Mechanisms of local and systemic defences in *Arabidopsis thaliana* in response to host and non-host strains of *Pseudomonas syringae*

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Julius-Maximilians-Universität Würzburg

vorgelegt von

Tatiana E. Mishina
aus Radovizkij (Russland)

Würzburg 2007

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: Prof. Dr. Martin J. Müller
Gutachter 1: Prof. Dr. Markus Riederer
Gutachter 2: Prof. Dr. Thomas Roitsch

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Contents

1. Introduction

1.1 Pathogen elicitors

Plants depend on an innate immune system to defend themselves against pathogens that grow epiphytically on their surface. Neither an acquired immune system nor a circulatory system comparable to mammals is known for plants. However, plants are able to restrict the development of disease caused by many pathogens. The restriction occurs due to recognition of the pathogen and activation of defences. All microbial signals that are perceived by plant cells and induce defence responses are considered as elicitors (Keen and Buergger, 1977).

Two types of elicitors are differentiated: general (or non-specific) elicitors, which do not significantly differ in their effect on different cultivars within a plant species and may therefore be involved in general resistance, and specific elicitors, which are special to the pathogen race or strain and function only in plant cultivars carrying a matching disease resistance gene. The latter are involved in specific resistance, which is specified through the development of cell death in the so-called hypersensitive response (HR).

General elicitors are also designated as pathogen-associated molecular patterns (PAMPs). Bacterial PAMPs include bacterial surface components and cytoplasmic molecules. Among exposed PAMPs recognized by plants are flagellin, a protein subunit that builds up the bacterial flagellum (Felix et al., 1999), lipopolysaccharides (LPS), and lipooligosaccharides. The latter two are abundant components of the outer membrane of Gram-negative bacteria (Newman et al., 1997; 2001). The elongation factor Tu (EF-Tu) and cold shock proteins (CSPs) are two examples for cytosolic PAMPs (Kunze et al., 2004; Felix and Boller, 2002). Variations between strains of *Pseudomonas syringae* in genes encoding PAMPs, for instance differences in flagellins, can determinate the outcome of a plant-*P. syringae* interaction (Takeuchi et al., 2003).

Specific elicitors belong to effectors proteins, which bacterial pathogens deliver into the plant apoplast or directly into the plant cell by the type III secretion system (Jin et al., 2003). The intended function of these effector proteins is to promote bacterial virulence. However, specific plant cultivars have developed the ability to recognize particular effector proteins by matching resistance proteins (R proteins). The effectors are in this case named avirulence (*avr*)-proteins. The avr-R-protein recognition event induces rapid defence reactions including the production of reactive oxygen species (ROS) and the HR (Lindgren et al., 1986). When both of these genetic determinants are present, host defence responses are triggered and pathogen colonization is limited. In some cases, mutation of an individual *avr* gene in a *P. syringae* strain can render the strain compatible (Tsiamis et al., 2000). In other cases, deletion of an *avr* gene does not cause that strain to

become compatible (Vinatzer et al., 2006). Interestingly, effector repertoires vary in size and in composition between strains (Greenberg and Vinatzer, 2003; Chang et al., 2005). Only 13 effectors are shared between the three sequenced strains *P. syringae* pv. *tomato* DC3000 *(Pst*), *P.s.* pv. *syringae* B728a (*Psy*) and *P.s.* pv. *phaseolicola* 1448a (*Psp*). The remaining approximately 40 effectors are ether unique to one of these strains or only shared between two of them (Vinatzer et al., 2006). These differences in effector repertoires are thought to be main determinants of host range in *P. syringae* (Alfano and Collmer, 2004). The type III secretion system (TTSS) is encoded by in-cluster-organised *hrp* (hypersensitive response and pathogenicity) genes. Mutation in the *hrp* genes leads to a failure of HR elicitation in resistant varieties of host and certain non-host plants, and to loss of pathogenicity in susceptible varieties. The *hrp*-genes are located on a 25kb stretch on the bacterial chromosome or on plasmids. The sequence and cluster structure of *hrp*-genes are similar between the different bacterial pathogens *P. syringae* pv. *syringae*, *Erwinia amylovara* and *Xanthomonas campestris* (Hueck, 1998). The *hrp* gene cluster contains the genes encoding for components of the specialized pilus system and secreted proteins called harpins. For instance, the *hrpA* gene encodes an extracellular protein that forms a pilus conduit, which delivers effectors across the plant cell wall into the cytoplasm (Li et al., 2002). HrpZ, by contrast, is a glycine-rich, cysteine-lacking harpin protein secreted into the apoplast (Kim and Beer, 1998; Jin et al., 2003). Harpins can induce the HR after infiltration into the plant. Although harpins from *P.s.* pv. *syringae* have been shown to interact with tobacco cell walls, the biological function of harpins and the means by which they elicit cell death is unknown (Hoyos et al., 1996).

1.2 Plant receptors

For the recognition of pathogen-associated patterns and effectors proteins, the plant has developed a battery of receptors (Fig. 1), which initiate defence responses and therefore constitute important components of the plants defence machinery (Jones and Takemoto, 2004). The receptors which recognize pathogen effectors are named resistance (R) proteins, and the corresponding genes R-genes. R-proteins can be located either extracellularly with a plasma membrane anchor or intracellularly. Three classes of R-protein have been distinguished according to the occurrence and organization of conserved protein domains (Cunha et al., 2006):

1. Cytoplasmic NB-LRR proteins with a central nucleotide binding site (NB) and an N-terminal leucine-rich repeat domain (LRR). The C-terminal LRR is involved in protein-protein interaction and recognition specificity. The NB exhibits similarity to the NOD domain of animal pattern recognition receptors (PRRs), which

intracellularly recognize PAMPs (Girardin et al., 2003). According to the nature of their N-terminus, NB-LRR proteins can be grouped into two categories. TIR-NB-LRR contain a TIR domain with homology to the Toll-like innate immunity receptors from *Drosophila melanogaster* or *Homo sapiens*, and CC-NB-LRR proteins contain a putative coiled-coil domain (CC) at their N-terminus.

2. LRR-RLK proteins with extracellular LRRs, a transmembrane and an intracellular receptor-like kinase (RLK) domain.
3. Receptor-like proteins (RLP) with extracellular LRRs and a membrane-spanning domain.

R-proteins from the first group explicitly recognize type III effector proteins. RPM1, RPS2 and RPS5 represent CC-NB-LRR proteins from Arabidopsis that indirectly recognize the *Pseudomonas* type III effectors AvrRpm1, AvrRpt2 and AvrPphB, respectively. Another *Arabidopsis* R-protein, RRS1-R, which belongs to TIR-NB-LRR group, recognizes the PopR2 type III effector from the vascular pathogen *Ralstonia solanacearum*. RRS1-R is a unique R-protein because of an extended carboxy-terminus that includes a nuclear localisation signal (NLS) and a WRKY domain, both characteristics of large family of plant transcription factors. RRS1-R associates directly with PopP2 and this interaction localizes RRS1-R to the nucleus where it may activate transcription of defence gene (Deslandes et al., 2003). Another R protein from tomato, Pto, is a Ser/Thr protein kinase that can recognize the AvrPto protein from *P. syringae* by direct physical interaction. For its R protein function, Pto requires Prf, an NBS-LRR protein which recognizes AvrPto indirectly.

The R-protein from the second group can recognize both effector proteins and PAMPs. An example of a plant receptor that activates defence reactions in response to PAMPs is the receptor like kinase FLAGELLIN-SENSING2 (FLS2) from Arabidopsis. FLS2 directly interacts with a conserved 22 amino acid stretch of bacterial flagellin and thus initiates defence responses (Gomez-Gomez et al., 2000; Chinchilla et al., 2006). Another LRR-RLK was identified as receptor for elongation factor EF-Tu (EFR; Zipfel et al., 2006). An example of an RLK that recognizes a pathogen-derived effector protein is Xa21 from rice, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* harbouring the AvrXa21 protein (Song et al., 1995).

Members of the RLP group of R-proteins also initiate defences in response to both PAMPs and effector proteins. The tomato EIX (ethylene-inducing xylanase) receptor recognizes a PAMP from within the fungal EIX protein (Rotblat et al., 2002; Ron and Avni, 2004). The tomato Cf-2 receptor confers resistance to isolates of the leaf mold *Cladosporium fulvum* which contain the Avr2 avirulence protein (Jones et al., 1993). Fig. 1 summarizes the localisation of known R-protein classes and corresponding elicitors.

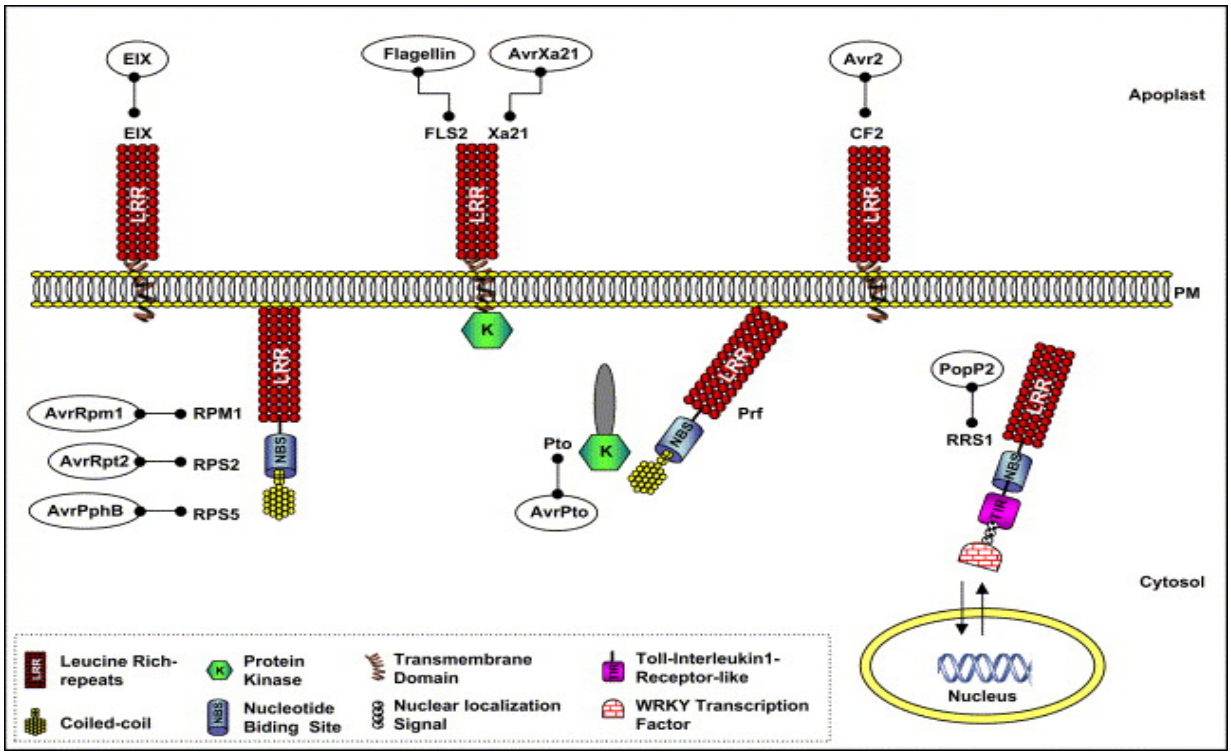


Fig. 1 *Schematic representation of plant R-proteins. Pathogen-derived elicitors are in circles and connected by lines to the names of their cognate R-proteins (adapted from Cunha et al., 2006).*

1.3 Plant defences

Plant can defend themselves efficiently against most phytopathogens. The outcome from a plant-pathogen interaction depends on the ability of the pathogen to overcome plant defences which rely on preformed resistance barriers, on induced responses after perception of pathogen elicitors, or on a combination of both (Thordal-Christensen, 2003).

1.3.1 Preformed defences

Preformed defences represent the first barriers which pathogens need to overcome to successfully colonise a plant. They can be structural or chemical in nature. Structural barriers comprise the plant cuticle with epicuticular wax layers and cutinized cell walls that prevents leaf penetration and invasion of most fungal and bacterial pathogens.

Phytoanticipins are preformed, low molecular weight antimicrobial compounds which are present in plants before contact with a pathogen, or which are produced after inoculation solely from pre-existing constituents. The phytoanticipins are known in many plant families, and typical examples are triterpenoid or steroid saponines, cyanogenic glycosides, and glucosinolates (Morrissey and Osbourn, 1999). Glucosinolates (GS) are

sulphur containing, secondary metabolites commonly occurring in cruciferous plants including Arabidopsis (Hogge et al., 1988). Upon tissue damage, GS are converted into breakdown products, some of which are known to inhibit microbial growth *in vitro* (Mithen et al.,1986; Kirkegaard et al., 1996; Manici et al., 1997). Arabidopsis plants expressing the cytochrome P450 gene CYP79D2 from cassava accumulate aliphatic isopropyl and methylpropyl GS and show enhanced resistance against the necrotrophic bacterium *Erwinia carotovora.* Moreover, expression of sorghum CYP79A1 or CYP79A2 leads to accumulation p-hydroxybenzyl or benzyl GS, respectively, which increases resistance towards *P.syringae* pv. *tomato* DC3000. This increase in resistance, however, results most probably not from direct toxicity of GS hydrolysis products, but from an indirect activation of the salicylic acid signalling pathway (Langlois-Meurinne et al., 2005). By contrast, the *gsm1-1* (TU1) mutant, which has reduced amounts of many aliphatic GS, shows no alteration in resistance towards *Pst* but is more susceptible to *Fusarium oxysporum* (Tierens et al., 2001). Up to now, the particular role of GS in resistance against *P. syringae* is not clear.

Saponins are surface active agents which damage fungal cell membranes. They are produced by several plant species including oat, tomato and different members of the *Caryophyllaceae* family. Some saponins, e.g. avenacin A-1 from oat, act as preformed toxic substances in plant tissue. In other cases, active saponines are rapidly produced from inactive precursor after tissue damage. Avenacin A-1 is a phytoanticipin in oat roots and acts as a protectant against black root rot disease caused by the fungal pathogen *Gaeumannomyces graminis*. The wheat root pathogen *G. graminis* var. *triciti* is not adapted to attack oat successfully and cannot cause disease on oat roots. The related oat root pathogen *G. graminis* var. *avenae* depends on its ability to detoxify avenacin A-1 to succeed in pathogenesis. A mutant oat line which no longer produces avenacin A-1 is susceptible to *G. graminis* var. *triciti*, demonstrating that the saponin directly protects wild-type oat from infection by the wheat root rot fungus. The latter lacks, in contrast to the oat pathogen, the enzyme avenacinase which detoxifies avenacin A-1 (Papadopoulou et al., 1999). Resistance of oat to *G. graminis* var. *triciti* represents a typical example for non-host resistance. Non-host resistance occurs when an entire plant species is resistant to a particular isolate of pathogen. It can result from successful passive defence or from active defence that is induced upon pathogen recognition.

1.3.2 Induced defence responses

Induced defenses are activated after perception of general or specific microbial elicitors. Similar to pre-formed defences, they comprise structural and chemical components.

1.3.2.1 Pre-invasion defence

Defence reaction activated after recognition of general elicitors (PAMPs) like flagellin and LPS contribute to basal resistance. This type of resistance combines defence reactions triggered before a pathogen enters a plant, and defence responses elicited after pathogen invasion. For instance, a defence reaction induced before bacterial invasion is stomata closure (Mellotto et al., 2006). *P.syringae* is a non-invasive bacterial pathogen that penetrates into the apoplast only through natural openings like stomata or wounds. To gain access to the extracellular spaces of the leaf interior, bacteria move on the leaf surface toward open stomata. On their way, bacteria lose part of their flagellar subunits, which plants recognize by the FLS2 receptor and thereupon induce stomatal closure. Some bacteria including *P.syringae* pv. *tomato* and *P.s.* pv. *glycinea* have developed the ability to overcome the structural barrier imposed by closed stomata. They produce coronatine, a structural mimic of the phytohormone jasmonic acid (JA) and its precursor 12-oxophytodienoic acid (OPDA), which play a role in wound responses, fruit abscission and senescence. Coronatine promotes the reopening of stomata through interaction with a key component of the JA signalling pathway, allowing entry of bacteria into leaf apoplastic spaces (Melloto et al., 2006).

Having gained entry into the plant interior, pathogens are often confronted with preformed barriers like antimicrobial phytoanticipins (see 1.3.1) and with induced defence response. The formation of papillae represents an inducible structural defence, which is activated by general elicitors (Gomez-Gomez et al., 1999). Papillae are local cell wall fortifications formed on the inner side of plant cell walls at sites of pathogen contact. They are composed of callose, phenolics, hydroxyproline-rich glycoproteins and other cell wall material (Hauck et al., 2003). Papilla formation represents an important defence mechanism to prevent fungal invasion. An example of papilla-based resistance, which is unusual because it provides complete protection, is *mlo*-resistance of barley to the host pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). MLO is a negative regulator of papilla formation, and a mutation in this gene makes barley resistant to *Bgh* (Freialdenhoven et al., 1996). In the non-host interaction of Arabidopsis with *Bgh*, about 80% of the germinated conidia undergoing attempted penetration are stopped in association with papillae. Three penetration mutants, *penetration1* (*pen1*), *pen2* and *pen3*, were isolated in genetic screens for mutations in Arabidopsis resulting in decreased penetration resistance

to *Bgh*. *PEN1* encodes a plasma-membrane resident syntaxin, which is a member of the super-family of SNARE (soluble N-ethylmaleimide-sensitive factor) domain proteins. PEN1 has been suggested to have both a basal secretory function and a specialized defence-related function, being required for polarized secretion events that give rise to papilla formation (Assaad et al., 2004). The barley *ROR2* gene is a functional homologous to Arabidopsis *PEN1* (Collins et al., 2003). Barley *ror* mutants, like Arabidopsis *pen*, show significantly reduced penetration resistance to *Bgh* (Freialdehoven et al., 1996). This demonstrates a close link between non-host and basal resistance. Arabidopsis *PEN2* and *PEN3* encode a glycosyl hydrolase and an ATP binding cassette transporter protein, respectively. They might be involved in generation and transport of toxic substances to sites of attempted fungal invasion (Lipka et al., 2005; Stein et al., 2006).

Cell-wall based defences are also important for resistance against bacterial pathogens. The non-pathogenic *hrp* mutant strain *Pst hrcC*, which is defective in type III secretion, and the bean pathogen *P. syringae* pv. *phaseolicola* (*Psp*), for which Arabidopsis represents a non-host plant, induce papilla formation in Arabidopsis at sites of bacterial contact with the cell wall (Hauck et al., 2003; Soylu et al., 2005). This cell wall-based defence might impede water and nutrient flow out of plant cells into the apoplast and thus prevent bacterial feeding and multiplication. Interestingly, virulent, disease causing pathogens have developed the ability to repress cell-wall based defences. For instance, *Pst* inhibits papilla formation by delivering adequate effector proteins into the plant cell through type III secretion. Hauck et al. (2003) have demonstrated that heterologous expression of a single bacterial TTSS effector in Arabidopsis, AvrPto, is sufficient to repress papilla formation. Moreover, a similar set of genes has been activated by virulent *Pst* and heterologous AvrPto expression in Arabidopsis (Hauck et al., 2003). These findings highlight the importance of bacterial TTSS effectors in suppression of pre-invasive defences and in the promotion of bacterial virulence.

1.3.2.2 Post-invasion defence

In plant-bacteria interactions, post-invasion defences are induced after the pathogen has entered the plant apoplastic space and managed to establish a functional TTSS. Some delivered effectors proteins can be recognized by a corresponding plant R-protein (see 1.1 and 1.2), and this leads to so called specific or gene-for-gene resistance. The pathogen carrying the recognized effector or 'avirulence protein' is named avirulent. An example for such an incompatible interaction is the encounter of *Pst* strains harbouring the AvrRpm1 effector (*Pst avrRpm1*) with Arabidopsis accession Col-0 which possess the Rpm1 resistance protein (Bisgrove et al, 1994). Gene-for-gene resistance is characterized

by fast mobilization of a battery of defence responses including the oxidative burst and the hypersensitive response. The HR results in the rapid appearance of dry necrotic lesions at infection sites (Keen, 1990). Induction of the HR can take place not only by contact with avirulent pathogens, but in some cases also by interaction with non-host pathogens. For instance, the *P. syringae* pv. *pisi* effector AvrPpiA1, which is recognized by an R protein in pea, also induces an HR in the nonhosts Arabidopsis and bean (Dangl et al., 1992).The *P.syringae* pv. *phaseolicula* causes HR-like lesions in the non-host plant tobacco but not in Arabidopsis (Lu et al., 2001; Oh et al., 2006). This suggests that bacteria use TTSS-mediated translocation of effectors also in non-host plants which might but not necessarily does lead to an HR response. The HR is generally thought to deprive pathogens of nutrients and water, to confine them to initial infection sites, and therefore to restrict pathogen growth and spread.

Among the earliest changes observed after inoculation with avirulent bacterial pathogens are changes in ion fluxes over the plant plasma membrane and the so-called oxidative burst, i.e. production of ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2; Levine et al., 1994). These two processes can be consecutive, and one might be causative for the other. In elicitor-treated parsley cells, ion fluxes (Ca^{2+} and H^+ influx, K^+ and Cl^- efflux) triggers O_2^- production (Jabs et al., 1997). In Arabidopsis, a transient increase in cytosolic Ca^{2+} directly precedes a *Pst avrRpm1*-induced oxidative burst and represents a prerequisite for ROS production and the HR (Grant et al., 2000). Complementarily, the production of ROS is a trigger for rapid Ca^{2+} influx in soybean cells which leads to HR cell death (Levine et al., 1994; 1996). Another study using tobacco cell cultures indicates that neither ion fluxes nor the oxidative burst contribute to hypersensitive cell death (Dorey et al., 1999).

However, ROS are generated rapidly and transiently after challenge at sites of infection (Jabs et al. 1997). Recently, it has been shown that nitric oxide (NO) works synergistically with ROS to promote the HR (Delledonne et al., 1998; 2001). NO potentiates the ROS-mediated induction of HR cell death and can function independently of ROS to induce defence related genes (Delledonne et al., 1998). NO is well known as a signalling molecule in the immune, nervous and vascular system of vertebrates, and is generated in animal cells by NO synthase through L-arginine to L-citrulline conversion (Schmidt and Walter, 1994). Whether a similar reaction takes place in plants is far from clear. Although in plants and fungi, NO synthase activity was detected (Ninnemann and Maier, 1996), no NOS-like enzyme has been unequivocally identified until now. The AtNOS1 protein from Arabidopsis has been considered as a plant NOS for some years (Guo et al., 2003), but recent evidence suggests that this enzyme does not exhibit NOS activity (Zemojtel et al., 2006). Many recent reports suggest a regulatory function of NO in physiological processes, such as guard cell abscisic acid signalling (Desikan et al., 2002),

regulation of iron homeostasis (Graziano et al., 2002), execution of programmed cell death in barley aleurone layers (Beligni et al., 2002), root organogenesis (Pagnussat et al., 2002), and wound signalling (Orozco-Cardenas and Ryan, 2002). In the majority of cases, the evidence was obtained from studies based on pharmacological experiments, including exogenous application of NO donors, NO scavengers or inhibitors of mammalian NO synthase (Delledonne et al., 1998; Durner et al. 1998). In pharmacological approaches, side effects cannot be excluded and therefore involvement of NO in all described processes should be regarded with caution (Floryszak-Wieczorek et al., 2006). In addition, a widely-used method for NO detection, which is based on a fluorescence increase of diaminofluorescein (DAF) derivatives (e.g. Foissner et al, 2000), has been questioned because of possible non-specific signals (Planchet and Kaiser, 2006). For instance, changes in DAF fluorescence are not necessarily indicative for NO production, but may also reflect NO oxidation and/or production of other DAF-reactive compounds (Balcerczyk et al., 2005). The chemiluminescence method seems to be more reliable for NO detection.

The function of ROS in plant defence has been investigated in detail. They can act directly as antimicrobial agents (Peng and Kuc, 1992) or indirectly as second messenger in the activation of defence genes (Jabs et al., 1997). They also play an important role in the execution of hypersensitive cell death after pathogen recognition (Levine et al., 1994). The HR is accompanied by biosynthetic production of salicylic acid (SA). SA can potentiate ROS production and plant cell death in response to pathogens or fungal elicitors (Shirasu et al., 1997; Dempsey et al., 1999). Expression of phenylalanine ammonium lyase (PAL), the key enzyme of phenylpropanoid biosynthesis, is induced in leaves a few hours after pathogen treatment, suggesting that PAL might be involved in pathogen-induced SA biosynthesis (Dorey et al., 1997; Mauch-Mani and Slusarenko, 1996). However, Arabidopsis *pal1* and *pal2* mutants still accumulate SA after *P. syringae* infection (TE Mishina and J Zeier, unpublished results). Instead, they show reduced lignin content and are deficient in several phenolic lignin precursors indicating that PAL is predominantly involved in lignin biosynthesis (Rohde et al., 2004). Wildermuth et al. (2001) demonstrated the existence of an alternative biosynthesis pathway for pathogen-induced SA biosynthesis in Arabidopsis. Mutation in the *SALICYLIC ACID INDUCTION-DEFICIENT2 (SID2)* gene which encodes the chloroplast-localized isochorismate synthase1 (ICS1) leads to abolishment of pathogen-induced SA accumulation, suppression of defense gene expression, and enhances susceptibility to *P. syringae* pv. *maculicula (Psm)* and *Pst* (Nawrath and Métraux, 1999; Wildermuth et al., 2001). Exogenous application of SA analogs complement the *sid2* defects in *PATHOGENESIS-RELATED-1* (*PR-1*) expression (Nawrath and Métraux, 1999). These findings demonstrate the involvement of ICS1 in pathogen-induced SA biosynthesis and suggest

that SA produced by ICS1 is involved in signal transduction leading to induction of pathogens-related (PR) gene expression and resistance.

PR and other defence proteins are induced after interaction of plants with pathogens. Whereas some of them exhibit direct antimicrobial activity, the function of others still awaits elucidation. Based on their primary structure, PR proteins can be classified into distinct families. Tabelle1 summarizes the main properties of 12 families of well characterized antimicrobial proteins (Broekaert, et al.,1995; 1997). PR-2-type proteins are β-1,3-glucanases, PR-3-, PR-8-, PR-11- and possibly also PR-4-type proteins hydrolyse chitin or related substrates. PR-5-type proteins have been proposed to affect particular membrane binding sites (Thevissen et al., 1997; Yun et al., 1997). For PR-1-type proteins, no enzymatic activity has been described until now. In Arabidopsis and probably also other plant species, expression of *PR-1* is closely associated with and strongly dependent on SA signalling (Nawrath and Métraux, 1999). PR proteins have been discovered in every plant species investigated so far, and they can be expressed in any plant organ. In most cases, PR proteins are encoded by small gene families usually counting from 5 to over 50 members (Linthorst, 1991), and different members of gene families can be differently expressed depending on the external stimulus (Tornero et al., 1997). Induction of distinct sets of PR genes has been observed upon infection of plant with various types of plant pathogens.

AMP family	*Typical size (kb)*	*Enzymatic properties*	*Proposed microbial target*
PR-1-type	*15*	*N.E.A.R*[a]	*Unknown*
PR-2-type	*30*	*ß-1,3-glucanase*	*ß-1,3-glucan*
PR-3-type	*25-30*	*Chitinase*	*Chitin*
PR-4-type	*15-20*	*Chitinase (?)*[b]	*Chitin (?)*
PR-5-type	*25*	*Thaumatin-like*	*Membrane (membrane proteins)*
PR-8-type	*28*	*Chitinase*	*Chitin (?)*
PR-11-type	*40*	*Chitinose*	*Unknown*
Hevein-type AMPs	*3-5*	*N.E.A.R*	*Unknown*
Thionins (Thi2.1)	*5*	*N.E.A.R*	*Membrane (phospholipids)*

Table 1 *Key properties of the main families of pathogenesis-related, antimicrobial proteins (AMP) and peptides. (Adapted from Broekaert, et al., 1997; [a]no enzymatic activity reported; [b]question mark indicates lack of direct evidence)*

Recognition of pathogen-derived elicitors leads to activation of different signal transduction cascades which result in expression of PR and other defence genes. Several secondary signalling molecules whose synthesis is increased in response to elicitors and which are involved in activation of PR gene have been identified. These include H_2O_2 and

other ROS which can activate genes only immediately surrounding the infection site (Lamb and Dixon, 1997; Levine et al., 1994), jasmonic acid (Creelman and Mullet, 1997), ethylene (Boller, 1991), abcisic acid (Zeevaart and Creelman, 1988), and salicylic acid which can be transported over longer distances and activate PR gene expression in tissue distant from initial infection (Mölders et al., 1996; Shulaev et al., 1995). Many aspects of SA long-distance signalling are not fully understood. It still unclear whether SA is transported in unmodified form, as a glycosidic derivate, as methyl salicylate, and whether it is accompanied by other mobile signals (Shulaev et al., 1995; 1997; Durner et al., 1997). To which secondary signal a particular PR gene responds depends primary on the presence and relative position of binding sites for transcription factors in its promoter (Somssich, 1994). Several components of the signal transduction cascade leading to PR gene induction have been identified by mutagenesis and transgenic approaches.

SA is a central defence metabolite and several defence signalling cascades are SA-dependent. The Arabidopsis ICS1 mutant *sid2* (see above), and transgenic plants overexpressing the bacterial salicylate hydroxylase NahG, which rapidly converts SA to catechol (Hunt et al., 1997), are both deficient in pathogen-induced *PR-1* expression, indicating that SA is triggering this process. Plants that are nonresponsive to SA were identified in several mutant screens and found to have mutations in the same gene, *NPR1* (*NON-EXPRESSER OF PR GENES1*; Cao et al., 1994; Delaney et al. 1994; Glazebrook et al., 1996; Shah et al., 1997). Although SA accumulates in *npr1* mutants to levels higher than in wild-type after pathogen infection, they are not able to induce pathogen- or SA-mediated PR gene expression. Thus, NPR1 acts downstream of SA. The NPR1 protein contains an ankyrin-repeat domain, which in other proteins mediates protein-protein interactions. In further experiments, it has been discovered that both localisation and the state of NPR1 depend on the amount of SA in the plant cell. When SA levels are low, NPR1 exists in an oligomeric form in the cytoplasm. When SA levels increase after pathogen infection, the redox environment of the cell becomes more reductive and the NPR1 oligomers dissociate into monomers (Mou et al., 2003). The NPR1 monomers enter the nucleus and interact with TGA-type transcription factors (Fan and Dong, 2002). The TGA transcription factors bind a DNA element known as *as-1*, which is present in various plant and viral genes including the *PR-1* promoter (Lam and Lam, 1995). TGAs 2, 5 and 6 are required for the activation *PR-1* expression by SA (Zhang et al., 2003; Fig. 2b).

ENHANCED DISEASE SUSCEPTIBILITY5 (EDS5; SID1) is a further gene involved in SA biosynthesis discovered by a mutant screen. Arabidopsis *eds5* mutants have a similar phenotype than *sid2* indicating that EDS5 is needed for SA biosynthesis after pathogen attack (Nawrath and Métraux, 1999). Cloning of the *EDS5* gene shows that it encodes a MATE family transporter that may be involved in transport of intermediates for SA biosynthesis (Nawrath et al., 2002). EDS5 is also expressed after external

application of SA to leaves suggesting a possible positive feedback regulation. Pathogen-induced *EDS5* expression is not altered in *sid2* mutants indicating that EDS5 acts upstream of SID2 in the regulation of SA biosynthesis.

Two other Arabidopsis genes, *PHYTOALEXIN DEFICIENT4* (*PAD4*) and *EDS1* are required for full accumulation of SA and *EDS5* expression in response to pathogens (Falk et al., 1999; Zhou et al., 1998), indicating that PAD4 and EDS1 act upstream from EDS5 and SID2 in SA signalling (Nawrath et al., 2002). PAD4 and EDS1 encode proteins with similarities to triacyl-glycerol lipases that interact with each other, but so far no evidence exists that lipase activity is needed for the function of either proteins (Feys et al., 2001; Falk et al., 1999). Because SA induces expression of *EDS1* and *PAD4*, and both EDS1 and PAD4 are required for accumulation of SA to wild-type levels, the existence of a positive feedback loop between SA and PAD4/EDS1 has been suggested (Falk et al., 1999; Fig. 2b). The *pad4* mutant was previously isolated as camalexin deficient mutant (Glazebrook and Ausubel, 1994), but further experiments have shown that *PAD4*, like *EDS1*, encodes a regulatory factor for basal defence. *Eds1* and *pad4* mutants exhibit defects in defence responses, including camalexin biosynthesis, SA accumulation and PR gene expression. They show enhanced susceptibility when infected with virulent strains of *P.syringae* or with avirulent strains that are recognized by TIR-NBS-LRR-type of resistance proteins, but are not impaired in defences triggered by avirulent strains which activate R proteins of the CC-NBS-LRR class (Zhou et al., 1998; Wiermer et al., 2005).

Using yeast two-hybrid analysis, Feys et al. (2001) showed that EDS1 and PAD4 interact with each other and proposed two distinct functions of EDS1 in specific resistance mediated by TIR-NBS-LRR type of receptors. The first one is upstream of PAD4 and triggers early plant defence. The second one requires PAD4 to potentiate plant defences through SA signalling. Another component in this signalling pathway, SENESCENCE-ASSOCIATED GENE101 (SAG101), has been recently identified as an additional EDS1 interacting partner. The PAD4 and SAG101 proteins fail to accumulate in *eds1* mutants, suggesting that EDS1 might act as a kind of scaffold for PAD4 and SAG101 activities. PAD4, EDS1 and SAG101 play important roles not only in specific and basal defence, but also contribute to non-host resistance (Wiermer et al., 2005). This supports the idea that different types of defences share common signalling compounds, and that the same defence pathways can be activated through different elicitors resulting in activation of similar responses such as SA accumulation and PR gene expression.

The TIR-NBS-LRR-type receptors RPP2, RPP4 and RPP5 require the EDS1/PAD4/SAG101 complex for activation of defence responses. By contrast, the CC-NBS-LRR-type R genes RPM1, RPS2 and RPS5 require another signalling component, *NON-RACE SPECIFIC DISEASE RESISTANCE1* (*NDR1*) for signal translation (Aarts et al., 1998). *NDR1* encodes putative glycosylphosphatidylinositol (GPI)-anchored protein,

which is located at the plasma membrane (Coppinger et al., 2004). This suggests that part of the function of NDR1 is to hold R-proteins close to the membrane. Other R-protein including RPP7 and RPP8 neither require EDS1/PAD4 nor NDR1 for defence activation, indicating the existence of third independent pathway (Glazebrook, 2001; Fig. 2a).

Although SA is the signalling compound triggering *PR-1* expression (Fig. 2b), the signals necessary for induction of other PR genes are not definitely determined. The observation that expression of *PR-2* and *PR-5* is abolished in *NahG* overexpressing plants might lead to the assumption that *PR-2* and *PR-5* expression is SA-dependent. However, the fact that *PR-2* and *PR-5* are still expressed in *sid2* mutants after pathogen infection suggests that an SA-independent signalling pathway that does not operate in *NahG* plants is active in *sid2* mutant and leads to *PR-2* and *PR-5* gene expression (Nawrath and Métraux, 1999).

PR-3, *PR-4* and the defensin *PDF1.2* are induced after application of methyl jasmonate (Thomma et al., 1998). Jasmonic acid is another defence and signalling compound accumulating after pathogen invasion. Some components of the JA signalling cascade have been identified in Arabidopsis by mutagenesis approaches. The *jar1* (*jasmonic acid insensitive1*) mutant was identified as a mutant insensitive to JA (Staswick et al., 1992). *JAR1* encodes a JA-amino acid synthetase that can form conjugates between JA and several amino acids including isoleucine. The isoleucine conjugate may be the active form of JA (Staswick et al., 2004). Moreover, all known activities of JA in Arabidopsis require the function of *CORONATINE-INSENSITIVE1* (*COI1*). The predicted amino acid sequence of the COI1 protein contains 16 leucine-rich repeats and an F-box motif. It has similarity to the F-box proteins *Arabidopsis* TIR1, human Skp2, and yeast Grr1, which appear to function by targeting repressor proteins for removal by ubiquitination (Xie et al., 1998). Many JA-regulated genes, for instance *PDF1.2*, require ethylene (ET) as a secondary messenger in addition to JA. Hence, JA and ET are often considered as co-regulators that regulate common signalling pathways (Kunkel and Brooks, 2002). ET is perceived in Arabidopsis by a family of five receptors (ETR1, ETR2, ERS1, ERS2 and EIN4) which all share similarities with bacterial two-component regulators (Chang and Stadler, 2001). Dominant ethylene-insensitivity mutations in *ETR1* have been found to abolish ET binding, allowing the mutant ETR1 receptor to escape from inactivation by the hormone and leads to constitutive receptor signalling (Shaller and Bleecker, 1995). Some evidence indicates that the SA and JA/ET pathways influence each other. Most of this cross-talk consists of mutual repression. For instance, the *mpk4* mutant, which is defective in the mitogen activated protein (MAP) kinase 4, shows constitutive activation of SA-dependent signalling but fails to express the JA-dependent *PDF1.2* gene (Petersen et al., 2000), suggesting either that a block in JA signalling relieves the suppression of SA signalling or that the activation of SA signalling blocks JA signalling. On the other hand,

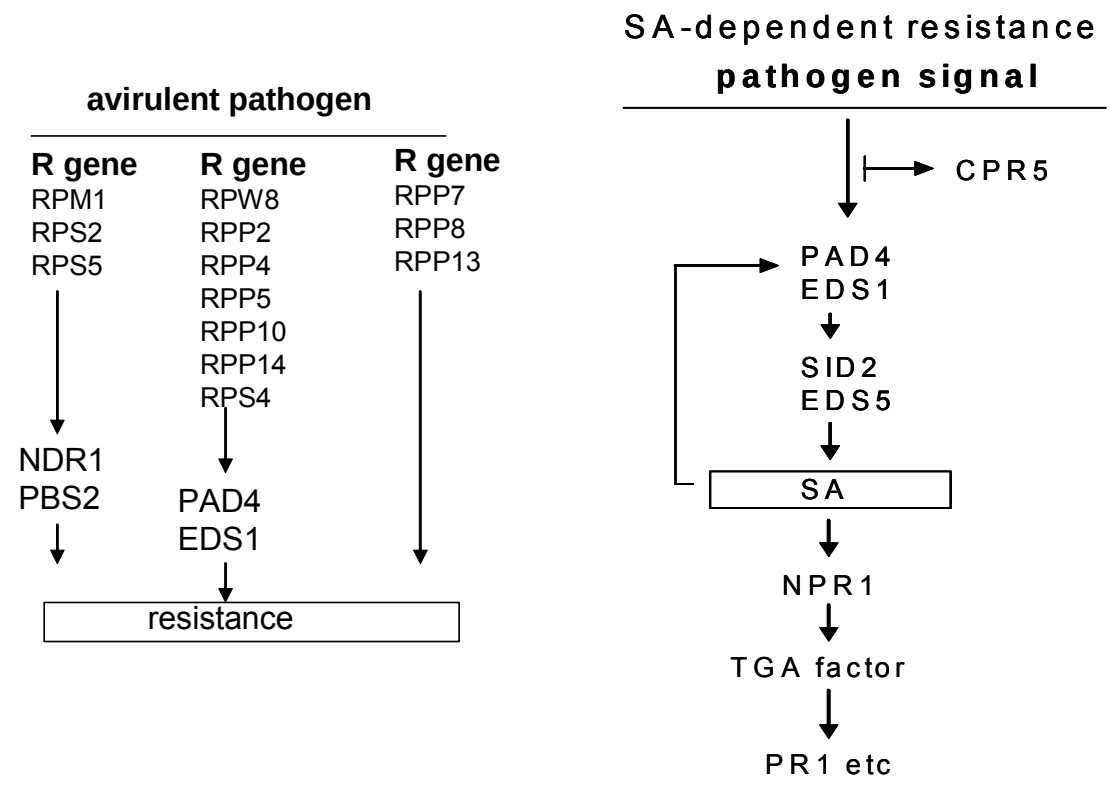


Fig. 2 *a) gene-for-gene resistance* *b) SA-dependent defence signalling*

Camalexin accumulation does not take place after treatment of Arabidopsis with exogenous SA or JA. Moreover, expression of SA- and JA/ET-dependent genes are similar in *A. brassicicola* treated *pad3* mutants and wild type plants, and camalexin accumulation is not decreased in mutants defective in synthesis or perception of these defence-related signalling molecules. These data indicate that little crosstalk exists between regulation of camalexin biosynthesis and SA- or JA/ET-dependent signalling pathways (Thomma et al., 1999).

1.4 Systemic resistance responses

After a local pathogen infection, plants do not only induce defence responses at the site of inoculation, but also develop increased resistance in tissue distant from initial pathogen attack. This phenomenon is known as systemic acquired resistance (SAR). SAR is believed to be triggered by HR-inducing or necrotizing pathogens, and once established, it confers long-lasting, broad-spectrum resistance to subsequent infections. Accumulation of SA and increased expression of PR genes in leaves distant from pathogen attack are characteristic features of SAR (Durrant and Dong, 2004).

Mutants defective in SA biosynthesis like *sid2, pad4* and *NahG* transgenic plants fail to develop SAR after pathogen infection, indicating that SA is a necessary compound

for SAR establishment. Constitutively enhanced resistance and elevated levels of SA accompanied by spontaneous lesion formation is observed in the constitutive defence mutants *cpr1, cpr5* and *cpr6* (Durrant and Dong, 2004). This might indicate that cell death is a trigger for induction of SA biosynthesis and establishment of SAR. However, the existence of other constitutive defence signalling mutants like *mpk4*, *snc1*, or *dnd1*, which all exhibit elevated SA levels without forming spontaneous HR lesions (Petersen et al., 2000; Zhang et al., 2003; Yu et al., 1998), shows that the HR is not required for the activation of SA biosynthesis.

After a plant-pathogen encounter, SA accumulation in non-inoculated, systemic leaves is a reliable characteristic of SAR. Previous studies in cucumber using isotope-labelled SA showed that SA in systemic leaves was both imported from the infected leaves and synthesized de novo in distant leaves (Métraux et al., 1990). Grafting experiments in tobacco between wild-type and *NahG*-expressing rootstocks and scions suggest that SA might not be the SAR long-distance signal but that its accumulation in systemic leaves is required for SAR establishment (Vernooij et al., 1994).

More recent studies indicate that lipids might function as mobile SAR signals. The *dir1* (*defective in induced resistance1*) mutant exhibits wild-type-like local defence responses, functional SA metabolism and normal response to SA and INA, but is not able to develop SAR. Experiments with petiole exudates show that *dir1* is deficient in signal generation in infected leaves or in signal translocation from those leaves, but is still capable to perceive signals originating from wild-type petiole sap. As *DIR1* codes for a putative lipid transfer protein, the DIR1 protein might act as a chaperone for a lipid-derived SAR signal generated in inoculated leaves (Maldonado et al., 2002). Further evidence for lipid-based SAR signalling comes from the characterisation of *eds1* and *pad4* mutants (Falk et al 1999; Jirage et al., 1999). In *eds1* and *pad4* plants, SAR cannot be induced, even when a normal HR is elicited by the attacking pathogen (Durrant and Dong, 2004). In local tissue, PAD4 and EDS1 influence the expression of each other. *PAD4* expression decreases more strongly in *eds1* mutants than *EDS1* expression in *pad4* (Feys et al., 2001). External application of SA induces *PAD4* and *EDS1* expression. These findings indicate that EDS1 and PAD4 function in a positive feedback loop which amplifies their own expression and increases production of SA after infection (Durrant and Dong, 2004). The similarities of PAD4 and EDS1 to lipases suggest that lipid metabolites may be involved in regulating the synthesis and/or accumulation of SA in local and systemic tissue (Durrant and Dong, 2004). Another gene necessary for SAR establishment is *SFD1* (*SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY1*), which encodes a dihydroxyacetone phosphate reductase involved in glycerolipid synthesis. *Sfd1* mutants are compromised in SA accumulation and *PR-1* gene expression in systemic leaves after infection with an avirulent strain of *P. syringae* (Nandi et al., 2004). All these data indicate

that lipid-derivatives may act as mobile SAR signals, although no such molecule has been unequivocally identified until now.

Another important component in SAR signal transduction is NPR1. Although *npr1* mutants exhibit strongly elevated SA levels in pathogen-inoculated leaves, systemic leaves fail to accumulate SA after infection, resulting in a compromised SAR response. Moreover, the induction of *PR-1* is diminished in local and systemic leaves. Functional studies have shown that accumulation of NPR1 in the nucleus after treatment with SAR inducers is essential for PR gene expression in local and systemic leaves (Kinkema et al., 2000).

NPR1 is also involved in a resistance response designated as induced systemic resistance (ISR), which is induced upon root colonisation by plant growth-promoting rhizosphere bacteria. Like SAR, ISR confers increased resistance to further infection (Ton et al., 2002), but it is independent from SA and PR gene induction (Pieterse et al., 1996). The *jar1 (jasmonate resistant1)* and *etr1 (ethylene response1)* mutants are not able to develop induced systemic resistance, indicating that the signal for ISR establishment is JA/ET-dependent. ET can induce ISR in *jar1* mutant suggesting that the requirement for JA lies upstream of ET in ISR signalling (Pieterse et al., 2001). NPR1 is required at the point downstream of JAR1 and ETR1 in ISR signalling (Pieterse et al., 1998). Simultaneous induction of SAR and ISR leads to additive resistance effects against *P.syringae* (van Wees et al., 2000). Therefore, NPR1 is able to act in SAR and ISR signal pathways simultaneously.

1.5 Pathogen effectors that suppress plant immunity

Plants have developed a variety of mechanisms to protect themselves against pathogen infection. In return, to be able to cause disease, pathogenic microorganisms must have evolved strategies to overcome plant immunity.

Basal resistance triggered by PAMPs can be suppressed by several proteins which bacteria deliver into the plant cell through type III secretion (Table 2). For example, AvrPto in Arabidopsis suppresses papilla formation induced by perception of flagellin. Moreover, when the bacterial effectors AvrPto or AvrRpt2 are heterologously expressed in Arabidopsis, growth of the TTSS-defective *P. syringae* strain *Pst hrcC* is significantly enhanced, suggesting that plant defences induce by this mutant strain are suppressed by both AvrRpt2 and AvrPto (Hauck et al., 2003; Chen et al., 2004). The *P. syringae* effectors AvrE and HopM1 are similarly able to suppress basal defences in Arabidopsis, i.e. callose deposition and cell wall fortifications (Debroy et al., 2004). Such features are important for successful colonisation of the host plant.

Bacterial pathogens that overcome the barriers of non-host resistance in a particular plant species can be formally classified as virulent or avirulent. After inoculation of Arabidopsis leaves with virulent strains of *P. syringae*, bacteria are able to heavily multiply for several days before producing visible symptoms in terms of water-soaked, necrotic lesions (compatible interaction). Avirulent bacteria translocate TTSS-effector proteins in host plant cells which are recognized through matching R proteins, and the outcome is an HR (incompatible interaction; Alfano and Collmer, 1996).

Defence function	Type III effector	Previous name	References
Supression of papilla formation	AvrPto1 AvrE1 HopM1 AvrRpm1 AvrRpt2	AvrPto AvrE HopPtoM1 AvrRpm1 AvrRpt2	Hauck et al.,2003 DebRoy et al., 2004 Kim et al., 2005
Inducuction of JA-responsive genes	AvrB1 AvrRpt2 HopA1 HopD1 HopK1 HopX1 HopAO1	AvrB AvrRpt2 HopPsyA HopPtoD1 HopPtoK AvrPphE HopPtoD2	He et al.,2004
Altering ethylene responses	AvrPto1 HopAB2	AvrPto AvrPtoB	Cohn et al.,2005
Supression of cell death induced by specific disease resistance gene	AvrRpm1 AvrRpt2 HopAB2 AvrB2 HopF2	avrRpm1 AvrRpt2 AvrPtoB AvrPtoC AvrPphE	Orth et al., 2000 Reuber et al., 1996 Abramovitch et al., 2003 Tsiamis et al.,2000
Suppression of cell death in non-host plant	HopN1 HopAB2 HopAO1	HopPtoN AvrPtoB HopPtoD2	Lopez-Solanilla et al., 2004 Abramovitch et al., 2003 Jackson et al., 1999

Table 2 *Pseudomonas syringae type III effectors that alter basal and specific defence functions (adapted from Grant et al., 2006).*

Rpm1 and Rps2 are both constituents of the Arabidopsis Col-0 repertoire of R proteins, and they confer HR-based resistance to *P. syringae* by recognizing the effectors AvrRpm1 and AvrRpt2, respectively (Liester et al., 1999). AvrRpm1 induces the HR more quickly than AvrRpt2. When both avirulence genes are present in *P. syringae*, only the

slower AvrRpt2-dependent HR develops (Mackey et al., 2003). AvrRpt2 and AvrRpm1 can also inhibit PAMP induced signalling by manipulation of the RIN4 protein and presumably associated proteins (Belkhadir et al., 2004). RIN4 is a negative regulator of basal defence mechanisms stimulated by flg22. The *rin4* mutant responds to flg22 with enhanced callose deposition (Kim et al., 2005). Present evidence indicates that pathogens have developed different mechanisms to overcome plant defences by targeting distinct plant proteins. Table 2 summarises type III effector proteins from *P. syringae* which are known to alter plant defence.

The enzymatic activity of several type III effector proteins is known (Table 3). Some type III effector proteins show (cysteine) protease activity and target host proteins to modify their normal cellular or defence function. For example, the AvrPphB cysteine protease from *Psp* targets the Arabidopsis PBS1 kinase, a protein involved in R protein-mediated defence (Shao et al., 2003). HopZ2 from *P. syringae* and AvrBsT from *Xanthomonas campestris* show protease activity but a specific target has not been identified so far (Orth et al., 2000). Several TTSS-effectors also interfere with phosphorylation events in plant signalling. The HopPtoD2 effector protein from *Pst* exhibits tyrosine phosphatase activity and targets MAP kinase pathways, which play important roles in plant defence signal transduction (Zhang and Klessig, 2001). Table 3 depicts further type III effectors with known or predicted enzymatic function.

Function	Organism	Type III effector	Previous name	References
Papaine-like cysteine protease, YopT-like	*P. syringae*	HopC1 HopN1 HopAR1	HopPtoC HopPtoN AvrPphB	Buell et al., 2003 Lopez-Solanilla et al., 2004 Schecher et al., 2004
Staphopain cysteine protease	*P. syringae*	AvrRpt2	AvrRpt2	Coaker et al., 2005
YopJ-like SUMO protease	*P. syringae* *X. campestris*	HopZ2 AvrXv4 AvrBsT AvrRxv	AvrPpiC AvrXv4 AvrBsT AvrRxv	Arnild et al., 2001 Roden et al., 2004
Protein tyrosine phosphatase	*P. syringae*	HopAO1	HopPtoD2	Espinosa et al., 2003
Ubiquitin E3 ligase	*P. syringae*	HopAB2	AvrPtoB	Janjusevic et al., 2006

Table 3 *Type III effectors with known or predicted enzyme function (adapted from Grant et al., 2006).*

Some pathogens also manipulate the hormonal state of the plant to suppress defence responses. Many *P. syringae* strains produce the phytotoxine coronatine (COR) which is a chemical mimic of JA and activates JA-dependent defence signalling. As the JA and SA signalling pathways predominantly interact with each other in an antagonistic manner (Kunkel et al., 2002), SA-dependent defences, which are essential for resistance against *P. syringae*, are repressed by coronatine (Brooks et al., 2005).

1.6 The Pseudomonas-Arabidopsis interaction as a model system to dissect plant defence mechanisms

In the 1980s, *Pseudomonas syringae* was the first pathogen demonstrated to infect the model crucifer *Arabidopsis thaliana* and cause disease symptoms in the laboratory (Dong et al., 1991; Whalen et al., 1991). Since then, the Arabidopsis-*Pseudomonas* interaction has developed into a widely used pathosystem to study the molecular principles of bacterial virulence, plant defence and disease resistance in plants. This system is useful for many reasons:

- Depending on the *P. syringae* strain or Arabidopsis accession, three principal outcomes of a plant-pathogen interaction can be studied: non-host resistance, gene-for-gene resistance, and compatibility (virulence, basal resistance).
- The genomes of Arabidopsis and different *P. syringae* strains (pv. *tomato* DC3000, pv. *syringae* B728a, pv. *phaseolicola* 1448A) are completely sequenced.
- Both plant and pathogen are amenable to genetic manipulation.
- All the advantages that come along with the model plant Arabidopsis can be deployed for functional analyses of plant defence responses (e.g. availably of gene-specific knockout mutants, natural variation, publicly available whole genome expression information; see below).
- *P. syringae* is a pathogen of commercially important plants like bean and soybean.

1.6.1 *Arabidopsis thaliana* as model plant

Arabidopsis thaliana is a small flowering plant belonging to the mustard (*Brassicacea*e) family (Fig. 3), which includes cultivated species such as cabbage and radish. It does not have major agronomic significance, but because of its small genome it offers many advantages for basic research in functional genetics and molecular biology. The whole genome, which is located on 5 chromosomes, has been completely sequenced in 2000 by The Arabidopsis Genome Initiative (AGI). *Arabidopsis* has become an important model

plant also because of its rapid life cycle, its seed productivity and ability to be cultivated in different growth conditions. Because of easy transformation with *Agrobacterium tumefaciens*, large collections of T-DNA and transposon mutants have been generated. This allows to apply forward and/or reverse genetic approaches to determine the function of each of the approximately 20000 genes.

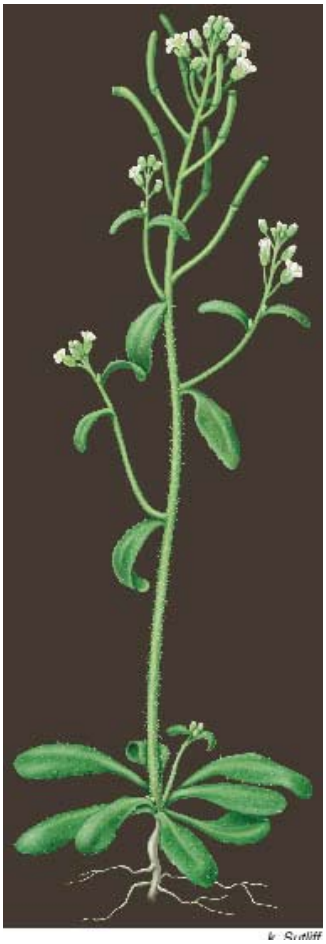


Fig. 3
Phenotype of a flowering Arabidopsis thaliana plant

Apart from the availability of mutant collections, *Arabidopis thaliana* has more then 750 natural accessions. They are very variable in terms of form and development (leaf shape, hairiness) and physiology (flowering time, disease resistance). This variation is useful to investigate complex genetic relations, such as plant responses to the environment and evolution of morphological traits. Because of the variability in secondary metabolite composition between ecotypes, they are also useful tools to study the importance of plant metabolites in plant-insect or plant-pathogen interactions. For example, the composition and amount of glucosinolates and their breakdown products varies between different Arabidopsis accessions (Haughn et al., 1991; Kliebenstein et al., 2001), and this influences herbivory of the cabbage looper *Trichoplusia ni* (Lambrix et al., 2001). The most widely used Arabidopsis ecotypes are Columbia (Col-0), Landsberg (Ler-0), Wassilewskija (Ws) and C24. The Col-0 genome has been sequenced and the majority of available mutants have Columbia as background. Wassilewskija (Ws) present a natural mutant of the flagellin receptor FLS2 (Gomez-Gomez and Boller, 2000). Differences between ecotypes are powerful tools for the investigation of many physiological processes.

1.6.2 *Pseudomonas syringae* as a pathogen for Arabidopsis

Pseudomonas syringae is Gram-negative bacterium with polar flagella (Agrios, 1997). It is a non-invasive, extracellular pathogen which colonizes the host intercellular spaces outside the plant cell wall (Fig. 4).

Several *P. syringae* strains belonging to the pathovars *tomato*, *maculicola*, *pisi*, and *atropurpurea* are able to infect Arabidopsis (Crute et al., 1994). The virulent strains *P. syringae* pv. *tomato* DC3000 (*Pst*) and *P. syringae* pv. *maculicola* ES4326 (*Psm*) were

isolated and widely used to infect Arabidopsis. After discovery of the avirulence genes *avrRpt2* and *avrRpm1* and the corresponding R-genes *RPS2* and *RPM1*, it was also feasible to study defined gene-for-gene interactions within this pathosystem (Dong et al., 1991; Whalen et al.,1991; Dangl et al.,1992). The presence of *avrRpt2* and *avrRpm1* in *Pst* or *Psm* converted the virulent strains into avirulent ones allowing a direct comparison between compatible and incompatible interactions. The study of non-host interaction is also possible within this pathosystem. *P.syringae* pv. *glycinea* (*Psg*) and *P.syringae* pv. *phaseolicola* (*Psp*), which infect bean and soyabean, respectively, are not able to multiply or cause disease symptoms in Arabidopsis leaves, thus representing useful tools to study non-host plant-bacteria interactions.

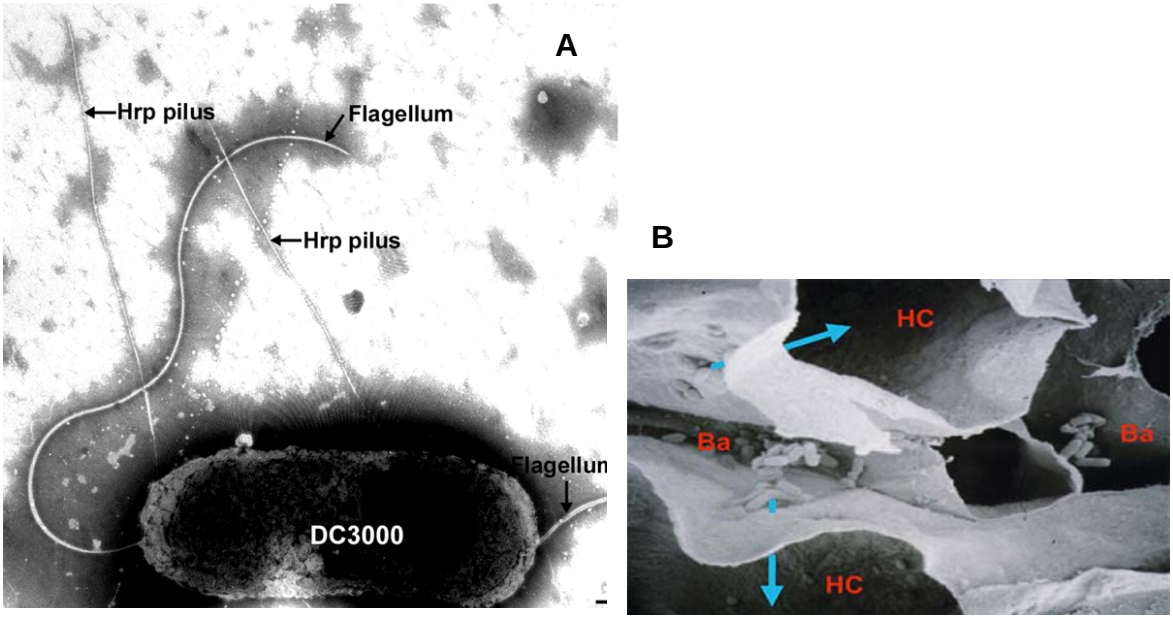


Fig. 4 *a) Transmission electron microscopic image of the phytopathogenic bacterium Pseudomonas syringae pv. tomato DC3000. b) Scanning electron microscopic image of a cross section of an Arabidopsis leaf infected with Pst. Adapted from Katagiri et al., (2002).*

P. syringae produces toxins which induce chlorosis and lesions in the infected host tissue. *Pst*, *Psm*, and *Psg* produce coronatine (see 1.5). By contrast, the bean pathogen *Psp* poduces phaseolotoxin as phytotoxin.

cpr5 and *cpr6 (constitutive expression of PR genes 5 and 6)* mutants show constitutively high expression of *PR-1* and *PDF1.2*, suggesting that SA and JA/ET signalling pathways may share common activating signals (Glazebrook, 2001). Recently, it has been shown that PAD4 and EDS1 might be these common components, because they act downstream of MAPK4 in regulation of SA/JA signals pathways (Brodersen et al., 2006).

A big body of evidence collected in last years indicate that SA- and JA/ET dependent pathways are important for defences against different kinds of pathogens. According to their lifestyles, plant pathogens can be divided into biotrophs and necrotrophs. Biotrophs feed on living host tissue, whereas necrotrophs kill tissue and feed on the remains (Glazebrook, 2005). Arabidopsis mutants defective in SA signalling like *sid2* and *npr1* suffer enhanced susceptibility to the biotrophic bacterial pathogen *P.syringae* and to the biotrophic oomycete *Peronospora parasitica*, but exhibit wild-type-like resistance to the necrotrophic fungus *Alternaria brassicicola*. Conversely, the JA signalling-defective *coi1* mutants are compromised in resistance to *A. brassicicola* but still show resistance to *P. parasitica* (Thomma, et al., 1998). Such observations suggest that SA-triggered defences confer resistance against biotrophic pathogens, and JA/ET signalling activates defences are primarily effective against necrotrophs (Glazebrook, 2005).

In contrast to pre-existing phytoanticipins, phytoalexins represent low molecular weight antimicrobial compounds which are synthesized by plants not until pathogen or elicitor contact has occurred (Kuć, 1995). Although the main Arabidopsis phytoalexin camalexin, a sulphur-containing indole derivative, strongly accumulates in leaves after infection with both biotrophic *P. syringae* and necrotrophic *A. brassicicola*, its protective role appears to be restricted to necrotrophic pathogens. Camalexin inhibits growth of *P. syringae* and *Cladosporium cucumerinum in vitro* (Tsuji et al., 1992). However, *pad3* mutants which are defective in camalexin accumulation, do not show a higher susceptibility to avirulent or virulent strains of *P. syringae*, but are more susceptible than wild-type to infection by *A. brassicicola* (Glazebrook et al., 1994; Thomma et al., 1999). PAD3 encodes the cytochrome P450 CYP71B15 which catalyses the final step in camalexin synthesis, the decarboxylation of dihydroxycamalexic acid to camalexin (Zhou et al., 1999; Schuhegger et al., 2006).

1.7 Leaf senescence

Leaf senescence represents the final step in leaf development. It progresses in an age-dependent manner, but its induction can be accelerated or delayed by various internal and external factors, including light conditions, nutrient supply and environmental stress (Smart, 1997). The senescence process is characterized by dramatic metabolic and physiological changes and is accompanied by changes in expression of a large number of genes. One group of them, the so-called senescence-associated gene (SAGs), have been isolated from various plant species (Biswal and Biswal, 1999). During the progress of senescence, expression of PR genes, accumulation of salicylic acid, and increased production of ROS has been observed (Morris et al., 2000). The latter processes are triggered also during the hypersensitive response indicating similarities between senescence and the HR, which are both forms of plant programmed cell death (Heath, 2000).

Nitric oxide might be one of the common signals involved in both physiological processes. NO participates in regulation of the HR triggered by pathogen infection (Delledonne et al., 1998). On the other hand, NO emission from Arabidopsis plants decrease significantly when plants mature and leaves start to senescence (Magalhaes et al., 2000). A similar tendency has been observed for fruit maturation and floral senescence (Leshem et al., 1998). In addition, exogenous NO application counteracts leaf senescence caused by ABA and methyl jasmonate in rice (Hung and Kao, 2003; 2004). In plants, NO can be produced enzymatically or non-enzymatically by reduction of nitrite (Stöhr et al., 2001; Rockel et al., 2002; Bethke et al., 2004). The enzymatic production of NO out of nitrite is mediated by nitrate reductase (NR; Kaiser et al., 2002). Interestingly, several factors, among them cytokinin, light and nitrate treatment, stimulate expression or activity of NR (Crawford, 1995), enhance the *in planta* production of NO (Planchet et al., 2006) and delay the progress of senescence (Smart 1994). Above findings and the observation that Arabidopsis mutant plants with decreased levels of endogenous NO exhibit accelerated senescence indicate a role for NO as negative regulator of leaf sensecence (Guo and Crawford, 2005).

Arabidopsis *CPR5* represents another link between the HR and senescence. The *cpr5* mutant has been identified because of its constitutive expression of defence response and spontaneous cell death (Bowling et al., 1997). The *CPR5* gene is allelic to *HYS1* which is expressed in early stages of dark-induced and age-dependent leaf senescence. In *hys1* mutant plants, expression of several SAGs occurs earlier as in wild-type plants. The *HYS1/CPR5* gene encodes a novel membrane protein that has a nuclear localisation signal, suggesting a function in signal transduction (Yoshida et al., 2002). However, some SAGs are senescence-specific and not expressed during HR (Weaver et

al., 1998). For instance, *SAG12* is exclusively induced during later stages of senescence and not during defence-related cell death responses. This is exemplified in the *acd11 (accelerated cell death11)* mutant, which is defective in a sphingosin transfer protein and does not show an age-dependent cell death phenotype (Brodersen et al., 2002). Thus, despite several similarities, HR-related cell death can be separated from age-dependent leaf senescence at the molecular level.

2. Aims of the work

Interactions of plants with phytopathogenic bacteria might result in resistance or disease. The molecular interplay determining the respective outcome is multi-layered and complex. Although several components of plant defence against bacterial pathogens have been identified, many of the molecular principles involved in plant resistance to bacterial pathogens are still to be elucidated. This work aimes to contribute to a better understanding of the molecular mechanisms underlying plant defence at the levels of specific resistance, non-host resistance and systemic acquired resistance. For this purpose, the Arabidopsis-*Pseudomonas syringa*e model interaction has been utilised as an experimental system.

One focus of this work was to study the function of nitric oxide in plant-pathogen interactions using a transgenic approach. Therefore, the defence behaviour of two types of previously generated, transgenic Arabidopsis plants with either enhanced or attenuated endogenous levels of NO was analysed after challenge with avirulent, virulent or non-host *P. syringae* strains. These plants involved Arabidopsis plants expressing an *Escherichia coli* nitric oxide dioxygenase (NOD), which degrades NO and decreases endogenous NO levels in plants, and Arabidopsis plant expressing a bacterial NO synthase from *Deinococcus radioduras* (deiNOS), which exhibit enhanced NO production. Furthermore, based on the observation that the NO-deficient, NOD expressing plants show an accelerated leaf senescence phenotype, the role of NO during leaf senescence was addressed in this work.

Another aim of this work was to examine molecular events associated with non-host resistance against bacteria, a research area that had only marginally been investigated in the past. It was examined which factors restrict the growth of the non-adapted *P. syringae* strains *Psg* and *Psp* in Arabidopsis leaves, which plant defence responses are induced after bacterial inoculation, and which signalling pathways are involved in induction of these defence responses.

During the progress of the work, it became apparent that non-host bacteria do not only trigger local defence responses, but also systemic defence reactions. Based on this finding, factors and events necessary to trigger SAR in inoculated leaf tissue were specified. Especially, possible roles of necrotic lesions and PAMPs in SAR induction were examined.

The proximate goal was to determine new molecular components contributing to local and systemic resistance in Arabidopsis and to characterize their function. To identify previously uncharacterized defence genes, local and systemic resistance responses of selected Arabidopsis T-DNA insertion lines with defects in candidate gene that are highly up-regulated by *P. syringae* infection were analysed. The role of one candidate gene, the

flavin-dependent monooxygenase FMO1, during systemic acquired resistance was experimentally specified.

3. Own research

3.1 Summaries of publications and manuscripts

3.1.1 The role of nitric oxide in plant responses to pathogens and in senescence

Nitric oxide has been shown to act as a signal in the immune, nervous, and vascular system in vertebrates (Schmidt and Walter, 1994). Recent pharmacological experiments suggest that nitric oxide is also involved in many physiological processes in plants. NO seems to be an important signalling compound contributing to the development of the hypersensitive response. Inhibitors of NO synthesis as well as NO scavengers are able to block the HR induced by avirulent *P. syringae* in soybean cell cultures and in Arabidopsis plants (Delledonne et al., 1998). Furthermore, NO induces expression of a set of defence genes, such as *PR-1*, *PAL* and chalcone synthase (CHS), together with SA accumulation (Durner et al., 1998).

Most of the evidence about the role of NO in plant physiology and defence relies on the action of pharmacological substances, which are either NO releasing chemicals or inhibitors of mammalian NOS, in plants. In **publication 1 (Zeier et al., 2004)**, we report a complementary, genetic approach to investigate the role of NO in the interaction of Arabidopsis with the avirulent *P. syringae* strain *Pst avrB*. We generated transgenic Arabidopsis plants expressing the flavohemoglobin Hmp, which acts as an NO dioxygenase (NOD) in *E. coli*, under the control of an inducible promoter system. NOD expressing plants show a significant reduction of plant NO emission, and leaf extracts from NOD plants degrade given amounts of NO significantly faster than wild-type plants. Moreover, we quantified by DAF2-DA staining that production of NO in response to *Pst avrB* is lower in the transgenic line than in wild-type. The defence responses most strikingly affected in the NO-deficient Arabidopsis plants were the pathogen-induced oxidative burst and induced expression of the *PAL1* gene, which were both attenuated. However, NOD plants still showed accumulation of SA, expression of *PR-1*, and an HR, albeit to a slightly lower extent than in wild-type. We also attempted to remove NO from the pathogen side by expressing the flavohemoglobin HmpX from *Erwinia chrysanthemi*, a functional analog of *E. coli* Hmp, in *Pst avrB*. Remarkably, concomitant removal of NO at infection sites by plant and pathogen further reduced plant defence responses in the incompatible interaction, including delay in *PR-1* expression and a diminished HR. In conclusion, the results from this genetic approach are in-line with a function of NO as a defence compound in the incompatible Arabidopsis-*P. syringae* interaction. NO is capable to ensure prolonged H_2O_2 levels during the oxidative burst, possibly by inhibiting

antioxidant enzymes, and activates expression of key phenylpropanoid pathway genes involved in plant defence.

In **manuscript 2 (Mishina et al., 2007b)**, we studied the interaction of different *P. syringae* strains with Arabidopsis plants (cNOS) constitutively expressing the NO synthase subunit deiNOS from *Deinococcus radiodurans*. We have measured enhanced emission of NO in cNOS plants when compared with wild-type plants, indicating an NO producing capacity of deiNOS in Arabidopsis. Contrary to our expectations, cNOS plants do not show increased disease resistance, but exhibit increased susceptibility towards non-adapted, virulent and avirulent *P. syringae* strains. Pathogen-induced accumulation of SA, camalexin, and several transcripts of *PAL1* and *PR-1* were not altered in cNOS plants, and a wild-type like HR developed in these plants. However, expression of a specific subset of *PR* genes including *PR-2* and *PR-5* were attenuated in cNOS plants, and a diminished oxidative burst occurred after pathogen infection. Additionally, cNOS plants showed a strongly attenuated SAR response. We conclude that a continuous overproduction of NO in the transgenic cNOS lines does not constitutively activate plant defence responses, but partially interferes with several defences to attenuate plant disease resistance.

In **publication 3 (Mishina et al., 2007a)**, we address the role of NO during leaf senescence. We observed that the NO-deficient Hmp plants (see publication1) undergo a senescence-like process several days after activation of the NOD. This NOD-induced senescence effect occurs faster in older than in younger leaves and is associated with a massive switch in gene expression, followed by changes in levels of several leaf metabolites, and visible leaf yellowing. Out of eight senescence-associated genes, seven are up-regulated during NOD-induced senescence, including the senescence-specific marker gene *SAG12*. In addition, expression of some defence genes and the ethylene biosynthesis gene *ACS6* is increased. When externally fumigating the NOD-expressing plants with NO gas in the low ppm range or applying environmental conditions that stimulate endogenous NO production via nitrate reductase, i.e. nitrate feeding, high light treatment and cytokinin application, the observed senescence effect is attenuated or delayed. Metabolic changes during NOD-induced senescence include accumulation of salicylic acid, γ-tocopherol and camalexin, all of which also occur during natural senescence. Moreover, fumigation of Arabidopsis wild-type plants with NO delays dark-induced senescence of individual leaves. Our data indicate that NOD expression and subsequent NO-deficiency in transgenic Hmp plants triggers a process with many similarities to natural senescence at the molecular and phenotypic level, and suggests that NO acts as negative regulator of leaf senescence.

3.1.2 Non-host resistance in Arabidopsis against *P. syringae*

The resistance mechanisms of plants against non-adapted bacterial pathogens are not well investigated. In experiments summarized in **manuscript 4 (Mishina and Zeier, 2007b)**, we analyse the interaction of Arabidopsis with two non-adapted *P. syringae* strains, *Psg* and *Psp*, and provide a direct comparison with compatible and incompatible interactions of Arabidopsis with host *Pst* strains. We found that non-adapted bacteria induce salicylic acid accumulation and PR gene expression at sites of inoculation, and that induction of these SA-associated defences is dependent on a functional type III secretion system. The defence signalling pathways activated by non-host bacteria are similar to those activated by host bacteria and include SA, NPR1, NDR1, PAD4, and EDS1. Between the two non-host strains *Psg* and *Psp*, however, we also observed activation of different signalling components, which indicates that for each strain, distinct type III effector proteins are translocated and recognized by the plant. Nevertheless, induction of SA-associated defences does not directly contribute to non-host resistance against *P. syringae*, because mutants defective in those signalling pathways do not exhibit enhanced susceptibility against *Psg* or *Psp*. In contrast to the numbers of a host *Pst* mutant strain that is defective in type III secretion, numbers of non-adapted bacteria rapidly decline after inoculation, suggesting that a pre-formed toxic barrier exists in Arabidopsis which cannot be overcome by the non-adapted *Psg* and *Psp* strains. We thus examined a possible contribution of the glucosinolate/myrosinase system to bacterial non-host resistance. However, although different survival rates of non-adapted bacteria in leaves of Arabidopsis mutants and accessions with distinct glucosinolate composition and hydrolysis exist, our data cannot fully prove a participation of mustard oils to non-host resistance against bacteria. However, we have observed early, TTSS-independent up-regulation *PAL1* and *BCB*, two lignin biosynthesis genes which might be involved in papilla formation or other kinds of cell wall fortification, upon inoculation with non-adapted bacteria. Importantly, Arabidopsis *PAL1* knockout lines permit significantly higher survival of non-adapted bacteria in leaves than wild-type plants, demonstrating a functional importance of *PAL1* up-regulation. Thus, our data indicate that an early inducible, cell wall-based defence mechanism contributes to bacterial non-host resistance.

3.1.3 Molecular determinants triggering systemic acquired resistance in Arabidopsis

A widely assumed dogma in the SAR-field is that systemic acquired resistance is triggered by infection with HR-inducing or other necrotizing pathogens. In **publication 5 (Mishina and Zeier, 2007a)**, we show that non-host bacteria, which do not cause an HR or visible disease symptoms after inoculation, not only induce defence response at inoculation sites, but also in tissue distant from initial inoculation. In contrast to induction of local SA-associated responses, these systemic responses are independent of a functional TTSS. We show that the systemic resistance response triggered locally by symptomless plant-bacteria interactions is mechanistically identical to SAR. First, inoculation with non-adapted or TTSS-deficient *P. syringae* strains not only elevates SA levels and PR gene expression at inoculation sites, but also in systemic tissue. Second, increased systemic expression of the flavin-dependent monooxygenase gene *FMO1*, which is closely associated with SAR (publication 6; Mishina and Zeier, 2006), occurs after inoculation with those strains. And third, a whole set of SAR-deficient Arabidopsis mutants, including the SA biosynthesis pathway mutants *sid2* and *npr1* and the defence signalling mutants *ndr1*, *eds1*, *pad4*, and *fmo1* do not show the observed resistance response. By contrast, systemic resistance triggered by non-host bacteria is different to induced systemic resistance (ISR), because it is established in the jasmonate pathway mutant *jar1*. When SAR is induced with different concentrations of virulent or avirulent host bacteria, we have observed that the magnitude of certain defence reaction at inoculation sites, including SA accumulation, *PR-1* expression, and camalexin production, but not the amount of tissue necrosis or JA accumulation, correlate with the magnitude of SAR induction in distant leaves. The finding that avirulent, virulent, non-host and TTSS-deficient bacteria are all capable to trigger SAR prompted us to test whether common structural elements present in all these bacteria were involved in this process. We thus tested the SAR-eliciting capacity of flagellin and lipopolysaccharides (LPS), two typical PAMPs. We found that local applications of both flagellin and LPS are able to trigger SAR. As it is the case for non-adapted bacteria, different SAR-deficient Arabidopsis mutants fail to express PAMP-induced systemic resistance. Flagellin-induced SAR is dependent on a functional FLS2 receptor, because the natural *fls2* mutant Ws-0 does not show the response, and LPS-triggered SAR is dependent on a lipid A part of the LPS molecule. In summary, our data show that bacterial PAMPs are important determinats that trigger SAR in Arabidopsis, and that tissue necrosis or an HR at inoculation sites are dispensable for SAR activation.

3.1.4 Identification new defence components: the flavin-dependent monooxygenase FMO1 as an essential component of SAR in Arabidopsis

Several components of the signal transduction network that control the activation of defence responses in plants have been identified using the Arabidopsis genetic system. We used gene expression information from publicly available microarray experiments to select candidate genes possibly contributing to local and systemic disease resistance in Arabidopsis. After choosing genes strongly up-regulated after *P. syringae* infection, we selected corresponding, putative Arabidopsis T-DNA insertion lines and experimentally verified the occurrence of T-DNA insertion by PCR. Homozygous gene knockout lines were then inoculated with virulent and avirulent *P. syringae* strains to examine a possible impairment in local and systemic disease resistance.

This strategy revealed that a functional *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) gene is essential for SAR establishment in Arabidopsis (**publication 6; Mishina and Zeier, 2006**). Inoculation of Arabidopsis leaves with avirulent or virulent *P. syringae* pv. *maculicola* (*Psm avrRpm1* and *Psm*, respectively) induces expression of *FMO1* both at inoculation sites and in distant, untreated leaves. The SAR response triggered by *Psm* or *Psm avrRpm1* is completely abrogated in *fmo1* mutant plants, and this is associated with a failure of those plants to accumulate salicylic acid and to express various defence genes in distant leaves. In contrast to systemic responses, the *fmo1* mutation does not critically affect defence responses induced by *Psm avrRpm1* at the site of pathogen attack. At inoculation sites, *FMO1* expression is independent of SA accumulation and signalling through NPR1 and NDR1, but depends on the EDS1/PAD4 defence pathway. Importantly, pathogen-induced expression of *FMO1* in non-inoculated, systemic leaves closely correlates with the capability of different Arabidopsis lines to develop SAR. Thus, the SAR-defective SA-pathway mutants *sid2* and *npr1*, and the defense mutant *ndr1* do not exhibit systemic up-regulation of *FMO1*. Based on these results, we propose the existence of an amplification loop operating in leaves distant from pathogen attack. According to this model, FMO1, ROS, salicylic acid and the defense regulators NPR1 and NDR1 cooperatively act in amplifying incoming signals in order to realize defense responses at the systemic level and SAR.

3.2 Listing of publications and manuscripts (chronological order)

Zeier J, Delledonne M, **Mishina T**, Severi E, Sonoda M, Lamb C (2004) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. **Plant Physiol** 136: 2875-2886 (**publication 1**).

Mishina TE, Zeier J (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. **Plant Physiol** 141: 1666-1675 (**publication 6**).

Mishina TE, Lamb C, Zeier J (2007a) Expression of a nitric oxide degrading enzyme induces a senescence program in Arabidopsis. **Plant Cell Environ** 30: 39-52 (**publication 3**).

Mishina TE, Zeier J (2007a) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. **Plant J**, in press (**publication 5**).

Mishina TE, Zeier J (2007b) Interactions of Arabidopsis with non-adapted Pseudomonas syringae strains: possible determinants of bacterial non-host resistance. Submitted (**manuscript 4**).

Mishina TE, Sonoda M, Fraaß V, Kaiser WM, Zeier J (2007b) Heterologous expression of a nitric oxide synthase in Arabidopsis enhances plant NO production and attenuates local and systemic resistance towards bacterial pathogens. Submitted (**manuscript 2**).

3.3 Reprints of publications and manuscripts

PUBLICATION 1

Zeier J, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C

Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions.

Plant Physiol (2004) 136: 2875-2886

Genetic Elucidation of Nitric Oxide Signaling in Incompatible Plant-Pathogen Interactions[w]

Jürgen Zeier*, Massimo Delledonne, Tatiana Mishina, Emmanuele Severi, Masatoshi Sonoda, and Chris Lamb

John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, United Kingdom (J.Z., E.S., C.L.); Dipartimento Scientifico e Tecnologico, Università degli Studi di Verona, 37134 Verona, Italy (M.D., E.S.); and Julius-von-Sachs-Institut für Biowissenschaften, Lehrstuhl für Botanik II, Universität Würzburg, D–97082 Wurzburg, Germany (J.Z., T.M., M.S.)

Recent experiments indicate that nitric oxide (NO) plays a pivotal role in disease resistance and several other physiological processes in plants. However, most of the current information about the function of NO in plants is based on pharmacological studies, and additional approaches are therefore required to ascertain the role of NO as an important signaling molecule in plants. We have expressed a bacterial nitric oxide dioxygenase (NOD) in Arabidopsis plants and/or avirulent *Pseudomonas syringae* pv *tomato* to study incompatible plant-pathogen interactions impaired in NO signaling. NOD expression in transgenic Arabidopsis resulted in decreased NO levels in planta and attenuated a pathogen-induced NO burst. Moreover, NOD expression in plant cells had very similar effects on plant defenses compared to NOD expression in avirulent Pseudomonas. The defense responses most affected by NO reduction during the incompatible interaction were decreased H_2O_2 levels during the oxidative burst and a blockage of Phe ammonia lyase expression, the key enzyme in the general phenylpropanoid pathway. Expression of the NOD furthermore blocked UV light-induced Phe ammonia lyase and chalcone synthase gene expression, indicating a general signaling function of NO in the activation of the phenylpropanoid pathway. NO possibly functions in incompatible plant-pathogen interactions by inhibiting the plant antioxidative machinery, and thereby ensuring locally prolonged H_2O_2 levels. Additionally, albeit to a lesser extent, we observed decreases in salicylic acid production, a diminished development of hypersensitive cell death, and a delay in pathogenesis-related protein 1 expression during these NO-deficient plant-pathogen interactions. Therefore, this genetic approach confirms that NO is an important regulatory component in the signaling network of plant defense responses.

Plants have evolved several mechanisms to defend themselves from bacterial or fungal invasion. The rapid recognition of pathogenic microbes is based on the interaction of products from a pathogen-derived avirulence gene and a plant-derived resistance gene and represents a prerequisite to specific resistance in incompatible plant-pathogen interactions (Flor, 1956). The multicomponent defense responses associated with specific resistance include a burst of reactive oxygen intermediates (ROI; Lamb and Dixon, 1997), transcriptional activation of defense genes encoding phenylpropanoid pathway enzymes, lytic and antimicrobial pathogenesis-related (PR) proteins (Lamb et al., 1989), increase of intracellular levels of salicylic acid (SA; Malamy et al., 1990; Métraux et al., 1990), and development of the hypersensitive response (HR). The HR results in the rapid appearance of a dry, necrotic lesion at the infection site that is clearly delimited from surrounding healthy tissue and is thought to contribute to the limitation of pathogen spread (Keen, 1990).

One of the earliest events following pathogen recognition is a burst of oxidative metabolism leading to the generation of superoxide (O_2^-) and subsequent accumulation of H_2O_2 (Lamb and Dixon, 1997). These ROI are directly protective and drive the oxidative cross-linking of cell wall structural proteins (Brisson et al., 1994). The H_2O_2 originating from the oxidative burst induces some plant genes involved in cellular protection and defense such as gluthathione *S*-transferase (GST) and is necessary for the initiation of host cell death following the HR (Levine et al., 1994).

Recent pharmacological experiments indicate that nitric oxide (NO), which acts as a signal in the immune, nervous, and vascular system in vertebrates (Schmidt and Walter, 1994), also plays an important role in plant disease resistance. Generation of NO by chemical NO donors augments the induction of hypersensitive cell death by H_2O_2 in soybean (*Glycine max*) suspension cultures (Delledonne et al., 1998, 2001). Likewise, inhibitors of NO synthesis as well as NO scavengers are able to block the HR induced by avirulent *Pseudomonas syringae* in soybean cell cultures and in Arabidopsis plants. Compared to ROI, NO induces a complementary set of plant defense genes, including two key enzymes of the phenylpropanoid pathway, namely Phe ammonia lyase (PAL) and chalcone synthase (CHS). Furthermore, NO-treated tobacco (*Nicotiana tabacum*) cells were shown to induce the pathogenesis-related protein 1 (PR-1) together

* Corresponding author; e-mail zeier@botanik.uni-wuerzburg.de; fax 49 (0)931 8886235.

[w]The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.042499.

with an accumulation of SA (Durner et al., 1998), a key molecule for the expression of systemic acquired resistance (Gaffney et al., 1993). Moreover, the molecular components of NO signaling in plants appear to be similar to those in animals, regarding the involvement of NO producing NO synthases (NOS; Chandok et al., 2003; Guo et al., 2003) and cGMP as a second messenger (Clark et al., 2000).

As an increasing number of recent reports suggests, a regulatory function of NO in plants seems to be essential in other physiological processes, including guard cell abscisic acid signaling (Desikan et al., 2002; Garcia-Mata and Lamattina, 2003), regulation of iron homeostasis (Graziano et al., 2002; Murgia et al., 2002), execution of programmed cell death in barley (*Hordeum vulgare*) aleurone layers (Beligni et al., 2002), root organogenesis (Pagnussat et al., 2002), and wound signaling (Orozco-Cardenas and Ryan, 2002). However, despite the recent identification of a pathogen-inducible NOS (Chandok et al., 2003), assumptions of NO function in plants emerging from all these studies are almost exclusively based on pharmacological studies, i.e. either exogenous application of NO donors, NO scavengers, and inhibitors of mammalian NOS or detection of NO by essentially indirect methods using fluorescent dyes or photometric indicator molecules (Delledonne et al., 1998; Foissner et al., 2000). If indeed the application of pharmacological compounds reflects a physiological NO situation without exerting nonspecific side effects is far from clear, and additional experimental approaches are therefore desirable.

We report here a novel genetic approach to manipulate NO levels in planta, which has been used to gain a better understanding of the function of NO in the signaling network underlying incompatible plant-pathogen interactions. We first generated transgenic Arabidopsis plants overexpressing the *Escherichia coli hmp* gene encoding NO dioxygenase (NOD), a flavohemoglobin capable of converting NO to nitrate by use of NAD(P)H and O_2 (Vasudevan et al., 1991; Gardner et al., 1998; Poole and Hughes, 2000). In this way, we attempted to directly reduce the levels of NO in plant cells. We then compared the defense responses of NO-deficient plants and wild-type Arabidopsis following challenge with avirulent *P. syringae* pv *tomato* (*Pst*) bacteria. Additionally, we employed avirulent *Pst* expressing the *hmpX* gene from *Erwinia chrysanthemi* [*Pst*(*avr-hmpX*)] (Favey et al., 1995) that encodes a highly similar NOD to lower NO levels specifically at the side of pathogen infection. We then challenged wild-type Arabidopsis and *hmp*-expressing Arabidopsis plants with *Pst*(*avr-hmpX*) to study the effect of NO removal at both the plant and the pathogen side.

RESULTS

Arabidopsis Plants Expressing a Bacterial NOD

For the production of Arabidopsis ecotype Col-0 plants expressing a functional NOD, the *hmp* coding sequence from *E. coli* (Vasudevan et al., 1991) was cloned into the dexamethasone (DEX)-based pTA7001-inducible vector system (Aoyama and Chua, 1997). After Agrobacterium-mediated transformation of wild-type Col-0 plants with the vector construct, homozygous T3 plants segregating for a single T-DNA insertion were used for further experiments. Upon treatment with 3 μM DEX, the various lines expressed the *hmp* transgene at different levels (data not shown). We selected one of the lines that expressed *hmp* to a higher degree, designated hmp8, for more detailed analysis. In hmp8 plants, *hmp* transcripts accumulated at 5 h after spraying rosette leaves with DEX (Fig. 1A). Transcriptional levels increased 1 d after DEX treatment and remained nearly constant for at least 1 week. Western-blot analysis with antibodies raised against *E. coli* Hmp revealed the production of a full-size Hmp (43 kD) protein in planta (Fig. 1B). The kinetics of Hmp protein expression was similar to transcriptional *hmp* induction.

The functional effects of Hmp in transgenic plants were first investigated by studying the capability of isolated leaf protein extracts to degrade NO. Whereas wild-type plants and noninduced hmp8 plants showed highly similar degradation kinetics for NO (Fig. 1C), plant extracts from the DEX-induced hmp8 line significantly accelerated the degradation of NO (Fig. 1D).

In leaves, NO can be produced from nitrite by nitrate reductase, and this NO production is measurable as emission by chemiluminescence (Rockel et al., 2002). A temporary rise of NO emission resulting from increased nitrate reductase activity was detected when dark-adapted plants were transferred to light. When the light source was switched off again, a light-off peak caused by transient nitrite accumulation resulted (Kaiser et al., 2002). Both the light-induced NO emission and the NO light-off peak were significantly lower in DEX-treated hmp8 plants compared to the wild type (Fig. 1E).

P. syringae Expressing a Bacterial NOD

To expand our genetic approach to study NO deficiency in incompatible plant-pathogen interactions, we transformed *Pst* (*avrB*) with the *hmpX* gene from *E. chrysanthemi*. Like Hmp from *E. coli*, *E. chrysanthemi* HmpX represents a NOD (M. Delledonne and R. Poole, unpublished data). The expression of HmpX in the bacterium should decrease the concentration of the diffusible molecule NO specifically at the site of pathogen infection. In this way, we were able to study plant-pathogen interactions in which NO is simultaneously removed at the infection site from both the plant and the pathogen side.

Plant Defense Responses under NO-Deficient Conditions

Pst carrying the *avrB* avirulence gene is recognized by Arabidopsis ecotype Col-0 carrying the Rpm1

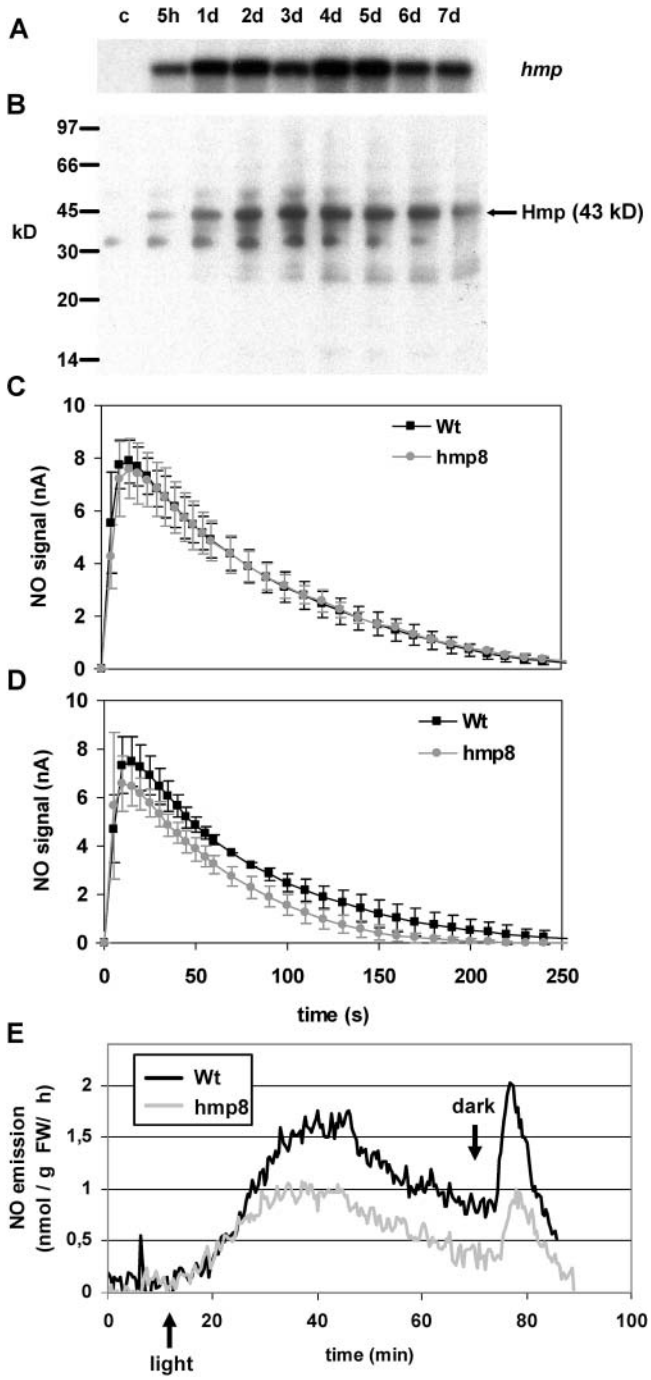


Figure 1. Expression of *E. coli hmp* in transgenic Arabidopsis as a functional NOD. A, Northern-blot analysis illustrating time-dependent accumulation of *hmp* transcripts after treatment of hmp8 plants with 3 μM DEX (c, no DEX). B, Western blot demonstrating the appearance of correctly sized Hmp protein in leaf extracts; time course as in A. C and D, Electrochemically measured degradation kinetics of 10 μM NO in leaf extracts of wild-type and hmp8 transgenic Arabidopsis. Error bars represent the SDs of five independent measurements. C, Hmp8 plants without DEX-induced transgene expression. D, Wild-type and hmp8 plants 1 d after DEX treatment. NO concentrations of less than 1 μM were reached for wild-type, noninduced hmp8, and DEX-induced hmp8 plants at 197 s, 202 s, and 131 s, respectively. E, NO emission from intact wild-type and hmp8 plants 1 d after DEX treatment. Plants were first incubated in the dark for 10 min and then illuminated for 60 min to cause nitrate reductase-dependent NO emission. The light was switched off again, which gave rise to a characteristic light-off peak (see "Results"). Experiments were repeated three times with similar results.

resistance gene (Bisgrove et al., 1994). The recognition results in the oxidative burst, a production of ROI like H_2O_2 and O_2^- at the site of infection, induction of defense and cellular protectant genes, development of the HR, and a limitation of pathogen growth in comparison to isogenic virulent *Pst*.

NO Production

To investigate whether NO is produced during the infection of Col-0 plants with avirulent bacteria, we infiltrated DEX-treated wild-type leaves with the NO-sensitive, cell-permeable fluorescent dye 4,5-diaminofluorescein diacetate (DAF2-DA; Foissner et al., 2000) 3 h after challenge with 2×10^6 colony-forming units (cfu) mL^{-1} *Pst* (*avrB*). Inside the area of pathogen infiltration, various brightly green fluorescing cell groups were discernible 1 h after DAF2-DA treatment (Fig. 2C, white stars), whereas $MgCl_2$-infiltrated control leaves only showed a weak fluorescence when treated with the fluorophore for the same period of time (Fig. 2B). Pathogen infiltration without fluorophore treatment did not cause any fluorescence (Fig. 2A). As compared to wild-type leaves, DEX-induced hmp8 plants challenged with *Pst* (*avrB*) and subsequently treated with DAF2-DA showed a markedly reduced fluorescence at the site of pathogen infiltration (Fig. 2D). A similar reduction of DAF2-DA fluorescence was observed when wild-type leaves were challenged in the presence of 100 μM CPTIO, a NO scavenging compound (data not shown). These results suggest that NO is produced during the earlier stages of the Arabidopsis-*Pst* (*avrB*) interaction and that this NO burst is attenuated in NOD-expressing hmp8 plants.

Oxidative Burst

To assess the accumulation of ROI in response to infection with an avirulent pathogen, DEX-treated leaves from wild-type and hmp8 plants were infiltrated with 2×10^6 cfu mL^{-1} *Pst* (*avrB*) and stained with diaminobenzidine (DAB), a histochemical reagent that forms a reddish-brown precipitate upon contact with H_2O_2 (Thordal-Christensen et al., 1997). Wild-type leaves showed a strong production of H_2O_2 at the infection site 4 h after pathogen challenge (Fig. 3). By contrast, leaves from hmp8 plants showed significantly lower levels of H_2O_2 compared to the wild type when challenged with *Pst* (*avrB*). Moreover, when DEX-treated leaves from hmp plants where challenged with *Pst* (*avrB/hmpX*) bacteria, the suppression of H_2O_2 levels during the oxidative burst was even more pronounced.

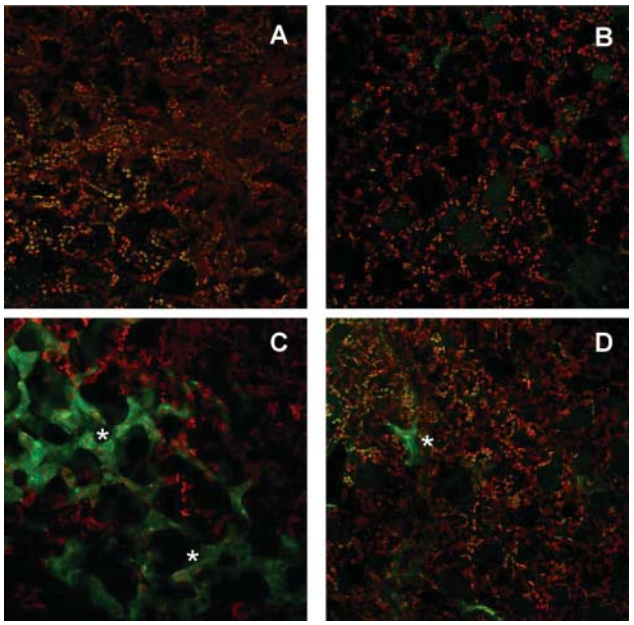


Figure 2. Pathogen-induced DAF2-DA fluorescence as a measure for NO production in DEX-treated wild-type and hmp8 plants. Leaves were pretreated with *Pst* (*avrB*) or $MgCl_2$ for 3 h and subsequently infiltrated with 10 μM DAF2-DA or control buffer (10 mM Tris/KCl, pH 7.2). Infiltrated leaf areas were analyzed 1 h later by confocal laser scanning microscopy. DAF2-DA fluorescence (green) was recorded using a channel with a 505- to 530-nm band-pass filter, and autofluorescence of chloroplast (red) was captured with a channel equipped with a 560-nm long-pass filter. A, Treatment of a wild-type Arabidopsis leaf with *Pst* (*avrB*) and control buffer. B, Wild-type Arabidopsis-$MgCl_2$ and DAF2-DA. C, Wild-type Arabidopsis-*Pst* (*avrB*) and DAF2-DA. D, Hmp8-*Pst* (*avrB*) and DAF2-DA. Seven independent samples were recorded for each condition, and representative leaf areas are shown.

This observation prompted us to test whether the reduced H_2O_2 levels were a consequence of less H_2O_2 production or, once produced, an effect of increased H_2O_2 degradation. We infiltrated equal amounts of the H_2O_2-generating system Glc/Glc oxidase into leaves of wild-type and hmp8 plants and performed DAB staining 1 h after infiltration. Again, wild-type leaves showed stronger staining patterns with respect to induced hmp8 leaves (Fig. 4), suggesting that the action of Hmp increased the ability of the plants to degrade H_2O_2.

Defense Gene Expression

We next examined whether the expression of three typical defense-related genes, GST, PAL, and PR-1, was affected in the hmp8 line (Fig. 5). GST functions in cellular protection, and *gst* transcripts are induced during the oxidative burst (Levine et al., 1994; Delledonne et al., 2001). *Gst* transcripts accumulated 4 to 10 h after DEX-treated wild-type plants were challenged with *Pst* (*avrB*). Similar induction kinetics were observed when DEX-induced hmp8 plants were challenged with *Pst* (*avrB*) or *Pst* (*avrB/hmpX*). Only in the latter case was the amount of gene induction slightly diminished, presumably reflecting the extremely low H_2O_2 levels during the oxidative burst.

PAL catalyzes the first step in phenylpropanoid biosynthesis and possibly initiates the synthesis of lignin, antibiotics, and SA. A strong induction of *pal* transcripts occurred 4 h after pathogen infection in wild-type plants challenged with *Pst* (*avrB*) (Fig. 5). This strong induction of *pal* was highly suppressed in NO-deficient interactions, i.e. when DEX-induced

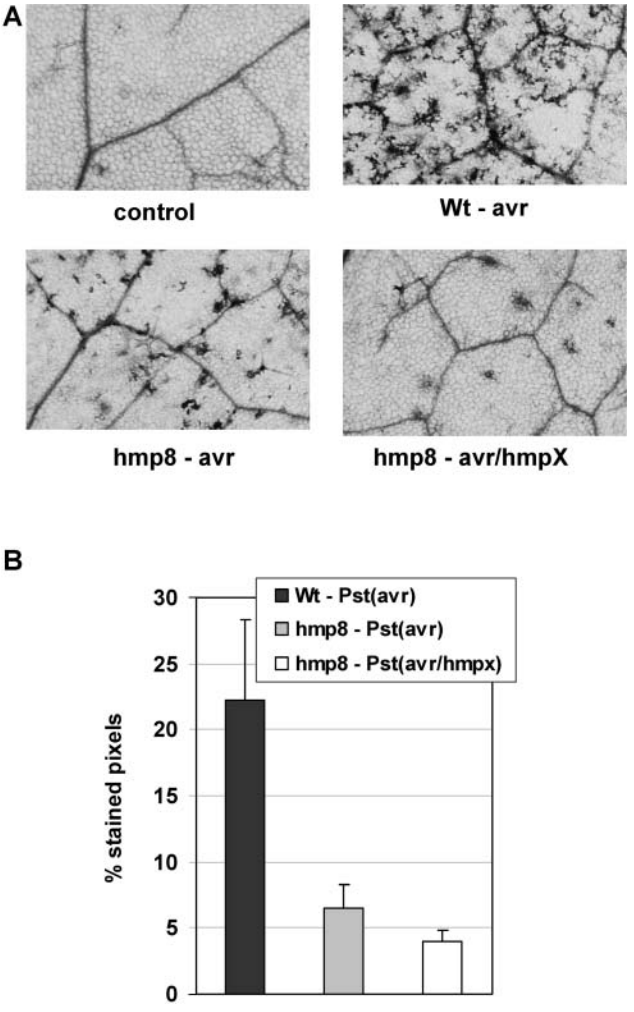


Figure 3. DAB staining of Arabidopsis leaves to assess H_2O_2 accumulation during the oxidative burst in DEX-treated wild-type and hmp8 transgenic plants. Solutions of *Pst* (*avrB*) were infiltrated into Arabidopsis leaves, and DAB staining was initiated 4 h after infection. A, Staining patterns of representative, $MgCl_2$-infiltrated wild-type or hmp8 leaves (control), *Pst* (*avrB*)-infected wild-type Arabidopsis leaves (Wt-avr), *Pst* (*avrB*)-infected hmp8 plants (hmp8-avr), and *Pst* (*avrB/hmpX*)-infected hmp8 plants (hmp8-avr/hmpX) 4 h after the respective treatment (100-fold magnification). B, Quantification of DAB staining in *Pst* (*avrB*)-infected wild-type and hmp8 leaves. The percentage of stained pixels inside the infiltration area was assessed as described in "Materials and Methods." Values are shown as the mean ± SD of at least five leaves from different plants. Experiments were repeated three times with similar results.

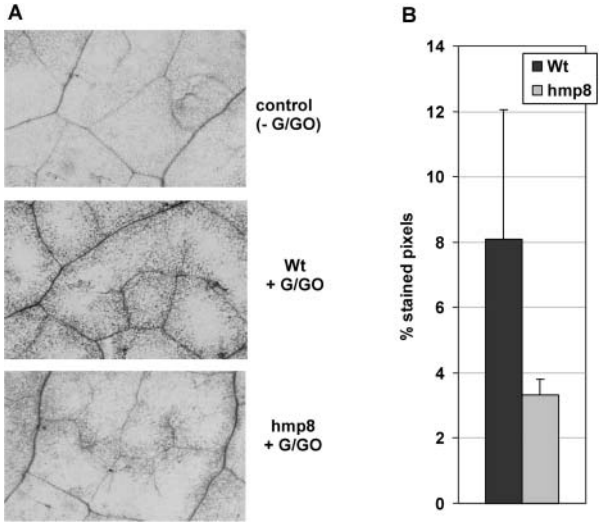


Figure 4. DAB staining of Arabidopsis leaves to assess their capability to degrade H_2O_2 in DEX-treated wild-type and *hmp* transgenic plants. Solutions of 2.5 mM Glc/2.5 units mL^{-1} Glc oxidase were infiltrated into leaves and DAB staining was performed 1 h after infiltration. A, Staining patterns of representative leaves inside the infiltrated area (100-fold magnification). B, Percentage of stained pixels inside the infiltration zone. Values are shown as the mean ± SD of at least five leaves from different plants. Experiments were repeated three times with similar results.

hmp8 plants where challenged either with *Pst* (*avrB*) or *Pst* (*avrB/hmpX*).

Transcriptional induction of the antimicrobial PR-1 protein occurred 10 h after challenge of both wild-type and hmp8 plants with *Pst* (*avrB*) (Fig. 5). *PR-1* induction was delayed, albeit not fully suppressed, when hmp8 plants were challenged with *Pst* (*avrB/hmpX*).

The finding that pathogen-induced *pal* expression is blocked under NO-deficient conditions prompted us to test whether NO could act as a general signal for the activation of the phenylpropanoid pathway. Another stimulus activating this pathway is UV light (Chappell and Hahlbrock, 1984), and we examined the UV-induced expression of PAL and CHS, the key enzyme for flavonoid biosynthesis, in DEX-treated wild-type and hmp8 plants (Fig. 6). Whereas wild-type plants strongly expressed *chs* and *pal* after 24 h of UV treatment, this UV-induced gene expression was markedly attenuated in hmp8 plants, indicating that NO is required for phenylpropanoid pathway activation by different environmental stimuli.

Development of Hypersensitive Cell Death

When leaves from wild-type plants were challenged with *Pst* (*avrB*) at concentrations of 5×10^6 cfu mL^{-1}, a dry, colorless lesion limited to the site of pathogen infiltration developed within 2 d in at least 5 out of 7 leaves (Fig. 7A). These macroscopic symptoms characteristic of hypersensitive cell death developed to the same extent when noninduced hmp8 plants were used (data not shown). However, when infected with *Pst* (*avrB*), DEX-treated hmp8 plants showed a more chlorotic lesion that was reminiscent of the symptoms caused by isogenic virulent *Pst* in about 50% of leaves (Fig. 7B). Infection of induced hmp8 plants with *Pst* (*avrB/hmpX*) further increased this percentage. In this case, virtually every challenged leaf developed chlorotic symptoms (Fig. 7C).

To characterize the HR development in more detail, we performed Trypan blue staining of infected leaves 24 h after bacterial inoculation (Fig. 8). Blue-stained dead cells or patches of dead cells appeared to a similar extent at the sites of *Pst* (*avrB*) infiltration in DEX-treated wild-type leaves and nontreated hmp8 leaves. In DEX-treated hmp8 leaves, the density of dead cells was reduced when challenged with *Pst* (*avrB*), and *Pst* (*avrB/hmpX*) challenge led to a dramatic reduction of HR lesions.

SA Levels and Bacterial Growth

SA plays a central role in the activation of plant defense responses, and a relationship between NO and SA signaling pathways has been discussed (Klessig et al., 2000). To determine the effects of NO on SA levels in the *Pst* (*avrB*)-Col-0 interaction, we determined the levels of SA and SA glucoside (SAG) in our system 8 h after pathogen infection (Fig. 9). DEX-treated wild-type and hmp8 plants infiltrated with 10 mM $MgCl_2$ had SA levels of about 120 ng g^{-1} leaf fresh weight and SAG contents of about 600 ng g^{-1}. Upon *Pst* (*avrB*) challenge, SA levels increased in wild-

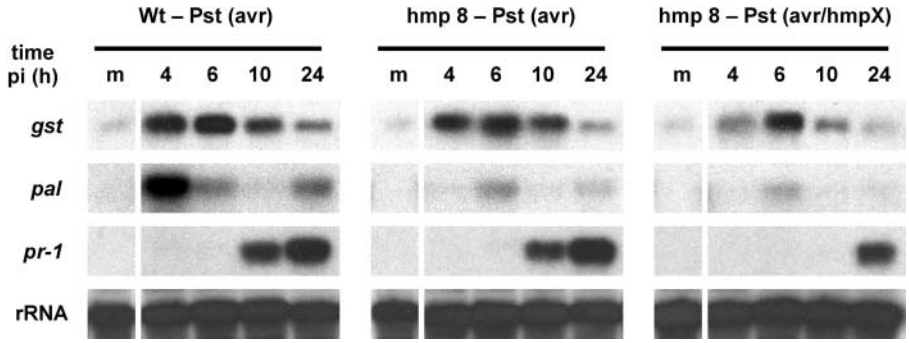


Figure 5. Expression of defense and cellular protectant genes in wild-type and hmp8 transgenic plants after *Pst* (*avrB*) challenge. Three parallel leaf samples were collected at the indicated times after infection for RNA extraction and northern-blot analysis. $MgCl_2$ (m)-infiltrated leaves were collected 4 h after infection. Experiments were repeated three times with similar results.

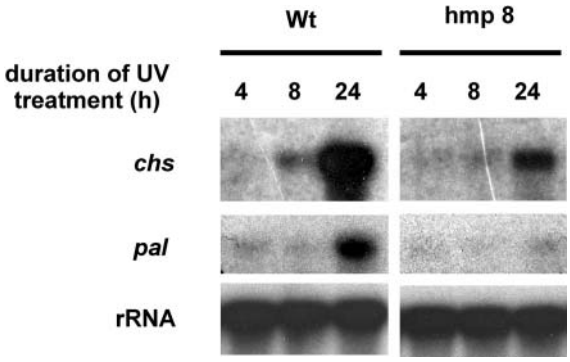


Figure 6. Expression of PAL and CHS in DEX pretreated wild-type and hmp8 plants after UV exposure. Three parallel leaf samples were collected at the indicated times after the beginning of UV treatment. Experiments were repeated three times with similar results.

type plants about 4-fold to 500 ng g^{-1}, and SAG levels reached values of 1,700 ng g^{-1}, representing a 3-fold increase. In DEX-treated hmp8 plants, the SA content in *Pst* (*avrB*)-challenged leaves showed a small, albeit statistically not significant, decrease to about 80% of the wild-type value (Fig. 9A). A similar trend was observed for the glucoside (Fig. 9B). Again, the most striking effect was detected when induced hmp8 plants were infiltrated with *Pst* (*avrB/hmpX*). Here, SA and SAG were reduced to about 50% of the wild type-Pst (avrB) values, reflecting a pathogen-induced increase of only about 2-fold (SA) and 1.5-fold (SAG), respectively.

To test whether these changes of defense responses in our genetically different pathosystems affected bacterial growth in planta, we determined the number of colony-forming bacteria in the apoplast 2 d after leaf inoculation (Table I). Compared to wild-type plants, bacterial growth was slightly, but statistically insignificantly, enhanced in DEX-treated hmp8 plants when challenged with *Pst* (*avrB*). A more pronounced growth enhancement was detected when DEX-treated hmp8 plants were challenged with *Pst* (*avrB/hmpX*). However, this enhancement did not reach the extent of growth found in the compatible interaction of wild-type plants and the isogenic virulent *Pst* strain (Table I).

DISCUSSION

Pharmacological methodologies in different laboratories using mainly mammalian NOS inhibitors, NO scavengers, and NO-releasing systems have implicated a pivotal role for NO in plant disease resistance (Delledonne et al., 1998; Durner et al., 1998). Moreover, the recent report that activity suppression of a pathogen-inducible NOS in tomato (*Lycopersicon esculentum*) increases susceptibility to *P. syringae* demonstrates the involvement of a NO-generating enzyme in plant defense (Chandok et al., 2004). This plant pathogen-inducible NOS represents a variant form of the P protein of the Gly decarboxylase complex that shares some biochemical characteristics with animal NOS, such as sensitivity to inhibitors (Chandok et al.,

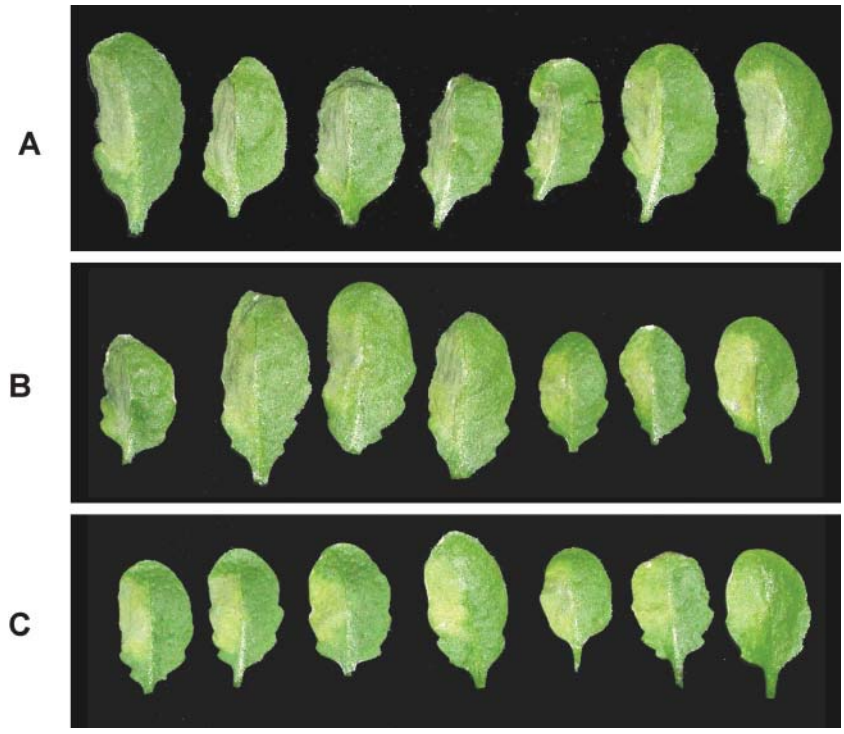


Figure 7. Macroscopic HR symptoms 2 d after infiltration of DEX-pretreated wild-type and hmp8 plants with avirulent Pseudomonas. Bacteria were infiltrated into the left side of leaves. Seven parallels are shown for each condition. A, Wild-type plants-*Pst* (*avrB*). B, hmp8 plants-*Pst* (*avrB*). C, hmp8 plants-*Pst* (*avrB/hmpX*).

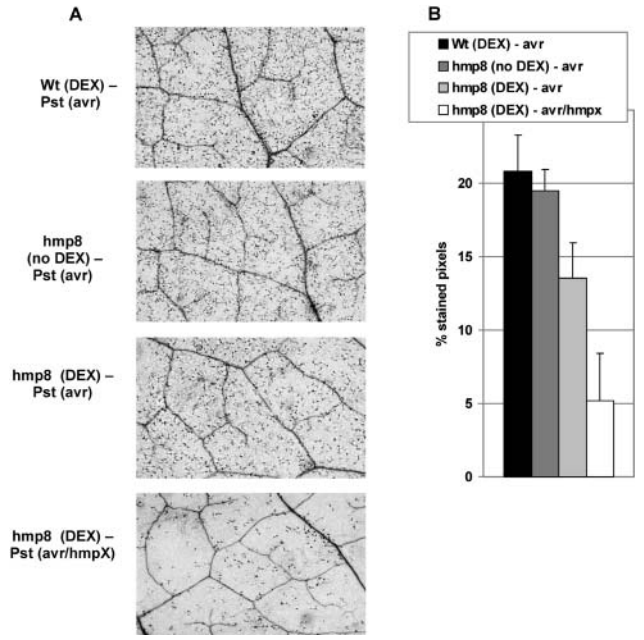


Figure 8. Microscopic cell death after pathogen challenge of DEX-pretreated wild-type and hmp8 plants. Trypan blue staining was performed 24 h after infection. A, Staining patterns of representative leaves inside the infiltrated area (100-fold magnification). B, Percentage of stained pixels inside the infiltration area. Values are shown as the mean ± SD of at least six leaves from different plants. Experiments were repeated three times with similar results.

2003), indicating the validity of the data obtained with the use of pharmacological approaches. However, it cannot be fully excluded that pharmacological compounds not only interfere with the metabolic pathway of interest but also have nonspecific effects. The widely used NADPH oxidase inhibitor diphenylene iodonium, for instance, has been shown to be a potent inhibitor of mammalian NOS and other flavoproteins (Stuehr et al., 1991; Bolwell, 1999). To generalize and broaden the knowledge of NO function in plant defense, we employed a genetic approach to interfere with NO signaling by expressing a NOD in transgenic Arabidopsis plants as well as in avirulent *P. syringae*.

Transgenic Arabidopsis plants were produced that express the Hmp protein from *E. coli*, and whole-plant NO emission was evaluated. The emission was shown to be markedly reduced in Hmp-expressing plants, and leaf extracts from transgenic plants degraded NO significantly faster than extracts from control plants. These findings demonstrate that Hmp is a functional NOD in planta (Fig. 1). Using the NO-sensitive fluorescence indicator DAF2-DA, we furthermore showed that NO is produced during the incompatible interaction of Arabidopsis and *Pst* (*avrB*) and that this NO burst is attenuated in NOD-expressing plants (Fig. 2). We also produced avirulent Pseudomonas expressing HmpX, a similar NOD from *E. chrysanthemi*. Biochemical experiments suggest that HmpX is located in the periplasm and represents a functional NOD in transformed Pseudomonas (R. Poole and M. Delledonne, unpublished data).

With these genetic tools, we examined the hypersensitive disease resistance response when NO accumulation was attenuated by the action of NODs in two different surroundings. We could generally state that the removal of NO from the plant resulted in strikingly similar tendencies compared to NO removal from the pathogen side. When comparing the interaction of hmp8 plants with *Pst* (*avrB*) on the one hand and the interaction of wild-type plants with *Pst* (*avrB/hmpX*) on the other hand, we found very similar tendencies (data not shown). Moreover, when combining the two genetically modified systems, i.e. the interaction of hmp8 plants with *Pst* (*arvB/hmpX*), we observed additive effects in all examined defense responses.

We first detected significantly lower H_2O_2 levels during the oxidative burst in NO-deficient interactions, and removal of NO from both the plant and the pathogen side had an additive effect (Fig. 3). Less H_2O_2 staining in the presence of NOD was also obvious

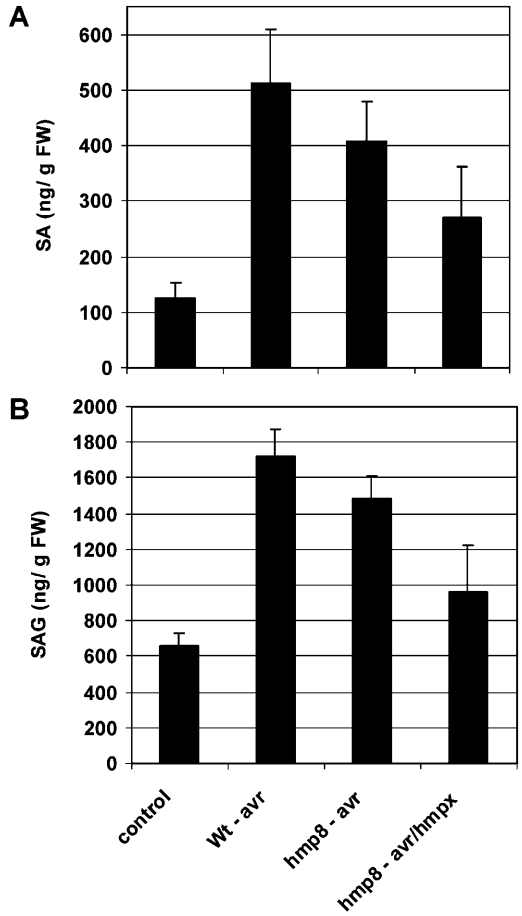


Figure 9. SA contents of wild-type and hmp8 transgenic plants after challenge with avirulent *Pst* (± *hmpX*). Leaf samples were collected 8 h postinfection. Leaves were pretreated with DEX for 16 h. Bars indicated mean values of three independent measurements. Control, $MgCl_2$-infiltrated plants. A, Free SA. B, SAG.

Table I. *Bacterial growth of different Pst in Arabidopsis leaves of wild-type Arabidopsis and hmp8 transgenic plants*

Leaves were pretreated with DEX 16 h before pathogen inoculation, infiltrated with 10^6 cfu mL^{-1} Pst, and harvested 1 h and 2 d after inoculation. Values $\times 10^4$ represent means of cfu per cm^2 (±SD), each from five sets of three leaf discs. Experiments were repeated twice with similar results. vir, Virulent; avrB, avirulent; avrB/hmpX, avirulent expressing hmpX.

Time	Wild Type vir	Wild Type avrB	hmp8 avrB	hmp8 avrB/hmpX
1 h	2.33 (±1.14)	2.2 (±0.67)	2.00 (±0.76)	1.57 (±0.77)
2 d	2,700 (±361)	152 (±47)	170 (±53)	267 (±76)

when plants were infiltrated with Glc/Glc oxidase, a H_2O_2-generating system (Fig. 4), indicating that the in planta capability to degrade H_2O_2 was increased by the action of the NOD. This effect might be due to the NO-degrading function of the NOD or possibly by direct degradation of H_2O_2 by NOD. Because the rate of H_2O_2 degradation was identical in leaf extracts from wild-type and hmp8 plants (data not shown), we conclude that direct H_2O_2 degradation through NOD does not take place. Rather, a factor differing in hmp8 and wild-type plants but not in the corresponding extracts might account for the different observation in intact plants and extracts, respectively. This factor might be the concentration of NO, which is delivered by the intact plant continuously but not necessarily by extracts. Following this interpretation, the higher in planta H_2O_2 degradation capability of NO-deficient Hmp plants suggests an inhibitory effect of NO toward H_2O_2-degrading enzymes. In fact, the predominant H_2O_2 scavenging enzymes are catalase and ascorbate peroxidase. Mammalian catalase is reversibly inhibited by NO (Brown, 1995), and it has been shown in vitro that NO inhibits both tobacco catalase and ascorbate peroxidase (Clark et al., 2000). In accordance to previous reports (Delledonne et al., 1998; Foissner et al., 2000), our data reveal that a local burst of NO coincides with the oxidative burst at the site of pathogen infection. Therefore, it is conceivable that this NO burst locally contributes to maintain more sustained and higher H_2O_2 levels that can then either act directly as an antimicrobial oxidant or indirectly by triggering various defense responses (Lamb and Dixon, 1997).

The most striking differences in defense gene expression concerned the induction of *pal* transcripts, which was significantly attenuated in hmp plants and in the presence of avirulent Pseudomonas expressing HmpX (Fig. 5). This observation confirms pharmacology-based findings demonstrating a reduction of *pal* expression by NOS inhibitors in soybean cells and *pal* induction by NO donors and recombinant NOS in soybean and tobacco, respectively (Delledonne et al., 1998; Durner et al., 1998). In addition, the UV-induced expression of *pal* and *chs*, the first committed enzyme in anthocyanin biosynthesis, was strongly repressed in hmp plants (Fig. 6). Based on this experimental evidence, NO appears to play a pivotal regulatory role in the signaling processes leading to expression of phenylpropanoid pathway genes.

PAL is the key enzyme for the general phenylpropanoid pathway, and possible outcomes are lignin, anthocyanin, and/or SA biosynthesis. However, despite the strong repression of *pal*, SA levels only showed a 20% reduction in the presence of Hmp in plants or HmpX in bacteria (Fig. 9). This supports the findings that in Arabidopsis, SA is produced by alternative routes, e.g. by the isochorismate pathway (Wildermuth et al., 2001). The observed induction of *pal* in the Pseudomonas-Arabidopsis pathosystem might then feed alternative processes like lignification or the production of other phenylpropanoid compounds to a significant extent. If SA is the causative agent of *PR-1* induction (Yalpani et al., 1991; Uknes et al., 1992), the rather weak attenuation of SA induction might explain the fact that in these cases the expression of *PR-1* is not affected (Fig. 5). The levels of SA might still be above a threshold value necessary for full pathogen-induced *PR-1* expression. In the double experiment in which NO is reduced from both the pathogen and the plant, however, SA was reduced to 50% of the usual value and *PR-1* expression was clearly delayed, although not fully suppressed. It is worth noting that the expression of *PR-1* is also up-regulated in plants challenged with virulent pathogens but slower than in the corresponding incompatible interaction (de Torres et al., 2003). Compared to pharmacological experiments with tobacco and soybean cells (Delledonne et al., 1998; Durner et al., 1998), our results demonstrate a similar tendency of NO involvement in SA and PR-1 production and a strong regulatory role of NO toward the synthesis of phenylpropanoid pathway enzymes like PAL.

When NO is scavenged by Hmp or HmpX alone, dry lesion development is delayed but not eliminated (Fig. 7), and the appearance of microscopic HR lesions is only moderately suppressed (Fig. 8). However, when NO is scavenged by the simultaneous action of Hmp and HmpX, the macroscopic dry HR lesions are yellowish with less pronounced symptoms, and the microscopic HR lesions are significantly reduced (Fig. 8). Therefore, HR lesion development is clearly affected by a reduced NO content in Arabidopsis, and the correlation with SA levels suggests a mediatory role of SA in these processes. This is in accordance with findings that SA is required for induction of the HR in response to bacterial pathogens in soybean (Tenhaken and Rubel, 1997) and that SA is needed for cell death initiation in Arabidopsis *lsd* mutants (Weymann et al., 1995). The execution of hypersensitive cell death in soybean cells challenged with avirulent *P. syringae* is strongly diminished by NO scavenging compounds and NOS inhibitors (Delledonne et al., 1998). Furthermore, a poised production of ROI and NO is necessary to trigger the HR, and NO together with H_2O_2, but not O_2^-, are

indicated as the essential players in this process (Delledonne et al., 2001). One possible mechanism for this cooperation could be that NO ensures maintenance of high, persistent H_2O_2 levels that are necessary to trigger the HR. The threshold H_2O_2 levels for HR development in soybean cells is about 6 μM (Levine et al., 1994), and in our experiments, H_2O_2 levels may not have significantly fallen below a comparable threshold value in the cases of Hmp or HmpX action alone. In the double experiment, however, when H_2O_2 levels showed the strongest decrease (Fig. 3), the H_2O_2 threshold might not have been exceeded and, consequently, a significant reduction in HR development could be observed. A similar reasoning could explain why a significant induction of *gst* transcription still took place under NO deficiency, even in the double experiment (Fig. 5). For *gst* expression, H_2O_2 threshold levels have been shown to be below the HR value, which are around 2 μM in soybean cells (Levine et al., 1994).

Blockage of the accumulation of NO by NO scavengers or mammalian NOS inhibitors has previously been shown to enhance bacterial growth of avirulent Pseudomonas in Arabidopsis leaves, although to a lower extent in comparison to the growth of virulent strains (Delledonne et al., 1998). In this work, NO scavenging by NOD led to a similar, albeit weaker, tendency of bacterial growth enhancement of avirulent *Pst* (Table I). Obviously, the removal of NO in these and other recent experiments is not sufficient for a strong growth enhancement of avirulent pathogens, despite the apparent effects on progression of the described defense responses. This behavior is in some respects reminiscent of the *dnd* (defense, no death) class of Arabidopsis mutants that show the ability to limit pathogen growth in a gene-for-gene resistance without developing an HR (Yu et al., 1998). Dnd1 encodes a cyclic nucleotide-gated ion channel (Clough et al., 2000). In mammals, cyclic nucleotides like cGMP or cADP-Rib are second messengers closely associated with NO signaling (Galione and White, 1994; Schmidt and Walter, 1994), and this has been suggested for plants as well (Pfeiffer et al., 1994; Durner et al., 1998; Clarke et al., 2000). It will be of interest to investigate if generation of NO leads to activation of Dnd1, which contributes to the development of symptoms characteristic of the HR.

The complete scavenging of a highly diffusible and reactive molecule like NO is difficult to achieve for a single protein, and the (physiologically active) reaction of NO with other cellular molecules might to a certain extent still compete with the NOD reaction. This is also reflected by the fact that Hmp-overexpressing plants still emitted about one-half the amount of gaseous NO than wild-type plants (Fig. 1E). Therefore, not all cellular NO-mediated effects might have been fully suppressed by this transgenic approach. Compared to the very similar salicylate hydroxylase (NahG) strategy applied by Gaffney et al. (1993), which addressed the role of the far less reactive signaling compound SA in disease resistance, these data have some limitations due to the physicochemical properties of NO. In general, the possibility that protein overexpression in plants leads to side effects not associated with the physiological process under investigation cannot be fully excluded. For instance, it was demonstrated recently that effects on plant defense responses observed by overexpression of NahG might not necessarily result from a lack of SA accumulation but could partly be a consequence of the presence of the SA degradation product catechol (van Wees and Glazebrook, 2003). Because Hmp converts NO, O_2, and NAD(P)H to nitrate in equistoichiometric amounts, it is conceivable that physiological side effects resulting from oxygen and NAD(P)H consumption or nitrate accumulation exist in hmp-overexpressing plants. Such side effects, however, should be minimal in hmp plants considering the comparatively low NO levels produced in plants (Fig. 1E) and taking into account the use of an inducible vector system, which restricted the action of Hmp to a small experimental window. The parallel tendencies observed in two different genetic backgrounds, i.e. the action of Hmp in Arabidopsis and HmpX in Pseudomonas, further support that the described effects on plant defenses were a direct consequence of NO degradation rather than a result of indirect effects caused by NOD on plant metabolism. Besides its NOD activity, however, it was shown that Hmp-overexpressing *E. coli* strains are capable of generating ROI (Poole and Hughes, 2000). We can rule out that this metabolic activity took place in transgenic hmp plants during the course of our pathogen and UV experiments due to the findings that transcriptional up-regulation of *gst*, a sensitive marker for ROI production (Levine et al., 1994), and a positive DAB reaction could not be detected in uninfected but DEX-induced hmp plants (Figs. 3 and 5). The latter statement, however, could not be maintained when hmp plants were exposed to DEX for longer periods of time. About 4 d after transgene induction, we detected elevated levels of ROI in leaves of hmp plants by DAB staining and the expression of *gst* and *pr-1* in the absence of pathogens (data not shown). Obviously, a new physiological situation in hmp plants appeared when plants perpetually accumulated Hmp protein, either caused by the constant removal of NO or by an emerging ROI-generating activity. Interestingly, in a recent transgenic approach, the constitutive overexpression of an alfalfa (*Medicago sativa*) hemoglobin with putative NO scavenging properties in tobacco similarly resulted in increased basal ROI levels, besides elevated pathogen-induced SA levels and reduced disease symptoms after *P. syringae* infection (Seregélyes et al., 2003). These findings indicate that a temporally controlled expression rather than constitutive or prolonged overexpression of certain transgenes like (flavo) hemoglobins might be crucial in overexpression studies. The use of the inducible vector system permitted us to use a window of 3 to 4 d after transgene expression to perform pathogen and phys-

iological experiments without undesirable side effects like ROI production.

MATERIALS AND METHODS

Generation of Hmp-Overexpressing Arabidopsis

To generate transgenic Arabidopsis overexpressing the *Escherichia coli hmp* gene, pTA7001, a dexamethasone-inducible expression system, was used (Aoyama and Chua, 1997). The *hmp* coding sequence was generated by PCR with the primers 5′-CGG*CTCGAG*ATGCTTGACGCTCAAACCATC-3′ and 5′-GG*ACTAGT*ACGCGGCAATTTAAACCGCGTC-3′ using a full-length hmp clone as a template, which was kindly provided by A.M. Gardner (University of Cincinnati). The PCR product was subcloned into pGEMT-Easy (Promega, Madison, WI), sequenced, and introduced into pTA7001 by the use of 5′-*Xho*I and 3′-*Spe*I restriction sites. The construct was transformed into *Agrobacterium tumefaciens* (strain GV3101) and the latter used for plant transformation of Arabidopsis ecotype Col-0 by the floral dip method (Clough and Bent, 1998). After transformation, seeds were harvested from T0 plants and surface sterilized, and positive transformants were selected on phytagar plates supplemented with Murashige minimal organics medium (Life Technologies, Paisley, UK) containing 15 μg L^{-1} hygromycin.

Homozygous T3 plants from single insert lines were used for all experiments, and plants were grown at 22°C under a 9-h-light/15-h-dark cycle. For transgene induction, hmp plants were sprayed with a solution of 3 μM DEX in 0.01% Tween 20. Control experiments were performed with wild-type Col-0 plants treated with 3 μM DEX in 0.01% Tween 20 and/or hmp transgenic plants solely sprayed with 0.01% Tween 20. Pathogen infiltrations followed 16 h after DEX/Tween 20 treatment.

Growth of Plant-Pathogens and Infection

Pseudomonas syringae pv *tomato* carrying the avirulence gene *avrB* were transformed with a pRK415 broad host vector (Keen et al., 1988) carrying the complete coding sequence of hmpX (Favey et al., 1995) under control of the Lac promoter, which is constitutively active in Pseudomonas. Detailed description of the construction of pRK415-hmpX and maintenance in Pseudomonas is not provided (M. Boccara, C. Mills, J. Zeier, C. Anzi, C. Lamb, R. Poole, and M. Delledonne, unpublished data).

Pst strains were grown overnight at 28°C in King's B medium containing the appropriate antibiotics (concentrations: rifampicin 50 μg L^{-1}, kanamycin 50 μg L^{-1}, tetracycline 15 μg L^{-1}). Bacteria were pelleted, washed three times with 10 mM $MgCl_2$, resuspended, and diluted in 10 mM $MgCl_2$ to the desired concentration (generally 2×10^6 cfu mL^{-1}, for symptom development 5×10^6 cfu mL^{-1}, for bacterial growth 10^6 cfu mL^{-1}). The bacterial solutions were infiltrated from the abaxial side into one-half of a sample leaf using a 1-mL syringe without a needle. Control (mock) inoculations were performed with 10 mM $MgCl_2$. Macroscopic symptoms were documented 2 d after infection. Bacterial growth was assessed by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL of 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium containing Rifampicin, and quantifying colony numbers after 2 to 3 d.

UV Treatment of Arabidopsis Plants

Five-week-old Arabidopsis wild-type and hmp8 plants were pretreated with DEX for 16 h and placed into a growth chamber equipped with UV-A light-emitting black light tubes (Phillips TL 8 W/08; Eindhoven, The Netherlands).

Histochemical Staining and Quantification of H_2O_2 Levels and Microscopic HR Lesions

DAB and Trypan blue staining were performed as described by Thordal-Christensen et al. (1997) and Koch and Slusarenko (1990), respectively. Exogenous H_2O_2 was generated by infiltrating 10 μL of 2.5 mM D-Glc and 2.5 units mL^{-1} *Aspergillus niger* Glc oxidase (Calbiochem, San Diego) in 20 mM Na phosphate buffer, pH 6.5, into Arabidopsis leaves.

For quantification of the number of stained pixels inside the infected leaf area, the histogram function of Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA) was used. Microscopic photographs were reduced to grayscale mode, and all pixels inside the infiltration zone with a gray tone value <125 were quantified. To account for background staining, the corresponding value for an area of equal size inside the noninfected opposite side of the leaf was subtracted from the latter value and the result divided by the total amounts of considered pixels to yield the relative number of stained pixels in percentage.

RNA and Protein Analysis

Total RNA was isolated from Arabidopsis leaves using Trizol reagent (Life Technologies) following the manufacturer's instructions. RNA-blot hybridization (Levine et al., 1994) was performed with probes of the Arabidopsis *gst*, *pal*, and *PR-1* genes: ATGST (GenBank accession no. U70672), ATPAL1A (X62747), and ATHRPRP1A (M90508). Equal loading was verified by gel staining with ethidium bromide and by hybridization with an rDNA probe.

For protein extraction, three leaves were homogenized with 1 mL of extraction buffer (15 mM HEPES, 40 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.6). The mixture was centrifuged for 30 min at 19,000*g* and 4°C. The supernatant constituted the protein extract. Protein samples were subjected to SDS-PAGE on 10% (w/v) polyacrylamide (Sambrook et al., 1989) and electroblotted to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia, Little Chalfont, UK). Probing and detection of western blots were performed as described in the ECL western-blotting detection kit (Amersham). The primary antibody raised against *E. coli* Hmp was kindly provided by Robert Poole (Sheffield, UK) and used at a dilution of 1:3,000. A goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma, St. Louis) was used as the secondary antibody with a dilution of 1:10,000.

Determination of SA Levels

Measurements of SA and SAG essentially followed the protocol of Raskin et al. (1989). Briefly, 0.15 g of frozen leave tissue was homogenized in 1 mL of 90% methanol and extracted for 10 min at 40°C. The mixture was centrifuged for 5 min at 14,000*g*, and the pellet was extracted for another 10 min at 40°C with 100% methanol. Supernatants from both extractions were combined and dried under a gentle stream of N_2 at 40°C. The residue was resuspended in 1.5 mL of 0.1 M HCl, and 100 ng of *o*-anisic acid was added as an internal standard. After centrifugation for 10 min at 14,000*g*, the aqueous solution was extracted three times with 2 mL of cyclopentane/ethylacetate (1:1). The extracts were combined and the solvent removed under N_2 at 40°C. The residue was dissolved in 50 μL of methanol and passed through a solid-phase extraction column filled with ODS-H optimal packing material (Capital HPLC, Broxburn, UK) using 6 mL of methanol. The eluate was dried under N_2 at 40°C, dissolved in 100 μL of methanol, and used for HPLC analysis. For detection of SAG, the aqueous, acidic phase from the first extraction step was heated to 100°C for 30 min to convert the glucoside to free SA. The above protocol was repeated starting with addition of the internal standard.

HPLC analysis was performed using an ODS-H optimal column (10 × 2.1 mm, Capital HPLC) on a Shimadzu (Columbia, MD) LC-5A chromatograph. For separation, a linear gradient from 95% of H_2O/BuOH/HOAc (98.3/1.2/0.5) to 90% acetonitrile/BuOH/HOAc (98.3/1.2/0.5) in 20 min and flow rate of 0.7 mL min^{-1} was applied. For detection, a Waters (Milford, MA) 474 scanning fluorescence detector with an excitation wavelength of 300 nm was used. The emission wavelength was switched at 7 min elution time from 365 nm to 405 nm to ensure highest sensitivities for *o*-anisic and SA, respectively.

NO Degradation Assay

The kinetics of NO degradation were measured electrochemically using an Iso-NO meter (World Precision Instruments, Sarasota, FL). A saturated, 2 mM NO solution was prepared by bubbling 10 mL of NO gas through 5 mL of HEPES buffer (see above). Protein extracts were prepared as described above using six fully grown Arabidopsis leaves and 1 mL of HEPES-extraction buffer (without dithiothreitol). For NO degradation measurements, 1 mL of plant extract was supplemented with 10 μL of 10 mM NADH and the temperature of the solution kept at 24°C in a water bath. An Iso-NO electrode was calibrated according to the manufacturer's instructions and submerged into the protein solution inside a gas-tight vial. Under stirring, 5 μL of NO solution was added, and the time-dependent changes of the NO signal were recorded.

NO Emission Measurements

Rosette leaves were cut from root parts of Arabidopsis plants and immediately floated on deionized water. The leaves from three different plants were placed in a transparent lid container with 2 L of air volume. A constant flow of measuring gas (NO-free air conducted through a custom-made charcoal column) of 1.5 L/min was pulled through the container and subsequently through the chemiluminescence detector (CLD 770 AL ppt; Eco-Physics, Dürnten, Switzerland) by a vacuum pump connected to an ozone destroyer. Light was provided by a 400 W HQi-lamp (Schreder, Winterbach, Germany) above the container. The quantum flux density could be adjusted at 100 μmol m^{-2} s^{-1} photosynthetic active radiation by a polyester sieve (pore size is 210 μm) on the lid of the container. Air temperature in the container was usually about 23°C in the dark and 23°C to 25°C under light conditions.

NO Detection using DAF2-DA Fluorescence

Arabidopsis leaves were treated with 2×10^6 cfu mL^{-1} *Pst* (*avrB*) or 10 mM $MgCl_2$ as described above, and 3 h later, 10 μM DAF2-DA (Sigma) dissolved in 10 mM Tris/KCl, pH 7.2, was infiltrated into the pretreated leaf areas. One hour after DAF2-DA infiltration, leaf areas were analyzed microscopically using a Zeiss Axioskop 2 fluorescence microscope equipped with a confocal laser scanner (LSM 5 PASCAL; Zeiss, Oberkochen, Germany). Leaves were excited with an argon laser (488 nm). DAF2-DA fluorescence was recorded using a channel with a 505- to 530-nm band-pass filter, and autofluorescence of chloroplast was captured with a channel equipped with a 560-nm long-pass filter.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers X58872, X75893, U70672, X62747, and M90508.

ACKNOWLEDGMENTS

We thank Robert Poole (Sheffield, UK) and Anne M. Gardner (Cincinnati) for kindly providing hmp antibodies and the full-length hmp clone, respectively. Werner M. Kaiser (Würzburg, Germany) is gratefully acknowledged for providing the opportunity to perform NO-emission experiments. J.Z. is a Fellow of the Alexander-von-Humboldt Foundation. The corresponding fellowship supervision by Robert Chow (Edinburgh, UK) is also gratefully acknowledged.

Received March 11, 2004; returned for revision May 26, 2004; accepted June 21, 2004.

LITERATURE CITED

Aoyama T, Chua N-H (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J **11:** 605–612

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL (2002) Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. Plant Physiol **129:** 1642–1650

Bisgrove SR, Simonich MT, Smith NM, Sattler A, Innes RW (1994) A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. Plant Cell **6:** 927–933

Bolwell GP (1999) Role of active oxygen species and NO in plant defence responses. Curr Opin Plant Biol **2:** 287–294

Brisson LF, Tenhaken R, Lamb C (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell **6:** 1703–1712

Brown GC (1995) Reversible binding and inhibition of catalase by nitric oxide. Eur J Biochem **232:** 188–191

Chandok MR, Ekengren SK, Martin GB, Klessig DF (2004) Suppression of pathogen-inducible NO synthase (iNOS) activity in tomato increases susceptibility to *Pseudomonas syringae*. Proc Natl Acad Sci USA **101:** 8239–8244

Chandok MR, Ytterberg AJ, van Wijk KJ, Klessig DF (2003) The pathogen-inducible nitric oxide synthase (iNOS) in plants is a variant of the P protein of the glycine decarboxylase complex. Cell **113:** 469–482

Chappell J, Hahlbrock K (1984) Transcription of plant defence genes in response to UV light or fungal elicitor. Nature **311:** 76–78

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ (2000) NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. Plant J **24:** 667–678

Clark D, Durner J, Navarre DA, Klessig DF (2000) Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. Mol Plant Microbe Interact **13:** 1380–1384

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Clough SJ, Fengler KA, Lippok B, Smith Jr RK, Yu I-c, Bent AF (2000) The Arabidopsis *dnd1* ''defence, no death'' gene encodes a mutated cyclic nucleotide-gated ion channel. Proc Natl Acad Sci USA **97:** 9323–9328

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585–588

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive response. Proc Natl Acad Sci USA **98:** 13454–13459

Desikan R, Griffiths R, Hancock JT, Neill SJ (2002) A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **99:** 16319–16324

de Torres M, Sanchez P, Fernandez-Delmond I, Grant M (2003) Expression profiling of the host response to bacterial infection: the transition from basal to induced defence responses in RPM1-mediated resistance. Plant J **33:** 665–676

Durner J, Wendehenne D, Klessig DF (1998) Defence gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci USA **95:** 10328–10333

Favey S, Labesse G, Vouille V, Boccara M (1995) Flavohaemoglobin HmpX: a new pathogenicity determinant in *Erwinia chrysanthemi* strain 3937. Microbiology **141:** 863–871

Flor HH (1956) The complementary genetic systems in flax and flux rust. Adv Genet **8:** 29–54

Foissner I, Wendehenne D, Langebartels C, Durner J (2000) *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J **23:** 1–10

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Galione A, White A (1994) Ca^{2+} release induced by cyclic ADP-ribose. Trends Cell Biol **4:** 431–436

Garcia-Mata C, Lamattina L (2003) Abscisic acid, nitric oxide and stomatal closure—is nitrate reductase one of the missing links? Trends Plant Sci **8:** 20–26

Gardner PR, Gardner AM, Martin LA, Salzman AL (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc Natl Acad Sci USA **95:** 10378–10383

Graziano M, Benigni MV, Lamattina L (2002) Nitric oxide improves internal iron availability in plants. Plant Physiol **130:** 1852–1859

Guo F-Q, Okamoto M, Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science **302:** 100–103

Kaiser WM, Weiner H, Kandlbinder A, Tsai CB, Rockel P, Sonoda M, Planchet E (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. J Exp Bot **53:** 875–882

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. Annu Rev Genet **24:** 447–463

Keen NT, Tamaki S, Kobayashi D, Trollinger D (1988) Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene **70:** 191–197

Klessig DF, Durner J, Zhou JM, Kumar D, Navarre R, Zhang S, Shah J, Wendehenne D, Du H, Trifa Y, et al (2000) NO and salicylic acid signalling in plant defence. Proc Natl Acad Sci USA **97:** 8849–8855

Koch E, Slusarenko AJ (1990) Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell **2:** 437–445

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275

Lamb CJ, Lawton MA, Dron M, Dixon RA (1989) Signals and transduction mechanisms for activation of plant defences against microbial attack. Cell **56:** 215–224

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. Cell **79:** 583–593

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic acid. A likely endogenous signal in the resistance response of tobacco to viral infection. Science **250:** 1002–1004

Métraux J-P, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **250:** 1004–1006

Murgia I, Delledonne M, Soave C (2002) Nitric oxide mediates iron-induced ferritin accumulation in *Arabidopsis*. Plant J **30:** 521–528

Orozco-Cardenas ML, Ryan CA (2002) Nitric oxide negatively modulates wound signalling in tomato plants. Plant Physiol **130:** 487–493

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. Plant Physiol **129:** 954–956

Pfeiffer S, Janistyn B, Jessner G, Pichorner H, Ebertmann R (1994) Gaseous nitric oxide stimulates guanosine-3′5′-cyclic monophosphate (cGMP) formation in spruce needles. Phytochemistry **36:** 259–262

Poole RK, Hughes MN (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol Microbiol **36:** 775–783

Raskin I, Turner IM, Melander WR (1989) Regulation of heat production in the inflorescences of an Arum lily by endogenous salicylic acid. Proc Natl Acad Sci USA **86:** 2214–2218

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase *in vivo* and *in vitro*. J Exp Bot **53:** 103–110

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schmidt HHHW, Walter U (1994) NO at work. Cell **78:** 919–924

Seregélyes C, Barna B, Hennig J, Konopka D, Pasternak TP, Lukács N, Fehér A, Gábor V, Horváth GV, Dudits D (2003) Phytoglobins can interfere with nitric oxide functions during plant growth and pathogenic responses: a transgenic approach. Plant Sci **165:** 541–550

Stuehr DJ, Fasehun OA, Kwon NS, Gross SS, Gonzalez JA, Levi R, Nathan CF (1991) Inhibition of macrophage and endothelial cell nitric oxide synthase by diphenyleneiodonium and its analogs. FASEB J **5:** 98–103

Tenhaken R, Rubel C (1997) Salicylic acid is needed in hypersensitive cell death in soybean but does not act as a catalase inhibitor. Plant Physiol **115:** 291–298

Thordal-Christensen H, Zhang Z, Wei YD, Collinge DB (1997) Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J **11:** 1187–1194

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J (1992) Acquired resistance in *Arabidopsis*. Plant Cell **4:** 645–656

van Wees SC, Glazebrook J (2003) Loss of non-host resistance of *Arabidopsis* NahG to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J **33:** 733–742

Vasudevan SG, Armarego WL, Shaw DC, Lilley PE, Dixon NE, Poole RK (1991) Isolation and nucleotide sequence of the hmp gene that encodes a haemoglobin-like protein in *Escherichia coli* K-12. Mol Gen Genet **226:** 49–58

Weymann K, Hunt M, Uknes S, Neuenschwander U, Lawton K, Steiner H-Y, Ryals J (1995) Suppression and restoration of lesion formation in Arabidopsis *lsd* mutants. Plant Cell **7:** 2013–2022

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565

Yalpani N, Silverman P, Wilson TM, Kleier DA, Raskin I (1991) Salicylic acid is a systemic signal and an inducer of pathogenesis-related proteins in virus-infected tobacco. Plant Cell **3:** 809–818

Yu I-c, Parker J, Bent AF (1998) Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis *dnd1* mutant. Proc Natl Acad Sci USA **95:** 7819–7824

MANUSCRIPT 2

Mishina TE, Sonoda M, Fraaß V, Kaiser WM, Zeier J

Heterologous expression of a nitric oxide synthase in Arabidopsis enhances plant NO production and attenuates local and systemic resistance towards bacterial pathogens.

(2007b) Submitted

Heterologous expression of a nitric oxide synthase in Arabidopsis enhances plant NO production and attenuates local and systemic resistance towards bacterial pathogens

Tatiana E. Mishina[1], Masatoshi Sonoda[1, 2], Verena Fraaß[3], Werner M. Kaiser, and Jürgen Zeier*

Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, Julius-von-Sachs-Platz 3, D-97082 Würzburg, Germany

[1] These authors contributed equally to this work

[2] Present address: Faculty of Horticulture, Chiba University, Matsudo, Japan

[3] Present address: Instrumental Analysis and Bioanalysis, Saarland University, D-66041 Saarbrücken, Germany

*Correspondence to:

Dr. Jürgen Zeier

Julius-von-Sachs-Institute of Biological Sciences

Julius-von-Sachs-Platz 3

D-97082 Würzburg

Germany

Tel. (0)931 888 6218

Fax (0)931 888 6235

e-mail: zeier@botanik.uni-wuerzburg.de

KEY WORDS

Plant defence, systemic acquired resistance, nitric oxide, oxidative metabolism, pathogenesis-related proteins

ABBREVIATIONS

L-Arg, L-arginine; Col-0, Arabidopsis ecotype Columbia, cPTI, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl; cPTIO; 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl-3-oxide; cNOS, constitutive NO synthase; DAF-2DA, 4,5-diaminofluorescein diacetate; deiNOS, *Deinococcus radiodurans* NOS; HR, Hypersensitive response; JA, jasmonic acid; L-NAME, N-nitro-L-arginine-methylester; NO, nitric oxide; NOS, nitric oxide synthase; NOR-3, (±)-(E)-Ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexene-amide; PAL, phenylalanine ammonia lyase; *Pst*; *Pseudomonas syringae* pv. *tomato* DC3000; *Pst avrRpm1*, *Pst* carrying the avrRpm1 avirulence protein; *Psg*, *P. syringae* pv. *glycinea* race 4; PR, pathogenesis-related; ROS, reactive oxygen species; SA, salicylic acid; SAG, SA glucoside; SNP, sodium nitroprusside; SNO, S-nitrosothiol; SAR, systemic acquired resistance; TMV, tobacco mosaic virus; THF, tetrahydrofolate; Wt, wild type

ABSTRACT

Nitric oxide (NO) has been implicated in various aspects of plant-pathogen interactions, and positive regulation of several defence responses has emerged as a key function of NO in plant disease resistance. In order to investigate the effects of elevated endogenous NO production on plant defence responses, we heterologously expressed a bacterial NOS protein from *Deinococcus radiodurans* (deiNOS) in Arabidopsis. Arabidopsis plants constitutively expressing *deiNOS* (cNOS) exhibited increased levels of NO emission when compared with wild-type plants, indicating a functional expression of *deiNOS* and elevated NO production *in planta*. Remarkably, cNOS plants did neither exhibit increased disease resistance nor constitutive activation of defence responses, but were more susceptible towards virulent, avirulent and non-host strains of the bacterial pathogen *Pseudomonas syringae*. cNOS lines were not or only slightly impaired in salicylic acid (SA) biosynthesis or expression of the defence genes *PAL* and *PR-1*. Similarly, accumulation of jasmonic acid (JA), synthesis of the phytoalexin camalexin, and development of the hypersensitive cell death response in incompatible interactions were only marginally affected by *deiNOS* expression. In contrast, expression of a specific subset of SA- and JA-independent pathogenesis-related proteins (*PR-2, PR-5*) was significantly attenuated in different cNOS lines, and biologically induced systemic acquired resistance (SAR) was markedly reduced. In addition, both basal and pathogen-induced *GST1* transcript levels were reduced in cNOS plants, reflecting an alteration in oxidative metabolism. Taken together, our data show that constitutive expression of a bacterial NO synthase in Arabidopsis does not constitutively trigger plant defences, but partially interferes with specific defence responses to attenuate several forms of disease resistance.

RUNNING TITLE

NO and plant disease resistance

INTRODUCTION

After recognizing its importance as a signal in the mammalian immune system, the gaseous radical nitric oxide (NO) has been implicated as a mediator of defence responses also in plants (Mur et al., 2006; Delledonne, 2005). Rises in plant NO production or NO synthase (NOS) activity after pathogen contact have been detected in various incompatible interactions, for instance after inoculation of soybean, Arabidopsis and tobacco with different avirulent strains of *Pseudomonas syringae* (Delledonne et al., 1998; Clarke et al., 2000; Conrath et al., 2004; Mur et al., 2006), after infection of tobacco with tobacco mosaic virus (TMV), or during the response of pepper to an incompatible isolate of *Phytophtora capsici* (Durner et al., 1998; Requena et al., 2005). The temporal production of NO in incompatible interactions closely coincides with or precedes the production of reactive oxygene species (ROS; oxidative burst), and increases in NO levels or NOS activity in adequate compatible interactions are either lower or absent. Pathogen-elicited NO production has been implicated as a critical component in the initiation of the hypersensitive cell death response (HR; Delledonne et al., 1998; Clarke et al., 2000; Pedroso et al., 2000), and synergistic interactions between NO and ROS are likely to play a key role during this process (Delledonne et al., 2001; Zeier et al., 2004a). Independently from ROS, NO has been shown to positively regulate the production of salicylic acid (SA), up-regulation of the SA-dependent pathogenesis-related protein 1 (*PR-1*), and induction of further defence genes like *PAL* (phenylalanine ammonia lyase), *PAD4* (phytoalexin-deficient 4), *GST1* (glutathione-S-transferase 1), and *AOX1* (alternative oxidase 1) [Durner et al., 1998; Delledonne et al, 1998; Huang et al., 2002a; Zeier et al., 2004a]. In addition, phytoalexin accumulation in potato and soybean has been demonstrated to be mediated by NO (Noritake et al., 1996; Modolo et al., 2002).

An involvement of NO in non-host and basal resistance was implicated more recently. The non-host HR of tobacco induced by *P. syringae* pv. *phaseolicola (Psp)* is preceded by NO formation, and cell death is delayed by the NOS inhibitor L-NAME or the NO scavenger cPTIO (Mur et al., 2005). Papilla-based non-host resistance of barley to *Blumeria graminis* was found to be associated with a burst of NO, and application of the NO scavenger cPTIO increased the penetration frequency of the non-host fungus (Prats et al., 2005). Moreover, bacterial lipopolysaccharides, typical cell surface components of gram-negative bacteria, provoke NO formation, enhance NOS activity, and elicit defence gene induction in Arabidopsis (Zeidler et al., 2004). As these responses are attenuated in the putative Arabidopsis NO synthase mutant *Atnos1* (Guo et al., 2003), and *Atnos1* plants are more susceptible towards virulent *P. syringae*, NO was considered an essential component of basal defence towards bacteria (Zeidler et al.,

2004). However, recent experiments failed to confirm the biochemical function of AtNOS1 as a plant NOS (Zemojtel et al., 2006; Crawford et al., 2006). These findings not only question the involvement of NO in basal resistance, but also clarify that, despite of considerable efforts, an NO synthase in plants still awaits identification. Considering the lack of a plant NOS-like enzyme, it is astonishing that effects caused by inhibitors of mammalian NO synthases are still taken as evidence for an involvement of NO in plant processes. NOS inhibitors predominantly involve L-arginine derivatives, which in principle are able to block any L-Arg-dependent event in plants (Planchet and Kaiser, 2006).

Thus, although considerable data suggesting NO to function as a positive regulator of plant defences has accumulated during the past years, considerable ambiguities about this role and the applied methods exist. This problem is further exemplified by difficulties of NO detection in plants and inconsistent results concerning the cryptogein-mediated HR in tobacco. Using the frequently employed DAF-methodology for fluorometric NO determination, a cryptogein-induced NO burst has been monitored (Foissner et al., 2000). However, changes in DAF fluorescence are not necessarily indicative for NO production, but may also reflect NO oxidation and/or production of other DAF-reactive compounds (Balcerczyk et al., 2005). Moreover, cryptogein-triggered NO production in tobacco has not been confirmed by the chemiluminescence methodology, which allows a more sensitive NO detection than DAF application (Planchet et al., 2006). Application of the NO scavenger cPTIO attenuates cryptogein-induced cell death in tobacco (Lamotte et al., 2004), supporting the putative role of NO as a pro-death signal in plants (Delledonne et al., 1998). However, cPTI, the reaction product of NO and cPTIO, inhibits the HR without scavenging NO, indicating that cPTIO-based experiments have to be reconsidered (Planchet et al., 2006). Alongside, the validities of several studies using sodium nitroprusside (SNP), a NO donating compound that has been widely used in plant NO research, have to be questioned, as SNP additionally releases bioactive and cytotoxic cyanide (Bethke et al., 2006).

In light of the difficulties of accurate NO detection in plants and the unequivocal side effects of widely employed pharmacological compounds scavenging or releasing NO (Planchet and Kaiser, 2006), it turns out that different approaches have to be combined to better understand the involvement of NO in plant defence. In this work, we pursue the role of NO in disease resistance in a transgenic strategy. To increase *in planta* levels of NO, we generated transgenic Arabidopsis plants constitutively expressing a bacterial NO synthase (cNOS). Given the putative positive regulatory action of NO in plant defence, we expected such plants to be activated or strengthened in distinct NO-dependent defence pathways. We show here that cNOS plants, although exhibiting enhanced *in planta* NO production, do not posses enhanced

defence capacity, but are more susceptible towards virulent, avirulent and non-host strains of the bacterial pathogen *Pseudomonas syringae*. Additionally, cNOS plants display a decreased ability to mount systemic acquired resistance. This decrease in disease resistance is accompanied with the weakening of specific defence responses, including up-regulation of SA- and JA-independent defence genes and an alteration of oxidative metabolism.

MATERIALS AND METHODS

Generation of Arabidopsis constitutively expressing *deiNOS*

The *deiNOS* coding sequence was generated by PCR with the primers 5'-GGTACCGGATGAGTTGCCCCGC-3' and 5'-CTGCAGGCCTTTATCGTGGGGTAAC -3' using genomic DNA isolated from *Deinococcus radiodurans* strain 20539 (obtained from the German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany) as a template. The PCR product was subcloned into pGEMT-Easy (Promega) and the correct *deiNOS* sequence confirmed. The *deiNOS* coding region was then cloned into pCHF2 by use of the introduced 5'-KpnI- and 3'-PstI-restriction sites. pCHF2 is a pPZP221-based (Hajdukiewicz et al., 1994) plant expression vector carrying three copies of the cauliflower mosaic virus 35S promoter and a pea ribulose 1,5-bisphosphate carboxylase/oxygenase terminator (Jarvis et al., 1998). The pCHF2::deiNOS construct was transformed into *Agrobacterium tumefaciens* (strain GV3101), and plant transformation of *Arabidopsis thaliana* ecotype Col-0 was performed by the floral dip method (Clough and Bent, 1998). Seeds were harvested from T0 plants, surface sterilized, and positive transformants were selected on phytagar plates supplemented with Murashige minimal organics medium (Life Technologies) containing 70 $\mu g\ l^{-1}$ gentamycin sulfate. Homozygous T3 plants from single insert cNOS lines were selected and used for further experiments.

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 and cNOS plants were grown in a mixture of soil (Fruhstorfer Pflanzenerde, Archut, Germany), vermiculite and sand (9:1:1) in a controlled environment chamber (J-66LQ4, Percival, Boone, IA) with a 9 h day / 15 h night cycle and a relative humidity of 70 %. The photon flux density and growth temperature during the day period constituted 70 $\mu mol\ m^{-2}\ s^{-1}$ and 22°C, respectively, and growth temperatures were set to 18 °C during the night period.

NO emission measurements

NO emission from leaves was measured by chemiluminescence detection (Rockel et al., 2002). Rosette leaves were cut from root parts of *Arabidopsis* plants and immediately floated on deionized water. The leaves from five different plants were placed in a transparent lid container with 2 l air volume. A constant flow of measuring gas (NO-free air conducted through a custom-made charcoal column) of 1.5 l/min was pulled through the container and subsequently through the chemiluminescence detector (CLD 770 AL ppt, Eco-Physics, Munich, Germany) by a vacuum pump connected to an ozone destroyer. All measurements were performed under a light intensity of 150 µmol m^{-2} s^{-1} provided by a 400 W HQi-lamp (Schreder, Winterbach, Germany).

Growth of bacteria and plant inoculation

Pseudomonas syringae pv. *tomato* strain DC3000 with (*Pst avrRpm1*) and without (*Pst*) a plasmid carrying the avrRpm1 avirulence gene were provided from S. Berger, and *P. syringae* pv. *glycinea* strain A29-2 race 4 (*Psg*) was obtained from C. Lamb. Bacteria were grown at 28°C in King's B medium containing the appropriate antibiotics. Overnight log phase cultures were pelleted, washed three times with 10 mM $MgCl_2$, resuspended, and diluted in 10 mM $MgCl_2$. For experiments regarding gene induction, metabolite accumulation, or development of hypersensitive cell death, final dilutions of OD_{600} = 0.005 were used for all bacteria. For bacterial growth experiments, the following final concentrations were used: *Pst*: OD_{600} = 0.002, *Pst avrRpm1*: OD_{600} = 0.005, *Psg*: OD_{600} = 0.1. The bacterial suspensions were pressure-infiltrated into leaf areas covering about 80% of a sample leaf with a needle-less 1 ml syringe. Control treatments were performed by infiltrating adequate leaf areas with 10 mM $MgCl_2$. Macroscopic symptoms were documented 5 days after infection. Bacterial growth was assessed by homogenising disks originating from infiltrated areas of 3 different leaves in 1 ml 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28 °C for two days.

All pathogen experiments depicted in the figures were repeated at least twice with similar results.

Characterization of systemic resistance responses

Three lower leaves of a given plant were first infiltrated with a suspension of avirulent *Pst avrRpm1* (OD = 0.02) to induce SAR, or with 10 mM $MgCl_2$ as a control. Two days later, non-treated, upper leaves were either harvested for SA determination and gene expression analysis,

or plants were inoculated on three upper leaves with virulent *Pst* (OD 0.002). Growth of *Pst* in upper leaves was assessed another 3 days later as described above.

Quantification of microscopic HR lesions

The extent of microscopic HR lesion formation was assessed by Trypan blue staining, light microscopy and quantification of stained cells essentially as described by Zeier et al. (2004a).

Northern blot analysis

Total RNA was isolated from frozen leaves using peqGOLD RNAPure^{TM} reagent (peqLab, Erlangen, Germany) following the manufacturer's instructions. Two leaves from different plants of the same treatment were collected for each sample. 1 µg of total RNA was loaded on formaldehyde-agarose gels, separated by electrophoresis and blotted to nylon-membranes (Hybond-N, Amersham). RNA blot hybridisation was performed with specific, ^{32}P-labelled DNA-probes generated by PCR using appropriate oligonucleotide primers. The probes represented the following Arabidopsis genes: *SID1* (Arabidopsis annotation At4g39030), *PAL1* (At2g37040), *GST1* (At1g02930), *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-4* (At3g04720), *PR-5* (At1g75040),*THI2.1* (At1g72260).

RT-PCR analysis

Determination of *FMO1* (At1g19250) expression was performed by semi quantitative RT-PCR as described in detail by Mishina and Zeier (2006). The following primers were used for the amplification of cDNA derived from *18S* rRNA and *FMO1* mRNA, respectively: 5'-AAACGGCTACCACATCCAAG-3' (*18S*-forward), 5'-ACCCATCCCAAGGTTCAACT-3' (*18S*-reverse), 5'-CTTCTACTCTCCTCAGTGGCAAA-3' (*FMO1*-forward), 5'-CTAATGTCGT-CCCATCTTCAAAC-3' (*FMO1*-reverse).

Gas chromatographic determination of salicylic acid, jasmonic acid, and camalexin levels

Salicylic acid, jasmonic acid, and camalexin levels in leaves were quantified by a modified vapor-phase extraction method and GC/MS analysis as detailed in Mishina and Zeier (2006).

RESULTS

Arabidopsis thaliana plants expressing a bacterial NO synthase

To study the effect of elevated endogenous NO levels on plant defence and disease resistance, we aimed to heterologously express a functional NOS in Arabidopsis. NO synthases catalyse the production of NO from L-arginine (Crawford, 2006). Whereas mammalian NOS requires the redox cofactor tetrahydrobiopterin for catalysis, a pteridin derivative that has not yet been identified in plants (Kohashi, 1980), some bacterial NOS enzymes like deiNOS from the gram-positive bacterium *Deinococcus radiodurans* are able to use tetrahydrofolate (THF) to support NO formation (Adak et al., 2002). THF is a soluble coenzyme present in both microorganism and plants (Sahr et al., 2005), and we thus selected the *D. radiodurans NOS* gene (*deiNOS*) as a promising candidate for functional expression in Arabidopsis.

We cloned the coding region of *deiNOS* into pCHF2, a pPZP221-based plant expression vector that carries the cauliflower mosaic virus 35S promoter and a pea ribulose 1,5-bisphosphate carboxylase/oxygenase terminator (Hajdukiewicz et al., 1994; Jarvis et al., 1998). After Agrobacterium-mediated transformation of Arabidopsis ecotype Col-0 plants, transgenic T3 lines homozygous for the *deiNOS* transgene and segregating for a single T-DNA insert were selected. Northern blot analysis of three of those lines (designated as cNOS1, cNOS2, and cNOS3) showed that the *deiNOS* gene was constitutively expressed in leaf tissue (Fig. 1A). To functionally characterize *deiNOS* expression in the transgenic lines, NO production in cNOS and wild-type (Wt) plants was compared. Plant NO production can be estimated by measuring NO emission in purified air with chemiluminescence detection, a methodology more specific and sensitive than most other procedures currently used for NO quantification (Rockel et al., 2002; Planchet et al., 2006). As detected by chemiluminescence, Wt plants showed emission rates of 1.4 (±0.5) pmol NO g^{-1} fresh weight (FW) min^{-1}. Compared with that value, the different cNOS lines exhibited 1.5 to 4-fold higher rates of NO emission (Fig. 1B), indicating that constitutive expression of *deiNOS* in cNOS lines increased NO production *in planta* by the action of a functional *D. radiodurans* NOS.

deiNOS expression in Arabidopsis attenuates basal, specific, and non-host resistance towards *Pseudomonas syringae*

deiNOS expression in Arabidopsis resulted in alterations of the plant phenotype during vegetative growth. Plant growth of the different cNOS lines proved to be retarded when compared with Wt plants, and the rosette appeared more compact and bushy in cNOS lines

(Fig. 2A, B). Assuming a positive regulatory role of NO in plant defence, we at first speculated that the retarded growth phenotype might be a consequence of constitutive expression of defence responses and disease resistance, as many constitutive Arabidopsis defence mutants exhibit reduced growth (Bowling et al., 1997; Yu et al., 1998).

To directly assess disease resistance of cNOS plants towards distinct classes of plant pathogens, we inoculated leaves of cNOS1 (highest NO emission, Fig. 1B), cNOS3 (medium NO emission), and Wt plants (lowest NO emission) with different strains of the bacterial pathogen *Pseudomonas syringae*. *P. syringae* pv. *tomato* DC3000 (*Pst*) is virulent to Arabidopsis (Whalen et al., 1991), and pressure infiltration of *Pst* into leaves of wild-type Col-0 plants led to vigorous multiplication of bacteria inside the plant apoplast. Surprisingly, when leaves of cNOS and Wt plants were inoculated with the same initial titer of *Pst,* bacterial multiplication was about five times higher in both cNOS lines than in Wt plants (Fig. 3A). Thus, in contrast to our expectation, cNOS expression markedly decreased basal resistance to virulent *Pst*.

We next investigated whether specific and non-host resistance was influenced by *deiNOS* expression in the Arabidopsis-Pseudomonas pathosystem. *Pst* carrying the avrRpm1 avirulence protein (*Pst avrRpm1*) is recognized by Col-0 through the Rpm1 resistance protein and consequently elicits an HR (Bisgrove et al., 1994). Similar to the isogenic virulent *Pst* strain, bacterial multiplication of avirulent *Pst avrRpm1* was significantly higher in either of the cNOS lines than in Wt plants (Fig. 3B). In contrast to the *P. syringae* pv. *tomato* strains, the soybean pathogen *P. syringae* pv. *glycinea* race 4 (*Psg*; Keen and Buzzel, 1991; Delledonne et al., 1998) is not adapted to substantially grow in Arabidopsis and consequently represents a non-host pathogen to Col-0. When quantifying bacteria 3 days after infiltration of leaves with a high inoculum of *Psg*, we observed a twenty-fold and nine-fold higher bacterial number in leaves of cNOS1 and cNOS3 than in leaves of Wt plants, respectively. Consequently, expression of *deiNOS* not only attenuated basal disease resistance, but also led to a reduction of specific and non-host resistance in Arabidopsis.

To better understand the observed decrease in disease resistance in cNOS plants, we examined various defence responses that are typically induced by *P. syringae* at the site of pathogen ingress.

Salicylic acid- and jasmonic acid-related defence pathways are functional in cNOS plants

Salicylic acid is a well-characterized defence signal that accumulates in Arabidopsis in response to *P. syringae* infection. SA accumulation is required for the establishment of disease resistance against various *P. syringae* strains (Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). In $MgCl_2$-treated control plants, expression of the SA biosynthesis gene *SID1* was low in both Wt and cNOS (Fig. 5A, B), and levels of free SA constituted about 0.05 to 0.1 µg per g fresh weight (FW; Fig. 4A). After leaf inoculation with *Pst avrRpm1*, *SID1* was up-regulated between 4hpi and 10hpi in Wt plants, and a similar expression pattern was observed in the two cNOS lines (Fig. 5A, B). At 10h after treatment with the avirulent pathogen, levels of free SA increased about 10-fold in both Wt and cNOS1, and to a somewhat higher extent in cNOS3 (Fig. 4A). Moreover, a marked accumulation of the SA glucoside (SAG) was evident in all lines under investigation (Fig. 4B). The SA pathway culminates in the up-regulation of SA-inducible defence genes like the pathogenesis-related (PR) gene *PR-1*. Induction of *PR-1* by *Pst avrRpm1* inoculation occurred in a similar manner in Wt and both cNOS lines starting from 10hpi (Fig. 5A, B). When Wt or cNOS plants were infected with virulent *Pst*, enhanced *SID1* expression was observed starting from 6hpi, but up-regulation was more pronounced in Wt than in cNOS (Fig. 6A, B). Consistently, *Pst* triggered accumulation of SA and up-regulation of *PR-1* to a somewhat higher extent in Wt than in the two cNOS lines (Fig. 4, Fig. 6).

Jasmonic acid represents another signaling compound that is produced in plants after contact with *P. syringae*. A pronounced accumulation of JA was observed in Wt, cNOS1, and cNOS3 plants at 10h after inoculation with *Pst avrRpm1*, and JA levels declined again at 24hpi (Fig. 7A). JA production was similar in Wt and cNOS1, yet a tendency to slightly higher values existed in cNOS3. The JA-inducible defence gene *PR-4* (hevein-like, *HEL*) exhibited a comparable expression pattern after inoculation with the avirulent strain in all lines under investigation (Fig. 5A, B). However, up-regulation of *PR-4* by virulent *Pst* was faster in Wt than in both cNOS1 and cNOS3 (Fig. 6A, B).

Taken these findings together, we can state that SA- and JA-dependent defence pathways are still functional in cNOS. Avirulent *Pst avrRpm1* provokes induction of these pathways in cNOS plants in a wild-type like manner, whereas responses to virulent *Pst* are attenuated in cNOS. Moreover, *deiNOS* expression does not constitutively activate the SA or JA defence pathways, neither at the level of SA or JA synthesis, nor at the level of gene expression.

Camalexin accumulation, induction of the phenylpropanoid pathway and the hypersensitive cell death response are not affected by *deiNOS* expression

Further characteristic plant responses to pathogen challenge include synthesis of antimicrobial phytoalexins and activation of phenylpropanoid pathway genes. The indole derivative camalexin represents the major phytoalexin in Arabidopsis and is produced in response to elictor or pathogen treatment (Tsuji et al., 1992; Zhou et al., 1998). Camalexin was essentially absent in $MgCl_2$-treated control leaves of Wt or cNOS plants, substantially started to accumulate by inoculation with *Pst avrRpm1* at 10hpi, and reached an average of about 6.6 µg g^{-1} FW in Wt, 4.1 µg g^{-1} FW in cNOS1, and 11.1 µg g^{-1} FW in cNOS3 at 24hpi (Fig. 7B). The differences between Wt and each cNOS line, however, were not statistically significant. Moreover, expression of the key phenylpropanoid pathway gene phenylalanine ammonia lyase 1 (*PAL1*) was enhanced similarly by *Pst avrRpm1* in both Wt and cNOS plants at 4 to 6hpi (Fig. 5A, B).

As nitric oxide has been implicated in cell death development during the HR (Delledonne et al., 1998; Zeier et al., 2004a), we next investigated the capability of cNOS plants to execute hypersensitive cell death in response to *Pst avrRpm1* by trypan blue staining of pathogen-treated leaves (Fig. 8). At 24hpi, inoculated leaf areas of Wt plants exhibited a considerable amount of stained cells, indicating hypersensitive cell death. A distinct amount of HR also developed in inoculated areas of cNOS lines (Fig. 8). Moreover, a spontaneous cell death development in the absence of a pathogen did not occur in cNOS plants. However, when comparing the formation of HR symptoms 3d after treatment with *Pst avrRpm1*, we observed that inoculated areas of Wt leaves predominantly collapsed to produce colorless, dry lesions (Fig. 2C), whereas inoculated leaf areas of cNOS plants exhibited a yellowish appearance that is more reminiscent of disease symptoms usually observed during infections with virulent bacteria (Fig. 2D).

Compromised disease resistance in cNOS plants is associated with attenuated expression of a specific set of PR-genes, and partly with an altered oxidative metabolism

The extensive similarities between defence responses in Wt and cNOS plants observed so far could not conclusively explain the respective differences in disease resistance. We therefore tested whether further defence responses might be affected by *deiNOS* expression. A burst of ROS after recognition of avirulent pathogens (‘oxidative burst’) represents a hallmark of incompatible plant-pathogen interactions (Lamb and Dixon, 1997). Pathogen-induced ROS contribute to trigger hypersensitive cell death at infection sites and drive expression of cellular

protectant genes in neighboring plant tissue (Levine et al., 1994). The glutathione-S-transferase gene *GST1* is a typical example of a gene induced by ROS, and serves as a reliable marker for the oxidative burst (Alvarez et al., 1998; Zeier et al., 2004b). In Wt, pronounced basal *GST1* transcription occurred in uninfected control plants, and *Pst avrRpm1* treatment markedly enhanced these basal levels starting from 4hpi. Strikingly, both basal and *Pst avrRpm1*-induced *GST1* levels were substantially attenuated in cNOS1 plants (Fig. 5A). Although basal *GST1* expression levels were also reduced in cNOS3, the pathogen-induced expression pattern of *GST1* was similar in Wt and cNOS3 (Fig. 5B).

Moreover, we examined expression of the pathogenesis-related proteins *PR-2* and *PR-5*, which are both expressed independently of the SA or JA defence pathways (Nawrath and Métraux, 1999). Basal levels of *PR-2* and *PR-5* transcripts proved to be significantly lower in both cNOS lines than in Wt (Figs. 5, 6). Similarly, when leaves were inoculated with avirulent *Pst*, we observed a strongly attenuated induction of *PR-2* and *PR-5* in cNOS1 or cNOS3 compared with Wt plants (Fig. 5A, B). A marked reduction of *PR-2* and *PR-5* up-regulation was also observed when cNOS were infected with virulent *Pst* (Fig. 6A, B). Thus, *deiNOS* expression in Arabidopsis obviously interfered with the expression of a specific subset of pathogenesis-related proteins.

Systemic acquired resistance and systemic defence responses are compromised in cNOS plants

After a localized inoculation with virulent or avirulent *P. syringae*, Arabidopsis gradually develops systemic acquired resistance (SAR) at the whole plant level (Cameron et al., 1994; Zeier et al., 2004b). To investigate whether expression of *deiNOS* affects SAR, we infiltrated leaves of Wt or cNOS plants with HR-inducing *Pst avrRpm1* or a $MgCl_2$ control solution in a primary treatment (leaves designated as ‘primary leaves’), and performed a secondary or challenge infection two days later in rosette leaves located straight above the primary leaves (‘secondary’ or ‘systemic leaves’). Bacterial growth of virulent *Pst* in the challenge infection was scored another three days later.

Pretreatment of Wt or cNOS plants with 10 mM $MgCl_2$ allowed *Pst* to extensively grow during the challenge infection (Fig. 9A, dark bars), and conspicuous disease symptoms in terms of leaf yellowing developed in secondary leaves (Fig. 9B and D, white arrows). Growth difference between Wt and cNOS plants observed in $MgCl_2$ pretreated plants thereby reflected differences in local resistance described above (Fig. 3A). When Wt plants were pretreated with

Pst avrRpm1 instead of $MgCl_2$, we observed an eight-fold lower growth of *Pst* during the subsequent challenge infection in systemic leaves (Fig. 9A), and disease symptoms caused by the virulent strain were markedly attenuated (Fig. 9C, white arrows). These results document that SAR successfully developed in Wt plants. In cNOS1, by contrast, pre-inoculation of primary leaves with *Pst avrRpm1* instead of $MgCl_2$ still allowed vigorous growth of *Pst* in secondary leaves, providing only a non-significant, 1.8-fold increase in resistance to a subsequent *Pst* infection (Fig. 9A). Additionally, disease symptoms in secondary leaves of $MgCl_2$- and *Pst avrRpm1*-pretreated cNOS1 plants appeared similarly pronounced (Fig. 9D and E). Thus, SAR proved to be largely abolished in cNOS1 plants. In cNOS3, pre-inoculation with avirulent bacterial only led to 2.4-fold growth reduction of *Pst* during the challenge infection, demonstrating that SAR was substantially attenuated in this line as well (Fig. 9A).

The establishment of SAR is strongly associated with salicylic accumulation and enhanced defence gene expression in systemic tissue (Ryals et al., 1996). Consistently, Wt plants responded to a local *Pst avrRpm1* inoculation with a seven-fold elevation of SA levels in systemic leaves 2d after treatment, and increased expression of the SA biosynthesis gene *SID1* (Fig. 10A and B). Moreover, enhanced transcript levels of the pathogenesis-related genes *PR-1* (SA-inducible), *THI2.1* (JA-inducible), *PR-2*, and *PR-5* (both SA- and JA-independent) were detected in systemic leaves of *Pst avrRpm1*-pretreated Wt plants (Fig. 10B). By contrast, in both cNOS lines, inoculation with *Pst avrRpm1* in primary leaves did neither lead to a substantial SA accumulation nor to a marked systemic enhancement of *PR-1* expression. Moreover, pathogen-induced systemic expression of *SID1*, *THI2.1*, *PR-5*, and *PR-2* was virtually absent in cNOS1 and strongly attenuated in cNOS3. These experiments demonstrate that *deiNOS* expression severely compromises the establishment of SAR and the induction of systemic defence responses in Arabidopsis.

The flavin-containing monooxygenase FMO1 has recently been identified as a central component of SAR in Arabidopsis (Mishina and Zeier, 2006). Wt plants characteristically accumulate *FMO1* in systemic tissue after local contact with SAR-inducing pathogens, whereas SAR-defective defence mutants fail to do so, revealing a close correlation of SAR establishment and systemic *FMO1* expression. We therefore tested whether SAR attenuation in cNOS was paralleled by a reduction in induced systemic *FMO1* expression. Whereas systemic accumulation of *FMO1* was observed in Wt plants inoculated with *Pst avrRpm1*, up-regulation of *FMO1* in systemic leaves of both cNOS1 and cNOS3 did not occur (Fig. 10C).

DISCUSSION

Nitric oxide has been implicated as a positive regulator of various defence responses in plants. However, overlap of responses triggered by distinct NO treatments or different NO releasing compounds is astonishingly low. For instance, in Arabidopsis cells, induction of the defence genes *PR-1* and *PAD4* takes place with the NO donor NOR-3, but not with gaseous NO (Huang et al., 2002a; Huang et al., 2002b), and application of different NO donors results in distinct effects on ferritin regulation, programmed cell death and cellular redox state in Arabidopsis or tobacco (Murgia et al., 2004). Moreover, several difficulties in detecting and reliably quantifying NO in plant tissue exist, and the use of pharmacological compounds releasing or scavenging NO unequivocally bear side effects (see introduction; Planchet and Kaiser, 2006).

In order to investigate whether plants with elevated NO levels would exhibit increased defence capacity and disease resistance, we heterologously expressed a NOS enzyme from *Deinococcus radiodurans* (deiNOS) in Arabidopsis, which is able to use the plant-occurring coenzyme THF as a cofactor. Using chemiluminescence, a sensitive and specific methodology to detect gaseous NO produced by plants (Rockel et al., 2002), we have quantified significantly higher NO emission rates in all *deiNOS* expressing lines compared with wild-type plants. deiNOS bears 34% identity to the oxygenase domain of mammalian NO synthases (NOS_{oxy}), and is able to produce NO upon interaction with a separate reductase protein. Although the native *D. radiodurans* reductase is not yet identified, deiNOS is able to accept electrons from mammalian NOS reductase domains to produce NO (Adak et al., 2002). Possible candidates for bacterial deiNOS redox partners are sulfite reductase flavoproteins (Zemojtel et al., 2003). For the presence of deiNOS activity in cNOS plants, we have to postulate the existence of a corresponding redox partner *in planta*. Arabidopsis cytochrome reductases might be potential candidates to reduce the heterologuously expressed deiNOS protein, because they bear ~30% identity to bacterial sulfite reductases and contain both NADPH and flavin nucleotide binding domains.

Although we have no direct evidence for such redox interactions between deiNOS and a reductase protein *in planta*, plants constitutively expressing *deiNOS* show higher levels of NO emission than Wt plants, indicating the functionality of deiNOS and elevated NO production in cNOS plants. Despite this elevated NO production in cNOS plants, we did neither observe constitutive accumulation of the phytoalexin camalexin or of salicylic acid, nor enhanced expression of *PAL*, *PR-1*, or other defence genes. Thus, at first glance, our results are not consistent with the notion that NO represents a positive regulator of plant defence responses. However, in cNOS plants, elevated levels of NO are a consequence of a continuously acting

NOS, whereas exogenous application of NO donors, NO gas, or NOS preparations gives rise to a sudden increase in NO levels. Therefore, it seems that a gradient in NO production rather than continuous elevation of NO is necessary to trigger plant defence responses.

Beyond the absence of constitutively activated defence pathways, we found that cNOS plants are even more susceptible to virulent, avirulent, and non-host strains of *P. syringae* than Wt plants. A similar loss of basal, specific, and non-host resistance has recently demonstrated to be associated with increased S-nitrosothiol (SNO) formation in Arabidopsis (Feechan et al., 2005). S-nitrosylation of cysteine thiols has emerged as a central mechanism for the transduction of NO bioactivity. However, some human diseases like multiple sclerosis or stroke are characterized by increased levels of protein S-nitrosylation (Foster et al., 2003). Elevated SNO levels might result from NO overproduction but also from impaired SNO breakdown. Regarding the latter, mutations in the S-nitrosoglutathione reductase gene *AtGSNOR*1, which encodes for an enzyme that catalyses the reductive degradation of SNOs, enhances SNO levels and compromises disease resistance in Arabidopsis (Feechan et al., 2005). Overproduction of NO in cNOS plants is likely to result in increased cellular SNO levels via N_2O_3 formation (Foster et al., 2003), and this suggests that disease resistance might be impaired by similar molecular events in cNOS and *atgsnor1* mutant plants.

Loss of local resistance in cNOS1 is associated with a marked attenuation in basal and pathogen-induced expression levels of the glutathione-S-transferase gene *GST1*. *GST1* has been shown to be a reliable marker of ROS production (Levine et al., 1994; Alvarez et al., 1998), indicating that basal ROS levels as well as ROS accumulation after pathogen attack are significantly reduced in cNOS1. Plant NADPH oxidases represent a pivotal source of ROS during development and in response to biotic stress (Torres and Dangl, 2005). They catalyse the reduction of molecular oxygen to superoxide, which in turn dismutates to hydrogen peroxide by action of superoxide dismutase (Alscher et al., 2002). Alternatively, O_2^- can react with NO in a fast, diffusion-limited reaction to produce peroxynitrite (Huie and Padmaja, 1993). This suggests that an excess of NO in cNOS1 plants is able to reduce basal and pathogen-induced ROS levels by scavenging O_2^-, and this would consequently suppress ROS-mediated signaling and defence responses, which might finally contribute to the observed attenuated disease resistance response (Fig. 3). Comparable antioxidant capacities of NO have been described in several studies. For instance, NO donors protect from oxidative damage caused by methylviologen herbicides and counteract programmed cell death in barley aleurone layers (Beligni and Lamattina, 1999; Beligni et al., 2002).

cNOS3 plants exhibit lower NO emission rates than cNOS1 (Fig. 1B). Thus, following the above reasoning, the capacity to suppress ROS-induced responses should be lower in cNOS3 than in cNOS1. This assumption is consistent with the observed *GST1* expression patterns: basal *GST1* expression, resulting from relatively low constitutive or developmental ROS production, was reduced in cNOS3, yet *GST1* expression in response to *Pst avrRpm1*, arising from the massive ROS accumulation during the oxidative burst, was not substantially affected. Consequently, ROS-induced defence pathways might still be functional in cNOS3 but suffer severe attenuation in cNOS1. The different capabilities to initiate ROS-induced defences might in turn account for the finding that disease resistance towards *Pst avrRpm1* is significantly higher in cNOS3 than in cNOS1 (Fig. 3B).

Besides affecting the oxidative burst, expression of *deiNOS* did not substantially influence a number of other defence reactions characteristic for the incompatible Arabidopsis-Pseudomonas interaction. These responses include production of the defence signals salicylic acid and jasmonic acid, up-regulation of SA- and JA-inducible PR genes, accumulation of the phytoalexin camalexin, and induction of phenylpropanoid pathway genes. However, a strikingly consistent observation regarding both cNOS1 and cNOS3 represents the reduced basal and pathogen-induced expression of *PR-2* and *PR-5*. Expression of both PR-genes is not regulated by SA- and JA-signaling (Nawrath and Métraux, 1999). This indicates that *deiNOS* expression affects specific defence signaling pathways, i.e. at least one SA- and JA-independent pathway, rather than attenuating the plants defence capacity in a non-specific way. *PR-2* bears sequence homology to β-1,3-glucanases, whereas *PR-5* encodes for a thaumatin-like protein. Both PR proteins have antimicrobial potential, are secreted to the extracellular space and are closely associated with local and systemic acquired resistance (Uknes et al., 1992). Attenuated expression of a subset of PR genes including *PR-2* and *PR-5* is thus likely to contribute to the enhanced disease susceptibility in cNOS plants.

Nitric oxide has been implicated in the execution of the plant hypersensitive cell death response (Delledonne et al., 1998). Pharmacological experiments have established that NO acts in combination with hydrogen peroxide to trigger the HR in suspension cultured soybean cells challenged with avirulent bacteria. A balance model has been proposed in which a fine-tuned ratio between ROS and NO is a prerequisite for the capability of cells to undergo the HR (Delledonne et al., 2001). However, experiments using a tobacco nitrite reductase antisense-line (‘line 271’, Morot-Gaudry-Talarmain et al., 2002), which exhibits a 100-fold higher NO production than Wt plants, have demonstrated that a large excess of NO did not interfere with the cryptogein-mediated HR in tobacco (Planchet et al., 2006). In the present work, we

observed a comparable development of microscopic HR lesions in cNOS1, cNOS3 and Wt plants (Fig. 8). Although overproduction of NO compared with the respective Wt was not that extensive in any of the cNOS lines than in tobacco line 271, our data indicate that microscopic lesion development during the HR is quite robust towards varying NO levels in Arabidopsis. In a previous transgenic approach, we have expressed a NO degrading dioxygenase (NOD) in Arabidopsis, a flavohemoglobin that markedly reduced plant NO levels (Zeier et al., 2004a). Compared with Wt plants, a small reduction in the number of microscopic HR lesions was observed in NOD plants after challenge with avirulent *P. syringae*. Thus, moderate modulations of endogenous NO levels in Arabidopsis do only marginally affect the formation of microscopic HR lesions. Having said that, the absence of typically dry HR lesions in cNOS at the macroscopic level suggests that, compared with Wt, avirulent bacteria encounter a different plant environment during the course of the HR in cNOS (Fig. 1C, D).

The present data also demonstrates that Arabidopsis expressing *deiNOS* exhibit an attenuated systemic acquired resistance response. During the establishment of SAR, a feedback loop in uninfected, systemic tissue, which amplifies an incoming long-distance signal generated at the site of pathogen attack, has been proposed (Mishina and Zeier, 2006). ROS, salicylic acid, and the flavin-dependent monooxygenase FMO1 represent central constituents of this defence amplification loop (Alvarez et al., 1998; Shirasu et al., 1997; Mishina and Zeier, 2006), and removal of one of these components is likely to abolish SAR. Like several SAR-defective defence mutants (Mishina and Zeier, 2006), cNOS plants fail to systemically express *FMO1*. As *FMO1* has been shown to be up-regulated by superoxide (Olszak et al., 2006), we propose that scavenging of O_2^- by NO could be casual for the lack of systemic SA and *FMO1* transcript accumulation, and finally for the attenuated SAR response in cNOS.

In summary, we show that transgenic Arabidopsis plants exhibiting enhanced endogenous NO production are impaired in mobilizing specific defence pathways, which results in attenuated local and systemic disease resistance. Our data remarkably parallel recent findings that Arabidopsis *atgsnor1* mutants, which possess elevated S-nitrosothiol contents, are compromised in basal, specific, and non-host resistance (Feechan et al., 2005). These results indicate that elevated NO levels or metabolic changes associated therewith (e.g. SNO formation) are able to negatively influence plant defence responses. Considering the existence of numerous reports implicating a positive regulatory role of NO in disease resistance, nitric oxide obviously has the potential to act as a double-edged sword, promoting plant defence responses in some situations, and compromising them in others. A similar double-faced behaviour of NO is known in animal biology (Cirino et al., 2002), and whether NO serves as a

defence promoting or suppressing agent in plants might dependent on quantitative, temporal and spatial aspects of its production.

ACKNOWLEDGMENTS

We thank the Deutsche Forschungsgemeinschaft (SFB 567) for financial support.

LITERATURE CITED

Adak S, Bilwes AM, Panda K, Hosfield D, Aulak KS, McDonald JF, Tainer JA, Getzoff ED, Crane BR, Stuehr DJ. Cloning, expression, and characterization of a nitric oxide synthase protein from *Deinococcus radiodurans*. Proc Natl Acad Sci USA 2002; 99:107-12.

Alscher RG, Erturk N, Heath LS. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 2002; 53:1331-41.

Alvarez ME, Pennell RI, Meijer, P-J, Ishikawa A, Dixon RA, Lamb C. Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell 1998; 92:773-84.

Balcerczyk A, Soszynski M, Bartosz G. On the specificity of 4-amino-5-methylamino-2',7'-difluorofluorescein as a probe for nitric oxide. Free Radic Biol Med 2005; 39:327-35.

Beligni MV, Lamattina L. Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants. Nitric Oxide 1999; 3:199-208.

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL. Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. Plant Physiol 2002; 129:1642-50.

Bethke PC, Libourel IG, Reinohl V, Jones RL. Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. Planta 2006; 223:805-12.

Bisgrove SR, Simonich MT, Smith NM, Sattler A, Innes, RW. A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. Plant Cell 1994; 6:927-33.

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell 1997; 9:1573-84.

Cameron RK, Dixon RA, Lamb CJ. Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J 1994; 5:715-25.

Cirino G, Santagada V, Caliendo G. Nitric oxide related therapeutic phenomenon: a challenging task. Curr Pharm Des 2002; 8:233-39.

Clarke A, Desikan R, Hurst RD, Hancock JT, Neill SJ. NO way back: nitric oxide and programmed cell death in *Arabidopsis thaliana* suspension cultures. Plant J 2000; 24:667-78.

Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 1998; 16:735-43.

Conrath U. Non-invasive online detection of nitric oxide from plants and some other organisms by mass spectrometry. Plant J 2004; 38:1015-22.

Crawford NM. Mechanisms for nitric oxide synthesis in plants. J Exp Bot 2006; 57:471-78

Crawford NM, Galli M, Tischner R, Heimer YM, Okamoto M, Mack A. Response to Zemojtel et al: Plant nitric oxide synthase: back to square one. Trends Plant Sci 2006; 11:526-27.

Delledonne M, Xia Y, Dixon RA, Lamb C. Nitric oxide functions as a signal in plant disease resistance. Nature 1998; 394:585-88.

Delledonne M, Zeier J, Marocco A, Lamb, C. Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive response. Proc Natl Acad Sci USA 2001; 98:13454-59.

Delledonne M. NO news is good news for plants. Curr Opin Plant Biol 2005; 8:390-96.

Durner J, Wendehenne D, Klessig DF. Defence gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci USA 1998; 95:10328-33.

Feechan A, Kwon E-J, Yun B-W, Wang Y, Pallas, JA, Loake GJ. A central role for S-nitrosothiols in plant disease resistance. Proc Natl Acad Sci USA 2005; 102:8054-59.

Foissner I, Wendehenne D, Langebartels C, Durner J. *In vivo* imaging of an elicitor-induced nitric oxide burst in tobacco. Plant J 2000; 23:1-10.

Foster MW, McMahon TJ, Stamler JS. S-nitrosylation in health and disease. Trends Mol Med 2003; 9:160-8.

Guo F-Q, Okamoto M, Crawford NM. Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science 2003; 302:100-3.

Hajdukiewicz P, Svab Z, Maliga P. The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol 1994; 25:989-94.

Huang X, von Rad U, Durner J. Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in Arabidopsis suspension cells. Planta 2002a; 215:914-23.

Huang X, Kiefer E, von Rad U, Ernst D, Foissner I, Durner J. Nitric oxide burst and nitric oxide-dependent gene induction in plants. Plant Physiol Biochem 2002b; 40:625-31.

Huie RE, Padmaja S. The reaction of NO with superoxide. Free Radic Res Commun 1993; 18:195-199.

Jarvis P, Chen L-J, Li H-m, Peto CA, Fankhauser C, Chory J. An Arabidopsis mutant defective in the plastid general protein import apparatus. Science 1998; 282:100-3.

Keen NT, Buzzel RI. New disease resistance genes in soybean against *Pseudomonas syringae* pv. *glycinea*: Evidence that one of them interacts with a bacterial elicitor. Theor Appl Genet 1991; 81:133-38.

Kohashi M. Isolation of six unconjugated pteridines from soybean (*Glycine max* L. Tsurunoko) seeds. J Biochem (Tokyo) 1980; 87:1581-86.

Lamb C, Dixon RA. The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 1997; 48:251-75.

Lamotte O, Gould K, Lecourieux D, Sequeira-Legrand A, Lebrun-Garcia A, Durner J, Pugin A, Wendehenne D. Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein. Plant Physiol 2004; 135:516-29.

Levine A, Tenhaken R, Dixon RA, Lamb C. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. Cell 1994; 79:583-93.

Mishina TE, Zeier J. The *Arabidopsis* flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant Physiol 2006; 141:1666-75.

Modolo LV, Cunha FQ, Braga MR, Salgado I. Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp *meridionalis* elicitor. Plant Physiol 2002; 130:1288-97.

Morot-Gaudry-Talarmain Y, Rockel P, Moureaux T, Quillere I, Leydecker MT, Kaiser WM, Morot-Gaudry JF. Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. Planta. 2002; 215(5):708-15

Mur LAJ, Santosa IE, Laarhoven LJJ, Holton NJ, Harren FJM, Smith AR. Laser photoacoustic detection allows *in planta* detection of nitric oxide in tobacco following challenge with avirulent and virulent *Pseudomonas syringae* pathovars. Plant Physiol 2005; 138:1247-58.

Mur LAJ, Carver TLW, Prats E. NO way to live; the various roles of nitric oxide in plant-pathogen interactions. J Exp Bot 2006; 57:489-505.

Murgia I, de Pinto MC, Delledonne M, Soave C, De Gara L. Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. Journal Plant Physiol 2004; 161:777-83.

Nawrath C, Métraux JP. Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell 1999; 11:1393-04.

Nawrath C, Heck S, Parinthawong N, Métraux JP. EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in *Arabidopsis*, is a member of the MATE-transporter family. Plant Cell 2002; 14:275-86.

Noritake T, Kawakita K, Doke N. Nitric oxide induces phytoalexin accumulation in potato tuber tissues. Plant Cell Physiol 1996; 37:113-16.

Olszak B, Malinovsky FG, Brodersen P, Grell M, Giese H, Petersen M, Mundy J. A putative flavin-containing mono-oxygenase as a marker for certain defense and cell death pathways. Plant Sci 2006; 170:614-23.

Pedroso MC, Magalhaes JR, Durzan D. Nitric oxide induces cell death in *Taxus* cells. Plant Sci 2000; 157:173-80.

Planchet E, Sonoda M, Zeier J, Kaiser WM. Nitric oxide (NO) as an intermediate in the cryptogein-induced hypersensitive response - a critical re-appraisal. Plant Cell Environ 2006; 29:59-69.

Planchet E, Kaiser WM. Nitric oxide production in plants. Plant Signal Behav 2006; 1:46-51

Prats E, Mur LAJ, Sanderson R, Carver TLW. Nitric oxide contributes both to papilla-based resistance and the hypersensitive response in barley attacked by *Blumeria graminis* f. sp *hordei*. Mol Plant Pathol 2005; 6:65-78.

Requena ME, Egea-Gilabert C, Candela ME. Nitric oxide generation during the interaction with *Phytophthora capsici* of two *Capsicum annuum* varieties showing different degrees of sensitivity. Physiol Plant 2005; 124:50-60.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM. Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. J Exp Bot 2002; 53:103-10.

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD. Systemic acquired resistance. Plant Cell 1996; 8:1809-19.

Sahr T, Ravanel S, Rébeillé F. Tetrahydrofolate biosynthesis and distribution in higher plants. Biochem Soc Transact 2005; 33:758-62.

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defence mechanisms. Plant Cell 1997; 9:261-70.

Torres MA, Dangl JL. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol 2005; 8:397-03.

Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC. Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *syringae*. Plant Physiol 1992; 98:1304-09.

Uknes S, Mauch-Mani B, Moyer M, Potter S, Williams S, Dincher S, Chandler D, Slusarenko A, Ward E, Ryals J. Acquired resistance in Arabidopsis. Plant Cell 1992; 4:645-56.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM. Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 2001; 414:562-65.

Whalen MC, Innes RW, Bent AF, Staskawicz BJ. Identification of *Pseudomonas syringae* pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. Plant Cell 1991; 3:49-59.

Yu I-c, Parker J, Bent AF. Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proc Natl Acad Sci USA 1998; 95:7819-24.

Zeidler D, Zahringer U, Gerber I, Dubery I, Hartung T, Bors W, Hutzler P, Durner J. Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc Natl Acad Sci USA 2004; 101:15811-16.

Zeier J, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C. Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. Plant Physiol 2004a; 136:2875-86.

Zeier J, Pink B, Mueller MJ, Berger S. Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. Planta 2004b; 219:673-83.

Zemojtel T, Wade RC, Dandekar T. In search of the prototype of nitric oxide synthase. FEBS Lett 2003; 554:1-5.

Zemojtel T, Frohlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, Durner J. Plant nitric oxide synthase: a never-ending story? Trends Plant Sci 2006; 11:524-25.

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J. PAD4 functions upstream from salicylic acid to control defence responses in *Arabidopsis*. Plant Cell 1998; 10:1021-30.

Figure legends

Figure 1. Functional expression of *D. radiodurans deiNOS* in Arabidopsis. (A) Northern blot analysis demonstrating constitutive expression of *deiNOS* in leaf tissue of different cNOS lines. (B) NO-emission from rosette leaves of Wt and different cNOS lines as detected by chemiluminescence. Mean values (± SD) of at least three independent samples are given. Asterisks denote statistically significant differences between Wt and cNOS lines (*: $P < 0.05$, **: $P < 0.005$; Student's t-test).

Figure 2. Representative phenotypes of 5 week old wild-type Col-0 (A) and cNOS1 (B) plants (note the different magnifications). (C) Macroscopic HR of a Wt leaf infiltrated with avirulent *Pst avrRpm1* (OD = 0.005) 5 days after inoculation. (D) Macroscopic symptoms of a cNOS1 leaf treated with *Pst avrRpm1* 5dpi.

Figure 3. Quantification of bacterial growth to assess basal, specific, and non-host resistance of Wt and cNOS plants towards *P. syringae*. (A) Growth of virulent *Pst* in leaves 3 days after inoculation with a bacterial suspension of OD = 0.002. (B) Growth of *Pst avrRpm1* in leaves 3 days post inoculation (OD = 0.005). (C) Numbers of the non-host bacterium *Psg* in leaves 3 days after infiltration of a bacterial suspension (OD = 0.1). Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from 7 parallel samples consisting each of 3 leaf disks. Asterisks denote statistically significant differences between Wt and cNOS (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; Student's t-test).

Figure 4. Accumulation of salicylic acid at sites of pathogen inoculation in Wt, cNOS1, and cNOS3 plants in response to *Pst avrRpm1* and *Pst* (OD = 0.005 for each pathogen). Control samples were treated with 10 mM $MgCl_2$. Leaves were collected 10 h post treatment. (A) Free salicylic acid (SA) levels. (B) Glucosid-bound salicylic acid levels (SAG). Mean values (± SD) of three independent samples are given.

Figure 5. Expression of defence-related genes in leaves of Wt and cNOS plants challenged with avirulent *Pst avrRpm1* (OD = 0.005), as assessed by northern blot analysis. Numbers indicate hours post inoculation (hpi). Control leaves (c) were treated with 10 mM $MgCl_2$ (4h). (A)

Comparison between Wt and cNOS1. (B) Different experiment comparing responses of Wt and cNOS3.

Figure 6. Expression of defence-related genes in leaves of Wt and cNOS plants infected with virulent *Pst* (OD = 0.005), as assessed by northern blot analysis. Numbers indicate hours post inoculation (hpi). Control leaves (c) were treated with 10 mM $MgCl_2$ (4h). (A) Comparison between Wt and cNOS1. (B) Different experiment comparing responses of Wt and cNOS3.

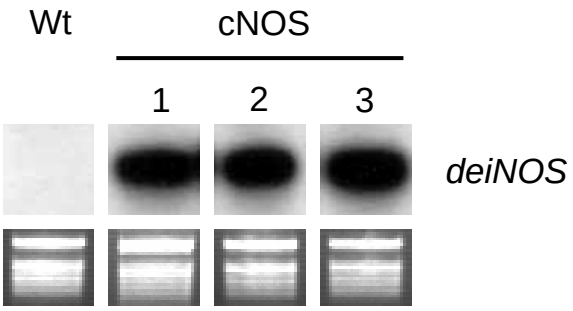
Figure 7. Accumulation of jasmonic acid (JA) and the phytoalexin camalexin in leaves of Wt, cNOS1, or cNOS3 plants inoculated with *Pst avrRpm1* (OD = 0.005). Control plants were infiltrated with 10 mM $MgCl_2$. All samples were collected 10 h after treatment. (A) Jasmonic acid (JA) levels. (B) Camalexin accumulation. Mean values (± SD) of three independent samples are given.

Figure 8. Microscopic cell death after *Pst avrRpm1* inoculation of Wt and cNOS plants. Trypan blue staining was performed 24 h after treatment. (A) Staining patterns of representative leaves of Wt and cNOS1 inside the infiltration zone (100-fold magnification). (B) Percentage of dead cells inside infiltrated areas. Values are shown as the mean ± SD of at least 5 leaf areas from different plants. Dark bars: infiltration with 10 mM $MgCl_2$, dark bars: *Pst avrRpm1*-infiltration.

Figure 9. Systemic acquired resistance (SAR) in Wt and cNOS. (A) Bacterial growth of virulent *Pst* in systemic leaves of pre-treated plants to assess SAR. Plants were infiltrated with $MgCl_2$ or *Pst avrRpm1* (OD = 0.02) in three primary leaves (1°), and two days later, three systemic leaves (2°) located directly above the primary leaves were inoculated with *Pst* (OD = 0.002). Bacterial growth in 2° leaves was assessed three days (3dpi) after *Pst* infection. Bars represent mean values (± SD) of colony forming units (cfu) per square centimetre from 7 parallel samples consisting each of 3 leaf disks. A 'SAR-factor' representing the ratios of mean values from $MgCl_2$- and *PstavrRpm1*-treatments is given for each line. (B-E) Symptoms of leaves (white arrows) challenged with *Pst* during the 2° infection, two days after 1° treatment. (B) Wt plant, 1° treatment: $MgCl_2$. (C) Wt plant, 1° treatment: *Pst avrRpm1*. (D) cNOS1 plant, 1° treatment: $MgCl_2$. (E) cNOS1 plant, 1° treatment: *Pst avrRpm1*.

Figure 10. Systemic defence responses in Wt and cNOS plants. Primary leaves of plants were treated as described in Fig. 9, and untreated, systemic leaves were harvested two days later for analysis. (A) Systemic accumulation of salicylic acid (SA). Bars represent mean values (± SD) of three independent samples. Each sample consisted of six leaves from two different plants. Dark bars: $MgCl_2$-treatment, light bars: *Pst avrRpm1* inoculation. (B) Systemic expression of defence-related genes, assessed by northern blot analysis (m: $MgCl_2$-treatment, i: *Pst avrRpm1* inoculation). (C) Systemic expression of the flavin-dependent monooxygenase gene *FMO1*, as assessed by RT-PCR analysis (m: $MgCl_2$-treatment, i: *Pst avrRpm1* inoculation). 18S ribosomal RNA was amplified for each sample as an internal control.

A


Wt
cNOS
1
2
3
deiNOS

B

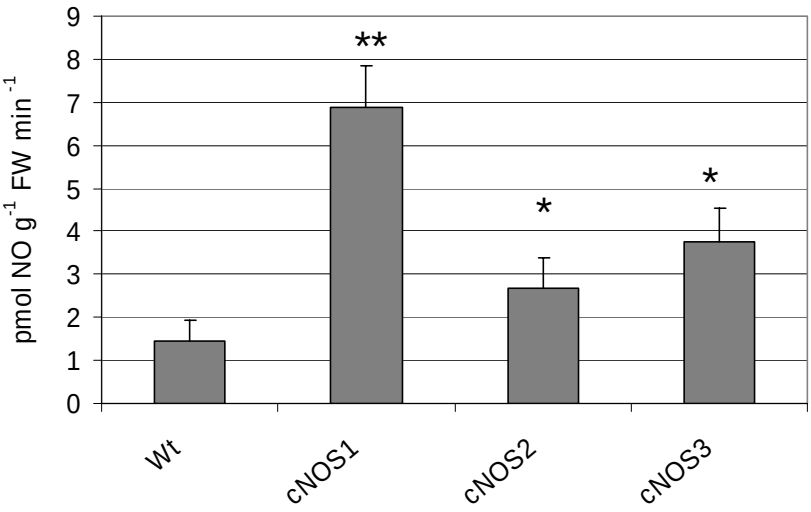

pmol NO g-1 FW min-1
0
1
2
3
4
5
6
7
8
9
**
*
*
Wt
cNOS1
cNOS2
cNOS3

Fig. 1

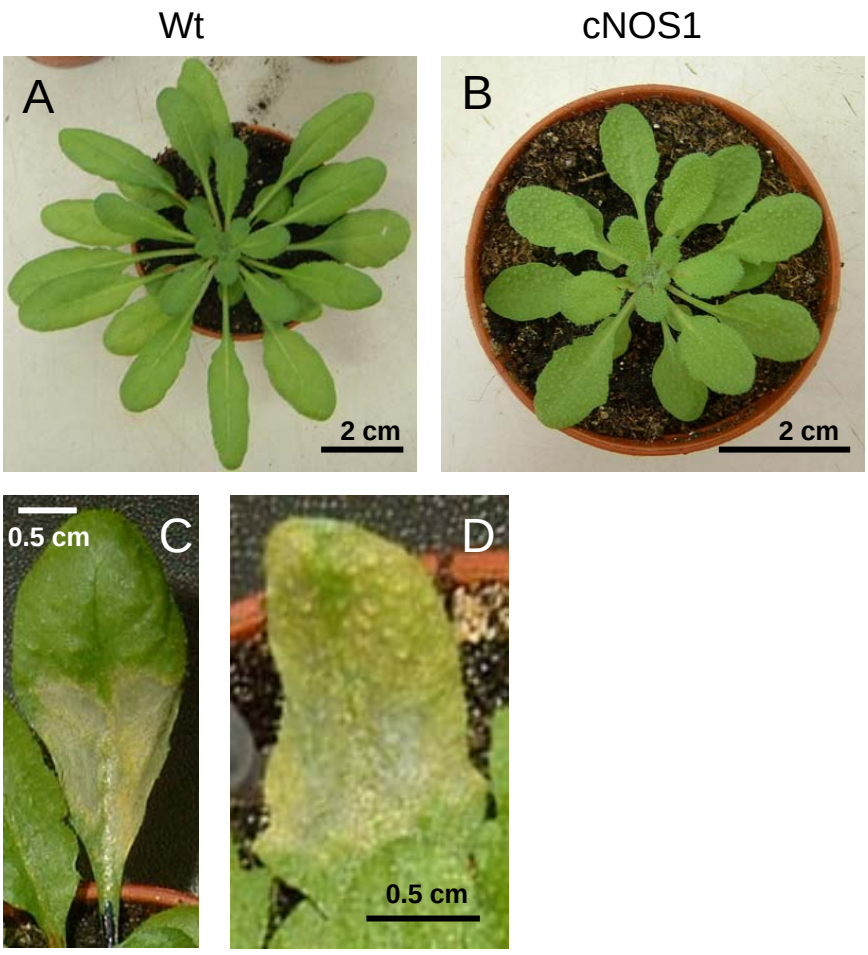


Fig. 2

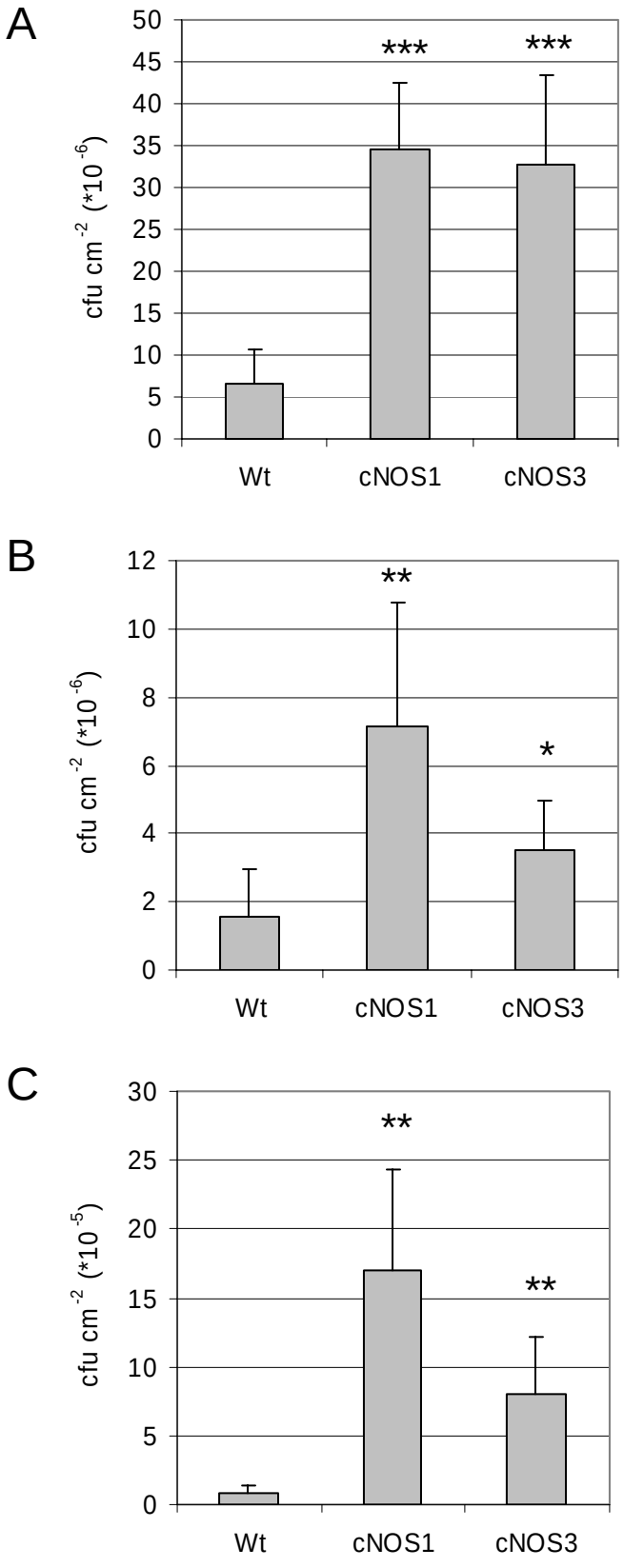

A
50
45
40
35
30
25
20
15
10
5
0
cfu cm-2 (*10-6)
***
***
Wt
cNOS1
cNOS3
B
12
10
8
6
4
2
0
cfu cm-2 (*10-6)
**
*
Wt
cNOS1
cNOS3
C
30
25
20
15
10
5
0
cfu cm-2 (*10-5)
**
**
Wt
cNOS1
cNOS3

Fig. 3

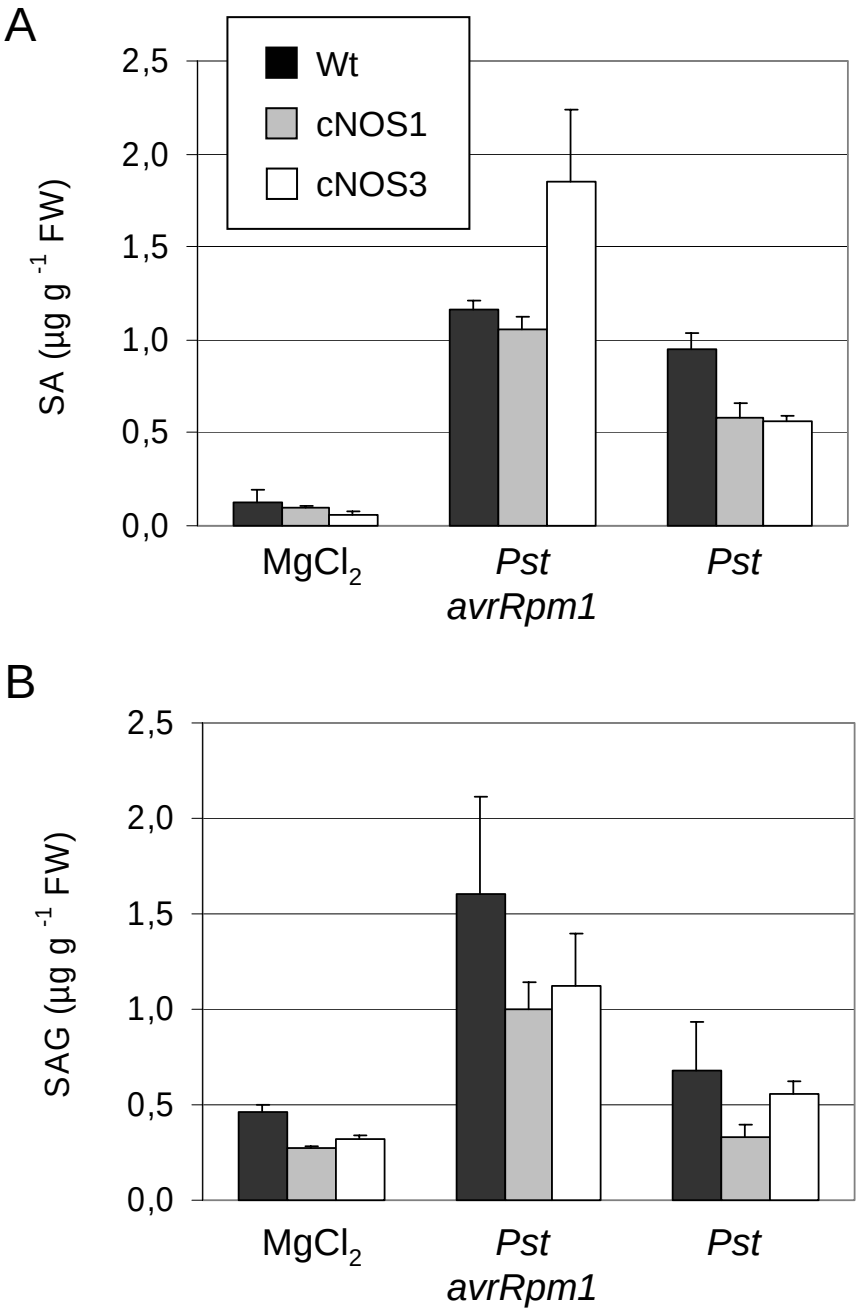

A
Wt
cNOS1
cNOS3
SA (µg g -1 FW)
2,5
2,0
1,5
1,0
0,5
0,0
MgCl2
Pst avrRpm1
Pst
B
SAG (µg g -1 FW)
2,5
2,0
1,5
1,0
0,5
0,0
MgCl2
Pst avrRpm1
Pst

Fig. 4

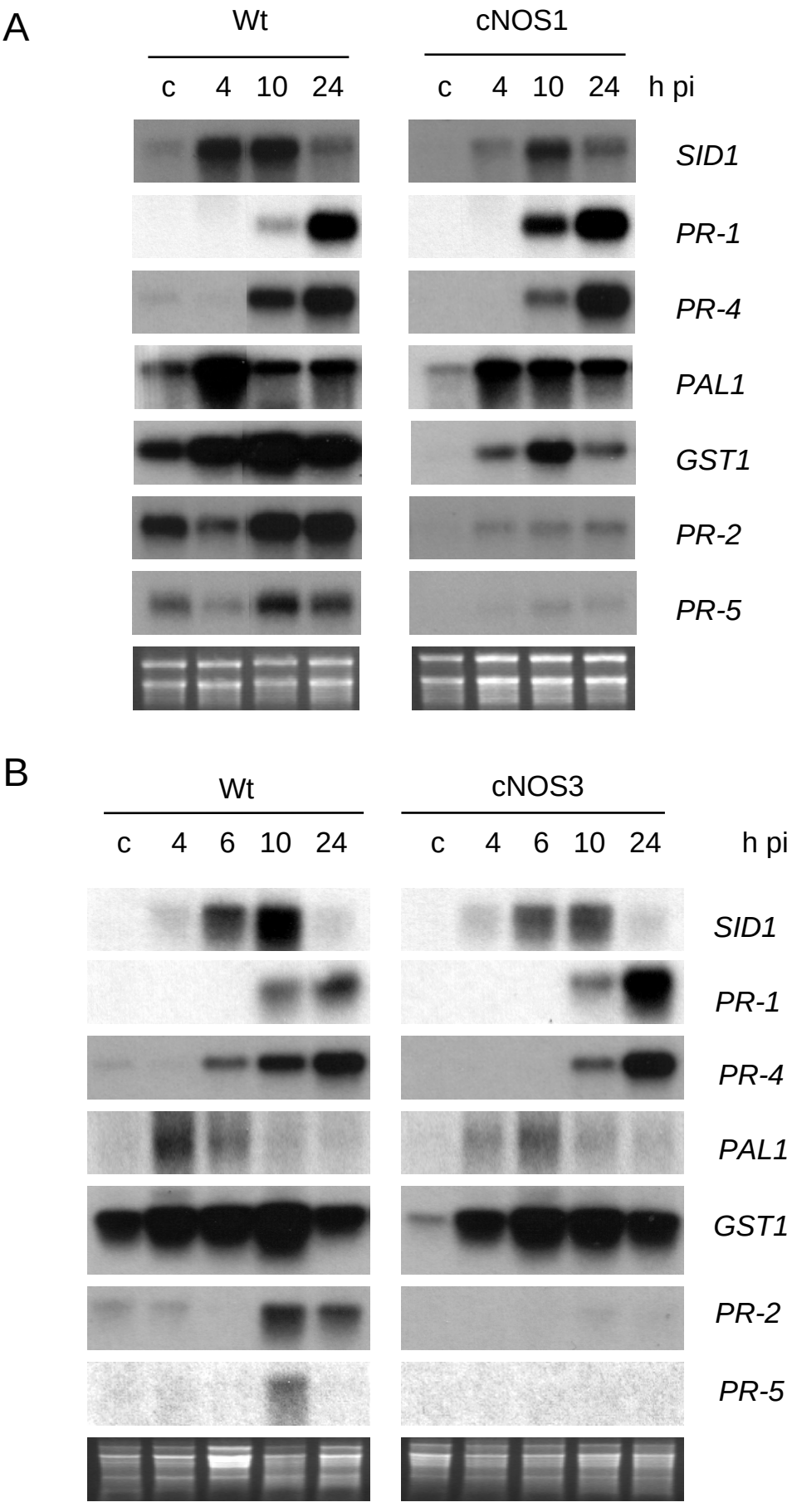

A
Wt
cNOS1
c 4 10 24
c 4 10 24 h pi
SID1
PR-1
PR-4
PAL1
GST1
PR-2
PR-5
B
Wt
cNOS3
c 4 6 10 24
c 4 6 10 24 h pi
SID1
PR-1
PR-4
PAL1
GST1
PR-2
PR-5

Fig. 5

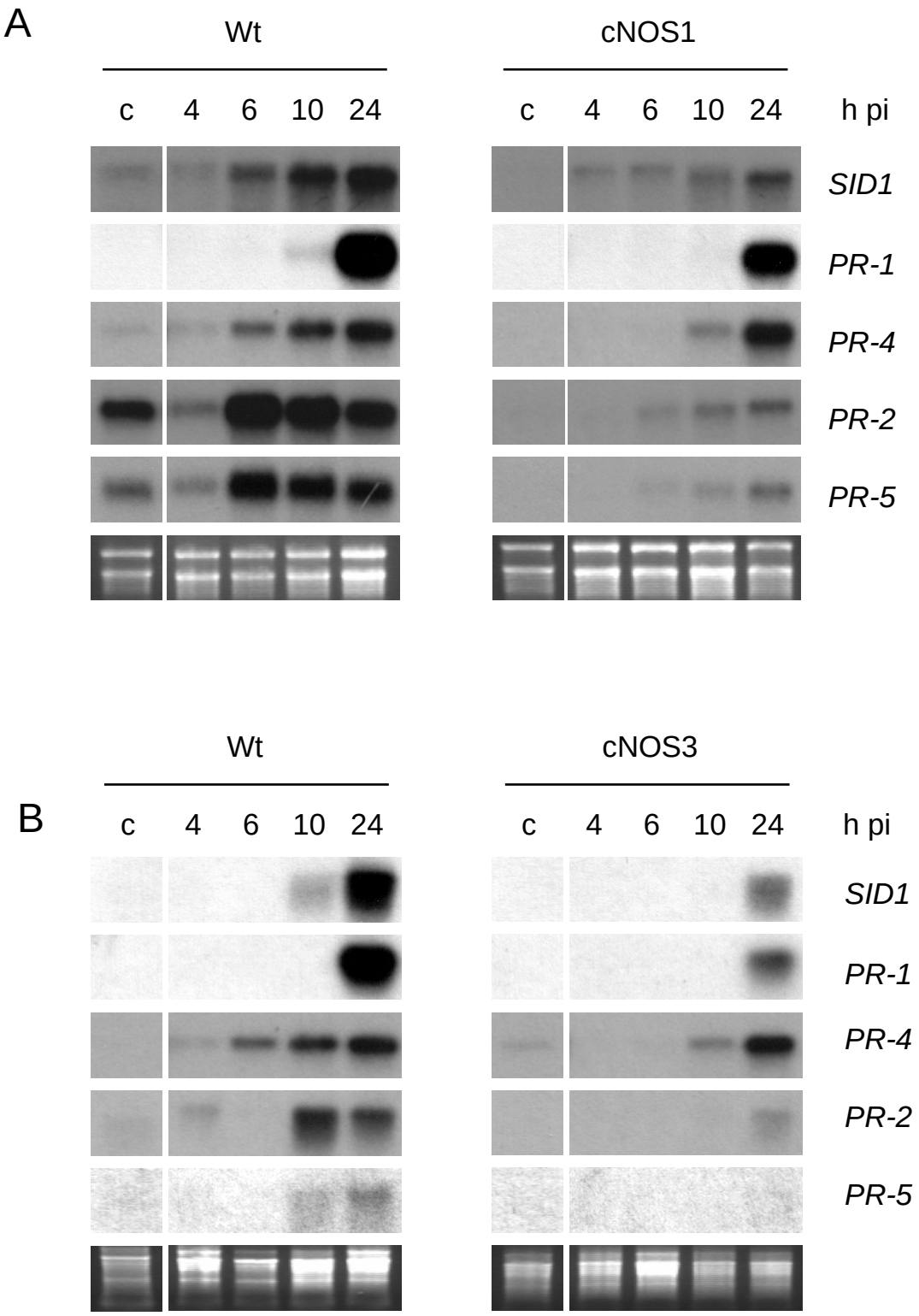
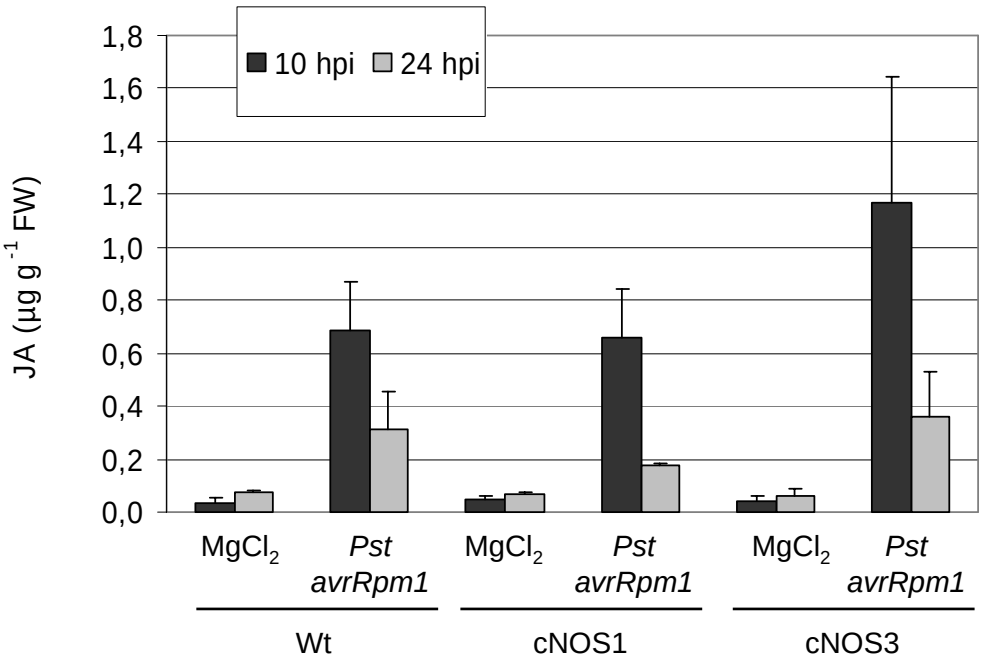

A
Wt
cNOS1
c 4 6 10 24
c 4 6 10 24
h pi
SID1
PR-1
PR-4
PR-2
PR-5
B
Wt
cNOS3
c 4 6 10 24
c 4 6 10 24
h pi
SID1
PR-1
PR-4
PR-2
PR-5

Fig. 6

A


10 hpi
24 hpi
JA (µg g-1 FW)
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
MgCl2
Pst avrRpm1
MgCl2
Pst avrRpm1
MgCl2
Pst avrRpm1
Wt
cNOS1
cNOS3

B

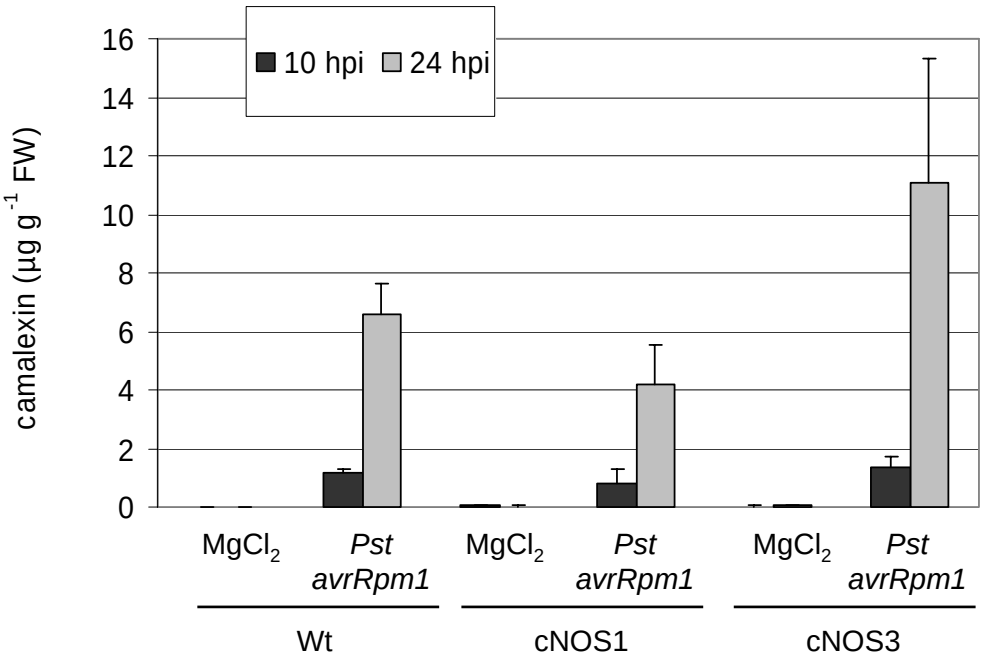
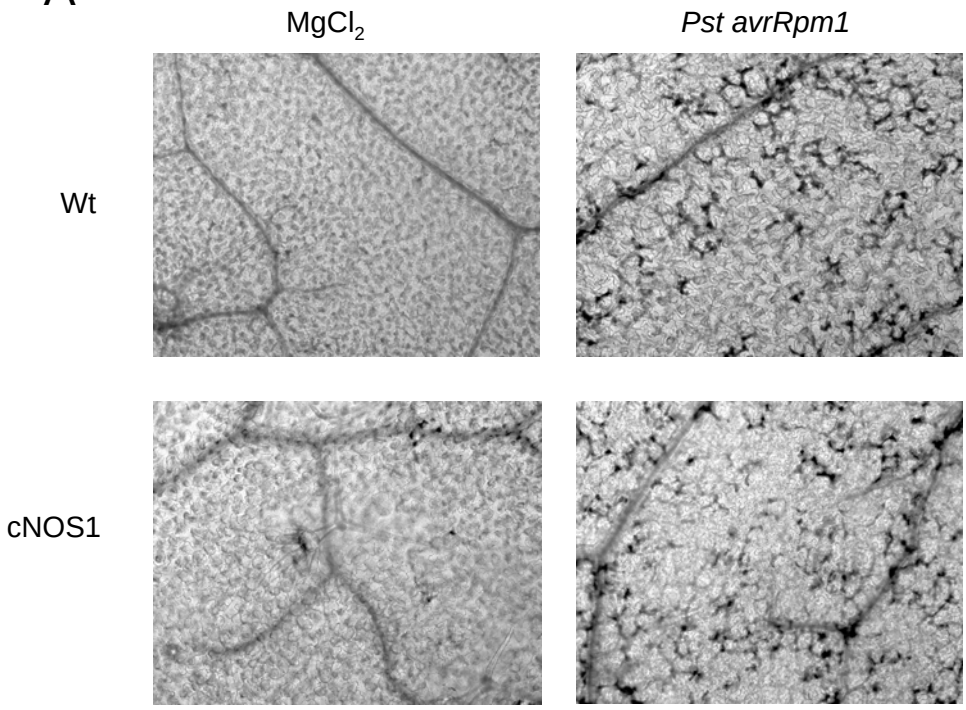

10 hpi
24 hpi
camalexin (µg g-1 FW)
16
14
12
10
8
6
4
2
0
MgCl2
Pst avrRpm1
MgCl2
Pst avrRpm1
MgCl2
Pst avrRpm1
Wt
cNOS1
cNOS3

Fig. 7

A


MgCl2
Pst avrRpm1
Wt
cNOS1

B

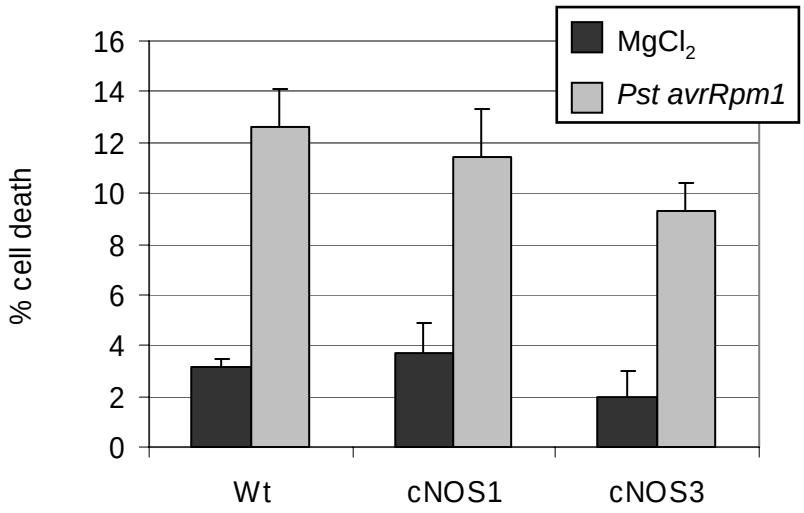

MgCl2
Pst avrRpm1
% cell death
16
14
12
10
8
6
4
2
0
Wt
cNOS1
cNOS3

Fig. 8

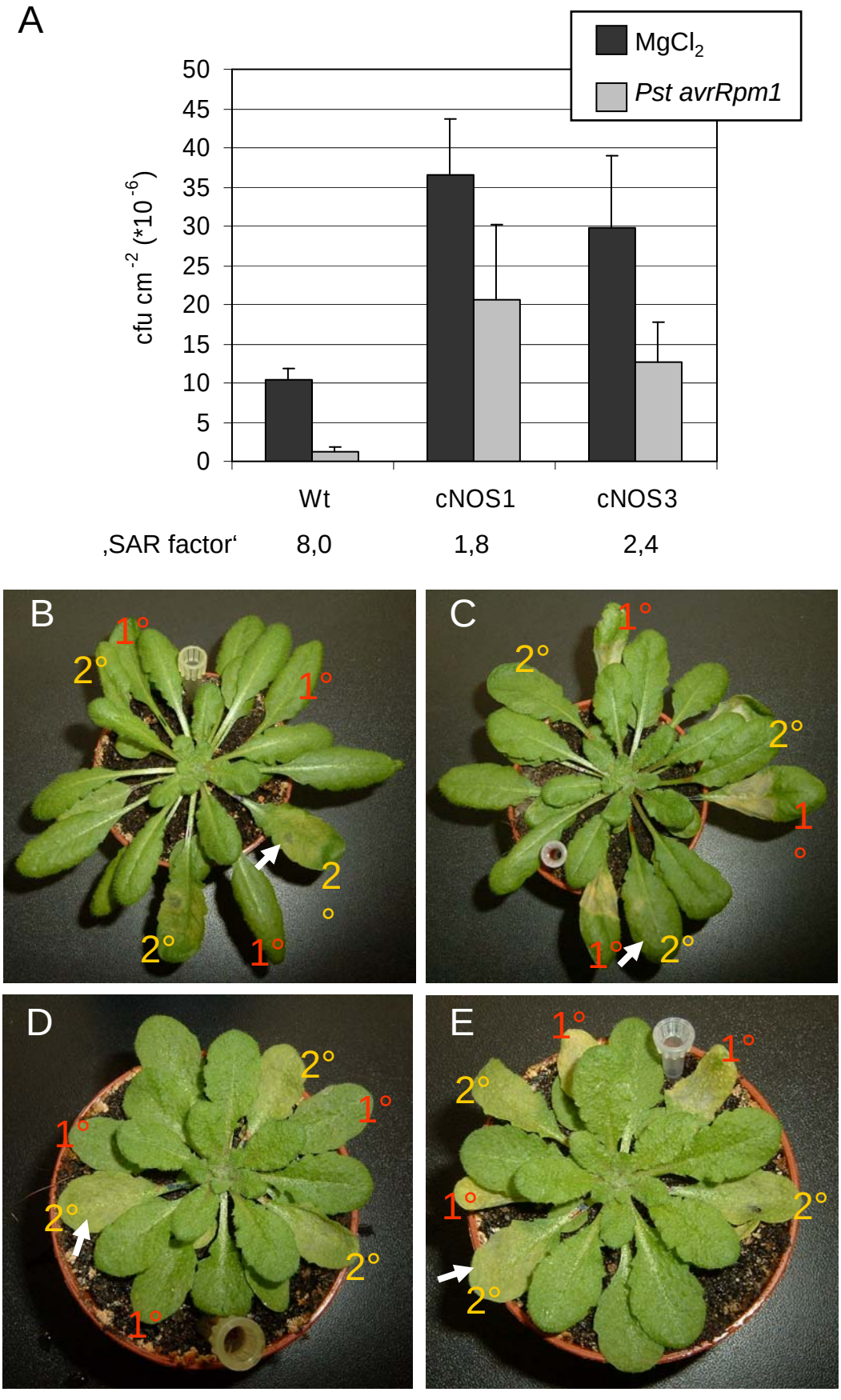

A
MgCl2
Pst avrRpm1
cfu cm-2 (*10-6)
50
45
40
35
30
25
20
15
10
5
0
Wt
cNOS1
cNOS3
‚SAR factor‘
8,0
1,8
2,4
B
C
D
E
1°
2°

Fig. 9

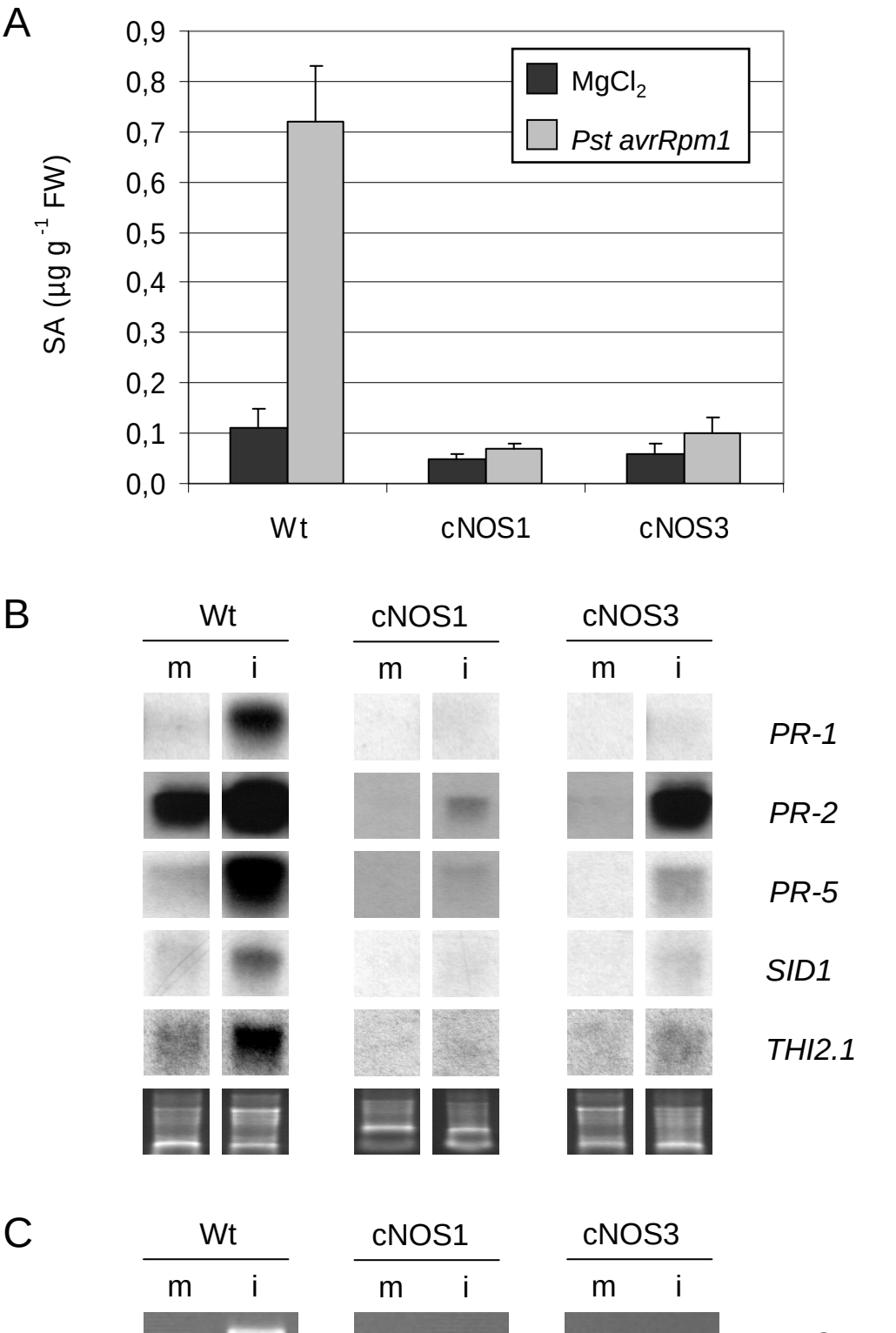

A
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
SA (µg g-1 FW)
MgCl2
Pst avrRpm1
Wt
cNOS1
cNOS3
B
Wt
cNOS1
cNOS3
m
i
PR-1
PR-2
PR-5
SID1
THI2.1
C
FMO1
18S RNA

Fig. 10

PUBLICATION 3

Mishina TE, Lamb C, Zeier J

Expression of a nitric oxide degrading enzyme induces a senescence program in Arabidopsis.

Plant Cell Environ (2007a) 30: 39-52

Plant, Cell and Environment (2007) **30**, 39–52 doi: 10.1111/j.1365-3040.2006.01604.x

Expression of a nitric oxide degrading enzyme induces a senescence programme in *Arabidopsis*

TATIANA E. MISHINA[1], CHRIS LAMB[2] & JÜRGEN ZEIER[1]

[1]*Julius-von-Sachs-Institute of Biological Sciences, Department of Botany II, University of Würzburg, Julius-von-Sachs-Platz 3, D-97082 Würzburg, Germany and* [2]*John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK*

ABSTRACT

Nitric oxide (NO) has been proposed to act as a factor delaying leaf senescence and fruit maturation in plants. Here we show that expression of a NO degrading dioxygenase (NOD) in *Arabidopsis thaliana* initiates a senescence-like phenotype, an effect that proved to be more pronounced in older than in younger leaves. This senescence phenotype was preceded by a massive switch in gene expression in which photosynthetic genes were down-regulated, whereas many senescence-associated genes (SAGs) and the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene *ACS6* involved in ethylene synthesis were up-regulated. External fumigation of NOD plants with NO as well as environmental conditions known to stimulate endogenous NO production attenuated the induced senescence programme. For instance, both high light conditions and nitrate feeding reduced the senescence phenotype and attenuated the down-regulation of photosynthetic genes as well as the up-regulation of SAGs. Treatment of plants with the cytokinin 6-benzylaminopurin (BAP) reduced the down-regulation of photosynthesis, although it had no consistent effect on SAG expression. Metabolic changes during NOD-induced senescence comprehended increases in salicylic acid (SA) levels, accumulation of the phytoalexin camalexin and elevation of leaf γ-tocopherol contents, all of which occurred during natural senescence in *Arabidopsis* leaves as well. Moreover, NO fumigation delayed the senescence process induced by darkening individual *Arabidopsis* Columbia-0 (Col-0) leaves. Our data thus support the notion that NO acts as a negative regulator of leaf senescence.

Key-words: camalexin; cytokinin; high light; nitrate; nitric oxide dioxygenase; salicylic acid; senescence-associated genes; γ–tocopherol.

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; BAP, 6-benzylaminopurin; CAB, chlorophyll *a* and *b* binding protein; DAF-2DA, 4,5-diaminofluorescein diacetate; DEX, dexamethasone; GC/MS, gas chromatography/mass spectrometry; GST, glutathione-S-transferase; IAN, indoleacetonitrile; NO, nitric oxide; NOD, nitric oxide dioxygenase; NOS, nitric oxide synthase; NR, nitrate reductase; PCR, polymerase chain reaction; PPFD, photosynthetic photon flux density; ppm, parts per million; PR, pathogenesis related; RBCS, small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; ROS, reactive oxygen species; SA, salicylic acid; SAG, senescence-associated gene; WT, wild type.

Correspondence: J. Zeier. Fax: +49 931 888 6235; e-mail: zeier@botanik.uni-wuerzburg.de

INTRODUCTION

Leaf senescence, the final stage of leaf development, constitutes a coordinated degeneration programme during which nutrients are mobilized from the senescing leaf to other plant parts (Buchanan-Wollaston 1997). The senescence process is characterized by a sharp decline in photosynthetic capacity, chlorophyll degradation, visible leaf yellowing and a decrease in total RNA and protein contents. Moreover, leaf cells undergo various changes in cell structure, metabolism and gene expression (Nooden, Guiamet & John 1997; Zimmermann & Zentgraf 2005). Many genes called SAGs are up-regulated during senescence, whereas photosynthetic genes are actively down-regulated during this process (Lohman *et al*. 1994; Buchanan-Wollaston 1997; Gepstein *et al*. 2003).

Leaf senescence is controlled by various internal and external factors including leaf age, light conditions, nutrient supply and environmental stress (Smart 1994). Plants may integrate these different factors through endogenous signalling molecules in order to decide whether the senescence process should be executed or not. The plant hormones ethylene and cytokinin have been recognized to influence plant senescence, the former as a senescence-promoting factor, and the latter as a senescence-delaying factor (Gan & Amasino 1995; Grbic & Bleecker 1995). More recently, NO, a mediator of various plant developmental and (patho) physiological processes (Neill, Desikan & Hancock 2003; Crawford & Guo 2005; Mur, Carver & Prats 2006), has been implicated in plant senescence and maturation. For instance, the temporal progress of fruit maturation and floral senescence is associated with a significant decrease in NO emission, and application of NO donating compounds retards flower senescence and extends the post-harvest life of fruits and vegetables (Leshem, Wills & Ku 1998). Similarly, NO emission from *Arabidopsis* plants decreases significantly when plants mature and leaves start to senesce (Magalhaes, Monte & Durzan 2000). In addition, exogenous

NO counteracts the promotion of leaf senescence caused by ABA and methyl jasmonate in rice (Hung & Kao 2003, 2004).

To date, two major biochemical means of NO production have been identified in plants. On one hand, NO can be produced through enzymatic or non-enzymatic reduction from nitrite (Stöhr *et al*. 2001; Rockel *et al*. 2002; Bethke, Badger & Jones 2004). In fact, a major source of NO in plants originates from nitrite mediated by the action of nitrate reductase (NR; Yamasaki & Sakihama 2000; Kaiser *et al*. 2002). Interestingly, several factors, among them cytokinin, light and nitrate treatment, do simultaneously stimulate the expression or activity of NR (Crawford 1995; Yu, Sukumaran & Márton 1998), enhance the *in planta* production of NO (Magalhaes *et al*. 2000; Tun, Holk & Scherer 2001; Planchet *et al*. 2006) and retard the progress of plant senescence (Smart 1994). On the other hand, NO can be generated by NOS from L-arginine, and a corresponding plant NOS gene (*AtNOS1*) has been cloned and characterized in *Arabidopsis* (Guo, Okamoto & Crawford 2003). The AtNOS1 protein is localized to mitochondria, and is involved in ABA-induced stomatal closure, the control of flowering and defense responses towards bacterial lipopolysaccharide elicitors (Guo *et al*. 2003; He *et al*. 2004; Zeidler *et al*. 2004; Guo & Crawford 2005). Moreover, NOS activity appears to represent one enzymatic means to influence plant senescence, as dark-induced leaf senescence occurs more rapidly in *Atnos1* knockout mutants compared with WT plants (Guo & Crawford 2005). In addition, a NOS-like activity of pea peroxisomes is down-regulated during the senescence process in pea leaves (Corpas *et al*. 2004).

In an attempt to study the (patho)physiological effects of reduced endogenous NO levels in plants, we recently expressed a bacterial NOD under the control of an inducible promoter in *Arabidopsis* (Zeier *et al*. 2004a). We observed that the resulting NO-deficient plants undergo a senescence-like process several days after activation of the NOD. Here we show that this NOD-induced senescence process shares many similarities with natural senescence at the molecular level, and report effects of exogenous NO treatment on the progression of NOD- and dark-induced senescences. Our results support the hypothesis that NO acts as a negative regulator of leaf senescence.

MATERIALS AND METHODS

Plant material, plant growth and NOD induction

Arabidopsis plants expressing the bacterial flavohemoglobin Hmp [Columbia-0 (Col-0) background] were generated by *Agrobacterium*-mediated plant transformation using the DEX-inducible expression vector pTA7001 (Aoyama & Chua 1997) as described in Zeier *et al*. (2004a). WT Col-0 plants originated from the Nottingham Arabidopsis Stock Centre (NASC). Plants were grown on a mixture of soil (Fruhstorfer Pflanzenerde; Archut, Lauterbach-Wallenrod Germany), vermiculite and sand (9:1:1) at 22 °C under a 9 h light/15 h dark cycle and a relative humidity of 70%. The photon flux density of the day period was 70 μmol m^{-2} s^{-1} provided by cool-white fluorescent bulbs (F32T8; Percival Scientific, Boone, IA). Growth temperatures were set to 22 °C during the day period and to 18 °C during the night. For NOD induction, the Hmp plants were sprayed with a solution of 3 μM DEX in 0.01% Tween 20. Control samples were obtained by spraying WT Col-0 or homozygous transgenic lines harbouring the empty vector pTA7001 with the same DEX solution, or by treating Hmp plants solely with 0.01% Tween 20. If not otherwise stated, 5–6-week-old plants were used for experiments. Leaves were numbered in ascending order according to their time of appearance, and leaves 8–10 were defined as 'old', whereas leaves 16–18 were designated as 'young'. All experiments were conducted at least twice with similar results.

Nitrate, high light and cytokinin treatments

Nitrate feeding experiments were performed by watering plants with a 50 mM KNO_3 solution instead of using water for control plants. Plants were watered 3 d before and 2 d after DEX treatment. For high light illumination, the photon flux density was adjusted to 500 μmol m^{-2} s^{-1} after DEX treatment of plants, and plants exposed to the usual illumination of 70 μmol m^{-2} s^{-1} served as controls. Cytokinin treatment was performed by spraying rosettes with a solution of 50 μM BAP in 0.01% Tween 20 both 4 h before and 2 d after DEX treatment. One experimental set-up consisted of at least 5 plants per treatment, and representative phenotypes of each case were depicted in the figures.

Natural and dark-induced senescence

For the examination of natural senescence, 10-week-old Col-0 plants, which generally contained several senescing leaves, were taken for experiments. As described in Fig. 8, selected leaves were grouped into three categories: non-senescent (NS), early senescent (S2, 10–20% of the leaf area exhibits visible leaf yellowing) and late senescent (S4, 50–80% of the leaf area exhibits visible leaf yellowing; Lohman *et al*. 1994).

For experiments considering dark-induced senescence, 8-week-old Col-0 plants harbouring in sum 40–45 leaves were examined. Older, attached rosette leaves (leaves 10–12) initially without any visible signs of senescence were individually darkened by coverage with a black, appropriate-sized piece of paperboard. Leaves were further examined 10 d after continuous darkening.

NO fumigation

NO fumigation was performed by continuously flushing plants situated in a glass chamber with diluted NO gas (4 ppm NO in air). A NO/nitrogen mixture (1% NO in nitrogen 5.0; Messer Griesheim, Darmstadt, Germany) was used as a source gas. The final flow rate composition was adjusted using two separate mass flow controllers (model F-201C-FA-11-V; Bronkhorst, Ruurlo, the Netherlands) for both the NO/nitrogen gas mixture and compartment air, as well as an electronic control system (E-7000, Bronkhorst).

Plants situated in a second glass chamber flushed with pure air were used as controls. The overall flow rate constituted 1 mL min^{-1} in both cases.

RNA analysis and determination of gene expression

Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (PeqLab, Erlangen, Germany) following the manufacturer's instructions. For each sample, two leaves from different plants of the same treatment were used. One microgram of total RNA was loaded on formaldehyde-agarose gels, separated by electrophoresis and blotted to nylon membranes (Hybond-N, Amersham, Freiberg, Germany). RNA blot hybridization was performed with specific, ^{32}P-labelled DNA probes generated by PCR using appropriate oligonucleotide primers. The probes represented the following *Arabidopsis* genes: *CAB* (*Arabidopsis* annotation At3g54890), *RBCS* (At5g38410), *RNS2* (At2g39780), *SAG12* (At5g45890), *SAG13* (At2g29350), *SAG14* (At5g20230), *SAG15* (At5g51070), *SAG20* (At3g10980), *SAG21* (At4g02380), *CCH* (At3g56240), *ACS6* (At4g11280), *PR-1* (At2g14610), *GST1* (At1g02930). Each northern blot analysis was repeated at least twice with similar results.

Determination of metabolite levels

The determination of SA, γ-tocopherol, camalexin and IAN levels in leaves was performed by a modified vapour-phase extraction method (Schmelz *et al*. 2004).

Briefly, 150 mg frozen leaf tissue was homogenized with 600 μL of extraction buffer (H_2O : 1-propanole : HCl = 1.0:2.0:0.005). After addition of internal standards (D_4-salicylic acid, dihydrojasmonic acid, indolepropionic acid; 100 ng each) and 1 mL of methylene chloride, the mixture was shaken thoroughly and centrifuged at 20 500 g for phase separation. The lower, organic phase was then removed, dried over Na_2SO_4 and treated with 2 μL of 2 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich, Taufkirchen, Germany) for 5 min at room temperature to convert carboxylic acids into their corresponding methyl esters. After stopping the methylation reaction with 2 M acetic acid in hexane, the sample was subjected to a vapor phase extraction procedure (Schmelz *et al*. 2004) using a volatile collector trap packed with Super-Q absorbent (VCT-1/4X3-SPQ; Analytical Research Systems, FL, USA). The final evaporation temperature was set to 220 °C, and samples were eluted from the collector trap with 1 ml methylene chloride. Finally, the sample volume was reduced to 50 μL in a stream of nitrogen, and the sample was subjected to GC/MS analysis.

For determination of SA glucoside levels, the upper, aqueous phase resulting from the centrifugation step previously described was supplemented with 100 ng D_4-salicylic acid for internal standardization, and was heated to 100 °C for 30 min to convert the SA glucoside to free SA. After cooling, the aqueous solution was extracted three times with 2 mL cyclohexane/ethyl acetate (1:1), and the combined organic extracts were dried over Na_2SO_4. After removal of the organic solvent under a stream of nitrogen, the residue was dissolved in 400 μL of methylene chloride/methanol (3:1), methylated and subjected to vapor phase extraction as previously described.

The sample mixture (2 μL) was separated on a gas chromatograph (GC 6890 N; Agilent Technologies, Waldbronn, Germany) equipped with fused silica capillary column (DB-1; Fisons, Folsom, CA, USA) and combined with a 5975 mass spectrometric detector (Agilent Technologies). For quantitative determination of metabolites, peaks originating from selected ion chromatograms were integrated. The area of a substance peak was related to the peak area of the corresponding internal standard (SA–D_4-salicylic acid; jasmonic acid–dihydrojasmonic acid, camalexin/γ-tocopherol–indolepropionic acid), and experimentally determined correction factors for each substance/standard pair were considered.

Determination of leaf chlorophyll contents

Frozen *Arabidopsis* leaves were ground to a fine powder in liquid nitrogen and extracted with 80% acetone in 2.5 mM sodium phosphate buffer (pH 7.8). Extracts were centrifuged at 10 500 g at 4 °C for 10 min, and dilutions of the supernatant were used for a photometric chlorophyll assay. Absorbances at 663.6 and 646.6 nm were determined and the chlorophyll content was estimated according to Porra, Thompson & Kriedemann (1989).

RESULTS

In order to study the function of NO in plant–pathogen interactions, we recently generated *Arabidopsis* Col-0 plants expressing the bacterial flavohemoglobin Hmp (Zeier *et al*. 2004a). Hmp functions as a NOD in *Escherichia coli*, converting NO to nitrate by using NAD(P)H and O_2 (Vasudevan *et al*. 1991; Gardner *et al*. 1998; Poole & Hughes 2000). Leaf extracts from transgenic plants overexpressing the Hmp protein degraded NO significantly faster than extracts from control plants. Moreover, *in planta* NO production, as assessed by application of the NO-sensitive fluorescence indicator DAF2-DA, and NO emission from intact plants, as demonstrated by chemiluminescence detection, were reduced in Hmp transgenics compared to control plants (Zeier *et al*. 2004a). These findings demonstrated the functionality of the NOD in transgenic Hmp plants.

Expression of Hmp in plants was realized by the pTA7001 DEX-inducible promoter system (Aoyama & Chua 1997) allowing the production of the NOD protein at defined stages of development. When 5-week-old, short-day-grown plants were treated with DEX, the *HMP* transgene, originally absent in untreated plants, was expressed as early as 5 h after treatment, and transcript levels were evenly expressed for at least 1 week (Fig. 2a). Appearance of the Hmp protein revealed a similar kinetics (Zeier *et al*. 2004a). About 4 d after the beginning of NOD expression, we noticed that

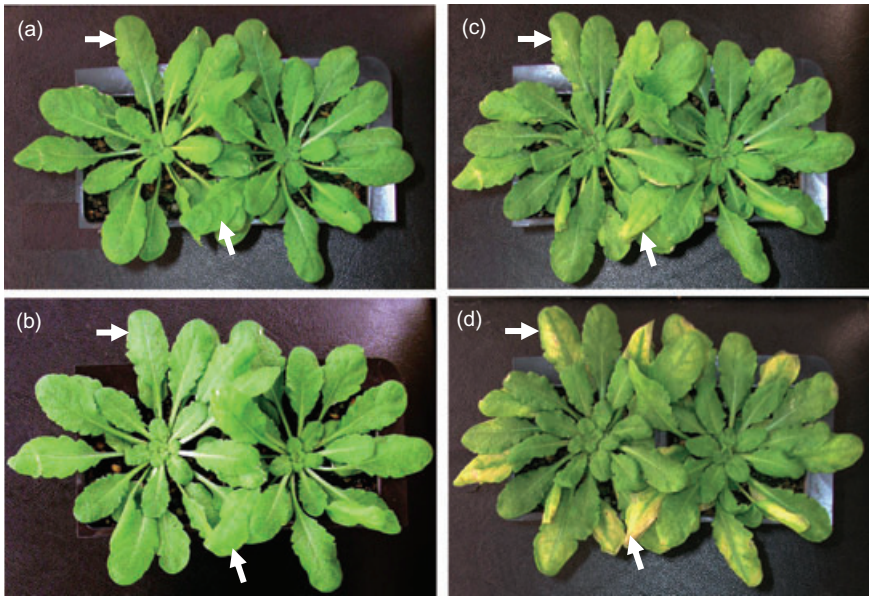


Figure 1. Heterologous expression of the flavohemoglobin Hmp, an *Escherichia coli* NOD, triggers a senescence-like process in *Arabidopsis*. Phenotype of transgenic Hmp plants at days 0 (a), 2 (b), 4 (c) and 6 (d) after induction of the NOD with 3 μM DEX. Arrows exemplarily indicate the development of the same two leaves during the yellowing process.

older leaves exhibited signs of leaf yellowing, a phenotype that was clearly discernible 6 d after transgene induction (Fig. 1a–d). At day 6 after treatment, some younger leaves also started to show visible signs of leaf yellowing, although the effect was markedly less pronounced than in older leaves (Fig. 4a). In contrast to Hmp plants, WT or pTA7001 empty vector plants treated with DEX did not exhibit visible signs of leaf yellowing (Fig. 3 a–c).

Leaf yellowing as a consequence of chlorophyll degradation represents the first visible symptom of senescence (Quirino *et al*. 2000). The yellowing phenotype observed in Hmp plants therefore prompted us to investigate whether the events occurring after NOD induction would bear resemblance to senescence at the molecular level. During natural senescence and senescence induced by artificial treatments, like shading of detached leaves, many photosynthetic genes are actively down-regulated (Lohman *et al*. 1994). When following the expression patterns of the typical photosynthesis-related genes *CAB* and *RBCS* [small subunit of ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco)], we found that both genes were down-regulated at 3 and 4 d after NOD induction, respectively (Fig. 2b). This decline in expression of photosynthetic genes just occurred before visible signs of leaf yellowing were discernible (Fig. 1).

In addition to the down-regulation of photosynthesis, leaf senescence is characterized by an increase in expression of a multitude of genes that are often referred to as SAGs (Buchanan-Wollaston 1997; Gepstein *et al*. 2003). We checked the timing of expression of eight typical SAGs previously used to investigate senescence phenomena in plants at the molecular level (Taylor *et al*. 1993; Lohman *et al*. 1994; Weaver *et al*. 1998; Miller, Arteca & Pell 1999). Expression of seven of these genes, the ribonuclease gene *RNS2*, the senescence-specific cysteine protease gene *SAG12*, the short-chain alcohol dehydrogenase *SAG13*, the blue-copper-binding gene *SAG14*, the *SAG15* or *ERD1* (early responsive to dehydration) gene, the *SAG 20* gene (unknown function) and the *SAG21* gene encoding for a late embryogenesis abundant protein were abruptly enhanced at days 3–4 after induction of the NOD transgene and continued to be strongly expressed during the remaining period of investigation (Fig. 2c). The timing of up-regulation of these SAGs coincided with the timing of down-regulation of the photosynthetic genes *CAB* and *RBCS* (Fig. 2b). The copper chaperone *CCH*, however, was not altered to the same extent as other SAGs during the course of NOD expression. The up-regulation of SAGs as well as the down-regulation of photosynthetic genes, as observed in DEX-treated Hmp plants, did not occur when WT plants or pTA7001 empty vector control plants were treated with DEX (Fig. 3d–f).

Ethylene functions as a senescence-promoting factor and regulates the timing of leaf senescence in *Arabidopsis* (Smart 1994; Grbic & Bleecker 1995). Its production rises when plants start to senesce (Aharoni, Lieberman & Sisler 1979; Magalhaes *et al*. 2000), and expression of the ACC synthase gene *ACS6* involved in ethylene biosynthesis is enhanced during ozone-induced senescence in *Arabidopsis* (Miller *et al*. 1999). In Hmp plants, expression of the *ACS6* gene was up-regulated at 3–4 d after NOD induction with a similar kinetic pattern than most SAGs (Fig. 2d). As certain defense-related genes expressed in response to pathogen attack are up-regulated during leaf senescence (Hanfrey, Fife & Buchanan-Wollaston 1996; Quirino *et al*. 2000), we also followed the expression of two typical defense genes in Hmp plants. Expression of both the *PR-1* gene encoding the PR protein 1 and the GST gene *GST1*, which is up-regulated during the oxidative burst following attack of avirulent bacteria (Levine *et al*. 1994; Zeier *et al*. 2004b), proved to be increased by the same time after NOD induction than *ACS6* and most of the SAGs tested (Fig. 2d). In

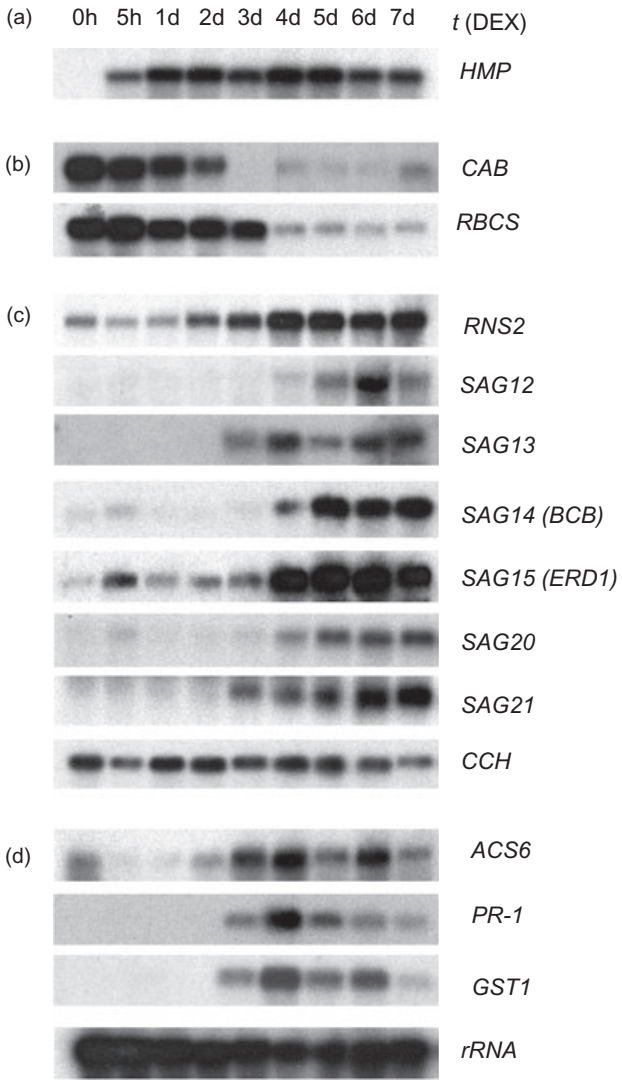


Figure 2. Northern blot analysis illustrating gene expression in leaves of Hmp plants at different times (*t*) after NOD activation with DEX. (a) Accumulation of the *HMP* transgene at indicated times after DEX application. (b) Down-regulation of the photosynthetic genes *CAB* and *RBCS* during the NOD-induced senescence process. (c) Expression patterns of different SAGs at indicated times after NOD induction. (d) Expression patterns of the ACC synthase gene *ACS6* and the defense-related genes *PR-1* and *GST1*. *ERD1*, early responsive to dehydration gene; BCB, blue-copper-binding gene.

contrast to the latter, both *PR-1* and *GST1* expression decreased again at days 6 and 7 after transgene induction, respectively.

As previously stated, visible leaf yellowing in response to NOD expression occurred faster and was more pronounced in old than in young Hmp leaves (Fig. 4a). To further characterize this difference at the molecular level, we compared expression of *SAG12*, *SAG14* and *CAB* in old and young leaves (Fig. 4b,c). *SAG12* expression proved to be more pronounced in older than in younger leaves, especially at days 6 and 7 after NOD induction. In addition, whereas up-regulation of *SAG14* in younger leaves was evident at day 3 after DEX induction of the *NOD* transgene, it occurred already at day 2 in older leaves. In contrast, the kinetics of *CAB* down-regulation was similar in young and old leaves, as the switch in gene expression occurred in both cases at day 3 after NOD induction.

The findings that the expression of a NO degrading enzyme in *Arabidopsis* and the concomitant decrease in plant NO levels (Zeier *et al*. 2004a) lead to a yellowing phenotype and changes in gene expression similar to senescence prompted us to test whether the observed senescence-like effect could be inhibited by exogenous application of NO. We therefore fumigated DEX-treated Hmp plants continuously with 4 ppm of NO gas in a glass chamber and compared the development of the senescence-effect with DEX-induced Hmp plants situated in a control chamber. Six days after NOD induction, control plants exhibited a clear yellowing in older leaves, whereas the yellowing effect in adequate leaves of NO-treated plants appeared markedly attenuated (Fig. 5a). Additionally, whereas *CAB* expression in leaves of Hmp controls was clearly diminished from day 4 after transgene expression, *CAB* was almost equally expressed throughout the experiment in leaves of DEX-induced Hmp plants fumigated with NO (Fig. 5b). Similarly, the observed increase in *SAG12* and *SAG14* expression in Hmp plants was strongly attenuated by external NO application (Fig. 5b). These findings suggest that the senescence-like effect in Hmp plants was a result of NO deficiency that could be compensated by external NO.

We next investigated whether external conditions known to favour NO production *in planta* would affect the progress of the senescence-like phenotype in Hmp plants. Reduction of nitrite by NR represents a major enzymatic plant NO source (Yamasaki & Sakihama 2000; Kaiser *et al*. 2002). Consequently, factors like nitrate feeding, high light treatment or cytokinin application, all of which stimulate NR activity (Crawford 1995; Yu *et al*. 1998), were able to enhance NO formation in plants (Magalhaes *et al*. 2000; Tun *et al*. 2001; Planchet *et al*. 2006). Watering Hmp plants with a solution of 50 mM nitrate before DEX-treatment markedly reduced the development of leaf yellowing in comparison with control plants (Fig. 6a,b). A similar effect was observed when plants were kept under high light conditions (500 μmol m^{-2} s^{-1}) during the experiment instead of the usual illumination at 70 μmol m^{-2} s^{-1} (Fig. 6a,c). Both nitrate and high light treatments weakened the NOD-induced expression of *SAG14* and the concomitant down-regulation of *CAB* (Fig. 7). In addition, a reduction in *SAG12* expression was observed under these conditions. Thus, both treatments strikingly attenuated the senescence-like process in Hmp plants. External application of cytokinins or autoregulated endogenous cytokinin production is known to retard plant senescence (Gan & Amasino 1995; Lim, Woo & Nam 2003). Leaf application of the cytokinin BAP diminished NOD-induced leaf yellowing, reduction of *CAB* expression and expression of *SAG12* in Hmp plants. However, BAP treatment had no effect on the course of

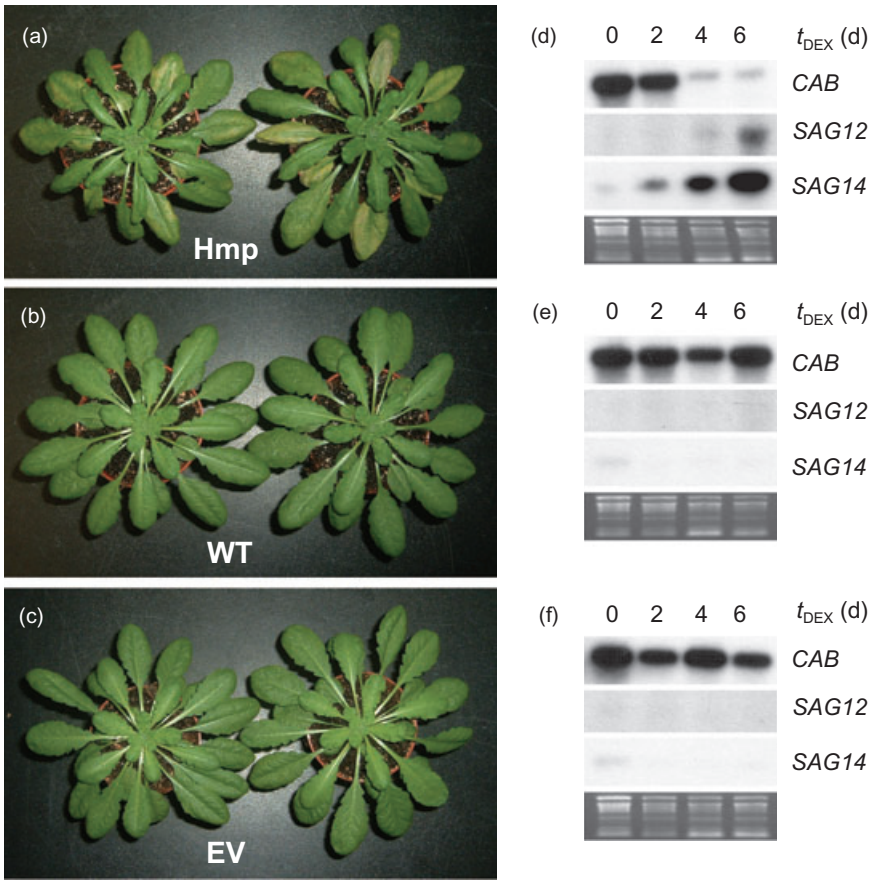


Figure 3. (a–c) Phenotype of Hmp (a), WT (b) and pTA7001 empty vector (EV) plants (c) 6 d after treatment with 3 μM DEX. (d–f) *CAB* and SAG expression in leaves of Hmp (d), WT (e) and EV plants (f) at indicated times (0, 2, 4 and 6 d) after treatment with 3 μM DEX. t_{DEX}, time after DEX treatment.

SAG14 expression and promoted the development of necrotic lesions in leaves (Figs 6d & 7).

To further characterize the inducible senescence-like phenotype in Hmp plants, we compared metabolic changes appearing after NOD induction to those occurring during natural senescence (Figs 8 & 9). Therefore, we classified discrete leaves of 10-week-old, short-day-grown *Arabidopsis* Col-0 plants into the three categories NS, S2 and S4 according to Lohman *et al*. (1994), and analyzed leaf extracts of each category by GC/MS (Fig. 8a). It had been demonstrated that levels of the defense signal SA rise during senescence (Morris *et al*. 2000). In agreement with these findings, we found that total SA contents were significantly higher in the S4 stage than in the earlier developmental stages, whereas most of the SA existed in the glucoside-bound form (Fig. 8b,c). During the induced senescence-like process in Hmp plants, we observed a massive increase in free SA levels at about 4 d after NOD induction (Fig. 9a) with a parallel strong augmentation of SA glucoside levels (data not shown). Recently, it has been demonstrated that concentrations of the chloroplastically located, antioxidant tocopherols rise during leaf senescence, a tendency that proved to be especially pronounced for γ-tocopherol (Holländer-Czytko *et al*. 2005). γ-Tocopherol was not detectable in non-senescent leaves, but contents steadily increased from the S2 to the S4 stage in naturally senescing leaves (Fig. 8d). A similar increase of γ-tocopherol was detected during the induced senescence phenotype of Hmp plants, although the quantities of the antioxidant metabolite were lower than during the S4 stage of natural senescence (Fig. 9b). Interestingly, the metabolic switch favouring γ-tocopherol accumulation again took place about 4 d after NOD induction, and the effect was more pronounced in older than in younger leaves. In addition, we found that the indole derivative camalexin, the predominant phytoalexin in *Arabidopsis* (Tsuji *et al*. 1992), both accumulates during natural senescence and NOD-induced senescence (Figs 8e & 9c). A similar effect was found for indole carboxylic acid, a possible intermediate in camalexin biosynthesis (data not shown). In contrast, leaf concentrations of the indole glucosinolate degradation product IAN decreased during natural senescence (Fig. 8f). A comparable decrease was not found during the induced senescence process in Hmp plants, although it appeared that older leaves generally exhibited lower concentrations of IAN than younger leaves (Fig. 9d). In summary, however, we can state that many metabolic changes occurring during natural senescence were also detectable during the NOD-induced senescence process.

In the next experiment, we studied the effect of external NO application on dark-induced senescence in 8-week-old, short-day-grown *Arabidopsis* plants. Leaf senescence in *Arabidopsis* is induced by darkening of individual rosette leaves rather than by darkening of the whole plant (Weaver

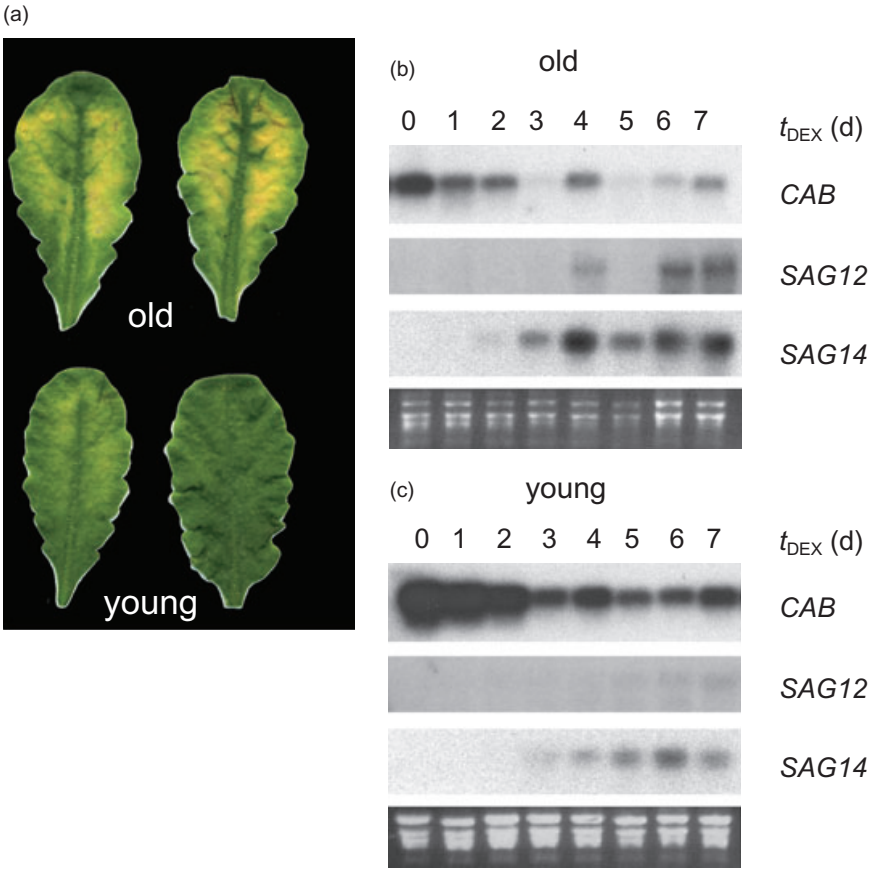


Figure 4. (a) Yellowing phenotype of representative old (leaves 10–12; leaves numbered from their time of appearance in ascending order) and young leaves (leaves 18–20) from 5-week-old Hmp plants. (b,c) Expression patterns of *CAB*, *SAG12* and *SAG14* during the NOD-induced senescence process (b) in old and (c) in young leaves of Hmp plants. t_{DEX}, time after DEX treatment.

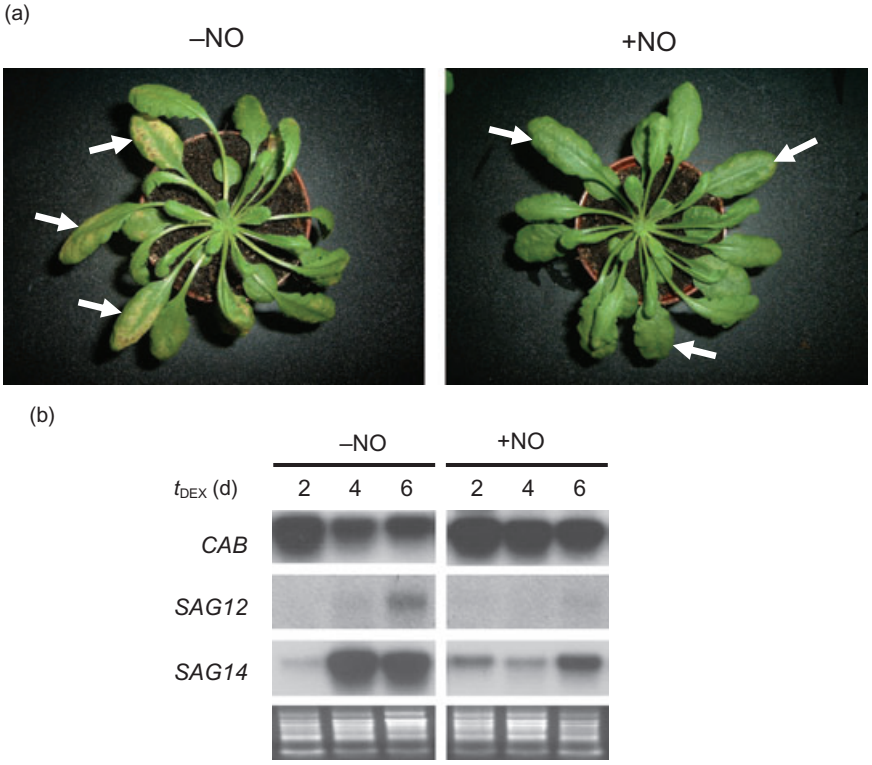


Figure 5. Fumigation of Hmp plants with NO gas (4 ppm) attenuates NOD-induced senescence. (a) Phenotype of Hmp plants 6 d after NOD induction. Left: representative plant fumigated with air during the course of the experiment (–NO); right: representative plant treated with 4 ppm NO gas (+NO). Arrows indicate leaves of similar age. (b) Expression patterns of *CAB*, *SAG12* and *SAG14* in older leaves of Hmp plants fumigated with air (left) or NO (right) after NOD induction. t_{DEX}, time after DEX treatment.

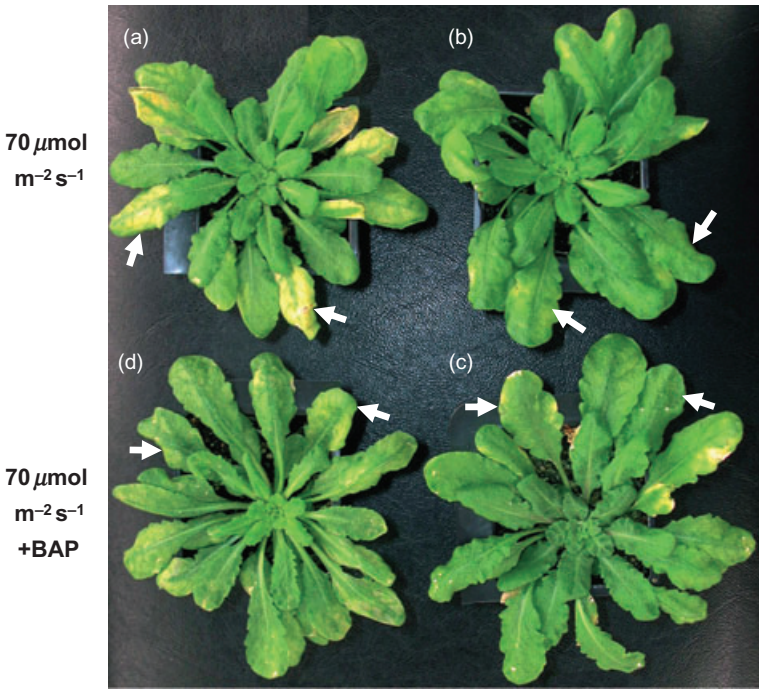


Figure 6. NOD-induced yellowing is attenuated by nitrate feeding, high light treatment and cytokinin application. (a) Hmp plant 6 d after DEX treatment, kept under usual light conditions (PPFD = 70 μmol m^{-2} s^{-1}); (b) same as (a), but plant was watered with a solution of 50 mM nitrate both 3 d before and 2 d after DEX treatment; (c) same as (a), but plant was kept under high light conditions (PPFD = 500 μmol m^{-2} s^{-1}) after DEX application; (d) same as (a), but plant was sprayed with 50 μM BAP both 4 h before and 2 d after DEX treatment. Arrows indicate leaves of similar age. Representative plants of each treatment are shown.

& Amasino 2001). We therefore darkened individual, older rosette leaves having no apparent signs of senescence (leaf numbers 9–12 of plants harbouring 40–45 rosette leaves), and placed different plants into two adequate glass chambers. One of those chambers was continuously fumigated with diluted NO gas (4 ppm NO in air), whereas the other was flushed with compartment air as a control. Ten days after leaf darkening, pronounced yellowing was observed in covered leaves of control plants, and this was associated with a strong decline in total chlorophyll content (Fig. 10a,b). In comparison, darkened leaves of plants fumigated with NO exhibited a lesser degree of yellowing, and the total chlorophyll content in these leaves proved to be higher than in darkened control leaves (Figs. 10a,b). Moreover, leaf darkening of control plants led to a strong decline in expression of *CAB*, and to an increase in *SAG12* and *SAG14* expressions (Fig. 10c). NO fumigation both attenuated the down-regulation of *CAB* and the up-regulation of *SAG12.* In contrast, the increase of *SAG14* was more pronounced in darkened leaves of NO-treated plants compared with leaves of control plants (Fig. 10c). This up-regulation of *SAG14*, however, was also observed in uncovered and non-senescing leaves fumigated with NO (data not shown).

DISCUSSION

Similarities and differences between NOD-induced and natural senescence

Induced expression of the *E. coli* flavohemoglobin Hmp in *Arabidopsis*, an enzyme functioning as a NOD (Vasudevan *et al*. 1991; Gardner *et al*. 1998; Poole & Hughes 2000), reduced both the *in planta* detection of NO as well as the emission of NO from plants, and furthermore increased the NO degrading capacity of leaf extracts (Zeier *et al*. 2004a). We show here that NOD expression triggers a senescence-like yellowing process in Hmp plants (Fig. 1), and this NOD-induced senescence exhibits many similarities to natural senescence at the molecular level, including down-regulation of photosynthetic genes, up-regulation of different SAGs (Figs 2 & 3) and metabolic increases in the levels of SA, γ-tocopherol and camalexin (Figs 7 & 8).

The cysteine protease gene *SAG12* is known to be expressed exclusively during senescence, and its expression is therefore used as a senescence-specific marker (Weaver *et al*. 1998; Zimmermann & Zentgraf 2005).

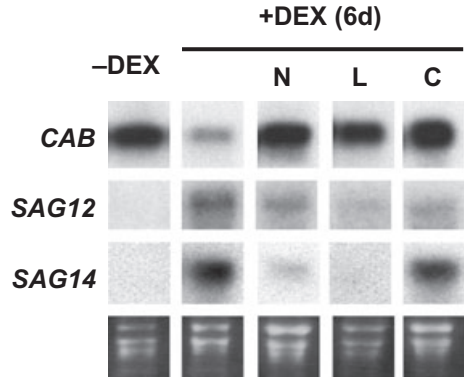


Figure 7. Influence of nitrate feeding (N), high light treatment (L) and cytokinin application (C) on expression of *CAB*, *SAG12* and *SAG14* 6 d after NOD induction (+DEX) in leaves of Hmp plants. The left column (–DEX) depicts gene expression without NOD induction. For details, see legend to Fig. 6.

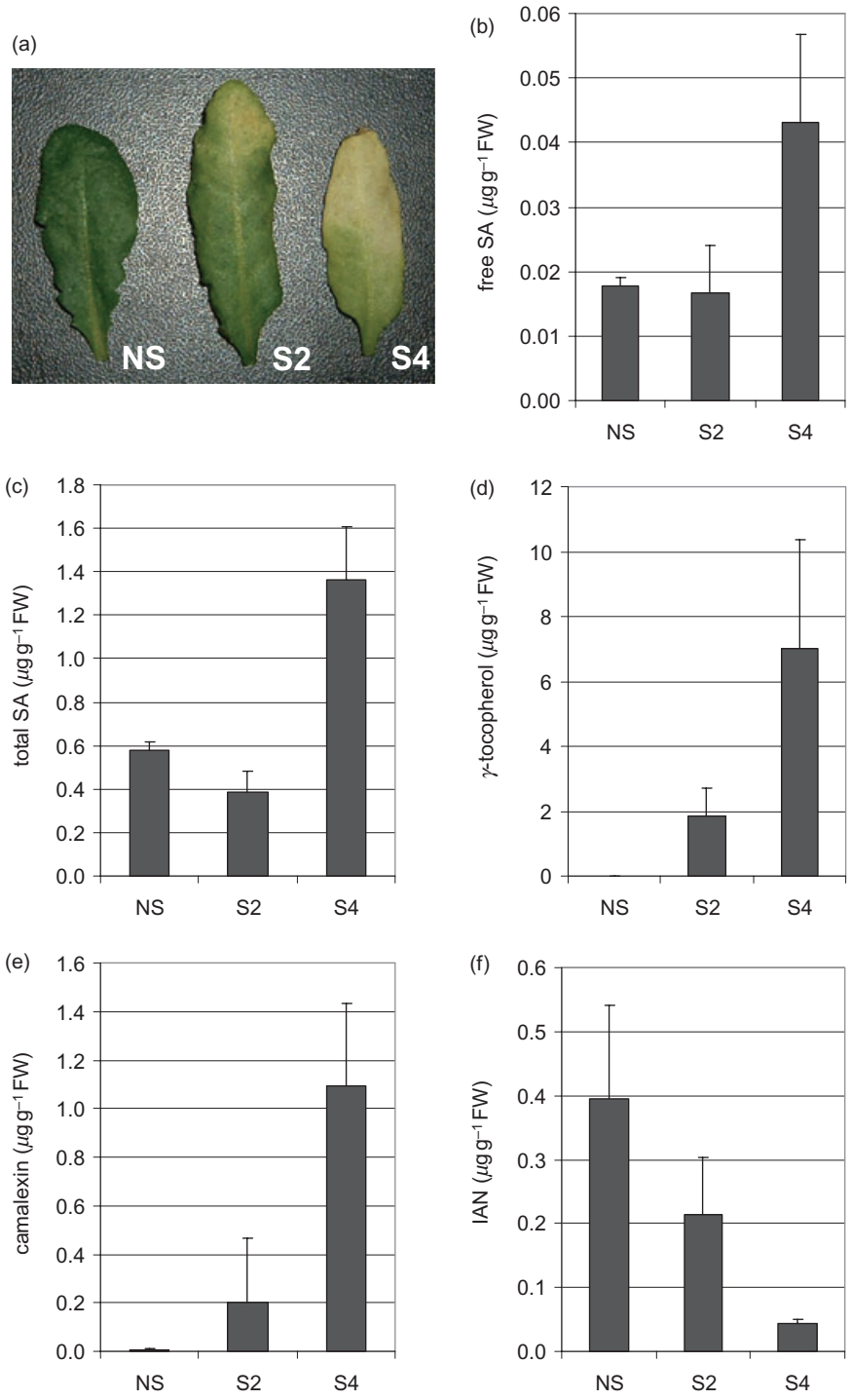


Figure 8. Leaf metabolite levels during natural senescence. (a) Leaves of 10-week-old, short-day-grown *Arabidopsis* Columbia-0 (Col-0) plants were classified according to Lohman *et al.* (1994) into the categories 'non-senescent' (NS), early senescent (S2, 10–20% of leaf area exhibits visible leaf yellowing) and late senescent (S4, 50–80% leaf area exhibits visible leaf yellowing). Levels of (b) free SA, (c) total SA (free + glucoside bound), (d) γ-tocopherol, (e) camalexin and (f) IAN in leaves of the categories NS, S2 and S4 [microgram per gram fresh weight (FW)]. Bars represent mean values of at least four independent samples; SDs are indicated by error bars.

The up-regulation of *SAG12* during the NOD-induced senescence phenotype indicates a high similarity of the process to natural senescence. Moreover, resemblance of NOD-induced senescence to natural senescence manifests itself in the finding that seven out of eight SAGs tested in this study were clearly up-regulated after NOD induction. Other analogies emerge when looking more closely at the relative kinetics of expression of particular SAGs. For instance, *SAG13* is generally expressed shortly before visible senescence begins, while *SAG12* shows no detectable expression until yellowing is discernible (Lohman *et al.* 1994; Weaver *et al.* 1998). This is consistent with the senescence process in Hmp plants, as *SAG13* is already expressed at day 3 after NOD induction, whereas *SAG12* expression starts not until days 4–5 (Fig. 2).

Enhanced biosynthesis of ethylene and elevated levels of ROS represent further hallmarks of natural senescence (Aharoni *et al.* 1979; Magalhaes *et al.* 2000; Zimmermann &

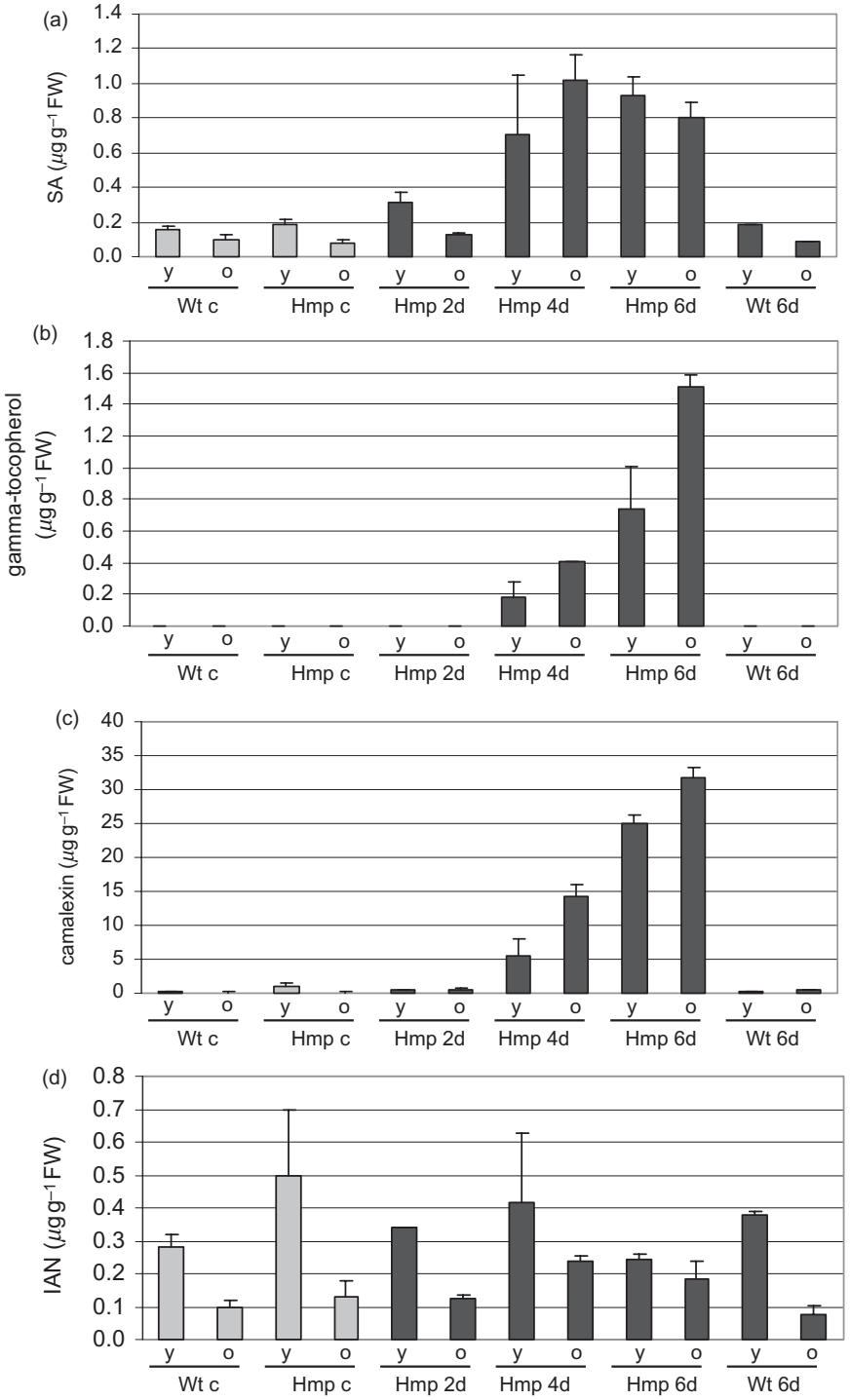


Figure 9. Leaf metabolite levels during NOD-induced senescence. Young (y) and old (o) leaves (see legend to Fig. 4) were separately examined. (a) Leaf levels of free SA, (b) γ-tocopherol, (c) camalexin and (d) IAN [microgram per gram fresh weight (FW)]. Samples originate from WT Columbia-0 (Col-0) (Wt) or transgenic Hmp plants treated either with a control solution (c, light bars) or with 3 μM DEX (dark bars). The duration of DEX treatment in days is indicated. Bars represent mean values of at least five independent samples; error bars indicate SDs.

Zentgraf 2005). Although we did not measure ethylene production directly, we found that expression of the ACC synthase gene *ACS6*, which is involved in ethylene biosynthesis and is up-regulated during ozone-induced senescence (Miller *et al*. 1999), increased during NOD-induced senescence (Fig. 2). Similarly, enhanced expression of the GST gene *GST1* and rises in γ-tocopherol levels, both suitable markers for the detection of elevated ROS levels (Levine *et al*. 1994; Zeier *et al*. 2004b; Holländer-Czytko *et al*. 2005), occur at the transition to the senescence phenotype at 3–4 d after NOD induction (Figs 2 & 9). The latter data and the finding that we detected increased H_2O_2 levels by diaminobenzidine staining in leaves of Hmp plants from about 4 d after NOD induction (Zeier *et al*. 2004a) demonstrate that enhanced production of ROS takes place during the course of NOD-induced senescence.

We furthermore showed that levels of the defense metabolites SA and camalexin are enhanced during both

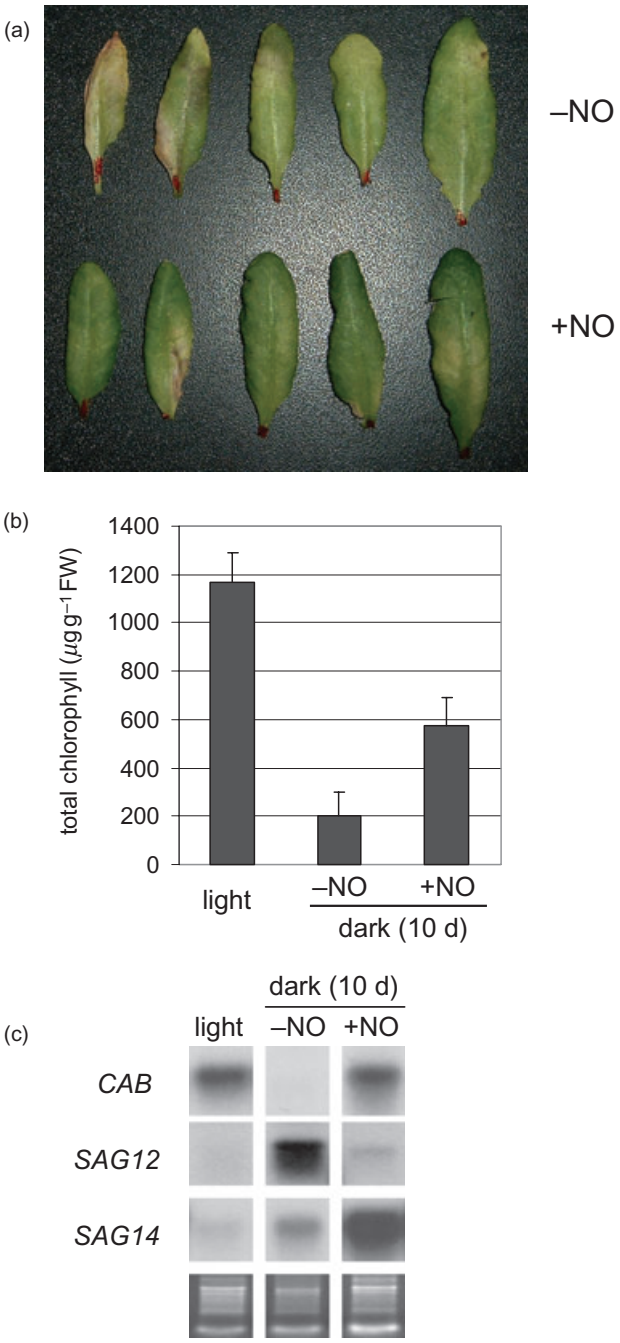


Figure 10. NO fumigation delays dark-induced senescence. Older, attached rosette leaves (leaves 10–12) without any visible signs of senescence from 8-week-old, short-day-grown WT *Arabidopsis* [(accession Columbia-0 (Col-0)] plants harbouring in sum 40–45 leaves and initially lacking any signs of visible senescence were examined during the experiment. Leaves were individually darkened by coverage with a black, appropriate-sized piece of paperboard. (a) Appearance of leaves after a dark period of 10 d. Upper row (–NO): air-flushed leaves; lower row: leaves treated with 4 ppm of NO gas. (b) Total chlorophyll contents of uncovered control leaves (light), and leaves darkened for 10 d and either fumigated with air (–NO) or with 4 ppm of NO gas. Bars represent mean values of five independent leaf samples; SDs are indicated by error bars. (c) Expression of *CAB*, *SAG12* and *SAG14*, with the same treatments as in (b).

NOD-induced and natural senescences (Figs 7 & 9; Morris *et al*. 2000), and the same holds true for expression of the defense-related gene *PR-1* (Fig. 2; Hanfrey *et al*. 1996). To our knowledge, this is the first demonstration that camalexin, the major phytoalexin in *Arabidopsis* (Tsuji *et al*. 1992), is produced during senescence. Camalexin is predominantly protective against different necrotrophic pathogens (Thomma *et al*. 1999; Ferrari *et al*. 2003). Further, the simultaneous activation of the SA pathway and concomitant expression of *PR-1* represent both effective defenses against biotrophic pathogens (Wildermuth *et al*. 2001). This demonstrates that distinct defense pathways are activated during senescence, which are possibly able to provide broad-spectrum resistance for the senescing leaf to different kinds of pathogens.

Although many similarities between NOD-induced and natural senescences exist, it has to be emphasized that the observed NOD-induced phenotype clearly represents an accelerated senescence process. Thus, it is not astonishing that we also find molecular differences between both senescence events. In contrast to natural senescence (Fig. 8; Quirino, Normanly & Amasino 1999), we could not detect a decrease in leaf levels of the glucosinolate degradation product IAN during NOD-induced senescence (Fig. 9). Moreover, the quantitative changes of several metabolites during the two processes differ significantly, as SA and camalexin elevation prove to be higher, and γ-tocopherol increases are lower in NOD-induced senescence compared with the S4 stage of natural senescence (Figs 8 & 9). Possibly, these differences occur as a consequence of the nature of the decline in leaf NO content (see subsequent discussion), which is certainly much more gradual during natural senescence.

NO as a negative regulator of leaf senescence

During the last years, several studies in different laboratories have indicated a negative regulatory role of NO in plant senescence and maturation (Leshem *et al*. 1998; Magalhaes *et al*. 2000; Hung & Kao 2004; Guo & Crawford 2005). The findings that leaf yellowing, the down-regulation of *CAB* and SAG expression during NOD-induced senescence are attenuated by exogenous application of NO (Fig. 5) substantiates that the senescence phenotype in Hmp plants results from NO deficiency. The *Arabidopsis* mutant *Atnos1* impaired in NOS-mediated NO production exhibits a comparable phenotype. The first true leaves of *Atnos1* mutants are yellowish and fail to fully green, and attached and detached leaves of *Atnos1* mutant plants show strongly accelerated dark-induced senescence compared with leaves of WT plants (Guo *et al*. 2003; Guo & Crawford 2005). Obviously, a prolonged lowering of endogenous NO levels by NOD in Hmp plants or a diminished NO production as

a consequence of NOS failure in *Atnos1* mutants is sufficient to induce or promote leaf senescence, respectively. During natural ageing of *Arabidopsis*, plant NO emission decreases continuously and reaches a minimum value during senescence (Magalhaes *et al*. 2000). Considering the processes observed in NO-deficient Hmp and *Atnos1* plants, we speculate that during leaf development, NO levels might fall below a certain threshold that in turn contributes to the induction of natural senescence.

We have also found that several molecular events occurring during dark-induced leaf senescence, that is, chlorophyll loss, down-regulation of *CAB* and up-regulation of *SAG12*, can be retarded by fumigating plants with NO gas in the low ppm range (Fig. 10). This finding confirms previous publications reporting negative regulatory properties of NO in senescence and maturation. Thus, application of NO-releasing chemicals or NO gas extended the post-harvest life of fruits and vegetables and retarded the senescence of flowers (Leshem *et al*. 1998). Further, NO donors counteracted methyl jasmonate and ABA promoted senescence of rice leaves (Hung & Kao 2003, 2004), and attenuated dark-induced leaf senescence in *Arabidopsis Atnos1* mutants (Guo & Crawford 2005). When applying higher NO concentrations in our experiments (≥10 ppm), however, a significant portion of the darkened leaves exhibited leaf damage and dessication. This emphasizes the dose-dependent action of NO as a double-edged sword (Colasanti & Suzuki 2000), providing anti-senescent properties at lower and damaging effects at higher concentrations. Moreover, fumigation with 4 ppm NO gas did not attenuate dark-induced up-regulation of *SAG14*, yet enhanced expression of the gene in darkened and light-exposed leaves. As *SAG14* is known to be up-regulated not only during senescence but also in response to dehydration and darkness (Weaver *et al*. 1998), this possibly indicates that NO fumigation affects gene expression events independent of senescence.

Internal and external factors influencing NOD-induced senescence

The integration and balance of internal and external factors is thought to be important in controlling the induction of leaf senescence (Yoshida 2003). Leaf age represents a significant internal variable influencing senescence. For instance, Weaver & Amasino (2001) have shown that dark-induced senescence in individual leaves occurs more rapidly and strongly in older leaves than in younger ones. Consistently, we have found that NOD-induced senescence proceeds faster and is more pronounced in older than in younger leaves (Fig. 4). In analogy to the finding that older plants exhibit lower NO emission than young ones (Magalhaes *et al*. 2000), the capacity of NO production in leaves is likely to decrease with increasing age. Constant removal of NO by NOD would then have more severe consequences on NO-mediated processes in older leaves, and this may explain the faster induction of the NOD-induced senescence phenotype in these leaves.

The NOD-induced senescence effect is not only attenuated by NO fumigation but also by high light treatment, nitrate feeding and with restrictions, by cytokinin application (Figs 5 & 6). Although cytokinins are frequently able to block visible senescence, it is known that they may not be sufficient to delay all symptoms associated with leaf senescence (Nam 1997). In addition, application of cytokinins in higher concentrations has been shown to induce programmed cell death in plants (Carimi *et al*. 2003). This is consistent with our findings that BAP application failed to block *SAG14* expression during NOD-induced senescence and caused the development of necrotic lesions.

NO emission is generally much more pronounced in light-situated plants as compared with darkened plants, and higher light intensities give rise to stronger NO emission signals than lower light levels (Magalhaes *et al*. 2000; Planchet *et al*. 2006). Similarly, nitrate-grown plants emit considerably higher amounts of NO than plant grown on ammonium (Planchet *et al*. 2006), and cytokinins give rise to increased NO releases in plant cell cultures (Tun *et al*. 2001). Light, nitrate and cytokinin are thus capable to stimulate endogenous NO production, and an internal NO generation caused by the previous treatments may have consequently counterbalanced the NO diminishing action of NOD in Hmp plants. Considering the postulated anti-senescent properties of NO, this in turn would explain the observed attenuation of NOD-induced senescence. NR may play a key role in mediating these effects, as NR expression and activity are known to be positively regulated by the three treatments applied (Crawford 1995; Yu *et al*. 1998), and at the same time, NR represents a major NO source in plants (Yamasaki & Sakihama 2000; Kaiser *et al*. 2002). A similar scenario would be feasible in plant development, during which light conditions, nitrogen nutrition and other environmental factors could be integrated through cytokinin action, NR activity and NO levels to influence the regulation of natural senescence.

Mechanisms through which NO may counteract plant senescence

NO has been recognized to possess both pro- and antioxidant effects in plants, and this antagonism may be based on the relative ratios of ROS and NO levels in different physiological situations. For instance, NO acts in concert with ROS to trigger the hypersensitive response elicited by avirulent pathogens, whereby a fine balance of NO and H_2O_2 levels appears essential (Delledonne *et al*. 2001). By inhibition of antioxidant enzymes like catalase and ascorbate peroxidase, NO may contribute to elevated ROS levels and oxidative stress under certain circumstances (Clark *et al*. 2000). Consistently, until about 2 d after NOD induction in the NO-deficient Hmp plants, a pathogen-induced oxidative burst is clearly attenuated, and as compared to WT plants, a significantly faster H_2O_2 degrading capacity in Hmp leaves exists (Zeier *et al*. 2004a). This suggests that during the oxidative burst, NO ensures prolonged H_2O_2 levels at the site of pathogen challenge.

Contrastingly, NO has been shown to act as an antioxidant in other situations. NO donors protect from oxidative damage caused by methylviologen herbicides, and counteract ROS-mediated programmed cell death in barley aleurone layers (Beligni & Lamattina 1999; Beligni *et al*. 2002). During ABA- and jasmonate-induced senescence in rice, NO-releasing substances prevent increases in H_2O_2 levels and lipid peroxidation (Hung & Kao 2003, 2004). Accelerated dark-induced senescence in *Atnos1* mutants is accompanied with increased ROS levels and protein oxidation (Guo & Crawford 2005). Moreover, as we detect higher ROS levels at 3–4 d after NOD induction in Hmp plants, a negative correlation between NO and ROS levels in these plants exists during the onset of the senescence phenotype. Thus, a conceivable action of NO in prohibiting certain aspects of senescence may be to avoid ROS accumulation. As a free radical, NO reacts with superoxides in a diffusion-limited reaction to form peroxynitrite (Huie & Padmaja 1993), and a subsequent fast isomerization of this toxic compound to a harmless end product like nitrate represents a possible mechanism to reduce ROS levels and cell damage through oxidative stress.

Although oxidative stress and ozone application induce various SAGs, these treatments do not initiate expression of the specific senescence marker *SAG12* (Miller *et al*. 1999; Navabpour *et al*. 2003). Thus, ROS elevation is not sufficient to trigger a full and coordinated execution of the natural senescence programme, and attenuation of *SAG12* expression by NO (Figs 5 & 10) must therefore occur by ROS-independent mechanisms. The plant hormone ethylene is capable of promoting senescence in plants (Smart 1994; Grbic & Bleecker 1995), and ethylene levels rise when plants start to senesce (Aharoni *et al*. 1979; Magalhaes *et al*. 2000). Considering the negative correlation of ethylene and NO emission during plant ageing (Magalhaes *et al*. 2000) and the up-regulation of ACC synthase during NOD-induced senescence, it is feasible that falling NO levels also contribute to senescence regulation by initiating ethylene biosynthesis.

REFERENCES

Aharoni N., Lieberman M. & Sisler H.D. (1979) Patterns of ethylene production in senescing leaves. *Plant Physiology* **64,** 796–800.

Aoyama T. & Chua N.-H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant Journal* **11,** 605–612.

Beligni M.V. & Lamattina L. (1999) Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants. *Nitric Oxide* **3,** 199–208.

Beligni M.V., Fath A., Bethke P.C., Lamattina L. & Jones R.L. (2002) Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiology* **129,** 1642–1650.

Bethke P.C., Badger M.R. & Jones R.L. (2004) Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* **16,** 332–341.

Buchanan-Wollaston V. (1997) The molecular biology of leaf senescence. *Journal of Experimental Botany* **48,** 181–199.

Carimi F., Zottini M., Formentin E., Terzi M. & Lo Schiavo F. (2003) Cytokinins: new apoptotic inducers in plants. *Planta* **216,** 413–421.

Clark D., Durner J., Navarre D.A. & Klessig D.F. (2000) Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Molecular Plant-Microbe Interactions* **13,** 1380–1384.

Colasanti M. & Suzuki H. (2000) The dual personality of NO. *Trends in Pharmacological Sciences* **21,** 249–252.

Corpas F.J., Barroso J.B., Carreras A., *et al*. (2004) Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants. *Plant Physiology* **136,** 2722–2733.

Crawford N.M. (1995) Nitrate: nutrient and signal for plant growth. *Plant Cell* **7,** 859–868.

Crawford N.M. & Guo F.Q. (2005) New insights into nitric oxide metabolism and regulatory functions. *Trends in Plant Science* **10,** 195–200.

Delledonne M., Zeier J., Marocco A. & Lamb C. (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive response. *Proceedings of the National Academy of Sciences of the USA* **98,** 13454–13459.

Ferrari S., Plotnikova J.M., De Lorenzo G. & Ausubel F.M. (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires EDS4 and PAD2, but not SID2, EDS5 or PAD4. *Plant Journal* **35,** 193–205.

Gan S. & Amasino R.M. (1995) Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270,** 1986–1988.

Gardner P.R., Gardner A.M., Martin L.A. & Salzman A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proceedings of the National Academy of Sciences of the USA* **95,** 10378–10383.

Gepstein S., Sabehi G., Carp M.-J., Hajouj T., Nesher M.F.O., Yariv I., Dor C. & Bassani M. (2003) Large-scale identification of leaf senescence-associated genes. *Plant Journal* **36,** 629–642.

Grbic V. & Bleecker A.B. (1995) Ethylene regulates the timing of leaf senescence in *Arabidopsis*. *Plant Journal* **8,** 595–602.

Guo F.-Q. & Crawford N.M. (2005) *Arabidopsis* nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *Plant Cell* **17,** 3436–3450.

Guo F.-Q., Okamoto M. & Crawford N.M. (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* **302,** 100–103.

Hanfrey C., Fife M. & Buchanan-Wollaston V. (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. *Plant Molecular Biology* **30,** 597–609.

He Y., Tang R.-H., Hao Y., *et al*. (2004) Nitric oxide represses the *Arabidopsis* floral transition. *Science* **305,** 1968–1971.

Holländer-Czytko H., Grabowski J., Sandorf I., Weckermann K. & Weiler E.W. (2005) Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in *Arabidopsis* under stress conditions. *Journal of Plant Physiology* **162,** 767–770.

Huie R.E. & Padmaja S. (1993) The reaction of NO with superoxide. *Free Radical Research Communications* **18,** 195–199.

Hung K.T. & Kao C.H. (2003) Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. *Journal of Plant Physiology* **160,** 871–879.

Hung K.T. & Kao C.H. (2004) Nitric oxide acts as an antioxidant and delays methyl jasmonate-induced senescence of rice leaves. *Journal of Plant Physiology* **161,** 43–52.

Kaiser W.M., Weiner H., Kandlbinder A., Tsai C.B., Rockel P., Sonoda M. & Planchet E. (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. *Journal of Experimental Botany* **53,** 875–882.

Leshem Y.Y., Wills R.B.H. & Ku V.V.-V. (1998) Evidence for the function of the free radical gas – nitric oxide (NO) – as an

endogenous maturation and senescence regulating factor in higher plants. *Plant Physiology and Biochemistry* **36,** 825–833.

Levine A., Tenhaken R., Dixon R.A. & Lamb C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. *Cell* **79,** 583–593.

Lim P.O., Woo H.R. & Nam H.G. (2003) Molecular genetics of leaf senescence in *Arabidopsis*. *Trends in Plant Science* **8,** 272–278.

Lohman K.N., Gan S., John M.C. & Amasino R.M. (1994) Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiologia Plantarum* **92,** 322–328.

Magalhaes J.R., Monte D.C. & Durzan D. (2000) Nitric oxide and ethylene emission in *Arabidopsis thaliana*. *Physiological and Molecular Biology of Plants* **6,** 117–127.

Miller J.D., Arteca R.N. & Pell E.J. (1999) Senescence-associated gene expression during ozone-induced leaf senescence in Arabidopsis. *Plant Physiology* **120,** 1015–1023.

Morris K., Mackerness S.A., Page T., John C.F., Murphy A.M., Carr J.P. & Buchanan-Wollaston V. (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant Journal* **23,** 677–685.

Mur L.A.J., Carver T.L.W. & Prats E. (2006) NO way to live; the various roles of nitric oxide in plant-pathogen interactions. *Journal of Experimental Botany* **57,** 489–505.

Nam H.G. (1997) The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology* **8,** 200–207.

Navabpour S., Morris K., Allen R., Harrison E., A.-H.-Mackerness S. & Buchanan-Wollaston V. (2003) Expression of senescence-enhanced genes in response to oxidative stress. *Journal of Experimental Botany* **54,** 2285–2292.

Neill S.J., Desikan R. & Hancock J.T. (2003) Nitric oxide signalling in plants. *New Phytologist* **159,** 11–35.

Nooden L.D., Guiamet J.J. & John I. (1997) Senescence mechanisms. *Physiologia Plantarum* **101,** 746–753.

Planchet E., Sonoda M., Zeier J. & Kaiser W.M. (2006) Nitric oxide (NO) as an intermediate in the cryptogein-induced hypersensitive response – a critical re-evaluation. *Plant, Cell & Environment* **29,** 59–69.

Poole R.K. & Hughes M.N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. *Molecular Microbiology* **36,** 775–783.

Porra R.J., Thompson W.A. & Kriedemann P.E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochimica et Biophysica Acta* **975,** 384–394.

Quirino B.F., Normanly J. & Amasino R.M. (1999) Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Molecular Biology* **40,** 267–278.

Quirino B.F., Noh Y.-S., Himelblau E. & Amasino R.M. (2000) Molecular aspects of leaf senescence. *Trends in Plant Science* **5,** 278–282.

Rockel P., Strube F., Rockel A., Wildt J. & Kaiser W.M. (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *Journal of Experimental Botany* **53,** 103–110.

Schmelz E.A., Engelberth J., Tumlinson J.H., Block A. & Alborn H.T. (2004) The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. *Plant Journal* **39,** 790–808.

Smart C.M. (1994) Gene expression during leaf senescence. *New Phytologist* **126,** 419–448.

Stöhr C., Strube F., Marx G., Ullrich W.R. & Rockel P. (2001) A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. *Planta* **212,** 835–841.

Taylor C.B., Bariola P.A., delCardayre S.B., Raines R.T. & Green P.J. (1993) RNS2: a senescence-associated RNase of *Arabidopsis* that diverged from the S-RNases before speciation. *Proceedings of the National Academy of Sciences of the USA* **90,** 5118–5122.

Thomma B.P., Nelissen I., Eggermont K. & Broekaert W.F. (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant Journal* **19,** 163–171.

Tsuji J., Jackson E.P., Gage D.A., Hammerschmidt R. & Somerville S.C. (1992) Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv. *syringae*. *Plant Physiology* **98,** 1304–1309.

Tun N.N., Holk A. & Scherer G.F. (2001) Rapid increase of NO release in plant cell cultures induced by cytokinin. *FEBS Letters* **509,** 174–176.

Vasudevan S.G., Armarego W.L., Shaw D.C., Lilley P.E., Dixon N.E. & Poole R.K. (1991) Isolation and nucleotide sequence of the hmp gene that encodes a haemoglobin-like protein in *Escherichia coli* K-12. *Molecular and General Genetics* **226,** 49–58.

Weaver L.M. & Amasino R.M. (2001) Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. *Plant Physiology* **127,** 876–886.

Weaver L.M., Gan S., Quirino B. & Amasino R.M. (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology* **37,** 455–469.

Wildermuth M.C., Dewdney J., Wu G. & Ausubel F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* **414,** 562–565.

Yamasaki H. & Sakihama Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. *FEBS Letters* **468,** 89–92.

Yoshida S. (2003) Molecular regulation of leaf senescence. *Current Opinion in Plant Biology* **6,** 79–84.

Yu X., Sukumaran S. & Márton L. (1998) Differential expression of the *Arabidopsis Nia1* and *Nia2* genes: cytokinin-induced nitrate reductase activity is correlated with increased *Nia1* transcription and mRNA levels. *Plant Physiology* **116,** 1091–1096.

Zeidler D., Zähringer U., Gerber I., Dubey I., Hartung T., Bors W., Hutzler P. & Durner J. (2004) Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Sciences of the USA* **101,** 15811–15816.

Zeier J., Delledonne M., Mishina T., Severi E., Sonoda M. & Lamb C. (2004a) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. *Plant Physiology* **136,** 2875–2886.

Zeier J., Pink B., Mueller M.J. & Berger S. (2004b) Light conditions influence specific defence responses in incompatible plant–pathogen inter-actions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta* **219,** 673–683.

Zimmermann P. & Zentgraf U. (2005) The correlation between oxidative stress and leaf senescence during plant development. *Cellular and Molecular Biology Letters* **10,** 515–534.

Received 3 April 2006; received in revised form 13 July 2006; accepted for publication 19 August 2006

MANUSCRIPT 4

Mishina TE, Zeier J

Interactions of Arabidopsis with non-adapted *Pseudomonas syringae* strains: possible determinants of bacterial non-host resistance.

(2007b) Submitted

Interactions of Arabidopsis with non-adapted *Pseudomonas syringae* strains: possible determinants of bacterial non-host resistance

Tatiana E. Mishina and Jürgen Zeier*

Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, Julius-von-Sachs-Platz 3, D-97082 Würzburg, Germany

Abstract

Although interactions of plants with virulent and avirulent host pathogens are under intensive study, relatively little is known about plant interactions with non-adapted pathogens and the molecular events underlying non-host resistance. Here we show that two *Pseudomonas syringae* strains for which Arabidopsis is a non-host plant, *P. syringae* pv. *glycinea* (*Psg*) and *P. syringae* pv. *phaseolicola* (*Psp*), induce salicylic acid (SA) accumulation and pathogenesis-related gene expression at inoculation sites, and that induction of these defences is largely dependent on bacterial type III secretion. The defence signalling components activated by non-adapted bacteria resemble those initiated by host pathogens, including SA, NPR1, NDR1, PAD4 and EDS1. However, some differences in individual defence pathways induced by *Psg* and *Psp* exist, suggesting that for each strain, distinct sets of type III effectors are recognized by the plant. Although induction of SA-related defences occurs, it does not directly contribute to bacterial non-host resistance, because Arabidopsis mutants compromised in SA signalling and other classical defence pathways do not permit enhanced survival of *Psg* or *Psp* in leaves. The finding that numbers of non-adapted bacteria in leaf extracellular spaces rapidly decline after inoculation suggests that they fail to overcome toxic or structural defence barriers preceding SA-related responses. Consistent with this hypothesis, rapid, TTSS-independent up-regulation of the lignin biosynthesis genes *PAL1* and *BCB*, which might contribute to an early-induced, cell wall-based defence mechanism, occurs in response to non-adapted bacteria, and knockout of *PAL1* permits increased leaf survival of non-host bacteria. Moreover, different survival rates of non-adapted bacteria in leaves from Arabidopsis accessions and mutants with distinct glucosinolate composition or hydrolysis exist. Possible roles for early inducible, cell wall-based defences and the glucosinolate/ myrosinase system in bacterial non-host resistance are discussed.

Abbreviations

cfu, colony-forming units; Col, Columbia; HR, hypersensitive response; JA, jasmonic acid; Ler, Landsberg erecta; LPS, lipopolysaccharides; OD, optical density; PAL, phenylalanine ammonia lyase; PAMPs, pathogen-associated molecular patterns; PR, pathogenesis-related; *Psg*, *Pseudomonas syringae* pv. *glycinea* race 4; *Psm*, *Pseudomonas syringae* pv. *maculicola* ES4326; *Psp*, *Pseudomonas syringae* pv. *phaseolicola* NPS4008; *Pst*, *Pseudomonas syringae* pv. *tomato* DC3000; R, resistance; SA, salicylic acid; SAG, salicylic acid glucoside; SAR, systemic acquired resistance; TTSS, type III secretion system

Introduction

The phenomenon that an entire plant species is immune to a particular pathogen is termed non-host resistance (Nürnberger and Lipka 2005). Non-host resistance occurs when a pathogen is poorly adapted to the basic physiology of a plant, cannot build up appropriate infection structures or is unable to overcome the plants basal defence machinery (Thordal-Christensen et al. 2003). Plant defences underlying non-host resistance are not sufficiently understood at the molecular level, although it is emerging that they are both constitutive and inducible in nature.

Recent studies addressing the interaction between barley powdery mildew and *Arabidopsis thaliana* have established that a sequential action of pre- and post-invasive defences ensures non-host resistance in Arabidopsis towards non-adapted phytopathogenic fungi (Ellis 2006). The pre-invasion barrier requires at least three genetically distinct loci, *PENETRATION1* (*PEN1*), *PEN2*, and *PEN3*, and provides penetration resistance against the barley powdery mildew fungus. *PEN1* encodes a membrane-associated syntaxin possibly functioning in vesicle secretion and formation of cell wall reinforcing papillae at sites of attempted fungal ingress (Collins et al. 2003). *PEN2* and *PEN3* code for a glycosyl hydrolase and an ATP binding cassette transporter protein, respectively, which might be involved in generation and transport of a toxic substance at infection sites (Lipka et al. 2005, Stein et al. 2006). Thus, structural and chemical barriers appear to constitute this first line of defence. Non-host powdery mildew infections that escape pre-invasive defences result in haustorium formation but are later stopped by a post-haustorial hypersensitive response (HR). This post-invasion barrier is dependent on a functional *EDS1*/*PAD4* defence signalling complex, which is also involved in basal resistance to restrict dissolute disease development during compatible host–pathogen interactions. Simultaneous blockage of pre- and post-invasion defence components eventually enables epiphytic growth of the barley powdery mildew on the non-host plant Arabidopsis (Lipka et al. 2005, Stein et al. 2006).

In contrast to the epidermis-penetrating powdery mildew, the phytopathogenic bacterium *Pseudomonas syringae* enters internal plant tissue through open stomata or wounds (Katagiri et al. 2002). To successfully colonize the plant apoplast, *P. syringae* uses a *hrp* gene encoded type III secretion system (TTSS) to deliver virulence effectors into the plant cell which contribute to pathogen multiplication and disease development by suppression of basal plant defence responses (Alfano and Collmer 2004). The latter are triggered by invariant pathogen-associated molecular patterns (PAMPs) which include bacterial flagellin, lipopolysaccharides and elongation factor TU (Ingle et al. 2006). In some cases, recognition of one or several TTSS effectors by appropriate resistance (R) proteins renders the interaction incompatible, allowing the plant to more vigorously defend itself through initiation of an HR and associated defence responses which restrict but not fully abrogate bacterial multiplication (Lamb and Dixon 1997). The Arabidopsis-Pseudomonas interaction has been widely used in the past as a model system to study the molecular

mechanisms of such compatible and incompatible host-bacterium interactions. Appropriate strains capable to multiply in Arabidopsis leaves and causing disease symptoms or an HR include *P. syringae* pv. *tomato* DC 3000 (*Pst*) and *P. syringae* pv. *maculicola* ES4326 (*Psm*; Whalen et al. 1991, Dong et al. 1991). Other *P. syringae* strains, i.e. the TTSS-deficient *Pst hrpA*$^-$ or the pathovars *glycinea* (*Psg*) and *phaseolicola* (*Psp*), do not excessively multiply in Arabidopsis extracellular spaces and interactions with leaves of the model crucifer remains symptomless (Mishina and Zeier 2007). These less adapted strains are apparently not able to overcome the barriers of non-host resistance.

Interactions of plants with non-adapted phytopathogenic bacteria and the molecular events underlying bacterial non-host resistance are poorly understood. For instance, only one Arabidopsis gene required for bacterial non-host resistance, the glycerol kinase *NHO1*, has been described so far (Kang et al. 2003). Inducible cell wall-based defence responses including papilla formation and callose deposition at sites of inoculation, however, have been considered crucial for resistance against non-adapted bacteria. These relatively early responses might restrict water and nutrient flow towards the apoplast and thus prevent bacterial feeding and multiplication (Hauck et al. 2003, Soylu et al. 2005, Truman et al. 2006). Some reports have demonstrated that interactions of non-adapted bacterial pathogens with plants also induce defence gene expression at inoculation sites (Jakobek and Lindgren 1993, Lu et al. 2001, Truman et al. 2006). Moreover, these symptomless interactions result in systemic acquired resistance (SAR) induction in Arabidopsis (Mishina and Zeier 2007).

The present study aims to provide a detailed analysis of the *P. syringae* - Arabidopsis non-host interaction. We show that numbers of non-adapted *Psg* and *Psp* bacteria rapidly decline in extracellular spaces of Arabidopsis leaves, possibly due to the failure to overcome a chemical or structural defence barrier. Nevertheless, non-host *P. syringae* strains induce salicylic acid (SA) accumulation and expression of various pathogenesis-related (*PR*) genes. A functional TTSS is required for a large part of those inducible responses. By contrast, early up-regulation of genes involved in lignin biosynthesis is independent of TTSS secretion. Blockage of classical signalling pathways in Arabidopsis defence mutants does not abrogate the non-host defence barrier. However, significant differences in survival rates of non-adapted bacteria in leaves from Arabidopsis accessions and mutants with distinct glucosinolate composition and hydrolysis exist. Moreover, knockout lines of the lignin biosynthesis gene *PAL1* also permit increased survival of non-host bacteria. Possible barrier roles for inducible cell wall fortifications and the glucosinolate/myrosinase system in non-host resistance of Arabidopsis against bacterial pathogens are discussed.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. plants were grown on a mixture of soil (Fruhstorfer Pflanzenerde, Archut, Germany), vermiculite and sand (9:1:1) in a controlled environment chamber (J-66LQ4, Percival) with a 9 h day (photon flux density 70 µmol m^{-2} s^{-1}) / 15 h night cycle and a relative humidity of 70 %. Growth temperatures were set to 22 °C during the day period and to 18 °C during the night. 5-6 weak old plants were used for experiments.

A. thaliana ecotypes Col-0 (NASC ID: N1092), Ler-0 (NW20), Ws-0 (N1602), C24 (CS909), Ep-0 (N1142), Wa-1 (N1586), Bu-0 (N1006), Da-0 (N1098), Ra-0 (1480), Bch-0 (N956), and Dr-0 (N1114) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). If not otherwise stated, the *A. thaliana* ecotype Col-0 was used as wild-type in all experiments. The following Arabidopsis (defence) mutants were used in this study: *sid2-1* (Nawrath and Métraux 1999), *npr1-2* (NASC ID: N3801), *ndr1* (Century et al. 1995), *pad3-1* (Glazebrook and Ausubel 1994), *pad4-1* (Glazebrook et al. 1997), *eds1-2* (Ler-0 background; Aarts et al. 1998), *pal1-2* (C24 background; Rohde et al. 2004), *gsm1-1* (NASC ID: N2226), and TU3 (N2228; Haughn et al. 1991). The *pal1-1* and *pal2-1* mutants represent Salk T-DNA insertion lines SALK_000357 and SALK_092252, respectively (Col-0 background). Homozygous insertion mutants were identified by PCR, using a gene specific and a T-DNA specific primer according to Alonso et al. (2003), and used for experiments.

Growth of plant pathogens and infection

P. syringae pv. *tomato* DC3000 (*Pst*) and the corresponding avirulent strains carrying the plasmid pLAFR3::avrRpm1 (*Pst avrRpm1*) were obtained from C. Lamb (Maldonado *et al*., 2002). *Pst hrpA*$^{-}$ (Roine et al. 1997), *P. syringae* pv. *glycinea* race 4 (*Psg*; Staskawicz et al. 1984), and *P. syringae* pv. *phaseolicola* NPS4008 ± *hrpR* (*Psp* and *Psp hrpR*$^{-}$; Grimm et al. 1995) were obtained from S. Berger, C. Lamb, and W. Aufsatz, respectively. Strains were grown overnight at 28°C in King's B medium containing the appropriate antibiotics (Zeier et al. 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final concentration of OD 0.005 (defence responses) or OD 0.002 (bacterial growth) for host strains, and to OD 0.1 for non-adapted or TTSS-deficient strains. The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1 ml syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed by homogenising disks originating from infiltrated areas of 3 different leaves in 1 ml 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28 °C for two days.

Northern blot analysis

Total RNA was isolated from frozen leaves using peqGOLD RNAPure^{TM} reagent (peqLab, Erlangen, Germany) following the manufacturer's instructions. For each sample, two leaves from different plants of the same treatment were used. 2 µg of total RNA was loaded on formaldehyde-agarose gels, separated by electrophoresis and blotted to nylon-membranes (Hybond-N, Amersham). RNA blot hybridisation was performed with specific, ^{32}P-labelled DNA-probes generated by PCR using appropriate oligonucleotide primers. The probes represented the following Arabidopsis genes: *SID1* (Arabidopsis annotation At4g39030), *PAL1* (At2g37040), *BCB* (At5g20230), *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-4* (At3g04720), *PR-5* (At1g75040).

Determination of defence metabolites

The determination of SA, SAG, jasmonic acid, and camalexin levels in leaves was realised by a modified vapor-phase extraction method and subsequent gas chromatographic/mass spectrometric analysis as described in detail in Mishina and Zeier (2006).

Reproducibility of experiments and statistical analyse*s*

All pathogen experiments and the respective bacterial growth analyses, metabolite determinations and gene expression analyses depicted in the figures were conducted three times with similar results. Statistical analyses were performed utilising Student's *t*-test.

Results

Inoculations of Arabidopsis leaves with non-adapted *P. syringae* strains are characterized by a rapid decline of initial bacterial numbers

Depending on their ability to multiply in the plant apoplast, bacterial pathogens can be divided into adapted (host) and non-adapted (non-host) bacteria. The *P. syringae* pathovar *tomato* DC3000 (*Pst*) is well-adapted to Arabidopsis accession Col-0 and rapidly propagates in leaves causing water-soaked lesions (Fig. 1). *Pst* host strains carrying the avirulence protein AvrRpm1 (*Pst avrRpm1*) are rapidly recognized by Arabidopsis Col-0 through the RPM1 resistance protein and elicit a hypersensitive response (HR; Bisgrove et al. 1994). Although development of the HR diminishes growth of bacteria by a factor of 10-20, it cannot completely abolish bacterial multiplication (Fig. 1). By contrast, bacteria not sufficiently adapted to Arabidopsis, such as the *P. syringae* pathovars *glycinea* (*Psg*) or *phaseolicola* (*Psp*), which represent soybean and bean pathogens, respectively (Staskawicz et al. 1984, Lu et al. 2001), are not able to propagate in Col-0 leaves and do not cause disease symptoms (Mishina and Zeier 2007). After infiltrating leaves with relatively high inoculum concentrations (OD_{600} = 0.1), numbers of *Psg* or *Psp* were drastically reduced after 1dpi and

then essentially remained at a constant low level (Fig. 1). Comparatively, initial numbers of the mutant host strain *Pst hrpA*$^-$, which is defective in establishment of a functional type III secretion system and does not cause disease symptoms in Col-0 (Roine et al. 1997, Mishina and Zeier 2007), more gradually decreased after infiltration. Obviously, *Pst hrpA*$^-$ is able to survive for longer periods of time in extracellular spaces of Col-0 leaves than *Psg* or *Psp*, indicating a fundamental difference between interactions of Arabidopsis with non-adapted bacterial strains and with mutant strains of adapted bacteria that are defective in type III secretion.

Induction of SA-dependent and related defence responses after inoculation with non-host bacteria is largely dependent on type III secretion

We directly compared defence activation by host and non-host *P. syringae* strains and examined the influence of type III secretion on defence responses through inoculation of Col-0 leaves with *Pst*, *Pst avrRpm1*, *Pst hrpA*$^-$, *Psg*, *Psp*, and *Psp hrpR*$^-$ combined with determination of defence metabolite accumulation and defence-related gene induction at 24 hpi. To compensate differences in bacterial multiplication (Fig. 1), we used a higher inoculum concentration for non-adapted and TTSS-deficient strains (OD 0.1) than for host *Pst* and *Pst avrRpm1* (OD 0.005).

When the virulent and avirulent host strains *Pst* and *Pst avrRpm1* were infiltrated in Col-0 leaves, strong accumulation of the defence signal salicylic acid (SA), the corresponding glucoside (SAG) and the phytoalexin camalexin occurred at 24 hpi (Figs. 2, 3B). The avirulent but not the virulent strain also caused synthesis of the stress hormone jasmonic acid (JA; Fig. 3A). Moreover, increased expression of the SA-biosynthesis gene *SID1* and the pathogenesis-related (PR) genes *PR-1*, *PR-2*, *PR-4*, and *PR-5* was induced by both *Pst* and *Pst avrRpm1* (Fig. 4). The TTSS-defective mutant host strain *Pst hrpA*$^-$ failed to induce camalexin and JA accumulation, and when compared to *Pst*, SA synthesis and *PR* gene expression were substantially reduced, albeit not fully abrogated. This indicates that Arabidopsis responses to host *P. syringae* strains are largely dependent on secretion of type III effectors. To a minor extent, however, the SA pathway can still be activated by TTSS-independent elicitation events.

Although the non-adapted *Psg* and *Psp* strains did not provoke symptom development or an HR (Mishina and Zeier 2007), they caused significant elevation of SA levels and induction of *SID1*, *PR-1*, *PR-2*, *PR-4*, and *PR-5* in Col-0 leaves at 24 hpi (Figs. 2, 4). Overall responses to *Psg* and *Psp* inoculation, however, proved to be lower than responses to infection with *Pst* host strains. Particularly, *Psg* and *Psp* did not induce JA or camalexin accumulation in Col-0 leaves (Fig. 3), and induction of *PR-4* expression occurred to comparatively low levels (Fig. 4). Remarkably, the TTSS-deficient non-host strain *Psp hrpR*$^-$ caused SA accumulation and *PR* gene expression to a lesser extent that *Psp*. These findings indicate that the TTSS of *P. syringae* is functional in non-host plants, and that

TTSS-secreted effectors markedly contribute to the induction of SA-associated and kinetically related defence responses also in non-host plant-bacteria interactions.

Induction of early, cell wall-associated defence responses is TTSS-indepen-dent and absent in compatible interactions

Phenylalanine ammonia lyase (PAL) represents the key enzyme of phenylpropanoid biosynthesis and is involved in lignin formation (Rohde et al. 2004). *PAL1* transcripts are rapidly up-regulated in the incompatible *P. syringae*-Arabidopsis host interaction (Zeier et al. 2004). Consistently*, Pst avrRpm1* strongly induces *PAL1* expression at 4hpi (Fig. 4). A similar early increase in *PAL1* transcripts was observed after inoculation of both *Psg* and *Psp* non-host strains. Additionally, both *hrp* mutant strains, *Pst hrpA*$^{-}$ and *Psp hrpR*$^{-}$, triggered *PAL1* expression in a comparable manner. Infection with the virulent *Pst* strain, however, did not cause increased expression of *PAL1* (Fig. 4). Another Arabidopsis protein involved in lignin biosynthesis is the blue copper binding protein (BCB; Ezaki et al. 2005). Similar to the expression characteristics of *PAL1*, we found that *BCB* transcripts were rapidly up-regulated by all utilized pathogens except the compatible *Pst* strain (Fig. 4). These data suggest that both non-host and avirulent host bacteria trigger the induction of an early defence mechanism that potentially contributes to cell wall fortifications through lignification at inoculation sites. In contrast to SA-associated defences, this cell wall-based defence is not dependent on TTSS secretion. Moreover, virulent strains might have a mechanism to actively suppress the induction of this early response.

Non-host and host bacteria trigger similar defence signalling pathways

The finding that non-host bacteria induce TTSS effector-mediated SA accumulation and *PR* gene expression prompted us to test whether similarities exist between defence signalling pathways initiated by adapted and non-adapted bacterial pathogens. To do so, we examined *Psg*- and *Psp*-triggered defence responses in Arabidopsis mutant lines known to be impaired in specific or basal resistance against host bacteria.

Due to a mutational defect in the SA biosynthesis gene *ISOCHORISMATE SYNTHASE1* (*ICS1*), *sid2* plants do not accumulate SA and *PR-1* upon contact with avirulent and virulent *Pst* and thus exhibit enhanced susceptibility towards these pathogens (Nawrath and Métraux 1999, Wildermuth et al. 2001). Similar to *Pst*, the *Psg* and *Psp* non-host strains do not trigger SA biosynthesis in *sid2* (Fig. 5A). Moreover, the absence of SA biosynthesis induction results in a failure of expression of the SA-dependent *PR-1* gene after contact with both host and non-host bacteria (Fig. 5B). The same holds true for the *non-expressor of PR-1* (*npr1*) mutant (Fig. 5B), which is impaired in signalling events downstream of SA (Durrant and Dong 2004). However, *Psg*- or *Psp*-induced accumulation of free SA was even more pronounced in *npr1* than in wild-type plants (Fig. 5A), a tendency that has been previously observed with *Pst* inoculation (Delaney et al. 1995). In contrast to

SA, SAG levels were elevated to a similar extent in wild-type and *npr1* after inoculation with the different host and non-host *P. syringae* strains (data not shown).

Other defence signalling components involved in basal or specific resistance in Arabidopsis are *NDR1* (*NON-RACE SPECIFIC RESISTANCE1*), *PAD4* (*PHYTOALEXIN-DEFICIENT4*), and *EDS1* (*ENHANCED DISEASE SUSCEP-TIBILITY1*; Century et al. 1995; Wiermer et al. 2005). Compared to the wild-type, *Psg*- or *Psp*-triggered SA accumulation was strongly reduced in *ndr1* and *pad4* mutant plants (Fig. 5A), coinciding with the absence of *PR-1* induction after inoculation with both non-host strains in these mutants. By contrast, host *Pst* or *Pst avrRpm1* still caused increased *PR-1* expression in *ndr1* and *pad4* (Fig. 5B). In *eds1* mutant plants, responses to *Psg*- and *Psp*-inoculation were not uniform. Like the host *Pst* strains, *Psg* triggered a marked accumulation of SA and enhanced *PR-1* expression, whereas *Psp* only induced a minor increase in SA levels and failed to elevate *PR-1* transcription (Fig. 5A, B).

Some distinctions within defence initiation by *Psg* and *Psp* are also obvious when expression of the JA-associated *PR-4* gene (*HEVEIN-LIKE1*; Thomma et al., 1998) in different defence mutants is considered (Fig. 6A). Whereas *PR-4* expression in response to *Psp*, like with *Pst* and *Pst avrRpm1*, is unchanged or even potentiated in SA-signalling mutants *sid2* and *npr1*, an intact SA signalling pathway seems necessary for full *PR-4* expression after *Psg* inoculation. Moreover, functional *EDS1* is required for *Psg*- and *Pst avrRpm1*-, but not for *Psp*- or *Pst*-triggered induction of *PR-4*. Up-regulation of the thaumatin-like gene *PR-5* occurs in virtually every mutant-pathogen combination (Fig. 6B). When compared to wild-type, however, *PR-5* expression is attenuated in *pad4* mutants inoculated with *Pst avrRpm1*, *Psg*, and *Psp*. Complementary, *eds1* mutants exhibit a potentiated or accelerated *PR-5* induction when inoculated with *Pst avrRpm1*, *Pst*, and *Psg*, but not after encountering *Psp*.

Together, these findings indicate that non-host bacteria activate plant defences by utilizing signalling pathways that play a central role in resistance to host bacteria. However, when comparing the initiation of specific defence responses through *Pst avrRpm1*, *Pst*, *Psg* or *Psg* in more detail, similarities and differences in induced defence signalling pathways exist. For instance, *Psg* and *Pst avrRpm1* elicit similar *EDS1*-related defence signalling, whereas clear differences in this respect between the two non-host strains *Psg* and *Psp* exist (Figs. 5, 6). It is also noteworthy that, in contrast to the described varations in SA accumulation and *PR* gene expression, up-regulation of the early-responsible genes *PAL1* and *BCB* took place in every of the different defence signalling mutants in a similar manner than in wild-type (data not shown).

Non-host resistance in Arabidopsis towards *P. syringae* is not compromised when classical host resistance pathways are blocked

Inducible defence responses act as an integral part of basal and specific resistance towards adapted *P. syringae* strains (Glazebrook 2005). For instance, the SA signalling-defective mutants *sid2* and *npr1* are compromised in both specific resistance towards avirulent and basal resistance towards virulent bacterial strains (Nawrath and Métraux 1999). Moreover, *eds1* and *pad4* signalling mutants exhibit enhanced susceptibility to virulent bacteria and are defective in specific resistance towards avirulent bacteria that activate the TIR-NBS-LRR type of resistance proteins (Wiermer et al. 2005). By contrast, *ndr1* shows attenuated gene-for-gene resistance triggered by R proteins of the CC-NBS-LRR-type (Aarts et al. 1998). To examine whether induction of the SA pathway (Figs. 2, 4) and defence signalling mediated by *NDR1*, *EDS1*, or *PAD4* (Figs. 5, 6) contributes to bacterial non-host resistance in Arabidopsis, we quantified bacterial numbers in the respective mutant strains three days after inoculation with *Psg* or *Psp* (Fig. 7). Survival of *Psg* or *Psp* in leaves of *sid2* or *npr1* mutant plants was virtually identical to survival in Col-0 leaves indicating that a blockage of the SA signalling pathway is not sufficient to break bacterial non-host resistance. Further, bacterial numbers at 3dpi were similar in leaves of *ndr1*, *pad4*, *eds1* and the respective wild-type (Col-0 or Ler-0). Moreover, the *pad3* mutant that is unable to accumulate the phytoalexin camalexin (Glazebrook and Ausubel 1994), as well as the ethylene and jasmonate signalling mutants *etr1* and *jar1* (Chang et al. 1993, Staswick et al. 1992) did not allow increased survival of the non-adapted bacteria in the leaf apoplast (Fig. 7; data not shown). However, significant differences of bacterial survival rates existed in extracellular spaces of the Arabidopsis Col-0 and Ler-0 accessions (Fig. 7). Thus, although non-host *P. syringae* induces defences when inoculated in Arabidopsis leaves, blockage of those inducible responses do not compromise non-host resistance. Rather, other factors varying in different accessions might influence the interaction of Arabidopsis and non-host bacteria.

Survival rates of non-host bacteria differ in Arabidopsis accessions and mutants with altered glucosinolate hydrolysis

The observed different survival rates of non-host *P. syringae* in Col-0 and Ler-0 ecotypes prompted us to include further Arabidopsis accessions in our study. Whereas ecotype Ler-0 allowed significantly higher survival rates of *Psg* than Col-0, Ws and C24 exhibited increased non-host resistance to this bacterial strain (Fig. 8A). A similar, albeit less pronounced tendency was observed for survival of *Psp* in these accessions (Fig. 8B). Survival rates in different Arabidopsis ecotypes did not negatively correlate with the magnitude of SA elevation in inoculated leaves (Fig. 8D). For instance, although survival rates of *Psg* in Ler-0 is higher than in Col-0, we detected a stronger accumulation of SA in inoculated Ler-0 leaves than in Col-0 leaves. By contrast, resistance of the four different ecotypes against host *Pst* was closely associated with the capability of those accessions to

induce SA biosynthesis (Fig. 8C). Col-0 plants, which accumulate lowest amounts of SA after *P. syringae* inoculation, permit significantly higher growth of *Pst* than the remainder accessions. Moreover, the C24 ecotype, which already shows relatively high constitutive SA levels, restricts growth of *Pst* most efficiently.

Our hitherto findings indicate that SA-associated and kinetically similar inducible defence pathways play a minor role during bacterial non-host resistance. One explanation might be that bacteria must be able to overcome constitutive defence barriers before these inducible responses will become effective. In Arabidopsis and other *Brassicaceae*, the glucosinolate/myrosinase system constitutes a major pre-formed chemical defence arsenal that protects plants against generalist herbivores (Wittstock and Halkier 2002). In this defence system, myrosinase enzymes cleave the thioglucoside linkage of non-toxic glucosinolates to produce toxic mustard oils which include isothiocyanates, nitrils, thiocyanates, epithionitrils and other hydrolysis products. In Arabidopsis ecotypes, natural variation exists both on the level of glucosinolate (i.e. side chain) composition and in the nature of the preferred hydrolysis reaction. According to the chemical nature of their predominant hydrolysis products, Arabidopsis accessions have been grouped into six categories (Lambrix et al. 2001). To examine whether variations in mustard oil composition influence bacterial non-host resistance in Arabidopsis, we have inoculated accessions belonging to three different categories, in which methylsulfinylalkyl isothiocyanates (Col-0, Ep-0, Wa-1), methylsulfinylalkyl nitrils (Bu-0, Da-0), or 4-hydroxybutyl nitrils (Ler-0, Ra-0, Bch-0, Dr-0) represent the predominant hydrolysis products, with the non-adapted *Psg* strain and scored bacterial numbers 1dpi (Fig. 9A). Survival rates of *Psg* were not statistically significant in all tested members of the methylsulfinylalkyl isothiocyanate category which includes Col-0. Moreover, *Psg* survival in the two members of the methylsulfinylalkyl nitril group was similar to the Col-0 group. In the 4-hydroxybutyl nitril category, three out of four accessions (Ler-0, Ra-0, Bch-0) permitted a statistically higher survival of *Psg* in the leaf apoplast than in the Col-0 ecotype. However, when compared to Col-0, non-host resistance against *Psg* was not attenuated in the forth member of this group, Dr-0.

To further examine the role of glucosinolates and their degradation products in bacterial non-host resistance, we have determined survival rates of *Psg* and *Psp* in leaves of two Col-0 glucosinolate biosynthesis mutant lines, *gsm1-1* (TU1) and TU3. Both mutant lines exhibit greatly reduced amounts of several aliphatic glucosinolates in leaves. *Gsm1-1* is defective in alkyl glucosinolates bearing butyl, pentyl and hexyl core groups, whereas TU3 shows reduced amounts of glucosinolates with heptyl and octyl core groups. By contrast, *gsm1-1* possesses elevated contents indolyl glucosinolates (Haughn et al. 1991). Whereas survival rates of *Psg* were significantly lower in both *gsm1-1* and TU3 than in Col-0, a higher bacterial number of *Psp* at 1dpi was observed for both mutants in comparison with the Col-0 wild-type. Together, these data suggest that the composition of glucosinolates and their respective hydrolysis products might influence survival rates of non-adapted bacteria in

Arabidopsis leaves, and that different *P. syringae* strains can cope differently with a specific glucosinolate composition.

PAL1 knockout lines exhibit attenuated non-host resistance against *P. syringae*

We have finally investigated whether a blockage in lignin biosynthesis, which contributes to the postulated early, cell wall-associated defence response, would alter bacterial non-host resistance in Arabidopsis. Therefore, *pal1* and *pal2* T-DNA knockout lines were obtained from the Salk collection [SALK_000357 (*pal1-1*) and SALK_092252 (*pal2-1*), respectively], and homozygous insertion lines were selected. Out of those, *pal1-1* permitted significantly higher survival rates in response to *Psg* than the Col-0 wild-type (Fig. 9D). A similar difference was obtained when a previously characterized *pal1* mutant in the C24 background (Rohde et al. 2004; in the present work designated as *pal1-2*), was compared with the respective wild-type (Fig. 9D). These data indicate that rapidly induced expression of *PAL1* (Fig. 4) contributes to non-host resistance against *P. syringae*.

Discussion

The present study provides a direct comparison of three distinct outcomes during the *Arabidopsis – Pseudomonas* model interaction: 1) the compatible, disease-causing host interaction of *Pst* and Arabidopsis Col-0, 2) the incompatible host interaction of *Pst avrRpm1* and Col-0 that results in an HR, and 3) the symptomless non-host interaction between *Psg* or *Psp* and Col-0. Both host interactions result in strong accumulation of SA and its glucoside, *PR* gene expression, and accumulation of the phytoalexin camalexin in Col-0 leaves at 24hpi (Figs. 2 to 4). The avirulent strain *Pst avrRpm1* additionally causes early induction of lignin biosynthesis genes and, later on, JA accumulation, which appears to be a good measure for necrotic cell death (Mishina and Zeier 2007). *Pst avrRpm1* is more restricted in bacterial multiplication than the virulent *Pst* strain because of an earlier and more vigorous defence programme that includes the HR and a strong oxidative burst (Lamb and Dixon 1997). Non-adapted *Psg* or *Psp* strains are not able to multiply in Arabidopsis apoplastic spaces. However, albeit to a lesser extent than host *Pst* strains, they induce SA accumulation and *PR* gene expression, but not JA production, camalexin accumulation or an HR. (Figs. 2-4; Mishina and Zeier 2007). Moreover, like avirulent *Pst avrRpm1*, they activate an early transcription of the lignin biosynthesis genes *PAL1* and *BCB* (Fig. 4).

Non-host resistance against bacteria and other microbes has been grouped into two categories (Mysore and Ryu 2004). Type I non-host resistance does not produce any visible symptoms, whereas type II non-host resistance results in hypersensitive cell death. The examined interactions between Arabidopsis and the soybean pathogen *Psg* or the bean pathogen *Psp* can thus be grouped into the type I category. We have shown that the triggered SA and *PR* gene accumulation at inoculation sites is largely dependent on a TTSS.

This finding, together with the fact that the type II non-host HR also requires type III secretion (Deng et al. 1998), emphasizes that non-adapted bacteria build up a functional TTSS also in non-host plants and deliver effector proteins into the plant cell. The TTSS effectors subsequently might interact with cellular targets to initiate SA-associated defence responses. *P. syringae* translocates a cocktail of more then 30 effectors (Joardar et.al. 2005), and depending on effector composition in different strains, distinct defence pathways are likely to be activated in the plant cell. For instance, *Psp* requires intact *EDS1* signalling to initiate SA accumulation and *PR-1* induction, whereas *Psg* activates the SA pathway independently of *EDS1* (Fig. 5). Both strains, however, require *ICS1*, *NPR1*, *NDR1*, and *PAD4* to fully initiate the SA-associated defence responses.

As SA accumulation and *PR* gene expression are rather weakly induced in *Psp hrpR*$^-$ - and *Pst hrpA*$^-$-inoculated leaves (Figs. 2, 4), the contribution of PAMPs to trigger these local defences is small in comparison with the inducing capacity of TTSS effectors. We have recently found that non-adapted bacteria induce systemic defence responses and SAR in Arabidopsis (Mishina and Zeier 2007). In contrast to the induction of defences at inoculation sites, the induction of SAR by non-host bacteria proved to be virtually independent of type III secretion. On the other hand, we have shown that bacterial PAMPs like flagellin and lipopolysaccharides (LPS) markedly participate in SAR activation (Mishina and Zeier 2007). Thus, a fundamental difference seems to exist in the nature of bacterial molecules that trigger local and systemic SA-associated defence responses in Arabidopsis.

Beside this work, few studies have demonstrated that symptomless interactions of non-adapted bacterial pathogens with plants result in induction of SA-related defences. Thus, a TTSS-deficient *P. syringae* pv. *tabaci* strain triggers defence gene induction in bean without causing an HR (Jakobek and Lindgren 1993), and a non-host *Psp* strain induces SA synthesis and *PR* gene expression in Arabidopsis (van Wees and Glazebrook 2003, Lu et al. 2001). Induction of SA-dependent *PR* gene expression has been previously reported to contribute to bacterial non-host resistance because *Psp* is able to propagate in leaves of SA-deficient Arabidopsis plants expressing the bacterial salicylate hydroxylase NahG (Lu et al. 2001). However, loss of non-host resistance in NahG plants has been shown to occur due to accumulation of the SA conversion product catechol (van Wees and Glazebrook 2003). In line with the latter finding, our results indicate that activation of SA signalling and inducible defence pathways mediated by *EDS1*, *PAD4*, *NDR1*, or *NPR1* does not directly contribute to bacterial non-host resistance (Fig. 7). Further, neither jasmonate signalling nor phytoalexin (camalexin) accumulation represent defence means that are used to inactivate non-adapted bacteria in Arabidopsis leaves (Figs. 3, 7). Yet which factors instead do contribute to the non-host barrier in Arabidopsis?

Bacterial flagellin, as well as TTSS-deficient *P. syringae* strains induce papillae formation at inoculation sites (Gomez-Gomez et al. 1999, Hauck et al. 2003). Papillae are cell wall appositions composed of lignin, callose, hydroxyproline-rich glycoproteins and other

materials. These cell wall fortifications at sites of attempted bacterial ingress might restrict water and nutrient flow towards the apoplast and thus prevent bacterial feeding and multiplication (Soylu et al. 2005). A recent Arabidopsis genome-wide microarray analyses revealed that a common set of 96 genes displays early, sustained up-regulation in treated leaves by TTSS-deficient bacteria (*Pst hrpA*$^-$, *Pst hrcC*$^-$), non-host bacteria (*Psp*), and PAMPs (flagellin, LPS). This set includes genes coding for leucine rich repeat receptor proteins and phenylpropanoid biosynthesis enzymes, suggesting that early defence signalling and lignin deposition at inoculation sites play major roles during bacterial non-host resistance (Truman et al. 2006). Consistently, our data highlight the importance of an early-inducible, cell-wall based defence. We have shown that, one hand, the lignin biosynthesis genes like *PAL1* and *BCB* are rapidly up-regulated by non-adapted and TTSS-deficient bacteria, and on the other hand, that Arabidopsis *pal1* knock-out lines permit enhanced survival rates of *Psg* in leaf apoplastic spaces (Figs. 4, 9D). *PAL1* expression after recognition of bacteria might contribute to the lignification of papillae and therefore to the effectiveness of these cell wall appositions to restrict feeding and propagation of non-adapted and TTSS-deficient bacteria. Suppression of these cell wall alterations by appropriate TTSS effectors could be one means by which virulent host strains manage to acquire nutrients and multiply in Arabidopsis leaves (Fig. 4; Hauck et al. 2003, Truman et al. 2006).

In several plant species, antifungal phytoanticipins contribute to non-host resistance against non-adapted fungal pathogens, and the enzymatic detoxification of those compounds represents an important virulence strategy of adapted fungi (Morrissey and Osbourn 1999). The observation that bacterial numbers of the non-adapted *Psg* and *Psp* strains rapidly decline after inoculation suggest that a preformed toxic principle, which contributes to non-host resistance against these bacteria, is present in Arabidopsis extracellular spaces (Fig. 1). Interestingly, bacterial numbers of the TTSS-deficient *Pst hrpA*$^-$ host strain decline less rapidly than those of *Psg* or *Psp* indicating that the *Pst* strain might tolerate the postulated toxic barrier independently of TTSS secretion. In Arabidopsis and other *Brassicaceae*, the glucosinolate/myrosinase system constitutes a pre-formed defence barrier against generalist herbivores (Wittstock and Halkier 2002). Whether this system is operative in defence against bacterial pathogens is less-well established. A recent report shows that transgenic Arabidopsis plants with altered glucosinolate profiles, i.e. plants with elevated levels of p-hydroxybenzyl or benzyl glucosinolates, exhibit increased resistance against virulent *Pst*. However, this effect is most likely triggered by enhanced SA-related defence responses in those plants rather than by a direct toxic effect of glucosinolate hydrolysis products (Brader et al. 2006). In leaves from Arabidopsis accession Col-0, the glucosinolate breakdown product 4-methyl-sulphinylbutyl isothiocyanate (4-MSB-ITC) represents a major phytoanticipin, exhibiting strong *in vitro* toxicity against *P. syringae* (Tierens et al. 2001). Yet the *gsm1-1* mutant, which is deficient in both alkyl glucosinolates

and 4-MSB-ITC, does not show increased susceptibility against virulent *Pst*, indicating that adapted bacterial pathogens might have developed strategies to cope with the presence of antibacterial glucosinolate-derived compounds (Tierens et al. 2001).

The non-adapted *Psp* strain exhibits enhanced survival rates in leaves of *gsm1-1* compared with Col-0, and this is also true for the TU3 mutant whose leaves are deficient in longer chain aliphatic glucosinolates (Fig. 9C; Haughn et al. 1991). This indicates that aliphatic glucosinolates and their respective hydrolysis products might contribute to non-host resistance against *Psp*, which is presumably unable to tolerate those compounds. By contrast, *Psg* shows better survival in Col-0 than in *gsm1-1* or TU3 leaves (Fig. 9B), suggesting that the mutant glucosinolate composition, which is characterized by enhanced levels indolyl glucosinolates, acts more harmful to this particular strain. Thus, chemically distinct mustard oils might exert different toxicity effects on different *P. syringae* strains. Moreover, as three out of for Arabidopsis accessions belonging to the Ler-0 category permit a significantly prolonged leaf survival of *Psg* than other ecotypes (Fig. 9A), 4-hydroxybutyl nitrils might be less toxic to *Psg* than other mustard oils. On the other hand, the low survival rates of *Psg* in leaves of Dr-0 exemplify that glucosinolate composition and hydrolysis are not the only factors contributing to bacterial non-host resistance. Thus, although measured differences in survival rates of non-adapted bacteria in leaves of Arabidopsis mutants and accessions with distinct glucosinolate composition and hydrolysis exist, further experiments are required to fully prove the suggested function of mustard oils as phytoanticipins against bacterial pathogens in Arabidopsis.

The overall differences between detected survival rates of non-host bacteria in the lines under investigation were relatively small in comparison with the extensive multiplication of adapted bacteria in Arabidopsis. Thus, bacterial non-host resistance is not completely overcome by a single mutation or variation in one biochemical trait, but like non-host resistance against fungal invaders, consists of multiple layers of defence (Lipka et al. 2005, da Cunha et al. 2006). Our results support the idea that an early-inducible, cell wall-based mechanism constitutes one of these defence layers. Although non-host strains are able to induce SA-related defence responses in the plant, the latter will only be effective in cases when all upstream defence layers will be blocked or overcome. Analyses of mutants concomitantly blocked in early barriers and SA-related defences are therefore necessary to further unravel the molecular principles underlying bacterial non-host resistance.

Acknowledgements

This research was financially supported by the Deutsche Forschungsgemeinschaft (SFB 567).

References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two R gene-mediated signalling pathways in Arabidopsis. Proc Natl Acad Sci USA 95: 10306-10311

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: Double agents in bacterial disease and plant defence. Annu Rev Phytopathol 42: 385-414

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geralt M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653-657

Bisgrove SR, Simonich MT, Smith NM, Sattler A, Innes RW (1994) A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. Plant Cell 6: 927-933

Brader G, Mikkelsen MD, Halkier BA, Tapio Palva E (2006) Altering glucosinolate profiles modulates disease resistance in plants. Plant J 46: 758-767

Century KS, Holub EB, Staskawicz BJ (1995) *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc Natl Acad Sci USA 92: 6597-6601

Chang C, Kwok SF, Bleecker AB, Meyerowitz EM (1993) Arabidopsis ethylene-response gene *ETR1*: similarity of product to two-component regulators. Science 262: 539-544

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrink E, Qiu JL, Hückelhoven R, Stein M, Freialdenhoven A, Somerville SC, Schulze-Lefert P (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature 425: 973-977

da Cunha L, McFall AJ, Mackey D (2006) Innate immunity in plants: a continuum of layered defences. Microbes Infect 8: 1372-1381

Delaney TP, Friedrich L, Ryals JA (1995) Arabidopsis signal transduction mutant defective in chemically and biologically induced disease resistance. Proc Natl Acad Sci USA 92: 6602-6606

Deng WL, Preston G, Collmer A, Chang CJ, Huang HC (1998) Characterization of the *hrpC* and *hrpRS* operons of *Pseudomonas syringae* pathovars *syringae*, *tomato*, and *glycinea* and analysis of the ability of *hrpF*, *hrpG*, *hrcC*, *hrpT*, and *hrpV* mutants to elicit the hypersensitive response and disease in plants. J Bacteriol 180: 4523-4531

Dong X, Mindrinos M, Davis KR, Ausubel FM (1991) Induction of Arabidopsis defence genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell 3: 61-72

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42: 185-209

Ellis J (2006) Insights into nonhost disease resistance: can they assist disease control in agriculture? Plant Cell 18: 523-528

Ezaki B, Sasaki K, Matsumoto H, Nakashima S (2005) Functions of two genes in aluminium (Al) stress resistance: repression of oxidative damage by the *AtBCB* gene and promotion of efflux of Al ions by the *NtGDI1* gene. J Exp Bot 56: 2661-2671

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci USA 91: 8955-8959

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Hammerschmidt R, Ausubel FM (1997) Phytoalexin-deficient mutants of Arabidopsis reveal that *PAD4* encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics 146: 381-392

Glazebrook J (2005) Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43: 205-227

Gomez-Gomez L, Felix G, Boller T (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J 18: 277-284

Grimm C, Aufsatz W, Panopoulos NJ (1995) The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. Mol Microbiol 15: 155-165

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defence in susceptible Arabidopsis plants. Proc Natl Acad Sci USA 100: 8577-8582

Haughn GW, Davin L, Giblin M, Underhill EW (1991) Biochemical genetics of plant secondary metabolites in *Arabidopsis thaliana*: the glucosinolates. Plant Physiol 97: 217-226

Ingle RA, Carstens M, Denby KJ (2006) PAMP recognition and the plant-pathogen arms race. Bioessays 28: 880-889

Jakobek JL, Smith JA, Lindgren PB (1993) Suppression of bean defence responses by *Pseudomonas syringae*. Plant Cell 5: 57-63

Joardar V, Lindeberg M, Jackson RW, Selengut J, Dodson R, Brinkac LM, Daugherty SC, Deboy R, Durkin AS, Giglio MG, Madupu R, Nelson WC, Rosovitz MJ, Sullivan S, Crabtree J, Creasy T, Davidsen T, Haft DH, Zafar N, Zhou L, Halpin R, Holley T, Khouri H, Feldblyum T, White O, Fraser CM, Chatterjee AK, Cartinhour S, Schneider DJ, Mansfield J, Collmer A, Buell CR (2005) Whole-genome sequence analysis of *Pseudomonas syringae* pv. *phaseolicola* 1448A reveals divergence among pathovars in genes involved in virulence and transposition. J Bacteriol 187: 6488-6498

Kang L, Li J, Zhao T, Xiao F, Tang X, Thilmony R, He S, Zhou JM (2003) Interplay of the Arabidopsis nonhost resistance gene *NHO1* with bacterial virulence. Proc Natl Acad Sci USA 100: 3519-3524

Katagiri F, Thilmony R, He SY (2002) The *Arabidopsis thaliana*-P*seudomonas syringae* interaction. In: Somerville CR, Meyerowitz EM (eds) The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD, pp 1-35

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48: 251-275

Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ, Gershenzon J (2001) The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. Plant Cell 13: 2793-2807

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defences both contribute to nonhost resistance in Arabidopsis. Science 310: 1180-1183

Lu M, Tang X, Zhou JM (2001) Arabidopsis *NHO1* is required for general resistance against *Pseudomonas* bacteria. Plant Cell 13: 437-447

Maldonado AM, Doerner P, Dixon RA, Lamb C, Cameron RC (2002) A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature 419: 399-403

Mishina TE, Zeier J (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant Physiol 141: 1666-1675

Mishina TE, Zeier J (2007) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant J, in press

Morrissey JP, Osbourn AE (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol Mol Biol Rev 63: 708-724

Mysore KS, Ryu CM (2004) Nonhost resistance: how much do we know? Trends Plant Sci 9: 97-104

Nawrath C, Métraux JP (1999) Salicylic acid induction-deficient mutants of Arabidopsis express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell 11: 1393-1404

Nürnberger T, Lipka V (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol 6: 335-345

Rohde A, Morreel K, Ralph J, Goeminne G, Hostyn V, De Rycke R, Kushnir S, Van Doorsselaere J, Joseleau JP, Vuylsteke M, Van Driessche G, Van Beeumen J, Messens E, Boerjan W (2004) Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell 16: 2749-2771

Roine E, Wei W, Yuan J, Nurmiaho-Lassila EL, Kalkkinen N, Romantschuk M, He SY (1997) Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. Proc Natl Acad Sci USA 94: 3459-3464

Soylu S, Brown I, Mansfield JW (2005) Cellular reactions in Arabidopsis following challenge by strains of *Pseudomonas syringae*: from basal resistance to compatibility. Physiol Mol Plant Pathol 66: 232-243

Staskawicz BJ, Dahlbeck D, Keen NT (1984) Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. Proc Natl Acad Sci USA 81: 6024-6028

Staswick PE, Su W, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA 89: 6837-6840

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18: 731-746

Thomma BP, Nelissen I, Eggermont K, Broekaert, WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J 19: 163-171

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol 6: 351-357

Tierens KF, Thomma BP, Brouwer M, Schmidt J, Kistner K, Porzel A, Mauch-Mani B, Cammue BP, Broekaert WF (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. Plant Physiol 125: 1688-1699

Truman W, de Zabala MT, Grant M (2006) Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. Plant J 46: 14-33

van Wees SC, Glazebrook J (2003) Loss of non-host resistance of Arabidopsis NahG to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J 33: 733-742

Whalen MC, Innes RW, Bent AF, Staskawicz BJ (1991) Identification of *Pseudomonas syringae* pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. Plant Cell 3: 49-59

Wiermer M, Feys BJ, Parker JE (2005) Plant immunity: the EDS1 regulatory node. Curr Opin Plant Biol 8: 383-389

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414: 562-565

Wittstock U, Halkier BA (2002) Glucosinolate research in the Arabidopsis era. Trends Plant Sci 7: 263-270

Zeier J, Pink B, Mueller MJ, Berger S (2004) Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. Planta 219: 673-683

Figure legends

Fig. 1. Bacterial numbers of virulent (*Pst*), avirulent (*Pst avr Rpm1*), and TTSS-deficient (*Pst hrpA*$^-$) host *P. syringae* strains, as well as of non-adapted strains (*Psg* and *Psp*) in Arabidopsis Col-0 leaves at different times after inoculation (OD 0.002 for *Pst* and *Pst avrRmp1*; OD 0.1 for *Pst hrpA*$^-$, *Psg* and *Psp*). Three disks from inoculated areas from different leaves of the same plant were homogenized for one sample. Data points represent mean values (± SD) of 5 parallel samples originating from different plants.

Fig. 2. Accumulation of (A) salicylic acid (SA) and (B) SA glucoside (SAG) in Col-0 leaves challenged with different *P. syringae* strains (OD 0.005 for *Pst* and *Pst avrRpm1*; OD 0.1 for *Pst hrpA*$^-$, *Psg*, *Psp* and *Psp hrpR*$^-$). Control samples were treated with a 10 mM $MgCl_2$ solution. All samples were collected 1d post inoculation/treatment. Bars represent mean values (± SD) of three independent samples, each consisting of six leaves from different plants. Levels are given in µg per g leaf fresh weight (FW). Asterisks denote values with statistically significant differences to the respective $MgCl_2$ control (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; Student's t-test).

Fig. 3. Accumulation of (A) jasmonic acid and (B) camalexin in Col-0 leaves challenged with different *P. syringae* strains (OD 0.005 for *Pst* and *Pst avrRpm1*; OD 0.1 for *Pst hrpA*$^-$, *Psg*, *Psp* and *Psp hrpR*$^-$). Control samples were treated with a 10 mM $MgCl_2$ solution. All samples were collected 1d post inoculation/treatment. Bars represent mean values (± SD) of three independent samples, each consisting of six leaves from different plants. Levels are given in µg per g leaf fresh weight (FW). Asterisks denote values with statistically significant differences to the respective $MgCl_2$ control (*: $P < 0.05$, **: $P < 0.01$; Student's t-test).

Fig. 4. Expression of defence-related genes in Col-0 leaves inoculated with different *P. syringae* strains or infiltrated with 10 mM $MgCl_2$ (for details, see legend of Fig. 2). Numbers indicate hours post inoculation (hpi). Samples consisting of two leaves from different plants were collected at the indicated times after infiltration for RNA extraction and northern blot analysis.

Fig. 5. Accumulation of salicylic acid and transcripts of the SA-dependent pathogenesis-related gene *PR-1* in leaves of Arabidopsis wild-type plants and defence mutants inoculated with host *Pst* and *Pst avrRpm1* (OD 0.005 each) and non-host *Psg* and *Psp* (OD 0.1 each). Control samples were treated with a solution of 10 mM $MgCl_2$. (A) free salicylic acid (SA) levels (10 hpi); (B) transcript accumulation of *PR-1*, as assessed by gel blot analysis (2 µg

of total RNA were loaded on gels for each sample). Samples were collected at 4, 6 and 24h post inoculation.

Fig. 6. Expression of the pathogenesis-related genes *PR-4* (A) and *PR-5* (B) in leaves of Arabidopsis wild-type plants and defence mutants inoculated with different *P. syringae* strains or infiltrated with 10 mM $MgCl_2$ (for details see legend of Fig. 2). For gel blot analyses, 2 µg of total RNA were used for each sample. Samples were collected at 4, 6, and 24h post inoculation.

Fig. 7. Bacterial numbers of non-adapted *Psg* (A) and *Psp* (B) in leaves of Arabidopsis wild-type plants and defence mutants at 3d post inoculation (OD 0.1). Three disks from inoculated areas from different leaves of the same plant were homogenized for one sample. Bars represent mean values (± SD) of 5 parallel samples originating from different plants. Asterisks denote lines with statistically significant differences to the Col-0 accession (*: $P < 0.05$, **: $P < 0.01$; Student's t-test). No significant differences in bacterial numbers were detected at day 0 (1hpi) for each line (data not shown).

Fig. 8. (A-C), Bacterial numbers of *Psg* (A), *Psp* (B), and *Pst* (C) in leaves of Arabidopsis accessions Col-0, Ws-0, C24 and Ler-0 at 3d post inoculation (OD 0.1 for *Psg* and *Psp*; OD 0.002 for *Pst*). Bars represent mean values (± SD) of 5 parallel samples originating from different plants. Asterisks denote lines with statistically significant differences to the Col-0 accession (*: $P < 0.05$, **: $P < 0.01$; ***: $P < 0.001$; Student's t-test). No significant differences in bacterial numbers were detected at day 0 (1hpi) for each accession in (A), (B), and (C) (data not shown).

(D) Accumulation salicylic acid (SA) in leaves inoculated with *Psg* (OD 0.1). Control samples were treated with a 10 mM $MgCl_2$ solution. All samples were collected 1d post inoculation/treatment. Bars represent mean values (± SD) of three independent samples, each consisting of six leaves from different plants. Asterisks denote values with statistically significant differences to the respective Col-0 treatment (**: $P < 0.01$; Student's t-test).

Fig. 9. (A) Bacterial numbers of *Psg* in leaves of different Arabidopsis accessions with methylsulfinylalkyl isothiocyanates (dark grey bars), methylsulfinylalkyl nitrils (light grey bars), or 4-hydroxybutyl nitrils (medium grey bars) as main glucosinolate breakdown products (Lambrix et al. 2001) at 1d post inoculation (OD 0.1).

(B, C) Bacterial numbers of *Psg* (B), and *Psp* (C) in leaves of Col-0, *gsm1-1* (TU1), and TU3 plants at 1d post inoculation (OD 0.1).

(D) Bacterial numbers of *Psg* in leaves of wild-type, *pal1*, and *pal2* mutant plants at 3d post inoculation (OD 0.1; *pal1-1* and *pal2-1* are in background Col-0; *pal1-2* is in background C24).

Bars represent mean values (± SD) of 5 parallel samples originating from different plants. Asterisks denote lines with statistically significant differences to the Col-0 accession (*: $P < 0.05$, **: $P < 0.01$; Student's t-test). No significant differences in bacterial numbers were detected at day 0 (1hpi) for each accession in (A), (B), and (C) (data not shown).

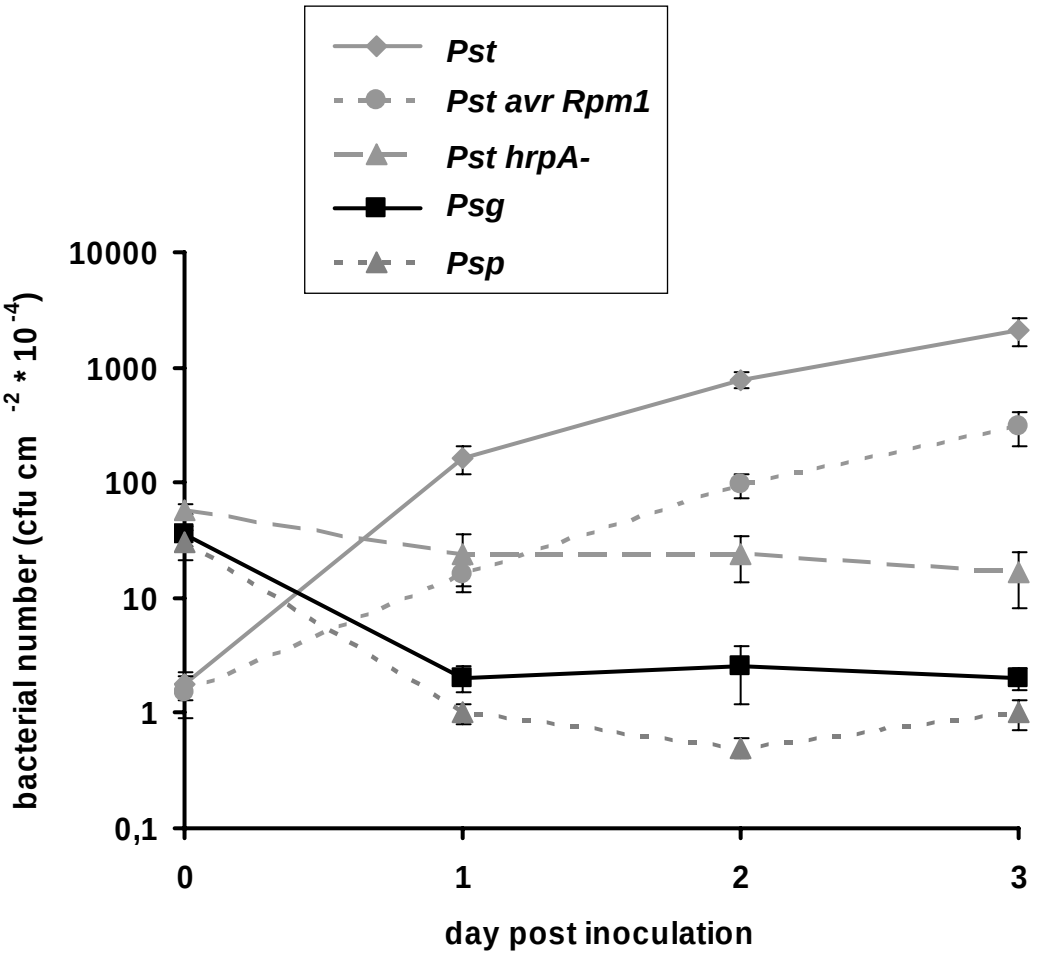
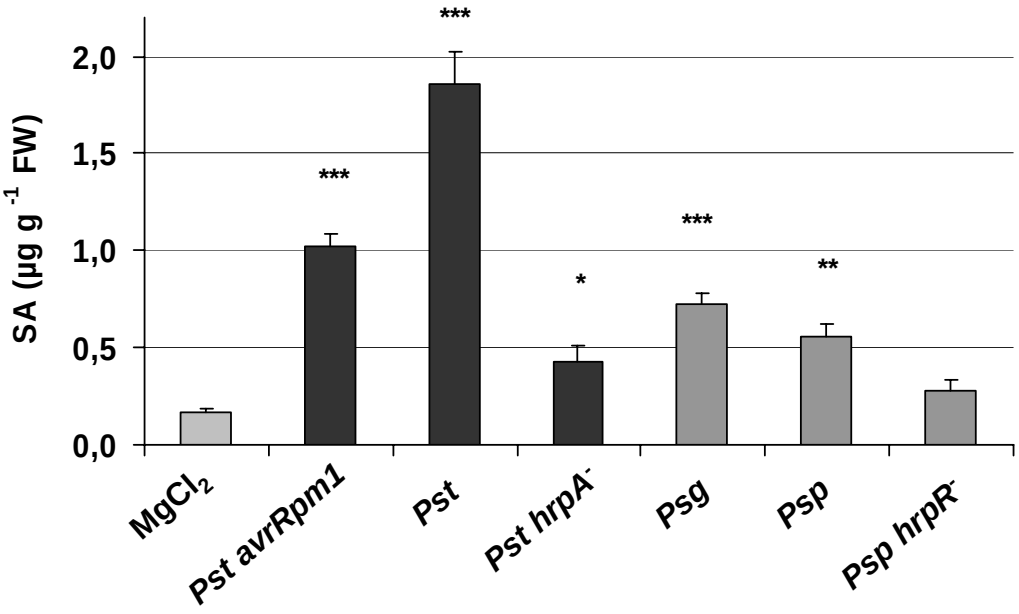

Pst
Pst avr Rpm1
Pst hrpA-
Psg
Psp
10000
1000
100
10
1
0,1
bacterial number (cfu cm -2 * 10-4)
0
1
2
3
day post inoculation

Fig. 1

A


SA (µg g -1 FW)
2,0
1,5
1,0
0,5
0,0
***
***
*
***
**
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-

B

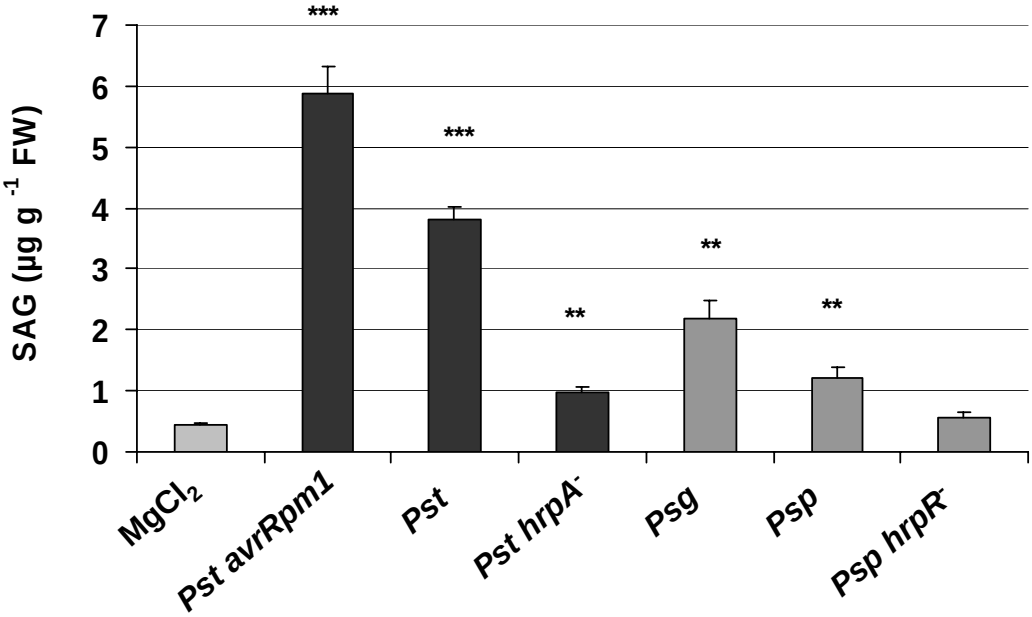
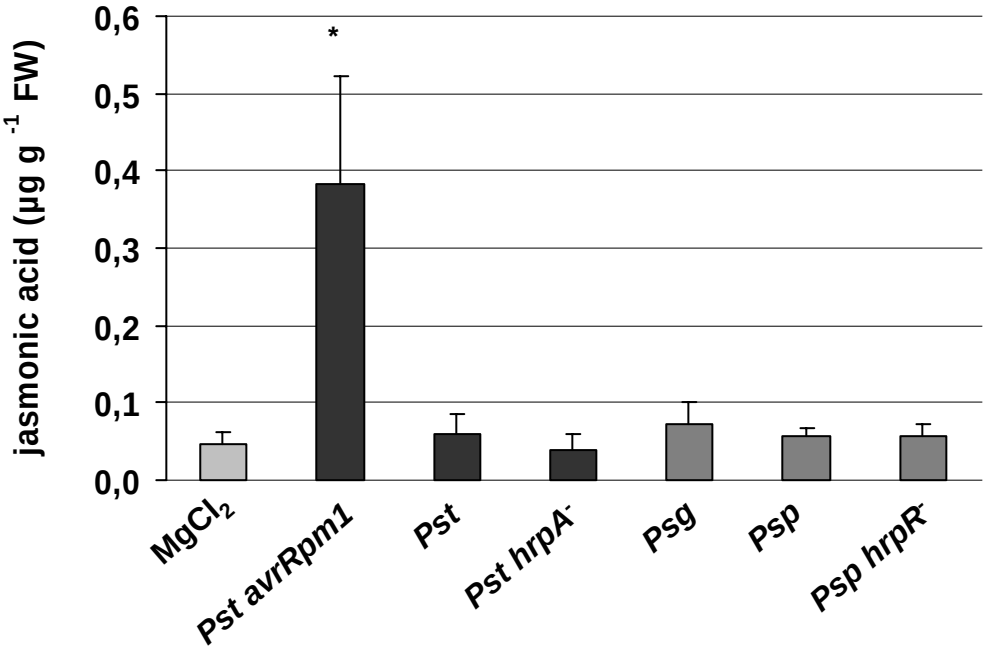

SAG (µg g -1 FW)
7
6
5
4
3
2
1
0
***
***
**
**
**
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-

Fig. 2

A


jasmonic acid (µg g -1 FW)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-

B

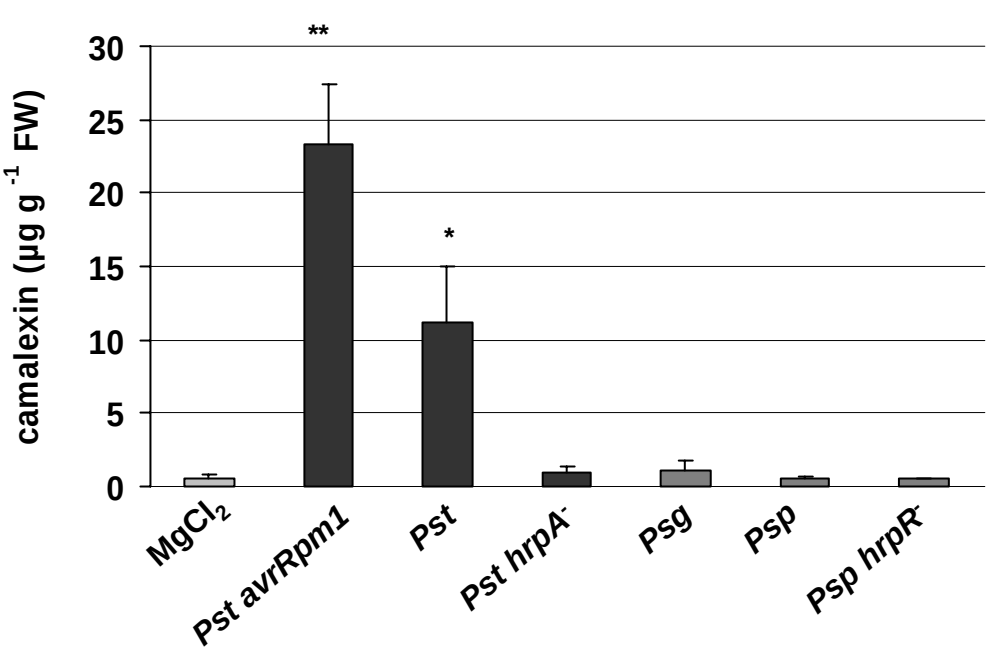

camalexin (µg g -1 FW)
30
25
20
15
10
5
0
**
*
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-

Fig. 3

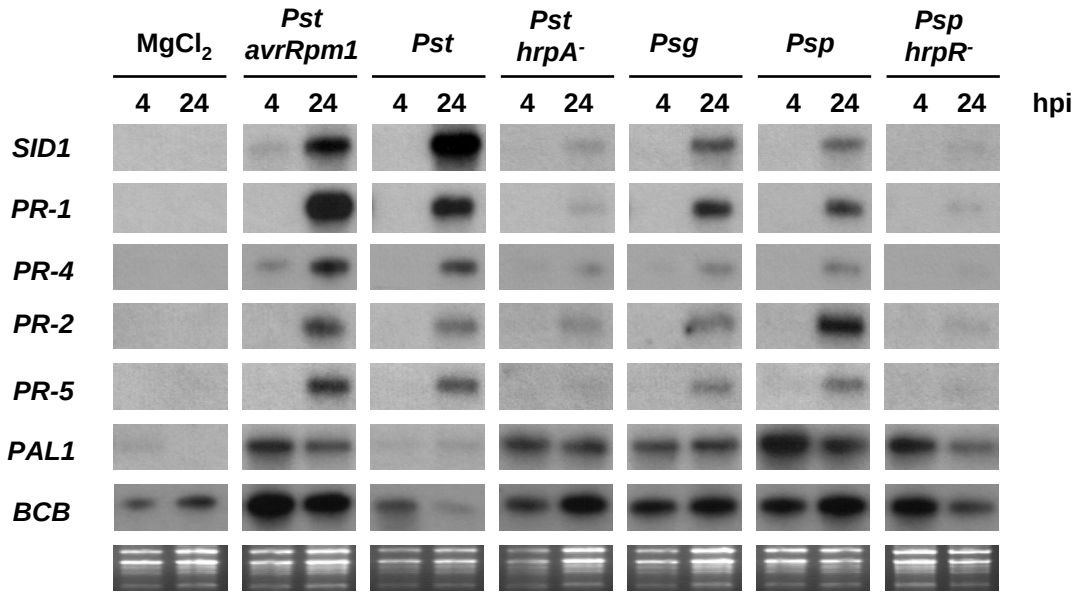
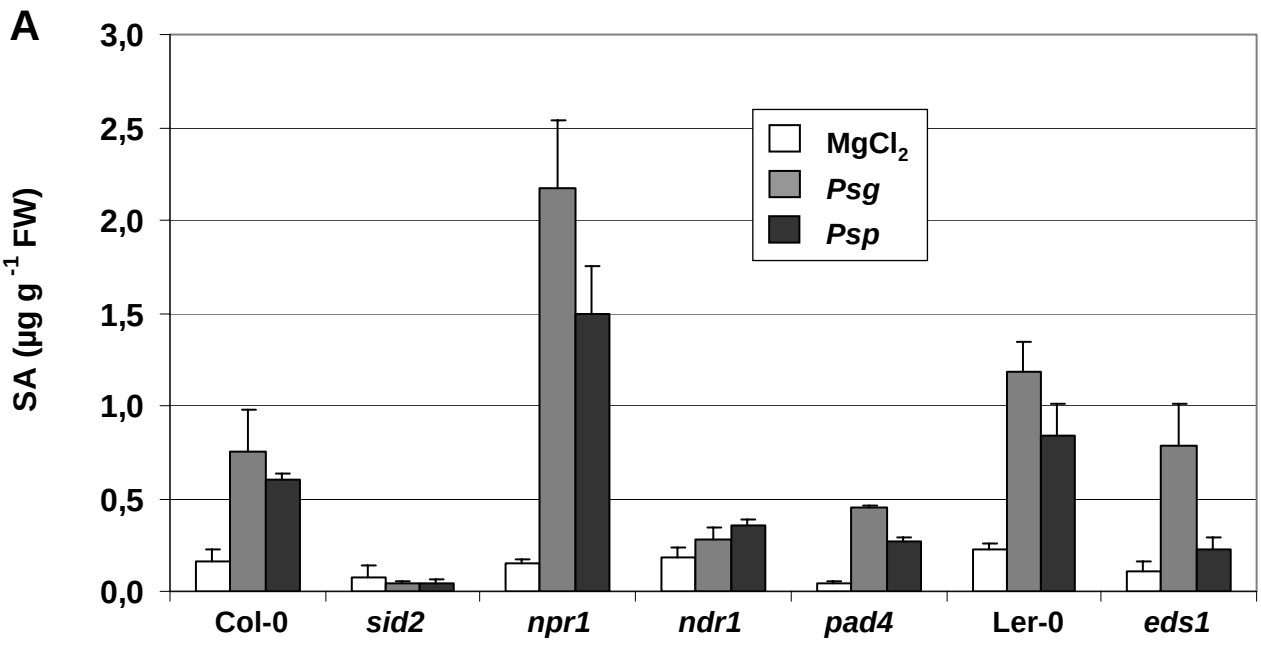

MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-
4 24 4 24 4 24 4 24 4 24 4 24 4 24
hpi
SID1
PR-1
PR-4
PR-2
PR-5
PAL1
BCB

Fig. 4


A
SA (µg g -1 FW)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
MgCl2
Psg
Psp
Col-0
sid2
npr1
ndr1
pad4
Ler-0
eds1

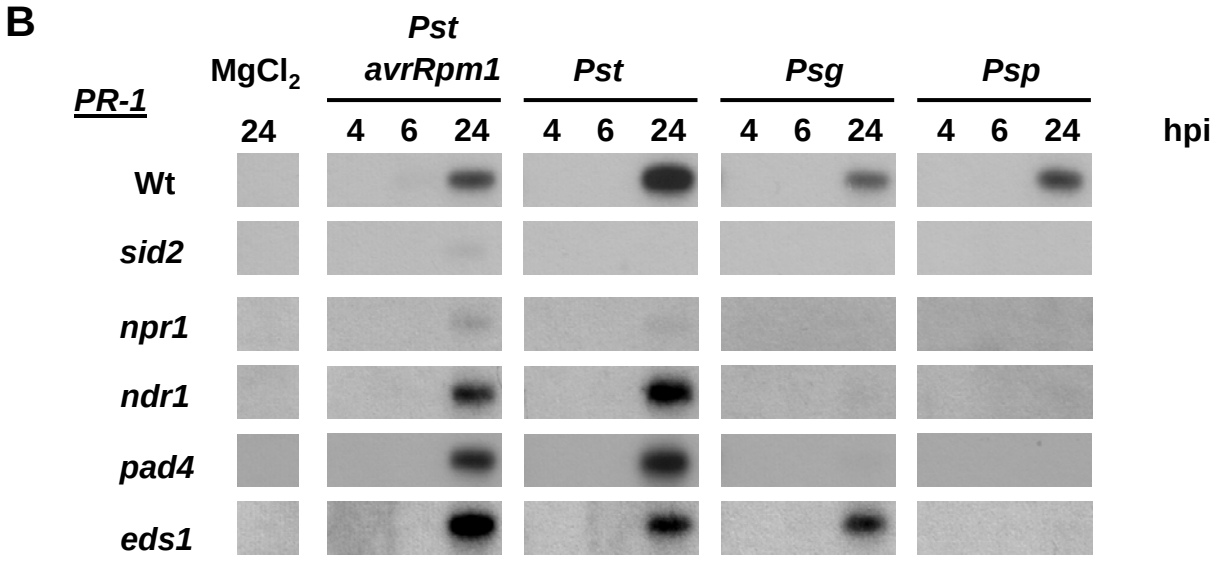
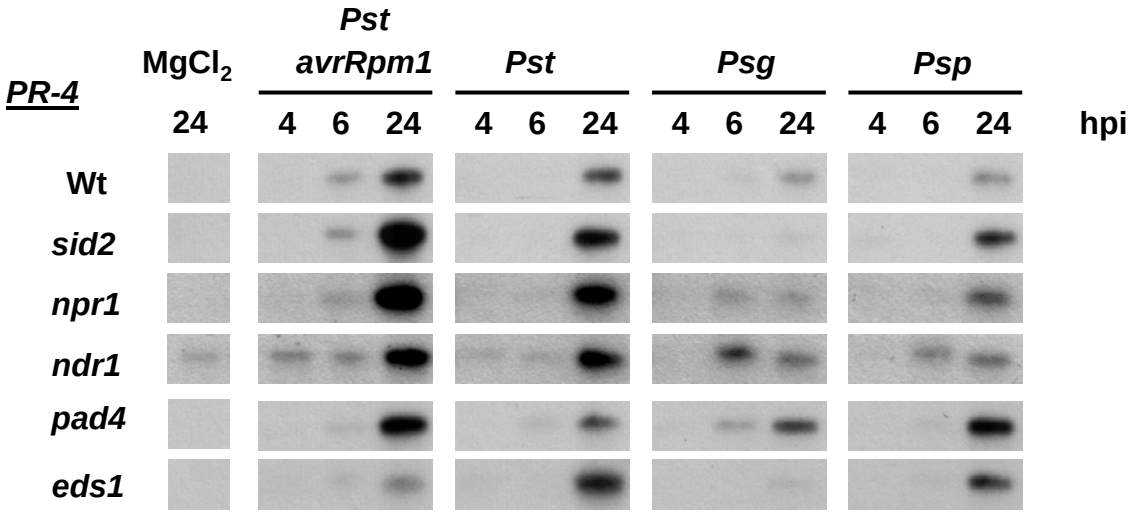

B
PR-1
MgCl2
Pst avrRpm1
Pst
Psg
Psp
24
4 6 24 4 6 24 4 6 24 4 6 24
hpi
Wt
sid2
npr1
ndr1
pad4
eds1

Fig. 5

A


PR-4
MgCl2
Pst avrRpm1
Pst
Psg
Psp
24
4 6 24 4 6 24 4 6 24 4 6 24
hpi
Wt
sid2
npr1
ndr1
pad4
eds1

B

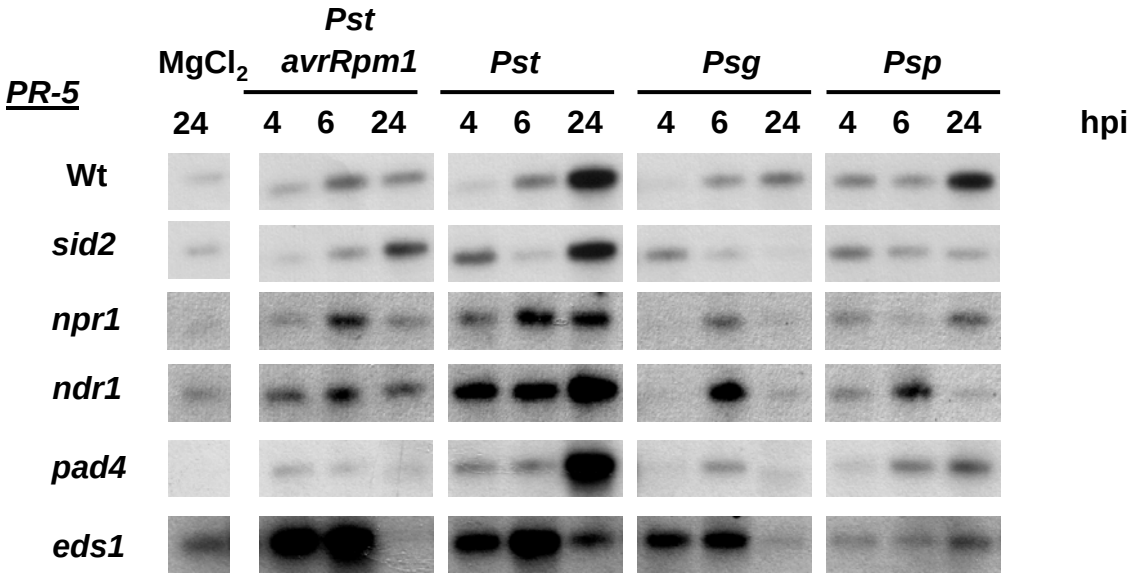

PR-5
MgCl2
Pst avrRpm1
Pst
Psg
Psp
24
4 6 24 4 6 24 4 6 24 4 6 24
hpi
Wt
sid2
npr1
ndr1
pad4
eds1

Fig. 6

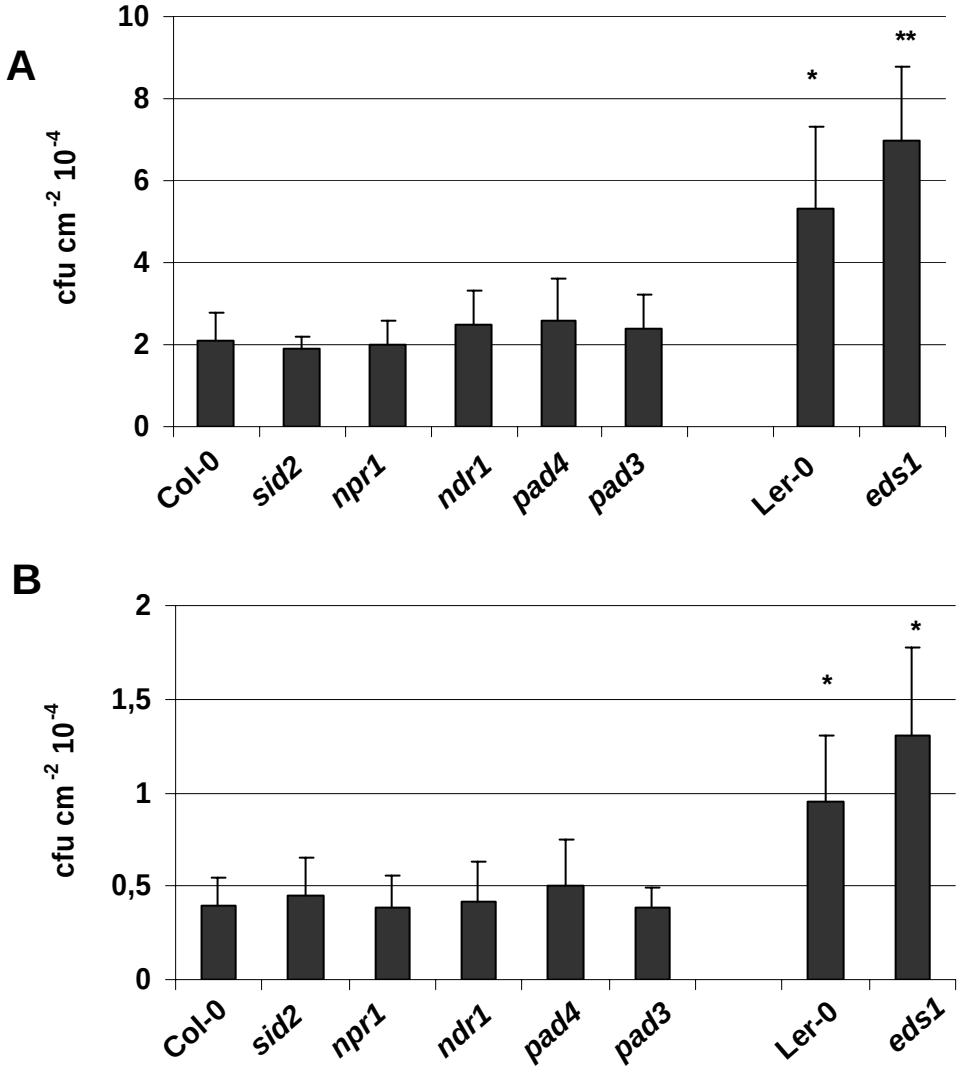

A
cfu cm -2 10-4
10
8
6
4
2
0
*
**
Col-0
sid2
npr1
ndr1
pad4
pad3
Ler-0
eds1
B
cfu cm -2 10-4
2
1,5
1
0,5
0
*
*
Col-0
sid2
npr1
ndr1
pad4
pad3
Ler-0
eds1

Fig. 7

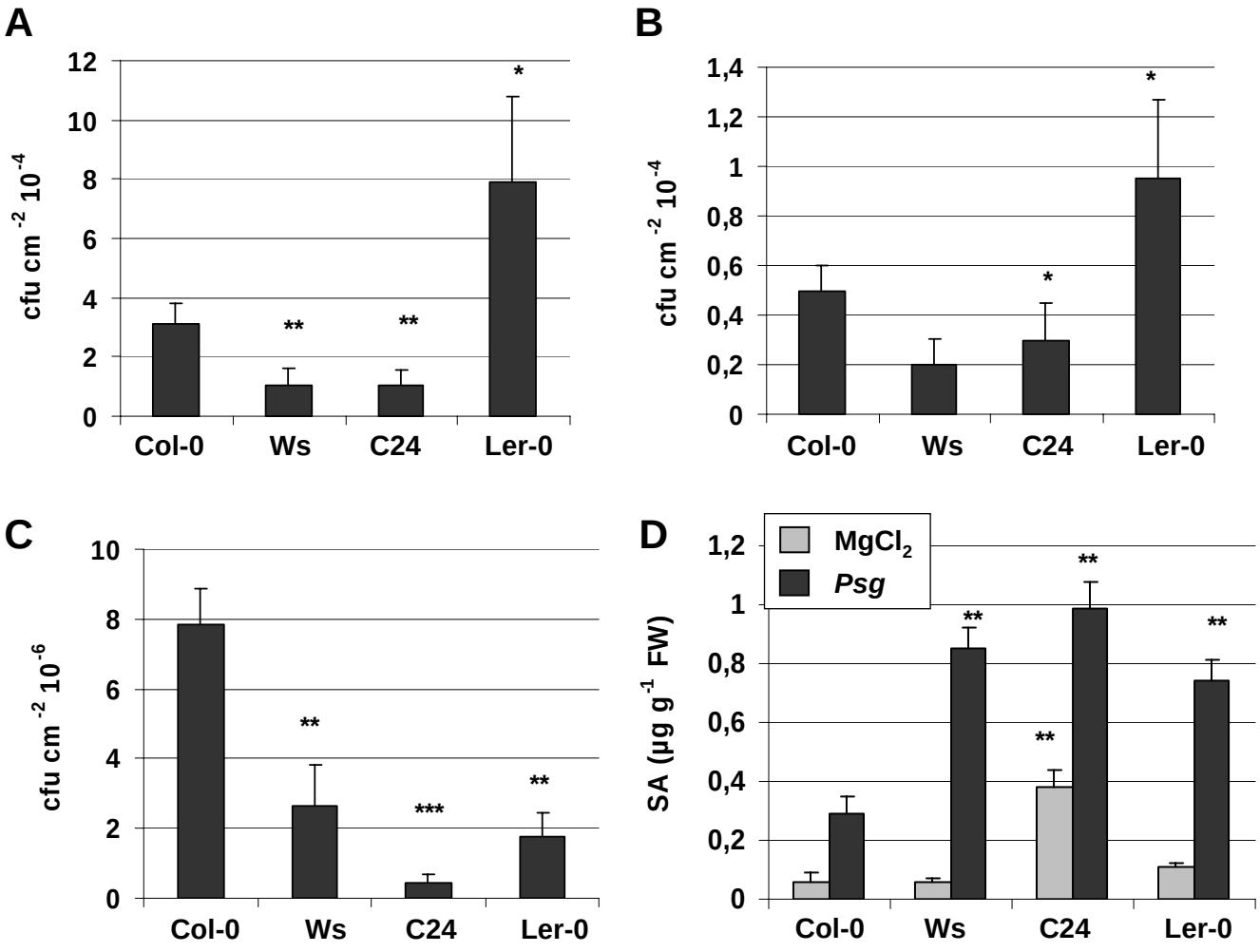

A
cfu cm -2 10-4
12
10
8
6
4
2
0
*
**
**
Col-0
Ws
C24
Ler-0
B
cfu cm -2 10-4
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
*
Col-0
Ws
C24
Ler-0
C
cfu cm -2 10-6
10
8
6
4
2
0
**
***
**
Col-0
Ws
C24
Ler-0
D
SA (µg g-1 FW)
MgCl2
Psg
1,2
1
0,8
0,6
0,4
0,2
0
**
**
**
**
Col-0
Ws
C24
Ler-0

Fig. 8

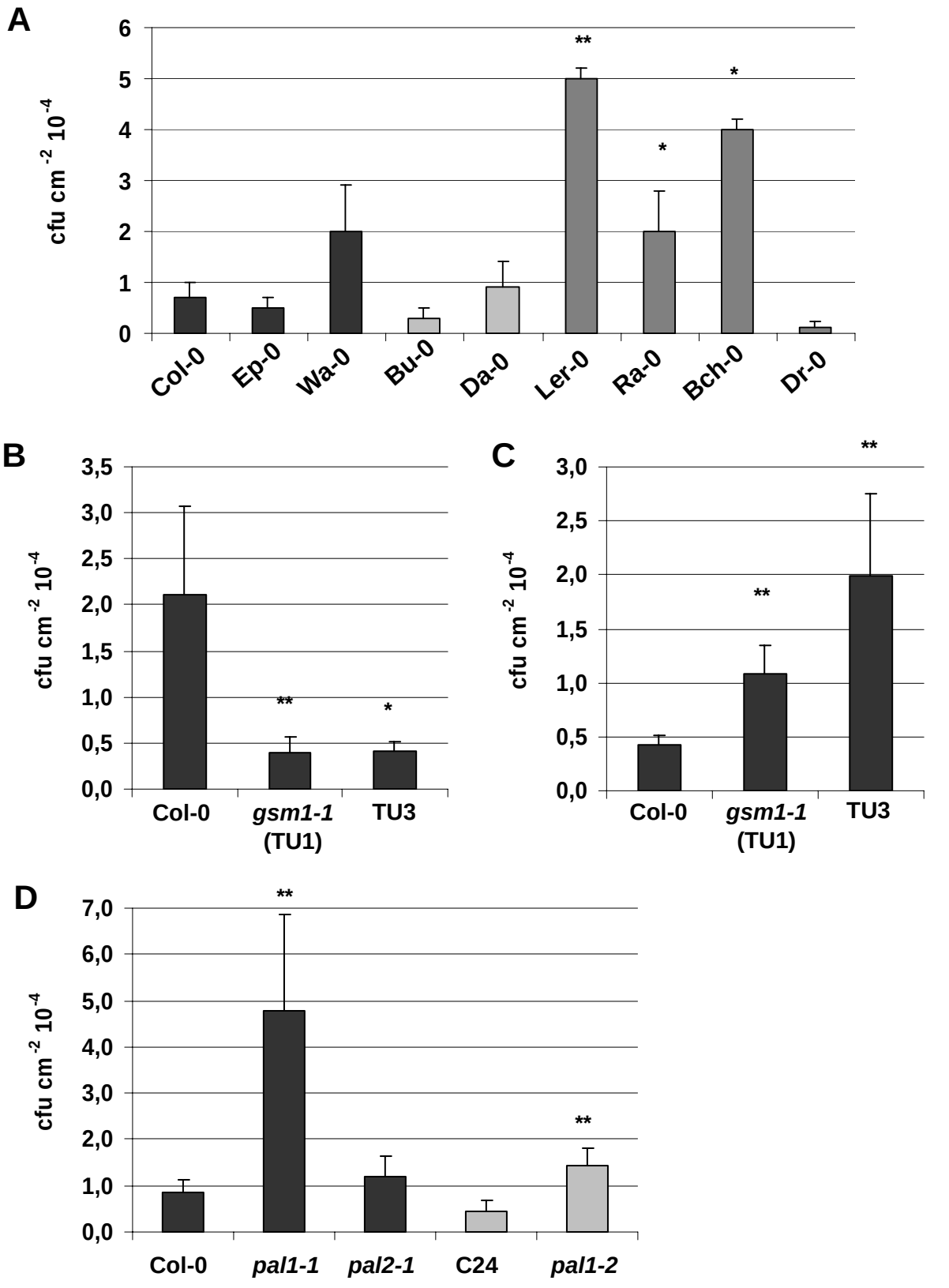

A
cfu cm -2 10-4
6
5
4
3
2
1
0
**
*
*
Col-0
Ep-0
Wa-0
Bu-0
Da-0
Ler-0
Ra-0
Bch-0
Dr-0
B
cfu cm -2 10-4
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
*
Col-0
gsm1-1 (TU1)
TU3
C
cfu cm -2 10-4
3,0
2,5
2,0
1,5
1,0
0,5
0,0
**
**
Col-0
gsm1-1 (TU1)
TU3
D
cfu cm -2 10-4
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
**
**
Col-0
pal1-1
pal2-1
C24
pal1-2

Fig. 9

PUBLICATION 5

Mishina TE, Zeier J

Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis.

Plant J (2007a) in press

The Plant Journal (2007) doi: 10.1111/j.1365-313X.2007.03067.x

Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis

Tatiana E. Mishina and Jürgen Zeier*

Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, Julius-von-Sachs-Platz 3, D-97082 Würzburg, Germany

Received 1 November 2006; revised 6 January 2007; accepted 12 January 2007.
*For correspondence (fax + 49 931 888 6235; e-mail zeier@botanik.uni-wuerzburg.de).

Summary

1 **Systemic acquired resistance (SAR) is usually described as a phenomenon whereby localized inoculation with a necrotizing pathogen renders a plant more resistant to subsequent pathogen infection. Here we show that *Pseudomonas syringae* strains for which *Arabidopsis thaliana* represents a non-host plant systemically elevate resistance although the underlying interactions neither trigger a hypersensitive response nor cause necrotic**
2 **disease symptoms. A similar enhancement of systemic resistance was observed when elicitor-active preparations of two typical bacterial pathogen-associated molecular patterns (PAMPs), flagellin and lipopolysaccharides (LPS), were applied in a localized manner. Several lines of evidence indicate that the observed systemic resistance responses are identical to SAR. Localized applications of non-adapted bacteria, flagellin or LPS elevate levels of the SAR regulatory metabolite salicylic acid (SA) and pathogenesis-related (*PR*) gene expression not only in treated but also in distant leaves. All treatments also systemically increase expression of the SAR marker gene *FLAVIN-DEPENDENT MONOOXYGENASE 1*. Further, a whole set of SAR-deficient Arabidopsis lines, including mutants in SA biosynthesis and signalling, are impaired in establishing the systemic resistance response triggered by non-host bacteria or PAMPs. We also show that the magnitude of defence reactions such as SA accumulation, *PR* gene expression or camalexin accumulation induced at sites**
3 **of virulent or avirulent *P. syringae* inoculation but not the extent of tissue necrosis during these interactions determines the extent of SAR in distant leaves. Our data indicate that PAMPs significantly contribute to SAR initiation in Arabidopsis and that tissue necroses at inoculation sites are dispensable for SAR activation.**

Keywords: Arabidopsis, systemic acquired resistance (SAR), flagellin, lipopolysaccharides (LPS), non-host bacteria, necrosis.

Introduction

Plants have evolved a number of strategies to defend themselves against microbial pathogens, both at the site of attempted ingress and in tissue distant from initial inoculation. Pre-formed structural and chemical barriers, as well as inducible plant responses triggered by invariant pathogen-associated molecular patterns (PAMPs), constitute the basis
4 of non-host resistance (Nürnberger and Lipka, 2005). To spread inside a plant and cause disease, a pathogen must be adapted to the particular plant species in order to avoid, tolerate and/or suppress the multi-faceted defences underlying non-host resistance. Phytopathogenic bacteria have evolved a type III secretion system (TTSS) delivering effector proteins into the plant cell to modify host responses (Nomura *et al.*, 2006). These effectors actively inhibit PAMP-triggered defence reactions and thus contribute to plant susceptibility (Li *et al.*, 2005). Even in compatible interactions that result in disease, PAMP-induced defences limit the spread of pathogens to a certain extent. This so-called basal resistance is compromised in various Arabidopsis *eds* (enhanced disease susceptibility) mutants that allow enhanced growth of a variety of virulent pathogens (Glazebrook *et al.*, 1996; Parker *et al.*, 1996). However, recognition of type III effectors through an adequate set of resistance (R) proteins still allows a host plant to effectively defend itself in an encounter with a well-adapted pathogen, resulting in specific or gene-for-gene resistance. Such incompatible

T P J | 3 0 6 7 | B
Journal Name | Manuscript No.
Dispatch: 27.2.07 | Journal: TPJ | CE: Bharathy
Author Received: | No. of pages: 14 | PE: Kajalakshmi

interactions involve a massive production of reactive oxygen species (ROS; oxidative burst), early defence gene induction and hypersensitive death of invaded plant cells (hypersensitive response, HR) (Lamb and Dixon, 1997).

PAMPs or 'general elicitors' include flagellin, lipopolysaccharides (LPS) and elongation factor Tu (EF-Tu) from Gram-negative bacteria and chitin, β-glucans and ergosterol from fungi (Ingle *et al.*, 2006; Kunze *et al.*, 2004). Flagellin is a protein subunit of the bacterial flagellum that is required for motility of bacterial pathogens. A 22 amino acid motif in the N-terminal region of flagellin (flg22) binds to the Arabidopsis receptor kinase FLS2 and elicits various defence responses including H_2O_2 production, pathogenesis-related (*PR*) gene induction and callose deposition through a MAP kinase cascade (Asai *et al.*, 2002; Chinchilla *et al.*, 2006; Felix *et al.*, 1999). Lipopolysaccharides (LPS) are indispensable cell surface components of Gram-negative bacteria associated with the outer membrane of the cell envelope. Structurally, LPS consists of a lipid part called lipid A, a core oligosaccharide and an O-polysaccharide part (Erbs and Newman, 2003). LPS from various bacterial sources trigger H_2O_2 production in tobacco (Meyer *et al.*, 2001), *PR* gene expression in tobacco and Arabidopsis (Coventry and Dubery, 2001; Zeidler *et al.*, 2004), and prime pepper leaves to more quickly mobilize several plant defence responses upon infection (Newman *et al.*, 2002). Pre-treatment of leaves with flagellin or LPS preparations renders those leaves more resistant to successional pathogen attack (Graham *et al.*, 1977; Newman *et al.*, 2002; Zipfel *et al.*, 2004).

Plants are also able to express induced defence responses at sites remotely located from initial infection, resulting in enhanced systemic resistance to subsequent pathogen encounter. These systemic resistance responses are generally grouped into two broad categories, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Grant and Lamb, 2006). SAR is considered as a response to a pathogen that causes a necrotic lesion, whereby lesion formation might be the result of a hypersensitive response during an incompatible interaction, or a consequence of successful infection in the course of a compatible interaction (Durrant and Dong, 2004; Hammerschmidt, 1999). The resistance conferred by SAR is long-lasting and effective against a broad pathogen spectrum, including viruses, bacteria, oomycetes and fungi. Establishment of SAR is dependent on a functional salicylic acid (SA) signalling pathway and is closely associated with systemic SA accumulation and systemic expression of a set of *PR* and other defence genes. Characterization of several SAR-deficient Arabidopsis mutants has shed light on the molecular regulation and the signalling requirements of SAR (Durrant and Dong, 2004; Grant and Lamb, 2006; Mishina and Zeier, 2006). Unlike SAR, ISR develops in response to colonization of plant roots by certain plant growth-promoting rhizosphere bacteria, and depends on jasmonic acid (JA) and ethylene signalling (Pieterse *et al.*, 1998; Ton *et al.*, 2002).

The *Arabidopsis thaliana–Pseudomonas syringae* pathosystem has been successfully used to dissect the molecular principles underlying local and systemic disease resistance in plants (Cameron *et al.*, 1994; Dong *et al.*, 1991; Whalen *et al.*, 1991). In contrast to interactions with virulent disease-causing and avirulent HR-producing 'host bacteria', interactions of Arabidopsis with non-adapted ('non-host') bacterial strains have been studied in much less detail. In the present study, we show that inoculation of leaves with *P. syringae* strains for which Arabidopsis represents a non-host plant enhances resistance at the systemic level although the interaction does not result in necrotic lesion development. Increased systemic resistance also develops when plants are locally treated with flg22 peptide or bacterial LPS. These symptomless systemic resistance responses are associated with systemic elevation of salicylic levels and *PR* gene expression, and experiments with various SAR-deficient Arabidopsis mutants revealed that the response is mechanistically identical to SAR. We also show that the levels of several active defence responses induced at sites of virulent or avirulent *P. syringae* inoculation rather than the extent of tissue necrosis determine the magnitude of SAR. These findings are in contrast to the general concept of SAR being a consequence of lesion formation in response to necrotizing or HR-inducing pathogens, but highlight a central role for PAMP recognition and induced defence responses in inoculated plant tissue during SAR induction.

Results

Non-adapted P. syringae *strains induce systemic resistance in Arabidopsis without necrotic lesion formation*

When infiltrated into the extracellular spaces of Arabidopsis leaves, the virulent *P. syringae* pathovars *tomato* DC3000 (*Pst*) and *maculicola* ES4326 (*Psm*) are able to rapidly multiply in the plant apoplast and cause water-soaked lesions that, depending on the inoculum concentration, exhibit a yellowish or necrotic appearance (Figure S1b) (Dong *et al.*, 1991; Whalen *et al.*, 1991). By contrast, inoculation of Arabidopsis with the *P. syringae* pathovars *glycinea* (*Psg*) or *phaseolicola* (*Psp*), soybean and bean pathogens, respectively (Lu *et al.*, 2001; Staskawicz *et al.*, 1984), results in a rapid initial decline in bacterial number (data not shown), and visible disease symptoms do not develop (Figure S1d,e). Nevertheless, these non-host bacteria induce salicylic acid accumulation and *PR* gene expression at the inoculation site when relatively high bacterial densities are applied (T.E. Mishina and J. Zeier, unpublished results).

Pst or *Psm* host strains that harbour the avirulence protein AvrRpm1 are rapidly recognized by Arabidopsis Col-0

through the RPM1 resistance protein, and consequently elicit an HR (Bisgrove *et al.*, 1994; Figure S1a,g). These avirulent HR-inducing strains are well known to induce SAR in Arabidopsis. The HR, however, is not a pre-requisite for SAR induction because virulent *P. syringae* strains causing necrotic disease symptoms instead of an HR (Figure S1b,g) are also capable of triggering SAR (Cameron *et al.*, 1994; Mishina and Zeier, 2006). To quantitatively compare the efficiency of SAR induction between avirulent and virulent strains, lower leaves ('primary leaves') of a given plant were treated with $MgCl_2$, *Pst avrRpm1* or *Pst* in a primary inoculation, and 2 days later, a secondary or challenge infection with virulent *Psm* was performed in upper leaves ('systemic leaves'). When *Pst avrRpm1* at an optical density (OD) of 0.02 was used for SAR induction, growth of *Psm* in systemic leaves at day 3 post-infection was reduced about fivefold compared with the $MgCl_2$ control treatment (Figure 1a). Application of the same OD of *Pst* in the primary inoculation caused a significantly higher growth reduction after challenge infection (about 15-fold; Figure 1a), demonstrating that rapidly multiplying, virulent bacteria more efficiently initiate SAR than avirulent bacteria whose growth is more restricted due to the hypersensitive cell death response.

In order to test whether the ability to trigger enhanced systemic resistance is dependent on type III secretion of effector proteins, we treated plants with the *Pst hrpA*$^-$ mutant strain that is defective in establishment of the TTSS (Roine *et al.*, 1997). To compensate for the inability of *Pst hrpA*$^-$ to multiply *in planta*, we used a fivefold higher inoculum density (OD 0.1) than for SAR experiments with *Pst* or *Pst avrRpm1*. Although *Pst hrpA*$^-$ neither caused any visible symptoms or an HR at inoculation sites (Figure S1c,g), it was able to initiate a significant systemic resistance response in Arabidopsis (Figure 1a). We next investigated whether the symptomless non-host interactions between *Psg* or *Psp* with Col-0 (Figure S1d,e) also led to an enhancement of resistance in non-treated, systemic leaves. Trypan blue staining revealed that *Psg* inoculation (OD 0.1) did not enhance the number of cells undergoing the HR inside the treated area when compared with a $MgCl_2$ control infiltration, whereas *Psp* inoculation at the same OD slightly increased microscopic HR lesion formation. The latter, however, was not comparable with the large increase in cells undergoing the HR after treatment with the avirulent strain *Pst avrRpm1* (Figure S1g). Similar to *Pst hrpA*$^-$ pre-treatment, local inoculation with both *Psg* and *Psp* significantly enhanced systemic resistance to subsequent infection (Figure 1a). This increase in systemic resistance was not dependent on functional type III secretion because the *Psp hrpR*$^-$ strain lacking the ability to build up a TTSS (Grimm *et al.*, 1995) conferred resistance in a similar manner as *Psp* (Figure 1a).

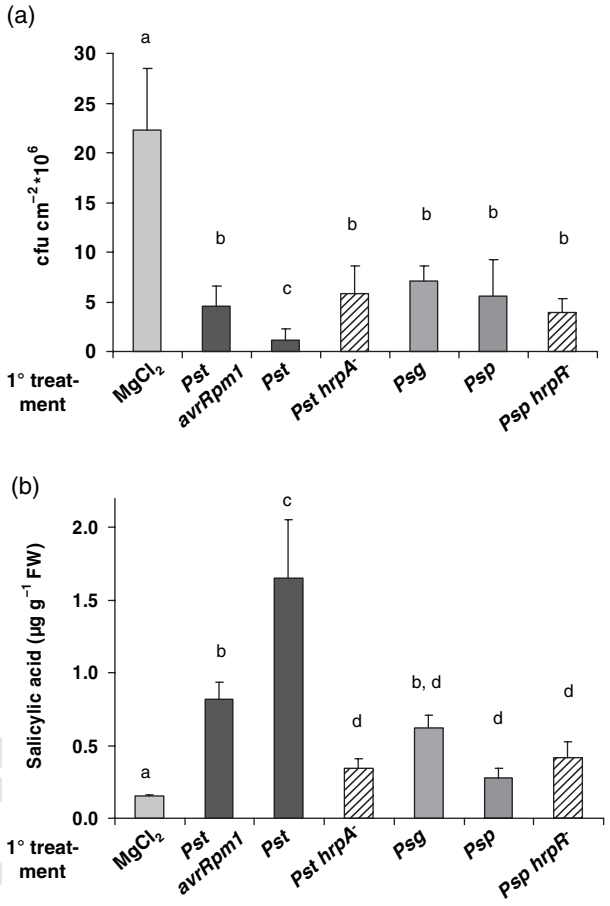


Figure 1. Systemic resistance and systemic salicylic acid accumulation induced in Arabidopsis by virulent, avirulent, non-host and TTSS-deficient *P. syringae* strains.

(a) Growth quantification of *Psm* during a secondary infection in upper leaves to assess systemic resistance induced by a primary treatment in lower leaves. Col-0 plants were pre-treated with 10 mM $MgCl_2$ or inoculated with various *P. syringae* strains [*Pst avrRpm1* (OD 0.02), *Pst* (OD 0.02), *Pst hrpA*$^-$ (OD 0.1), *Psg* (OD 0.1), *Psp* (OD 0.1), *Psp hrpR*$^-$ (OD 0.1)] in three lower (primary) leaves (1° treatment), and 2 days later, three upper leaves located directly above the primary leaves were challenged with *Psm* (OD 0.002). Bacterial growth in upper leaves was assessed 3 days post-infection (3 dpi). Bars represent mean values (±SD) of colony-forming units (cfu) per cm^2 from five to seven parallel samples each consisting of three leaf disks.

(b) Systemic accumulation of salicylic acid (SA). Primary leaves of Col-0 plants were treated as described in (a), and untreated upper leaves were harvested 2 days later for analysis. SA levels are given in microgram per gram leaf fresh weight (FW). Bars represent mean values (±SD) of three independent samples.

In (a) and (b), values for bars bearing different letters are significantly different at $P < 0.05$. Independent experiments were repeated twice with similar results.

Systemic resistance triggered by non-host bacteria is mechanistically identical to SAR

SAR is generally described as a response to necrotizing pathogens (Durrant and Dong, 2004; Hammerschmidt, 1999). Two characteristic features of SAR that distinguish this form of systemic resistance from ISR are accumulation

of salicylic acid and enhanced expression of a number of *PR* genes at the systemic level (Ryals *et al.*, 1996). As expected, necrotizing and SAR-inducing *Pst avrRpm1* and *Pst* both trigger a marked elevation of SA levels in non-treated, distant Col-0 leaves at 2 days post-inoculation (dpi) (Figure 1b). Similar to bacterial growth reduction (Figure 1a), systemic SA accumulation proved to be quantitatively more pronounced in response to the virulent than the avirulent strain. Moreover, *Pst avrRpm1* and *Pst* both triggered a strong induction of the SAR-associated defence genes *PR-1*, *PR-2*, *PR-4* and *PR-5* (Figure 2a). Significant systemic increases in SA levels and *PR* gene expression at 2 dpi were also observed when plants were treated with the non-host strains *Psg* and *Psp*, or the TTSS-deficient *Pst hrpA*$^-$ and *Psp hrpR*$^-$ strains, although overall systemic defences triggered by these symptomless interactions proved to be weaker than those induced after infection with *Pst* or *Pst avrRpm1* (Figures 1b and 2a). The flavin-dependent monooxygenase FMO1 has recently been identified as a central SAR component. Systemic expression of *FMO1* has been shown to reflect a molecular SAR characteristic, because it is closely associated with the ability of various Arabidopsis lines to establish SAR (Mishina and Zeier, 2006). Systemic *FMO1* expression not only occurred after localized contact with necrotizing *Pst* strains, but also after inoculation with non-adapted and TTSS-deficient *P. syringae* strains, further emphasizing the mechanistic similarity between systemic resistance induced by host and non-host bacteria (Figure 2b).

To investigate the molecular mechanisms underlying the observed symptomless systemic resistance response in more detail, we tested whether *Psg* or *Pst hrpA*$^-$ would be able to enhance systemic resistance in SAR-deficient Arabidopsis lines. The requirement of a functional SA signalling pathway for SAR in Arabidopsis is reflected by the inability of the SA biosynthesis mutant *sid2*, the SA-deficient *NahG* transgenics and the SA-insensitive *npr1* mutant to establish SAR (Delaney *et al.*, 1994; Durrant and Dong, 2004; Wildermuth *et al.*, 2001). As expected, SAR responses triggered by *Pst avrRpm1* or *Pst* in Col-0 were completely abolished in *sid2*, *NahG* and *npr1* plants. Similarly, we did not detect an increase in systemic resistance when these lines were treated with *Pst hrpA*$^-$ or *Psg*, demonstrating a requirement for SA signalling during the symptomless systemic resistance response (Figure 3a–c and Figure S2a). Other SAR-defective mutants include the defence mutant *ndr1* (non-race-specific disease resistance; Century *et al.*, 1995) and the *fmo1* mutant (Mishina and Zeier, 2006). Again, enhanced systemic resistance was not observed with either necrotizing or non-necrotizing *P. syringae* strains (Figure S2b and Figure 3d). Moreover, in contrast to their respective wild-type lines Col-0 and Ler-0, the *pad4* and *eds1* mutants, which are both compromised in basal resistance (Wiermer *et al.*, 2005), did not or only weakly showed SAR after contact with necrotizing *Pst avrRpm1* or *Pst*, and the symptomless interactions with *Pst hrpA*$^-$ or *Psg* also failed to increase systemic resistance in these mutants (Figure 3a,e–g). In camalexin-deficient *pad3* mutants and jasmonate-insensitive *jar1* mutants, however, SAR was triggered by *Pst* (±*avrRpm1*), and symptomless systemic resistance was induced by *Pst hrpA*$^-$ and *Psg* (Figure S2c and Figure 3h). This demonstrates that enhancement of systemic resistance by necrotizing and non-necrotizing *P. syringae* was independent of both camalexin accumulation and JA signalling. Together, the obvious similarities between systemic resistance induction by necrotizing and non-necrotizing pathogens indicate that non-host and TTSS-deficient *P. syringae* strains, such as necrotizing *Pst avrRpm1* and *Pst*, induce SAR in Arabidopsis.

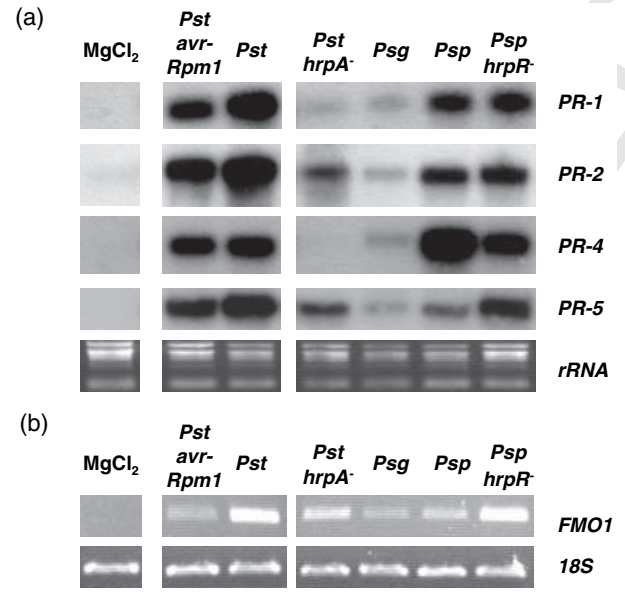


Figure 2. Systemic expression of defence-related genes in Col-0 plants inoculated with virulent, avirulent, non-host and TTSS-deficient *P. syringae*. Primary leaves were treated as described in Figure 1, and untreated upper leaves were harvested 2 days later for analysis.
(a) Systemic expression of *PR* genes as assessed by Northern blot analysis. Blots hybridized with specific ^{32}P-labelled DNA probes were exposed to X-ray films for 24 h.
(b) Systemic expression of the flavin-dependent monooxygenase gene *FMO1* as determined by RT-PCR analysis (30 PCR cycles). *18S* rRNA was amplified as a control (25 PCR cycles).

Active defence responses due to bacterial interaction with living plant tissue rather than necrotic lesion formation contribute to SAR initiation

To further examine the significance of necrotic lesion development during SAR initiation, we infiltrated primary leaves of Arabidopsis Col-0 plants with various inoculum densities of virulent *Psm*. Two days after infiltration with

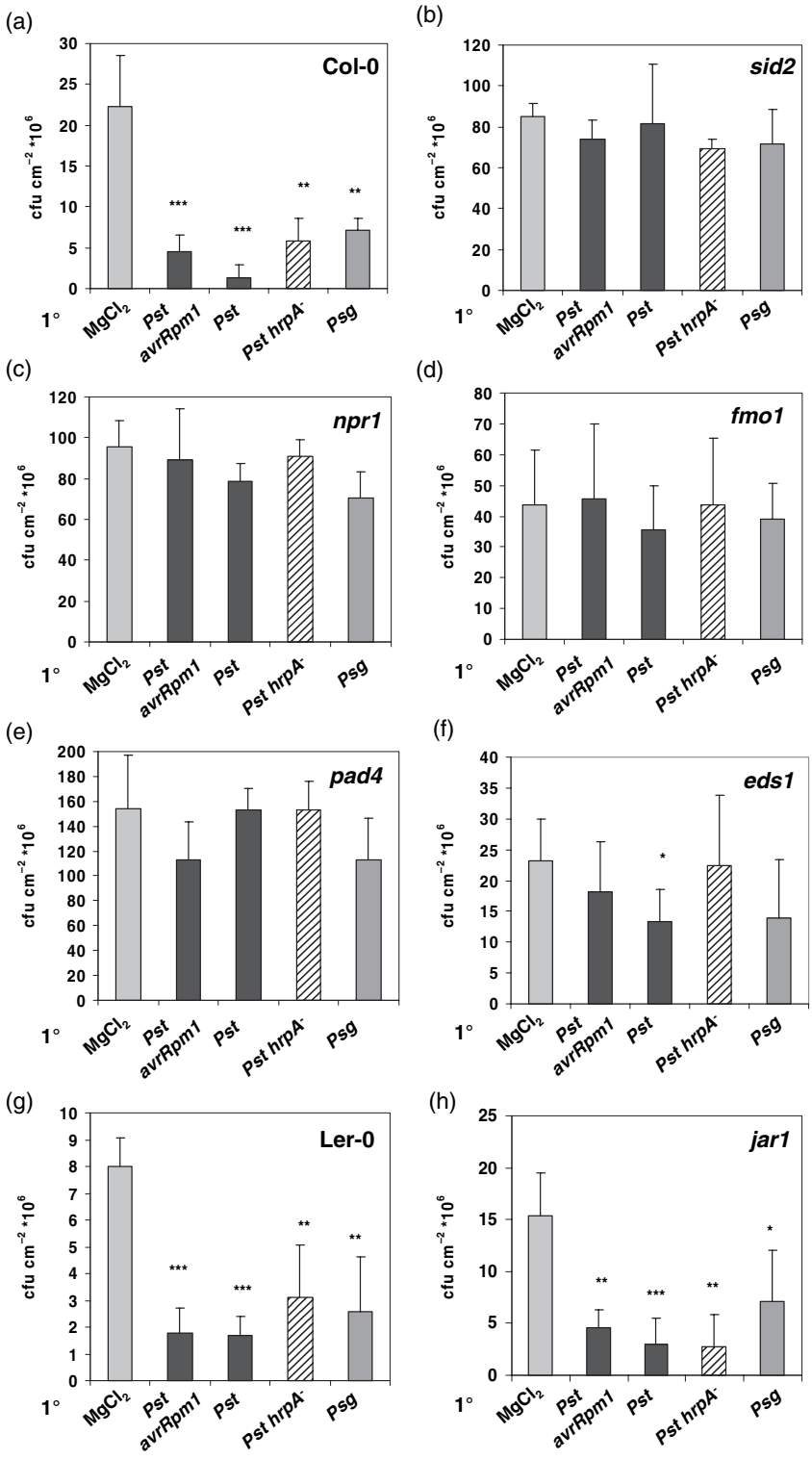


Figure 3. Systemic resistance induced by TTSS-deficient and non-host *P. syringae* is lost in SAR-deficient Arabidopsis lines.
(a–h) Growth quantification of virulent *Psm* during a secondary infection in upper leaves to assess systemic resistance in various Arabidopsis lines after a primary treatment in lower leaves. All mutant/transgenic lines have background Col-0, except *eds1* (Ler-0 background). Bars represent mean values (±SD) of colony-forming units (cfu) per cm^2 from five to seven parallel samples each consisting of three leaf disks. 1°, nature of primary treatment (for details, see legends to Figure 1). Asterisks denote values with statistically significant differences relative to the respective $MgCl_2$ control (*P < 0.05, **P < 0.01, ***P < 0.001; Student's t test).

bacterial ODs of 0.0002, 0.002, 0.02 or 0.2, treated leaf areas exhibited slight yellowing, medium yellowing, pronounced yellowing combined with necrosis, or strong necrotic tissue collapse, respectively (Figure 4a). At this time (2 dpi), we removed treated leaves from plants and performed challenge inoculations with low doses of *Psm* in upper, previously untreated leaves to determine the magnitude of acquired resistance that had developed in the latter within

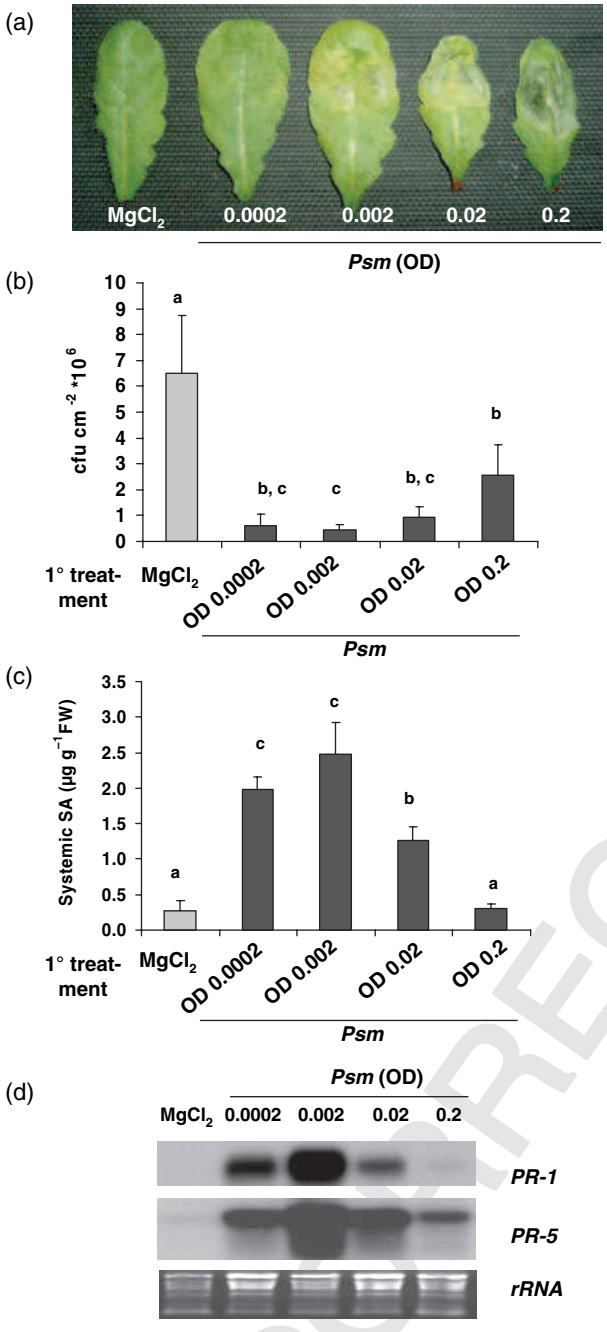


Figure 4. The efficiency of SAR in Arabidopsis is dependent on the bacterial inoculum density.
SAR and systemic defence responses develop most prominently when lower or medium inoculi levels are used that do not result in extensive tissue necrosis.
(a) Appearance of leaves infiltrated with $MgCl_2$ or with various ODs of virulent *Psm* 2 days after treatment. The numbers show the ODs applied. About 60% of each leaf area was infiltrated. Treated leaves were cut from plants at 2 days post-inoculation for visual documentation.
(b) Efficiency of the SAR response after treatment of plants with various ODs of *Psm*. Three lower leaves per plant were treated as indicated (1° treatment). Infiltrated leaves were removed at 2 dpi, and secondary inoculations with low doses (OD 0.002) of *Psm* in upper previously untreated leaves were performed. The magnitude of SAR was determined by quantifying bacterial numbers of *Psm* in upper leaves at 3 dpi. Bars represent mean values (±SD) of colony-forming units (cfu) per cm^2 from five to seven parallel samples each consisting of three leaf disks. Values for bars bearing different letters are significantly different at $P < 0.05$.
(c) Salicylic acid accumulation in upper leaves 2 days after treatment of lower leaves with various ODs of *Psm*. SA levels are given in microgram per gram FW. Bars represent mean values (±SD) of three independent samples. Values for bars bearing different letters are significantly different at $P < 0.05$.
(d) Defence gene expression in upper leaves 2 days after treatment of lower leaves with various ODs of *Psm*.

the first 2 days after primary inoculation. Although infiltrating *Psm* at the lowest inoculum (OD 0.0002) only resulted in weak yellowing symptoms without necrosis, we observed a strong SAR response, a marked elevation of SA levels in systemic leaves and a considerable increase in systemic *PR-1* or *PR-5* expression (Figure 4b–d). SAR efficiency and systemic defence responses increased to a maximum when the inoculum was increased to OD 0.002. However, a further inoculum increase to OD 0.02, which resulted in pronounced symptom development and tissue necrosis at the infection site, lowered both SAR efficiency and systemic SA or *PR* gene elevation (Figure 4b–d). This tendency was even more striking with the highest bacterial inoculum (OD 0.2) used for SAR induction. Here, a strong necrotic tissue collapse during the first 2 days after inoculation was accompanied with only a modest SAR response and weak *PR* gene expression, and systemic SA levels did not differ significantly from those of $MgCl_2$-treated control plants (Figure 4b–d).

We next characterized the extent of defence metabolite production at sites of infection, i.e. in primary leaves that we had removed from plants at day 2 post-inoculation in the above SAR experiment. Levels of free SA were significantly elevated by *Psm* irrespective of the inoculum OD, and the highest levels of free SA accumulated after treatment with the most concentrated OD of 0.2 (Figure 5a). However, simultaneous quantification of SA glucoside (SAG) revealed that total SA production at inoculation sites underwent a similar curve to SA accumulation in distant leaves or SAR efficiency, exhibiting the highest value at the medium OD of 0.002 (Figures 4b,c and 5b). A comparable tendency was obvious for camalexin accumulation in inoculated tissue (Figure 5c). By contrast, jasmonic acid production seemed to be positively correlated with the extent of symptom severity and leaf necrosis, as it accumulated to highest values after inoculation with ODs 0.02 and 0.2 (Figure 5d). Accumulation of the JA precursor 12-oxo-phytodienoic acid (OPDA) followed a similar trend (data not shown). *Psm*-induced expression of *PR-1* and *PR-5* at infection sites, however, followed a tendency comparable with total SA production, being more pronounced with lower than with higher inoculum densities (Figure 5e).

When the same experiment was conducted using varying ODs of avirulent *Psm avrRpm1* instead of virulent *Psm* for primary inoculation, similar relationships between inoculation density, tissue necrosis resulting from the HR, SAR

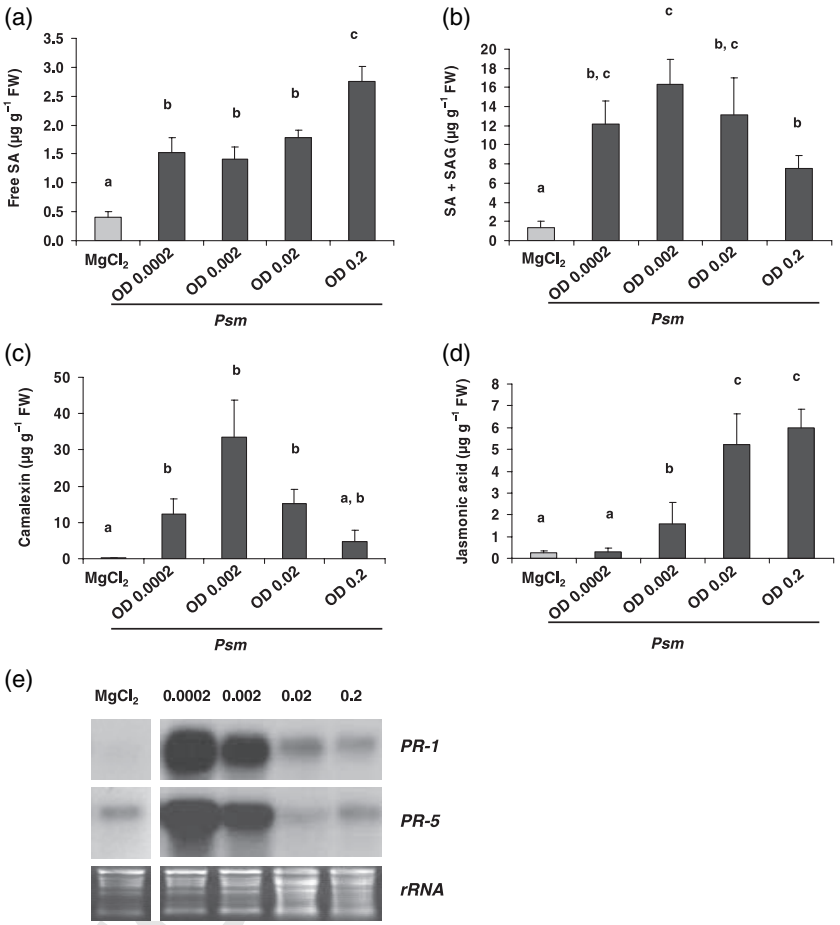


Figure 5. Magnitude of defence responses at inoculation sites relative to the *Psm* inoculum density at 2 dpi (leaf samples originate from the experiment described in Figure 4).
(a–d) Accumulation of defence signalling and antimicrobial compounds in leaves infected with various ODs of *Psm*. Control samples were treated with 10 mM $MgCl_2$. (a) Free salicylic acid levels. (b) Total salicylic acid (free plus glucosidic form). (c) Accumulation of the phytoalexin camalexin. (d) Jasmonic acid content.
(e) Defence gene expression in leaves inoculated with various ODs of *Psm* (2 dpi).

efficiency and local defence responses were observed (data not shown). Together, these experiments demonstrate that the magnitude of SAR induction and the extent of systemic defence responses are not related to the severity of symptoms and the development of necrotic lesions at the site of inoculation but rather correlate with the extent of initiated defence responses such as salicylic acid production, camalexin accumulation and *PR* gene expression in infected tissue.

Local treatment with flagellin or LPS induces SAR in Arabidopsis

The finding that avirulent, virulent, non-host and TTSS-deficient *P. syringae* strains are all able to trigger SAR in Arabidopsis prompted us to test whether common structural components or general elicitors would contribute to SAR induction. When heat-killed bacteria were infiltrated into Arabidopsis leaves at high optical densities, a significant enhancement in resistance of distant leaves to *Psm* challenge infection occurred, indicating that pre-existing structural components of bacteria are indeed able to trigger a SAR response (Figure 6a). We next investigated whether flagellin, a well-characterized PAMP involved in basal and non-host resistance, could trigger systemic resistance in various Arabidopsis ecotypes. When 200 nM of flg22, a peptide corresponding to the elicitor-active epitope of flagellin (Gomez-Gomez *et al.*, 1999), was infiltrated into lower leaves of Col-0 or Ler-0 plants, distant leaves experienced a marked increase in resistance in both ecotypes (Figure 6b). By contrast, enhancement of systemic resistance by flg22 was not observed in the Ws-0 ecotype, a natural *fls2* mutant insensitive to flagellin (Figure 6b). On the other hand, *Psm* was still capable of triggering SAR in Ws-0 plants, indicating that factors independent of flagellin perception contribute to biologically induced SAR. flg22-treated leaves exhibited a significant increase in SA and SAG levels 2 days after treatment, although this elevation of SA(G) was by far not as pronounced as SA(G) accumulation in response to *Psm* infection (Figure 7a,b). Moreover, the systemic resistance response induced by flg22 was associated with a small but significant increase of total SA levels in systemic leaves, whereas most of the SA was present in the glucosidic form at 2 dpi (Figure 7c,d). flg22 treatment also resulted in increased expression of *PR-1*, *PR-5* and *FMO1* in systemic leaves (Figure 7e, f). However, *Psm* enhanced systemic

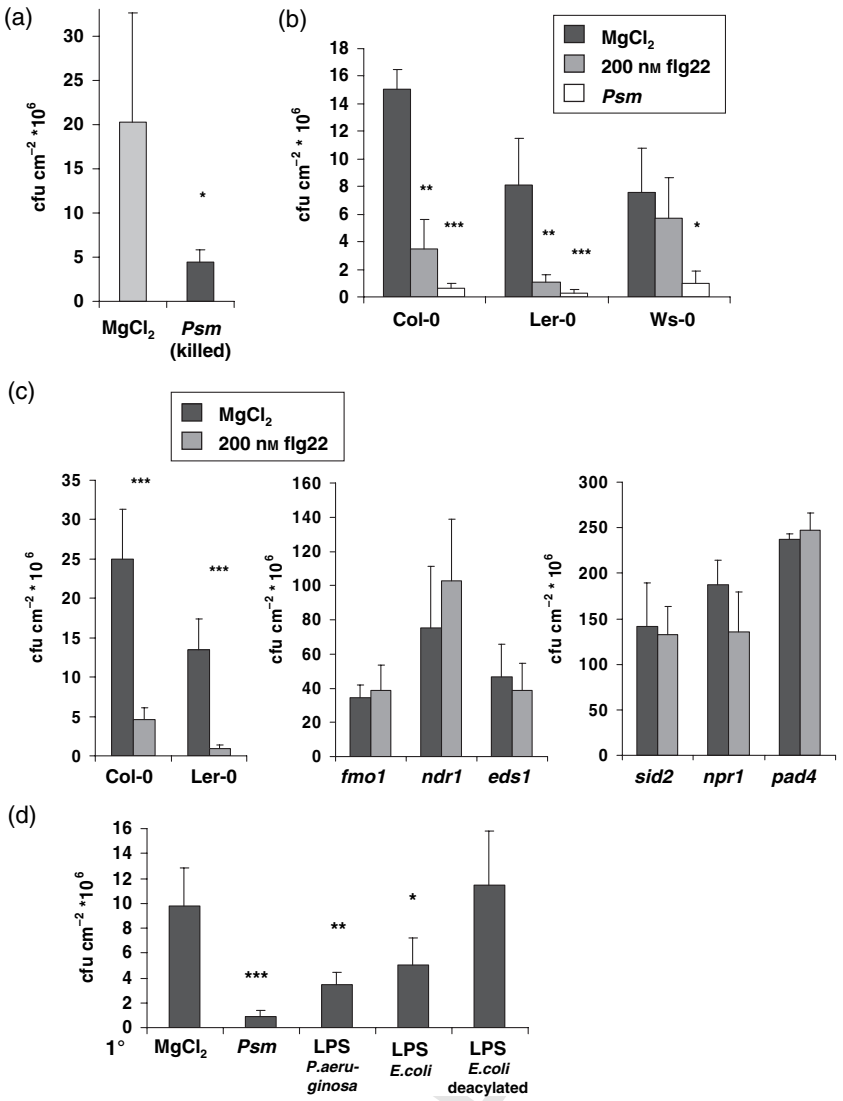


Figure 6. flg22 peptide and lipopolysaccharides induce SAR in Arabidopsis.
(a) Pre-treatment with heat-killed *Psm* (OD 2) enhances systemic resistance in Col-0 plants.
(b) Infiltration of 200 nM flg22 peptide, the elicitor-active fragment of flagellin, in lower leaves enhances resistance to subsequent infection in upper leaves in Col-0 and Ler-0, but not in Ws-0 plants. SAR induction by *Psm* (OD 0.02) in the same experimental set-up is shown for comparison.
(c) Loss of flg22-induced systemic resistance in SAR-deficient Arabidopsis mutants.
Bacterial growth assays were performed as described in the legend to Figure 1.
(d) Infiltration of purified LPS from *P. aeruginosa* or *E. coli* (100 μg ml^{-1}), but not of deacylated LPS, in lower leaves of Col-0 plants enhances resistance in upper leaves to subsequent infection. For comparison, SAR induction by *Psm* (OD 0.02) was conducted in the same experimental set-up.
Asterisks denote values with statistically significant differences relative to the respective $MgCl_2$ control (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Student's *t* test).

defence gene expression, as well as SA accumulation, to a much greater extent than flg22 treatment (Figure 7c–e). Similar to systemic resistance triggered by non-host bacteria, flagellin-induced systemic resistance was absent in the SAR-deficient mutants *sid2*, *npr1*, *ndr1*, *fmo1*, *eds1* and *pad4* (Figure 6c). Together, these data indicate that locally confined treatment with flagellin induces SAR in Arabidopsis.

We finally examined a possible involvement of lipopolysaccharides during SAR initiation in Arabidopsis. When gel-purified LPS preparations from *Pseudomonas aeruginosa* or *Escherichia coli* at a concentration of 100 μg ml^{-1} were infiltrated into primary leaves, systemic leaves exhibited significantly increased resistance to *Psm* infection 2 days after treatment. This systemic resistance response was not observed when a de-esterified LPS preparation lacking the lipid A part of the molecule was used (Figure 6d). LPS also triggered accumulation of free and glucosidic SA, both in treated and in systemic leaves (Figure 7a–d). Moreover, systemic expression of *PR-1*, *PR-5* and *FMO1* were increased by LPS application to a similar extent as by flg22 treatment (Figure 7e,f). The LPS-induced systemic resistance response did not develop in the SAR-defective mutants *sid2*, *npr1*, *ndr1*, *fmo1*, *eds1* and *pad4*, indicating that like flg22, LPS contributes to the induction of SAR in Arabidopsis (Figure S3).

Discussion

Systemic acquired resistance is a phenomenon whereby disease resistance to subsequent microbial infection is induced at the whole-plant level by a localized pathogen inoculation. Although the parameters critical for the induction of SAR are poorly understood, the development of tissue necrosis at the site of initial inoculation is considered a common and necessary feature for SAR activation (Durrant and Dong, 2004; Sticher *et al.*, 1997). This necrotic lesion

LOW RESOLUTION FIG

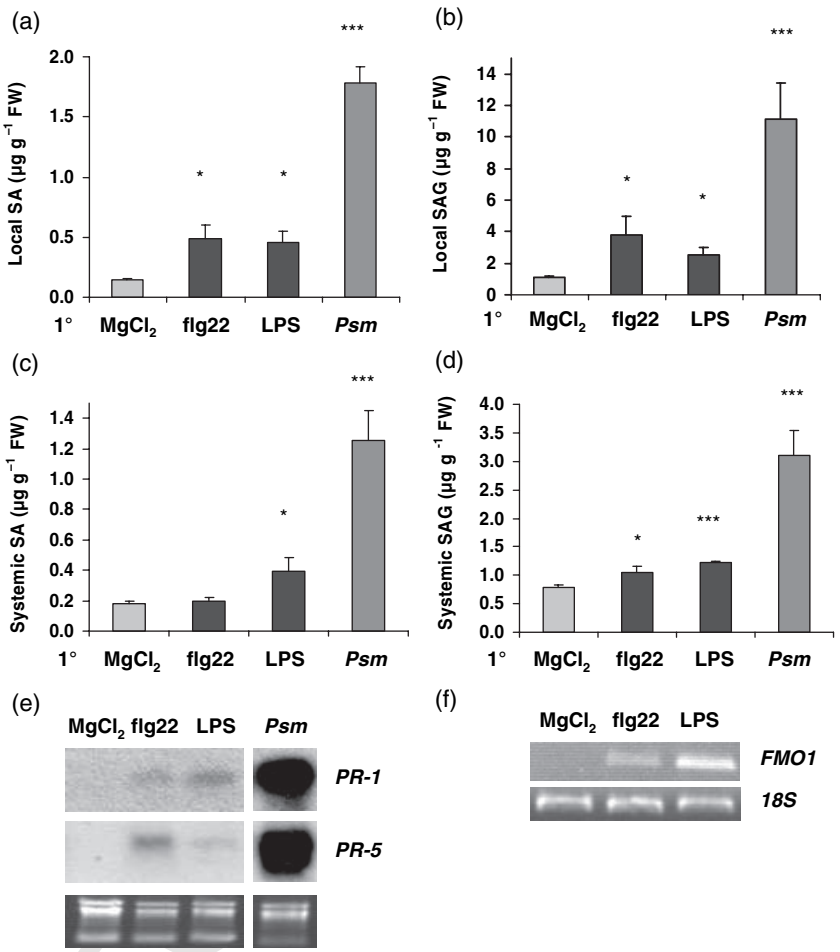


Figure 7. Defence responses triggered by flg22 and LPS treatment in Col-0 plants.
(a–d) Levels of free SA and glucosidic SA (SAG). Levels of (a) free SA and (b) SAG in leaves treated with flg22, LPS (*P. aeruginosa*) and Psm (0.02) at day 2 post-infection. (c) SA and (d) SAG levels in non-treated, systemic leaves at 2 days post-infiltration. SA(G) levels are given in microgram per gram FW. Bars represent mean values (±SD) of three independent samples. Asterisks denote values with statistically significant differences relative to the respective $MgCl_2$ control (*$P < 0.05$, ***$P < 0.001$; Student's *t* test).
(e,f) Defence-related gene expression in systemic leaves after local treatment with flg22, LPS (*P. aeruginosa*) or *Psm*.
(e) Expression of *PR* genes as determined by Northern blot analysis (exposure time 48 h).
21 (f) Expression of *FMO1*, assessed by RT-PCR (32 cycles). *18S* rRNA was amplified as a control (25 PCR cycles).

formation might result from an HR caused by avirulent pathogens, or originate from disease symptom development following successful infection by virulent pathogens (Cameron *et al.*, 1994; Hammerschmidt, 1999). In the present work, we have focused on the issue of SAR initiation within the Arabidopsis–*Pseudomonas* model pathosystem, which has been widely used to study systemic resistance phenomena in plants (Cameron *et al.*, 1994; Maldonado *et al.*, 2002; Pieterse *et al.*, 1998; Zeier, 2005; Zeier *et al.*, 2004). We show that non-adapted or TTSS-deficient *P. syringae* strains are able to induce SAR without producing tissue necroses or an HR, indicating that a necrotizing response is dispensable for SAR initiation. Our data also reveal that the magnitude of active defence responses at the site of primary inoculation rather than leaf necrosis correlates with SAR efficiency in distant leaves. Moreover, we demonstrate that flagellin and LPS, two typical bacterial PAMPs, significantly contribute to SAR induction in Arabidopsis.

Several lines of evidence indicate that the observed systemic resistance responses induced by non-adapted bacteria, flagellin or LPS are identical to SAR. First, their localized application not only elevates salicylic acid levels in treated leaves, but also causes SA accumulation in untreated, distant leaves (Figures 1 and 7). Such pathogen- or elicitor-triggered increases in local and systemic SA levels are a major characteristic of SAR (Sticher *et al.*, 1997). In addition, these treatments result in enhanced systemic expression of defence genes such as *PR-1* or *PR-5* that are systemically activated during SAR (Figures 2a and 7e; Ryals *et al.*, 1996). Second, increased systemic expression of the flavin-dependent monooxygenase gene *FMO1*, a molecular event that is closely associated with the SAR-induced state (Mishina and Zeier, 2006), occurs after treatment of Arabidopsis with non-adapted bacteria, flagellin and LPS (Figures 2b and 7f). Third, a whole set of SAR-deficient Arabidopsis mutants used in this study fail to express both SAR and systemic resistance induced by non-host bacteria, flagellin or LPS (Figures 3 and 6c and Figures S2 and S3). These mutants include the SA pathway mutant *sid2* that has a defect in the SA biosynthesis enzyme isochorismate synthase 1 (Wildermuth *et al.*, 2001), and *npr1*, which is not able to transduce the SA signal into downstream responses such as *PR* gene expression (Durrant and Dong, 2004). Further, mutational

defects in the defence signalling component NDR1, encoding a plasma membrane-localized signalling component required for several *R* gene-mediated signalling pathways (Century *et al.*, 1995), and defects in the flavin-dependent monooxygenase FMO1, compromise both SAR induced by necrotizing pathogens (Mishina and Zeier, 2006; Shapiro and Zhang, 2001) and the symptomless systemic resistance response described here. Moreover, mutations in EDS1 and its interacting partner PAD4, which are both required for basal resistance against virulent *P. syringae* strains (Wiermer *et al.*, 2005), result in strongly attenuated systemic resistance responses after contact with necrotizing and non-necrotizing *P. syringae*, as well as after treatment with flagellin and PAMPs. Finally, leaf infiltration of non-adapted *P. syringae* enhances systemic resistance in the jasmonate-insensitive *jar1* mutant, demonstrating that this acquired resistance response is mechanistically different from ISR, a JA-dependent but SA-independent process triggered by colonization of roots with growth-promoting rhizobacteria (Pieterse *et al.*, 1998).

In comparison with plant responses triggered by adapted bacterial pathogens, the molecular events induced by non-adapted bacteria are poorly defined. Only one Arabidopsis gene involved in bacterial non-host resistance, the glycerol kinase gene *NHO1*, has been identified so far (Kang *et al.*, 2003). However, inducible responses certainly represent an integral part of defence against non-host bacteria at inoculation sites. For instance, plant cell-wall alterations such as papilla formation and callose deposition might restrict water and nutrient flow towards the apoplast and thus prevent bacterial feeding and multiplication (Hauck *et al.*, 2003; Truman *et al.*, 2006). Some reports demonstrate that interactions of non-adapted bacterial pathogens with plants also result in locally
11 induced defence gene expression (Jakobek *et al.*, 1993; Lu *et al.*, 2001; Truman *et al.*, 2006). Both the TTSS-deficient *Pst hrpA*$^-$ and non-host bacteria (*Psg*, *Psp*) used in the present study enhance salicylic acid production and *PR* gene expression in inoculated Arabidopsis leaves (T.E. Mishina and J. Zeier, unpublished results). Thus, locally induced defences triggered by non-adapted pathogens and PAMPs are closely associated with the observed SAR response. Local defences are also related to SAR triggered by adapted bacteria, as we have found that the amount of total SA production, camalexin synthesis and *PR* gene expression at sites of *Psm* or *Psm avrRpm1* inoculation positively correlate with the magnitude of systemic resistance induction (Figures 4 and 5). Our data indicate the importance of SA-related defence pathways at the inoculation site for SAR induction, although a number of experiments argue against a direct role for SA as a long-distance SAR signal (Sticher *et al.*, 1997). However, one SA-derived compound with putative capacity to act as a mobile SAR signal is methyl salicylate (Forouhar *et al.*, 2005). By contrast, local JA accumulation correlates with tissue necrosis rather than SAR induction, suggesting that JA or JA-related oxylipins do not play a major role in SAR long-distance signalling.

As inoculations with either virulent, avirulent or non-host bacteria result in SAR establishment, common molecular themes must exist that induce SAR independently of resistance protein-mediated recognition events,
12 irrespective of the hypersensitive response, and independently of necrotic lesion formation. PAMPs such as bacterial flagellin and LPS represent such shared structural elements, occurring in both host and non-host *P. syringae* strains. We have shown that localized application of elicitor-active flg22 peptide, which corresponds to the most conserved domain of flagellin, and purified preparations of LPS trigger SAR in Arabidopsis (Figures 6 and 7), indicating that bacterial PAMPs not only play important roles in non-host and basal resistance, but also contribute to the initiation of systemic acquired resistance. Flagellin and LPS are well known to induce defence responses in treated leaf or cell culture tissue. Perception of flagellin by the receptor kinase FLS2 significantly contributes to basal resistance against *Pst*, and pretreatment of whole plants with flagellin renders them more resistant against subsequent pathogen attack (Zipfel *et al.*, 2004). Flagellin recognition initiates a mitogen-activated protein (MAP) kinase signalling cascade and activates WRKY transcription factors, resulting in upregulation of defence genes (Asai *et al.*, 2002; Navarro *et al.*, 2004). Its application provokes rapid extracellular medium alkalization in suspension-cultured cells (Felix *et al.*, 1999), triggers H_2O_2 production in Arabidopsis leaves and induces callose deposition and *PR* gene expression in seedlings (Gomez-Gomez *et al.*, 1999). However, flagellin treatment does not initiate hypersensitive cell death (Gomez-Gomez and Boller, 2002). Our finding that localized flagellin application induces SAR in Arabidopsis ecotypes Col-0 and Ler-0 with a functional FLS2 receptor but not in the natural *fls2* mutant Ws-0 extends the importance of flagellin perception in disease resistance from the local to the systemic level, and provides a further example that SAR can be initiated by elicitors that do not cause an HR. As Arabidopsis MAP kinase 4 functions as a negative regulator of the SA pathway (Petersen *et al.*, 2000), MAP kinase signalling is likely to represent a molecular link between flagellin perception and SAR.

The finding that *P. syringae* is able to cause SAR in Ws-0 highlights the fact that defence pathways independent of flagellin perception contribute to SAR initiation. Consistently, we have shown that LPS, a further general elicitor from bacteria, evoke SAR in Arabidopsis. Deacylated LPS lack the capability to induce SAR, confirming previous

results that the presence of the lipid A part is required for elicitor activity of LPS (Graham *et al.*, 1977). Like flagellin application, LPS treatment triggers H_2O_2 production, supporting the view that ROS might mediate a systemic signalling network contributing to SAR (Alvarez *et al.*, 1998). In line with our data showing that LPS enhance SA accumulation and *PR* gene expression in distant leaves, LPS have been demonstrated to induce systemic defence gene expression in tobacco and Arabidopsis (Coventry and Dubery, 2001; Zeidler *et al.*, 2004). Based on the finding that
13 LPS-treated pepper leaves more quickly mobilize production of antibacterial hydroxycinnamoyl-tyramine during subsequent pathogen infection (Newman *et al.*, 2002), it is possible that LPS-induced SAR results from increased antimicrobial activity through PR protein accumulation and/or is based on priming. However, with regard to SA accumulation, we have not found a priming response during LPS-induced SAR (data not shown). A further PAMP potentially contributing to *P. syringae*-induced SAR might be elongation factor Tu, because recognition of EF-Tu by the Arabidopsis receptor kinase EFR results in increased ROS production, ethylene synthesis and enhanced resistance of pre-treated leaves (Kunze *et al.*, 2004; Zipfel *et al.*, 2006). In addition, harpin, a glycine-rich acidic protein produced by Gram-negative plant pathogenic bacteria, represents an extracellular elicitor that is capable of inducing systemic defence gene expression and SAR in plants (Dong *et al.*, 1999).

We have shown that rapidly multiplying virulent bacteria induce a stronger SAR response than avirulent or non-host bacteria (Figure 1). The magnitude of SAR might thus be governed by the number of bacterial cells in the inoculated leaf, which in turn would determine the SAR-eliciting capacity through the number of PAMPs and other extracellular elicitors present in the leaf apoplast. PAMPs could contribute to the generation of endogenous SAR signals by activation of pattern recognition receptor-mediated responses. Alternatively, PAMP molecules themselves could act as long-distance signals that move out of pathogen-infected or PAMP-treated leaves and are systemically distributed to directly initiate defence responses in systemic tissue. Whether such a translocation of PAMPs takes place and whether sufficient amounts of PAMPs would systemically accumulate to noticeably enhance resistance remains to be determined.

Tissue necrosis that develops in response to infection with host bacteria might either be a side-effect of rapid bacterial multiplication and associated toxin production, or a consequence of an HR, but, according to our data, is not related to SAR initiation. The observation that necrotic tissue collapse does not necessarily result in SAR, e.g. tissue necrosis inflicted after contact with cold or very hot objects (Sticher *et al.*, 1997), and the existence of Arabidopsis mutants such as *dnd1* (defence-no-death), which exhibit enhanced disease resistance without cell death development after infection (Yu *et al.*, 1998), further supports this statement. On the contrary, an early necrotic collapse of infected leaves might even counteract SAR (Figure 4), presumably by disrupting the vascular tissue necessary for long-distance transport of systemic signals. Finally, the SAR-inducing capacity of PAMPs highlights the fact that many overlapping principles between basal resistance, non-host resistance and SAR exist, both at the level of elicitor perception and during downstream signalling.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. plants were grown in an autoclaved
14 mixture of soil (Fruhstorfer Pflanzenerde), vermiculite and sand
15 (9:1:1) in a controlled environment chamber (J-66LQ4, Percival) at 70% relative humidity, with a 9 h day period (photon flux density 70 μmol m^{-2} sec^{-1}, temperature 21°C) and a 15 h night period (temperature 18°C). For these experiments, 5 to 6-week-old, naïve and unstressed plants showing a uniform appearance were used.

A. thaliana ecotypes Col-0, Ler-0 and Ws-0 were obtained from the Nottingham Arabidopsis Stock Centre (lines N1092, NW20 and N1602, respectively). Unless otherwise stated, Col-0 was used as wild-type in all experiments. The following mutant or transgenic lines were used: *sid2-1* (Nawrath and Métraux, 1999), *NahG* (Delaney *et al.*, 1994), *npr1-2* (NASC line N3801), *ndr1* (Century *et al.*, 1995), *fmo1* (Mishina and Zeier, 2006), *pad3-1* (Glazebrook and Ausubel, 1994), *pad4-1* (Glazebrook *et al.*, 1997), *eds1-2* (Aarts *et al.*, 1998) and *jar1-1* (Staswick *et al.*, 1992).

Culture of bacteria and plant infection

P. syringae pv. *tomato* DC3000 (*Pst*), *P. syringae* pv. *maculicola* ES4326 (*Psm*) and the corresponding avirulent strains carrying the plasmid pLAFR3::avrRpm1 (*Pst avrRpm1*, *Psm avrRpm1*) originated
16 from C. Lamb (Maldonado *et al.*, 2002). *Pst hrpA*$^-$ (Roine *et al.*, 1997), *P. syringae* pv. *glycinea* race 4 (*Psg*; Staskawicz *et al.*, 1984) and *P. syringae* pv. *phaseolicola* NPS4008 ± *hrpR* (*Psp* and *Psp hrpR*$^-$; Grimm *et al.*, 1995) were obtained from S. Berger, C. Lamb and W. Aufsatz, respectively. Strains were grown overnight at 28°C in King's B medium containing the appropriate antibiotics (Zeier *et al.*, 2004). Overnight log-phase cultures were pelleted, washed three times with 10 mM $MgCl_2$, resuspended and diluted to the desired final concentration. The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1 ml syringe without a needle.

flg22 and LPS treatments

The flg22 peptide (QRLSTGSRINSAKDDAAGLQIA) corresponding to the elicitor-active domain of bacterial flagellin (Gomez-Gomez
17 *et al.*, 1999) was synthesized by Perbio Science. Aqueous flg22 aliquots were diluted in 10 mM $MgCl_2$ to a final concentration of 200 nM. Chromatographically purified LPS preparations from *E. coli* (L3024) and *P. aeruginosa* (L8643), as well as deacylated *E. coli* LPS (L3023), were purchased from Sigma-Aldrich (http://www.sigma-aldrich.com/), and diluted in 10 mM $MgCl_2$ to a final concentration of 100 μg ml^{-1}. Elicitor preparations were infiltrated into sample leaves as described above.

Characterization of systemic resistance responses

Three lower leaves of a given plant were first infiltrated with the various bacterial suspensions, elicitor preparations or a 10 mM $MgCl_2$ control solution. If not otherwise stated, a bacterial density of OD 0.02 was used for virulent and avirulent (*Pst* ± *avrRpm1* or *Psm* ± *avrRpm1*) strains. Non-host and TTSS-deficient strains (*Psg*, *Psp*, *Psp hrpR*$^-$, *Pst hrpA*$^-$) were inoculated at OD 0.1. Two days after the primary infiltration, non-treated upper leaves were either harvested for SA determination and gene expression analysis, or plants were inoculated on three upper leaves with virulent *Psm* (OD 0.002). Growth of *Psm* in upper leaves was assessed 3 days later by homogenizing disks originating from infiltrated areas of three different leaves in 1 ml 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium and counting colony numbers after incubating the plates under standardized conditions at 28°C for 2 days. To ensure the uniformity of the experiments, initial bacterial numbers (1 h post-inoculation; hpi) in leaves were also quantified. No significant differences in bacterial numbers were generally detected at 1 hpi for comparable bacterial growth experiments (Figures 1a, 3, 4b and 6, and Figures S2 and S3; data not shown). Bacterial numbers at 1 hpi amounted to 5.5 (±2.9) × 10^3 cfu cm^{-2}.

Quantification of microscopic HR lesions

The extent of microscopic HR lesion formation was assessed by Trypan blue staining, light microscopy and quantification of stained cells as previously described (Zeier *et al.*, 2004).

Analysis of gene expression

Analysis of defence gene expression was performed as described by Mishina and Zeier (2006). Expression levels of *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-4* (At3g04720) and *PR-5* (At1g75040) were determined by Northern blot analysis, and *FMO1* (At1g19250) expression was analysed by RT-PCR.

Determination of defence metabolites

Determination of SA, SAG, jasmonic acid, OPDA and camalexin levels in leaves was realised by a modified vapor-phase extraction method and subsequent gas chromatographic/mass spectrometric analysis as described in detail by Mishina and Zeier (2006).

Reproducibility of experiments and statistical analyses

All pathogen/elicitor experiments and the respective bacterial growth analyses, metabolite determinations and gene expression analyses depicted in the figures were conducted three times with similar results or tendencies. Statistical analyses were performed utilizing Student's *t* test for comparison of two data sets, and using ANOVA analysis (Fisher's LSD test) to analyse multiple data sets from comparable treatments.

Acknowledgements

We thank Werner Aufsatz, Chris Lamb and Susanne Berger for providing *P. syringae* strains, and Dirk Becker for allocating flg22 peptide. Donation of Arabidopsis mutant seeds by Volker Lipka and Juriaan Ton is gratefully acknowledged. This research was financially supported by the Deutsche Forschungsgemeinschaft (SFB 567).

Supplementary Material

The following supplementary material is available for this article online:

Figure S1. Development of macroscopic disease symptoms and microscopic HR cell death in Arabidopsis Col-0 leaves inoculated with various *P. syringae* strains.

Figure S2. Systemic resistance induced by TTSS-deficient and non-host *P. syringae* is lost in SAR-deficient Arabidopsis lines.

Figure S3. Loss of systemic resistance induced by *P. aeruginosa* LPS in SAR-deficient Arabidopsis mutants.

This material is available as part of the online article from http://www.blackwell-synergy.com.

References

Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E. (1998) Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two R gene-mediated signalling pathways in *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **95**, 10306–10311.

Alvarez, M.E., Pennell, R.I., Meijer, P.-J., Ishikawa, A., Dixon, R.A. and Lamb, C. (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell*, **92**, 773–784.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M. and Sheen, J. (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature*, **415**, 977–983.

Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A. and Innes, R.W. (1994) A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. *Plant Cell* **6**, 927–933.

Cameron, R.K., Dixon, R.A. and Lamb, C.J. (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* **5**, 715–725.

Century, K.S., Holub, E.B. and Staskawicz, B.J. (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. *Proc. Natl Acad. Sci. USA* **92**, 6597–6601.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G. (2006) The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* **18**, 465–476.

Coventry, H.S. and Dubery, I.A. (2001) Lipopolysaccharides from *Burkholderia cepacia* contribute to an enhanced defensive capacity and the induction of pathogenesis-related proteins in *Nicotianae tabacum*. *Physiol. Mol. Plant Pathol.* **58**, 149–158.

Delaney, T.P., Uknes, S., Vernooij, B. *et al.* (1994) A central role of salicylic acid in plant disease resistance. *Science*, **266**, 1247–1250.

Dong, X., Mindrinos, M., Davis, K.R. and Ausubel, F.M. (1991) Induction of Arabidopsis defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *Plant Cell* **3**, 61–72.

Dong, H., Delaney, T.P., Bauer, D.W. and Beer, S.V. (1999) Harpin induces disease resistance in Arabidopsis through the systemic acquired resistance pathway mediated by salicylic acid and the *NIM1* gene. *Plant J.* **20**, 207–215.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42**, 185–209.

Erbs, G. and Newman, M.-A. (2003) The role of lipopolysaccharides in induction of plant defence responses. *Mol. Plant Pathol.* **4**, 421–425.

Felix, G., Duran, J.D., Volko, S. and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**, 265–276.

Forouhar, F., Yang, Y., Kumar, D. *et al.* (2005) Structural and biochemical studies identify tobacco SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc. Natl Acad. Sci. USA* **102**, 1773–1778.

Glazebrook, J. and Ausubel, F.M. (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. *Proc. Natl Acad. Sci. USA* **91**, 8955–8959.

Glazebrook, J., Rogers, E.E. and Ausubel, F.M. (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics*, **143**, 973–982.

Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R. and Ausubel, F.M. (1997) Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. *Genetics*, **146**, 381–392.

Gomez-Gomez, L. and Boller, T. (2002) Flagellin perception: a paradigm for innate immunity. *Trends Plant Sci.* **7**, 251–256.

Gomez-Gomez, L., Felix, G. and Boller, T. (1999) A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *Plant J.* **18**, 277–284.

Graham, T.L., Sequeira, L. and Huang, T.S. (1977) Bacterial lipopolysaccharides as inducers of disease resistance in tobacco. *Appl. Environ. Microbiol.* **34**, 424–432.

Grant, M. and Lamb, C. (2006) Systemic immunity. *Curr. Opin. Plant Biol.* **9**, 414–420.

Grimm, C., Aufsatz, W. and Panopoulos, N.J. (1995) The *hrpRS* locus of *Pseudomonas syringae* pv. *phaseolicola* constitutes a complex regulatory unit. *Mol. Microbiol.* **15**, 155–165.

Hammerschmidt, R. (1999) Induced disease resistance: how do induced plants stop pathogens? *Physiol. Mol. Plant Pathol.* **55**, 77–84.

Hauck, P., Thilmony, R. and He, S.Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. *Proc. Natl Acad. Sci. USA* **100**, 8577–8582.

Ingle, R.A., Carstens, M. and Denby, K.J. (2006) PAMP recognition and the plant–pathogen arms race. *BioEssays*, **28**, 880–889.

Jakobek, J.L., Smith, J.A. and Lindgren, P.B. (1993) Suppression of bean defense responses by *Pseudomonas syringae*. *Plant Cell* **5**, 57–63.

Kang, L., Li, J., Zhao, T., Xiao, F., Tang, X., Thilmony, R., He, S. and Zhou, J.M. (2003) Interplay of the *Arabidopsis* nonhost resistance gene *NHO1* with bacterial virulence. *Proc. Natl Acad. Sci. USA* **100**, 3519–3524.

Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G. (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* **16**, 3496–3507.

Lamb, C. and Dixon, R.A. (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

Li, X., Lin, H., Zhang, W., Zou, Y., Zhang, J., Tang, X. and Zhou, J.M. (2005) Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. *Proc. Natl Acad. Sci. USA* **102**, 12990–12995.

Lu, M., Tang, X. and Zhou, J.M. (2001) Arabidopsis *NHO1* is required for general resistance against *Pseudomonas* bacteria. *Plant Cell* **13**, 437–447.

Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C. and Cameron, R.C. (2002) A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. *Nature*, **419**, 399–403.

Meyer, A., Puhler, A. and Niehaus, K. (2001) The lipopolysaccharides of the phytopathogen *Xanthomonas campestris* pv. *campestris* induce an oxidative burst reaction in cell cultures of *Nicotiana tabacum*. *Planta*, **213**, 214–222.

Mishina, T.E. and Zeier, J. (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. *Plant Physiol.* **141**, 1666–1675.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T. and Jones, J.D. (2004) The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant Physiol.* **135**, 1113–1128.

Nawrath, C. and Métraux, J.P. (1999) Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. *Plant Cell* **11**, 1393–1404.

Newman, M.A., von Roepenack-Lahaye, E., Parr, A., Daniels, M.J. and Dow, J.M. (2002) Prior exposure to lipopolysaccharide potentiates expression of plant defenses in response to bacteria. *Plant J.* **29**, 487–495.

Nomura, K., Debroy, S., Lee, Y.H., Pumplin, N., Jones, J. and He, S.Y. (2006) A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science*, **313**, 220–223.

Nürnberger, T. and Lipka, V. (2005) Non-host resistance in plants: new insights into an old phenomenon. *Mol. Plant Pathol.* **6**, 335–345.

Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J. (1996) Characterization of *eds1*, a mutation in Arabidopsis suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. *Plant Cell* **8**, 2033–2046.

Petersen, M., Brodersen, P., Naested, H. *et al.* (2000) *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. *Cell*, **103**, 1111–1120.

Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J. and van Loon, L.C. (1998) A novel signaling pathway controlling induced systemic resistance in Arabidopsis. *Plant Cell* **10**, 1571–1580.

Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.L., Kalkkinen, N., Romantschuk, M. and He, S.Y. (1997) Hrp pilus: an *hrp*-dependent bacterial surface appendage produced by *Pseudomonas syringae* pv. *tomato* DC3000. *Proc. Natl Acad. Sci. USA* **94**, 3459–3464.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell* **8**, 1809–1819.

Shapiro, A.D. and Zhang, C. (2001) The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. *Plant Physiol.* **127**, 1089–1101.

Staskawicz, B.J., Dahlbeck, D. and Keen, N.T. (1984) Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. *Proc. Natl Acad. Sci. USA* **81**, 6024–6028.

Staswick, P.E., Su, W. and Howell, S.H. (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proc. Natl Acad. Sci. USA* **89**, 6837–6840.

Sticher, L., Mauch-Mani, B. and Métraux, J.P. (1997) Systemic acquired resistance. *Annu. Rev. Phytopathol.* **35**, 235–270.

Ton, J., De Vos, M., Robben, C., Buchala, A., Métraux, J.P., Van Loon, L.C. and Pieterse, C.M. (2002) Characterization of

Arabidopsis enhanced disease susceptibility mutants that are affected in systemically induced resistance. *Plant J.* **29**, 11–21.

Truman, W., de Zabala, M.T. and Grant, M. (2006) Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. *Plant J.* **46**, 14–33.

Whalen, M.C., Innes, R.W., Bent, A.F. and Staskawicz, B.J. (1991) Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. *Plant Cell* **3**, 49–59.

Wiermer, M., Feys, B.J. and Parker, J.E. (2005) Plant immunity: the EDS1 regulatory node. *Curr. Opin. Plant Biol.* **8**, 383–389.

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature*, **414**, 562–565.

Yu, I.-c., Parker, J. and Bent, A.F. (1998) Gene-for-gene disease resistance without the hypersensitive response in *Arabidopsis* 20 *dnd1* mutant. *Proc. Natl Acad. Sci. USA* **95**, 7819–7824.

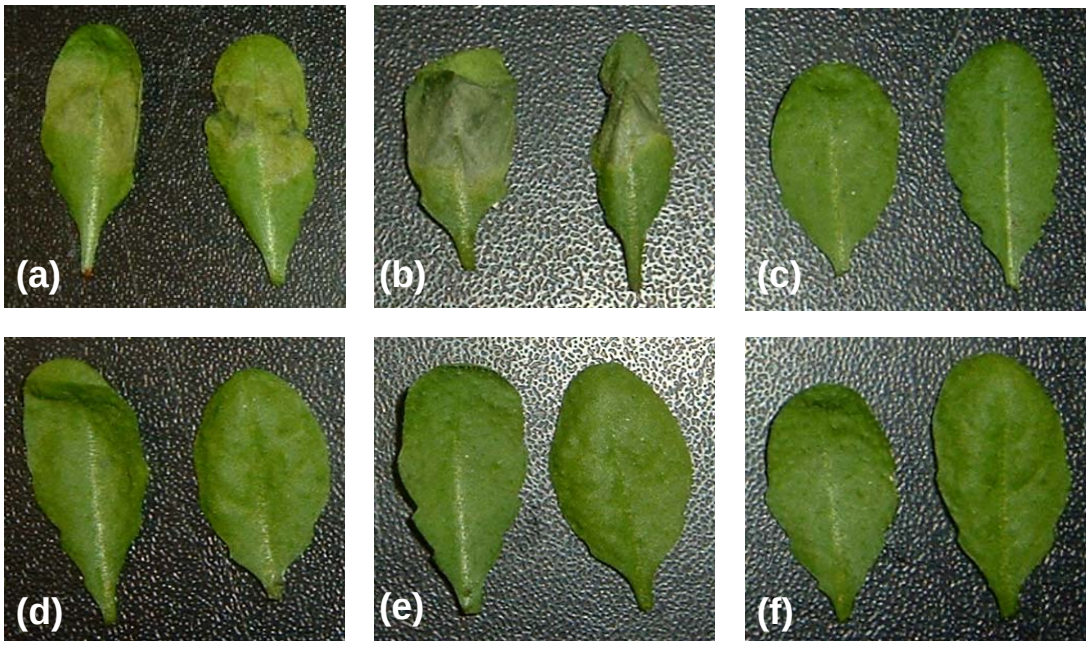
Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P. and Durner, J. (2004) Innate immunity in *Arabidopsis thaliana*: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc. Natl Acad. Sci. USA* **101**, 15811–15816.

Zeier, J. (2005) Age-dependent variations of local and systemic defence responses in *Arabidopsis* leaves towards an avirulent strain of *Pseudomonas syringae*. *Physiol. Mol. Plant Pathol.* **66**, 30–39.

Zeier, J., Pink, B., Mueller, M.J. and Berger, S. (2004) Light conditions influence specific defence responses in incompatible plant–pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. *Planta*, **219**, 673–683.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G. and Boller, T. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature*, **428**, 764–767.

Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller, T. and Felix, G. (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell*, **125**, 749–760.


(a)
(b)
(c)
(d)
(e)
(f)

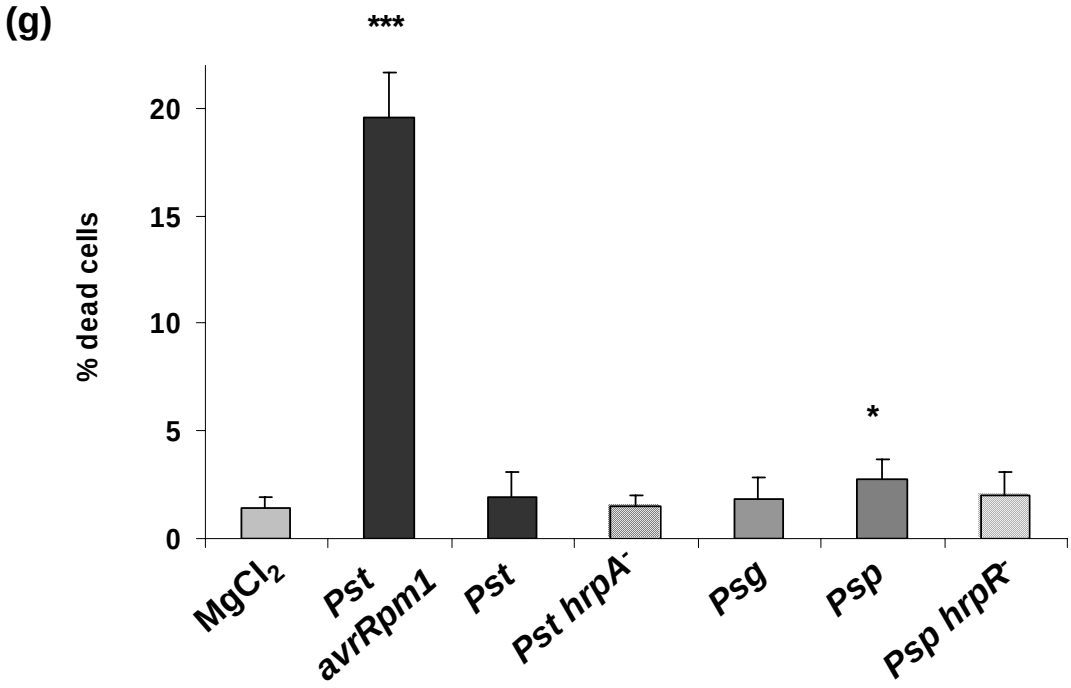

(g)
***
*
% dead cells
0
5
10
15
20
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
Psp
Psp hrpR-

Fig. S1

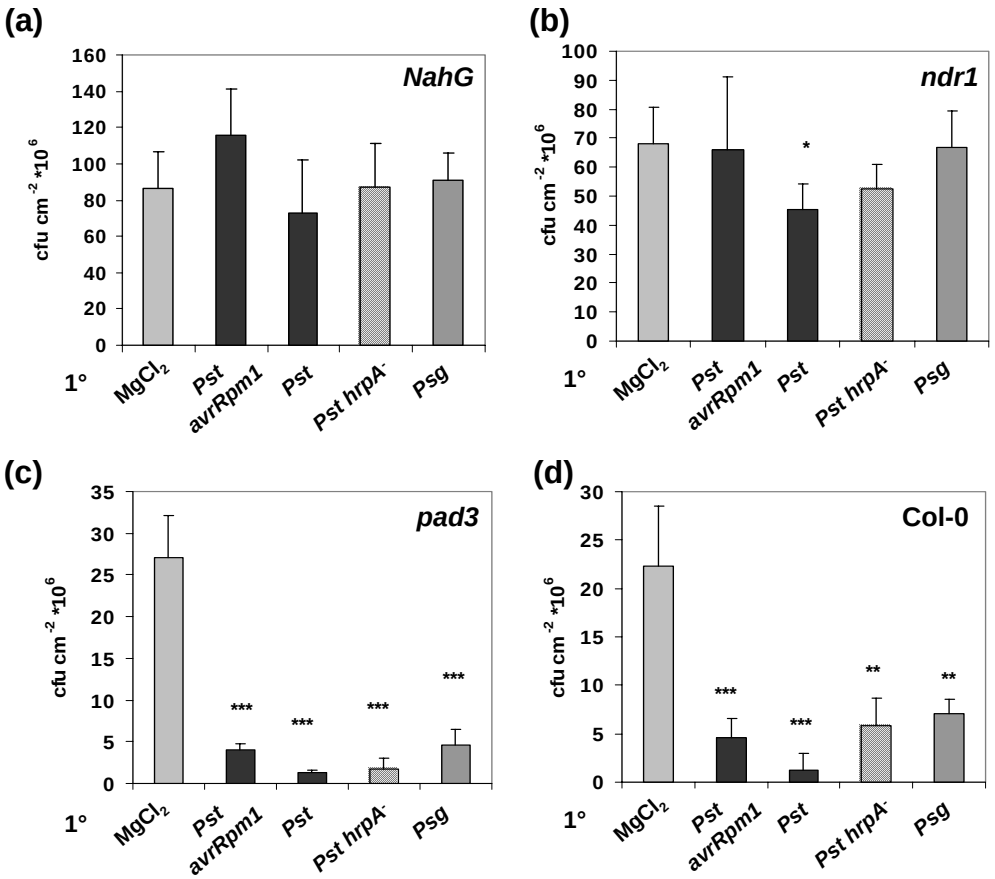

(a)
NahG
cfu cm -2 *10 6
160
140
120
100
80
60
40
20
0
1°
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
(b)
ndr1
cfu cm -2 *10 6
100
90
80
70
60
50
40
30
20
10
0
*
1°
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
(c)
pad3
cfu cm -2 *10 6
35
30
25
20
15
10
5
0
***
***
***
***
1°
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg
(d)
Col-0
cfu cm -2 *10 6
30
25
20
15
10
5
0
***
***
**
**
1°
MgCl2
Pst avrRpm1
Pst
Pst hrpA-
Psg

Fig. S2

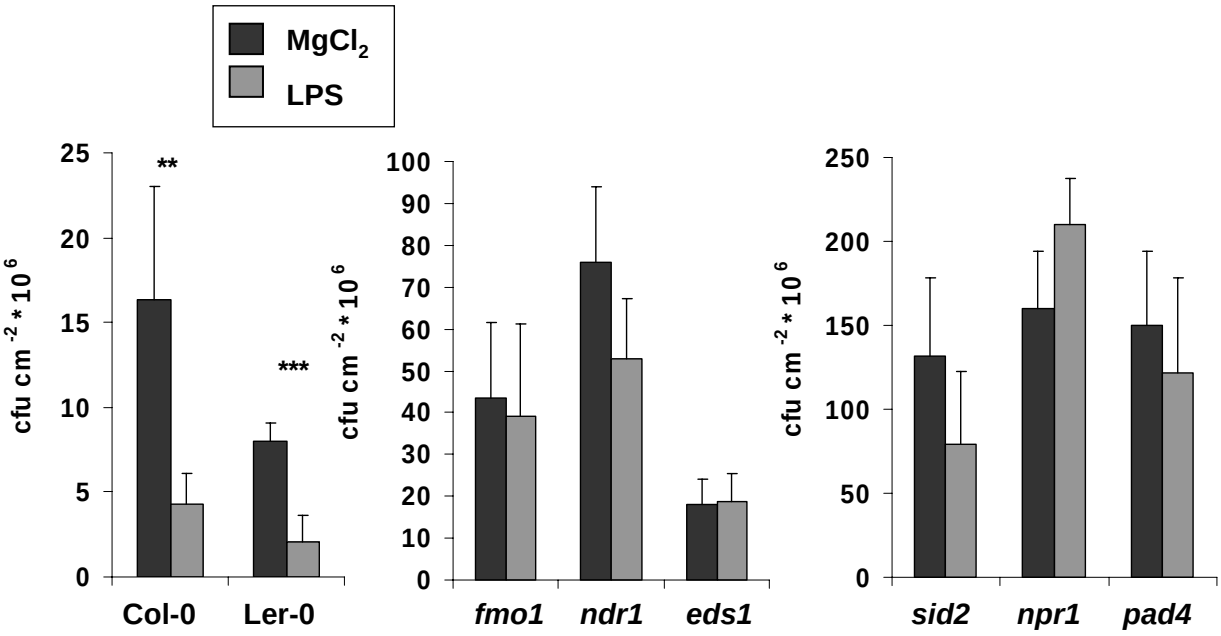

MgCl2
LPS
cfu cm -2 * 10 6
25
20
15
10
5
0
**
***
Col-0
Ler-0
cfu cm -2 * 10 6
100
90
80
70
60
50
40
30
20
10
0
fmo1
ndr1
eds1
cfu cm -2 * 10 6
250
200
150
100
50
0
sid2
npr1
pad4

Fig. S3

PUBLICATION 6

Mishina TE, Zeier J

The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance.

Plant Physiol (2006) 141: 1666-1675

The Arabidopsis Flavin-Dependent Monooxygenase FMO1 Is an Essential Component of Biologically Induced Systemic Acquired Resistance[1][OA]

Tatiana E. Mishina and Jürgen Zeier*

Julius-von-Sachs-Institute of Biological Sciences, University of Würzburg, D–97082 Wuerzburg, Germany

Upon localized attack by necrotizing pathogens, plants gradually develop increased resistance against subsequent infections at the whole-plant level, a phenomenon known as systemic acquired resistance (SAR). To identify genes involved in the establishment of SAR, we pursued a strategy that combined gene expression information from microarray data with pathological characterization of selected Arabidopsis (*Arabidopsis thaliana*) T-DNA insertion lines. A gene that is up-regulated in Arabidopsis leaves inoculated with avirulent or virulent strains of the bacterial pathogen *Pseudomonas syringae* pv *maculicola* (*Psm*) showed homology to flavin-dependent monooxygenases (FMO) and was designated as *FMO1*. An Arabidopsis knockout line of *FMO1* proved to be fully impaired in the establishment of SAR triggered by avirulent (*Psm avrRpm1*) or virulent (*Psm*) bacteria. Loss of SAR in the *fmo1* mutants was accompanied by the inability to initiate systemic accumulation of salicylic acid (SA) and systemic expression of diverse defense-related genes. In contrast, responses at the site of pathogen attack, including increases in the levels of the defense signals SA and jasmonic acid, camalexin accumulation, and expression of various defense genes, were induced in a similar manner in both *fmo1* mutant and wild-type plants. Consistently, the *fmo1* mutation did not significantly affect local disease resistance toward virulent or avirulent bacteria in naive plants. Induction of *FMO1* expression at the site of pathogen inoculation is independent of SA signaling, but attenuated in the Arabidopsis *eds1* and *pad4* defense mutants. Importantly, *FMO1* expression is also systemically induced upon localized *P. syringae* infection. This systemic up-regulation is missing in the SAR-defective SA pathway mutants *sid2* and *npr1*, as well as in the defense mutant *ndr1*, indicating a close correlation between systemic *FMO1* expression and SAR establishment. Our findings suggest that the presence of the FMO1 gene product in systemic tissue is critical for the development of SAR, possibly by synthesis of a metabolite required for the transduction or amplification of a signal during the early phases of SAR establishment in systemic leaves.

Plants generally possess multiple layers of defense to restrict the growth of potentially pathogenic microorganisms. Preformed mechanical or chemical barriers constitute an effective first line of defense against nonadapted or nonhost pathogens (Thordal-Christensen, 2003). Host pathogens that are able to overcome this first barrier provoke a whole set of inducible reactions. In specific or gene-for-gene resistance, plants rely on the presence of resistance gene products, which recognize matching avirulence factors from the pathogen to induce a multitude of protective responses (Dangl and Jones, 2001). Avirulent pathogens thus trigger rapid production of reactive oxygen species (ROS), accumulation of the defense signals salicylic acid (SA) and/or jasmonic acid (JA), increased expression of various defense-related genes, production of phytoalexins, and hypersensitive death of challenged cells (Kuć, 1995; Lamb and Dixon, 1997). Some of these responses also occur, albeit delayed, after infection with virulent pathogens, which manage to escape resistance protein recognition. Induced defenses thus limit the extent of pathogen spread not only in incompatible interactions to ensure specific resistance, but also in compatible interactions to centrally contribute to basal resistance (Parker et al., 1996).

Plant defense responses are initiated not only locally at the site of pathogen attack, but also in tissue distant from the site of infection (Cameron et al., 1994). These systemic resistance responses are generally subdivided into two broad categories, systemic acquired resistance (SAR) and induced systemic resistance. SAR develops in response to a pathogen that causes a necrotic lesion either as a consequence of a hypersensitive response (HR) or as a result of disease symptom development in the course of a compatible interaction (Hammerschmidt, 1999). Plants exhibiting SAR are generally resistant to a broad range of different pathogens. Establishment of SAR is dependent on the SA pathway and associated with both systemic increase of SA levels and systemic expression of pathogenesis-related (PR) genes (Ryals et al., 1996). By contrast, induced systemic resistance, a response to colonization of plant roots by certain rhizosphere bacteria, is dependent on JA and ethylene signaling (Pieterse et al., 2002).

The molecular mechanisms underlying SAR are under intensive study. The capability of plants to accumulate SA is known to be indispensable for SAR, as Arabidopsis (*Arabidopsis thaliana*) SA biosynthesis mutants

[1] This work was supported by the Deutsche Forschungsgemeinschaft (SFB 567).

* Corresponding author; e-mail zeier@botanik.uni-wuerzburg.de; fax 49–931–8886235.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jürgen Zeier (zeier@botanik.uni-wuerzburg.de).

[OA] Open Access articles can be viewed online without a subscription.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.106.081257.

SA induction deficient 1 and 2 (*sid1* and *sid2*) and transgenic plants expressing the SA-degrading enzyme NahG are SAR defective (Gaffney et al., 1993; Nawrath and Métraux, 1999; Wildermuth et al., 2001; Nawrath et al., 2002). SA activates the SAR regulatory protein nonexpressor of PR genes (NPR1) through redox changes, which in turn drives systemic expression of antimicrobial PR proteins and facilitates their secretion by up-regulating protein secretory pathway genes (Mou et al., 2003; Wang et al., 2005). Several recent studies indicate that components of entirely distinct biochemical origin are necessary to realize SAR. Lipid metabolism turned out to play a central role in SAR signaling, as SAR is specifically compromised in Arabidopsis *defective in induced resistance 1* (*dir1*) and *suppressor of fatty acid desaturase deficiency 1* (*sfd1*), which bear mutations in a lipid transfer protein and a dihydroxyacetone phosphate reductase, respectively (Maldonado et al., 2002; Nandi et al., 2004). In addition, a peptide signal system mediated by the Asp protease constitutive disease resistance 1 (CDR1) appears to be essential for SAR long-distance signaling in Arabidopsis (Xia et al., 2004), and thiamine (vitamin B1) is capable of inducing SAR in a SA-dependent manner (Ahn et al., 2005). Moreover, ROS mediate a systemic signaling network that contributes to SAR (Alvarez et al., 1998). The complexity of systemic resistance regulation is further reflected by the fact that SAR establishment is subject to environmental and developmental plasticity. For instance, initiation of SAR has been demonstrated to occur in a light-dependent manner and the mechanisms of realizing SAR differ under variable light regimes (Zeier et al., 2004). Furthermore, leaf age influences the capability of initiating and executing SAR (Zeier, 2005).

Molecular events triggered by the primary infection process play a key role in SAR initiation. To identify uncharacterized genes involved in SAR establishment, we have selected Arabidopsis candidate genes up-regulated by SAR-inducing pathogens at the inoculation site, as indicated in microarray experiments publicly available from the Nottingham Arabidopsis Stock Centre (NASC) and from The Arabidopsis Information Resource (TAIR). T-DNA knockout lines corresponding to candidate genes were subsequently checked for an impaired SAR phenotype. This strategy revealed that the Arabidopsis *flavin-dependent monooxygenase 1* (*FMO1*) gene is essential for the establishment of SAR and systemic defense responses provoked both by an avirulent (*Psm avrRpm1*) and a virulent (*Psm*) strain of *Pseudomonas syringae*. By contrast, *FMO1* did not critically influence defense responses at the site of pathogen attack during these interactions.

RESULTS

FMO1 Is Expressed in Response to Virulent and Avirulent *P. syringae*

Gene expression profiling from two independent microarray datasets indicated that expression of the Arabidopsis *FMO1* gene (At1g19250) is increased 12 h post leaf infection of Arabidopsis ecotype Columbia (Col-0) with virulent *P. syringae* pv *tomato* DC3000 (*Pst*; Fig. 1, A and B). Inoculation with the isogenic avirulent *Pst avrRpm1* strain, which is recognized by Col-0

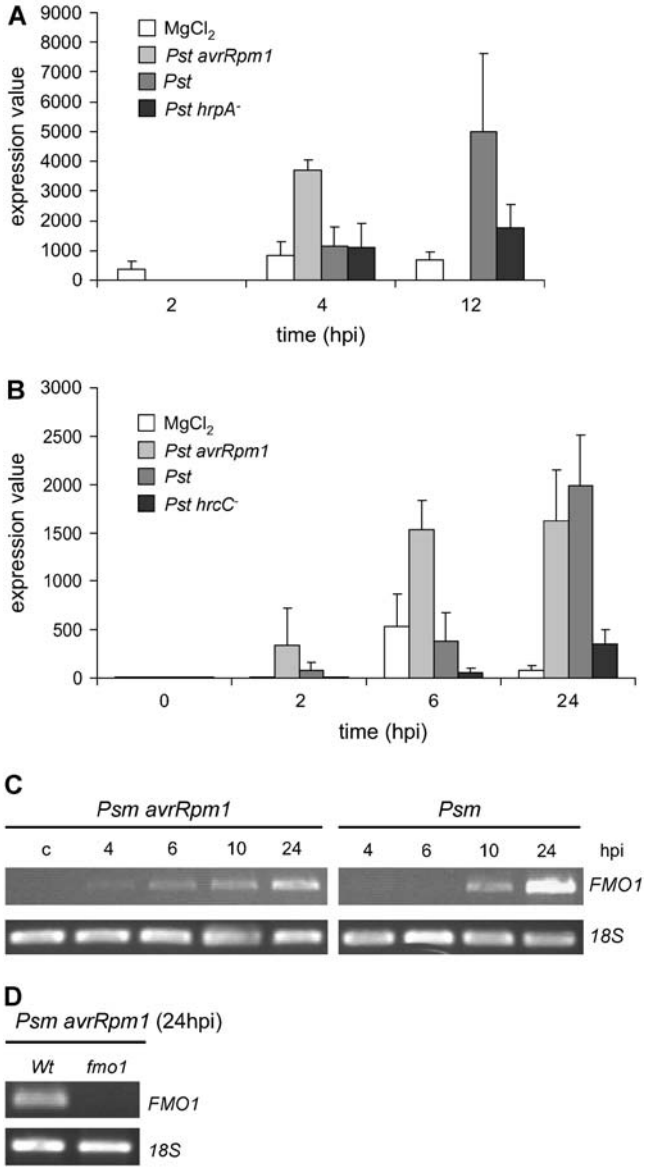


Figure 1. A and B, Expression levels of *FMO1* (At1g19250) in Arabidopsis leaves challenged with *Pst* according to microarray analyses. Means (±SD) of Affymetrix expression values originating from three independent replicates are given. The data were normalized according to the Affymetrix MAS 5.0 scaling protocol. A, Expression data from the NASC array *NASCARRAYS-59*: impact of type III effectors on plant defense responses. B, Expression data from TAIR (*TAIR-ME00331*: response to virulent, avirulent, type III secretion system-deficient and nonhost bacteria). C, RT-PCR analysis of *FMO1* expression triggered by *Psm* (virulent strain) and *Psm avrRpm1* (avirulent strain). Numbers indicate hpi. Control leaves (c) were infiltrated with 10 mM $MgCl_2$ for 24 h. 18S rRNA was amplified to standardize the transcript levels of each sample. D, Expression of *FMO1* in leaves of wild-type and *fmo1* mutant plants (T-DNA insertion line SALK_026163) 24 h after inoculation with *Psm avrRpm1*.

through the RPM1 resistance protein and consequently elicits an HR (Bisgrove et al., 1994), leads to earlier induction of *FMO1* expression, starting from about 4 h postinfection (hpi; Fig. 1, A and B). Upregulation of *FMO1* by *Pst* is largely dependent on a functional bacterial type III secretion system because the type III secretion-defective *Pst hrpA*$^-$ or *Pst hrcC*$^-$ strains only weakly induce its expression (Fig. 1, A and B). To experimentally verify the microarray data, we inoculated Col-0 with *Psm* ES4326, another virulent pathogen that induces similar defense responses as *Pst* (Dong et al., 1991). Reverse transcription (RT)-PCR analysis revealed that *FMO1* transcripts started to increase at about 6 hpi with avirulent *Psm avrRpm1* and at 10 hpi with virulent *Psm*, confirming that *FMO1* expression is triggered by host bacteria and that avirulent strains provoke an earlier transcription when compared with virulent strains (Fig. 1C).

Experiments investigating the kinetics of SAR establishment in the Arabidopsis-*Psm* interaction indicated that pathogen-treated primary leaves start to initiate SAR at least 1 d postinoculation. Moreover, the avirulent strain activated SAR faster than the virulent strain (data not shown). This tendency correlated with the expression pattern of *FMO1* in inoculated leaves (Fig. 1) and we thus postulated that FMO1 might play a role during SAR induction in primary leaves. A SALK insertion line (SALK_026163) harboring a T-DNA insertion in exon 4 of the *FMO1* gene in the Col-0 background was obtained from the NASC to examine whether FMO1 contributes to SAR establishment. In contrast to Col-0 plants, *fmo1* mutant plants failed to express *FMO1* after inoculation with *Psm* (Fig. 1D), demonstrating the knockout of *FMO1*.

SAR Is Compromised in *fmo1* Mutants

To investigate the biological induction of SAR, leaves of a given plant were treated with *Psm avrRpm1* or *Psm* in a primary inoculation (designated as primary leaves) and 2 d later a secondary or challenge infection with virulent *Psm* was performed in rosette leaves located just above the primary leaves (systemic leaves). Bacterial growth was scored in systemic leaves 3 d later. After treatment of primary leaves with a control solution of $MgCl_2$, growth of *Psm* during the challenge infection was vigorous in both wild-type and *fmo1* mutant plants (Fig. 2A). When primary leaves of wild-type plants were preinoculated with *Psm avrRpm1* or *Psm*, we observed a significant reduction of bacterial growth in the subsequent challenge infection in systemic leaves, demonstrating the establishment of SAR in both cases. In marked contrast, SAR did not develop in *fmo1* mutant plants because growth of *Psm* in systemic leaves proved to be equally pronounced in plants pretreated in primary leaves with $MgCl_2$, *Psm avrRpm1*, or *Psm* (Fig. 2A).

Systemic accumulation of SA and enhanced expression of defense genes in systemic leaves are characteristic features of SAR (Ryals et al., 1996). When primary

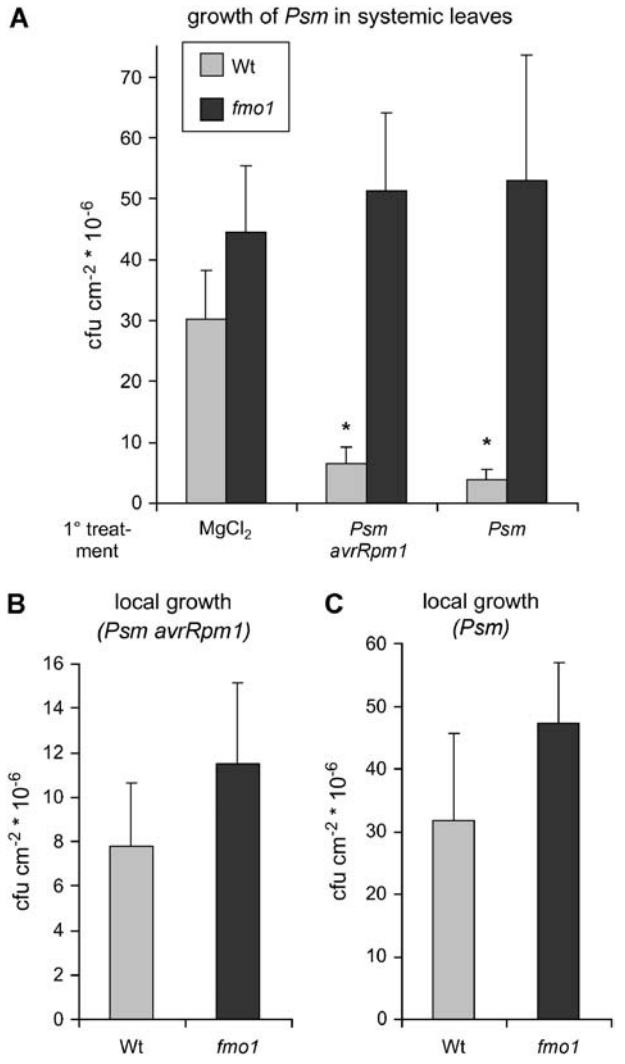


Figure 2. A, Bacterial growth quantification of *Psm* in systemic leaves to assess SAR in wild-type and *fmo1* mutant plants. Five-week-old Arabidopsis plants were pretreated with $MgCl_2$, *Psm avrRpm1*, or *Psm* (OD = 0.02 for each pathogen) in three primary leaves (1° treatment), and 2 d later, three systemic leaves located directly above the primary leaves were inoculated with *Psm* (OD = 0.002). Bacterial growth in systemic leaves was assessed 3 d (3 dpi) after infection of systemic leaves. Bars represent mean values (±SD) of colony-forming units per square centimeter from seven parallel samples each consisting of three leaf discs. Asterisks denote pathogen treatments with statistically significant differences to the respective $MgCl_2$ control ($P < 0.001$; Student's *t* test). Light bars, Wild-type plants; dark bars, *fmo1* plants. B and C, Quantification of bacterial growth to assess local resistance. B, Growth of *Psm avrRpm1* in leaves 3 d after inoculation with a bacterial suspension of OD = 0.005. C, Growth of *Psm* in leaves 3 d after inoculation (OD = 0.002). In both B and C, no statistical differences between the wild type and *fmo1* existed ($P > 0.05$; Student's *t* test). In addition, to ensure the uniformity of the experiments, initial bacterial numbers (1 hpi) were quantified. No significant differences in bacterial numbers were detected at 1 hpi for comparable treatments in A, B, and C (data not shown).

leaves of wild-type plants were treated with *Psm avrRpm1* or *Psm*, systemic leaves exhibited about 5- and 7-fold higher levels of free SA, respectively, compared to naive plants that were pretreated with $MgCl_2$ solution only (Fig. 3A). Additionally, both avirulent

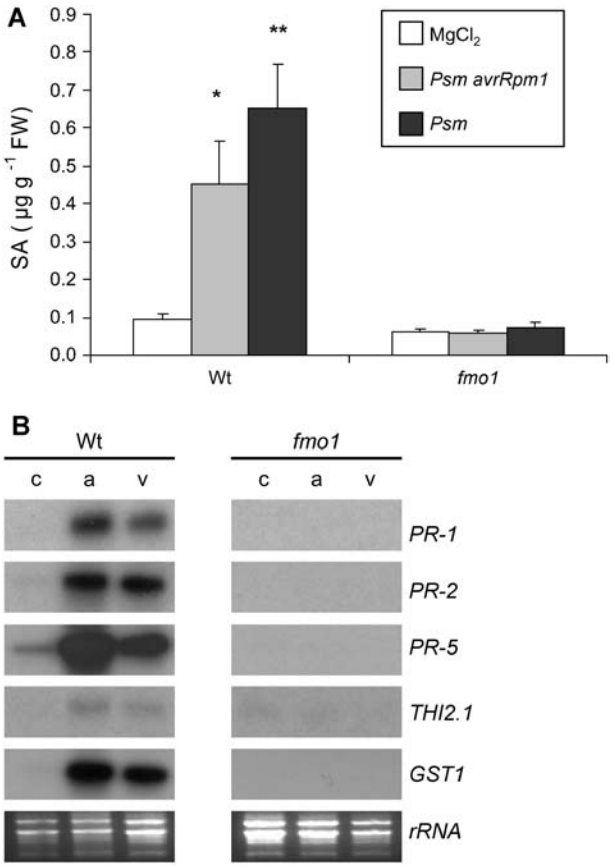


Figure 3. Systemic defense responses in wild-type and *fmo1* plants. Primary leaves of 5-week-old plants were treated as described in Figure 2A and untreated systemic leaves were harvested 2 d later for analysis. A, Systemic accumulation of SA. Bars represent mean values (±SD) of three independent samples. Each sample consisted of six leaves from two different plants. Asterisks denote pathogen treatments with statistically significant differences to the respective $MgCl_2$ control (*, $P < 0.02$; **, $P < 0.005$; Student's *t* test). White bars, $MgCl_2$ treatment; gray bars, *Psm avrRpm1* inoculation; black bars, *Psm* inoculation. B, Systemic expression of defense-related genes assessed by northern-blot analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; v, *Psm* inoculation).

and virulent bacteria triggered systemic expression of the SA-inducible defense gene *PR-1* (Nawrath and Métraux, 1999), the jasmonate-dependent thionin gene *THI2.1* (Epple et al., 1995), and the SA- and JA-independent defense genes *PR-2* and *PR-5* in the wild type (Fig. 3B). Moreover, the *glutathione S-transferase* gene *GST1*, a reliable marker for ROS production during plant-pathogen interactions (Levine et al., 1994; Alvarez et al., 1998), was systemically up-regulated in wild-type plants inoculated with *Psm* or *Psm avrRpm1* (Fig. 3B). Unlike the wild type, *fmo1* mutant plants exhibited neither elevated systemic SA levels after a local infection with *Psm* or *Psm avrRpm1* nor increased systemic expression of any of the defense genes under examination (Fig. 3).

Local Resistance in *fmo1* Mutants Is Similar to Wild-Type Plants

To examine whether the loss of SAR in *fmo1* mutants was associated with compromised local disease resistance in the *P. syringae*-Arabidopsis interaction, we determined bacterial growth of *Psm avrRpm1* and *Psm* in naive plants. Bacterial multiplication of both avirulent and virulent isolates was similar in wild-type and *fmo1* mutants (Fig. 2, B and C). In some experiments, a slightly enhanced growth tendency of *fmo1* mutant plants could be observed for *Psm* or *Psm avrRpm1*, but this tendency was not statistically significant. These results indicate that specific or basal disease resistance in the examined interactions is not compromised in *fmo1*.

To further address this issue, we investigated typical defense responses that are induced by *Psm avrRpm1* in Col-0 wild-type plants at inoculation sites. SA and JA are well-characterized signaling molecules accumulating during incompatible interactions. Up-regulation of the SA biosynthesis gene *SID1* occurred in a similar manner in both wild-type and *fmo1* plants starting 4 hpi (Fig. 5). Accordingly, local SA accumulation in *fmo1* closely resembled SA elevation in wild-type plants at 10 hpi (Fig. 4A). Levels increased from about 0.15 $\mu g\ g^{-1}$ fresh weight in control leaves to about 1.5 $\mu g\ g^{-1}$ fresh weight in inoculated leaves. Likewise, *Psm avrRpm1* induced accumulation of JA to a comparable extent in wild-type and *fmo1* plants (Fig. 4B). Further downstream in these pathways, SA and JA trigger the expression of distinct sets of PR genes (Reymond and Farmer, 1998). Again, striking similarities were obvious in the expression patterns of the SA-inducible *PR-1* gene and the JA-inducible *PR-4* gene after *Psm avrRpm1* inoculation (Fig. 5). Moreover, *Psm avrRpm1*-induced transcription of the SA- and JA-independent defense genes *PR-2* and *PR-5* was detected in both wild type and *fmo1*, yet to a somewhat higher extent in the mutant. These data indicate that, at the site of pathogen inoculation, FMO1 is neither required for the execution of SA- and JA-dependent defense pathways nor for the accomplishment of pathways independent of these defense signals.

Increased production of secondary metabolites represents a further characteristic response to host pathogens. The indole derivative camalexin constitutes the major phytoalexin in Arabidopsis and accumulates in response to elicitor and pathogen treatment (Tsuji et al., 1992; Zhou et al., 1998). Camalexin was essentially absent in noninoculated leaves, but accumulated substantially in *Psm avrRpm1*-treated leaves already at 10 hpi (Fig. 4C). Again, no significant difference between wild type and *fmo1* existed. Moreover, expression of Phe ammonia lyase (PAL), the key enzyme of phenylpropanoid biosynthesis, is up-regulated upon infection with avirulent Pseudomonas (Zeier et al., 2004). *PAL1* transcripts were elevated at 4 to 6 hpi in both wild-type and *fmo1* leaves, indicating that the phenylpropanoid pathway is initiated independently from *FMO1*.

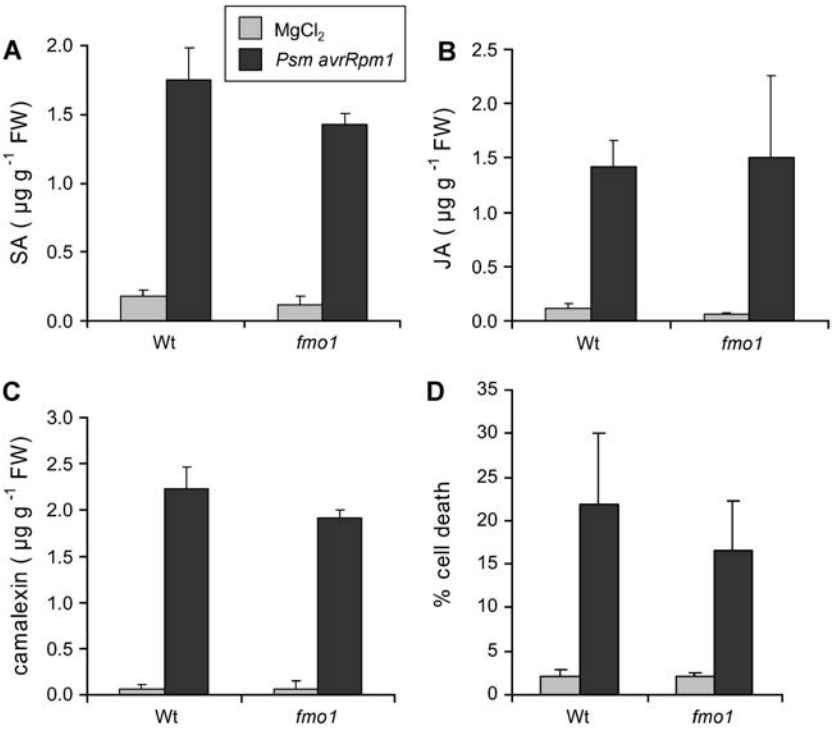


Figure 4. Local defense responses in wild-type and *fmo1* plants. A to C, Accumulation of signaling and antimicrobial compounds in leaves challenged with *Psm avrRpm1* (OD = 0.005). Control samples were treated with 10 mM $MgCl_2$. All samples were collected 10 h post treatment. A, SA levels. B, JA content. C, Accumulation of the phytoalexin camalexin. Mean values (±SD) of three independent samples are given. No statistical differences between equally treated wild-type and *fmo1* plants existed for each metabolite ($P > 0.05$; Student's *t* test). D, Quantification of microscopic HR lesions in leaves inoculated with *Psm avrRpm1* that were stained with trypan blue 24 hpi. Bars represent mean values (±SD) of dead cells in infiltrated areas from at least seven independent leaf samples. Light bars, Areas infiltrated with 10 mM $MgCl_2$; dark bars, *Psm avrRpm1*-infiltrated areas.

The oxidative burst at the site of pathogen ingress and the subsequent hypersensitive cell death response represent hallmarks of incompatible plant-pathogen interactions (Lamb and Dixon, 1997). During the oxidative burst, ROS are produced that contribute to triggering the HR in infected cells and driving expression of protective genes in neighboring tissue (Levine et al., 1994). The expression of *GST1* is triggered by ROS produced during the oxidative burst (Alvarez et al., 1998; Zeier et al., 2004). Enhanced *GST1* expression was observed from 4 to 10 h after *Psm avrRpm1* inoculation in wild-type leaves and a similar pattern was evident in *fmo1* mutant leaves. *FMO1* has recently been described as a marker gene for cell death pathways in plants (Olszak et al., 2006). To investigate whether FMO1 contributes to hypersensitive cell death lesion formation upon infection with *Psm avrRpm1*, we performed trypan blue-staining experiments with inoculated leaves (Zeier et al., 2004). At 24 hpi, wild-type plants exhibited a considerable amount of stained cells inside the pathogen-treated leaf area (Fig. 4D) and similar staining patterns were observed in inoculated *fmo1* mutant leaves. Thus, FMO1 does not play a critical role in the regulation of the oxidative burst or the hypersensitive cell death response at the site of pathogen attack.

Local and Systemic Expression of *FMO1* in Arabidopsis Defense Mutants

The SA-signaling pathway is essential for the full establishment of local and systemic disease resistance in the Arabidopsis-*P. syringae* interaction (Nawrath and Métraux, 1999). To examine whether expression of *FMO1* is dependent on SA signaling and SA-related defense pathways, we checked the pathogen-induced up-regulation of *FMO1* in different Arabidopsis defense mutants (Fig. 6). *Psm avrRpm1* or *Psm* induced *FMO1* expression to a similar extent in the SA biosynthesis mutant *sid2*, in the SA-insensitive mutant *npr1*, and in wild-type plants at the site of inoculation (Fig. 6A), demonstrating that local *FMO1* expression

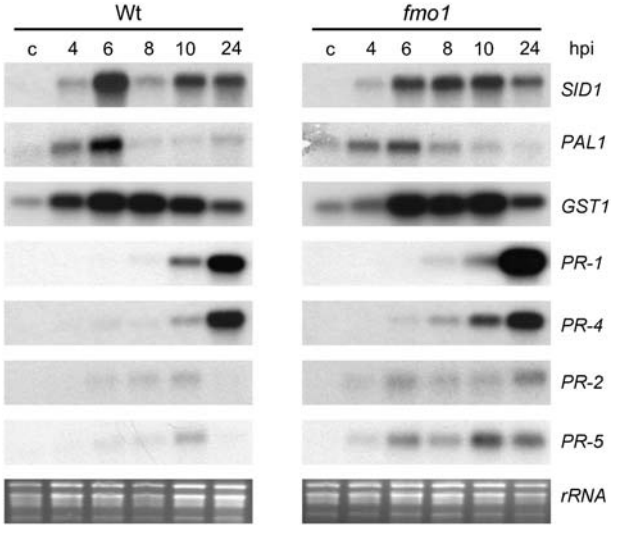


Figure 5. Local defense responses in wild-type and *fmo1* plants. Expression of defense-related genes in leaves challenged with *Psm avrRpm1* (OD = 0.005), as assessed by northern-blot analysis. Numbers indicate hpi. Control leaves (c) were treated with 10 mM $MgCl_2$ (4 h).

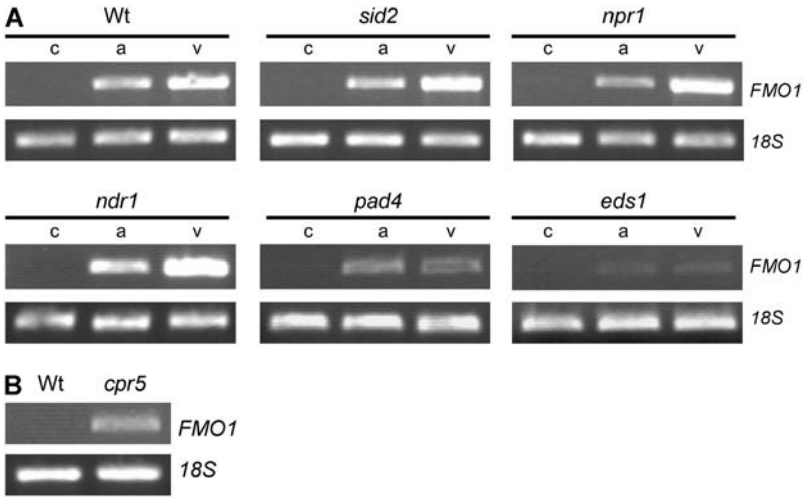


Figure 6. A, Expression of *FMO1* at the site of pathogen inoculation in wild-type plants and Arabidopsis defense mutants (24 hpi) as assessed by RT-PCR analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; v, *Psm* inoculation; OD = 0.005 for each pathogen). B, Levels of *FMO1* transcripts in untreated leaves of wild-type and *cpr5* mutant plants.

does not require SA signaling. Additionally, *FMO1* was strongly expressed in the *no disease resistance 1* (*ndr1*) defense mutant (Century et al., 1995) upon *P. syringae* infection. In the defense-signaling mutants *phytoalexin-deficient 4* (*pad4*; Zhou et al., 1998) and *enhanced disease susceptibility 1* (*eds1*; Parker et al., 1996), pathogen-induced FMO1 expression was clearly attenuated or entirely suppressed, respectively. Moreover, the *constitutive expression of PR 5* (*cpr5*) mutant that constitutively exhibits resistance in both an *npr1*-dependent and -independent manner (Bowling et al., 1997) exhibited marked constitutive *FMO1* expression (Fig. 6B).

To further analyze the function of FMO1 during SAR, we tested whether *FMO1* is systemically expressed upon local *Psm avrRpm1* inoculation (Fig. 7). The pathogen-induced SAR response was associated with an up-regulation of *FMO1* in systemic leaves of wild-type plants (Fig. 7, A and B). In SA-signaling mutants *sid2* and *npr1*, however, SAR was fully compromised and *FMO1* failed to be expressed systemically. The *ndr1* mutant constitutes a further SAR-deficient mutant, and systemic expression of *FMO1* was not enhanced upon *Psm avrRpm1* infection. The *eds1* mutation, by contrast, did not abolish *Psm avrRpm1*-triggered SAR, and systemic *FMO1* up-regulation still took place in *eds1*, albeit to a lesser extent than in the wild type. Moreover, the camalexin-deficient mutant *pad3* exhibited a wild-type-like SAR response and showed a systemic *FMO1* expression pattern similar to the wild type. Thus, establishment of SAR closely correlated with systemic elevation of *FMO1* transcript levels in the lines under investigation and, in contrast to the expression characteristics at the site of pathogen attack (Fig. 6A), systemic *FMO1* expression was dependent on an intact SA-signaling pathway.

DISCUSSION

SAR can be activated in many plant species by necrotizing pathogens and, once established, it confers long-lasting resistance toward a broad spectrum of different pathogens (Durrant and Dong, 2004). SAR turns out to be under complex molecular regulation because several components of entirely distinct biochemical origin are necessary for its induction in Arabidopsis (Nawrath and Métraux, 1999; Maldonado et al., 2002; Nandi et al., 2004; Xia et al., 2004; Wang et al., 2005). We show here that FMO1, whose expression is induced by virulent and avirulent strains of *P. syringae* both at the site of pathogen ingress and in systemic tissue, constitutes a further component essential for the successful activation of SAR in Arabidopsis because *fmo1* knockout mutants proved to be totally compromised in the activation of systemic defense responses and the establishment of SAR (Figs. 2A and 3).

Currently, a central role for FMO1 in plant disease resistance is emerging. In fact, the *FMO1* gene has recently been recognized by distinct approaches to be involved in plant defense (Bartsch et al., 2006; Koch et al., 2006; Olszak et al., 2006). *FMO1* was demonstrated to be up-regulated in Arabidopsis *acd11*, a mutant exhibiting constitutively activated SA-, PAD4-, and EDS1-dependent defenses and spontaneous HR lesions (Brodersen et al., 2002; Olszak et al., 2006). In addition, it was shown that *FMO1* expression is enhanced in the runaway cell death *lesion-simulating disease 1* (*lsd1*) mutant (Dietrich et al., 1997), but not in the constitutive defense-signaling mutants *ctr1*, *cev1*, *mpk4*, and *cpr6* that do not develop spontaneous cell death (Olszak et al., 2006). Thus, *FMO1* was suggested as a marker gene for certain forms of defense and cell death. In a screen for genes whose expression depends on EDS1 and PAD4, Bartsch et al. (2006) showed the requirement of functional FMO1 in the execution of basal resistance against a virulent isolate of the oomycete pathogen *Hyaloperonospora parasitica* and of specific resistance against *H. parasitaca* isolate Noco2 or *P. syringae* carrying the *avrRps4* avirulence gene. Moreover, an activation-tagging approach identified an Arabidopsis line constitutively overexpressing *FMO1*,

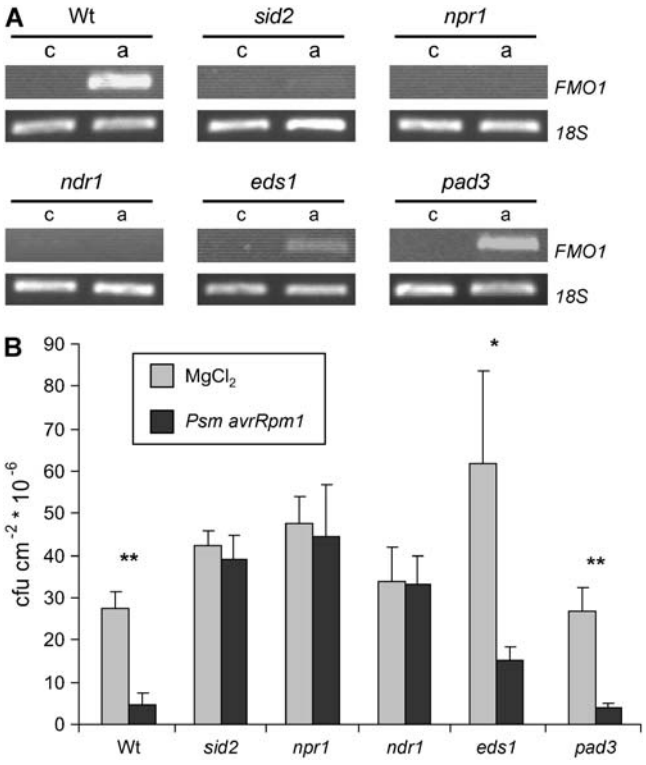


Figure 7. Correlation of systemic *FMO1* expression and SAR establishment. A, Systemic expression of *FMO1* in wild-type plants and Arabidopsis defense mutants in response to *Psm avrRpm1* as assessed by RT-PCR analysis (c, $MgCl_2$ treatment; a, *Psm avrRpm1* inoculation; OD = 0.02). For further details, see legend to Fig. 3. B, Growth quantification of *Psm* in systemic leaves (3 dpi) to assess SAR induced by *Psm avrRpm1* in wild-type and Arabidopsis defense mutants. For further details, see legend to Fig. 2A. Asterisks denote lines with statistically significant differences between plants pretreated with $MgCl_2$ and *Psm avrRpm1* (*, $P < 0.01$; **, $P < 0.001$; Student's *t* test).

which is characterized by enhanced disease resistance against *P. syringae* (Koch et al., 2006).

Basal resistance is triggered by a multitude of relatively unspecific pathogen-associated molecular patterns to limit the growth of virulent pathogens to a certain extent (Nürnberger and Lipka, 2005). By contrast, resistance gene-mediated resistance is based on specific recognition events and two subclasses of resistance proteins with distinct signaling requirements are generally distinguished, depending on the presence of either an N-terminal coiled-coil domain (CC-NB-LRR) or a domain with similarity to the Drosophila Toll and mammalian interleukin-1 receptors (TIR-NB-LRR; Aarts et al., 1998). We have demonstrated that, at the site of pathogen inoculation, basal resistance against virulent *Psm* and specific resistance against *Psm avrRpm1* are not compromised in *fmo1* mutants (Fig. 2, B and C). Moreover, various characteristic defense responses locally triggered by *Psm avrRpm1*, including oxidative burst, accumulation of SA, JA, and camalexin, and expression of defense genes as well as the hypersensitive cell death response, are not significantly altered in *fmo1* (Figs. 4 and 5). In line with these findings, Bartsch et al. (2006) showed that resistance to *Pst avrRpm1* is not affected in *fmo1* mutants, using the same T-DNA insertion line (SALK_026163). Because the AvrRpm1 avirulence protein is recognized by RPM1, a CC-NB-LRR-type resistance protein, we conclude that basal resistance to *P. syringae* and specific resistance mediated by CC-NB-LRR receptors are largely FMO1 independent. By contrast, resistance to *Pst avrRps4* has been reported to be attenuated in *fmo1* and therefore FMO1 is required for specific resistance against *P. syringae* mediated by TIR-NB-LRR resistance proteins (Bartsch et al., 2006). The contribution of FMO1 to basal resistance against *H. parasitica*, specific resistance against *Pst avrRps4*, and SAR triggered by *Psm* reveals that overlapping molecular principles exist in distinct kinds of resistance within different pathosystems.

Our finding that FMO1 represents a critical component of SAR in Arabidopsis is further underlined by recent work demonstrating that constitutive overexpression of *FMO1* leads to enhanced disease resistance toward *P. syringae* (Koch et al., 2006). FMO1 might function in the induction of SAR in inoculated tissue, in the propagation of a mobile signal to distant tissue, in the perception of this long-distance signal in systemic tissue, or in the potentiation of defense responses in systemic tissue. Induced expression of *FMO1* in inoculated leaves is attenuated in *eds1* and *pad4* mutants, confirming that FMO1 contributes to the EDS1/PAD4 pathway in local defense signaling (Bartsch et al., 2006). In contrast, FMO1 expression is not affected in *sid2*, *npr1*, and *ndr1* mutants, demonstrating that local *FMO1* induction is independent of the SA-signaling pathway and NDR1-mediated signaling (Fig. 6). However, in contrast to the wild type, these three mutants fail to express *FMO1* systemically (Fig. 7A), and this is associated with a loss of SAR (Fig. 7B). In the *eds1* mutant, pathogen-induced *FMO1* expression is abolished at the site of inoculation, yet still observable in systemic tissue, and a significant SAR response is established in *eds1*. Thus, the failure to systemically rather than locally up-regulate *FMO1* transcription correlates with the development of SAR in all investigated lines. Additionally, *fmo1* mutants, despite exhibiting unaltered local defenses, are totally compromised in any of the examined systemic responses (Fig. 3). These include systemic SA accumulation, systemic expression of SA-dependent and -independent PR genes, as well as up-regulation of *GST1*, a reliable marker for ROS generation (Levine et al., 1994). Moreover, *FMO*1 transcripts are up-regulated in the absence of a pathogen in defense of the mutant *cpr5*, which exhibits constitutive disease resistance (Fig. 6B; Bowling et al., 1997). Taking these findings together, we propose a model in which the presence of FMO1 in systemic tissue is critical for the realization of SAR. A metabolite generated by FMO1 might be necessary during the early phase of SAR establishment in systemic leaves, presumably for the transduction or amplification of a long-distance signal originating from primary leaves.

Feedback loops, including SA and ROS, exist to amplify plant defense responses (Shirasu et al., 1997) and oxidative microbursts in systemic tissue have been shown to mediate a reiterative signal network during SAR (Alvarez et al., 1998). Moreover, superoxide has been demonstrated to induce *FMO1* expression (Olszak et al., 2006). We thus propose that FMO1 contributes to a signal amplification loop involving ROS, SA, NPR1, and NDR1 that is required to potentiate SAR responses in systemic tissue.

Although SA represents a central and necessary signaling component for the establishment of SAR, there are controversial data as to whether it functions as a mobile signal that moves from infected leaves to systemic tissue. $^{18}O_2$ feeding experiments in tobacco mosaic virus-infected tobacco (*Nicotiana tabacum*) demonstrate that about 60% to 70% of the SA detected in systemic leaves originates from inoculated tissue, with the remainder resulting from de novo synthesis (Shulaev et al., 1995). Similarly, ^{14}C-labeling experiments in cucumber (*Cucumis sativus*) plants inoculated with tobacco necrosis virus showed that SA accumulation in systemic leaves results both from transport and from de novo synthesis (Mölders et al., 1996). Although SA transport from inoculated to systemic tissue is feasible in these species, SA does not necessarily represent the SAR long-distance signal. In cucumber, removal of pathogen-treated leaves led to systemic resistance induction before a rise in SA levels was detectable in petiole exudates of inoculated leaves (Rasmussen et al., 1991). Moreover, grafting experiments using transgenic tobacco expressing the salicylate hydroxylase NahG indicate that SA is not the long-distance signal during SAR, but it is required for signal transduction in systemic tissue (Vernooij et al., 1994). Considering Arabidopsis, Kiefer and Slusarenko (2003), by applying ^{14}C-SA to rosette leaves, have demonstrated that exogenous SA is able to move from source to sink tissue. On the other hand, we have shown here that Pseudomonas-infected *fmo1* mutant plants locally accumulate wild-type levels of SA, whereas no SA accumulation occurs systemically (Figs. 3A and 4A). A similar trend is observed in the SAR-defective mutants *ndr1* and *npr1* (Fig. 7B; T.E. Mishina and J. Zeier, unpublished data). This indicates that systemic accumulation of SA that is normally observed during biologically induced SAR in Arabidopsis is not due to transport of SA produced at the site of infection, but is largely caused by de novo synthesis in systemic tissue in which the above proposed feedback loop, including FMO1 and SA, might operate.

Mammalian FMO either contribute to oxidative xenobiotic metabolism or catalyze the oxygenation of endogenous metabolites, i.e. biogenic amines (Krueger and Williams, 2005). Besides *FMO1*, the only plant FMO genes characterized so far represent Arabidopsis *YUCCA* and its petunia (*Petunia hybrida*) ortholog *FLOOZY*, which are involved in auxin biosynthesis (Zhao et al., 2001; Tobena-Santamaria et al., 2002). YUCCA has been demonstrated to catalyze the hydroxylation of the amino group in tryptamine. A challenging future task represents the identification of the putative metabolite generated by FMO1 and the clarification of its role in disease resistance.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) L. Heynh. plants were grown in a mixture of soil (Fruhstorfer Pflanzenerde), vermiculite, and sand (9:1:1) in a controlled environment chamber (J-66LQ4; Percival) with a 9 h day (photon flux density 70 μmol m^{-2} s^{-1})/15 h night cycle and 70% relative humidity. Growth temperatures were set to 22°C during the day and 18°C during the night.

Arabidopsis ecotype Col-0 was used as the wild type in all experiments. The *fmo1* line represents the Salk T-DNA insertion line SALK_026163 in the Col-0 background. Homozygous insertion mutants were identified by PCR, using a gene-specific and a T-DNA-specific primer (Alonso et al., 2003), and used for experiments. Further, the following Arabidopsis defense mutants were used in this study: *sid2-1* (Nawrath and Métraux, 1999), *npr1-2* (NASC ID no., N3801), *ndr1* (Century et al., 1995), *pad3-1* (Glazebrook and Ausubel, 1994), *pad4-1* (Glazebrook et al., 1997), *eds1-2* (Aarts et al., 1998), and *cpr5-2* (Bowling et al., 1997).

Growth of Plant Pathogens and Infection

Pseudomonas syringae pv *maculicola* ES4326 lacking (*Psm*) or carrying (*Psm avrRpm1*) the *avrRpm1* avirulence gene were grown at 28°C in King's B medium containing the appropriate antibiotics (Zeier et al., 2004). Overnight log phase cultures were washed three times with 10 mM $MgCl_2$ and diluted to a final optical density (OD) concentration of 0.02, 0.005, or 0.002. The bacterial suspensions were infiltrated from the abaxial side into a sample leaf using a 1-mL syringe without a needle. Control inoculations were performed with 10 mM $MgCl_2$. Bacterial growth was assessed by homogenizing discs originating from infiltrated areas of three different leaves in 1 mL 10 mM $MgCl_2$, plating appropriate dilutions on King's B medium, and counting colony numbers after incubating the plates at 28°C for 2 d.

All pathogen experiments depicted in the figures were repeated at least twice with similar results.

Characterization of Systemic Resistance Responses

Plants were first infiltrated into three lower leaves with a suspension of *Psm* or *Psm avrRpm1* (OD = 0.02), or with 10 mM $MgCl_2$ as a control. Two days after the primary inoculation, nontreated upper leaves were harvested for SA determination and gene expression analysis or plants were inoculated on three upper leaves with virulent *Psm* (OD = 0.002). Growth of *Psm* in upper leaves was scored 3 d later.

Quantification of Microscopic HR Lesions

The extent of microscopic HR lesion formation was assessed by trypan blue staining, light microscopy, and quantification of stained cells as described by Zeier et al. (2004).

Northern-Blot Analysis

Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (peqLab) following the manufacturer's instructions. For each sample, two leaves from different plants of the same treatment were used. One microgram of total RNA was loaded on formaldehyde-agarose gels, separated by electrophoresis, and blotted on nylon membranes (Hybond-N; Amersham). RNA-blot hybridization was performed with specific ^{32}P-labeled DNA probes generated by PCR using appropriate oligonucleotide primers. The probes represented the following Arabidopsis genes: *SID1* (Arabidopsis annotation At4g39030), *PAL1* (At2g37040), *GST1* (At1g02930), *PR-1* (At2g14610), *PR-2* (At3g57260), *PR-4* (At3g04720), *PR-5* (At1g75040), and *THI2.1* (At1g72260).

RT-PCR Analysis

One microgram of extracted RNA was treated with DNase I (Fermentas) for 30 min at 37°C to remove genomic DNA, the DNase inactivated by incubation at 70°C for 5 min in the presence of 2.5 mM EDTA, and cDNA synthesized in a final reaction volume of 20 μL at 42°C for 1 h using random primer mix, ribonuclease inhibitor (RNaseOUT; Invitrogen), and reverse transcriptase (SuperScript II; Invitrogen). After another enzyme inactivation step for 15 min at 70°C, the cDNA mixture was diluted in water (1:10) and 3 to 10 μL of the final dilution used in a 30-μL RT-PCR reaction (3 μL for 18S rRNA, 10 μL for *FMO1*). The following primers were used for the amplification of cDNA derived from 18S rRNA and *FMO1* mRNA, respectively: 5′-AAACGGCTACCACATCCAAG-3′ (18S-forward), 5′-ACCCATCCCAAGGTTCAACT-3′ (18S-reverse), 5′-CTTCTACTCTCCTCAGTGGCAAA-3′ (*FMO1*-forward), and 5′-CTAATGTCGT-CCCATCTTCAAAC-3′ (*FMO1*-reverse). The PCR reaction was performed as follows: 95°C for 10 min, 25 (18S) or 30 (*FMO1*) cycles of 92°C for 60 s, 60°C for 90 s, 72°C for 90 s, and a final extension step at 72°C for 5 min. Ten microliters of each PCR reaction were visualized by agarose gel electrophoresis with ethidium bromide staining.

Gas Chromatographic Determination of SA, JA, and Camalexin

The determination of SA, JA, and camalexin levels in leaves was performed by a modified vapor-phase extraction method (Schmelz et al., 2004). Briefly, 150 mg of frozen leaf tissue were homogenized with 600 μL of extraction buffer (water:1-propanole:HCl = 1:2:0.005). After addition of internal standards (D_4-SA, dihydrojasmonic acid, and indolepropionic acid; 100 ng each) and 1 mL of methylene chloride, the mixture was shaken thoroughly and centrifuged at 14,000 rpm for phase separation. The lower, organic phase was then removed, dried over Na_2SO_4, and treated with 2 μL of 2 M trimethylsilyldiazomethane in hexane (Sigma-Aldrich) for 5 min at room temperature to convert carboxylic acids into their corresponding methyl esters. After stopping the methylation reaction with 2 M acetic acid in hexane, the sample was subjected to a vapor-phase extraction procedure using a volatile collector trap packed with Super-Q absorbent (VCT-1/4X3-SPQ; Analytical Research Systems). The final evaporation temperature was set to 200°C, and samples were eluted from the collector trap with 1 mL methylene chloride. Finally, the sample volume was reduced to 50 μL in a stream of nitrogen, and the sample was subjected to gas chromatography-mass spectrometry analysis. The sample mixture (2 μL) was separated on a gas chromatograph (GC 6890 N; Agilent Technologies) equipped with a fused silica capillary column (DB-1; Fisons), and combined with a 5975 mass spectrometric detector (Agilent Technologies). For quantitative determination of metabolites, peaks originating from selected ion chromatograms were integrated. The area of a substance peak was related to the peak area of the corresponding internal standard (SA/D_4-SA; JA/dihydrojasmonic acid, camalexin/indolepropionic acid). Experimentally determined correction factors for each substance/standard pair were considered.

ACKNOWLEDGMENTS

We thank Volker Lipka and Juriaan Ton for the donation of Arabidopsis mutant seeds, and Michael Rostás, Markus Riederer, and Volker Lipka for proofreading the manuscript. We are grateful to M. de Torres Zabala, M. Grant, B. Kemmerling, and T. Nürnberger for publicly sharing their microarray data.

Received April 2, 2006; revised June 1, 2006; accepted June 1, 2006; published June 15, 2006.

LITERATURE CITED

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signalling pathways in Arabidopsis. Proc Natl Acad Sci USA **95:** 10306–10311

Ahn I-P, Kim S, Lee Y-H (2005) Vitamin B1 functions as an activator of plant disease resistance. Plant Physiol **138:** 1505–1515

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana.* Science **301:** 653–657

Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92:** 773–784

Bartsch M, Gobbato E, Bednarek P, Debey S, Schultze JL, Bautor J, Parker JE (2006) Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the nudix hydrolase NUDT7. Plant Cell **18:** 1038–1051

Bisgrove SR, Simonich MT, Smith NM, Sattler A, Innes RW (1994) A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. Plant Cell **6:** 927–933

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. Plant Cell **9:** 1573–1584

Brodersen P, Petersen M, Pike HN, Olszak B, Skov-Petersen S, Oedum N, Joergensen LB, Brown RE, Mundy J (2002) Knockout of Arabidopsis ACCELERATED-CELL-DEATH11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. Genes Dev **16:** 490–502

Cameron RK, Dixon RA, Lamb CJ (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana.* Plant J **5:** 715–725

Century KS, Holub EB, Staskawicz BJ (1995) NDR1, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc Natl Acad Sci USA **92:** 6597–6601

Dangl JL, Jones JDG (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826–833

Dietrich RA, Richberg MH, Schmidt R, Dean C, Dangl JL (1997) A novel zinc finger protein is encoded by the Arabidopsis *LSD1* gene and functions as a negative regulator of plant cell death. Cell **88:** 685–694

Dong X, Mindrinos M, Davis KR, Ausubel FM (1991) Induction of Arabidopsis defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. Plant Cell **3:** 61–72

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol **42:** 185–209

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol **109:** 813–820

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc Natl Acad Sci USA **91:** 8955–8959

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Hammerschmidt R, Ausubel FM (1997) Phytoalexin-deficient mutants of Arabidopsis reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics **146:** 381–392

Hammerschmidt R (1999) Induced disease resistance: how do induced plants stop pathogens? Physiol Mol Plant Pathol **55:** 77–84

Kiefer IW, Slusarenko AJ (2003) The pattern of systemic acquired resistance induction within the Arabidopsis rosette in relation to the pattern of translocation. Plant Physiol **132:** 840–847

Koch M, Vorwerk S, Masur C, Sharifi-Sirchi G, Olivieri N, Schlaich N (2006) A role for a flavin-containing monooxygenase in pathogen resistance in Arabidopsis. Plant J (in press)

Krueger SK, Williams DE (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther **106:** 357–387

Kuć J (1995) Phytoalexins, stress metabolism, and disease resistance in plants. Annu Rev Phytopathol **33:** 275–297

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol **48:** 251–275

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. Cell **79:** 583–593

Maldonado AM, Doerner P, Dixon RA, Lamb C, Cameron RC (2002) A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature **419:** 399–403

Mölders W, Buchala A, Métraux J-P (1996) Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants. Plant Physiol **112:** 787–792

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113:** 935–944

Nandi A, Welti R, Shah J (2004) The *Arabidopsis thaliana* dihydroxyacetone phosphate reductase gene SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. Plant Cell **16:** 465–477

Nawrath C, Heck S, Parinthawong N, Métraux JP (2002) EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE-transporter family. Plant Cell **14:** 275–286

Nawrath C, Métraux JP (1999) Salicylic acid induction-deficient mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11:** 1393–1404

Nürnberger T, Lipka V (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol Plant Pathol **6:** 335–345

Olszak B, Malinovsky FG, Brodersen P, Grell M, Giese H, Petersen M, Mundy J (2006) A putative flavin-containing mono-oxygenase as a marker for certain defense and cell death pathways. Plant Sci **170:** 614–623

Parker JE, Holub EB, Frost LN, Falk A, Gunn ND, Daniels MJ (1996) Characterization of *eds1*, a mutation in Arabidopsis suppressing resistance to *Peronospora parasitica* specified by several different RPP genes. Plant Cell **8:** 2033–2046

Pieterse CMJ, Van Wees SCM, Ton J, Van Pelt JA, Van Loon LC (2002) Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana.* Plant Biol **4:** 535–544

Rasmussen JB, Hammerschmidt R, Zook MN (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae.* Plant Physiol **97:** 1342–1347

Reymond P, Farmer EE (1998) Jasmonate and salicylate as global signals for defense gene expression. Curr Opin Plant Biol **1:** 404–411

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell **8:** 1809–1819

Schmelz EA, Engelberth J, Tumlinson JH, Block A, Alborn HT (2004) The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. Plant J **39:** 790–808

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defence mechanisms. Plant Cell **9:** 261–270

Shulaev V, Leon J, Raskin I (1995) Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell **7:** 1691–1701

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. Curr Opin Plant Biol **6:** 351–357

Tobena-Santamaria R, Bliek M, Ljung K, Sandberg G, Mol JNM, Souer E, Koes R (2002) FLOOZY of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. Genes Dev **16:** 753–763

Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC (1992) Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv *syringae.* Plant Physiol **98:** 1304–1309

Vernooij B, Friedrich L, Morse A, Reist R, Kolditzjawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959–965

Wang D, Weaver ND, Kesarwani M, Dong X (2005) Induction of protein secretory pathway is required for systemic acquired resistance. Science **308:** 1036–1040

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565

Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, Dixon RA, Lamb C (2004) An extracellular aspartic protease functions in Arabidopsis disease resistance signalling. EMBO J **23:** 980–988

Zeier J (2005) Age-dependent variations of local and systemic defense responses in Arabidopsis leaves towards an avirulent strain of *Pseudomonas syringae.* Physiol Mol Plant Pathol **66:** 30–39

Zeier J, Pink B, Mueller MJ, Berger S (2004) Light conditions influence specific defence responses in incompatible plant-pathogen interactions: uncoupling systemic resistance from salicylic acid and PR-1 accumulation. Planta **219:** 673–683

Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291:** 306–309

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) PAD4 functions upstream from salicylic acid to control defence responses in Arabidopsis. Plant Cell **10:** 1021–1030

4. Discussion

4.1 The role of NO in plant defence and senescence

Nitric oxide has been recognized as a positive regulator of several plant defence responses (Mur et al., 2005). Evidence for a physiological function of NO in plants is largely based on studies applying pharmacologically active substances like NO donors, NO scavengers, or mammalian NOS inhibitors to plants or cell suspensions (Delledonne et al., 1998; Durner et al., 1998). Remarkably, application of different NO donors like SNP, SNAP, or NOC-18, respectively, results in diverging cellular responses (Murgia et al., 2004), suggesting that in addition to their NO releasing capacity, other factors dictate their physiological action. Affirmatively, SNP has been shown to release cytotoxic cyanide in addition to NO, which deeply questions the applicability of this widely used NO donor (Bethke et al., 2006).

Using an alternative approach, we have generated transgenic Arabidopsis plants with modulated NO levels. To reduce plant NO levels, we have expressed an NO dioxygenase (NOD) under control of an inducible promoter, and to increase plant NO production, we have constitutively expressed the *D. radiodurans* NO synthase (deiNOS). According to NO emission measurements via chemiluminescence detection and NO degradation assays, we could confirm that, compared with the wild-type, NOD expressing plants exhibit decreased NO levels, and deiNOS expressing plants (cNOS) produce increased levels of NO.

In both NOD and cNOS plants, we observed reduced levels of ROS after inoculation with bacterial pathogens. Obviously, either increased or decreased plant NO levels can lead to a reduction of ROS levels. This apparent contradiction might be explained through a dual role of NO in regulation of ROS homeostasis in plants. On one hand, the presence of NO in cells can contribute to increased ROS levels. NO might support the elevation of H_2O_2 levels by reversibly inhibiting the H_2O_2 degrading enzymes ascorbate peroxidase (APX) and catalase (CAT; Clarck et al., 2000). NO can also promote the production of O_2^- by inhibition of the last enzyme in the mitochondrial respiratory electron transport chain, cytochrom c oxidase (Zottini et al., 2002). On the other hand, if NO is present in elevated amounts, it can react with O_2^- and generate peroxynitrite (ONOO-), which may isomerase to nitrate. Alternatively, peroxynitrite can further scavenge NO via the formation of nitrogen dioxide (Daiber et al., 2002). Thus, the interplay between ROS and NO is complex and might also depend on the physiological situation and the subcellular location.

Delledonne et al. (1998) present pharmacological evidence that NO, in combination with ROS, is required for cell death triggered by avirulent bacterial pathogens. NADPH-oxidase is activated after pathogen infection and generates superoxide, which serves as a substrate for superoxide dismutase (SOD) converting O_2^- to H_2O_2 (Lamb and Dixon, 1997). A balance between ROS and NO and the cooperation of H_2O_2 and NO is crucial for HR induction (Delledonne et al., 2001). In NOD overexpressing plants, we observed a small reduction in the number of microscopic HR lesions triggered by avirulent *P. syringae*. In contrast, cNOS plants showed HR cell death to a similar extent than wild-type plants after pathogen inoculation. This suggests that the development of hypersensitive cell death is quite robust towards varying NO level in Arabidopsis. Moreover, experiments using a tobacco nitrite reductase antisense-line ("line-271", Morot-Gaudry-Tamarmain et al., 2002), which exhibits a 100-fold higher NO production than wild-type, show that even large amounts of endogenous NO do not affect the cryptogein-mediated HR in tobacco (Planchet et al., 2006).

NO has been reported to act as a secondary messenger in signal transduction pathways activating plant defence responses. For instance, treatments of soybean cells wit the NO donor SNP triggered *PAL* expression in soybean (Delledonne et al., 1998), and application of a mammalian NOS preparation induced *PAL* and *PR-1* expression in tobacco (Durner et al., 1998). Consistently, we have observed reduced expression of the defence gene *PAL1* in NOD-plants after pathogen challenge. In cNOS plants with constitutively elevated NO levels, we expected a constitutive, or at least increased expression of *PAL1* after pathogen challenge. However, cNOS plants do not exhibit altered constitutive or pathogen-induced *PAL1* expression. Apparently, a constant elevation of NO is not sufficient as a trigger to induce defence genes. Rather, as in the case of NO donor application or pathogen treatment, a sudden increase in NO levels might trigger defence gene expression.

Although we have observed attenuation of some defence responses in NOD plants, disease resistance in terms of bacterial growth was not significantly reduced in these plants. In addition, beyond the absence of constitutively activated defences, cNOS plants are more susceptible to virulent, avirulent and non-host strains of *P.syringae*. A comparable effect was observed in *Atgsnor1* mutant plants, which are defective in S-nitrosoglutathione reductase. This leads to increased protein S-nitrosylation (Feechan et al., 2005). S-nitrosylation of cysteine thiols has been suggested to represent a central mechanism for the transduction of the NO signal (Grün et al., 2006). Increased protein S-nitrosylation might also result from NO overproduction in cNOS plants. The enhanced formation of S-nitrosylated proteins in *Atgsnor1* or cNOS might interrupt signalling cascades due to conformational changes of proteins and attenuation of defence response. For instance, in cNOS plants, we observed reduced expression of the SA-independent

defence genes *PR-2* and *PR-5*, which might contribute to the decreased resistance phenotype in cNOS.

Besides S-nitrosylating proteins and peptides, NO can form metal nitrosyls by binding to the heme moieties or other metal-containing prosthetic groups of proteins. Moreover, it can react with tyrosine residues and thereby nitrate proteins (Pfeilschifter et al., 2001). Both types of reactions might represent further NO-dependent regulatory mechanisms in plants.

Arabidopsis mutants for which an altered NO production is described include *Atnos1* and *nox1* (Guo et al., 2003; He et al., 2004). According to the relatively unspecific DAF-method (Planchet and Kaiser, 2006), *Atnos1* mutants show impaired NO production in response to abscisic acid and bacterial LPS preparations, and they are more sensitive to virulent *P. syringae* strains when bacteria are spray-inoculated on the leaf surface (Guo et al., 2003; Zeidler et al., 2004). Although AtNOS1 has been provisionally considered as a plant NO synthase (Guo et al., 2003), recent evidence suggests that this enzyme does not exhibit NOS activity (Zemoijtel et al., 2006). This indicates that the defects caused by the *Atnos1* mutation are not well understood and might not or only indirectly be related to an action of NO. A similar statement can be made for the NO-overproducer mutant *nox1*, which is mutated in the *CUE1* gene encoding for a chloroplast phosphoenolpyruvate/ phosphate translocator. The mutation in *CUE1* leads to a dramatic change in general plant metabolisms, among others L-arginine accumulation, and as a possible side effect, enhanced NO formation (He et al., 2004).

The lack of natural mutations which are specifically impaired in NO production severely limit the functional study of NO in plants. Moreover, the global alterations of NO levels due to NO donors or NO scavengers most certainly do not represent the spatially and temporarily defined changes of NO concentrations occurring in many natural biological processes like plant-pathogen interactions. Unspecific biochemical reactions of NO which do normally not happen in nature might be the consequence. Similar restrictions might hold true for the use of transgenic plants used in the current study. Additionally, the difficulties to reliably detect NO in intact plant tissue represent a serious problem. Thus, to further understand the role of NO in plant-pathogen interactions, the real sources and targets of NO on one hand, and the development of new NO detections systems on the other hand, are necessary.

Nevertheless, exogenous application of NO to whole plants might have defined action on other physiological effects. For instance, treatment with NO donors or NO gas extends the post-harvest life of fruits and vegetables, and retards the senescence of flowers. Moreover, the temporal progress of fruit maturation and floral or leaf senescence is associated with a continuous decrease in NO emission (Leshem et al., 1998; Magalhaes et al., 2000). These findings are inline with the hypothesis that a continuous

drop in endogenous NO levels might serve as a trigger for the induction of leaf senescence. In NOD overexpressing plants, we observed induction of a senescence phenotype about 3-4 days after expression of the transgene, which results in a lowering of NO levels. This phenotype exhibits strong similarity to natural and dark-induced senescence. For example, *SAG12*, which is specifically induced during natural senescence (Weaver et al., 1998), is up-regulated during NOD-induced senescence, and many metabolic changes are shared between both processes. Importantly, exogenous application in the low ppm range to NOD expressing plants delayed the induced senescence process, substantiating that the induced phenotype is due to lowering of NO levels in these plants. An accelerated senescence phenotype is also induced in another NO-deficient Arabidopsis line, the *Atnos1* mutant (Guo and Crawford, 2005).

An unequivocal source of NO in plants is nitrate reductase which in a side reaction reduces nitrite to NO (Rockel et al., 2002). Several factors stimulating NR activity, i.e. nitrate feeding, high light treatment and cytokinin application, also lead to an enhanced production of NO (Maghalaes et al., 2000; Tun et al., 2001; Planchet et al., 2006). The same conditions, as we observed in our experiments, delayed NOD-induced senescence. Therefore, during natural senescence, light conditions, nitrogen supply, and cytokinin action might be integrated through NR-derived NO production to control the onset of senescence.

The natural senescence process is associated by increased levels of ROS, and this is also true for NOD-induced senescence. Indeed, increased ROS levels might be one of the factors triggering senescence (Zimmermann and Zentgraf, 2005). A possible action of NO in delaying senescence might be to attenuate ROS accumulation. For instance, NO donors protect plants from oxidative damage caused by methylviologen herbicides (Beligni and Lamattina, 1999). During ABA- and jasmonate-induced senescence in rice, NO donors prevent increases in H_2O_2 levels and lipid peroxidation (Hung and Kao, 2003; 2004). Hence, NO seems to act as an antioxidant preventing the accumulation of ROS and therefore delaying senescence.

4.2 Non-host resistance in Arabidopsis against *P. syringae*

In the Arabidopsis-*Pseudomonas syringae* pathosystem, three types of interactions can be discriminated: compatible, incompatible and non-host interactions. After entering the leaf apoplastic spaces through stomata, the bacterial pathogens need to overcome different layers of defence and achieve nutrient uptake to multiply and colonize the plant (Thordal-Christensen, 2003).

Preformed antimicrobial substances (phytoanticipins) might constitute one of the earliest defence layers. According to our data, the number of non-adapted *Psg* and *Psp* bacteria rapidly decline after inoculation. By contrast, host bacteria like *Pst* or *Psm* are able to heavily multiply in the apoplast. We thus suggest the presence of a toxic barrier that contributes to resistance against non-adapted bacteria. Obviously, host bacteria developed the ability to overcome such a toxic barrier. This ability might not depend on type III secretion, because the TTSS-deficient, non-pathogenic *Pst hrpA*$^-$ mutant host strain is able to survive for longer periods of time in the Arabidopsis apoplast than non-adapted *Psg* or *Psp*. We hypothesized that the glucosinolate/myrosinase defence system that is present in all members of the *Brassicaceae* family could provide toxic hydrolysis products to counteract non-host *P. syringae*. These mustard oils are known to possess antimicrobial activity *in vitro* (Tierens et al., 2001). Although we have detected different survival rates of non-adapted bacteria in leaves of Arabidopsis mutants and accessions with distinct glucosinolate composition or hydrolysis, a clear correlation between bacterial survival and a particular mustard oil composition was not observed. Thus, our data cannot provide final evidence for a defence function of glucosinolate hydrolysis products against bacterial pathogens.

Inducible cell wall-based defences possibly represent another early defence layer against non-adapted bacteria. Papillae are cell wall fortifications composed of callose, lignin and other cell wall material. Formation of papillae was observed in response to leaf inoculation with non-adapted or TTSS-deficient bacteria, in response to bacterial flagellin, and at fungal infection sites (Soylu et al., 2005; Bestwick, 1995). For proper formation of papillae, callose synthase and lignin biosynthesis genes should be activated. In accordance, we have shown that *PAL1* and *BCB*, which are both involved in lignin biosynthesis (Rohde et al., 2004; Ezaki et al., 2005), represent genes that are early induced in the Arabidopsis-*P. syringae* non-host interaction. It has been suggested that papillae restrict the flow of nutrients and water towards the apoplast and thus might restrict bacterial feeding and multiplication. A further conceivable role is to prevent the attachment of bacteria to the plant cell wall which is possibly required for bacterial multiplication. Unattached bacteria might not reach a critical mass for quorum sensing that presumably is necessary for pathogenesis (Müller and Bassler, 2001). Virulent bacteria are able to repress papilla formation due to delivery of appropriate type III effector proteins into the plant cell (Hauck et al., 2003). Considering the lignification of papillae, our observation that virulent *Pst* both suppressed *PAL1* and *BCB* expression represents a possible mechanism by which virulent bacteria avoid effective cell wall fortification. By contrast, non-adapted bacteria and TTSS-deficient mutants strains are unable to suppress *PAL1* and *BCB* expression. Our finding that *pal1* knockout mutants permit higher survival rates of *Psg* than wild-type plants provides direct evidence that induction of the general

phenylpropanoid pathway, and presumably, lignification of papillae, contributes to non-host resistance against *P. syringae*.

Another inducible defence layer that occurs to later times after infection than the above described cell-wall based defence consists of SA accumulation and induction of PR genes. We have shown that, similar to host bacteria, non-host bacteria induce these defences primarily through type III secretion and to a lesser extent through general elicitors. For defence induction by the two non-host strains *Psg* and *Psp*, several common signalling components, including ICS1, NPR1, NDR1, and PAD4 are required. However, specific differences also exist. For example, *Psp* triggers SA accumulation and *PR-1* induction through EDS1 signalling, whereas *Psg* induces the SA pathway independently of EDS1. This indicates that the two strains translocate a different cocktail of effector proteins into the plant cell which results in activation of distinct defence signalling pathways. Although non-host strains induce these SA-related defences, blockage of these defence pathways, i.e. in *sid2*, *npr1*, *ndr1*, *eds1* or *pad4* mutants, does not compromise non-host resistance (manuscript 4; van Wees and Glazebrook, 2003). The fact that SA-induced defences do not directly contribute to bacterial non-host resistance is also illustrated by our finding that survival rates in leaves of different Arabidopsis accessions do not correlate with the magnitude of SA accumulation in inoculated leaves. For instance, Ler-0 permitted a significantly higher leaf survival of *Psg* than Col-0, although SA accumulated to higher amounts in the former ecotype. By contrast, resistance to *Pst* and SA accumulation closely correlated in different ecotypes, underlying the importance of the SA pathway to restrict growth of virulent bacteria. Consistently, SA pathway mutants exhibit enhanced disease susceptibility to virulent *P. syringae* (Glazebrook, 2005).

Taken together, our data indicate the importance of an early-inducible, cell wall-based defence in non-host resistance of Arabidopsis against bacterial pathogens, and the existence of a pre-formed chemical defence barrier. Later, SA-related defence pathways are induced by non-host bacteria, but they do not come into effect because former obstacles are not overcome by the non-adapted pathogens.

4.3 Molecular determinants triggering SAR in Arabidopsis

Systemic acquired resistance (SAR) is a phenomenon whereby disease resistance to subsequent microbial infection is induced at the whole plant level by localized pathogen inoculation. The local necroses which result from an HR caused by avirulent pathogens or which originate from disease symptom development following successful infection by virulent pathogens have been thought to be necessary for SAR establishment (Sticher et al., 1997; Durrant and Dong, 2004; Hammerschmidt ,1999). In our studies, we showed

that non-adapted or TTSS-deficient *P. syringae* strains, and also flagellin and LPS, two typical bacteria PAMPs, are able to induce SAR without producing tissue necrosis or an HR. The observation that tissue collapse not necessarily results in SAR, for instance tissue necrosis inflicted after contact with cold or very hot objects (Sticher et al., 1997), and the existence of Arabidopsis mutant like *dnd1* (*defence-no-death1*), which exhibit enhanced disease resistance without cell death development after infection (Yu et al., 1998) are in accordance with our finding that tissue necrosis is dispensable for SAR induction. Moreover, a classical gene-for-gene recognition event that occurs after inoculation with avirulent pathogens is not necessary for SAR induction, because virulent, non-adapted and TTSS-deficient *P. syringae* strains all trigger SAR.

Several lines of evidence indicate that the observed systemic resistance responses triggered by non-pathogenic strains of *P. syringae* and PAMPs are identical to SAR. The local application of non-pathogenic bacteria or PAMPs induces accumulation of SA in systemic leaves which is a major characteristics of SAR (Sticher et al., 1997). All SAR-deficient Arabidopsis mutants we used in our studies fail to express both SAR and systemic resistance induced by non-host bacteria or PAMPs. In addition, increased systemic expression of the flavin-containing monooxygenase gene *FMO1*, which is closely associated with the SAR-induced state (publication 6), was observed also after treatment with non-adapted bacteria, flagellin or LPS. Finally, non-adapted bacteria or PAMPs were able to induce systemic resistance in the jasmonate-insensitive *jar1* mutant, demonstrating that the observed response is mechanically different from induced systemic resistance (ISR), for which JA-dependent signalling is necessary. ISR can be triggered in roots by colonization with growth-promoting rhizobacteria (Pieterse et al., 1998).

We have observed that amounts of total SA and camalexin accumulation but not the extent of tissue necroses at sites of *Psm* or *Psm avrRpm1* infection positively correlate with the magnitude of SAR. This indicates that SA- or camalexin-associated defence responses but not processes related with tissue necrosis are involved in generation of a mobile signal that is transferred over longer distances from the inoculated leaf to non-infected plant parts to trigger SAR. A good candidate for a mobile SAR signal is methyl salicylate (MeSA; Kumar and Klessig, 2003; Forouhar et al., 2005). A part of the SA generated in inoculated leaves might be converted to MeSA, which is translocated to systemic tissue and finally reconverted to signal-competent SA. We can exclude a role for camalexin as a SAR signal because the camalexin biosynthesis mutant *pad3* exhibits a clear SAR response, and the Arabidopsis phytoalexin is only accumulating in inoculated but not in distant leaves. Recent work by Truman et al. (2007) proposes jasmonate as a mobile SAR signal. This is in contradiction to our results, because JA is not produced in many cases where SAR is observed, e.g. after PAMP treatment and inoculation with non-host bacteria. Furthermore, when SAR was induced by varying optical densities of *Psm*, a

close correlation of JA accumulation and tissue necrosis was observed, but no correlation of JA production and the extent of SAR existed. Moreover, we never detected increased levels of JA in distant leaves of SAR-induced plants. Finally, the JA insensitive mutant *jar1* exhibited a clear SAR response in our experiments. We identified the bacterial PAMPs flagellin and LPS, which trigger a set of local defence reaction (Felix et al., 1999; Coventry and Dubery, 2001), as SAR-inducing components. This opens the interesting possibly that PAMP molecules themselves might act as long-distance signals which move out of pathogen-infected leaves and distribute systemically to directly initiate defense responses in systemic tissue. Whether such a translocation of PAMPs takes place and whether sufficient amounts of PAMPs would systemically accumulate to noticeably enhance resistance remains to be determined.

4.4 Identification new defence components: the flavin-dependent monooxygenase FMO1 as an essential component of SAR in Arabidopsis

To identify uncharacterized gene involved in defence and SAR establishment we have selected Arabidopsis candidate genes up-regulated in leaves treated with *P. syringae*, as indicated in microarray experiments publicly available from the Nottingham Arabidopsis Stock Centre (NASC) and from The Arabidopsis Information Resource (TAIR). Arabidopsis T-DNA knockout lines corresponding to selected candidate genes were analyzed for impaired defence or SAR phenotypes.

We identified the *fmo1* knockout line that shows no significant alteration in local response to Ps*m avrRpm1* and *Psm*, but is totally compromised in systemic defence responses and acquired resistance, demonstrating that *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE1*) is a key component for SAR establishment in Arabidopsis. An Arabidopsis line constitutively overexpressing *FMO1*, identified by an activation-tagging approach, shows enhanced disease resistance against *P. syringae* (Koch et al., 2006). This indicates that overexpression of *FMO1* is sufficient to constitutively activate a SAR equivalent state in non-inoculated plants. In *sid2, npr1*and *ndr1* mutant plants, which are all SAR-defective, *FMO1* is expressed in leaves inoculated with *P. syringae*, indicating that local expression of *FMO1* is independent from SA- and NDR1-mediated signalling pathways. Strikingly, these mutants fail to express *FMO1* systemically. On the other hand, all tested Arabidopsis lines that are able to activate SAR also express FMO1 systemically upon local pathogen treatment. This close correlation between the ability of Arabidopsis lines to establish SAR and induced expression of *FMO1* in systemic leaves indicates that the presence of FMO1 in systemic tissue is essential for SAR development. The fact that systemic but not local defence responses triggered by the avirulent *P. syringae* strain *Psm*

avrRpm1, i.e. SA accumulation and PR gene expression, are compromised in *fmo1* knockout mutants support this idea. Expression of *FMO1* in inoculated leaves is attenuated in *eds1* and *pad4* mutants, indicating that *FMO1* contribute to the EDS1/PAD4 pathway in local defence signalling (Bartsch et al., 2006). The *eds1* mutant shows an attenuated SAR response and decreased *FMO1* expression in systemic leaves after pathogen treatment, but systemic responses are not completely absent in this mutant.

FMO1 expression is up-regulated in the *acd11* mutant, which constitutively expresses SA-, PAD4-, and EDS1-dependent defences and exhibits a spontaneous HR (Brodersen et al., 2002; Olszak et al., 2006). The same is true for the runaway cell death mutant *lesion-simulating disease1 (lsd1*), but not for the constitutive defence-signalling mutants *ctr1,cev1,mpk4* and *cpr6* that do not develop spontaneous cell death. Moreover, expression of *FMO1* was observed during senescence. *FMO1* has thus been suggested as a marker gene for plant cell death (Olszak et al., 2006). Our data do not support this conclusion, because *FMO1*-up-regulation in distant leaves during SAR is not accompanied with increased HR cell death, and up-regulation of FMO1 also takes place in the constitutive defence mutant *cpr5* which does not exhibit a spontaneous HR.

Bartsch et al. (2006) have shown that local FMO1 expression is required for basal defence against a virulent isolate of the oomycete pathogen *Hyaloperonospora parasitica* and for specific resistance against *H. parasitica* isolate Noco2 or *P. syringae* carrying the *avrRps4* avirulence gene. Our data show that defences at the sites of *Psm avrRpm1* inoculation are not compromised in the *fmo1* knockout line. In addition, various characteristic local defence responses triggered by *Psm avrRpm1*, including the oxidative burst, accumulation of SA, JA and camalexin, and expression of defence genes as well as hypersensitive cell death, are not significantly altered in *fmo1.* Because the AvrRpm1 avirulence protein is recognized by the RPM1 resistance protein, which belongs to the CC-NB-LRR class of receptors, we suggested that basal resistance to virulent strains of *P. syringae*, and specific resistance mediated by CC-NB-LRR receptors are *FMO1*-independent. In contrast, specific resistance to *Pst avrRps4*, which is mediated by TIR-NB-LRR receptors, is *FMO1*-dependent (Bartsch et al., 2006). The involvement of *FMO1* in basal resistance against *H. parasitica*, in specific resistance mediated by the TIR-NB-LRR R-protein class and in SAR reveals overlapping molecular principles on different resistance levels.

ROS seem to play an important role in the regulation of *FMO1* transcription, because superoxide-generating compounds trigger FMO1 expression (Olszak et al., 2006). ROS production represents one of the earliest systemic responses after pathogen challenge in Arabidopsis (Alvarez et al., 1998). Taking these information and our results as a basis, we propose the existence of a signal amplification loop occurring in distant, non-inoculated leaves after localized, biological SAR induction. Here, FMO1 acts in

concert with other defence components including ROS, SA, NPR1 and NDR1 to amplify incoming mobile SAR signals generated at infection sites and potentiate defence responses in systemic tissue and thus enable SAR establishment.

The analysis of enzymatic activity of the FMO1 monooxygenase and the determination of its substrate and reaction product is now necessary to better understand the processes involved in SAR establishment. Mammalian flavin-dependent monoogygenases either contribute to oxidative xenobiotic metabolism or catalyze the oxygenation of endogens metabolites. Their substrates are primarily N- or S-containing substances (Krueger and Williams, 2005). The single yeast FMO ensures correct protein folding at the endoplasmic reticulum by conversion of aminothiols to their corresponding disulfides, thus providing a suitable oxidizing environment at the ER (Suh et al., 1999). In plants, a family of approximately 30 FMO-like genes exists (Neumann et al., 2002). The only plant FMO proteins that have been biochemically characterised so far represent Arabidopsis YUCCA and its petunia (petunia hybrida) ortholog FLOOZY, which are involved in auxin biosynthesis (Zhao et al., 2001; Tobena-Santamaria et al., 2002). YUCCA has been demonstrated to be involved in the hydroxylation of the amino group to tryptamine. This suggests that FMO1 might be involved in hydroxylation processes of amines or thiols.

5. Summary and perspectives

NO has been described as an important component involved in the development of the hypersensitive reaction (Delledonne et.al., 1998). Furthermore, NO induces expression of a set of defence gene, such as *PR-1*, *PAL1* and chalcone synthase (*CHS*), and accumulation of SA (Durner et al., 1998). In this study, transgenic plants with altered NO levels were used to study the role of NO in plant defence. Arabidopsis plants which, due to expression of a bacterial NO dioxygenase, exhibit lower levels of NO than wild-type plants, show several weakened defence response, including the oxidative burst and expression of phenylpropanoid pathway genes. By contrast, constitutive expression of a bacterial NO synthase in Arabisopsis results in increased levels of endogenous NO. However, these plants do not show constitutively activated defence responses, but suffer from increased susceptibility to various strains of *P. syringae*. This might indicate that a gradient in NO production rather than constitutive elevation of NO is necessary to trigger plant defence responses. Nevertheless, NO seems to be important for regulation of the oxidative state in plant cells. This function of NO is important during leaf senescence. The data of the present work indicate that NO acts as senescence-delaying factor during plant development.

The molecular action of NO in plants and signalling cascades in which NO is involved as second messenger are still poorly understood. Experiments addressing the selective quantification of NO in intact plant tissue, the identification of NO-target proteins as well as the function of NO-modified biomolecules might help to understand the role of NO in plants.

Non-host resistance consists of several layers of defence that include preformed compounds existing in plants before pathogen infection and induced defences which the plant activates after recognition of a pathogen. The role of inducible defences in preventing multiplication of non-adapted bacteria is not clear. Our experiments suggest that to restrict non-adapted bacterial growth, pre-formed antimicrobial compounds and an early inducible cell wall-based defence might play an important role in Arabidopsis leaves. Upon inoculation with non-adapted bacteria, we have observed early, TTSS-independent up-regulation of *PAL1* and *BCB*, two lignin biosynthesis genes which might be involved in papilla formation or other kinds of cell wall fortification. Moreover, Arabidopsis *pal1* knockout lines permit significantly higher survival of non-adapted bacteria in leaves than wild-type plants, suggesting a functional importance of *PAL1* up-regulation. Although non-host bacteria, like host bacteria, induce accumulation of SA and PR gene expression in a TTSS-dependent manner, SA-dependent or JA/ET-dependent defences do not directly contribute to non-host resistance. Moreover, non-adapted bacteria activate similar defence signalling pathways as do host bacteria. However, because of varieties in effector

protein composition between different non-adapted bacterial strains, the activated signalling pathways might also include different compounds. The Arabidopsis ecotype Ler-0 is more susceptible to a non-adapted strain of *P. syringae* than ecotype Col-0. Although differences in glucosinolate content and composition between those ecotypes exist, they are probably not a major reason for the observed difference in non-host resistance.

To further understand the mechanisms underlying non-host resistance, the generation of double or triple mutants with deficits in both cell wall-based defences and SA-dependent signal cascades is necessary. Moreover, the study of genome polymorphism and composition of secondary metabolites between Ler-0 and Col-0 can shed new light into the mechanisms of non-host resistance against bacterial pathogens. Additionally, experiments addressing papilla formation and callose biosynthesis in Ler-0 and Col-0 could help to further elucidate bacterial non-host resistance.

Our data indicate that localized contact of Arabidopsis leaves with non-adapted bacteria, type III secretion-defective *P. syringae* strains and bacterial pathogen-associated molecular patterns (PAMPs) induce systemic acquired resistance (SAR) at the whole plant level. This finding contrasts the general belief that an HR or other leaf necroses are required for SAR induction. The observed symptomless systemic response was abolished in all SAR-deficient mutants tested in this study, but was intact in the *jar1* mutant, which is compromised in induction of ISR, indicating that non-host bacteria and PAMPs induce SAR in a mechanistically similar way than host bacteria. In addition, our data show that the extent of SA accumulation or PR gene expression induced at sites of virulent or avirulent *P. syringae* inoculation rather than the amount of tissue necroses or jasmonate accumulation determine the magnitude of SAR. The fact that systemic responses were also triggered after local treatment with type III secretion-defective *P. syringae* strains and bacterial PAMPs indicate that induction of SAR is TTSS-independent. Instead, recognition of general elicitors like flagellin and LPS play an important role in activation of the SAR process.

To broaden the concept of PAMP-based SAR initiation, further general elicitors from bacteria and fungal pathogens should be tested for their capability to induce SAR. Screens for mutants with deficiency in SAR activation by individual PAMPs can help to identify new components involved in the SAR signalling cascade. Possible functions of PAMPs as mobile systemic signals should be tested in future experiments.

By selection of candidate genes whose expression is up-regulated in *Arabidopsis* leaves infected with avirulent and virulent *P. syringae* and pathophysiological analyses of corresponding T-DNA knockout lines, *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*) was identified as a key SAR regulator. SAR triggered by *P. syringae* is completely abolished in *fmo1* mutant plants, and pathogen-induced expression of *FMO1* in systemic

leaves is closely correlated with the capability of different Arabidopsis lines to develop SAR. According to our findings, we have proposed that the FMO1 acts in signal amplification in non-inoculated, systemic leaves to trigger SAR.

Experimental verification of the postulated potential amplification cycle underlying SAR should be tested in future experiments. The generation of transgenic lines expressing *FMO1*::*GFP* will provide useful information about the cellular localization of the FMO1 protein. Moreover, a comparative metabolomic analysis using SAR-induced wild-type, *fmo1* knockout and *FMO1* overexpressing lines can be used to identify substrates and reaction products of the FMO1 monooxygenase. As the single yeast FMO (yFMO) provides oxidizing equivalents at the ER for correct protein folding, expression of FMO1 in *yfmo* mutant yeast combined with protein activity assays might indicate whether FMO1 exhibits functional similarities with yeast FMO, e.g. in assuring proper folding of ER-targeted proteins essential for SAR establishment. Identification of further genes involved in activation of systemic resistance and biochemical characterization of the corresponding proteins can help to understand the SAR process in more detail.

6. Zusammenfassung und Ausblick

Stickstoffmonooxid (NO) wird als wichtige Signalkomponente bei der Entwicklung der Hypersensitiven Reaktion beschrieben. Außerdem wird NO eine Rolle als Signalmolekül bei der Expression von Abwehrgenen wie *PR-1*, *PAL1* oder Chalkonsynthase (*CHS*) und bei der Akkumulation von Salicylsäure zugeordnet (Durner et al., 1998). In der vorliegenden Arbeit wurden transgene Pflanzen mit veränderten endogenen NO-Spiegeln verwendet, um die Rolle von NO in Pflanze-Pathogen-Interaktionen zu untersuchen. Arabidopsis-Pflanzen, die aufgrund der Expression einer NO Dioxygenase erniedrigte NO-Gehalte aufweisen, zeigen nach einem Angriff avirulenter Pathogene einen abgeschwächten *oxidative burst* und eine reduzierte Expression von Genen des Phenylpropanbiosyntheseweges. Weitere Experimente mit transgenen Pflanzen, die eine bakterielle NO-Synthase exprimieren, legen nahe, dass eine konstitutive Erhöhung der NO-Spiegel nicht zu einer konstitutiv verstärkten Pathogenabwehr führt. Möglicherweise ist eine graduelle Steigerung der NO-Gehalte nach Pathogenkontakt für die Induktion pflanzlicher Abwehrreaktionen erforderlich. Im Gegenteil, die NOS-exprimierenden Pflanzen waren anfälliger gegen bakterielle Pathogene als Wildtyp-Pflanzen und zeigten eine abgeschwächte SAR-Reaktion. Die Ergebnisse deuten auch darauf hin, dass NO eine wichtige Rolle bei der Regulation des Redoxstatus in der Pflanzenzelle spielt. Diese Funktion von NO ist wichtig beim Seneszenzvorgang. Entsprechend der Ergebnisse dieser Arbeit kann NO als negativer Regulator der Blattseneszenz angesehen werden.

Die Wirkungsweise von NO auf molekularer Ebene und die Signalkaskaden, in die NO involviert ist, sind immer noch nicht ausreichend verstanden. In zukünftigen Experimenten wird es notwendig sein, die selektive Quantifizierung von NO in intaktem Pflanzengewebe zu gewährleisten, die Proteintargets von NO zu identifizieren und die Struktur und Funktion NO-modifizierter Biomoleküle zu entschlüsseln, um die Rolle von NO in Pflanze-Pathogen-Wechselwirkungen besser verstehen zu lernen.

Die Nichtwirtsresistenz beruht auf mehreren Verteidigungsebenen, welche konstitutive und induzierte Komponenten beinhalten. Die Bedeutung induzierter Abwehrreaktionen für die Nichtwirtsresistenz gegen bakterielle Pathogene ist nicht vollständig klar. Die Daten der vorliegenden Arbeit legen nahe, dass das Wachstum von Nichtwirtsbakterien in Arabidopsis-Blättern durch vorgebildete toxische Substanzen und durch induzierte Zellwandverstärkungen gehemmt wird. Nichtwirtsbakterien verursachen eine schnelle Induktion der Expression der Ligninbiosynthesegene *PAL1* und *BCB*, die unabhängig vom Typ III-Sekretionssystem ist und möglicherweise zur Papillenbildung beiträgt. Darüber hinaus ist die Überlebensrate der Nichtwirtsbakterien in den extrazellulären Räumen der Arabidopsis *pal1*-Mutante höher als in Wildtyp-Pflanzen, was die funktionelle Bedeutung der *PAL1*-Expression bei der Nichtwirtsresistenz verdeutlicht.

Außerdem zeigen die Experimente, dass Nichtwirtsbakterien in ähnlicher Weise wie Wirtsbakterien die Akkumulation von Salicylsäure und die Expression von PR-Genen induzieren. Die Induktion dieser Abwehrkomponenten ist abhängig von einem intakten Typ III-Sekretionssystem. Die Signalwege, auf denen nach Kontakt mit Nichtwirtsbakterien und Wirtsbakterien Abwehrreaktionen induziert werden, sind ähnlich. Es wurden jedoch zwischen zwei verschiedenen Nichtwirtsstämmen auch unterschiedliche Signalwege aktiviert, was möglicherweise auf ein unterschiedliches Repertoire von TypIII-Effektoren der beiden Stämme zurückgeführt werden kann. Trotz der Aktivierung dieser induzierten Abwehr zeigen Experimente mit klassischen Abwehrmutanten, dass SA- und JA-abhängige Abwehrreaktionen nicht direkt zur Nichtwirtsresistenz gegen *P. syringae* beitragen. Weiterhin zeigt diese Arbeit, dass die Nichtwirtsresistenz des Arabidopsis-Ökotyps Col-0 effektiver ist als die des Ler-0-Ökotyps, obwohl bei letzterem die Resistenz gegen virulente Bakterien höher ist. Diese Unterschiede scheinen nicht mit der unterschiedlichen Glucosinolatzusammensetzung der beiden Ökotypen im Zusammenhang zu stehen.

Um das Verständnis der Nichtwirtsresistenz von Arabidopsis gegenüber *P. syringae* zu verbessern, können in zukünftigen Experimenten Doppel- und Triplemutanten hergestellt werden, die gleichzeitig Defekte in der zellwandabhängigen Abwehr (Lignin- und Callosebiosynthese) und in klassischen, SA-abhängigen Abwehrreaktionen aufweisen. Auch können Analysen des Genom-Polymorphismus und der Zusammensetzung von Sekundärmetaboliten in den Ökotypen Ler-0 und Col-0 zu einem besseren Verständnis der Nichtwirtsresistenz führen.

Die Resultate dieser Arbeit zeigen, dass ein lokaler, symptomfreier Kontakt von Arabidopsis-Blättern mit Nichtwirtsbakterien, TTSS-defiziente Bakterien und allgemeine bakterielle Elicitoren (PAMPs) wie Flagellin und Lipopolysaccharide die systemisch erworbene Resistenz innerhalb der Gesamtpflanze hervorrufen. Die symptomlose systemische Resistenzreaktion findet in SAR-defizienten Mutanten nicht statt, wird jedoch in der Jasmonat-insensitiven *jar1*-Mutante, die keine ISR-Reaktion ausbilden kann, beobachtet. Durch Behandlung von Arabidopsis-Blättern mit unterschiedlichen Inokuli von virulenten oder avirulenten *P. syringae*-Stämmen wurde auch eine deutliche Korrelation des Ausmaßes der SAR-Induktion mit der Höhe der SA-Akkumulation oder der PR-Genexpression, aber nicht mit der Nekrosenbildung oder der JA-Produktion, am Infektionsort festgestellt. Diese Ergebnisse verdeutlichen, dass nicht die Hypersensitive Reaktion oder Gewebenekrosen, sondern möglicherweise die Stärke bestimmter Abwehrreaktionen am Ort der Inokulation zur Auslösung der SAR beitragen. Die Befunde, dass die systemische Resistenz auch durch PAMPs und durch TTSS-defekte *P. syringae*-Stämme erhöht wird, verdeutlicht die wichtige Rolle von allgemeinen Elicitoren bei der SAR-Induktion.

In künftige Experimenten kann untersucht werden, ob verschiedene PAMPs die SAR in synergistischer Weise induzieren und ob allgemeine Elicitoren pilzlicher Herkunft SAR auslösen können. Weiterhin können die molekulare Prozesse spezifiziert werden, die stromabwärts von PAMP-Erkennungsprozessen für die SAR-Ausbildung notwendig sind. In weiteren Experimenten könnte die Hypothese überprüft werden, ob einzelner PAMPs als mobile SAR-Langstreckensignale fungieren können.

Durch phytopathologische Charakterisierung von T-DNA-Knockout-Linien, die Defekte in Genen aufweisen, welche in Arabidopsis nach einer *P. syringae*-Infektion aufreguliert werden, konnte das *FLAVIN-DEPENDENT MONOOXYGENASE1* (*FMO1*)-Gen als notwendige Komponente der SAR in Arabidopsis identifiziert werden. So bleiben die im Wildtyp induzierten systemischen Abwehrreaktionen und die Erhöhung der systemischen Resistenz nach lokaler Inokulation mit *P. syringae* in *fmo1*-Knockout-Pflanzen vollständig aus. Weiterhin korreliert die systemische Expression des FMO1-Gens eng mit der SAR-Induktion. So gibt es bei allen Abwehrmutanten, die keine SAR nach Kontakt mit *P. syringae* ausbilden können, keine *FMO1*-Expression in distalen Blättern inokulierter Pflanzen. Umgekehrt verhält es sich mit Arabidopsis-Linien, die die SAR ausbilden. Die erhaltenen Ergebnisse deuten darauf hin, dass FMO1 eine wichtige Komponente eines Signalverstärkungszyklus darstellt, der in nichtinfizierten, systemischen Teilen der Pflanze wirkt, um die SAR zu ermöglichen.

In künftigen Experimenten soll der postulierte Amplifizierungsmechanismus experimentell verifiziert werden. Die Konstruktion von transgenen Linien, die ein *FMO1:GFP*-Fusionsprodukt exprimieren, kann Informationen über die zelluäre Lokalisation des FMO1-Proteins liefern. Weiterhin können vergleichende Analysen der chemischen Zusammensetzung von Blattextrakten der *fmo1* Knockout-Linien, von *FMO1*-Überexprimierern und von Wildtyp-Pflanzen zur Aufklärung der biochemischen Reaktion beitragen, die die FMO1-Monooxygenase katalysiert. In Anlehnung an die Funktion von yFMO, die die einzige Flavin-abhängige Monooxygenase der Hefe darstellt, kann überprüft werden, ob FMO1 die korrekte Faltung von Proteinen am endoplasmatischen Retikulum vermittelt. Schließlich kann durch die Identifizierung weitere SAR-Gene nach der beschriebenen Strategie und durch funktionelle Charakterisierung der zugehörigen Proteine das Verständnis der SAR-Reaktion auf molekularer Ebene weiter verbessert werden.

7. References

Aarts N, Metz M, Holub E, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signalling pathways in Arabidopsis. *Proc Natl Acad Sci USA* 95: 10306-10311.

Abramovitch RB, Kim YJ, Chen S, Dickman MB, Martin GB (2003) Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. *EMBO J* 22: 60–69

Agrios GN (1997) Plant Pathology (San Diego: Academic Press).

Alfano JR, Collmer A (1996) Bacterial pathogens in plants: life up against the wall. *Plant Cell* 8: 1683-1698.

Alfano JR, Collmer A (2004) Type III secretion system effector proteins: Double agents in bacterial disease and plant defense. *Annu Rev Phytopathol* 42: 385-414.

Alvarez ME, Pennell RI, Meijer P-J, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92: 773-784.

Assaad FF, Qiu JL, Youngs H, Ehrhardt D, Zimmerli L, Kalde M, Wanner G, Peck SC, Edwards H, Ramonell K, Somerville CR, Thordal-Christensen H (2004) The PEN1 syntaxin defines a novel cellular compartment upon fungal attack and is required for the timely assembly of papillae. *Mol Biol Cell* 15: 5118-5129.

Balcerczyk A, Soszynski M & Bartosz G (2005) On the specificity of 4-amino-5-methylamino-2',7'-difluorofluorescein as a probe for nitric oxide. *Free Radic Biol Med* 39: 327–335.

Bartsch M, Gobbato E, Bednarek P, Debey S, Schultze JL, Bautor J, Parker JE (2006) Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monoxygenase FMO1 and the nudix hydrolase NUDT7. *Plant Cell* 18: 1038-1051.

Beligni MV, Lamattina L (1999) Nitric oxide counteracts cytotoxic processes mediated by reactive oxygen species in plant tissues. *Planta* 208: 337–344.

Beligni MV, Fath A, Bethke PC, Lamattina L, Jones RL (2002) Nitric oxide acts as an antioxidant and delays programmed cell death in barley aleurone layers. *Plant Physiol*. 129: 1642-1650.

Belkhadir Y, Nimchuk Z, Hubert DA, Mackey D and Dangl JL (2004) Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial Type III effectors AvrRpt2 or AvrRpm1. *Plant Cell* 16: 2822–2835.

Bestwick CS, Bennett MH, Mansfield JW (1995) Hrp Mutant of Pseudomonas syringae pv phaseolicola Induces Cell Wall Alterations but Not Membrane Damage Leading to the Hypersensitive Reaction in Lettuce. *Plant Physiol* 108: 503-516.

Bethke PC, Badger MR, Jones RL (2004) Apoplastic synthesis of nitric oxide by plant tissues. *Plant Cell* 16: 332-341.

Bethke PC, Libourel IG, Jones RL (2006) Nitric oxide reduces seed dormancy in Arabidopsis. *J Exp Bot* 57: 517-26.

Bisgrove SR, Simonich MT, Smith NM, Sattler NM and Innes RW (1994) A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. *Plant Cell* 6: 927–933.

Biswal B, Biswal UC (1999) Leaf senescense: physiology and molecular biology. *Curr Sci* 77: 775-782.

Boller T (1991) Ethylene in pathogenesis and disease resistance. In: *The Plant Hormone Ethylene, A.K. Mattoo and J.C. Suttle, eds., CRC Press, Boca Raton*, pp. 293-314.

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X (1997) The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *Plant Cell* 9:1573-84.

Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Odum N, Jorgensen LB, Brown RE, MundyJ (2002) Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes Dev* 16: 490-502.

Brodersen P, Petersen M., Nielsen HB, Zhu SJ, Newman M A, Shokat K M, Rietz S, Parker J and Mundy J (2006) Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. *Plant Journal* 47: 532-546.

Broekaert WF, Terras FRG, Cammue BPA, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol* 108: 1353-1358.

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, De Samblanx GW and Osborn RW (1997) Antimicrobial peptides from plants. *Crit Rev Plant Sci* 16: 297-323.

Brooks DM, Bender CL and Kunkel BN (2005) The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. *Mol Plant Pathol* 6: 629–639.

Cao H, Bowling SA, Gordon S and Dong X (1994) Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *Plant Cell* 6: 1583–1592.

Chang C, Stadler R (2001) Ethylene hormone receptor action in Arabidopsis. *Bioessays* 23: 619-627.

Chang JH, Goel AK, Grant SR, Dangl JL (2004) Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria. *Curr Opin Microbiol* 7: 11-8.

Chang JH, Urbach JM, Law TF, Arnold LW, Hu A, Gombar S, Grant SR, Ausubel FM, Dangl JL. (2005) A high-throughput, near-saturating screen for type III effector genes from Pseudomonas syringae. *Proc Natl Acad Sci U S A* 102: 2549-2554.

Chen AP, Kloek A, Cuzick W, Moeder D, Tang RW, Innes DF, Klessig JM, McDowell and. Kunkel BN (2004) The Pseudomonas syringae type III effector AvrRpt2 functions downstream or independently of SA to promote virulence on *Arabidopsis thaliana*. *Plant J*. 37: 494–504.

Chinchilla D, Bauer Z, Regenass M, Boller T and Felix G (2006) The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18: 465–476.

Clark D, Durner J, Navarre DA, Klessig DF (2000) Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. *Mol Plant-Microbe Interact* 13: 1380-1384.

Cohn JR, Martin GB. (2005) Pseudomonas syringae pv. tomato type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato. *Plant J* 44: 139–154

Collins NC, Thordal-Christensen H, Lipka V, Bau S, Kombrinh E, Qiu J-L, Huclelhoven R, Stein M, Freialdenhoven A, Somerville SC, et al. (2003) SNARE-protein-mediated disease resistance at the plant cell wall. *Nature* 425: 973–977.

Coppinger JP, Repetti P, Day B, Dahlbeck D, Mehlert A and Staskawicz B (2004) Overexpression of the plasma membrane-localized NDR1 protein results in enhanced bacterial disease resistance in Arabidopsis thaliana. *Plant J* 40: 225–237.

Coventry HS, Dubery IA (2001) Lipopolysaccharides from Burkholderia cepacia contribute to an enhanced defensive capacity and the induction of pathogenesis-related proteins in Nicotiana tabacum. *Physiol Mol Plant Pathol* 58: 149–158.

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. *Plant Cell* 7: 859-68.

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonates in plants. *Annu Rev Plant Physiol Plant Mol Biol* 48: 355-381.

Crute IJ, Beynon J, Dangl E, Holub B, Mauch-Mani B et al., (1994) Microbial pathogenesis of Arabidopsis. In Arabidopsis, edited by Meyerowitz EM, Somerville CR *Cold Spring Harbor Laboratory Press, New York*. pp. 705–747.

da Cunha L, Aidan J McFall , David Mackey (2006) Innate immunity in plants: a continuum of layered defenses. *Microbes Infect* 23:166976-167074 .

Daiber A, Frein D, Namgaladze D, Ullrich V. (2002) Oxidation and nitrosation in the nitrogen monoxide/superoxide system. *J Biol Chem* 277: 11882–11888.

Dangl JL, Ritter C, Gibbon MJ, Mur LA, Wood JR, Goss S, Mansfield J, Taylor JD, Vivian A (1992) Functional homologs of the Arabidopsis RPM1 disease resistance gene in bean and pea. *Plant Cell* 4: 1359–1369.

DebRoy S, Thilmony R, Kwack YB, Nomura K and He SY (2004) A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proc Natl Acad Sci USA* 101: 9927–9932.

Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. *Nature* 394: 585-588.

Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. *Proc Natl Acad Sci USA* 98: 13454-13459.

Delaney T P, Friedrich L, Kessmann H, Uknes S, Vernooij B, Ward E, Weymann K and Ryals J (1994) The molecular biology of systemic acquired resistance. In: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 3, M. J. Daniels, J. A. Downie and Anne E. Osbourn, (eds.*). Kluwer Academic Publishers, Dordrecht, Netherlands*, pp. 339-347.

Dempsey DA, Shah J and Klessig DF (1999) Salicylic acid and disease resistance in plants. *Crit Rev Plant Sci* 18: 547–575.

Desikan R, Griffiths J, Hancock J and Neill S (2002) A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. *PNAS* 99: 16314 - 16318.

Deslandes L, Olivier J, Peeters N, Feng DX, Khounlotham M, Boucher C, Somssich I, Genin S, Marco Y (2003) Physical interaction between RRS1-R, a protein conferring resistance to

bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proc Natl Acad Sci U S A* 100: 8024-8029.

Dong X, Mindrinos M, Davis KR, Ausubel FM (1991) Induction of Arabidopsis defense genes by virulent and avirulent Pseudomonas syringae strains and by a cloned avirulence gene. *Plant Cell* 3: 61–72.

Dorey S, Kopp M, Geoffroy P, Fritig B, Kauffmann S (1999) Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. *Plant Physiol* 121: 163-172.

Dorey S, Baillieul F, Pierrel MA, Saindrenan P, Fritig B, Kauffmann S (1997) Spatial and temporal induction of cell death, defense genes, and accumulation of salicylic acid in tobacco leaves reacting hypersensitively to a fungal glycoprotein elicitor. *Mol Plant-Microbe Interact* 10: 646-655.

Durner J, Shah J and Klessig DF (1997) Salicylic acid and the induction of defense genes. *Trends Plant Sci* 2: 266-274.

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. *Proc Natl Acad Sci USA* 95: 10328-10333.

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42: 185-209.

Ezaki B, Sasaki K, Matsumoto H, Nakashima S (2005) Functions of two genes in aluminium (Al) stress resistance: repression of oxidative damage by the AtBCB gene and promotion of efflux of Al ions by the NtGDI1gene. *J Exp Bot* 56: 2661-271.

Falk BJ, Feys LN, Frost JDG, Jones MJ, Daniels and Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. *Proc Natl Acad Sci USA* 96: 3292-3297.

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. *Plant Cell* 14: 1377-89.

Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ (2005) A central role for S-nitrosothiols in plant disease resistance. *Proc Natl Acad Sci USA* 102: 8054-8059.

Felix G, Duran JD, Volko S, Boller T (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J 18:265-76.

Felix G, Boller T (2002) Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. J Biol Chem 278: 6201-6208.

Feys BJ, Moisan LJ, Newman MA, Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. *EMBO J* 20: 5400-5411.

Floryszak-Wieczorek J, Milczarek G, Arasimowicz M, Ciszewski A (2006) Do nitric oxide donors mimic endogenous NO-related response in plants? *Planta* 224: 1363-1372.

Foissner I, Wendehenne D, Langebartels C, Durner J (2000) In vivo imaging of an elicitor-induced nitric oxide burst in tobacco. *Plant J* 23: 817-824.

Forouhar F, Yang Y, Kumar D, Chen Y, Fridman E, Park SW, Chiang Y, Acton TB, Montelione GT, Pichersky E, Klessig DF, Tong L (2005) Structural and biochemical studies identify tobacco

SABP2 as a methyl salicylate esterase and implicate it in plant innate immunity. *Proc Natl Acad Sci USA* 102: 1773-1778.

Freialdenhoven A, Peterhansel C, Kurth J, Kreuzaler F, Schulze-Lefert P (1996) Identification of Genes Required for the Function of Non-Race-Specific mlo Resistance to Powdery Mildew in Barley. *Plant Cell* 8: 5-14.

Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ and Sansonetti PJ (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem* 278: 8869–8872.

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of Arabidopsis thaliana and characterization of their interactions with bacterial pathogens. *Proc Natl Acad Sci USA* 91: 8955 8959.

Glazebrook J, Rogers EE, Ausubel FM (1996) Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. *Genetics* 143: 973-982.

Glazebrook J (2001) Genes controlling expression of defense responses in Arabidopsis--2001 status. *Curr Opin Plant Biol* 4: 301-308.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol* 43: 205-227.

Gómez-Gómez L, Felix G and Boller T (1999) A single locus determines sensitivity to bacterial flagellin in Arabidopsis thaliana. *Plant J* 18: 277–284.

Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Mol Cell* 5: 1003-1011.

Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *Plant J* 23: 441-450

Grant SR, Fisher EJ, Chang JH, Mole BM and Dangl JL (2006) Subterfuge and manipulation: Type III effector proteins of phytopathogenic bacteria. *Annu. Rev. Microbial.* 60: 425-429.

Graziano M, Beligni MV, Lamattina L (2002) Nitric oxide improves internal iron availability in plants. *Plant Physiol* 130: 1852-1859.

Greenberg JT, Vinatzer BA (2003) Identifying type III effectors of plant pathogens and analysing their interaction with plant cells. *Current Opinion in Microbiology* 6: 20-28.

Grün S, Lindermayr C, Sell S, Durner J (2006) Nitric oxide and gene regulation in plants. *J Exp Bot* 57: 507-516.

Guo F-Q and NM Crawford (2005) Arabidopsis nitric oxide synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. *Plant Cell* 17: 3436-3450.

Guo F-Q, Okamoto M and Crawford NM (2003) Identification of a plant nitric oxide synthase gene involved in hormonal signaling. *Science* 302: 100-103.

Hammerschmidt R (1999) Induced disease resistance: how do induced plants stop pathogens? *Physiol Mol Plant Pathol* 55: 77-84.

Hauck P, Thilmony R, He SY (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proc Natl Acad Sci USA* 100: 8577-8582.

Haughn GW, Davin L, Giblin M, Underhill EW (1991) Biochemical genetics of plant secondary metabolites in Arabidopsis thaliana: the glucosinolates. *Plant Physiol* 97: 217-226.

He Y, Tang R-H, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F-Q, Fiorani F, Jackson RB, Crawford NM, Pei Z-M (2004) Nitric Oxide Represses the Arabidopsis Floral Transition. *Science* 305: 1968 – 1971.

Heath MC (2000) Nonhost resistance and nonspecific plant defenses. *Curr Opin Plant Biol* 3: 315-319.

Hogge LR, Reed DW, Underhill EW, Haughn GW (1988) HPLC separation of glucosinolates fromleaves and seeds of Arabidopsis thaliana and their identification using thermospray liquidchromatography/mass spectrometry. *J Chromatogr Sci* 26: 551-556.

Hoyos M E, Stanley CM, He SY, Pike S, Pu X-A, Novacky A (1996) The interaction of harpinPss with plant cell walls. *Mol Plant-Microbe Interact* 9: 608-616.

Hueck CJ (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62: 379-433.

Hung KT, Kao CH (2003) Nitric oxide counteracts the senescence of rice leaves induced by abscisic acid. *J Plant Physiol* 160: 871–879.

Hung KT, Kao CH (2004) Nitric oxide acts as an antioxidant and delays methyl jasmonate-induced senescence of rice leaves. *J Plant Physiol* 161: 43-52.

Hunt MD, Delaney TP, Dietrich RA, Weymann KB, Dangl JL and JA Ryals (1997) Salicylate-independent lesion formation in Arabidopsis lsd mutants. *Mol Plant-Microbe Interact* 10: 531-536.

Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D (1997) Elicitor-stimulated ion fluxes and O2- from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci USA* 94: 4800-4805.

Jin Q, Thilmony R, Zwiesler-Vollick J, He SY (2003)Type III protein secretion in Pseudomonas syringae. *Microbes Infect* 5: 301-10.

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM and Glazebrook J (1999) Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signalling. *Proc Natl Acad Sci USA* 96: 13583-13588.

Jones DA, Dickinson MJ, Balint-Kurti PJ, Dixon MS and Jones JDG (1993) Two complex resistance loci revealed in tomato by classical and RFLP mapping of the Cf-2, Cf-4, Cf-5 and Cf-9 genes for resistance to Cladosporium fulvum. *Molecular Plant Microbe Interactions* 6: 348-357.

Jones DA, Takemoto D (2004) Plant innate immunity—Direct and indirect recognition of general and specific pathogen-associated molecules. *Curr Opin Immunol* 16: 48–62.

Kaiser WM, Weiner H, Kandlbinder A, Tsai CB, Rockel P, Sonoda M, Planchet E (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. *J Exp Bot* 53: 875-882.

Katagiri F, Thilmony R, He SY (2002) The *Arabidopsis Thaliana-Pseudomonas Syringae* Interaction. *American Society of Plant Biologists* doi: 10.1199/tab.0039.

Keen NT and Bruegger B (1977) Phytoalexins and chemicals that elicit their production in plant. *ACS Symp Ser* 62: 1–26.

Keen NT (1990) Gene-for-gene complementarity in plant-pathogen interactions. *Annu Rev Genet* 24: 447-463.

Kim JF, Beer SV (1998) HrpW of Erwinia amylovora, a new harpin that contains a domain homologous to pectate lyases of a distinct class. *J Bacteriol* 180: 5203-5210.

Kim M-G, da Cunha L, Belkhadir Y, DebRoy S, Dangl J L and Mackey D (2005) Two Pseudomonas syringae type III effectors inhibit RIN4-regulated basal defense in Arabidopsis. *Cell* 121: 749-759.

Kinkema M, Fan W, Dong X (2000) Nuclear localization of NPR1 is required for activation of PR gene expression. *Plant Cell* 12: 2339-2350.

Kirkegaard JA, Wong PTW, Desmarchelier JM (1996) In vitro suppression of fungal root pathogens of cereals by Brassica tissues. *Plant Pathol* 45: 593-603.

Klibenstein DJ, Lambrix VM, Reichelt M, Gershenzon J and Mitchell-olds T (2001) Gene duplication in the diversification of secondary metabolism: tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in Arabidopsis. *Plant Cell* 13: 681–693.

Koch M, Vorwerk S, Masur C, Sharifi-Sirchi G, Olivieri N, Schlaich N (2006) A role for a flavin-containing monooxygenase in pathogen resistance in Arabidopsis. *Plant J* 47: 629-639.

Krause M, Durner J (2004) Harpin inactivates mitochondria in Arabidopsis suspension cells. *Molecular Plant–Microbe Interactions* 17:131–139.

Krueger SK, Williams DE (2005) Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. *Pharmacol Ther* 106: 357-387.

Kuć J (1995) Phytoalexins, stress metabolism, and disease resistance in plants. *Annu Rev Phytopathol* 33: 275-297.

Kumar D, Klessig DF (2003) High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. *Proc Natl Acad Sci USA* 100: 16101–16106.

Kunkel BN, Brooks DM (2002) Cross talk between signaling pathways in pathogen defense. *Curr Opin Plant Biol* 5: 325-331.

Kunze G, Zipfel C, Robatzek S, Niehaus K, Boller T, Felix G (2004) The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *Plant Cell* 16: 3496-507.

Lam E, Lam YK (1995) Binding site requirements and differential representation of TGF factors in nuclear ASF-1 activity. *Nucleic Acids Res* 23: 3778–3785.

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* 48: 251-275.

Lambrix V, Reichelt M, Mitchell-Olds T, Kliebenstein DJ and Gershenzon J (2001) The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences Trichoplusia ni herbivory. *Plant Cell* 13: 2793–2807.

Langlois-Meurinne M, Gachon CM, Saindrenan P (2005) Pathogen-responsive expression of glycosyltransferase genes UGT73B3 and UGT73B5 is necessary for resistance to Pseudomonas syringae pv tomato in Arabidopsis. *Plant Physiol* 139:1890-1901.

Levine A, Tenhaken R, Dixon RA, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive response. *Cell* 79: 583-593.

Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Curr Biol* 6: 427-437.

Leshem YY, Willis RBH and Ku V-V V (1998) Evidence for the function of the free radical gas – nitric oxide (NO) – as an endogenous maturation and senescence regulating factor in higher plants. *Plant Physiology and Biochemistry* 36: 825–833.

Li CM, Brown I, Mansfield J, Stevens C, Boureau T, Romantschuk M, Taira S (2002) The Hrp pilus of *Pseudomonas syringae* elongates from its tip and acts as a conduit for translocation of the effector protein HrpZ. *EMBO J* 21: 1909-1915.

Leister RT, Ausubel FM, Katagiri F (1996) Molecular recognition of pathogen attack occurs inside of plant cells in plant disease resistance specified by the Arabidopsis genes RPS2 and RPM1. *Proc Natl Acad Sci U S A* 93: 15497-502

Lindgren PB, Peet RC and Panopoulos NJ (1986) Gene cluster of Pseudomonas syringae pv. phaseolicola controls pathogenecity on bean plants and hypersensivity on nonhost plant.

J *Bacteriol* 168: 512-522.

Linthorst HJM (1991) Pathogenesis-related proteins of plant Crit Rev Plant Sci 10:123-150.

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. *Science* 310: 1180-1183.

Lu M, Tang X and Zhou JM (2001) Arabidopsis NHO1 is required for general resistance against Pseudomonas bacteria. *Plant Cell* 13: 437-447.

Mackey D, Holt III BF, Wiig A and Dangl JL (2002) RIN4 interacts with Pseudomonas syringae Type III effector molecules and is required for RPM1-mediated disease resistance in Arabidopsis. *Cell* 108: 743–754.

Mackey D, Belkhadir Y, Alonso JM, Ecker JR, Dangl JL (2003) Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. *Cell* 112(3): 379-389.

Magalhaes JR, Monte DC, Durzan D (2000) Nitric oxide and ethylene emission in Arabidopsis thaliana. *Physiological and Molecular Biology of Plants* 6: 117–127.

Maldonado AM, Doerner P, Dixon RA, Lamb C, Cameron RC (2002) A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature* 419: 399-403.

Manici LM, Lazzeri L, Palmieri S (1997) In vitro fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. *J Agri Food Chem* 45: 2768–2773.

Mauch-Mani B, Slusarenko AJ (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of Arabidopsis to Peronospora parasitica. *Plant Cell* 8: 203-212.

Melotto M, Underwood W, Koczan J, Nomura K, He SY (2006) Plant stomata function in innate immunity against bacterial invasion. *Cell* 126: 969-980.

Métraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, et al. (1990) Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science* 250:150-156.

Meuwly P, Buchala AJ, Mölders W, Métraux JP (1995) Local and systemic biosynthesis of salicylic acid in infected cucumber plants. *Plant Physiology* 109: 1107-1114.

Mithen RF, Lewis BG, Fenwick GR (1986) In vitro activity of glucosinolates and their products against Leptosphaeria maculans. *Trans Br Mycol Soc* 87: 433–440.

Mölders W, Buchala AJ and Métraux JP (1996) Transport of salicylic acid in infected cucumber plants. *Plant Physiol*. 112: 787-792.

Morot-Gaudry-Talarmain Y, Rockel P, Moureaux T, Quilleré I, Leydecker M-T, Kaiser WM, Morot-Gaudry J-F (2002) Nitrite accumulation and nitric oxide emission in relation to cellular signaling in nitrite reductase antisense tobacco. *Planta* 215: 708-715.

Morris K, MacKerness SA, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J* 23: 677-685.

Morrissey JP, Osbourn AE (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiological Molecular Biological Revs* 63: 708–724.

Mou Z, Fan W, Dong X (2003) Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. *Cell* 27: 935–944.

Miller MB, Bassler BL (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* 55: 165-199.

Mur LA, Santosa IE, Laarhoven LJ, Holton NJ, Harren FJ, Smith AR (2005) Laser photoacoustic detection allows *in planta* detection of nitric oxide in tobacco following challenge with avirulent and virulent *Pseudomonas syringae* pathovars. *Plant Physiol* 138: 1247-1258.

Murgia I, de Pinto MC, Delledonne M, Soave C, De Gara L (2004) Comparative effects of various nitric oxide donors on ferritin regulation, programmed cell death, and cell redox state in plant cells. *J Plant Physiol* 161: 777-783.

Nandi A, Welti R, Shah J (2004) The Arabidopsis thaliana dihydroxyacetone phosphate reductase gene SUPPRESSOR OF FATTY ACID DESATURASE DEFICIENCY 1 is required for glycerolipid metabolism and for the activation of systemic acquired resistance. *Plant Cell* 16: 465–477.

Nawrath C, Pharintawong N, Heck S and Métraux J-P (2002) EDS5, an essential component of SA-dependent signalling for disease resistance in Arabidopsis, is a member of the MATE-transporter family. *Plant Cell* 14: 275-286.

Nawrath C and Métraux J-P (1999) Salicylic acid induction-deficient (sid) mutants of Arabidopsis express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. Plant Cell 11: 1393-1404.

Naumann C, Hartmann T, Ober D (2002) Evolutionary recruitment of a flavin-dependent monooxygenase for the detoxification of host plant-acquired pyrrolizidine alkaloids in the alkaloid-defended arctiid moth Tyria jacobaeae. *Proc Natl Acad Sci U S A* 99: 6085-6090.

Newman MA, Daniels MJ, Dow JM (1997) The activity of lipid A and core components of bacterial lipopolysaccharides in the prevention of the hypersensitive response in pepper. *Mol Plant-Microbe Interact* 10: 926–928.

Newman M-A, Dow JM, Daniels MJ (2001) Bacterial lipopolysaccharides and plant–pathogen-interactions. *European Journal of Plant Pathology* 107: 95–97.

Ninnemann H, Maier J (1996) Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidiation of Neurospora crassa. *Photochem Photobiol* 64: 393-398.

Oh SK, Lee S, Chung E, Park JM, Yu SH, Ryu CM, Choi D (2006) Insight into Types I and II nonhost resistance using expression patterns of defense-related genes in tobacco. *Planta* 223: 1101-1107.

Olszak B, Malinovsky FG, Brodersen P, Grell M, Giese H, Petersen M, Mundy J (2006) A putative flavin-containing mono-oxygenase as a marker for certain defense and cell death pathways. *Plant Sci* 170: 614-623.

Orozco-Cardenas ML, Ryan CA (2002) Nitric oxide negatively modulates wound signaling in tomato plants. *Plant Physiol* 130: 487-493.

Orth K, Xu Z, Mudgett MB, Bao ZQ, Palmer LE, Bliska JB, Mangel WF, Staskawicz B, Dixon JE (2000) Disruption of signaling by Yersinia effector YopJ, a ubiquitin-like protein protease . *Science* 290: 1594-1599.

Takeuchi K, Taguchi F, Inagaki Y, Toyoda K, Shiraishi T, Ichinose Y (2003) Flagellin glycosylation island in *Pseudomonas syringae pv. glycinea* and its role in host specificity. *J Bacteriol* 185: 6658-6665.

Thevissen K, Osborn RW, Acland DP, Broekaert WF (1997) Specific, high affinity binding sites for an antifungal plant defensin on Neurospora crassa hyphae and microsomal membranes. *J Biol Chem* 272: 32176-32181.

Thomma BP, Nelissen I, Eggermont K, Broekaert WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. *Plant J* 19: 163-171.

Thomma BPHJ, Eggermont K, Penninckx IAMA, Mauch-Mani B, Vogelsang R, Cammue BPA, Broekaert WF (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in arabidopsis are essential for resistance to distinct microbial pathogens. *Proc Natl Acad Sci U S A* 95: 15107-15111.

Thordal-Christensen H (2003) Fresh insights into processes of nonhost resistance. *Curr Opin Plant Biol* 6: 351-357.

Tierens KF, Thomma BP, Brouwer M, Schmidt J, Kistner K, Porzel A, Mauch-Mani B, Cammue BP, Broekaert WF (2001) Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. *Plant Physiol*125: 1688-1699.

Ton J, Van Pelt JA, Van Loon LC, Pieterse CM (2002) Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in Arabidopsis. *Mol Plant Microbe Interact* 15: 27-34.

Tobena-Santamaria R, Bliek M, Ljung K, Sandberg G, Mol JNM, Souer E, Koes R (2002) FLOOZY of petunia is a flavin mono-oxygenase-like protein required for the specification of leaf and flower architecture. *Genes Dev* 16: 753-763.

Tornero P, Gadea J, Conejero V, Vera P (1997)Two PR-1 genes from tomato are differentially regulated and reveal a novel mode of expression for a pathogenesis-related gene during the hypersensitive response and development. *Mol Plant Microbe Interact* 10: 624-634.

Truman W, Bennett MH, Kubigsteltig I, Turnbull C, Grant M (2007) Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates *Proc Natl Acad Sci U S A* 104:1075-1080.

Tsiamis G, Mansfield JW, Hockenhull R, Jackson RW, Sesma A, Athanassopoulos E, Bennett MA, Stevens C, Vivian A, Taylor JD, Murillo J (2000) Cultivar-specific avirulence and virulence functions assigned to avrPphF in *Pseudomonas syringae pv. phaseolicola*, the cause of bean halo-blight disease. *EMBO J* 19: 3204-3214.

Tsuji J, Jackson EP, Gage DA, Hammerschmidt R & Somerville SC (1992) Phytoalexin accumulation in Arabidopsis thaliana during the hypersensitive reaction to *Pseudomonas syringae pv. syringae. Plant Physiology* 98: 1304–1309.

Tun NN, Holk A & Scherer GF (2001) Rapid increase of NO release in plant cell cultures induced by cytokinin. *FEBS Letters* 509:174–176.

Pagnussat GC, Simontacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. *Plant Physiol* 129: 954-956.

Papadopoulou K, Melton RE, Leggett M, Daniels MJ, Osbourn AE (1999) Compromised disease resistance in saponin-deficient plants. *Proc Natl Acad Sci U S A* 96: 129231293-8.

Peng M, Kuc J (1992) Peroxidase-generated hydrogen peroxide as a source of antifungal activity in vitro and on tobacco leaf disks. Phyta J 82: 718-722.

Petersen M, Brodersen P, Naested H, Andreasson E, Lindhart U, Johansen B, Nielsen HB, Lacy M, Austin MJ, Parker JE, Sharma SB, Klessig DF, Martienssen R, Mattsson O, Jensen AB, Mundy J (2000) Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. *Cell* 103:1111-1120.

Pfeilschifter J, Eberhardt W, Beck KF (2001) Regulation of gene expression by nitric oxide. *Pflugers Archiv* 442: 479–486.

Pieterse CM, van Wees SC, Hoffland E, van Pelt JA, van Loon LC (1996) Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *Plant Cell* 8: 1225-1237.

Pieterse CMJ, van Wees SCM, van Pelt JA, Knoester M, Laan R, Gerrits N, Weisbeek PJ and van Loon LC (1998). “A novel signaling pathway controlling induced systemic resistance in Arabidopsis.” *Plant Cell* 10: 1571-1580.

Pieterse CMJ, van Pelt JA, van Wees SCM, Ton J, Léon-Kloosterziel K, Keurentjes JJB, Verhagen BWM, Knoester M, van der Sluis I, Bakker PAHM, van Loon LC (2001) Rhizobacteria-mediated induced systemic resistance: triggering, signalling and expression. *European Journal of Plant Pathology* 107: 51–61.

Planchet E, Kaiser WM (2006) Nitric Oxide Production in Plants: Facts and Fictions. *Plant Signal Behav* 1: 46-51.

Reuber TL, Ausubel FM (1996) Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. *Plant Cell* 8:241-249.

Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J Exp Bot* 53: 103–110.

Rohde A, Morreel K, Ralph J, Goeminne G, Hostyn V, De Rycke R, Kushnir S, Van Doorsselaere J, Joseleau JP, Vuylsteke M, Van Driessche G, Van Beeumen J, Messens E, Boerjan W.(2004) Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana

reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. *Plant Cell* 16: 2749-2771.

Ron M and Avni A (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. *Plant Cell* 16: 1604–1615.

Rotblat B, Enshell-Seijffers D, Gershoni JM, Schuster S, Avni A.(2002) Identification of an essential component of the elicitation active site of the EIX protein elicitor. *Plant J* 32: 1049-1055.

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (sai1) of Arabidopsis thaliana, identified in a selective screen utilizing the SA-inducible expression of the tms2 gene. *Mol Plant-Microbe Interact* 10: 60-78.

Shaller GE and Bleecker AB (1995) Ethylene binding sites generated in yeast expressing the Arabidopsis ETR1 gene. *Science* 270: 1809-1811.

Shao F, Golstein C, Ade J, Stoutemyer M, Dixon JE, and Innes RW (2003) Cleavage of Arabidopsis PBS1 by a bacterial type III effector. *Science* 301: 1230–1233.

Shapiro AD, Zhang C (2001) The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in Arabidopsis. *Plant Physiol* 127:1089-1101.

Shirasu K, Nakajima H, Rajasekhar VK, Dixon RA, Lamb C (1997) Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. *Plant Cell* 9: 261-270.

Schmidt HHHW, Walter U (1994) NO at work. Cell 78: 919-924.

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatos A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. *Plant Physiol* 141: 1248–1254.

Shulaev V, Silverman P, Raskin I (1997) Airborne signalling by methyl salicylate in plant pathogen resistance. *Nature* 385: 718-721.

Shualev V, Leon J & Raskin I (1995) Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? *The Plant Cell* 7: 1692-1701.

Smart CM (1994) Gene expression during leaf senescence. *New Phytol* 126: 419-448.

Somssich IE (1994) Regulatory elements governing pathogenesisrelated (PR) gene expression. In *Results and Problems in Cell Differentiation*, Vol. 20 (Nover, L., ed.). Berlin: SpringerVerlag, pp. 163–179.

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH, Fauquet C, Ronald P (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* 270: 1804-1806.

Soylu S, Brown I, Mansfield JW (2005) Cellular reactions in Arabidopsis following challenge by strains of Pseudomonas syringae: from basal resistance to compatibility. *Physiological and Molecular Plant Pathology* 66: 232–243.

Staswick PE, Su W, Howell S (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an Arabidopsis thaliana mutant. *Proc Natl Acad Sci USA* 89: 6837-6840.

Staswick PE, Tiryaki I (2004) The Oxylipin Signal Jasmonic Acid Is Activated by an Enzyme That Conjugates It to Isoleucine in Arabidopsis. *Plant Cell* 16: 2117-2127.

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. *Plant Cell* 18: 731-746.

Sticher L, Mauch-Mani B and Métraux JP (1997) Systemic acquired resistance. *Annu Rev Phytopathol* 35: 235-270.

Stöhr C, Strube F, Marx C, Ullrich WR, Rockel P (2001) A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite*. Planta* 212: 835–841.

Suh JK, Poulsen LL, Ziegler DM, Robertus JD. (1999) Yeast flavin-containing monooxygenase generates oxidizing equivalents that control protein folding in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 96: 2687-91.

Vernooij B, Friedrich L, Morse A, Reist R, Kolditzjawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. *Plant Cell* 6: 959-965.

Vinatzer BA, Teitzel GM, Lee M-W, Jelenska J, Hotton S, Fairfax K, Jenrette J, and Greenberg JT (2006) The Type III effector repertoire of Pseudomonas syringae B728a and its role in survival and disease on host and non-host plants. *Molecular Microbiology* 62: 26-44.

Weaver LM, Gan S, Quirino B, Amasino RMA (1998) Comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Mol Biol* 37:455-469.

van Wees SCM, De Swart EAM, Van Pelt JA, Van Loon LC and Pieterse CMJ (2000) Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in Arabidopsis thaliana. *Proc Natl Acad Sci USA* 97: 8711-8716.

van Wees SC and Glazebrook J (2003) Loss of non-host resistance of Arabidopsis NahG to *Pseudomonas syringae pv. phaseolicola* is due to degradation products of salicylic acid. *Plant J* 33: 733–742.

Van Wees SC, Chang HS, Zhu T, Glazebrook J (2003) Characterization of the early response of Arabidopsis to Alternaria brassicicola infection using expression profiling. *Plant Physiol* 132: 606-617.

Whalen MC, Innes RW, Bent AF, Staskawicz BJ (1991) Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell* 3: 49–59.

Wiermer M, Feys BJ, Parker JE (2005) Plant immunity: the EDS1 regulatory node. *Curr Opin Plant Biol* 8: 383-389.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414: 562-565.

Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science* 280: 1091–1094.

Yoshida S, Ito M, Nishida I, Watanabe A (2002) Identification of a novel gene HYS1/CPR5 that has a repressive role in the induction of leaf senescence and pathogen-defence responses in Arabidopsis thaliana. *Plant J* 29: 427-437.

Yu I-c, Parker J, Bent AF (1998) Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. *Proc Natl Acad Sci USA* 95: 7819-7824.

Yun D-J, Zhao Y, Pardo JM, Narasimhan ML, Damsz B, Lee H, Abad LR, D'Urzo MP, Hasegawa PM, Bressan RA (1997) Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. *Proc Natl Acad Sci USA* 94: 7082-7087.

Zhang Y, Goritschnig S, Dong X, Li X (2003) A Gain-of-Function Mutation in a Plant Disease Resistance Gene Leads to Constitutive Activation of Downstream Signal Transduction Pathways in suppressor of npr1-1, constitutive 1. *Plant Cell* 15: 2636-2646.

Zhang S, Klessig DF (2001) MAPK cascades in plant defense signaling. *Trends Plant Sci* 6: 520–527.

Zhang Y, Tessaro MJ, Lassner M and Li X (2003) Knockout analysis of Arabidopsis transcription factors TGA2, TGA5, and TGA6 reveals their redundant and essential roles in systemic acquired resistance. *Plant Cell* 15: 2647–2653.

Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* 291: 306-309.

Zhou N, Tootle TL, Tsui F, Klessig DF, Glazebrook J (1998) PAD4 functions upstream from salicylic acid to control defense responses in Arabidopsis. *Plant Cell* 10: 1021–1030.

Zhou N, Tootle TL, Glazebrook J (1999) Arabidopsis PAD3, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. *Plant Cell* 11: 2419-2428.

Zeevaart JAD, Creelman RA (1988) Metabolism and physiology of abscisic acid. *Annu Rev Plant Physiol Plant Mol Biol* 39: 439-473.

Zeidler D, Zähringer U, Gerber I, Dubey I, Hartung T, Bors W, Hutzler P, Durner J (2004) Innate immunity in Arabidopsis thaliana: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proc Natl Acad Sci USA* 101: 15811-15816.

Zemojtel T, Frohlich A, Palmieri MC, Kolanczyk M, Mikula I, Wyrwicz LS, Wanker EE, Mundlos S, Vingron M, Martasek P, Durner J (2006) Plant nitric oxide synthase: a never-ending story? *Trends Plant Sci* 11: 524-525.

Zimmermann P, Zentgraf U (2005) The correlation between oxidative stress and leaf senescence during plant development. *Cellular and Molecular Biology Letters* 10: 515–534.

Zipfel C, Kunze G, Chinchilla D, Caniard A, Jones JD, Boller T, Felix G (2006) Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* 125:749-760.

Zottini M, Formentin E, Scattolin M, Carimi F, Lo Schiavo F, Terzi M (2002) Nitric oxide affects plant mitochondrial functionality in vivo. *FEBS Letters* 515: 75–78.

8. Abbreviations

ABA	abscisic acid
ACS6	1-aminocyclopropane-1-carboxylate synthase
CEV1	constitutive expression of *VSP1*
COR	coronatine
CTR1	constitutive triple response 1
CUE1	*CAB* unexpressed 1
DNA	deoxyribonucleic acid
DND1	defence-no-death 1
ET	ethylene
Flg22	22-amino-acid, elicitor-active flagellin peptide
FLS2	FLAGELLIN-SENSITIVE 2
GFP	green fluorescent protein
hrp genes	hypersensitive response and pathogenicity genes
H_2O_2	hydrogen peroxide
HR	Hypersensitive response
ICS1	isochorismate synthase
ISR	induced systemic resistance
JA	jasmonic acid
LPS	lipopolysaccaride
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
NADPH oxidase	nicotinamide adenine dinucleotide phosphate-oxidase
NB-LRR	nucleotide binding, leucine-rich repeat
NO	nitric oxide
NOC-18	1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene
NR	nitrate reductase
O_2^-	superoxide
PR	pathogenesis-related
PRR	pattern recognition receptor
PCR	polymerase chain reaction
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SAG	senescence-associated gene
SNAP	S-nitroso-N-acetyl-D,L-penicillamine
SNC1	suppressor of *npr1-1*, constitutive 1
SNP	sodium nitroprusside
T-DNA	transposon-DNA

9. Supplement

9.1 List of publications

Zeier J, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C (2004) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. Plant Physiol 136: 2875-2886.

Mishina TE, Zeier J (2006) The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant Physiol 141: 1666-1675.

Mishina TE, Lamb C, Zeier J (2007) Expression of a nitric oxide degrading enzyme induces a senescence program in Arabidopsis. Plant Cell Environ 30: 39-52.

Mishina TE, Zeier J (2007) Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant J, in press.

Mishina TE, Zeier J (2007) Interactions of Arabidopsis with non-adapted *Pseudomonas syringae* strains: possible determinants of bacterial non-host resistance. Submitted.

Mishina TE, Sonoda M, Fraaß V, Kaiser WM, Zeier J (2007) Heterologous expression of a nitric oxide synthase in Arabidopsis enhances plant NO production and attenuates local and systemic resistance towards bacterial pathogens. Submitted.

9.2 Poster Presentations

Mishina TE, Zeier J. Physiological plasticity of inducible defence responses in Arabidopsis. 15th International conference on Arabidopsis research, Berlin (Germany), 11-14 July 2004.

Mishina TE, Fraaß V, Zeier J. Analysis of the roles of nitric oxide and cGMP in plant defence. Annual Main Meeting of the Society for Experimental Biology, Barcelona (Spain), 11-15 July 2005.

Mishina TE , Sonoda M, Zeier J Nitric oxide in plant defence and senescence. 1st Plant NO Group Meeting, Verona (Italy), 28-29 August 2006.

Mishina TE, Sonoda M, Planchet E, Fraaß V, Kaiser W, Zeier J. Nitric oxide: a positive regulator of plant defence responses? Symposium 'Non-specific and specific innate and acquired plant resistance. Budapest (Hungary), 31 August - 3 September 2006.

9.3 Oral presentations

Mishina TE, Zeier J (2006) Inducible defence responses in Arabidopsis triggered by non-host bacteria. Meeting of the German Phytomedical Society (DPG), Arbeitskreis Wirt-Parasit-Beziehungen, Berlin (Germany) 16-17 March 2006.

9.4 Curriculum Vitae

Personal Data

Name	Tatiana Egorovna Mishina
Date of Birth	1.04.1976
Nationality	Russian

Educational Background

09/1983 – 06/1993	schooling (Radovizky, Moscow area, Russia)
09/1993 – 05/1994	studies of biology, pedagogical Institute Orehovo-Zyevo (Moscow area, Russia)
09/1994 – 07/1999	studies of agriculture, crop breeding and genetics; Agricultural Academy, Moscow
05/1998 – 09/1998	practical training in ecological agriculture, ("Logo" exchange program, Bielefeld, Germany)
10/1998	practical training ,*Cell culture and meristem maintenance*', Bundesforschungsanstalt für Landwirtschaft, Braunschweig;
07/1999	high-school diploma (degree with honours); Agricultural Academy, Moscow diploma thesis: *Development of methods for A. tumefaciens and A. rhizogenes mediated transformation of potato*
09/1999 – 03/2002	scientific employee at the Scientific Institute of Plant Physiology, Moscow in the lab of Prof. Dr. G.A. Romanov; *Cryo-preservation of potato meristem*; *molecular cloning and transformation of tobacco to investigate the initiation of flowering*
04/2002 – 03/2004	scientific employee at IPK Gatersleben and Humboldt University, Berlin (lab of Prof. Dr. Bernhard Grimm).; '*Role of Arabidopsis HAP transcription factors in abiotic stress responses*'
from 04/2004	scientific employee at the Department of Botany II, Julius-von-Sachs-Institute, Würzburg in the lab of Dr. Jürgen Zeier; '*Mechanisms of local and systemic defences in Arabidopsis thaliana in response to host and* *non-host strains of Pseudomonas syringae*'

9.5 Acknowledgements

I would like to thank Dr. Jürgen Zeier for the opportunity to perform my work in his group and for his excellent supervision, his helpful suggestions and his great support during all my time here in Würzburg.

I would like to thank Martina Geuecke and Thomas Griebel for helpful assistance during my work and contributing to the pleasant climate in our laboratory.

Additionally, I thank the members of Prof. Dr. W. Kaiser's group, in particular Elisabeth Planchet and Maria Lesch, for assistance in nitric oxide measurements.

I thank the people of the Department of Botany II and the Julius-von-Sachs-Institute supporting me during my work.

I wish to express my gratitude to Prof. Dr. Markus Riederer and Prof. Dr. Thomas Roitsch for reviewing this work.

I would like to address many thanks to the DFG for financial support.

Especially, I would like to thank my parents for all what they have done and do for me.

Erklärung

Hiermit erkläre ich, die vorliegende Dissertation selbst angefertigt und nur die angegebenen Quellen und Hilfsmittel verwendet zu haben.

Weiterhin erkläre ich, dass die vorliegende Dissertation weder in gleicher noch ähnlicher Form einem anderen Prüfungsverfahren vorgelegt wurde.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Julius-Maximilians-Universität Würzburg.

Würzburg, den 5. 3. 2007

……..

Tatiana E. Mishina