IMMUNE SIGNALING IN *ARABIDOPSIS THALIANA* UPON PERCEPTION OF BACTERIAL AND VIRAL MOLECULAR PATTERNS WITH A SPECIAL EMPHASIS ON ROOTS

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SUMMARY

In order to recognize a vast variety of attackers, plants possess a plethora of sophisticated detection systems. Perception of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by the plant pattern recognition receptors (PRRs) leads to subsequent initiation of defense responses, a process collectively referred to as pattern-triggered immunity (PTI). PTI has been extensively studied in plant leaves, especially of the model organism *Arabidopsis thaliana*, whereas the mechanisms underlying PTI in roots so far attracted less attention. However, since a vast number of plant pests are soil-borne and attack roots in order to propagate and colonize whole plants, understanding the mechanisms underlying basic defense at the root level is of high interest for the development of new tools to combat root pathogens of crop plants.

It has been demonstrated that recognition of flg22, the conserved epitope of the bacterial flagellin protein, leads to tissue-specific defense responses in roots. In order to investigate the cause for this tissue-specific induction of downstream responses, several approaches were employed during the course of this work. By studying the cellular localization of the PRR recognizing flg22, FLAGELLIN-SENSING 2 (FLS2), we were able to depict an expression map of *FLS2* in wild-type Arabidopsis plants. Our study revealed that *FLS2* was expressed in a highly tissue-specific manner in roots and shoots and that the *FLS2* promoter activity was inducible upon environmental stimuli as well as during developmental processes, changing not only in intensity in expressing tissues but also in tissue-specificity. These results indicate an important role of the tissue-specific PRR localization in immunity mechanisms.

In a parallel study, we expressed *FLS2* under the control of several root tissue-specific promoters, which allowed us to analyze the competence of these tissues to detect flg22. Unexpectedly, all investigated root tissues were able to perceive externally applied flg22. In fact, PTI responses could be activated in intact roots as well as in dissected roots, suggesting that the peptide is able to penetrate through the different tissue layers. Remarkably, the expression level of the receptor was not the major parameter determining the magnitude of the immune response output. Thus, we postulated that perception of flg22 by certain tissues leads to stronger PTI responses potentially indicating why plants restrict immune receptor accumulation to tissue-specific locations possibly in order to balance the outcome of the defense activation.

Due to the fact that many developmental or immunity processes in plants depend on systemic communication between different plant organs and that beneficial root microbes are known to prime and enhance resistance in aerial plant tissues, we hypothesized that MAMP perception by roots might induce a signaling event from roots to shoots. In order to address the potential existence of such systemic alarm signals, various methods were implemented. However, we encountered several technical limitations mainly concerning elicitor diffusion. Therefore, we focused on the development of an improved application method for studying systemic root-to-shoot signaling in Arabidopsis plants. Our system proved suitable to perform systemic signaling analysis and revealed that at the transcriptional level no systemically activated defense gene modifications were detectable in distal shoots of root-treated plants in our conditions.

Like root pathogens, also viruses constitute a major threat in agro-economy and are responsible for immense crop losses. The basal defense response against viruses is thought to be mediated by RNA silencing, a process by which viral replication intermediates are cleaved and degraded by the plant silencing machinery through the recognition of virus-derived small RNAs. Intriguingly, a recent study conducted in our lab demonstrated a role of BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1), a coreceptor of several PRRs involved in immunity and development, in antiviral defense. These results indicated that PTI may also contribute to antiviral resistance but the exact recognition process remained elusive. Because dsRNA produced during viral replication has been shown to act as a PAMP in animals, we decided to test whether dsRNA is perceived as a viral PAMP *in planta* as well. We found that natural as well as synthetic dsRNA is indeed perceived as a PAMP by Arabidopsis, leading to the activation of typical PTI responses. Remarkably, dsRNA application also promoted protection of Arabidopsis plants against viral infection.

Taken together, this study provides new insights into the recognition mechanisms of bacteria- and virus-associated molecular patterns by different plant organs and contributes to elucidate the molecular defense strategy of plants against agriculturally important diseases.

LIST OF ABBREVIATIONS

Aa	Amino acid
At	*Arabidopsis thaliana*
BAK1	Brassinosteroid insensitive 1-associated kinase
BR	Brassinosteroid
BRI1	Brassinosteroid insensitive 1
BIK1	Botrytis-induced kinase 1
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementing DNA
CDPK	Calcium-dependent protein kinases
CERK1	Chitin elicitor receptor kinase 1
Col-0	Columbia-0 ecotype
CS	Casparian strip
DAMP	Damage-associated molecular pattern
DCL	Dicer-like
Dpi	Day(s) post inoculation
DNA	Deoxyribonucleic acid
Ds	Double-stranded
DZ	Differentiation zone
EFR	Elongation Factor TU Receptor
EF-Tu	Elongation factor thermo unstable
elf18/26	18/26-amino acid peptide of the N-terminus of EF-Tu
ER	Endoplasmic reticulum
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EZ	Elongation zone
flg22	22-amino acid peptide of the N-terminus of flagellin
FLS2	Flagellin-sensing 2
FRK1	Flagellin responsive kinase 1
GFP	Green fluorescent protein
GUS	β-glucuronidase
HAMP	Herbivore-associated molecular pattern
HR	Hypersensitive response
ISR	Induced systemic resistance
JA	Jasmonic acid
LPS	Lipopolysaccharides
LR	Lateral root
LRR	Leucine-rich repeat
LysM	Lysin motif
LYK	LysM receptor kinase

MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MS	Murashige & Skoog medium
MZ	Meristematic zone
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nucleotide binding
Nb	*Nicotiana benthamiana*
OG	Oligogalacturonides
ORMV	Oilseed rape mosaic virus
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pep	Danger peptide
PEPR	Pep-receptor
PER5	Peroxidase 5
PGN	Peptidoglycan
PGPR	Plant growth promoting rhizobacteria
PM	Plasma membrane
poly(I:C)	Polyinosinic-polycytidylic acid (synthetic analog of dsRNA)
poly(I)	Polyinosinic acid (synthetic analog of ssRNA)
PR	Pathogenesis-related
PRR	Pattern recognition receptor
Pro	Promoter
Pto	*Pseudomonas syringae pv tomato*
PTI	Pattern/PAMP-triggered immunity
QC	Quiescent center
qRT	Quantitative real time PCR
R gene/protein	Resistance gene/protein
RBOH	Respiratory-burst oxidase homologue
RLK	Receptor-like kinase
RLP	Receptor-like protein
RLU	Relative light unit
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SERK	Somatic embryogenesis receptor kinase
SGI	Seedling growth inhibition
siRNA	Short interfering RNA
SOBIR1	Suppressor of BIR1-1
Ss	Single-stranded
TLR	Toll-like receptor
UBQ	Ubiquitin

1 GENERAL INTRODUCTION

Plants convert light energy, carbon dioxide and water into chemical energy fixed as sugar, thereby making carbon available for other organisms. While they are the primary producers in the food chain, plants are constantly attacked by a vast number of feeding enemies. Furthermore, as sessile organisms, plants are continuously exposed to ever-changing environmental conditions in nature. Nevertheless, only a small fraction of biotic attacks results in a successful infection and plants prevail in or even dominate most environments. Indeed, they are resistant to many foes due to the evolution of an efficient, multilayered defense system controlling constitutive and inducible responses (Thordal-Christensen, 2003; Jones and Dangl, 2006; Howe and Jander, 2008).

The first line of plant defense is composed of structural and physiological barriers such as a waxy cuticle covering the leaf surface, thorns, trichomes, as well as a robust cell wall. The numerous structural polymers forming the cell wall, such as lignin, cellulose or suberin provide the plant with an extremely efficient barrier, which prevents microbes from entering host tissues (Thordal-Christensen, 2003). In addition to these mechanical barriers, plants possess a chemical defense shield supplied by the constitutive or inducible production of repellent molecules or antimicrobial compounds. The secretion of these potentially harmful secondary metabolites deters most organisms from attacking the plant (Thordal-Christensen, 2003). Occasionally, some microbes are able to overcome these primary obstacles and colonize the plant tissues. In such cases, they have to face a highly sophisticated plant immune system. Plants specifically recognize molecules derived from the invading organism or from already attacked plant cells leading to a set of immediate and long-term local and systemic defense responses (Boller and Felix, 2009; Dangl et al., 2013).

In the following chapter, I will describe the initiation and integration of defense signaling pathways against biotic invaders and discuss the most important mechanisms and paradigms of these signaling systems. Subsequently, I will focus on the knowledge about plant roots and their immunity because defense responses induced in roots, within the scope of the basal resistance against soil-borne pathogens, are poorly known compared to the shoot's immune responses (Okubara and Paulitz, 2005). As roots are the organs the most subjected to microbial interactions, understanding the molecular mechanisms underlying root immunity is crucial.

1.1 Plant innate immunity

1.1.1 Pattern-triggered immunity

Plants lack the somatic adaptive immunity mechanisms of vertebrates, involving mobile circulating defender cells like macrophages specialized in enemy recognition and destruction. Thus, they rely solely on innate immune responses. Each plant cell is individually able to detect putative "danger", initiate signaling cascades to induce defense responses and alert other plant cells or tissues of the imminent attack (Schilmiller and Howe, 2005; Jones and Dangl, 2006). Figure 1.1 provides an overview of the cellular components involved in plant immunity. Potential pathogens can be detected by membrane-bound receptors, so-called pattern recognition receptors (PRRs), which recognize essential patterns exposed by the invading organism, called microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) (Medzhitov and Janeway, 2000; Boller and Felix, 2009). Plant PRRs share remarkable similarities with mammalian TOLL-LIKE RECEPTORS (TLR), recognizing pathogens at the cell surface (Medzhitov, 2001; Hopkins and Sriskandan, 2005). In addition, plant PRRs can detect endogenous self-modified molecules, the damage-associated molecular patterns (DAMPs) (Newman et al., 2013). PRR activation upon MAMP or DAMP perception subsequently initiates downstream signaling and basal defense responses leading to non-host resistance (Zipfel et al., 2004; Boller and Felix, 2009). This first level of immunity is referred to as pattern-triggered immunity (PTI) (Ausubel, 2005; Jones and Dangl, 2006).

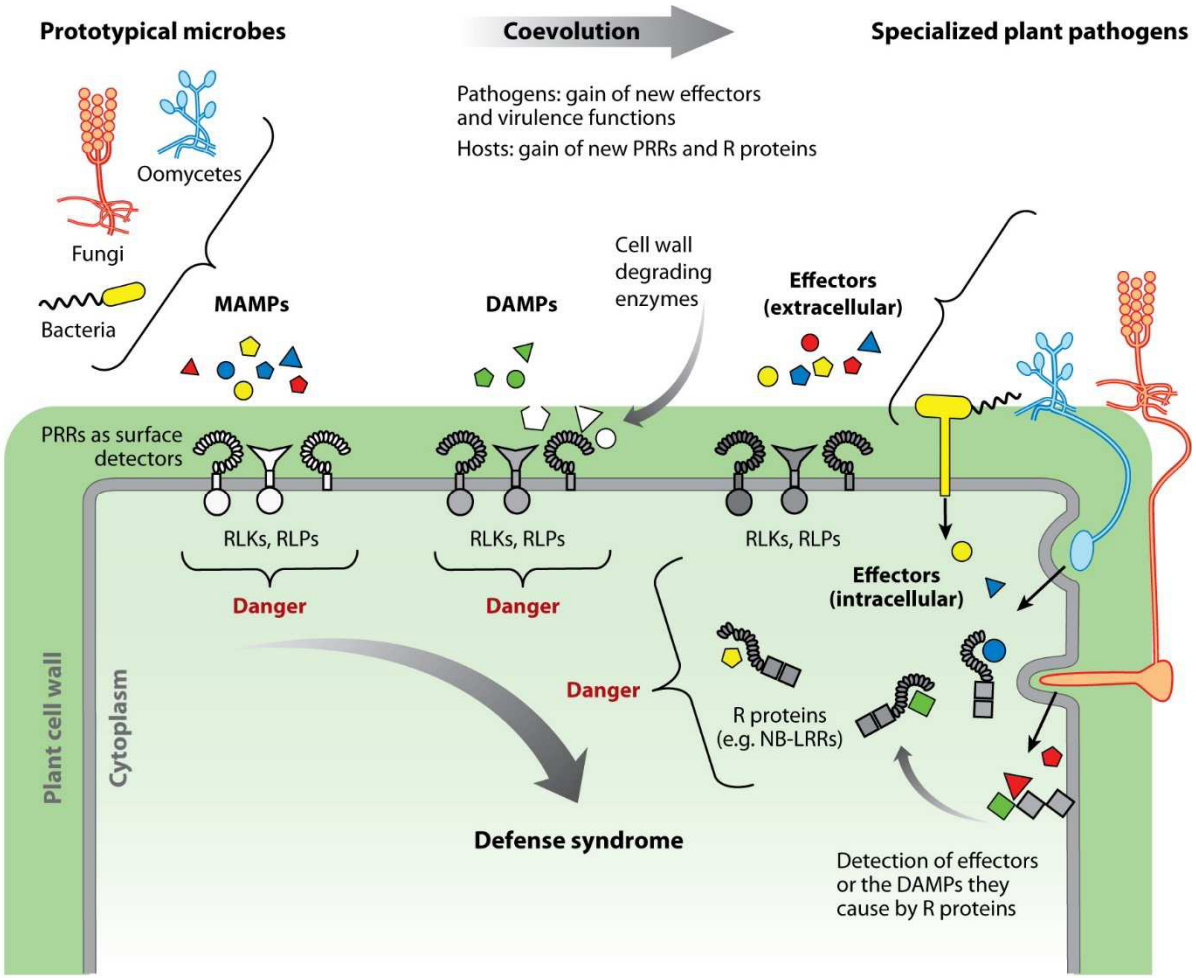

Fig. 1.1 Overview of danger perception by a plant cell. Perception of extracellular microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs) alerts cells to dangers. In the course of coevolution, pathogens gained effectors as virulence factors to inhibit MAMP signaling. In turn, plants evolved new immune receptors, called resistance (R) proteins in order to perceive effectors and reestablish immunity. A partially conserved panel of defense responses is induced upon perception of MAMPs, DAMPs and effectors by PRRs and R proteins, respectively. RLK, receptor-like kinase; RLP, receptor-like protein; NB-LRR, nucleotide binding-site-leucine-rich repeat. Adapted from Boller and Felix (2009).

1.1.2 Effector-triggered susceptibility

Although PTI is in general very efficient, successful pathogens have evolved strategies to overcome this defense system by injecting virulence effectors across the plant cell wall, which inhibit specific steps of PRR signaling (Fig. 1.1). This way, the effector activity contributes to plant pathogenesis, in a process known as effector-triggered susceptibility (ETS) (Jones and Dangl, 2006; Boller and He, 2009; Dodds and Rathjen, 2010). For example, the plant pathogenic strain *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000 uses the type III secretion system, which is widely spread in pathogenic bacteria, to introduce effectors into the host's

cytoplasm (Abramovitch et al., 2006; Cunnac et al., 2009). In general, a virulent bacterium delivers about 15-30 type III effectors, some of which can promote pathogen virulence by directly counteracting PTI. This is the case for the AvrPto and AvrPtoB effectors from *Pseudomonas*, which directly interact with PRRs involved in MAMP detection to inhibit the initiation of downstream signaling processes (Göhre et al., 2008; Shan et al., 2008). Other effectors do not directly target PTI signaling or MAMP recognition. For instance, HopU1 interferes with some RNA-binding proteins to directly activate transcriptional reprogramming in host cells and improve microbial survival (Fu et al., 2007) whereas other effectors have been shown to target the hormonal integration of defense responses (da Cunha et al., 2007). Effectors are also present in fungi and can be trans-located into plant cells through the haustorial interface where they interfere with the host's immune system (Panstruga and Dodds, 2009).

In the case of viruses, these intracellular pathogens use the host translation machinery to produce their effectors. The only known viral effectors today act as suppressors of RNA silencing, the primary defense response against viruses (Zvereva and Pooggin, 2012). Viral silencing suppressor proteins are distinct among members of the different viral families, which indicates their independent evolution (Chapman et al., 2004; Pumplin and Voinnet, 2013). Since a viral PAMP has not yet been identified, it is unclear whether viral effectors also target the classic PTI signaling pathways. However, there is growing evidence that viruses have evolved effectors, which function to suppress innate immune responses and RNA silencing (Zvereva and Pooggin, 2012; Kørner et al., 2013).

1.1.3 Effector-triggered immunity

During evolution, plants have evolved strategies to counteract the activity of effectors. This adaptation is referred to as effector-triggered immunity (ETI), a second layer of defense formerly known as "gene-for gene" resistance (Flor, 1971). Plants are able to either recognize the presence of effectors by direct binding or indirectly by detecting their activity in the host cells (Boller and He, 2009). For this recognition step, they use another type of immune receptors called resistance R proteins, which are intracellular nucleotide binding leucine-rich repeat (NB-LRR) proteins (Jones and Dangl, 2006) having striking similarities with animal proteins also involved in immunity (Inohara and Nuñez, 2003; Rairdan and Moffett, 2007; Fig. 1.1).

Upon effector recognition, R proteins get activated and mediate usually very strong defense responses. Although some of the downstream ETI events partially overlap with PTI responses, the stronger ETI responses are frequently accompanied by a hypersensitive response (HR) (Boller and Felix, 2009). HR is characterized by cell death development in the infested and surrounding tissues and is rarely observed in response to MAMPs (Greenberg and Yao, 2004; Jones and Dangl, 2006; Truman et al., 2006; Tsuda and Katagiri, 2010). Generally, these initial steps of plant immunity strongly resemble the system of innate immunity in animals (Medzhitov and Janeway, 2000; Boller and Felix, 2009). The cellular components involved in ETI are also shown in Figure 1.1.

PTI is mainly based on the recognition of highly conserved microbial structures, which are difficult for the microbes to modify without affecting their virulence. In contrast, effectors are not essential for microbial survival (Dangl and Jones, 2001). Thus, whereas PTI targets microbes in general, ETI is rather an evolutionary dynamic process including constant adaptation and alternations in plant and pathogen structures in order to be one step ahead of the opponent. Consistent with the hypothesis of co-evolution, effectors are extremely diverse with little amino acid (aa) similarity among them and recognized in a highly specific fashion by the host R proteins, which are present in particular plant cultivars (White et al., 2000; Jones and Dangl, 2006; Niehl and Heinlein, 2009). This ongoing evolutionary arms race between plants and pathogens in order to achieve or avoid recognition is nicely visualized in the "zigzag" model demonstrated in Figure 1.2, proposed by Jones and Dangl (Jones and Dangl, 2006).

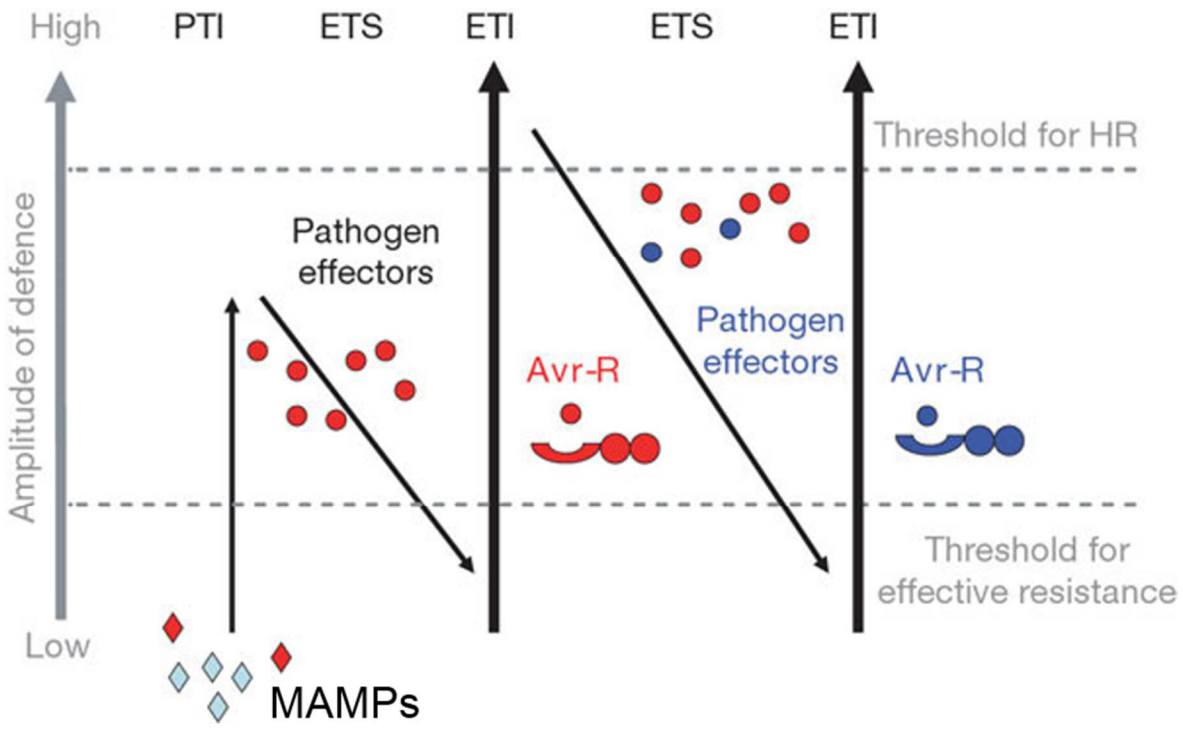

Fig. 1.2 "Zigzag" model elaborated by Jones and Dangl (2006). This figure describes the evolutionary basis of effector-triggered susceptibility (ETS) and effector-triggered immunity (ETI). Detection of microbe-associated molecular patterns (MAMPs) by the plant leads to pattern-triggered immunity (PTI). In turn, PTI is evaded by the ability of certain adapted pathogens to produce effectors (Avr-R), which interfere with PTI, leading to ETS. The recognition of these pathogen-specific effectors by plant R proteins can activate an enhanced immune response, referred to as ETI. ETI is often an amplified version of PTI, might passing a threshold for induction of hypersensitive cell death (HR). Pathogen isolates that have lost the primary effectors (red) and possibly gained new effectors through horizontal gene flow (in blue) may suppress ETI. In response, plants might evolve new receptor alleles recognizing one of the newly acquired effectors, which results again in ETI. This ongoing gain and evasion of detection constitutes the paradigm of Jones' and Dangl's "zigzag" model of ETS/ETI. Adapted from Jones and Dangl (2006).

1.2 Elicitors of non-host resistance

In order to detect the many potentially harmful organisms and initiate PTI, plant PRRs specifically recognize conserved molecules derived from invading organisms or from already attacked plant cells (Boller and Felix, 2009; Dangl et al., 2013). Several structural components of the microbial cell wall have been shown to elicit defense responses in plants, including peptidoglycan (PGN) (Gust et al., 2007), β-glucans (Klarzynski et al., 2000), bacterial lipopolysaccharides (LPS) (Newman et al., 1995; Meyer et al., 2001), and fungal polysaccharides such as chitin fragments (Felix et al., 1993; Miya et al., 2007). However, the nature of molecular patterns identified as triggers of plant immunity is diverse and their number is constantly increasing. The following subchapters will address the most significant molecular patterns described to act as elicitors of non-host resistance. An overview of the so far characterized MAMPs/DAMPs and their corresponding PRRs is presented in Table 1.1.

1.2.1 Bacterial MAMPs

One of the best characterized MAMPs active in plants and animals is the protein flagellin, forming helical filaments that constitute the bacterial flagellum (Felix et al., 1999; Wyant et al., 1999; Smith et al., 2003). As the flagellum is the main bacterial motility organ it has a strong impact on bacterial virulence (Taguchi et al., 2008).

The N- and C-terminal sequences of flagellin are conserved whereas the middle part, exposed to the outside, is highly variable. The epitope shown to be sufficient for significant defense elicitation in plants, at nanomolar concentrations, is a highly conserved 22-aa sequence present in the N-terminus of the protein, called flg22 (Felix et al., 1999). Despite differences in specificities and efficiencies, flg22 has been observed to act as a MAMP in most plants species (Felix et al., 1999; Albert et al., 2010a). Additionally, different epitopes of flagellin were recently identified and shown to modulate the induction of PTI responses in different plant species. These include flgII-28 which is only active in solanaceaeous species (Cai et al., 2011) and CD2-1, the C-terminal region of flagellin, eliciting PTI responses in rice (Katsuragi et al., 2015). Beside leading to several typical PTI responses, flg22 perception has been shown to strongly enhance disease resistance to the pathogen *Pto* DC3000 (Zipfel et al., 2004).

Importantly, microbial cell wall and structural components are not the only MAMPs recognized. Bacterial cold-shock proteins and the elongation factor thermo unstable (EF-Tu)

are recognized in tobacco and *Brassicaceae,* respectively, although these proteins are soluble and localized to the cytosol of microbes (Felix and Boller, 2003; Kunze et al., 2004).

EF-Tu is the most abundant bacterial protein, conserved over a wide variety of species and shows 90% sequence homology between hundred bacterial species (Kunze et al., 2004). EF-Tu is essential for the elongation of the aa chain during protein synthesis and therefore plays a crucial role in bacterial mRNA translation (Steitz, 2008). Comparably to flg22, plants are able to perceive a highly conserved 18- or 26-aa sequence present at the N-terminus of the protein, exhibiting its highest elicitor activity when N-acetylated. This short peptide, elf18 or elf26, is sufficient to induce a defense response comparable to full-length EF-Tu, but in contrast to flg22, which is active in most tested plant species, it is known to be recognized only by *Brassicaceae* (Kunze et al., 2004). However, a recently identified 50-aa epitope derived from the central region of the EF-Tu bacterial protein, termed EFa50, has been shown to be fully active as MAMP in rice (Furukawa et al., 2014). Like in the case of flg22, this result represents one more line of evidence for convergent evolution of MAMP perception systems in plants.

1.2.2 Fungal MAMPs

Plants mainly sense fungal microbes through the perception of chitin fragments. Chitin is the main structural component of the fungal cell wall and is a long-chain polymer of a N-acetylglucosamine, a derivative of glucose (Felix et al., 1993; Shibuya et al., 1993). Further known fungal MAMPs include ergosterol, a component of fungal cell membranes (Granado et al., 1995) and xylanase, an enzyme able to degrade hemicelluloses, one of the major components of the plant cell wall (Hanania and Avni, 1997). β-glucans, which are cell wall components of certain fungi, oomycetes and bacteria are also sensed as MAMPs by several plant species as legumes and tobacco (Klarzynski et al., 2000). However, the list of characterized fungal MAMPs is currently increasing. For instance, the toxin cerato-platanin BCSpL1 from *Botrytis cinerea* was recently shown to induce defense responses in tobacco (Frías et al., 2013; Klemptner et al., 2014). Additionally, fungal endo-polygalacturonases, a class of secreted pectinases, are recognized as MAMPs in Arabidopsis (Zhang et al., 2014). Interestingly, necrosis and ethylene-inducing peptide 1 (Nep1)-like proteins (NLPs), secreted by a wide range of plant-associated microorganisms including fungi, oomycetes and bacteria, have been shown recently to trigger immunity in Arabidopsis (Oome et al., 2014).

1.2.3 Endogenous DAMPs

In addition to MAMPs, plants can recognize different DAMPs, which are endogenous molecules or fragments of damaged cells and tissues that can act as elicitors of defense responses. These DAMPs are either signals actively synthesized by plants, or passively produced upon damage of plant structures (Yamaguchi and Huffaker, 2011).

For instance, AtPep1 is an inducible peptide originating from a longer precursor protein (*PROPEP*) in Arabidopsis in response to microbe infection and other stresses (Huffaker et al., 2006). AtPep1 is thought to be recognized by the plant to amplify PTI through a positive feedback loop (Yamaguchi et al., 2006; Huffaker and Ryan, 2007), just like the tomato peptide systemin, which induces PTI responses in solanaceous plants (Ryan et al., 2002; Marmiroli and Maestri, 2014). Similarly, systemins derive from a longer precursor protein (Prosystemin) via so far unknown cleavage mechanisms induced upon herbivore and pathogen detection (Ryan and Pearce, 2003).

On the other hand, oligogalacturonides (OGs) would be an example of passively emerged signals that can elicit defense responses (Ferrari et al., 2013). OGs are sugar polymers derived from the pectin component of plant cell walls upon degradation by pathogens or damage associated to herbivory (Ferrari et al., 2013). Both, exogenously applied and *in-vivo*-released OGs have been shown to act as DAMP signals to trigger immunity in Arabidopsis (Brutus et al., 2010; Benedetti et al., 2015). Interestingly, the release of ATP by cell damage has recently been shown to serve as a DAMP in Arabidopsis. Extracellular ATP is perceived by a plasma membrane-localized receptor leading to downstream immune responses (Choi et al., 2014; Tanaka et al., 2014a).

DAMPs are generally recognized by surface located receptors of the PRR-type (Newman et al., 2013). Since the discovery of the first of these DAMP receptors, PEP RECEPTOR 1 (PEPR1) and PEP RECEPTOR 2 (PEPR2) recognizing AtPep1 in Arabidopsis (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010) several DAMP/PRR pairs have been characterized (Table 1.1; Zipfel, 2014). Nevertheless, DAMPs are often recognized in a plant family-specific manner and broadening the current knowledge to other plant species could render interesting new results about recognition and signaling specificities.

1.2.4 Viral PAMPs

Plant viruses, as all viruses, are obligate intracellular pathogens. As they do not have the molecular machinery to replicate, they are dependent on host-specific proteins throughout their life cycle, from virus accumulation to intracellular, local, and systemic movement (Nelson, 2005). Both, RNA and DNA viruses take advantage of these plant proteins, which are normally involved in host-specific activities like the mRNA processing and translation machinery (Thivierge, 2005). Viruses are transmitted through vector organisms like insects or nematodes, which feed on the plant host tissue and therefore allow virus infection of wounded plant cells (Andret-Link and Fuchs, 2005). As viruses are intracellular pathogens they are thought to be recognized by intracellular receptors. Recently, for instance, a NB-domain LRR (NLR) class of receptors has been shown to recognize different viral components and initiate diverse signaling processes that induce programmed cell death and ETI in infected cells and restrict virus spread in various plants (Padmanabhan and Dinesh-Kumar, 2014).

However, until now, no viral PAMP inducing PTI was identified, and the primary plant defense against viruses is thought to be mainly based on RNA silencing (Ding and Voinnet, 2007; Ruiz-Ferrer and Voinnet, 2009; Llave, 2010). RNA silencing is an evolutionary conserved, sequence-specific mechanism that regulates gene expression and chromatin states and represses invasive nucleic acids such as transposons, transgenes and viruses (Vaucheret, 2006; Ding and Voinnet, 2007; Matzke et al., 2009). Furthermore, recent studies ascribe an additional role of RNA silencing in plant defenses against non-viral pathogens (Navarro et al., 2008; Weiberg et al., 2013). Moreover, increasing evidence indicates that PTI also contributes to plant defense against viruses. Indeed, the innate immune system is involved in defense responses against viruses in animal cells where viral components such as double-stranded (ds) RNA, single-stranded (ss) RNA and DNA are sensed by three classes of receptors (Arpaia and Barton, 2011; Bonardi et al., 2012; Berke et al., 2013; Peisley and Hur, 2013). As expected, these receptors are predominately intracellular and soluble or located in the endomembrane system (Rathinam and Fitzgerald, 2011; Jensen and Thomsen, 2012). In plants, viral pathogens induce similar immune reactions as non-viral microbes, including several features of PTI as, for instance, the induction of systemic acquired resistance (SAR) in uninfected tissues upon viral infections (Whitham et al., 2003) or the induction of defense gene expression (Love et al., 2005; Hu et al., 2011; Love et al., 2012; Kørner et al., 2013), suggesting that indeed viruses are recognized by PTI. In line with this hypothesis, the PRR coreceptor BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1) was shown to play

a role in resistance against diverse RNA viruses. BAK1 functions there as a coreceptor for a PRR recognizing a viral PAMP or a virus-induced DAMP leading to viral resistance (Kørner et al., 2013).

1.2.5 Herbivore-associated molecular patterns

The detection of herbivorous insects by PRRs is believed to be achieved by two different mechanisms. Either the presence of a DAMP caused by chewing insects or an insect-derived pattern may be detected. To date, several herbivore-associated molecular patterns (HAMPs) have been identified (Mithöfer and Boland, 2008). Among the first ones identified was a fatty aa conjugate, called volicitin, which was isolated from oral secretions of the beet armyworm (Turlings et al., 1993; Alborn, 1997). Additionally, bruchins, fatty acid molecules derived from insect eggs and inceptines, produced in the insect gut by degradation of plant-derived ATPases, have been shown to elicit defense responses in pea and cowpea (Doss et al., 2000; Schmelz et al., 2006). Whereas the exact recognition mechanisms of HAMPs remain to be investigated, several observations indicate that as for PTI, HAMP perception is mediated by membrane-bound receptors (Truitt et al., 2004; Maischak et al., 2007). Thus, similarities between MAMP and HAMP perception are anticipated.

1.2.6 Nematode-associated molecular patterns

Plant-parasitic nematodes are highly abundant in different environmental systems and can infect a broad range of host plants. They are extremely harmful to agriculture and difficult to control (Bird et al., 2009). Until only recently, nematode- or plant-derived compounds resulting from nematode attack that activate basal host defenses had not been identified. However, nematodes were shown to induce defense responses in, for example, tomato and Arabidopsis (Goverse and Smant, 2014). In fact, a very recent publication characterized ascarosides, an evolutionarily conserved family of nematode pheromones, as the first nematode-associated molecular patterns (NAMPs). Application of picomolar to micromolar concentrations of ascr#18, the major ascaroside in plant-parasitic nematodes, led to the activation of conserved immune responses in Arabidopsis and resulted in enhanced resistance to a broad-spectrum of pathogens and pests in Arabidopsis, tomato, potato and barley (Manosalva et al., 2015). Furthermore, membrane-bound PRRs have been shown to mediate

highly specific resistances to cyst nematodes strongly indicating that also NAMPs or plant-derived compounds produced upon nematode attack can be perceived by plant PRRs (Cai et al., 1997; Lozano-Torres et al., 2012).

1.2.7 Alterations of MAMPs

As most MAMPs are structures essential for microbial survival, they cannot easily be modified and exchanged by the microbe in order to evade recognition. Nevertheless, there are known cases of evasion due to evolutionary adaptation of MAMPs (Felix et al., 1999; Kunze et al., 2004; Andersen-Nissen et al., 2005). For example, structural differences and variations in the lipid A domain of LPS have been shown to affect MAMP recognition by plant cells (Pel and Pieterse, 2013). On the other hand, the root pathogen *Ralstonia solanacearum* causing bacterial wilt produces modified flagellin molecules with alternations in the aa sequence that do not trigger defense responses in Arabidopsis (Pfund et al., 2004; Sun et al., 2006). MAMPs can also be masked by other mechanisms. In the case of flagellin post-translational modifications such as glycosylation, down-regulation of protein biosynthesis, modulation of flagella content upon colonization by, for instance, proteases, expression of multiple, alternative flagellin types as well as shedding or complete lack of flagella have been found (Trdá et al., 2015). Furthermore, also beneficial microorganisms use this sort of "camouflage" to avoid recognition and induction of defense responses and to establish symbiotic interactions with their hosts (Felix et al., 1999).

Such modifications indicate an evolutionary need of plant interacting microorganisms to avoid recognition by the plant immune system and are interesting examples of the dynamism in these associations.

Table 1.1 Overview of the characterized MAMPs/DAMPs and PRRs identified so far.

Ligand	„Epitope“	Responsive plants	Receptor	Extracellular domain	Intracellular domain	Reference
Bacterial MAMPs						
Cold-shock protein	CSP22, RNP-1	*Solanaceae*	Unknown			(Felix and Boller, 2003)
Elongation factor Tu	elf18 EFa50	*Brassicaceae* Rice	AtEFR Unknown	LRR	Non-RD RK	(Kunze et al., 2004; Zipfel et al., 2006; Furukawa et al., 2014)
Flagellin	flg22 flgII-28 CD2-1	Most plants *Solanaceae* Rice	FLS2 Unknown Unknown	LRR	Non-RD RK	(Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006; Cai et al., 2011; Katsuragi et al., 2015)
Harpin	Unknown	Various plants	Unknown			(Engelhardt et al., 2009)
Lipopolysacharides	Lipid A	Arabidopsis, pepper, tobacco	LORE	B-type lectin S-domain	RD RK	(Erbs and Newman, 2011; Ranf et al., 2015)
Peptidoglycan	GlcNAc-X-GlcNAc	Arabidopsis	LYM1 LYM3	LysM	RLP/GPI	(Gust et al., 2007; Erbs et al., 2008; Willmann et al., 2011; Mesnage et al., 2014)
Superoxide dismutase	Unknown	Tobacco	Unknown			(Watt et al., 2006)
eMAX	Unknown	Arabidopsis	ReMAX	LRR	RLP	(Jehle et al., 2013a)
Fungal/Oomycetal MAMPs						
β-glucans	Branched hepta-β-glucosides linear oligo-β-glucosides	Legumes, tobacco	Unknown Unknown			(Umemoto et al., 1997; Fliegmann et al., 2004)
Cellulose binding elicitor lectin (CBEL)	Cellulose binding domain	Tobacco, Arabidopsis	Unknown			(Séjalon-Delmas et al., 1997; Khatib et al., 2004; Gaulin et al., 2006)
Chitin	Chitooligosaccharides polymers ≥ 4 residues	Arabidopsis, tomato, wheat, rice	OsCERK1 OsCEBiP AtCERK1 AtLYK5	LysM	RK RLP/GPI RD RK Non-RD RK	(Felix et al., 1993; Shibuya and Minami, 2001; Okada et al., 2002; Kaku et al., 2006; Shimizu et al., 2010; Cao et al., 2014)
Ergosterol	Unknown	Tomato, tobacco	Unknown			(Granado et al., 1995)
Invertase	N-glycosylated peptide	Tomato	Unknown			(Basse and Boller, 1992; Basse et al., 1993)

Necrosis inducing proteins	Nlp20	Several dicotyledones	RLP	LRR	RLP	(Mattinen et al., 2004; Böhm et al., 2014)
Transglutaminase	Pep13	Parsley, potato	Unknown			(Nürnberger et al., 1994; Brunner et al., 2002; Fellbrich et al., 2002)
Endopolygalacturonases	Unknown	Arabidopsis	RBPG1/ AtRLP42	LRR	RLP	(Zhang et al., 2014)
Xylanase	TKLGE pentapeptide	Tobacco, tomato	LeEIX2	LRR	RLP	(Bailey et al., 1990; Hanania and Avni, 1997; Rotblat et al., 2002; Ron and Avni, 2004)
Cerebroside	Unknown	Rice, tomato, chilli, pearl millet	Unknown			(Koga et al., 1998; Umemura et al., 2000; Umemura et al., 2002)
Sclerotinia culture filtrate elicitor1 (SCFE1)	Unknown	Arabidopsis	RLP30	LRR	RLP	(Zhang et al., 2013)
Elicitin	INF1	Tobacco, tomato, potato	StELR	LRR	RLP	(Baillieul et al., 2003; Domazakis et al., 2014; Du et al., 2015; Peng et al., 2015)
Endogenous DAMPs						
Oligogalacturonides/ pectin fragments	9 ≤ polymers ≥ 16 residues	Various plants	WAK1	EGF	RD RK	(Hahn et al., 1981; Brutus et al., 2010)
AtPeps	AtPep 1-8 AtPep 1-2	Arabidopsis	PEPR1 PEPR2	LRR	RD RK	(Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010; Bartels et al., 2013)
PrePIP1	PIP1	Arabidopsis	RLK7	LRR	RD RK	(Hou et al., 2014)
Prosystemin	Systemin	*Solanaceae*	Unknown			(Pearce et al., 1991; Felix and Boller, 1995)
Cutin	Monomers	Arabidopsis, barley, rice, potato	Unknown			(Schweizer et al., 1994; Schweizer et al., 1996a; Schweizer et al., 1996b)
Extracellular ATP		Arabidopsis	DORN1	L-type lectin	RD RK	(Choi et al., 2014)

Abbreviations: At, *Arabidopsis thaliana;* ATP, adenosine triphosphate; CEBiB, chitin elicitor binding protein; CERK, chitin elicitor receptor kinase 1; DORN1, does not respond to nucleotides 1; EGF, epidermal growth factor; Eix2, ethylene-inducing xylanase 2; ELR, elicitin-response receptor; eMAX, enigmatic MAMP of Xanthomonas; GPI, glycosylphosphatidyl inositol anchored; INF1, major secreted elicitin from *Phytophthora infestans;* Le, *Lycopersicum esculentum*; LORE, lectin S-domain-1 receptor-like kinase; LRR, leucine-rich repeat; LYK5, lysin motif receptor kinase; LysM, lysin motif; Nlp, necrosis and ethylene-inducing peptide 1 (Nep1)-like protein; Os, *Oryza sativa*, rice; Pep13, *Phytophthora*-derived oligopeptide elicitor; PEPR, pep receptor; PrePIP, PAMP-induced secreted peptides; RBPG1, responsiveness to botrytis polygalacturonases1; ReMAX, receptor of eMAX; RLP, receptor-like protein; RLK, receptor-like kinase; RK, receptor kinase; St, *Solanum tuberosum;* WAK1, wall-associated kinase 1.

1.3 Pattern recognition receptors (PRRs)

1.3.1 General structural characteristics of PRRs

So far, all plant PRRs perceiving MAMPs and DAMPs are known to be surface-localized, membrane-bound receptors, which can be classified in either receptor-like kinases (RLKs) or receptor-like proteins (RLPs). Typically, a RLK contains a divergent extracellular domain providing ligand binding specificity, a membrane-spanning domain and an intracellular kinase domain, which is absent in RLPs (Macho and Zipfel, 2014). In the Arabidopsis genome, the *RLK* gene family includes more than 600 members, represents 60% of all kinases present and encompasses 2.5% of the coding region (Shiu and Bleecker, 2003). Besides their role in danger detection, several RLKs have been shown to be involved in other physiological processes like growth, development, and reproduction (Shiu and Bleecker, 2001; Shiu and Bleecker, 2003; Shiu et al., 2004). In addition, 57 RLPs have been identified so far, which play a role in a variety of physiological processes, including defense (Tör et al., 2009; Jehle et al., 2013a).

RLKs are classified into 21 structural classes by the characteristic structure of their ectodomain, consisting of either leucine-rich repeats (LRRs), lysine motifs (LysMs), lectin motifs, or epidermal growth factor (EGF)-like domains. With more than 235 members, the LRR-RLKs, typically binding to proteins or peptides such as bacterial flagellin, EF-Tu or endogenous Pep peptides (Chinchilla et al., 2006; Zipfel et al., 2006; Yamaguchi et al., 2006), are the largest subgroup of transmembrane RLKs in Arabidopsis. PRRs with other domains than LRR have been shown to recognize carbohydrate-containing molecules, such as fungal chitin, bacterial PGN, extracellular ATP or plant cell wall-derived OGs (Table 1.1; Kaku et al., 2006; Miya et al., 2007; Brutus et al., 2010; Choi et al., 2014).

According to their intracellular kinase domain, RLKs are further grouped into RD and non-RD kinases (Dardick and Ronald, 2006; Tör et al., 2009). RD indicates a specific motif presented by a conserved arginine (R) residue in front of an aspartate (D) in the catalytic loop of the kinase domain. Since the kinase activity of non-RD kinases is considerably weaker than that of RD kinases (Schwessinger et al., 2011), it seems that non-RD ligand-binding RLK PRRs rely on the association and complex formation with a strong RD kinase immediately after ligand binding in order to amplify phosphorylation of the kinases and initiate signaling (Dardick et al., 2012). This observation is to some extent similar to what has been reported for RLPs, which completely lack a kinase domain (Müller et al., 2008; Bleckmann et al., 2010; Zhu et al., 2010; Lee et al., 2012).

1.3.2 Examples of LRR-RLK PRRs

FLAGELLIN-SENSING 2 (FLS2) is by far the best studied RLK PRR in plants. This receptor is responsible for the recognition of the bacterial MAMP flg22 and was first identified and characterized in Arabidopsis (Gómez-Gómez and Boller, 2000). Orthologues of the FLS2 protein have been identified in tomato, tobacco, rice and grapevine (Hann and Rathjen, 2007; Robatzek et al., 2007; Takai et al., 2008; Trdá et al., 2014). Furthermore, proteins with a high degree of conservation can be identified *in silico* from outputs of genome sequencing projects of ricinus, maize, poplar and other plant species.

FLS2 is an LRR-RLK with an extracellular domain comprised of 28 aa long stretches rich in leucine residues (LRRs) and a non-RD serine/threonine kinase domain linked to the extracellular domain by a single-pass plasma membrane-spanning domain segment (Gómez-Gómez and Boller, 2000). The cytoplasmic kinase activity is required to initiate signaling in the cytoplasm (Schulze et al., 2010; Schwessinger et al., 2011), while the extracellular LRR domain is dedicated to binding of the flg22 peptide (Chinchilla et al., 2006; Sun et al., 2013b). Recent functional and binding studies using chimeric receptors obtained by a domain swapping approach between the LRR domain of tomato and Arabidopsis FLS2, have specifically defined potential LRRs involved in flg22 recognition (Dunning et al., 2007; Helft et al., 2011; Mueller et al., 2012a). Further studies revealed that binding of the flg22 N-terminal part is required for association to the receptor whereas the C-terminal part of flg22 is necessary for activation of immune responses (Meindl et al., 2000; Sun et al., 2013b).

The LRR-RLK ELONGATION FACTOR-TU RECEPTOR (EFR), specifically recognizing elf18 shares high structural similarity with FLS2. Although EFR encompasses only 21 LRRs, it has been demonstrated that chimeras of EFR and FLS2 are functional and can be used for studying receptor function (Albert et al., 2010b). The same study showed that different, noncontiguous parts of the ectodomain from EFR are required to form a functional ligand binding site. Therefore, although the exact mechanism of elf18 binding remains unclear, it could mimic the association of flg22 to FLS2 where a part of the peptide is required for binding and the other for response activation.

The perception of AtPeps is achieved by two highly homologous, membrane-localized LRR-RLKs, termed PEPR1 and PEPR2 (Yamaguchi et al., 2006; Krol et al., 2010; Yamaguchi et al., 2010). In contrast to PEPR1, which is able to recognize all eight AtPeps present in Arabidopsis, PEPR2 can only detect AtPep1 and AtPep2 (Bartels et al., 2013). The expression

of the receptors is induced upon wounding, MAMP treatment and jasmonic acid (JA) application, similarly to what has been reported for FLS2 (Mersmann et al., 2010; Yamaguchi et al., 2010; Bartels et al., 2013).

1.3.3 Molecular mechanisms controlling PRR activation

1.3.3.1 Model of FLS2 activation by flg22

1.3.3.1.1 Oligomerization/Complex formation

Recognition and binding of flg22 by FLS2 leads to the instantaneous (≤ 5 seconds) association with the LRR-RLK BAK1, indicating that FLS2 and BAK1 already exist in close proximity at the plasma membrane (Chinchilla et al., 2007; Heese et al., 2007; Schulze et al., 2010). BAK1 is the only characterized coreceptor of several RD and non-RD RLKs and has been extensively studied. It was originally discovered to positively regulate and dimerize with BRASSINOSTEROID INSENSITIVE 1 (BRI1), the receptor for the plant hormone brassinosteroid (BR) (Wang et al., 2001; Li et al., 2002; Nam and Li, 2002). Indeed, BAK1 is not only required for full responsiveness to BR but also for signal transduction in response to multiple MAMPs and unknown cell death pathways (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). A structural study based on cocrystalization of the FLS2 and BAK1 ectodomains in complex with flg22 revealed that the flg22 bound to the C-terminal part of the FLS2 ectodomain directly interacts with the BAK1 ectodomain stabilizing the FLS2-BAK1 complex (Sun et al., 2013b). A similar activation mechanism has been recently reported for BRI1 and BAK1 (Santiago et al., 2013; Sun et al., 2013a).

BAK1, also called SERK3, belongs to the subfamily of the SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES (SERKs) containing five members, and shows a rather short, extracellular domain composed of only five LRRs and a typical characteristic serine and proline rich region adjacent to the plasma membrane. Apart from FLS2, also EFR is a well-known interaction partner for BAK1 (Zipfel et al., 2006).

Although BAK1 seems to have a predominant role in FLS2 activation in Arabidopsis (Chinchilla et al., 2007), other SERKs have potentially redundant functions as interaction partners for PRRs, like EFR or PEPRs (Roux et al., 2011). However, also RD RLKs, such as BRI1 or PEPR1 and PEPR2 rely on BAK1 and other SERKs for full responses (Li et al., 2002; Nam and Li, 2002; Krol et al., 2010; Roux et al., 2011; Gou et al., 2012). Thus, this implies that SERK proteins are critical for the function of both, RD and non-RD RLKs.

1.3.3.1.2 Cross phosphorylation

Association of FLS2 and BAK1 leads to the immediate (≤ 15 seconds) de novo phosphorylation of both receptors (Schulze et al., 2010). The cross phosphorylation of both receptors is thought to initiate a cascade of cellular events leading to the activation of downstream signaling pathways (Chinchilla et al., 2007; Schulze et al., 2010). EFR and PEPR1/PEPR2 also recruit BAK1 indicating that heterodimerization might represent a common feature in LRR-RLK signaling responses (Schulze et al., 2010). Interestingly, mutations that impair complex formation between FLS2 or EFR and BAK1 abolish the phosphorylation of both proteins and the initiation of downstream signaling (Sun et al., 2013b). Nevertheless, the weaker kinase activities of both, FLS2 and EFR are still required for flg22- and elf18-triggered responses, respectively (Schwessinger et al., 2011; Cao et al., 2013).

1.3.3.1.3 BIK1 dissociation

The receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE 1 (BIK1) might associate with FLS2 and BAK1 in absence of flg22 (Lu et al., 2010; Zhang et al., 2010). After flg22 perception, BIK1 gets phosphorylated by BAK1 and in turn phosphorylates both, FLS2 and BAK1 followed by dissociation from the FLS2-BAK1 complex. BIK1 phosphorylation by BAK1 also occurs after elf18 and AtPep1 perception and BIK1 interacts with EFR and PEPR1 (Zhang et al., 2010; Liu et al., 2013). Interestingly, BIK1 also interacts with CERK1 and may therefore represent a convergent signaling element between BAK1-dependent and BAK1-independent PRR complexes (Zhang et al., 2010). RLCKs emerge as direct substrates of PRR complexes and key positive regulators of PTI signaling in order to link PRR activation with downstream intracellular signaling (Lu et al., 2010; Zhang et al., 2010; Liu et al., 2013).

An important role for BIK1 and its close paralog PBL1 (PBS (AvrPphB SUSCEPTIBLE)-LIKE 1) in plant immunity is supported by the finding that both RLCKs are required for PTI activation and flg22-mediated resistance to *Pto* DC3000 (Lu et al., 2010; Zhang et al., 2010; Laluk et al., 2011; Liu et al., 2013; Li et al., 2014). The mechanism of PRR receptor activation using the example of FLS2 is demonstrated in Figure 1.3.

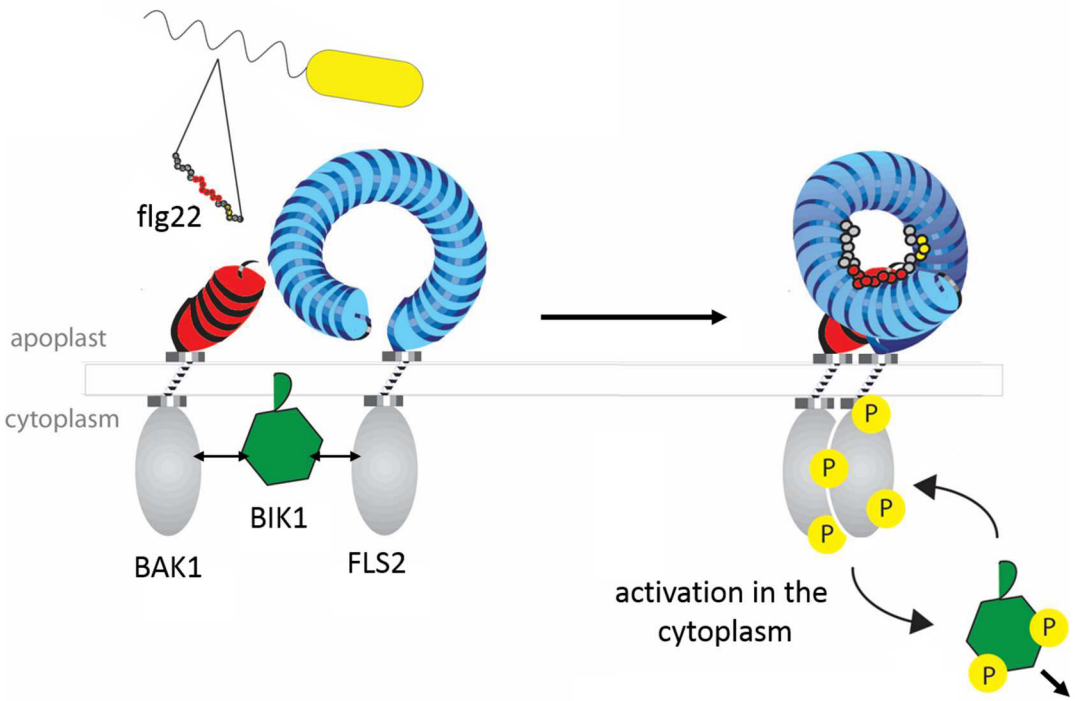

Fig. 1.3 Model of PRR receptor activation. Model of flagellin signaling adapted from Delphine Chinchilla. In the absence of flg22, BIK1 might associate with FLS2 and/or BAK1 in an inactive state. Upon flg22 binding to FLS2, flg22 induces FLS2 and BAK1 association and phosphorylation. The activated complex then phosphorylates BIK1, which in turn trans-phosphorylates the FLS2-BAK1 complex. The fully active complex may further phosphorylate BIK1 and other substrates. BIK1 then is likely released from the FLS2-BAK1 complex to activate downstream intracellular signaling.

1.3.3.2 Molecular mechanisms of other PRRs: RLPs mode of action

As LRR-RLPs lack a kinase domain, it is anticipated that their activation relies on the interaction with kinases in order to form a signaling-competent receptor complex (Jones et al., 1994; Joosten and de Wit, 1999; Rivas and Thomas, 2005). This model was confirmed for several LRR-RLPs involved in plant development. For instance, the RLP TOO MANY MOUTH (TMM), which regulates stomatal patterning, was shown to interact with the LRR-RLK ERECTA (Lee et al., 2012) whereas the RLP CLAVATA 2 (CLV2), involved in meristem maintenance, forms a complex with the transmembrane kinase CORYNE as well as with the LRR-RLK CLV1 to activate downstream signaling (Müller et al., 2008; Bleckmann et al., 2010; Zhu et al., 2010; Lee et al., 2012).

However, until recently, a transmembrane RLK interacting with RLPs involved in disease resistance had not been identified. Lately, the LRR-RLK SOBIR1 (SUPPRESSOR OF BIR1-1) was found to interact specifically with LRR-RLPs involved in plant immunity and development to regulate their function (Gao et al., 2009; Liebrand et al., 2013). A further study confirmed the importance of SOBIR1 for the function of other RLPs as, for instance, RLP30

involved in resistance against fungal pathogens (Zhang et al., 2013; Liebrand et al., 2014). In Arabidopsis, the activity of REMAX, the RLP receptor for the proteinaceous MAMP eMax (enigmatic MAMP from *Xanthomonas*) present in different *Xanthomonas* species also requires the presence of functional SOBIR1 (Jehle et al., 2013b). Moreover, other investigations indicated that SOBIR1 could be required and/or even function as a scaffold protein for the accumulation of LRR-RLP-containing complexes. Additionally, it is speculated that the process of association between RLPs and adaptor kinases (like SOBIR1) is possibly important for proper subcellular localization, stability and functionality of these receptors (Liebrand et al., 2014).

The number of LRR-RLPs shown to interact with SOBIR1 is continually increasing and the requirement for SOBIR1 homologues in LRR-RLP function appears to be widely conserved among the eudicots (Liebrand et al., 2014). Up to now, SOBIR1 was found to interact specifically with LRR-RLPs in contrast to BAK1 and other members of the SERK family, which interact with RLKs and RLPs and are required for their function (Liebrand et al., 2014). Currently, it is speculated that RLP/adaptor complexes might function as a bimolecular receptor equivalent to RLKs that also sometimes require BAK1 function as shown by a couple of studies (Zhang et al., 2013; Gust and Felix, 2014) and depicted in Figure 1.4.

1.3.3.3 Molecular mechanisms of other PRRs: Chitin perception as an example

FLS2 is often used as a model to study activation of PRRs but the mechanisms of receptor activation differ between PRRs. For instance, perception of chitin does not require BAK1 to initiate chitin-triggered signaling in Arabidopsis (Heese et al., 2007; Gimenez-Ibanez et al., 2009). The chitin receptor was first reported in rice with the identification of the CHITIN-ELICITOR BINDING PROTEIN (CEBiP), which contains an extracellular LysM domain but lacks an intracellular kinase domain (Kaku et al., 2006). CEBiP forms a complex with the rice CHITIN-ELICITOR RECEPTOR KINASE 1 (OsCERK1) to mediate PTI in response to chitin (Shimizu et al., 2010; Hayafune et al., 2014). In addition to a LysM domain, OsCERK1 has an active, intracellular kinase domain. In Arabidopsis, the homologue of OsCERK1 was identified as a chitin binding RD-RLK indispensable for chitin induced defense responses (Miya et al., 2007; Wan et al., 2008; Petutschnig et al., 2010). Furthermore, it has been shown that binding of chitin to CERK1 rapidly induces *in vivo* phosphorylation of CERK1 at multiple residues in the juxtamembrane and kinase domain (Petutschnig et al., 2010). The kinase domain seems to be required for this chitin-dependent *in vivo* phosphorylation as well as for

early defense responses and downstream signaling. However, recent studies indicated that AtLYK5, a LYSM RECEPTOR KINASE, is also required for chitin-induced AtCERK1 homodimerization and phosphorylation (Cao et al., 2014). AtLYK5 binds to chitin with a much higher affinity than AtCERK1 as tested by isothermal titration calorimetry. In this new model, AtLYK5 is the primary receptor for chitin, forming a chitin-inducible complex with AtCERK1 to induce plant innate immunity (Fig. 1.4; Cao et al., 2014).

Although several PRRs exhibit different molecular pathways for MAMP-induced activation and initiation of signaling it can be generally concluded that receptor homodimerization or oligomerization and subsequent phosphorylation are common mechanisms for ligand-mediated receptor activation.

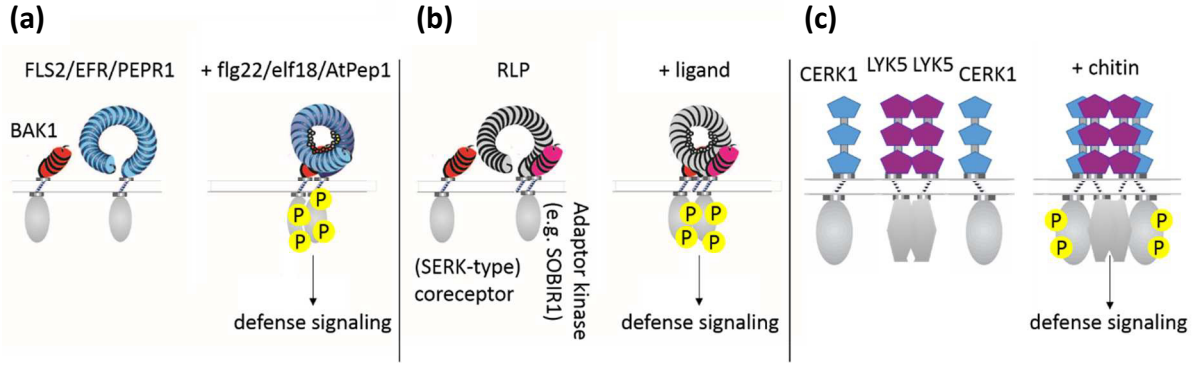

Fig. 1.4 Model of PRR activation involving RLK and RLP complexes. (**a**) Upon ligand binding, RLKs (e.g. FLS2, EFR or PEPR1) undergo association with a coreceptor (like BAK1) that brings the cytoplasmic domains of the complexed partners in proximity, allowing (trans)-phosphorylation and activation of intracellular signaling. (**b**) RLPs lacking a cytoplasmic kinase domain undergo association with adaptor kinases. For LRR-RLPs that associate with SOBIR1, this event might occur by interaction of their LRR domains, by ionic interaction of their oppositely charged juxtamembrane domains and/or by helix-helix interactions of their transmembrane domains. RLP/adaptor complexes then function as bimolecular receptors, equivalent of RLKs. Upon binding of their ligands the RLP/adaptor complexes, much like RLKs, undergo complex formation with coreceptors, notably of the BAK1/SERK-type. (**c**) Chitin perception is another well-known example of PRR activation. In Arabidopsis, in absence of trigger, the receptor kinase AtLYK5 is present as a homodimer not associated with CERK1. After chitin elicitation, AtCERK1 and AtLYK5 form a possible tetramer to mediate chitin signaling, concomitantly to AtCERK1 phosphorylation. In this model, AtLYK5 serves as a chitin perception receptor, while AtCERK1 is responsible for chitin signaling transduction to complement the lack of kinase activity of AtLYK5. Part **a** and **b** of the figure have been modified after Delphine Chinchilla, part **c** has been adapted from Cao et al. (2014).

1.4 Plant responses upon microbe recognition

Immediately after the detection of a biotic threat or an exogenously applied MAMP, plants activate a set of short and long-term responses as well as downstream signaling cascades in order to react against the attacking microbe. Most of these direct immune responses have been thoroughly studied in the context of microbial pathogen detection and PTI and can be used as robust readouts for innate immunity responses. In the following section, the most important plant responses to biotic invaders will be presented with a special focus on the recognition of bacterial elicitors, mainly flg22, by FLS2. Figure 1.5 illustrates the spatial and temporal arrangement of the direct, cellular immune responses.

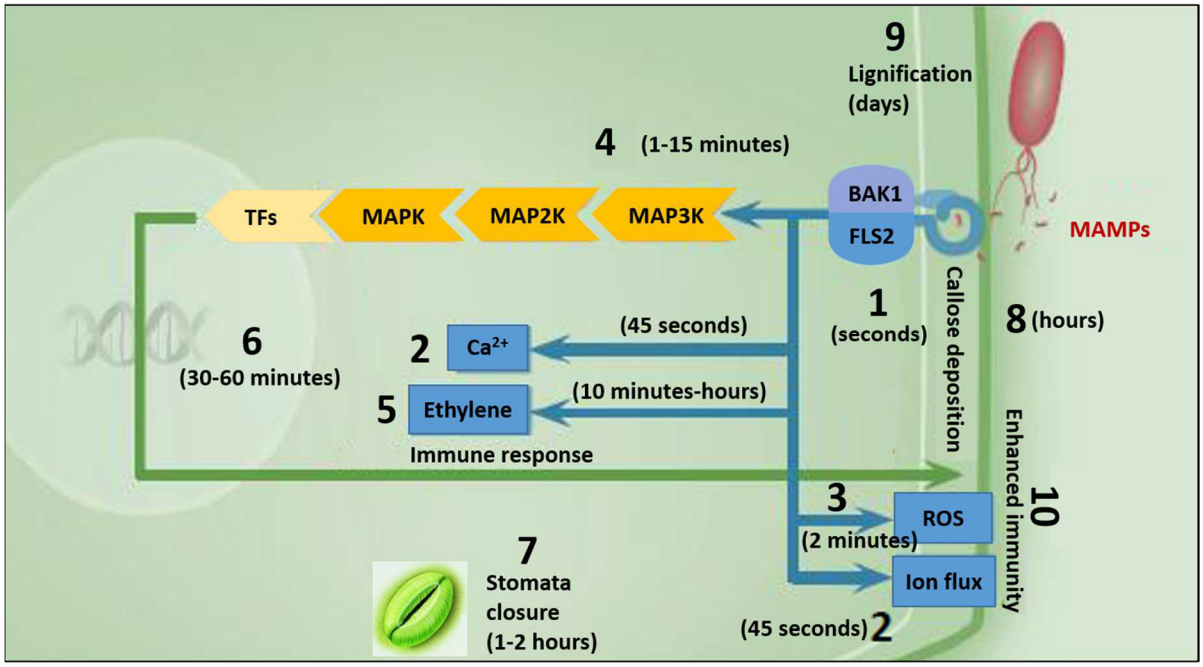

Fig. 1.5 Spatial and temporal assembly of direct, cellular PTI responses. Extracellular ligands are perceived by plasma membrane-localized PRRs. Upon this recognition event, many PRRs interact with coreceptors (such as FLS2 with BAK1) to initiate kinase activation, potentially leading to cross-phosphorylation followed by phosphorylation of downstream targets (**1**, seconds). Subsequently, a set of immediate, quantitatively measurable immune responses is induced by the plant cell. These responses include initiation of cross-membrane ion fluxes (**2**, 45 seconds), the production of reactive oxygen species (ROS) (**3**, 2 minutes), the activation of MAPK cascades (**4**, 1-15 minutes), the biosynthesis of ethylene (**5**, 10 minutes-hours), some transcriptional changes of defense genes in the nucleus (**6**, 30-60 minutes), stomata closure (**7**, 1-2 hours) as well as the deposition of callose between the plasma membrane and the cell wall at a later stage (**8**, hours) and lignification of the cell wall (**9**, days). Altogether, these responses lead to enhanced immunity against pathogen attack (**10**). Figure is adapted from Sebastian Bartels and Dominik Klauser, Plant Science Center.

1.4.1 Ion fluxes

Within 45 seconds upon perception of MAMPs, ion fluxes across the plasma membrane get initiated, following opening of ion channels with influxes of H^+, K^+ and Ca^{2+} cations into the cell and a Cl^- anion efflux. This event can best be measured by an alkalinization of the liquid growth medium of plant cell suspension cultures (Boller, 1995). Furthermore, the increase of intracellular Ca^{2+} is of particular interest as Ca^{2+} is known to be a second messenger in various cellular processes (Lecourieux et al., 2006). In particular, four CALCIUM-DEPENDENT PROTEIN KINASES (CDPKs) have been identified as Ca^{2+} sensors regulating innate immunity in Arabidopsis (Boudsocq et al., 2010).

1.4.2 Production of ROS

The production of ROS at the cell surface by the NICOTINAMIDE ADENINE DINUCLEOTIDE PHOSPHATE (NADPH) oxidase is another detectable early process linked to plant defense. ROS can be measured around 2-10 minutes after MAMP application with a luminol-based assay (Chinchilla et al., 2007). Plasma membrane-bound NADPH oxidases, referred to as RESPIRATORY-BURST OXIDASE HOMOLOGUES (RBOH), are the main producers of ROS (Torres et al., 2002; Torres et al., 2006). Recently it was confirmed that RBOHD, the NADPH oxidase responsible for the MAMP-induced ROS burst (Nühse et al., 2007; Zhang et al., 2007), exists in complex with EFR and FLS2 (Kadota et al., 2014). In the same complex, BIK1 interacts and regulates by phosphorylation the activity of the NADPH oxidase upon MAMP perception in a calcium independent manner (Li et al., 2014). This study further reports that the phosphorylation of specific residues of RBOHD by BIK1 is critical for the MAMP-induced ROS burst and the onset of antibacterial immunity. In addition to the regulation by BIK1, other regulatory processes including calcium-mediated mechanisms target RBOHD.

In plant defense, ROS are considered to have an antimicrobial activity. This is achieved either by directly targeting the membranes of invasive microbes, as reported for animal phagocytes and lymphocytes (Apel and Hirt, 2004) or in an indirect manner. For example, ROS production induces the strengthening of the cell wall through oxidative cross linking of glycoproteins and induces intracellular signaling pathways such as the synthesis of the defense hormone salicylic acid (SA) and the activation of MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs) (O'Brien et al., 2012). Moreover, it has been proposed that the activation of SAR is associated

with the systemic propagation of the oxidative burst to alert uninfected tissues (Lamb and Dixon, 1997; Nühse et al., 2007). Indeed, the fact that some bacterial effectors directly block the ROS production machinery further supports the role of ROS in intracellular signaling processes (Göhre et al., 2008; Gimenez-Ibanez et al., 2009; Miller et al., 2009).

1.4.3 Activation of MAPK cascades

The rapid activation of a MAPK cascade (≤ 2 minutes) is a key element in signaling in many eukaryotic organisms: It serves as a hub in order to transduce external stimuli into an intracellular response (Dong et al., 2002). In general, a MAPK cascade starts with the phosphorylation of a MAP kinase kinase kinase (MAPKKK), which in turn transfers a phosphate group to a target MAPKK, which then phosphorylates a MAPK. Ultimately, MAPKs can directly target various nuclear or cytoplasmic proteins for phosphorylation including transcription factors to induce transcriptional reprogramming. In the case of MAMP perception, the phosphorylated transcription factors either control the expression of defense genes involved in PTI or negatively regulate PTI (Asai et al., 2002; Boudsocq et al., 2010; Rasmussen et al., 2012). Therefore, MAPK pathways might provide a precise control of plant defense responses (Zhang and Klessig, 2001). In Arabidopsis, four main MAPKs are activated during PTI responses by double phosphorylation: MPK3, MPK4, MPK6 and MPK11 (Asai et al., 2002; Rasmussen et al., 2012). The phosphorylation of MAPKs is a transient response that can be detected 1-15 minutes after MAMP application by Western blot constituting an useful readout for immediate immune responses (Nühse et al., 2000; Suarez-Rodriguez et al., 2007).

1.4.4 Ethylene production

Upon MAMP detection, the aminocyclopropane-1-carboxylic acid (ACC) synthase, which is the rate limiting enzyme involved in ethylene (ET) biosynthesis, gets activated and ET is produced within several hours, which can be detected by gas chromatography (Oetiker et al., 1997; Krol et al., 2010). The gaseous phytohormone ET serves several functions in plants. It is involved in some plant development processes as fruit ripening or flowering, as well as in abiotic and biotic stress adaptations (Johnson and Ecker, 1998; Love et al., 2005; Love et al., 2007). Furthermore, its role in defense has been shown to be crucial in several different events. ET has been shown to be a critical factor required for the FLS2 receptor accumulation, flg22-

induced ROS production as well as for callose deposition at the plasma membrane (Clay et al., 2009; Boutrot et al., 2010; Mersmann et al., 2010).

1.4.5 Transcriptional changes

Transcriptomic studies revealed that already after 30 minutes of flg22 treatment more than 1000 genes were up-regulated and around 200 down-regulated in Arabidopsis (Navarro et al., 2004; Zipfel et al., 2004). A similar pattern of gene regulation was also observed upon elf18 or chitin treatment, indicating that MAMP signaling converges at an early stage (Ramonell et al., 2002; Zipfel et al., 2006). Induced genes include those coding for PATHOGENESIS RELATED (PR) proteins, transcriptional regulators and kinases or phosphatases. Interestingly, over 100 of the roughly 600 *RLK* genes present in the Arabidopsis genome are also MAMP-induced, including those coding for the PRRs FLS2 and EFR, indicating a positive feedback loop of PTI activation (Zipfel et al., 2004).

1.4.6 Receptor endocytosis

Within one hour, flg22 treatment leads to vesicle-mediated endocytosis from the cell membrane and degradation of the FLS2 receptor. Endocytosis can be observed by microscopy of fluorescent protein-tagged receptors. FLS2 is one of the first examples of plant RLKs shown to undergo ligand-induced endocytosis and subsequent degradation (Robatzek et al., 2006; Göhre et al., 2008; Lu et al., 2011; Beck et al., 2012b; Choi et al., 2013). Later, similar observations were reported for LeEIX2 (Ron and Avni, 2004; Bar and Avni, 2009a) and the Xa21 receptor, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6 in rice (Chen et al., 2010). In mammalian cells, receptor-mediated endocytosis was reported to be important for the response to pathogens, for instance in the case of the TLRs (Husebye et al., 2006). Likely widely spread in PRR signaling, the exact contribution of endocytosis to PTI remains unknown. Potential roles include desensibilization of the cells via removal of ligand-bound receptors from the site of perception or recycling of the receptor for resensibilization of the cells (Beck et al., 2012a; Smith et al., 2014).

1.4.7 Stomatal closure

Stomata are microscopic pores in the epidermis of aerial organs of plants, which ensure the gas exchange and transpiration, both required for photosynthesis and water homeostasis. They are also used as entry points for microbes (Melotto et al., 2008). As stomata close 1 to 2 hours after recognition of bacteria, fungi or MAMPs, thereby preventing the entry of microbes and host tissue colonization, they have been assigned a function in the early phases of innate immunity (Melotto et al., 2006; Sawinski et al., 2013). The physiological importance of stomatal closure is supported by the fact that, for instance, *fls2* receptor mutants show a decreased resistance to *Pto* DC3000 infection only when bacteria are sprayed onto the leaf surface but not when infiltrated into the leaf, a condition which does not involve stomata function (Zipfel et al., 2004). The stomatal closure can be assessed and quantified by microscopy.

1.4.8 Callose deposition

The synthesis and deposition of callose between the plant cell wall and the plasma membrane during the relatively early stages of pathogen invasion serves as an effective barrier induced at the sites of attack. Callose is an amorphous high-molecular weight β-1-3 glucan polymer, which not only provides a matrix in which antimicrobial compounds can accumulate as chemical defenses to the infection site, but also reinforces the cell wall to limit penetration by pathogens (Boller and Felix, 2009; Luna et al., 2011). In Arabidopsis leaves, callose-containing cell-wall appositions can be easily visualized under the microscope by fixing and staining with aniline blue after 16 hours of MAMP treatment (Gómez-Gómez et al., 1999).

1.4.9 Lignification

Lignin is one of the most abundant biopolymers on earth and is resistant to degradation by most microorganisms (Vance et al., 1980). Lignin is composed of aromatic alcohols known as monolignols and is an integral part of the secondary cell walls of plants. Accumulation of lignin or lignin-like phenolic compounds was shown to occur in a variety of plant-microbe interactions at the sites of attempted penetration (Bhuiyan et al., 2009). Lignification not only renders the cell wall more resistant to mechanical pressure but makes the cell wall more water resistant and thus, less accessible to cell wall-degrading enzymes. Furthermore, it restricts

diffusion of enzymes and toxins into the host and in the case of viruses, lignification around local lesions is thought to serve as a barrier for viral spread (Vance et al., 1980). Additionally, lignification may be an important part of defense as this process is associated with the production of activated oxygen species. Furthermore, the monolignol lignin precursors also have antifungal activity and it has been proposed that they function as phytoalexins, (antimicrobial compounds) in flax (Ride and Pearce, 1979). Lignin accumulation can be detected by histochemical color reactions after several days of MAMP treatment or by the use of fluorescence-tagged monolignols (Tobimatsu et al., 2013).

1.4.10 Seedling growth inhibition

A prolonged treatment of seedlings with MAMPs can lead to strong inhibitory effects of seedling growth in a concentration-dependent manner (Gómez-Gómez et al., 1999; Zipfel et al., 2006). The exact molecular details underlying this event remain unclear. One possibility could be a shift in the limited resource allocation from growth to defense by the plant due to the perception of danger signals at the cost of plant fitness (Walters and Heil, 2007; Boller and Felix, 2009). Moreover, a complex signaling network including hormonal pathways described in the next paragraph, might also contribute to the arrested seedling growth.

1.4.11 Modifications in phytohormone concentrations

Plants produce a wide array of hormones interacting in complex networks in order to balance diverse growth and developmental processes but also biotic and abiotic stress responses. ET, JA and SA have been shown to play a central role in the regulation of plant immune responses (Bari and Jones, 2009). In addition, other plant hormones, such as auxins (Kazan and Manners, 2009), abscisic acid (Ton et al., 2009), cytokinins (Walters and McRoberts, 2006), BR (Nakashita et al., 2003) and several others that have been described to regulate plant development and growth processes, have recently emerged as regulators of plant immunity (Bari and Jones, 2009). Commonly, infection of plants with pathogens results in the production of various plant hormones, which activate and enhance defense signaling but also spread the danger signal to healthy tissues (Adie et al., 2007; Robert-Seilaniantz et al., 2007; Pieterse et al., 2009). Depending on the type of attacker and the plant species, the hormonal integration of the defense response can differ remarkably in plants.

In general, pathogens can be divided into two groups determined by their feeding specification. Pathogens with a biotrophic life cycle are reliant on living cells to obtain their nutrients. In contrast, necrotrophic pathogens kill their host cells early using lytic enzymes and phytotoxins to feed from the dead material. Often pathogens switch between biotrophic and necrotrophic life styles and thus, it is difficult to classify them. In this case, they are referred to as hemi-biotrophic pathogens, such as *Pto* DC3000 (Glazebrook, 2005). In turn, plants have evolved specialized sensing and long-term signaling strategies according to the life style of the invasive pathogen. Generally, SA plays a crucial role in plant defense against biotrophic or hemi-biotrophic pathogens in addition to its well-known role in SAR (Grant and Lamb, 2006; Vlot et al., 2008). In pathogen-challenged tissues of plants, SA levels increase and exogenous applications result in the induction of a large set of defense related genes, commonly referred to as *PATHOGENESIS-RELATED* (*PR*) genes and enhanced resistance to a broad range of pathogens (Dong, 2004; Moore et al., 2011). Defense against biotrophs often includes a local HR and SA-dependent cell death, which limit the spread of the pathogen by elimination of its nutrient resource (Glazebrook, 2005). Interestingly, a recent study revealed that SA accumulation also causes increased levels of PRRs and coreceptors, which potentiates the responsiveness of plants to MAMPs, implying a dynamic effect of SA on the regulation and function of PRRs (Tateda et al., 2014).

In contrast, necrotrophic pathogens and herbivorous insects usually induce the accumulation of JA and ET (Bari and Jones, 2009). Although SA and JA/ET defense pathways are mutually antagonistic, evidences of synergistic interactions have also been reported, indicating that the defense signaling network activated by the plant depends on the type of pathogen and its mode of pathogenicity (Schenk et al., 2000; Kunkel and Brooks, 2002; Beckers and Spoel, 2006; Mur et al., 2006). JA levels increase locally in response to pathogen infections and tissue damage (Lorenzo and Solano, 2005; Wasternack, 2007) and perception of JA isoleucine, leads to the liberation of transcription factors for JA-responsive genes in the nucleus (Feys et al., 1994; Fonseca et al., 2009; Yan et al., 2009). As mentioned previously in Chapter 1.4.4, ET production is induced upon pathogen attack and plays an important role in the activation of *PR* genes, the synthesis of secondary metabolites and the strengthening of the cell wall (Broekaert et al., 2006). Similarly to SA and SAR, JA/ET signaling activation can also lead to a systemic priming of tissues referred to as induced systemic resistance (ISR). ISR is induced by wounding and herbivore feeding, as well as by plant growth promoting microbes (Howe and Schaller, 2008; Pieterse et al., 2014).

The balance of the hormonal cross-talk strongly influences the outcome of plant-pathogen interactions and potentially the establishment of effective systemic immunity. Interactions between defense signaling pathways is an important mechanism for regulating immune responses against various types of pathogens. Thus, it is not surprising that hormone signaling pathways are targeted by pathogens to disturb and evade plant defense responses (Denancé et al., 2013). Although several components regulating the cross-talk between SA, JA and ET pathways have been identified, the complex underlying molecular mechanisms still remain poorly understood.

1.5 Root-microbe interactions

Root defense responses to pathogens have received much less attention than leaf responses for several reasons. First of all, disease symptoms in aerial plant parts are more visible and therefore easier to assess. Furthermore, as plants are usually grown in soil, technical factors make roots difficult to access, observe, isolate and wash without causing damage. Additionally, many microbes from the rhizosphere, the biologically active zone surrounding the roots, are almost impossible to cultivate in the laboratory (Singh et al., 2004). Ultimately, many leaf pathogens are generally not considered to be root pathogens like, for instance, the *Pseudomonas* pathovars. This situation represents a practical experimental problem as most genetic and genomic tools have been developed for well-studied leaf pathogens but not root pathogens. Nevertheless, plant roots are constantly exposed to a multitude of soil microbes, including pathogens and symbionts. Some root diseases are extremely severe and cause considerable worldwide losses of vegetables and overall crop yield (Krupa, 2012). Thus, it is of high importance to understand the molecular mechanisms underlying defense responses against pathogens at the root level in order to develop new strategies for plant protection.

The following section provides an overview of the Arabidopsis root anatomy and structure and highlights the tissues and zones most vulnerable for pathogen infection.

1.5.1 The root

Roots absorb water and nutrients, which is facilitated by providing a large surface area further increased by the presence of root hairs. Additionally, roots conduct water and nutrients and anchor the plant body to the soil. Besides this, roots provide mechanical support, are used for the storage of water and photosynthetic products and participate in gas exchange. The root penetration by unwanted toxic compounds and infection by soil-borne pathogens needs to be avoided while water and mineral need to be taken up. Because roots maintain this dual role in nutrient and water uptake and protection, they are often compared to inverted guts (Waisel et al., 2002).

Interactions of roots with microbes have been proposed to be tissue-specific and highly complex (Millet et al., 2010). Therefore, in order to investigate root-microbe interactions, it is essential to consider the root architecture. The root consists of several tissue layers with different specific functions, and exposed to the soil and the soil microbiota in various manners.

1.5.1.1 Root tissue layers

1.5.1.1.1 Epidermis

At the outer root surface lies the epidermis (Fig. 1.6). Epidermal cells are tightly linked to each other providing mechanical strength and protection to the plant. Thus, the epidermis forms a boundary between the plant and the external environment and serves several other functions. It regulates gas exchange, protects the plant from water loss while forming the first layer that interacts with microbes, including pathogens and beneficials. Furthermore, the epidermis secretes metabolic compounds and is able to absorb water and mineral nutrients (Dolan et al., 1993).

1.5.1.1.2 Cortex/Endodermis

Directly below the epidermis lies a cortical region. The cortex is a band of parenchymal cells with storage function. For instance, it stores carbohydrates or essential oils and tannins and borders the innermost cortical layer, the endodermis. The endodermal cells exhibit specialized cell wall modifications forming a belt surrounding the endodermal cells in the longitudinal direction, called Casparian strip (Fig. 1.6b). Chemically, the Casparian strip cell walls contain lignin polymers. These polymers form a diffusion barrier where the passive, apoplastic flow of water and nutrients from the soil solution across the cortex to the central cylinder is forced

to become symplastic or transcellular (through polarly localized influx and efflux carriers) and thus remains under control. Figure 1.6 shows a picture of the root transversal section (a), as well as a schematic representation of the water and nutrient flow into the stele (b). At a secondary stage of endodermal differentiation, suberin, a hydrophobic aliphatic polyester highly resistant to chemical and enzymatic degradation, accumulates between the plasma membranes and primary cell walls of endodermal cells. The presence of suberin blocks or reduces the transport of nutrients across the plasma membranes of suberized cells. This barrier significantly affects radial uptake of water and dissolved nutrients, and radial loss of oxygen (Geldner, 2013). Figure 1.7 shows the appearance of suberin lamellae at lager stages of development, closing off endodermal cells and forcing nutrients to undergo a longer symplastic passage through plasmodesmata. Furthermore, the modified cell wall structures form a significant physical barrier against microorganisms. Importantly, cell wall degrading enzymes, typically secreted by pathogens during the infection of plant tissues, cannot easily degrade these chemically distinct endodermal cell walls (Geldner, 2013). In line with this observation, fungi and also *Ralstonia solananacearum* are unable to efficiently penetrate the endodermal cell layer, indicating that the endodermis is an important colonization barrier, maybe principally due to the formation of suberin (Bishop et al., 1983; Vasse, 1995; Parniske, 2008). In summary, the endodermis mainly acts as a selectivity filter for microbes and the transport of water and solutes between the root and soil interfaces.

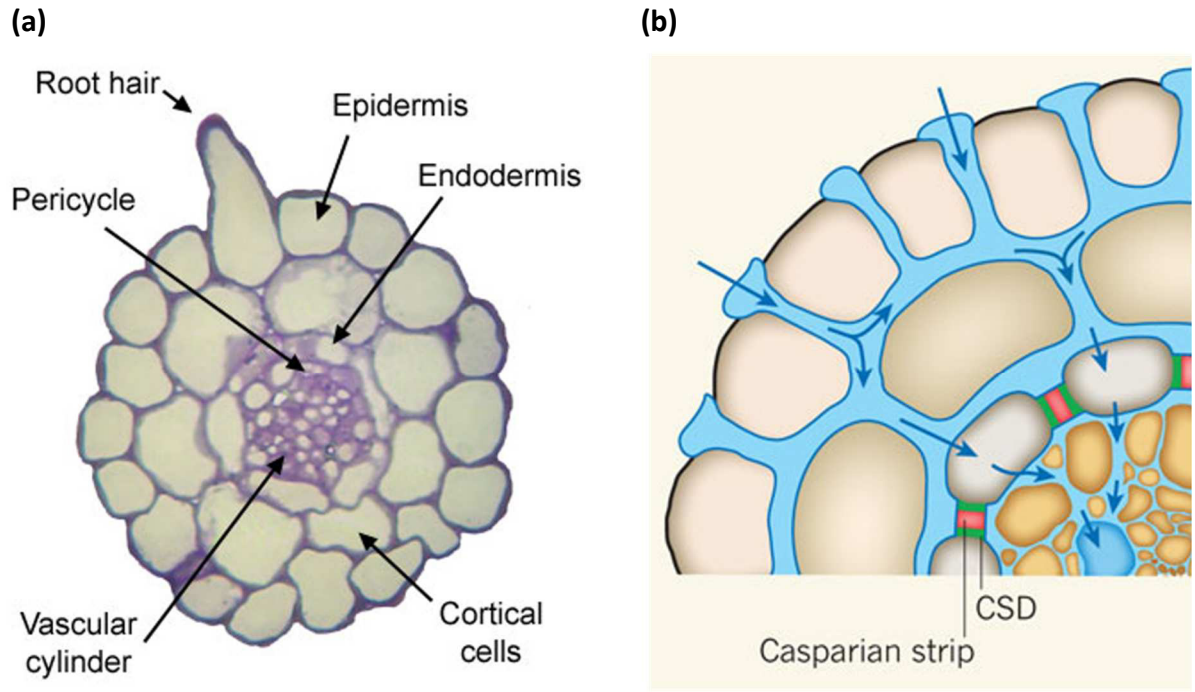

Fig. 1.6 The root cross-section and its water/nutrient flow. (**a**) Picture of the Arabidopsis root cross-section after staining with toluidine for visualization of the cell wall. From the root surface to its center epidermal tissues with emerging root hair surrounding the cortical cell layer, the endodermis and the vascular cylinder are shown. The cylinder is composed of three tissues, the pericycle, the xylem and the phloem. This figure has been adapted from http://www.ccrc.uga.edu/~mao/ultrast/root/root.htm. (**b**) Scheme of the upper-left quarter of a root cross-section showing the flow of water and solutes (depicted as blue arrows) through the cell-wall space. The transverse endodermal cell walls that connect cortical and vascular cell walls are impregnated by a cell-wall thickening, the Casparian strip (shown in red). The plasma membrane domain of differentiated endodermal cells in direct contact with the strip is called the Casparian strip domain (CSD, shown in green). Because of the Casparian strip, water and solutes cannot cross transverse cell walls and are redirected to the plasma membrane and the interior of endodermal cells. The Casparian strip acts as selective diffusion barrier, before releasing water and selected solutes into the vascular system (shown in yellow). The figure was first published by Grebe (2011).

1.5.1.1.3 Vascular Cylinder

The stele, or vascular cylinder, includes all of the tissues subjacent to the endodermis: the pericycle and the vascular tissues, xylem and phloem. The outer layer of the vasculature, adjacent to the endodermis is the pericycle. The pericycle cells retain the ability to divide throughout their life. Lateral (branch) roots emerge from a localized division of pairs of pericycle cells that originate from three cell files adjacent to the xylem pole (De Smet et al., 2007; Lucas et al., 2008). The formation of lateral roots requires breaking all endodermal and epidermal barriers adjacent to the initiated root and could therefore constitute a possible entry site for pathogens into the vasculature. The vascular tissue is organized in roots within a single central vascular cylinder. There, the vascular tissue is organized into strands called vascular bundles, each containing xylem and phloem. The xylem transports water and minerals within

the primary plant body, whereas the phloem conducts nutrients produced in the shoot to the roots. The xylem and phloem cells form a similar longitudinal continuum throughout the plant. In brief, the primary vascular system serves three functions. First, the phloem conducts photosynthates from the green stems and leaves to non-green areas (usually roots, lateral meristems, and shoot apical meristems) to promote growth and development. Second, the xylem provides a water-conducting system and a mechanical support as a result of the rigid lignified cell walls. Third, fibres, cells with thickened walls, provide the plant with additional support and stability (Lucas et al., 2013).

1.5.1.2 Developmental root zones

In the longitudinal section of a root, three main zones are visible. The zone of cell division is the lowest one where the root cap protects the apical meristem from rocks, dirt and pathogens and facilitates movement of the root through the soil. Cells are continuously released from the outer surface of the root cap. Interestingly, the root cap seems to perceive and process many environmental stimuli and mediates the direction of root growth accordingly (Hasenstein et al., 1988; Ishikawa and Evans, 1990; Okada and Shimura, 1990; Fortin and Poff, 1991; Takahashi, 1997; Eapen et al., 2003).

The root apical meristem contains a pool of stem cells, which surrounds an organizing center called “quiescent center” (QC) (Bennett and Scheres, 2010). The QC is the heart of the root meristem, it has very little mitotic activity itself but functions to maintain the stem cells in their undifferentiated state. Recent findings indicate that the QC may also act as a reservoir to replenish stem cells (Heidstra and Sabatini, 2014). The apical meristem initial cells give rise to the three primary meristems (Fig. 1.7): 1) the protoderm, which stands around the outside of the stele and develops into the epidermis, 2) the procambium, which lies inside the protoderm and develops into primary xylem and phloem and also produces the pericycle and 3) finally, the ground meristem, which develops into the cortex, composed of parenchyma, collenchyma and sclerenchyma cells (Bennett and Scheres, 2010).

Once the cells have reached a certain distance from the meristem and perceived changes in the concentrations of growth regulators such as hormones, cells stop dividing and begin to elongate rapidly (Fig. 1.7). Thereby, they push their apical neighbor cells farther away from the meristem to the elongation zone. As in the elongation zone the epidermis and endodermis have not fully established yet, this zone represents a risky area with regard to pathogen

invasion. Once elongated, these cells start to differentiate and meet their fate in the zone of differentiation or specialization. This zone can be recognized by the appearance of root hairs in the epidermis and lignification of the xylem (Geldner, 2013). Root hairs are outgrowths from root epidermal cells and increase the root surface and diameter. They have functions in nutrient acquisition, plant anchorage and microbe interactions (Grierson et al., 2014).

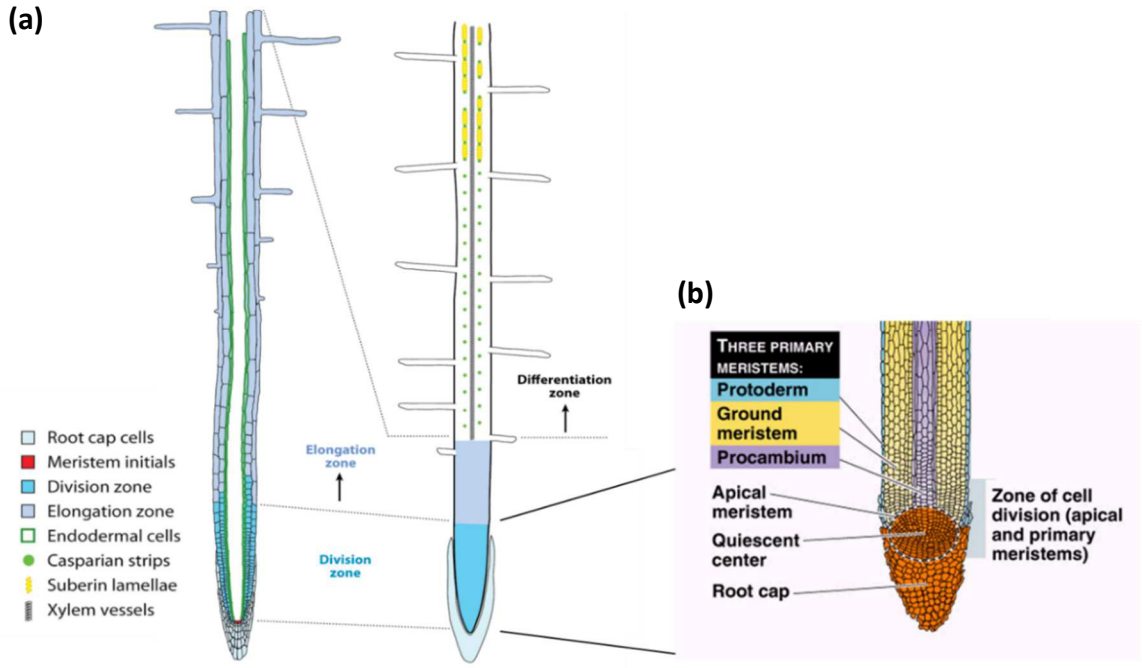

Fig. 1.7 Detailed cellular scheme of the root longitudinal axis including a division zone magnification. (**a**) The division zone (blue) includes the meristem initials (red) and the root cap cells (light blue) and is followed by the elongation zone (grey). The path of the endodermal cell lineage is shown in green (left panel). In the differentiation zone (white), the appearance of the Casparian strip is depicted by green dots, concomitant with xylem vessels (dark grey) and later, the patchy appearance of suberin lamellae in yellow (right panel). This figure is adapted from Geldner (2013). In (**b**) a magnification of the root division zone with the root cap, depicting the quiescent center surrounded by the apical meristem, producing the three primary meristems (protoderm, ground meristem and procambium) is shown. This part of the figure has been adapted from Reece and Campbell (2011).

1.5.2 The root microbiota

The rhizosphere is defined as the area around a plant root that is inhabited by a unique population of microorganisms influenced by the chemicals released from plant roots. It is enriched in dead cells, root secretions and harbors diverse bacterial and fungal taxa (Lundberg et al., 2012). A subset of these microorganisms even enter the root and live as endophytes (Buée et al., 2009). The microbiome in the rhizosphere is extremely dense and diverse; one gram of soil is estimated to contain up to 10^{11} microbial cells, which can be pathogenic or beneficial (Egamberdieva et al., 2008). Beneficial microbes provide the host roots with

nutrients or protection (Friesen et al., 2011; Berendsen et al., 2012). Additionally, a vast amount of rhizosphere microbes prime local and systemic resistance in the plant and lead to ISR, making plants more resistant for subsequent attack by pathogens (Pieterse et al., 2014). The fine-tuning of defenses, activated through priming instead of a direct activation of resistance, is an important mechanism and a critical step to improve plant resistance while saving fitness costs (Selosse et al., 2014). Thus, plant roots have to sharply distinguish between friends and foes at a very local level (Antolín-Llovera et al., 2014). Yet, very little is known about how roots perceive and defend themselves against attackers.

In respect of exposure to microbes, the root is often compared to mammalian skin, respiratory and especially gut epithelia. Like for roots, the number of potential pathogens is vast in animal tissue. It is estimated that 10^{14} indigenous bacteria from more than 1000 species are present in the human colon (Whitman et al., 1998). However, this abundant microflora does not elicit over-inflammation in the intestinal mucosa under physiological conditions (Kazmierczak et al., 2001; Bantel et al., 2002). In a similar manner, miscellaneous environmental microbes present around gut epithelia do not over-activate host immune responses whereas in the meantime more deeply buried tissues are still sufficiently sensitive (McClure and Massari, 2014; Selosse et al., 2014). Furthermore, beneficial root as well as gut microbes protect the host by competing for space and food with potential invaders and prime host defenses (Clemente et al., 2012; Ramírez-Puebla et al., 2013).

1.5.2.1 Beneficial root microbes

The most important beneficial root microbes include mycorrhizal fungi, nitrogen fixing bacteria and plant growth promoting rhizobacteria (PGPR). Mycorrhiza are fungi which form a symbiosis with plant roots. Whereas the symbiotic fungi provide water and mineral nutrients such as phosphate to the plant, the host in turn supplies the fungi with mainly carbohydrates (Parniske, 2008). Additionally, mycorrhizal fungi produce antimicrobial compounds, prime the plants for defense responses and compete with pathogens for ecological niches and are therefore believed to protect the plant against different kinds of pathogens (Morgan et al., 2005). The arbuscular mycorrhizal symbiosis is probably the most widespread terrestrial symbiosis (Fitter, 2005) and is formed by 70-90% of land plant species with fungi that belong to a monophyletic phylum, the *Glomeromycota* (Hibbett et al., 2007).

For nitrogen fixing bacteria, the interaction between *Rhizobium* and roots of legume plants is the best studied case. In a specialized plant organ, the root nodule, plants feed bacteria with carbohydrates, whereas the symbiont converts by fixation the atmospheric nitrogen into ammonium, an organic form of nitrogen available for the plant (Long, 1989).

The establishment of both, mycorrhizal and rhizobial associations, is achieved through a specific chemical communication between the microorganisms present in the rhizosphere and the host plant root (Oldroyd, 2013). Strigolactones and flavonoids are released by the plant root as signals for the arbuscular mycorrhizal fungi and rhizobia, respectively. In turn, fungi and rhizobia produce lipo-chitoolisaccharides called mycorrhizal factors (Myc factors) and nodulation factors (Nod factors). Those microbial signals are recognized in the root cells by specific LysM receptors to activate a symbiosis signaling pathway. The perception of Myc factors is required for root colonization by the symbiotic fungi. Nod factors promote the organogenesis of root nodules, as well as their infection by rhizobia (Oldroyd, 2013). Interestingly, Myc and Nod factors are closely related to pathogen-derived chitin (lipo)-chitooligosaccharides perceived by highly similar LysM receptors. Despite structural similarities between the microbial signaling molecules and the receptors involved in their recognition, each LysM receptor shows a strong ligand specificity, which allows plant cells to initiate appropriate signaling pathways resulting in very different outputs, symbiosis and defense (Antolín-Llovera et al., 2014). Moreover, some studies indicate that effectors secreted by symbiotic microbes may play a role in suppressing defense responses triggered by LysM receptors to allow the establishment of symbiosis (Yang et al., 2010a; Kloppholz et al., 2011; Plett et al., 2011; Wang et al., 2012a). More research is needed to further evaluate the importance of effectors in the process of symbiosis establishment (Tóth and Stacey, 2015).

Despite the fact that Arabidopsis is the main model organism to study plant-microbe interactions, it is not the optimal model to investigate symbiosis because it is unable to establish symbiotic associations with either mycorrhizal fungi or *Rhizobium* (Lionetti and Métraux, 2014). However, Arabidopsis can be used to examine the interaction between PGPRs and roots. In general, growth promotion by PGPRs occurs through niche exclusion in the rhizosphere, the production of antimicrobial compounds, as well as metabolites that enhance plant growth, induction of plant defense, competition for and sequestering of nutrients (Pieterse et al., 2014). Besides these local mechanisms, induction of ISR is a further positive effect of PGPRs (Whipps, 2001; Pieterse et al., 2014). Although many bacteria from the genus *Pseudomonas* are successful foliar pathogens, they are generally not described as root

pathogens. Yet, they have been shown to colonize roots and several *Pseudomonas* strains are promoting plant growth by protecting roots against potential pathogens (Haas and Défago, 2005). Some plant growth promoting *Pseudomonas* species have been shown to activate ISR against a broad spectrum of fungal and bacterial pathogens by priming the activation of defense genes in leaves (Pieterse et al., 2014). This process is mediated by JA and ET signaling and requires the transcriptional regulator NPR1, a key regulator of SA signaling (Pieterse, 1998). Other PGPRs include *Bacillus* strains, which are also able to colonize the root surface and control plant disease (Kloepper et al., 2004). Due to their plant growth promoting characteristics, PGPRs are considered as biocontrol agents.

1.5.2.2 Root pathogens

Generally, root interactions with beneficial microbes have been extensively investigated compared to root-pathogen interactions. However, several groups of soil microorganisms cause root diseases. Studying soil-borne plant pathogens is particularly challenging since they are often able to survive in soil for many years and each crop may be susceptible to several species. Various systematic groups can affect plant roots but the major groups are oomycetes, fungi, bacteria, protists and nematodes. Furthermore, a few soil-borne viruses affect vegetable crops (Quentin et al., 2012).

One economically important soil-borne root pathogenic bacterium is *Ralstonia solanacearum*, responsible for the bacterial wilt disease in a wide host range and for immense yield losses worldwide (Hayward, 1991). *Ralstonia* enters to the root by attaching to the root epidermis, especially at the root elongation zone and the junction between main and lateral roots (Vasse, 1995). It penetrates into the intercellular spaces of the root cortex from where it reaches the vasculature. There, it colonizes and spreads from the xylem vessels, which become plugged, to the leaves and infects the rest of the plant (Digonnet et al., 2012).

A further major root pathogenic bacterium is *Agrobacterium tumefaciens*, which is responsible for the crown gall disease and the hairy root disease. It colonizes the root vasculature of a large variety of plants and therefore presents a great concern for crop production (Escobar and Dandekar, 2003).

Finally, the most significant root pathogenic bacteria are the filamentous *Streptomyces* (Loria et al., 2003; Okubara and Paulitz, 2005). Scab-causing *Streptomyces* have a wide host range and infect diverse underground plant tissues. Infection of roots results in root stunting,

browning, and seedling death (Loria et al., 2006). Harmful *Streptomyces* species infect roots through penetration with specialized infection hyphae and produce a toxin inhibiting cellulose deposition and causing necrosis of root tissue (Stingl et al., 2005).

The majority of successful root pathogens includes damaging oomycete pathogens such as *Phytophthora* and *Pythium* as well as fungal pathogens like, for instance, *Fusarium*. Most of them are necrotrophs, feeding on dead plant tissue after killing the roots using toxins, peptide effectors or enzymes that trigger host cell lysis and death (Okubara and Paulitz, 2005). Examples are the oomycete *Pythium*, the basidiomycete *Rhizoctonia* and the ascomycete *Sclerotinia,* often causing root and crown rot diseases responsible for huge crop losses (Quentin et al., 2012). Others, like *Phytophthora sojae*, are hemibiotrophs and form haustoria or feeding structures to extract nutrients from living cells (Mendgen and Hahn, 2002; Tyler, 2007). Additionally, some oomycetes such as *Phytophthora* or *Pythium* and fungi as *Rhizoctonia* species can also cause damping off diseases, responsible for seed or seedling lethality before or after germination (Quentin et al., 2012; Krupa, 2012). In contrast to biotrophic pathogens, the majority of root necrotrophic pathogens can infect a wide range of plant species and does not appear to have closely coevolved with a specific host what makes them especially ecologically threatening (Quentin et al., 2012). For instance, almost all fruit and nut trees, as well as most ornamental trees and shrubs can develop *Phytophthora* rot. Tomatoes, peppers, eggplant, and other vegetable crops can also be affected by *Phytophthora* rot (Hansen et al., 2012).

In addition, plant-parasitic nematodes and biotrophic protists from the class *Phytomyxea* attack roots and infect their hosts (Quentin et al., 2012). For instance, the protist *Plasmodiophora brassicae* causes clubroot disease in cruciferous plants, and is an emerging severe threat to the production of Brassica crops (Hwang et al., 2012). The two most economically damaging groups of nematodes are root-knot nematodes (*Meloidogyne spp.*) and the cyst nematodes, mainly represented by the two genera *Globodera* and *Heterodera* (Krupa, 2012). Nematodes are able to induce dramatic morphological and physiological changes in host roots: infected roots undergo a developmental switch that results in the formation of aberrant root structures forming permanent feeding sites. These feeding sites, called galls, consist of cells within the vascular cylinder, which become completely reorganized, hypertrophied and metabolically highly active, serving as food sources throughout the nematode life cycle (Davis et al., 2008; Bird et al., 2009; Gheysen and Mitchum, 2009).

An overview about these agricultural most harmful root pathogens and the crop diseases caused by them are summarized in the table below.

Table 1.2 Summary of the most ecologically important root pathogens and their associated diseases.
The table has been adapted according to the data from Krupa (2012) and www.viralzone.com.

Root pathogen	Disease	Affected plant species
Bacteria		
Ralstonia solanacearum	Bacterial wilt	Wide host range (tomato, pepper, eggplant, potato…)
Agrobacterium tumefaciens/rhizogenes	Crown gall/hairy root disease	Many dicotyledonous (grapevine, radish, tobacco, apple…)
Streptomyces	Common scab	Root crops (potato, radish, parsnip, beet, carrot…)
Fungi		
Fusarium	Root rot	Soybean, barley, banana…
Rhizoctonia	Root rot, damping off diseases	Wide host range (soybean, potato, cereals, sugar beet, cucumber…)
Sclerotinia	Root rot, damping off diseases	> 400 plant species (beans, carrots, celery, lettuce, radish, potato, tomato, peas…)
Verticillium	Vascular wilt	> 300 eudicot species (cotton, tomato, potato, oilseed rape, eggplant, pepper…)
Oomycetes		
Phytophthora	Root rot, damping off diseases	Almost all fruit and nut trees, ornamental trees and shrubs, tomato, pepper, soybean, eggplant and other vegetables
Pythium	Root rot, damping off diseases	Extremely wide host range (crops, grasses, weeds, vegetables)
Nematodes		
Meloidogyne spp (root-knot nematodes)	Root-knot galls	> 2000 plant species (field crops, pasture and grasses, horticultural, ornamental and vegetable crops)
Globodera/*Heterodera* (cyst nematodes)	Growth retardation, root damage, early plant senescence	Narrow and specialized host range of each species (cabbage, pea, soybean, carrot, sugarbeet, potato…)
Protists (*Phytomyxea)*		
Plasmodiophora brassicae	Clubroot	Only *Brassicaceae* (cabbage, broccoli, cauliflower, brussels sprouts, radish…)
Viruses		
Secoviridae (Nepovirus)	Chlorosis, systemic necrosis	Cowpea, tobacco, grapevine, beet, bean, tomato, rice…
Potyviridae	Chlorosis, systemic necrosis	Stone fruit crops, potato, tobacco, tomato…
Virgaviridae (Tobravirus)	Chlorosis, systemic necrosis	Tobacco, pea, pepper, bean, potatoes…

Fighting root pathogens is generally difficult because root diseases are often only visible when they have already become systemically established what makes them complicated if not impossible to control. Furthermore, exogenous applications of protecting treatments are tedious (Abawi and Widmer, 2000; Krupa, 2012). Therefore, understanding the exact mechanisms used by both, plants and pathogens during their interactions is important in order to develop efficient molecular tools to combat root pathogens.

1.5.3 Defense mechanisms in roots

Despite the remarkable advances in the knowledge of plant immune responses in leaves, the molecular interactions taking place in roots remain largely unexplored. However, plant roots should have developed defensive strategies that are clearly different from those of aerial organs, as they lack a robust cuticula and extracuticular barriers, like waxes and trichomes (Valkama et al., 2004). Roots have to interact with their environment directly via the apoplastic space in order to conduct water and mineral uptake for the plant, and thus, cannot be protected the same way as leaves. Finally, they have to sharply distinguish between "good and bad" in order to fend off pathogenic soil-microbes while maintaining the capacity to establish positive interactions with beneficial microbes (Antolín-Llovera et al., 2014).

1.5.3.1 PTI responses in roots

Roots are well-capable of responding to elicitors produced by soil-borne microbes and defense responses have been demonstrated in this organ (Attard et al., 2010; Millet et al., 2010; Jacobs et al., 2011). For instance, investigation of transgenic seedlings expressing the *β-glucuronidase* (*GUS*) reporter gene under the control of promoters of MAMP-responsive genes revealed that flg22, chitin and PGN triggered strong responses in Arabidopsis roots. Furthermore, callose deposition was detected upon MAMP treatment in roots (Millet et al., 2010). The same study demonstrated that flg22 induced the production and exudation of camalexin in roots, a well-studied antimicrobial compound. Accumulation of ROS and defense gene transcripts has also been documented for roots of flg22-treated seedlings (Jacobs et al., 2011). Remarkably, root border-like cells, emerging and individually released from the root cap into the rhizosphere, have recently been ascribed a role in perception and activation of defense responses (Plancot et al., 2013). In Arabidopsis and flax (*Linum usitatissimum*) these cells were shown to autonomously sense flg22 and PGN. Both MAMPs triggered in

detached border-like cells a rapid production of ROS accompanied by modifications in the extensin distribution within the cell wall (Plancot et al., 2013). It is assumed that extensins can be cross-linked by hydrogen peroxide in order to increase the mechanical strength of the cell wall (Kieliszewski and Lamport, 1994; Ribeiro et al., 2006; Pereira et al., 2011). Remarkably, even MAMP-triggered callose deposition and overexpression of genes involved in the plant immune response were observed in isolated root border-like cells of Arabidopsis (Plancot et al., 2013). However, as these cells are released from the roots, their contribution to plant protection against microbes remains largely elusive. Presumably, they form a protective shield around the root due to their fortified cell walls.

R gene-mediated resistance has only been described for a few root-pathogen examples (Segal et al., 1992; Ori et al., 1997) and the typical HR seen in leaves has not been described in roots. Several explanations have been proposed for this observation. First, some HR components might be missing in the root. Secondly, plants might actively suppress HR in roots, although the reasons for this suppression are unknown (Millet et al., 2010). In summary, these data indicate that different defense responses take place in roots but the exact, underlying events are poorly characterized.

1.5.3.2 Root defense responses and beneficial microbes

Surprisingly, at initial steps, beneficial microbes are also recognized as potentially harmful invaders by the plant (Pel and Pieterse, 2013). For example, rhizobial bacteria or PGPRs are recognized as a threat, which elicits PTI responses in their hosts (Kouchi et al., 2004; Lohar et al., 2006; Bakker et al., 2007; Van Wees et al., 2008; Zamioudis and Pieterse, 2012). Consistently, the establishment of mutualistic relationships between beneficial microbes and plants has been shown to rely on an active interference of the microbe with the host immune system (Zamioudis and Pieterse, 2012). For instance, many beneficial root microbes were shown to actively suppress hormone-dependent PTI responses in Arabidopsis roots, thereby allowing the establishment of symbiosis (Jacobs et al., 2013; Cole et al., 2014; Plett et al., 2014). Additionally, other root beneficial interaction partners as, for instance, the symbiont *Rhizobium meliloti* or the PGPR *Burkholderia phytofirmans*, evade PRR recognition by variations in their flg22 sequence, which allows the establishment of a beneficial interaction (Felix et al., 1999; Trdá et al., 2015). However, beneficial and pathogenic microbes are often perceived by highly similar receptors (Antolín-Llovera et al., 2014). For example, the structurally closely related pathogen-derived chitin and symbiotic (lipo)-chitooligosaccharides

are perceived by almost identical LysM-RLKs, but trigger defense and symbiosis signaling, respectively. Hence, the plant has to be capable to differentiate between foe and friend by employing similar or even identical receptors for both stimuli. Several mechanisms might contribute to ensure that the appropriate signaling pathway is initiated. As LysM-RLKs have been shown to assemble into alternative heterocomplexes in a ligand-dependent manner it is suggested that coreceptors play a crucial role in determining the response pathway initiated (Antolín-Llovera et al., 2012). Besides the respective coreceptors, also different downstream response components might be recruited to a receptor complex or differentially activated, allowing the induction of various types of signaling pathways upon the initial activation of the same LysM-RLKs (Antolín-Llovera et al., 2014). Several studies indicate that the discrimination process critically depends on the abundance and localization of PRRs at the cell surface. Their activity and abundance is believed to be regulated by tissue-specific expression or via ubiquitination, endocytic trafficking and/or recycling mechanisms (Antolín-Llovera et al., 2014). Additionally, the function of RLKs has been shown to be controlled by cleavage of their ectodomain, generating a truncated and rapidly degraded RLKs fragment (Antolín-Llovera et al., 2014). Undoubtedly, robust mechanisms need to be available in plants to control signaling output.

1.5.3.3 Hormones in root defense

In general, little is known about the importance of hormones in root defense. JA and ET signaling are known to be important players against root necrotrophic pathogens (Turner et al., 2002; Wang et al., 2002; Devoto and Turner, 2003; Gutjahr and Paszkowski, 2009). SA has been shown to accumulate in roots upon root colonization by fungal pathogens (Balmer et al., 2013) and is involved in resistance against root-knob nematodes (Wubben et al., 2008). Additionally, root treatment with SA activates a number of genes involved in plant defense (Badri et al., 2008). Moreover, SA-mediated plant defense pathways have been shown to control nodule formation and mycorrhiza colonization, indicating that SA is involved in the control of biotrophic plant-microbe interactions at the root level (Herrera Medina, 2003; Gutjahr and Paszkowski, 2009). Abscisic acid has been assigned a potential role in fine tuning hormonal pathways in roots (Erb et al., 2009; Nahar et al., 2011).

1.5.3.4 Toxic products

As leaves, roots can produce and secrete an equally rich or even higher variety of natural toxic products or secondary metabolites, which have a prominent function in the protection against predators and microbial pathogens (Kaplan et al., 2008b; Rasmann and Agrawal, 2008). Furthermore, often volatile secondary metabolites such as terpenoids, fatty acid derivatives, phenyl propanoids or benzenoids are produced in roots upon herbivore attack to attract natural enemies of the predator (Ali et al., 2010; Hiltpold et al., 2010; Rasmann et al., 2011; Turlings et al., 2012). The recruitment of these "soldier microbes" by the plant during defense signaling can strongly alter the composition and quantity of the rhizosphere microbial communities. Through the production of antimicrobial and insecticidal compounds, these microbes help the plant to suppress pathogen or herbivore attacks (Carvalhais et al., 2013).

1.5.3.5 The role of tissue-specificity in root defense

Two zones in the root are particularly susceptible to pathogen entry due to discontinuities in the protective root barriers: the elongation zone and the sites of lateral root emergence. Indeed, Millet and colleagues showed that MAMP-responsive promoter-*GUS* reporter lines exhibited induced GUS activity in the elongation zone after MAMP treatment, indicating the presence of active defense responses in this zone (Millet et al., 2010). Thus, it is well possible that this root entry site requires locally a high-sensitive, even constitutively active perception and defense machinery. Furthermore, this defense machinery might be restricted in localization to these specific points in order to avoid constitutive activation of induced resistance mechanisms, which can negatively impact plant fitness and growth (Heil, 2002; Heil and Baldwin, 2002). In line with this idea, plants exposed to long flg22 treatment show reduction in growth, possibly due to a trade-off between immune and hormonal pathways as mentioned above (Gómez-Gómez et al., 1999; Navarro et al., 2006; Lozano-Durán et al., 2013; Chapter 1.4.10). Interestingly, a tomato *R* gene (*RESISTANCE GENE I-2*) was shown to be locally expressed at the basis of emerging lateral root primordia, where potential pathogens are expected to infect the root (Mes et al., 2000). Similarly, a compartmentalization of PRR receptors at very restricted localizations could be important to avoid the induction of unnecessary defense responses but this hypothesis remains to be investigated.

The matter of tissue-specificity is of special interest given that the innate immune system, PRRs and/or their responses may need to be modulated depending on the accessibility of that tissue from the outside compartment. Interestingly, this strategy of reducing receptor

accessibility or activation is known in animal cells. As an example, the TLR9, the PRR for microbial DNA, has been shown to exhibit distinct transcriptional responses depending on the location of its activation by the MAMP, at either the apical or the basolateral surface domains of polarized intestinal epithelial cells (Lee et al., 2006). The polarity of this cellular mechanism may explain how intestinal epithelial cells avoid inflammation in a microbe-rich environment under steady-state conditions. Whether a similar system also occurs in plant roots is not clear. Nevertheless, it is proposed that localization of plant immune receptors in specific tissues, cells or subcellular locations might play an important role in order to avoid over-stimulation of immune responses (Faulkner and Robatzek, 2012).

Taken together, our current knowledge indicates that tissue-specific defense responses might play an important role to balance and regulate immunity in roots.

1.5.3.6 Systemic signaling between roots and shoots

Root colonization by some PGPRs, but also plant growth promoting fungi (PGPFs) and symbiotic arbuscular mycorrhizal fungi, can prime the whole plant body for enhanced defense against a broad spectrum of pathogens, including fungi, bacteria, as well as some herbivorous insects and viruses (van Loon, 2007; Lugtenberg and Kamilova, 2009; Shoresh et al., 2010; Cameron et al., 2013). In particular, root colonization by PGPRs has been shown to prime distant tissues for stronger and faster defense response in case of a pathogen attack. This rhizobacteria-mediated plant growth promoting effect has been described as ISR by van Loon et al. (1998). It is widely assumed that this priming effect limits energy costs for plant defense. Although the exact mechanism underlying the elicitation of ISR remains unclear, increasing evidence suggests that MAMPs or microbial elicitors are involved in this process (Bakker et al., 2007). Indeed, root application of flagella from *Pseudomonas putida* WCS358 triggered ISR against *Pseudomonas syringae* in Arabidopsis (Meziane et al., 2005). As a *P. putida* mutant lacking flagella was still able to induce ISR, the perception of MAMPs other than flagellin is sufficient to elicit this response. Consistently, it was shown that treatment with LPS from *Pseudomonas fluorescens* WCS417r also triggers ISR against *P. syringae* in Arabidopsis (Van Wees et al., 1997). Further bacterial determinants described to induce resistance include: biosurfactants, N-acyl-homoserine lactones, N-alkylated benzylamines, antibiotics, exopolysaccharides and siderophores (van Loon et al., 1998; De Vleesschauwer and Höfte, 2009).

SAR is defined as a "whole-plant" enhanced resistance state that occurs following a localized exposure to a pathogen. SAR has mainly been studied with regard to leaf pathogens; however, root pathogens like the fungus *Colletotrichum,* the tobacco necrosis virus or the oomycete *Peronospora tabacina* have been shown to induce SAR in systemic leaves (Jenns, 1979; Tuzun and Kuć, 1985). Since then, the exact characterization of SAR-inducing root pathogens has received sparse attention. Only recently, it has been shown that root infection with nematodes was able to elicit SAR in shoots of tomato plants (Molinari et al., 2014). Additionally, it was acknowledged that an attack by root herbivory may also result in systemic induction of defense compounds in the shoot (van Dam et al., 2003; Bezemer and van Dam, 2005; Kaplan et al., 2008a). In contrast to SAR, ISR induced by beneficial microbes is often regulated through SA-independent mechanisms. Nevertheless, several PGPRs have been shown to trigger the SA-dependent SAR pathway (Choudhary et al., 2007; Pieterse et al., 2014). These results open an interesting field of study that will lead to a deeper understanding of signaling in plants in the future.

1.6 Aims of this thesis

Plant pathogenic microorganisms are responsible for immense yield losses in agriculture and the development of new technologies to engineer plant resistance is of general agro-economical concern. As the role of roots in plant defense has been neglected in the past although soil-borne pathogens constitute a major threat for crop plants, the predominant aim of my thesis was to increase our knowledge about the specific molecular mechanisms underlying MAMP perception and immunity in roots.

The functions of immune receptors, their interactions and downstream signaling processes have been widely studied in leaves. In roots, it has been previously shown that defense reactions against MAMPs are orchestrated in a highly tissue-specific manner. From this observation, several hypotheses which were to be tested in this thesis arose: 1) the immune receptors themselves or downstream signaling components could be expressed or localized in a tissue-specific manner, which would explain why only certain tissues have the capacity to respond; 2) only certain tissues could be able to perceive externally applied MAMPs, due to their accessibility to the elicitor.

To shed light on the regulation of immune responses in roots, I first investigated the autonomous competence of roots to initiate typical defense reactions upon flg22 perception (Chapter 2). In a second approach, in collaboration with Martina Beck from the group of Silke Robatzek, Sainsbury Laboratory, Norwich, UK, I analyzed the spatial activity of the *FLS2* promoter in wild-type plants in order to elucidate whether the observed lack of defense responses in certain tissues correlates with the expression level of the PRR (Chapter 2.1).

In parallel, to determine whether all root tissues have the capacity to perceive flg22 and transmit flg22 recognition by FLS2 to activate downstream signaling, I expressed *FLS2* in the Arabidopsis *fls2* mutant background under the control of several tissue-specific promoters and measured PTI responses in roots upon flg22 application. This strategy allowed me to compare and quantify the nature and intensity of the immune responses initiated in the various types of tissues perceiving the elicitor. The evaluation of tissue-specific immune responses could further help to understand the biological attribution/relevance of the tissues in the immune response (Chapter 2.2).

Another important part of this thesis was the generation of new tools that will be used in the future to broaden our understanding of immunity in plants. In this respect, I produced Arabidopsis transgenic lines expressing nuclear-targeted fluorescent reporter proteins under

the control of promoters of MAMP-induced marker genes (*Prom:NLS-3xVenus*). These lines were used in collaboration with the Sinergia project members from the group of Niko Geldner, University of Lausanne, and the group of Jean-Pierre Métraux, University of Fribourg, to elucidate tissue-specific downstream activation of immune responses upon MAMP recognition (Appendix). Furthermore, I aimed at developing a method to study systemic signaling from roots to shoots, which enables a local treatment of roots and avoids contact of the elicitor with the aerial part of the plant in axenic conditions (Chapter 3).

A second aim of my thesis was to understand the role of PTI in viral immunity. Viral pathogens cause extensive crop losses in agriculture but whether plants recognize viruses via PTI is just beginning to be elucidated. Although RNA silencing is considered to be the main antiviral defense mechanism, a recent study conducted in our lab indicated a possible role of BAK1 in the perception of a viral pattern or a virus-induced plant elicitor. The development of crop plants recognizing viral PAMPs may lead to cultivars resistant to a broad range of viruses.

As dsRNA produced during viral reproduction has been shown to be perceived by animal innate immune receptors and to induce innate immune responses, dsRNA could also be perceived as viral PAMP in plants. Therefore, in collaboration with Annette Niehl, University of Basel, I studied whether dsRNA is perceived as a *bona fide* PAMP in Arabidopsis. Additionally, the potential of dsRNA to protect plants against viral infection was examined (Chapter 4).

2 TISSUE-SPECIFIC *FLAGELLIN-SENSING 2* (*FLS2*) EXPRESSION IN ARABIDOPSIS ROOTS

2.1 Expression patterns of *FLAGELLIN-SENSING 2* map to bacterial entry sites in plant shoots and roots

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Statement

Expression of the flagellin receptor *FLS2* is regulated in a cell/tissue-specific and stress-induced manner that correlates with sites of bacterial infection. The vasculature expresses *FLS2* and responds to flagellin.

2.1.1 Abstract

Pathogens can colonize all plant organs and tissues. To prevent this, each cell must be capable of autonomously triggering defense. Therefore, it is generally assumed that primary sensors of the immune system are constitutively present. One major primary sensor against bacterial infection is the FLAGELLIN-SENSING 2 (FLS2) pattern recognition receptor (PRR). To gain insights into its expression pattern, we monitored the *FLS2* promoter activity in respective GUS reporter lines. Our data show that *pFLS2:GUS* activity is highest in cells and tissues vulnerable for bacterial entry and colonization, such as stomata, hydathodes and lateral roots. GUS activity is also high in the vasculature and by monitoring Ca^{2+} responses in the vasculature we found that this tissue contributes to the flg22-induced Ca^{2+} burst. The *FLS2* promoter is also regulated in a tissue- and cell type-specific manner and is responsive to hormones, damage, and biotic stresses. This results in stimulus-dependent expansion of the *FLS2* expression domain. In summary, we have created a tissue- and cell type-specific map of *FLS2* expression correlating with prominent entry sites and target tissues of plant bacterial pathogens.

2.1.2 Introduction

Plant pathogens use a variety of different strategies to invade their hosts, which are tightly associated to the lifestyle of the pathogen as well as to plant development (Faulkner and Robatzek, 2012). The general aim of a pathogen is to invade and access plant tissues where it can find nutrients for its own development. Bacterial phytopathogens typically try to reach the apoplastic space between cells where they can multiply and reprogram host metabolism by the injection of bacterial effectors into the extra- and intracellular space. During a susceptible interaction, as observed between *Arabidopsis thaliana* and *Pseudomonas syringae*, the gram-negative bacterium enters the host tissue (typically leaves) via natural openings (stomata) or wound sites, from where it propagates in the apoplastic spaces causing water soaked, chlorotic (and later also necrotic) lesions (Preston, 2000).

Lacking a circulatory system and specialized immune cells, plants depend on the ability of every cell to recognize potentially pathogenic microbes and initiate immunity. For this, plants exploit cell surface-localized pattern recognition receptors (PRRs), which allow the detection of conserved microbial molecules, so called microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Boller and Felix, 2009). In the case of immunity against

bacterial pathogens, a major PRR is the receptor kinase FLAGELLIN-SENSING 2 (FLS2) which recognizes bacterial flagellin through its conserved elicitor-active epitope flg22 (Gómez-Gómez et al., 1999). Studies show that flg22 triggers defense responses in whole seedlings, leaves, and roots (Zipfel et al., 2004; Millet et al., 2010; Jacobs et al., 2011). This suggests that the receptor is expressed in these tissues, which is consistent with findings of mRNA expression studies and FLS2-GFP imaging (Gómez-Gómez and Boller, 2000; Robatzek et al., 2006). These observations generally imply that defense components like FLS2 might be constitutively expressed, but this might lead to an unwanted activation of defense responses which can negatively impact plant processes such as growth. A typical response, which can be observed for plants that are exposed long-term to flagellin, is the reduction in plant growth, due to a defined trade-off between immune and hormonal signaling (Gómez-Gómez et al., 1999; Navarro et al., 2006; Navarro et al., 2008; Lozano-Durán et al., 2013).

Publicly available gene expression data (Arabidopsis eFP browser: http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Faulkner and Robatzek, 2012) revealed that *FLS2* is not expressed at similar levels throughout the plant. For example, *FLS2* does not have measurable expression in root cells, despite flg22 triggering some defense responses in this organ (Millet et al., 2010; Jacobs et al., 2011). In leaves, FLS2 exhibits a more specific cellular function since flg22 perception seems to play a predominant role in stomatal immunity (Zipfel et al., 2004; Zeng and He, 2010). Recent studies showed that *FLS2* transcriptional activation depends on ethylene (ET) signaling involving binding of the transcription factors ETHYLENE-INSENSITIVE 3 (EIN3) and ETHYLENE-INSENSITIVE3-LIKE 1 (EIL1) (Boutrot et al., 2010; Mersmann et al., 2010), and is positively regulated by its own ligand and other microbe-associated molecular patterns (MAMPs) (Zipfel et al., 2004; Zipfel et al., 2006). These observations indicate that *FLS2* expression is under spatio-temporal control, but the extent to which the transcription of *FLS2* is regulated remains unknown.

Here, we demonstrate that the *FLS2* promoter is active in a cell type- and tissue-specific manner and is up-regulated in response to hormones and stress. Using transgenic Arabidopsis plants producing β-glucuronidase (GUS) under the control of the *FLS2* promoter, we detected *GUS* activity in all organs, of which highest levels were found in hydathodes, stomata and the vasculature, representing prominent entry sites and target tissues of bacteria in plants. Tissue-specific Ca^{2+} measurement shows the vasculature is responsive to flg22. Detail imaging furthermore revealed that FLS2 is present in roots but restricted to outgrowing lateral roots (LR) and the inner central cylinder, suggesting a specific role for FLS2 in these tissues.

Hormones, wounding, abiotic and biotic stress can differentially activate *pFLS2:GUS* in specific tissue layers. Altogether, this study provides a detailed expression map of a major plant immune receptor and reveals spatio-temporal control of the *PRR* promoter activity for optimal plant defenses upon pathogen attack.

2.1.3 Results

2.1.3.1 *FLS2* is highly expressed in stomata, hydathodes and wound sites in leaves

To investigate the promoter activity of *FLS2* at the tissue level, we generated transgenic *Arabidopsis thaliana* lines containing the putative promoter sequences of the *FLS2* gene fused to *GUS*. We used a ~900 base pair (bp) genomic sequence upstream of the start codon of *FLS2* (Fig. S2.1), which was sufficient to fully complement an *fls2* mutant expressing the FLS2-GFP fusion protein (Zipfel et al., 2004). *In silico* motif analysis of the promoter sequence 900 bp upstream of AT5G46330 revealed the presence of a TATA box motif and several *cis*-elements such as W-boxes, known binding sites of WRKY transcription factors (Fig. S2.1). Two binding sites in the region were previously shown to be occupied by EIN3 and EIL1, transcription factors of the ET pathway mediating *FLS2* expression (Boutrot et al., 2010).

Monitoring GUS accumulation in the *pFLS2:GUS* lines during plant development, we could confirm that the *FLS2* promoter exhibited expression in all organs examined (Fig. S2.2). In two-day old seedlings, a clear blue staining could be detected in the developing cotyledons and root. In older seedlings, a prominent staining occurred additionally at the vascular tissue of cotyledons and hypocotyl (Fig. S2.2). At later stages of plant development, stipules, small leaf-like appendage at the bases of leaves, as well as floral and reproductive organs including petals, stamen and the dehiscence zone in mature siliques conferred a clearly visible *pFLS2:GUS* expression (Fig. S2.2).

As FLS2-mediated immunity is predominantly studied in the Arabidopsis interaction with the leaf infecting pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000), we focused on the basal *pFLS2:GUS* expression in different leaf developmental stages (Fig. S2.3a). In cotyledons and the first pair of true leaves, the promoter expression showed a homogenous pattern throughout the leaf tissue with higher expression levels in the vascular tissue and hydathodes (Fig. 2.1a,c,e). In younger leaves, GUS staining exhibited a more patchy distribution throughout the leaves (Fig. 2.1b; Fig. S2.3b), but continuously showed a strong staining in hydathodes (Fig. 2.1d). At the cellular level, *pFLS2:GUS* expression was

significantly visible in mesophyll and phloem, as well as in epidermal cells, such as in the guard cells of the stomata (Fig. 2.1e,g). Notably, the mesophyll cells underneath the stomatal openings, forming the sub-stomatal cavity, had clear promoter activity as revealed by cross sectioning of leaf tissues (Fig. 2.1f).

The sub-stomatal expression pattern is correlated to cells exposed to early invasion of bacteria, which enter the apoplastic space underneath stomata. To visualize the entry of bacteria in Arabidopsis leaves, we incubated Col-0 plants with a GFP transformed *Pto* DC3000 strain (Fig. S2.4a). The GFP-labelled bacteria were clearly visible at epidermal cells and within the openings of stomata (Fig. S2.4a). Bacterial accumulation was often detectable in the intercellular space of mesophyll cells directly underneath stomata (Fig. S2.4a). We next tested whether the presence of bacteria on the leaf surface would have an influence on the *FLS2* promoter activity. Overnight incubation of 14-18 days-old plants with *Pto* DC3000 led to a strong visible GUS staining in stomatal guard cells in leaves and the hypocotyl (Fig. 2.1h; Fig. S2.3d).

Bacteria also take advantage of wound sites and cracks in the epidermis to enter plant tissues, and we therefore investigated the influence of wounding on the *FLS2* promoter activity. In general, in young leaves the *pFLS2:GUS* activity was very low without stimuli (Fig. 2.1b). By contrast, wounding of leaves led to up-regulation of the promoter around the wound sites (Fig. 2.1i; Fig. S2.3c), which was not obvious in cotyledons and 1st true leaves (Fig. S2.3c). All these findings reveal that high levels of *FLS2* expression in leaves occur in cells and tissues that represent natural entry sites of bacteria, or can become entry sites due to wounding.

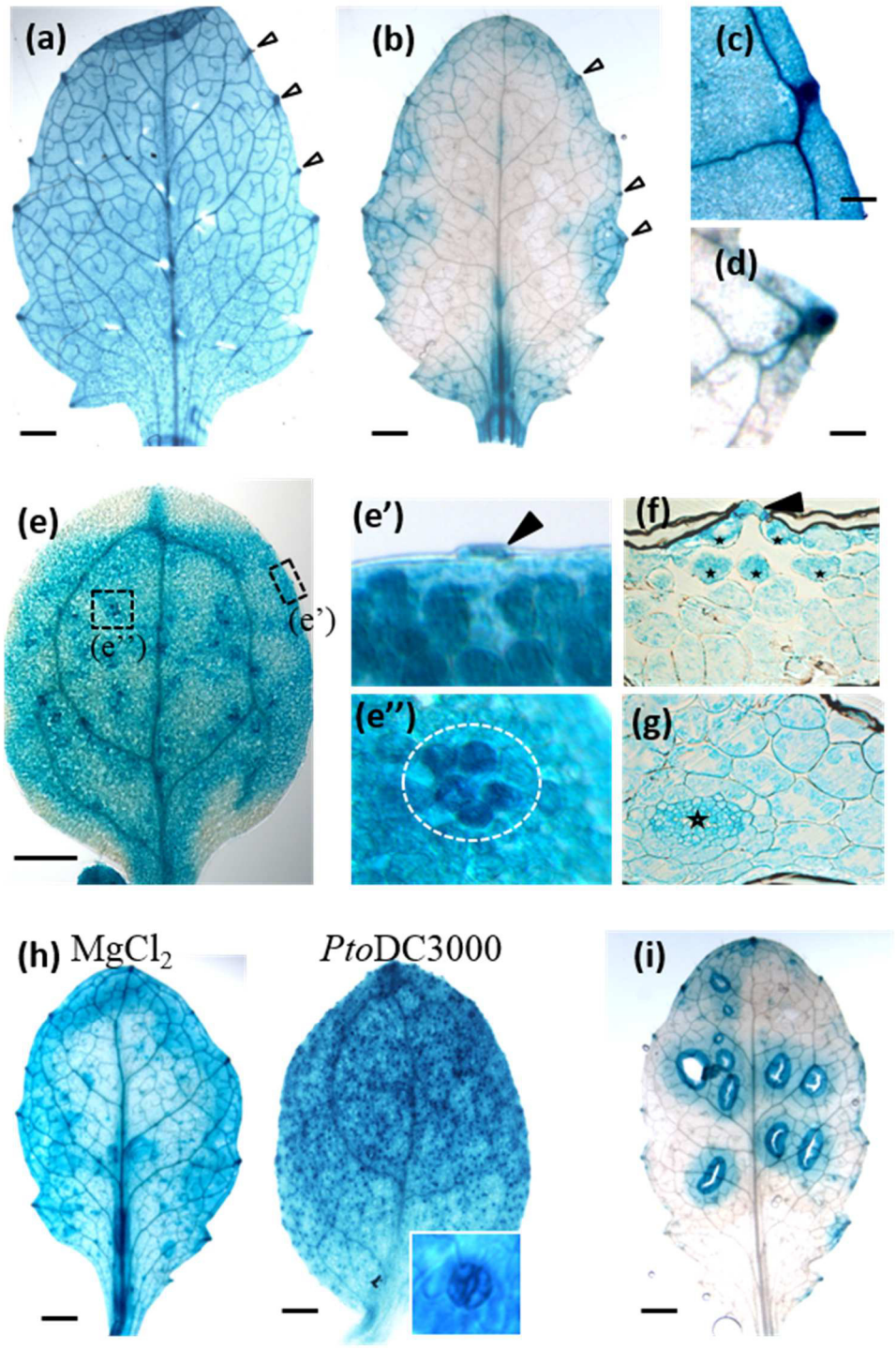


Fig. 2.1 *FLS2* is differentially activated in leaves. Representative images of *pFLS2:GUS* expression. (**a**) 1st pair of true leaves. (**b**) 2nd pair of true leaves. Arrows show strong expression in hydathodes from (**c**) cotyledons and (**d**) 2nd pair of true leaves. (**e**) Promoter activity in cotyledons; dashed boxes show expression (**e'**) in stomata (arrow) and (**e''**) group of mesophyll cells (circle). Cross section of cotyledons (**f**) shows guard cell expression (arrow) and high GUS staining in mesophyll cells surrounding the stomatal cavity (asterisks); (**g**) shows high expression in leaf veins (asterisk) and mesophyll. (**h**) *Pto* DC3000 increases promoter activity in stomata from 1st pair of true leaves compared to mock ($MgCl_2$) treatment. Inset shows an enlarged stoma. (**i**) Wound-induced GUS staining in 2nd pair of true leaves. (**a**, **b**, **e**, **h**, **i**) bar = 1mm, (**c**, **d**) bar = 0.1mm.

2.1.3.2 *FLS2* shows specific expression patterns and flg22 responses in roots

In roots, the *pFLS2:GUS* lines showed a basal expression in the root vascular cylinder starting at the root differentiation zone; no *GUS* expression could be observed in the root meristematic zone (Fig. 2.2a,b). Under sterile conditions, the highest expression was restricted to the inner cellular layers of the root, the vascular cylinder (Fig. 2.2b). In root cross sections, a pronounced accumulation of GUS precipitate was observed in cells inside of the endodermis (Fig. 2.2c) and this expression maxima correlated with a high accumulation of the *pFLS2:*FLS2-GFP fusion protein in the stele as revealed by co-staining the roots with the apoplastic tracer propidium iodide, of which uptake is blocked at the endodermis (Alassimone et al., 2010; Fig. 2.2d). These observations are consistent with the accumulation of the native FLS2 protein in roots as revealed by immunoblot analysis (Fig. 2.2g). This basal expression pattern of FLS2 in roots may protect the plant from bacterial infections of the vasculature and ultimately colonization throughout all tissues.

One of the earliest responses to MAMPs is a transient and rapid (within seconds) increase of free intracellular Ca^{2+} ($[Ca^{2+}]_i$), which subsequently (within minutes) declines to steady-state $[Ca^{2+}]_i$ levels (Blume et al., 2000; Ranf et al., 2008). This $[Ca^{2+}]_i$ $^+$ increase was shown to be crucial for many of downstream responses. To test whether the vasculature tissue is sensitive to flg22 stimulation, we took advantage of GAL4-mediated vascular enhancer trap line KC274 expressing the Aequorin (AEQ) specifically in the vasculature (Martí et al., 2013). Treatment with flg22 induced a rapid increase in $[Ca^{2+}]_i$ in both the vasculature-specific KC274;UAS aequorin line and in the line, in which aequorin was expressed constitutively under the control of CaMV35S promoter (Fig. 2.2f). The magnitude of the reported flg22-induced increase in $[Ca^{2+}]_i$ was greater when aequorin was targeted specifically to the vasculature tissue in KC274 (Fig. 2.2f) suggesting that FLS2 in the vasculature mediates a typical early flg22 response and indicates that this tissue contributes to the source of the MAMP-induced $[Ca^{2+}]_i$ burst in plants.

To gain further insights into the functional relevance of FLS2 presence in roots, we studied the phosphorylation of mitogen-activated protein kinases (MAPKs) upon flg22 elicitation. Immunoblot analysis revealed a specific flg22-induced activation of MAPK in root tissue of Col-0 but not *fls2* mutants demonstrating that FLS2 in roots activates similar signaling responses as shown for leaf tissues (Fig. 2.2h).

We further explored the root's response to flg22 elicitation on a more global scale, and performed whole transcriptome expression analysis. Sterile grown seedlings (*Ler*) were mock and flg22 treated and roots were harvested after 30 minutes. ATH1 microarray expression analysis revealed flg22-regulated genes overlapping with those identified from whole seedling expression analysis (Zipfel et al., 2004), but also identified about 75 genes specifically up-regulated in roots (Fig 2I). 53 of these genes showed more than 2.5 fold induction after flg22 treatment (Table S2.1: http://jxb.oxfordjournals.org/content/65/22/6487/suppl/DC1). Sixty-five of these genes have their highest expression values during root development (eFP Browser http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), which confirmed the enrichment for root-specific processes (Table S2.1: http://jxb.oxfordjournals.org/content/65/22/6487/suppl/DC1). These genes exhibited specific gene transcriptional changes in roots after flg22 with roles in hormone and stress signaling, like auxin- and ET-mediated pathways (AT1G59500, AT5G65600, AT1G72360, AT5G46080), root and lateral root (LR) development (AT4G31500, AT5G13080), or signaling and defense pathways (AT2G17060, AT3G21650) (Table 2.1). Taken together, our data show that not only *FLS2* promoter activity is present in roots, but also the functional protein. Root-specific activation of FLS2 reveals a subset of genes, which are specifically enriched after flg22 treatment, pointing at additional functions of this receptor in roots.

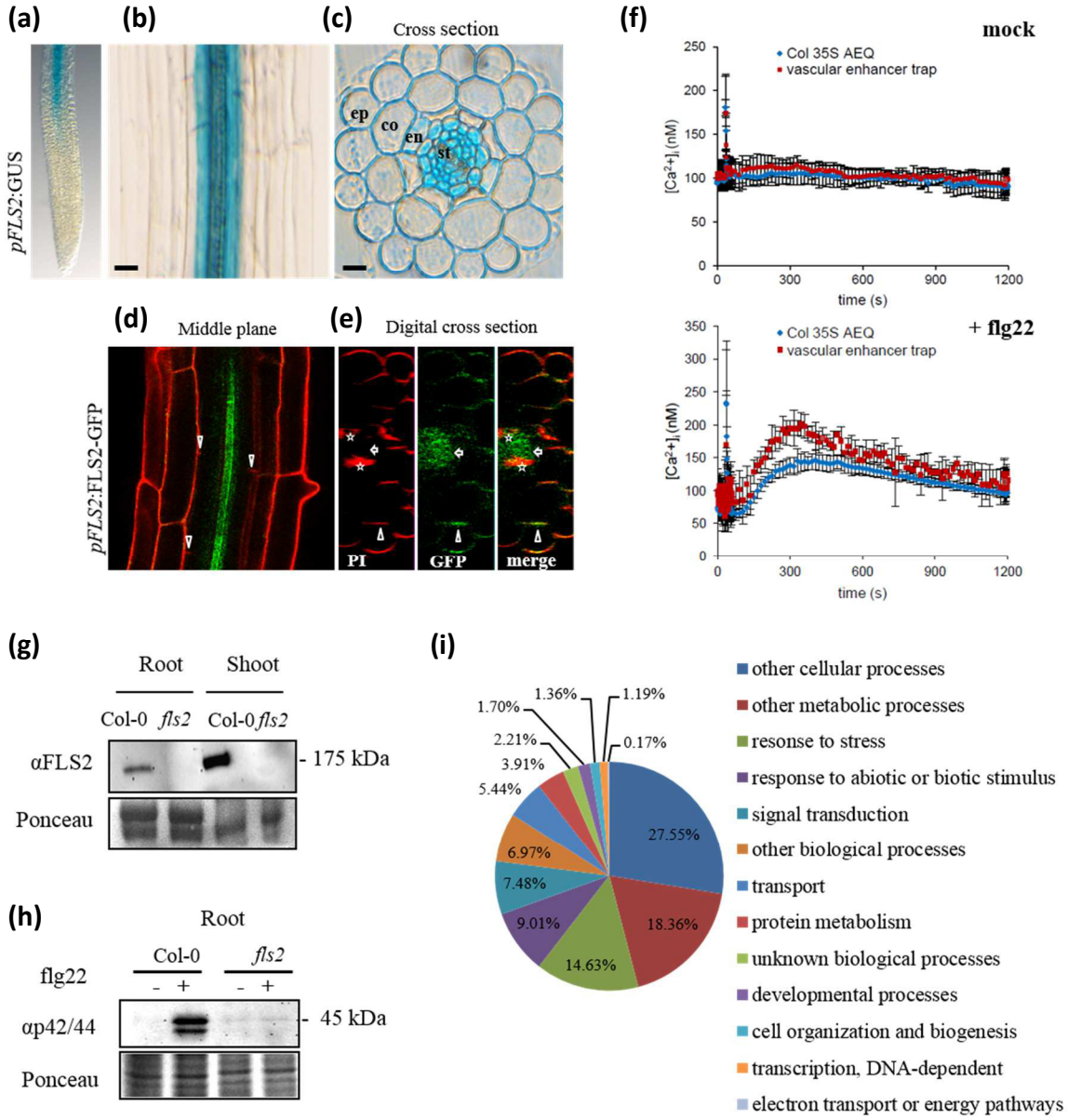


Fig. 2.2 Roots exhibit specific *FLS2* expression patterns and tissue specific responsiveness to flg22. In sterile-grown roots eight days after germination of *pFLS2:GUS*, the promoter activity is not present in root tips (**a**), but shows a high expression in the root stele (**b**) as revealed by root cross section (**c**); bar = 10 µm. (**d**) Confocal micrographs of *pFLS2:*FLS2-GFP show accumulation of GFP signal in inner part of the stele (arrow heads point to inhibited uptake of propidium iodide at endodermis; bar = 10 µm. (**e**) Digital cross section with plasma membrane localization of FLS2-GFP at cortex cells (arrow heads) and in the root cylinder (block arrow). Autofluorescence of xylem marked with asterisks. (**f**) Changes in $[Ca^{2+}]$ values in mock-treated control (water, 35 seconds) or in response to flg22 (100 nm, 35 seconds) in 35S:AEQ seedlings and the vasculature enhancer trap line KC274. Luminescence was measured over 1200 seconds. Data are presented as means ± SD, n=4 (mock), n=6 (flg22). (**g**) Immunoblot of detected FLS2 protein in roots and shoots. Samples were enriched for glycosylated proteins using ConA. (**h**) Immunoblot detection of phosphorylated MAPKs present in Col-0 after 1 µM flg22 (10 minutes) treatment but not in *fls2*. (**i**) GO ontology of enriched genes specifically up regulated in L*er* roots after flg22 treatment (10 µM, 30 minutes).

Table 2.1 Flg22-induced genes in roots: candidates with maximum expression in roots.

	Gene	Flg22 fold induction	Maximum expression level	Annotation	Biological process
Hormone and stress signaling	AT1G59500	**6.67**	3915.41 (1)	GH3.4; indole-3-acetic acid amido synthetase	auxin homeostasis, response to auxin stimulus
	AT5G65600	**5.3**	1132.13 (2)	legume lectin family protein / protein kinase family protein	protein phosphorylation, response to ethylene stimulus
	AT1G08050	**4.46**	2268.52 (2)	zinc finger (C3HC4-type RING finger) family protein	MAPK cascade,abscisic acid mediated signaling pathway, cell communication
	AT5G11920	**4.2**	8344.6 (2)	AtcwINV6 (6-&1-fructan exohydrolase)	carbohydrate metabolic process, regulation of hydrogen peroxide metabolic process
	AT1G15670	**4.05**	14417.2 (2)	kelch repeat-containing F-box family protein	negative regulation of cytokinin mediated signaling pathway
	AT5G67340	**3.94**	3905.65 (2)	armadillo/beta-catenin repeat family protein	ER-nucleus signaling pathway, MAPK cascade, negative regulation of defense response
	AT1G72360	**3.76**	11466.3 (2)	ethylene-responsive element-binding protein	cellular response to ethylene stimulus, regulation of transcription
	AT3G28580	**3.76**	8339.31 (2)	AAA-type ATPase family protein	response to abscisic acid stimulus, response to ethylene stimulus
	AT5G46080	**3.62**	890.14 (2)	protein kinase family protein	ethylene biosynthetic process, protein phosphorylation
	AT5G01550	**3.08**	1208.89 (2)	LECRKA4.2 (LECTIN RECEPTOR KINASE A4.1);	abscisic acid mediated signaling pathway, protein phosphorylation, response to chitin
	AT3G13100	**2.66**	2294.74 (2)	ATMRP7; ATPase	response to other organism, salicylic acid biosynthetic process
Root development	AT4G31500	**3.44**	17621.3 (2)	CYP83B1 (CYTOCHROME P450 MONOOXYGENASE)	adventitious root development, callose deposition in cell wall during defense response
	AT1G67980	**3.42**	1164.11 (2)	CCoAMT; caffeoyl-CoA O-methyltransferase	lignin biosynthetic process
	AT3G45960	**2.83**	1535.55 (2)	ATEXLA3 (*Arabidopsis thaliana* expansin-like a3)	plant-type cell wall loosening, plant-type cell wall organization
	AT5G13080	**2.58**	3789.39 (2)	WRKY75; transcription factor	cellular response to phosphate starvation, lateral root development, response to ethylene stimulus
Signaling/Defense	AT2G17060	**3.79**	561.85 (2)	disease resistance protein (TIR-NBS-LRR class)	defense response, signal transduction
	AT4G28350	**3.66**	1223.16 (2)	lectin protein kinase family protein	defense response to fungus, protein phosphorylation, response to chitin
	AT1G64400	**3.09**	2202.11 (2)	long-chain-fatty-acid-CoA ligase	defense response to insects, fatty acid biosynthetic process
	AT3G21650	**2.74**	900.65 (2)	serine/threonine protein phosphatase 2A (PP2A)	signal transduction

(1) Lateral root
(2) Root

2.1.3.3 *FLS2* is highly expressed in emerging lateral roots

In soil, roots are exposed to a variety of microorganisms, both pathogenic and beneficial. Interestingly when plants were grown under non-sterile conditions, we observed an up-regulation of the *FLS2* promoter expression in the endodermis and cortex cells but not in epidermal cells showing that the *pFLS2:GUS* expression in roots is not restricted to the vascular cylinder but can expand at least to the cortical cell layer (Fig. S2.5a,b).

This expansion of the promoter activity to different tissues became also apparent during the developmental process of LR growth. The *pFLS2:GUS* lines exhibited significant staining in the LR primordia and outgrowing LR (Fig. 2.3a-d). When reaching a certain developmental stage, the promoter activity was restrained again in the vascular cylinder of the developed LR and no staining was found in the tip of the LR, similarly as observed for the primary root tip (data not shown). Outgrowing LRs provide prominent entry points of bacterial pathogens as the outgrowth from the pericycle to the outer epidermis is accompanied by epidermal cracks, where bacteria can easily attach and gain access to root tissues (Fig. S2.4; Dong and Iniguez, 2003; Tyler and Triplett, 2008). Thus, similar to leaves promoter activity can be found in cells vulnerable for bacterial infection.

2.1.3.4 Flg22 regulates lateral root growth and auxin distribution

Long-term treatment with flg22 leads to inhibition of root growth in wild-type seedlings (Gómez-Gómez et al., 1999). We extended this study and observed that the flg22-dependent inhibition of root growth (Fig. S2.5c) was accompanied with a reduced number of LR (Fig. 2.3e,f). As LR initiation is strongly dependent on auxin accumulation in the cells primed for LR outgrowth (Dubrovsky et al., 2008), we analyzed whether flg22 treatment might interfere with auxin distribution and maxima during root and LR growth. We treated DR5-GFP (auxin-responsive GFP) lines with flg22 and found that the auxin maxima in the LR primordia are reduced after 72 hours of flg22 treatment compared to the mock-treated control line (Fig. 2.3g). In addition, we observed in the flg22-treated DR5-GFP seedlings GFP signals in the root epidermal cells, which were not present in control lines (Fig. 2.3g). Thus, these data showed that flg22 does influence auxin distribution in a cell type-specific manner. The ectopic up-regulation of auxin in the epidermal cells as well as the down regulation of auxin in the LR primordia might contribute to the flg22-dependent inhibition of root and LR growth. This correlates with the identification of AT1G59500 and AT1G68765 from our transcriptome data set, which are known auxin-responsive genes (The Arabidopsis Information Resource

(TAIR)), and is in agreement with previous studies showing that auxin and auxin-responsive genes are also regulated by flg22 (Navarro *et al.,* 2006, Zipfel *et al.,* 2004). Our findings are also consistent with reduced DR5-GUS expression in roots and inhibition of auxin-mediated adventitious root growth when stimulated with oligogalacturonides (OGs), components of the plant cell wall known to trigger plant defenses similar to MAMPs (Savatin et al., 2011).

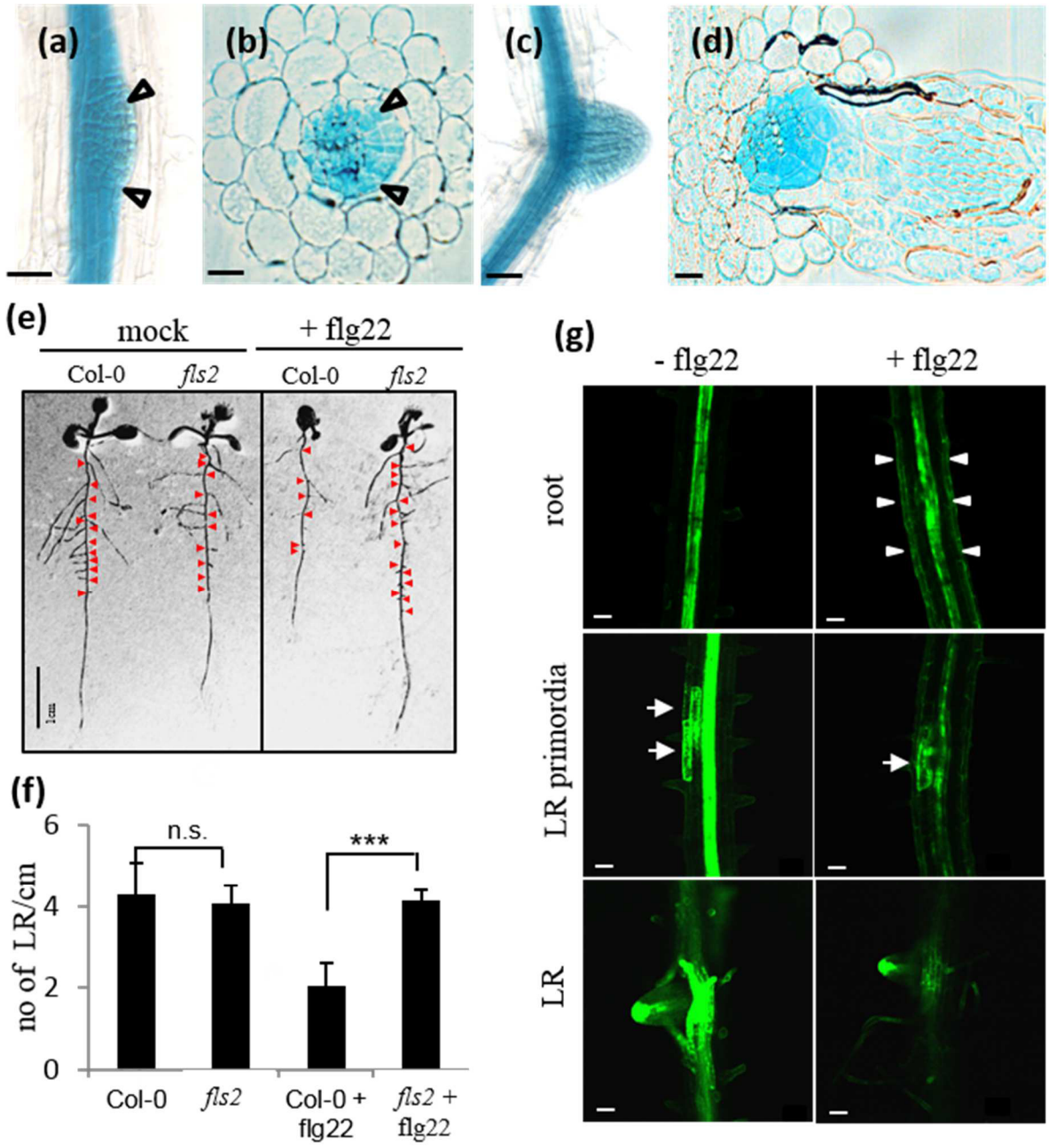


Fig. 2.3 Flg22 affects growth of *FLS2*-expressing lateral roots and auxin distribution. (**a**) *pFLS2:GUS* seedlings (ten days after germination) show prominent GUS staining in outgrowing lateral roots (LR) (arrows); bar = 50 µm. (**b**) Cross section of LR outgrowth (arrows); bar = 10 µm. (**c**) Promoter activity is present in developed LR; bar = 50 µm. (**d**) Cross section of developed LR; bar = 10 µm. (**e**) Picture depicts 12 days after germination Col-0 and *fls2* seedlings with and without flg22 (1 µM) treatment, red arrows point at LR. (**f**) Graph shows quantification of LR per cm root length in Col-0 and *fls2* seedlings with and without flg22 treatment (1 µM); bars represent average of three independent experiments, error bars represent SD, statistical significance represented with a Student's t-test (p-value > 0.001). (**g**) Confocal micrographs show roots of DR5-GFP transgenic seedling roots (ten days after germination) incubated for 72 hours with or without flg22 (1 µM); arrowheads point to GFP signals in epidermal cells of flg22 treated seedlings; middle and bottom panels depict different developmental stages of lateral root formation along the axis of ten days-old roots; arrows point to DR5-GFP signals marking LR primordia; bar = 50 µm.

2.1.3.5 Hormones and stress signals regulate *FLS2* expression in different root tissues

Pattern-triggered immunity (PTI) is highly regulated by the action of phytohormones like salicylic acid (SA), ethylene (ET), and jasmonate (JA) (Bari and Jones, 2009). In this context we studied the effect of different hormones and abiotic stresses, which are known to play important roles in PTI responses for their effect on *FLS2* promoter activity. In mock-treated roots, *pFLS2:GUS* expression was visible in the root late elongation zone, as described above (Fig. 2.2a; Fig. 2.5). Additionally we observed in ~20% of the control roots a distinct GUS staining in root cap cells directly underneath the root meristem (Fig. 2.4a). Incubation with flg22 led to an increased *FLS2* promoter activity in the root tip starting at the transition zone extending to cortical cells in the differentiation zone (Fig. 2.4a,b). When treated with SA, *pFLS2:GUS* roots showed a strong blue staining in the vasculature, which started closely after the meristematic zone (Fig. 2.4a), but did not extent to the cortex and also not to the differentiation zone (data not shown). Treatment with H_2O_2 or the ET precursor 1-aminocyclopropancarbonic acid (ACC) provoked an almost uniform promoter activity in the root cap, root meristem and root epidermal cells (Fig. 2.4a,b). However, ACC induced *pFLS2:GUS* activity in the vasculature to a much higher extent compared to H_2O_2 or mock treatment (Fig. 2.4b).

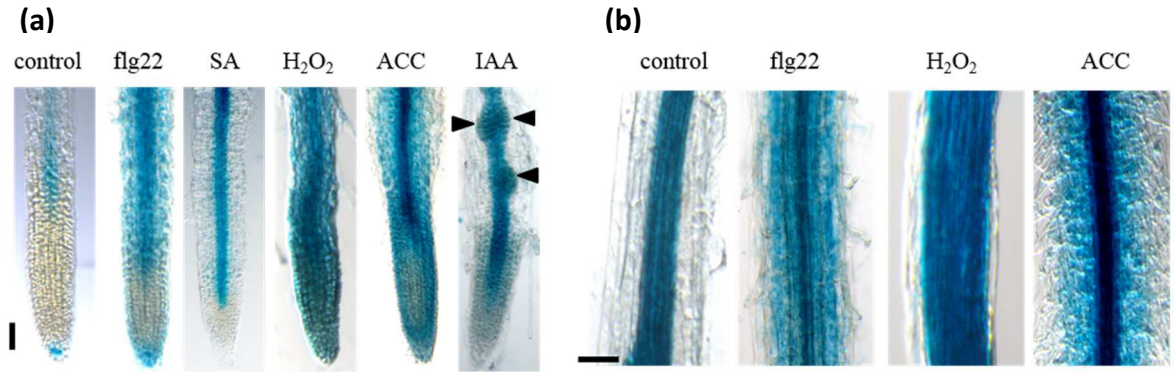


Fig. 2.4 Induced *FLS2* expression in roots is regulated in a tissue-dependent manner. (**a**) Promoter activity in the root tip of *pFLS2:GUS* seedlings (eight days after germination) after treatment with flg22 (10 µM), SA (50 µM), H_20_2 (1 mM), ACC (10 µM) and IAA (10 µM). (**b**) Promoter activity in root differentiation zone after flg22 (10 µM), H_20_2 (1 mM) and ACC (10 µM) treatment; (**a**, **b**) bar = 100 µm.

We also tested whether the promoter of *FLS2* is auxin-responsive. The emergence of LR primordia becomes highly induced by incubation with the auxin analogue indol-3-acetic acid (IAA), which exhibited clear *FLS2* promoter activity (Fig. 2.4a). However, GUS accumulation was specific to LR primordia and the vasculature in IAA-treated roots and no GUS staining in cortex cells was observed. These experiments revealed that flg22, SA, H_2O_2 and ET all

influence the expression activity of the *FLS2* promoter, but the responses are specific to different tissue layers in the root (Fig. 2.4). In summary, our study identifies an unexpected level of tissue-dependent *FLS2* expression regulation in response to a variety of different stresses (Fig. 2.5).

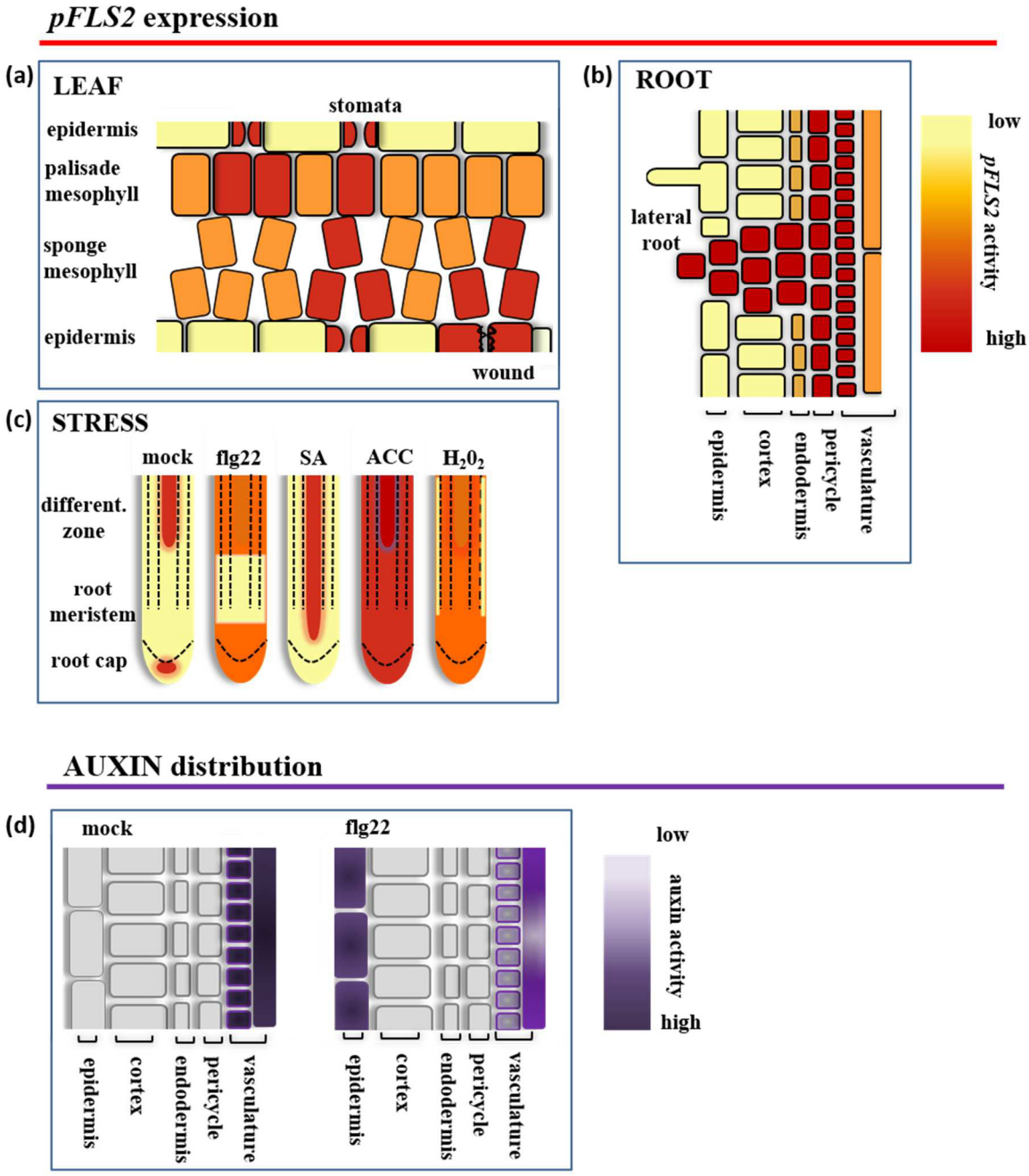


Fig. 2.5 Model summarizing *FLS2* cell-type and tissue-specific expression patterns. Cartoon depicts (**a**) promoter activity of *FLS2* in leaves (**b**) and roots; (**c**) stress responsiveness of the promoter in roots; and (**d**) flg22-dependent ectopic up-regulation of auxin in root epidermal cells.

2.1.4 Discussion

The prevailing view in plant immunity is that all plant cells are capable of pathogen perception and initial defense responses. This would require constitutive expression of at least the primary sensors of the immune system. Based on plant-scale expression analysis, *FLS2* was found in all plant organs including flowers, leaves, stems and roots (Gómez-Gómez and Boller, 2000; and this study). However, cell type-specific responses might play an important role in the context of how plants initiate defense responses against potentially invasive pathogens, but not fend off beneficial microbes that are often needed for plant growth especially in nutrient-low conditions (Bulgarelli et al., 2013). It was therefore proposed that the cellular and tissue location of immune components is essential to mount the appropriate defense responses, and that they should be best located at putative entry sites of pathogens to efficiently inhibit their invasion (Faulkner and Robatzek, 2012). In this study, we followed the *FLS2* promoter activity and identified that while *FLS2* is generally expressed in all tissues, there are remarkable differences in the level of the expression regulated in a cell type-specific and developmental manner. In addition, the *FLS2* promoter activity is responsive to several hormones playing roles in plant immunity such as SA and ET, which themselves are induced upon flg22 elicitation (Felix et al., 1999; Tsuda et al., 2008). Consistently, these and our observations show that *FLS2* expression in positively feedback regulated to fine-tune the immune response.

2.1.4.1 Prominent entry sites of potential pathogens are guarded by high *FLS2* expression

Hydathodes are pores at the leaf margin that are continuous with the xylem. Hydathodes are targeted by pathogenic bacteria such as *Xanthomonas campestris* pv. *campestris*, as points of access into plant tissue (Hugouvieux *et al.*, 1998)**.** The stomatal pores (Zeng and He, 2010) represent another prominent entry route of bacterial pathogens. Stomata close upon MAMP perception to restrict pathogen entry and successful pathogens secrete effectors such as HopM1, syringoline and coronatine that inhibit the closure and/or actively induce re-opening (Melotto et al., 2006; Schellenberg et al., 2010; Zeng and He, 2010; Lozano-Durán et al., 2014). Both cell types are characterized by a high promoter activity of *FLS2* compared to the surrounding mesophyll cells (Fig. 2.1c) suggesting that cells at tissue entry points are particularly well equipped to detect invading pathogens. Further, the mesophyll cells forming the sub-stomatal cavity also exhibit a higher *FLS2* promoter activity (Fig. 2.1f; Fig. 2.5). Previous data show that FLS2 mediates immunity at the level of stomatal entry (Zipfel et al.,

2004; Zeng and He, 2010). In agreement, stomatal expression of *FLS2* is enhanced upon bacterial infection. GUS staining is more intense in guard cells relative to surrounding cells, indicating a guard cell-specific regulation of *FLS2* promoter activity (Fig. 2.1h). Although we cannot rule out the possibility that the prominent GUS staining at hydathodes might be unspecific, *FLS2* expression at this location is consistent with the fact that hydathodes mark the end points of the vasculature, another tissue exhibiting high *FLS2* expression and importantly, responsive to flg22. The overall patterns of *pFLS2:GUS* expression we observe (Fig. 2.5a-c) are in agreement with publicly available expression data (eFPBrowser; Faulkner and Robatzek, 2012).

Wounds and cracks in the epidermal layers represent sites of vulnerability with respect to pathogen infection. The bacterial colonization beyond these primary infection sites is dependent on secreted effectors such as syringoline promoting distant tissue colonization (Misas-Villamil et al., 2013). The *FLS2* promoter is responsive to wounding in leaves (Fig. 2.1i; Fig. S2.3c) suggesting that cells at these sites might depend on higher FLS2 levels to fend off pathogen invasion of neighboring tissues. This is consistent with a previous study, which revealed that higher protein levels of FLS2 contribute to more flg22 binding and are positively associated with reduced *Pto* DC3000 proliferation (Vetter et al., 2012).

Plants have also “natural” wounds, which occur during the emergence of LR. These manifest as ruptures in the epidermal cell layer around the LR meristem. Detailed observations of bacterial colonization of roots led to the assumption that bacteria use these LR emergence sites as entry routes in the roots (Dong and Iniguez, 2003; Tyler and Triplett, 2008; Fig. S2.4b). Although in developed roots *FLS2* expression was not present in the meristem, the *FLS2* promoter exhibited a strong activity in the LR primordia and outgrowing LR (Fig. 2.3a-d). These observations indicate the *FLS2* expression is highly dynamic and regulated in a cell-type and development-dependent manner (Fig. 2.5b,c). Considering that LRs do not possess a root cap, which can also function as a MAMP-reactive physical barrier to the root meristem (Plancot et al., 2013), it might be essential for a plant to guard the LR meristem.

2.1.4.2 The vasculature is a tissue with high *FLS2* expression

Evidently, the vasculature provides excellent means for pathogens to spread throughout the plant. Together with the vasculature being rich in nutrients and water, this makes the vasculature a very attractive target tissue for pathogens. In plant interactions with a fungal pathogen, strong lignification of vascular bundles is associated with a compromised infection (Tanaka et al., 2014b). One significant observation of our study is the defined and high activity of the *FLS2* promoter in the root stele, which is correlated with a high abundance of the FLS2-GFP fusion protein (Fig. 2.2b-e). Interestingly, high promoter activity in vascular tissue were also found for *PEPR1* and *PEPR2*, receptors associated with damage-elicited responses and immunity (Bartels et al., 2013). In addition, we observed that the vasculature contributes to the flg22-induced increase in $[Ca^{2+}]_I$ (Fig. 2.2f). It has been described that flg22 induces the production of lignin (Schenke et al., 2011) but whether lignification is part of the FLS2-mediated immunity to prevent colonization and spread through the vasculature remains to be addressed. In the leaf, *Pseudomonas* bacteria colonize distant tissues along the vasculature (Misas-Villamil et al., 2011) whereas in the root, the bacterial pathogen *Ralstonia solanacearum* directly utilize plant xylem vessels to move through the plant (Digonnet et al., 2012). It is tempting to speculate that the absence of elicitor-active flagellin promotes the infection success of *Ralstonia solanacearum* bypassing FLS2-mediated defenses in the vasculature (Pfund et al., 2004).

While *FLS2* expression is restricted to the stele under normal conditions, expression can be expanded to the cortex under certain stresses (Fig. 2.4; Fig. 2.5c) and it is shown that roots are sensitive to flg22 initiating typical defense responses (Millet et al., 2010; Jacobs et al., 2011; this study). It is possible that low expression of *FLS2* in the root cortex allows the colonisation of this tissue by beneficial bacteria without triggering defense. High constitutive expression of *FLS2* in the stele might provide an additional barrier to bacterial invasion of the vascular tissue beyond the cortex, and stress induced expansion of this zone of expression might reflect increased vulnerability of the tissue. Flg22-dependent gene induction was quite specifically activated in the elongation zone, whereas flg22-induced callose deposition was observed over the entire root length (Millet et al., 2010). However, whether these immune response are initiated in epidermal cells, cortex cells or inner cylinder cells needs to be addressed in future.

2.1.4.3 Auxin-mediated root development is responsive to flg22

The long-term incubation with flg22 is known to inhibit root growth (Gómez-Gómez et al., 1999) and this inhibition of root growth is accompanied by a reduced development of LR (Fig. 2.3e,f). Auxin, an important plant hormone involved in the regulation of root cell elongation and LR outgrowth, is found to be ectopically up-regulated in the epidermal cells of flg22-treated roots while down-regulated in the LR primordia (Fig. 2.3g; Fig. 2.5d). This is in agreement with studies describing a flg22-dependent antagonism for auxin activity, which leads to a rapid down-regulation of auxin- responsive genes and contributes to plant resistance against bacteria (Navarro et al., 2006). Ectopic up-regulation of auxin in root epidermal cells was also described to be involved in ET-dependent root growth arrest (Růzicka et al., 2007). As ET production is triggered by flg22 (Felix et al., 1999) it might be possible that these hormones are together integrated in the flg22-induced inhibition of root growth, with a possible outcome being that flg22 reduces putative bacterial entry points at LR.

This interplay between the flg22 responses and hormone signaling is also reflected at the level of the *FLS2* promoter activity, as seen by the influence of IAA and ACC on the expression of *FLS2*. ACC treatment as well as the high induction around wound sites is consistent with a direct control of *FLS2* transcription by ET signaling (Boutrot et al., 2010; Mersmann et al., 2010). Altogether, these findings show a positive regulation of *FLS2* expression by hormones (ET and SA) and small signaling molecules such as ROS, which are produced upon flg22 trigger (Bari and Jones, 2009). This positive transcriptional regulation might be important to deliver newly synthesized receptors to the plasma membrane since activated FLS2 is removed from the plasma membrane by endocytosis and degradation (Robatzek et al., 2006; Göhre et al., 2008; Smith et al., 2014).

2.1.5 Concluding remark

We show that the *FLS2* promoter activity maps to vulnerable tissues targeted by bacteria for entry and colonization in plants. These findings will be useful to understand the tissue- and cell type-specific role of FLS2 in immune signaling, and will aid to develop strategies to enhance plant resistance by targeting defense to relevant tissues.

2.1.6 Materials and methods

2.1.6.1 Plant materials and growth conditions

The following *Arabidopsis thaliana* transgenic plants were used in this study (accession Columbia-0, if not otherwise indicated): *fls2* (Zipfel et al., 2004) *DR5:*GFP (Benková et al., 2003; courtesy of J. Friml). For microscopy, stress treatments and developmental studies, seedlings were grown for six to eight days on sterile 1x Murashige and Skoog plates supplemented with 1% sucrose and 0.8% phytoagar (w/v) under 16 hours light at 22°C. For Ca^{2+} measurement, the Col-0 35S:AEQ and GAL4-mediated vascular enhancer trap line KC274 was used (Martí et al., 2013). Seeds were surfaced sterilized and sown on half-strength 0.5 x MS medium with 0.8% agar (w/v). Seedlings were grown in long day conditions at 19°C, light intensity 50 μmol m-2 s-1 (Sanyo MLR30 growth cabinet) for 12 days. For non-sterile conditions used in developmental studies, plants were grown for 2-8 weeks on soil in controlled environments (12 hours light, 22°C and 60% humidity).

2.1.6.2 Gene constructs and plant transformation

The promoter of *FLS2* (988bp) was used from *pFLS2:FLS2-GFP* (Robatzek et al., 2006) and fused to the *GUS* gene, which was isolated from pGUS Topo via BamHI and HindIII restriction digest and inserted into *pFLS2*:pCAMBIA2300 resulting in *pFLS2:GUS*-pCAMBIA2300. Col-0 plants were transformed by the floral dipping method (Clough and Bent, 1998). Transformants were selected for kanamycin resistance. Our experiments were repeated in two independent transgenic lines of the T3 generation.

2.1.6.3 GUS staining

All samples were processed according to the method described by Block and Debrouwer (1992), with 1 mM of 5-bromo-4-chloro-3- indolyl-D-glucuronide (X-Gluc) in staining buffer [0.1 M NaH2PO4, 0.1 M Na2HPO4, 10mM EDTA, 2 mM FeK3(CN)6, 2mM FeK4(CN)6 x3 H2O, pH 7.0, 0.1% v/v Triton X-100] at room temperature for 2-18 hours. Samples were fixed and destained with EtOH/Acetic Acid (*v/v* 50%). Specimen were examined using the Leica M165 FC stereomicroscope.

2.1.6.4 Embedding and sectioning

Tissue was fixed in 2.5% glutaraldehyde or 4% paraformaldehyde, followed by washing in an ethanol series 30%, 50%, 70%, 90%, 100%, for 30 minutes each. Pre-infiltration of the tissue was done for two hours with 50:50 (*v/v*) ethanol: *Technovit®7100* (Heraeus-Kulzer, Germany) base liquid. The preparation solution (*Technovit®7100.* see embedding protocol*)* was infiltrated and tissue samples were left for polymerization. Samples were sectioned in 10 µm thickness by using an ultramicrotome Ultracut E (Reichert-Jung/ Germany).

2.1.6.5 Microscopy

Standard confocal laser microscopy was performed using the laser point scanning microscope Leica SP5. GFP/ Propidium Iodide was excited using the 488 nm argon laser, and fluorescence emissions were captured between 500 and 550 nm for GFP and between 580 and 640 nm for Propidium Iodide. Seedlings were incubated for 20 min in 10 µg/ml Propidium Iodide solution.

2.1.6.6 Stress treatments

The chemicals were diluted in ½ MS media to their respective working solutions: 10 µM flg22 (10 mM in dH_2O), 50 µM SA (100 mM in DMSO), 1 mM H_20_2 (1.5 M), 10 µM ACC (10mM dH_20), 10 µM IAA (100 mM dH_20). ½ MS was used as mock treatment. For each treatment, seedlings (8-10 days after germination) were transferred from agar plates and incubated in respective solutions for 48 hours under 16 hours light at 22°C, followed by GUS staining. For bacterial stress and wound treatments, detached leaves of three to four weeks-old soil-grown plants were used. Detached leaves were submerged in 10 mM $MgCl_2$ (mock) or with *Pto* DC3000 (OD 0.1) in 10 mM $MgCl_2$ solution, slightly shaking for 24 hours at RT. Wound stress was inflicted by a sharp needle on ten detached leaves mounted on ½ MS agar and left on plates for 4-6 hours at RT before staining. All stress treatments were done with at least ten seedlings or ten leaves of the two independent T3 transgenic lines at the same developmental stage. Images show representative results of three biological repetitions.

2.1.6.7 Ca^{2+} measurements

Seedlings grown for 12 days were supplied with ½ MS liquid medium supplemented with 20 µM coelenterazine (Nanolight) and incubated overnight in dark at room temperature. Luminescence measurements were performed using plate reader FLUOstar OPTIMA (BMG LABTECH). Luminescence from single wells was measured over 35 seconds and flg22 (EZBiolab) dissolved in ½ MS was injected to a final concentration of 100 nM and measured at 15 seconds intervals for 1200 seconds. Mock treatment (water, 35 seconds) was performed with the same conditions. At the end of the experiment the remaining aequorin pool was discharged by treatment with final concentration of 1M $CaCl_2$ in 10% (v/v) ethanol. Luminescence values were converted to estimates of $[Ca^{2+}]_i$ according to Fricker *et al.* (1999).

2.1.6.8 LR growth analysis

Col-0 and *fls2* mutants were germinated on 1 x MS plates and transferred three days after germination in liquid 1 x MS media without or with 1 µM flg22. After six days, the root length and number of lateral roots were determined.

2.1.6.9 Immunoblot and ConA precipitation

100 mg root tissue of seedlings (Col-0) vertically grown for two weeks on 1 x MS plates were homogenized in 0.2 ml of cold IP buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% (v/v) Nonidet P40 and protease inhibitor cocktail) and incubated for one hour at 4°C followed by centrifugation step (10,000 g for ten minutes, three times). The supernatant was incubated for one hour at 4°C with Concanavalin A (ConA)-sepharose beads (Amersham Biosciences) to enrich samples for glycosylated proteins. This was used as FLS2 is highly glycosylated (Häweker et al., 2010) and weakly detectable in root total extracts. The beads were collected and washed three times with ice-cold IP buffer. After denaturation in SDS-PAGE sample loading (0.35 M Tris-HCl pH 6.8; 30% [v/v] glycerol; 10% [v/v] SDS; 0.6 M dithiothreitol; and 0.012% [w/v] bromophenol blue), proteins retained on the beads were eluted by SDS-PAGE sample loading buffer and separated by 7% SDS-PAGE. FLS2 was detected by immunoblot analyses with anti-FLS2 antibodies (Chinchilla et al., 2006).

2.1.6.10 MAPK activation in roots

12 root systems of two week-old plants were placed in water and left over night. For MAPK detection in intact roots, 12 seedlings were placed overnight on split Petri dishes in order to treat roots separately of shoots. The following day, 1 µM flg22 was added to root tips for ten minutes and roots were dissected from shoot tissue immediately prior to freezing in liquid nitrogen. Tissue (50 mg per sample) was shock frozen in liquid nitrogen and ground into fine powder before addition of 50 µL SDS- extraction buffer (0.35 M Tris-HCl pH 6.8; 30% [v/v] glycerol; 10% [v/v] SDS; 0.6 M dithiothreitol; and 0.012% [w/v] bromophenol blue). Total proteins were separated by electrophoresis in 12% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufacturer's instructions (Bio-Rad). Transferred proteins were detected with Ponceau-S. Polyclonal primary antibodies against phospho-p44/42 MAPK (Cell Signaling Technologies) were used, with alkaline phosphatase-conjugated anti-rabbit as secondary antibodies. Signal detection was performed using CDP*st*ar (Roche).

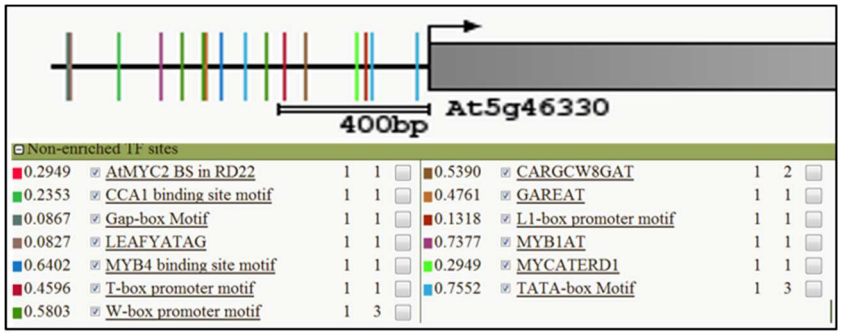
2.1.6.11 Microarray

Landsberg erecta (ecotype *Ler*) seedlings and *fls2-17* (Zipfel et al., 2004) were grown in liquid culture under constant shaking in 1 x MS media for 21 days. Plants were mock or flg22 (10 µM, 30 minutes) treated, roots were harvested and stored at -80ºC for sample preparation. Experimental conditions for RNA extraction, microarray hybridizations, and statistical analyses were performed as in Zipfel et al. (2004).

2.1.7 Acknowledgements

We thank Kim Findlay and Elaine Barclay (the BioImaging Support, John Innes Centre, Norwich UK) for technical help with the Microtome and Christine Faulkner for critically reading the manuscript. M.B. was supported by a grant from the Deutsche Forschungsgemeinschaft. Research in the S.R. laboratory is supported by the Gatsby Charitable Foundation and a grant from the European Research Council (ERC).

2.1.8 Supplemental figures



At5g46330		
Motifs		
AtMYC2 BS in RD22		**CACATG**
189 -184	(-)	CATGTG
CARGCW8GAT		**CWWWWWWWWG**
329 -320	(+)	CATAAATAAG
329 -320	(-)	CATAAATAAG
CCA1 binding site motif		**AAMAATCT**
826 -819	(-)	AGATTGTT
GAREAT		**TAACAAR**
593 -587	(+)	TAACAAA
Gap-box Motif		**CAAATGAA**
962 -955	(-)	TTCATTTG
L1-box promoter motif		**TAAATGYA**
164 -157	(-)	TACATTTA
LEAFYATAG		**CCAATGT**
953 -947	(+)	CCAATGT
MYB1AT		**WAACCA**
710 -705	(+)	AAACCA
MYB4 binding site motif		**AMCWAMC**
550 -544	(-)	GTTTGTT
MYCATERD1		**CATGTG**
189 -184	(+)	CATGTG
T-box promoter motif		**ACTTTG**
381 -376	(+)	ACTTTG
TATA-box Motif		**TATAAA**
484 -479	(+)	TATAAA
29 -24	(+)	TATAAA
151 -146	(-)	TTTATA
W-box promoter motif		**TTGACY**
601 -596	(+)	TTGACC
432 -427	(+)	TTGACT
655 -650	(-)	AGTCAA
EIN3 binding sites		**ATGTATCT**
325-317		ATGAATaT
304-297		AcATACAT
285-278		ATGAATaT

Fig. S2.1 Prediction of *FLS2* promoter motifs 1000bp upstream of At5g46330. (**a**) Visualization of promoter motifs 1000 bp upstream of ATG. (**b**) Details of predicted promoter motifs 1000 bp upstream of ATG. Prediction with Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences (O'Connor et al., 2005; http://www.bioinformatics2.wsu.edu/Athena).

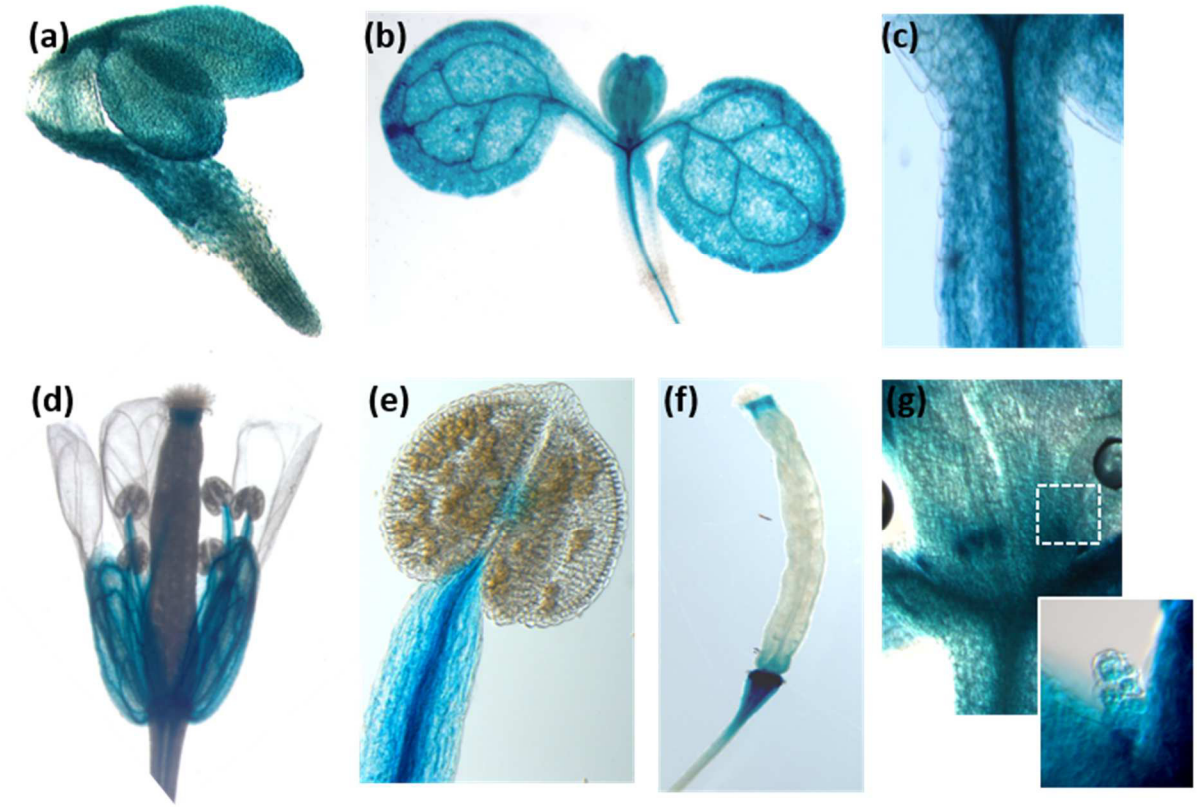


Fig. S2.2 *FLS2* promoter activity during plant development. *GUS* is ubiquitously expressed in (**a**) two days-old seedling, in (**b**) eight days-old seedling cotyledons and (**c**) hypocotyl; and highly expressed in (**d**) flower sepals and (**e**) stamen, in (**f**) pod dehiscence zone in mature siliques and (**g**) stipules.

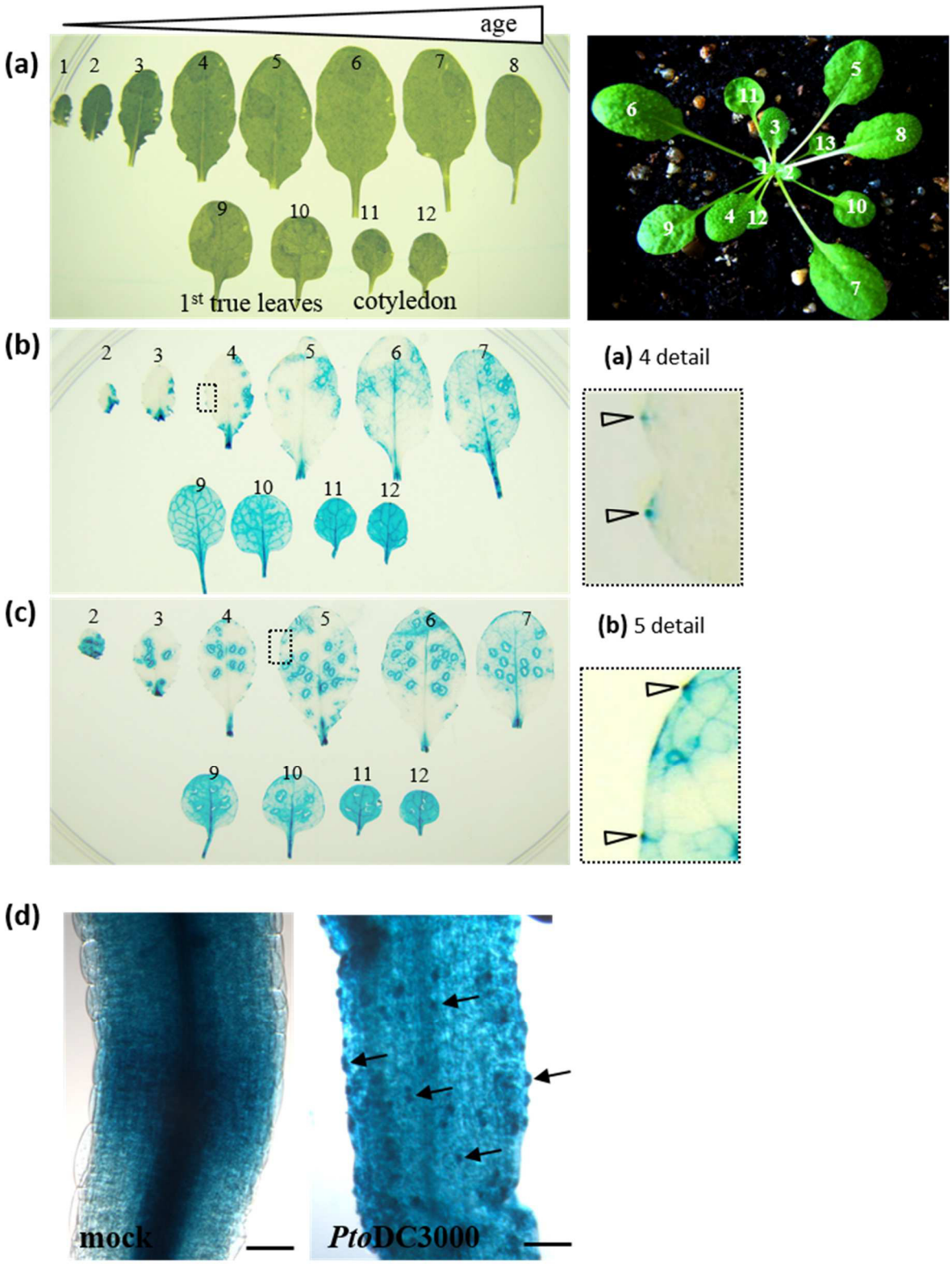


Fig. S2.3 *FLS2* promoter activity during leaf development, wound stress and biotic stress. (**a**) Definition of different leaf stages; (**b**) GUS staining of *pFLS2:GUS* transgenic plants in different leaf stages. Insert shows hydathodes. (**c**) Wound-induced promoter activity in different leaf stages. Insert shows hydathodes. (**d**) Hypocotyl after *Pto* DC3000 incubation with enhanced promoter activity in stomata (arrows), compared to mock treated (10mM $MgCl_2$) hypocotyl; bar = 100 µm.

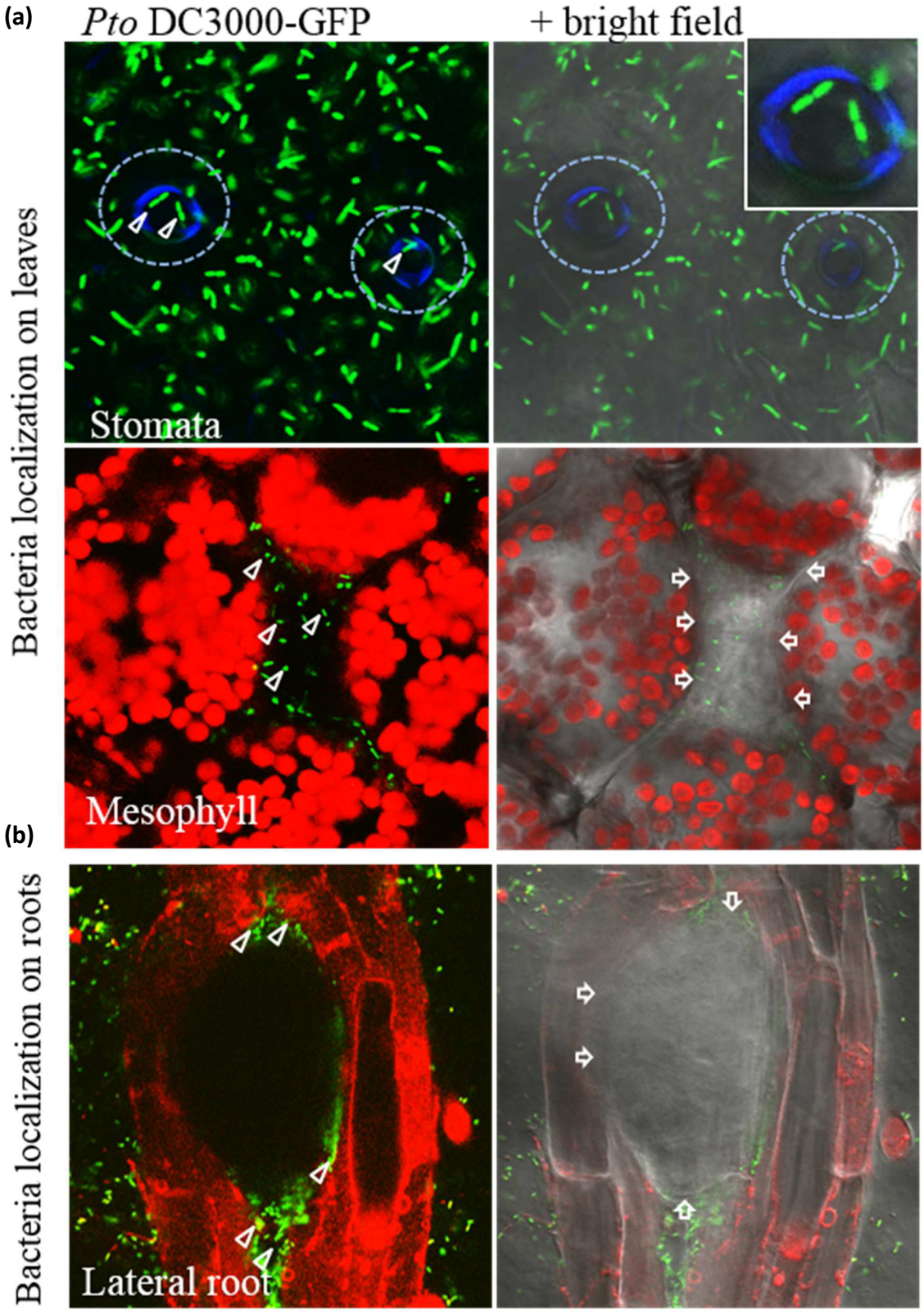


Fig. S2.4. *Pto* DC3000-GFP localization on leaves and roots. (**a**) Confocal micrographs showing *Pto* DC3000-GFP (green) on Col-0 leaves, upper panel shows bacteria on epidermis, encircled are stomata, lower panel shows bacteria (arrow heads) in the apoplast of mesophyll cells (block arrows); chloroplast autofluorescence is represented in red. (**b**) Confocal micrographs showing DC3000-GFP (green) bacteria on Col-0 roots stained with propidium iodide (red); note the accumulation of bacteria (arrow heads) at outgrowing LR (block arrows).

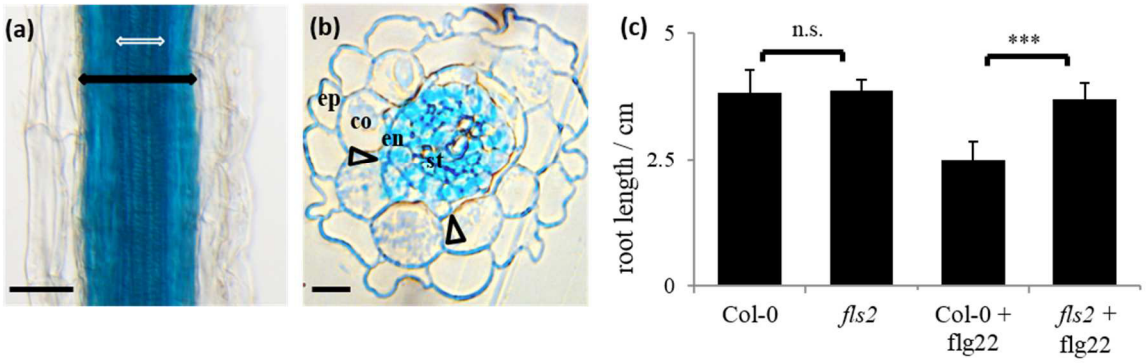


Fig. S2.5 *FLS2* promoter activity in non-sterile grown roots and flg22 dependent inhibition of root growth. (**a**) *pFLS2:GUS* activity in 14 days-old seedlings grown on soil, white arrow shows inner vasculature, black arrows mark expansion of GUS staining, bar = 50 µm. (**b**) Cross section of roots grown on soil, arrows mark expanded GUS activity in endodermis, bar = 10 µm. (**c**) Graph depicts root length of 12 days-old Col-0 or *fls2* treated with 1 µM flg22 or without (bars represent average of 3 independent experiments, error bars represent SD, statistical significance represented with Student's t-test (p-value >0.001).

2.2 Tissue-specific *FLS2* expression in roots restores immune responses in Arabidopsis *fls2* mutants

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2.2.1 Summary

- The flagellin receptor of Arabidopsis, At-FLAGELLIN-SENSING 2 (FLS2), has become a model for mechanistic and functional studies on plant immune receptors. Responses to flagellin or its active epitope flg22 have been extensively studied in Arabidopsis leaves. However, the perception of microbe-associated molecular patterns (MAMPs) and the immune responses in roots are poorly understood.
- Here, we show that isolated root tissue is able to induce pattern-triggered immunity (PTI) responses upon flg22 perception, in contrast to elf18 (the active epitope of elongation factor thermo unstable (EF-Tu)). Making use of *fls2* mutant plants and tissue-specific promoters, we generated transgenic Arabidopsis lines expressing *FLS2* only in certain root tissues. This allowed us to study the spatial requirements for flg22 responses in the root.
- Remarkably, the intensity of the immune responses did not always correlate with the expression level of the *FLS2* receptor, but depended on the expressing tissue, supporting the idea that MAMP perception and sensitivity in different tissues contribute to a proper balance of defense responses according to the expected exposure to elicitors.
- In summary, we conclude that each investigated root tissue is able to respond to flg22 if FLS2 is present and that tissue identity is a major element of PTI in roots.

2.2.2 Introduction

Like animals, plants possess a multilayered defense system of innate immunity conferring resistance against pathogens. Plant cells are able to recognize conserved epitopes of microbe-derived molecules called microbe-associated molecular patterns (MAMPs) such as bacterial flagellin (Felix et al., 1999) and bacterial elongation factor Tu (EF-Tu) (Kunze et al., 2004). Other MAMPs include lipopolysaccharides (Newman *et al.* 1995; Meyer *et al.*, 2001) and peptidoglycans (Gust et al., 2007) as well as chitin, a major component of the fungal cell wall (Felix et al., 1993; Miya et al., 2007). Perception of MAMPs by pattern recognition receptors (PRRs) involves the induction of a set of immediate and long-term responses that are collectively referred to as pattern-triggered immunity (PTI) (Boller and Felix, 2009), leading to an enhanced resistance against the invading pathogen (Zipfel et al., 2004; Zipfel et al., 2006). In Arabidopsis leaves, the initial recognition of the minimal active epitope of flagellin, flg22, by the receptor kinase FLAGELLIN-SENSING 2 (FLS2) is one of the best studied immunity pathways (Boller and Felix, 2009; Schwessinger and Ronald, 2012). Characteristic physiological downstream responses of FLS2 signaling such as the production of reactive oxygen species (ROS) in the apoplast (Torres et al., 2006), the activation of ET biosynthesis (Spanu et al., 1994), the change in ion fluxes across cellular membranes (Felix et al., 1999) and the phosphorylation of mitogen-associated protein kinases (MAPKs) (Nühse et al., 2000) have been studied extensively in aerial plant tissues as part of plant immunity (Boller and Felix, 2009).

However, although roots express the *FLS2* receptor and respond to treatment of seedlings with elicitors (Gómez-Gómez and Boller, 2000; Robatzek et al., 2006; Millet et al., 2010; Jacobs et al., 2011; Beck et al., 2014), it remains unknown whether the root itself is capable of sensing the pathogen to induce defense responses or if the induction of defense in the root depends on the transport of a signal from the shoot. Moreover, little is known about how and where potential threats and beneficial interactions are perceived and distinguished within roots in order to implement an adequate response (Millet *et al.*, 2010; Attard *et al.*, 2010; Lakshmanan *et al.*, 2012; Wang *et al.*, 2012b).

The Arabidopsis root structure is defined by specialized radially organized tissue layers (Dolan et al., 1993). The epidermis constitutes the physical barrier between the soil and the root cortex cells. The underlying endodermal cells surround the pericycle and the vascular tissues and provide a second barrier for solutes: the Casparian strip. Lateral roots emerge from the pericycle and break through the outer tissue layers and therefore the endodermal and epidermal

barriers. Unlike the root apex and meristem, which are coated by the root cap, the site of cell elongation is relatively unprotected since epidermal and endodermal tissues are locally not yet fully differentiated (Dolan et al., 1993). Bacterial root pathogens such as *Ralstonia solanacearum* can exploit such natural weaknesses in the root architecture as entry points to cross the root physical barriers (Digonnet et al., 2012; Faulkner and Robatzek, 2012). Once a pathogen has colonized the vasculature tissue, it can propagate throughout the whole plant and cause severe damage (Digonnet et al., 2012).

To shed light on the mechanisms governing pathogen perception and defense activation, we tested isolated roots for their ability to induce PTI responses after flg22 treatment, thereby circumventing any interference of systemic defense signaling from the shoot. To investigate the root tissues susceptible to elicitation and the efficiency of PTI responses in specific root tissues, we studied flg22 perception and defense pathway activation in Arabidopsis transgenic lines expressing *FLS2* under the control of tissue-specific epidermal, endodermal and pericycle promoters in the *fls2* mutant background.

Our results clearly demonstrate that local expression of *FLS2* in roots leads to flg22-dependent downstream MAPK phosphorylation, the production of reactive oxygen species (ROS) and the induction of defense marker genes in isolated roots. Additionally, flg22 perception in any of the tested tissues is sufficient to trigger PTI responses but the intensity of the induced response appears to depend on the tissue in which the signal originates rather than on the expression level of the receptor, suggesting that tissue identity is a major factor of PTI in roots.

2.2.3 Results

2.2.3.1 Perception of flg22 in isolated Arabidopsis roots

As it is known that the recognition of MAMPs by plants activates the innate immune system and one of the earliest defense responses is the production of ROS (Torres et al., 2006), we assessed the ROS production following treatment with flg22 in isolated wild-type Arabidopsis roots over time. The results clearly demonstrate that wild-type roots are able to autonomously perceive flg22 and produce ROS shortly after treatment (Fig. 2.6a). We observed that elf18 treatments did not elicit ROS production or MAPK activation in root tissues (Fig. S2.7b), while treatment of roots with flg22 caused phosphorylation of MPK3 and MPK6 already at concentrations as low as 1 nM (Fig. S2.a). The response of isolated roots to flg22 was completely abolished in the *fls2* mutant, indicating the need for a specific recognition through the FLS2 receptor (Fig. 2.6a; Fig. S2.6; Fig. S2.).

To further demonstrate that the root is able to induce PTI responses independently of the shoot, isolated root tissues were treated with flg22 and the expression of three defense marker genes was analyzed by real-time quantitative reverse transcription PCR (qRT-PCR). The selected genes under investigation were *FRK1*, encoding a flg22-induced receptor-like kinase (Asai et al., 2002), *WRKY11*, a transcription factor reported to be induced in the elongation zone of seedling roots after flg22 treatment of whole seedlings (Millet et al., 2010) and *PER5*, a peroxidase superfamily protein known to be involved in responses to oxidative stress (Tognolli et al., 2002), which has been shown to be induced upon flg22 treatment (Boudsocq et al., 2010). Indeed, *PER5* was strongly induced in isolated roots by flg22 and AtPep1 treatment but not by elf18 treatment and is thus suitable as a MAMP-responsive marker gene in roots (Fig. S2.). All genes investigated were up-regulated in isolated wild-type root tissues three hours after flg22 treatment, whereas no induction was detectable in *fls2* mutant roots (Fig. 2.6b). Our findings demonstrate that roots are able to respond to flg22 and to induce PTI responses in the absence of signals from the shoots. These results are consistent with the findings of previous studies showing that *FLS2* is expressed in wild-type Arabidopsis roots (Gómez-Gómez and Boller, 2000; Robatzek et al., 2006; Beck et al., 2014) and that flg22 treatment can trigger PTI responses in roots (Millet et al., 2010; Jacobs et al., 2011).

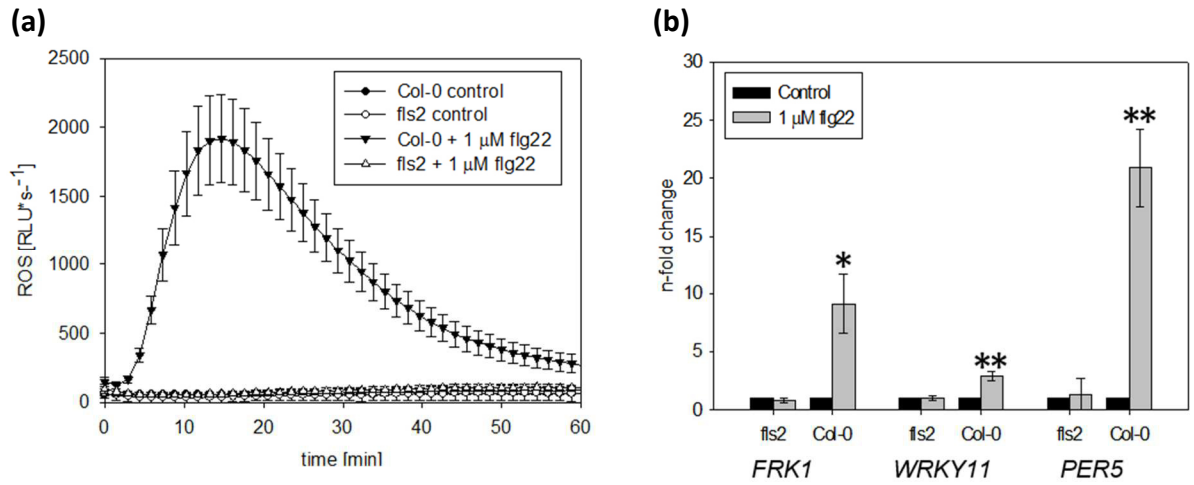

Fig. 2.6 Induced PTI responses in isolated root systems. (**a**) ROS production in isolated three week-old roots of wild-type *Arabidopsis thaliana* seedlings, treated with 1 µM flg22 or without elicitor. Graphs display averages of 12 replicates. Error bars show SE of the mean. (**b**) Induction of marker gene transcription in isolated two week-old root tissues treated with 1 µM flg22 or buffer without peptide (control). Transcript levels of *FRK1, WRKY11* and *PER5* were measured by qRT-PCR and first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The bars represent the mean of three biological replicates. Error bars show ± SE of the mean. Significant differences with respect to the control according to Student's *t*-test are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$. RLU, relative light units.

2.2.3.2 Expression of *FLS2* under tissue-specific promoters

In order to study the response to flg22 of Arabidopsis plants expressing *FLS2* in specific root tissues, transgenic plant lines were generated in the *fls2* mutant background, which express *FLS2-GFP* under the control of different tissue-specific promoters. The following six promoters were selected for this purpose.

The promoter of *UBQ10* (Czechowski et al., 2005), which is one of five polyubiquitin genes in Arabidopsis, was used as a control to drive expression of *FLS2* in nearly all tissues of Arabidopsis. For expression of *FLS2* in the pericycle, the promoter of *LBD16* (Goh et al., 2012), encoding a lateral organ boundaries (LOB)-domain protein, was used. Furthermore, the endodermal promoters *ELTPpro* and *SCRpro* were used. ELTP serves as a lipid transfer protein and its promoter drives strong expression in differentiating root endodermal cells (Roppolo and Vermeer, personal communication). In contrast, SCR encodes a member of a protein family having similarities to DNA binding proteins and is expressed in cortex/endodermal initial cells, the quiescent center and in the endodermal cell lineage (Di Laurenzio et al., 1996). To control expression in the central and lateral root cap, the root epidermis and in root hair cells, we employed the promoter of *PGP4* (Terasaka and Blakeslee, 2005), which encodes an auxin efflux transmembrane transporter.

Next, we used the promoter of *WEREWOLF* (Lee and Schiefelbein, 1999), a myeloblastosis (MYB)-related protein known to be active in non-hair root epidermal cells and in the lateral root cap.

The specificity of the promoters was studied by investigating the spatial GFP accumulation in FLS2-GFP transgenic lines using confocal microscopy (Fig. 2.7). Several lines of each construct were analyzed and the line showing strongest GFP signal was selected for further analysis. Moreover, a line expressing *FLS2-GFP* under the control of its own endogenous promoter *FLS2pro* (Robatzek et al., 2006) was included. In line with our previous study, FLS2-GFP signal was mainly restricted to the vasculature when expressed under the *FLS2* promoter (Fig. 2.7; Beck *et al.*, 2014; Chapter 2.1).

As expected, the promoter of the *UBQ10* gene drove *FLS2-GFP* expression in all tissues. The *LBD16* promoter led to tissue-specific expression of *FLS2-GFP* in the pericycle of the early differentiation zone whereas the endodermal promoters *ELTPpro* and *SCRpro* (Di Laurenzio et al., 1996) were strongly active and drove high *FLS2-GFP* expression in the mature endodermis and the young endodermal cells. The GFP signal under the control of the epidermal promoter *WERpro* was detectable in the epidermis of the differentiation zone but the expression was lower in more differentiated tissues. In the *PGP4pro:FLS2-GFP* line, the FLS2-GFP signal was also present in the root cap and the epidermis. GFP expression in the elongation zone was only detectable in the *SCRpro*, *PGP4pro* and *WERpro:FLS2-GFP* lines (Fig. 2.7). Furthermore, the tissue-specific expression did not change or expand after flg22 treatment or dissection of roots in any of the generated lines (Fig. S2.9), supporting the conclusion that these promoters are suitable for use in studying the effect of tissue-specific FLS2 localization after stress treatments. In contrast, *FLS2-GFP* under the control of the endogenous *FLS2* promoter showed slight expansion towards outer tissues after dissection and treatment of roots with flg22 as previously reported (Fig. S2.9; Beck *et al.*, 2014). Moreover, none of the lines displayed any noticeable phenotype (Fig. S2.10).

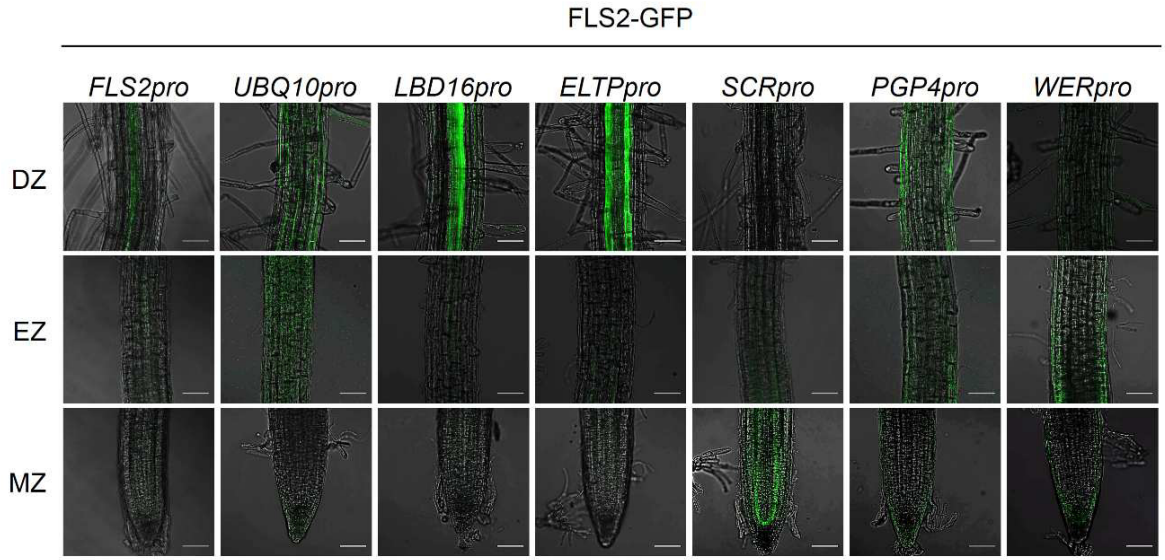

Fig. 2.7 FLS2-GFP accumulation under the control of tissue-specific promoters. Fluorescence microscopy of axenically grown five day-old transgenic Arabidopsis seedlings illustrates position-dependent GFP accumulation. Images show overlays of differential interference contrast and UV light pictures. The upper panel shows GFP expression in the early root differentiation zone (DZ), the middle panel that in the elongation zone (EZ) and the lower panel that in root tips and the meristematic zone (MZ). Bars, 100 µm.

In addition, the FLS2 transcript and protein level in the transgenic lines was investigated by qRT-PCR and Western blot (Fig. 2.8). The results clearly show that all lines expressed *FLS2* but that the expression level differed between the various lines. Protein and transcript levels correlated, except for the *UBQ10pro:FLS2-GFP* line, which exhibited lower protein accumulation levels compared to transcript levels indicating that protein accumulation is modulated at the posttranscriptional level in this line. Since *FLS2* expression in the different lines was higher and more specific in the lower parts of the roots, we performed all our assays excluding the older, mature root parts.

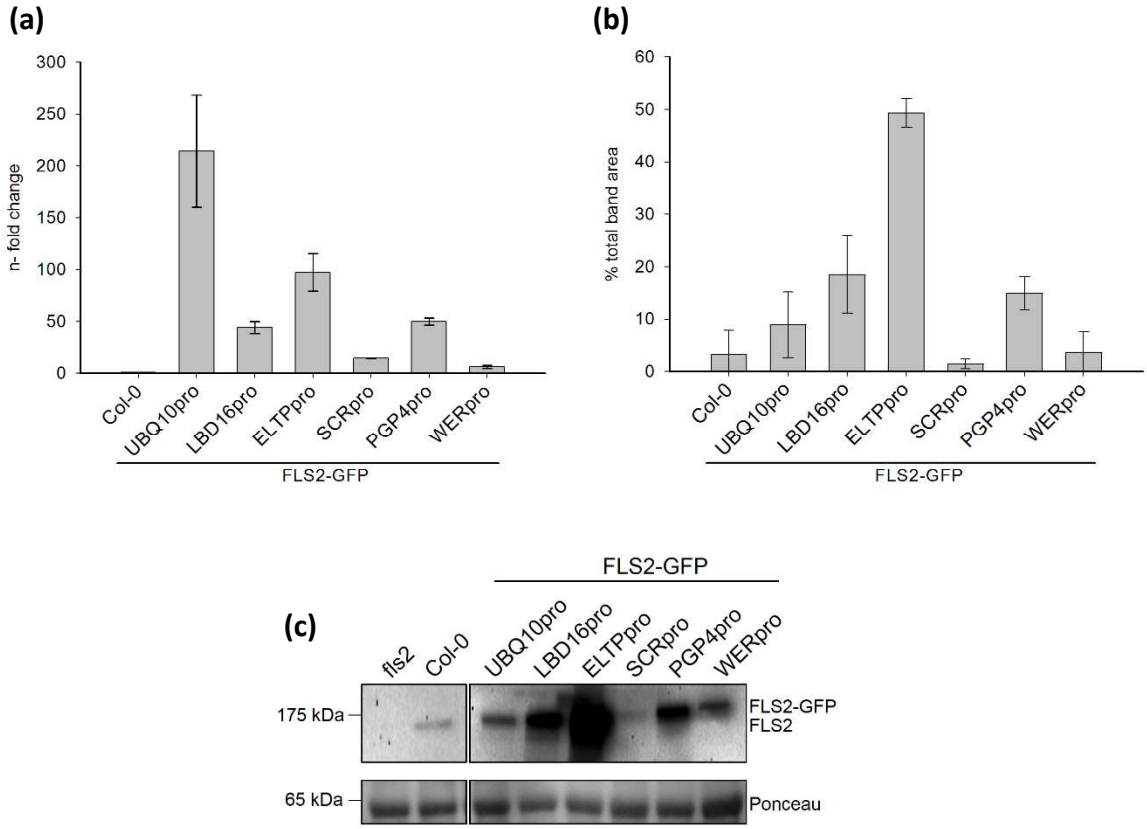

Fig. 2.8 Expression level of FLS2 under the control of tissue-specific promoters. (**a**) Transcript levels of *FLS2* in untreated, isolated roots of all transgenic Arabidopsis lines, measured by qRT-PCR. Data were first normalized to the reference gene *UBQ10* before calculation of expression relative to that of the wild-type Col-0. The bars represent the mean of three independent biological replicates. Data show mean values ± SE. (**b**) Quantification of band intensity as a percentage of total band area x intensity in two biologically independent Western blots. Data show mean values ± SD. (**c**) Immunoblot to detect FLS2 protein in transgenic seedling roots under tissue-specific promoters. Glycoproteins were extracted and precipitated with concanavalin A beads, and FLS2 was detected by Western blot. Ponceau staining was used to visualize proteins.

2.2.3.3 PTI responses in isolated root systems expressing *FLS2* in a specific root tissue

To study whether flg22 perception in a specific root tissue is sufficient to induce PTI responses, we investigated the production of ROS in roots of the transgenic *FLS2*-expressing lines in response to flg22. Our results showed that the *FLS2pro:FLS2-GFP* line over-expressed *FLS2* in comparison to the wild-type Col-0 and since this overexpression correlated with an increased responsiveness of the line to flg22 (Fig. S2.11), we decided to use Col-0 as control for the study of PTI responses in our lines.

All transgenic lines showed a clear production of ROS comparable to the wild-type root systems upon flg22 treatment (Fig. 2.9a). The reaction to a different stimulus, the endogenous elicitor *At*Pep1, was undistinguishable in the different lines indicating a similar responsiveness of the transgenic plants. However, perception of flg22 by the *UBQ10pro:FLS2-GFP* line and

the *LBD16pro:FLS2-GFP* line resulted in markedly stronger ROS production than perception of flg22 by any of the other lines (Fig. 2.9a). Such stronger responses did not correlate with expression levels since FLS2 accumulated more in the *ELTPpro:FLS2-GFP* line (Fig. 2.8).

To further analyze the ability of the different root tissues to sense flg22, the activation of MAPKs after ten minutes of flg22 treatment was investigated in isolated roots of our transgenic lines. All tissues showed the ability to induce MAPKs, except the *SCRpro:FLS2-GFP* line (Fig. 2.9b). Since *FLS2* expression in this line was restricted to the young endodermal tissues at the root tip and MAPKs might get activated only very locally upon flg22 treatment, we dissected root tips of this line to repeat the MAPK assay. MAPKs were indeed activated upon flg22 treatment in dissected root tips (Fig. 2.9c). These results demonstrate that all investigated root cells are "prepared" to perceive flg22 if *FLS2* is expressed and thus, the response machinery is present in these cells.

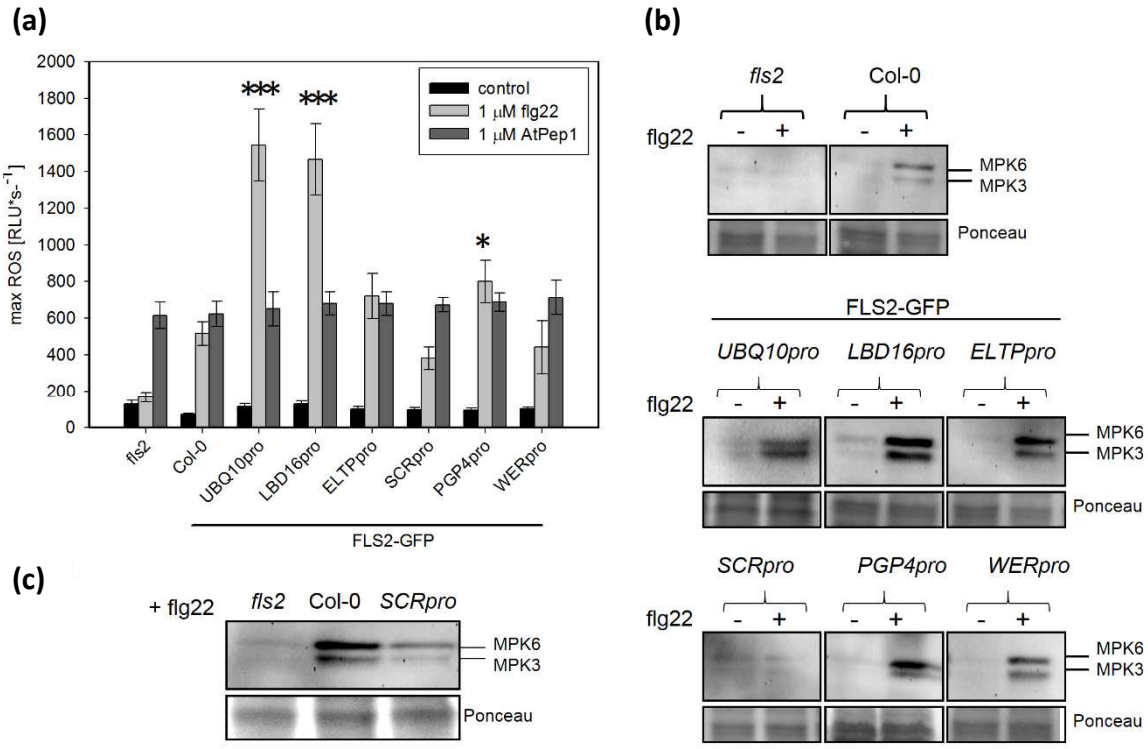

Fig. 2.9 PTI responses in tissue-specific *FLS2*-expressing isolated root systems. (**a**) ROS production in isolated root systems of Arabidopsis transgenic lines and wild-type Col-0 plants treated with 1 µM flg22, 1 µM AtPep1 or water as a control. Columns represent averages of the peak values of ROS production of 24 replicates. Error bars show ± SE of the mean. Significant differences with respect to Col-0 according to Student's *t*-test are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$. (**b**, **c**) MAPK activation detected by Western blot after ten minutes of flg22 treatment in (**b**) two week-old isolated root systems of Col-0 and transgenic lines and (**c**) dissected root tips. Ponceau staining was used as a loading control. Experiments represent one of four independent replicates with similar results. RLU, relative light units.

As the MAPK cascade is known to control a set of specific genes including *FRK1* (Asai et al., 2002), we investigated the expression of this marker gene by qRT-PCR. In addition, the transcriptional changes of *WRK11* and *PER5,* already used before, was examined. The expression of all three marker genes was highly up-regulated in all lines already one hour after elicitation of the isolated root systems with flg22 (Fig. 2.10). After three hours of elicitation the induction was in general slightly decreasing and was nearly abolished in all lines after eight hours of treatment. Thus, flg22 perception by roots induces a transient accumulation of transcripts for all MAMP-induced genes tested.

Interestingly, all lines were able to at least restore wild-type signaling output for every gene with the only exception of *PER5* in the *SCRpro:FLS2-GFP* line (Fig. 2.10b). This observation might be attributed, again, to the use of half root systems instead of dissected root tips in the assay; *PER5* might only be induced locally or weakly after perception of flg22 in young meristematic cells.

Remarkably, flg22 perception in the lines expressing *FLS2* under the control of the *LBD16* and *PGP4* promoters led to an induction of all three marker genes approximating that by the *UBQ10pro:FLS2-GFP* line, which perceives flg22 ubiquitously (Fig. 2.10; Table 2.2). In general, lines expressing *FLS2* in the endodermis (*ELTPpro:FLS2-GFP* and *SCRpro:FLS2-GFP*) caused a lower defense gene induction than *PGP4pro* and *LBD16pro:FLS2-GFP* lines. Noteworthy, the expression level of the receptor has been shown to be the responsible for the strength of the immune response (Gómez-Gómez & Boller, 2000). We also observed, using different lines from the same construct that changes in *FLS2* expression levels (not associated with changes in localization) correlated with the intensity of MAPK activation and defense gene induction (Fig. S2.12). Therefore, differences in *FLS2* expression could explain the lower defense gene induction observed in the *SCRpro:FLS2-GFP* line but such a phenotype associated to the highly expressing *ELTPpro:FLS2-GFP* line suggests an important role of the specific tissue perceiving flg22 on the intensity of the immune response (Fig. 2.10; Fig. 2.8; Table 2.2).

Given that the *FLS2* expression level is likely not the only determinant of the response intensity, differences in the *FRK1* and *WRKY11* induction in the *PGP4pro:FLS2-GFP* and the other epidermal line *WERpro:FLS2-GFP* may be due to more restrictive expression of *FLS2* in the *WERpro:FLS2-GFP* compared to the *PGP4pro:FLS2-GFP* line. In contrast to *PGP4pro:FLS2-GFP*, *WERpro:FLS2-GFP* is not expressing *FLS2* in older epidermal root tissues and in root hair cells. Surprisingly, and in support of the idea that *FLS2* expression

level does not always determine the intensity of the response, *PER5* was highly induced in the *WERpro:FLS2-GFP* line and in the *PGP4pro:FLS2-GFP* line (Fig. 2.10b). Thus, *PER5* may be in general highly induced upon flg22 perception at the epidermis or especially the root cap. This might indicate that the nature of the elicitated responses varies in different tissues. The induction of all three genes upon flg22 treatment in the *UBQ10pro:FLS2-GFP* line, that expresses *FLS2* in all tissues, was twice as much as in the wild-type. This effect could be attributed to the presence of FLS2 in all tissues in this line.

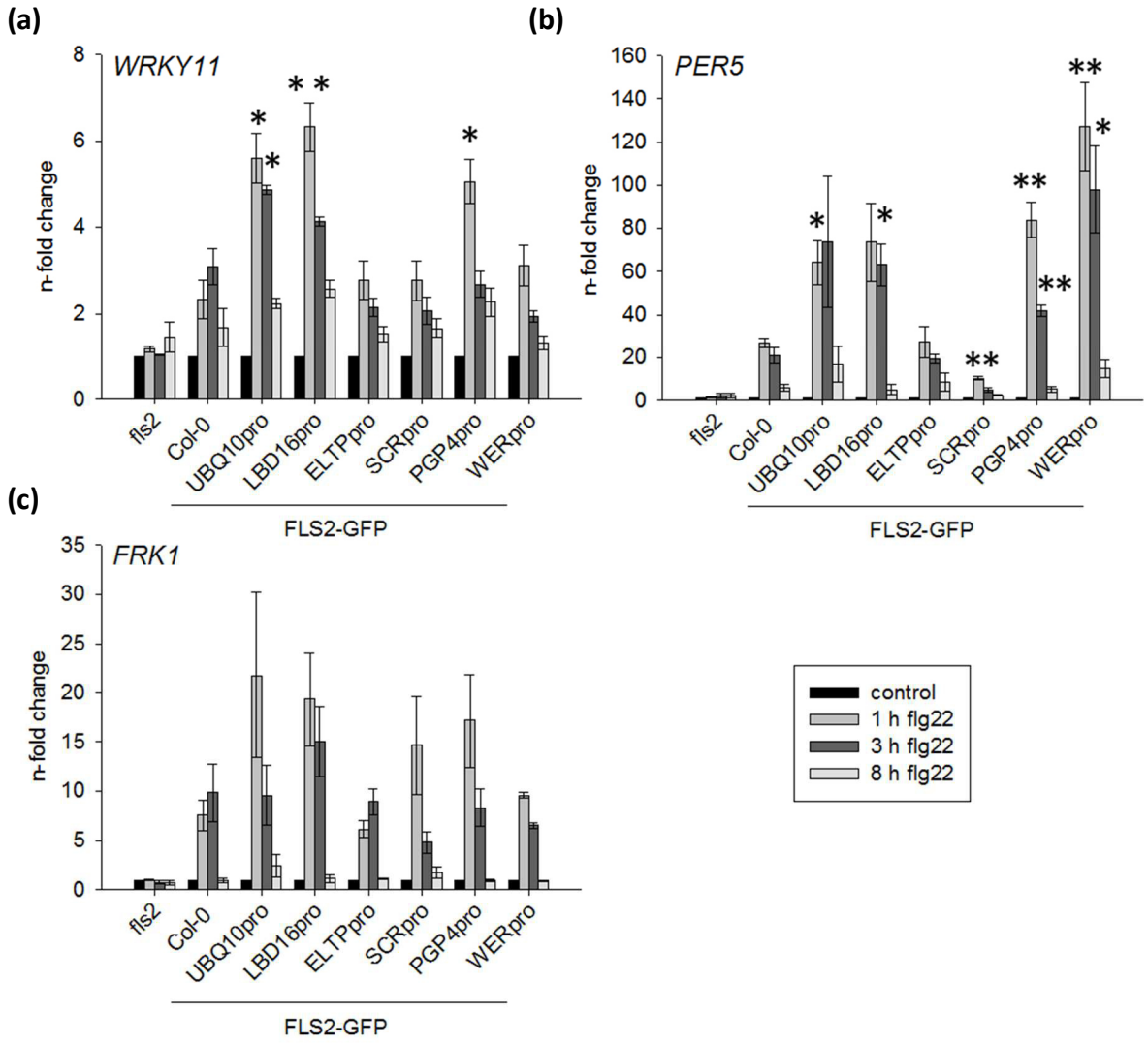

Fig. 2.10 Defense marker gene induction in transgenic seedling roots after flg22 treatment. Transcript accumulation of three MAMP-induced genes was measured by qRT-PCR in isolated Arabidopsis root tissues treated with 1 µM flg22 or without peptide (control) for 1, 3 and 8 hours. The analysis shows (**a**) *WRKY11*, (**b**) *PER5* and (**c**) *FRK1* induction in all lines. Transcript levels of the indicated genes were normalized to that of the reference gene *UBQ10* and then expression relative to that of the control was calculated. Data represent mean values of three biological replicates ± SE. Significant differences with respect to Col-0 according to Student's *t*-test are indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$.

Table 2.2 Summary table of pattern-triggered immune responses in relation to the accumulation level of FLAGELLIN-SENSING 2 (FLS2) in roots of Arabidopsis transgenic lines expressing *FLS2* under the control of the indicated promoters.

Promoter:	% FLS2:	% MAPK:	% ROS:	% *PER5*:	% *WRKY11*:	% *FRK1*:
SCRpro	44	18	74	38	119	194
Col-0	100	100	100	100	100	100
WERpro	111	114	86	475	134	127
UBQ10pro	273	126	299	240	240	289
PGP4pro	458	119	155	313	217	227
LBD16pro	567	120	284	275	271	256
ELTPpro	1512	71	140	102	119	81

All immune response induction values in roots of the transgenic Arabidopsis lines were relativized as a percentage of Col-0 induction values. MAPK, mitogen-associated protein kinase; ROS, reactive oxygen species; *PEROXIDASE SUPERFAMILY PROTEIN 5* (*PER5*); *WRKY DNA-BINDING PROTEIN 11* (*WRKY11*)*; FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*).

2.2.3.4 Penetration of flg22 through protective root barriers

The results of our experiments demonstrate that each root tissue can respond to flg22 if FLS2 is present. However, in our assays with the root systems, roots were not intact and thus it is not clear whether flg22 reached the corresponding tissues through the cutting sites or by penetrating the epidermal and endodermal barriers. Therefore, MAPK assays were repeated with intact plants floating only the root systems in a flg22 solution. The results show that all lines could perceive flg22 after ten minutes in absence of root damage (Fig. 2.11). This analysis indicates that flg22 diffuses across protective root barriers as the epidermis and the Casparian strip into the endodermal and vasculature tissues, possibly through natural openings, for instance, at the elongation zone or at the site of emerging lateral roots.

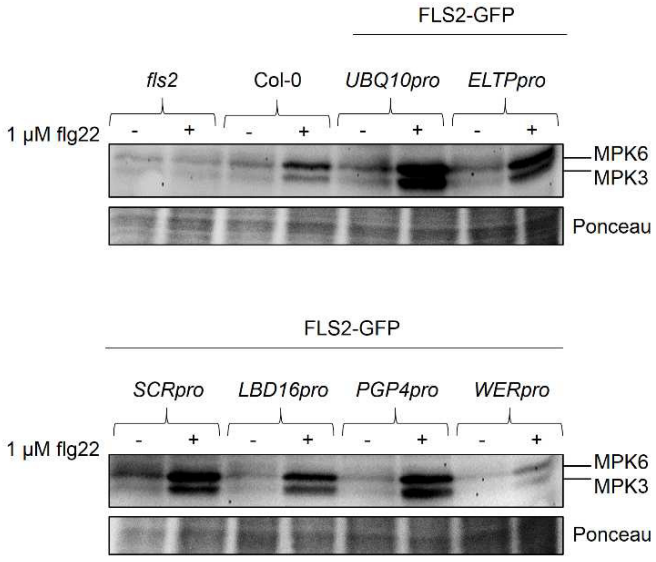

Fig. 2.11 Activation of MAPKs in intact roots. Immunodetection of MAPK phosphorylation in intact roots of two week-old Arabidopsis seedlings treated locally for ten minutes with 1 µM flg22 was performed. Ponceau staining was used as a loading control.

2.2.4 Discussion

Although previous studies indicated that *FLS2* is constitutively expressed in roots (Gómez-Gómez and Boller, 2000; Robatzek *et al.*, 2006) and that PTI responses can be detected in this organ (Millet et al., 2010; Jacobs et al., 2011; Cannesan et al., 2012), it has never been clarified whether these immune responses occur upon autonomous perception of flg22 in the root or rather upon MAMP perception in the shoot eliciting a systemic response (Millet et al., 2010; Cannesan et al., 2012; Lakshmanan et al., 2012). In this study, we tested isolated roots for their ability to trigger PTI responses upon flg22 perception, hence excluding any possible interference of the shoot. Our data show that isolated roots of wild-type Arabidopsis plants perceive flg22 independently of the shoot and induce all the hallmarks of PTI responses, such as the production of ROS and the induction of defense marker genes (Fig. 2.6), demonstrating that the shoot is not required for root immune responses.

But do all root tissues have the capacity to respond to flagellin provided the receptor FLS2 is present? Surprisingly, all the generated, tissue-specific *FLS2*-expressing lines were able to sense flg22 leading to MAPK activation (Fig. 2.9b,c), ROS production (Fig. 2.9a) and strong transcriptional induction of marker genes (Fig. 2.10). Thus, all the examined root tissues had the capacity to induce PTI responses when the receptor was present, although the intensity of the response differed between the lines.

Moreover, the induction of MAPKs in all the lines in intact root systems after flg22 treatment suggests that flg22 is able to penetrate through the protective epidermal and endodermal root barriers (Fig. 2.11). Remarkably, even the *LBD16pro:FLS2-GFP* line expressing *FLS2* in the pericycle was able to perceive flg22 in intact root systems, indicating that the barriers of the epidermis and the Casparian strip in the endodermis did not block access of flg22 to the central root stele (Fig. 2.11). We cannot completely rule out the possibility that undetectable levels of *FLS2-GFP* expression might take place in other tissues leading to the observed activation of defense responses. Nevertheless, we believe that the strong MAPK activation observed in the *LBD16pro:FLS2-GFP* line would most probably require higher levels of *FLS2* expression.

Current opinion regarding plant immunity is that perception and activation of initial defense responses take place in a cell-autonomous manner, which assumes that the perception and response machinery would have to be jointly expressed in each cell. Since ectopic expression of *FLS2* in the different tissues was enough to render them responsive to flg22, we believe that the downstream elements necessary for PTI are already present in all cells. But on the other hand, it is known that the constant activation of the immune system negatively influences other plant processes. For example, it has been observed that long-term flg22 treatment leads to a severe inhibitory effect in seedlings growth (Heil & Baldwin, 2002; Heil, 2002). Thus, it has been speculated that high expression of immune receptors in the outer, epidermal cells of roots, which are constantly exposed to MAMPs, would be disadvantageous for a plant not only because of constant PTI signaling but also in order to allow colonization and interaction with beneficial bacteria (Faulkner and Robatzek, 2012). Furthermore, it is known that a beneficial microbiota is usually present in association with plant (leaf surface and rhizoplane) and animal (skin, gut and respiratory epithelia) systems and does not lead to overactivation of host immune responses (Kubinak & Round, 2012; McClure & Massari, 2014; Selosse *et al.*, 2014). Therefore, perception of potential pathogens through MAMPs such as bacterial flagellin may require a particularly precise fine-tuning and regulation in order to avoid unnecessary alarm.

Nevertheless, our results clearly show that expression of *FLS2* in the epidermis leads to strong activation of PTI responses after flg22 treatment (Fig. 2.9; Fig. 2.10). Especially remarkable is the strong defense gene induction in the line *PGP4pro:FLS2-GFP* (Fig. 2.10). These findings support the hypothesis that in natural conditions plants might need to restrict the expression of MAMP receptors to tissue-specific locations, especially at putative pathogen entry sites, in order to efficiently inhibit pathogen invasion and suppress or regulate constitutive activity of PTI signaling. Such a hypothesis was recently supported by evidence

from the field of animal biology, where tissue-specific regulation was shown to be involved in the activity of Toll-like receptors in epithelial cells ensuring that an immune response is only mounted when bacteria penetrate the host epithelial layer (Lee et al., 2006; Lundin et al., 2008; Abreu, 2010; Kubinak and Round, 2012).

In fact, our findings confirm those of our recent study showing that the *FLS2* promoter activity is mainly present in the root stele and expands to the cortex and epidermal region after different stress treatments (Fig. S2.9; Beck *et al.*, 2014; Chapter 2.1). The perception of MAMPs in the pericycle or vasculature could therefore be important for the plants, especially because many pathogenic bacteria as *Ralstonia* and *Pseudomonas syringae* pv. *tomato* (*Pto*) DC3000 use the plant vasculature to move throughout the plant and often colonize xylem vessels of its host plants to multiply inside and gain access to nutrients (Digonnet et al., 2012; Misas-Villamil et al., 2013). Furthermore, plants have natural "wounds" in their physical barriers, manifested as epidermal cracks at the site of emerging lateral roots or at the elongation zone. It is widely assumed that bacteria use these natural entry sites to colonize the root vasculature (Dong and Iniguez, 2003; Tyler and Triplett, 2008; Chapter 2.1; Beck et al., 2014). Thus, the appearance of MAMPs in the pericycle or vasculature might indicate a potential threat and a strong, localized PTI response in this tissue could be favorable for the plant. This speculation is in agreement with recent findings indicating the existence of cell type and tissue-specific responses in roots to pathogens (Millet et al., 2010; Cannesan et al., 2012) especially at the elongation zone, because there the protective root cap is absent and epidermal and endodermal barriers have not fully evolved yet, meaning that MAMPs could easily penetrate into the vasculature. Moreover, the promoters of *PEPR1* and *PEPR2*, two PRRs involved in recognition of endogenous Arabidopsis peptides playing a role in plant immunity, have been shown to be highly active in the vascular tissue (Bartels et al., 2013). In addition, flg22 is known to induce the production of lignin which probably also prevents colonization and spread of bacteria through the vasculature (Schenke et al., 2011).

In order to determine the importance of flg22 perception in the inner tissues, the *LBD16* promoter was employed as *FLS2* expression under this promoter is restricted to the pericycle. Indeed, we observed that ectopic expression of *FLS2* in the pericycle cells led to enhanced PTI responses in isolated root systems compared to the wild-type (Fig. 2.9; Fig. 2.10). It is known that changes in *FLS2* expression can correlate with variations in defense responses (Fig. S2.11; Fig. S2.12; Gómez-Gómez & Boller, 2000). However, our observations cannot be fully explained by the difference in *FLS2* expression, as the *LBD16pro:FLS2-GFP* line expressed

FLS2 to a lower extent than the *ELTPpro:FLS2-GFP* line, but reacted stronger (Table 2.2). Based on these findings, we think that the nature of the tissue perceiving flg22 has a major impact on the intensity of the initiated defense response. In particular, we suggest that PTI responses at the inner root tissues, behind the Casparian strip, could be more pronounced than at the outer, epidermal or endodermal root tissues, since constitutive activation of PTI responses at the latter would severely affect plant fitness (Heil, 2002; Heil and Baldwin, 2002).

In conclusion we show that all root tissues are able to perceive flg22 leading to the induction of PTI responses if FLS2 is present. Furthermore, our data suggest that the intensity of the response depends on the tissue type perceiving the signal. Our tissue-specific *FLS2*-expressing lines will provide a helpful tool to illuminate local and systemic signaling pathways in plant tissues and will help to gain further insight into the complexity of the plant immune network.

2.2.5 Materials and methods

2.2.5.1 Plant material

Plant materials used were wild-type *Arabidopsis thaliana* L. Heynh cultivar 6 Columbia (Col-0) and the mutant *fls2* (Zipfel et al., 2004) (SALK_062054C). Seeds were surface sterilized and germinated on half-strength MS plates (0.5 x Murashige and Skoog basal medium (Phytotechnology Laboratories) containing 1% sucrose and 0.8% phytoagar) for five days in a plant growth chamber (24 hours of photoperiod, 20°C). Seedlings were then transferred to 24 well plates containing 1 mL of half-strength MS medium (one seedling per well) and further grown in the same conditions for ten days. For confocal analysis, seedlings were grown vertically for five days in square Petri dishes containing half-strength MS medium.

2.2.5.2 Elicitor peptides

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), AtPep1 (ATKVKAKQRGKEKVSS-GRPGQHN) and elf18 (ac-SKEKFERTKPHVNVGTIG) were obtained from EZBiolabs and diluted in water to a final concentration of 1 µM for all assays.

2.2.5.3 Construction of transgenic lines

Vectors containing promoter regions of *UBIQUITIN10* (*UBQ10*, AT4G05320), *EMBRYO LIPID TRANSFER PROTEIN* (*ELTP*, AT2G48140), *SCARECROW* (*SCR*, AT3G54220), *LATERAL ORGAN BOUNDARIES-DOMAIN* (*LBD16*, AT2G42430), *P-GLYCOPROTEIN 4 (PGP4*, AT2G47000) and *WEREWOLF* (*WER*, AT5G14750) had been obtained previously in Niko Geldner's lab at the University of Lausanne. The promoter regions of *UBQ10*, *ELTP* and *SCR* were cloned into the Gateway vector pDONR^{TM}P4-P1R (http://gateway.psb.ugent.be). *LBD16pro*, *WERpro* and *PGP4pro* were cloned by blunt end cloning into a modified pUC57 vector. The full coding sequence of *FLS2* fused to *GFP* was amplified from a pCambia vector (Robatzek et al., 2006) and cloned into the gateway vector pDon207 (http://gateway.psb. ugent.be). Using multistep gateway cloning, pDon201/modified pUC57 vector containing the promoter regions and the vector pDon207 containing the *FLS2-GFP* region were combined, substituted with LR clonase and transferred to the multisite vector pH7m24GWB (http://gateway.psb.ugent.be). Transgenic lines were obtained by dip-inoculation with agrobacteria and selection on hygromycin. Several transgenic lines were analyzed and the strongest *FLS2-GFP* expressing line of each construct was chosen for further studies. PTI assays were performed with a pool of at least 12 seedlings from the T2 generation.

2.2.5.4 Microscopy

The GFP expression in the promoter:FLS2-GFP expressing plants was examined in five day-old seedlings using a Zeiss LSM700 upright point scanning confocal microscope with a 488 nm excitation mirror and fluorescence emissions were captured between 500 and 550 nm to record images. Images were processed using the LSM image browser (Carl Zeiss Microscop GmbH Jena, Germany) and Photoshop CS5 software packages (Adobe Systems, Basel, Switzerland).

2.2.5.5 ConA precipitation

For the detection of FLS2 in roots, 100 mg of root tissue from seedlings grown for two weeks in liquid MS was frozen in liquid nitrogen and homogenized in 0.2 ml of cold IP buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% (w/v) octylphenoxypolyethoxyethanol (Nonidet P-40) and protease inhibitor cocktail (Sigma-Aldrich)). After incubation for one hour at 4°C with gentle shaking, this preparation was centrifuged three times at 10,000 g for ten minutes. The

supernatant containing the solubilized proteins was incubated one hour at 4°C with concanavalin A-sepharose beads (Sigma-Aldrich). The beads were collected and washed three times with ice-cold IP buffer. After denaturation in SDS-buffer, proteins retained on the beads were separated by SDS-PAGE 7% and analyzed by Western blot and immunodetection with anti-FLS2 antibodies (Chinchilla et al., 2006). Band intensity was analyzed with the Image J gel analysis tool (http://imagej.net) and quantified as percentage of wild-type Col-0 band area x intensity in two biologically independent Western blots.

2.2.5.6 Measurement of reactive oxygen species

For ROS assays, root systems (the isolated lower half of the root) of two plants grown in liquid MS were placed into each well of a Lia White 96-well plate (Greiner Bio-One) in 0.1 mL water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase (Sigma-Aldrich) and luminol (Sigma-Aldrich) was added to a final concentration of 10 µg/mL and 100 µM, respectively. Luminescence was measured directly after addition of elicitor peptides in a MicroLumat LB96P plate reader (Berthold Technologies) for one hour.

2.2.5.7 MAPK phosphorylation

12 root systems of two week-old plants were placed in water and left over night. For MAPK detection in intact roots, 12 seedlings were placed overnight on split Petri dishes in order to treat roots separately of shoots. The following day, 1 µM flg22 was added to root tips for ten minutes and roots were dissected from shoot tissue immediately prior to freezing in liquid nitrogen. Tissue (50 mg per sample) was shock frozen in liquid nitrogen and ground into fine powder before addition of 50 µL SDS- extraction buffer (0.35 M Tris-HCl pH 6.8; 30% [v/v] glycerol; 10% [v/v] SDS; 0.6 M dithiothreitol; and 0.012% [w/v] bromophenol blue). Total proteins were separated by electrophoresis in 12% SDS-polyacrylamide gel and electrophoretically transferred to a polyvinylidene fluoride membrane according to the manufacturer's instructions (Bio-Rad). Transferred proteins were detected with Ponceau-S. Polyclonal primary antibodies against phospho-p44/42 MAPK (Cell Signaling Technologies) were used, with alkaline phosphatase-conjugated anti-rabbit as secondary antibodies. Signal detection was performed using CDP*st*ar (Roche). MAP kinase activation was estimated using band intensity as percentage of mock treated control and then relativized to the wild-type Col-0 from 3 biologically independent Western blots using the Image-J gel analyzing tool.

2.2.5.8 Determination of gene expression

Root tissue of two week-old seedlings was collected and left in water over night. After treatment with 1 µM flg22 for 1, 3 and 8 hours, material was frozen and ground in liquid nitrogen. RNA from 50 mg tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with recombinant DNase according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of RNA with oligo (dT) primers using the avian myeoloblastosis virus (AMV) reverse transcriptase according to the manufacturer's instructions (Promega). Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed in a 96-well format using a LightCycler®480 System (Roche Applied Science). Normalized expression to the reference gene *UBIQ10* (AT4G05320) was calculated using the qGene protocol (Muller et al., 2002). The gene-specific primers used were as follows: *UBQ10* (AT4G05320) with UBQ_fw (GGCCTTGTATAATCCCTGATGAATAAG) and UBQ_rv (AAAGAGATAACAGGAAC-GGAAACATAG), *FRK1* (AT2G19190) with FRK1_fw (TGCAGCGCAAGGACTAGAG) and FRK1_rv (ATCTTCGCTTGGAGCTTCTC), *WRKY11* (AT4G31550) with WRKY11_fw (AGGAGAGCACCGTCATAACC) and WRKY11_rv (AGCCGAGGCAAACACTAAAT), *PER5* (AT1G14550) with AT1G14550_fw (TCTCAATGCTTCTTGTTCCG) and AT1G14550_rv (CTAGATCCAATGCTGCCAGA) and *FLS2* (AT5G46330) with FLS2_fwd (ACTCTCCTCCAGGGGCTAAGGAT) and FLS2_rv (AGCTAACAGCTCTCCAGGGAT-GG).

2.2.6 Acknowledgements

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2.2.7 Supplemental figures

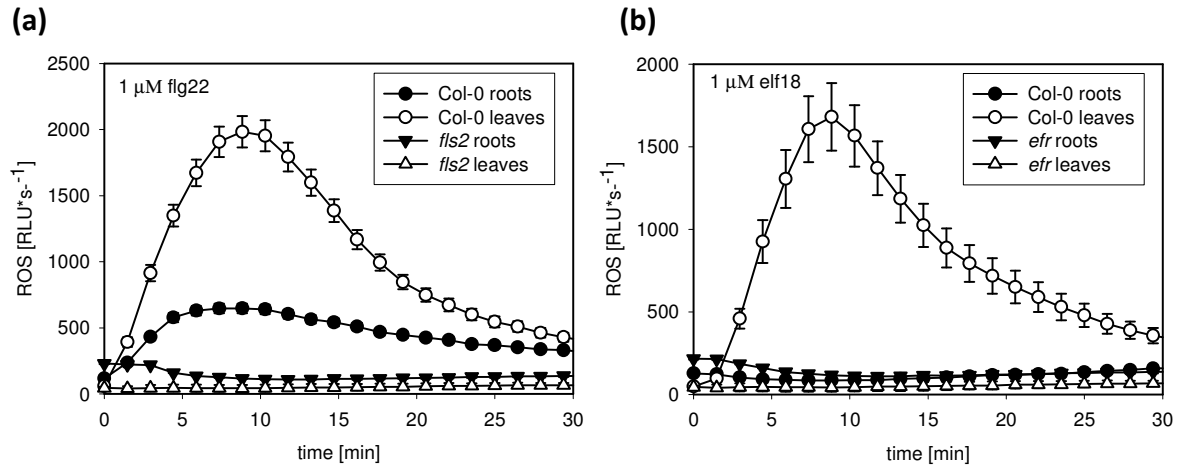

Fig. S2.6 Flg22 but not elf18 leads to the production of ROS in isolated Col-0 roots. ROS production in isolated roots or leaves of three week-old Arabidopsis seedlings in response to 1 µM flg22 (**a**) or elf18 (**b**). Graphs display averages of 12 replicates. Error bars represent ± SE of the mean. The experiment was repeated three times with similar results.

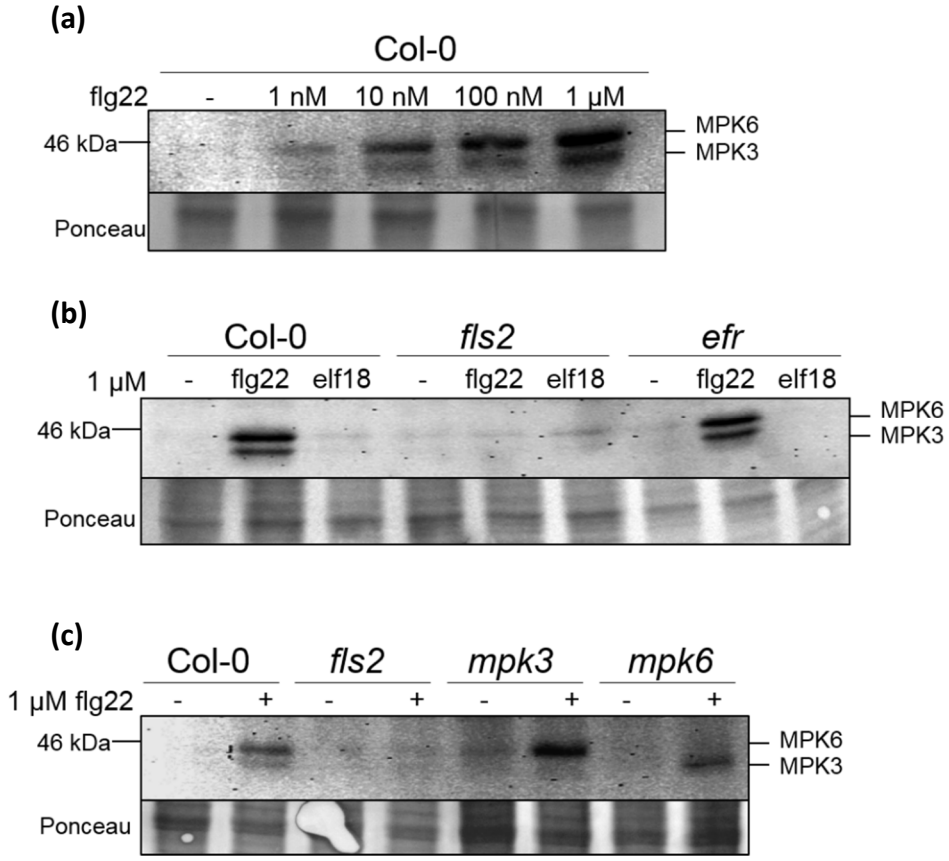

Fig. S2.7 Flg22 but not elf18 induces MPK3 and MPK6 phosphorylation in isolated roots. MAPK activation detected by Western blot in two week-old root systems after ten minutes using (**a**) Arabidopsis Col-0 plants in response to different flg22 concentrations, (**b**) Col-0, *efr* and *fls2* mutant plants in response to flg22 or elf18 (1 µM) and (**c**) Col-0, *fls2*, *mpk3* and *mpk6* mutant plants in response to 1 µM flg22.

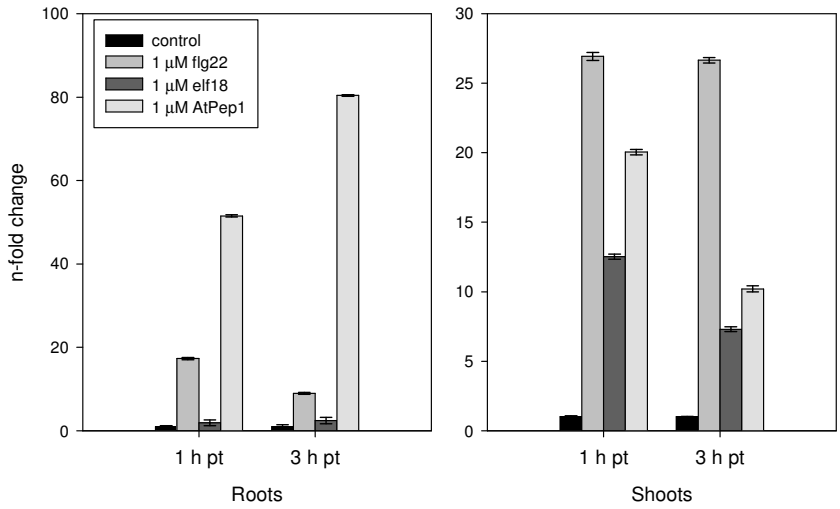

Fig. S2.8 *PER5* can be used as MAMP-induced marker gene. Analysis of *PER5* transcription in isolated two week-old Arabidopsis roots (left panel) or shoots (right panel) treated with 1 µM flg22, elf18 or AtPep1 or without peptide (control) for 1 and 3 hours. Transcript level of the indicated gene was normalized to the reference gene *UBQ10* and then relative expression to the control was calculated. The bars represent mean of three technical replicates. Error bars show ± SE of the mean. Results from one out of two independent biological replicates with similar induction patterns are shown.

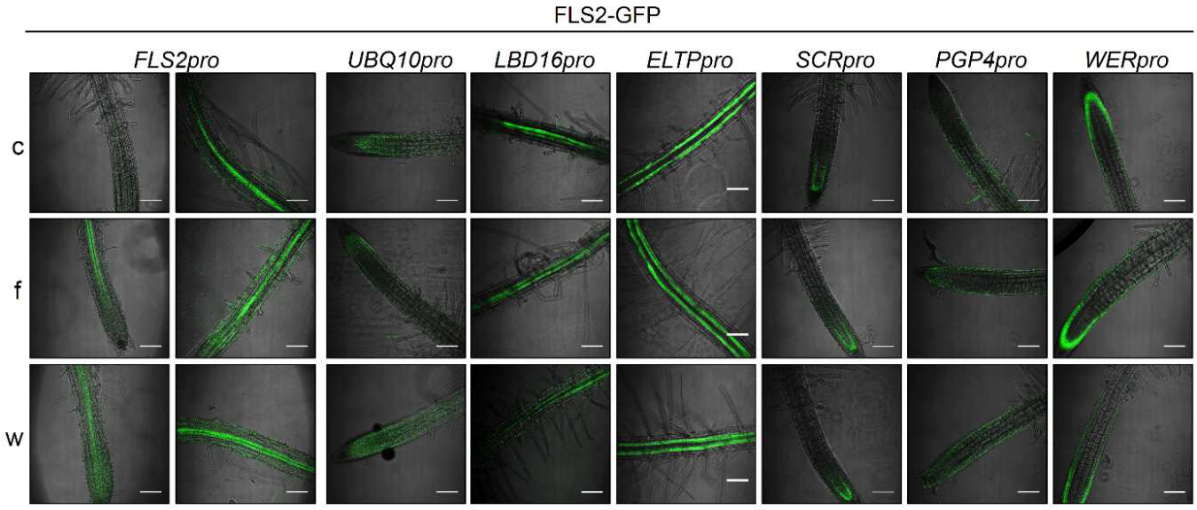

Fig. S2.9 GFP expression in roots of transgenic lines after flg22 treatment and wounding. GFP expression in roots of transgenic Arabidopsis lines expressing *FLS2-GFP* under the control of the stated promoter was investigated by fluorescence microscopy 12 hours after control (c), 1 µM flg22 treatment (f) and wounding/cut (w) of five day-old Arabidopsis seedlings. Images present overlay of light and UV pictures of one representative out of five biological replicates. Bar corresponds to 100 µm.

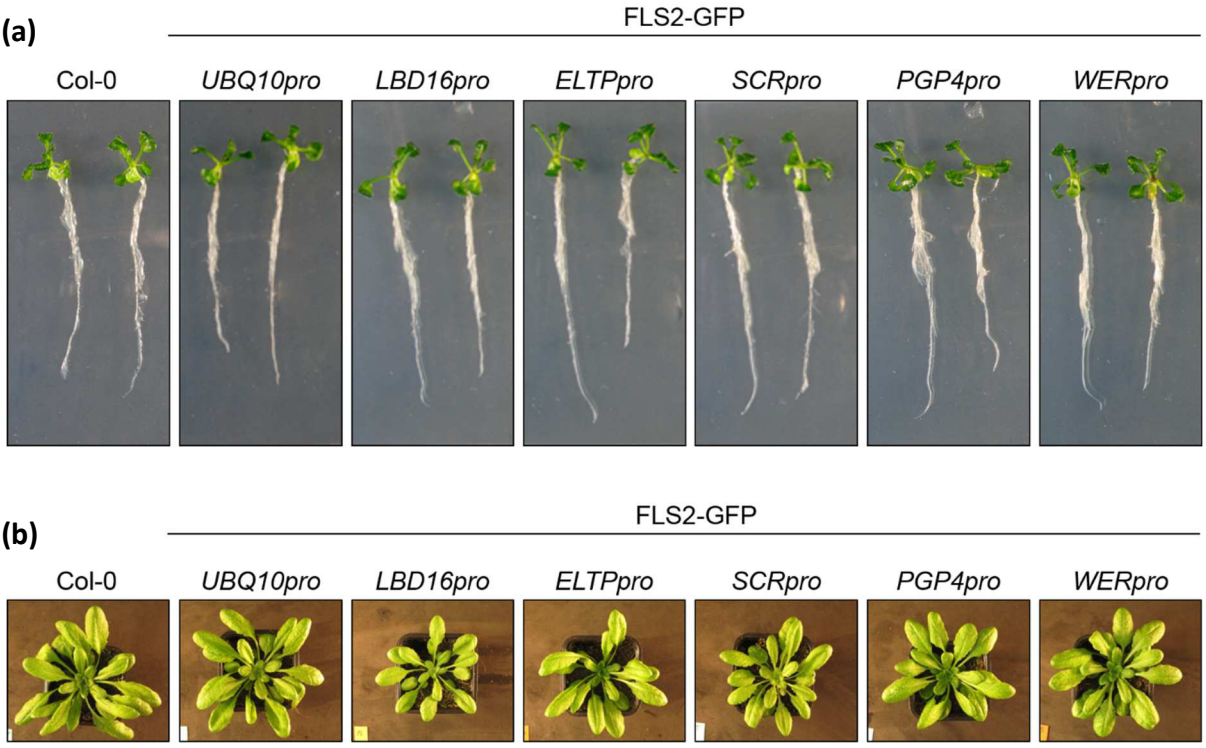

Fig. S2.10 Phenotypes of transgenic lines. Transgenic Arabidopsis T2 lines selected on hygromycin were grown under sterile conditions in liquid MS solution for ten days (**a**) or placed to soil and grown for five weeks (**b**). No differences compared to the wild-type Col-0 plants were observed.

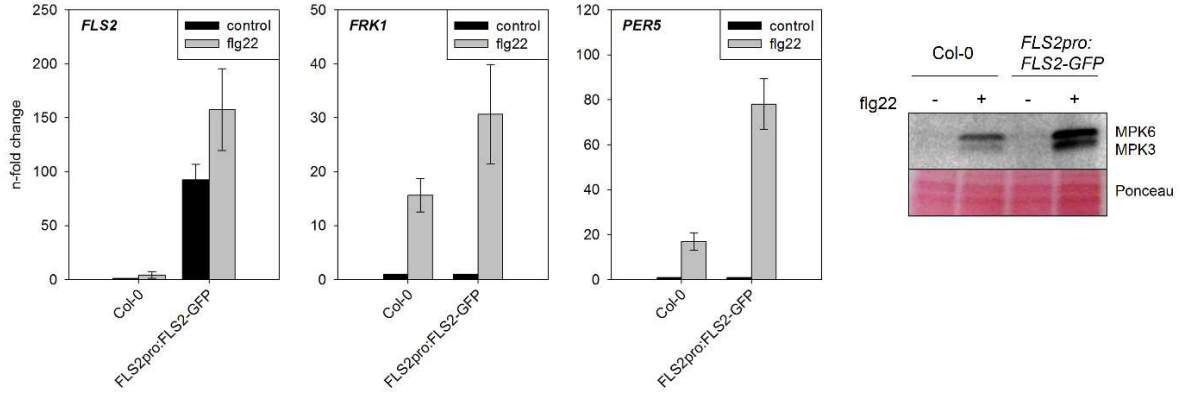

Fig. S2.11 *FLS2pro:FLS2-GFP* line shows altered PTI responses and *FLS2* expression. Isolated roots of Arabidopsis Col-0 and *FLS2pro:FLS2-GFP* were treated for one hour with 1 µM flg22 or solvent (control). Transcript level of the indicated gene was normalized to the reference gene *UBQ10* and relative expression to the Col-0 control was calculated in the case of *FLS2* (left panel). For *FRK1* and *PER5*, relative expression was calculated to the corresponding untreated control. Bars represent the mean of two independent experiments with three technical replicates each (middle panels). Error bars show ± SD. MAPK induction in isolated roots was analyzed by Western blot after ten minutes treatment with flg22 (right panel). One experiment out of two with similar results is shown.

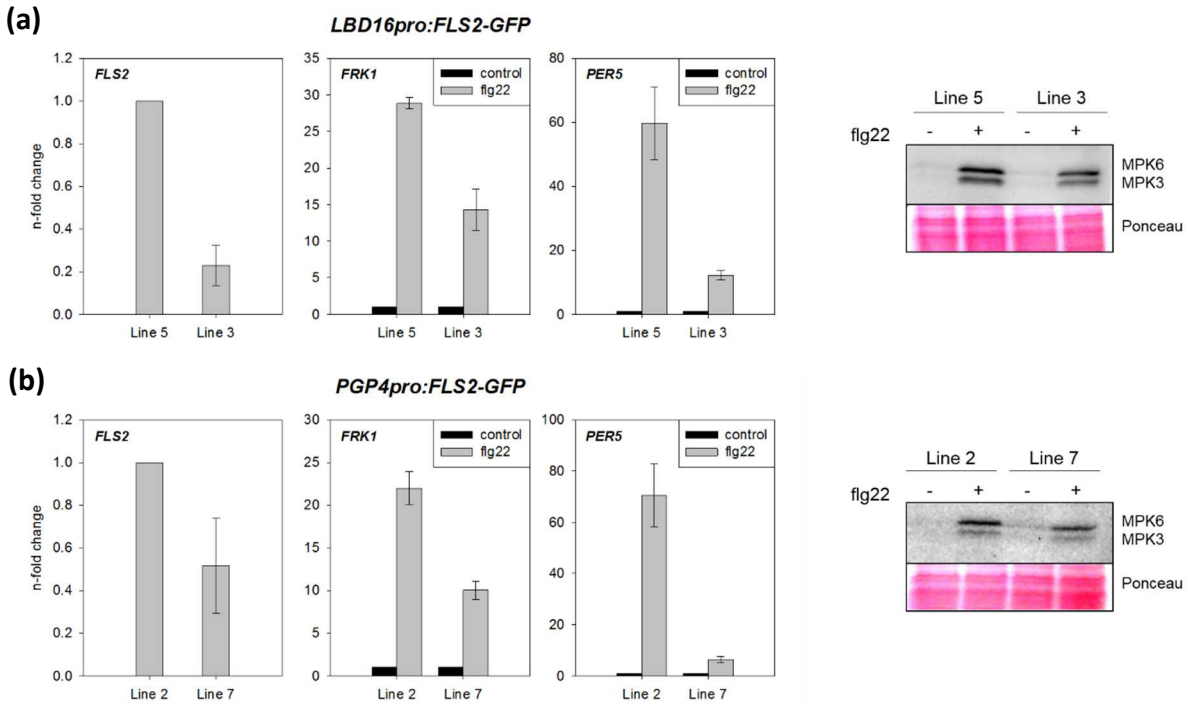

Fig. S2.12 Analysis of PTI responses in different *LBD16pro:FLS2-GFP* and *PGP4pro:FLS2-GFP* lines. Two independent Arabidopsis lines expressing different amounts of *FLS2-GFP* were selected for the *LBD16pro:FLS2-GFP* construct (**a**) and the *PGP4pro:FLS2-GFP* construct (**b**). *FLS2* expression levels were analyzed by qRT-PCR, transcript levels were normalized to the reference gene *UBQ10* and relative expression to the higher expressing line is shown (left panels). Expression of *FRK1* and *PER5* was assessed in the different lines after treatment with 1 µM flg22 for one hour. Transcript levels of the indicated gene were normalized to the reference gene *UBQ10* and relative expression to the control is presented (middle panels). Bars represent the mean of two independent experiments with three technical replicates each. Error bars show ± SD. MAPK induction in isolated roots of the tested lines was analyzed by Western blot after ten minutes treatment with 1 µM flg22 (right panels). One experiment out of two with similar results is shown.

3 DEVELOPMENT OF A METHOD TO STUDY POTENTIAL ROOT-TO-SHOOT SIGNALING IN ARABIDOPSIS

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3.1 Abstract

Plants perceive microbes through the recognition of microbe-associated molecular patterns (MAMPs) by plasma membrane-bound pattern recognition receptors (PRRs). Recognition leads to the activation of signaling cascades, which result in local and systemic defense responses. While systemic signaling from leaf-to-leaf has been investigated upon pathogen attack, root-to-leaf signaling in response to pathogens or MAMPs has received little attention. We here tested whether local MAMP application to roots of Arabidopsis leads to systemic defense gene induction in shoots and tested several experimental systems for their suitability to study root-to-shoot signaling. During our initial experiments, we often observed movement of elicitor traces along the plant and hence, conclude that caution should be taken when studying systemic signaling in plants. To reduce the risk of contamination by movement of the elicitor itself, we developed a novel method relying on local treatment of roots in Petri dishes. In our improved system, defense marker genes were not induced in shoots upon local application of the MAMP flg22 and the damage-associated molecular pattern (DAMP) AtPep1 to roots at different time points, indicating that systemic root-to-shoot signaling upon MAMP perception does not involve transcript changes of typical defense marker genes.

3.2 Introduction

Plant defense responses are known to depend on the recognition of conserved microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRR) (Boller and Felix, 2009). Perception of MAMPs as non-self results in local and systemic signaling and downstream defense responses that contribute to growth restriction of microbial pathogens and can establish durable resistance to a large number of pathogens in the whole plant (Conrath et al., 2006; Boller and Felix, 2009; Dodds and Rathjen, 2010; Zipfel, 2014). One of the best-studied elicitors of plant immunity is the bacterial MAMP flagellin, which is recognized by the PRR FLAGELLIN-SENSING 2 (FLS2) (Felix et al., 1999; Chinchilla et al., 2006; Boller and Felix, 2009; Robatzek and Wirthmueller, 2013). Other well-known elicitors of defense in *Arabidopsis thaliana* are elf18 which is perceived by the PRR ELONGATION FACTOR-TU RECEPTOR (EFR) or the damage-associated molecular pattern (DAMP) AtPep1, perceived by the PRRs PEP RECEPTOR 1 (PEPR1) and PEP RECEPTOR 2 (PEPR2) (Macho and Zipfel, 2014).

One important downstream event upon the activation of immunity in plants is the generation of long-distance mobile alarm signals (Shah, 2009). Although the exact nature of these signals remains elusive (Dempsey and Klessig, 2012), it is known that perception of such mobile alarm signals in distant tissues and organs not exposed to microbes can lead to the establishment of a long-lasting (several weeks) systemic resistance, active against a broad range of pathogens. This systemic resistance can consist of a primed state, in which the plant reacts faster and more efficiently to subsequent pathogen challenge (Conrath et al., 2006; Conrath, 2011), or of systemic acquired resistance (SAR), which typically involves the induction of *PATHOGENESIS-RELATED* (*PR*) genes and relies on a functional salicylic acid (SA) pathway (Métraux et al., 2002; Durrant and Dong, 2004). SA is believed to fulfill a dual role in SAR signaling; either SA directly activates *PR* gene expression or, alternatively, low doses of SA that do not activate defense genes directly prime the tissue for potentiated defense gene expression upon subsequent pathogen infection (Métraux et al., 2002; Durrant and Dong, 2004; Conrath et al., 2006; Shah, 2009; Fu and Dong, 2013; Gruner et al., 2013). Up to now, SAR has been mainly studied in leaves (leaf-to-leaf signaling) (Dempsey and Klessig, 2012; Fu and Dong, 2013) and systemic signaling between roots and shoots during defense has received much less attention. This is surprising given that roots are constantly exposed to soil-borne microbes and their MAMPs.

Despite poorly investigated systemic communication between roots and shoots upon pathogen perception by roots, many studies have shed light on root-to-root as well as root-to-shoot signaling upon the interaction of plant roots with beneficial microbes. Studies on root-to-root communication mainly focused on the importance of coordination during the establishment of symbiosis between plants and soil microbes (Kosslak and Bohlool, 1984; Sargent et al., 1987; van Brussel et al., 2002; Kassaw and Frugoli, 2012; Laguerre et al., 2012). With respect to root-to-shoot signaling, it has been demonstrated that root colonization by non-pathogenic soil microbes often leads to increased plant resistance, a phenomenon which was named induced systemic resistance (ISR) (Schmidt, 1979; van Loon et al., 1998; Huang et al., 2014; Pieterse et al., 2014). ISR has been associated with stronger cellular responses upon immune activation in aerial tissue of root-colonized plants compared to those observed in aerial tissue of non-colonized plants (van Loon et al., 1998; Conrath et al., 2006; Pozo and Azcón-Aguilar, 2007). Interestingly, rhizobacteria-mediated induced systemic resistance typically involves the jasmonic acid (JA) and/or ethylene (ET) signaling pathway (Pieterse et al., 2000). It has also been shown that microbial root colonization by, for instance, bacterial and fungal symbionts can lead to systemic changes in the expression of defense and stress-related genes (Liu et al., 2007; van de Mortel et al., 2012). For example, colonization of roots with the fungal symbiont *Piriformospora indica* is known to trigger changes in the expression of defense-related transcripts in distant roots, indicating the movement of immunity-related signals (Pedrotti et al., 2013). However, in most cases, root colonization by beneficial microbes has not been linked to specific changes in shoot gene expression, even when an increased resistance to pathogens was observed (Verhagen et al., 2004). In contrast, whether the perception of pathogens and/or MAMPs in roots leads to direct systemic defense gene induction in shoots has never been investigated. We have recently demonstrated that isolated root tissue was able to perceive flg22 and the DAMP AtPep1 (Wyrsch et al., 2015; Chapter 2.2). Hence, to investigate systemic root-to-shoot signaling in plant-microbe interactions, we here studied plant defense gene expression in shoots upon local application of MAMPs and DAMPs to roots. To be able to reliably determine systemic signaling events, we first developed an application method, which ensures the perception of the elicitor specifically by roots and avoids that the elicitor itself moves to aerial tissues through diffusion. With this improved system, none of the analyzed marker genes for systemic defense signaling was systemically induced in shoots upon local MAMP/DAMP application to roots after up to 24 hours in our conditions.

3.3 Results

3.3.1 Local root treatments in spliced Petri dishes

A direct application of solutions to the soil is often used in the case of microbial inoculation but as we were aiming at studying a potential systemic response upon local MAMP addition to roots, we preferred to use an axenic system, where plants would be exposed to MAMPs for the first time and only little and controlled amounts of elicitor would be needed. Therefore, Arabidopsis plants were grown in agar plates for two weeks and then transferred to spliced Petri dishes containing a filter paper and 2 mL water in each compartment (Fig. 3.1). The following day, roots were treated with a control solution, flg22 or elf18. Elf18 was included as a diffusion control because this MAMP was shown not to be recognized in roots, most probably due to lack of *EFR* expression in these organs (Millet et al., 2010; Wyrsch et al., 2015). By contrast, elf18 can clearly elicit immune responses in shoots (Kunze et al., 2004). After 3, 12 and 24 hours of treatment, root and shoot tissues were harvested separately and gene expression was analyzed by real-time quantitative reverse transcription PCR (qRT-PCR). For this, the induction of the PTI marker genes *FRK1*, encoding a *FLG22-INDUCED RECEPTOR-LIKE KINASE* (Asai et al., 2002) and *PER5,* a *PEROXIDASE SUPERFAMILY PROTEIN* known to be involved in responses to oxidative stress (Tognolli et al., 2002) and induced upon flg22 treatment, were investigated (Boudsocq et al., 2010; Wyrsch et al., 2015; Chapter 2.2). However, the experiment, repeated several times, gave inconsistent results. In eight cases, transcripts of PTI marker genes clearly accumulated in shoots upon root treatment with flg22, whereas in seven cases there was no difference compared to the control treatment (data not shown). Such variation could also be observed with elf18 treatments. The latter result indicates the movement of the peptide to the shoot. This effect might be due to capillary or other forces along the vasculature of the stele or along the apoplast of the cortex and may result in the perception of the elicitor by the shoot.

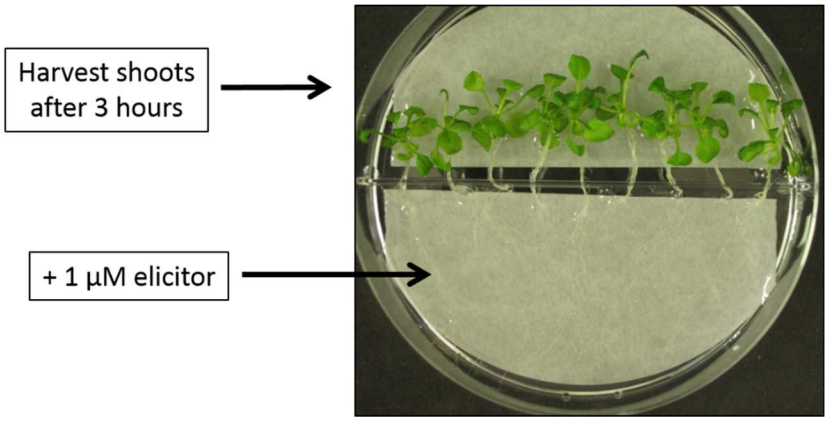

Fig. 3.1 System using spliced Petri dishes. Three week-old Arabidopsis plants grown in axenic conditions were placed into a spliced Petri dish containing 2 mL water and a filter paper in each compartment. The following day, elicitors were added at a final concentration of 1 µM to the root compartment. After three hours, root and shoot tissues were harvested separately.

3.3.2 Visualization of liquid diffusion between compartments

In order to evaluate the tightness of our experimental system, the solution loaded into the root compartment was stained with bromophenol blue. Surprisingly, we were able to observe a clear movement of the stained solution along the root surface, up to the shoot compartment in this system already after three hours (Fig. 3.2a). Remarkably, sealing off the junctions between the roots and the aerial tissues with vaseline and including a wider space filled with vaseline between roots and shoots did not avoid the quick movement of the dyed solution towards the shoot (Fig. 3.2b,c). Whether the solution only moved upwards through capillary forces at the outside of the root or in the apoplast or was actively taken up by and moved within the root, remains to be investigated.

In summary, our data show that with the tested system we cannot exclude elicitor movement and subsequent perception in the shoot. As very low concentrations of flg22 suffice to induce PTI responses (Mueller et al., 2012b), any movement of the MAMP-containing solution towards the shoot compartment can have undesired effects and strongly affect result interpretation.

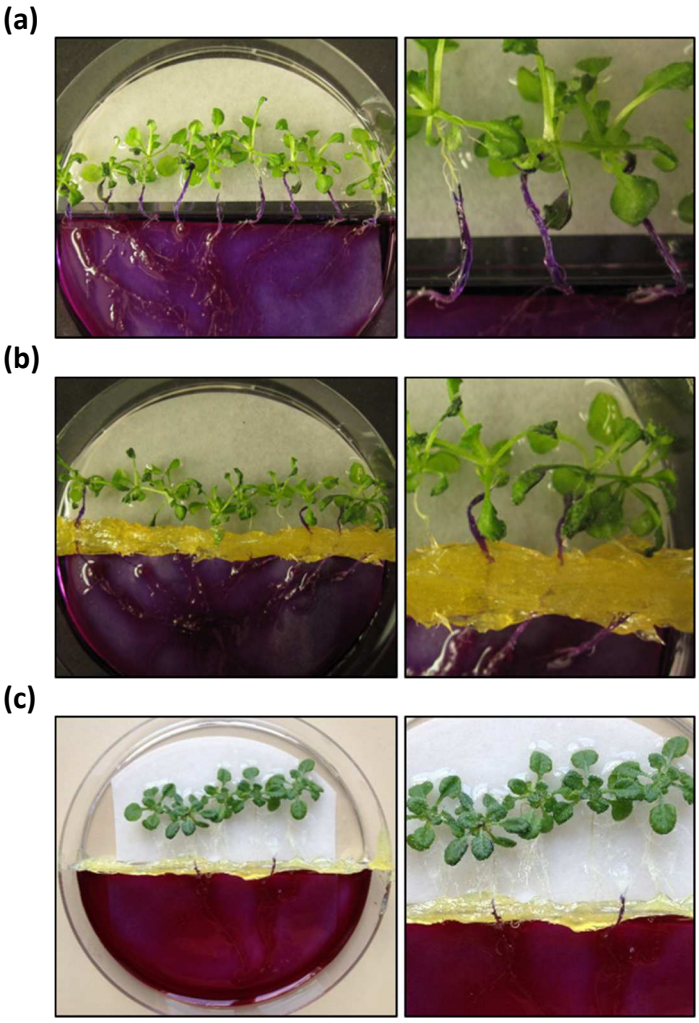

Fig. 3.2 Analysis of elicitor movement using bromophenol blue. The root compartment was stained with bromophenol blue in order to observe the movement of the solution along the Arabidopsis seedlings. The time point shown here is three hours. (**a**) System with spliced Petri dishes, (**b**) improved system using vaseline to seal the junction between roots and shoots, (**c**) improved system with vaseline including a wider space between roots and shoots. Movement along, and possibly inside the root was observed in all cases.

3.3.3 An improved treatment system avoids shoot elicitation and reveals no transcript changes in shoots upon local MAMP/DAMP applications to roots

We now attempted to establish a more robust system including a physical diffusion barrier between roots and shoots. For this, strong agar (2%) was poured in both compartments and an aerial stripe was created by dissecting the agar (Fig. 3.3a). Plants grown vertically in agar were transferred to this system and roots were treated locally with flg22, elf18 or AtPep1 the following day. AtPep1 was included because its perception by roots was known to elicit stronger local responses than flg22 (Wyrsch et al., 2015; Chapter 2.2). To restrict the dose of liquid on the agar surface, a higher concentration of the elicitors (10 µM) in a smaller volume was distributed on the root (three spots of 10 µl per root). After 3, 12 and 24 hours, root and shoot tissues were harvested separately and gene expression was analyzed by qRT-PCR.

After three hours of treatment, an increase in *PER5* transcripts was detected in elicited root tissues, indicating that they responded to the local application of flg22 and AtPep1 (Fig. 3.3b). As expected, no induction was observed upon elf18 treatment.

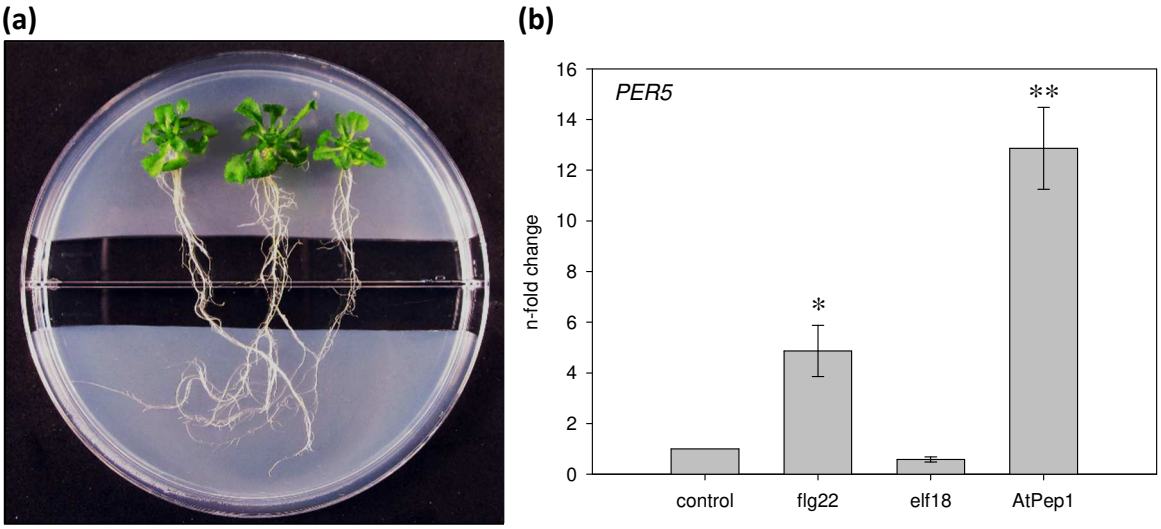

Fig. 3.3 An improved method allows induction of *PER5* in roots upon local treatment. (**a**) Representative picture of the improved system to study systemic root to shoot signaling using a spliced Petri dish containing 2% bactoagar in each compartment. An agar stripe was dissected in order to reduce the leakiness of the system. Three week-old plants grown vertically on agar were carefully transferred to the system. (**b**) Transcript levels of *PER5* were measured by qRT-PCR in roots three hours after treatment with the elicitors as indicated (10 µM, three spots of 10 µL per root). *PER5* expression was first normalized to that of the constitutive gene *UBQ10*, before calculating its expression relative to that of the control. Error bars show ± SE of the mean of three biological replicates with each performed with three technical replicates. Significant differences with respect to the control were calculated using a Student's t-test with: *, $P < 0.05$; **, $P < 0.01$.

By contrast, *PER5* and *FRK1* transcript levels remained unchanged in shoot tissue at different time points (Fig. 3.4). This result suggested that *FRK1* and *PER5* transcript levels are not systemically induced at the tested time points. Moreover our result proved that this system was suitable to study the possible systemic effect of root MAMP/DAMP application on shoot transcriptomes.

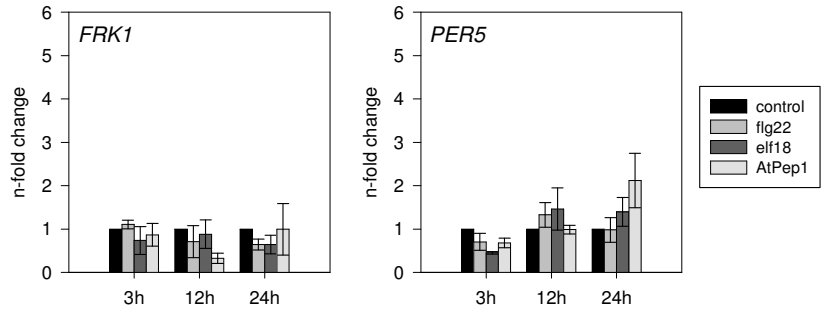

Fig. 3.4 Transcript accumulation of PTI marker genes analyzed in shoots of root-elicited plants. Transcrit levels of PTI marker genes *FRK1* and *PER5* were analyzed in shoots upon treatment of roots with the indicated elicitor (10 µM, three spots of 10 µL per root) for 3, 12 and 24 hours by qRT-PCR. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The error bars show ± SE of the mean of three biological replicates. No significant differences were found compared to the control according to Student's t-test.

Next, SA-responsive genes were analyzed for their transcriptional changes in shoot tissues upon MAMP/DAMP treatments of roots (Gaffney et al., 1993). These genes include *PATHOGENESIS-RELATED GENE 1* (*PR1*), *PATHOGENESIS-RELATED GENE 5* (*PR5*) and *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*). *PR1* gene expression is known to be induced in response to a variety of pathogens and is a useful molecular marker for SAR (Uknes et al., 1992; Delaney et al., 1994). Expression of this gene is SA-dependent (Durner et al., 1997; Kombrink and Somssich, 1997). The *PR5* gene family was shown to be induced by fungal infection and had antifungal activity (Kombrink and Somssich, 1997). *PR5* transcription was induced in response to activation of the SAR pathway in Arabidopsis and, therefore, is used as a marker for SAR-dependent defense triggering (Kawamura et al., 2009). *EDS5* is an essential component of SA-dependent signaling for disease resistance. Apart from flg22, its expression has been demonstrated to be induced by SA, pathogens and UV-C light and *eds5* mutants are SA-deficient (Nawrath et al., 2002).

However, in our conditions, none of the investigated SAR markers was significantly induced in shoots upon treatments of roots with flg22, elf18 or AtPep1 at any time point (Fig. 3.5).

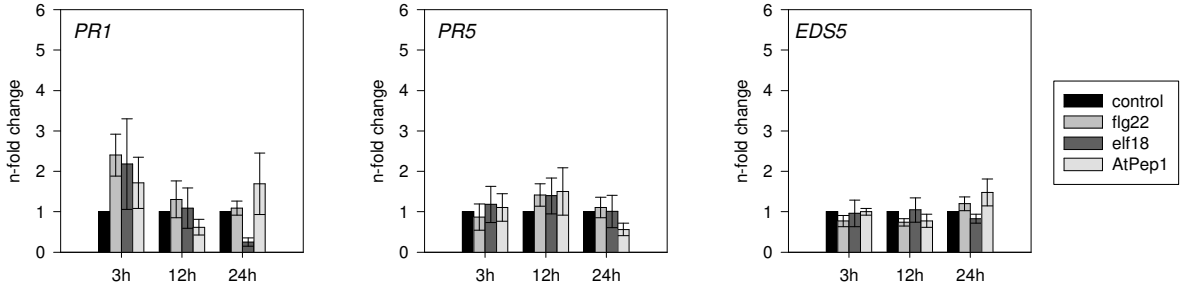

Fig. 3.5 Transcript levels of SA-responsive marker genes analyzed in shoots of root-elicited plants. Transcript levels of SA-responsive marker genes *PR1*, *PR5* and *EDS5* were analyzed in shoots upon treatment of roots with the indicated elicitor (10 µM, three spots of 10 µL per root) for 3, 12 and 24 hours by qRT-PCR. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The error bars show ± SE of the mean of three biological replicates. No significant differences were found compared to the control according to Student's t-test.

We further investigated the effects of root MAMP/DAMP applications on the transcript level of JA or ET-responsive genes, including the *LIPOXYGENASE 2* and 3 (*LOX2*, *LOX3)* genes and the plant *DEFENSIN 1.2* (*PDF1.2)* gene.

Genes from the *LOX* family encode dioxygenases involved in JA synthesis in plants (Schaller and Stintzi, 2008). *LOX2* is a *13-lipoxygenase* gene (Bannenberg et al., 2009), known to catalyze the first step of the JA biosynthetic pathway and *LOX2* mRNA accumulates rapidly after JA induction and flg22 treatments (Beckers and Spoel, 2006). The closely related *LOX3* is induced upon pathogen infection and exposure to methyl jasmonate (Melan et al., 1993; Vellosillo et al., 2007); therefore we also used it as a marker for JA signaling. The *PDF1.2* gene encodes a small protein with anti-fungal activity and is used as an ET-dependent marker for systemic resistance triggered by PGPRs (Brodersen et al., 2006; Bari and Jones, 2009) and has also been shown to be transcriptionally up-regulated upon flg22 treatment (Kawamura et al., 2009). Despite our efforts, we could not show any significant change in the expression of the JA- or ET-responsive genes in shoot tissue upon local root MAMP/DAMP application (Fig. 3.6).

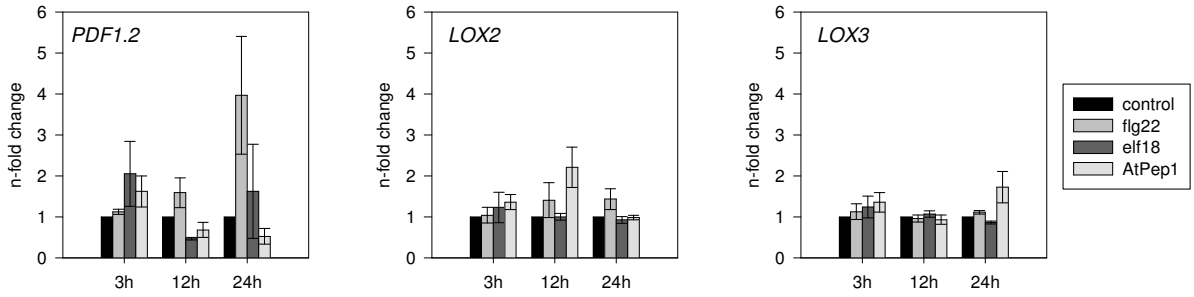

Fig. 3.6 Transcript levels of JA/ET-responsive marker genes analyzed in shoots of root-elicited plants. Transcript levels of JA/ET-responsive marker genes *PDF1.2*, *LOX2* and *LOX3* were analyzed in shoots upon treatment of roots with the indicated elicitor (10 µM, three spots of 10 µL per root) for 3, 12 and 24 hours by qRT-PCR. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The error bars show ± SE of the mean of three biological replicates. No significant differences were found compared to the control according to Student's t-test.

We then tested if other defense marker genes were systemically induced in locally MAMP-treated plants. The well-characterized secondary metabolites camalexin as well as glucosinolates exhibit antimicrobial activity. Furthermore, genes involved in the biosynthesis pathways for these compounds were shown previously to display transcriptional changes in roots and shoots upon root colonization by PGPRs (van de Mortel et al., 2012). Therefore, *CYP71A12*, encoding a cytochrome P450, which catalyzes the conversion of indole-3-acetaldoxime to indole-3-acetonitrile during camalexin biosynthesis, was included in our analysis (Nafisi et al., 2007). *CYP71A12* has been shown to be induced in roots upon MAMP treatments (Millet et al., 2010). *MYB51*, encoding a transcription factor essential for the regulation of indole-glucosinolate biosynthesis (Gigolashvili et al., 2007) was also found to be induced in Arabidopsis roots upon MAMP treatments (Millet et al., 2010). It was previously reported that flg22-elicited callose deposition in Arabidopsis cotyledons is dependent on the biosynthesis of indol-3-ylmethylglucosinolate, which is in turn dependent on *MYB51* (Clay et al., 2009). However, in our study, *CYP71A12* and *MYB51* gene activity was not significantly induced in shoots of root-treated plants at any of the investigated time points (Fig. 3.7).

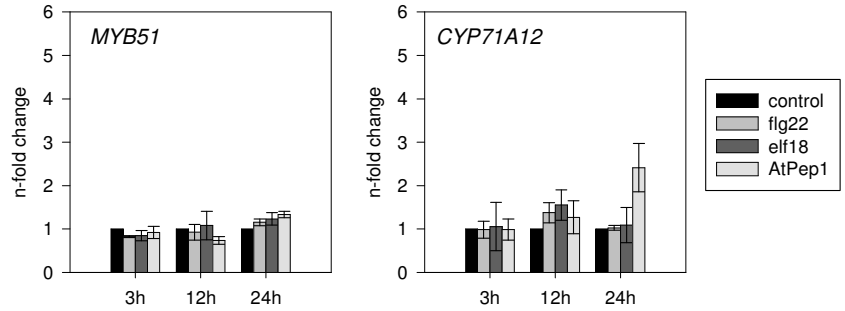

Fig. 3.7 Transcript levels of secondary metabolite marker genes analyzed in shoots of root-elicited plants. Secondary metabolite marker genes *MYB51* and *CYP71A12* were analyzed for their transcriptional change in shoots upon treatment of roots with the indicated elicitor (10 µM, three spots of 10 µL per root) for 3, 12 and 24 hours by qRT-PCR. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The error bars show ± SE of the mean of three biological replicates. No significant differences were found compared to the control according to Student's t-test.

3.4 Discussion

In order to study root-to-shoot signaling upon local MAMP and DAMP perception by roots, we developed a system using spliced Petri dishes. However, our evaluation revealed that diffusion of the solution between the two, separated compartments occurred within or along the roots (Fig. 3.2). Thus, the observed, systemic transcript induction of defense-related genes may result from the perception of small concentrations of diffused elicitors by shoot-localized PRRs. We believe that local elicitor applications should be performed carefully as minimal traces of elicitors can move throughout or alongside plants by capillary forces and be perceived in distal organs. Our results indicate that treatments of roots, for instance, by soil applications, dip-inoculation methods but also foliar spray-treatments cannot completely rule out the possibility that the stimulus is perceived by an untreated distal organ. Although experiments performed with microbial colonization might allow a better separation between roots and shoots due to colonization preferences or attraction of microbes to specific tissues through, for instance, root exudates, we think that generally caution with these systems should be taken. Notably, we recommend considering the passive diffusion of elicitor treatments when working with systemic signaling between plant organs to interpret results.

In order to allow the study of long-distance systemic signals from roots to shoots, we set out to improve the system. For this purpose, we included a more robust aerial barrier separating root and shoot tissues, and applied elicitors on agar in order to minimize the passive flow of soluble MAMPs/DAMPs. This system proved to be reliable for local elicitor applications and allowed us to study the effect of restricted MAMP/DAMP treatments (Fig. 3.3a). We were

able to demonstrate that roots responded locally to the application of flg22 and AtPep1 (Fig. 3.3b) whereas no induction in the expression of defense genes was observed in shoot tissues upon root treatments, indicating that the elicitors were not perceived in shoot tissues at concentrations sufficient to induce a response (Fig. 3.4-Fig. 3.7). Although eight of the ten selected genes have been shown to be involved in SA- or JA/ET-mediated systemic defense signaling pathways in plants, none of these genes analyzed was significantly induced in the shoots of the root-treated plants during the course of our experiment (Fig. 3.4-Fig. 3.6). The possibility remains that different genes could be induced, which we did not analyze. To overview the Arabidopsis leaf transcriptome upon MAMP treatment of roots, microarray or RNAseq experiments could be performed. Furthermore, different and additional time points may be considered as the induction of these genes after temporally more extended root treatments might still be observed. Usually, SAR becomes induced several days after perception of a biotic stress (Cameron et al., 1994). Our improved system is currently not optimal to study longer incubation periods as we observed that the plants started to dry after 24 hours.

Considering the diffusion of elicitors, grafting could provide a more advantageous and secure method to study long-distance signaling between plant organs upon long-time treatments. In the last decade, suitable grafting protocols have been developed for Arabidopsis, allowing the study of long-range signals from a molecular perspective (Bainbridge et al., 2014). However, grafting has been reported to be time-consuming and causing a wounding stress to the plant (Kumari et al., 2015). Therefore, we decided not to perform grafting experiments.

With respect to MAMP/DAMP-induced systemic resistance, it will be interesting in the future to test whether MAMP or DAMP treatments of roots leads to a primed state of the aerial tissues. We believe that our newly developed experimental system is quick, easy and reproducible and therefore suitable to study immediate priming effects. However, the exact readouts and treatment times remain to be investigated. Preliminary experiments revealed that under our conditions, none of the selected marker genes was induced more strongly in flg22-treated shoots upon MAMP or DAMP pretreatments of roots (Fig. S3.1). Interestingly, our preliminary results do not agree with previous studies, which show that upon local application of flagella from *Pseudomonas fluorescens* to Arabidopsis roots systemic resistance is induced (Meziane et al., 2005).

Finally, we hypothesize that systemic signals may move throughout the plant but are perceived only in specific recipient tissues in the shoot, where they induce cell-autonomous PTI responses. That might be a reason why we could not detect any systemic induction with our system as we investigated the whole shoots. If so, defense marker genes could be induced only in the vascular tissues, for instance, in companion cells and our system would not allow us to detect some transcriptional changes due to a dilution effect when harvesting whole shoots. In line with this idea, we found that *PR1* and *CYP71A12* were slightly induced upon flg22 or AtPep1 treatment, respectively, indicating a tendency for defense gene induction (Fig. 3.5, Fig. 3.7). In order to circumvent these problems, we propose that transgenic lines expressing either β-glucuronidase (GUS) or a fluorescent protein fused to a nuclear localizing sequence (for instance *Venus3xNLS*) (Vermeer et al., 2014) under the control of MAMP- or hormone-responsive promoters could be analyzed with the system described above. Promoter activity in the shoot could then be analyzed in a more sensitive manner upon local root treatments. As we hypothesized that a phloem-translocated signal would not be perceived by old source leaves but only by the smallest, young sink leaves, defense marker gene induction might be analyzed exclusively in sink leaves. Finally, we believe that an investigation at the protein level could lead to new information about systemic signaling from root-to-shoot upon local MAMP treatments. In support, previous studies revealed that local virus infection and wounding of plants lead to systemic induction of proteins, but only marginal transcript changes (Niehl et al., 2013).

In conclusion, we provide here a simple, rapid and useful method to study systemic signaling from roots to shoots in Arabidopsis that avoids elicitor movement along plant surfaces and/or within roots often observed in other systems. Therefore, we believe that our method is reliable to study systemic responses upon local applications of signals related to immunity, development or abiotic stress. As we could not detect any significant modification in marker gene expression in shoots upon MAMP/DAMP treatment of roots, MAMP/DAMP-induced root-to-shoot signaling likely does not involve transcript changes.

3.5 Materials and methods

3.5.1 Plant growth conditions

Plants used were wild-type Arabidopsis (*Arabidopsis thaliana* L. Heynh cultivar 6 Columbia). Seeds were surface-sterilized and germinated on MS plates (half-strength 0.5 x Murashige and Skoog basal medium (Phyto-technology Laboratories) containing 1% sucrose and 0.8% phytoagar) for five days in a plant growth chamber (24 hours of photoperiod, 20°C). Seedlings were then transferred to square Petri dishes containing MS medium and agar and further grown vertically in the same conditions for 14 days. For assays, plants were carefully taken out from the plates and transferred to the spliced Petri dishes containing 2% of bactoagar and left overnight in the same conditions before treatment with elicitor.

3.5.2 Staining with bromophenol blue

100 µl of water containing ~0.01% of bromophenol blue was added to the 2 mL water in the compartment containing the roots of a spliced Petri dish. Vaseline was included in order to seal the junction between roots and shoots. After three hours, plants were photographed.

3.5.3 Determination of gene expression

Root and shoot tissues were collected after treatment of roots with 10 µM elicitor for 3, 12 and 24 hours. Material was frozen and ground in liquid nitrogen. RNA from 50 mg tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with recombinant DNase according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of RNA with oligo (dT) primers using the Avian Myeloblastosis Virus reverse transcriptase according to the manufacturer's instructions (Promega). Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed in a 96-well format using a LightCycler®480 System (Roche Applied Science). Normalized expression to the reference gene *UBQ10* was calculated using the qGene protocol (Muller et al., 2002). All primer sequences and accession numbers of genes analyzed by qRT-PCR are listed in Table S3.1.

3.5.4 Elicitor treatments

Peptides, flg22 (QRLSTGSRINSAKDDAAGLQIA), *At*Pep1 (ATKVKAKQRGKEKV-SSGRPGQHN) and elf18 (ac-SKEKFERTKPHVNVGTIG), were obtained from EZBiolabs and applied at a final concentration of 1 µM in the first system using Petri dishes. For local root treatments, the elicitors were dissolved in 0.4% agarose at a concentration of 10 µM and applied as spots of 10 µl on roots (three spots per root).

3.6 Supplemental figure

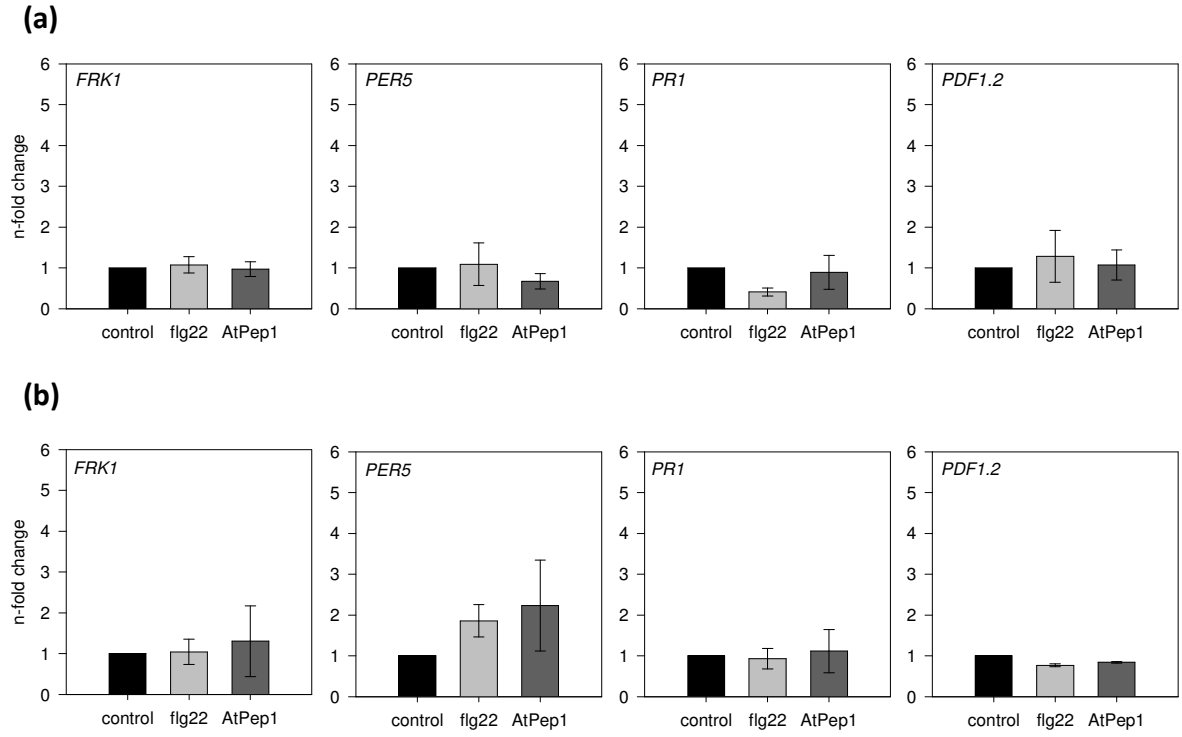

Fig. S3.1 Flg22-elicited transcript induction in shoots after pretreatment of roots with flg22 and AtPep1. Marker gene induction was investigated in shoots of root-treated seedlings after secondary application of flg22 to shoots. (**a**) Roots were treated for 12 hours with the indicated elicitors or without elicitor (control) in the optimized system. For secondary challenge, all leaves were sprayed with 1 µM flg22 after 12 hours and shoot samples harvested 45 minutes later. (**b**) Roots were treated for six hours with the indicated elicitors or without elicitor (control) in the optimized system. Subsequently, seedlings were washed and transplanted to soil. For secondary challenge, flg22 was sprayed at a concentration of 1 µM to all leaves after six days and shoot tissue was harvested 45 minutes later. (**a** and **b**) Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The error bars show ± SE of the mean of three biological replicates. No statistically significant differences were found compared to the control according to a Student's t-test.

3.7 Supplemental information: Development and assessment of new methods for studying root-to-shoot signaling

3.7.1 Abstract

In the course of this thesis, I have developed and tested several methods for their suitability to study the effect of local MAMP applications to roots on shoot transcript changes. However, most methods failed due to several technical problems. Particularly, I was not able to exclude the possibility that root-applied elicitors were perceived by shoot tissue due to movement of the elicitors. Here, I would like to give a short overview about the different methods additionally tested during my thesis to study root-to-shoot signaling and describe their advantages and drawbacks. All four approaches have been developed with the hope to restrict the movement of root-applied elicitors towards shoot tissue and to ensure local elicitor perception.

3.7.2 Method I: Transgenic plants expressing *FLS2* under root-specific promoters

3.7.2.1 Method

Transgenic plants expressing *FLS2* only locally in the root under root tissue-specific promoters (Wyrsch et al. 2015; Chapter 2.2 of this thesis) were used.

3.7.2.1.1 Expected advantages

The system ensures a local perception of elicitors only in tissues where *FLS2* is expressed and hence, excludes unspecific flg22 perception. Furthermore, we consider this system to be faster than, for instance, grafting of *fls2* mutant scions with wild-type rootstocks, which additionally often causes stress for a plant.

3.7.2.1.2 Drawbacks

Not all promoters are root-specific, which may cause unspecific perception of flg22 by the shoot-expressed *FLS2*. Additionally, the absence of *FLS2* expression in certain root tissues could be problematic as, for example, flg22 perception by the vasculature might be important for systemic root-to-shoot signaling.

3.7.2.2 Results and discussion

First, shoots of two week-old seedlings were investigated for their ability to perceive flg22 because we hypothesized that FLS2-GFP accumulation, although undetectable by confocal microscopy, might occur in shoots of the transgenic lines. Therefore, the *FLS2* expression level in shoots was measured by qRT-PCR (Fig. S3.2a) and its functionality assessed by ROS production upon flg22 treatment in leaf discs of the corresponding transgenic lines (Fig. S3.2b).

Although several of the selected promoters were specific for some distinct root tissues (Wyrsch et al., 2015; Chapter 2.2), all lines showed *FLS2* transcript accumulation in the shoot when compared to those of wild-type levels (Fig. S3.2a). The *UBQ10pro*, *SCRpro* and *PGP4pro:FLS2-GFP* lines showed enhanced transcript levels when compared to wild-type levels whereas the *LBD16pro, WERpro* and *ELTPpro:FLS2-GFP* lines accumulate *FLS2* transcript levels comparable to wild-type levels (Fig. S3.2a). When isolated leaf tissue was investigated for its ability to perceive flg22, we were able to detect ROS accumulation in the lines showing higher *FLS2* expression than Col-0, except for the *SCRpro:FLS2-GFP* expressing line. *WERpro* and *ELTPpro:FLS2-GFP* lines did not show ROS accumulation although expressing *FLS2* at equal levels to those found in wild-type (Fig. S3.2b). This observation could be attributed to the fact that *FLS2* is expressed only weakly in certain aerial tissues which might not be accessible by the elicitor or do not contribute to the ROS production in leaves. The absence of ROS production in the *SCRpro:FLS2-GFP* line could be explained by the fact that this line expressed *FLS2* only in the shoot meristem (Wysocka-Diller et al., 2000), but not in the leaf discs used in this analysis.

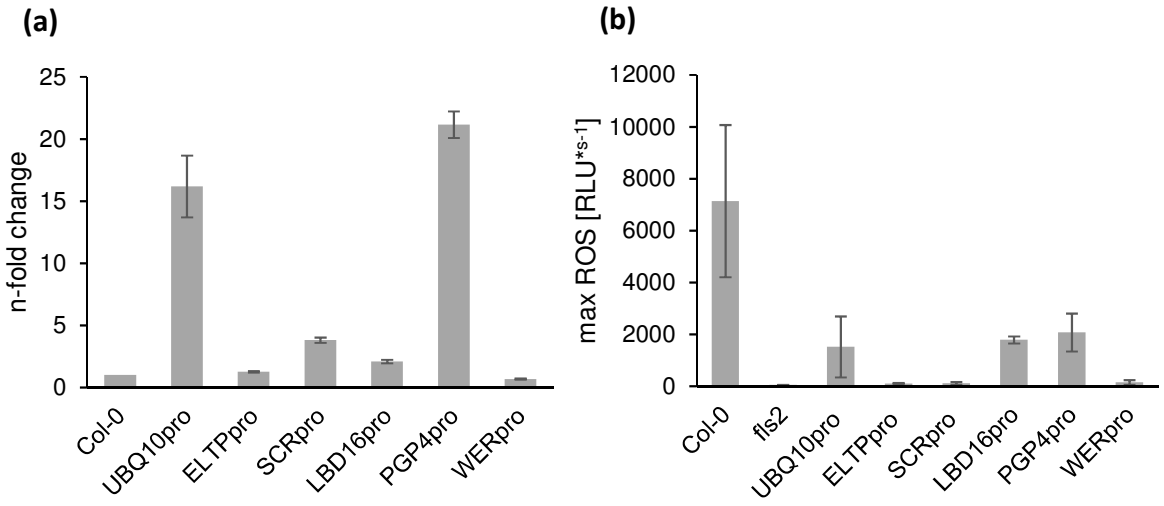

Fig. S3.2 Analysis of *FLS2* expression and functionality in the shoots of transgenic lines. (**a**) Shoots of two week-old Arabidopsis *fls2* mutants expressing *FLS2-GFP* under the indicated, "root-specific" promoters were analyzed for *FLS2* expression by qRT-PCR. All lines showed *FLS2* expression in shoots when compared to wild-type Col-0. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the wild-type control. Values show mean of three technical replicates ± SE. (**b**) Maximal ROS production in leaf discs of transgenic lines upon1 µM flg22 treatment. Columns represent mean values of maximal ROS production in three biological replicates with each performed with at least eight technical replicates Error bars show ± SE of the mean. RLU = reactive light units.

All lines were analyzed for their ability to induce *FRK1* transcription upon flg22 treatment of roots in spliced Petri dishes (as described in Chapter 3.3.1). In fact, all lines showed enhanced *FRK1* transcript levels when compared to the ones of shoots of *fls2* plants treated locally on roots with flg22 (Fig. S3.3). Nevertheless, the *FRK1* transcript accumulation was weaker in the *ELTPpro*, *SCRpro*, *PGP4pro* and *WERpro:FLS2-GFP* lines when compared to wild-type levels, which could be due to several reasons. First, it is possible that the low level of shoot-expressed *FLS2* observed in lines *ELTPpro* and *WERpro:FLS2-GFP* (Fig. S3.2a) is responsible for the weaker *FRK1* transcript levels in these lines. Second, *FLS2* might only be expressed in these lines in shoot tissues which are less accessible or responsive to diffused, shoot perceived flg22. Third, it is possible that the nature of the root tissue perceiving flg22 decides for the intensity of the systemic *FRK1* transcript induction in the shoot. We have shown previously that the intensity of PTI responses in roots varied depending on the type of tissue where flg22 was perceived (Wyrsch et al., 2015; Chapter 2.2). Thus, the tissue identity detecting flg22 could play an important role also in systemic signaling. It needs to be considered that the lines *ELTPpro*, *SCRpro*, *PGP4pro* and *WERpro:FLS2-GFP* express *FLS2* only in the meristem, epidermis or endodermis. Thus, the absence of *FLS2* expression in the

stele in these lines correlates with the absence of a systemic response in the shoot. Hence, we propose that flg22 perception in the stele or pericycle may be important in order to obtain a systemic signal, as PTI responses have been shown to be strongly induced in this tissue (Wyrsch et al., 2015; Chapter 2.2). In agreement with this idea, the wild-type, the *UBQ10pro* and *LBD16pro:FLS2-GFP* lines, all expressing *FLS2* in the pericycle and/or stele (Chapter 2.2; Fig. 2.7) show a strong systemic *FRK1* induction in shoots upon root flg22 treatments (Fig. S3.3). However, as the *FRK1* induction also correlated with the *FLS2* expression level in shoots, except for the *PGP4pro:FLS2-GFP* line (Fig. S3.2a; Fig. S3.3), it is impossible to determine whether the shoot-localized *FLS2* perceiving diffused flg22 is responsible for the *FRK1* induction or whether the *FRK1* transcription is indeed induced due to a systemic signal originating from roots. In view of these results, we decided not to investigate these lines in more detail.

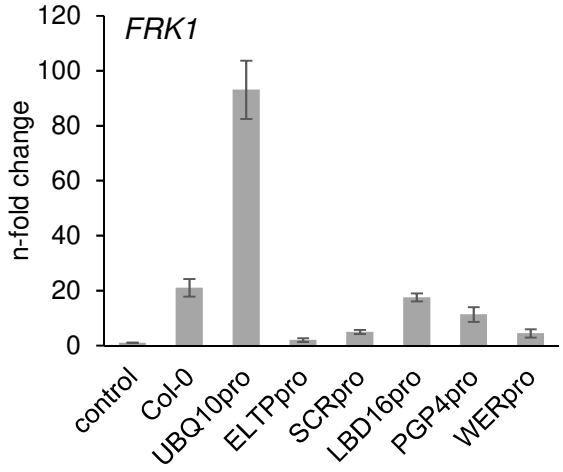

Fig. S3.3 Systemic *FRK1* induction in shoots of root-treated transgenic plants. *FRK1* induction in shoots upon 1 µM flg22 treatment of roots for three hours in spliced Petri dishes. Control is flg22-treated *fls2* mutant. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. Data show mean values of three technical replicates ± SE.

3.7.2.3 Outlook

To obtain further specificity of *FLS2* expression, three more root-specific promoters, which were shown to express *FLS2* only in the root (Christ et al., 2013), were cloned in front of the *FLS2-GFP* construct and transferred by stable transformation in *fls2* plants. For this purpose, the *HH32* (AT5G54310) promoter, controlling the accumulation of a late embryogenesis abundant protein-related/LEA protein-related, shown to be expressed in the root cap, columella and lateral root cap was selected. Furthermore, the *HH29* (AT5G43520) promoter, controlling the accumulation of a DC1 domain-containing protein, active in the epidermis of

the root meristem, cortex, epidermis and endodermis of the mature part of the root and the *HH24* (AT3G11550) promoter, controlling an integral membrane family protein (CASP2) expressed in the pericycle and endodermis of the mature part of the root were cloned. These promoters are described to express *FLS2* in almost all root tissues and *HH24* even strongly in the root pericycle (Christ et al., 2013). Moreover, to ensure expression of *FLS2* in the vasculature, which might be important to generate a systemic signal, we additionally expressed *FLS2-GFP* under the control of the *WOL/CRE1* promoter (Mähönen et al., 2000). These lines can further be investigated for systemic transcript induction upon root application of flg22.

In future, these new tools should be useful to further analyze systemic MAMP signaling between roots and shoots.

3.7.3 Method II: Generation and characterization of transgenic plants secreting flg22 autonomously

3.7.3.1 Method

Transgenic Arabidopsis plants which produce and secrete flg22 in the apoplast were generated. For this, the amino acid sequence of *Pto* DC3000 flg22 was reverse translated using the biophp translation tool (http://www.biophp.org/minitools/protein_to_dna/demo.php) and edited with the codon usage of Arabidopsis. The sequence was cloned and fused to the basic chitinase signal sequence in its N-terminus (*flg22_sec*), which had been shown to promote the secretion of GFP into the apoplast of *N. benthamiana* plants (Su et al., 2004). *Flg22_sec* was cloned under the control of the chimeric transactivator XVE (Zuo et al., 2000) to allow conditional *flg22_sec* expression upon estradiol treatment. Arabidopsis Col-0 plants were stably transformed with the corresponding plasmid. As control, we included the same construct but lacking the sequence coding for the two last amino acids of *flg22*. This shortened version, flg22-Δ2, was shown to act as an antagonist of flg22 as it is recognized by the receptor FLS2 without triggering immune responses (Bauer et al., 2001). As further control, we generated plants lacking the functional flg22 receptor FLS2 (*fls2*) expressing *flg22_sec*.

3.7.3.1.1 Expected advantages

This system allows the induction of flg22 only locally in specific tissues upon local estradiol treatment.

3.7.3.1.2 Drawbacks

In addition to the secreted flg22, the locally applied estradiol might also move along the plant, especially as it is dissolved in ethanol.

3.7.3.2 Results and discussion

Col-0 plants were transformed with *flg22_sec* and *flg22-Δ2_sec* and *fls2* plants with *flg22_sec*. Independent lines were isolated and tested in the T2 generation. Seeds were germinated on agar plates without antibiotic selection and one seedling was placed into each well of a 24-well plate either containing 10 µM of estradiol or ethanol as control. Pictures were taken after eight days of treatment and one representative line for each construct has been selected for further investigation (Fig. S3.4). The phenotype of the *flg22_sec* expressing Col-0 plants clearly showed that estradiol treatment led to a growth inhibition phenotype comparable to long-term flg22 treatment. However, the fact that this phenotype was also detectable in ethanol-treated plants might indicate that the XVE transactivator was slightly leaky. As flg22 can induce PTI responses already at nanomolar concentrations (Mueller et al., 2012b), we suggest that over this long-term treatment, a relatively small amount of secreted flg22 could lead to the observed phenotype. By contrast, estradiol treatment did not elicit any change in phenotype in the transgenic Col-0 plants expressing *flg22-Δ2_sec* or the *fls2* plants expressing *flg22_sec* when compared to the control treatment (Fig. S3.4b,c). These results support the idea that the observed growth inhibition phenotype in *flg22_sec* expressing Col-0 plants indeed results from intact flg22 secretion and perception (Fig. S3.4a).

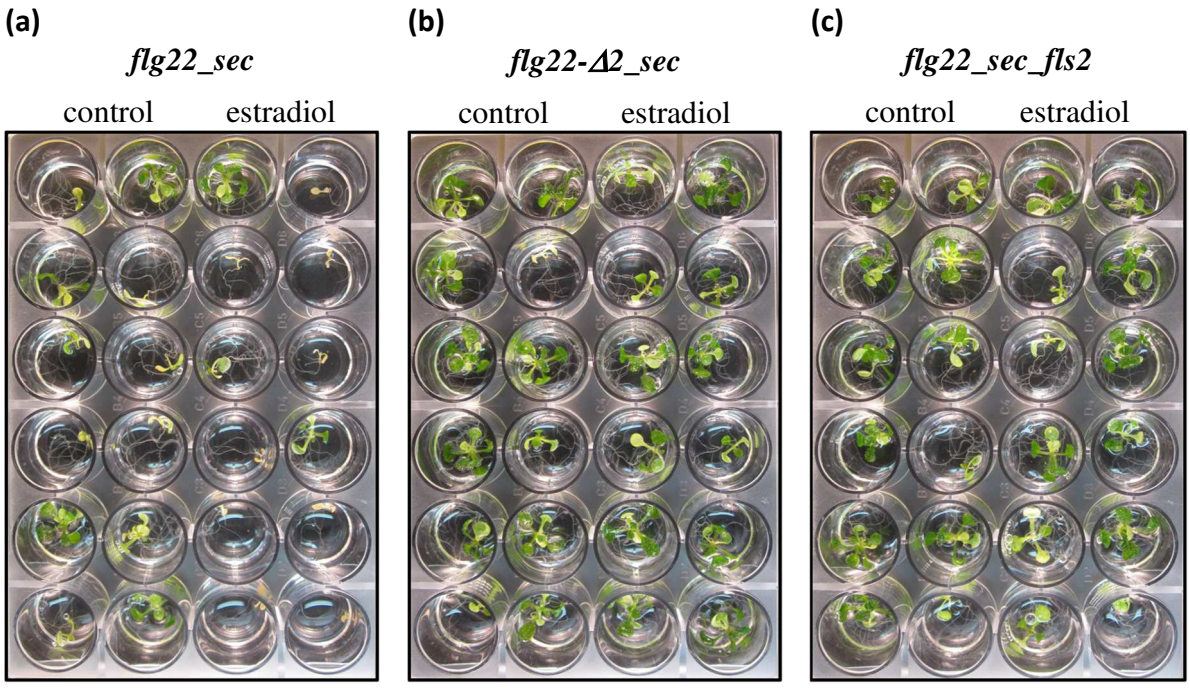

Fig. S3.4 Growth phenotype of transgenic Arabidopsis lines expressing *flg22_sec* or *flg22-Δ2_sec*. Growth phenotype of transgenic Arabidopsis T2 wild-type lines transformed with estradiol-inducible *flg22_sec* (**a**) and *flg22-Δ2_sec* (**b**) or *fls2* mutant lines transformed with estradiol-inducible *flg22_sec* (**c**). Seedlings were treated for eight days with 10 µM estradiol and photographed.

To test whether estradiol-induced flg22 secretion led to the activation of PTI in transgenic plants, two week-old seedlings grown in liquid MS media (without preselection on hygromycin) were treated with 10 µM estradiol for 24 hours. Total RNA was extracted from a pool of 12 T2 seedlings and *FRK1* transcript accumulation was measured by qRT-PCR and compared to the one measured for the ethanol control treatment. Our data showed that all lines expressed the *flg22_sec* or *flg22-Δ2_sec* construct (Fig. S3.5b). Interestingly, the results indicated that only wild-type plants expressing the *flg22_sec* construct showed enhanced *FRK1* transcript accumulation upon estradiol application. The seedlings expressing the *flg22_Δ2_sec* or the *fls2* plants expressing *flg22_sec* remained unresponsive to the estradiol application (Fig. S3.5a). Remarkably, these data indicate that active flg22 is produced and secreted into the apoplast *in planta.*

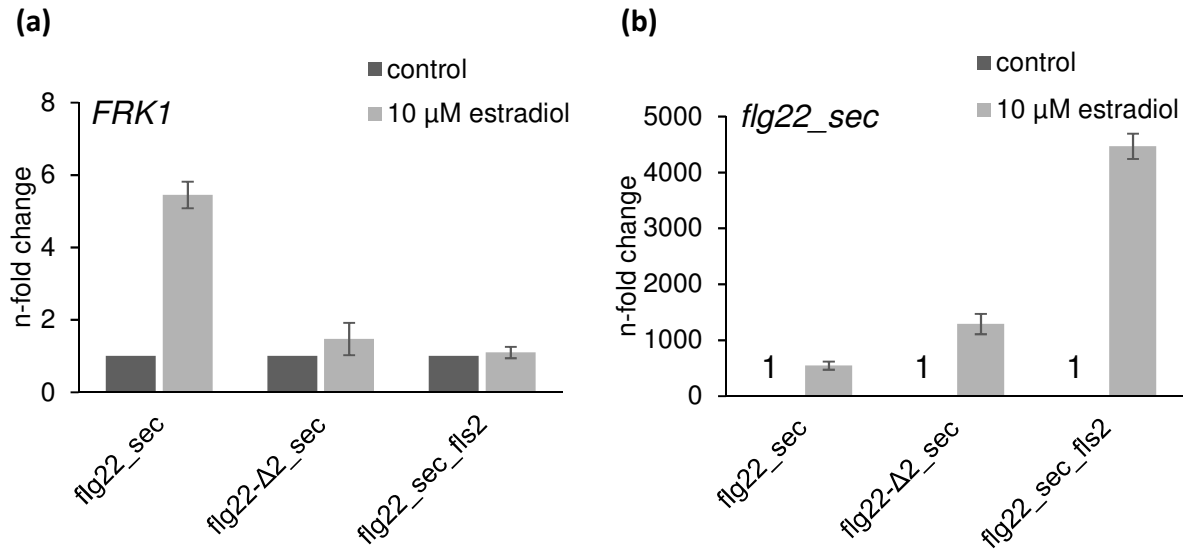

Fig. S3.5 Transcript levels of *FRK1* and *flg22_sec* in transgenic seedlings. 12 two week-old segregating T2 seedlings were treated for 24 hours with 10 µM estradiol and analyzed for the levels of transcripts of *FRK1* (**a**) and *flg22_sec* or *flg22-Δ2_sec* (**b**). The control treatment is ethanol. Expression of *flg22_sec* and *flg22-Δ2_sec* was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the background expression level. Data show mean values of two biological replicates ± SD.

Although the results implied that flg22 was secreted by transgenic Arabidopsis seedlings, several factors need to be considered when applying the method for investigation of systemic signaling. The most problematic point might constitute the treatment time. The plants were treated for 24 hours in our system, thus, when applying estradiol locally to roots to trigger local flg22 secretion for such a long period, the risk of estradiol diffusion towards the shoot tissue may increase. Hence, shorter treatment conditions might be used but we first need to study the exact kinetics of the flg22 production and secretion by plants.

Although our results indicate that flg22 is actively secreted by intact cells, its secretion cannot easily be detected in intact seedlings. Note that we did not include a tag in the *flg22_sec* construct as this could inhibit secretion of the small peptide into the apoplast. Hence, we cannot rule out the possibility that flg22 overexpression in cells might be toxic leading to the observed growth inhibition phenotype (Fig. S3.4). Flg22 might be stuck in the endoplasmatic recticulum or other organells instead of being secreted into the apoplast. Therefore, in order to prove that flg22 is indeed secreted and active, we have tried to perform classical apoplastic extractions with *flg22_sec* transformed Col-0 plants after estradiol treatment. With this apoplastic extractions, other plant samples were treated and analyzed for increased ethylene production. However, we faced several technical problems mainly due to the buffer conditions, weak ethylene production of treated plants or insufficient extract (data not shown). Thus, we

rely on destructive methods such as ROS or qRT-PCR assays as readouts for the activity of the peptide, which do not simultaneously prove its secretion. Finally, it needs to be considered that the actively, *in planta* secreted flg22 might also be moving inside the vascular system or at the plant's surface. Thus, systemic PTI induction might result from this diffusion and shoot perception of the secreted flg22. Due to these assumptions, more work needs to be performed to specifically characterize the transgenic lines.

3.7.3.3 Outlook

Our data indicate that flg22 can be produced and secreted *in planta*. In view of these results, we think that the newly generated transgenic plants might be used to study different effects of flg22 secretion. Nevertheless, we encountered a problem when using them for investigating systemic signaling as we believe that locally applied estradiol might move along the plant. Nevertheless, we produced root tissue-specific flg22 secreting lines and the use of root tissue-specific promoters under the control of the estradiol-inducible transactivator XVE would further allow to induce flg22 only locally in the root and could help to study the effects of tissue- or organ-specific flg22 perception. Remarkably, the group of our Sinergia project partner Niko Geldner from the University of Lausanne succeeded to produce such an inducible root-specific promoter, which could now be fused to the *flg22_sec* construct. In addition, the applied strategy and cloning method could be employed to analyze the effects of *in planta* secretion of additional peptides such as endogenous AtPeps but also microbe-derived molecules. In addition, the system may help to produce other peptides as, for instance, for pharmaceutical use which could be gained by purification of apoplastic washes.

3.7.4 Method III: Transgenic plants expressing *FLS2* under the control of an estradiol-inducible transactivator

3.7.4.1 Method

A homozygous line expressing *FLS2* under the estradiol-inducible transactivator XVE (Zuo et al., 2000) in the *fls2* background, kindly provided by Delphine Chinchilla, was used. Upon local estradiol treatment, *FLS2* expression could be induced only locally in roots ensuring specific flg22 perception.

3.7.4.1.1 Expected advantages

This method provides the advantage that *FLS2* expression can be induced in roots only. Hence, the possibility that root-applied flg22 is perceived by *FLS2* expressed in shoots can be excluded.

3.7.4.1.2 Drawbacks

The root-applied estradiol and/or flg22 might still move along the plant. Furthermore, *FLS2* expression was induced only weakly in roots upon estradiol application.

3.7.4.2 Results and discussion

Transgenic plants were grown vertically on agar for three weeks and placed on the spliced Petri dishes overnight (Chapter 3.3.1). The following day, roots were treated with either 10 µM estradiol or ethanol as control. After five hours, flg22 was added to the root compartment at a final concentration of 1 µM and root and shoot tissues were harvested separately three hours later. The shoot tissue was then analyzed for PTI marker gene transcript accumulation by qRT-PCR. The genes under investigation were PTI markers (*FRK1*, *PER5* and *NDR1/HIN1-LIKE 10 (NHL10)*) as well as a marker for the SA signaling pathway *(PR1*) used also in Chapter 3. Our data indicate that flg22 was perceived locally by root tissues because the PTI markers *PER5* and *FRK1* were induced even if *FLS2* was only weakly expressed (Fig. S3.6a).

Nevertheless, no systemic defense gene induction was detectable in the aerial tissues (Fig. S3.6b). The analysis remains preliminary and in future, it will be important to include other markers such as additional hormonal signaling markers. Furthermore, the kinetics of the gene

transcript accumulation need to be investigated. It has been previously suggested that the various genes exhibited their maximal induction values at different time points of MAMP treatment (Domínguez-Ferreras et al., 2015; Chapter 2.2.3.3; Fig. 2.10). Therefore, it is important to perform time course experiments.

The slight induction of the *FLS2* transcript levels in the shoot further indicates that estradiol might have induced *FLS2* expression in this tissue due to its diffusion towards the shoot (Fig. S3.6a). Surprisingly, this up-regulation in *FLS2* expression was not sufficient to perceive flg22 in shoot tissues as no other genes tested were up-regulated in the shoot. Eventually, flg22 did not diffuse towards aerial tissues, possibly due to the shorter treatment period of only three hours compared to the five hours estradiol treatment. However, this indicates a potentially increased diffusion risk when analyzing longer treatment conditions.

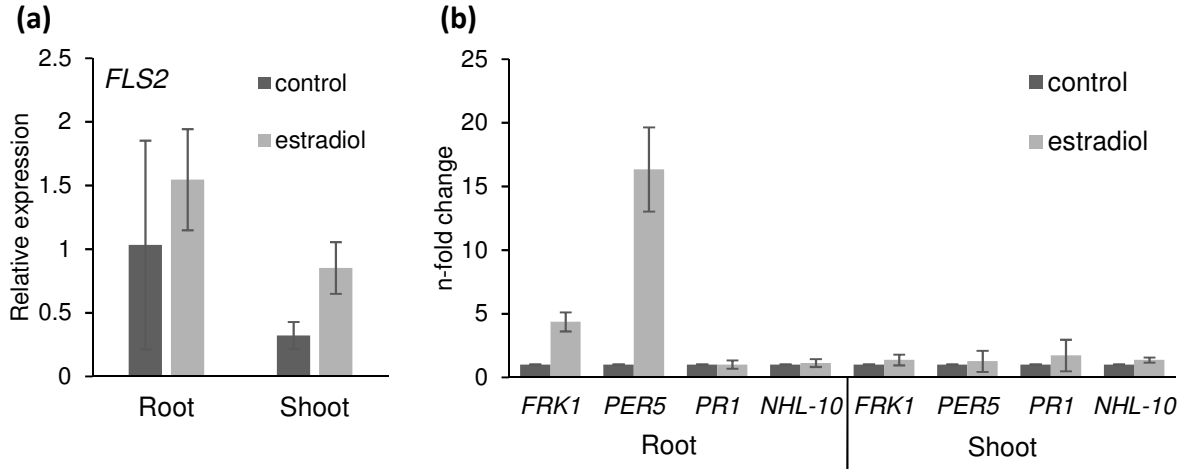

Fig. S3.6 *FLS2* and marker gene transcript accumulation in root-treated plants. Plants expressing *FLS2* under the control of the estradiol-inducible transactivator XVE were treated locally on roots with 10 µM estradiol or ethanol as control for five hours. Subsequently, 1 µM flg22 was added to roots for three more hours in spliced Petri dishes. (**a**) Relative expression levels of *FLS2* normalized to the reference gene *UBQ10* expression in roots and leaves are shown. (**b**) Fold change of marker gene expression in roots and shoots analyzed by qRT-PCR. Expression was first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. (**a** and **b**) Data show mean values of two biological replicates ± SD.

3.7.4.3 Outlook

Unfortunately, this system does not yet allow to exclude elicitor and/or estradiol diffusion. Nevertheless, it provides the advantage to quickly analyze and exclude *FLS2* expression in shoots by qRT-PCR or Western blot. Furthermore, it poses the possibility to study systemic marker gene induction under different treatment conditions as, for instance, period of induction. It is possible that the transcription levels of the genes analyzed or other genes are

induced in distal organs upon longer flg22 root treatments. Furthermore, extended estradiol treatments might increase the level of *FLS2* expression in roots but in the meantime also increase the risk of elicitor and estradiol diffusion. Thus, although in theory the system provides the advantage to exclude unspecific *FLS2* expression, we nevertheless believe that it is quite time-consuming and risky as two factors might move along the plant (estradiol and flg22).

3.7.5 Other methods reducing the contact between roots and shoots

In general, the different systems tested so far indicated that the PTI marker genes *FRK1* and *PER5* were not induced systemically in shoot tissues when flg22 was perceived specifically by roots. This observation helped us to control the reliability of our systems and we decided to continue working with wild-type plants, which also facilitates our experiments as they require less controls and treatments and instead allow analyzing more time points and genes. Therefore, we aimed at developing a growing method to simultaneously germinate a high number of plants, allowing a direct treatment of roots and in the meantime reduce the risk of elicitor diffusion.

3.7.5.1 Methods

3.7.5.1.1 Method A: Plants grown in tip boxes containing agar-filled tips

Tip boxes were filled with liquid MS medium without sucrose. The tips were filled with medium containing agar and one sterilized seed was placed on top of each tip for germination (Fig. S3.7).

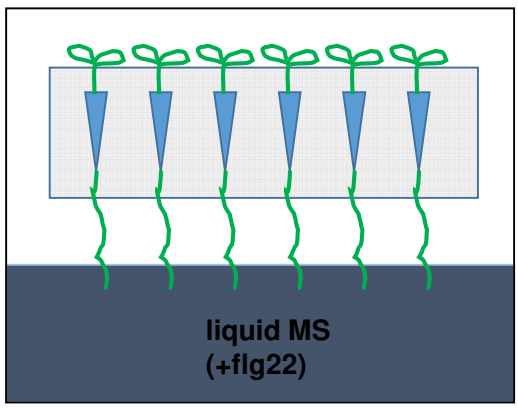

Fig. S3.7 Schematic representation of the growing system with tip boxes. Seeds were germinated in tips containing agar. The bottom of the tip box was filled with liquid MS medium.

3.7.5.1.2 Method B: Plants grown on impermeable foam floating on liquid MS medium

Impermeable polyether foam (Jaece, Identi-Plugs, L800-A) was cut into 1-2 mm discs and autoclaved. One disc was placed in each well of a 24-well plate containing 1 mL MS. A small drop of agar was added onto each disc and two sterilized seeds were placed on top (Fig. S3.8).

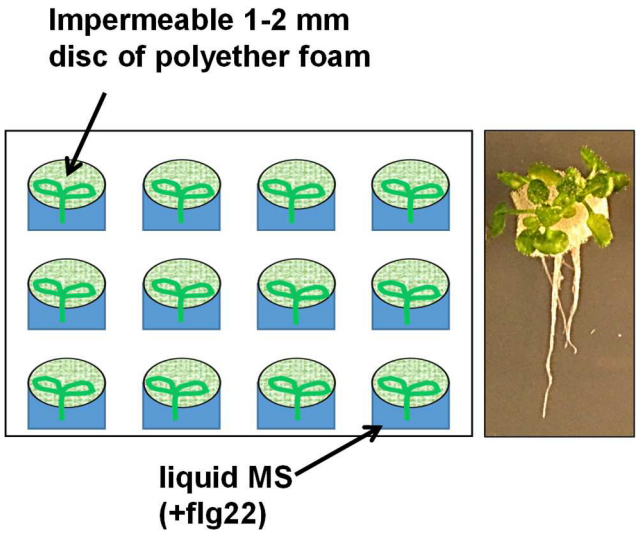

Fig. S3.8 Illustration of the growing system with impermeable foam. Seeds were germinated on an impermeable foam cylinder floating on liquid MS medium in 24-well plates.

3.7.5.1.3 Method C: Plants grown on styrofoam ships containing a hole filled with agar

Styrofoam rings were cut containing a small hole in each ring. The styrofoam was sterilized in ethanol overnight. After drying, one ring per well was added into a 24-well plate and the hole was filled with MS containing agar. Rings were floating on 1 mL liquid MS media and one seedling per ring was added onto the agar-containing hole (Fig. S3.9).

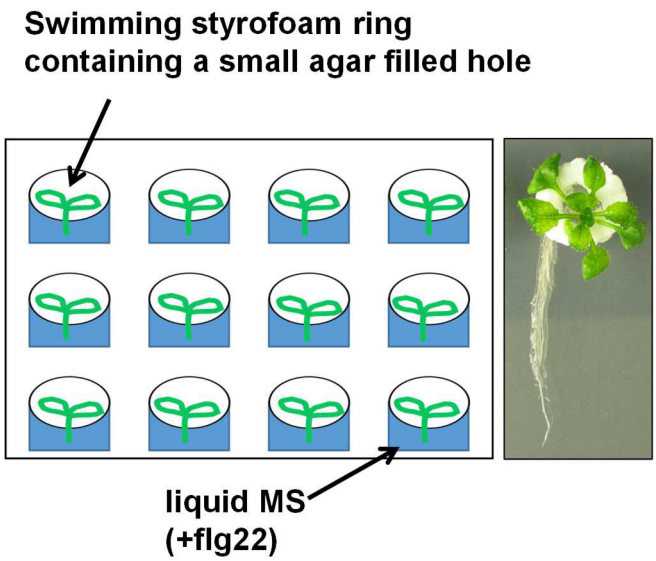

Fig. S3.9 Illustration of the growing system with styrofoam rings. Seeds were germinated on agar-filled holes of a styrofoam ring floating on liquid MS medium in 24-well plates.

3.7.5.1.4 Expected advantages

All systems were meant to reduce the risk of elicitor diffusion between roots and shoots. They could be quickly set up and allow the immediate treatment of roots without handling the plants. The movement of the solution from roots to shoots could be observed by staining of the elicitor solution in which the roots were placed. Furthermore, all systems allowed growing a high number of plants.

3.7.5.1.5 Drawbacks

In all cases, we faced problems of low-rate germination, high contamination with fungi and/or bacteria and leakiness as none of the systems was impermeable to bromophenol blue staining (data not shown). Therefore, the systems were not further employed.

3.7.6 Conclusion

Although several of the methods described above have the potential to be further employed in order to study systemic root-to-shoot signaling or other signaling aspects, we continued our studies using wild-type Arabidopsis plants treated in the optimized system described in Chapter 3.3.3.

3.7.7 Supplemental materials and methods

3.7.7.1 Plant growth conditions

Plant materials used were wild-type Arabidopsis (*Arabidopsis thaliana* L. Heynh cultivar 6 Columbia) and the mutant *fls2* (Zipfel et al., 2004) (SALK_062054C). Unless stated otherwise, seeds were surface-sterilized and placed on MS (0.5 x Murashige and Skoog basal medium (Phyto-technology Laboratories) containing 1% sucrose and 0.8% phytoagar). After stratification for two days at 4°C, they were placed in a plant growth chamber with continuous light for two weeks (24 hours of photoperiod, 20°C).

3.7.7.2 Measurement of reactive oxygen species

For ROS assays, leaf discs of two week-old plants grown in liquid MS were placed into each well of a Lia White 96-well plate (Greiner Bio-One) in 0.1 mL water and kept in the dark overnight. For elicitation and ROS detection, horseradish peroxidase (Sigma-Aldrich) and luminol L012 (Sigma-Aldrich) was added to a final concentration of 10 µg/mL and 100 µM, respectively. Luminescence was measured directly after addition of elicitor peptides in a MicroLumat LB96P plate reader (Berthold Technologies) for 30 minutes.

3.7.7.3 Peptides and solutions

Peptides of flg22 (QRLSTGSRINSAKDDAAGLQIA), *At*Pep1 (ATKVKAKQRG KEKVSSGRPGQHN) and elf18 (ac-SKEKFERTKPHVNVGTIG) obtained from EZBiolabs were dissolved to a 1 mM final concentration in water. β-estradiol (Sigma, E2758) was prepared as a stock solution at a final concentration of 10 mM in ethanol (100%).

3.7.7.4 Determination of gene expression

Root or shoot tissue of two week-old seedlings was frozen and ground in liquid nitrogen. RNA from 50 mg tissue was extracted using the NucleoSpin RNA plant extraction kit (Macherey-Nagel) and treated with recombinant DNase according to the manufacturer's recommendations. Per PCR reaction, complementary DNA was synthesized from 10 ng of RNA with oligo (dT) primers using the Avian Myeloblastosis Virus reverse transcriptase according to the manufacturer's instructions (Promega). Quantitative real-time reverse transcription- PCR (qRT-PCR) was performed in a 96-well format using a LightCycler®480

System (Roche Applied Science). Normalized expression to the reference gene *UBQ10* was calculated using the qGene protocol (Muller et al., 2002). All primer sequences and accession numbers of genes analyzed by qRT-PCR are listed in Table S3.1.

3.7.7.5 Cloning of *flg22_sec*

The published secretion signal of an Arabidopsis vacuolar basic chitinase (Haseloff et al., 1997) was amplified from genomic DNA (BasChiSec). A Sma1 restriction site was integrated at the C-terminal end (Table S3.1). The purified sequence was introduced into the pDon207 vector by BP cloning according to standard methods (GATEWAY® Invitrogen). The amino acid sequence of *Pto* DC3000 flg22 was reverse translated (http://www.biophp.-org/minitools/-protein_to_dna/demo.php) and edited with the codon usage of Arabidopsis (http://www.kazusa.or.jp/codon/; http://eu.idtdna.com/CodonOpt). The cloning of *flg22_sec* and *flg22-Δ2_sec* was performed with overlapping primer sequences (Table S3.1) containing a stop codon and the overlap was performed at a melting temperature of 66°C. The purified sequence was introduced into the by Sma1 digested pDon207 vector, containing the basic chitinase signal sequence, by blunt end cloning. Then, the fused sequence was introduced by LR cloning into the pMDC7 plasmid containing the chimeric transactivator XVE (Zuo et al., 2000). The plasmid was kindly provided by Nam-Hai Chua (Rockefeller University, NY, USA). XVE is a fusion of the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E; ER). The transactivating activity of the chimeric XVE factor is strictly regulated by estrogens allowing a rapid induction of gene expression in response to estradiol treatment (Zuo et al., 2000).

Table S3.1 Primer sequences for qRT-PCR and cloning of *flg22_sec*/flg22-*Δ2_sec*.

Gene name	ID	forward primer	reverse primer
qRT-PCR			
UBQ10	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAG
FRK1	AT2G19190	TGCAGCGCAAGGACTAGAG	ATCTTCGCTTGGAGCTTCTC
PER5	AT1G14550	TCTCAATGCTTCTTGTTCCG	CTAGATCCAATGCTGCCAGA
PR1	AT2G14610	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG
PR5	AT1G75040	GGAACTCTCGCCGGTCAAG	TTTGAATTGACTCCAGGTGCTTC
PDF1.2	AT5G44420	TGTTCTCTTTGCTGCTTTCGACGC	TGTGTGCTGGGAAGACATAGTTGC
LOX2	AT3G45140	CTATGGAATCTTCGTAAGACTCATG	CGGCTGAACTTAGCTCTAATGCATA
LOX3	AT1G17420	CGGATAGAGAAAGAGATTGAGAAAAGGAAC	AGGTACACCTCTACACGTAACACCAGG
EDS5	AT4G39030	CTCTTGGACCGGGAACAGTA	GTGCGCTTCTTTCTTGTCC
CYP71A12	AT2G30750	GATTATCACCTCGGTTCCT	CCACTAATACTTCCCAGATTA
MYB51	AT1G18570	ACAAATGGTCTGCTATAGCT	CTTGTGTGTAACTGGATCAA
NHL10	AT2G35980	TTCCTGTCCGTAACCCAAAC	CCCTCGTAGTAGGCATGAGC
FLS2	AT5G46330	ACTCTCCTCCAGGGGCTAAGGAT	AGCTAACAGCTCTCCAGGGATGG
flg22_sec	-	TGCCTCCACAAAAAGAAAACCA	TAGACAGTCTTTGGGGCTCG
Cloning			
BasChiSec	-	GGGGACAAGTTTGTACAAAAAAGCAGGCTTG ATGCCTCCACAAAAAGAAAACATAG	GGGGACCACTTTGTACAAGAAAGCTGGGTCCC CGGGCTCGGCCGAGGATAATGATAG
flg22	-	CAAAGACTGTCTACTGGATCTAGAATTAACTCT GCTAAGGATGATGCT	CTAAGCAATTTGCAGTCCAGCAGCATCATCCTT AGCAGA
flg22-Δ2	-	GATCTAGAATTAACTCTGCTAAGGATGATGCT	AAGTTTAAACGCCACGTGTTGCAGTCCAGCAG CATCATC

4 DOUBLE-STRANDED RNAS ARE PERCEIVED AS PATHOGEN-ASSOCIATED MOLECULAR PATTERNS AND INDUCE ANTIVIRAL IMMUNE RESPONSES IN PLANTS

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Manuscript in preparation

4.1 Abstract

Plants possess a sophisticated layered immune system for defense against invading pathogens. The first layer of active defense in plants is pattern-triggered immunity (PTI) that relies on the perception of conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs). Although believed to be specific for microbes, it was recognized recently that PTI also restricts virus infection in plants. However, the nature of the viral or infection-induced PTI elicitors and the underlying signaling pathways are still unknown. Here we show that *in-vitro*-generated double-stranded (ds)RNA and its synthetic analogue, polyinosinic-polycytidylic acid (poly(I:C)) induce typical PTI responses such as the activation of the MITOGEN-ACTIVATED PROTEIN KINASES (MPKs) 6 and 3, ethylene synthesis, defense gene expression and seedling growth inhibition in *Arabidopsis thaliana,* suggesting that dsRNA represents a *bona fide* PAMP *in planta.* Consistently, treatment with dsRNA induced antiviral resistance. During virus infection dsRNAs occur as viral replication intermediates and are known to trigger antiviral RNA silencing through interaction with the dsRNA-processing dicer-like proteins DCL*4* and DCL2. *dcl2,4* mutants exhibited constitutive expression of PTI-related genes and still responded to dsRNA and its analogue by MAPK activation and ethylene synthesis, indicating that DCLs play no role in the perception of the dsRNA elicitor but rather act as negative regulators of PTI. Interestingly, poly(I:C) treatment significantly reduced *DCL1* and *DCL4* expression. Hence, dsRNA-induced PTI and RNA silencing represent two defense layers that undergo mutual regulation and may act in a concerted balance during viral attack.

4.2 Significance statement

The first layer of the plant immune system is based on receptor-mediated recognition of conserved microbial patterns to ward off microbial pathogenic invaders. However, whether plants also recognize virus-associated molecular patterns to induce antiviral immunity remains largely unexplored. In animals, viral double-stranded (ds)RNAs associated with viral replication induce signaling cascades to establish an antiviral state. Here, we tested whether dsRNAs also induce signaling and immune responses in plants. We found that elicitation of Arabidopsis with dsRNA induces immune signaling and dsRNA treatment protects plants against virus infection. Furthermore, this immune response is different from antiviral silencing and cross-talk may exist between dsRNA-mediated antiviral immunity and antiviral silencing. Hence, our data identify dsRNA as elicitor inducing antiviral pattern-triggered immunity in plants.

4.3 Introduction

Antiviral defense in plants is known to involve RNA silencing (Ding and Voinnet, 2007; Incarbone and Dunoyer, 2013; Pumplin and Voinnet, 2013; Szittya and Burgyán, 2013). During antiviral silencing, viral double-stranded (ds)RNA replication intermediates are specifically recognized by the plant dicer-like proteins and cleaved into short-interfering (si)RNA duplexes. Viral siRNAs are then loaded into Argonaute-containing RNA-induced silencing complexes (RISCs) and guide the complex to the complementary viral messenger RNA or viral genomic RNA for cleavage. However, recently, it was shown that plants recognize viruses also by pattern-triggered immunity (PTI) (Yang et al., 2010b; Kørner et al., 2013). PTI is a well-known antimicrobial defense response triggered by specific recognition of conserved microbe- or pathogen associated molecular patterns (MAMPs or PAMPs) such as bacterial flagellin, lipopolysaccharides, or fungal chitin by pattern recognition receptors (PRRs) at the plasma membrane. Upon stimulation of a PRR, a downstream signaling cascade is activated, involving changes in ion fluxes across the plasma membrane, a burst of reactive oxygen species, activation of the MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs), specifically MPK6, MPK3 and MPK4, and activation of hormone signaling (Boller and Felix, 2009; Pieterse et al., 2012; Frei dit Frey et al., 2014). These signaling events ultimately lead to cell wall strengthening, defense gene expression, and the production of antimicrobial compounds. To counteract these defense responses, microbial pathogens have evolved

effectors, which are released into infected cells and inhibit the signaling cascade at different steps (Tsuda and Katagiri, 2010; Zhang and Zhou, 2010; Weiberg et al., 2013; Doehlemann et al., 2014; Macho and Zipfel, 2015), thus leading to effector-triggered susceptibility (ETS). As part of the continuous evolutionary arms race, some plants developed resistance proteins capable of recognizing the effectors and reestablishing resistance. This type of immune response is called effector-triggered immunity (ETI) (Cui et al., 2015).

A role of PTI in antiviral defense was recently demonstrated in Arabidopsis by showing that mutants in the PRR coreceptor kinase BAK1/SERK3 (for BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1, also named SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 3) exhibit increased susceptibility to different RNA viruses and that Arabidopsis plants respond to crude extracts from virus-infected plants, but not to purified virions, in a BAK1-dependent manner (Kørner et al., 2013). Consistently, also the BAK1-related SERK-family members BKK1/SERK4 and NIK1 (NUCLEAR SHUTTLE PROTEIN (NSP)-INTERACTING KINASE) have been shown to be involved in virus resistance (Fontes et al., 2004; Yang et al., 2010b; Zorzatto et al., 2015). Together, these data implicate PTI-associated kinases in antiviral defense and suggest that viral or virus-induced elicitors are present in crude plant extract of virus-infected cells. However, up to now, the molecular nature of these elicitors has remained elusive.

Here, we explored whether dsRNAs are recognized as genuine PAMPs in plants. The production of dsRNA is a hallmark of viral replication and represents a conserved molecular pattern associated with virus infection. In vertebrates, specific, membrane-bound PRRs of the Toll-like receptor family (TLR3, TLR7 and TLR9) and intracellular retinoic acid inducible gene-1 (RIG-1)-like receptors perceive viral dsRNA, ssRNA and DNA (Arpaia and Barton, 2011; Berke et al., 2013; Brencicova and Diebold, 2013; Peisley and Hur, 2013). Perception leads to downstream signaling, global suppression of protein synthesis and establishment of the antiviral state. We show that dsRNA and its synthetic analogue poly(I:C) induce typical PTI responses in plants, including induction of MPK6 and MPK3, ethylene (ET) synthesis and defense gene expression. Moreover, poly(I:C) treatment induced seedling growth inhibition, a phenotype associated with activation of plant immunity. Consistent with the hypothesis that dsRNAs are perceived as viral PAMPs in plants, dsRNA treatment of plants protected against virus infection. We also show that the two main antiviral dicer-like proteins DCL2 and DCL4 (Deleris et al., 2006; Ziebell and Carr, 2009; Garcia-Ruiz et al., 2010) are likely not the dsRNA receptors conferring PTI. However, defense-related gene expression as well as expression of

the salicylic acid (SA)-dependent systemic acquired resistance (SAR) marker *PR1* was significantly induced in *dcl2,4* mutants, and treatment with the dsRNA analogue poly(I:C) reduced *DCL* expression levels in wild-type. Our data identify dsRNA as a molecular pattern recognized in plants for the establishment of PTI and indicate negative cross-talk between RNA silencing and immunity.

4.4 Results

4.4.1 dsRNAs induce PTI resonses in plants

To investigate whether dsRNA represents a genuine PAMP in plants we applied *in-vitro*-synthesized 746 bp-long dsRNA derived from a DNA encoding GFP, or the synthetic dsRNA analogue and TLR3 agonist poly(I:C) to Arabidopsis leaf discs and analyzed MAPK activation on immunoblots probed with antibodies against phosphorylated MAPKs. Application of dsRNA and poly(I:C) resulted in MPK6 and MPK3 activation, as did application of the known bacterial elicitor flg22, but application of siRNA did not (Fig. 4.1a). The specific signal for phosphorylated MAPKs remained when the poly(I:C) elicitor was treated with proteinase K, but it disappeared when the poly(I:C) elicitor was degraded by RNAse (Fig. 4.1b), indicating that the elicitor was indeed of RNA nature. Activity of proteinase K and RNAse were confirmed by digestion of flg22 with proteinase K and analysis of MAPK activation by immunoblot, and analysis of RNAse-digested poly(I:C) on agarose gels, respectively (Fig. S4.1). To confirm a role of MPK6 and MPK3 in dsRNA-mediated signaling, we tested MAPK activation in *mpk6* and *mpk3* mutant plants elicited with poly(I:C). Absence of the MPK3-specific signal in *mpk3* mutants and of the MPK6-specific signal in *mpk6* mutants upon elicitation confirmed that dsRNA-signaling indeed involved activation of MPK6 and MPK3 in Arabidopsis shoots and roots (Fig. S4.2). To further analyze the dsRNA-mediated signaling cascade, we investigated ET production, another well-known marker of the PTI response (Boller and Felix, 2009). Poly(I:C) treatment led to a significant increase of ET synthesis as did the positive control flg22 (Fig. 4.1c), thus suggesting that dsRNA acts as a *bona fide* elicitor inducing downstream PTI responses in plants.

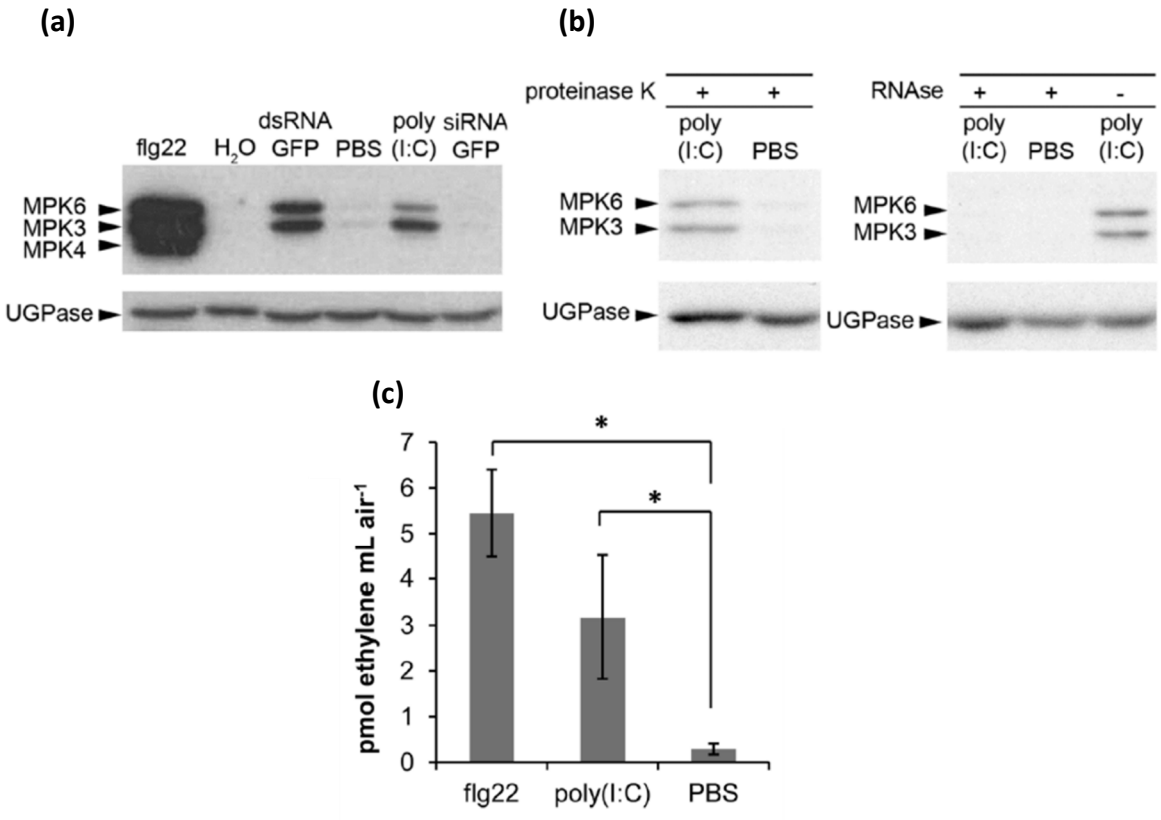

Fig. 4.1 Detection of MAPK phosphorylation and ethylene production upon dsRNA treatment of Arabidopsis leaves. (**a**) Immunoblot to detect MAPK phosphorylation in Arabidopsis leaf discs treated with flg22, GFP-dsRNA, poly(I:C), GFP siRNA, PBS, or water. The blot was probed with an antibody against phosphorylated MAPKs. (**b**) Immunoblot to detect MAPK phosphorylation in Arabidopsis leaf discs treated with proteinase K-treated poly(I:C) or PBS, or with RNAse A/T1-digested poly(I:C), RNAse A/T1-digested PBS, or non-digested poly-(I:C). Blots were probed with an antibody against phosphorylated MAPKs. (**a** and **b**) As equal loading control, immunoblots were re-probed with anti-UGPase antibody. (**c**) Ethylene production by Arabidopsis leaf strips treated with flg22, poly(I:C), PBS or water. Data represent mean values of n=6 biological replicates ± SE. Significant differences compared to control are marked by asterisks ($P < 0.05$, Student's t-test).

We also tested whether poly(I:C) treatment induced seedling growth inhibition, a characteristic late PTI response, and observed significantly reduced root growth in seedlings grown in poly(I:C)-containing medium compared to control plants (Fig. 4.2).

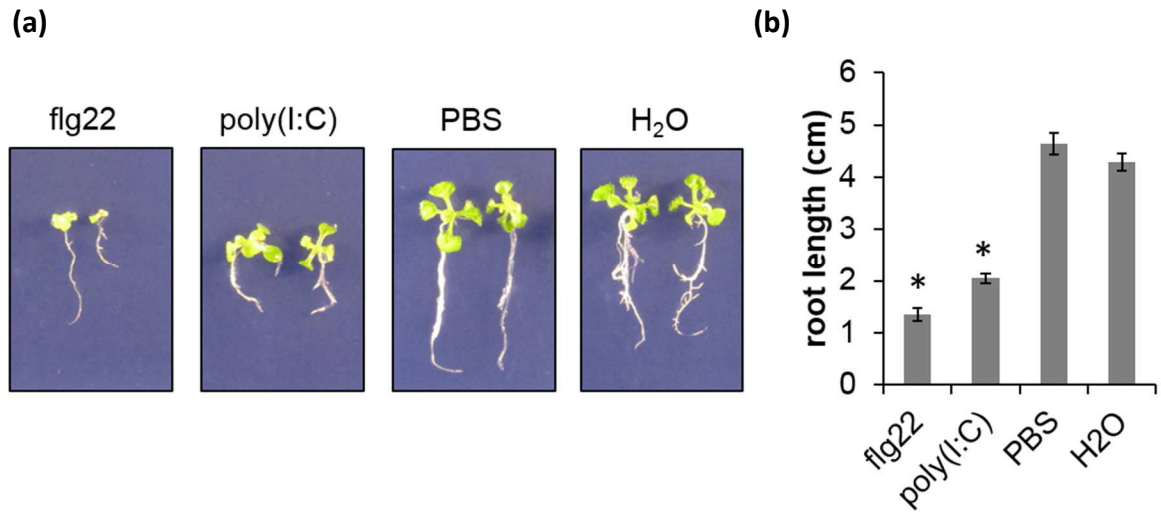

Fig. 4.2 Seedling growth inhibition upon poly(I:C) treatment. (**a**) Photographs of representative Arabidopsis seedlings after treatment with flg22, poly(I:C), PBS, or water for eight days. (**b**) Root length of Arabidopsis seedlings treated with flg22, poly(I:C), PBS, or water for eight days. Data represent mean ± SE of n = 8 individual seedlings. Asterisks indicate significant differences ($P < 0.05$, Student's t-test) to water and PBS controls, respectively.

Next, we investigated whether poly(I:C) treatment resulted in the induction of PTI-responsive gene expression. We selected PTI-responsive candidate genes from publicly available gene expression data (Genevestigator, Hruz et al., 2008; materials and methods 4.6.8). Among the genes induced upon flg22 and poly(I:C) treatment were classical PTI-response genes such as *PROPEP3* and *ANTIFUNGAL PROTEIN*; however, we also found enhanced expression levels of the ROS-related genes *RBOHD* and *ANIONIC PEROXIDASE*, of the SA-dependent *EDS5*, and of the lipid- and jasmonic acid-signaling genes *PHOSPHOLIPASE A 2A* and *LIPOXYGENASE 3* (Table 4.1).

Table 4.1 Expression of pattern-triggerd immunity (PTI)-related genes upon poly(I:C) and flg22 treatment.

gene name†	poly(I:C) fold change vs. PBS*	flg22 fold change vs. PBS*
PROPEP3	**6.99 ± 0.62**	**230.94 ± 0.41**
ANTIFUNGAL PROTEIN	**29.08 ± 0.45**	**26.20 ± 0.30**
RBOHD	**1.75 ± 0.29**	**1.98 ± 0.22**
POD	**3.62 ± 0.39**	**18.25 ± 0.37**
EDS5	**15.92 ± 0.67**	**3.20 ± 0.30**
PLPA2	**2.62 ± 0.60**	**14.59 ± 0.41**
LOX3	**9.09 ± 0.64**	**3.70 ± 0.62**
BAK1	**1.39 ± 0.12**	**5.74 ± 0.26**
SOBIR1	**1.84 ± 0.12**	**1.45 ± 0.20**
BRI1-LIKE KINASE 3	**3.30 ± 0.36**	**2.71 ± 0.40**
AtCRK4	**2.19 ± 0.37**	**4.13 ± 0.16**
SERK1	1.61 ± 0.43	**2.60 ± 0.35**
SERK2	**0.56 ± 0.28**	1.04 ± 0.21
SERK4	**1.57 ± 0.35**	**3.80 ± 0.32**
SERK5	**0.68 ± 0.23**	1.28 ± 0.25
PR1	0.78 ± 1.29	1.40 ± 1.31
PR5	2.45 ± 0.81	0.89 ± 0.53
PDF1.2	1.30 ± 0.62	0.81 ± 0.58

*Averages of four biological replicates ± coefficient of variation are shown. Significant ($P < 0.05$ Student's t-test) gene expression changes compared to PBS-treated controls are marked in bold. †*PROPEP3, ELICITOR PEPTIDE 3 PRECURSOR; RBOHD, RESPIRATORY BURST OXIDASE HOMOLOG D; POD, ANIONIC PEROXIDASE; EDS5, ENHANCED DISEASE RESISTANCE 5; PLPA2, PHOSPHOLIPASE A 2A; LOX3, LIPOXYGENASE 3; BAK1, BRI1-ASSOCIATED RECEPTOR KINASE 1; SOBIR1, SUPPRESSOR OF BIR1-1/EVERSHED; AtCRK4, CYSTEINE-RICH RECEPTOR LIKE KINASE 4; SERK, SOMATIC EMBRYOGENESIS LIKE KINASE; PR, PATHOGENESIS-RELATED GENE; PDF1.2, PLANT DEFENSIN 1.2*.

We also investigated the expression of different signaling-related kinases: the regulators of PTI signaling *SOBIR1*, *BAK1*/*SERK3* and the other BAK1-related SERK family members, *BRI1-LIKE KINASE 3*, and *AtCRK4*. Except for *SERK1*, which was only induced upon flg22 treatment, and of *SERK2* and *SERK5*, which exhibited significantly reduced expression upon poly(I:C) treatment and no significantly altered expression upon flg22 treatment, the expression of all kinases was induced upon elicitor treatment. Interestingly, and consistent with our analysis of publicly available expression data (Genevestigator, Hruz et al., 2008), the typical pathogenesis-related genes *PR1* and *PR5*, associated with SAR, as well as the ET- and jasmonate-responsive *PDF1.2* gene were not induced upon treatment with flg22 or poly(I:C) at the tested time point.

Our recently published data showed that *bak1* mutants were more susceptible to different RNA viruses (Kørner et al., 2013), hence implicating PTI in antiviral defense. To test whether BAK1 would be involved in the perception of poly(I:C), we measured MAPK activation and ET synthesis in *bak1* mutants. *bak1* mutants still induced MAPK phosphorylation and ET synthesis upon dsRNA treatment (Fig. S4.3), indicating that suppression of *BAK1* expression alone is not sufficient to impair dsRNA signaling.

Taken together, these results suggest that dsRNA and its synthetic analogue poly(I:C) are recognized as *bona fide* PAMPs in Arabidopsis and induce typical PTI responses including the activation of MPK6 and MPK3, ET synthesis, PTI-related response gene expression and seedling growth inhibition.

4.4.2 Poly(I:C) protects plants against virus infection

As the production of long dsRNA is typically associated with viral replication (den Boon et al., 2010; Romero-Brey and Bartenschlager, 2014) the recognition of dsRNA replication intermediates via PTI might represent an antiviral defense mechanism operating against viruses in plants. Hence, we tested whether the application of poly(I:C) protected plants against the Arabidopsis-infecting tobamovirus *Oilseed rape mosaic virus* (ORMV). Application of poly(I:C) together with the virus resulted in significantly reduced accumulation of virus in poly(I:C)-treated leaves compared to flg22-treated or PBS-control-treated leaves at four days post infection (dpi) (Fig. 4.3a). Consistently, when the remainder of the plants was allowed to continue growing after sampling of the treated and virus-infected leaves, PBS and flg22-treated plants developed disease symptoms, while poly(I:C)-treated plants did not (Fig. 4.3b). Importantly, we did not obtain antiviral protection when the single-stranded molecule poly-inosinic acid (poly(I)) was used as control (Fig. 4.3c). Together, these results suggest that the PTI responses induced upon poly(I:C) treatment confer antiviral immunity.

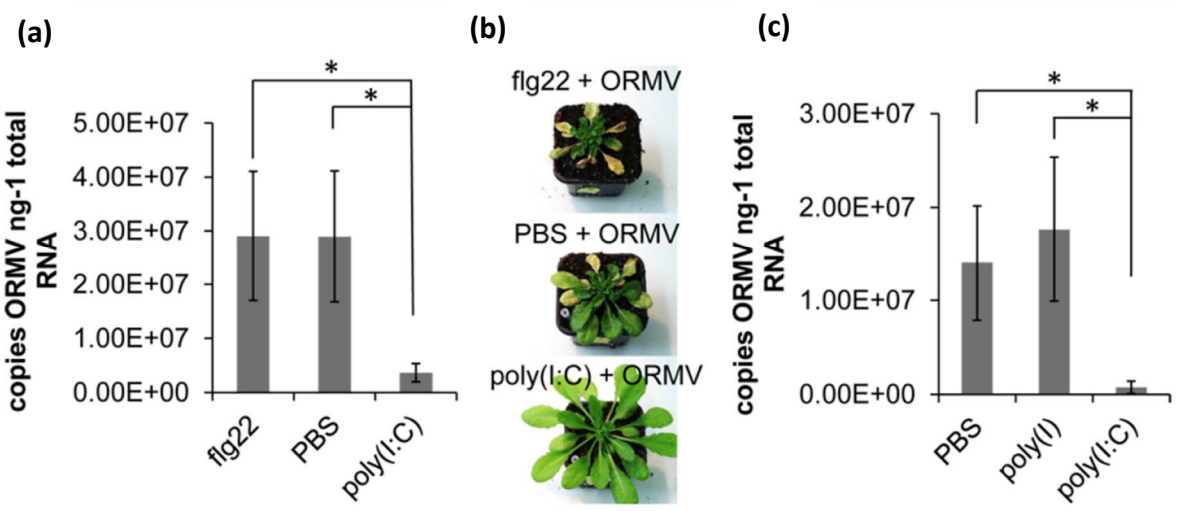

Fig. 4.3 Plant protection by poly(I:C). (**a**) Quantification of viral genomic RNA in Arabidopsis leaves treated with flg22, poly(I:C), or PBS and infected with ORMV for four days. Data represent averages of eight biological replicates of individual leaves ± SE. Asterisks mark significant differences ($P < 0.05$, Student's t-test). (**b**) Representative photographs of disease symptoms developed by Arabidopsis plants infected with ORMV after treatment with flg22, PBS, or poly(I:C) 23 dpi. The infected leaves were removed at four dpi and the remainder of the plants allowed to continue growing for an additional 19 days. (**c**) Quantification of viral genomic RNA in Arabidopsis leaves treated with PBS, poly(I), or poly(I:C) and infected with ORMV for four days. Data represent averages of eight biological replicates ± SE. Asterisks mark significant differences ($P < 0.05$, Student's t-test).

4.4.3 Cross-talk between antiviral silencing and PTI

As dsRNA produced during viral replication is typically recognized by the plant RNA silencing machinery to induce antiviral silencing, we investigated putative cross-talk between antiviral silencing and antiviral PTI. We tested whether mutants deficient in the main antiviral DICER-LIKE PROTEINS, DCL2 and DCL4 (Deleris et al., 2006; Ziebell and Carr, 2009; Garcia-Ruiz et al., 2010) were impaired in dsRNA-mediated PTI. We found that MAPKs were still activated in *dcl2,4* mutants upon dsRNA and poly(I:C) treatment (Fig. 4.4a) and *dcl2,4* mutants still showed significantly enhanced ET production upon elicitation by poly(I:C) (Fig. 4.4b). These findings suggested that DCL2 or DCL4 are not directly involved in dsRNA perception for PTI signaling. By contrast, *dcl2,4* mutants exhibited induction of immunity-related gene expression compared to Col-0. In particular, *dcl2,4* mutants showed significantly increased expression of the SA-dependent defense-related gene *PR1*, the kinases *SOBIR1* and *BRI1-LIKE KINASE 3*, as well as *PHOSPHOLIPASE A 2A* and the plasma membrane NADPH oxidase *RBOHD* under control conditions (Fig. 4.4c). These observations indicate that DCL2 and DCL4, the main antiviral dsRNA-processing enzymes, might represent negative regulators of immunity. This is consistent with our finding that DCL2 or DCL4 themselves

are likely not the dsRNA receptor during PTI. In agreement with this, we found that *DCL1* and *DCL4* expression was significantly reduced in wild-type plants treated with the dsRNA elicitor poly(I:C) (Fig. 4.4d).

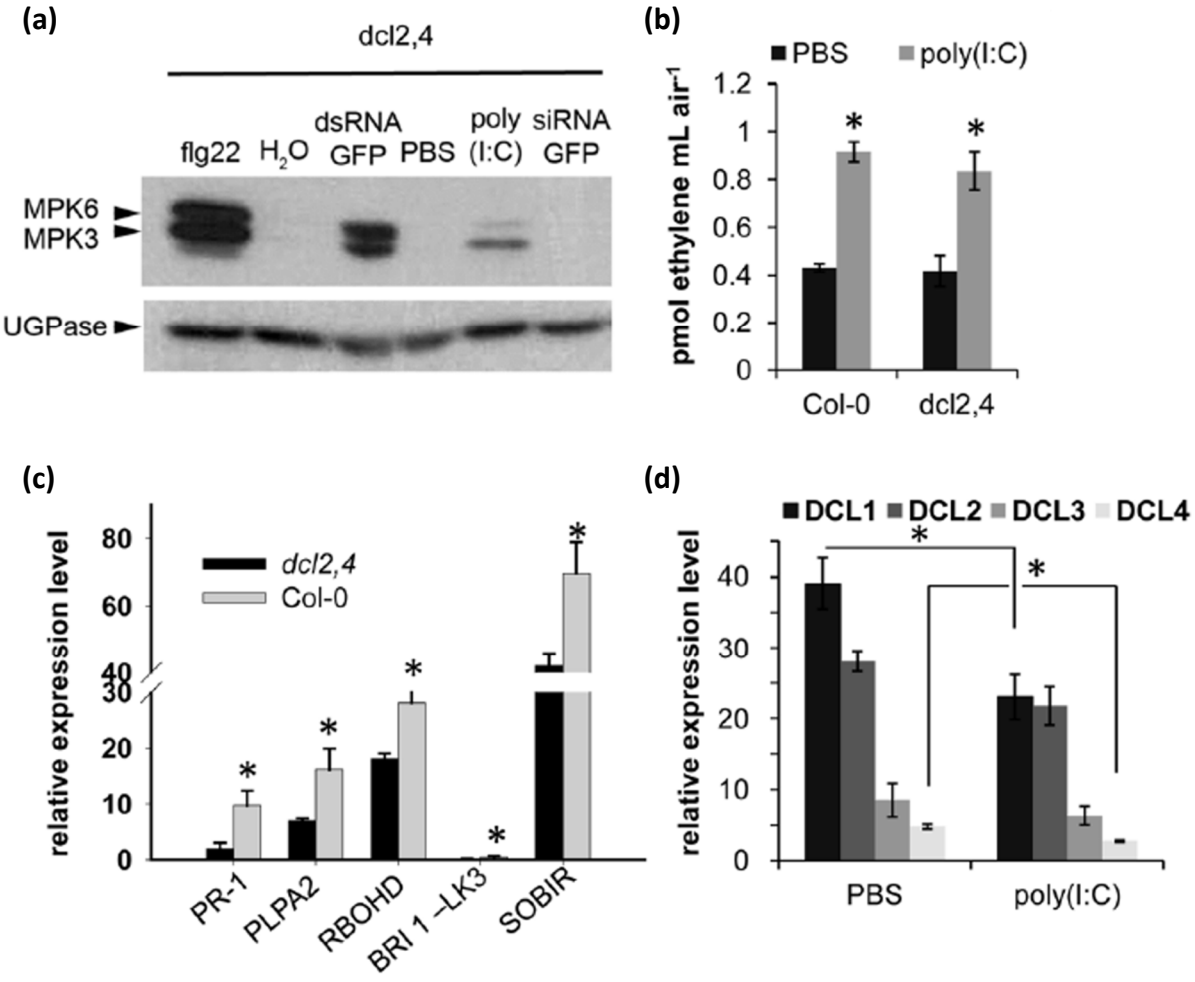

Fig. 4.4 Defense-responses in *dcl2,4* mutants. (**a**) Immunoblot to detect MAPK phosphorylation in Arabidopsis *dcl2,4* double mutant leaf discs treated with flg22, GFP-dsRNA, poly(I:C), GFP siRNA, PBS, or water. The blot was probed with an antibody against phosphorylated MAPKs. As equal loading control, the same immunoblot was re-probed with anti-UGPase antibodies. (**b**) Ethylene production by Arabidopsis Col-0 or *dcl2,4* leaf strips treated with poly(I:C) or PBS. Data represent mean values of n=6 biological replicates ± SE. Significant differences compared to control are marked by asterisks ($P < 0.05$, Student's t-test). (**c**) Quantification of PTI-related gene expression in leaf discs of Col-0 or *dcl2,4* mutant Arabidopsis plants treated with PBS. Data are mean values of n=4 biological replicates ± SE. Relative expression levels normalized to reference gene expression are shown. Significant ($P < 0.05$ Student's t-test) gene expression changes compared to WT are marked by asterisks. *PR1, PATHOGENESIS-RELATED GENE 1; PLPA2, PHOSPHOLIPASE A 2A; RBOHD, RESPIRATORY BURST OXIDASE HOMOLOG D; BRI1-LK3, BRI1-LIKE KINASE 3; SOBIR1, SUPPRESSOR OF BIR1-1/EVERSHED.* (**d**) Quantification of *DCL* expression in leaf discs of Col-0 WT plants treated with poly(I:C) or PBS. Data are mean values of n = 4 biological replicates ± SE. Relative expression levels normalized to reference gene expression are shown. Significant ($P < 0.05$ Student's t-test) gene expression changes are marked by asterisks.

4.5 Discussion

In animal systems, virus-derived dsRNA is sensed by membrane-bound TLR3 or intracellular RIG-1-like dsRNA receptors, which leads to the induction of the antiviral state (Arpaia and Barton, 2011; Berke et al., 2013; Brencicova and Diebold, 2013); however, whether these conserved molecules associated with virus infection are also recognized by PRRs in plants and induce PTI remained elusive. Here, we show that dsRNA can elicit typical PTI responses in plants and that dsRNA treatment protects against virus infection. Our recent data identified the PRR-coreceptor kinase BAK1 as a positive regulator of antiviral immunity in plants and provided evidence that virus- or infection-derived elicitors are recognized in plants and confer antiviral PTI (Kørner et al., 2013). Although our current experiments did not reveal a non-redundant function of BAK1 in dsRNA-mediated PTI, the involvement of BAK1 in antiviral PTI suggested the involvement of membrane-localized receptors in the recognition of virus-derived, or host-derived, infection-induced elicitors. Future experiments will reveal whether different isoforms of the SERK family -to which BAK1 belongs- function redundantly in dsRNA-mediated signaling, or whether BAK1 is rather involved in the recognition of a different, yet unidentified viral PAMP.

The membrane-localized dsRNA-sensing animal TLR3 receptor localizes to the plasma membrane and endosomal compartments (de Bouteiller et al., 2005; Itoh et al., 2008; Vercammen et al., 2008b; Qi et al., 2012). It binds extracellular dsRNAs from dying or virus-infected cells in the environment, and recognizes intracellularly produced dsRNA during virus infection via infection-induced membrane fusion during autophagy (Brencicova and Diebold, 2013). TLR3 signaling is associated with endosomal compartments and allows the cells to distinguish between self and non-self (Matsumoto et al., 2014). Interestingly, virus replication in plants usually occurs on cellular membranes and involves extensive membrane rearrangements (Laliberté and Sanfaçon, 2010), the induction of ER-stress (Ye et al., 2011; Niehl et al., 2012), and the degradation of viral suppressors of RNA silencing (VSRs) by autophagy (Derrien et al., 2012; Nakahara et al., 2012). In analogy to the animal system, these membrane-associated processes could lead to recognition of dsRNA produced intracellularly during plant virus replication by membrane-bound PRRs in membrane fusion events. That plant receptor proteins can signal from endosomes during immunity and development has been shown (Geldner et al., 2007; Bar and Avni, 2009b; Sharfman et al., 2011; Irani et al., 2012). However, as viruses are obligate intracellular pathogens, dsRNA may also be sensed by soluble, intracellular receptors in plants. Interestingly, similarity searches reveal that plant

dicers are related to the intracellular vertebrate dsRNA helicases of the RIG-1 family. Hence, dicer proteins could be candidates for receptors playing a role in recognizing virus-derived dsRNA during antiviral PTI as well as during RNA silencing. The existence of two dsRNA-triggered antiviral defense pathways, antiviral silencing and antiviral PTI, may be supported by the recent identification of an RNA-interference-based antiviral mechanism in mammalian cells (Li et al., 2013; Maillard et al., 2013). However, the fact that dsRNA still triggers PTI responses in *dcl2,4* double mutants does not support the hypothesis that dicers are directly involved in dsRNA sensing during dsRNA-mediated PTI. Nevertheless, the finding that *dcl2,4* double mutants exhibited induced expression of immunity-related genes and that poly(I:C) treatment suppressed the expression of *DCLs* suggests the existence of negative cross-talk between RNA silencing and plant immunity. However, as PTI induces changes in response gene expression similar to those observed during ETI, it is difficult to assign the negative regulation of immunity by dicers specifically to one of these processes. Interestingly, in agreement with our observations, recent data identified the RNA-dependent RNA polymerase RDR6 as negative regulator of PTI and ETI (Boccara et al., 2014). Moreover, RNA silencing has been shown to act as key negative regulator of resistance-gene expression (Yi and Richards, 2007; Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Boccara et al., 2014). Interference of plant pathogens with RNA silencing is a conserved strategy employed by different pathogens to enhance virulence, as demonstrated by the action of viral silencing suppressors (Incarbone and Dunoyer, 2013; Pumplin and Voinnet, 2013), bacterial silencing suppressing effectors (Navarro et al., 2008) and fungal effector siRNAs selectively silencing specific host immunity genes (Weiberg et al., 2013). In this sense, the activation of PTI and ETI may represent a failsafe mechanism of the host to combat pathogen infection once RNA silencing gets disturbed.

In conclusion, the finding that dsRNA is recognized as an elicitor inducing typical PTI responses in plants defines a novel antiviral defense response that operates in addition to RNA silencing in plants. It remains unknown how dsRNA is perceived by the plant. Hence, as a next step, it will be important to identify the plant dsRNA receptor. This will allow to further illuminate the mechanism of dsRNA sensing in the elicitation of antiviral PTI and to investigate the role and regulation of antiviral PTI in relation to RNA silencing. Dissecting the PTI-based and RNA silencing-based antiviral immune system in plants and the differential efficiency of these immune systems in specific plant tissues or during specific developmental stages will lead to a better understanding of phenomena like limited host range of viruses,

phloem-restriction of several plant viruses, exclusion of plant viruses from meristems, or recovery from virus disease symptoms. Moreover, dsRNA-mediated antiviral PTI may be economically exploited to develop new crop protection strategies. Due to the absence of protection strategies except for resistant cultivars and preventive growing conditions, plant virus disease causes extensive crop losses worldwide. In times of climate change and increasing population pressure the development of crop plants primed for virus defense becomes increasingly important.

4.6 Materials and methods

4.6.1 Plant material

Arabidopsis Col-0 wild-type, *bak1-4* (Kemmerling et al., 2007), *bak1-5* (Schwessinger et al., 2011), and *dcl2,4* (Deleris et al., 2006) mutants were grown from seeds on soil at 21°C with 12/12 hours light/dark cycles for five to eight weeks. For seedling growth inhibition assays, Arabidopsis Col-0 seeds were surface sterilized and germinated on half-strength 0.5 x MS medium containing 1% sucrose and 0.8% agar for five days in a growth chamber at 20°C under continuous light. Then, seedlings were transferred to 24-well plates containing 1 mL of liquid half-strength MS-medium with 1% sucrose and kept at 20°C and continuous light for an additional eight days.

4.6.2 dsRNA synthesis and purification

746 bp-long dsRNAs derived from GFP DNA were produced *in vitro* using the Replicator RNAi kit (Finnzymes) following the manufacturer's instructions. The GFP4 sequence was amplified by PCR from plasmids using primers against the 5' and 3'-end of the GFP sequence and flanked by the T7 promoter and Phi6 replicase promoter sequences, respectively using the Replicator RNAi kit (Finnzymes). High-fidelity PCR was performed with an annealing temperature of 58°C for five cycles and subsequently with an annealing temperature of 62°C for 25 cycles. PCR products were purified by centrifugation through DNA-purification columns (Macherey-Nagel), and 500 ng of purified PCR product was used for dsRNA synthesis using the Replicator RNAi kit (Finnzymes) according to the manufacturer's recommendations. dsRNA synthesis was allowed to proceed at 35°C overnight. dsRNA was purified by stepwise LiCL precipitation in 2M LiCl to precipitate ssRNA and 4M LiCl to

precipitate dsRNA. dsRNA was washed with 70% ethanol, dried and resuspended in ultrapure water. The integrity and concentration of purified dsRNA was analyzed on 1% agarose gels and absorption measurement at 260 nm.

4.6.3 Elicitor preparations and dilution

Poly(I:C) with PBS buffer salts was purchased from Sigma and has an average length of approximately 350 to 850 base pairs (bp). It was reconstituted in ultrapure water. Poly(I) was purchased from Sigma and reconstituted in water. GFP siRNA duplexes were ordered from microsynth (http://www.microsynth.ch) and reconstituted in water. Flg22 peptides were purchased from EZBiolabs and stock solutions prepared in PBS.

4.6.4 Proteinase K digest and RNAse A/T1 treatment of poly(I:C)

To ensure the absence of any proteinaceous elicitor contamination, a solution of 50 µL 10 mg/mL poly(I:C) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), PBS, or 50 µL of a 1 µM solution of flg22 (EZBiolabs) were digested with 1 µL proteinase K (Invitrogen) at 37°C overnight. Non-proteinase K-treated flg22 was kept under the same conditions as control. For degradation of poly(I:C) with RNAse A/T1 (Fermentas), 10 mg/mL poly(I:C) in PBS or PBS were diluted 1:1 with ultrapure water and incubated in the presence of 25 µg/mL RNAse A and 62.5 U/mL RNAse T1 at 30°C overnight. As control, one poly(I:C) sample was kept under the same conditions without addition of RNAse. Efficiency of the RNAse digest was analyzed on a 1% agarose gel and the samples were purified by sequential LiCl precipitation. After washing with 70% ethanol, the poly(I:C) pellet was air dried and resuspended in 1 x PBS.

4.6.5 ORMV purification

ORMV-infected *N. benthamiana* leaves were homogenized to fine powder in liquid N_2 and virions extracted using butanol/chloroform and subsequent precipitation of the virions from the aqueous phase using 4% polyethylene glycol 8000 in the presence of 1% NaCl. Virions were resuspended in 10 mM sodiumphosphate-buffer pH7.4 and virion concentration estimated from absorbance at 260 nm.

4.6.6 Analysis of PTI responses

4.6.6.1 MAPK phosphorylation

Arabidopsis leaf discs were excised with a cork borer and incubated overnight in 24-well plates containing 300 µL deionized, ultra-pure water (two leaf discs per well). 3 µL 100 µM flg22 (EZBiolabs), 50µL 1.5 µg/µL GFP dsRNA, 15 µL 10 µg/µL poly(I:C) (Sigma), 7.5 µL 40 µM GFP siRNA (Microsynth), 15 µL 1 x PBS, or 15 µL deionized, ultra-pure water, respectively, were added to the leaf discs the next day. For immunoblots with and without proteinase K treatment, flg22 was applied to a final concentration of 150 nM. After vacuum-infiltration in a desiccator for 10 min and subsequent incubation for additional 20 min, leaf discs were frozen in liquid nitrogen. Proteins were extracted by grinding in liquid nitrogen, addition of 2 x sample loading buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 10% 2-mercapto-ethanol, 4% SDS, and 0.02% bromophenol blue) and boiling for 5 min at 95°C. Total proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skimmed milk for two hours and probed with primary antibodies against phosphor-p44/42 MAPK (Cell Signaling Technologies) and HRP-labeled secondary antibodies for luminescence detection (Lumilight Plus, Roche).

4.6.6.2 Ethylene production

To measure ET production, leaf strips were kept in ultrapure water over night. The next day, poly(I:C) or flg22 was added to a final concentration of 500 ng/µL and 1 µM, respectively. Equal volumes of PBS or water, respectively, were added to control samples. ET production was measured by gas chromatography as described previously (Felix et al., 1999) after 3.5 hours.

4.6.6.3 Seedling growth inhibition

For seedling growth inhibition assays, sterile five days-old old Arabidopsis Col-0 seedlings (four to six biological replicates per experiment) were transferred to 24-well plates containing liquid half-strength MS medium supplemented with 1% sucrose (two seedlings per well) and poly(I:C) added to a final concentration of 500 ng/µL. Control seedlings were treated with 1 µM flg22, PBS or ultrapure water. The effect of treatment on seedling growth was documented

on photographs at eight days after treatment. For quantitative analysis, length of the roots of the seedlings was measured using a ruler.

4.6.6.4 Virus infection

Rosette leaves of five week-old Arabidopsis Col-0 plants were gently rubbed with 4 µl of 10 mg/mL poly(I:C) or poly(I), 4 µL PBS or 4 µL of a 10 µM flg22 solution and 50 ng purified ORMV virions in the presence of celite as abrasive. Immediately after treatment, remaining elicitors, buffers, and virions were washed off the leaf surface. At four dpi, the treated leaves were sampled for analysis of virus accumulation by qRT-PCR. The plants from which the inoculated leaves were removed were maintained for further two to three weeks for the analysis of disease symptom development. To exclude that poly(I:C) treatment during plant protection experiments interfered with the proper readout for RNA concentration in absorbance measurements, a subset of the RNA samples used to quantify virus titer upon poly(I:C), PBS and flg22 treatment was DNAse digested, cDNA made using oligo(dT) primers, and expression of five different housekeeping genes (Czechowski et al., 2005) determined using qRT-PCR as described below (Table S4.1).

4.6.7 Analysis of virus accumulation

To quantify ORMV accumulation, total RNA was extracted from elicitor-treated and ORMV-infected leaves using the Tri-reagent (Sigma), following the manufacturer's protocol. Approximately 50 ng of total RNA were analyzed in duplicates by qRT-PCR using specific primers amplifying a fragment in the ORMV RNA-dependent RNA polymerase region and an ORMV-specific 6-carboxyfluorescin labeled probe. Primer and probe sequences are described by Mansilla *et al.*, (2009). The 15 µl qRT-PCR mix to detect ORMV contained 0.5 µM primer, 0.25 µM FAM-ORMV probe, 0.15 µl Superscript III (Invitrogen), 0.175 µl RNAse Out (Invitrogen), 7.5 µl 2x LC 480 Master Mix (Roche) and 50 ng total RNA. Reverse transcription was performed for 30 minutes at 50°C. PCR conditions after denaturation for 10 min at 95°C were 40 cycles of 95°C for 2 seconds, 55°C for 10 seconds and 60°C for 15 seconds. Copies of viral RNA per ng total RNA were calculated in reference to ORMV standard curves with known concentrations of viral RNA.

4.6.8 Selection of PTI-responsive genes

To investigate whether poly(I:C) treatment resulted in the induction of PTI-responsive gene expression we selected candidate genes by analysis of early flg22-responsive genes in a publicly available gene expression dataset (GSE17382, (Boudsocq et al., 2010)). CEL files of the GSE1782 experiment were downloaded and gene expression analyzed using ROBIN (Lohse et al., 2010). After selection of a subset of flg22-responsive genes we visualized their general induction in PTI, hence, upon treatment with different microbial elicitors using Genevestigator (Hruz et al., 2008).

4.6.9 Analysis of gene expression by qRT-PCR

Leaf discs treated with poly(I:C) (500ng/µL final concentration), PBS, and flg22 (1µM final concentration) were sampled three hours after treatment and frozen in liquid N_2. Samples were ground to fine powder in liquid N_2 and total RNA extracted using Tri-reagent according to the manufacturer's protocol. After DNAse digestion (Promega RQ1 DNAse kit) for 30 minutes at 37°C, 1 µg of total RNA was reverse-transcribed using oligo(dT) primers and superscript III (Invitrogen). qRT-PCR with primers for immunity-related genes and the reference genes *GAPDH*, *ACTIN 2* and *UBIQUITIN 10* (Czechowski et al., 2005) was performed with four biological replicates (two leaf discs per replicate) for each treatment. Relative gene expression levels were quantified using the $2^{\Delta CT}$ method. 10 µl PCR reactions contained 1 µl cDNA, 0.5 µM forward and reverse primer, and 5 µl Sybr green master mix (Roche). PCR was performed in a 480II light cycler (Roche) with the conditions 5 minutes denaturation at 95°C and 40 cycles at 95°C for 10 seconds, 60°C for 15 seconds and 72°C for 15 seconds. Threshold cycle (CT) values for immunity-related genes and *DCL* isoforms were normalized to CT-values obtained for the housekeeping genes *GAPDH*, *ACTIN 2* and *UBIQUITIN 10* (Czechowski et al., 2005), yielding ΔCT values. $2^{-\Delta CT}$ values representing relative expression levels, their mean values and standard error (SE) were calculated. Ratios between $2^{-\Delta CT}$ values for poly(I:C) or flg22 and PBS treatment, respectively or between *dcl2,4* and WT yielded relative fold changes. Coefficients of variation were calculated by dividing the SD of the $2^{-\Delta CT}$ values by the $2^{-\Delta CT}$ mean value, and subsequently calculating the error of the ratio by using the formula $cv = \sqrt{(cv_1^2 + cv_2^2)}$. Significant differences were determined by Student's t-test. All primer sequences and accession numbers of genes analyzed by qRT-PCR are listed in Table S4.2.

4.7 Acknowledgements

This study was supported by the Swiss National Science Foundation, grants 31003A_140694 to M. H. and 3100A_136278 to T.B.

4.8 Supplemental figures

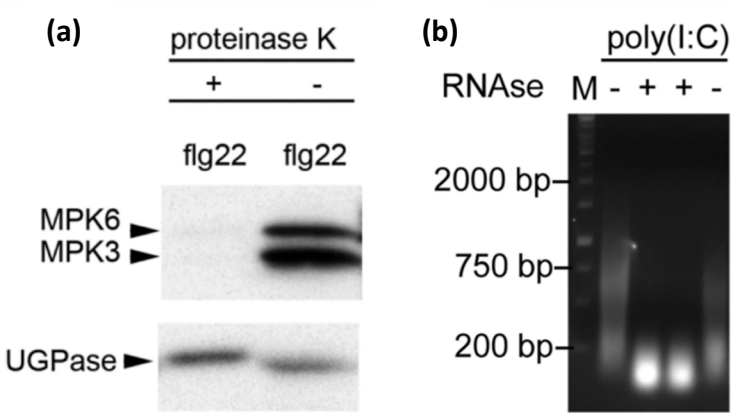

Fig. S4.1 Controls for poly(I:C) treatment with proteinase K and RNAse A/T1. (**a**) Immunoblot to detect MAPK phosphorylation in Arabidopsis leaf discs treated with proteinase K-digested or undigested flg22. The blot was probed with an antibody against phosphorylated MAPKs. To test for proteinase K activity, a solution of flg22 was digested with proteinase K overnight, or kept without addition of enzyme under the same conditions. The next day, the solutions were used to elicit Arabidopsis leaf discs. Arabidopsis proteins were extracted and used for immunoblot analysis using antibodies against phosphorylated MAPKs and UGPase, respectively. (**b**) Agarose gel electrophoresis of RNAse A/T1-digested or undigested poly(I:C). Solutions of poly(I:C) were digested with RNAseA/T1 for one hour, or kept without addition of enzyme under the same conditions. The RNAse-treated or untreated samples were analyzed on a 1% agarose gel, stained with ethidium bromide and photographed under UV light.

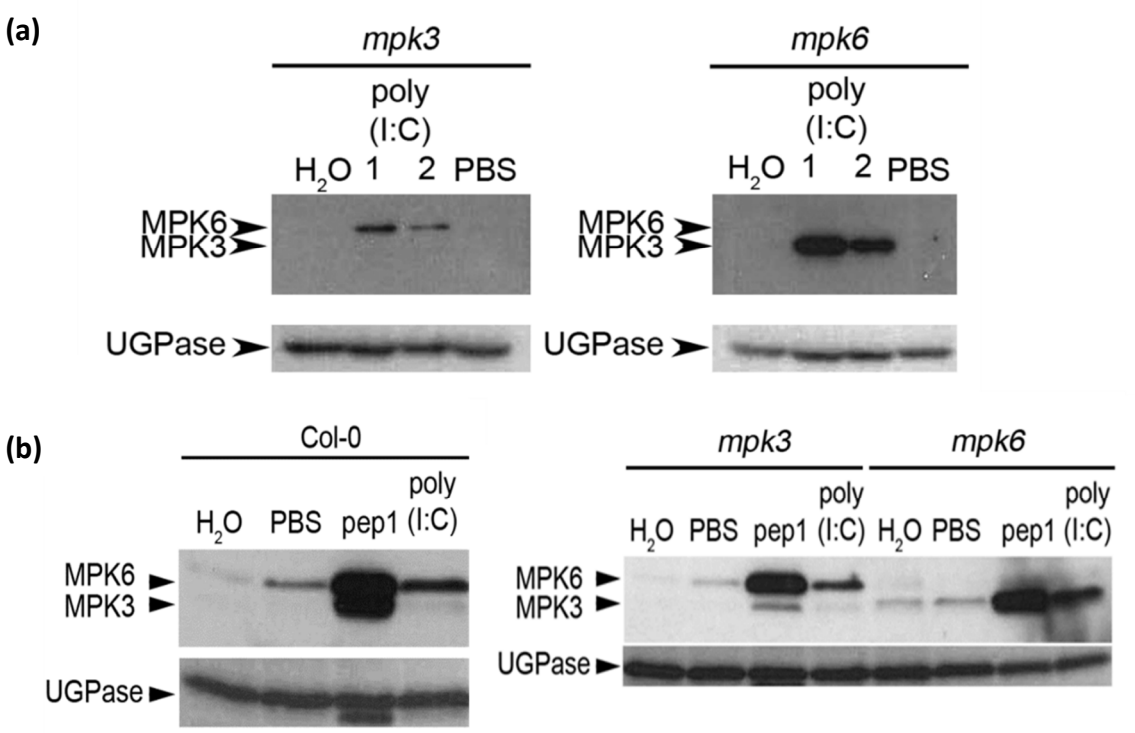

Fig. S4.2 dsRNA-signaling involves phosphorylation of MPK3 and MPK6 in roots and shoots. (**a**) Immunoblot to detect MAPK phosphorylation in Arabidopsis *mpk3* and *mpk6* mutant leaf discs treated with 500 ng/µL poly(I:C) (two biological replicates), water or PBS using an antibody against phosphorylated MAPKs. (**b**) Immunoblot to detect MAPK phosphorylation in Arabidopsis wild-type, *mpk3* and *mpk6* mutant isolated roots treated with 500 ng/µL poly(I:C) or water, PBS and AtPep1 as a control using an antibody against phosphorylated MAPKs. As equal loading control, the same blots were reprobed with anti-UGPase antibodies.

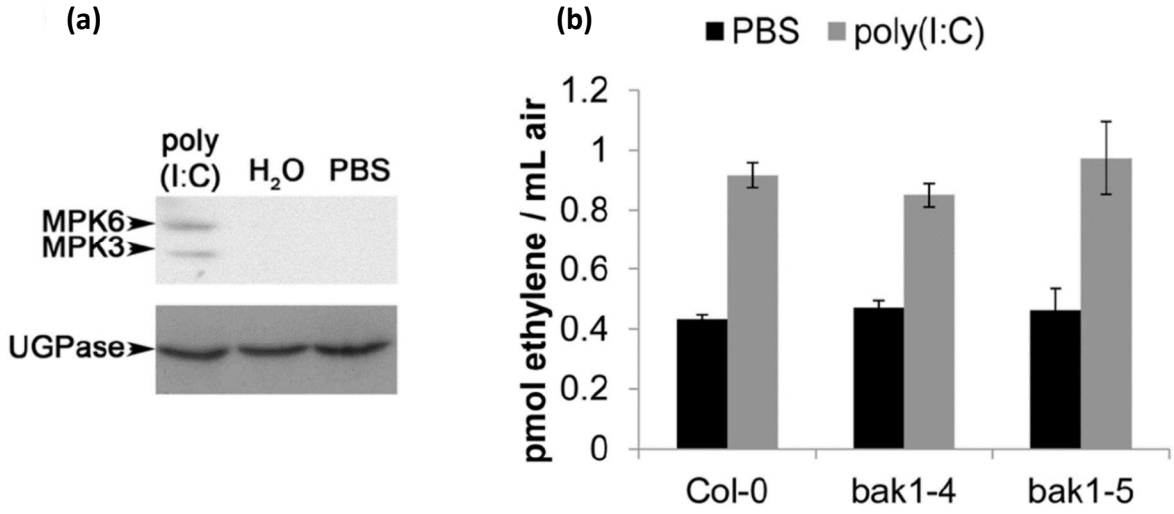

Fig. S4.3 *bak1* mutants still respond to poly(I:C)-treatment by MAPK activation and ethylene production. (**a**) Immunoblot to detect MAPK phosphorylation in Arabidopsis *bak1-4* mutant leaf discs treated with 500 ng/µL poly(I:C), water, or PBS using an antibody against phosphorylated MAPKs. As equal loading control, the same blot was reprobed with anti-UGPase antibodies. (**b**) Ethylene production by Arabidopsis Col-0, *bak1-4* or *bak1-5* mutant leaf strips treated with PBS or 500 ng/µL poly(I:C) for 3.5 hours. The Col-0 samples shown as reference were analyzed together with *bak1* mutants and are the same than in Figure 4.4b of the main manuscript text. Mean values ± SE of six biological replicates are shown. Poly(I:C) treatment resulted in significant ($P < 0.05$, Student's t-test) production of ethylene compared to PBS controls in all three Arabidopsis genotypes.

Table S4.1 Housekeeping gene expression in plant protection experiments.

	PBS		poly(I:C)		flg22	
Gene name	mean CT	SD	mean CT	SD	mean CT	SD
EXPRESSED PROTEIN	24.84	0.28	24.64	0.37	24.84	0.56
GAPDH	21.60	0.42	20.94	0.25	21.33	0.58
UBIQUITIN 10	18.99	0.27	18.86	0.25	18.97	0.52
TIP	24.78	0.32	24.70	0.28	24.88	0.60
ACTIN	20.99	0.40	21.52	0.82	20.93	0.23

Expression of five different housekeeping genes was analyzed in samples used for virus accumulation in plant protection experiments. Average CT-values ± SD of four biological replicates are shown. cDNAs of poly(I:C), PBS-treated or flg22-treated and ORMV-infected leaves were analyzed by quantitative RT-PCR using primers against *EXPRESSED PROTEIN, GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE, UBIQUITIN 10, TIP41-LIKE FAMILY PROTEIN,* and *ACTIN 2*.

Table S4.2 Primer sequences.

Gene name	ID	forward primer	reverse primer
EXPRESSED PROTEIN	AT4G33380	CGTCCACAAAGCTGAATGTG	CGAAGTCATGGAAGCCACTT
GAPDH	AT1G13440	TTGGTGACAACAGGTCAAGCA	AAACTTGTCGCTCAATGCAATC
UBIQUITIN 10	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG	AAAGAGATAACAGGAACGGAAACATAGT
TIP	AT4G34270	GTGAAAACTGTTGGAGAGAAGCAA	TCAACTGGATACCCTTTCGCA
ACTIN	AT3G18780	CTTGCACCAAGCAGCATGAA	CCGATCCAGACACTGTACTTCCTT
PROPEP3	AT5G64905	TCACACAGCGAGGAAGATGA	CCTTTTCCTGAACTTGGCGT
ANTIFUNGAL PROTEIN	AT3G02840	GTTTTGAAAGATTCGCGGCG	AAGAGAAGCCGTTGATCCCA
RBOHD	AT5G47910	GTCGTTGCAACACGCTAAGA	GGGATGTTGAACAGCGATCT
POD	AT1G14540	GAGATTCCACCAACGCGTTT	AGAGATCGTTGAGGCTAGCC
EDS5	AT4G39030	CTCTTGGACCGGGAACAGTA	GTTGCGCTTCTTTCTTGTCC
PLPA2	AT2G26560	AACTTCAGCTGCCCCTACAT	TAGCTGCAACTCCACCATCA
LOX3	AT1G17420	GGCCGTGGTTGATACTTTGT	GACGGCTGTTGTCTCTCTCC
BAK1	AT4G33430	TGCAGTTCCAGACAGAGGTT	CAAGTTTCTGTGAACCGCCA
SOBIR	AT2G31880	GCGATTGATCCGAAGTTGAT	AGGCGATCTTCAGAACCAGA
BRI1-LIKE KINASE 3	AT3G13380	TCTGCACCATAGCTGCATTC	CAGCCTTGCCATACCAAAAT
AtCRK4	AT5G24430	TCCTATATCCGTGCCTCACC	TAAGGGATAGGCCTCCGTCT
SERK1	AT1G71830	GCGACGAAGAAAGCCACTAG	CATCACTCGCCACTTGTAGC
SERK2	AT1G34210	AGGAATTTGAGGCGGTGGTA	ACTCAGGAGCAATGTGTCCA
SERK4	AT2G13790	CTCGACTGGGTGAAAGAGGT	ATCAGCTGCTCCACTTCTGT
SERK5	AT2G13800	AGAACGTCCACCATCACAGT	TACCACCGCCTCAAATTCCT
PR1	AT2G14610	CCCACAAGATTATCTAAGGGTTCAC	ATGCAGTGGGACGAGAGGG
PR5	AT1G75040	GGAACTCTCGCCGGTCAAG	TTTGAATTGACTCCAGGTGCTTC
PDF1.2	AT5G44420	CACCCTTATCTTCGCTGCTC	GCACAACTTCTGTGCTTCCA
DCL1	AT1G01040	TACACAAGCCTTCCTCCTGG	GCAACGCGAGCAAAATTCTC
DCL2	AT3G03300	CCAGGTCAATCATCTGCAGC	TACAACATCGGCCACACTCT
DCL3	AT3G43920	CCAATCGTGAGGCTGTGATG	AAGCAATTCCACGACTGCAG
DCL4	AT5G20320	TCGGGAAATATCAGCGACGA	TTTGTTCCGACCAGTTGCAG

5 GENERAL DISCUSSION

5.1 The underestimated role of roots in PTI

At present, the agricultural sector faces dramatic losses due to pests and diseases. As many significant crop pests are root pathogens (Chapter 1.5.2.2; Table 1.2) and root colonization by microorganisms has a major impact on plant growth (Pieterse et al., 2014), understanding the molecular processes controlling defense responses in roots is important in order to develop new technologies to maintain or increase crop yields. During the last years, we contributed to illuminate the mechanisms of MAMP and DAMP signaling in roots.

Although it had been known at the beginning of this thesis that MAMPs elicited defense responses in roots, it was not clear whether the root by itself was able to detect MAMPs (Attard et al., 2010; Millet et al., 2010). In our study we have shown that isolated root tissue is able to perceive flg22 and activate downstream PTI responses (Chapter 2). In addition, our data demonstrate that AtPep1 and chitin, but not elf18, are perceived by dissected roots (Chapter 2.2; Appendix). The immune responses triggered are comparable to leaf responses although slight differences in kinetics and amplitude occur. Furthermore, our data reveal that the intensity of the PTI responses depends on the types of MAMPs/DAMPs applied. It may be interesting to see whether the strength of PTI responses correlates with the levels of PRR expression in roots. In support of this idea, PEPR1 and PEPR2 have been shown to be strongly expressed in roots (Bartels et al., 2013). Consistently, root responses to AtPep1 are stronger when compared to those induced by MAMPs, which suggests a potentially important role for DAMP signaling in roots (Chapter 2.2; Appendix). As already discussed in Chapter 2, we believe that due to the permanent contact of roots with soil-borne microorganisms, defense responses against MAMPs are locally restricted in roots to avoid their overstimulation, deleterious for establishing positive interactions with beneficial microbes and for plant fitness. Instead, disruption of root tissues and the release/activation of DAMP signaling through actively invading pathogens might indicate a severe threat situation for the plant and therefore, reactions against AtPep1 may be strong. Interestingly, a similar hypothesis has been proposed in the field of animal biology (Lafferty and Cunningham, 1975; Matzinger, 2002). This model proposes that danger or alarm signals originating from injured cells, such as those exposed to pathogens, toxins or mechanical damage, activate stronger responses in cells of the same kind than do signals originating from other cells. However, whether this model can be also applied to plant immunity remains to be determined.

As another goal of this thesis, I have successfully adapted different assays well-established for the analysis of leaf PTI responses, to root assays (Chapter 2.2). These methods can now be employed to investigate root responses to signals associated to immunity, development or abiotic stresses.

Interestingly, in the course of this study, we could identify *PER5* as strong transcriptional marker for defense induction in roots (Chapter 2.2). Although it has been ascribed a role in response to oxidative stress (Tognolli et al., 2002), little information about PER5 is present in the literature. We believe that PER5 might also be involved in other processes related to changes of the cell wall composition like lignification and the functional characterization of this gene will be an interesting topic for further studies.

Another significant advance made during this thesis is the characterization of dsRNA as a viral PAMP, active not only in leaves but also in isolated roots (Chapter 4; Fig. S4.2). Based on these results, we assume that an enigmatic dsRNA receptor exists in roots, which helps plants to defeat viruses and their transmission vectors (fungi, nematodes or bacteria) present in soil (Roberts, 1950; Kimura, 2008).

Recently, it has been shown for beneficial and pathogenic microorganisms that root infection relies on an active suppression of PTI (Trdá et al., 2015). For instance, the beneficial fungus *Piriformospora indica* has been shown to suppress root immunity triggered by various MAMPs in Arabidopsis (Jacobs et al., 2011). Similarly, the phytotoxin coronatine, a JA-isoleucine mimic produced by *Pseudomonas syringae* pathovars, was shown to be involved in the active suppression of MAMP responses in roots (Millet et al., 2010). These findings support indirectly the importance of pattern recognition in root immunity. But the impact of MAMP and more importantly DAMP signaling on root colonization by microbes is only beginning to be elucidated and should be explored further (Trdá et al., 2015).

5.2 The *FLS2* expression level is not the major factor determining the intensity of root PTI responses

The former study by Millet and colleagues does not distinguish whether the pattern of MAMP responses observed in the root elongation zone upon flg22 treatment is due to a localized penetration of the externally applied peptide or to the localization of the perception and/or response machinery in this zone (Millet et al., 2010). In an attempt to discriminate between these two possibilities, we studied whether the expression pattern of *FLS2* correlated with the spatial induction of defense responses. We prove that *FLS2* is mainly expressed in vasculature tissues in wild-type roots (Chapter 2; Fig. 2.2; Fig. 2.7; Fig. S2.9) and not at the outer, epidermal surface, which would be disadvantageous for the plant encountering zillions of MAMPs in its soil environment which, in turn, would stimulate constant defense responses in this tissue.

As studying the promoter activity of PRRs alone does not allow us to conclude whether all tissues can respond to the cognate elicitors, we took a second approach with transgenic Arabidopsis lines expressing *FLS2* only in certain root tissues. With these lines we aimed at elucidating whether all tissues have the ability to perceive externally applied flg22 and initiate PTI signaling and whether the intensity of the downstream response activated differs between the types of tissues perceiving the elicitor (Chapter 2.2). Using isolated roots of these transgenic plants, we reveal that *FLS2* expression in each of the investigated, specific tissues is sufficient to render the roots responsive to flg22.

Upon treatment of roots of intact, transgenic seedlings our MAMP-response measurements clearly show that externally applied flg22 is perceived by inner root tissues and that the flg22 response system is active there (Chapter 2; Fig. 2.2f; Fig. 2.11). This finding also implies that the peptide quickly diffuses within roots. To complete this analysis, we propose to study the movement of this elicitor throughout the plant using fluorescence-labeled flg22 peptides like TAMRA-flg22 (Underwood and Somerville, 2013). Alternatively, we have generated transgenic lines secreting flg22 autonomously in certain root tissues (Chapter 3.7.3; Method II). Crossings between these plants and the tissue-specific *FLS2*-expressing lines will provide a tool to address flg22 movement in plants. Such investigations will be helpful to understand peptide mobility and are of general interest as peptide signaling is an important mechanism in intraplant, intertissue and intercellular communication (Boller, 2005).

Moreover, we show that differences in the intensity of the PTI output occur depending on the type of tissue perceiving flg22 (Chapter 2.2). We conclude that the observed variations in the activity of the downstream responses result from distinct specificities associated to the perceiving tissues and not from the differences in the tissue accessibility. These results indicate that in general strong PTI responses are induced if flg22 is sensed by the pericycle or epidermal tissues. This observation further supports the idea that *FLS2* expression needs to be limited to inner tissues in wild-type plants as its expression in the epidermis, even at a low level, renders this tissue strongly responsive (Chapter 2.2).

However, our results do not fully explain yet why reactions against MAMPs were stronger in the elongation zone than in other root zones, as reported by Millet *et al.* (2010). In order to study the correlations between the detected, enhanced marker gene induction upon MAMP treatments in the elongation zone and the *FLS2* expression level in more detail, we propose to use promoters which limit the transgene *FLS2* expression in the different longitudinal root zones instead of radial root tissues. This approach might help to gain further insight into the regulation of MAMP perception and immune signaling in the root.

Remarkably, our data clearly show that each tissue is able to respond to flg22 if FLS2 is present (Chapter 2.2). Thus, the response machinery seems to be present in each tissue although the expression of the PRR varies depending on the tissue. This observation indicates that the coreceptor *BAK1* is expressed ubiquitously in the root. In support, SERKs, including BAK1, are strongly detectable on Western blots in crude root material (Appendix; Fig. A1a). Previous studies did indicate the presence of BAK1 in certain root tissues (Bücherl et al., 2013) and Northern blot analysis with total RNAs isolated from various tissues of wild-type Arabidopsis plants using a BAK1-specific cDNA probe showed ubiquitous *BAK1* expression (Nam and Li, 2002). Our data further support the presence of functional BAK1 in the analyzed root tissues.

5.2.1 Model for flg22-elicited responses in roots

Combining the data of the two studies described in Chapter 2, I propose a model in which outer root tissues of wild-type plants accumulate no or only low amounts of FLS2 under "normal" conditions, probably due to the reasons discussed above. However, when bacteria are able to penetrate and reach the vasculature, vigorous PTI responses may get activated, as supported by our observation that detection of flg22 by inner root tissues leads to strong immune responses (shown in Chapter 2). In addition, flg22 perception induces an up-regulation of the *FLS2* promoter activity in outer tissues including the epidermis, associated with an increase in *FLS2* expression in these tissues (Chapter 2; Fig. 2.4; Fig. 2.5c; Fig. S2.9). Hence, upon subsequent flg22 recognition, the presence of FLS2 in these outer tissues may allow strong PTI reactions, which could restrict bacterial colonization and prevent subsequent microbial infection. In line with this idea, we demonstrate that flg22 perception by the epidermis triggers strong PTI responses (Chapter 2.2). However, the identity of the tissues in which the measured PTI responses take place upon activation of FLS2 in a specific tissue cannot be determined from the data presented in this thesis. A different strategy (Chapter 5.3.1; Fig. 5.2) will help to discriminate whether PTI responses occur in the same cells in which flg22 is detected or whether a signal propagates from flg22-perceiving cells to initiate PTI responses in other cells.

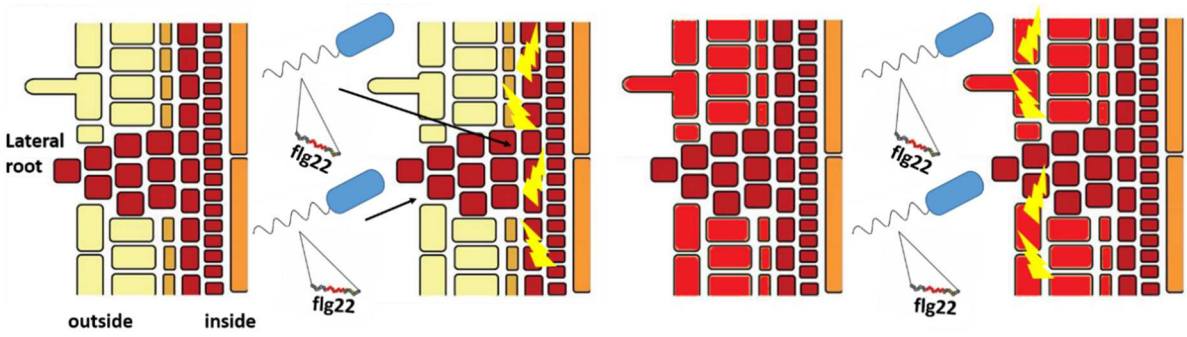

Fig. 5.1 Model for flg22-elicited responses in roots. *FLS2* expression (red) is restricted to inner tissues and at the site of emerging lateral roots in absence of active MAMPs. Upon perception of flg22 exposed by bacteria (blue), strong defense reactions get activated (yellow flashes). *FLS2* expression is up-regulated in outer, epidermal tissues, which are now able to initiate defense reactions upon further flg22 perception, possibly leading to increased resistance to pathogenic bacteria.

The model described above does not integrate the different developmental zones of the longitudinal root axis. Nevertheless, two hypotheses are proposed to explain the strong, tissue-specific activation of downstream signaling responses in the elongation zone observed by Millet et al. (2010). On the one hand, the tissue-specific expression of *FLS2* could be responsible for the restricted activation of PTI responses in only certain tissues. *FLS2* expression is inducible in wild-type roots and its expression pattern is influenced by changing developmental and environmental conditions, strongly indicating a crucial role of the tissue-specific PRR compartmentalization pattern (Chapter 2).

On the other hand, assuming that *FLS2* is expressed ubiquitously at low levels in all root tissues in wild-type plants, the tissue-specific activation of downstream defense responses might be explained by variances in the response capacity of different cells, which may be caused by the availability of downstream signaling components. Consistent with this scenario, our results presented in Chapter 2.2 suggest that the intensity of the downstream PTI response does not necessarily correlate with the *FLS2* expression level. Furthermore, we did neither detect enhanced *FLS2* expression in the elongation zone nor did we find exclusive expression of *FLS2* in this region (Chapter 2; Fig. 2.2; Fig. 2.4; Fig. 2.5c; Fig. 2.7; Fig. S2.9). Hence, young, developing tissues in the elongation zone may have a comparatively higher capacity to directly respond to MAMPs or to perceive and respond to signals propagating from cells transmitting MAMP perception than other tissues. Alternatively, the activation of downstream responses might be inhibited in cells outside of the elongation zone through processes like cleavage, ubiquitination or rapid endoctytosis of receptor complex or downstream components (Antolín-Llovera et al., 2014). These mechanisms would ensure the activation of PTI responses locally at the elongation zone, where they are important in order to prevent pathogen entry while saving energy costs, which would probably result from ubiquitious PTI activation.

5.2.2 Biological aspects of tissue-specific RLK accumulation

Tissue-specific expression of PRRs appears to be a common phenomenon in Arabidopsis. For example, the PRRs *PEPR1* and *PEPR2* have been shown to be expressed in specific root tissues (Bartels et al., 2013). It is still unclear whether this regulation is meant to facilitate accessibility to the ligand or to limit energy costs, thus avoiding unnecessary responses which could inhibit other processes like root development or symbiotic interactions. In development, tissue-specific expression of RLKs has been documented by several studies (Clark et al., 1993; Yokoyama et al., 1998). For instance, the enigmatic systemin receptor is assumed to localize specifically to the phloem cells in the vicinity to the parenchyma cells, expressing the ligand prosystemin (Hind et al., 2010). In this case, this compartmentalization may avoid unspecific or inappropriate binding of systemin to highly similar receptors known to have systemin binding capacity. A new study revealed the tissue-specific accumulation of a RLK modulating CLV3/EMBRYO SURROUNDING REGION (CLE) peptide-triggered growth inhibition in Arabidopsis roots (Shimizu et al., 2015). As peptides can act as long-distance signals in plants (Boller, 2005), their tissue-specific effects might need to be regulated by a spatial accumulation of their corresponding receptors. Taken together, these examples provide evidence that tissue-specific compartmentalization of RLKs might fulfill several functions in plants and is a quite common mechanism. Similarly, specific spatial expression of TLRs in animal cells has been shown to differentially modulate innate immunity (Muzio et al., 2000; Iwasaki and Medzhitov, 2004; Mogensen, 2009; Hu and Pasare, 2013).

In the future, the study of the compartmentalization pattern of other immune receptors will help to increase our knowledge about the role of tissue-specificity in regulating PTI.

Using the current set of tools for FLS2, I here propose to run several approaches to complete our analysis. First, the numerous generated and characterized tissue-specific *FLS2*-expressing lines (Chapter 2.2) can now be analyzed with regard to bacterial colonization. It is well possible that colonization by pathogenic bacteria like *Pseudomonas syringae* or *aeruginosa* as well as beneficial bacteria such as *Bacillus subtilis* FB17 or plant growth promoting *Pseudomonas* strains, is impaired in lines expressing high amounts of *FLS2* in the epidermis. Thus, studying the resistance of the transgenic lines against one or several such strains could generate interesting new results about the tissue relevance in these interactions. Nevertheless, several experimental limitations should be considered. First of all, we observed that root treatments of *fls2* mutant plants with *Pseudomonas* extracts induce MAPK phosphorylation (Appendix; Fig. A1b). This result indicates the presence of other MAMPs in the bacterial

extracts recognized by roots. In addition, the FLS2 accumulation in shoots of certain transgenic lines (Chapter 3.7.2; Method I; Fig. S3.2; Fig. S3.3) may complicate the interpretation of results obtained with tissue-specific treatments because elicitors might move throughout or along the plant as discussed in Chapter 3. Nevertheless, such experiments remain interesting to determine the impact of certain root tissues on bacterial colonization and plant resistance.

One of the most important root pathogens, *Ralstonia solanacearum*, produces a flagellin with a mutated sequence on the flg22 epitope, which is not recognized by AtFLS2 as a consequence of its evolutionary evasion (Felix et al., 1999; Bauer et al., 2001; Pfund et al., 2004). Therefore, it would be meaningless to investigate our lines with regard to *Ralstonia* colonization. Mutating the flg22 epitope sequence of the *Ralstonia* flagellin to a classical flg22 sequence might constitute an option to study the effect of tissue-specific FLS2 accumulation on *Ralstonia* infection. Alternatively, other PRRs recognizing bacterial MAMPs could be exploited to address disease resistance in roots. To test this idea, the effect of increased, tissue-specific *EFR* expression, usually not or only marginally present in roots (Millet et al., 2010; Ranf et al., 2011; Wyrsch et al., 2015; Chapter 2), on bacterial root colonization could be investigated. In future, (tissue-specific) expression of *EFR* in roots might provide a helpful tool to increase resistance against soil-borne bacteria, as shown in other systems (Lacombe et al., 2010; Holton et al., 2015; Schoonbeek et al., 2015).

An important question which remains to be addressed is whether the same expression patterns of PRRs and regulations exist for roots of crop plants. Moreover, it has to be mentioned that Arabidopsis contains a “taproot” system with the central root being the strongest and most dominant root. Thus, it remains elusive whether the same observations also count for “fibrous” root systems of monocotyledons.

Nonetheless, we believe that the tissue-specific expression of PRRs in roots, for instance in the pericycle, could be sufficient to support resistance against soil-borne pathogens and would avoid constitutive PTI activity resulting from ubiquitous PRR expression and related trade-offs.

5.3 MAMP- and DAMP-triggered downstream responses differ in localization and intensity patterns in roots

Although we conclude that each analyzed root tissue has the capacity to respond to flg22 if FLS2 is present, our study did not yet allow us to determine in which tissues the measured PTI responses take place. In order to wire off the downstream immune responses in roots at the cellular resolution, we analyzed transgenic Arabidopsis plants expressing the YFP-derived fluorophore Venus under the control of MAMP-responsive promoters (Appendix). The Venus reporter contains a nuclear localization signal sequence that facilitates the detection of the fluorescent signal in the nucleus of single cells. Hence, these Venus reporter lines (referred to hereafter as *Prom:NLS-3xVenus* constructs) allow the detection of the fluorescent signal upon elicitor treatment at a cell- and tissue-specific level.

Our data reveal that the intensity of the transcriptional downstream responses initiated in roots depends on the type of molecular pattern applied. In addition, different elicitors activate markers in specific, not necessarily overlapping root tissues. For example, the promoter activity of the indolic glucosinolate biosynthetic pathway and flg22-triggered callose deposition marker (Gigolashvili et al., 2007; Clay et al., 2009) *MYB51* is induced upon flg22 treatments in the root epidermis, whereas AtPep1 treatment leads to its induction mainly in inner tissues (Appendix; Fig. A3). There are several feasible explanations for this observation. First, the peptide AtPep1, naturally produced in plants, may be generally more present in the vasculature as it might be distributed throughout the vascular system. But both synthetic elicitors were applied externally and flg22 is also quickly distributed and perceived even in the pericycle in intact roots (Chapter 2.2; Fig. 2.11). Second, the high accumulation of PEPR2 in inner tissues (Bartels et al., 2013) may mediate the strong activation of the *MYB51* promoter in the vasculature. However, *FLS2* expression is also present at high levels in the vasculature (Chapter 2; Fig. 2.2; Fig. 2.7; Fig. S2.9) but flg22 application mainly induces *MYB51* promoter activity in the outer tissues (Appendix; Fig. A3). Thus, we speculate that distinct elicitors induce downstream signaling responses differing in nature and amplitude in the various root tissues as a result of discrepancies in receptor levels and/or mechanisms of downstream signaling. I propose the hypothesis that, in nature, active tissue disruption by invading microbes might induce Pep signaling, leading to strong PTI responses in the vasculature, the most important tissue to protect as it is often colonized by microbes to facilitate their distribution. This speculation again supports an important role of DAMP signaling in root

immunity. Nonetheless, flg22 might be perceived by the same tissues as AtPep1 but leads to a stronger induction of downstream PTI responses in the outer tissues in order to defeat newly invading bacteria.

In contrast to AtPep1- and flg22-mediated responses, chitin treatment elicits the *MYB51* promoter activity very weakly, only in the mature root differentiation zone (Appendix; Fig. A3). This observation is in agreement with the results from a previous study, showing that callose deposition and the promoter activation of marker genes was observed in the root elongation zone in response to flg22, while chitin elicited callose deposition and promoters of marker genes in the root differentiation zone (Millet et al., 2010). One possible explanation for this finding could be that this less soluble polymer does not penetrate the tissues as efficiently as soluble peptides. However, the fact that the *MYB51* promoter activity was observed in distinct developmental root zones upon chitin treatments and peptide treatments, respectively, also opens the question whether plants evolved tissue and/or MAMP-specific innate immune responses that depend on the nature of the attacking microorganism. Furthermore, also the kind of the downstream response activated may directly depend on the type of MAMP applied. It is possible that marker genes other than those analyzed in our study would be more strongly induced upon chitin but not upon flg22 treatment and thus, might show a MAMP-specific expression pattern. Although several previous studies revealed a large overlap of up-regulated genes in chitin- and flg22-treated Arabidopsis seedlings (Ramonell et al., 2002; Wan et al., 2008), transcriptome data comparing flg22- and chitin-elicited gene induction specifically in roots are missing.

Variations in the nature, the cellular localization and the intensity levels of activated downstream responses upon perception of distinct kinds of defense elicitors in roots may reflect an adaptation of plants to the alternative invasion strategies of different pathogens. Although highly dissimilar, fungi and nematodes, in contrast to bacteria, both synthesize chitin (Tachu et al., 2008) and can directly penetrate the epidermal cell layers of the roots (Singh and Singh, 2005). Activation of *MYB51* upon perception of chitin at the differentiation zone may therefore reflect a response of the plant to a particular microbial invasion strategy.

But how can plants adapt their defense strategy to the invasion pathways of different types of pathogens? As mentioned before, this could be achieved by a tissue-specific accumulation of PRRs. For instance, the PRRs LYK5 or CERK1, involved in chitin perception/signaling might accumulate in other root tissues than FLS2 or PEPR1/PEPR2, maybe in more tissues of the differentiation zone. This could explain the observed spatial differences between the

flg22/AtPep1- and chitin-elicited downstream responses. However, as already discussed, we believe that the intensity, localization and/or nature of the downstream response does not necessarily correlate with the expression level or abundance of the PRR in specific cells (Chapter 2.2). It rather relies on differences in the response capacities of MAMP-perceiving cells, probably determined by the factors described before such as the availability of downstream components or the presence of response-suppressive mechanisms in specific cells (Chapter 5.2.1). Furthermore, signals potentially transmitted by MAMP-perceiving cells could also vary in their nature, depending on the type of molecular pattern initially perceived. The capacity of distal cells to perceive and respond to these different, propagated signals may in addition contribute to differences in the nature, amplitude and cellular localization of activated downstream responses.

5.3.1 Flg22-triggered downstream marker gene induction does not spatially correlate with *FLS2* expression patterns

Although expression of *FLS2* mainly localizes to inner root tissues (Chapter 2; Fig. 2.2; Fig. 2.7; Fig. S2.9), we could demonstrate by using the *Prom:NLS-3xVenus* lines that downstream responses are primarily initiated in outer, epidermal root tissues upon flg22 treatments (Appendix; Fig. A3; Fig. A4). Hence, the spatial activity of the downstream PTI responses and the *FLS2* expression levels do not always correlate. Rather, the results indicate the induction of PTI responses in cells different from those perceiving the elicitor. But considering that *FLS2* expression could also be present in outer tissues in these treatment conditions (Chapter 2; Fig. 2.4; Fig. 2.5; Fig. S2.9), it remains uncertain whether the marker genes are induced systemically or only in tissues of elicitor perception.

Working with wild-type plants does not allow us to define the tissue recognizing the elicitor and the one triggering the downstream PTI response. To simultaneously study the spatial expression of the *FLS2* receptor and downstream marker gene activation, crosses between the lines expressing *FLS2* under tissue-specific promoters or the endogenous *FLS2* promoter and the MAMP-responsive *Prom:NLS-3xVenus* lines have been made. The analysis of these *Prom:FLS2-GFP* x *Prom:NLS-3xVenus* lines will help us to investigate whether the downstream responses observed result from signaling between the different tissues or are only activated in tissues where the elicitor is perceived. By using these newly generated lines, we could, for instance, determine if the promoter activity of *PER5* or *MYB51* is induced in the

epidermal tissues if *FLS2* expression is restricted to the pericycle or whether only the perceiving tissues are capable to respond. This approach is illustrated as a model in the figure below.

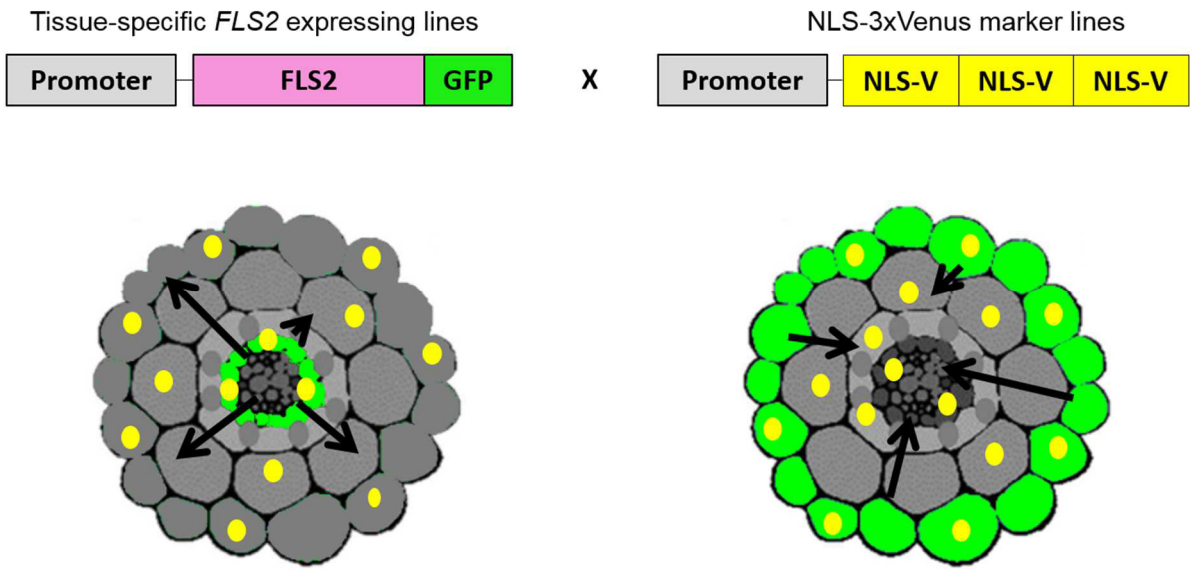

Fig. 5.2 Model illustrating the use of *Prom:FLS2-GFP* and *Prom:NLS-3xVenus* crossed lines. The crossed lines expressing *FLS2* in a tissue-specific manner and the marker *Prom:NLS-3xVenus* will help to analyze in which cells markers get activated (yellow) upon flg22 perception if *FLS2* expression (green) is restricted to certain tissues (here depicted in the pericycle in the left panel and the epidermis in the right panel). Arrows indicate the movement of a hypothetical short-range signal upon MAMP perception inducing responses in distal cells.

Additionally, the *NLS-3xVenus* construct under the control of the endogenous *FLS2* promoter might enable a more sensitive observation of the *FLS2* promoter activity at a better resolution than the markers used so far (Vermeer et al., 2014). As reported in Chapter 2.1, we have never been able to state whether *FLS2* was expressed in each tissue as the signal detection limit was high when using *FLS2pro:GUS* and *FLS2pro:GFP* lines. This approach would help us to clarify if *FLS2* is present in the epidermis and allow studying the mechanisms of *FLS2* activation in roots in more detail.

5.4 Defense gene expression in root-to-shoot signaling

Research on systemic signaling upon pathogen infection has until now mainly focused on events in the above-ground plant parts, thereby neglecting the root system. To fill this gap, we were interested to study the effect of local MAMP/DAMP application to roots on the distal defense gene induction in leaves. As a major outcome of our study, we noticed that frequently used systems to study systemic signaling in plants should be applied carefully. Often, peptide mobility cannot be excluded and several studies do not sufficiently take into account this phenomenon. Furthermore, as already mentioned, several lines of evidence indicate that peptides can function as potential long-distance signals in plants and might spread quickly throughout a plant, rendering studies on systemic effects upon organ-specific applications complicated to interpret (Boller, 2005). In support, a recent study provided evidence that a root-secreted peptide acts as a long-distance signal *in planta* and is perceived by shoot-expressed receptors (Tabata et al., 2014). Therefore, in order to exclude that the root-applied peptides themselves are perceived by shoot tissues, we developed an improved method to study systemic root-to-shoot signaling in Arabidopsis, which allows a controlled application of elicitors to roots.

Our study reveals that none of the genes investigated is significantly altered in shoots 3, 12 or 24 hours after root treatments in our conditions (Chapter 3.3.3). As longer treatment periods exceed the capacities of our system, causing strong drought stress on the roots exposed to air, we were not able to determine whether the short treatment time is responsible for the absence of transcriptional changes. To analyze transcript levels at later time points, I propose to transfer the seedlings after treatment in the system into soil.

Minimal transcriptional changes might only appear locally in specific tissues as, for instance, in the vasculature. Hence, the more sensitive system with the *Prom:NLS-3xVenus* lines described in the Appendix and Chapter 5.3 might be used to investigate systemic responses at a local cellular resolution. Micro-dissection of specific (shoot) tissues and analysis of gene expression in these cells upon MAMP or DAMP perception in the root would constitute an alternative approach but is more tedious. Moreover, other responses, such as ROS production or stomata closure could be investigated in shoots of root-treated plants to evaluate if a systemic effect exists there.

Infection of roots by PGPRs, mycorrhiza, herbivores, as well as abiotic factors has been proven to impact aboveground defenses and affect leaf metabolism and resistance (Kaplan et al., 2008a; Pieterse et al., 2014). However, MAMP treatments of roots probably only elicit organ-specific, local responses but no signal in the shoot. A direct metabolism change in shoots might not be required and biologically relevant for defense against soil-borne pathogens.

Another possibility is that plants may only be primed at the whole plant level upon local perception of MAMPs/DAMPs. It has previously been speculated that this type of induced resistance takes place without a direct induction of active defense mechanisms in plants (Conrath, 2011). With respect to regulations of gene expression, epigenetic reprogramming processes such as histone modifications or changes in DNA methylation patterns could occur in locally root-challenged plants. These mechanisms may provide a possibility and/or prerequisite for quick gene expression changes upon exposure of an unchallenged tissue to a subsequent stress trigger (Fu and Dong, 2013). Furthermore, it is well possible that instead of transcriptional changes, modifications in protein abundance and stability take place upon MAMP applications to roots. Indeed, virus infection and wounding were shown to produce changes in distal leaves at the protein level (Niehl et al., 2013). Other studies have identified distinct changes in the xylem sap proteome in response to pathogenic and symbiotic plant-microbe interactions, mainly affecting PR and anti-fungal proteins in different plant species (Young et al., 1995; Hilaire et al., 2001; Alvarez et al., 2006; Subramanian et al., 2009). For example, abundance of PR5 increased in the xylem sap of tomato in response to infection with the vascular wilt fungus *Fusarium oxysporum* (Rep et al., 2002). As proteins can also be regulated at the (post-) translational level, transcript changes may not always be directly translatable into protein abundances or activities (Niehl et al., 2013). Thus, studying proteomics, enzyme activity or secondary metabolism changes in shoots upon root treatments could reveal the presence of a systemic defense response. The system optimized during this thesis (Chapter 3.3.3) should be helpful to complete this analysis.

To test if MAMPs or DAMPs applied locally to roots cause priming events, shoots could subsequently be inoculated by a virulent pathogen such as *Pto* DC3000 as a second stimulus. A faster and/or stronger defense reaction or an enhanced disease resistance state upon bacterial infection might indicate a primed state of the plant immunity mechanism.

Supplementary, root-to-shoot signaling might require the production of volatile compounds. These signals can be transmitted by air and can even promote a tritrophic effect through the attraction of natural enemies of the predator (Heil, 2008) and thus, do not directly modify the transcriptional state of the plant defense mechanism.

Apart from that, the possible occurrence of systemic signals other than of chemical nature should be considered. Indeed, such long-distance signals as, for instance, changes in the electrophysiological membrane potentials have been shown to take place in plants upon herbivore feeding and wounding (Mousavi et al., 2013; Salvador-Recatalà et al., 2014).

5.5 A novel role for dsRNAs in PTI

Viruses can infect virtually all species of cultivated and wild plants and induce symptoms reducing crop growth, quality and yield. The extent of these crop losses is enormous and can reach up to several billions of US dollars of money lost (Hull, 2002; Cembali et al., 2003). On an agricultural scale, crop protection against viral diseases relies on usage of genetically resistant cultivars, cross protection, chemical treatment of viral transmission vectors and general preventive measures. However, currently implemented strategies are not sufficiently effective to prevent viral disease outbreaks (Nicaise, 2014). Therefore, understanding the mechanisms underlying antiviral defense in plants is of great interest and should help to develop new resistant cultivars or find compounds protecting plants against viral infections. In Chapter 4, we demonstrate that dsRNA is perceived as PAMP by Arabidopsis. Double-stranded RNA is produced during virus replication and we assume that its perception is controlled by a PRR receptor, likely localized to the cytoplasm or intracellular membrane compartments. We show that dsRNA application can elicit many of the typical MAMP responses such as the production of ET, activation of MAPKs and defense gene induction, collectively leading to an arrest in seedling growth (Chapter 4; Fig. 4.1; Fig. 4.2; Table 4.1). The absence of a ROS burst upon application of the dsRNA analogue poly(I:C) (Appendix; Fig. A6a) and the differences in the magnitude of gene induction (Table 4.1) upon flg22 and poly(I:C) treatments, respectively, indicate that the dsRNA-elicited PTI pathway might differ from the one elicited by other MAMPs downstream of receptor activation. However, it is also possible that the production of ROS is just too weak to be measurable and the exact signaling transduction pathway remains to be investigated more deeply.

Moreover, our data provide evidence that the PTI pathway initiated by dsRNA perception interacts with the RNA silencing pathway and that complex regulations work during a viral infection (Chapter 4; Fig. 4.4). These interesting observations imply a negative cross-talk between RNA silencing and plant immunity but the exact mechanisms of this interference remain elusive and should be further investigated.

It is known from animal systems that PRRs can signal from intracellular membrane systems and that the dsRNA-binding receptor TLR3 localizes to the plasma membrane and endosomal compartments (de Bouteiller et al., 2005; Itoh et al., 2008; Vercammen et al., 2008a; Qi et al., 2012). By analogy, membrane-bound plant receptor proteins have been shown to be able to signal from endosomes during immunity and development (Geldner et al., 2007; Bar and Avni, 2009b; Sharfman et al., 2011; Irani et al., 2012). However, as viruses are obligate intracellular pathogens, dsRNA may also be sensed by soluble, intracellular receptors in plants as a perception strategy. Obviously, our work opens the exciting perspective to identify the enigmatic receptor binding to dsRNA in plants and to study its cellular location and role in limiting virus infection in plants.

To assess the role of the dsRNA-activated PTI pathway in defense and its interaction with other responses such as RNA silencing, it is once again important to identify more signaling components involved in this process. To do so, several approaches have been proposed and started. First of all, a screen of Arabidopsis EMS-mutagenized seeds has been initiated, which could identify mutants insensitive to dsRNA. As dsRNA was shown to induce a strong inhibition of root growth upon temporally extended treatments (Chapter 4; Fig. 4.2), this effect is being employed as readout to identify insensitive mutants. In parallel, we started to analyze different accessions of *A. thaliana* for their ability to perceive the elicitor. It has been shown previously that the use of recombinant inbred lines (RILs) is a successful strategy to identify signaling components and allows exact identification of genes involved in signaling processes (Jehle et al., 2013a; Zhang et al., 2014). Thus, we aim at analyzing RILs between accessions not responsive to dsRNA and Col-0 in order to identify the actors of dsRNA perception and signaling. Thereupon, different approaches of biochemistry and cell biology could be instigated on these proteins to study their cellular localization and dynamics upon elicitation, the receptor-ligand interactions and their biological relevance in viral resistance.

Actually, in our study we have already shown that pretreatment of Arabidopsis plants with dsRNA renders them more resistant to further viral infection (Chapter 4; Fig. 4.3). Future investigations will reveal whether dsRNA signaling can be used in order to protect plants

against other pathogens such as bacteria, fungi or nematodes. The dsRNA-mediated antiviral PTI may be economically adapted to develop new crop protection strategies. This step could be achieved by generating transgenic crop plants expressing the dsRNA receptor. Upon this modification, plants, which are usually not responsive to the molecule, will probably gain the ability to detect and respond to the presence of dsRNA leading to increased resistance to infection with viral or microbial pathogens.

In addition, application of dsRNA could constitute a novel method of antiviral and possibly other disease control. As some preliminary data show that also *Nicotiana benthamiana* perceives dsRNA (Appendix; Fig. A6b), we believe that this molecule could be used to vaccinate crop plants. However, such studies on antiviral or antimicrobial resistance remain to be performed.

It is anticipated that the dsRNA elicitor can be optimized in size and chemical composition. Smaller molecules could increase the intensity of the PTI responses or resistance effects, as such a modified molecule might be better able to pass through the cell wall. These variations in the size of the dsRNA molecule could be achieved by, for instance, sonification and identification of an optimized version of the molecule might enable the utilization of smaller concentrations. In the long-term, this would help to exploit dsRNA as a cheaper, more feasible application in the field.

5.6 Concluding remarks and perspectives

During the course of this thesis, I have shown together with colleagues from England that *FLS2* is regulated in a highly tissue-specific manner in Arabidopsis roots and its expression localizes to tissues vulnerable for pathogen entry. Furthermore, I provide evidence ascribing an important role in fine-tuning the intensity of the downstream PTI response to the identity of the root tissue that transmits flg22 perception. Generally, the *FLS2* expression level is not the major element determining the magnitude of the defense response. Intriguingly, the flg22 peptide is seemingly able to penetrate the root tissues since it can be perceived by all tissues analyzed. The ubiquitous competence of all root tissues to induce PTI if *FLS2* is expressed further indicates that the necessary response machinery, including coreceptors, is present in each tissue. Moreover, differences between MAMP- and DAMP-triggered PTI responses are observed in roots, varying not only in the amplitude of the response but also with respect to the cellular localization. In general, DAMP signaling in roots may fulfill an important role in resistance against invaders.

Hence, multiple factors appear to contribute to a tissue-specific activation of root PTI responses. These factors mainly include the PRR localization/compartmentalization pattern and the differences in the capacity of specific types of tissues to respond to MAMPs or to propagated signals. These parameters may collectively control and balance the multilayered aspects and highly complex organization of the plant immune system in roots.

The transgenic Arabidopsis lines that I generated during my thesis proved suitable for studying the response competence of specific root tissues to flg22 and can be further applied to determine the contribution of each tissue to resistance against bacterial colonization. Crosses between the lines with localized expression of the *FLS2* receptor gene and PTI response markers, respectively, will help to untangle PTI signaling in a spatio-temporally resolved manner in roots. The use of these lines will further provide information about intercellular, intertissue and even systemic communication during PTI. In summary, the transgenic lines will help to unravel the role of tissue-specificity in PTI and in future, tissue-specific expression of PRRs in certain root tissues might be used to generate plants with higher resistance to soil-borne pathogens.

Interestingly, I have shown that no transcriptional modifications occur in shoots upon root treatments with MAMPs/DAMPs. The methods I developed provide a possibility to further assess systemic root-to-shoot signaling in Arabidopsis and to determine the importance of roots in whole plant immunity but also in the context of development or in regard to abiotic effects.

Simultaneously, the results I obtained in collaboration with coworkers in Basel confirmed that PTI is critical in antiviral immunity and identified dsRNA as the first viral PAMP active on roots and shoots in Arabidopsis. In addition, our data indicate that interactions and probably negative cross-talk of plant signaling networks take place in antiviral defense and are part of the complex aspects of immunity. Furthermore, dsRNA application promotes antiviral resistance against subsequent viral infection in Arabidopsis. Accordingly, its potential to protect crop plants against viral and/or microbial diseases will be further explored and may be established as a promising protective agent in agriculture.

In summary, this work provides new insights into the spatio-temporal regulation of elicitor perception, into the function, regulation and interaction of defense responses and helps to understand and further investigate the molecular mechanisms and physiological processes which lead to resistance against plant pathogens. Hence, this thesis generates knowledge with the aim to develop new means for crop protection in agriculture.

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APPENDIX

Accumulation of SERKs and activation of MAPKs upon application of crude bacterial extract in Arabidopsis roots

The presence of the FLS2 signaling partner, BRASSINOSTEROID INSENSITIVE1 (BRI1)-ASSOCIATED RECEPTOR KINASE 1 (BAK1), was confirmed in roots by Western blot (Fig. A1a). MAPK activation in roots was elicited by crude cell extracts of *Pseudomonas syringae* pv *tomato* (*Pto*) DC3000. The activity of these extracts on *fls2* mutant roots indicate that other MAMPs presented by *Pseudomonas* are recognized by Arabidopsis roots (Fig. A1b).

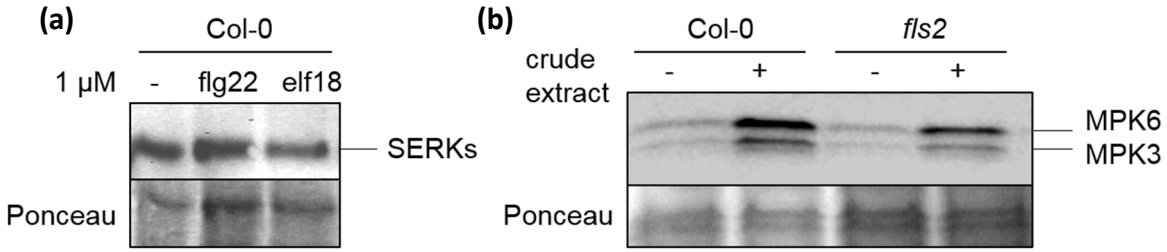

Fig. A1 Accumulation of SERKs and activation of MAPKs upon application of crude bacterial extract in Arabidopsis roots. (**a**) SERKs are strongly detectable in isolated root tissue of Arabidopsis seedlings. Isolated roots of two week-old Col-0 plants were treated with 1 µM elicitor peptide flg22, elf18 or water as a control for 15 minutes. Proteins were extracted and samples analyzed for SERK accumulation. Western blot was probed with antibodies against SERKs (Schulze et al., 2010). Ponceau staining shows equal loading control. (**b**) Crude *Pto* DC3000 extracts induce MAPK phosphorylation in isolated roots of Arabidopsis wild-type and *fls2* mutants. Bacteria grown in YEB medium were washed three times with water, resuspended in water and sonicated for 20 minutes. The cellular debris was removed by centrifugation and the supernatant was filter-sterilized (0.45 µm filters, Millipore). The obtained cell-free extract was diluted 1:10 in water and added to isolated root tissue of two week-old Arabidopsis plants. The tissue was harvested after 15 minutes and tested for MAPK phosphorylation by Western blot. Water-treated roots were used as control (Kunze et al., 2004).

Downstream PTI responses in Arabidopsis roots upon MAMP/DAMP treatments

In close collaboration with our Sinergia project partners, Silke Lehmann from the group of Jean-Pierre Métraux (University of Fribourg) and the group of Niko Geldner (University of Lausanne), we investigated the differences and characteristics of downstream PTI responses in Arabidopsis roots upon applications of distinct molecular patterns.

The accumulation of reactive oxygen species (ROS) is an early response of plant cells towards MAMPs such as flg22 or elf18. While this reaction has repeatedly been described for leaf tissue and seedlings, we compared the oxidative burst in Arabidopsis roots after treatment with 1 µM flg22, the chitin heptamer chitin7 and AtPep1 using a luminol-based assay (Fig. A2a). A significant increase in ROS accumulation was observed for all three elicitors, with flg22 and AtPep1 triggering a stronger response than chitin7. The peak of luminescence consistently appeared somewhat later in treatments with flg22 compared to samples treated with chitin7 and AtPep1.

Following elicitor perception, the phosphorylation of MAPKs transduces the signal towards downstream components. This activation of MAPKs also occurs in roots after treatment with 1 µM flg22, chitin7 and AtPep1 (Fig. A2b). The analysis of the respective receptor mutants *fls2*, c*erk1-2* and *pepr1/2* demonstrated that also in roots the activation of MAPKs by elicitor molecules depends on the previously described LRR-RLKs. In this assay, the exposure to AtPep1 led to the highest amount of phosphorylated MAPKs while lower levels were observed after treatment with flg22 and chitin7.

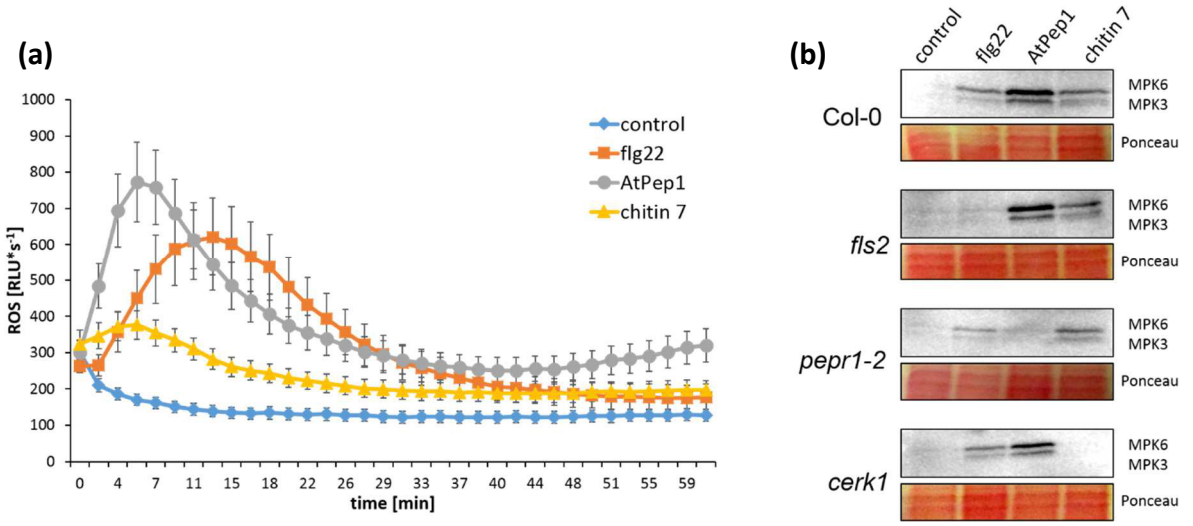

Fig. A.2 PTI responses upon flg22, AtPep1 and chitin treatments in isolated Arabidopsis roots. (**a**) ROS production in isolated three week-old roots of wild-type *Arabidopsis thaliana* seedlings, treated with 1 µM flg22, AtPep1, chitin hexamer (chitin 7) or control (0.5 x MS medium) was measured in a luminol-based assay (Chapter 2.2.5.6) for the duration of one hour. Graph shows mean values ± SE of 12 biological replicates. The experiment was repeated four times independently. RLU, relative light units. (**b**) MAPK activation was detected by Western blot as described previously (Chapter 2.2.5.7) after ten minutes of flg22, AtPep1, chitin7 or control treatments in two week-old isolated root systems of Col-0 wild-type, *fls2*, *pepr1-2* and *cerk1* mutants. Ponceau staining was used as a loading control. The pictures represent one of four independent replicates with similar results.

In order to elucidate the spatial and cellular induction patterns of downstream PTI responses upon elicitor treatments, we generated Arabidopsis wild-type plants, which express the YFP-derived fluorophore Venus (3xVenus) under the control of several defense-related promoters (*Prom:NLS-3xVenus* constructs), respectively. The pGreen229NLS3xmVenus plasmid (Vermeer et al., 2014) was used to enable *Prom:NLS-3xVenus* expression and the Venus reporter contains a nuclear localization sequence that facilitates the detection of the fluorescent signal at a cell-specific level. The selected promoters were representative for early induced MAMP genes, markers for hormonal pathways as well as markers for ROS and wounding. However, in this chapter, I will focus on the root PTI markers *PER5*, *MYB51* and *WRKY11*. *MYB51pro* and *WRKY11pro,* which have previously been shown to be induced in Arabidopsis roots upon MAMP treatments (Millet et al., 2010). *PER5* has been characterized as a strong transcriptional marker for PTI in Arabidopsis during my thesis (Wyrsch et. al. 2015; Chapter 2.2.7; Fig. S2.8).

Homozygous lines expressing the indicated constructs, respectively, were investigated for enhanced Venus fluorescence protein signal upon elicitor treatments by confocal microscopy. Our experiments revealed that the promoter activities were differentially induced upon flg22,

chitin7 and AtPep1 treatments. Remarkably, and consistent with the results obtained after measuring early PTI responses upon elicitor treatment in roots (see paragraph above) the intensity of the promoter activity depended on the type of MAMP/DAMP applied. The DAMP AtPep1 induced stronger *WRKY11* and *MYB51* promoter activity, whereas the MAMPs flg22 and chitin7 led to moderate expression of these promoters (Fig. A3).

Moreover, we did not only observe variations in the intensity of the response but also tissue-specific effects of the promoter activation (Fig. A3; Fig. A4). Activities of promoters were distinctly regulated in longitudinal developmental root zones as well as transversal tissue layers. Chitin7 induced promoter activity mainly restricted to the differentiation zone, whereas flg22 and AtPep1 treatment led to strong transcriptional changes also in the elongation zone (Fig. A4). Furthermore, whereas flg22 generally elicited the promoters of the PTI marker genes more in epidermal, outer tissues and cells, AtPep1 induced promoter activities more in inner, vasculature tissues, except for *PER5pro* (Fig. A3; Fig. A4). These results indicate that, despite similar downstream responses may get activated in roots upon elicitation with different elicitors, the intensity and localization patterns of the responses vary.

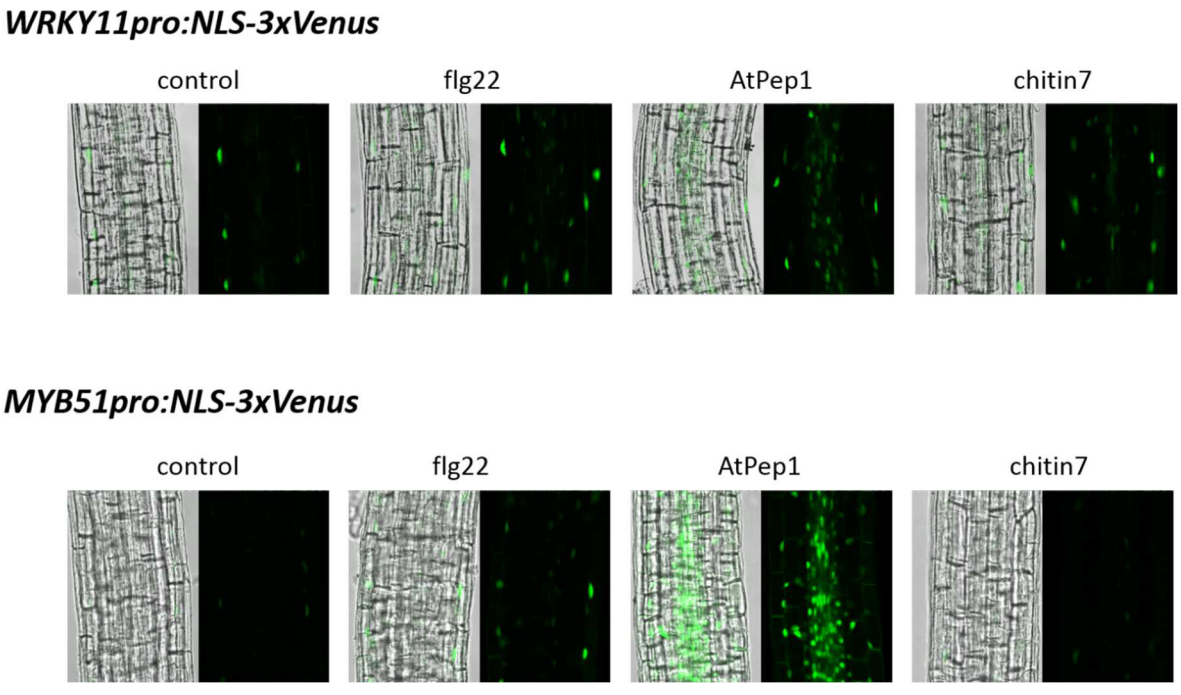

Fig. A3 *WRKY11pro:NLS-3xVenus* and *MYB51pro*:*NLS-3xVenus* induction in transgenic Arabidopsis roots. *WRKY11pro:NLS-3xVenus* and *MYB51pro:NLS-3xVenus* induction was analyzed by confocal microscopy in five day-old transgenic Arabidopsis seedling roots upon six hours of treatment with the indicated elicitors (at 100 nM concentration) in the elongation zone. The pictures were taken by Silke Lehmann.

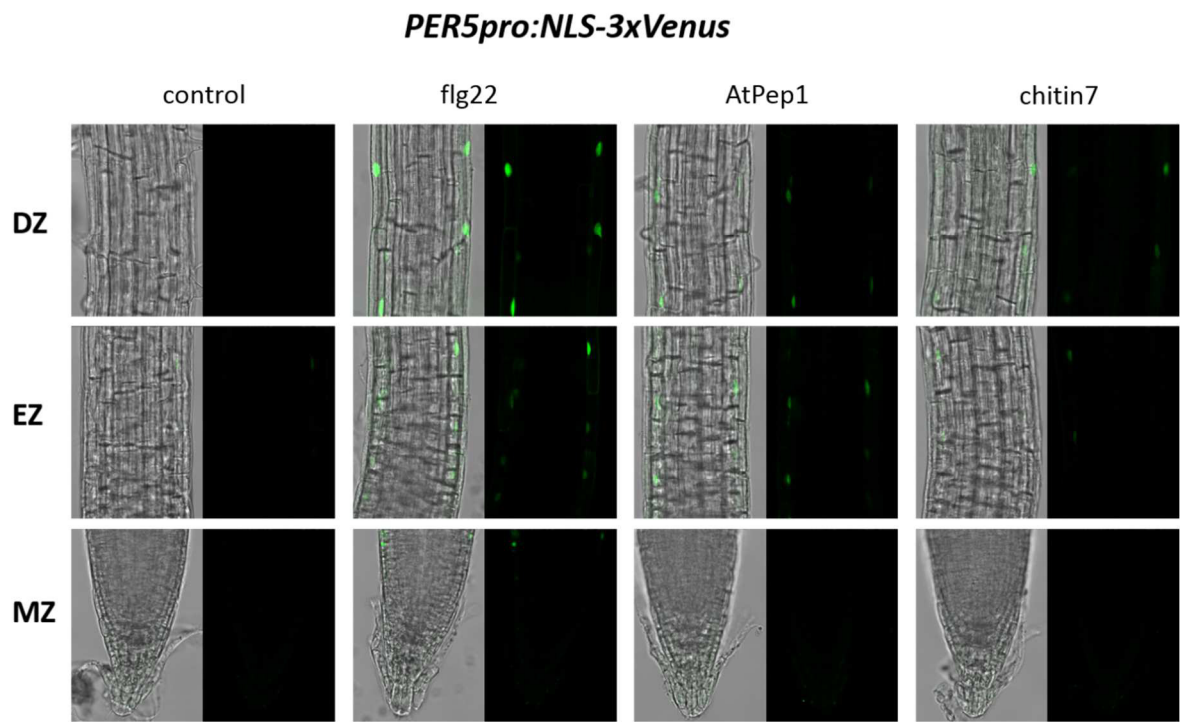

Fig. A4 *PER5pro:NLS-3xVenus* induction in transgenic Arabidopsis roots. *PER5pro:NLS-3xVenus* induction was analyzed by confocal microscopy in five day-old transgenic Arabidopsis seedling roots upon six hours of treatment with the indicated elicitors (at 100 nM concentration). DZ = differentiation zone, EZ = elongation zone, MZ = meristematic zone. The experiment was performed by Silke Lehmann.

When confirming the microscopy data by qRT-PCR, we were able to highlight the advantages of our generated, transgenic lines for accurate spatial determination of expression patterns of downstream responses upon MAMP perception. As whole roots were used for qRT-PCR assays, the induction differences measured by qRT-PCR do not necessarily reflect the observed differences in the expression distribution of marker genes along the longitudinal root axis or the radial cross section of the root (Fig. A3; Fig. A4; Fig. A5).

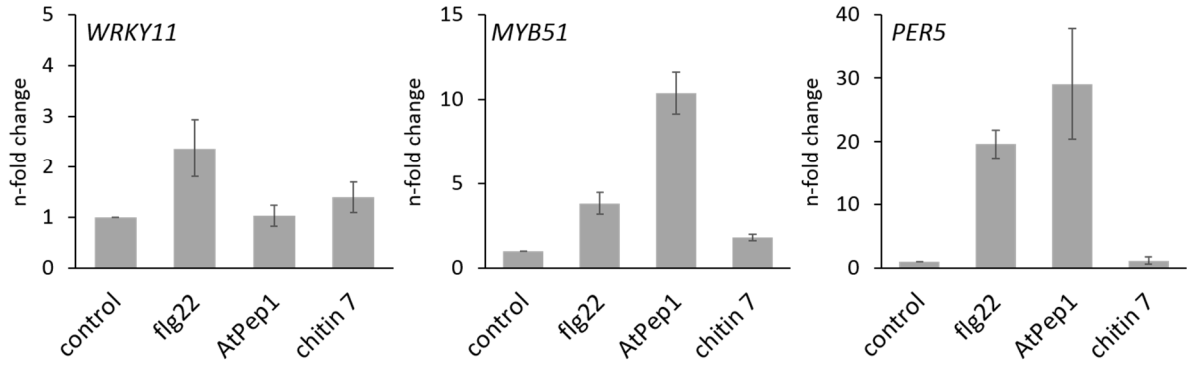

Fig. A5 Transcript levels of marker genes in Arabidopsis wild-type roots measured by qRT-PCR. Transcript levels of marker genes in isolated two week-old root tissues treated with 1 µM flg22, AtPep1, chitin7 or without peptide (control). Transcript levels of *WRKY11*, *MYB51* and *PER5* were measured by qRT-PCR and first normalized to that of the reference gene *UBQ10* before calculation of expression relative to that of the control. The bars represent the mean of four biological replicates. Error bars show ± SE of the mean. The exact protocol can be found in Chapter 2.2.5.8 of this thesis.

In summary, the tools generated in this study allow to gain more specific information about the intensity and localization patterns of marker gene induction in roots at a cellular resolution. Conclusively, this method presents a fast, non-destructive *in vivo* readout for transcriptional MAMP-triggered downstream responses.

ROS production in *Arabidopsis thaliana* and MAPK phosphorylation in *Nicothiana benthamiana* upon treatment of leaves with poly(I:C)

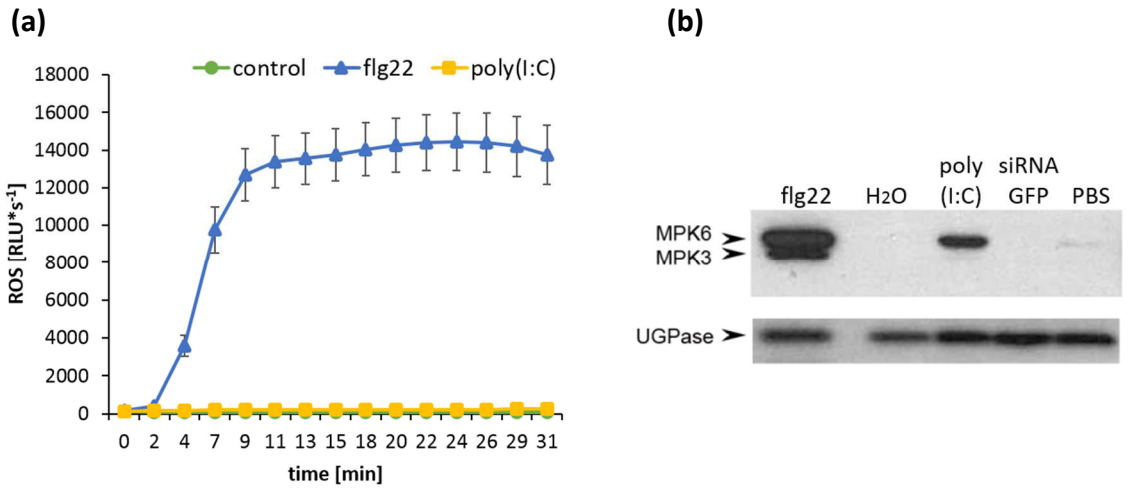

Fig. A6 ROS production in *Arabidopsis thaliana* and MAPK phosphorylation in *Nicothiana benthamiana* upon treatment of leaves with poly(I:C). (**a**) ROS production in leaf discs of Col-0 wild-type *Arabidopsis thaliana* seedlings, treated with flg22, poly(I:C) or control (PBS) was measured in a luminol-based assay (Chapter 2.2.5.6) for the duration of one hour. Graph shows mean values ± SE of 12 biological replicates. The experiment was repeated two times independently. RLU, relative light units. (**b**) Immunoblot to detect MAPK phosphorylation in *Nicothiana benthamiana* leaf discs treated with flg22, H_2O, poly(I:C), GFP siRNA, or PBS. The blot was probed with an antibody against phosphorylated MAPKs. As equal loading control, the immunoblot was re-probed with anti-UGPase antibody (for exact protocol see Chapter 4.6.6.1).