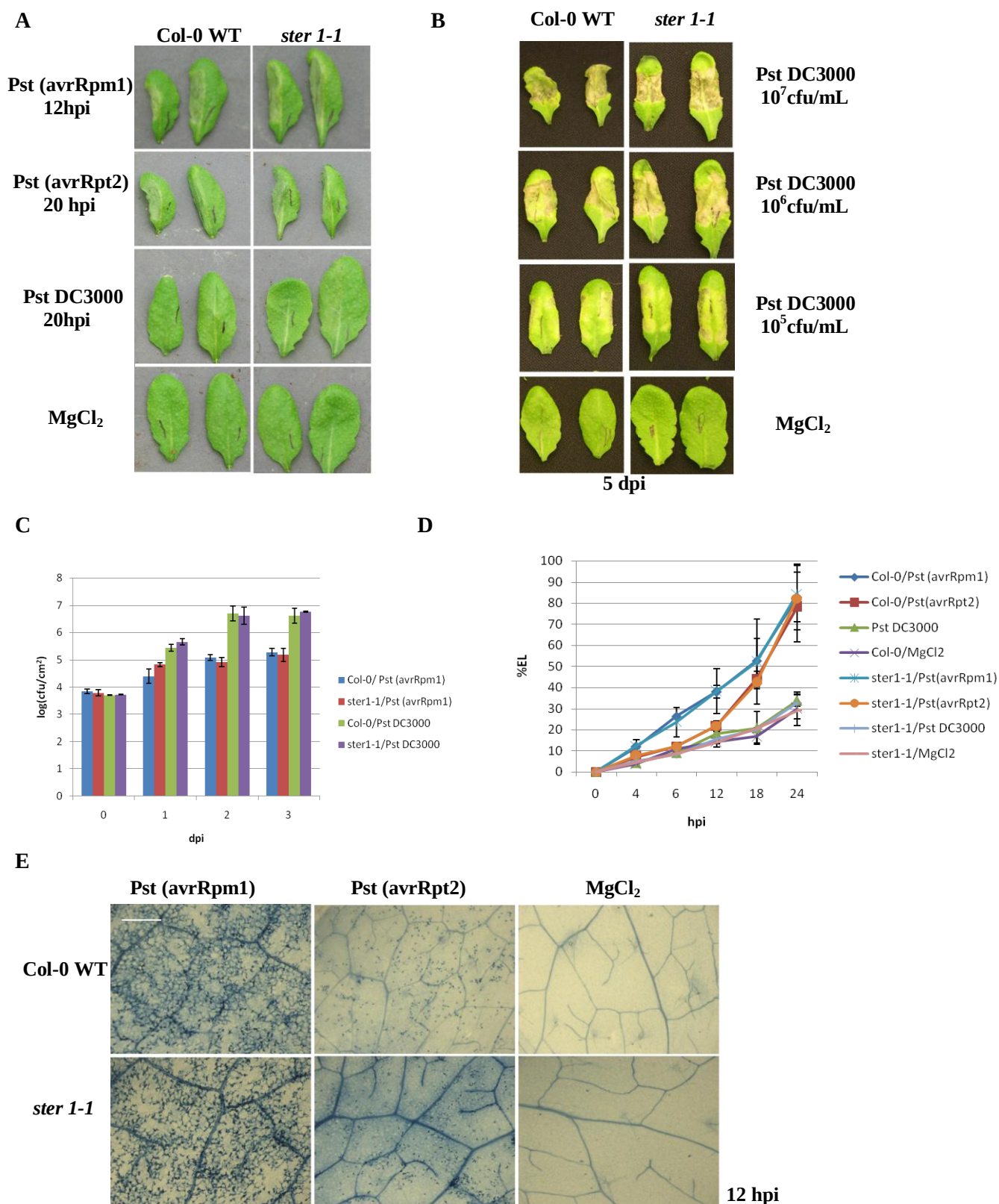


Figure 3.30. Phenotypic response to plant pathogens in the *ster1-1* mutant. (A) Col-0 and *ster1-1* phenotypic reactions to *Pst* (*avrRpm1*), *Pst* (*avrRpt2*) and *Pst* DC3000 (10⁸ cfu/mL) at indicated time-points; only the left half of the leaf was infiltrated; (B) Col-0 and *ster1-1* phenotypic reactions to indicated concentrations of *Pst* DC3000 5dpi; (C) Growth curve analysis of *Pst* (*avrRpm1*) and *Pst* DC3000 following Col-0 and *ster 1-1* infiltration. (D) Electrolyte leakage in Col-0 and *ster1-1* plants infiltrated with bacterial suspensions (10⁸ cfu/mL) as indicated; (E) Trypan-blue staining of leaves from Col-0 and *ster1-1* plants 12h after infiltration with bacterial suspensions (10⁸ cfu/mL) as indicated; 10mM MgCl₂ functioned as mock in all experiments except (C).

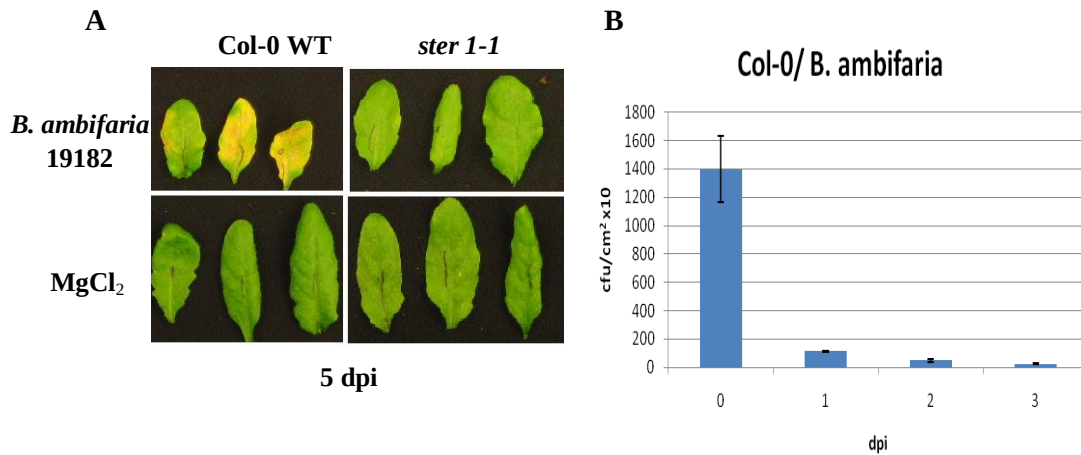


Figure 3.31. Infection of *ster 1-1* with *B. ambifaria* 19182. (A) Phenotypic reaction to *B. ambifaria* 19182 (10^8 cfu/mL); 10mM MgCl₂ functioned as mock (B) *B. ambifaria* 19182 growth curve in Col-0.



Figure 3.32 Infection of *ster 1-1* phenotypic with *B. cinerea*. Phenotypic response at 5dpi following *B. cinerea* (2×10^5 spores/mL in PDB) drop inoculation of the abaxial side of a rosette leaf (right); lesions remained largely contained within the droplet area on the adaxial side (middle); leaves were mock inoculated with PDB media.

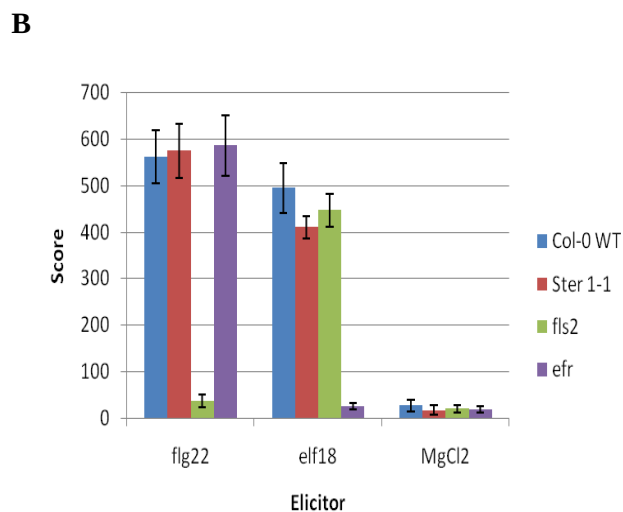
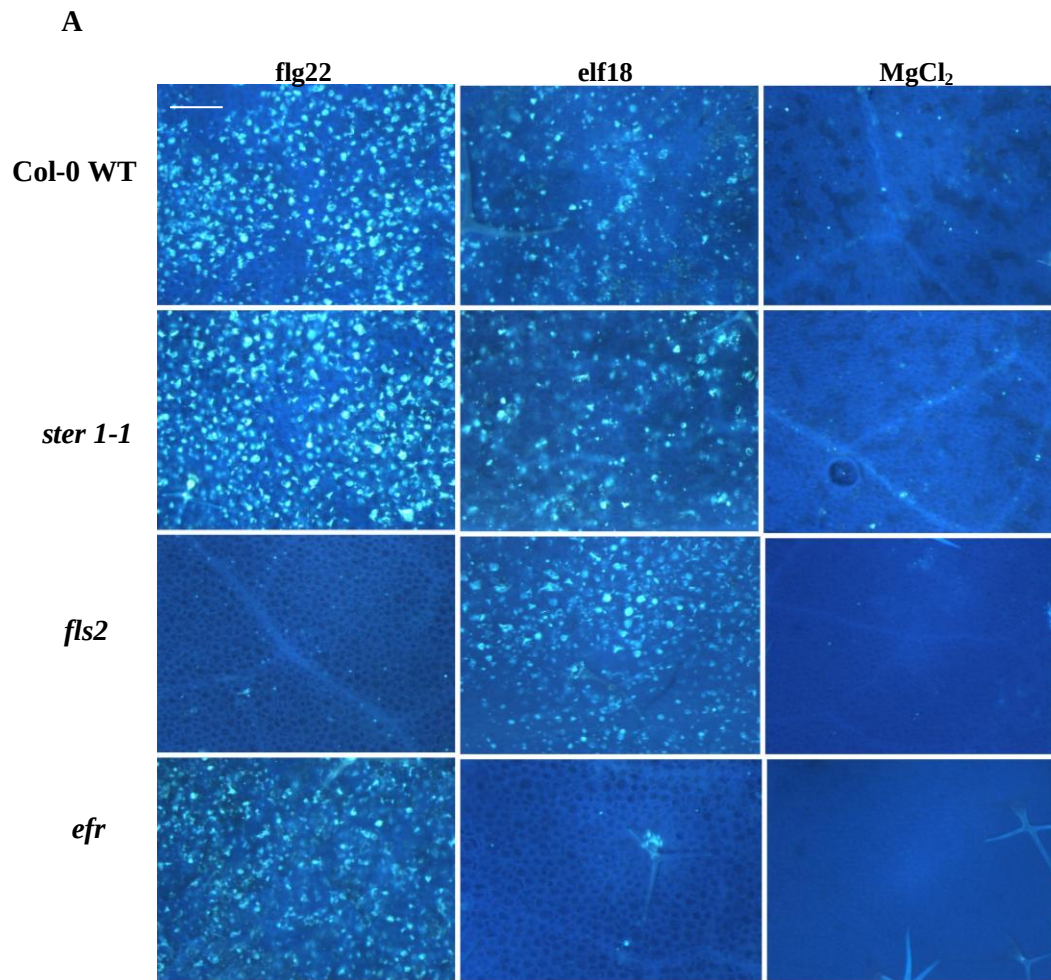


Figure 3.33. MAMP perception in the *ster1-1* mutant. (A) Aniline blue staining was performed to detect callose deposits. Representative photos are shown 24h after inoculation with 1 μ M flg22, 1 μ M elf18 or 10mM MgCl₂ (mock). Scale bar is 0.2mm; (B) Mean callose deposit counts of the wild-type and mutant plants. Magnification is 10x.

We also tested the phenotypic response to *Burkholderia ambifaria* strain 19182 originally isolated from pea rhizosphere (Figure 3.31A). This Gram negative rhizosphere organism stimulates plant growth and suppresses soilborne plant pathogenic *Pythium* spp (Bowers and Parke, 1993). *B. ambifaria* inoculation of *Arabidopsis* results in a chlorotic phenotype in wild-type Col-0, whereas *ster1-1* remains asymptomatic. In a similar fashion to *S. epidermidis*, this bacterium does not multiply *in planta* despite generating a pronounced phenotype (Figure 3.31B).

Phenotypic responses following infection with the necrotrophic fungus *Botrytis cinerea* led to similar chlorotic responses in wild-type Col-0 and *ster1-1* lines (Figure 3.32). An inoculum concentration of 2×10^5 spores/mL was found to be optimal for studying visual symptoms, as higher concentrations led to fast tissue collapse, while at lower concentrations experiments were not reproducible due to high phenotypic variation. At 5dpi, *B.cinerea* lesions were generally contained within the droplet application area, with a few leaves showing spreading lesions. By 7dpi, all leaves showed varied degrees of tissue collapse both for wild-type Col-0 and the mutant.

Additionally, responses of Col-0 and *ster1-1* to flagellin (or its bioactive peptide flg22) and Ef-Tu (or its bioactive peptide elf18), two well characterized MAMPs, were analysed by quantifying the number of callose deposits (Figure 3.33 A,B). The activity and specificity of these peptides was confirmed by assessing their ability to trigger callose deposition in the *fls2* and *efr* mutants, respectively (Figure 3.33A). After 24hpi, a high number of callose deposits were visible for flg22 and to a lesser extent for elf18 (Figure 3.33A). Similar scores were recorded for both Col-0 and *ster1-1* for both bioactive peptides, suggesting *ster1-1* is not impaired in the perception of these MAMPs (Figure 3.33B).

3.3.1.6. Discussion

STER1 is part of a cluster of 20 DUF26 RLK-encoding genes (CRKs) on Arabidopsis chromosome four. Some members of this cluster have already been implicated in plant defence (Czernic et al, 1999; Acharya et al, 2007). The characteristic C-X8-C-X2-C motifs and kinase active site are highly conserved in all members of this cluster. The novel DUF26 cysteine motif has recently been shown to contribute to structural stability by allowing for the formation of disulfide bridges (Miyakawa et al, 2009). Additionally, another

two cysteine residues outside the aforementioned structural motif are also completely conserved, in the extracellular domain of the CRKs suggesting they might participate in the establishment of intramolecular disulfide bridges as demonstrated for Gnk-2 (Miyakawa et al, 2009). In contrast with the extracellular domain, the intracellular kinase domain shows a high degree of conservation across STER1 homologues (35% sequence identity). Notably, most residues of the kinase active site are completely conserved within the CRKs and by comparison to other kinases (eg. FLS2, BAK1), suggesting STER1 is an active kinase. Similar to BAK1, STER1 is an RD kinase. To be more specific, kinases that are regulated by activation loop phosphorylation generally carry a conserved arginine (R) immediately preceding the invariant aspartate (D) required for catalytic activity (Dardick and Ronald, 2006). A survey of worm, fly, human and *Arabidopsis* kinomes found that most kinases known or predicted to function in PRR signalling are non-RD kinases, leading to the suggestion that non-RD catalytic loops are thought to be characteristic of PRRs (Dardick and Ronald, 2006). For example, *Arabidopsis* PRRs FLS2 and EFR are LRR-containing non-RD kinases; LRR motifs are traditionally associated with innate immunity and thought to bind proteinaceous ligands (Kobe and Kajava, 2001). Additionally, except for CERK1, all RLKs involved so far in plant immunity are LRR- kinases. As a DUF26-containing RD-kinase, STER1 is a novel RLK possibly involved in the recognition of a non-proteinaceous MAMP.

Results obtained from F1 and F2 analysis following inoculation with *S. epidermidis* 18888 suggested that asymptomatic accessions harbour a defect in *STER1*. The inability to amplify the whole gene from Cvi-0 or Tsu-1 genomic DNA points towards a possible rearrangement in this region. By contrast, sequencing data available from 1001genomes.org identified no base changes in asymptomatic accessions Cvi-0 and an early STOP codon in Tsu-1. While the latter finding is in accordance with STER1 controlling the response to *S. epidermidis* 18888, the case of Cvi-0 is less straightforward. No *STER1* RT-PCR product was detected in Cvi-0 compared to Col-0, suggesting additional elements required for STER1 expression are lacking or defective in Cvi-0.

A STER1-GFP fusion protein localized to the leaf cell plasma membrane and stomata, while microarray data indicated *STER1* is primarily expressed in rosette leaves and the hypocotyl. Additionally, publically available microarray data also indicated *STER1* is up-regulated in response to several MAMPs. This data is consistent with a role in defence, and expression patterns in plant tissues were confirmed using RT-PCR.

Ster1-1 does not appear to be impaired in the defence responses following inoculation with the bacterial *Pst* DC3000 or fungal *B. cinerea* plant pathogens. Tests conducted using *Pst* DC3000, as well as *Pst*(avrRpm1) and *Pst*(avrRpt2) ruled out any potential defects in basal resistance or HR, respectively. As many defence systems are redundant and eliminating one defence component might have a reduced impact on overall responses, assays aimed at identifying potential subtle differences in infection were also performed. Nevertheless, more sensitive assays such as electrolyte leakage and trypan-blue staining revealed no differences between the *ster1-1* mutant and Col-0. Inoculation with *B. cinerea* generated similar visual symptoms in both *ster1-1* and wild-type Col-0. However, tests with additional fungal pathogens are necessary before drawing a conclusion regarding *STER1* involvement in plant fungal resistance. Previous studies investigating the role of some CRKs in plant defence also failed to implicate these RLKs in plant resistance by using T-DNA mutants; instead over-expressor lines were instrumental in demonstrating a role for CRKs in defence (Chen et al, 2003; Chen et al, 2004; Acharya et al, 2007). Similarly, a *STER1* over-expressor should also be tested following bacterial and fungal plant pathogen treatments. Such a line has been generated and is currently being selected.

Unlike wild-type Col-0, *ster1-1* is asymptomatic when inoculated with a rhizosphere strain of the Gram negative bacterium *B. ambifaria*. Currently, reports on *Arabidopsis* responses to inoculation with *B. ambifaria* are lacking. So far, *B. ambifaria* has been shown to elicit disease symptoms on *Medicago sativa* (alfalfa) seedlings (Bernier et al., 2003). By contrast to growth data reported here, this Gram negative bacterium exhibited exponential growth in *M. sativa* over a 7 day period. In addition, a recent study reported the establishment of a novel tomato (*S. lycopersicum*) host model for *B. pseudomallei* study (Lee et al, 2010). This latter study also recorded that *Arabidopsis* showed a very weak phenotypic response 7dpi, although the authors did not clarify whether the symptoms were infection or senescence related. Moreover, whereas in this study *B. ambifaria* was syringe inoculated into *Arabidopsis* leaves, all *B. pseudomallei* infection procedures relied on root immersion in bacterial suspensions, thus rendering comparisons between the two studies difficult. Similar to *S. epidermidis*, *B. ambifaria* fails to multiply following infiltration suggesting that this non-adapted strain is unable to interfere with PTI. These findings could imply a common elicitor is recognised directly or indirectly via *STER1*. To be more specific, *STER1* could function as a receptor, but also as a co-receptor or signalling component that is essential for the elicitor response in this case.

Finally, tests aimed at quantifying responses to flagellin and EF-Tu showed no differences in the extent of callose deposition associated with either MAMP at 24 hpi and earlier time points (data not shown). As callose deposition is a common defence marker assessed in response to flg22 and elf18, it could be inferred that STER1 is not involved in the response to these MAMPs. Nevertheless, other parameters such as medium alkalinization, oxidative burst or PR1 expression should be investigated before drawing a definite conclusion.

3.3.2. Bacterial membrane-derived elicitors trigger defence responses in *Arabidopsis*

3.3.2.1. Introduction

STER1 is required for the response to both *S. epidermidis* 18888 and *B. ambifaria* 19182. Neither of these bacteria demonstrate an ability to grow *in planta*, suggesting they possibly trigger PTI. Taken together, these findings could suggest that a common elicitor is recognised by the plant, leading to the activation of a defence response which culminates in chlorosis or necrosis. Since *S. epidermidis* is a non-flagellated Gram-positive and *B. ambifaria* is a Gram-negative, PGN, a joint bacterial membrane component was considered a likely candidate.

PGN, a polymer of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues in beta-1-4 linkage cross-linked by short peptides, is an essential and unique component of the bacterial envelope. While PGN is the major constituent of the Gram positive cell wall, Gram negative bacteria harbour a relatively thin layer of PGN which is restricted to the periplasmic space. Recently, two distinct studies have researched *Arabidopsis* responses to Gram positive and Gram negative intact PGN, respectively (Gust et al, 2007; Erbs et al, 2008b). In both cases, the authors conclusively proved by researching a series of defence-associated parameters, that PGN can act as a MAMP. However, findings regarding the minimal structure which is required for recognition were contrasting, with the sugar backbone being required for Gram-positive bacteria (Gust et al, 2007), while mucopeptides seem to be the more active elicitors than intact PGN in the case of Gram negative bacteria (Erbs et al, 2008b).

Just as LPS, PGNs are orphan MAMPs, with a receptor yet to be identified. The PGN disaccharide backbone resembles that of chitin, the major component of the fungal cell wall. However, PGN and chitin seem to engage different perception systems (Gust et al, 2007; Gimenez-Ibanez et al, 2009b).

In addition to PGN, most *S. epidermidis* also harbour a surface exopolysaccharide, namely a β 1-6 poly N-acetylglucosamine homopolymer called PNAG or PIA (Mack et al, 1996). The biosynthesis of PNAG is under complex regulation and depends on the gene products of the *ica* (intracellular adhesion) locus, with membrane localized IcaA and IcaD proteins producing a chain from activated N-acetylglucosamine (GlcNAc) monomers which is exported via the IcaC protein (O’Gara et al, 2007; Gerke et al, 1998). Only one other *S. epidermidis* exopolysaccharide has been described, namely a sulfated 20 kDa polysaccharide (Karamanos et al, 1997). Although PNAG has not been tested in *Arabidopsis*, EPS from several Gram-negative bacteria, including xanthan from *Xanthomonas*, alginate from *Pseudomonas* and amylovoran from *Erwinia*, have long been associated with plant pathogenesis (Denny, 1995). Moreover, this extracellular polymer can also act as a virulence factor by disrupting specific defence responses such as calcium fluxes and callose deposition (Aslam et al, 2008; Yun et al, 2006).

Aim

The remaining goal of this project was to identify the *S. epidermidis* microbial features underlying the plant phenotypic response. In view of this, PGN from both *S. epidermidis* 18888 and *B. ambifaria* was isolated and tested in *Arabidopsis*. The minimal structure required for elicitation was also evaluated. Additionally, a series of defence parameters, as well as the ability of the elicitor to prime defence responses, were assessed following *Arabidopsis* elicitor inoculation.

3.3.2.2. STER1 is required for the *Arabidopsis* response to a non-proteinaceous *S.epidermidis* elicitor

Arabidopsis plants were treated with crude bacterial extracts in order to identify the existence of elicitors of the phenotypic response. Infiltration of *Arabidopsis* leaves with heat killed *S. epidermidis* cells results in a macroscopic reaction closely resembling the response triggered by live-cells (Figure 3.34 A).

This result suggests that a preformed molecule elicits the phenotypic reaction.

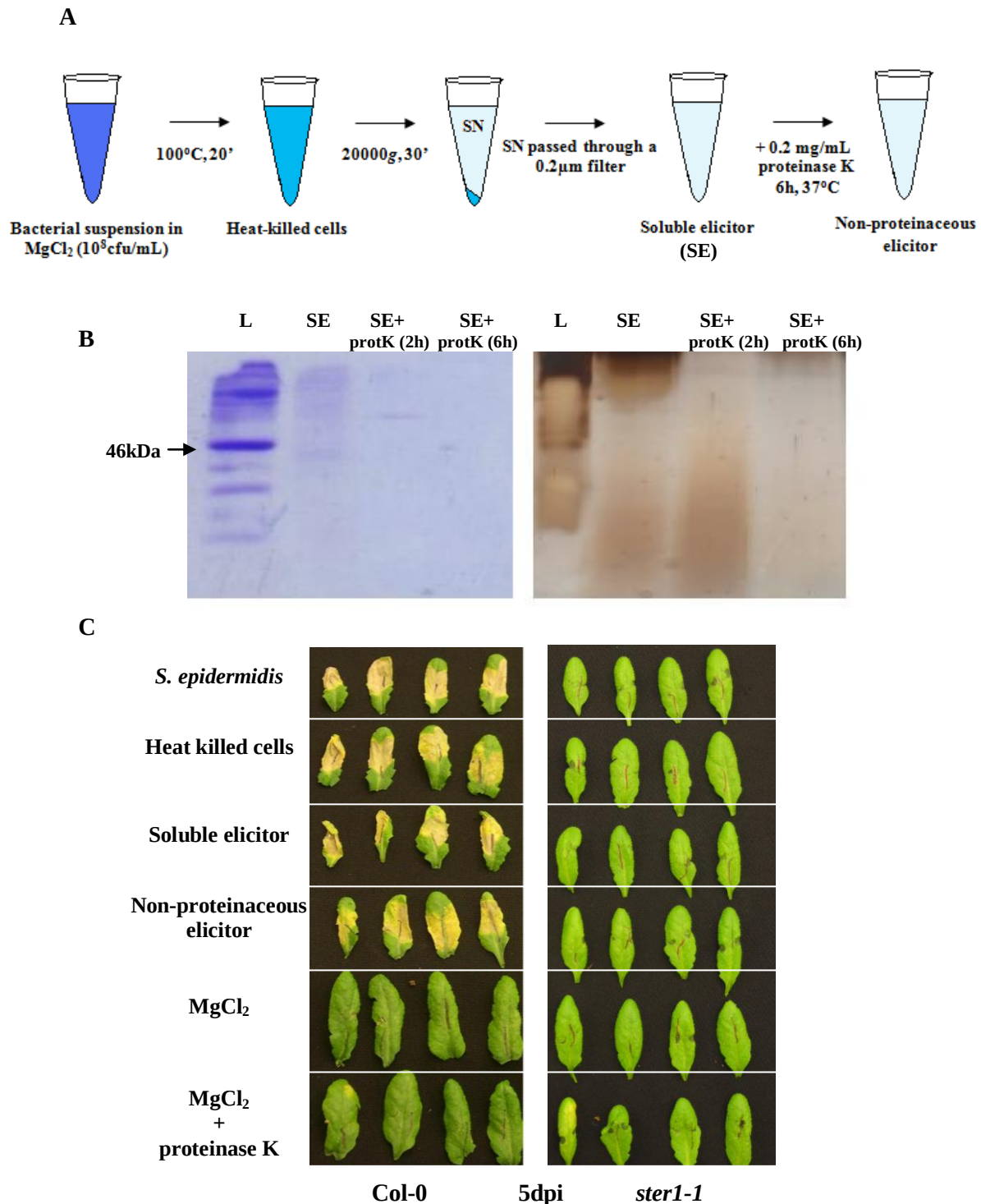


Figure 3.34. An *S. epidermidis* non-proteinaceous elicitor triggers a necrotic response in *Arabidopsis*. (A) Procedure employed for the isolation of the soluble elicitor fraction. A suspension of live cells was boiled for 20 minutes to generate the heat killed cells, centrifuged and the resulting supernatant (SN) was passed through an $0.2\mu\text{m}$ filter to ensure removal of all bacterial cells in order to generate the soluble elicitor; this fraction was treated with proteinase K to remove proteins and generate the non-proteinaceous elicitor fraction. (B) Soluble elicitor (SE) before and after proteinase (protK) treatment was run on 12% polyacrylamide gels and stained with Coomassie blue (left) and silver stained (right). A broad range prestained protein marker (NEB) was used as a ladder (L); (C) *Arabidopsis* accessions Col-0 and Cvi-0 were infiltrated with different bacterial extracts and phenotypes were observed 5dpi; 2 negative controls were used for comparison: a 10mM MgCl_2 solution and a 10mM MgCl_2 solution containing 0.2mg/mL proteinase K.

A soluble elicitor fraction, isolated from the heat-killed cell suspension, generated a chlorotic response in Col-0 but not in *ster1-1* (Figure 3.34 A,C). Treatment of the soluble elicitor with proteinase K did not alter the plant phenotypic response. Effective removal of proteins was confirmed by SDS-PAGE and silver staining (Figure 3.34 B). As a result, it would appear the elicitor-active component is not protein-based. Several carbohydrate MAMPs such as LPS, EPS and PGN have been previously shown to elicit plant innate immune responses, albeit not obvious macroscopic phenotypic reactions. Taking into consideration that the Gram positive *S. epidermidis* does not have an LPS layer and that Gram negative *B. ambifaria* also triggers a *STER1*-dependent plant response, PGN and EPS are potential elicitor candidates.

3.3.2.3. Bacterial membrane components trigger plant immune defences via *STER1*

Pure peptidoglycan was isolated from both *S.epidermidis* and *B.ambifaria*. The purification procedure yielded two fractions for *S. epidermidis*: a membrane fraction (MF) and pure PGN. MF (also called pre-HF fraction) is a precursor of the pure *S. epidermidis* PGN fraction (also called post-HF fraction). More specifically, the last step of the PGN purification procedure for Gram-positives relies on a mild acid hydrolysis of MF using hydrofluoric acid (HF), which removes secondary polysaccharides covalently bound to PGN. Plants were inoculated with elicitor solutions and phenotypes were observed 5dpi. PGN isolated from *B.ambifaria* generated a macroscopic response in *Arabidopsis* leaves comparable to live cells (Figure 3.35B). In contrast, pure *S. epidermidis* PGN did not elicit a phenotypic response in leaves (Figure 3.35A). Inoculation with MF resulted in a necrotic phenotype that visually matched the response seen for *S. epidermidis* suspensions. The response to *B. ambifaria* PGN was dose-dependent, with the chlorotic phenotype seen for 50µg/mL PGN gradually fading at lower PGN concentrations. At the same time, *S. epidermidis* MF concentrations as low as 5µg/mL trigger reactions matching the extent of the response seen with live cells. Next, pure PGN fractions from both *B. ambifaria* and *S. epidermidis* were treated with mutanolysin, an enzyme which hydrolyzes glycosidic linkages between disaccharide units of PGN, resulting in a mucopeptide mixture. Following HPLC purification, soluble mucopeptides were infiltrated into *Arabidopsis* and phenotypes were

compared to untreated PGN. Mutanolysin treatment abolished the plant response to *B. ambifaria* PGN, suggesting sugar chains longer than the disaccharide are required for the induced phenotype (Figure 3.35B). As expected, no activity was seen for the *S. epidermidis* PGN-derived muropeptides (Figure 3.35A). Importantly, *ster1-1* remained asymptomatic following inoculation with all tested elicitor fractions.

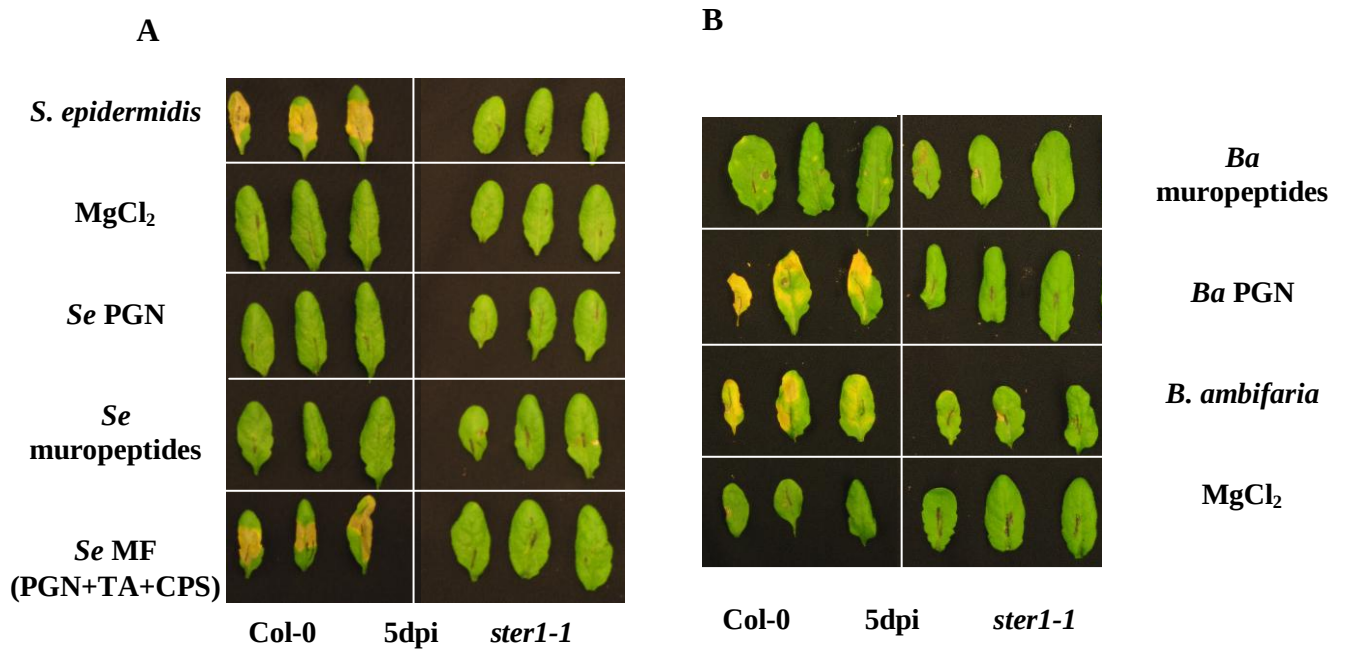


Figure 3.35. Bacterial membrane components trigger phenotypic responses in *Arabidopsis*. Col-0 and *ster1-1* were inoculated with (A) *S. epidermidis* derived elicitor fractions, (B) *B. ambifaria* derived elicitor fractions; Phenotypes were observed 5dpi; All elicitor solutions were prepared at a concentration of 50µg/mL. All bacterial suspensions were prepared at a concentration of 10⁸cfu/mL. *Se*, *S. epidermidis*; *Ba*, *B.ambifaria*; PGN, peptidoglycan; TA, teichoic acid; CPS, capsular polysaccharide

GC/MS analysis of *S. epidermidis* MF revealed it consists of PGN, teichoic acid (TA) and a capsular polysaccharide (CPS) different from PIA/PNAG. Pure TA (as confirmed by GC/MS) was isolated from *S.epidermidis* 18888 and inoculated into *Arabidopsis*. *S. epidermidis* TA did not elicit a response in *Arabidopsis* even at high concentrations (up to 200µg/mL) (Figure 3.36A). In order to further test possible PNAG contribution to the necrotic phenotype generated in *Arabidopsis*, three biofilm-producing *S. epidermidis* clinical isolates, 1457, 9142, and 8400, and their respective PIA-negative Δ *ica* (intracellular adhesion) mutants (Mack et al, 1999) were infiltrated in *Arabidopsis*. More than one *S. epidermidis* strain was included in this analysis as previous experiments have revealed not all

S. epidermidis strains cause severe phenotypes in *Arabidopsis* (Figure 3.6). Inoculation with the *S. epidermidis* Δ ica mutants reproduced the phenotype seen for the wild-type strains, confirming that PNAG does not contribute to the plant necrotic response, as suggested by GC-MS (Figure 3.36B). Taken together, these results suggest that the remaining MF component, namely CPS, is responsible for the necrotic plant response. Alternatively, prolonged MF exposure to HF might result in PGN hydrolysis/modification, which could potentially alter the extent of the phenotype.

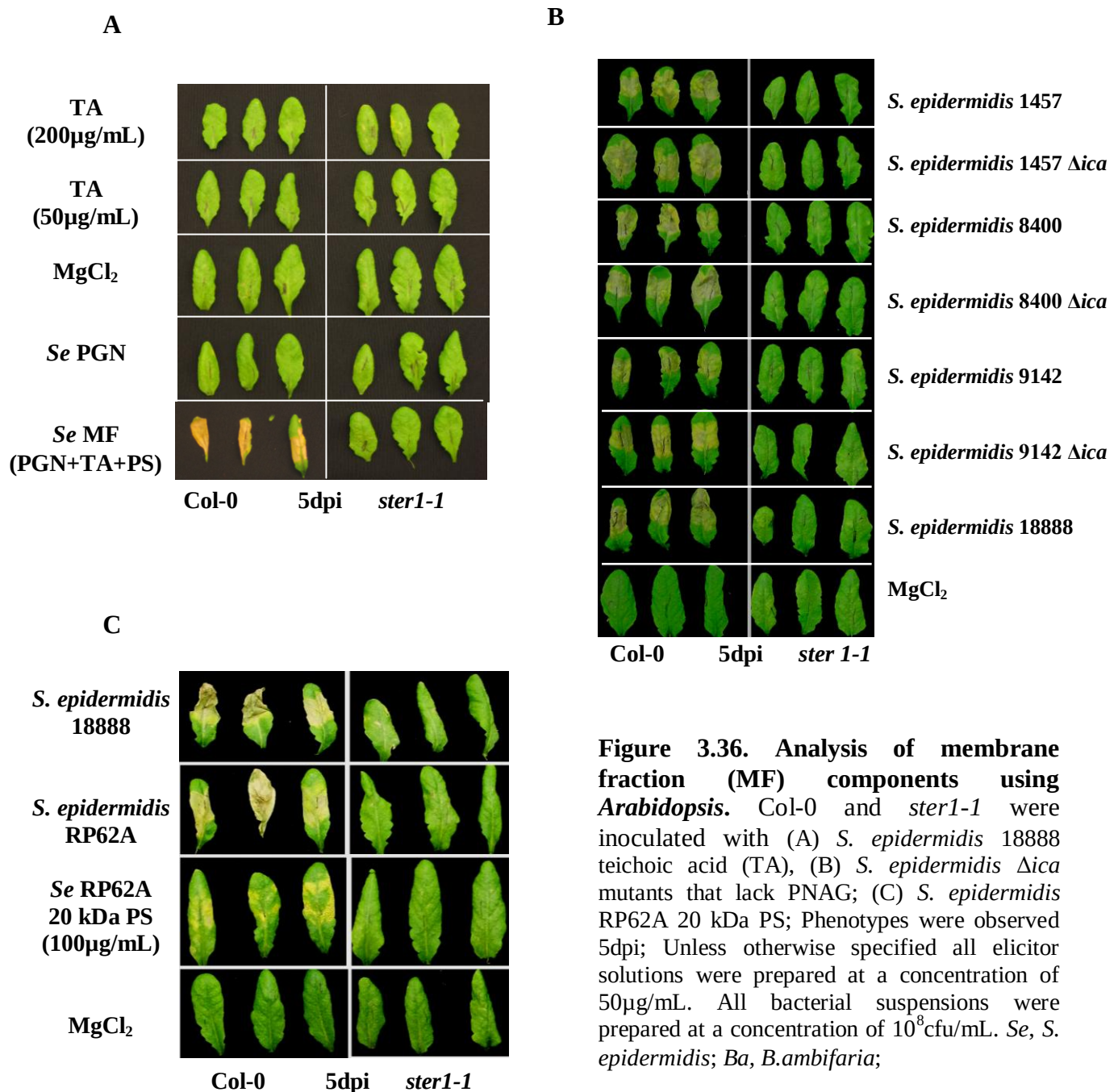


Figure 3.36. Analysis of membrane fraction (MF) components using *Arabidopsis*. Col-0 and *ster1-1* were inoculated with (A) *S. epidermidis* 18888 teichoic acid (TA), (B) *S. epidermidis* Δ ica mutants that lack PNAG; (C) *S. epidermidis* RP62A 20 kDa PS; Phenotypes were observed 5dpi; Unless otherwise specified all elicitor solutions were prepared at a concentration of 50µg/mL. All bacterial suspensions were prepared at a concentration of 10⁸cfu/mL. Se, *S. epidermidis*; Ba, *B.ambifaria*;

Additionally, the 20kDa polysaccharide (PS) isolated by Karamanos et al. (1997) originating from *S. epidermidis* RP62A was also infiltrated into *Arabidopsis* (Figure 3.36C). Although a high concentration was used (100µg/mL), this generated a mild chlorotic response compared to the live-cells. However, this chlorotic response was also STER1-dependent (Figure 3.36C).

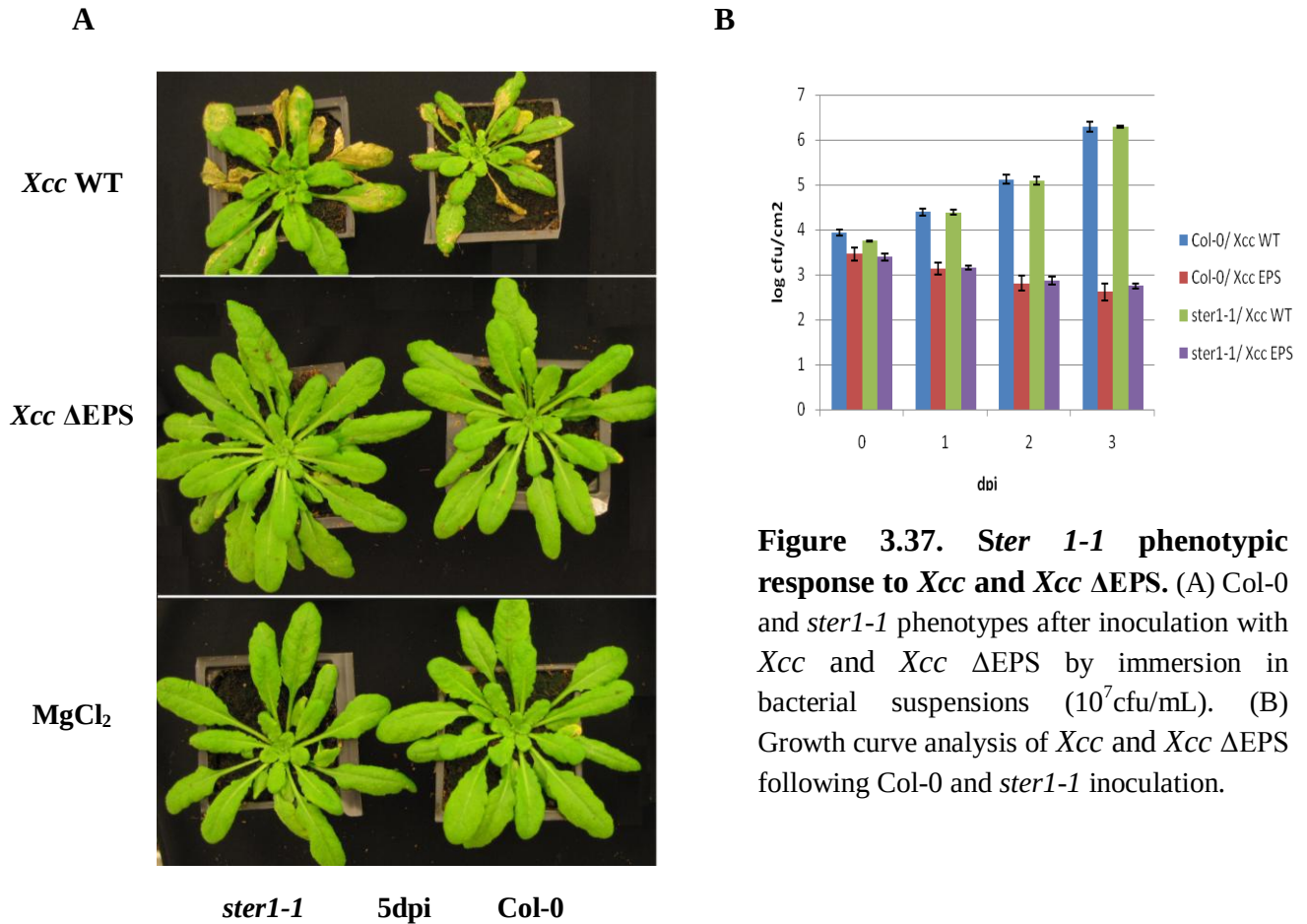


Figure 3.37. *Ster 1-1* phenotypic response to *Xcc* and *Xcc* ΔEPS. (A) Col-0 and *ster1-1* phenotypes after inoculation with *Xcc* and *Xcc* ΔEPS by immersion in bacterial suspensions (10^7 cfu/mL). (B) Growth curve analysis of *Xcc* and *Xcc* ΔEPS following Col-0 and *ster1-1* inoculation.

The plant pathogen *Xcc* and its EPS mutant were also tested to check for potential *ster1-1* defects in EPS perception. The *Xcc* 8397 *gum* ΔEPS mutant has been previously characterized and shown to produce no symptoms in *Arabidopsis* when compared to wild-type *Xcc* 8004 (Vojnov et al, 1998; Yun et al, 2006). As previously described, the *Xcc* EPS mutant was unable to elicit symptoms on Col-0 and to sustain growth *in planta* (Figure 3.37 A,B). However, both *Xcc* and *Xcc* ΔEPS displayed identical growth patterns and triggered

similar phenotypic responses following Col-0 and *ster 1-1* immersion in the bacterial suspensions (Figure 3.37 A,B). This result suggests *STER1* is required for *S. epidermidis* CPS-specific responses rather than in a more generalized EPS recognition pathway.

3.3.2.4. *STER1*-elicitor interplay

To test whether *Arabidopsis* exposure to PGN fractions and *S. epidermidis* MF leads to increased plant resistance, growth of pathogenic *Pst* DC3000 was studied following pretreatment of leaves with elicitor solutions. Wild-type Col-0 and the *ster1-1* mutant were pre-treated with membrane elicitor solutions and flg22 (Figure 3.38 A,B).

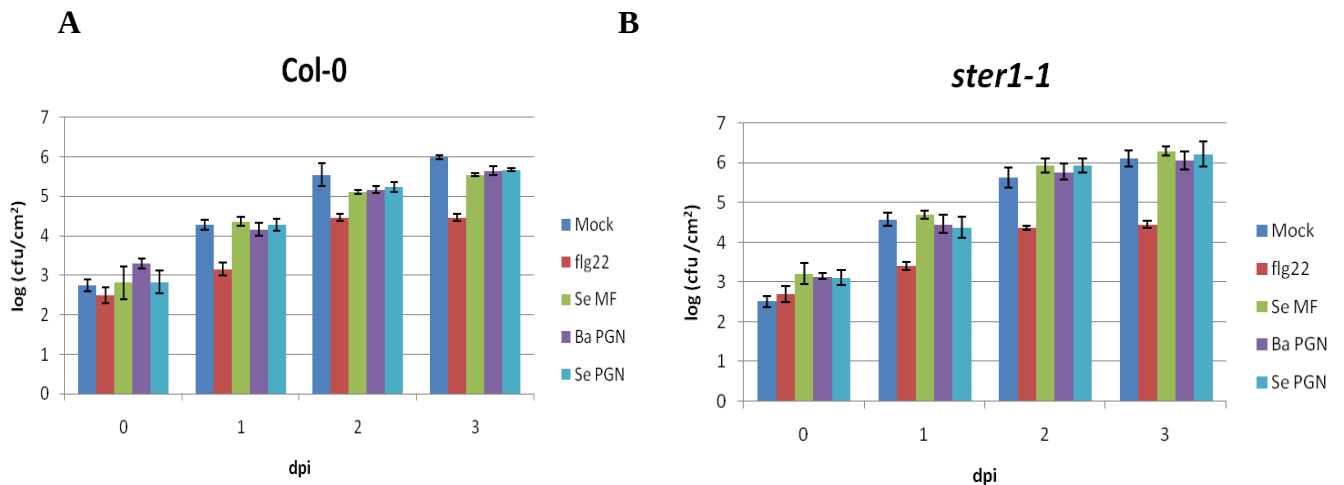
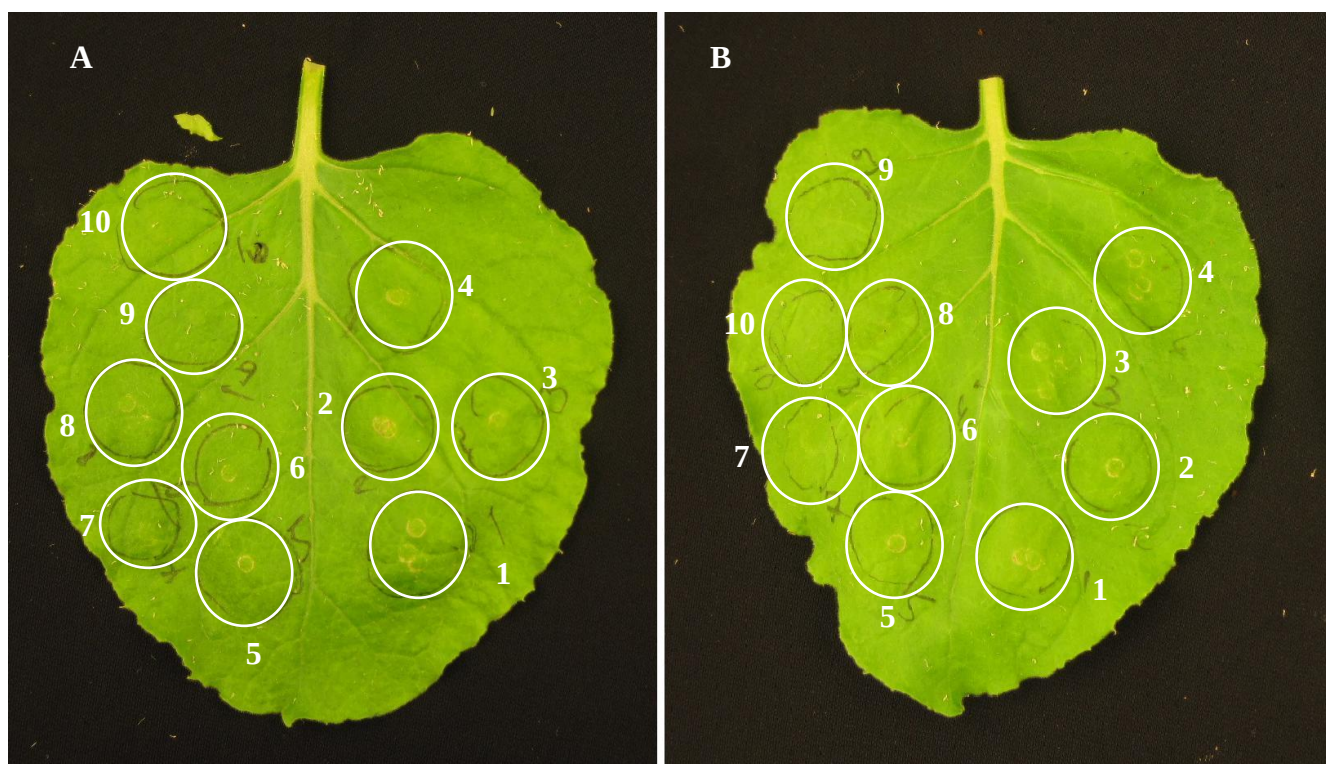


Figure 3.38. Pretreatment of Col-0 and *ster1-1* with membrane-derived elicitors restricts *Pst* DC3000 growth. *Arabidopsis* (A) Col-0 and (B) *ster1-1* plants were pretreated for 24h by leaf infiltration with different elicitor solutions as follows: 10mM MgCl₂ (mock), 1μM flg22, 5μg/mL Se MF, 50μg/mL Se and *Ba* PGN; Subsequently, leaves were infiltrated with 10⁵cfu/mL *Pst* DC3000 and bacterial growth was assessed every 24 hours;

In control wild-type and *ster1-1* plants that were mock-pretreated with MgCl₂, bacteria multiplied exponentially up to a titre of 10⁶ cfu/cm² by 3dpi. By contrast, starting with 1dpi, bacterial growth was strongly decreased in Col-0 plants pre-treated with flagellin

(1.5 log difference compared to mock treatment) as previously described (Zipfel et al, 2004) (Figure 3.38A). Starting with 2dpi, a mild reduction in bacterial growth was observed in plants pre-treated with the other elicitors (0.5 log difference compared to mock treatment). In conclusion, pre-treatment with the membrane derived-elicitors did arrest *Pst* DC3000 growth, but to a reduced level compared to flagellin. Additionally, pretreatment with TA and muropeptides did not have the same priming effect seen with MF and PGN fractions. At the same time, flagellin pre-treatment also restricted *Pst* DC3000 bacterial growth in *ster1-1* to the same levels as those seen for wild-type Col-0 (Figure 3.38B). However, bacterial growth was not arrested in *ster1-1* following treatment with *S. epidermidis* and *B.ambifaria* elicitor fraction. These experiments were repeated three times with similar results. Taken together, this data would suggest that the induction of resistance due to PGN/MF perception depends on a functional STER1.

Unlike *Arabidopsis*, *N. benthamiana* remains asymptomatic following inoculation with *S. epidermidis*, *B. ambifaria* and the associated MF and PGN elicitor fractions (Figure 3.39) . To examine whether this is due to a lack of functional STER1, a transient expression system using *Agrobacterium* was established in *N. benthamiana*. In view of this, *Agrobacterium* cells expressing a STER1-GFP fusion under a 35S promoter and those containing the empty vector (EV) (GFP only) were infiltrated in *N. benthamiana*; STER1 expression was confirmed through visualisation by confocal microscopy prior to elicitor infiltration. STER1 transient expression did not confer responsiveness in *N. benthamiana* to any of the elicitors and/or bacterial suspensions (Figure 3.39). Several variations of the procedure were attempted including different vector/ elicitor concentration combinations but *N. benthamiana* remained asymptomatic. As a negative result, this experiment was not very informative. However, this set of experiments might also indicate that either the *S. epidermidis* membrane-elicitor-related signalling pathway is not conserved in *N. benthamiana* or that STER1 is not the receptor in this case.



	FigA	FigB
1	35S:STER1-GFP+ <i>Se</i> MF	35S:STER1-GFP+ <i>Ba</i> PGN
2	EV+ <i>Se</i> MF	EV+ <i>Ba</i> PGN
3	35S:STER1-GFP+ <i>Se</i>	35S:STER1-GFP+ <i>Ba</i>
4	EV+ <i>Se</i>	EV+ <i>Ba</i>
5	35S:STER1-GFP	35S:STER1-GFP
6	EV	EV
7	<i>Se</i>	<i>Ba</i>
8	<i>Se</i> MF	<i>Ba</i> PGN
9	infiltration media	infiltration media
10	water	water

Figure 3.39. Co-infiltration of STER1 and membrane derived elicitors using a *N. benthamiana* transient expression system. Coexpression of STER1 and (A) *Se* and *Se* MF, (B) *Ba* and *Ba* PGN; Leaves were co-infiltrated with *Se* and *Ba* bacterial suspensions and associated elicitor fractions as listed in the table. Elicitors and bacterial suspensions were prepared in water at a concentration of 100µg/mL and 10⁸cfu/mL, respectively. Agrobacterium cells expressing a STER1-GFP fusion under a 35S promoter and those containing the empty vector (EV) were resuspended in infiltration media at an OD₆₀₀ of 1.0. Photographs were taken 5dpi.

3.3.2.5. Early plant defence responses to bacterial-membrane derived elicitors

PRRs link extracellular perception to intracellular signal transduction. Exposure to purified MAMP solutions will trigger immediate events at the plant plasma membrane, such as receptor internalization and ion fluxes, followed by extensive downstream signalling that ultimately restricts bacterial growth. Generally, early plant defence responses can be recorded within the first hour of the initial elicitor treatment.

Preliminary experiments aimed at studying STER1 localization following elicitor exposure have been performed. In this view, Col-0 was transformed with a construct encoding STER1 fused to GFP at the C-terminus under the control of a 35S promoter. Confocal microscopy revealed that the STER1-GFP fusion protein localizes to the plasma membrane. Initial experiments performed so far assessed the effect of both pure PGN and MF fractions on STER1-GFP localization by using 2-week old seedlings. Within 30 minutes of elicitor treatment, distinct fluorescently labelled vesicles occurred near the cell membrane initially and rapidly moved away from this site (Figure 3.40A). The phenomenon, consisting of vesicle trafficking along cytoplasmic strands, was transient and ceased after 45 minutes of treatment. No accumulation of STER1-GFP in vesicles was observed following flg22 treatment. As a whole, this preliminary data suggests STER1 participates strictly in PGN/MF perception.

However, at this stage more work is required in order to confirm STER1 dynamics upon exposure to varied elicitors. FLS2 endocytosis upon exposure to flg22 has been reported previously (Robatzek et al, 2006). The experiment was replicated using Ws2::FLS2-GFP seedlings and flg22 in an effort to compare the resulting RLK localization. FLS2-GFP internalization into vesicles similar to STER1-GFP was visible after 30 minutes of flagellin treatment, but not to the same extent as that seen by Robatzek et al (2006); nevertheless, the analysis was hampered by the low expression levels of FLS2-GFP which rendered comparisons difficult (Figure 3.40B). Overall, these results suggest the procedure needs to be further optimized to ensure reproducibility of the results; additionally, FLS2-GFP localization should also be observed following exposure to *S. epidermidis* 18888 and *B. ambifaria* elicitor fractions.

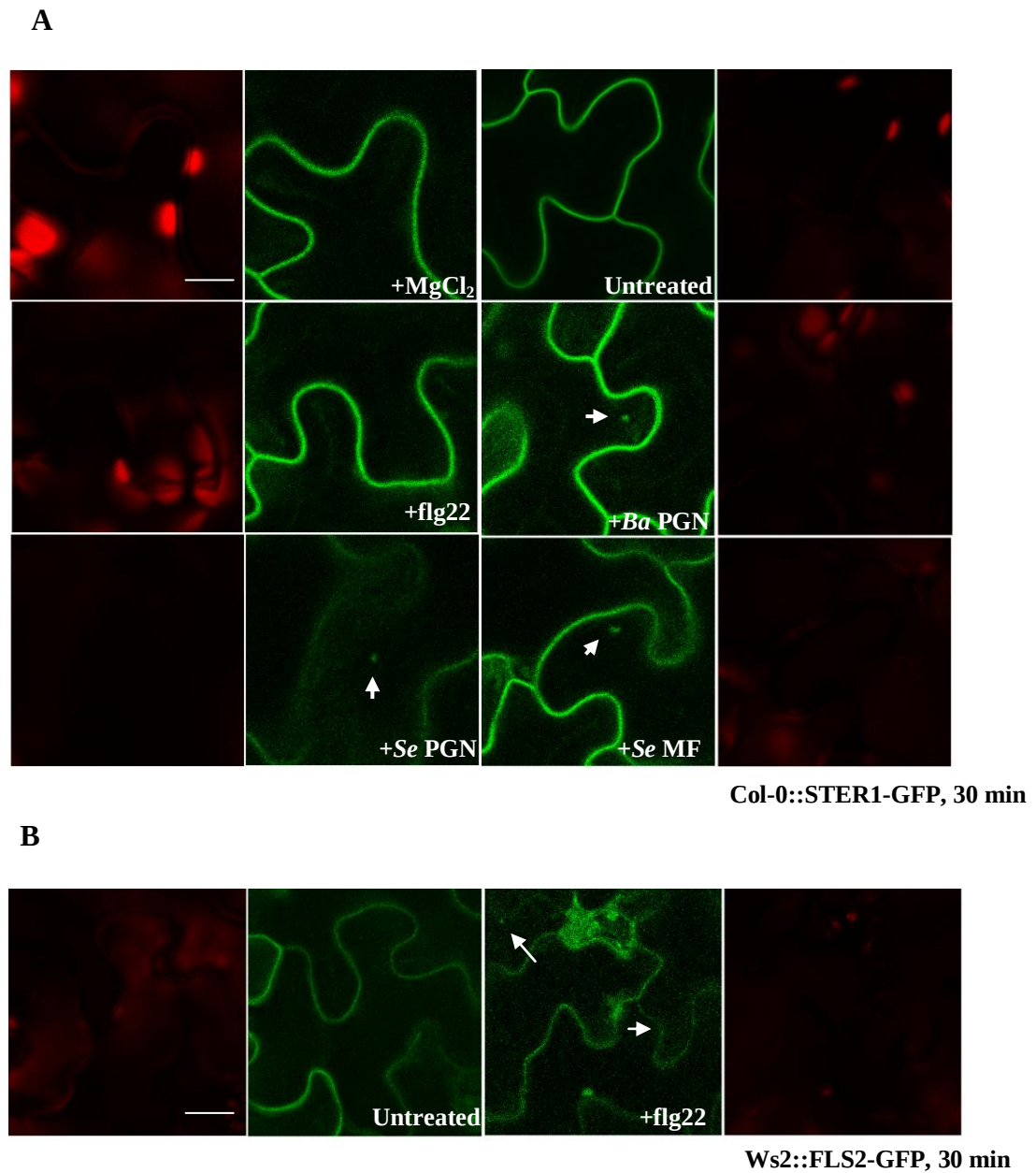


Figure 3.40. Dynamics of STER1 localization in response to bacterial membrane derived elicitors. Fluorescence micrographs show GFP signals in (A) Col-0::STER1-GFP and (B) Ws2::FLS2-GFP epidermal cells of 2-week old seedlings at 30 minutes following elicitor treatment as indicated; fluorescent vesicles are indicated by white arrows. Background autofluorescence of chloroplasts is shown in red on panels adjacent to GFP photos. Scale bar, 8 μ m. All membrane-derived elicitors were prepared at 100 μ g/mL, flg22 was used at 10 μ M and MgCl₂ at 10mM. *Se*, *S. epidermidis*; *Ba*, *B. ambifaria*.

Calcium functions as a second messenger in pathogen perception and changes in calcium levels are often monitored in response to MAMPs. Col-0 plants expressing cytoplasmic aequorin (Knight et al, 1996) were used to monitor changes in cytoplasmic calcium levels *in vivo* after elicitor treatment.

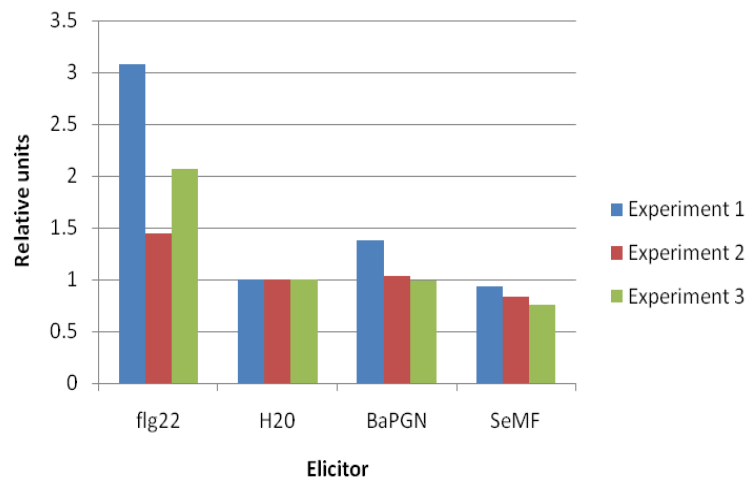


Figure 3.41. Induction of intracellular calcium flux by membrane-derived elicitors Cytoplasmic Ca^{2+} concentrations after exposure of 4-week old *Arabidopsis* leaves to 100mg/ml *Ba* PGN and *Se* MF and 5 μM flg22. Calcium values were recorded at 45 minutes after treatment and normalized by water values. Results for three experimental repeats are shown.

Initial experiments were aimed at assessing the effect of *B. ambifaria* PGN and *S. epidermidis* MF on intracellular calcium fluxes. Three different sets of experiments were performed using one batch of 4-week old *Arabidopsis* plants and results are presented as averages. Flg22 induced a rapid and strong increase in cytoplasmic calcium concentration, peaking after 2-3 minutes (data not shown). Figure 3.41 shows the calcium levels observed for different elicitors after 45 minutes in the three experiments. By comparison to the water control, flg22 appeared to induce an increase in cytoplasmic calcium, except for the second experiment when values were lower. Pure *B. ambifaria* PGN appeared to induce very slow calcium accumulation, with significant variation being seen between experiments. Interestingly, exposure to *S. epidermidis* MF seemed to result in a marked decrease in cytoplasmic calcium compared to the water control in all three experiments. However, more repeats are necessary in order to draw a definite conclusion on this aspect of defence, as not

enough data was available to estimate the error associated with each experiment. Additionally, different batches of plants grown at different times need to be analysed to confirm this initial data.

3.3.2.6. Late plant defence responses to bacterial-membrane derived elicitors

Apart from immediate responses, which can be assessed with the first hour of elicitor exposure, MAMP solutions also triggers late defence reactions such as callose deposition, expression of PR proteins and cell death starting with 24 hpi.

Exposure to flg22 leads to abundant callose deposition in both wild-type Col-0 (score of 753) and *ster1-1* (score of 743) at 24hpi, (Figure 3.42 A,B). As shown previously, *S. epidermidis* does not lead to the accumulation of callose deposits in Col-0 (Chapter 3.1, Figure 3.4). Similarly, infiltration with *S. epidermidis* MF and PGN, as well as *B. ambifaria* PGN triggered low levels of callose deposition (scores of 92, 46 and 57, respectively). By contrast, infiltration with *B. ambifaria* resulted in levels of callose comparable to flg22 in both Col-0 and *ster1-1*.

A statistically significant increase in number of callose deposits was observed repeatedly for *S. epidermidis* MF and *B. ambifaria* PGN in *ster1-1* (scores of 92 and 57, respectively) compared to wild-type Col-0 (scores of 55 and 25, respectively) and the MgCl₂ control (scores of 35 and 25, respectively).

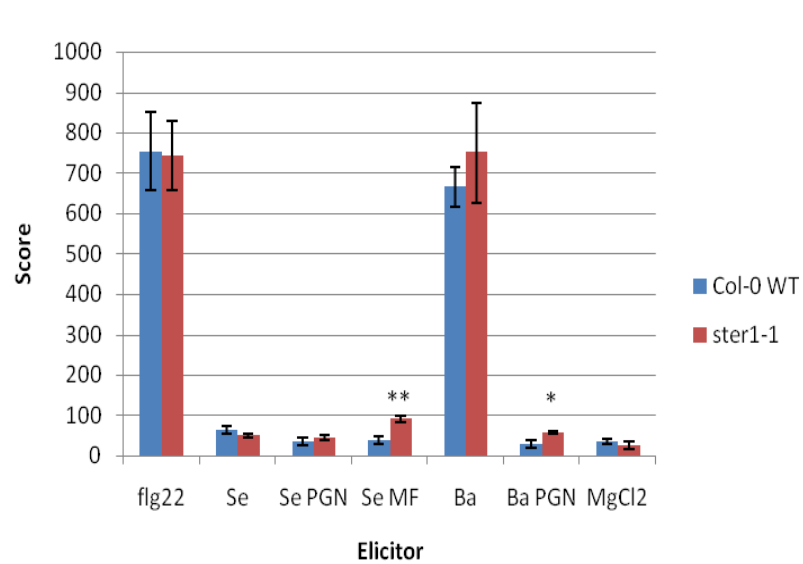
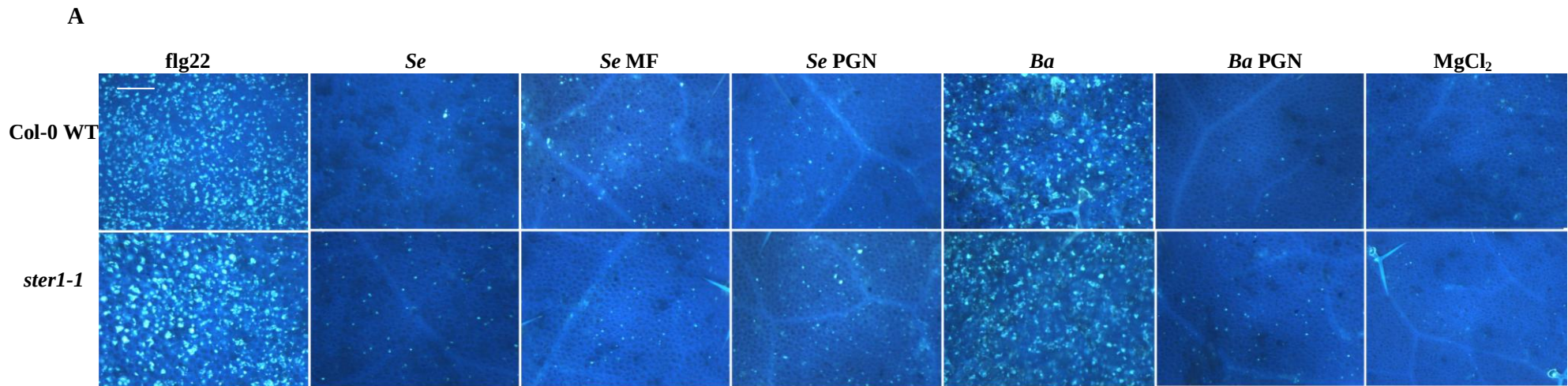


Figure 3.42. Callose deposition in response to membrane-derived elicitors. (A) Aniline blue staining to detect callose deposits in Col-0 WT and *ster1-1* 24hpi. Representative photos were taken following inoculation with 1 μ M flg22, 10⁸cfu/mL *S. epidermidis* (Se) and *B.ambifaria* (Ba), 50 μ g/mL elicitor solutions and 10mM MgCl₂ (mock). Scale bar is 0.2mm; (B) Mean callose deposit counts made in leaves of WT Col-0 and *ster 1-1* following inoculation with elicitors as indicated. Data sets marked with an asterisk are significantly different from the MgCl₂ control as assessed by the Student's t-test. Magnification is 10x.

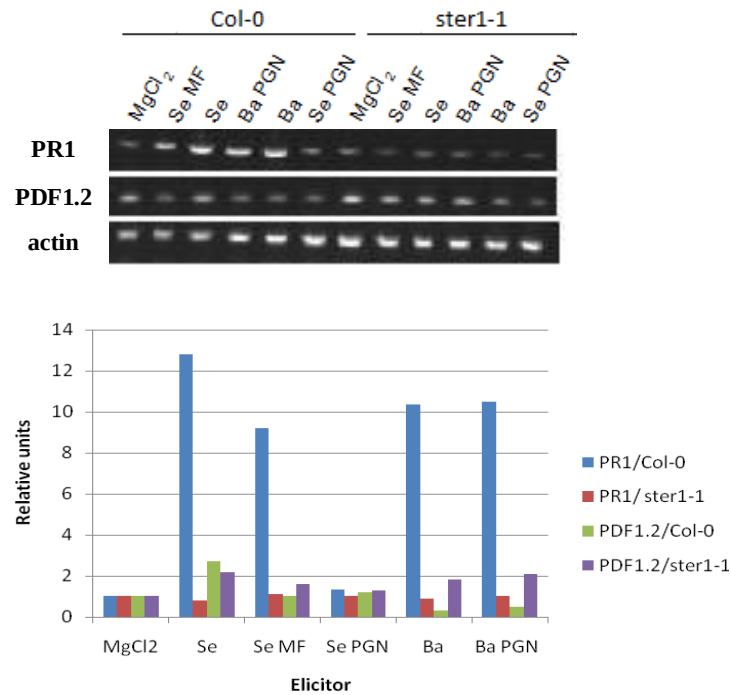
In order to assess marker gene expression in response to *S. epidermidis* and *B. ambifaria* elicitors, semi-quantitative RT-PCR analysis was performed. While PDF1.2 encoding a defensin is a commonly used marker gene for monitoring the JA-ET pathway, PR1 functions as a marker for the SA pathway. Previous experiments have implicated the SA-pathway in the response to *S. epidermidis* 18888 (Chapter 3.1, Figure 3.8). Additionally, PR1 was shown to be significantly up-regulated 24hpi; based on this initial observation, PR1 expression in response to elicitor treatment was quantified at this same time point. Treatment with *S. epidermidis* MF and *B. ambifaria* PGN induced high PR1 expression in wild-type Col-0 24hpi (Figure 3.43A). Unlike for MF, exposure to *S. epidermidis* pure PGN did not alter PR1 expression. PDF1.2 was not significantly up-regulated in response to *S. epidermidis*, suggesting the JA-ET pathway is not involved in plant defence responses in this case.

STER1 expression was also examined in Col-0 24hpi following elicitor treatment. Previous experiments have indicated STER1 is up-regulated 24 hours after *S. epidermidis* treatment (Figure 3.28) and elicitor-related expression was assessed for this same time-point. STER1 was highly up-regulated in response to *S. epidermidis* and *B. ambifaria* bacterial suspensions (Figure 3.43 B).

High levels of STER1 mRNA accumulation were also observed for the *B. ambifaria* PGN and *S. epidermidis* MF solutions. *S. epidermidis* pure PGN did induce STER1 expression, but to a lesser extent than the other elicitors.

Infiltration with *S. epidermidis* MF and *B. ambifaria* PGN resulted in cell-death in Col-0, but not in *ster1-1* (Figure 3.44A). The extent of cell death seen with these elicitors 24hpi matched the level of cell death induced by the corresponding bacterial suspensions. *S. epidermidis* PGN did not trigger cell death in Col-0 or *ster1-1*. No cell death was visible in *ster1-1* with the exception of Pst (*avrRpm1*)-associated HR. In an effort to quantify cell death, electrolyte leakage was measured over a time period of up to 96 hours (Figure 3.44 B,C). Very low electrolyte leakage (EL) values were recorded for the duration of the experiment for both *S. epidermidis* and *B. ambifaria* and their associated elicitor fractions in comparison with the positive control Pst (*avrRpm1*).

A



B

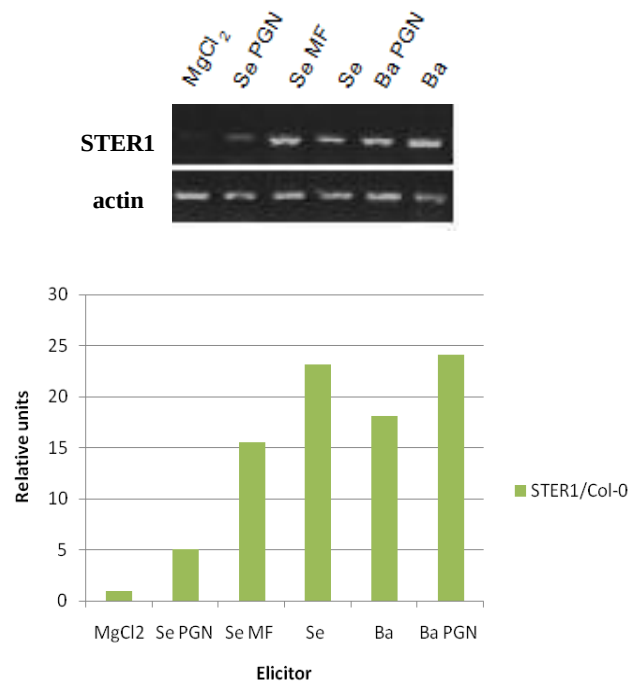


Figure 3.43. Membrane-derived elicitors induce *PR1* and *STER1* gene expression.

(A) Induction of *PR1* and *PDF1.2* genes in *Arabidopsis* Col-0 and *ster1-1* following elicitor treatment as indicated. (B) Induction of *STER1* in *Arabidopsis* Col-0 following elicitor treatment as indicated. RNA was extracted 24hpi following inoculation with 1 μ M flg22, 10^8 cfu/mL *S. epidermidis* (Se) and *B.ambifaria* (Ba), 10 μ g/mL elicitor solutions and 10mM $MgCl_2$ (mock); values were obtained by comparison to $MgCl_2$ -treated tissue following normalization to actin values;

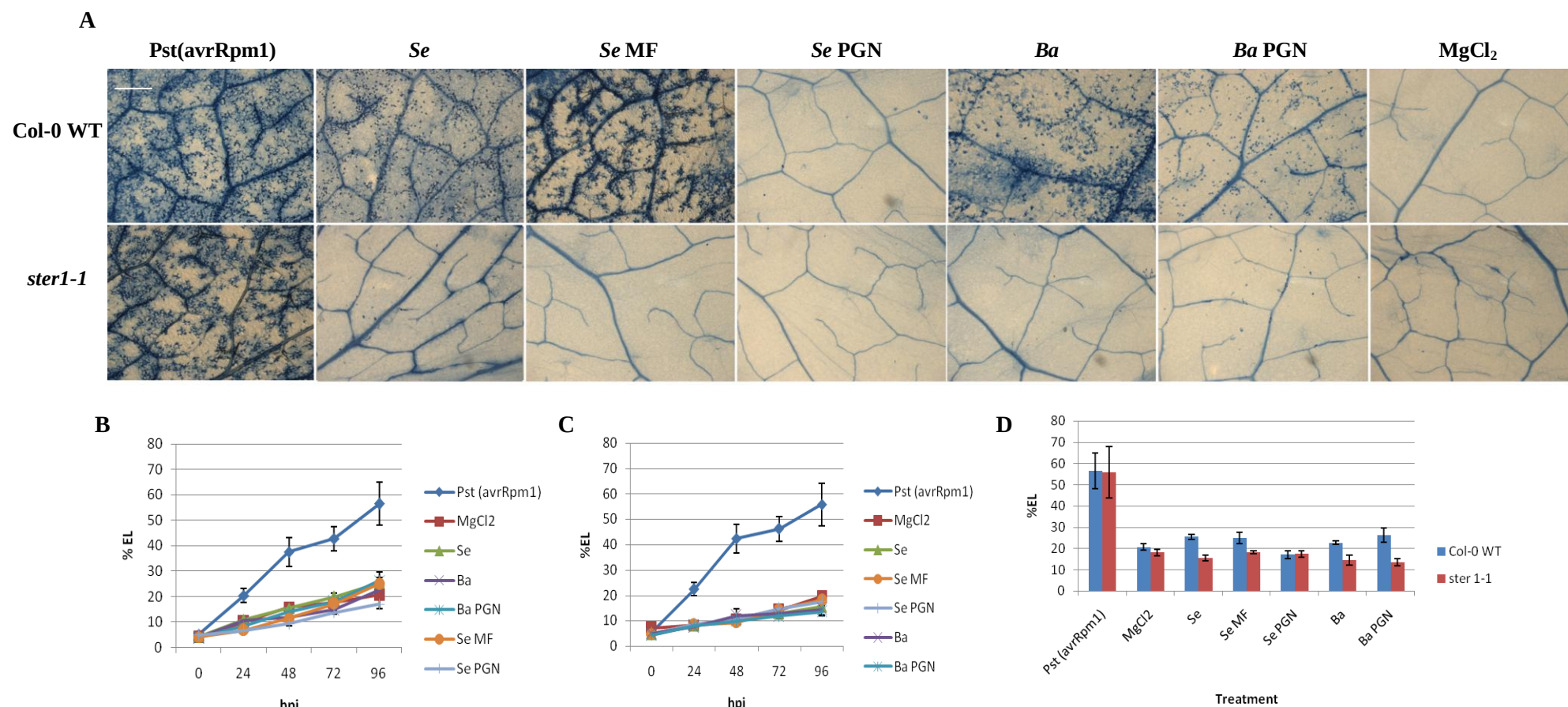


Figure 3.44. Membrane derived elicitors trigger cell death. (A) Distribution of microscopic cell death stained with trypan blue following treatments as indicated. Representative photos were taken 24hpi following inoculation with 10^7 cfu/mL Pst(avrRpm1), 10^8 cfu/mL *S. epidermidis*(Se) and *B. ambifaria* (Ba), 50 μ g/mL elicitor solutions and 10mM MgCl₂ (mock); No cell death was visible in *ster1-1* except for the positive control Pst (avrRpm1); Scale bar is 0.2mm; (B)Electrolyte leakage in Col-0 plants; (C) Electrolyte leakage in *ster1-1* plants; Plants were infiltrated with 10^6 cfu/mL Pst(avrRpm1), 10^8 cfu/mL *S. epidermidis*(Se) and *B. ambifaria* (Ba), 50 μ g/mL elicitor solutions and 10mM MgCl₂ (mock) and measurements were taken for 96h; (D) Comparison of electrolyte leakage values obtained for Col-0 and *ster1-1* 96hpi following different elicitor treatments as indicated. Values were expressed as percentage of electrolyte leakage (%EL) as described in Materials and Methods.

Small differences were visible between Col-0 and *ster1-1* at 96hpi (Figure 3.44D). More specifically, EL was generally higher in Col-0 than in *ster1-1*, except for the positive and negative controls (Pst (avrRpm1) and MgCl₂) and *S. epidermidis* PGN. Taken together, these results suggest the level of trypan blue staining does not correlate with EL.

Cell death is usually accompanied by ROS accumulation in *Arabidopsis*. Consequently, DAB staining was used to visualise in-situ peroxides. Nevertheless, a lot of variation was recorded within and between experiments and results have been inconclusive so far.

MAMPs such as flg22 and elf18 are known to cause seedling growth inhibition (Gomez-Gomez et al, 1999; Zipfel et al, 2006). Therefore we tested the effect of *S. epidermidis* PGN and MF and *B. ambifaria* PGN on wild-type Col-0 growth. It was found that very high elicitor concentrations up to 200µg/mL did not affect Col-0 seedling growth (data not shown).

3.3.2.7. Discussion

Although not considered as virulent as *S. aureus*, *S. epidermidis* expresses many morphological and secreted elements that could act as MAMPs or virulence factors. Most potential MAMPs reported so far, such as protective exopolymer PNAG, TA and matrix adhesion proteins, are involved in biofilm formation (Mack et al, 1996, Sadovskaya et al, 2005, Heilmann et al, 1997). The necrotic reaction seen in *Arabidopsis* subsequent to *S. epidermidis* inoculation appears to be a macroscopic defence response following PTI. Accordingly, treatment with boiled bacteria triggered responses similar to live cells, suggesting a pre-existing non-heat labile molecule is at the basis of the visual phenotype observed in *Arabidopsis*.

Additionally, a proteinase treatment with bacterial extracts suggested the elicitor(s) causing the necrotic reaction is not protein-based. Several carbohydrate have been reported so far in *Arabidopsis*, including *Xcc* LPS, EPS and PGN (Newman et al, 2007; Yun et al, 2006; Erbs et al, 2008b). For example, infiltration with 50µg/mL of either *Xcc* PGN or LPS results in PR1 up-regulation 24hpi (Silipo et al, 2005; Erbs et al, 2008b). As both a Gram-positive and a Gram-negative bacterium have been shown to elicit the same necrotic response in Col-0, but not in *ster1-1*, PGN was regarded a likely candidate elicitor. Subsequent tests

demonstrated elicitor activity resides with the *B. ambifaria* purified PGN and a PGN-containing *S. epidermidis* membrane fraction(MF), thereby confirming that elicitor activity is not protein-based.

Purification of PGN from *S. epidermidis* and *B. ambifaria* was undertaken to assess the biological activity of PGN and associated muropeptides. Additionally, an *S. epidermidis* membrane fraction (MF) was also included in the analysis. This fraction was obtained by omitting the hydrofluoric acid treatment step from the PGN purification procedure. GC/MS analysis confirmed both PGN preparations were pure. Additionally, the *S. epidermidis* MF fraction was shown to consist of PGN, teichoic acid (TA) and a capsular polysaccharide (CPS) different from PNAG. The purity of MAMPs is crucial and many analyses have been hampered by the presence of additional biologically-active compounds co-purified with the main elicitor (Girardin et al, 2003; Leulier et al, 2003). Infiltration with *S. epidermidis* MF and *B. ambifaria* PGN produced phenotypes mirroring those seen with the intact bacterial suspensions, while *S. epidermidis* PGN did not elicit a macroscopic response. *S. epidermidis* MF was subjected to a mild acid hydrolysis using hydrofluoric acid, a routine and well-established procedure, in order to remove TA and PS and generate pure PGN (deJonge et al 1992; Gust et al, 2007). Taken together, these results suggest either TA or CPS trigger the plant necrotic reaction or that the acid hydrolysis alters the PGN in some way. *Arabidopsis* infiltration with pure TA did not trigger a macroscopic response even at very high concentrations. Additionally, the main EPS isolated from *S. epidermidis*, PNAG, has been discounted through GC/MS analysis and the use of *S. epidermidis ica* mutants, which lack PNAG. The only other polysaccharide isolated so far from *S. epidermidis* (Karamanos et al, 1997) has also been tested in *Arabidopsis*. While this elicitor did generate a STER1-dependent chlorotic reaction, this phenotype did not match the phenotypes seen with live cells. This could suggest *S. epidermidis* CPS is different from the 20kDa PS and a *de novo* EPS purification procedure should be undertaken for *S. epidermidis* 18888.

This is the only report of a MAMP-related visual phenotype in plants. Previous studies using PGN from Gram-positive *S. aureus* (Gust et al, 2007) and Gram-negative *Xcc* and *A. tumefaciens* (Erbs et al, 2008b) did not report any macroscopic damage associated with elicitor treatments. By contrast, PGN monomers from several human pathogens, such as *Bordetella pertussis*, have been shown to cause damage to cells in culture reminiscent of disease pathology (Cloud-Hansen et al, 2006). In an effort to isolate the minimal active PGN epitope, pure PGN fractions were treated with mutanolysin, an enzyme which hydrolyzes

glycosidic linkages between disaccharide units of PGN. Mutanolysin treatment of *B. ambifaria* PGN abolished the phenotypic response, suggesting the elicitor activity resides with the conserved glycan backbone. This is in agreement with the findings of Gust et al (2007) for Gram-positive *S. aureus* PGN. However, Erbs et al (2008b) reported that muropeptides from Gram-negative plant pathogens were more active than intact PGN. The Gram-positive PGN peptide moiety differs from its Gram-negative counterpart in that it bears a key Lys residue in the stem peptide and an additional pentaglycine bridge. Consequently, it is premature to assess the minimum part required for activity in the absence of any structural data specific for *S. epidermidis* 18888 and *B. ambifaria*. The impact of the mutanolysin treatment on *S. epidermidis* PGN could not be assessed macroscopically as both fractions did not cause a phenotype in Col-0.

The ability of *S. epidermidis* MF and PGN and *B. ambifaria* PGN to act as MAMPs was assessed through several assays. Both elicitor fractions triggered changes in cytosolic calcium fluxes after 45 minutes, callose deposition, cell death and PR1 expression after 24 hours in Col-0 plants. *B. ambifaria* PGN generated little callose deposition by comparison to intact cells. This finding is not surprising as *B. ambifaria* also harbours flagella and LPS, which can act as potential PAMPs. Additionally, very low levels of electrolyte leakage were also recorded 96hpi. This was unexpected, considering the necrotic, severe response seen for *S. epidermidis* cells or MF fraction. A possible explanation for this could be that infiltration with *S. epidermidis* and *B. ambifaria* elicitors and bacterial suspensions does not generate membrane damage resulting in electrolyte leakage.

An apparent decrease in cytosolic calcium levels was recorded following exposure to *S. epidermidis* MF, possibly indicating calcium chelation. EPS has previously been reported to suppress innate immunity by calcium chelation (Aslam et al, 2008) and the lower levels of calcium seen in response to *S. epidermidis* MF might be due to the presence of CPS in this fraction. However, these experiments were performed only once and need to be repeated to ensure reproducibility. Additionally, the MF fraction is a mixture of PGN, TA and CPS and cytosolic calcium levels should also be assessed for pure *S. epidermidis* PGN and TA on their own to confirm a role for CPS in calcium chelation. Additionally, *ster1-1* plants expressing cytoplasmic aequorin should also be generated for comparison. The presence of CPS might also explain the low levels of callose deposition seen for *S. epidermidis* bacterial suspensions and MF. Similarly, Xcc EPS xanthan has been reported to suppress callose deposition (Yun et al, 2006). Nevertheless, *S. epidermidis* PGN also resulted in very low levels of callose

deposition rendering comparisons difficult. An *S. epidermidis* mutant or strain lacking CPS should be included in the panel for callose deposition comparisons. However, this strain will not be available until the identification of the CPS.

Unlike the biologically active *S. epidermidis* MF and *B. ambifaria* PGN fractions, *S. epidermidis* PGN did not induce cell death or PR1 up-regulation. Until recently, cell death was not associated with PTI but studies using flagellin from *Pseudomonas syringae* pv. *tabaci* have revoked this initial assumption (Naito et al, 2008). Similar to trypan blue data presented here, inoculation with pure flagellin resulted in cell death 24hpi.

In conclusion, reactions to all elicitor fractions were delayed when compared to flg22. This could be due to the fact that PGN and CPS are more readily perceived once bacterial cell lysis ensues.

Except for chitin, all carbohydrate MAMPs are orphan MAMPs, with a PGN receptor yet to be isolated. So far, the ubiquitous adaptor BAK1 has been implicated in PGN perception, whereas the likely candidate CERK1 has been discounted as a receptor (Shan et al, 2008; Gimenez-Ibanez et al, 2009b). The induction of the MAMP-specific early-defence gene FRK1 in response to *S. aureus* PGN was reduced in *bak1* mutants compared to wild-type Col-0 (Shan et al, 2008). Nevertheless, the authors used a commercial *S. aureus* PGN preparation and did not assess PGN purity. *Bak1*, *bkk1* and *cerk1* mutants produced phenotypes similar to the wild-type when infiltrated with *S. epidermidis*, indicating they are not involved in the phenotypic response. By contrast, *STER1* is required for several defence responses to *S. epidermidis* and *B. ambifaria* bacterial suspensions and MF and PGN elicitor solutions. A defect in *STER1* abolishes elicitor-induced cell death and PR1 expression.

Data gathered in this study suggests a role for *STER1* in PGN, and potentially CPS perception at receptor or signalling level. Firstly, *STER1* gene expression is highly induced in Col-0 following PGN and MF elicitor treatments. This result contrasts with previous reports regarding other RLKs, with FLS2 being induced only 3.5 times following flg22 treatment (Zipfel et al, 2004). Additionally, *STER1* is not listed among the genes up-regulated upon *S. aureus* PGN treatment (Gust et al, 2007). Secondly, PGN and MF fractions have been shown to prime the plant response to subsequent pathogen challenge in a *STER1*-dependent manner. Finally, *STER1* appears to localize to the intracellular vesicles following exposure to PGN, but not flg22, but more work is needed to confirm the validity of this result. Such an observation would be in accordance with previous reports indicating PRR internalization (Robatzek et al, 2006). However, all experiments were performed with a Col-0 line

expressing STER1-GFP prior to checking fusion protein functionality; a Cvi-0 line complemented with the same construct is being tested currently. STER1 appears to participate in Gram-negative, and to some extent Gram-positive PGN responses. Although *S. epidermidis* PGN does not appear to be involved in the phenotypic component of the response dependent on STER1, this elicitor does prime the immune response in Col-0 via STER1. Additionally, *S. epidermidis* PGN also triggers STER1 up-regulation and vesicular localization. These findings further emphasize a role for STER1 in general PGN perception. Although most plant pathogens are Gram negative, Gram positives can also infect plants and it would be advantageous for plants to maintain a PGN perception system for both types of PGN (Prithiviraj et al, 2005; Jha et al, 2005). The absence of PGN binding data coupled with the *N. benthamiana* transient expression results makes it difficult to assess whether STER1 acts as a PGN/CPS receptor, co-receptor or essential signalling component.

4. GENERAL DISCUSSION

In an environment that is rich in microbes, MAMP-triggered immunity (PTI) constitutes the primary plant defence response. More specifically, plants recognize conserved microbial features through PRRs and trigger responses aimed at restricting pathogen growth. The present study dealt with the isolation of STER1, a DUF26-containing RLK which participates in PGN and possibly specialized EPS-related signalling. Based on its ability to control elicitor-induced cell death, the pathogen-inducible STER1 is thought to function in disease resistance. Thus, upon the perception of a bacterial elicitor by its receptor, STER1 is probably activated and triggers signalling pathways leading to the activation of cell death in affected cells.

The DUF26 RLKs or CRKs form one of the largest protein superfamilies in *Arabidopsis*, with members displaying a novel C-X8-C-X2-C structural motif in their extracellular domain. CRKs are transcriptionally induced by oxidative stress, pathogen attack and application of SA (Wrzaczek et al, 2010; Chen et al, 2004; Acharya et al, 2007). A bioinformatic study aimed at identifying positively selected sites in the *Arabidopsis* RLK gene family found that 9 of the 12 groups under positive selection were in subfamilies that had either DUF26 or LRR receptor domains (Strain and Muse, 2005). Such findings not only emphasize the apparent significance of DUF26 proteins but also allow for the speculation that they might function in a multitude of important plant processes. Several CRKs are involved in defence regulation in *Arabidopsis*, with over-expression of CRK5 and CRK13 resulting in increased resistance to *Pst* DC3000 (Chen et al, 2003; Acharya et al, 2004). Additionally, over-expression of several CRKs by a chemically inducible promoter caused HR-like cell death (Chen et al, 2003; Chen et al, 2004; Acharya et al, 2007). In spite of increasing data on CRKs, DUF26 domains are yet to be assigned a clear function. The presence of four highly conserved cysteine residues in the extracellular domain may indicate that CRKs have a role in sensing changes of or may be modulated by redox changes in the apoplastic space during the activation of plant defence responses (Du and Chen, 2000; Wrzaczek et al, 2010). While it is true these residues may form disulfide bridges which could be potential targets for thiol redox regulation, these disulphide bonds may also participate in maintaining the three dimensional structure of the extracellular domain. Crystallization of ginkbilobin2 (Gnk-2), an antifungal protein from the gymnosperm *Ginkgo biloba* homologous to the DUF26 extracellular

domain, has confirmed the conserved cysteines form disulfide bridges which are thought to ensure the protein's structural stability (Miyakawa et al, 2009). Additionally, Gnk-2 displays a positively charged surface which was suggested to facilitate anti-fungal activity by associating with negatively charged components of the fungal surface (Miyakawa et al, 2009).

STER1 was mapped as the dominant gene controlling the response to *S. epidermidis*. Although Gram-positive bacteria have so far not been regarded as important plant pathogens, infection with *S. aureus* and *E. faecalis* caused typical bacterial disease symptoms in *Arabidopsis* which culminated in plant mortality (Prithiviraj et al, 2005; Jha et al, 2005). Leaf inoculation with *S. epidermidis* results in a necrotic response that is not associated with in planta bacterial growth, but caused by elicitor recognition. Similar to other findings (Gust et al, 2007; Willmann et al, 2010), this study demonstrates that *Arabidopsis* responds to Gram-negative PGN and possibly Gram-positive PGN through a novel PTI-like mechanism that involves *STER1*.

PGN displays characteristics that are consistent with a role as a MAMP, namely it is widely found in bacteria and structurally stable, with a conserved glycan backbone. Various evidence does indicate a role for PGN in plant pathogenicity. For example, PGN recycling and transport genes are up-regulated in *E. chrysanthemi* during plant infection, while mutation of the same genes reduces the virulence of *R. solanacearum* and *E. amylovora* (Cloud-Hansen et al, 2006). By contrast to previous reports, bacterial membrane-derived elicitors used in this study were toxic to *Arabidopsis* and resulted in macroscopic responses. PGN concentrations of 50µg/mL triggered plant immune responses, which is similar to concentrations required to stimulate *Drosophila* and mammalian immune systems (Kaneko et al, 2004). However, plant necrotic responses following *S. epidermidis* inoculation are not reproduced by pure PGN, but by a membrane fraction consisting of PGN, TA and CPS. Current results suggest only CPS component contributes significantly towards the necrotic plant response. Extracellular polysaccharides have been shown to inhibit responses such as calcium influx and the oxidative burst (Aslam et al, 2008). It is possible that the presence of CPS impairs some of the defence responses normally raised by PGN. As a result, the observed phenotypic reaction could be the result of a synergistic action of both PGN and CPS rather than a response triggered by an individual elicitor.

While Gram-positives have a thick PGN outer-layer, Gram-negative PGN is associated with the inner membrane and shielded by LPS. *S. epidermidis* PGN and CPS are

present on the bacterial surface and hence exposed to plant immune surveillance systems. However, it is less clear how *B. ambifaria* PGN is perceived; it is possible that PGN is either sensed due to the continuous turnover and release of the polymer or as a result of the degradation of bacterial cells by host defences (Park, 1995). Additionally, preliminary experiments indicate calcium influx is very low upon exposure to *B. ambifaria* PGN, as shown for other PGNs (Gust et al, 2007; Erbs et al, 2008b). A possible explanation for this could be that PGN is internalized before perception. In mammalian systems, NOD proteins recognize distinct muropeptides and function intracellularly (Rosentiel et al, 2008). Preliminary experiments have indicated STER1 localizes to intracellular vesicles following exposure to PGN, however it is unknown whether PGN is directly attached to the RLK at any point during plant perception.

Macroscopic elicitor responses were induced in *Arabidopsis* only by whole *B. ambifaria* PGN, with a mutanolysin treatment abolishing phenotypic reactions. This would suggest *Arabidopsis* has evolved a perception system for the glycan part of PGN which is highly conserved among bacteria. This contrasts with the situation in mammalian systems, where cytoplasmic NOD proteins recognize the PGN-derived peptides MDP and DAP (Girardin et al, 2003; Chamaillard et al, 2003). Additionally, *Drosophila* distinguishes between bacterial PGNs by exploiting the nature of the third residue in the peptide stem, namely lysine for Gram-positive bacteria and DAP for Gram-negative bacteria (Lim et al, 2006). In this case, a muropeptide dimer is sufficient to trigger the insect's innate immune system (Filipe et al, 2005). In the case of plant systems, previous reports have demonstrated that *S. aureus* whole PGN (Gust et al, 2007), as well as *Xcc* and *Agrobacterium* PGN-derived muropeptides (Erbs et al, 2008b) stimulate the *Arabidopsis* immune system, but a minimal PGN motif is yet to be characterized. Taken together, these data suggest that, although PGN functions as a MAMP in plants, insects and animals, each system has evolved a perception system for a distinct part of this complex molecule. In a similar fashion, the minimal active flagellar epitope differs between plants and animals; while *Arabidopsis* FLS2 recognizes the conserved flg22 peptide associated with the N-terminus, mammalian TLR5 senses a distinct peptide belonging to the hypervariable central region of flagellin (Zipfel and Felix 2005; Ramos et al, 2004).

In accordance with previous studies (Gust et al, 2007; Erbs et al, 2008b), elicitors used here were not as potent as flg22. This is not surprising considering the redundancy of MAMP perception systems in plants, as several features of a given class of microbes will be

recognized through several PRRs. It can be assumed that each MAMP triggers specific sets of both specific as well as generic cellular responses; additionally, strength, kinetics, and duration of the generated responses might differ. For example, there is only a small degree of overlap between genes up-regulated upon treatment with flg22, PGN or chitin (Ramonell et al, 2005; Gust et al, 2007). Similarly, animals activate specific responses to different MAMPs partly through selective usage of adaptor molecules linked to TLRs followed by differential activation of transcription factors (Akira et al, 2006). For instance, mammalian TLR2 participates in the recognition of tri-acylated lipoproteins when coupled to TLR1, while di-acylated lipoproteins signal through the TLR2-TLR6 complex (Jin et al, 2007; Takeuchi et al, 2001). Similarly, different MAMP perception systems in plants might rely on different classes of receptor molecules.

Interestingly, proteinaceous MAMPs such as flagellin and Ef-Tu are perceived by the same type of receptor (LRR-RLK) and induce nearly identical responses (Kunze et al, 2004; Zipfel et al, 2006). The identity of the plant PGN receptor(s) and its nature is still outstanding. Several PGN recognition molecules are known in animals including GPI anchored CD14, NODs, TLR2 and PGRPs (Dziarski et al, 1998; Rosentiel et al, 2008; Dziarski and Gupta, 2005; Dziarski and Gupta, 2006). Recently, the characterization of an *Arabidopsis* PGN receptor has been reported (Willmann et al, 2010). LYM3, a GPI-anchored protein with an extracellular domain consisting of two LysM domains, was shown to specifically bind Gram-positive and Gram-negative PGN and not other complex carbohydrate ligands. This finding is not consistent with plants maintaining a distinct perception system for Gram-positive and Gram-negative PGN similar to *Drosophila* (Royet et al, 2005). Further data supporting a role for LYM3 in PGN triggered plant defence include the fact that genetic inactivation of *LYM3* abolished PGN-induced plant responses and the increased susceptibility of *lym3* alleles to Pst DC3000 infection. However, as LYM3 lacks an intracellular domain, the expectation would be that it functions in combination with an RLK-type protein towards relaying the message from the extracellular matrix to the intracellular milieu. In a similar manner, LPS perception in mammals requires the participation of GPI-anchored CD14 alongside TLR4 (Shimazu et al, 1999). Interestingly, CD14 is also required together with TLR2 for PGN detection confirming a common element participates in the recognition of both carbohydrate MAMPs in mammals (Dziarski et al, 1998; Dziarski and Gupta, 2005). In plant systems, interactions between RLKs and proteins lacking a signalling domain have also been reported (LeEIX1/2 xylanase receptor of tomato) or suggested (rice CeBiP and soybean

GBP) (Ron and Avni, 2004; Kaku et al., 2006; Umemoto et al, 1997). Taking everything into account, a model for PGN perception involving LYM3 and STER1 can be proposed (Figure 4.1). In this case, STER1 might function as the RLK component of the PGN perception system. LYM3 could bind PGN and present it to STER1 directly or via another protein, thus triggering signalling cascades that result in a defence response. Preliminary experiments examining the responses of *lym3* mutants to *B. ambifaria* PGN and *S. epidermidis* MF have shown that the chlorotic/ necrotic phenotypes observed in wild-type plants are maintained in these mutants. However, other defence-associated parameters such as PR-1 induction also need to be investigated before drawing a conclusion on the role of LYM3 in the perception of the elicitor fractions used in this study.

Additionally, STER1 might also participate in *S. epidermidis* CPS perception. It is possible that CPS is also perceived through STER1 directly, via the positively charged surface of the CRK extracellular domain, or indirectly, via a different accessory protein. Correspondingly, mammalian TLR2 has been involved in both *S. aureus* PGN and *S. epidermidis* PIA perception (Dziarski and Gupta, 2005; Stevens et al, 2009). It is possible that CPS chelates Ca^{2+} and impedes other plant defences, prompting the suggestion the SA-dependent reaction to *S. epidermidis* is the result of a synergistic reaction between PGN and CPS. However, no clear conclusions can be drawn until the CPS has been purified and confirmed to cause the *S. epidermidis* phenotype on its own.

Ligand-induced multimerization has been proposed as a key event in the activation of several TLRs. The mammalian LPS recognition system relies on LPS interfacing with both MD-2 and TLR4. In the same way, *Drosophila* recognizes Gram-negative PGN through PGRPs; these proteins form dimers, with one molecule interacting with the tetrapeptide stem and the other binding the disaccharide moiety of the PGN molecule (Lim et al, 2006). Bioassays performed here have indicated that the glycan backbone is necessary for the STER1-dependent macroscopic response observed in *Arabidopsis* after infiltration with *B. ambifaria* PGN. On the other hand, LysM motifs commonly bind GlcNAc (Buist et al, 2008). As a result, it is conceivable LYM3 and STER1 interface through the PGN molecule, with STER1 perceiving MurNAc possibly together with some of the peptide stem residues in PGN, and LYM3 possibly binding the GlcNAc moiety of this complex molecule. Additionally, STER1 might also interact with LYM3 or another protein recruited to the recognition complex.

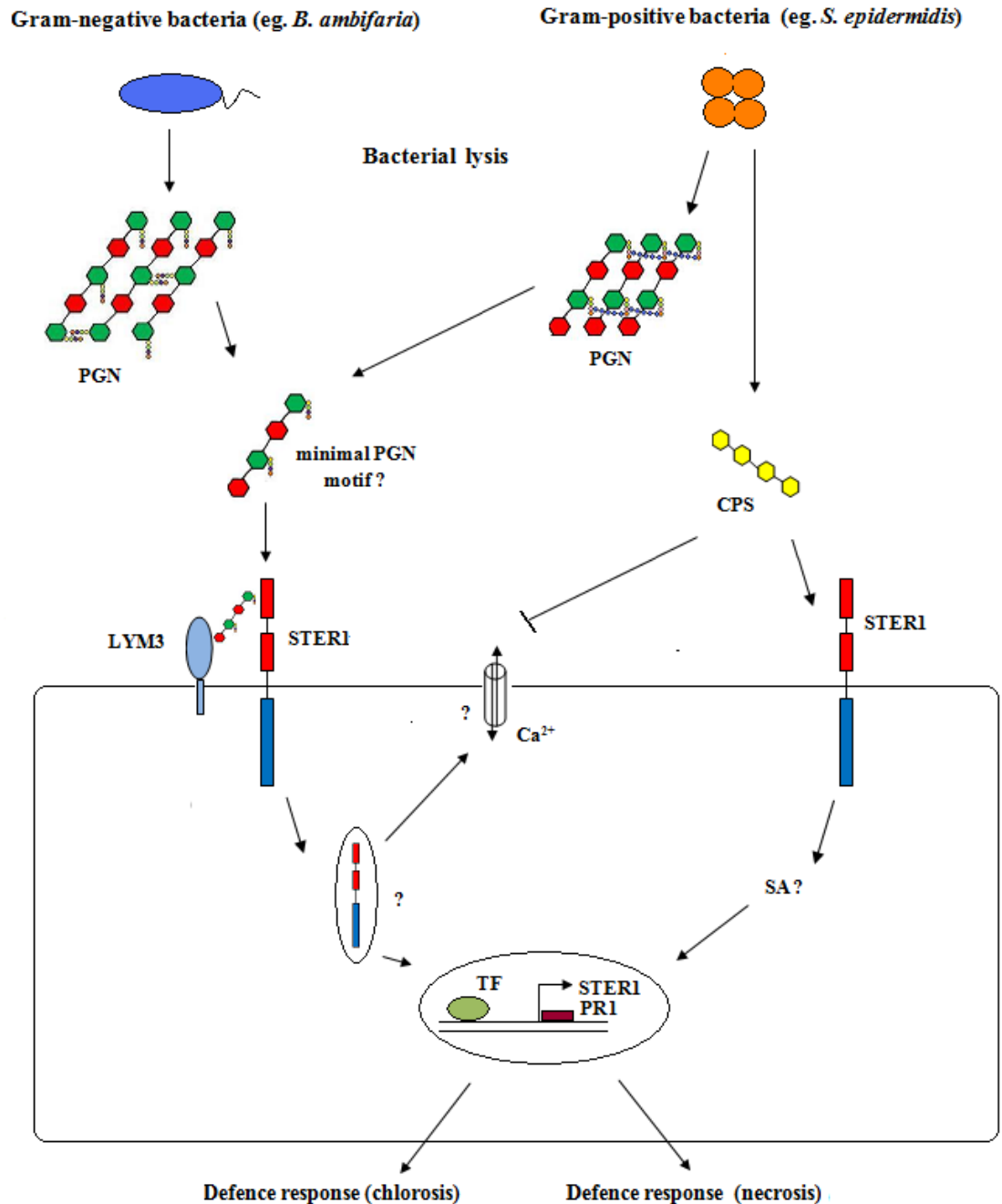


Figure 4.1. Model for STER1 function in MAMP perception. Once inside the apoplast, bacteria that are unable to proliferate *in planta* release MAMPs as a result of bacterial lysis. Gram-negative bacteria release PGN, a polymer consisting of MurNAc (green boxes), GlcNAc (red boxes) and peptide subunits. Gram-positive bacteria release PGN, which has an additional interpeptide bridge compared to Gram-negative PGN, and CPS, a glucose polymer (yellow boxes). Presumably, a minimal PGN motif is perceived by GPI-anchored LYM3 protein via the GlcNAc moiety and presented to STER1, which might internalize as part of PGN-related signalling. Further intracellular signalling leads to specialized transcription factor (TF) binding and up-regulation of STER1 and PR1 expression. In the case of Gram-positive bacteria, CPS is perceived directly by STER1 or an accessory protein and triggers plant defences consisting of up-regulation of STER1 and PR1 expression. CPS chelates Ca^{2+} and impedes other plant defences, prompting the suggestion the SA-dependent reaction to *S. epidermidis* is the result of a synergistic reaction between PGN and CPS. Infection with *B. ambifaria* triggers chlorosis, whereas infection with *S. epidermidis* triggers necrosis.

Structural data is not available at this stage for the isolated *B. ambifaria* and *S. epidermidis* PGN fractions. A study examining activities of *Xcc* and *A. tumefaciens* PGN-derived fractions in *Arabidopsis* revealed structural differences between the two distinct mucopeptide pools (Erbs et al, 2008b). While a change from an Ala to a Gly residue was seen for the subtle pathogen *A. tumefaciens*, PGN from the necrotroph *Xcc* lacked an acetyl group. It is tempting to speculate these variations underlie the different eliciting activities of *A. tumefaciens* and *Xcc* PGN fractions. Similarly, the apparent toxicity of *B. ambifaria* PGN by comparison to *S. epidermidis* PGN in *Arabidopsis* might be explained by structural data. Additionally, changes in *S. epidermidis* CPS structure might also lead to an altered response upon plant inoculation. Absence or modification of CPS might explain the varied responses seen in *Arabidopsis* following inoculation with different *S. epidermidis* strains. MAMP modifications have been shown to contribute towards the evasion of the plant immune response (Taguchi et al, 2009) and this might be the case here as well.

In conclusion, the present study has demonstrated STER1 is essential for PGN and, possibly, specialized CPS-related perception and/or signalling. It is yet unclear whether STER1 perceives MAMPs directly, or whether it perceives changes in the apoplastic redox state as suggested recently by Wraczek et al (2010). EPS have been shown to suppress ROS production following flg22 exposure (Aslam et al, 2008), so it is conceivable *S. epidermidis* CPS could act similarly in the apoplast, thus activating STER1-dependent defence responses. The question arises of how Cvi-0, a natural FLS2 mutant (Dunning et al, 2007), would be affected from also not expressing STER1. Exposure to *S. epidermidis* results in an exaggerated defence response in most accessions tested, in spite of this bacterium not proliferating inside the apoplast. In view of this, it could be inferred that Cvi-0 benefits from modulating the STER1 element and avoiding a metabolically costly defence response to this non-adapted pathogen. Since MAMP perception systems are redundant, with plants perceiving pathogen presence through several elements, lacking some MAMP signalling elements will not fully abolish PTI in Cvi-0 as it will detect bacterial presence through other PRRs.

5. FUTURE WORK

Findings presented in this study revealed the existence of STER1, a DUF26-RLK that functions in MAMP perception and/or signalling.

So far, whole *B. ambifaria* PGN was shown to trigger a chlorotic response in *Arabidopsis*. Nevertheless, the minimal PGN motif required for elicitation is yet to be identified. Synthetic organic chemistry devoted to the comprehension of this minimal structure is required to help further our knowledge of PGN perception. Furthermore, structural analysis of mucopeptides from *B. ambifaria*, *S. epidermidis* and well-studied plant pathogens will be performed in order to reveal the minimal inducing structure and the influence of chemical modifications. Similarly, CPS from several *Staphylococcus* strains should be purified and compared at the structural level. Presumably, variations in their composition or modifications will parallel differences in their defence eliciting abilities. Additionally, STER1 involvement in LPS signalling should also be investigated using LPS from *B. ambifaria* and other bacteria, as well as LPS mutants.

Fluorescent labelling of PGN and CPS could also provide a powerful tool to analyse the dynamics of their localisations and associations upon triggering the STER1 pathway. These labelled carbohydrates could be used in conjunction with the GFP-tagged version of STER1 generated in this study towards visually examining the STER1-PGN interaction.

Stable transgenic *Arabidopsis* lines that express a TAP-tagged STER1 under the control of a 35S promoter have been generated. *In planta* purification of native STER1-TAP from healthy and pathogen-challenged leaf material might allow for the identification of bound protein interactors or post-transcriptional modifications via mass-spectrometry that could be important for STER1-directed signal transmission. The results obtained from these experiments will be vital to understand signal relay through STER1 upon plant exposure to carbohydrate MAMPs.

Finally, the expression of the extracellular domain of STER1 is currently being attempted in *E.coli*. If the generated recombinant protein is soluble, crystallization assays will be initiated, as structural data on this domain could be instrumental in clarifying STER1 function.

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