Molecular components of PAMP-triggered oxidative burst in plant immunity

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Abbreviations

::	fused to (in context of gene fusion constructs)
% (v/v)	volume percent
% (w/v)	weight/volume percent
3’	downstream region (of a gene or sequence)
5’	upstream region (of a gene or sequence)
µ	micro
A	adenine
aa	amino acid
ABA	abscisinic acide
ACC	1-aminocyclopropane-1-carboxylic acid
APS	ammonium persulfate
ATP	adenosine trisphosphate
At, A.th.,	Arabidopsis thaliana
Arabidopsis	Arabidopsis thaliana
Arg	arginine
AVG	amino-vinyl glycine
avr	avirulence
°C	degrees Celsius
Ca^{2+}	calcium ions
cfu	colony formin units
cDNA	copy DNA
CEBiP	chitin oligosaccharide elicitor-binding protein
Col-0	Arabidopsis thaliana ecotype Columbia-0
C-terminus	carboxy terminus
dH_2O	de-ionized water
DMSO	dimethyl sulfoxide
DNA	desoxy ribonucleic acid
dNTPs	desoxyribonucleotides
dpi	days post inoculation
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetate
EFR	EF-Tu receptor
EMS	ethyl methane sulfonate, or methane sulfonic acid ethyl ester
ET	ethylene
ETI	effector-triggered immunity
flg	flagellin
FLS2	flagellin sensing receptor 2
G	guanine
h	hour
H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical
hpi	hours post inoculation
HR	hypersensitive response
HRP	horse radish peroxidase
JA	jasmonic acid
K	kilo
kb	kilo base
kD	kilo Dalton
l	liter
LRR	leucine-rich repeats
m	milli

M	molar (mol/l)
MAP4	mammalian microtubule-associated protein 4
MAPK	mitogen activated protein kinase
MeOH	methanol
min	minutes
mRNA	messenger RNA
n	nano
NADPH	Nicotinamide adenine dinucleotide phosphate
NASC	Nottingham Arabidopsis Stock Centre
Nb	Nicotiana benthamiana
Nt	Nicotiana tabacum
N-terminus	amino terminus
OD	optical density
Os	Oryza sativa
O2	oxygen
O_2^-	superoxide anion
P	probability value
p35S	promoter of Cauliflower mosaic virus promoter 35S
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
pH	negative logarithm of proton concentration
PRR	Pattern-recognition receptor
Pfu	Pyrococcus furiosus
PM	plasma membrane
PTI	PAMP-triggered immunity
pv.	pathovar
RT-PCR	reverse transcription-polymerase chain reaction
RbohD	Respiratory burst oxidase homologue D
RbohF	Respiratory burst oxidase homologue F
RbohC	Respiratory burst oxidase homologue C
RLK	receptor-like kinase
RLP	receptor-like protein
ROS	reactive oxygen species
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	seconds
SA	salicylic acid
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Taq	Thermophilus aquaticus
TBS	tris buffered saline
TBS-T	TBS with 0,5% Tween-20
TEMED	N,N,N',N'-Tetramethylethylenediamine
u	(enzymatic) unit
V	volt
v	volume
w	weight
WT	wild-type

Publications

Mersmann, S., Salomon, S., Vetter, M., and Robatzek, S. (2008) Selbst oder Nicht-Selbst–Pflanzliche Immunrezeptoren. BIOspektrum 14, 6, 593-596.

Goehre, V., Spallek, T., Haeweker, H., Mersmann, S., Mentzel, T., Boller, T., Torres, M., Mansfield, J.W., Robatzek, S. (2008) Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. Current Biology, 18, 1824-1832.

Mersmann, S., Rietz, S., Chinchilla, D., and Robatzek, S. (2009) Ethylene signaling functions in NADPH oxidase RbohD mediated oxidative burst required for plant immunity. (submitted)

Summary

Reactive oxygen species (ROS) are important molecules that are rapidly generated in response to abiotic and biotic stimuli and which regulate diverse physiological processes such as stomatal aperture and cell death. Plant immunity involves the detection of pathogen-associated molecular patterns (PAMPs) through cognate pattern recognition receptors (PRRs). Perception of PAMPs induces an extracellular oxidative burst, which requires the function of the NADPH oxidase *At*RbohD. However, little is known about the regulation of PAMP-elicited ROS and its role in plant PAMP-triggered immunity. We investigated ROS production mediated by the FLS2 receptor kinase responsible for the detection of bacterial flagellin (flg22) in Arabidopsis to elucidate components of early flg22 signaling. Rboh proteins are assumed to predominantly mediate extracellular ROS production in plants.

We observed *At*RbohD to be rate limiting for flg22-elicited ROS production. Moreover, bacterial multiplication monitored at early stages of infection with a disarmed but pathogenic *Pseudomonas syringae* strain indicated ROS accumulation important for plant immunity.

A forward genetic screen led to the isolation of *rio1* to *rio5* mutants, all of which were severely reduced in flg22-induced ROS production. *Rio1*, *rio2* and *rio3* carry mutations in *FLS2* and its co-receptor *BAK1*, respectively. The responsible gene mutations of *rio4* and *rio5* remain to be identified in a subsequent mapping approach.

A candidate gene approach revealed that flg22-stimulated oxidative burst was specifically inhibited in ethylene signaling mutants. Impaired ROS production in *etr1* and *ein2* mutants could be partially rescued by chemical interference with ethylene accumulation. Notably, wounding partially complemented the ROS reduced phenotype. Furthermore, accumulation of FLS2 but not BAK1 was in part dependent on ethylene signaling. Bacterial multiplication at early time points was significantly enhanced in ethylene signaling mutants indicating the importance of PAMP-triggered ROS production in plant defence responses.

Zusammenfassung

Reaktive Sauerstoff Spezies (ROS), deren Generierung durch abiotische und biotische Stimuli initiiert wird, sind für die Regulierung diverser physiologische Prozesse wichtige Moleküle, wie zum Beispiel der Öffnung von Stomata oder dem Zelltod. Die pflanzliche Immunität basiert auf der Erkennung von Pathogen assoziierten molekularen Mustern (PAMPs) durch Muster Erkennungs Rezeptoren (PRRs). Die Perzeption von PAMPs elizitiert einen extrazellulären oxidative Burst, der die Funktion der NADPH Oxidase *At*RbohD erfordert. Bisher ist wenig über die Regulierung PAMP-induzierten ROS, sowie dessen Rolle in der planzlichen Immunität bekannt. Die Rezeptor Kinase FLS2 perzeptiert und bindet bakterielles Flagellin (flg22) und vermittelt dadurch die Produktion von ROS. In dieser Arbeit untersuchen wir die FLS2 induzierte ROS Generierung, um Komponenten, die diese Signaltransduktion vermitteln, zu finden. Die Produktion extrazellulären ROS wird hauptsächlich durch Rboh Oxidasen bewerkstellingt und zeigt die Wichtigkeit dieser Proteine.

Unsere Untersuchung ergab, dass *At*RbohD die limitierende Oxidase für den flg22-vermittelen oxidativen Burst ist. Überdies deuten unsere Ergebnisse darauf hin, dass ROS, welche durch *At*RbohD generiert werden, entscheidend zur pflanzlichen Immunität beitragen, da die bakterielle Multiplikationsrate eines abgeschwächt pathogenen *Pseudomonas* Stammes in frühen Zeitpunkten der Infektion signifikant erhöht ist.

Mittels einer genetischen Untersuchung konnten wir die Mutanten *rio1* bis *rio5* isolieren, die alle eine Reduktion der ROS Produktion aufweisen. Weitere Untersuchungen ergaben, dass *rio1*, *rio2* und *rio3* Mutationen in entweder *FLS2* oder dessen Korezeptor *BAK1* tragen. Die Gene, die die Mutationen aufweisen, welche für die Phänotypen von *rio4* und *rio5* verantwortlich sind, konnten bisher nicht identifiziert werden. Das deutet darauf hin, dass *rio4* und *rio5* neue Komponenten der Signaltransduktion repräsentieren. Weitere genetische Verfahren sind erforderlich, um die Identität der betroffenen Gene zu analysieren.

In einem weiteren genetischen Ansatz mit bisher bekannten Mutanten konnten solche, die Ethylen insensitiv sind, als reduziert in der flg22-vermittelten ROS Produktion gefunden werden. Die Verminderung der ROS Generierung in *etr1* und *ein2* Mutanten konnte teilweise durch Ethylen-Biosynthese Inhibitoren aufgehoben werden. Interessanterweise wurde die Reduktion der ROS Generierung in verwundeten Blättern der *etr1* und *ein2* Mutanten nicht beobachtet. Die Akkumulierung von FLS2 aber nicht von BAK1 war anteilig von der Ethylen Signaltransduktion abhängig. Die bakterielle Multiplikationsrate in frühen Zeitpunkten der Infektion war in *etr1* und *ein2* Mutanten erhöht. Das impliziert die Relevanz der PAMP-vermittelter ROS Produktion in der pflanzlichen Immunität.

1 Introduction

1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are constantly produced and represent versatile molecules required for metabolic processes, signal transduction pathways, gene expression and responses to abiotic and biotic stress (Apel and Hirt, 2004; Pitzschke, 2006, Torres et al., 2006). Generation of ROS occurs in different intracellular compartments, predominantly chloroplasts but also peroxisomes and mitochondria (Apel and Hirt, 2004). Partial reduction of relatively unreactive ground state dioxygen gives rise to superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxy radical ($^{\cdot}OH$) generation, which herein is collectively referred to as ROS. The intrinsic cytotoxic character of ROS implies a tight control to balance an equilibrium in order to avoid oxidative damage of proteins, DNA and membrane lipids (Mittler et al., 2004). Enzymatic and non-enzymatic detoxification mechanisms thus are up-regulated upon ROS detection and ensure physiological ROS steady-state levels (Apel and Hirt, 2004). Abiotic and biotic stresses can perturb the physiological equilibrium between ROS production and scavenging leading to a rapid increase of intracellular and extracellular ROS levels. Moreover multicellular organisms intentionally produce ROS, which indicates the importance of these chemicals. Plants employ a rapid ROS induction to adjust signal transduction pathways in order to combat adverse environmental factors (Lamp and Dixon, 1997). A fast and transient increase in ROS, also termed “oxidative burst”, represents one of the most rapid responses upon abiotic and biotic challenge (Wojtaszek, 1997; Bolwell et al., 1998).

1.2 ROS generating systems

Chloroplasts represent a major source of ROS generation. During photosynthesis direct photoreduction of O_2 leads to formation of ROS (Stitt, 1991). Abiotic stresses, such as high light intensities and extreme temperatures result in changes in the redox status of chloroplasts and causes elevated intracellular ROS levels. This may induce photooxidative damage if ROS accumulation exceeds the capacity of antioxidant systems (Foyer, 1994; Foyer and Noctor, 2005). Besides plastids, plants posses many potential sources to generate ROS, which can synergistically feed into one particular response mechanism. ROS accumulation following ozone exposure is partly mediated in a chloroplast dependent manner, but is also generated by membrane bound components (Joo et al., 2005). ROS generating molecules include e.g. apoplastic peroxidases, amine oxidases or oxalat oxidases (Bolwell, 1998, Lherminier et al., 2009). However, plasma membrane resident homologs of

the mammalian phagocyte NADPH oxidase 2 (NOX 2), Rboh proteins, are assumed to predominantly mediate extracellular ROS production in plants (Desikan et al., 1998; Keller et al., 1998; Torres et al., 1998, Foreman et al., 2003; Kwak et al., 2003; Joo et al., 2005; Gapper and Dolan, 2006). Pharmacological studies have often indicated Rboh oxidases to contribute to ROS production, however, only recently their role was genetically confirmed (Torres et al., 2002).

The mammalian respiratory burst NADPH oxidase represents a multi protein complex. It encloses subunits $gp91^{phox}$ (NOX2) and $p22^{phox}$ that coexist in a heterodimer, the cytosolic proteins $p67^{phox}$, $p47^{phox}$ and $p40^{phox}$ pre-assembled in a heterotrimeric complex, and a small GTP-binding protein (Rac) (Wojtaszek, 1997; DeCoursey, 2003). ROS production is initiated upon assembling of the individual oxidase subunits, and in particular, the Rac protein serves as activator. ROS generation requires continuous phosphorylation events and persistent production of NADPH (DeCoursey and Ligeti, 2005). Complex disassembling terminates ROS accumulation and is presumptively mediated by changes in the cellular redox status and subsequent membrane depolarization. Moreover, dephosphorylation events and a rapid protein turn over are presumed to have attenuating functions (DeCoursey and Ligeti, 2005).

The Arabidopsis genome encodes 10 Rboh oxidases, of which *At*RbohC/RHD2, *At*RbohD, and *At*RbohF are best characterized, and were found to be induced upon abiotic and biotic stresses (Torres and Dangl, 2005; Sagi and Fluhr, 2006). These oxidases carry 6 predicted transmembrane-spanning domains containing an N-terminal extension that comprises EF-hand Ca^{2+} binding motifs and potential phosphorylation sites. At the C-terminus, Rboh proteins contain FAD and NADPH binding domains (Sagi and Fluhr, 2006; Kobayashi et al., 2007). N-terminal phosphorylation concomitant with Ca^{2+} binding to its EF-hand motifs activates plant oxidases (Ogasawara et al., 2008). An electron transport chain, using NADPH as a donor and oxygen as an acceptor, generates O_2^-, which rapidly converts into H_2O_2 and $^{\cdot}OH$ either enzymatically or spontaneously (Lamp and Dixon, 1997). In addition, heterotrimeric G-proteins and small G-proteins contribute to activation of plant Rboh proteins (Kawasaki et al., 1999; Morel et al., 2004; Joo et al., 2005, Wong et al., 2007). Termination of ROS production is dependent on Rac-GTPases (Wong et al., 2007). In rice, the active state of Rboh oxidases is determined by the interaction with Rac1. Elevated cytoplasmic Ca^{2+} levels that are probably generated through a H_2O_2- mediated Ca^{2+} influx, disrupt this interaction, which consequently terminates ROS accumulation.

1.3 ROS signaling

The redox state of chloroplasts serves as environmental sensor and integrates abiotic stimuli into physiological signaling events. Increased ROS, in particular H_2O_2, serves as a systemic signal. It activates protective mechanisms in neighbouring chloroplasts to induce and control systemic acquired acclimation to changing environments (Karpinski et al., 1999). Within a time frame of 5 min to 1 h ROS signaling also occurs across cell boarders (Joo et al., 2005). Upon ozone exposure the rapid increase in ROS production becomes first visible in guard cells. ROS accumulation then spreads to the periphery of nearby epidermal pavement cells, before it fully expands to cytoplasm and chloroplasts in epidermis cells surrounding stomata. Chloroplast resident enzymes as well as Rboh oxidases are suggested to be responsible for ROS signal propagation (Joo et al., 2005).

The stomatal complex is build by paired guard cells, which are morphologically distinct from general epidermal cells and are mechanistically separate from surrounding cells (Assmann and Wang, 2001; Franks and Farquhar, 2007). Guard cells react to external cues e.g. photosynthetic effective illumination, and respond to internal signals such as hormones and adapt stomata conductance accordingly (Lawson, 2009). ROS contributes to stomatal closure in an ABA-dependent manner (MacRobbie, 1998; Pei et al., 2000; Schroeder et al., 2001). In particular H_2O_2 but also nitric oxide (NO) are required for ABA-induced stomatal closure (Kwak et al., 2003; Desikan et al., 2002; Neill et al., 2003). In addition to a possible synergistic function of ROS and NO, NO is likely to interact with H_2O_2 giving rise to peroxynitrite ($ONOO^-$), another ROS assumed to have a role in plant defence responses (Delledonne et al., 2001, Neill et al., 2003). Stomatal closure is also induced upon plant perception of bacterial traits (Melotto et al., 2006), however, the underlying molecular mechanism yet remains to be elucidated.

Bacteria and yeast sense elevated production of ROS and utilize ROS as signaling molecules (D'Autéaux and Toledano, 2007). Likewise in plants, evidence arises that ROS serve as signaling molecules. In particular, H_2O_2 is thought to fulfil this function and to feeds into signaling pathways in a cell autonomous and/or non-autonomous manner (Desikan et al., 2001; D'Autéaux and Toledano, 2007, Miller et al., 2009). H_2O_2 represents a mild oxygen and can interact with thiol residues in proteins (Neil et al., 2003). Modifications of thiols, in turn, might mediate recognition of H_2O_2, implicating certain proteins as potential ROS sensors (Apel and Hirt, 2004, Desikan et al., 2005). Movement of H_2O_2 across membranes is presumed to be facilitated by aquaporins (Costet et al., 2002, Bienert et al., 2006, 2007).

H_2O_2 influences responses to abscisic acid (ABA) by activating plasma membrane located Ca^{2+} channels and modifies activity of phosphatases (Pei et al., 2000; Murata et al., 2001; Meinhard et al., 2001, 2002). Moreover, H_2O_2 stimulates mitogen-activated protein kinases (MAPKs; Desikan et al., 1999; Grant et al., 2000; Kovtun et al., 2000), increases expression of components involved in the phytochrome signaling pathway (Moon et al., 2003), activates serine/threonine kinases (Rentel et al., 2004) and interacts with two-component histidine kinases (Desikan et al., 2005). Microarray

analysis revealed more than 100 genes, whose transcript levels were upregulated in response to exogenous applied H_2O_2 (Desikan et al., 1998; Grant et al., 2000, Desikan et al., 2001). Interestingly, most genes code for components involved in stress responses or antioxidant mechanisms.

1.4 ROS-mediated processes

ROS is an important regulator of plant development (Rodriguez et al., 2002; Sagi et al., 2004). It contributes to cell wall loosening necessary for organ expansion in root growth, root-hair formation and pollen-tube growth (Foreman et al, 2003; Potocký et al., 2007). However, if organ formation and cell growth ceases, ROS contributes to processes that strengthen cell barriers conferring robustness e.g. to impede pathogen penetration (Gapper and Dolan, 2006). This dual role requires a spatial and temporal regulation of ROS.

Programmed cell death (PCD), initiated as part of plant growth and developmental programmes but also in response to environmental triggers such as pathogen attack, is often associated with a massive accumulation of ROS (Greenberg 1997; Pennell and Lamb, 1997; Van Breusegem and Dat, 2006). Relative rates of H_2O_2, O_2^- and $ONOO^-$ are suggested to drive PCD (Delledonne et al., 2001; Neill et al., 2006). In plant defence, rapid biphasic apoplastic accumulation of ROS is associated with pathogen-triggered PCD, potentially to prevent spread of infections (Doke, 1985; Apostol et al., 1989; Apel and Hirt, 2004). However, only the second, prolonged peak of ROS generation is required for PCD. Function of the first, comparatively weak peak, is often stated to be an unspecific event, but less well understood (Lamb and Dixon 1997). Noteworthy, PCD requires concomitant downregulation of detoxification mechanisms, extracellular ROS generation alone proved not to be sufficient to trigger cell death (Croft et al., 1990, Apel and Hirt, 2004).

ROS detoxification and scavenging is mainly mediated by superoxid dismutase, ascorbate peroxidase, catalase, glutathione peroxidase and peroxiredoxin, as well as ascorbic acid and glutathione, tocopherol, flavonoids, alkaloids and carotenoids (Mittler et al., 2004). ROS scavengers are present in almost all subcellular compartments, which is in agreement with differently localized sources of ROS generation and H_2O_2 lipid solubility. Scavenger transporters are central to maintain an equilibrium of the cellular redoxpotential (Foyer and Noctor, 2005).

1.5 Active plant immune responses

Unlike animals, plants solely rely on innate immunity, to combat bacterial infection (Hammond-Kosack and Parker, 2003). Phytopathogens can be separated into biotrophs and necrotrophs. Biotrophs gain nutrients from living host plant tissue, whereas necrotrophic pathogens access nutrients from dead or dying cells (Glazebrook, 2005). According to the life-style of pathogens plant defence responses need to be specifically adjusted to restrict colonization. For instance, PCD will be effective to combat biotrophic pathogens but will be less harmful to limit growth of necrotrophs.

The plant innate immune system is composed of several layers. In addition to passive physical barriers, the first layer of active defence is constituted by plasma membrane localized pattern recognition receptors (PRRs) that enable to distinguish between self and non-self. PRRs detect microbes on the basis of highly conserved constituents, so called pathogen-associated molecular patterns (PAMPs) and subsequently initiate PAMP-triggered immunity (PTI; Boller and Felix, 2009). PTI encloses rapidly induced and transient defence responses preventing host cell damage. However, pattern recognition not only refers to the detection of potential pathogens but is also assumed to account for perception of symbiotic traits (Zhao and Qi, 2008). In plant-symbiont interactions, PTI is attenuated and modified to allow colonization (Zhao and Qi, 2008).

Pathogenic microorganisms have evolved secretion systems to deliver effector proteins into plant cells in order to evade PTI and to successfully colonize plant tissues (Chisholm et al., 2006). Effector proteins promote pathogenicity by suppressing PAMP-mediated defences and render plants into susceptible hosts. To counteract bacterial effectors plants developed a second layer of plant innate immunity. A range of primarily intracellular plant immune receptors, resistance (R)-proteins, mediate detection of secreted effector proteins. Recognition thereby occurs in a plant-cultivar and strain-specific manner and induces effector-triggered immunity (ETI). The largest group of R-proteins encode nucleotide-binding site leucine-rich repeat (NB-LRR) class of proteins (Dangl and Jones, 2001). NB-LRRs can be subdivided in Toll-interleukin (IL)-1 receptors (TIR-NB-LRR) and coiled-coil domain containing R-proteins (CC-NB-LRR). ETI includes hypersensitive response (HR)-type PCD to restrict pathogen access to water and nutrients and to avoid further spread of infection (Dangl and Jones, 2001; Glazebrook, 2005; Chrisholm et al., 2006). Thus, in contrast to PTI, ETI causes plant cell damage.

Plant innate immunity also includes generation of plant stress hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene, which are under negative cross-talk and whose function contribute to resistance (Dong, 1998; Kunkel and Brooks, 2002; Glazebrook, 2005). Generation of stress hormones enables plants to acquire systemic resistance impeding secondary infections (Durrant and Dong, 2004). SA-mediated signaling confers this type of immunity mostly to biotrophic microbes. Induced systemic resistance, a responses to rhizobacteria, appears SA-independent and is mediated through jasmonate and ethylene responsiveness (Pieterse et al., 1998).

1.6 Receptors mediating PAMP-triggered defence responses

Most PRRs are membrane resident and belong to the group of single transmembrane receptor kinases (RKs) or receptor proteins (Hammond-Kosack and Jones, 1996; Gomez-Gomez and Boller, 2000; Shiu and Bleeker, 2001; Kunze et al., 2004). RKs build a large subgroup of molecules that represent 2.4% of the whole Arabidopsis protein coding genes (Shiu and Blecker, 2003). RKs comprise an N-terminal extracellular signal sequence, a transmembrane region and a C-terminal kinase domain. Whereas animals exhibit prevalence of receptor tyrosine kinases, in plants almost all receptor kinases carry serine/threonine specificity (Becraft, 2002). RKs can be classified due their extracellular domains, of which LRRs represent the most frequently found motif (Shiu and Bleeker, 2001). LRR-RKs contribute to plant growth and development, e.g. CLAVATA 1, which is involved in meristem development (Clark et al., 1996), ERECTA contributing to organ shape (Torii et al., 1996) as well as Brassinosteroid Insensitive 1 (BRI1) and BRI1-associated kinase 1 (BAK1) that mediate perception of the plant hormone brassinosteroid (Li and Chory, 1997). Yet, only few LRR-RKs have been functionally characterized. In Arabidopsis the Flagellin Sensing 2 (FLS2) and the EF-Tu-receptor (EFR) were identified to function as PRR (Zipfel et al., 2004; Zipfel et al., 2006). In rice Xa21 likewise act as PRR (Song et al., 1995).

PRRs posses specificity and sensitivity for respective ligand detection and target functional important, and highly conserved microbial pattern (Boller and He, 2009). FLS2 detects and physically interacts with flg22, the 22 amino acid elicitor active epitope of bacterial flagellin (Felix et al., 1999, Gomez-Gomez and Boller, 2000; Chinchilla et al, 2006). EFR mediates perception of elf18, which represents the active epitope of bacterial EF-Tu (Zipfel et al., 2006). Unlike flg22, which is perceived by most plant species, elf18 is only sensed by *Brassicaceae* (Felix et al., 1999; Kunze et al., 2004; Robatzek et al., 2007; Hann and Rathjen, 2007; Takai et al., 2008). However, recognized flagellin epitopes differ in a plant species-dependent manner (Bauer et al., 2001; Chinchilla et al., 2006).

Characteristic to PRRs RKs is the occurrence of a so called non-RD motif within the catalytic loop of the kinase domain, which implies co-receptor requirement (Dardick and Ronald, 2006). BAK1 comprises an active RD kinase and belongs to a group of five somatic embryogenesis-related kinases (SERKs; Shiu and Bleeker, 2003; Dardick and Roland 2006). BAK was first identified as component involved in hormone singnaling. It associates with BRI1 and reciprocal activation of both proteins induces brassinosteroid signaling (Russinova et al., 2004; Wang et al., 2008). However, BAK1 also functions in a brassinosteroid independent manner and controls host PCD in response to necrothrophic pathogens (Kemmerling et al., 2007). Moreover, *bak1* mutant plants show reduced responses to flg22 and elf18 (Chinchilla et al., 2007). Upon flg22 treatment, BAK1 rapidly forms an inducible complex with FLS2, required for full activation of subsequent defence responses. However, BAK1 appears to control signaling triggered by several different LRR-

RKs and presumptively represents a general regulatory adapter protein (Chinchilla et al., 2007; Kemmerling et al., 2007; Schwessinger and Zipfel, 2008).

The biological importance of FLS2 in plant immunity is demonstrated by Arabidopsis *fls2* mutant plants, which are clearly more susceptible than wild-type (WT) when pathogenic bacteria were spray inoculated onto leaf surfaces (Zipfel et al., 2004). Moreover, flg22 recognition mediates stomatal closure, which attributes FLS2 as a crucial component for pre-invasive immunity (Melotto et al., 2006). EFR likewise confers resistance to pathogens, which is illustrated by an enhanced susceptibility of *efr* mutant plants, when challenged with *Agrobacterium tumefaciens* (Kunze et al., 2004; Zipfel et al., 2006).

In addition to FLS2 and EFR, molecules exhibiting a different architecture or N-terminal organization were identified to operate as PRRs. Lysine motif (LysM) domains are essential for fungal chitin detection (Zhang et al., 2007). The Arabidopsis LysM-RK CERK1 mediates fungal chitin elicitor signaling (Miya et al., 2007). In rice, chitin detection is triggered by the LysM-receptor protein (RP) CEBiP (Kaku et al., 2006). Chitin serves as a classical PAMP and its detection triggers a plethora of defence responses (Felix et al., 1993, Eckard, 2008). Tomato LeEIX2 represents another RP, which detects a *Trichoderma viride* derived ethylene-inducing-xylanase (EIX; Ron and Avni, 2004).

1.7 PAMP mediated defence responses

PAMPs elicit an array of defence responses, which can be separated by their respective kinetics (Felix et al., 1993; Boller and Felix, 2009). Immediate early events occur within seconds to few minutes upon PAMP detection. Theses events include opening of ion channels to allow ion fluxes across the plasma membrane resulting in extracellular medium alkalinization. Changes in the membrane potential are following, probably facilitated by a change in ion distribution and activation of Ca^{2+} channels (Felix et al., 1999). Moreover, a MAP kinase cascade is rapidly stimulated (Felix et al., 1999; Asai et al., 2002). In response to flg22, *At*RbohD is phosphorylated and mediates immediate early ROS accumulation (Nühse et al., 2007, Benshop et al., 2007, Zhang et al., 2007). Further, NO generation follows PAMP detection (Melotto et al., 2006).

Early defence responses occur until 30 minutes upon PAMP detection and include induction of ethylene biosynthesis through activation of 1-amincyclopropane-1-carboxylate (ACC) synthase (ACS; Liu et al., 2004). Transcript levels of almost 1000 genes are induced including the PRRs themselves and the *BAK1* co-receptor, hence, establishing a positive feedback loop to accelerate or sustain PTI (Zipfel et al., 2004). A hallmark of FLS2 is its ligand dependent internalization. Within 30 minutes upon flg22 treatment FLS2 endocytosis is initiated (Robatzek et al., 2006).

Late PAMP responses represent callose deposition to strengthen plant cells and to counteract

bacterial entry. Moreover, a dose dependent arrest of seedling growth is initiated that probably reflects a physiological switch from growth to a defence program (Boller and Felix, 2009). Induction of late responses can last from hours to days upon PAMP stimulation.

Interestingly, the two different PAMP perception systems FLS2/flg22 and EFR/elf18, stimulate an almost identical set of defence responses. This indicates that common regulatory components mediate PRR elicitor signaling (Zipfel et a., 2004).

Perception of endogenous elicitors, so called danger associated molecular patterns (DAMPs), also contribute to innate immunity. DAMPs can constitute dissolved cell wall fragments, e.g. oligogalacturonides, which elicite a range of defence responses (D'Ovidio et al., 2004). DAMPs also represent components with a defined intracellular function, which when released outside of cells serve as danger signal (Rubartelli and Lotze, 2007), e.g. the release of the Arabidopsis Pep1 peptide from its precurser molecule PROPEP1 (Huffaker et al., 2006; Huffaker and Ryan, 2007). Protein processing is initiated upon cell damage or PAMP recognition. Pep1 initiates elicitor signaling through its LRR-RK PEPR1 and, moreover, serves as an endogenous amplifier of defence responses. It is also conceivable that such danger associated signaling occurs in response to volatile molecules, such as ROS.

1.8 Aim of thesis

ROS production represents one of the earliest responses upon pathogen attack and is indicated to serve as a major signal in diverse biological processes. Upon flg22 perception FLS2 elicits a rapid and transient production of ROS. Monitoring the generation of ROS enables therefore a quantitative and temporal resolution of PAMP-induced responses allowing molecular characterization of components, which contribute, induce or down-regulate PTI. Although, phosphatases may operate in ROS attenuation (Serrano et al., 2007), molecular components that link FLS2 activation to the various PAMP responses are little understood. To identify molecules important in FLS2 dependent ROS production we employed genetic analysis by a forward screen and a candidate approach. These approaches enabled characterization of molecular components regulating immediate early PAMP responses, and will further shed light on the biological relevance of the oxidative burst in PTI.

2 Material and Methods

2.1 Material

2.1.1 Plant Material

Arabidopsis wild-type and mutant lines used in this study are listed below in respective tables.

Table 1. Wild-type Arabidopsis accessions used in this study

Accession	Abbreviation	Original source
Columbia-0	Col-0	J. Dangl[a]
Landsberg-*erecta*-0	Ler-0	NASC[b]

[a] University of North Carolina, Chapel Hill, NC, USA
[b] Nottingham, UK

Table 2. Mutant and transgenic Arabidopsis lines used in this study

Mutant allele	Accession	description	Reference/Source
fls2	Col-0	T-DNA	Zipfel et al., 2004
bak1-3	Col-0	T-DNA	Chinchilla et al.,2007
pad4-1	Col-0	EMS	Glazebrook et al.,1997
rbohD	Col-0	*dSpm*	Torres et al., 2002
rbohF	Col-0	*dSpm*	Torres et al., 2002
35S::RbohD	Col-0	floral dipping	Torres et al., 2005
etr1-3	Col-0	EMS	Kieber et al., 1993
etr1-1	Col-0	EMS	Bleecker et al., 1988
ein2	Col-0	EMS	Guzmann,Ecker,1999
ctr1	Col-0	EMS	Kieber, et al., 1993
eto1	Col-0	EMS	Kieber et al., 1993
ein3	Col-0	EMS	Kieber et al., 1993
35S::ERF1	Col-0	vacuum infiltration	Solano et al., 1998
sid2-1	Col-0	EMS	Wildermuth et al2001
nudt7-1	Col-0	T-DNA	Bartsch et al., 2006
dnd1	Col-0	T-DNA	Clough et al., 2000
nutd7	Col-0	T-DNA	Bartsch et al., 2006
eds1	Col-0	FN	Bartsch et al., 2006
nahG	Col-0	floral dipping	Lawton et al., 1995
npr1-1	Col-0	T-DNA	Cao et al., 1997
abi1	Col-0	T-DNA	Amstrong et al.1995
abi2	Col-0	T-DNA	Koornneef et al., 1985
ost1	Col-0	FGT	Mustilli et al., 2002
ost2	Col-0	FGT	Mustilli et al., 2002
jar1	Col-0	EMS	Staswick et al., 2002
coi1	Col-0	EMS	Feys et al., 1994

2.1.2 Pathogens

Pseudomonas syringae pv. *tomato* (Pto) strain DC3000 ΔAvrPto/ΔAvrPtoB (Rif50, Kan50) lacking two effector proteins were used throughout this study (Rosebrock et al., 2007).

2.1.3 Oligonucleotides

Listed below are oligonucleotides used in this study that were synthesized by Invitrogen (Karlsruhe, Germany) or Operon Biotechnologies (Cologne, Germany). Working stocks were diluted to 10 pmol/ µl (=10 µM).

Primers used in this study

Primer	**Sequence (5' > 3')**
FLS2	CGCGGATCCTTATTCTTGGATCAGCCGCG
RbohD	ATGGCTCTCATATTGTTGCC
Actin	TGCGACAATGGAACTGGAATG

2.1.4 Chemicals

Laboratory grade chemicals and reagents were purchased from Sigma-Aldrich (Deisenhofen, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), and Serva (Heidelberg, Germany) unless otherwise stated.

2.1.5 Peptides

Peptides were synthesized by EZBiolab Inc. (Westfield IN, USA) with following sequences and 80 % purity:

flg22 – QRL STG SRI NSA KDD AAG LQI A

elf18 – AcSKE KFE RTK PHV NVG TIG

2.1.6 Antibiotics

Kanamycin (Kan) 50 mg/ml in dH_2O

Rifampicin (Rif) 100 mg/ml in DMSO

Stock solutions (1000x) were stored at -20° C. Aqueous solutions were sterile filtrated.

2.1.7 Media

Media were sterilized by autoclaving at 121° C for 20 min. For the addition of antibiotics and other heat labile compounds the solution or media were cooled down to 55° C. Heat labile compounds were sterilized using filter sterilization units prior to addition.

Pseudomonas syringae media

NYGA

Bactopepton 5.0 g/l

Yeast extract 3.0 g/l

Glycerol 20.0 ml/l

pH 7.0

For NYGA agar plates 1.5 % (w/v) bacto agar (Becton, Dickinson and Company, LePont de Claix, France) was added to the above medium.

***Arabidopsis thaliana* media**

MS (Murashige and Skoog) medium

MS powder including vitamins 4.4 g/l

Sucrose 10.0 g/l

pH 5.8

For MS plates 0.8 % (w/v) bacto agar (Becton, Dickinson and Company, LePont de Claix, France) was added. MS powder including nitch vitamins (MSN) were purchased from Duchefa (Haarlem, Netherlands).

2.1.8 Antibodies

Listed below are primary and secondary antibodies used for western blot analysis.

Primary antibodies	Source	Dilution	Reference
α-FLS2	rabbit polyclonal	1:5 000	V. Göhre[a]
α-BAK1	rabbit polyclonal	1:300	D. Chinchilla[b]

[a]Max Planck Institute for Plant Breeding Research, Carl-von-Linné-Weg 10, 50829 Cologne, Germany; synthesized by Eurogentec (Seraing, Belgium)

[b]University Basel, Hebelstr. 1, Basel 4056, Switzerland

Secondary antibodies

Antibody	Feature	Dilution	Source
rabbit anti-rabbit IgG-AP	Alkaline phosphatase, conjugated	1:30 000	SigmaAldrich, Deisenhofen, Germany

2.1.9 Buffers and solutions

Buffers and solutions used in this study are described below each method. If not otherwise stated, buffers were prepared in dH_2O and aqueous solutions were sterilized by autoclaving at 121°C for 20 min.

2.2 Methods

2.2.1 Maintenance and cultivation of Arabidopsis

Arabidopsis genotypes were grown on soil or H_2O moistened jiffy pellets (Jiffy Products International AS). For bacterial growth assays plants were grown for two weeks in a controlled environment growth chamber under short day conditions (10 h photoperiod, light intensity of approximately 200µE/m2s, 21°C during light period and 20°C during darkness, and 60% humidity). For classical leaf ROS assay (Felix et al., 1999) plants were additionally grown for two more weeks under greenhouse conditions.

2.2.2 Arabidopsis seed sterilization

Small quantities of Arabidopsis seeds were sterilized by ethanol treatment. Seeds were placed in columns (from DNA purification Kits, Qiagen, Hilden, Germany) and incubated in 70% ethanol for 3 min. After centrifugation for 1 min at max. speed, the flow-through was discarded. The seeds were washed a second time in 70 % ethanol for 3 min, centrifugated for 1 min and the flow-through discarded. Finally, seeds were incubated in absolute ethanol for 1 min, centrifugated for 1 min at max. speed, and the flow-through discarded. For drying the column was opened under a hood for ~ 5 min. Large quantities of Arabidopsis seeds were sterilized by chloride treatment. Seeds were transferred to Eppendorf tubes or Falcon tubes and placed with open lids in an exsiccator. Then 5 ml of fumy 37 % HCl were added to 100 ml 12 % sodiumhypochloride solution (chlorine bleach) in the exsiccator so that yellow-greenish vapours were forming and the solution was bubbling heavily. The lid of the exsiccator was closed immediately and vacuum was generated to get an air tight seal. The seeds were incubated for 3-5 h.

2.2.3 Maintenance of Pathogens

Pseudomonas syringae pv. *tomato* strains were streaked onto selective NYGA agar plates containing appropriate antibiotics from -80° C DMSO stocks. Streaked plates were incubated at 28° C for 48 h before using the bacteria for spray inoculation.

2.2.4 Pathogen infection assays and quantification

Bacterial infection assays were performed as described recently (Zipfel et a., 2004). *Pseudomonas syringae pv. tomato* (Pto) strain DC3000 ΔAvrPto/AvrPtoB (Rosebrock et al., 2007) was grown at 28°C for 48 h on solid NYGA-media supplemented with Rifampicin (50 µg/ml in DMSO) and Kanamycin (50 µg/ml in dH2O) for selection. Two weeks old seedlings were spray inoculated with PtoDC3000 ΔAvrPto/ΔAvrPtoB bacterial solution (OD600 = 0.2 equal to 108 cfu/ml in 10 mM MgCl2, Silwet L-77 0.01%) and transferred to a growth chamber (10 h photoperiod, 22°C and 65 % humidity). Samples were harvested 4 h, 24 h and 3 days after pathogen treatment. Therefore, seedlings were surface sterilized and ground in 10 mM MgCl2 with a microfuge tube plastic pestle. Subsequently, samples were vortexed and serially diluted 1:10 before plated on solid NYGA media containing antibiotics as described above. Plates were incubated at 28°C for 48 h and colony forming units were counted. Per Arabidopsis genotype 8 samples comprising each two seedlings were analyzed. Statistical analysis was calculated transforming data to the logarithms of the count to meet assumptions of ANOVA. Subsequently, a pairwise comparison (Tukey corrected) was performed. Statistical significant differences were observed when the p-value was <0.05.

2.2.5 Bioassays to monitor PAMP responses

2.2.5.1 ROS detection in seedlings

Sterile seeds of Arabidopsis genotypes were sown into sterile 96-well microtiter plates (CulturePlates-96, Perkin Elmer), one seed per well, using 100 µl liquid MSN growth media per well. Plants were grown for 14 days under short day condition in a light and climate controlled chamber before growth media were exchanged with a 10 nM flg22 containing solution. Upon 1h incubation flg22-solution was exhausted and 100 µl water were supplied for a 1 h incubation. ROS production was triggered by 100 nM flg22 applied in a mastermix containing 100 µl H_2O, 20 µM luminol (Fluka) and 1 µg horseradish peroxidase (Sigma-Aldrich). Luminescence was measured by a luminometer (Centro LB 960 microplate luminometer, Berthold Technologies). 13 measuring cycles per 96-well microtiter plate are performed over a total time of 40 minutes. Per well, H_2O_2-dependent luminescence of luminol (Keppler et al.,1989) was recorded for 2 seconds.

2.2.5.2 ROS detection in mature plants

ROS detection in *A. thaliana* leaf discs was performed as described recently (Felix et al., 1999). Briefly, plant leaves were cut into discs of equal sizes that were water incubated overnight. Slices were transferred into microtiter plates (CulturePlate-96 , Perkin Elmer) containing 50 µl H_2O. ROS production is triggered by 1 µM flg22 applied in a mastermix containing 50 µl H_2O, 20 µM luminol (Fluka) and 1 µg horseradish peroxidase (Sigma-Aldrich). Luminescence was measured as described 2.2.5.1.

2.2.5.3 Ethylene measurement

Measuring ethylene biosynthesis was deduced from a protocol previously described by Bauer et al., 2001. Seven 14 days old *in vitro* grown seedlings were transferred in glass tubes containing 1 ml H_2O. After addition of 2 µM aqueous flg22-solution vials were rapidly closed with rubber septa and placed horizontally on a shaker (100 rpm). Ethylene, accumulating in the air phase, was measured by gas chromatography (GC MS) 4 h after flg22 treatment (injection volume: 100 µl). The amount of ethylene generated was normalized against the void-volume and relative peak area was integrated.

2.2.5.4.Membrane depolarization

Epidermal peels of 5 week old plants were incubated in 5 mM KCl over night. The measuring capillary- and electrode were filled with 5 mM KCl and perfusion was started with 5 mM KCl. The measuring electrode was injected into leave cells and resting potential were hold stable for at least 2 min. Following, 5 min perfusion of a 10 nM flg22 solution containing 1 mg/ml BSA, 5 mM KCl were started. Membrane depolarization was measured for 20 min.

2.2.5.5 Arrest of seedling growth

Growth inhibition of seedlings was performed as described by Gomez-Gomez et al.,1999. Arabidopsis genotypes were grown on MSN plates for 7 days. Seedlings were transferred into 24-well plates (Grainer bio-one) pre-filled with 500 µl liquid MSN media with the final concentration of 100 nM and 1 µM flg22, respectively. After 7 days growth restriction was analyzed by determining the seedlings fresh wight.

2.2.5.6 Analysis of Callose deposition

Callose staining was performed as previously described (Gomez-Gomez et al., 1999). Callose deposition was analyzed in fully expanded leaves of 4- to 6-week-old *A. thaliana* plants. Leaves were syringae-infiltrated with H_2O, 2 µM flg22 peptide solution and harvested after 20-24 h. The leaves were cleared in acetic acid/ethanol 1:3 (v:v) over night, subsequently washed in H_2O and stained in aniline blue solution o/n. Stained material was mounted in 50 % glycerol and examined using ultraviolet epifluorescence with a Zeiss Axiophot2 fluorescence microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany). Aniline blue staining solution: 150 mM KH_2PO_4, 0.01% (w/v) aniline blue, pH9.5 (KOH pellets)

2.2.6 Biochemical Molecular biological methods

2.2.6.1 In-gel MAP kinase assay

Seedlings were grown on MSN plates for 7 days before transferred to 24-well plate pre-filled with 500 µl liquide MSN growth media. After 10 days wells were replenished with 2 ml MSN. After 3 h incubation flg22 was added to a final concentration of 100 nM per well and samples were harvested 5 min, 10 min, and 20 min upon treatment. Seedlings were dried and roots were cut before samples were frozen in liquid nitrogen. Frozen leaf tissue was ground in liquid nitrogen and 100 mg were solubilized in 100 µl of extraction buffer. After centrifugation at 14000 rpm for 20 min at 4°C, 30 µl of supernatant were mixed with 15 µl of loading buffer. Samples were boiled 5 min at 96°C and loaded on myelin basic protein (MBP) containing denaturing SDS-polyacrylamid gel. Proteins were separated by SDS-gel. Protein gels were washed, re-naturated and subsequently incubated with radioactively labelled ^{32}P-ATP. Several washing steps followed. Radiolabeling was monitored using a phosphor imager (Typhoon 8600 Phosphor imager und Image Eraser, Molecular Dynamics, Sunnyvale, USA). Image processing was performed with AdobePhotoshop8.0 (Adobe Systems Inc., San Jose, CA, USA).

11.25 % Separating Gels (2 mini gels):

Acrylamide: bis- (30 %:0.8 %)	3 ml
1.5 M Tris-HCl (pH 8.8)	2 ml
Water	2.4 ml
MBP (5 mg/ml)	0.4 ml
10 % SDS	0.08 ml
10 % APS	0.08 ml
TEMED	0.008 ml

Stacking gels (2 mini gels):

Acrylamide:bis- (30 %:0.8 %)	1 ml
0.5 M Tris-HCl (pH 6.8)	1 ml
Water	1.94 ml
10 % SDS	0.04 ml
10 % APS	0.17 ml
TEMED	0.005 ml

Extraction buffer (20 samples):

1 M Tris-HCl (pH 7.5)	150 µl
0.5 M EGTA	30 µl
0.5 M EDTA	30 µl
1 M DTT	6 µl
0.1 M AEBSF (Pefabloc)	6 µl
Protease Inhibitor for plants (SIGMA)	80 µl
1 M NaF	30 µl
1 M Na3VO4	15 µl
1 M ß-glycerophosphate	150 µl
H_2O	2503 µl
	3000 µl

Loading buffer:

0.5 M Tris-HCl (pH 6.8)	2.5 ml
100 % glycerol	6 ml
10 % SDS	3.2 ml
BPB	1 mg
Water	20 ml

Washing steps (2 mini gels):

Buffers	Buffer contents	Washing steps	
F	5ml 1M Tris-HCl (pH 7.5)	3 x 30 min, RT ,	45 rpm
	100µl 1M DTT		
	20µl 1M Na3VO4		
	1ml 1M NaF		
	0.1g BSA		
	2ml 10 % Triton X 100		
	@ 200 ml with H2O		
G	5ml 1M Tris-HCl (pH 7.5)	2 x 30 min, 4 °C,	45 rpm
	200µl 1M DTT	over night, 4 °C	
	20µl 1M Na3VO4		
	1ml 1M NaF		
	@ 200 ml with H2O		
H	2.5ml HEPES	1 x 30 min, RT,	45 rpm
	20µl 0.5M EGTA		
	400µl 3M MgCl2		
	100µl DTT		
	10µl 1M $Na3VO_4$		
	@ 100 ml with H2O		
Radioactivity	20ml buffer H	1 x 90 min, RT,	92 rpm
	40µl 100µM ATP		
	2µl y-^{32}P-ATP (5µCi/µl)		
	/ 2 mini gels		
1 % phosphoric	11.76 ml phosphoric acid (86%)	3 x shortly, RT, 15 ml	45 rpm
acid	@ 1 l with H_2O	6 x 30 min, RT, 50 ml	
H_2O		20 min, RT, 50 ml	45 rpm

2.2.6.2 Total protein extraction and Immunoblot analysis

Arabidopsis genotypes were cultivated, and, flg22/Mock-treatment was performed as described in 2.2.5.1. Frozen seedlings were ground in liquid nitrogen and 100 mg were mixed with 2x protein extraction buffer (100 mM Tris-HCl (pH 6.8), 2 % SDS) and immediately incubated on a thermo-shaker at 96°C for 10 min. Cell debris were pelleted by centrifugation (13000 rpm, 10 min) and 20 µl of 5x loading buffer (2.5% bromphenol blue, 20% glycerol, 4% SDS, 10% DTT, 200mM Tris-HCl pH 6.8) were applied to 80 µl of supernatant. Samples were boiled at 96°C for 5 min before 20 µl were loaded on denaturating 7% SDS-mini gels. SDS-Polyacrylamidgelelectrophoresis was performed as described by Laemmli, 1970. Semi-dry blotting of the gels onto a PVDF membrane (Imobilon, Milipore) was performed in BIO-RAD Trans-Blot SD Semi-Dry transfer cell. Membrane was following blocked with a 5% (w/v) milk TBS-t (140 mM NaCl, 2.5 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.05% Tween20) solution for 1 h. Subsequently, primary antibodies, α-FLS2 and α-BAK1, respectively, were applied for over night incubation at 4°C. Secondary alkaline phosphatase-coupled antibody was applied at least for 1 h. For detection blots were incubated with chemi-luminescence detection solution (CDP-Star, Roche Diagnostics GmbH) and light emission was documented on x-ray films (Hyperfilm, Amersham Pharmacia). For a protein loading control membranes were stained with coomassie dye solution (0.25% coomassie brilliant blue, 50% MeOH) for 5 min before imaging for documentation.

2.2.6.3 Total RNA extraction and Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Arabidopsis genotypes were cultivated, and, flg22/Mock-treatment was performed as described in 2.2.5.1.. 100 mg of frozen seedlings were ground. RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. All RNA extracts were adjusted to the same concentration with RNAse free H_2O. RT-PCR was carried out in two steps using SuperScript™ II RNase H- Reverse Transcriptase (Invitrogen) for first strand cDNA synthesis. Reaction mixture was incubated at 42°C for 50 min before enzyme was heat inactivated at 70°C for 15 min. For subsequent PCR reaction, 1µl of above RT-reaction was used as cDNA template.

2.2.6.4 Isolation of genomic DNA from Arabidopsis

Genomic DNA from Arabidopsis leaf tissue was isolated according to Edward's isolation protocol (Sambrook and Russel, 2001). Edwards buffer: 200 mM Tris/HCl (pH7.5), 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS

2.2.6.5 Polymerase Chain Reaction (PCR)

Standard PCR reactions were performed using Taq DNA polymerase (Amplicon) according to the manufacturer's instructions. All PCR reactions were carried out using a Peltier Thermal Cycler PTC-225 (GMI Inc.) and reaction mixture typically contained 2µl template DNA (genomic, plasmid) in a final volume of 20 µl. PCR program was adjusted to needs of applied primers and template DNA used.

3 Results

3.1.1 PAMP-triggered ROS production requires *At*RbohD important for immunity

ROS production in response to PAMPs is a transient event that is detectable approximately three minutes after elicitation, reaches its maximum at 10 - 15 minutes and declines to background levels after about 20 - 30 minutes. Monitoring ROS production over time was so far realized by excised leaves of adult plants (Felix et al., 1999). Here, we modified this assay to measure flg22-induced oxidative burst in whole seedlings. Individual seedlings did not uniformly generate ROS, therefore, ROS signatures were synchronized by a pre-treatment with a low dose of flg22, subliminal to trigger detectable ROS production. Following a recovery period an oxidative burst was provoked by addition of above threshold flg22 concentration (Suppl. Fig. 1). This procedure ensured a robust ROS performance and distinguished mutant oxidative burst signatures from WT ROS responses (Fig. 1A). While Col-0 WT exhibited a rapid increase of ROS production *fls2* mutants did not show any significant elevation of ROS, and *bak1* mutants were severely lowered in ROS production, which is in agreement with previous findings (Gomez-Gomez and Boller 2000; Chinchilla et al, 2007).

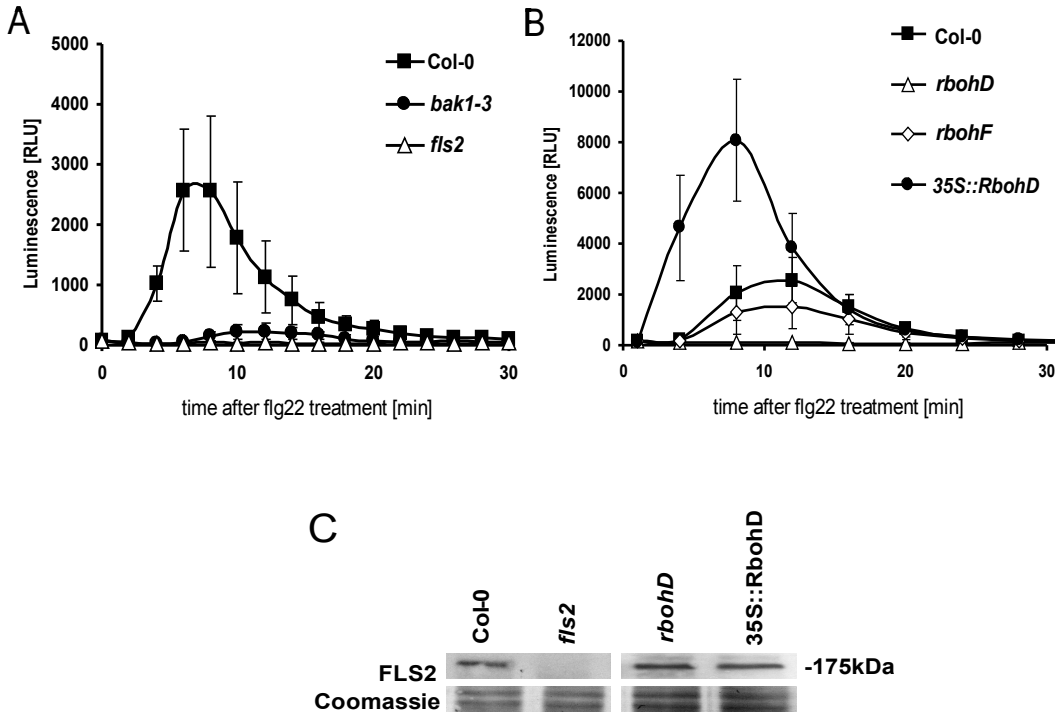


Fig. 1. Flg22-stimulated oxidative burst and FLS2 accumulation in seedlings. A, B) Induced ROS production was monitored in seedlings of the indicated genotypes over time. Depicted are average values (n=12); bars represent +/- SD. Similar results were obtained in multiple independent experiments. RLU, relative light units. C) FLS2 immunoblot analysis. Coomassie staining is shown for equal loading.

*At*RbohD and *At*RbohF were reported to regulate plant defence responses (Torres et al., 2002) and a recent report provided evidence that *At*RbohD alone is sufficient for the PAMP-triggered oxidative burst (Zhang et al., 2007). We tested both loss-of-function NADPH oxidases individually and our results confirmed that *AtrbohD*, but not *AtrbohF*, mutants are impaired in flg22-elicited ROS production (Fig. 1B). Moreover, Arabidopsis seedlings overexpressing *AtRbohD* accumulated clearly higher ROS levels (Fig. 1B; Suppl.Fig. 2). FLS2 steady-state levels remained unaltered in *AtrbohD* and 35S::*AtRbohD* lines (Fig. 1C), which indicates *At*RbohD as the essential and rate-limiting component of the flg22-induced oxidative burst.

Although a hallmark of PAMP responses, the role of early induced ROS accumulation in plant defence is still unclear. In addition to its antimicrobial properties, ROS might serve as signaling molecules promoting defence responses in either a cell autonomous or non-autonomous manner. To elucidate the contribution of ROS production in plant immunity, we investigated bacterial growth in *AtrbohD* and 35S::*AtRbohD* lines. Due to the rapid and transient nature of early PAMP responses, we reasoned that differences in pathogen proliferation might only become apparent at early infection time points and upon surface inoculation with a virulent but disarmed *Pseudomonas syringae* pv. *tomato* DC3000 (PtoDC3000 ΔAvrPto/ΔAvrPtoB) strain. We detected slightly but significantly enhanced bacterial proliferation in both *AtrbohD* loss-of-function mutants and 35S::*AtRbohD* overexpression lines (Fig.2). Enhanced susceptibility was not observed at late

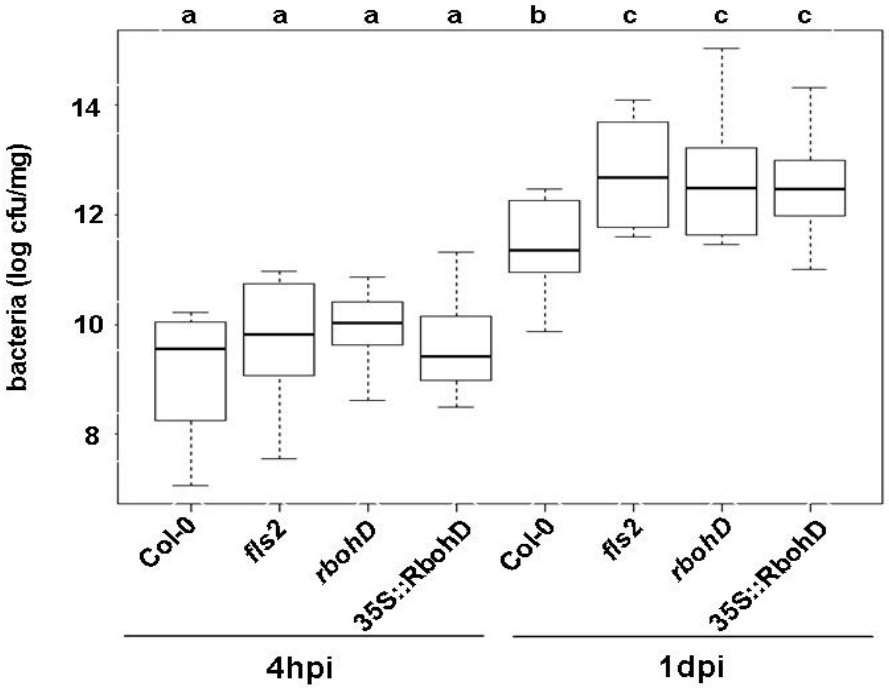


Fig. 2. Bacterial multiplication of PtoDC3000 ΔAvrPto/ΔAvrPtoB. Bacterial growth of indicated seedlings at 4 hours post infection (hpi) and 1 day post infection (dpi). Shown are average values of three independent experiments (n=16); bars represent +/-SD. Letters indicate significant differences $p < 0,05$.

time points (Suppl. Fig. 3). This provides evidence that PAMP-triggered ROS production by the action of *At*RbohD contributes to early stages of plant immunity. Since both loss of *AtrbohD* function and *AtRbohD* overexpression resulted in enhanced susceptibility, efficiency of plant defence seems to be affected by generally interfering with PAMP-triggered ROS levels.

3.1.2 Concluding remarks

We observed that ROS production of individual plants is subject to a strong natural variance under our experimental conditions, which is reflected by varying amounts of ROS produced upon elicitor treatment. Applying a low dose of flg22 as pre-stimulus led to synchronized ROS signatures probably by slightly inducing molecular components necessary for flg22 signaling (Asai et al., 2002; Zipfel et al., 2004).

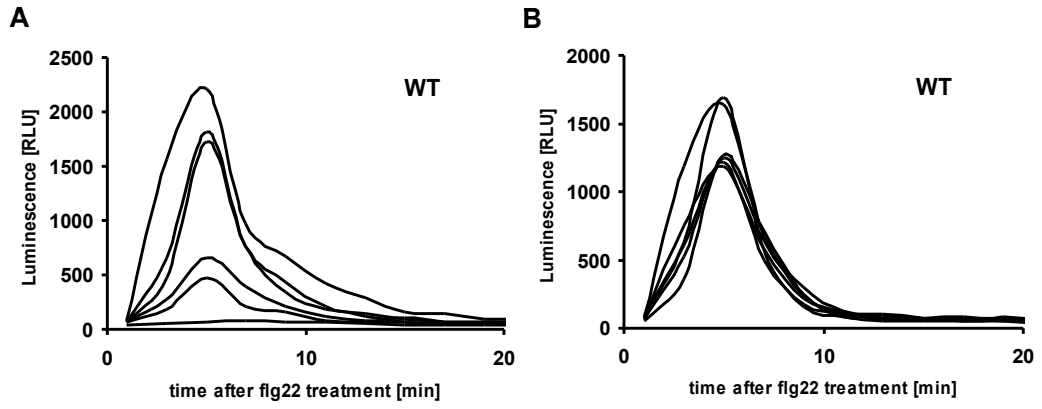
ROS derived from *At*RbohD and *At*RbohF appear to contribute to plant immunity as well as both ROS sources were reported to synergistically act in stomata function (Torres et al., 2002; Kwak et al., 2003; Zhang et al., 2007). Despite a functional overlap of *At*Rboh oxidases, in particular *At*RbohD is phosphorylated and activated upon flg22 detection (Nühse et al., 2007; Zhang et al., 2007). We tested *AtrbohD* and *AtrbohF* for their flg22 responses and could confirm *At*RbohD to be the responsible oxidases for the PAMP triggered oxidative burst. Further, we monitored ROS generation of *AtRbohD* overexpressing lines and observed that flg22-elicitation caused enhanced ROS production whereas FLS2 steady-state levels remained unaltered. This suggests *At*RbohD to control and regulate the precise amount of ROS generated upon elicitor detection and attributes this oxidase to be rate-limiting for the immediate early ROS response.

The contribution of ROS produced by *At*Rboh proteins to plant immune responses was little understood so far (Torres et al., 2002). Monitoring early time points following surface inoculation of virulent but disarmed pathogens, we observed significantly enhanced bacterial growth rates in *AtrbohD* mutant plants suggesting that extracellular ROS contributes to early stages of infection. Loss of ROS generation might impair signaling processes important for plant immunity. Surprisingly, we observed likewise enhanced bacterial multiplication rates in overexpressing *AtRbohD* plants indicating that not only a loss of ROS production but also enhance accumulation of ROS attenuates immune responses. Apparently, a dosage dependent generation of ROS is essential to properly induce signal transduction pathways that in turn antagonize bacterial multiplication. Thus, in loss of *AtrbohD* function or 35S::*AtRbohD* overexpressing lines the fine-tuning of defence responses requiring ROS signaling might be dis-balanced. This hypothesis is supported by pathogen induced phenotypes of mutant *OXI1*, a kinase assumed to contribute to PTI and ETI, and, required for activation of signaling MAP kinases MPK3 and MPK6 (Rentel et al., 2004). ROS generated by *At*RbohD partially drives expression of *OXI1*. Loss of *oxi1* function and *OXI1* overexpressing lines

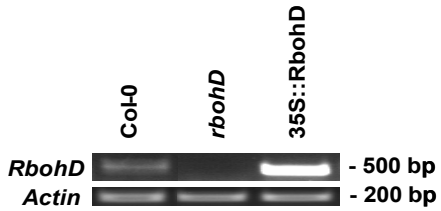
both exhibit enhanced susceptibility upon bacterial infection (Petersen et al., 2009). Given that *At*Rboh proteins act upstream of OXI1 it illustrates the importance of precisely regulated ROS accumulation and indicates ROS as signaling molecule required to activate defence pathways.

Moreover, driving the *AtRbohD* oxidase via the CaMV 35S promoter results in 40% increase of expressed protein (Torres et al., 2005). Hence, triggering an oxidative burst presumptively causes enhanced consumption of cofactors and ions. In addition, NADPH conversion results in cytoplasmic pH changes and acidification, which might affect the activity of signaling molecules (Pugin et al., 1997). Signal transduction pathways required to efficiently mount immune responses might be disordered or altered in *AtRbohD* overexpressing plants highlighting the precisely organized signaling machinery that mediates plant immunity.

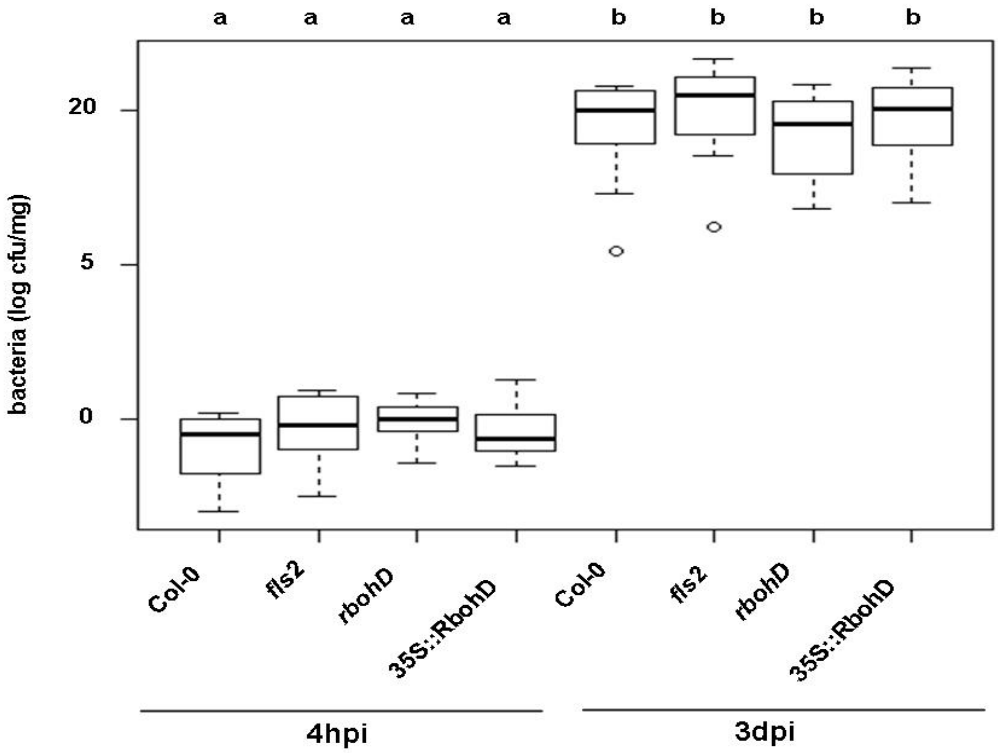
Supplementary figures



Suppl. Fig. 1. Synchronization of flg22 stimulated oxidative burst in seedlings. A) Induced ROS production without pre-stimulus application monitored over time. B) Induced ROS production with pre-stimulus application monitored over time. Depicted are ROS signatures of six individual seedlings; Similar results were obtained in multiple independent experiments. RLU, relative light units.



Suppl. Fig. 2. *RbohD* mutants and overexpression lines. *AtRbohD* transcript accumulation was analyzed by RT-PCR in the indicated genotypes. *Actin* is shown as a control. Results were obtained in three independent experiments.



Suppl. Fig. 3. Bacterial multiplication at prolonged time post infection. Bacterial growth of indicated seedlings infected with PtoDC3000 ΔAvrPto/ΔAvrPtoB at 1 and 3 day post infection (dpi). Shown are average values of three independent experiments (n=16); bars represent +/-SD. Letters indicate significant differences $p < 0,05$.

3.2.1 Identification of mutants reduced in ROS production

To identify molecular components that mediate FLS2 dependent ROS production, we conducted a genetic screen for Arabidopsis mutant that display altered ROS signatures in response to flg22 treatment. A gamma irradiated population was screened for candidates with altered ROS production. Out of 5000 M2 seeds 192 potential mutants were selected exhibiting either reduced or impaired ROS generation (Suppl.Fig.4). Re-screening of candidates, however, revealed a high variability in ROS production of M3 and M4 seedlings, hence, previous observed ROS phenotypes could not robustly be confirmed. We therefore inspected a pre-screened EMS-mutagenized population and adjusted our screening procedure accordingly.

Out of 20.000 mutagenized M2 seedlings, 600 were initially selected for impaired flg22-triggered inhibition of anthocyanin accumulation (kindly provided by Y. Saijo). Five out of initially 14 M2 candidates were confirmed in the M3 and M4 generation to exhibit a reduced flg22-elicited oxidative burst and were termed *reduced in oxidative burst* (*rio;* Fig. 3). A strong reduction in flg22-triggered ROS accumulation was observed in *rio1*. Impaired oxidative burst was observed in *rio2* to *rio5*. None of the selected candidates showed any obvious developmental alterations.

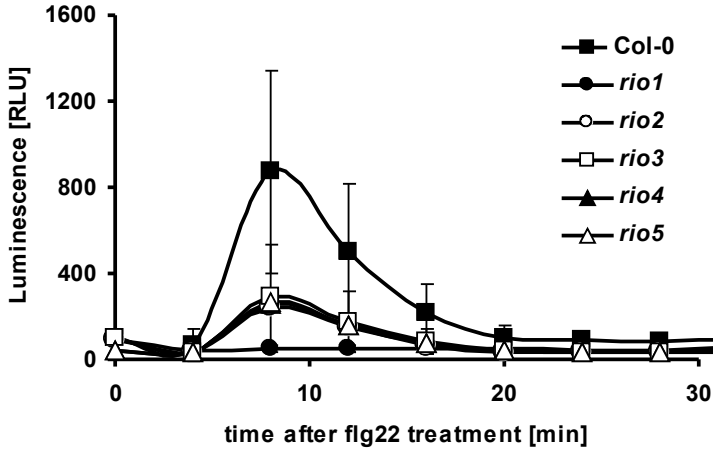


Fig. 3. Flg22-stimulated oxidative burst in *rio* mutants. Induced ROS production was monitored in seedling *rio* mutants. Depicted are average values (n=12); bars represent +/-SD. Similar results were obtained in multiple independent experiments. RLU, relative light units.

3.2.2 Flg22-dependent responses in *rio* mutants

To gain first insights into molecular components altered in *rio* mutants additional PAMP-induced responses were monitored (Table 3; Suppl. Fig. 5). We observed that flg22-elicited ROS production in mature leaves of *rio1* was impaired, whereas *rio2-5* showed reduced ROS accumulation in response to flg22. Upon elf18 application, mature leaves of all *rio* mutant responded with decreased ROS accumulation. This indicates that *rio* mutants were altered in molecular components that account for flg22- and elf18-mediated ROS production and were independent from developmental stages. Because *rio1* did not produce any ROS in response to flg22, other early PAMP responses were analysed and revealed WT-like membrane depolarization and ethylene induction in response to flg22 (Fig. 4A,B). Thus, *rio1* appeared to be particularly impaired in early PAMP-dependent ROS accumulation. Monitoring late flg22-elicited responses revealed that *rio1* displayed WT-like arrest of seedling growth as well as WT-like callose deposition. Likewise, *rio2-5* responded with callose deposition in a WT-like manner when treated with flg22. Analysing the arrest of seedling growth in *rio2* and *rio3,* these mutants displayed reduced sensitivity when 100 nM of flg22 were applied and appeared to be non-responsive to 1 µM of flg22. *Rio4* and *rio5* exhibited slightly decreased dose dependent fresh weight reduction when treated with 100 nM or 1 µM of flg22, respectively.

Table 3. PAMP triggered responses in *rio* mutants.

mutants "reduced in oxidative burst"	ROS in seedlings	ROS in mature plants		arrest of seedling growth	callose deposition in mature plants
	flg22	flg22	elf18	flg22	flg22
rio1	abolished	abolished	reduced	WT	WT
rio2	reduced	reduced	reduced	reduced	WT
rio3	reduced	reduced	reduced	reduced	WT
rio4	reduced	reduced	reduced	slightly reduced	WT
rio5	reduced	reduced	reduced	slightly reduced	WT

Obvious molecular candidates for the *rio* mutants represent *FSL2*, *BAK1* and *AtRbohD*, which were therefore sequence analysed. *Rio1*, *2* and *3* carried mutations within *FLS2* and *BAK1* respectively, whereas *rio4* and *rio5* displayed WT-like sequences for all genes tested. *Rio1* exhibits a G to A nucleotide exchange in position 280 bp of the *FLS2* coding region. This nucleotide exchange results in an amino acid change from Gly to Arg within the N-terminal domain of yet unknown

function, upstream of the LRR domain and downstream of the signal peptide (Gomez-Gomez and Boller, 2000). Subsequent immunoblot analysis revealed WT-like FLS2 accumulation in *rio1* (Fig. 4C). *Rio1* represents a new mutant *fls2* allele affected in a region that appears to be specifically required to mount flg22-mediated ROS production.

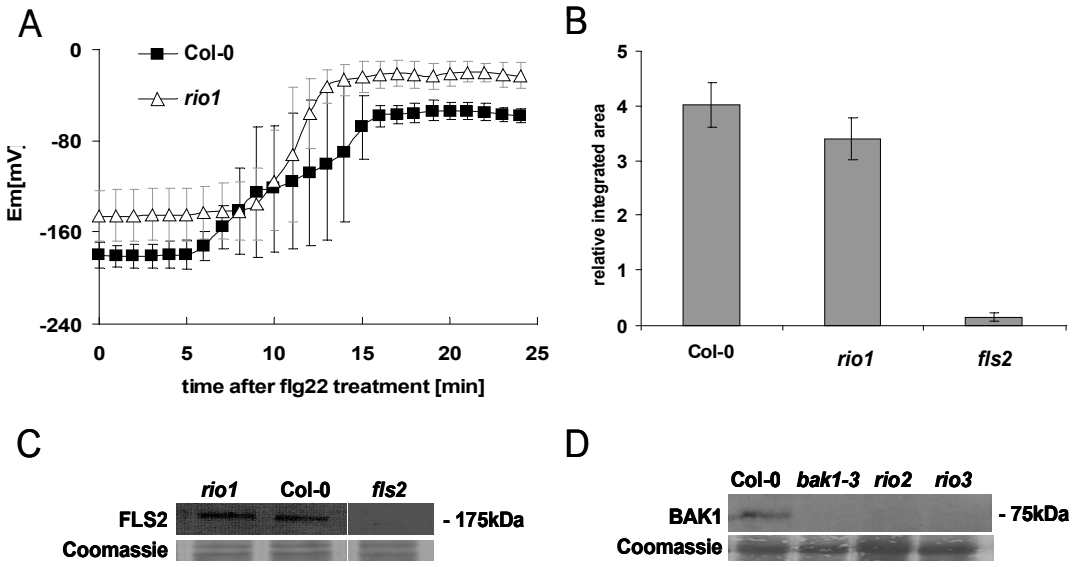


Fig. 4. Early flg22-triggered responses and immunoblot analysis in *rio* mutants. A) flg22-mediated membrane depolarization in *rio1*. Depicted are average values (n=3); bars represent +/-SD. B) flg22-triggered ethylene induction in mature *rio1* mutant leaves. Depicted are average values (n=3): bars represent +/-SD. C) FLS2 immunoblot analysis. Coomassie staining is shown for equal loading. D) BAK1 immunoblot analysis. Coomassie staining is shown for equal loading.

BAK1 genomic DNA sequence analysis of *rio2* revealed an G to A base change at position 955 bp that causes a Gly to Arg exchange. This mutation effects a donor sequence of the 4th GT-AG type intron in between regions coding for LRR domains. Mutations that alter this conserved donor sequence probably impair intron recognition that might result in aberrant splice products. In agreement, immunoblot analysis displayed no BAK1 protein accumulation in *rio2* mutants (Fig. 4D). Sequence analysis of *rio3* revealed an G to A base exchange in position 518 bp at the very N-terminus of the *BAK1* coding sequence. This mutation causes a pre-mature stop-codon and accordingly no BAK1 protein accumulation was detected in immunoblot analysis (Fig. 4D). Thus, flg22-triggered responses of *rio2* and *rio3* were similar reduced than *bak1-3* mutants (Chinchilla et al., 2007).

Sequence analysis of *rio4* and *rio5* mutants revealed WT-like *FLS2*, *BAK1* and *AtRbohD* sequences. Therefore, *rio4* and *rio5* likely encode new components of FLS2-activated and *At*RbohD-mediated ROS production.

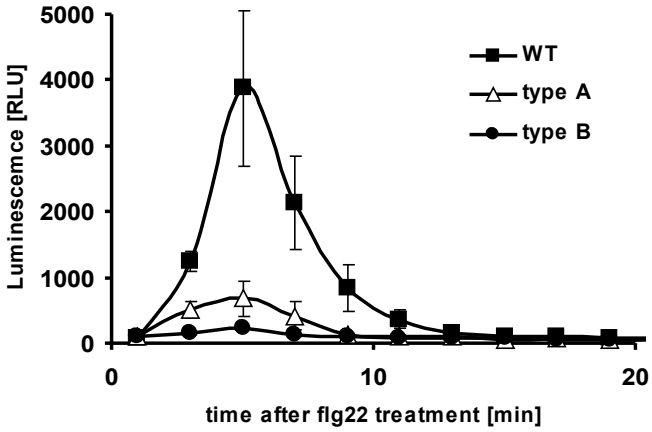
3.2.3 Concluding remarks

A population consisting of pooled mutagenized M2 seedlings were screened and candidates impaired in ROS production were selected. However, a high variability in ROS generation impeded robust confirmation of previous observed phenotypes, probably causes by a limited number of technical replicates. Using a population of mutagenized M2 plant lines, allowing an enlarged number of replicates, we could robustly identify candidates that exhibit altered ROS production. This indicates our method to be suitable to investigate ROS signatures.

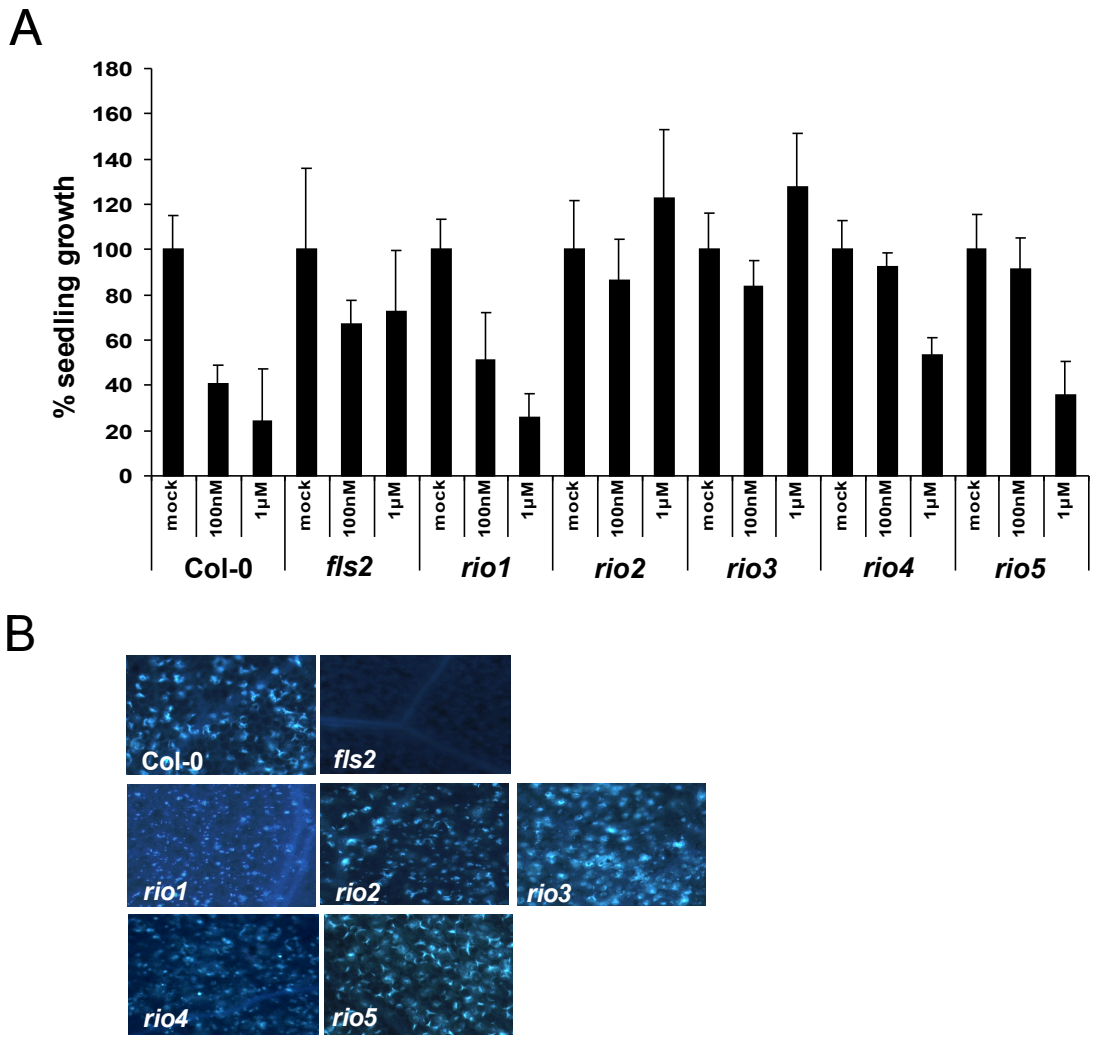
Three of the identified mutants correspond to established pathway components. Obvious molecular candidates represent *FLS2*, *BAK1* and *AtRbohD*. Distinct mutations in *FLS2* result in flg22-insensitivity and illustrate requirement of the FLS2 receptor for ROS production. The mutant alleles *fls2-17* and *fls2-24* are affected in flg22 elicitor binding, hence accumulation of flg22-elicited ROS is impaired (Gomez-Gomez et al., 2001). The *fls2* null allele similarly reflects the requirement of FLS2 for activating the flg22 response pathway (Chinchilla et al.,2006). The functional importance of BAK1 for stimulating PAMP-triggered responses is shown by loss of *bak1* function alleles. *Bak1-3* and *bak1-4* T-insertion lines are clearly impaired in early and late PAMP response signaling (Chinchilla et al., 2006). Both *bak1-3* and *bak1-4* alleles remain partial flg22 sensitive possibly resulting from functional redundancy with other SERK family members (Shiu and Bleecker, 2003; Chinchilla et al., 2007). In addition, complex formation of FLS2 and BAK1 proved to be essential for full activation of flg22-triggered defence responses (Chinchilla et al., 2007). *At*RbohD is essential for flg22-triggered ROS production exhibiting requirement of this oxidase for the FLS2 signaling pathway (Zhang et al., 2007).

Inspected population was pre-screened for flg22-mediated inhibition of anthocyanin, representing a late flg22 response before we screened for mutants exhibiting aberrant ROS generation. This might have increased the likelihood to identify *FLS2* and *BAK1* alleles and probably decreased the possibility to identify novel molecules involved in early defence responses. Sequence analysis revealed *rio1*, *rio2* and *rio3* to be mutated in *FLS2* or *BAK1*, respectively. None of the selected mutants exhibits defects in the *AtRbohD* sequence, assuming this screening approach not to be saturated or suggesting that due to the pre-screening procedure the possibility to identify early components, such as *At*RbohD, were reduced. However, identification of *rio4* and *rio5*, probably representing yet unknown components involved in flg22-elicited ROS signaling, disprove this assumption. Moreover, redundant action of several molecules contributing to the ROS pathway might have masked the identification of novel signaling components.

Supplementary figures



Suppl. Fig. 4. Flg22-stimulated oxidative burst in M2 mutant candidates. Induced ROS production was monitored in seedlings of a gamma irradiated population. 192 candidates were identified. Signatures were grouped into typeA (reduced ROS production) and typeB (impaired ROS production). Depicted are average values; bars represent +/-SD. RLU, relative light units.



Suppl. Fig. 5. Late flg22-triggered responses in *rio* mutants. A) Flg22-induced seedling growth arrest of the indicated genotypes was measured in the absence or presence of 100 nM or 1 µM flg22. Bars represent +/-SD. Similar results were obtained in three independent experiments. B) Callose deposition in *rio* mutants upon flg22 treatment. Similar result were obtained in two independent experiments.

3.3.1 ROS production in response to flg22 is diminished in ethylene insensitive mutants

Although flg22-induced resistance to bacterial infection appeared independent of individual hormone signaling pathways known to play key roles in plant defence (Zipfel et al, 2004), recent studies support a role for salicylic acid (SA) in PAMP-triggered immunity (Wang et al., 2009). To address the possible involvement of hormone, redox and kinase signaling pathways regulating individual PAMP responses, and, to dissect the oxidative burst, we monitored flg22-induced ROS production in a collection of known mutants (Suppl. Tab. 1). Most mutants tested were still able to mount an WT-like oxidative burst. However, slight changes in ROS signatures were observed in *nahG* plants and mutant ABA signaling components. *Rcd1*, a ROS-responsive lesion-mimic mutant and stress-induced ethylene biosynthesis regulating component (Overmayer et al., 2000) displayed reduced flg22-elicited ROS production. Further, a cyclic nucleotide-gated ion channel appears to contribute to flg22-triggered ROS generation, since *dnd1* mutant seedlings, known to be impaired in mounting HR, displayed reduced oxidative burst (Clough et al., 2000). Most severely, ethylene-insensitive mutants displayed a pronounced decrease in flg22-triggered ROS levels (Fig. 5A).

A strong allele of the ethylene receptor mutant, *etr1-1*, was almost non-responsive, whereas a partial insensitive allele, *etr1-3*, was strongly reduced in flg22-elicited oxidative burst. Additionally, flg22-mediated ROS production was nearly abolished in the ethylene-insensitive mutant *ein2-1*. Ethylene is a known component of plant immunity, and also accumulates upon flg22 treatment (Gomez-Gomez et al., 1999). Its perception is mediated by a family of membrane

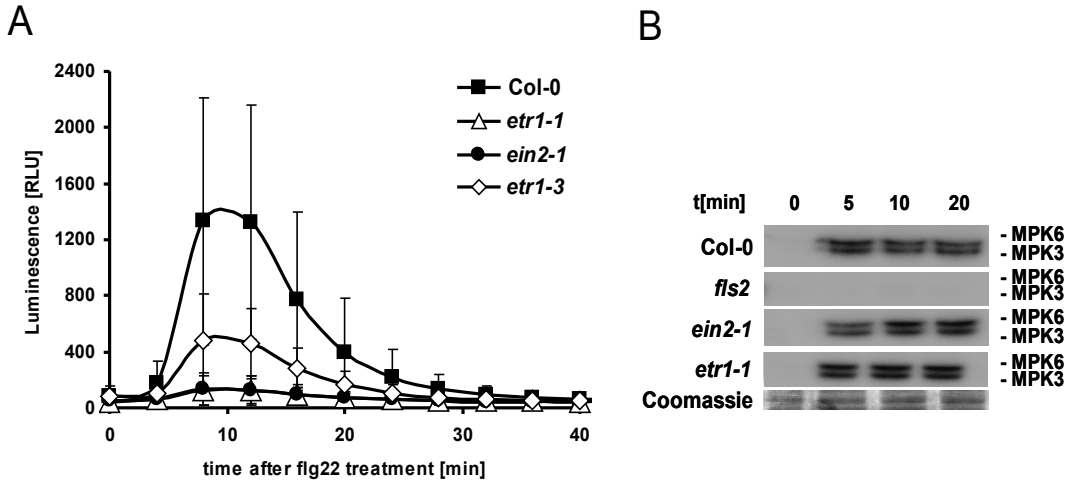


Fig. 5. Flg22/FLS2 function in ethylene-insensitive mutants. A) Induced ROS production was monitored in ethylene signaling mutants over time. Depicted are average values (n=12); bars represent +/-SD. Similar results were obtained in multiple independent experiments. RLU, relative light units. B) Flg22-induced MPK6 and MPK3 activation was determined by in gel MAP-kinase assays at the indicated time points and seedling genotypes.

receptors including ETR1, and leads to inactivation of the negative regulator CTR1, a Raf-like kinase (Wang et al., 2002). The downstream component EIN2 subsequently activates the transcription factor EIN3 driving transcriptional changes of ethylene responsive genes. Flg22-induced ROS production in loss-of-function mutants of *CTR1*, *EIN3* and *ETO1*, an ethylene biosynthesis regulatory protein, appeared undistinguishable from WT (Suppl. Tab. 1). These findings suggest that ethylene signaling is required for PAMP-induced ROS production. By contrast, flg22 activation of signaling MAP kinases MPK3 and MPK6 remained unaltered in *etr1-1* and *ein2-1* (Fig. 5B). The oxidative burst and MAP kinase activation represent early FLS2-mediated responses (Felix and Boller, 2009), and recently flg22-triggered callose deposition, a late PAMP response, was shown to depend on EIN2 function (Clay et al, 2009). When tested for flg22-elicited seedling growth arrest, however, *etr1-1*, *etr1-3* and *ein2-1* mutants displayed almost a WT-like dose-dependent response, indicative of distinct molecular mechanisms regulating individual flg22 responses (Suppl. Fig. 6).

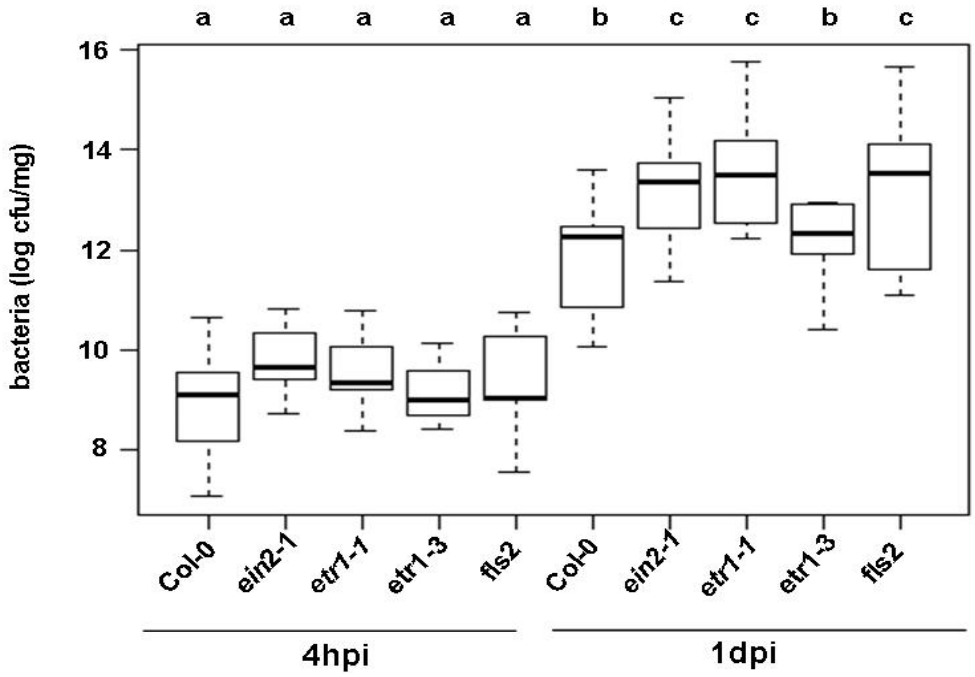


Fig. 6. Bacterial multiplication of PtoDC3000 ΔAvrPto/ΔAvrPtoB. Seedlings impaired in ethylene signaling were infected and growth was monitored at 4 hours post infection (hpi) and 1 day post infection (dpi). Shown are average values of three independent experiments (n=16); bars represent +/-SD. Letters indicate significant differences $p < 0,05$.

Ethylene performs diverse functions in plant-microbe interactions. It is known to play crucial roles for plant immunity towards necrotrophic fungi (Chagué et al., 2006). However, its contribution to bacterial infections remains unclear. Depending on infection conditions, unaltered, reduced or enhanced bacterial numbers were reported (Bent et al., 1992; Pieterse et al., 1998). We spray-infected our set of ethylene-insensitive mutants and observed that *etr1-1* mutants allowed significantly elevated bacterial multiplication compared to WT plants, which was less evident in *ein2-1* mutants (Fig. 6). This is in agreement with the recently reported increased susceptibility of *ein2-1* mutants (Clay et al, 2009). However, bacterial growth rates at later time points were similar between WT and ethylene-insensitive mutants (Suppl. Fig. 7). This suggests that ethylene may contribute to pre-invasive immunity through regulation of PAMP-triggered ROS production.

3.3.2 Ethylene and ethylene signaling controlling FLS2

Reduced generation of ROS in response to flg22 might be a result of low FLS2 or BAK1 abundance. We detected strongly reduced FLS2 steady-state levels in the *ein2-1* and *etr1-1* alleles, respectively (Fig. 7A), which could explain the compromised flg22-induced oxidative burst. Reduced FLS2 levels, however, were still sufficient to mediate flg22 activation of MAP kinases. Furthermore, the partial ethylene-insensitive allele, *etr1-3*, accumulated FLS2 at WT-like levels, suggesting that additional regulatory mechanisms underlie flg22-triggered ROS production. No differences in BAK1

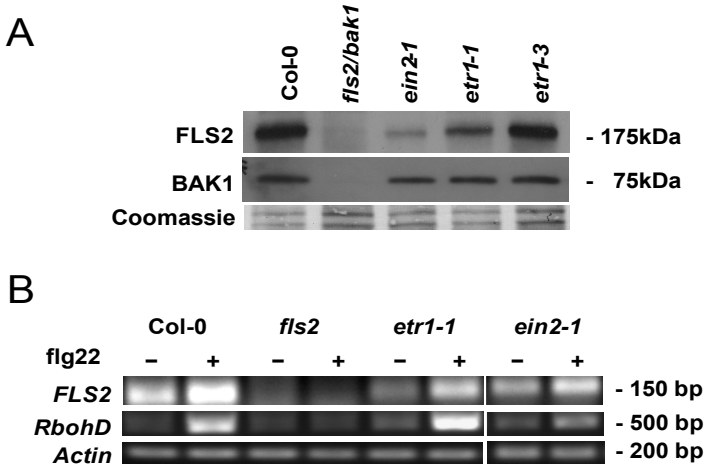


Fig. 7. FLS2 accumulation in ethylene-insensitive mutants. A) FLS2/BAK1 immunoblot analysis. Coomassie staining is shown for equal loading. B) RT-PCR analysis of *FLS2* and *AtRbohD* expression in mock and flg22 treated Col-0, *fls2*, *etr1-1* and *ein2-1* seedlings. *Actin* is shown as control. Results were obtained in two independent experiments.

abundance were observed in all three mutant alleles when compared to Col-0 WT seedlings (Fig. 7A). Differences in FLS2 protein levels between mutants appeared to correlate with differences in *FLS2* transcript accumulation (Fig. 7B). Likewise, induced *FLS2* transcript accumulation upon flg22 treatment appeared lower in ethylene- insensitive mutants when compared to Col-0 WT. *AtRbohD* transcript levels remained mostly unaltered in *etr1-1* and *ein2-1*, while flg22 induction of *AtRbohD* expression was reduced in *ein2-1* mutants. This indicates that the expression of *FLS2* (and to a lesser extent *AtRbohD*) is under the control of ethylene signaling, leading to a reduction of FLS2 steady-state levels in the absence of ethylene signaling.

It is known that ethylene signaling mutants exhibit enhanced endogenous ethylene levels compared to WT plants (Kende et al., 1993; Suppl. Fig. 8) We reasoned that these elevated ethylene levels might impair the PAMP-triggered oxidative burst. To test this hypothesis, we treated ethylene-insensitive mutants with AVG (aminovinyl glycine), a common ethylene synthesis inhibitor, which decreased ethylene levels (Fig. 8A; Suppl.Fig. 8). AVG pre-treated *etr1-1* and *ein2-1* mutant seedlings displayed a significant increase in flg22-stimulated ROS production compared to untreated mutant seedlings. However, a WT-like oxidative burst was not restored, which suggests that elevated ethylene levels in *etr1-1* and *ein2-1* are only partially contributing to compromised flg22-triggered ROS production. In addition, AVG pre-treatment did not cause an increase in FLS2 abundance (Suppl. Fig. 9), suggesting that the observed defect in flg22-triggered ROS production in ethylene signaling mutants is largely independent from high steady-state ethylene levels.

Defence gene expression and thus plant immune responses, are regulated by hormonal cross-talk between SA, ethylene and JA (Hammond-Kosack and Parker, 2003; Spoel et al., 2003). Ethylene action appears to be negative regulated by SA, and, in respect to ROS generation, SA were reported to down-regulate ROS detoxifying enzymes like ascorbate peroxidase and catalase (Klessig et al., 2000). SA pre-treated *etr1-1* and *ein2-1* seedlings showed a slight enhancement of the flg22-triggered oxidative burst (Fig. 8B), which suggests a role for SA in early PAMP-triggered defence responses through interaction with ethylene signaling pathways.

Ethylene is an important regulator of plant development and growth (Ecker, 1995). We therefore investigated flg22-triggered ROS production in fully expanded leaves of adult plants. Remarkably, in this assay *etr1-1*, *etr1-3* and *ein2-1* mutants produced WT-like levels of ROS (Fig. 9A, Suppl. Fig. 10). To exclude developmental differences we also monitored ROS production of seedling leaf discs. Likewise, all tested mutants displayed WT-like ROS levels upon flg22 treatment. FLS2 protein was present at almost similar levels in wounded *etr1-1*, *ein2-1* and Col-0 WT plants (Fig.9B). This suggests that wounding acts to reverse the compromised flg22-induced oxidative burst in ethylene signaling mutants, and hints that regulation of PAMP-triggered defence responses maybe stimulus dependent.

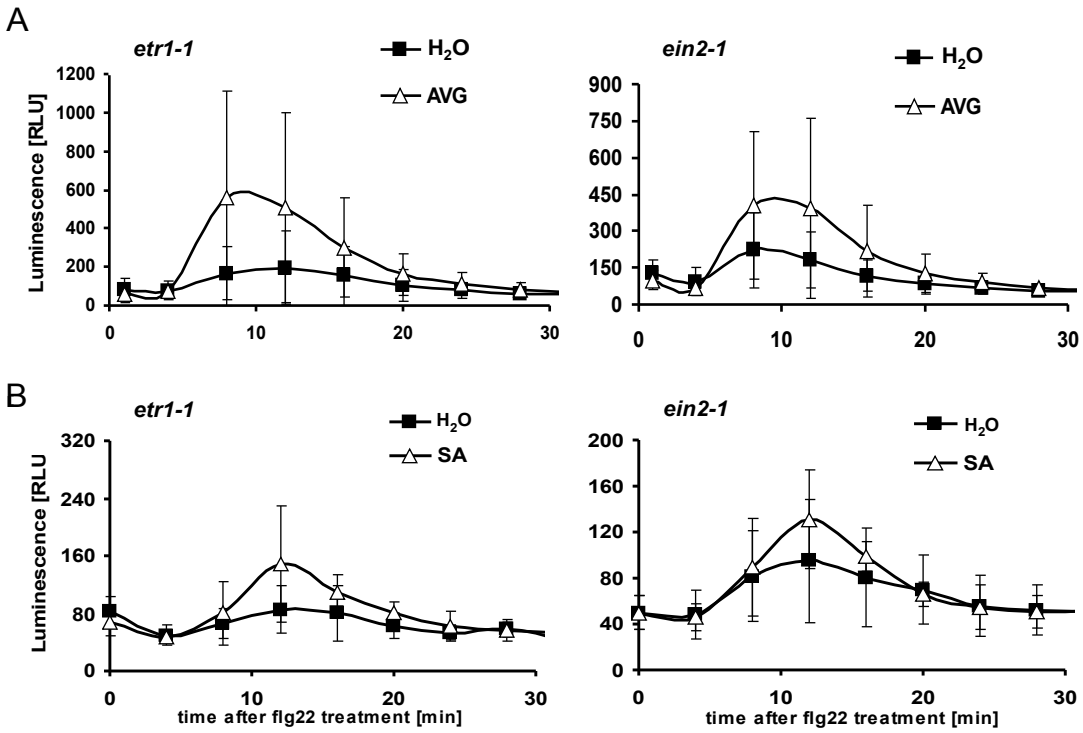


Fig. 8. Chemical interference of ethylene levels. A) AVG pre-treated *etr1-1* and *ein2-1* seedlings, respectively, were monitored for flg22-induced ROS production over time. B) SA pre-treated *etr1-1* and *ein2-1* seedlings, respectively, were monitored for flg22-induced ROS production over time. Each experiment included a mock treated mutant seedling control. Shown are average values (n=12); bars represent +/-SD. RLU, relative light units.

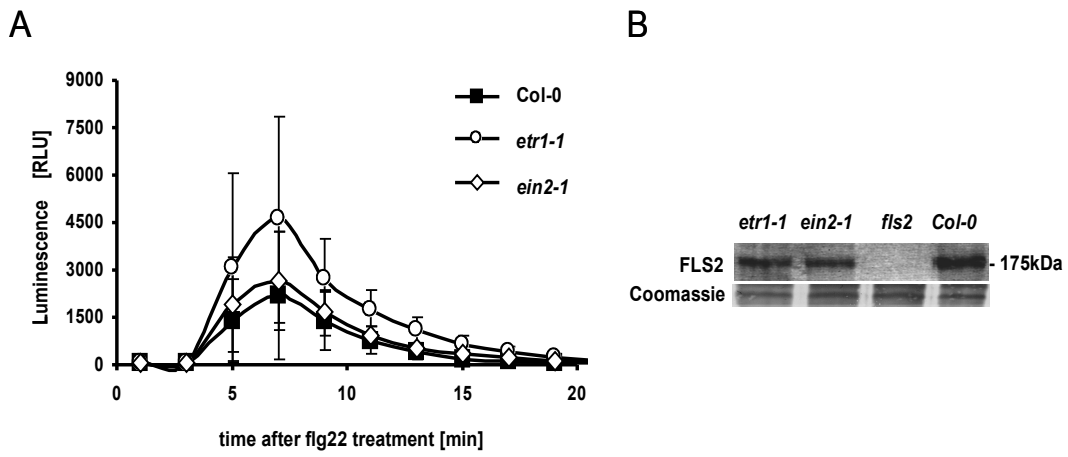


Fig. 9. Flg22-induced oxidative burst in leaf discs. A) Mature leaves of the indicated genotypes were excised into discs of approximately equal sizes and monitored for flg22-mediated ROS production. Depicted are average values (n=12); bars represent +/-SD. RLU, relative light units. B) FLS2 immunoblot analysis. Coomassie staining is shown for equal loading.

3.3.3 Concluding remarks

We tested a set of mutants with established roles in defence signaling for flg22-mediated ROS production. Besides slight changes in mutants affected in ABA signaling and *nahG* plants, most mutants tested displayed WT-like ROS production. This might partially be caused by a functional redundancy of molecular components and/or synergistic effects of several pathways that feed into the same cellular mechanism (Davis and Zhang, 1991; Alonso et al., 1999; Ghassemian et al., 2000). Analysis of double, triple or quadruble mutants will be required to circumvent this issue and to unmask further molecular players.

We identified ethylene signaling mutants to be affected in PAMP-triggered ROS accumulation. Strong mutant alleles, *etr1-1* and *ein2-1*, appear to be almost entirely impaired, while weak *etr1-3* mutant alleles exhibit reduced ROS accumulation. This suggests an important role for ethylene signaling in ROS production. We detected reduced FLS2 accumulation in *etr1-1* and *ein2-1* mutants indicative of a positive regulatory effect of ethylene signaling. Since we likewise observed lower flg22-induced *FLS2* transcript accumulation, we assume that ethylene-mediated transcriptional events control *FLS2* accumulation. However, the ethylene pathway appears to be required, but not sufficient to regulate *FLS2* gene expression. *Etr1-1* and *ein2-1* accumulate only low *FLS2* transcripts. Further, WT-like steady-state FLS2 proteins levels in *etr1-3* mutants do not result in WT-like ROS production, indicating that impaired ethylene signaling affects the flg22-triggered ROS response. Together, we assume yet unknown signaling events to additionally contribute to flg22-

elicited ROS production.

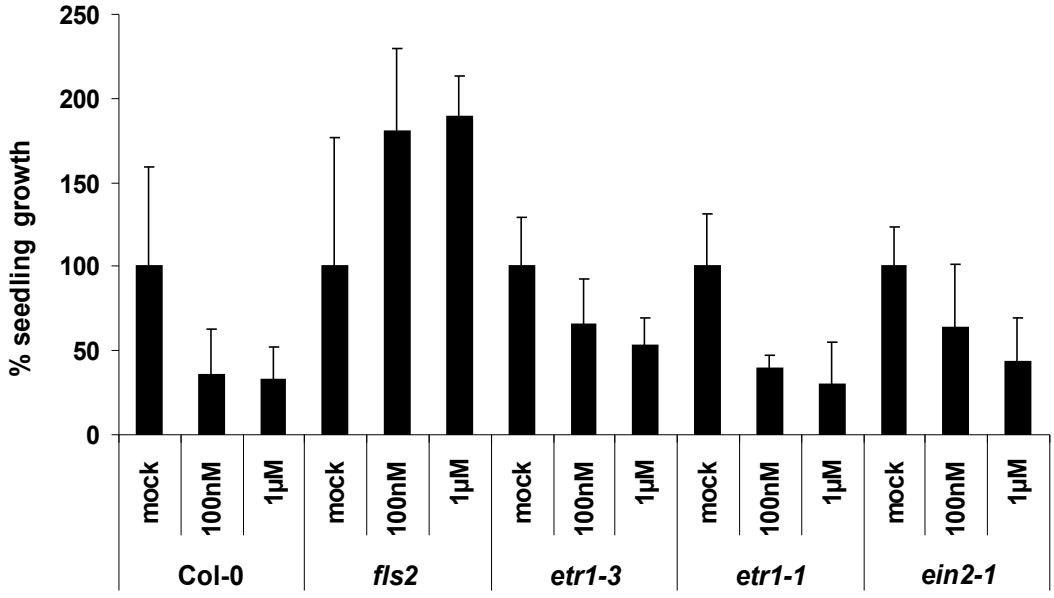
Our analysis revealed that the *etr1-1* and *ein2-1* mutants are more susceptible to bacterial infection when compared to WT. FLS2 is required for pre-invasive immunity, however, the underlying signal transduction pathway is little understood (Zipfel et al., 2004, Melotto et al., 2006). FLS2 steady-state protein accumulation is strongly reduced, and, ROS production is impaired in *etr1-1* and *ein2-1* mutants, supposedly causing enhanced susceptibility due to reduced activation of defence signaling.

Noteworthy, monitoring flg22-triggered oxidative burst in excised leaf material of whole seedlings, revealed substantial differences for the outcome of ethylene signaling mutants. ROS accumulation and FLS2 steady-state protein levels were almost reconstituted in response to wounding. Wound signaling is mediated by MAP kinase activation including MPK6 and involves ethylene (Schweighofer et al., 2007). Ethylene signaling is required for flg22-triggered dissociation of MPK6 and the ethylene response factor ERF104 (Nühse et al., 2000; Bethke et al., 2009). Overexpression of *ERF104* rendered seedlings to enhanced flg22-elicited growth arrest. It is therefore likely that wounding primes ethylene insensitive mutants competent to flg22-induced ROS production by at least partly utilizing the same signaling components. This implies that methods used to monitor PAMP-triggered ROS production have to be considered carefully.

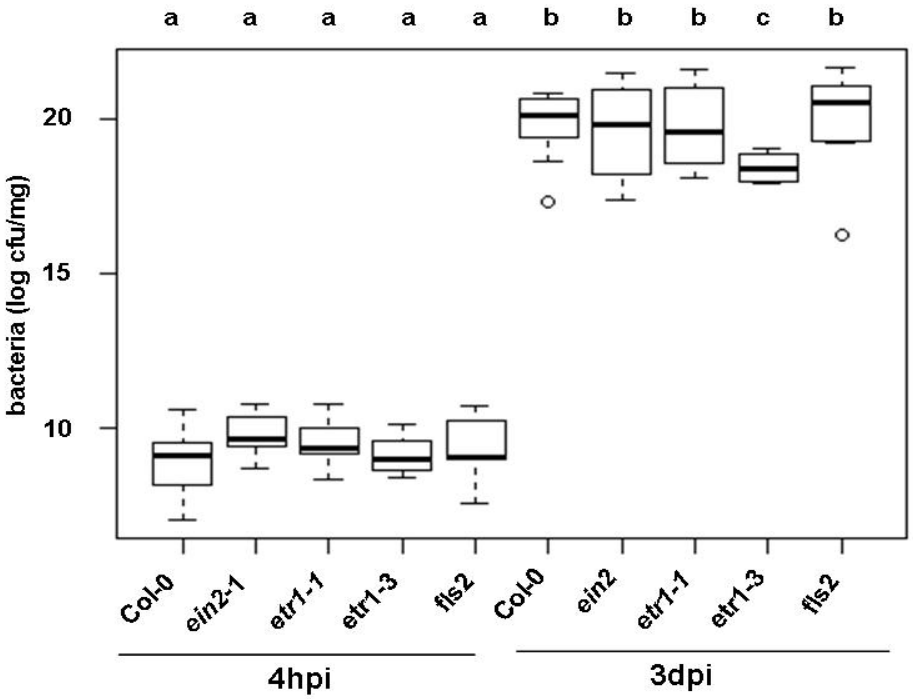
Supplementary files

Suppl. Tab.1: Overview of mutants with known roles in plant defence tested for flg22-ROS production."Y" WT-like ROS generation, "Y (+)" slightly enhanced ROS production, "Y (-)" slightly reduced ROS production, "N" no ROS production.

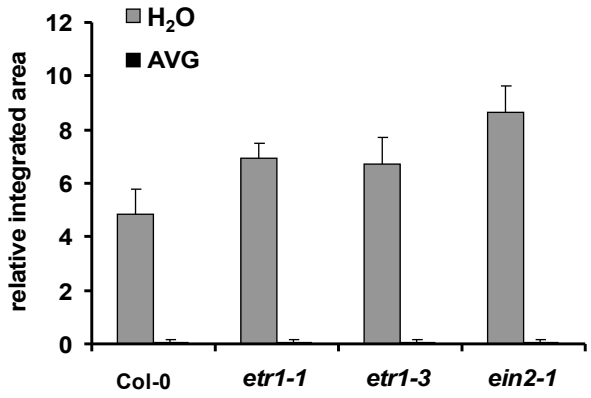
Mutant	ROS	Mutant	ROS
AtrbohD	**N**	***abi1***	**Y**
AtrbohF	**Y**	***abi2***	**Y**
AtRbohD/F	**N**	***ost1***	**Y (-)**
dnd1	**Y (-)**	***ost2***	**Y (-)**
rcd1	**Y (-)**	***jar1***	**Y**
mpk3	**Y**	***coi1***	**Y**
mpk6	**Y**	***gai***	**Y**
pmr4	**Y**	***etr1-3***	**Y (-)**
pen3-3	**Y (+)**	***etr1-1***	**N**
pen3-4	**Y (+)**	***ein2***	**N**
nutd7	**Y**	***ctr1***	**Y**
eds1	**Y**	***eto1***	**Y**
sid2	**Y**	***eto2***	**Y**
nahG	**Y (+)**	***ein3***	**Y**
npr1-1	**Y (+)**	**35S::ERF1**	**Y (-)**



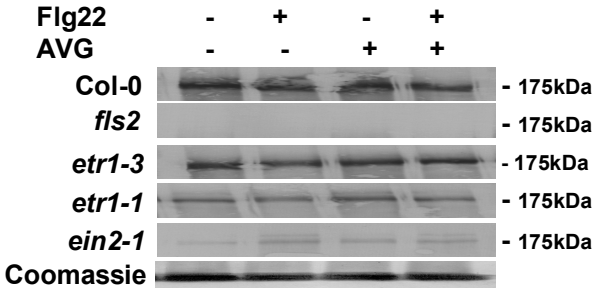
Suppl. Fig. 6. Flg22-induced growth arrest in seedlings of ethylene-insensitive mutants. Seedling growth of the indicated genotypes was measured in the absence or presence of 100 nM or 1 µM flg22. Bars represent +/-SD. Similar results were obtained in three independent experiments.



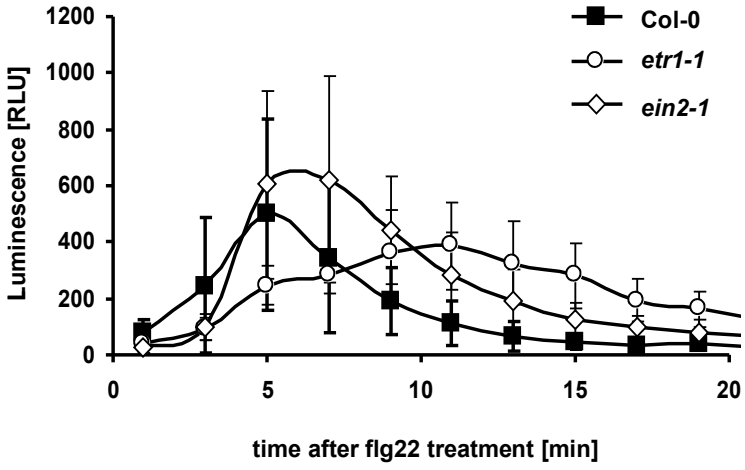
Suppl. Fig. 7. Bacterial multiplication at later times post infection. Bacterial growth in indicated seedlings infected with PtoDC3000 ΔAvrPto/ΔAvrPtoB at 1 and 3 day post infection (dpi). Shown are average values of three independent experiments (n=16); bars represent +/-SD. Letters indicate significant differences $p < 0,05$.



Suppl. Fig. 8. Steady-state and diminished accumulation of ethylene. Ethylene levels of mock and AVG treated Col-0, *etr1-1*, *etr1-3*, *ein2-1* seedlings were determined by GC-MS. Depicted are average values of relative integrated areas obtained in at least three independent experiments; bars represent +/-SD.



Suppl. Fig. 9. FLS2 accumulation in ethylene-insensitive mutants. FLS2 immunoblot analysis of the indicated genotypes and treatments. Coomassie staining is shown as loading control. Results were obtained in at least three independent experiments.



Suppl. Fig. 10. Flg22-induced oxidative burst in leaf discs. Seedlings leaves of the indicated genotypes were excised into discs of approximately equal sizes and monitored for flg22-mediated ROS production. Depicted are average values (n=12); bars represent +/-SD. RLU, relative light units.

4 Discussion

FLS2 activation results in a plethora of immune responses that include ROS production and accelerated induction of ethylene biosynthesis (Boller and Felix 2009). However, the contribution of a pathogen-induced oxidative burst to PAMP-triggered immunity was not established. Likewise, it was unclear whether individual PAMP-triggered responses interact with each other. This study provides more insight into molecular components that underlie PTI.

4.1 PAMP-triggered oxidative burst contributes to plant immunity

We confirm *At*RbohD as the responsible NADPH oxidase and rate-limiting component for flg22-elicited ROS production. Together with *At*RbohF, *At*RbohD mediates generation of ROS required for ABA regulated stomatal closure, and control the HR-type cell death during effector-triggered immunity (Torres et al., 2002; Kwak et al., 2003; Torres et al., 2005). Importantly, *At*RbohD appears sufficient to produce ROS upon application of extracellular reactive oxygen intermediates, which are known to trigger plant cell death (Torres et al., 2005; Wrzaczek et al., 2009). Our data show that *At*RbohD-mediated ROS production appears to be important for plant immunity, supposedly at early stages of the infection process.

Stomatal closure in response to ABA requires elevated cytosolic calcium concentration mediated by H_2O_2 activated calcium channels in Arabidopsis guard cells (Pei et al., 2000; Köhler et al., 2003). *AtrbohD/F* double mutants are impaired in stomatal conductance (Kwak et al., 2003), ABA induced ROS production is abolished and activation of plasma membrane resident Ca^{2+}-channels is diminished. However, exogenous application of H_2O_2 rescues this phenotype. PAMP-triggered responses are in particular relevant at the level of pre-invasive immunity (Melotto et al., 2006). Perception of PAMPs triggers stomatal closure and thereby prevents the entry of pathogens into plant tissues. ABA signaling as well as the guard cell specific OST1 kinase are mediators of PAMP-induced stomatal conductance. It is therefore likely that *At*RbohD functions in ROS-dependent flg22-induced stomatal closure, which is supported by a slight reduction of oxidative burst in *ost1* and *ost2* mutants when challenged with flg22 (Suppl. Tab. 1).

Genechip analysis revealed that *At*RbohD and *At*RbohF are expressed in guard cells. Moreover, their transcripts are up-regulated in response to ABA, pathogen- and fungal elicitors indicative of a role in plant immunity (Desikan et al., 1998; Yoshioka et al., 2001; Simon-Plas et al., 2002; Kwak et al., 2003). At early stages of infection loss of *AtrbohD* function allowed elevated growth of bacteria. Pathogen mediated early ROS generation might thus play a role in pre-invasive immunity probably through operating in stomatal conductance. Moreover, same phenotype was

observed for overexpression of *AtRbohD* and suggests that a general imbalance in induced ROS production perturbs the tight regulation and timing of PAMP responses. It is worth to note that function of COI1, previously identified as crucial component of PAMP-triggered stomatal closure (Melotto et al., 2006), or ABI1 and ABI2, key regulators of stomatal closure upon abiotic stress (Assmann et al., 2001), appeared dispensable for flg22-induced oxidative burst, indicating distinct genetic requirements for flg22 responses.

4.2 Molecular components of PAMP-triggered ROS

To date, only few molecular components, which constitute the flg22-triggered response pathway were functionally characterized. FLS2 physically interacts with flg22 and its stimulation upon ligand binding elicits a plethora of immune responses (Chinchilla et al., 2006). Full activation of these defense responses is dependent on ligand dependent interaction with the co-receptor BAK1 (Chinchilla et al., 2007). Rapid induction of MPK3 and MPK6 induces defense signaling and stimulates *At*RbohD and the ACS6, the rate limiting enzyme of ethylene biosynthesis (Liu and Zhang, 2004; Asai et al., 2002; Zhang et al., 2007).

Here, we identified *rio1* representing a novel *fls2* mutant allele. This variant caries a point mutation, which causes an amino acids exchange from Gly to Asp in position 72 aa at the very N-terminal region of yet unknown function (Gomez-Gomez et al.,2000). Importantly, accumulation of $FLS2^{G72R}$ was not altered. However, this mutant variant partially confers flg22 insensitivity. Flg22-triggered ROS production is impaired, whereas other early or late flg22-triggered responses occur in a WT-like manner. A Gly to Arg exchange probably results in a conformational change of FLS2, which in turn might hamper association with other proteins or with the ligand flg22 itself. As a consequence, the amount of activated FLS2 protein could be reduced. Decreased threshold levels of stimulated FLS2 might not be sufficient to trigger ROS production, but might meet requirements for mediating other responses. In addition, insertion of charged amino acids could likewise create other molecular interactions that probably compromise or alter established signaling pathways (Boyd and Beckwith, 1998). Several PAMPs trigger an almost identical set of responses, indicative of certain molecular components, which might be used in common (Nürnberger and Kemmerling, 2006; Boller and Felix, 2009). Interestingly, elf18-elicited ROS production appears reduced in $FLS2^{G72R}$, which implies that association with shared molecular components might be hampered in this mutant *fls2* allele.

We identified *rio2* and *rio3* carrying base pair changes in *BAK1*. Neither *rio2* nor *rio3* express BAK1 protein as a result of either aberrant splicing or an introduction of a premature stop-codon, respectively. Like *bak1-3* and *bak1-4*, *rio2* and *rio3* are reduced in early and late PAMP-triggered responses. BAK1 was demonstrated to be involved in EF-Tu elicited signaling and was

suggested to represent an adapter or co-regulator of various receptors (Chinchilla et al., 2007). In accordance, *rio2* and *rio3* proved to be reduced in elf18-triggered ROS generation. Moreover, BAK1 appeared not to be required for callose deposition explaining observed WT-like deposits in *rio2* and *rio3* mutants (Clay et al., 2009). Both, *rio4* and *rio5* mutants were only affected in PAMP-triggered ROS production and yet we could not attribute established pathway components responsible for this phenotype. Hence, we propose that responsible genes encode novel components of the important for immediate early oxidative burst.

Noteworthy, our analysis implies that individual PAMP responses can be uncoupled from each other. This is best illustrated in *rio1*, particularly diminished in early ROS production. *Rio2* to *rio5* are impaired in flg22-elicited ROS accumulation and arrest of seedling growth but mediate WT-like callose deposition. Previously, FLS2 variants carrying amino acid changes in the cytoplasmic domain were impaired in flg22-elicited ROS generation but only partially reduced in late PAMP responses (Robatzek et al., 2006). Thus, it is possible to generate FLS2 receptor variants, which are affected in some but not all PAMP responses.

4.3 Components of ethylene signaling regulate ROS production

We identified the mutants *etr1-1*, *etr1-3* and *ein2-1* to be diminished in flg22-triggered ROS, whereas *ctr1* and *ein3* mutant seedlings mounted WT-like ROS production. It is important to note that *etr1-1* and *ein2-1* represent dominant and ethylene-insensitive mutant alleles, which is not the case for the loss of function *ctr1* and *ein3* mutants (Kieber et al., 1993; McCourt 1999). Ethylene-insensitive mutants are known to accumulate elevated levels of endogenous ethylene (Kende et al., 1993). Chemical interference to reduce the ethylene steady-state levels could only partially recover the flg22-induced oxidative burst. Strong ethylene-insensitive alleles accumulate much less FLS2 protein levels, hence FLS2 expression in unstimulated plants depends at least partially on the amount of endogenous ethylene. Reduced FLS2 protein threshold levels seem not to be sufficient to trigger ROS production.

ETR1 and EIN2 represent positive regulators of FLS2 expression, probably, through downstream transcriptional events. Database analysis (ATTEB, http://atted.jp/) revealed that the FLS2 receptor is co-expressed with components mediating ethylene responses e.g. ERS1, CTR1 and ERF2. Additionally, *At*RbohD is co-expressed with ACS6, ERF1 and ERF2. In turn, the ETR1 receptor appears to be co-regulated with BAK1. These data uncover a transcriptional network connecting the flg22 receptor complex with components of the ethylene response pathway and the ROS generating enzyme.

Despite WT-like FLS2 accumulation, the weak *etr1-3* mutant allele exhibited clearly reduced flg22-triggered ROS production, suggesting additional regulatory components in between

ethylene signaling and oxidative burst. Moreover, reduced FLS2 levels in *etr1-1* and *ein2-1* mutants were still sufficient to mediate WT-like flg22 activation of MAP kinases. Individual flg22 responses might thus depend on different FLS2 threshold levels, but sensitivities of different response assays need to be considered. Thus, ethylene itself is required but not sufficient for WT-like ROS production. Ethylene activates plasma membrane Ca^{2+} channels (Zhao et al., 2007), and elevated cytosolic Ca^{2+} concentration serve as negative feedback mechanism for *Os*Rboh-generated oxidative burst (Wong et al., 2007). Further, H^{+}-ATPases are activated by ethylene (Vreeburg et al., 2005), which recently were noted to regulate stomatal closure in plant immunity (Liu et al., 2009). It is therefore possible that ethylene regulates ion fluxes and thereby ROS-mediated responses.

The ethylene-insensitive mutants were unable to accumulate callose deposits in response to flg22, however, other tested hormone signaling mutants reacted like WT (Clay et al., 2009). Interestingly, ethylene sensing appears to be required for flg22-induced ROS production and callose deposition but not for flg22-triggered MAP kinase activation, and induced resistance (Zipfel et al., 2004). Also, flg22-elicited seedling growth arrest was less pronounced in *ein2-1* mutants in the presence of poly (ADP-ribose) polymerase inhibition (Adams-Phillips et al., 2008). It is therefore evident that there are different genetic requirements for individual PAMP responses. However, genetic redundancy masking the importance of other than ethylene signalling pathways cannot be excluded.

The ETR1 receptor, which is expressed in guard cells, is discussed to be a point of convergence for ethylene and ROS signaling (Desikan et al., 2005, 2006). A role for EIN2 in ROS singaling was demonstrated in ozone-dependent accumulation of ROS (Overmyer et al., 2000). We therefore observed bacterial infections at the level of pre-invasive immunity, and revealed significantly enhanced bacterial growth rates in *etr1-1* and *ein2-1* mutants compared to WT. Increased susceptibility of ethylene-insensitive mutants was similarly reported when bacteria were surface inoculated (Pieterse et al., 1998; Clay et al., 2009). Both, ethylene signaling and PAMP-triggered ROS production contribute to early plant immunity, probably via flg22-dependent ROS-mediated stomatal closure. This, additionally, indicates ROS as a signaling molecule important for plant immunity.

4.4 Final remarks and perspectives

Our study provides evidence for a yet unknown interaction between flg22-induced ROS production and ethylene signaling. Ethylene is a hormone that itself inducibly accumulates in response to flg22. Obtained results further proposes that ethylene signaling controls the transient oxidative burst. Conjunction between ethylene induction and ROS accumulation underlies abiotic and biotic stress responses. The role of ethylene in pathogen-induced PCD has been evaluated in ethylene insensitive (never-ripe) tomatoes (Lund et al., 1999). Following infection these mutants were greatly reduced in PCD. Ethylene plays a critical role in H_2O_2 release during PCD and operates as a potentiator of ROS production (de Jong et al., 2002). Abiotic elicitors such as ozone up-regulates ethylene biosynthesis. If ethylene production or perception is blocked, the incidence of lesions is reduced, which suggested that both ROS and ethylene are involved in the induction of HR-type PCD upon ozone exposure (Overmeyer et al., 2000). We observed that a reciprocal interaction of flg22-induced ethylene production, and ROS generation and signaling also underlies PAMP-triggered responses. Interaction of ROS and ethylene might play a role in the proper timing and amplification of elicitor-mediated defence signaling possibly to fine tune plant innate immunity.

To date, the role of early and transient ROS production in plant immune responses is little understood. Our results provide evidence that the flg22-triggered oxidative burst contributes to stomatal conductance. Several pathogen-derived components, such as elf18 and lipopolysaccharide, cryptogein, oligogalacturonide and chitin rapidly induce a transient increase in ROS accumulation (Bottin et al., 1994; Pugin et al., 1997; Gommez et al., 1999; Meyer et al., 2001; Aziz et al., 2004; Kunze et al., 2004; Kaku et al., 2006), which suggests additional roles for ROS accumulation. Conceivably, similar to DAMPs (Ryan 2003/2005, Huffaker et al.,2006; Rubartelli et al., 2007), an oxidative burst could define a chemical danger signal initiating additional pathways for sustained defences. However, due to its short half-life time of 1 ms and a relative travelling distance of 1 µm (Möller et al., 2007) there is ongoing discussion about its *in vivo* signaling capacity. A recent study indicated that ROS produced by *At*RbohD can mediate rapid cell-to-cell communication over long distances (Miller et al., 2009). Rapid systemic signaling is accompanied by extracellular ROS generation and is induced by wounding, heat, cold, high-intensity light, and salinity stresses (Miller et al., 2009). Hence, accumulation of ROS could provide systemic redox changes to enable DAMP signaling (Rubatelli et al., 2007). Concomitant initiation of PAMP - and DAMP signaling is most likely required for sustained immune responses.

In mammals, the significance of ROS production is illustrated in host defence. Dysfunction of NOX2 results in enhanced susceptibility to bacteria and fungi and causes life-threatening infections (Bedard et al.,2007; Sumimoto, 2008). Plants likewise utilize Rboh proteins to intentionally produce ROS. In an incompatible plant-pathogen interaction rapid biphasic apoplastic accumulation of ROS is associated with PCD (Doke, 1983; Apostol et al., 1989, Baker et al., 1995; Lamb and Dixon 1997; Apel and Hirt, 2004). Moreover, plant Rboh proteins regulate plant

development. If Rboh expression is reduced chlorophyll content decreases and plants display altered morphology reminiscent of phenotypes caused through defects in hormone pathways (Foreman et al., 2003; Sagi et al., 2004). In agreement, ABA, auxin and ACC, the direct precursor of ethylene, were identified to positively regulate Rboh accumulation (Sagi et al., 2004). Rboh proteins are expressed in stomata, epidermal and mesophyll cells possibly to enable an ubiquitinously ROS singaling network (Desikan et al., 1998; Keller et a., 2001; Kwak et al., 2003, Joo et al., 2005, Miller et al., 2009). In contrast to animals, ROS generation by Rboh oxidases is not only an inducible, but also a constitutive active process (Sagi et al., 2004; Sagi and Fluhr, 2006). Signaling through ROS importantly contributes to stomatal closure, cell elongation and responses to pathogens (Neill et al., 2002; Torres et al., 2002; Kwak et al., 2003; Foreman et al., 2003). However, molecular mechanisms linking ROS to these physiological responses are less well understood.

H_2O_2-modulated gene expression in Arabidopsis and tobacco revealed two component signal transduction pathways to be up-regulated by exogenous application of ROS (Desikan et al., 2001; Vandenabeele et al., 2003). In yeast, two-component signaling systems operate as ROS sensors, likewise in plants, ETR1 functions as ROS sensor (Desikan et al., 2005). In particular, the Cys-Tyr65 mutation in the *etr1-1* mutants were identified to confer H_2O_2 insensitivity resulting in impaired stomatal conductance. Besides ethylene pathway components the cytokinin receptor family likewise represents two-component histidin kinases. Two signaling components (AHK1 and CRE1) complement the yeast *synthetic lethal of n-end rule 1* (*sln1*) redox-sensor and have been implicated in osmosensing (Verslues and Zhu, 2005, Phan Tran et al., 2007). Moreover, the hybrid kinase AHK5 was recently indicated to play a role in flg22-induced regulation of H_2O_2 production and stomatal closure (Desikan et al., 2008). Other kinases that could serve as H_2O_2 signaling mediators are cysteine rich repeat-RLKs (CRKs), which are implicated to act as extracellular redox-sensors, and contribute to plant immunity (Chen, 2001, Chen et al,. 2003, 2004). In addition, the cysteine rich kinase 13 (CRK13) is specifically activated in response to flg22, suggesting a role in PAMP-triggered immunity (Acharya et al., 2007).

H_2O_2 signaling could also be propagated through MAP kinase cascades, although, ROS dependent activation of MAPKs is controversially discussed. When tobacco cells are elicited with cryptogein, or parsley cells are treated with Pep13, MAP kinase activation appears to be independent of ROS accumulation (Garcia-Brugger et al., 2006). Alternatively, H_2O_2 activates OXI1 and ANP1, which are both required for full stimulation of MPK3 and MPK6 (Kovtun et al., 2000; Rentel et al., 2004). Moreover, flg22-elicited ROS accumulation is suggested to occur downstream of MAP kinase activation (Zhang et al., 2007), in contrast, elf18-mediated ROS accumulation seem to be independent of MPK3 and MPK6 activity (Sajio et al., 2009). Together, ROS appears not to be necessary to induce MAPKs, but appears to be able to stimulate these molecular components. ROS might thus contribute to a positive MAPK activation feedback loop. In agreement, a recent study reported that Rboh proteins and MAP kinases facilitate positive feedback regulation that contributes to ABA signalling (Lin et al., 2009).

*At*RbohD is phosphorylated in response to flg22-treatment and is required for flg22-elicited ROS accumulation (Benschop et al., 2007; Nühse et al., 2007; Zhang et al., 2007). Hence, it is conceivable that ROS production in response to flg22 is mediated by direct interaction with FLS2. Moreover, plasma membrane resident gp91phox (NOX2) actively assembles in endosomal vesicles following stimulation (Brown et al., 2003; Simonsen and Stenmark, 2001). Supporting the occurrence of a similar mechanism in plants, salt stress causes intracellular distribution of Rboh-mediated ROS production sites enclosed by endosomal membranes (Leshem et al., 2007). In agreement, wortmannin inhibits ABA-induced production of ROS in Arabidopsis guard cells (Park et al., 2003). Flg22-treatment mediates ROS production and stimulates FLS2 endocytosis (Robatzek et al., 2006), and thus *At*RbohD localization and identification of interacting proteins would be informative. In addition, gene expression profiles of untreated and elicited *AtrbohD* mutant plants will be necessary to better uncouple flg22 responses from ROS signaling. Finally, identification of the responsible genes for the mutants *rio4* and *rio5* as well as the search for more *rio* mutants will provide a better understanding of ROS signaling in PAMP-triggered plant immunity.

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Sophia Mersmann