Lehrstuhl für Phytopathologie der Technischen Universität München

ACTIVATION OF NITRIC OXIDE SYNTHASE AND INDUCTION OF DEFENSE GENES IN *ARABIDOPSIS THALIANA* BY BACTERIAL LIPOPOLYSACCHARIDES

DANA ZEIDLER

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SUMMARY

The aim of this study was to examine if Lipopolysaccharide [LPS] are novel elicitors of plant innate immunity using *Arabidopsis thaliana* as a model system.

LPS are the major outer membrane components of Gram-negative bacteria and consist of three distinct structural domains: O-antigen, core region and lipid A. They represent microbe-/pathogen-associated molecular patterns [PAMPs] in animal patho-systems and act as extremely potent stimulators of the mammalian and insect innate immunity. As for plants, the molecular mechanisms of signal transduction in response to LPS are not known.

Here is shown, that *Arabidopsis thaliana* reacts to LPS with a rapid burst of nitric oxide [NO] and reactive oxygen species [ROS], which are important hallmarks of innate immunity in animals. Fifteen LPS preparations [among them *Burkholderia cepacia, Pseudomonas aeruginosa,* and *Erwinia carotovora*] as well as lipoteichoic acid from Gram-positive *Staphylococcus aureus* were found to trigger NO-production in suspension cultured *Arabidopsis* cells as well as in leaves. NO was detected by confocal laser scanning microscopy in conjunction with the NO-sensitive fluorophore DAF-FM DA, by electron paramagnetic resonance [EPR], and by a nitric oxide synthase [NOS] assay.

NO biosynthesis in plants occurs either by nitrate reductase [NR] or by NOS. Because NR seemed not to be involved in LPS elicited NO, the source of NO was addressed by using T-DNA insertion lines for both known plant NOS. Interestingly, LPS did not activate the pathogen-inducible varP NOS, but AtNOS1, a distinct NOS previously associated with hormonal signaling in plants.

A prominent feature of LPS-treatment was activation of defense genes, which proved to be mediated by NO and salicylic acid. Northern analyses as well as transcriptional profiling using DNA microarrays revealed induction of defense and systemic acquired resistance [SAR] associated genes in local and systemic *Arabidopsis* leaves. We could show, that SAR gene induction resulted in a real resistance of LPS pre-treated plants against subsequent infection with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). In the atnos1 mutant was the LPS triggered gene induction nearly abolished. This fact could be repaired by treatment with a NO donor. Additionally, atnos1 mutants showed enhanced susceptibility to *Pst*.

Spread of LPS in the *Arabidopsis* plant during activation of plant defense response was monitored by using fluorescent-labeled LPS molecules from *Salmonella minnesota.* LPS were visible in middle-rip and minor veins of local as well as of systemic leaves.

In sum, perception of LPS and induction of NOS might contribute towards the activation of plant defense responses.

ABBREVIATIONS

ADP	**A**denine **di****p**hosphate
ADPRC	**ADP-r**ibosyl **c**yclase
As	**A**ctivator **s**equence
AtNOS1	***A****rabidopsis* ***t****haliana* **n**itric **o**xide **s**ynthase 1
Avr	**Av****ir**ulence
B. cepacia	***B****urkholderia cepacia*
BH_4	Tetra**h**ydro**b**iopterin
cADPR	**C**yclic **ADP r**ibose
CE	**C**apillary **e**lectrophoresis
CD14	**C**luster of **d**ifferentiation
CF	**C**ystic **f**ibrosis
cGMP	**C**yclic **G**uanylate **m**ono**p**hosphate
cPTIO	2-(4-**c**arboxy**p**henyl)**-**4,4,5,5-**t**etramethyl**i**midazoline-1-oxyl-3-**o**xide
CTAB	**C**etyl**t**rimethyl**a**mmonium **b**romide
CZE	**C**apillary **z**one **e**lectrophoresis
DAF-FM DA	4-**a**mino-5-**m**ethylamino-2′,7′-**di****f**luoro**f**luorescein **d**i**a**cetate
DEPC	**D**i**e**thyl **p**yro**c**arbonate
DETC	**D**i**e**thyldi**t**hio**c**arbamic acid
DF	**D**ilution **f**actor
DIG	**Dig**oxygenin
EDS	**E**nhanced **d**isease **s**usceptibility
eNOS	**E**ndothelial **NOS**
EPR	**E**lectron **p**aramagnetic **r**esonance
EPS	**E**xo**p**oly**s**accharide
ES	**E**ndomembrane **s**ystem
FAD	**F**lavin **a**denine **d**inucleotide
FMN	**F**lavin **m**ono**n**ucleotide
FW	**F**resh **w**eight
GM	**G**olgi **m**embrane
H_2DCFDA	2′,7′-**di****c**hlorodi**h**ydro**f**luorescein **d**i**a**cetate
HR	**H**ypersensitive **r**esponse
ICS	**I**so**c**horismate-**s**ynthase
IL	**I**nter**l**eukin
iNOS	**I**nducible **NOS**
ISR	**I**nduced **s**ystemic **r**esistance
JA	**J**asmonic **a**cid
jin	**J**asmonic acid **in**sensitive
Kdo	3-**d**eoxy-D-manno-**o**ct-2-ulopyranosonic acid
L,D-Hep	**L**-glycero-**D**-manno**hep**tose
L-Arg	**L-arg**inine
LBP	**L**PS-**b**inding **p**rotein
L-NMMA	N^G-Methyl-L-arginine acetate salt
L-NNA	N_ω-Nitro-L-arginine
Loc	**Loc**al
LPS	**L**ipo**p**oly**s**accharide

LRR	**L**eucine-**r**ich **r**epeat
LTA	**L**ipo**t**eichoic **a**cid
MAPK	**M**itogen-**a**ctivated **p**rotein **k**inase
MD-2	**M**yeloid **d**ifferentiation
MS	**M**urashige & **S**koog
N.A.	**N**ot **a**vailable
NADPH	β-**N**icotinamide **a**denine **d**inucleotide 2'-**p**hosphate
nahG	Plant mutant which carries a bacterial salicylate hydroxylase gene
ND	**N**ot **d**etermined
NIM1 = NPR1	**N**on **im**munity
NIMIN	**NIM**1 **in**teracting
nNOS	**N**euronal **NOS**
NO	**N**itric **o**xide
NOS	**N**itric **o**xide **s**ynthase
NPR1	**N**on-expressor of **PR** genes 1
NR	**N**itrate **r**eductase
PAD	**P**hyto**a**lexin **d**eficient
PAL	**P**henylalanine **a**mmonia **l**yase
PAMP	**P**athogen-**a**ssociated-**m**olecular-**p**attern
PCD	**P**rogrammed **c**ell **d**eath
PERK	**P**roline **e**xtension**-**like **r**eceptor **k**inase
PM	**P**lasma**m**embrane
POS	**P**olytechnische **O**ber**s**chule
PR	**P**athogenesis**-r**elated
PS	**P**oly**s**accharide
Pst	***P**seudomonas **s**yringae* pv. ***t**omato*
R gene	**R**esistance **gene**
RLK	**R**eceptor-**l**ike **k**inase
ROI	**R**eactive **O**xygen **I**ntermediate
ROS	**R**eactive **O**xygen **S**pecies
RR	**R**etrieval **r**ate
RT	**R**oom **t**emperature
SA	**S**alicylic **a**cid
SALK	Jonas **Salk**
SAR	**S**ystemic **a**cquired **r**esistance
SoA	**S**odium **a**zide
Sys	**Sys**temic
Taq	**T**hermo **aq**uaticus
TGA factor	Transcription factor with TGACGTt/g binding site
TIR	**T**oll-**i**nterleukin **r**eceptor
TLR	**T**oll-**l**ike **r**eceptor
TNF	**T**umor **n**ecrosis **f**actor
varP	**Var**iant **P** protein
WAK	**W**all**-a**ssociated **k**inase
WHY	**Wh**irl**y** transcription factor

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1 INTRODUCTION

1.1 PLANT INNATE IMMUNITY

Innate immunity constitutes the first line of defense against attempted microbial invasion, and it is a well-described phenomenon in vertebrates and insects. Recent pioneering work has revealed striking similarities between the molecular organization of animal and plant systems (Nuernberger et al., 2004). However, significant differences remain. For example, the immune system in vertebrates comprises innate and acquired immunity, both of which act in concert to protect the host from microbial attack (Medzhitov and Janeway, 2002; McGuinness, Dehal, and Pleass, 2003). Characteristic is the activation of acquired immunity by T and B lymphocytes. Such a clonal system of adaptive immunity and specialized cell types (macrophages, neutrophils, and dentritic cells), which as parts of blood system are the key players of animal immune system, are not found in plants. In contrast, plants are autonomously capable of detecting the presence of pathogens and of activating defense response at the level of each single cell (Jones and Takemoto, 2004; Nuernberger et al., 2004).

1.1.1 RECOGNITION OF INVADING PATHOGENS

The ability to discriminate between self and non-self is a key feature of all living organism, and forms the basis for the activation of innate defense mechanism (Nuernberger and Brunner, 2002). Generally, pathogen recognition and the subsequent activation of disease resistance responses in plants occur either at the non-cultivar-specific level [non-host resistance] or at the cultivar level [host-specific resistance] (Veronese et al., 2003).

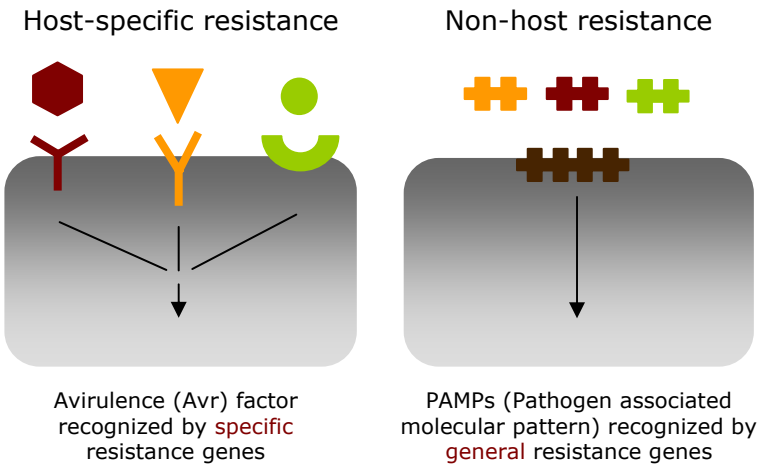

Figure 1-1: *Schematic model of the two types of pathogen recognition in plants.*

Plants detect invading pathogens either at the cultivar level by specific resistance genes or at non-cultivar level by general resistance genes. First type of resistance conforms to the gene-for-gene-hypothesis and is genetically determined by complementary pairs of pathogen encoded avirulence [avr] and plant resistance [R] genes. Non-host resistance is induced by invariant PAMPs that are characteristic of a whole class of microbial pathogens and are general elicitors.

Host-specific resistance is mediated by the familiar disease resistance [R] genes. R proteins determine the recognition of a specific molecule produced by pathogens. These elicitors of resistance response are called avirulence [Avr] proteins because their recognition by the corresponding R gene of the host results in the activation of a suite of defense mechanisms (Hammond-Kosack and Jones, 1996; Hammond-Kosack and Parker, 2003). This gene-for-gene recognition governs plant resistance against diverse classes of pathogens (Nimchuk et al., 2001). Non-host resistance is the most common form of disease resistance exhibited in plants. It generally seems to be under complex genetic control and can involve a multiplicity of defense factors (Heath, 2000a). Induced non-host resistance in plants is comparable to animal innate immunity and a large variety of microbe-associated products, which are referred as general elicitors triggers defense response in many plant species in non-host specific manner (Jones and Takemoto, 2004).

1.1.2 DEFENSE RESPONSE AGAINST MICROBIAL PATHOGENS

Plant-pathogen recognition causes the rapid activation of appropriate plant defense mechanisms (Schenk et al., 2000). This signal-transduction cascade leads to the production of endogenous signaling compounds that are able to activate genes involved in the production of antimicrobial effector molecules both at the side of infection and in tissues away [Fig. 1-1] (Thomma et al., 2001). Eventually, this can result in systemic acquired resistance [SAR], which is an induced state of immunity against subsequent infections caused by a broad spectrum of pathogens lasting from weeks to month (Ryals, Uknes, and Ward, 1994).

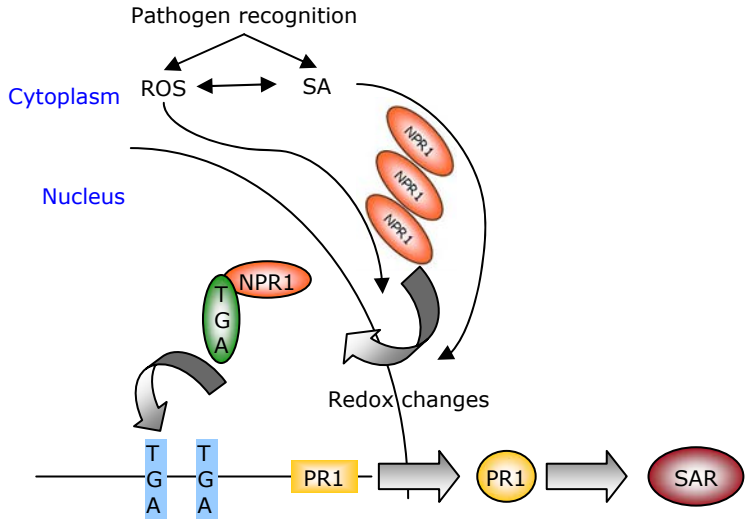

Figure 1-2: *The sequence from pathogen recognition to defense gene induction.*

Elevated levels of SA trigger increased NPR1 transcription and reduction of NPR1 oligomers. The resulting NPR1 monomers localize to the nucleus where they activate binding of TGA factors to TGA-boxes in the PR-1 promoter and SAR occurs. [Modified after (Durrant and Dong, 2004; Eulgem, 2005)].

The earliest reactions detectable are the opening of specific ion-channels and the formation of reactive oxygen species [ROS]. These initial transient reactions are prerequisites for further signal transduction events and are required for the onset of SAR (Somssich and Hahlbrock, 1998). Another essential signal for SAR across a range of plants is salicylic acid [SA], which accrues mainly in the isochorismate pathway during SAR (Wildermuth et al., 2001). SA in turn induces the translocation of NPR1 [non-expressor of PR genes] protein in the nucleus, which is also required for SAR development (Somssich, 2003). NPR1 acts as a modulator of PR gene expression by enhancing the DNA binding of TGA transcription factors to SA-responsive elements, which requires the SA-mediated reduction of NPR1 to a monomeric form (Mou, Fan, and Dong, 2003; Pieterse and Van Loon, 2004). TGA factors bind to activator sequence-1 [as-1] or as1-like promoter elements, which have found in several plant promoters activated during defense including *Arabidopsis* pathogenesis-related protein 1 [PR-1] a well characterized marker of defense response (Durrant and Dong, 2004). Further typical defense genes involved in SAR represent PR-2 and PR-5, which encode small secreted or vacuole-targeted proteins that have antimicrobial activities (Uknes *et al.*, 1992; Dong, 2004).

1.2 NITRIC OXIDE [NO]

Nitric oxide [NO] or nitrogen monoxide is a colorless gas formed by the combustion of nitrogen and oxygen as given by the reaction: energy + N_2 + O_2 → 2NO. Nitric oxide readily combines with oxygen or air to form nitrogen dioxide (NO_2), which can again be separated by ultraviolet light to produce nitric oxide and highly reactive oxygen atoms. These oxygen atoms combine with hydrocarbons producing noxious compounds that irritate the membranes of living organisms and destroy vegetation (Columbia-University-Press, 2005). Large amounts of nitric oxide are created by internal-combustion engines and manufacturing processes. Its quantity is greatly reduced by passing the oxide gas through a catalyst, thereby converting it back to its constituent nitrogen and oxygen gases. In the environment, nitric oxide is a precursor of smog and acid rain (Wellburn, 1990). Nitric oxide in minute amounts serves as a source of energy in certain bacteria. In the body, it serves as a chemical messenger with a wide range of functions (Columbia-University-Press, 2005).

1.2.1 NO IMMUNITY FUNCTION IN HUMAN BODY

NO is a molecule utilized throughout the animal kingdom as a signaling or toxic agent between cells (Mayer and Hemmens, 1997). Generated by many cell types in a variety of tissues in mammals, it acts as a vascular relaxing reagent, a neurotransmitter and an inhibitor of platelet aggregation (Moncada, Higgs, and Furchgott, 1997). In addition to these physiological roles, NO is produced during immune and inflammatory response

(Coleman, 2001). The potentially toxic effects of NO were recognized in the early phases of its discovery (Stamler, Singel, and Loscalzo, 1992). As a product of activated macrophages NO was found to be cytotoxic to certain tumor cells and also participate in the killing of a number of different microorganisms (Evans and Cohen, 1996). Another function of this molecule includes the modulation of cytokine response and the regulation of immune cell apoptosis (Schmidt and Walter, 1994). NO is known to affect the production of more than twenty cytokines by various immune cells (Bogdan, Rollinghoff, and Diefenbach, 2000). The role of NO in apoptosis is controversial. Indeed, it has been shown that NO can have both pro-and anti-apoptotic properties (Bruene, 2003). NO can prevent apoptosis in some cell lines, such as cardiac myocytes. On the other hand, exposure to NO donors also augmented the incidence of apoptosis (Blaise et al., 2005).

1.2.2 NO IN PLANTS

During the last few years NO has been detected in several plant species, and the increasing number of reports on its function in plants have implicated NO as a key molecular signal that participates in the regulation of several physiological processes (Romero-Puertas et al., 2004). NO was shown to be involved in plant signaling (Durner and Klessig, 1999), wounding (Orozco-Cárdenas and Ryan, 2002; Huang et al., 2004), programmed cell death (Clarke et al., 2000), root growth (Stoehr and Ullrich, 2002), leave expansion and seed germination (Beligni and Lamattina, 2000), senescence (Corpas et al., 2004), flowering (He et al., 2004), iron homeostasis (Graziano and Lamattina, 2005) and phytoalexine production (Noritake, Kawakita, and Doke, 1996). Additionally, NO plays a prominent role in plant defense against microbial pathogens by triggering resistance and by contributing to the local and systemic induction of defense genes (Delledonne, Polverari, and Murgia, 2003). The NO signaling pathway leading to defense gene expression in plants appears to be similar to that defined in animals [Fig. 1-2]. In animals, NO frequently acts through cGMP-dependent pathway (Landar and Darley-Usmar, 2003). In some type of animal cells, cGMP in turn activates ADP-ribosyl cyclase [ADPRC]. The resulting level of increased cyclic ADP ribose [cADPR] stimulates Ca^{2+} release into the cytoplasm (Galione and Churchill, 2000). In tobacco for example, NO induced a transient but dramatic increase in cGMP levels. Additionally cADPR activated PR1 and PAL expression which was dependent on calcium (Durner, Wendehenne, and Klessig, 1998; Klessig et al., 2000). The NO-induced regulation of defense genes probably involves the modulation of intracellular Ca^{2+} levels via the Ca^{2+} modulators cGMP and cADPR (Wendehenne, Durner, and Klessig, 2004). NO action in plants is also closely linked to salicylic acid. It seems to act, at least partially, through a salicylic acid-dependent signaling pathway (Metraux and Durner, 2004). Treatment of tobacco leaves with NO induced a significant increase in the endogenous SA required for defense gene induction (Wendehenne et al., 2001). A further signal is the activation of

MAP kinases [MAPK] through NO in both tobacco and *Arabidopsis*. The NO induced MAPK in tobacco can also be activated by SA and H_2O_2 (Neill et al., 2002). Thus, it seems to be that plants contain a functional NO signaling system whose components and targets are highly analogous to those identified in animals (Wendehenne, Durner, and Klessig, 2004).

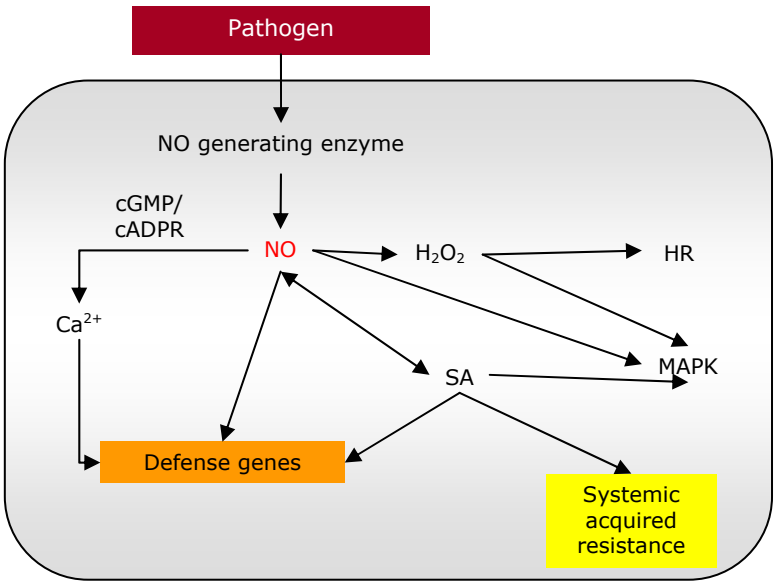

Figure 1-3: *NO functions in defense signaling.*

In response to pathogens or other elicitors of plant defenses, NO is produced by a NO generating enzyme. NO utilizes at least five pathways, whose interconnections are not well understood, [a] to elevate free cytosolic Ca^{2+} through cGMP and cADPR, [b] to induce the HR/cell death in cooperation with H_2O_2, [c] to induce SA production, which in turn enhances NO levels and facilitates local resistance and the development of systemic acquired resistance [SAR], [d] to induce the expression of defense genes through SA- and Ca^{2+}-dependent pathway[s], and [e] to activate MAP kinases [MAPK], which can also be activated through H_2O_2 or SA. [After (Wendehenne, Durner, and Klessig, 2004)].

1.2.3 NO BIOSYNTHESIS

In animal cells, biosynthesis of NO is primarily catalyzed by the nitric oxide synthase [NOS] enzyme (Stuehr, 1999). Three isoforms have been identified, named on the basis of the tissue source from which they were originally extracted: neuronal NOS [nNOS], inducible NOS [iNOS] in macrophages, and endothelial NOS [eNOS] (Wendehenne et al., 2001). These enzymes catalyze the oxygen- and NADPH-dependent oxidation of L-arginine to NO and citrulline in a complex reaction requiring Flavin adenine dinucleotide [FAD], Flavin adenine mononucleotide [FMN], tetrahydrobiopterin [BH_4], calcium and calmodulin (Alderton, Cooper, and Knowles, 2001; del Rio, Corpas, and Barroso, 2004). In plants, NO can be synthesized either by inorganic nitrogen pathways or by enzymatic catalysis. Slow and spontaneous liberation of NO can be observed with nitrite at neutral pH (Yamasaki, 2000). Another non-enzymatic pathway for NO synthesis occurs in the apoplast from barley seeds, where nitrite will be converted to NO (Bethke, Badger, and

Jones, 2004). A major advance in the understanding of NO functions in plants has been the identification of enzymes that catalyzes NO synthesis. Nitrate reductase [NR] was the first enzymatic source of NO to be identified. In addition to its role in nitrate reduction, NR catalyzes the reduction of nitrite to NO using NADPH as co-factor (Yamasaki and Sakihama, 2000; Lamotte et al., 2005). During the last few years, several groups have provided evidence for the existence of NOS-like activity in plants. A pathogen-inducible NOS [iNOS] has been identified in tobacco and *Arabidopsis* (Chandok et al., 2003). However, Klessig and some of his co-authors retracted the paper and a subsequent publication (Travis, 2004). But the discovery of another plant NOS, which is distinct from known animal proteins, was done in the same year. The enzyme was called *Arabidopsis thaliana nitric oxide synthase* 1 [AtNOS1] and the corresponding gene has sequence similarity to a gene from *Helix pomatia* [roman snail] (Guo, Okamoto, and Crawford, 2003). Knock-out of this gene in *Arabidopsis* resulted in reduced NO accumulation in roots and reduced NOS-activity in leave extracts. At present, AtNOS1 is the only known enzyme associated with arginine-dependent NOS activity in plants (Crawford and Guo, 2005).

Source	**Substrate**	**Co-factors**	**Cellular localization**	**Physiological process affected**	**Reference(s)**
Animal NOSs	L-Arg	heme NADPH FAD, FMN BH_4 calmodulin	cytosol, PM bound GM bound mitochondria	neuro-transmission immune response vasodilatation	(Mayer and Hemmens, 1997; Wendehenne et al., 2001)
Plant iNOS	L-Arg	Heme NADPH FAD BH_4 calmodulin	Chloroplasts (?)	defense responses to pathogens	(Chandok *et al.*, 2003; Chandok *et al.*, 2004; Lamotte *et al.*, 2004)
Plant AtNOS1	L-Arg	NADPH calmodulin	ND	ABA signaling growth and development fertility	(Guo, Okamoto, and Crawford, 2003)
Plant NR	nitrite	NADPH	cytosol	ABA signaling defense responses (?) photo inhibition	(Yamasaki, 2000; Desikan *et al.*, 2002; Rockel *et al.*, 2002; Yamamoto *et al.*, 2003)
Plant Ni-NOR	nitrite	cytochrome c	PM bound	ND	(Stoehr et al., 2001)
Non-enzymatic NO production	nitrite	Phenolics acidic pH	apoplast	ND	(Bethke, Badger, and Jones, 2004)

Table 1-1: *Enzymatic and non-enzymatic sources of NO in plants and animals.*

Abbreviations: BH_4, tetrahydrobiopterin; GM, Golgi membrane; ND, not determined; Ni-NOR, a 310-kDa plasma-membrane bound enzyme that catalyzes the reduction of nitrite to NO, PM, plasma membrane; (?), suggested but not demonstrated.

1.3 LIPOPOLYSACCHARIDES [LPS]

More than a century ago, Richard Pfeiffer, working in Robert Koch's laboratory, identified a heat resistant toxin in lysates of *Vibrio cholerae* causing a strong burst of ROS and NO and subsequent toxic shock in animals. Unlike the exotoxin, it was not secreted by the bacteria, so it was termed endotoxin (Caroff et al., 2002). Today is known, that not all endotoxins are toxic, just as bacteria are not all pathogenic. The endotoxins were soon shown to characterize the major group of Gram-negative bacteria, i.e., those having a second, outer membrane (Caroff and Karibian, 2003).

1.3.1 GENERAL STRUCTURE OF LPS

In the early part of the last century, it was becoming clear that endotoxin be composed of lipid and carbohydrate, and hence the term 'lipopolysaccharide' came into usage (Erridge, Bennett-Guerrero, and Poxton, 2002). Lipopolysaccharides are amphiphilic molecules present on the outer leaflet of Gram-negative bacteria (Gronow and Brade, 2001). Despite a great compositional variation depending on their particular bacterial origin, they all consist of a hydrophobic lipid component termed lipid A (Chaby, 2004), which is stabilized by divalent cations, and a hydrophilic polysaccharide [PS], extending outward from the bacterium [Fig. 1-3] (Chatterjee and Chaudhuri, 2004). The PS comprises generally of two distinct regions, a core oligosaccharide containing 10-12 sugars, and a polysaccharide chain of repeating units, the O-specific chain (Caroff et al., 2002).

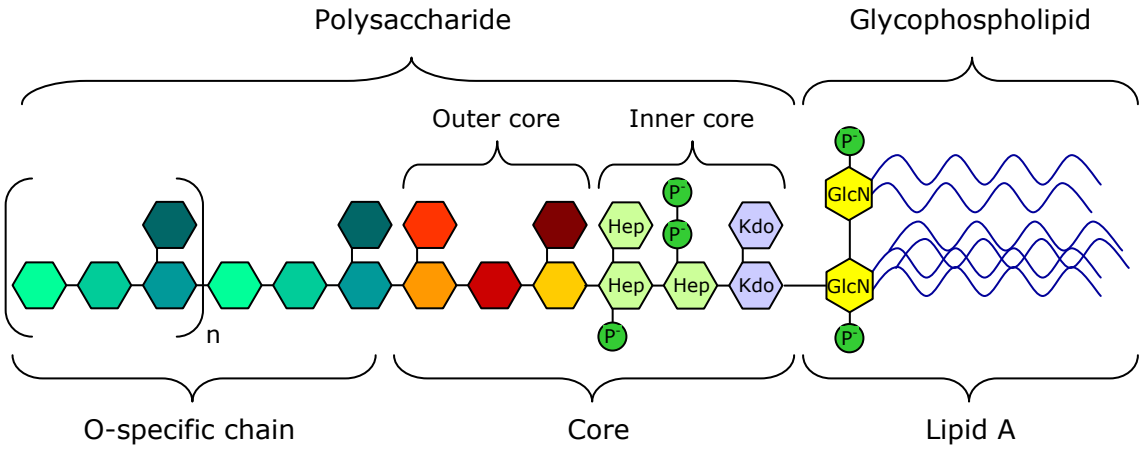

Figure 1-4: *Schematic representation of the chemical structure of enterobacterial LPS.*

Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; Hep, L-glycero-D-manno-heptose; P, phosphate; zigzag lines, fatty acids. [After (Caroff et al., 2002)].

Wild-type enterobacterial species with O-chains are termed 'smooth' [S] because of the morphology of their colonies. Mutants producing rough-looking colonies and lacking LPS O-chains are accordingly termed 'rough' [R] (Lerouge and Vanderleyden, 2002).

Variation in the number O-chain repeating units contributes greatly to LPS heterogeneity as observed on SDS gel electrophoresis as the classical ladder pattern (Haeffner-Cavaillon et al., 1999).The structures in the core part are limited in variability, with some regions being highly conserved between different strains and species. The outer core provide an attachment side for the O-chain and typically consists of common hexose sugars and is generally more variable than the inner core (Erridge, Bennett-Guerrero, and Poxton, 2002). Within a genus or family, the structure of the inner core tends to be well conserved, and typically contains residues of unusually sugars, such as 3-deoxy-D-Manno-oct-2-ulopyranosonic acid [Kdo] and L-glycero-D-mannoheptose [L, D-Hep]. The Kdo residue is the only component found in all known cores and seems to be a characteristic and essential sugar for the great majority of endotoxins (Raetz and Whitfield, 2002). In the 1980s free lipid A was prepared synthetically, that it was proven to be the endotoxic centre of the LPS (Tanamoto et al., 1984). Most lipid A molecules comprise of a phosphorylated disaccharide, to this structure are attached up to four acyl chains by ester or amide linkage (Holst et al., 1996).

1.3.2 LPS AND INNATE IMMUNITY IN HUMAN HOST

LPS act as extremely strong stimulators of innate or natural immunity in diverse eukaryotic species ranging from insect to humans, whereas the lipid A component is the primary immunostimulatory centre (Darveau, 1998). Then the conserved common architecture of the lipid A domain is a highly specific indicator or 'pathogen-associated molecular pattern [PAMP]' for infection by Gram-negative bacteria (Alexander and Rietschel, 2001). PAMPs represent conserved molecular patterns that are produced only by microbial pathogens, which are often shared by a large group of them and often be 'molecular signatures' of a pathogen class (Zipfel and Felix, 2005). These properties allow the recognition of self/non-self, a vast variety of microorganism and the immune system to choose the effector mechanism that is most efficient against a given class of pathogens (Medzhitov and Janeway, 2000). If LPS or lipid A came in the blood stream of human by infection or injury it will be recognized as PAMP and induce various host defense pathways such as production of ROS or NO [Fig. 1-4]. These cellular responses are dependent on the serum protein, LPS-binding protein [LBP] (Elsbach, 2000). The principal catalytic mechanism appears to reside in the ability of LBP to dissociate LPS aggregates into LPS monomers bound to LBP and deliver these to CD14 (Tobias, Tapping, and Gegner, 1999). CD14 is a glycoprotein, which sensitized host cells for LPS initiated cellular activation (Wright et al., 1990) and seems to possess an ability to discriminate between bacterial products and sort their signals to different toll-like receptors [TLR] (Fujihara et al., 2003). For innate immune response cells recognize PAMPs through TLRs, which play an essential role as pattern recognition receptors (Aderem and Ulevitch, 2000). The family of currently ten TLRs has been identified with a

wide range of ligand specificity including bacterial, fungal and yeast proteins (Cohen, 2002). Thus, TLR4 is the LPS receptor (Fitzgerald, Rowe, and Golenbock, 2004), but an additional cell-surface molecule, MD-2, has been identified that is required for activation of TLR4 (Shimazu et al., 1999). MD-2 is essential for correct intracellular distribution and LPS-recognition of TLR4 (Nagai et al., 2002). TLR4 is expressed most predominantly in immune cells, including macrophages and dendritic cells. Therefore, leads the activation of TLR4 to the production of classical pro-inflammatory cytokines such as tumor necrosis factor-α [TNF-α], interleukin [IL]-1, IL-6, IL-8 and IL-12 (Fujihara et al., 2003). These cytokines activate a second level of inflammatory cascades including cytokines, lipid mediators and reactive oxygen species (Cohen, 2002).

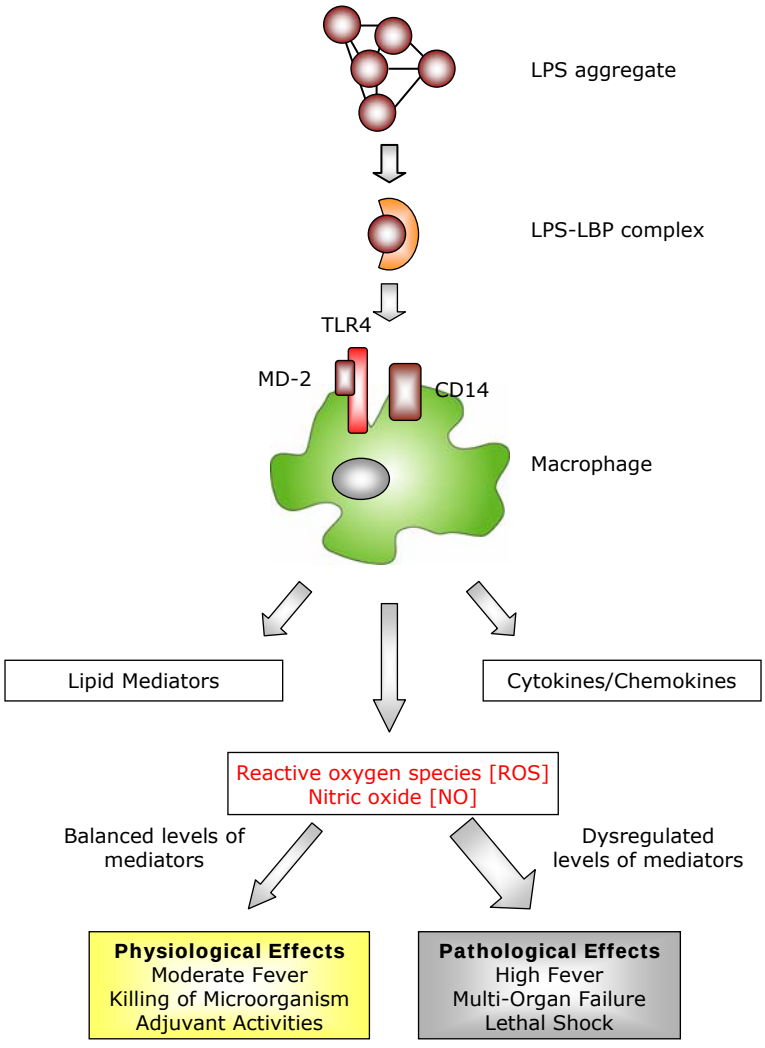

Figure 1-5: *Macrophage mediated activation of innate immunity by LPS.*

The central extracellular route for activation of peripheral monocytes or macrophages by endotoxically active LPS and the major inflammatory spectrum of cellular responses are schematically depicted. LPS-binding protein (LBP) catalyzes the transfer of monomerized LPS from aggregate structure or from intact Gram-negative bacteria to CD14 on the phagocyte surface that in turn initiates the release of a wide spectrum of endogenous mediators via TLR4-MD-2 complex. [After Alexander and Rietschel 2001; Cohen 2002].

In normal physiological immunoreactions, comparably low and balanced levels of these mediators lead to activation of general antimicrobial defense mechanism, but in case of pathological situations dysregulated and unbalanced levels of mediators may as well evoke dramatic and often life-threatening effects as observed in severe forms of sepsis (Alexander and Rietschel, 2001).

1.3.3 LPS AND PLANT-PATHOGEN INTERACTIONS

In contrast to the well documented effects of LPS on human host, much remains to be elucidated about the effect of LPS on plants. Non-pathogenic rhizobacteria can induce a systemic resistance in plants which is phenotypically similar to pathogen-induced systemic resistance [SAR] (van Wees et al., 1999). In the systemic protection of carnation against *Fusarium* wilt by *Pseudomonas fluorescens*, heat-killed bacteria or the purified LPS were as effective in inducing resistance as were live bacteria. This observation indicated that bacterial LPS acts as a determinant of resistance induction (Van Loon, Bakker, and Pieterse, 1998). Rhizobacteria-mediated induced systemic resistance [ISR] has been demonstrated against fungi, bacteria, and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato (van Peer, Niemann, and Schippers, 1991; Maurhofer et al., 1994; Liu, Kloepper, and Tuzun, 1995; Leeman et al., 1996; Pieterse and al., 1996; de Meyer and Hofte, 1997; Press et al., 1997). Another important role plays LPS in the development of symbiosis of nitrogen-fixing bacteria with their host plant (Fraysse, Couderc, and Poinsot, 2003). Soil bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*, are able to establish a symbiosis mainly with leguminous plants. The infection of legumes by the soil bacteria leads to the formation of a highly specialized structure, the root nodule, where biological N_2-fixation occurs (Hirsch, 1992). In addition to plant flavonoides and rhizobial nodulation factors, bacterial surface saccharides such as exopolysaccharides (EPS) and lipopolysaccharides form another important class of signal molecules for an effective symbiosis (Scheidle, Gross, and Niehaus, 2005). LPS released from the bacterial surface might function as a specific signal molecule, suppressing a pathogenic response in the host plant (Albus et al., 2001) and inform the plant to proceed with the symbiotic interaction and to develop a functional fixation zone (Mathis et al., 2005). Currently, strong efforts are being made to elucidate the molecular mechanisms of LPS in the stimulation of plant defense. One of the first findings of LPS effect on plant cells was its ability to prevent the hypersensitive response caused by avirulent plant-pathogenic bacteria (Sequeira, 1983). The origin of LPS seems thereby irrelevant and LPS-treatment leads to HR suppression in both host and non-host incompatible reactions (Newman, Daniels, and Dow, 1997; Newman et al., 2002). LPS from *B. cepacia* contribute induce the accumulation of pathogenesis-related proteins in *Nicotianae tabacum* after four days after treatment and could increase the membrane permeability of tobacco cells (Coventry

and Dubery, 2001). This enhanced permeability could lead to a trans-membrane flux of Ca^{2+} ions across the plasmalemma and to plant defense response, such as the LPS-triggered oxidative burst in tobacco (Meyer, Puehler, and Niehaus, 2001). LPS isolated from *B. cepacia* was found to trigger a rapid intracellular influx of Ca^{2+} into the cytoplasm, which is involved in LPS-induced oxidative burst and LPS-elicitation results in extracellular alkalinization (Gerber et al., 2004). A new study provide evidence that *B. cepacia* LPS has specific effects on reversible protein phosphorylation events underlying the perception systems involved in interaction of tobacco cells with LPS (Gerber and Dubery, 2004).

1.3.4 *BURKHOLDERIA CEPACIA*

Because of the use of LPS from *Burkholderia cepacia* for most experiments, this bacterium will be shortly characterized. *B. cepacia* was originally described by Walter H. Burkholder as the causative agent of 'sour skin' onion rot (Coenye and Vandamme, 2003). Due to its phenotypic characteristics including utilization of various carbon sources and the presence of polar flagella, this bacterium was first placed in the genus *Pseudomonas*. Based on biochemical, phenotypic, and genetic characteristics the genus name *Burkholderia* was given in honor of the bacteriologist Burkholder. The species name *cepacia* was chosen because of the association of this bacterium with onions, *caepa* being the genus name for onion (Coenye et al., 2001). The natural habitats of this bacterium are river sediments and the moist areas of soil around the roots of plants (Govan, Burns, and Speert, 1999). Interestingly, *B. cepacia* can not only cause plant diseases, like soft rot in alliums, but can also prevent them. Because of its ability to produce several antimicrobial compounds, it is an effective chemical fungicide. Another positive environmental effects are the promotion of plant growth and crop production by colonizing roots and fixing of nitrogen and the degradation of groundwater contaminants (Vinion-Dubiel and Goldberg, 2003).

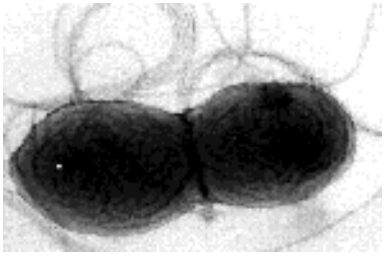

Figure 1-6: *A dividing cell of Burkholderia cepacia, which has been negatively stained with 1% (w/v) uranyl acetate to reveal the flagella.*

(Source: http://www.cbdn.ca/english/news_letters/julaug02.html)

But in contrast to its beneficial properties, *B. cepacia* is also known, as pathogen in human diseases. This bacterium is an opportunistic pathogen, which does not normally

infect healthy individuals but only those that are immunocompromised. Infections with *B. cepacia* typically occur in patients with cystic fibrosis [CF] (Evans, Poxton, and Govan, 1999). CF is a genetic disease, which causes the body to produce abnormally thick, sticky mucus that clogs the lungs and leads to life-threatening lung infections. On these people, the infections with *B. cepacia* often have an important impact on the morbidity and mortality, because the bacteria a frequently cultured in the blood leading to sepsis and pneumonia (Mahenthiralingam, Urban, and Goldberg, 2005).

1.4 GOALS OF THIS STUDY

The past few years have seen dramatic changes in our understanding of the molecular principles of disease resistance. A growing body of evidence indicates that some principles involved in innate immunity in mammalian and insect systems are strikingly similar to the molecular mechanisms underlying plant disease-resistance responses (Cohn, Sessa, and Martin, 2001). It has been proposed therefore, that innate immunity might be an evolutionarily ancient system of host defense (Nuernberger and Scheel, 2001). A key role in the activation of the innate immune system plays LPS, which are the major component of the outer membrane of gram negative bacteria (Heumann and Roger, 2002). LPS serves in many experimental systems as a prototypic model PAMP (Rietschel et al., 1996) and have been shown to activate the synthesis of antimicrobial peptides in Drosophila, as well as the production of immunoregulatory and cytotoxic molecules in humans (Alexander and Rietschel, 2001; Diks, van Deventer, and Peppelenbosch, 2001). One of the most important hallmarks of innate immunity activation by LPS is the induction of cellular mediators and antimicrobial defense mechanisms such as the production of nitric oxide (Nathan and Shiloh, 2000), a molecule whose importance in plant defense is just emerging (Wendehenne, Durner, and Klessig, 2004). In my study I wanted to investigate if LPS has also a stimulatory effect on plant immunity. First tests should therefore clarify the possibility of LPS to induce a NO burst in plants just like in animals. Further should be analyzed if plants respond to LPS with an activation of defense and/or defense-associated genes. In sum, it was my goal to elucidate whether LPS is a novel signaling molecule in plant-pathogen interactions.

2 MATERIAL

2.1 BUFFERS AND SOLUTIONS

Name	Contents		
Aminoallyl labeling mix (50x)	dATP	25	mM
	dCTP	25	mM
	dGTP	25	mM
	dTTP	15	mM
	aa-dUTP	10	mM
Ampicillin	Ampicillin	100	mg/ml
	Dissolve in ddH_20, sterile filter, store at -20°C		
Anode-Buffer (10x) for LPS gels	Tris	2	M
	Adjust to pH 8.9		
6-Benzylaminopurine stock for PS + MS medium	6-Benzylaminopurine	1	mg/ml
	Dissolve 100 mg in 3 ml 3N HCl by warming, fill up to 100 ml with ddH_2O.		
Buffer A	Magnesium chloride	2.5	mM
	Calcium chloride	1	mM
Carbonate buffer for CE (40 mM)	Sodium carbonate (41 mM)	10	%(v/v)
	Sodium bicarbonate (31 mM)	90	%(v/v)
Cathode-Buffer (10x) for LPS gels	Tris	1	M
	Tricine	1	M
	SDS	1	%(w/v)
CTAB-Buffer for DNA isolation	Sodium chloride	1.4	M
	Hexadecyltrimethylammonium bromide	2	% (w/v)
	PVP-40	1	% (w/v)
	Tris-HCl (pH 8.0)	100	mM
	EDTA (pH 8.0)	20	mM
Denhardt's solution (50x)	Ficoll® 400	1	% (w/v)
	Polyvinylpyrrolidone	1	% (w/v)
	Bovine serum albumin	1	% (w/v)
	Sterile filter, store at -20°C		
DEPC-H_2O	*Add 0.01% (v/v) DEPC to ddH_20, stirring over night, than autoclave or boiling for 15 min.*		
DETC-Buffer for EPR	Bovine serum albumin	1.8	% (w/v)
	Sodium hydrosulfite	1	M
	DETC	7	mM
	Iron sulfate	10	mM

Developer for Silver Stain	Citric Acid	0.005	%(w/v)
	Formaldehyde	0.05	%(v/v)
2,4-Dichlorophenoxyacetic acid stock for HaM medium	2,4-Dichlorophenoxyacetic acid	50	mg/ml
	Dissolve in absolute Ethanol, sterile filter, and store at -20°C		
2,4-Dichlorophenoxyacetic acid stock for PS medium	2,4-Dichlorophenoxyacetic acid	2	mg/ml
	Dissolve 100 mg in 200 ml Methanol.		
DNA-Loading-Buffer (5x)	TE	50	% (v/v)
	Glycerol	50	% (v/v)
	Xylene cyanole	40	mg
	Bromphenol blue	40	mg
DNase I buffer (10x)	Tris-HCl	400	mM
	Magnesium chloride	60	mM
	Adjust to pH 7.5		
EPR-Buffer	HEPES	50	mM
	DTT	1	mM
	Magnesium chloride	1	mM
	Adjust to pH 7.6		
Ethidium bromide		10	mg/ml
	Dissolve in DEPC-H_2O, store at 4°C.		
Extraction-Buffer for NOS assay	Sucrose	320	mM
	Tris-HCl (pH 7.4)	50	mM
	DTT	10	mM
	EDTA	1	mM
	PMSF	1	mM
	Leupeptin	1	µM
	Pepstatin	1	µM
Fix Solution for LPS gels using PRO-Q Emerald	Methanol	50	%(v/v)
	Acetic acid	5	%(v/v)
Gel-Buffer (3x) for LPS gels	Tris	1	M
	SDS	0.3	%(w/v)
HaM vitamin mix stock	Nicotinic acid	4	mM
	Pyridoxine hydrochloride	486	µM
	Thiamine hydrochloride	60	µM
	Sterile filter, store at -20°C.		
HPLC running buffer A	Sodium acetate	27	mM
	Citric acid	30	mM
	Adjust to pH 5.0		

HPLC running buffer B	Sodium acetate	27	mM
	Citric acid	30	mM
	Dissolve in 95% Methanol.		
HPLC sample buffer	Sodium acetate	27	mM
	Citric acid	30	mM
	Dissolve in 20% Methanol.		
Hybridization-Buffer for Microarray	Formamide	50	% (v/v)
	SSC	6	x
	Denhardt's	5	x
	SDS	0.5	% (w/v)
	Salmon sperm DNA (10 mg/ml)	1	% (v/v)
Iron-EDTA for HaM medium	Iron sulfate heptahydrate	5	mM
	EDTA	6.5	mM
	Dissolve in water, autoclave, and store dark.		
Iron-EDTA (200x) for PS medium	Iron sulfate heptahydrate	2.78	g
	Dissolve in 200 ml ddH_2O		
	Titriplex® III	3.72	g
	Dissolve in 200 ml ddH_2O		
	Add the Titriplex® III solution under permanent stirring to Iron sulfate heptahydrate solution. Fill up to 500 ml. Warm up to 60°C if necessary. Store at 4°C up to 2 months in the dark.		
Loading-Buffer (5x) for LPS gels	Glycerol	50	%(v/v)
	SDS	5	%(w/v)
	2-Mercaptoethanol	2.5	%(v/v)
	Tris, pH 6.8	50	mM
	Bromphenol blue	0.01	%(w/v)
MOPS (10x)	MOPS	200	mM
	Sodium acetate	50	mM
	EDTA	10	mM
	Adjust to pH 7.0		
MS macroelements (10x) for PS medium	Ammonium nitrate	200	mM
	Potassium nitrate	200	mM
	Calcium chloride dihydrate	30	mM
	Magnesium sulfate heptahydrate	15	mM
	Potassium dihydrogen phosphate	12.5	mM
	Potassium iodide	50	µM
	Store at 4°C up to 2 months		
MS microelements (10x) for PS medium	Manganese sulfate	1	M
	Boric acid	1	M
	Zinc sulfate heptahydrate	37	mM
	Sodium molybdate dihydrate	1	mM

	Copper sulfate	1.5	mM
	Cobalt chloride	2	mM
	Store at 4°C up to 2 months		
MS vitamins for PS medium	Nicotinic acid	406	µM
	Pyridoxine hydrochloride	243	µM
	Thiamine hydrochloride	30	µM
	myo-Inositol	55.5	mM
	Store at -20°C up to 2 months		
1-Naphthylacetic acid stock for MS medium	1-Naphthylacetic acid stock	1	mg/ml
	Dissolve 100 mg in 2 ml 1 N KOH by warming, fill up to 100 ml with ddH_2O		
Oxidizing Solution for LPS gels using PRO-Q Emerald	Oxidizing reagent	2.5	g
	Acetic Acid (3%)	250	ml
Oxidizing Solution for LPS gels using Silver Staining	Periodic Acid	0.7	%(w/v)
	Ethanol	40	%(v/v)
	Acetic Acid	5	%(v/v)
PBS	Sodium chloride	140	mM
	Sodium dihydrogen phosphate	3.2	mM
	Potassium chloride	2.7	mM
	Potassium dihydrogen phosphate	1.5	mM
	Adjust to pH 7.2		
PCR-Buffer (10x)	Tris	750	mM
	Ammonium sulfate	200	mM
	Magnesium chloride	15	mM
	TWEEN 20	0.1	% (v/v)
Phosphate buffer (1M KPO_4)	1M Dipotassium hydrogen phosphate	9.5	ml
	1M Potassium dihydrogen phosphate	0.5	ml
Phosphate wash buffer	KPO_4 (pH 8.5)	5	mM
	Ethanol	80	% (v/v)
PIC vitamins (100x) for MS medium	myo-Inositol	111	mM
	Thiamine hydrochloride	300	µM
	Nicotinic acid	800	µM
	Pyridoxine hydrochloride	500	µM
	Glycine	5.3	mM
	Adenine sulfate	22	mM
	Store at -20°C up to two months		
Pre-Hybridization-Buffer for microarray	SSC	6	x
	Bovine serum albumin	1	% (w/v)
	SDS	0.5	% (w/v)
	Salmon sperm DNA (10 mg/ml)	1	% (v/v)

Rifampicin	Rifampicin	50	mg/ml
	Dissolve in DMF, store at -20°C in glass vials.		
RNA-extraction buffer for the Resin method	Tris-HCl (pH 8.0)	100	mM
	EDTA	25	mM
	NaCl	2	M
	CTAB	2	% (w/v)
	Polyvinylpyrrolidone	2	% (w/v)
	Spermidine	0.5	% (w/v)
RNA Loading-Buffer (0.8 Vol)	Formamide	8.67	ml
	Formaldehyde	830	µl
	10x MOPS	500	µl
	Bromphenol blue	10	mg
Salicylic acid standard for HPLC	Salicylic acid (10µg/ml)	25	µl
	Methanol (100%)	50	µl
	HPLC running buffer A	400	µl
Salmon sperm DNA stock	Salmon sperm DNA	10	mg/ml
	Dissolve the ssDNA in water, autoclave, and store at -20°C. Before adding to hybridization buffer, ssDNA was denatured at 100°C for 2 min and cooled on ice.		
Separating Gel (12.5%)	Acrylamide/Bisacrylamide (29:1)	23.75	ml
	Gel Buffer (3x)	25	ml
	APS (30 %)	300	µl
	TEMED	50	µl
Separation buffer for CE	Carbonate buffer (20 mM)	50	%(v/v)
	SDS (20 mM)	50	%(v/v)
Silver nitrate staining solution	Ammonium Hydroxide (25 %)	4	ml
	Sodium Hydroxide (0.1 M)	56	ml
	After mixing 200 ml ddH_2O were added.		
	Silver nitrate (20 %)	10	ml
	Were added in drops under permanent stirring and the final volume was adjusted to 300 ml with ddH_2O.		
Sodium-Borohydride-Solution	Sodium borohydride	0.75	g
	PBS	200	ml
	Ethanol	75	ml
Stacking Gel (4%)	Acrylamide/Bisacrylamide (29:1)	4.5	ml
	Gel Buffer (3x)	15	ml
	APS (30 %)	300	µl
	TEMED	30	µl
Spotting-Solution	SSC	3	x
	Betaine	1.5	M

SSC (20x)	Sodium chloride	3	M
	Sodium citrate	300	mM
		Adjust to pH 7.4	
TAE-Buffer (50x)	Tris	2	M
	Acetic acid	5.71	% (v/v)
	EDTA	50	mM
TE-Buffer	Tris	10	mM
	EDTA	1	mM
Tetracycline	Tetracycline hydrochloride	10	mg/ml
	Dissolve in Ethanol, sterile filter, store at -20°C.		
Tri-Reagent	Ammonium thiocyanate	400	mM
	Guanidine thiocyanate	800	mM
	Sodium acetate	100	mM
	Glycerol	5	% (v/v)
		Adjust to pH 5.0.	
Washing-Buffer I for Northern Blots	SSC	2	x
	SDS	0.1	%(w/v)
Washing-Buffer II for Northern Blots	SSC	0.1	x
	SDS	0.1	%(w/v)
Wash solution for LPS gels using PRO-Q Emerald	Acetic acid	3	%(v/v)

2.2 MEDIUMS

2.2.1 PLANT CELLS

Name	Contents		
HaM medium (1l)	MS salts + MES (Duchefa)	4.8	g
	Sucrose	20	g
	myo-Inositol	100	mg
	2,4- Dichlorophenoxyacetic acid stock	10	µl
	Fe-EDTA stock	20	ml
	HaM vitamin mix stock	1	ml
	Agar (for plates)	12	g
		Adjust to pH 5.7.	
MS medium (1l)	MS salts (Sigma)	8.6	g
	PIC vitamins	10	ml
	Sucrose	60	g
	6-Benzylaminopurine stock	2	ml
	1-Naphthylacetic acid stock	6	ml
	Agar (for plates)	24	g
		Adjust to pH 5.7	

PS medium (1l)	MS macro elements stock	100	ml
	MS microelements stock	1	ml
	MS vitamins stock	10	ml
	Iron-EDTA stock	5	ml
	Sucrose	30	g
	2,4-Dichlorophenoxyacetic acid stock	1	ml
	6-Benzylaminopurine stock	1	ml
	Agar (for plates)	12	g
		Adjust to pH 6.0.	

2.2.2 BACTERIA

Name	Contents		
King's B medium (1l)	Peptone	20	g
	Glycerol	10	g
	di-Potassium hydrogen phosphate	1.5	g
	Magnesium sulfate heptahydrate	1.5	g
	Agar (for plates)	15	g
		Adjust to pH 7.2 – 7.4.	
LB glycine Medium	Tryptone	10	g
	Magnesium sulfate	50	mg
	Potassium dihydrogen phosphate	8.2	g
	Potassium phosphate dibasic	1.8	g
	Sodium citrate	0.5	g
	Ammonium sulfate	0.9	g
	Glycine	44	ml
	Sodium chloride	10	g
	Yeast extract	5	g
	Agar (for plates)	15	g
		Adjust to pH 8.0.	

2.3 CHEMICALS

2.3.1 SALTS AND SOLUTIONS

Name	CAS Number	Company
Acetic acid	64-19-7	Merck
Acrylamide/Bis Solution (29:1)		Bio-Rad
Adenine sulfate	321-30-2	Sigma
Agarose		Biozym
Agilent Stabilization and Drying Solution		Agilent
Aminoallyl-dUTP	109921-28-0	Sigma
Ammonium hydroxide solution	1336-21-6	Sigma
Ammonium nitrate	6484-52-2	Sigma
Ammonium persulfate	7727-54-0	Sigma
Ammonium sulfate	7783-20-2	Merck
Ammonium thiocyanate	1762-95-4	Sigma
Ampicillin	69-53-4	Sigma
Ampuwa®		Fresenius

Anti-Digoxigenin-AP, Fab fragments		Roche
6-Benzylaminopurine	1214-39-7	Sigma
Betaine	107-43-7	Sigma
Boric acid	10043-35-3	Sigma
Bovine serum albumin	90604-29-8	Merck
Bromphenol blue	115-39-9	Merck
Calcium chloride dihydrate	10035-04-8	Sigma
Calmodulin	73298-54-1	Sigma
Carboxy-PTIO	148819-94-7	Invitrogen
Chloroform	67-66-3	Merck
Citric Acid	77-92-9	Sigma
Cobalt chloride	7646-79-9	Sigma
Copper sulfate	7758-98-7	Sigma
CSPD, ready-to-use		Roche
Cyclohexane	110-82-7	Merck
Cy™3 Mono-Reactive Dye Pack		Amersham
Cy™5 Mono-Reactive Dye Pack		Amersham
DAF-2T		Alexis
DAF-FM diacetate	254109-22-3	Invitrogen
Deoxyribonuclease I (DNase I)		Amersham
DEPC	1609-47-8	Sigma
DETC	20624-25-3	Sigma
2,4-Dichlorophenoxyacetic acid	94-75-7	Sigma
DIG Easy Hyb Granules		Roche
DMF	68-12-2	Sigma
DMSO	67-68-5	Sigma
DNA, low molecular weight salmon sperm	9007-49-2	Sigma
dNTP-Set (dATP, dCTP, dGTP, dTTP)		Fermentas
DTT	3483-12-3	Sigma
EDTA	60-00-4	Sigma
Ethanol	64-17-5	Merck
Ethidium bromide	1239-45-8	Sigma
Ethyl acetate	141-78-6	Merck
Ficoll® 400	26873-85-8	Sigma
First strand buffer (5x)		Invitrogen
Formaldehyde	50-00-0	Sigma
Formamide	75-12-7	Sigma
Formic acid	64-18-6	Merck
GeneRuler™ DNA Ladder, 1kb		Fermentas
Glycine	56-40-6	Sigma
Glycerol	56-81-5	Sigma
GoldStar® DNA polymerase		Eurogentec
Guanidine thiocyanate	593-84-0	Sigma
H_2DCFDA	4091-99-0	Invitrogen
HEPES	7365-45-9	Sigma
Hexadecyltrimethylammonium bromide (CTAB)	57-09-0	Sigma
Human Cot-1 DNA®		Invitrogen
Hydrochloric Acid	7647-01-0	Sigma
Iron sulfate heptahydrate	7782-63-0	Sigma
Isopropanol	67-63-0	Bilgram
$_L$-[U-^{14}C]Arginine monohydrochloride		Amersham

Leupeptin	24125-16-4	Sigma
L-NMMA	53308-83-1	Sigma
L-NNA	2149-70-4	Sigma
LPS from *Pseudomonas aeruginosa*		Sigma
LPS from *Salmonella minnesota*, Alexa fluor™ 488 conjugate		Invitrogen
Magnesium chloride	7786-30-3	Merck
Magnesium sulfate heptahydrate	10034-99-8	Merck
Manganese sulfate	10034-96-5	Sigma
2-Mercaptoethanol	60-24-2	Sigma
Methanol	67-56-1	Merck
MOPS	1132-61-2	Sigma
MS salts including MES		Duchefa
myo-Inositol	87-89-8	Sigma
N,N,N′,N′-Tetramethylethylenediamine	110-18-9	Sigma
NADPH	2646-71-1	Sigma
1-Naphthylacetic acid	86-87-3	Merck
Nicotinic acid	59-67-6	Sigma
N-Lauroylsarcosine solution	137-16-6	Sigma
Oligo $(dT)_{12-18}$ Primer (0.5 µg/µl)		Invitrogen
Pepstatin	26305-03-3	Sigma
Periodic Acid	10450-60-9	Sigma
PicoGreen® dsDNA quantitation reagent		Invitrogen
PMSF solution	329-98-6	Sigma
Poly(vinylpolypyrrolidone)	25249-54-1	Sigma
Polyvinylpyrrolidone	9003-39-8	Sigma
Potassium chloride	7447-40-7	Merck
Potassium dihydrogen phosphate	7778-77-0	Merck
Potassium iodide	7681-11-0	Sigma
Potassium nitrate	7757-79-1	Sigma
Potassium phosphate dibasic	16788-57-1	Sigma
Pyridoxine hydrochloride	58-56-0	Sigma
RiboGreen® RNA quantitation reagent		Invitrogen
Rifampicin	13292-46-1	Serva
RNA Molecular Weight Marker I, DIG-labeled		Roche
RNase A (4 mg/ml)		Promega
RNase H		Amersham
RNaseOUT™ Recombinant Ribonuclease Inhibitor		Invitrogen
RNaseZAP		Sigma
Scintillation Fluid		Sigma
Silver nitrate	7761-88-8	Sigma
Sodium acetate	127-09-3	Sigma
Sodium bicarbonate	144-55-8	Sigma
Sodium borohydride	16940-66-2	Sigma
Sodium carbonate	497-19-8	Sigma
Sodium chloride	7647-14-5	Merck
Sodium citrate	68-04-2	Merck
Sodium dihydrogen phosphate	7558-80-7	Merck
Sodium dodecyl sulfate	151-21-3	Sigma
Sodium hydrosulfite	7775-14-6	Sigma
Sodium hydroxide	1310-73-2	Sigma
Sodium molybdate dihydrate	10102-40-6	Sigma
Spermidine	124-20-9	Sigma
SP300 DNA		Operon

SSPE Buffer (20x)		Sigma
Sucrose	57-50-1	Sigma
SuperScript™ II Reverse Transcriptase		Invitrogen
SYPRO® Ruby protein gel stain		Invitrogen
Tetracycline hydrochloride	64-75-5	Sigma
Thiamine hydrochloride	67-03-8	Sigma
Titriplex® III	6381-92-6	Merck
Tricine	5704-04-1	Sigma
Tris-HCl, pH 7.4	1185-53-1	Sigma
Trizma® base (Tris)	77-86-1	Sigma
Trizol®		Invitrogen
Trypan Blue	72-57-1	Sigma
TWEEN 20	9005-64-5	USB
Xylene cyanole	2650-17-1	Sigma
Zinc sulfate heptahydrate	7446-20-0	Sigma

2.3.2 LIPOPOLYSACCHARIDES, LIPOTEICHOIC ACID AND LIPID A

Source	Strain		Provenance
Burkholderia cepacia	LPS	ASP B 2D	I. Dubery
Erwinia carotovora	LPS		U. Zaehringer
Erwinia carotovora susp. *carotovora*	LPS	GSPD 436	U. Zaehringer
Escherichia coli	LPS	F515	U. Zaehringer
Escherichia coli	LPS	Nissle 1917	U. Zaehringer
Pseudomonas aeruginosa	LPS	F1	U. Zaehringer
Pseudomonas aeruginosa	LPS	F2	U. Zaehringer
Pseudomonas aeruginosa	Lipid A	PAC605	U. Zaehringer
Pseudomonas aeruginosa	LPS	commercial	Sigma
Pseudomonas fluorescens	LPS		U. Zaehringer
Pseudomonas fluorescens	Lipid A	271	U. Zaehringer
Pseudomonas plantarii	LPS		U. Zaehringer
Pseudomonas syringae	LPS		U. Zaehringer
Ralstonia solanacearum	LPS		U. Zaehringer
Salmonella minnesota	LPS	Alexa Fluor™ 488 conjugate	Invitrogen
Staphylococcus aureus	LTA		T. Hartung
Xanthomonas campestris pv. *begoniae*	LPS		U. Zaehringer

2.4 KITS

Name	Contents		
DIG Wash and Block Buffer Set (Roche)	Washing Buffer (10x)	500	ml
	Maleic acid buffer (10x)	500	ml
	Blocking solution (10x)	500	ml
	Detection buffer (10x)	100	ml
In situ Hybridization Kit Plus (Agilent)	2X Hybridization Buffer	2.5	ml
	25X Fragmentation Buffer	100	µl
	10% Triton X-102	4	ml
	10X Control Targets	for 500	µl
Nitric Oxide Synthase Detection Kit, Isotopic (Sigma)	Calcium chloride	300	µl
	Calmodulin	200	µl
	Elution Buffer	20	ml
	Equilibrated Resin	5	ml
	10X Homogenization Buffer	50	ml
	L-NMMA	40	µl
	Rat cerebellum extract	5x20	µl
	2X Reaction Buffer	1.25	ml
	Spin cups and holders	50	each
	Stop Buffer	20	ml
Nucleon PhytoPure™ Genomic DNA Extraction Kits (Amersham)	Reagent 1	31	ml
	Reagent 2	11	ml
	Phytopure Resin	6	ml
NucleoSpin® Plasmid (Macherey-Nagel)	Buffer A1	5	ml
	Buffer A2	5	ml
	Buffer A3	5	ml
	Buffer A4	2	ml
	Buffer AE	5	ml
	Buffer AW	6	ml
	NucleoSpin® Plasmid columns and collection tubes	50	each
	RNase A(lyophilized)	2	mg
PCR DIG Probe Synthesis Kit (Roche)	Control PCR primer mix	25	µl
	Control template	50	µl
	dNTP stock solution (10x)	125	µl
	Enzyme mix	30	µl
	PCR buffer with MgCl2 (10x)	1	ml
	PCR probe synthesis mix (10x)	125	µl
Pro-Q ® Emerald 300 Lipopolysaccharide Gel Stain Kit (Invitrogen)	Pro-Q Emerald 300 reagent	for 5	ml
	Pro-Q Emerald 300 staining buffer	250	ml
	Oxidizing reagent	2.5	g
	LPS standard from *E. coli* serotype 055:B5 (2.5 mg/ml)	25	µl
QIAquick PCR Purification Kit (Qiagen)	Buffer PB	30	ml
	Buffer PE	2x6	ml
	Buffer EB	15	ml
	Collection tubes and spin columns	50	each
R.E.A.L. Prep 96 Plasmid Kit (Qiagen)	QIAfilter™ 96 Plates	4	pieces
	Buffer R1	150	ml

Buffer R2	150	ml
Buffer R3	150	ml
RNase A	15	mg
Tape Pads	2	pieces
Square-Well Blocks	8	pieces
Lids	4	pieces

2.5 CELLS AND PLANTS

2.5.1 CELLS

Abbr. Name	Species	Eco Type	Function
Col. WT	*Arabidopsis thaliana*	Columbia	Wild type
Nt xanthii	*Nicotiana tabacum*	Xanthii	Wild type

2.5.2 PLANTS

Name	Species	Eco Type	Function	Provenance	Reference
Col. WT	*Arabidopsis thaliana*	Columbia	Wild type	Lehle Seeds	
nahG	*Arabidopsis thaliana*	Columbia	Expression of bacterial salicylate hydroxylase gene	D.F. Klessig	(Gaffney et al., 1993)
jin1	*Arabidopsis thaliana*	Columbia	Jasmonic acid insensitive mutant	S. Berger	(Berger, Bell, and Mullet, 1996)
atnos1	*Arabidopsis thaliana*	Columbia	Reduced Atnos1 activity	N.M. Crawford	(Guo, Okamoto, and Crawford, 2003)
varP	*Arabidopsis thaliana*	Columbia	Reduced variantP protein	Salk institute	(Zeidler et al., 2004)

2.6 CONSUMED MATERIAL

Name	Type	Company
Capillary	ID 75 µm, OD 375, 57 total length	Polymicro
Film	Chemiluminescent detection film Polaroid Film 667	Roche Carl Roth
Filter	Disposable filter 0.2 µm, 0.45 µm	Sartorius

Gasket slide	1 microarray/slide format	Agilent
HPLC column	RP-18 (150 x 4.6 mm. 5 µm, 120 A)	Bischoff
Membrane	Nylon Membrane, positively charged	Roche
Microarrays	*Arabidopsis* 1 Microarray *Arabidopsis* 3 Microarray	Agilent
Multiwell plate	F96 MicroWell™ Plates, black Thermo-Fast® 96 Non-Skirted MultiScreen Filter Plate	Nunc ABgene Millipore
Petri dish	Integrid™ Petri dish Petri Dish, 94 X 16 mm	Becton Dickinson Greiner
Pipette tip	Micro-Pipette tip 0.5-20 µl Pipette tip 21-200 µl, 201-1000 µl	Greiner Greiner
Reaction tube	Falcon™ Conical tube 15ml, 50 ml Safe-Lock reaction tube 0.5 ml, 1.5 ml, 2.0 ml	Becton Dickinson Eppendorf
Slide	CSS-100 silyated slide	TeleChem
Syringe	Single-use syringe 1ml Single-use syringe 20 ml	Sigma Sigma
Tape Sheets	AirPore Tape Sheets	Qiagen

2.7 INSTRUMENTS

Name	Type	Company
Autoclave	5075 EL	Tuttnauer
Balance	620S R180D U3600D	Sartorius Sartorius Sartorius
Camera	Polaroid DS34 Powershot G2	Polaroid Canon
Centrifuges	Beckman J2-21 Centrifuge 4K15C Centrifuge 5415 D Heraeus® Biofuge Universal	Beckman Coulter Sigma Eppendorf Kendro Hettich
Electrophoresis systems	Mini-vertical gel electrophoresis unit SE250 Model B2 EasyCast™ (12x14 cm) Mini Gel Electrophoresis System Model D3-14 Centipede™ (23x14 cm) Wide Gel Electrophoresis System P/ACE 5510 Capillary electrophoresis system	Hoefer Owl Owl Beckman Coulter
EPR instrument	Bruker ESP300 X-band spectrometer	Bruker

Exposition chamber	24x30 cm	Siemens
Film developer machine	Curix 60	Agfa
Flow Hood	Gelaire BSB 4A	Flow Laboratories
Fluorescent measurement	Fluostar Genios	BMG Tecan
Freeze-Dryer	Alpha 1/5	Christ
Fume Hood	CAPTAIR Filtersystem 804N	Captair
Gel caster	Multiple gel caster SE 215 External Gel Caster for B2 and D3	Hoefer Owl
HPLC system	Intelligent Pump L 6200A Auto-sampler Fluorescence-detector Chromato Integrator D 2500	Merck Spark Shimazu Merck
Hybridization chamber	Hybchamber Genemachine Legacy 6-Screw Chamber SureHyb	Geneworx Agilent Agilent
Hybridization oven	Hybrid 2000	Saur Laborbedarf
Incubator	BM 500 Friocell 111	Memmert MMM Medcenter
Magnetic stirrer with combined hot plate	IKA-Combimag Ret	Jahnke & Kunke
Microarray Robot	Biorobotics Microgrid II System	Geneworx
Microscopes	Zeiss Axiovert 100 M together with a confocal laser scanner LSM 510 Zeiss Axioskop	Carl Zeiss Carl Zeiss
pH measurement	pH electrode SenTix 21 pH Meter pH 523	WTW WTW
Power supply	Electrophoresis Power Supply E802	Carl Roth
Rotors	HFA 17.2 F45-24-11 1323 JA-10, JA-14, JA-20	Kendro Eppendorf Hettich Beckman Coulter
Scanner	Microarray Scanner Genepix™4000A	Biozym
Scintillation counter	LS 6000	Beckman Coulter
Shaker	Incubator Shaker G25 Combishaker KL 2	New Brunswick Carl Roth
Shaking water bath	GFL® Unitherm HB together with Unitwist	GFL UniEquip

Software	GenePix Pro 4.1 Acuity® 4.0 TAS Application Suite	Biozym Biozym Geneworx
Spectrophotometer	Ultrospec 3100 pro Ultrospec II	Amersham Biochrom
Thermal Cycler	PTC-100 Peltier Thermal Cycler PTC-200 Peltier Thermal Cycler	MJ Research MJ Research
Thermoblock	Thermomixer comfort Thermostat 5320	Eppendorf Eppendorf
Transilluminator	IL-350M	Bachofer
Ultra-pure water systems	Ultra Clear Direct	SG
UV crosslinker	Stratalinker 1800	Stratagene
Vacuum Concentrator Centrifuge	Univapo 150W	UniEquip
Vacuum Filtration	MultiScreen^{HTS} Vacuum Manifold	Qiagen

2.8 COMPANIES

Company	Location	
Abgene Germany	Hamburg	Germany
Agfa Deutschland Vertriebsgesellschaft mbH & Cie. KG	Cologne	Germany
Agilent Technologies GmbH & Co.KG	Waldbronn	Germany
AXXORA Deutschland GmbH	Gruenberg	Germany
Amersham Biosciences Europe GmbH	Freiburg	Germany
Bachofer Laboratoriumsgeräte	Reutlingen	Germany
Beckman Coulter GmbH	Krefeld	Germany
Becton Dickinson GmbH	Heidelberg	Germany
Bilgram Chemikalien	Ostrach	Germany
Biochrom Ltd	Cambridge	United Kingdom
Bio-Rad Laboratories GmbH	Munich	Germany
Biozym Scientific GmbH	Oldendorf	Germany
Bischoff Analysentechnik und -geräte GmbH	Leonberg	Germany
BMG Labtechnologies Inc.	Durham, NC	USA
Bruker BioSpin GmbH	Rheinstetten	Germany
Canon Deutschland GmbH	Krefeld	Germany
Captair Filtersystem GmbH	Duesseldorf	Germany
Carl Roth GmbH + Co. KG	Karlsruhe	Germany
Carl Zeiss AG	Oberkochen	Germany
Christ Gefriertrocknungsanlagen GmbH	Osterode	Germany
Duchefa Biochemie B.V.	Haarlem, RV	The Netherlands
Eppendorf AG	Hamburg	Germany
Eurogentec Deutschland GmbH	Cologne	Germany

Fermentas GmbH	St.Leon-Rot	Germany
Flow Laboratories GmbH	Meckenheim	Germany
Fresenius Kabi Deutschland GmbH	Bad Homburg	Germany
Geneworx AG	Oberhaching	Germany
GFL – Gesellschaft für Labortechnik mbH	Burgwedel	Germany
Greiner Bio-One GmbH	Frickenhausen	Germany
Helmut Saur Laborbedarf	Reutlingen	Germany
Hettich AG	Baech	Germany
Hoefer, Inc.	San Francisco, CA	USA
Invitrogen GmbH	Karlsruhe	Germany
Jahnke und Kunkel GmbH + Co.KG	Staufen	Germany
Kendro Laboratory Products GmbH	Langenselbold	Germany
Lehle Seeds	Round Rock, TX	USA
Machery-Nagel GmbH & Co. KG	Dueren	Germany
Memmert GmbH + Co.KG	Schwabach	Germany
Merck KgaA	Darmstadt	Germany
Millipore GmbH	Schwalbach	Germany
MJ Research, Inc.	Waltham, MA	USA
MMM Medcenter Einrichtungen GmbH	Planegg	Germany
New Brunswick Scientific Co., Inc.	Edison, NJ	USA
Nunc GmbH & Co. KG	Wiesbaden	Germany
Operon Biotechnologies, Inc.	Huntsville, AL	USA
Owl Separation Systems	Portsmouth, NH	USA
Polaroid GmbH	Offenbach	Germany
Promega GmbH	Mannheim	Germany
Qiagen GmbH	Hilden	Germany
Roche Diagnostics GmbH	Mannheim	Germany
Sartorius AG	Goettingen	Germany
Scientific Industries	Bohemia, NY	USA
Serva Electrophoresis GmbH	Heidelberg	Germany
SG Wasseraufbereitung und Regenerierstation GmbH	Barsbuettel	Germany
Sigma-Aldrich Chemie GmbH	Taufkirchen	Germany
Stratagene Corporate	La Jolla, CA	USA
Tecan Deutschland GmbH	Crailsheim	Germany
Telechem International	Sunnyvale, CA	USA
Tuttnauer Europe b.v.	Breda, GD	The Netherlands
UniEquip GmbH	Martinsried	Germany
USB Corporation	Cleveland, OH	USA
WTW Wissenschaftlich-Technische Werkstätten GmbH	Weilheim	Germany

3 METHODS

3.1 CELLS AND PLANTS

3.1.1 CELL CULTURE

Suspension cells were generally grown in the dark on a rotary shaker at 120 rpm and 27°C. Every week two gram cells were sub-cultured with a sifter in 40 ml fresh growth medium modified after Murashige & Skoog (Murashige and Skoog, 1962) in a 200 ml cell culture flask. For *Arabidopsis thaliana* cells HaM or PS Medium were taken, whereas *Nicotiana tabacum* var. *xanthii* cells were grown in MS Medium. All experiments were performed using cells in the logarithmic growth phase, 5-6 days after sub-culturing.

3.1.2 PLANT CULTURE

For plant breeding, soil was mixed with silica sand in a ratio of 4:1 and poured in 6-well plant pots. Soil was wetted with water; *Arabidopsis* seeds were sowed with a toothpick and incubated at 4°C for 3 days in the dark to synchronize germination. *Arabidopsis* plants were grown at 22°C in growth chambers programmed for a 14 hr light and 10 hr dark cycle. Five to six weeks-old plants were used for experimentation.

3.2 LPS TREATMENT

LPS preparation

Lipopolysaccharide, (1 mg/ml) were dissolved in water containing 2.5 mM $MgCl_2$ and 1 mM $CaCl_2$, shaken three hours on a mixer (Thermomixer comfort, Eppendorf) at 1,400 rpm and stored at 4°C until further use. Lipid A and Lipoteichoic acid were prepared in water (1 mg/ml). Working concentration for all experiments was 100 µg/ml. If there is no other description, experiments were performed with LPS from an endophytic strain of *Burkholderia cepacia* (ASP B 2D) isolated from *Asparagus officinalis* or for control with buffer A containing 0.25 mM $CaCl_2$, 0.1 mM $MgCl_2$.

Cells

LPS were added as stock solution of 1 mg/ml to cell culture to get a final concentration of 100 µg/ml. A control culture was treated with buffer A which is described above.

Plants

Lower *Arabidopsis* leaves were pressure inoculated with LPS or buffer A using a 1 ml syringe without a needle. Inoculated leaves were labeled and harvested after 4 hr, 8 hr, 24 hr and 48 hr and used for *local* analysis. Upper, not inoculated leaves were harvested at 24 hr and 48 hr and used for *systemic* analysis. Plant material was stored at -80°C until RNA preparation.

3.3 DETECTION OF NO

3.3.1 MICROSCOPY

The detection of NO is best done with NO-reactive fluorescent indicators in conjunction with fluorescence microcopy. This method allows bioimaging of NO, which is suitable for real-time analysis of intracellular NO (Kojima et al., 1998; Suzuki et al., 2002). Several studies have used the fluorescent probe DAF-2 DA for direct detection of NO in live plant cells and tissue, which advice the utility of this technique for NO detection after LPS treatment in *Arabidopsis* (Foissner et al., 2000; Pedroso, Magalhaes, and Durzan, 2000; Tun, Holk, and Scherer, 2001).

3.3.1.1 *ARABIDOPSIS* CELLS

For confocal laser scanning microscopy 100 µl cell suspension were placed on a coverslip bottom dish (Becton Dickinson GmbH), treated with 100 µg/ml LPS and/or for control only with 5 µM DAF-FM DA. To depict the time course of LPS-induced NO burst a Zeiss Axiovert 100M inverted microscope equipped with a confocal laser scanner (LSM 510, Zeiss) was used and dye emissions were recorded using a 505-530 nm band pass filter. First image for time course was obtained 2 min after treatment; remaining pictures were taken every minute for a time lapse of 6 min. Photographs were processed and analyzed using the Zeiss LSM 510 software. Microscope, laser and photomultiplier settings were held constant during the course of an experiment in order to obtain comparable data.

3.3.1.2 *ARABIDOPSIS* PLANTS

To analyze NO production by fluorescence microscopy, epidermal cell layers from the abaxial surface of leaves were peeled with a forceps, placed on a microscopy slide and treated with 100 µl LPS or 100 µl buffer A and with DAF-FM DA at a final concentration of 5 µM. Images were obtained after 10 min of treatment with a fluorescence microscope (Zeiss Axioskop, Carl Zeiss) and a digital camera (PowerShot G2, Canon) under bright field and fluorescence light. Dye emissions were recorded as described above. Autofluorescence of chloroplasts was captured with a 585 nm long pass filter.

3.3.2 SPECTROFLUOROMETRIC ASSAY

3.3.2.1 *ARABIDOPSIS* CELLS

To monitor the NO accumulation in LPS-treated *Arabidopsis* cells, the DAF-fluorescence was measured with a Genios plate reader (Tecan) with usual FITC excitation and emission filters.

Cells (100 µl) were placed in a black 96well microplate (Nunc), treated with LPS and/or (for control) 2 µM DAF-FM DA. NO production was estimated by measuring fluorescence intensity every min over time (30 min). The plate was rocked before measuring for 20

sec. Fluorescence was expressed as relative fluorescence units. The values are obtained by subtraction of cells autofluorescence.

3.3.2.2 QUANTITATION OF NO

Cells were treated with LPS from diverse strains, Lipid A or LTA. The NO production was determined during the first 30 min of treatment with a multiplate reader (Tecan) as described above and expressed as NO production per minute.

3.3.2.3 INHIBITION OF NO

All inhibitors (L-NNA 1 mM and 10 mM; SoA 5 mM 10 mM) were added to the cell suspension ten minutes before LPS treatment and cells were incubated on a rotary shaker (Combishaker KL2, Carl Roth) at 120 rpm in the dark. NO production was estimated with a multiwell plate reader (Tecan) as described above during 20 min of treatment and expressed as a percentage of the maximal NO production.

3.3.2.4 *ARABIDOPSIS* PLANTS

The lower epidermis of wild-type, variant P insertion-line and atnos1 mutant leaves was peeled with a forceps and pieces of ca. 3x3 mm were placed in a black multiwell plate (Nunc). The peels were treated with 50 µl LPS or 50 µl buffer A and with 20 µl DAF-FM DA (10 mM). Fluorescence intensity was determined as described above during 60 min of treatment.

3.3.3 ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY (EPR)

Electron paramagnetic resonance spectroscopy (EPR) is a common method for direct detection of NO in biological situations (Kleschyov et al., 2000). EPR imaging is considered to be the most effective and high specific technique available for observation of NO distribution and has also been used in plants for NO detection (Fodor et al., 2001; Nagano and Yoshimura, 2002).

For Electron Paramagnetic Resonance (EPR) analysis of NO, 500 µl cells were harvested 10 min after LPS (*Ralstonia solanacearum*) or buffer A treatment and were then incubated in a 0.6 ml EPR-Buffer at 37°C for 2 min (Thermostat 5320, Eppendorf). The mixture was centrifuged (model 5415D, Eppendorf) at 13,200g for 2 min. The supernatant was added to 300 µl of fresh made [Fe(II)$(DETC)_2$]-Solution (Tsuchiya et al., 1996) and incubated for two minutes at room temperature. EPR measurements were performed on a Brucker ESP300 X-band spectrometer under following conditions: room temperature; microwave power 20 mW; modulation amplitude 3G; scan rate ~2.5G/S; time constant, 164 ms. The centre peak of the 3-line NO-Fe$(DETC)_2$ signal was extracted and used for image construction.

3.3.4 NITRIC OXIDE SYNTHASE ACTIVITY ASSAY

Arabidopsis leaves were harvested at five, ten and twenty minutes after LPS or buffer A inoculation and frozen in liquid nitrogen. All used enamel ware and centrifuge tubes were precooled in liquid nitrogen and all subsequent steps were carried out at 4°C. One gram of frozen leaves was pound together with 50 mg of polyvinylpolypyrrolidone (PVPP) in liquid nitrogen using mortal and pestle (Guo, Okamoto, and Crawford, 2003). The fine powder was resuspended in 5 ml ice-cold extraction buffer and the homogenate was centrifuged at 18,000 g and 4°C for 60 minutes (model J2-21, Beckman Coulter). The supernatant was used to measure NOS activity with the NOS assay kit from Sigma. The reaction cocktail contained 20 µl 2X reaction buffer, 5 µl $CaCl_2$ (6 mM), 5 µl NADPH (10 mM), 4 µl Calmodulin (5000 U/ml), 10 µl leave extract, for inhibitor experiments 5 µl L-NMMA (250 µM) and 1 µl $_L$-[U-^{14}C]Arginine monohydrochloride (50 µCi/ml). After incubation at 37°C for 30 min (Thermostat 5320, Eppendorf), the reaction was terminated by adding 400 µl stop-buffer. Unreacted [^{14}C]Arginine was bound with 100 µl resin and removed with a spin cup by centrifugation at full speed for 30 s (model 5415D, Eppendorf). The flow through (600 µl) was transferred in 6 ml scintillation fluid and the radioactivity was quantitated in a liquid scintillation counter (LS 6000, Beckmann Coulter). The NOS activity was presented as pmol per mg fresh weight and minute.

3.4 DETECTION OF ROS PRODUCTION IN SUSPENSION CELLS

To analyze ROS production by fluorescence microscopy, tobacco or *Arabidopsis* suspension cells were incubated in 2',7'-dihydrodichlorofluoresceindiacetate (H_2DCF-DA) at a final concentration of 10 µM (added from a 10 mM stock in DMSO) for 10 min. Subsequently, the cells were transferred to a microscope slide. The slides were placed under the microscope (Zeiss Axioskop, equipped with standard FITC emission filters), and treated with LPS (100 µg/ml. Fluorescence pictures were taken with a Canon Powershot G2 digital camera (Gerber et al., 2004).

3.5 SCREENING OF DNA INSERTION LINES

The insertion line SALK_110091 should be investigated of homozygote insertion to get a plant with disturbed function of the varP gene (At4g33010). In this time, the varP gene was known as an pathogen inducible NOS in *Arabidopsis* and tobacco (Chandok *et al.*, 2003; Chandok *et al.*, 2004).

3.5.1 DNA ISOLATION WITH THE CTAB METHOD

This method (Murray and Thompson, 1980) was used for DNA extraction necessary for screening of insertion lines from SALK institute. Therefore a young small leave was grinded in a precooled reaction tube with a pipette tip and 250 µl of 2x CTAB-buffer were

added. After incubation for 15 to 30 min in a 65°C warmed shaking water bath (Unitherm with Unitwist, UniEquib), sample was mixed with 200 µl Chloroform by vortexing, followed by phase separation through centrifugation for 5 min at 14,000 rpm and 4°C (Heraeus Biofuge, Kendro). The supernatant was mixed with 600 µl absolute Ethanol and incubated for 20 min at -20°C. The DNA was precipitated by centrifugation for 15 min at 13,000 rpm and 4°C. The DNA pellet was washed with 70% Ethanol and after an additional centrifugation step and air drying for 5min, DNA was dissolved in 100 µl TE buffer and stored at -20°C for further use. For PCR an aliquot of 1 to 3 µl DNA was used.

3.5.2 DETECTION OF INSERTIONS WITH PCR

For this kind of PCR (Geelen et al., 2000; Javot et al., 2003), primers are necessary which span the possible insertion of the target gene to distinguish between wild-type, heterozygote and homozygote plants. The insertion sequence and position was obtained on website from SALK-Institute: HTTP://SIGNAL.SALK.EDU/CGI-BIN/TDNAEXPRESS. Primers were designed using the Primer3 Input software (HTTP://FRODO.WI.MIT.EDU/CGI-BIN/PRIMER3/PRIMER3_WWW.CGI). Therefore, whole sequence of interesting gene was aligned to all *Arabidopsis* coding sequences plus introns on following website: HTTP://MIPS.GSF.DE/PROJ/THAL/DB/SEARCH/SEARCH_FRAME.HTML. Parts of sequence with weak similarity to other genes before and after insertion were selected and applied for primer design. Forward and reverse primer have an approximately length of 20 bases, a melting temperature of 50 to 60 degrees and if possible no self complementary. An additionally primer directly located on insertion sequence was also used. Following primers were designed for target gene:

Primer	Wild-type screen	Mutant screen
Forward	aaccactgtgagtctcctttgc	gagaatgaaaggagtctaattttcc
Reverse	agaaaacaaacaaccaccaacg	ggttcacgtagtgggccatc (located on insertion)

Table 3-1: *Designed primer for the screen of the insertion line SALK_110091.*

Every sample was prepared for PCR as follows:

PCR buffer (10x)	2 µl
dNTP mix (10 mM each)	1.5 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq-DNA polymerase	0.5 µl
Template DNA	2 µl
Water (Ampuwa®)	up to 20 µl

DNA was amplified using the following cycling program:

Step	Temperature	Duration	Cycles
Initial denaturation	94°C	2:30 min	1
Denaturation	94°C	1 min	
Annealing	57°C	30 s	35
Extension	72°C	2:30 min	
Final extension	72°C	7 min	1
Storage	4°C	forever	1

Table 3-2: *Cycling program for the screen of the insertion line SALK_110091.*

3.5.3 ANALYSIS OF PCR PRODUCTS IN AGAROSE GELS

After PCR, products were analyzed in Agarose gels to see if a plant was homozygote. In wild-type plants a fragment is visible only after wild-type screen and in homozygote plants only after mutant screen, whereas heterozygote plants have both fragments.
DNA and RNA fragments can be separated in electric fields owing to its negatively charged phosphate residues. For a 1% DNA Agarose gel, 1.5g Agarose together with 150 ml 1x TAE-buffer was boiled for 3 to 5 min until the Agarose was completely dissolved. After the solution was cooled down to 60°C at RT, 5 µl of Ethidium bromide stock (10mg/ml) was added and mixture was transferred in a primed gel tray (Model B2 EasyCast, Owl) and an adequate comb was inserted. The gel was allowed to set and was then put in an electrophoresis chamber filled with 1x TAE-buffer. Gel was charged with whole amplified DNA samples, which were mixed with 4 µl 5x DNA-loading-buffer. For identifying fragment sizes, a 1 kb DNA ladder was loaded. Gel run was performed at 70 to 90 V for 1 to 2 hr. DNA was visualized with a UV-Transilluminator (IL-350M, Bachofer) at 302 nm and gel was photographed with a black-white Polaroid camera (Model DS34, Polaroid).

3.6 DNA-MICROARRAY

3.6.1 RNA-ISOLATION WITH THE TRIZOL METHOD

By the work with RNA the wearing of gloves and the cleaning of all used laboratory equipment with RNaseZAP is necessary to avoid RNase contamination and RNA degradation. For RNA isolation, plant material has pounded with mortal and pistil in liquid nitrogen, cells can be used as harvested. After that, 100 mg cells or pounded leaves were homogenized by vortexing with 1 ml TRIZOL® reagent or Tri-reagent and incubated for 5 min at room temperature. After the addition of 200 µl Chloroform, the tubes were shaken vigorously by hand for 15 s and incubated for 2 to 3 min at RT. Following centrifugation for 15 min, at 4°C and 14,000 rpm (Heraeus Biofuge, Kendro) the mixture separates into

a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase (~ 500 µl) was transferred in a fresh tube and mixed with 500 µl Isopropanol for RNA precipitation. Samples were incubated at RT for 10 min, followed by centrifugation for 10 min at 4°C and 14,000 rpm. RNA forms a gel-like pellet at the side and bottom of the tube. The supernatant was removed and pellet was washed with 1 ml of 75% Ethanol by vortexing samples and centrifuging probes for 5 min at 4°C. After discarding the supernatant, RNA-pellets were air-dried for 5 to 10 min. Probes were dissolved in 50 µl DEPC-treated ddH_2O and incubated for 10 min at 57 °C (Thermomixer comfort, Eppendorf). RNA was quantified by measuring 70 µl of a 500 fold dilution in a spectrophotometer at 260 nm (Ultrospec 3100pro, Amersham) and calculating concentration at follows:

$$C_{RNA\ [\mu g/ml]} = OD_{260nm} \times 40 \times DF$$

To determine RNA purification grade, the quotient of OD_{260}/OD_{280} was calculated and should be between 1.8 and 2.0 ideally. Values below indicate contamination of samples with either phenol and/or protein, whereas values above may indicate carbohydrates. OD ratio values A_{260}/A_{230} less than 2.0 may indicate sample impurity with polysaccharides.

3.6.2 FLUORESCENT PROBES

The given below protocol (Hasseman, 2001) of producing fluorescent probes was used for every kind of microarray.

3.6.2.1 cDNA SYNTHESIS

Probes were prepared by labeling of isolated RNA with aminoallyl labeled nucleotides via first strand cDNA synthesis followed by a coupling of aminoallyl groups either by Cyanine three or five (Cy3/Cy5) fluorescent molecules. Therefore, 20 µg of total RNA isolated with the TRIZOL method were used. Volume about 12 µl was evaporated, sample was mixed with 4 µl Oligo dT primer (0.5 µg/µl), incubated at 70°C for 10 min and snap-frozen in ice/water bath for 30 s. Following components were added per sample:

5x First strand buffer	6	µl
0.1 M DTT	3	µl
10x aminoallyl dNTP mix	3	µl
Superscript II RT (200 µl)	2	µl
RNaseOUT	1	µl

After incubation at 42°C over night (PTC-100 Peltier Thermal Cycler, MJ Research), RNA was hydrolyzed with the addition of 10 µl 1M NaOH and 10 µl 0.5 M EDTA and incubation at 65°C for 15 min.

3.6.2.2 PURIFICATION STEP I

The cDNA was purified of unincorporated aminoallyl dUTP and free amins using the Qiagen PCR Purification Kit. Sample was mixed with 300 µl buffer PB and transferred to a Qiaquick column. Column was placed in 2 ml collection tube and centrifuged at 13,000 rpm for 1 min (Model 5415D, Eppendorf). After every centrifugation step, the collection tube was emptied. For washing, 750 µl Phosphate wash buffer were added on the column and centrifuged at 13,000 rpm for 1 min. The wash step was repeated once and the column was centrifuged an additional minute at maximum speed. Column was transferred in a new 1.5 ml reaction tube and 30 µl Phosphate elution buffer were carefully added to the center of column membrane. After incubation of 1 min, the cDNA was eluted by centrifugation at 13,000 rpm for 1 min. This elution step was repeated once to get 60 µl purified cDNA.

3.6.2.3 DYE COUPLING

After drying sample in a speedvac (Univapo 150W, UniEquip), aminoallyl cDNA could be coupled to Cy Dye esters. Therefore, cDNA was resuspended in 4.5 µl 0.1 M Sodium Carbonate buffer and 4.5 µl of the appropriated NHS ester Cy Dyes were added. To avoid photobleeching of the Cy Dyes all reaction tubes were protected from light as much as possible. The reaction was incubated for 1 h in the dark with vortexing and spinning down every 15 min.

3.6.2.4 PURIFICATION STEP II

The uncoupled dye was removed by using the Qiagen PCR Purification Kit as follows. To the reaction 35 µl 100 mM NaOAc and 250 µl buffer PB were added. Sample was loaded on a spin column and centrifuged at 13,000 rpm for 1 min. cDNA was washed with 750 µl buffer PE and centrifugation at 13,000 rpm for 1 min. Column was dried with an additional centrifugation step at maximum speed for 1 min. Column was placed in a clean 1.5 ml reaction tube and 30 µl buffer EB was carefully added to the center of column membrane. After incubation at room temperature for 1 min, cDNA was eluted by centrifugation at 13,000 rpm for 1 min. This elution step was repeated once to get 60 µl of purified and labeled cDNA. Probes were stored at -80°C in the dark for further use.

3.6.3 IN-HOUSE ARRAYS

3.6.3.1 MICROARRAY PREPARATION

For the production (Huang, von Rad, and Durner, 2002) of cDNA microarrays, cDNA inserts of EST clones were amplified by PCR. Therefore, selected clones were transferred in 96 well plates containing 100 µl LB-glycerol medium with Ampicillin (100µg/ ml), plates were covered with AirPore Tape Sheets and incubated for 16 hours at 37°C. Plasmid DNAs were isolated using the Qiagen R.E.A.L. Prep 96 Plasmid Kit according the manufactures instructions. The 50-fold diluted plasmid DNA was amplified in 96 well plates (ABgene) with following M13 primers with a C6 amino modification to the 5' end:

Forward primer: 5'- GTA AAA CGA CGG CCA GT -3'
Reverse primer: 5'- GGA AAC AGC TAT GAC CAT G- 3'

Following components were added per sample:

PCR buffer (10x)	10 µl
dNTP mix (10 mM each)	3 µl
Forward primer	3 µl
Reverse primer	3 µl
Taq-DNA polymerase	0.5 µl
Template DNA	2 µl
Water (Ampuwa)	up to 100 µl

DNA was amplified using the following cycling program:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	
Annealing	52°C	30 s	30
Extension	72°C	2 min	
Final extension	72°C	5 min	1
Storage	4°C	forever	1

Table 3-3: *Cycling program for the amplification of cDNA for in-house microarrays.*

For efficient binding of the amplified clone inserts to the slides, it is essential to remove unincorporated nucleotides and primers from the reaction products. For purifying of cDNA 200 µl PCR products were loaded on 96 well multiscreen filter plates (Millipore). Plates were filtered with a Millipore vacuum manifold filtration system at a pressure of 15in (380 mM) Hg for 10 minutes or until the plates are dry. Each well was washed with 50µl

ddH_2O, this step was repeated once. The cDNA was resuspended in 50 µl ddH_2O by shaking vigorously for 10 min on a rotary shaker (Combishaker KL2, Carl Roth). The purified PCR products were transferred into a new 96 well plate, sealed with a Qiagen Tape Pads and stored at 4°C for future arraying. After lyophylization of 50 µl of purified amino-modified PCR products in a freeze-dryer (Alpha 1/5, Christ), the pellet was dissolved in 20 µl spotting solution. Using betaine can not only reduce evaporation, but improve the binding efficiency and the homogeneity of spotted DNA (Diehl et al., 2001). PCR products were arrayed from 384-well microarray plates onto silylated microscope slides (Telechem) using a MicroGrid DNA arraying robot (Biorobotics Microgrid II System, Geneworx) and corresponding software (TAS Application Suite, Geneworx). Thereafter printing slides are allowed to dry overnight in a slide box, because drying increases crosslinking efficiency. After drying unreacted aldehyde groups were blocked to reduce non-specific binding of labeled reactants (Schena et al., 1996). The printed slides were first rinsed twice in 0.1% SDS and then twice in ddH_2O for 2 min at room temperature with vigorous agitation to remove unbound DNA. To reduce free aldehyde groups, slides were washed in a fresh prepared Sodium-Borohydride-Solution for 5 min. DNA was denatured by transferring the slides into boiling ddH_2O for 2 min. The slides were then first rinsed twice for 1 min in 0.1% SDS and then twice for 1 min in ddH_2O.Finally, arrays were soaked briefly in Ethanol, air dried and stored at 4°C to further use.

3.6.3.2 HYBRIDIZATION AND SCANNING

Following reverse transcription, labeling and purification steps, the Cy3- and Cy5-labeled probes were hybridized to self-made microarrays. Therefore, arrayed cDNA were pre-hybridized with 80 µl pre-Hyb-Buffer for 45 min at 42°C under a coverslip. Slides were then washed thoroughly with ddH_2O and dried by centrifugation (Universal, Hettich). Meanwhile, the labeled probes were dried into speedvac (Univapo 150W, UniEquip) and each sample was dissolved in 100 µl Hybridization-Buffer. Probes were denatured at 95°C for 5 min (Thermomixer comfort, Eppendorf) and put on ice. Hybridization was performed using 20x21 mm^2 gene frames and hybridization chambers (Hybchamber Genemachines, Geneworx) at 42°C overnight. Subsequently, the chambers were opened in 3x SSC, arrays were washed for 5 min at low stringency (1x SSC), then for 5 min in 0.5x SSC, then briefly in 0.5x SSC, and finally for 15 sec in high-stringency wash buffer (0.1x SSC). Arrays were air-dried and scanned using a Microarray Scanner (GenePix 4000A, Biozym). To identify differentially expressed genes the GenePix Pro 4.1 software (Biozym) were used. Background fluorescence was calculated as the mean fluorescence signal of nontarget pixels around each gene spot. Less than a 2-fold difference between background and signal resulted in elimination of the corresponding spot. Induction or repression of a gene was defined as a minimum 1.5-fold change in its transcript level.

3.6.4 AGILENT I ARRAYS

Agilent ready-to-use *Arabidopsis* 1 Microarrays contains 16,000 of sequence verified 60-mer length oligonucleotide probes specific for genome wide expression profiling.

Fluorescent probes of LPS treated cells and of control cells were combined, dried in a speedvac (Univapo 150W, UniEquip) and resuspended in 93.75 µl of nuclease-free water. The hybridization mixture was prepared by adding 2.5 µl Deposition Control Targets and 3.75 µl Human Cot-1 DNA to the samples. The volume was brought to 200 µl by adding 100 µl 2x Deposition Hybridization Buffer. Samples were mixed by vortexing, incubated at 98°C for 2 min to denature cDNA (Thermomixer comfort, Eppendorf) and spun down at 14,000 rpm for 5 min (model 5415D, Eppendorf). Hybridization chamber (Legacy 6-Screw Chamber, Agilent) was prepared as follows before loading the samples. An array slide, which contains two microarrays, was placed with the "Agilent" labeled barcode facing up into the base. The slide was covered with a plastic backing and a gasket. On top of it came the small stainless steel cover, which was fixed with six screws. On each of the four ports a rubber septum was placed and pushed tightly into the ports using a flat metal edge. For each microarray, a 25-gauge needle was inserted into the opening of one of the septa installed in the chamber. The entire amount of one tube was slowly drawn up in a 1 ml syringe with a needle. The solution was slowly injected into the septum that doesn't have the needle in it. The needles were removed from the septa. These steps were repeated for the other microarray on the slide. Bubbles, which did form during loading, have to rotate freely in the hybridization chamber. The chamber was placed on rotator in a 60°C warmed hybridization oven (Hybrid 2000, Saur Laborbedarf) and was incubated over night with constant rotating for approximately 17 hr. Thereafter, hybridization chamber was placed in a dish containing wash solution I and opened. Slides were carefully placed in a slide rack in a second dish containing wash solution I and a stir bar. When all slides were submerged in wash solution I in the slide rack, they were washed with stirring for 5 min at moderate speed. The slide rack was transferred to a dish containing wash solution II and it was stirred for 2 min at moderate speed. Slides were dried by centrifugation for 2 min at 400 x g (model 4K15C, Sigma). Dried slides were scanned with a microarray scanner (GenePix 4000A, Biozym) and analyzed with GenePix Pro 4.1 and Acuity 4.0 (both Biozym).

3.6.5 AGILENT III ARRAYS

Dried fluorescent probes were resuspended in 200 µl of nuclease-free water. The cDNA was heat denatured by 98°C for 3 min (Thermomixer comfort, Eppendorf) and cooled to room temperature. The cDNA hybridization solution was prepared by adding 50 µl 10x Control targets and 250 µl 2x hybridization buffer to get a volume of 500 µl. Samples were mixed by careful pipetting up and down to avoid bubbles and spun briefly (model 5415D, Eppendorf). Hybridization chamber (Surehyb, Agilent) was prepared as follows

before loading the samples. A gasket slide was placed into chamber base with the label reading "Agilent" facing up. The hybridization mixture was slowly dispensed on gasket slide surface with pipette. A microarray with numeric barcode side facing up was carefully lowered on top of the gasket slide to get "sandwiched slides". The chamber cover was placed slipped with clamp assembly and fixed with a thumbscrew. Chambers were placed on rotator of a 60°C pre-heated hybridization over (Hybrid 2000, Saur Laborbedarf) and incubated for 17 hr. After hybridization the thumbscrew of the chamber was loosen and the chamber cover was removed. The "sandwiched slides" were placed in a dish containing wash solution I. The microarray was then quickly transferred to a second dish with wash solution I containing a slide rack and a stir bar. When all slides were collected in slide rack, microarrays were washed with constant stirring for 1 min. Slide rack was placed in a new dish with wash solution II and washed for 1 min. Slides were quickly transferred to wash solution III, an ozone scavenger containing Stabilization and Drying Solution, and washed for 30 s. Thereafter, the slide rack was removed very slowly and at constant speed during magnetic stirring. The dried slides were ready to be scanned in a microarray scanner.

3.7 NORTHERN BLOTS

3.7.1 ANALYSIS OF RNA IN AGAROSE GELS

To avoid RNase contamination, all used equipment was cleaned with RNaseZAP. RNA was analyzed in denaturing formaldehyde gels and an amount of 10 µg RNA isolated with the TRIZOL® method was used for Northern Blots. Therefore, 1.8 g Agarose was boiled together with 131 ml autoclaved ddH_2O for 3 to 5 min until the Agarose was completely dissolved. After the solution was cooled down to 60°C at RT, 15 ml 10x MOPS, 4.5 ml 37% Formaldehyde and 5 µl Ethidium bromide (10 mg/ml) were added. Mixture was transferred in gel tray, an adequate comb was inserted and gel was allowed to set. RNA samples were prepared at follows: all RNA samples were brought up to the same volume with DEPC-ddH_2O. The samples were mixed with the 0.8 fold volume of RNA-Loading-buffer incubated for 10 min at 65°C (Thermomixer comfort, Eppendorf) and transferred on ice. Gel were put in an electrophoresis chamber filled with 1x MOPS and samples were loaded. Electrophoresis was performed for 2 hr at 70 to 80 V. RNA was visualized with a UV-Transilluminator at 302 nm (IL-350M, Bachofer) and gel was photographed with a black-white Polaroid camera (model DS34).

3.7.2 GEL BLOT BY CAPILLARY TRANSFER

The gel was washed with 20x SSC for 20 min at gentle agitating on an orbital shaker (Combishaker KL2, Carl Roth) after run to remove formaldehyde. The RNA was blotted on a positively charged nylon membrane through capillary forces. Therefore, a blotting chamber was filled with 20x SSC, the first two whatman papers (from the bottom) have

to touch the buffer on both sides of the table. On these papers came the gel top-side down and was covered with nylon membrane, two Whatman papers and 15 to 20 cm paper towel. All covering papers and the membrane were cut at gel like size. Four pieces of parafilm were placed, on each side of the gel, to block buffer from transferring "outside" of gel. The paper towels were weight with one or two kilogram and blot was performed over night. On next day membrane was air dried; RNA was fixed with cross-linking in an UV crosslinker (Stratalinker, Stratagene) and membrane was shrink-wrapped in plastic foil for store at 4°C until further use.

3.7.3 PROBE LABELING AND HYBRIDIZATION

For the production of Northern probes, the PCR DIG Probe Synthesis Kit from Roche was used. Thus, the direct labeling of DNA fragments with digoxigenin (DIG) by PCR was possible. During this process highly sensitive probes were synthesized by incorporation of DIG-dUTP into the PCR product. The EST clones corresponding to At2g14610 (PR1), At3g57260 (PR2), At3g12500 (PR3), At3g04720 (PR4) and At1g75040 (PR5) served as templates for PCR. Samples were prepared as follows:

Reagent	Dig labeled probe	unlabeled DNA control
PCR buffer with $MgCl_2$ (10x)	5 µl	5 µl
PCR DIG labeling mix	5 µl	-
dNTP stock	-	5 µl
M13 Forward primer (10 µM)	1.5 µl	1.5 µl
M13 Reverse primer (10 µM)	1.5 µl	1.5 µl
Enzyme mix	0.75 µl	0.75 µl
Template	1 µl	1 µl
Water (Ampuwa®)	Up to 50 µl	Up to 50 µl

Table 3-4: *Preparation protocol for dig-labeled northern probes.*

PCR was performed under following conditions:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	30 s	35
Annealing	54°C	30 s	
Extension	72°C	1:30 min	
Final extension	72°C	5 min	1
Storage	4°C	forever	1

Table 3-5: *PCR conditions for the amplification of dig-labeled northern probes.*

To check the success of reaction, 5 µl probes were analyzed in a DNA agarose gel. The presence of DIG in DNA makes it run slower than unlabeled control. The probes were stored at -20°C until further use.
For hybridization, membrane in plastic foil was first incubated at 50°C for 30 min in 10 ml DIG Easy Hyb buffer on shaking water bath (Unitherm with Unitwist, UniEquib) followed by the addition of 3 µl heat denatured (95°C, 10 min) probe diluted in 1 ml DIG Easy Hyb buffer.

3.7.4 WASHING AND DETECTION

All washing steps were performed at room temperature with agitation (Combishaker KL2, Carl Roth). After hybridization over night, membrane was washed twice with 2x SSC, 0.1% SDS for 5 min, then twice with 0.1x SSC, 0.1% SDS at 50°C for 15 min and for 2 min in Washing buffer, followed by blocking for 30 min in 100 ml Blocking solution. After incubation for 30 min in Antibody solution membrane was washed twice for 15 min in Washing buffer and equilibrated for 3 min in Detection buffer. Membrane was placed RNA/DNA side face-up on plastic foil and covered with any drops of CSPD. The incubation was performed first for 5 min at room temperature and then for 10 min at 37°C, whereas the foil was sealed to prevent membrane desiccation. Finally membrane was exposed to luminescence detection film for 1 to 3 hr, which was developed using an automatic film processor (Curix 60, Agfa).

3.8 REAL-TIME PCR

Real-time PCR is used to measure accurately the different amounts of a target gene product present in independent samples. In this case of real-time RT-PCR, the samples are cDNA previously reverse transcribed from RNA preparations.

3.8.1 RNA-ISOLATION WITH THE RESIN METHOD

This protocol (Kiefer, Heller, and Ernst, 2000) describes a rapid RNA isolation method from plant and tissues rich in polyphenolics and polysaccharides without the use of phenol and the RNA is of sufficient quality for use in RT-PCR reactions.
Between 100 to 300 mg pounded leave material was mixed with 1 ml prewarmed (65°C) RNA-extraction-buffer and incubated at 65°C for 10 min (Unitherm HB together with Unitwist, UniEquib). After the addition of 500 µl chilled (-20°C) Chloroform, and 100 µl of Nucleon PhytoPure DNA extraction resin, samples were mixed by vortexing for 10 min at room temperature and 1,400 rpm (Thermomixer comfort, Eppendorf). After centrifugation for 10 min at 14,000 rpm and 4°C (Heraeus Biofuge, Kendro) supernatant was mixed with 500 µl Chloroform and centrifugation was repeated. RNA was precipitated by incubation of the supernatant with 0.5 volume of Isopropanol on ice for 15 min and centrifugation at fullspeed and 4°C for 10 min. The pellet was dissolved in 45 µl DNase-I-

buffer and incubated at 37°C for 20 min after mixing with 5 µl DNase I to digest genomic DNA. RNA was obtained by the addition of 2 volumes Ethanol and centrifugation. Pellet was washed with 1 ml 70% Ethanol and dissolved in 50 µl DEPC-treated water. Amounts and purity of RNA was determined as described above.

3.8.2 cDNA SYNTHESIS

To 5 µg RNA 1 µl oligo dT-primer and 1 µl dNTP mix (10 mM each) were added and the volume was brought up to 12 µl with water (Ampuwa®). After incubation at 70°C for 10 min following components were added per sample:

5x First strand buffer	4	µl
0.1 M DTT	2	µl
Superscript II RT (200 U/µl)	1	µl
RNaseOUT	1	µl

Synthesis of cDNA was performed at 42°C for 50 to 60 min followed by enzyme inactivation at 70°C for 15 min (PTC-200 Peltier Thermal Cycler, MJ Research). RNA was digested by 1 µl RNase H and 8 µl RNase A at 37°C for 20 min.

3.8.3 QUANTIZATION OF cDNA

Concentration of cDNA was determined using the Quant-iT™ RiboGreen® RNA Reagent, which is an ultra sensitive fluorescent nucleic acid stain for quantitating RNA and single stranded DNA in solution. For measurement, cDNA were diluted 100-fold in TE-buffer and dispensed in 100 µl aliquots in a black 96-well plate (Nunc). Aliquots were mixed with 100 µl of 2000 fold diluted Ribogreen Reagent (5 µl Ribogreen in 10 ml TE-buffer). Sample fluorescence was determined after an incubation of 2 to 5 min using a fluorescence microplate reader (Fluostar, BMG) and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). Amounts of cDNA were calculated using a standard curve.

3.8.4 PRIMER DESIGN FOR RT-PCR

Primers were designed using the Primer3 Input software (HTTP://FRODO.WI.MIT.EDU/CGI-BIN/PRIMER3/PRIMER3_WWW.CGI). Therefore, spliced sequence of interesting gene was aligned to all *Arabidopsis* coding sequences on following website: HTTP://MIPS.GSF.DE/PROJ/THAL/DB/SEARCH/SEARCH_FRAME.HTML. A part of sequence with weak similarity to other genes was selected and applied for primer design. Forward and reverse primer have an approximately length of 20 bases, a melting temperature of 50 to 60 degrees, as possible no self complementary and product sizes range between 300 to 500 bases. Following primers were designed for target genes:

Gene	Acc#	Forward primer	Reverse Primer	Annealing	Cycles	cDNA
PR 1	At2g14610	TTTTACTGGCTATTCTCGAT	TACCCCAGGCTAAGTTT	49°C	30	3 ng
PR 2	At3g57260	ATGTCTGAATCAAGGAGCTTAGCC	TGGGTCAGGGCCGTAGAG	53°C	30	1 ng
PR 3	At3g12500	GCCTCCACAAAAAGAAAACC	ACAAGCGGCATCATTCCTAT	55°C	35	5 ng
PR 4	At3g04720	TTTCTATAATCCGGCGCAGA	CAATGAGATGGCCTTGTTGA	53°C	35	5 ng
PR 5	At1g75040	CTAAGGAACAATTGCCCTACC	TTAAGCATGTCGGGGCAAG	53°C	35	1 ng
Atnos1	At3g47450	CCTGGAACCACCTTGGG	GCTCTCACCCTTGGGACTAC	53°C	35	5 ng

Table 3-6: *Designed primer and conditions for real-time PCR.*

3.8.5 DETECTION OF GENE EXPRESSION WITH PCR

PCR was performed using synthesized cDNA as template and PCR conditions were optimized for every gene. Following components were mixed per PCR sample:

PCR buffer (10x)	5 µl
$MgCl_2$ (25 mM)	3 µl
dNTP mix (10 mM each)	1 µl
Forward Primer (10 mM)	1 µl
Reverse Primer (10 mM)	1 µl
Goldstar® DNA polymerase	0.2 µl
cDNA (Tab. 3-6)	x ng
Water (Ampuwa)	up to 50 µl

Gene products were amplified with following program:

Step	Temperature	Duration	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	95°C	1 min	x (Table 3-6)
Annealing	x °C (Table 3-6)	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Storage	4°C	forever	1

Table 3-7: *Cycling programs for real-time PCR.*

3.8.6 QUANTITATING OF PCR PRODUCTS

To obtain the gene expression level, amounts of PCR products were determined using the Quant-iT™ PicoGreen® dsDNA Reagent. PCR products were diluted 100-fold in TE buffer and 100 µl of diluted sample were mixed with 100 µl of diluted Picogreen reagent (50 µl Picogreen in 10 ml TE-buffer). Sample fluorescence was determined after an incubation of 2 to 5 min using a fluorescence microplate reader (Fluostar, BMG) and standard

fluorescein wavelengths (excitation ~480 nm, emission ~520 nm). Amounts of cDNA were calculated using a standard curve.

3.9 DETERMINATION OF SALICYLIC ACID (SA) LEVELS

Free and conjugated SA was determined with some modification after a standard protocol (Meuwly and Métraux, 1993).

3.9.1 SAMPLE PREPARATION

Local and systemic leaves from LPS treated and control plants were pounded in liquid nitrogen and 100 mg were vortexed in 5 ml 100% Methanol. The obtained supernatant after centrifugation by 30,000 rpm for 10 min was decanted and pellet was mixed with 4 ml 100% Methanol. After anew centrifugation supernatants were pooled and evaporated. Pellet was resuspended with 2 ml Formic acid (20%) and acidified with 20 µl 32% HCl. An amount of 5 ml Cyclohexane/Ethyl acetate mixture (1:1) was added and the sample was centrifuged at 4,000 rpm for 8 min. The upper organic phase was transferred in a new flask and step was repeated using 3 ml Cyclohexane/Ethyl acetate mixture. Pooled organic phases containing the free SA were evaporated. Water phase was acidified with 1.3 ml 32% HCl and bound SA was hydrolyzed at 80°C for 1 hr. Released SA was extracted with Cyclohexane/Ethyl acetate as described above and evaporated.

3.9.2 SA QUANTITATION

Evaporated samples were suspended in 500 µl HPLC sample buffer and centrifuged at 15,000 rpm for 10 min. SA was determined using HPLC system equipped with an auto-sampler, a RP-18 Nucleosil-Column and a fluorescence detector (exsiccation 305 nm; emission 407 nm). HPLC was performed with 20 µl sample and the following program:

Time (min)	HPLC Running Buffer
0-20	100%A
20-25	to 100% B
25-30	100% B
35-40	to 100% A
40-45	100% A

Table 3-8: *HPLC program for SA determination.*

SA content in analyzed samples was calculated from area units of the HPLC chromatogram and via SA standards as follows:

$$c\ (\text{nmol/g FW}) = DF\ 25 \times RR \times 0.0724 \times \text{weighted sample (g)}^{-1} \times \text{area of sample peak} \times \text{area of standard peak}^{-1}$$

RR, retrieval rate: free SA = 1.3; conjugated SA = 1.43

3.10 BACTERIA GROWTH ASSAY

The classic phytopathological technique for quantifying bacterial virulence is an assay measuring bacterial multiplication within the host tissue. The assay was performed in the main as described in The *Arabidopsis* Book (Katagiri, Thilmony, and He, 2002).

3.10.1 BACTERIA PREPARATION

Pseudomonas syringae DC3000 pv. *tomato* bacteria are streaked out from a –80°C glycerol stock onto a plate of King's medium B complemented with Tetracycline (10 µg/ml) and Rifampicin (50 µg/ml) and were incubated for 2 days at 30°C (Friocell 111, MMM Medcenter). One colony was transferred in 15 ml liquid King's medium B and shaken for 24 hr at 300 rpm and 30°C on an incubator shaker (Model G25, New Brunswick). The whole pre-culture came in 400 ml medium and bacteria was allowed to growth for 1 day. Bacteria were centrifuged at 2,500 g and 4°C for 10 min (Model J2-21, Beckman Coulter) and washed twice with ddH_2O and renewed centrifugation. Concentration was determined by measuring 1 ml bacteria suspension against 1 ml water at 600 nm in a Spectrophotometer (Ultrospec II, Biochrom). Usual concentrations were for bacterial inoculation OD_{600}=0.0002 which correlates 10^5 colony-forming units/ml or for bacterial spraying OD_{600}=0.4 which correlates $5*10^8$ cfu/ml, respectively. Bacteria were inoculated as a water solution or sprayed as water solution complemented with 0.2% TWEEN.

3.10.2 PLANT TREATMENT

Syringe Infection

Two days before pathogen infection plants were either treated with LPS or for control with buffer A to induce a possible resistance. Three systemic leaves per plant were marked and pressure infiltrated with bacteria suspension using a 1 ml needleless syringe from the abaxial side.

Spraying Infection

This kind of infection was used to compare the susceptibility against pathogens of the wild-type plants and the atnos1 mutant. Three leaves per plant were marked and sprayed on all sides until there was imminent runoff. A normal spray bottle with the nozzle set to spray a fine mist was used.

3.10.3 BACTERIA COUNTING

Leaves were harvested and surface sterilized as follows: Whole leaves were removed from the host plant and placed in a 70% ethanol solution for 1 minute by gently mixing in the solution occasionally and dried on paper towels. The leaves were then rinsed in sterile distilled water for 1 minute and dried again. Leaf disks were excised from 3 leaves

per plant with a 0.5 ml Eppendorf tube, grounded with 1 ml ddH_2O using a mortal and a pestle and were then transferred in a sterile 1.5 ml Eppendorf tube. The samples were thoroughly vortexed to evenly distribute the bacteria within the water/tissue sample. A 100-µl sample was removed and diluted in 900 µl sterile distilled water. A serial 1:10 dilution series was created for each sample by repeating this process until a dilution of 10^5 was reached. Aliquots of 10 µl are dropped on solid King's medium B using square plates with 36 squares. Plates were incubated for 4 days at 30°C (Friocell 111, MMM Medcenter) until colonies became countable.

3.11 LPS MOBILIZATION

Fluorescent conjugates of LPS will help to follow LPS binding and transport processes. For example, in one study a BODIPY® FL derivative of LPS from *E. coli* strain LCD25 was used to measure the transfer rate of LPS from monocytes to high-density lipoprotein (HDL) (Kitchens et al., 1999). Another scientists utilized a BODIPY® FL derivative of LPS from *Salmonella minnesota* to demonstrate transport to the Golgi apparatus in neutrophils (Thieblemont and Wright, 1999; Vasselon et al., 1999). And a recently appeared work showed the endocytosis of fluorescent-labeled LPS in tobacco cells (Gross et al., 2005).

3.11.1 FLUORESCENCE MICROSCOPY

The lipopolysaccharides from *S. minnesota*, Alexa Fluor® 488 conjugate were used to investigate the LPS mobilization in *Arabidopsis* leaves. Therefore, 100 µg of lyophilized LPS were dissolved in 1 ml ddH_2O and incubated for 10 min at 37°C and 1,400 rpm (Thermomixer comfort, Eppendorf). Labeled leaves were pressure inoculated with a needleless syringe from the abaxial side and analyzed with a fluorescence microscope and a digital camera at indicated time-points.

3.11.2 CAPILLARY ELECTROPHORESIS (CE)

Capillary electrophoresis is an electrophoresis method performed in a capillary tube. It is the most efficient separation technique available for the analysis of both large and small molecules. The basis instrumental set-up consists of a high voltage power supply, a fused silica capillary, two buffer reservoirs, two electrodes and an on-column detector. Sample injection is accomplished by temporarily replacing one of the buffer reservoirs with a sample vial. A specific amount of sample is introduced by controlling either the injection voltage or injection pressure (Xu, 1996).

This method was used for the detection and analysis of fluorescence labeled LPS from *Salmonella minnesota* in direct treated *Arabidopsis* leaves and in systemic leaves.

3.11.2.1 SAMPLE PREPARATION

Leaves were pressure infiltrated with fluorescent-labeled LPS from *S. minnesota* as described above and incubated in the dark. After 1 hr, 6 hr and 24 hr 5-6 leaves per time-point were harvested and the middle-rips of direct treated leaves were cut out, whereas systemic leaves were used as a whole. Veins or whole leaves were pounded in liquid nitrogen using a mortal and pistil. Fine powder was dissolved in a concentration of 2 mg fresh-weight per µl ddH_2O. Mixture was incubated for 10 min at 37°C and 1,400 rpm (Thermomixer comfort, Eppendorf) followed by centrifugation at 13,200 rpm for 5 min (model 5415, Eppendorf). Supernatant was centrifuged again to remove all solid particles and stored at -80°C in the dark until use.

3.11.2.2 CAPILLARY ZONE ELECTROPHORESIS (CZE)

This method is the simplest form of CE. The sample is applied as a narrow zone, which is surrounded by separation buffer. As an electric field is applied, each component in the sample zone migrates according to its own apparent mobility.

CE measurements were performed with a Beckman P/ACE 5510 CE system, equipped with a fluorescence detector (exsiccation 488 nm; emission 520 nm), an auto-sampler, and a power supply. For data acquisition served a computer with corresponding software (Gold Software Version 8.10). An uncoated fused-silica capillary (75 µm ID, 375 µm OD, 50 cm length to detector, total length 57 cm), liquid cooled and filled with adequate buffer was used for separation. Capillary was washed before and between each run first with 0.1 M NaOH for 5 min and then with ddH_2O for 2 min. Finally, capillary was filled with Separation-Buffer, which was changed after every run. Samples were automatically applied by hydrodynamic injection for 2 to 5 sec and separation was performed at 32°C and 25 kV for 6 to 7 min.

3.12 ANALYSIS OF LPS IN SDS GELS

The separation of LPS is best done in SDS polyacrylamide gels, during which the heterogenous mixture of polymers separates into a characteristic ladder pattern (Palva and Makela, 1980).

This method was used for another possibility of detection of LPS mobilization in *Arabidopsis* leaves.

3.12.1 SAMPLE PREPARATION

Leaves were pressure inoculated with LPS from *B. cepacia* at a concentration of 2 mg/ml to come over the detection limit. Treated leaves were harvested after 1 hr, 6 hr and 24 hr. Samples of leave middle-rips were prepared as described above and stored at -20°C until use.

3.12.2 CASTING OF LPS-RESOLVING GELS

Gels were produced using a multiple gel caster (Model SE215, Hoefer), which was prepared by washing all components with a mild detergent and rinsing with ddH_2O. After placing the silicon rubber gasket and the filler plugs into the acrylic casting chamber, gel sandwiches were constructed. For each sandwich a notched aluminum plate, two spacers and one rectangular glass plate were used. One wax paper sheet came first in the casting chamber followed by a gel sandwich; this was repeated with all sandwiches. The face plate was laid on the gel caster and fixed with two spring clamps on each side. Separation gel solution was filled until the level of the notched plate and was overlaid with Ethanol. After solution had polymerized gel was finished by casting the stacking gel and inserting the combs. Gels were stored at 4°C in 1x Gel buffer wetted paper towels.

3.12.3 GEL ELECTROPHORESIS

For running LPS gels a Hoefer SE 250 small format vertical slab gel unit was used. The cast gel was set in the bottom of the lower chamber and the plate was centered so that the gasket sealed both sides. Gel was fixed with on spring clamp on each side so that the upper buffer chamber was formed, which was filled with Cathode Buffer. The Anode Buffer was used for the lower buffer chamber. Before loading the gel, 10 µl of LPS preparations were mixed with 2 µl of 5x Loading Buffer and heat denature at 100°C for 5 min (Thermomixer comfort, Eppendorf). Electrophoresis was done at 12 mA in the stacking gel and 25 mA in the separating gel until the dye had run about 10 cm.

3.12.4 GEL STAINING

3.12.4.1 STAINING WITH PRO-Q® EMERALD 300

The PRO-Q Emerald 300 dye reacts with periodate-oxidized carbohydrate groups, creating a bright green-fluorescent signal.

After LPS was separated by standard SDS-polyacrylamide gel electrophoresis, gel was immersed in 100 ml Fix Solution for 45 min. This step was repeated once and the gel was washed two times with 100 ml Wash Solution for 10 min. The carbohydrates were then oxidized with 25 ml Oxidizing Solution for 30 min. After washing the gel three times with 100 ml Wash Solution for 10 min, gel was stained in fresh prepared PRO-Q Emerald 300 Staining Solution for 2 hr. PRO-Q Emerald stain was visualized using a 300 nm UV transilluminator (IL-350M, Bachofer) and gel was photographed with a CCD camera (model DS34, Polaroid).

3.12.4.2 STAINING WITH SYPRO® RUBY PROTEIN GEL STAIN

The SYPRO Ruby protein gel stain is a ready-to-use, ultrasensitive, luminescent dye for detection of proteins separated by polyacrylamide gel electrophoresis. This dye could be used for detection of proteins after staining the LPS gel with PRO-Q Emerald. Therefore,

gel was incubated in 50 ml SYPRO Ruby protein gel stain over night with gentle agitation on an orbital shaker (Combishaker KL2, Carl Roth). Proteins were visualized using a 300 nm UV transilluminator (IL-350M, Bachofer) and documented with a CCD camera (model DS34, Polaroid).

3.12.4.3 SILVER STAINING

After staining the LPS gel with PRO-Q Emerald and SYPRO Ruby, the gel was additionally stained with silver nitrate to detect LPS and protein together.

LPS and proteins in the gel were oxidized two times in 100 ml Oxidizing Solution for 30 min. The gel was the washed three times with ddH_2O for 5 min and stained for 10 min in freshly prepared Silver Nitrate Staining Solution. After washing the gel again, the color was generated with 200 ml Developer. Reaction was stopped by exposure in 100 ml 10% Acetic Acid for 1 min followed by repeated washings in ddH_2O (Tsai and Frasch, 1982; Fomsgaard, Freudenberg, and Galanos, 1990).

4 RESULTS

4.1 LPS INDUCE A NO BURST IN *ARABIDOPSIS* CELLS

4.1.1 DETECTION OF NO BY FLUORESCENCE MICROSCOPY

Lipopolysaccharides (LPS) are strong inducers of mammalian innate immunity including NO production, and activation of iNOS by LPS is the most applied readout to analyze innate immune responses (Alexander and Rietschel, 2001). Since NO seems to be a key player in regulating plant defense response (Durner and Klessig, 1999), the potential of LPS to induce a NO burst in *Arabidopsis* suspension cells was examined. Real-time imaging of NO is best done with the fluorescent NO-indicator DAF-FM diacetate (DA) in combination with confocal laser scanning microscopy (Kojima *et al.*, 1998; Foissner *et al.*, 2000). Fig. 4-1 shows real-time imaging of NO production in *Arabidopsis* cells after loading with 5 µM DAF-FM DA and subsequent LPS treatment (100 µg/ml). Effective LPS concentrations were between 10 and 200 µg/ml, concentrations that are routinely applied by others (Meyer, Puehler, and Niehaus, 2001; Gerber et al., 2004). In order to characterize also the less active preparations shown by Fig. 4-4, a standard concentration of 100 µg/ml was used. LPS treatment resulted in a rapid burst of green fluorescence within a few minutes, indicative of NO production (lower row). Fluorescence depicted in control cells (upper row) shows basal NO.

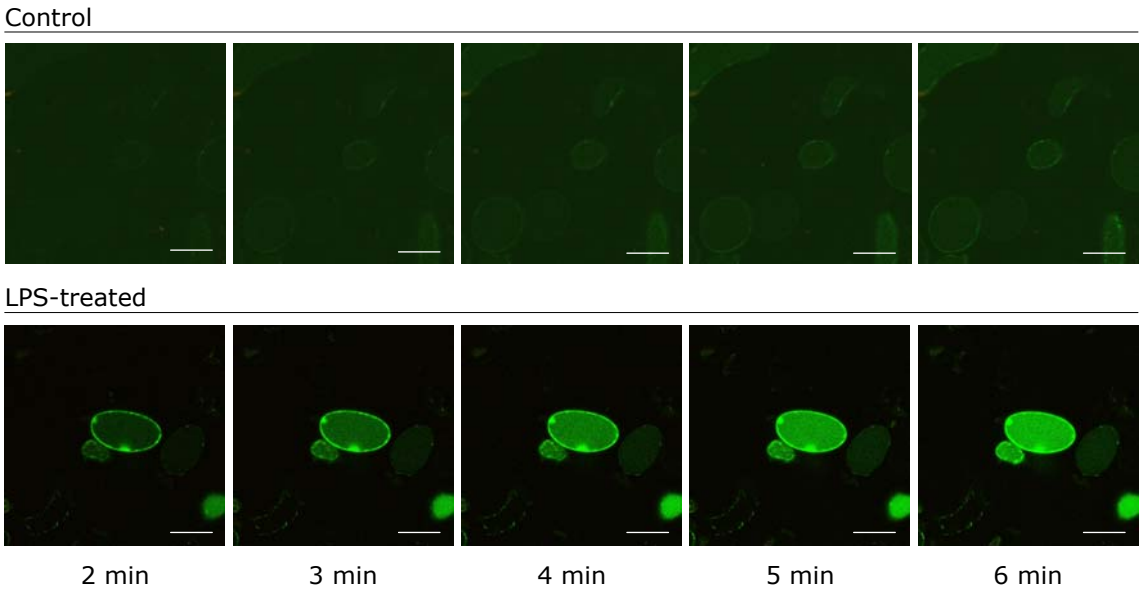

Figure 4-1: *Time course of the LPS-induced NO burst as detected by confocal laser scanning microscopy.*

Arabidopsis cells were loaded with 5 µM DAF-FM DA and treated with buffer A (upper row) or LPS (*Burkholderia cepacia*; 100µg/ml; lower row). Green fluorescence is indicative for NO (Scale bars, 25 µm).

4.1.2 DETECTION OF NO BY EPR

To specifically detect NO, the use of more than one technique is highly recommended. NO is characterized by its high reactivity and short life-time. These characteristics generate a need for its real time detection *in vivo* with the EPR method (Hirayama et al., 2003). This technique is highly specific for NO detection in plants and animals (Tsuchiya *et al.*, 1996; Yoshimura *et al.*, 1996; Huang *et al.*, 2004). The EPR study was performed using a spin trapping reagent, which turns the unstable NO radical to a relatively stable molecule. Therefore Diethyldithiocarbamate (DETC) and iron, which form a paramagnetic complex with NO (NO-Fe-$(DETC)_2$) were used (Jackson et al., 2001). Additionally we utilized $Na_2S_2O_4$ as a strong reductant to increase the sensitivity and stability of the EPR spectrum of the NO-Fe_2+$(DETC)_2$ complex (Tsuchiya et al., 1996).

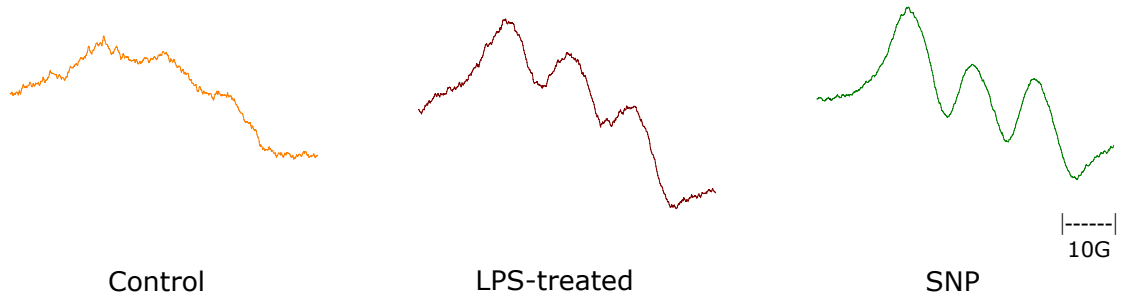

Figure 4-2: *LPS-induced increases of NO in Arabidopsis cells as detected by electron paramagnetic resonance (EPR).*

NO was detected by EPR using the spin trap Fe_2+$(DETC)_2$. Shown are a NO control (5 µM SNP in Hepes, right), an extract obtained from untreated *Arabidopsis* cells (left) and an extract from cells 10 min after LPS treatment (middle). The signals were recorded at identical EPR settings

The complex gives a characteristic three line EPR spectrum, which was extracted for image construction. It could clearly demonstrate a NO production 10 min after LPS (*Ralstonia solanacearum*) treatment (Fig. 4-2, middle) in *Arabidopsis* cells in comparing with control cells (Fig. 4-2, left). For better illustration an *in vitro* synthetic NO-Fe_2+$(DETC)_2$ spectrum is also shown (Fig. 4-2, right).

4.1.3 DETECTION OF NO BY FLUORESCENCE MEASUREMENT

To monitor the time course for NO accumulation in LPS-treated *Arabidopsis* cells, a spectrofluorometric assay was developed, using the NO-sensitive fluorophore DAF-FM diacetate and a multiwell plate reader. An increasing fluorescence indicative for NO production of LPS treated cells was observed, which was significant stronger as in control cells (Fig. 4-3). NO burst was already detected within 5 min of treatment and became stronger in course of 30 min. For an estimation of NO amount see 4.1.4.

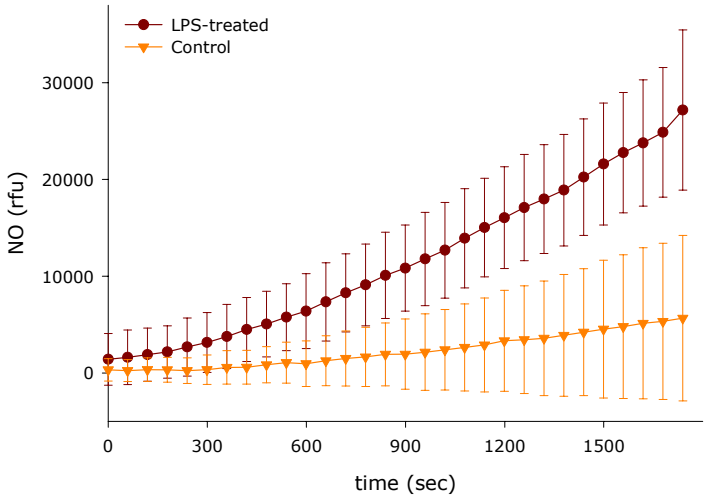

Figure 4-3: *Time course of the NO burst after LPS (B. cepacia) treatment.*

NO production was determined by measuring fluorescence intensity with a microplate reader. The values (relative fluorescence units) represent a mean of 25 independent experiments.

4.1.4 COMPARISON OF DIFFERENT LPS PREPARATIONS, LIPID A AND LTA

Many LPS preparations (also commercial ones) contain other bacterial components such as peptidoglycans that can stimulate animal cells independently of LPS (O'Neill, 2002).

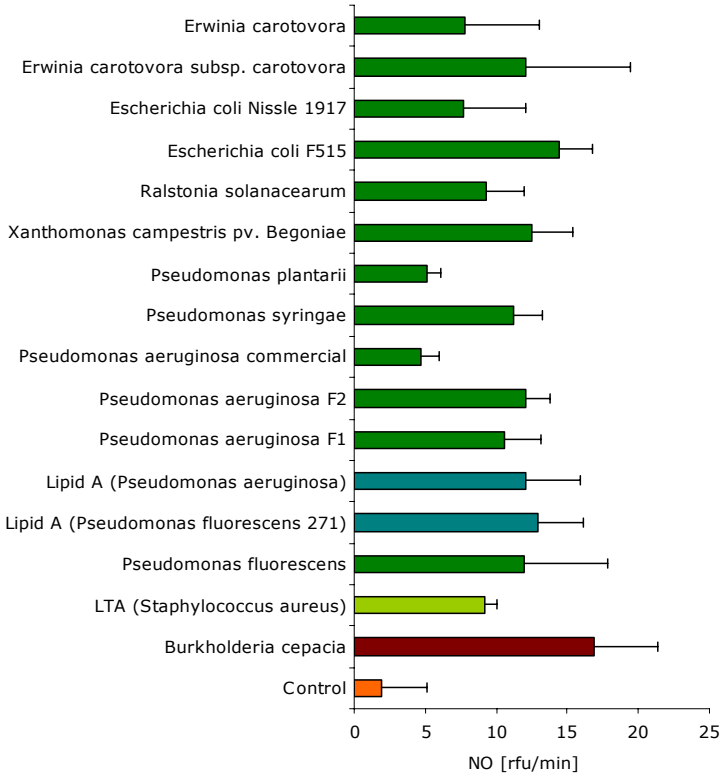

Figure 4-4: *Comparison of LPS-induced NO burst by diverse LPS preparations, Lipid A and LTA.*

Cells were treated with the same concentration (100 µg/µl) of LPS, Lipid A or LTA and/or 1 µM DAF-FM diacetate as described (Fig. 4-1 and 4-3). NO production was determined with a microplate reader (Fig. 4-3). Values are expressed as a NO production per minute and represent a mean of 10 independent experiments. Color code; orange, control; dark red, LPS used for most experiments; light green, lipoteichoic acid (LTA); green, LPS from different bacteria; blue, Lipid A.

As many as 18 different LPS batches (shown are 15) were used, from an array of plant- or animal-associated bacteria and prepared by several different laboratories. Here we show that LPS from *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Erwinia carotovora*, *Escherichia coli*, *Burkholderia cepacia* and others induce immediate production of NO in *Arabidopsis* cells (Fig. 4-4). *P. aeruginosa* and *B. cepacia* are opportunistic pathogens in cystic fibrosis patients. Other *B. cepacia* isolates, like the one shown have been used as biocontrol agents in agriculture. The strong response of *Arabidopsis* cells towards lipid A suggests that this component may be at least partially responsible for LPS perception by plants. In addition to LPS, *Arabidopsis* cells responded strongly to Lipoteichoic acid (LTA) from *Staphylococcus aureus* (Fig. 4-4). If calibrated against DAF-T (the fluorescent adduct of NO and DAF-FM) amount of produced NO within the cells can be calculated. Here, the control (orange) produced 0.06 nmol NO/g FW x min, and the highest induction was found after stimulation with *B. cepacia* LPS (dark red; 0.49 nmol/g FW x min).

4.1.5 NOS INHIBITOR REDUCES LPS TRIGGERED NO PRODUCTION

In plants, NO can be produced by NOS-like enzymes, or by nitrate reductase (NR) (Yamasaki and Sakihama, 2000; Chandok et al., 2003; Guo, Okamoto, and Crawford, 2003). To find out which NO-source becomes activated by LPS we resorted to a pharmacological approach. Therefore, *Arabidopsis* cell culture was first incubated with NOS (L-NNA) or NR (sodium azide) inhibitor, respectively, and then challenged with LPS. NO production was measured using the spectrofluorometric assay as described above (Fig. 4-3). The LPS induced burst of DAF-FM diacetate fluorescence in *Arabidopsis* cells was reduced dramatically (86 % by 1 mM and 99% by 10 mM) by L-NNA (Fig. 4-5).

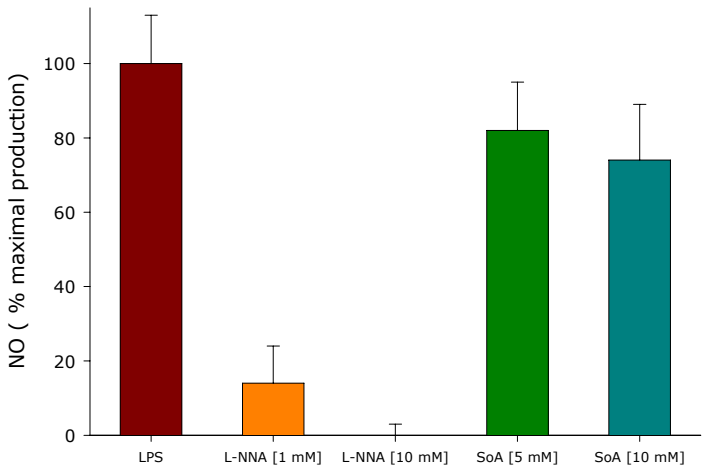

Figure 4-5: *Effects of NOS and NR inhibitors on LPS-induced NO burst.*

Arabidopsis cells were treated with LPS and analyzed for NO using 1 µM DAF-FM DA and a microplate reader. In case of inhibitor studies, cells were pre-treated for 10 min with N_{ω}-Nitro-L-arginine (L-NNA) or sodium azide (SoA) before addition of LPS. Values represent a mean of 5 independent experiments

L-NNA as NOS inhibitor is a methyl ester derivate of the NOS substrate L-arginine. NO can be also produced by plant nitrate reductase, which catalyzes the NADPH-dependent reduction of nitrite to NO (Yamasaki and Sakihama, 2000). To analyze if NR is responsible for NO production observed in response to LPS, we investigated the effect of sodium azide (SoA), a potent inhibitor of NR (Yamasaki and Sakihama, 2000). Figure 4-5 shows that the elicitor-induced NO burst was insensitive to SoA treatment, indicating that NR is not involved in NO synthesis.

4.2 LPS INDUCE A NO BURST IN *ARABIDOPSIS* LEAVES

To verify the data on the NO-burst in *Arabidopsis* suspension cells, the action of LPS in epidermal cells of *Arabidopsis* leaves was analyzed. Epidermal (abaxial) peels were loaded with DAF-FM diacetate and analyzed with fluorescence microscopy as described previously (Foissner et al., 2000; Huang et al., 2004).

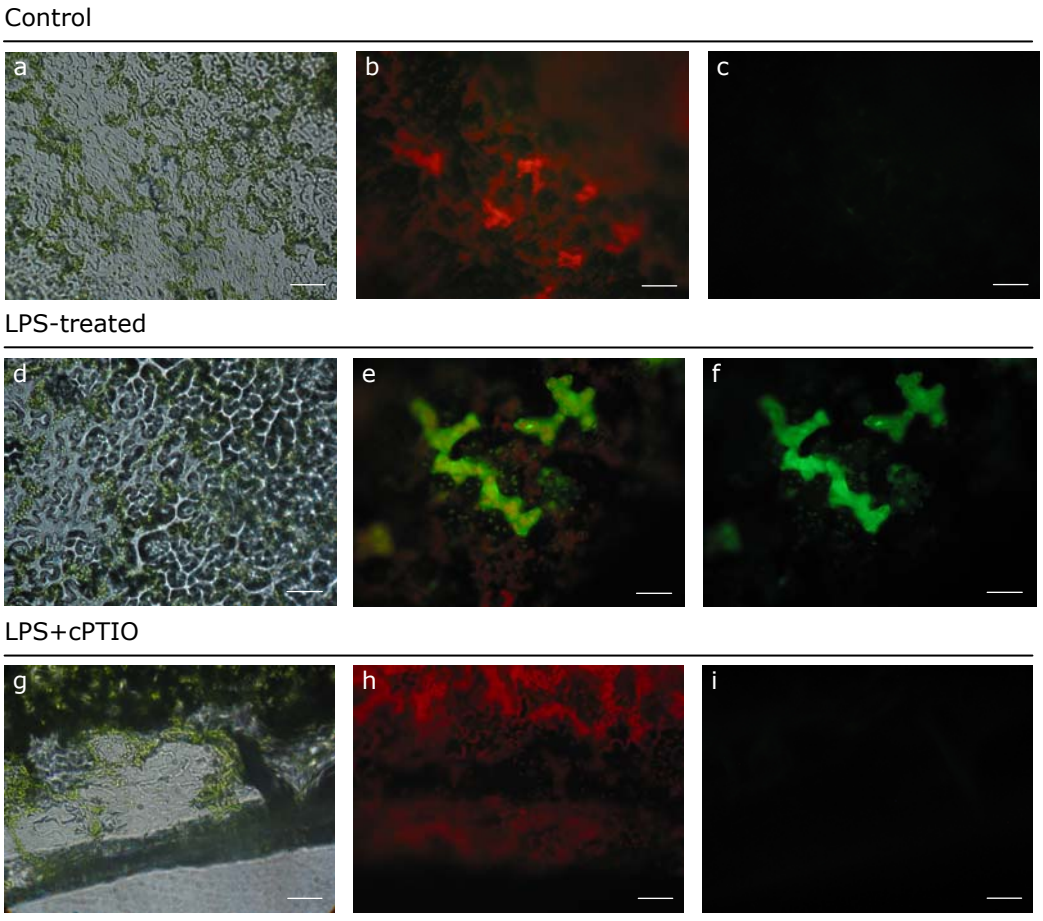

Figure 4-6: *Fluorescence microscopy of LPS-induced increases in intracellular DAF-FM DA signals in epidermal cells from Arabidopsis thaliana.*

The lower epidermis of *Arabidopsis* leaves was loaded with 1 µM DAF-FM DA in absence (upper row) or presence of LPS (*Burkholderia cepacia*; 100 µg/ml; middle row). The images were obtained 10 min after LPS treatment under bright field (a, d, and g), and under fluorescence light (green light filter, 505-530 nm; c, f and i). Chlorophyll fluorescence was captured with a long-pass filter (585 nm; b, e and h). Lower row shows a LPS-treated leaf coinfiltrated with the NO scavenger cPTIO (100 µM). (Scale bars, 100 µm).

LPS-induced NO production became apparent within few minutes (Fig. 4-6 middle row). The NO scavenger cPTIO (100 µM) suppressed the elicited bursts of fluorescence (Fig. 4-6 lower row). No green fluorescence and consequently no NO were detectable in peels of control leaves (Fig. 4-6 upper row).

4.3 LPS ACTIVATE NITRIC OXIDE SYNTHASE

4.3.1 MEASUREMENT OF NOS-ACTIVITY

To analyze the NO-burst in more detail, leave extracts were assayed for NOS activity using a conventional citrulline/arginine assay (Guo, Okamoto, and Crawford, 2003). Here, basal NOS activity was 3.6 pmol/mg x min. Immediately after LPS treatment, NOS activity could not be reduced by the NOS inhibitor L-NMMA. In contrast, the significantly increased NOS activity 20 min after LPS administration was clearly repressed (4-fold) by L-NMMA (Fig. 4-7). These results demonstrate that a NOS-like enzyme is involved in LPS induced NO production.

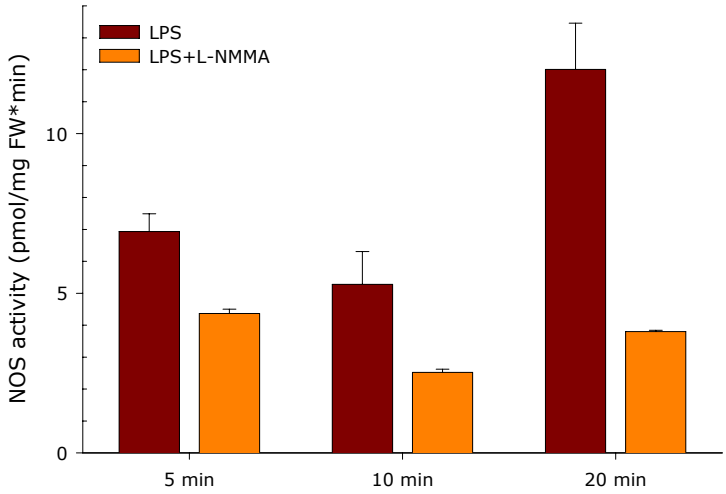

Figure 4-7: *NOS activity in wild-type Arabidopsis leave-extracts after LPS treatment.*

LPS treated and control leaves were harvested at different time-points and an extract of leave tissue was prepared. The NOS activity was determined with the NOS assay kit. Values represent a mean of 4 independent experiments.

4.3.2 MEASUREMENT OF NO IN NOS MUTANTS

To substantiate the data of LPS induced NO burst, the inhibitor experiments and the measurement of NOS activity; two *Arabidopsis* NOS mutants were tested for NO production after LPS treatment. During my experimental phase, two plant NOS were reported: The pathogen-inducible varP-iNOS, a variant of the P proteins of the glycine-decarboxylase-complex (Chandok et al., 2003) and the hormone-inducible atnos1, a plant homolog from the NOS of the snail *Helix pomatia* (Guo, Okamoto, and Crawford, 2003).

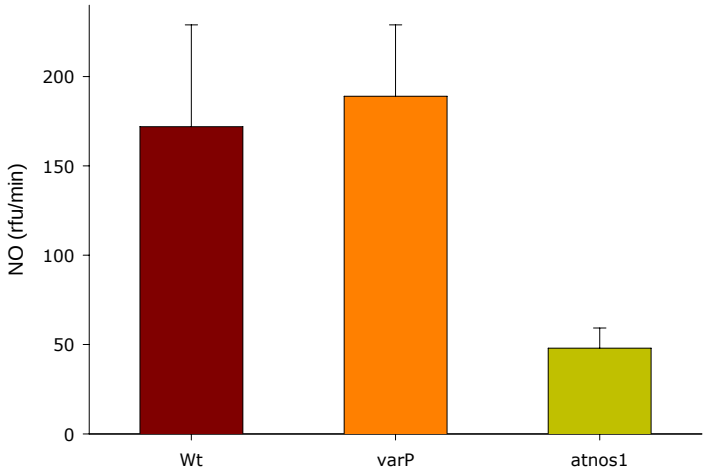

Figure 4-8: *LPS-induced NO in epidermal cells of Arabidopsis wild-type (WT), variantP-iNOS (varP) and atnos1 mutant plants.*

The NO burst was determined with a microplate reader during the first 60 minutes of treatment. The relative values represent a mean of 4 independent experiments.

The spectrofluorometric assay to detect NO accumulation (Fig. 4-3) was used to assay peels of wild-type, a varP-iNOS mutant (Salk T-DNA insertion line #110091) and atnos1 mutant plants (Guo, Okamoto, and Crawford, 2003). After stimulation with LPS, NO production in the varP-iNOS insertion line wild-type leaves was approximately as high as in wild-type plants, indicating that variant P is not involved in LPS triggered NO synthesis (Fig. 4-8). In contrast, in the atnos1 mutant the LPS induced NO was reduced by about 80%, suggesting that it is the AtNOS1 enzyme which is generating the LPS-stimulated NO.

4.3.3 ATNOS1 IS INVOLVED IN BACTERIAL RESISTANCE

Because NO is involved in disease resistance in plants (Delledonne et al., 1998; Wendehenne, Durner, and Klessig, 2004) we tested whether plants lacking NOS are more susceptible to pathogenic bacteria. We therefore infected *A. thaliana* wild-type and atnos1-mutant plants by spraying *Pseudomonas syringae* pv. *tomato* DC3000 bacteria onto leave surfaces. After 2 and 5 days disease symptoms were photographed and bacterial number inside the host tissue was determined. Under these conditions, *atnos1* plants showed a faster and much more severe development of disease symptoms than wild-type plants (Fig. 4-9, left). These stronger symptoms correlated with higher numbers of bacteria in *atnos1* leaves (Fig. 4-9, right). Thus, AtNOS1 is involved in bacterial disease resistance in *Arabidopsis thaliana.*

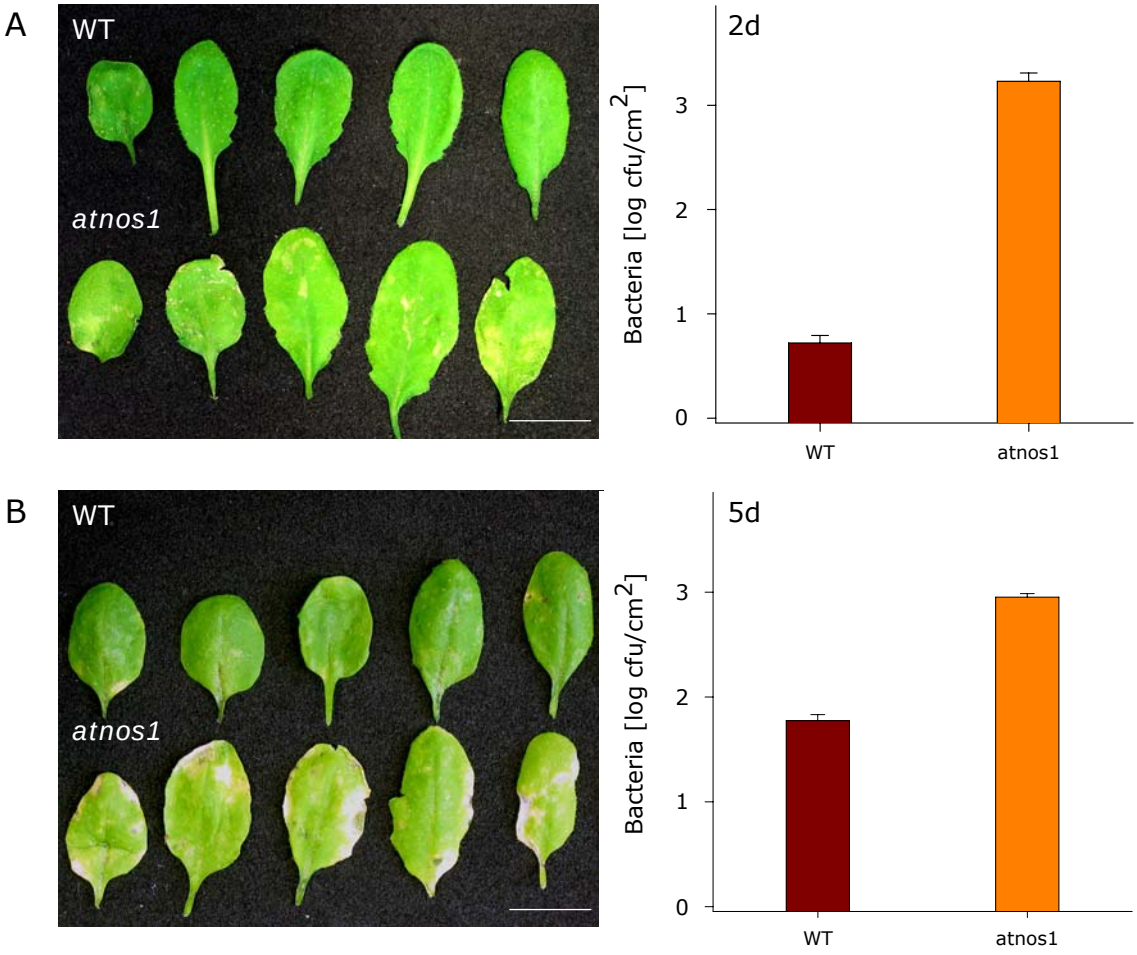

Figure 4-9: An *atnos1 mutant shows enhanced disease susceptibility against Pst DC3000.*

Wild-type and *atnos1* mutant plants were sprayed with *Pst* DC3000 bacteria or with water (data not shown) and photographed 2 [A] and 5 days [B] later, respectively. Left: symptoms after 2 and 5 days in a series of leaves. The bar graphs indicate the number of *Pst* DC3000 bacteria extracted from wild-type and *AtNOS1* mutant plants 2 and 5 days after infection, respectively. Scale bar, 1 cm.

4.4 LPS INDUCE AN OXIDATIVE BURST IN PLANT CELL CULTURES

In the mammalian immune system, NO often function together with reactive oxygen intermediates (ROI), for example in macrophage killing of bacteria and tumor cells (Schmidt and Walter, 1994; Nathan, 1995). Owing to this fact and because of the known important role of ROI in plant pathogen defense (Doke et al., 1996), the potential of LPS to induce an oxidative burst in plant cells was investigated. Tobacco and *Arabidopsis* suspension cells were loaded with H_2DCF-DA (10 µM), placed on a microscopic slide featuring an incubation well and analyzed with epifluorescence microscopy. Fig. 4-10 indicates a bright-field image of a cell cluster after loading with H_2DCF-DA and subsequent incubation in fresh loading buffer, but before elicitation with LPS (100 µg/ml). LPS addition during image acquisition resulted in a rapid burst of fluorescence, indicative of a massive ROI production.

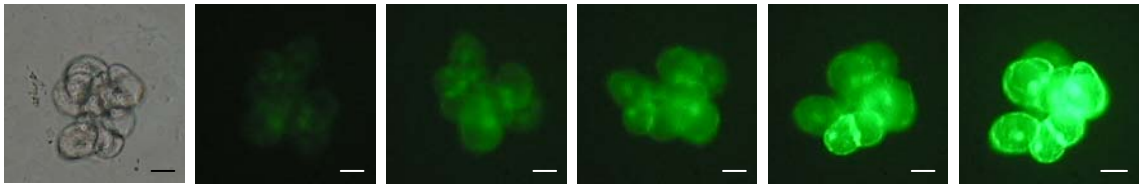

Figure 4-10: *Time course of the oxidative burst as detected by intracellular H_2DCF-DA fluorescence in tobacco cells after LPS stimulation.*

Tobacco suspension cells were loaded with H_2DCF-DA (10 µM), washed, treated with LPS (100 µg/ml) and examined by fluorescence microscopy. Shown are a bright-field image of a cell cluster, and a time lapse for the first 9 min of the oxidative burst. Scale bar, 25 µm.

The addition of LPS to cell culture of *Arabidopsis* resulted in a massive production of reactive oxygen species (ROS; Fig. 4-11). Cells were loaded with H_2DCF-DA (10 µM) and subsequently triggered with LPS (100 µg/ml). Green fluorescence is indicative for ROS production and became already visible after 1 min of treatment.

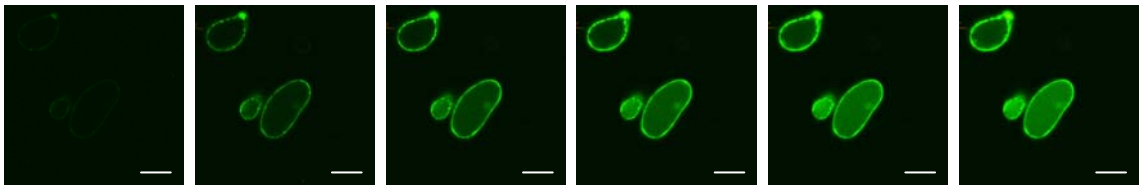

Figure 4-11: *Development of ROS in Arabidopsis cells after LPS stimulation as detected by intracellular H_2DCF-DA fluorescence.*

Arabidopsis suspension cells were loaded with H_2DCF-DA (10 µM), followed by LPS treatment (100 µg/ml). Pictures were obtained by laser scanning microscopy. Shown is a time lapse for the first 5 min of treatment. Scale bar, 25 µm.

4.5 LPS INDUCE DEFENSE GENES

Host defense becomes apparent not only by triggering production of reactive oxygen and NO, but also in induction of defense genes (Zipfel et al., 2004). Almost no data are available for gene induction by LPS in plants. However, LPS pre-treatment enhanced the expression of several genes involved in defense upon subsequent bacterial infection (Newman et al., 2002). This finding, together with the results on NO production after LPS-treatment, asked for an analysis for alterations in plant gene expression after LPS-stimulation.

4.5.1 GLOBAL TRANSCRIPTONAL PROFILING OF LPS INDUCED GENES

To get a global overview of LPS induced gene expression, *Arabidopsis* cell cultures were exposed to LPS for one day, cells were harvested at six different time-points (0.5 hr, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr) and total RNA reverse transcribed to cDNA from control and LPS-treated cells were used as probes for Agilent *Arabidopsis*-I-Microarrays. This array consists of approximately 16,000 genes. LPS affected the expression of ~970 genes; this corresponds to 6% of all on the Agilent chip existing oligonucleotides. A

global representation of the changes in gene expression is depicted in Fig. 4-12. To determine whether LPS regulated different expression of only particular classes of genes, a functional classification of LPS induced genes was performed. Approximately 250 genes could not be sorted in a functional category. The classification was achieved using the MIPS (HTTP://MIPS.GSF.DE/), TIGR (HTTP://WWW.TIGR.ORG/) and TAIR (HTTP://ARABIDOPSIS.ORG/) databases. The broad spectrum of gene functions shown in Fig. 4-12 is distributed over the whole *Arabidopsis* genome. The most number of increased genes were found after 0.5 hr, 2 hr and 24 hr. Most genes are involved in metabolism, transcription, transport and defense processes. The amounts of decreased transcripts are generally weaker, but with a higher accumulation after 1 hr of treatment.

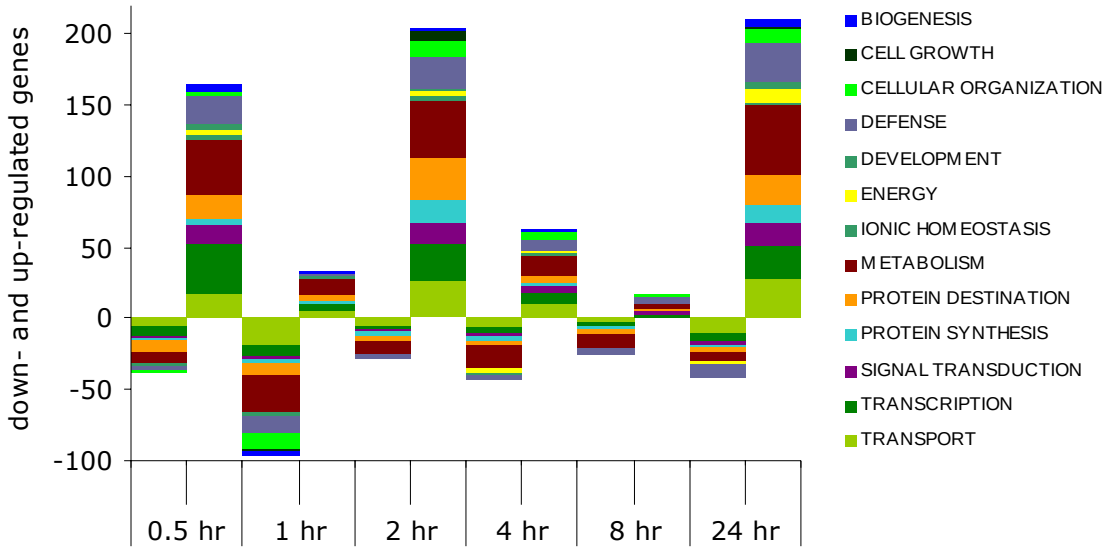

Figure 4-12: *Analysis of global transcriptional changes classed by function in Arabidopsis cell culture in response to LPS treatment.*

At the indicated time points after LPS treatment, total RNA was isolated, reverse transcribed and hybridized to the microarray. A complete data set is presented in the Supplement (Tab. 9-1). Genes were classed by function and presented as down- or up-regulated if more than 2.0-fold increased or repressed.

4.5.2 PROFILE OF LOCAL AND SYSTEMIC GENE ACTIVATION BY LPS TREATMENT

To verify the array data obtained with LPS-treated *Arabidopsis* cell culture (sections 4.5.1 and 4.5.2) and to investigate systemic processes, gene expression after LPS elicitation was examined in wild-type plants using Agilent *Arabidopsis* III full genome microarrays (~28,000 genes). Leaves were inoculated with LPS (100 µg/ml) and harvested at indicated time-points. Thereby were directed treated leaves termed as local and the leaves about as systemic leaves. After isolating RNA, reverse transcription and fluorescence labeling, probes were hybridized to microarray. LPS treatment resulted in a regulation of 1,895 genes which corresponds 7% of the *Arabidopsis* genome.

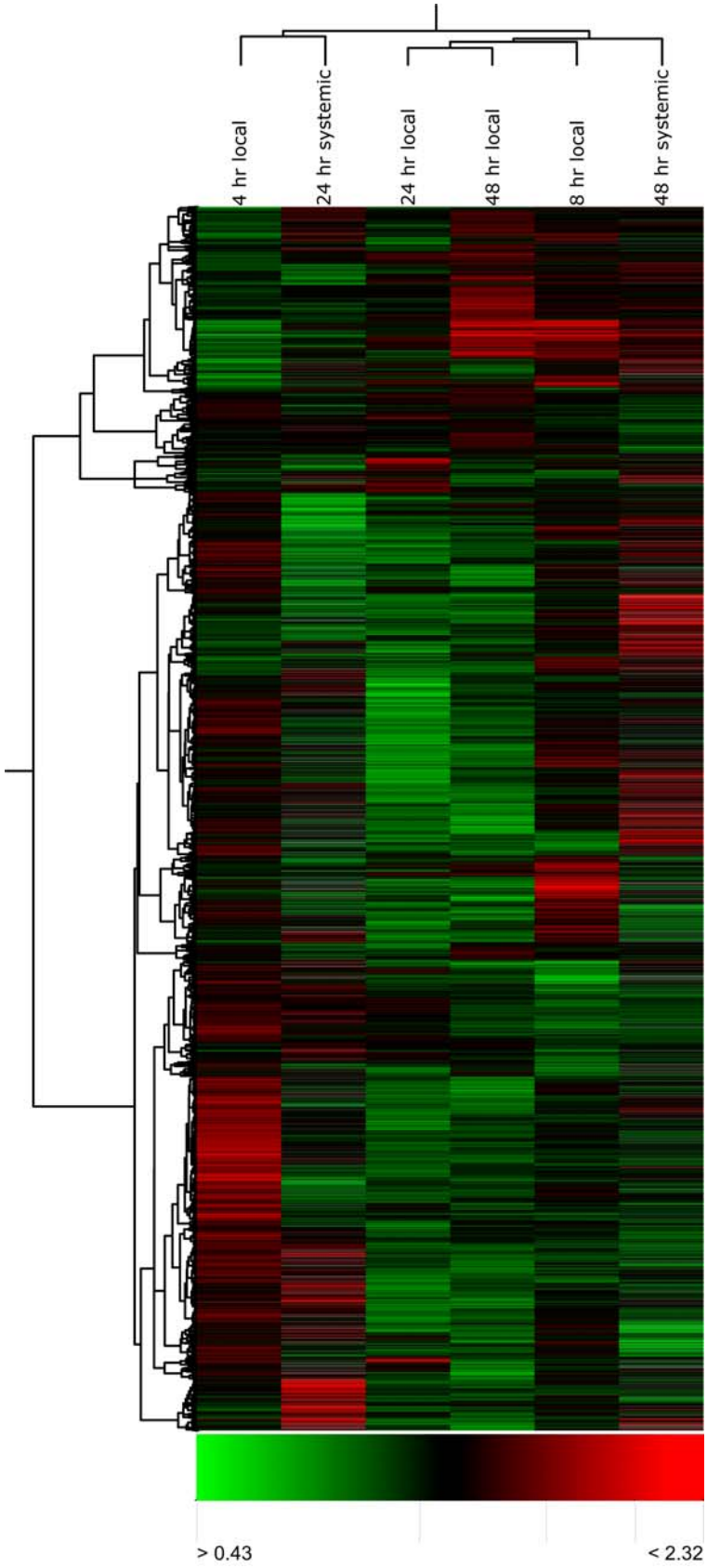

Figure 4-13: *Hierarchical clustering of LPS-induced transcripts in Arabidopsis wild-type leaves.*

LPS-inoculated leaves were harvested at indicated time-points (on the top), RNA was isolated, and reverse transcribed and probed to full genome arrays from Agilent. Each column represents data from 4 microarrays including two independent biological repeats. Red indicates expression above and green expression below the median value; black represents expression at the median. A complete data set and analysis are presented in the Supplement (Tab. 9-2).

The LPS-responsive transcripts were ordered by hierarchical clustering by using standard correlation coefficient as distance metric (Fig. 4-13). Most genes exhibit a single upward or downward wave of expression over the time of observation; the number of up-regulated genes (1,647) is more than two-fold higher as the number of down-regulated genes (695). Several kinetic patterns are visible. One group of genes exhibits a maximal deviation from baseline at 4 hr in the local and at 24 hr in the systemic leaves. A second group shows slightly later response that peaks at 8 hr in the local and at 48 hr in the systemic tissue. A third group, consisting mainly of reduced genes, shows a more protracted time course, responding maximally by 24-48 hr.

Generally, most increased genes were observed after 4 (490), 8 (434) and 48 (430) hours in local leaves. Great numbers of decreased genes (293) were found after 24 hr. The response to LPS treatment was somewhat weaker in the systemic tissue, which also resulted in a lower number of LPS regulated genes (Fig. 4-14).

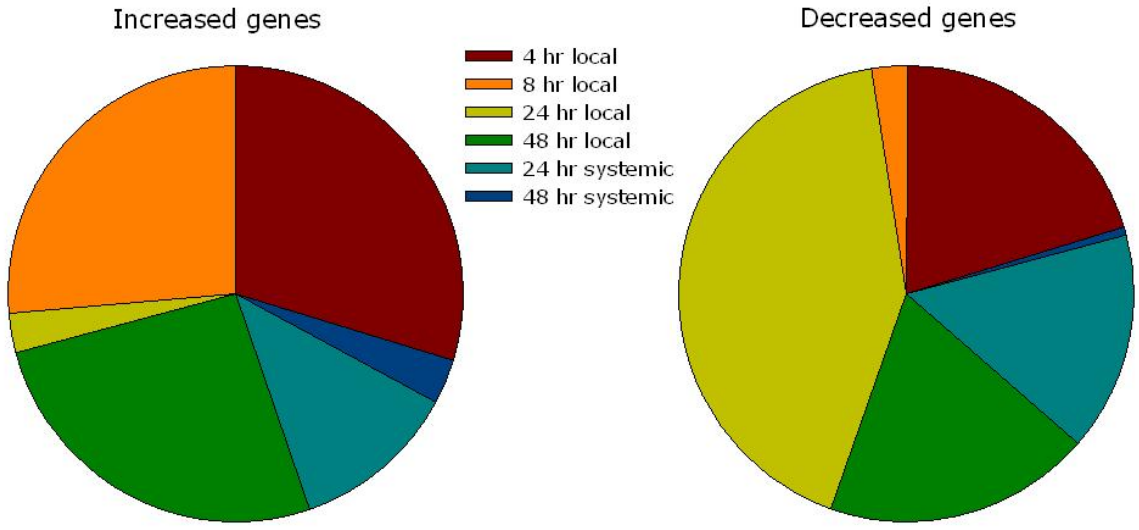

Figure 4-14: *Proportional representation of each time-point among LPS induced genes whose expression increased (left) or decreased (right) in response to LPS.*

After performing of microarray experiment as described above, LPS responsive genes were characterized as increased (up to 1.5 fold activation) or decreased (up to 1.5 fold deactivation) and counted per indicated time-point.

4.5.3 LPS ACTIVATE GENES REQUIRED FOR SYSTEMIC AQCUIRED RESISTANCE

Many genes with direct role in host defense showed significant changes in expression to LPS in *Arabidopsis* cell culture (see Fig. 4-12). Several classical systemic acquired resistance (SAR) markers were classed in this group, including PR -1, PR-2 and PR-5. In leaves of LPS treated plants, a coordinate induction of genes which act in SAR signaling pathway was observed. Development of SAR is dependent on SA, which is synthesized from chorismate by isochorismate-synthase (ICS; (Wildermuth et al., 2001)). LPS treatment induces three genes with isochorismate-synthase activity including the Isochorismate synthase I (At1g74710) with maximum expression after 8 hr. EDS5

(enhanced disease susceptibility) is also required for SA accumulation and might be involved in moving SA or a phenolic precursor out of the plastid after synthesis (Metraux, 2002). Induction of this gene could be measured after 8 and 48 hr in local tissue. SA synthesis also requires EDS1 and PAD4 (phytoalexin deficient), which have a feed back function for amplifying their own expression and increases SA signaling (Nawrath et al., 2002). Both genes were induced after 48 hr of LPS elicitation. Downstream of SA in SAR signal transduction acts NPR1 (non-expressor of PR-genes), this protein is essential for regulating SA dependent gene-expression. NPR1 interacts differentially with members of the TGA class of bZIP transcription factors and regulates their DNA binding activity (Despres et al., 2003). One of six members of the NPR1 and one of ten members of the TGA bZIP family were regulated through LPS.

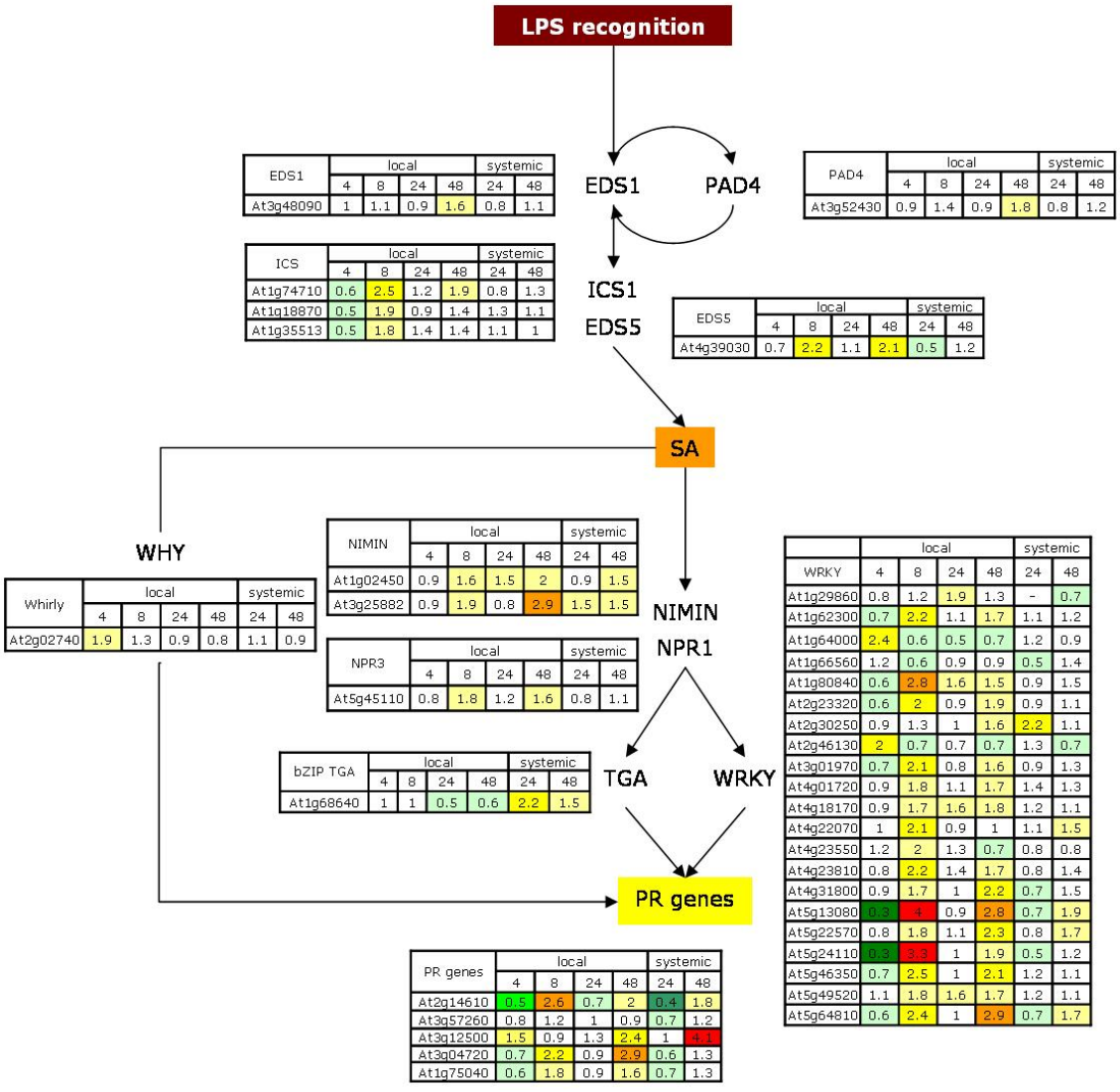

Figure 4-15: *Functional mapping of LPS affected genes to the systemic acquired resistance pathway.*

Upon LPS recognition, activation of PAD4, ICS1 and EDS5 triggers increased levels of SA. This leads to an induction of several transcription factors, such as NIMIN, NPR1, TGA, WRKY and WHY, which resulted in expression of PR-genes, known markers of SAR. White boxes are indicative for no or to 1.5-fold activation. Genes are highlighted in light yellow (higher than 1.5-to less than 2.0-fold activation), yellow (2.0- to less than 2.5-fold activation), orange (2.5- to less than 3.0-fold activation) and red (3.0-fold or more activation). Greenish colors indicate repression.

Another parts of the SAR pathway are NIMIN (NIM interacting; NIM=NPR1) proteins via physical interaction with NPR1 and two of three identified NIMIN proteins response to LPS treatment (Weigel et al., 2001). A further group of transcription factors for SAR signaling represent WRKY factors, which are also coregulators of PR gene transcription (Maleck et al., 2000). This family contains 76 members and 21 of them are affected by LPS. Candidates for regulators of NPR1-independent PR gene expression and resistance are the Whirly (WHY) family of transcription factors, which were induced by SA treatment and comprise three members (Desveaux et al., 2004). LPS elicitation induces two of them. Finally, this signaling cascade leads to the expression of the PR genes 1 to 5, which are good characterized markers for plant defense (van Loon and van Strien, 1999).

4.5.4 LPS INDUCE RECEPTOR-LIKE KINASES

Recognition of invading pathogens is the first step in activation of defense mechanism. Both animals and plants use leucine-rich repeats (LRRs)-containing receptors to detect specific pathogenic molecules (Dievart and Clark, 2004). In animals, specific cellular recognition of LPS/lipid A occurs through binding of LPS by CD 14 and MD-2, and transmission of the signal by TLR4, a transmembrane protein consisting of LRRs and a Toll/IL receptor domain (TIR;(O'Neill, 2002; Vasselon and Detmers, 2002).

Category	Number	Plants	Cells	Equal
family members	610	71	47	16
Subfamilies:	17	10	12	9
C-lectin	1	-	-	-
CR4-like	8	3	1	1
CrRLK1-like	18	-	2	-
DUF26	45	12	8	4
extensin-like	5	-	-	-
L-lectin	46	8	3	1
LRK10-like	13	-	1	-
LRR	232	29	12	5
LysM	4	-	1	-
PERK-like	19	3	1	-
RKF-like	2	-	-	-
SD	40	1	3	1
Thaumatin	3	1	-	-
TAKL	11	2	1	-
URK 1	2	-	-	-
WAK-like	25	5	2	1
RLCK	118	5	9	1
N.A.	18	2	3	2

Table 4-1: *Comparison of LPS induced receptor-like kinases in Arabidopsis plants and cell culture.*

All known *Arabidopsis* receptor-like kinases were classed in subfamilies according (Shiu and Bleecker, 2001a) and induced or repressed RLKs were counted in both test-systems. Gene expression was determined with Agilent arrays (I for cells, 3 for plants). Induction or repression of genes was calculated as described in Table 4-2. The abbreviations for the extracellular domains stand for: C-lectin, C-type lectin; CR4L, Crinkly4-like; CrRLK1, *Catharanthus roseus* RLK1; DUF 26, domain of unknown function 26; L-lectin, Legume lectin; LRKL, wheat LRK10-like; LRR, leucine-rich repeat, the numbers refer to the number of repeats; LysM, lysine motif; RLCK, receptor-like cytoplasmic kinase; PERK, Proline Extensin-like Receptor Kinase; SD, S-locus glycoprotein-like domain; TAKL, thylakoid-associated kinase like; URK, unknown receptor kinase; WAKL, wall-associated kinase like; N.A., not available.

A LPS recognition receptor has not yet been detected in plants, but the genome of *Arabidopsis* contains numerous putative receptors featuring toll-interleukin (TIR) domains (Dangl and Jones, 2001). For example, the receptor-like kinase (RLK) FLS2 that binds flagellin exhibits a structural similarity to *Drosophila* Toll and mammalian TLR5 (Toll like receptor), both of them are LRR-type receptors involved in mediating the innate immune responses in animals (Felix et al., 1999; Gomez-Gomez and Boller, 2002). Thus, the superfamily of receptor-like kinases in *Arabidopsis* could possibly contain a LPS receptor and was closely examined. RLKs belong to a large family with at least 610 members that represent nearly 2.5% of the *Arabidopsis* protein coding sequences (Shiu and Bleecker, 2001b). The RLK superfamily was completely represented on the Agilent III microarray (testing of plants LPS response) and with 564 genes on the Agilent I array (testing of cells response). LPS activate 102 receptor kinases genes in *Arabidopsis* plants and cell culture, 71 in plants, 47 in cells and 16 equal in both systems (Tab. 4-1). That adds up to approximately 4-5% of all LPS induced genes in plants as well as in cells. Responding RLKs were distributed over 13 of 17 subfamilies. Most regulated RLKs belong to the LRR-family in plants and cells, respectively. This subfamily represents with 232 members the largest group and five genes are found affected in both models.

Name	Subfamily	Location	Plants						Cells					
			local				systemic							
			4hr	8hr	24hr	48hr	24hr	48hr	0.5hr	1hr	2hr	4hr	8hr	24hr
At2g28250	-	ES, KF	1.96	0.99	0.66	0.81	0.82	1.04	0.33	0.96	0.87	1.93	1.9	3.78
At5g46080	-	ES	0.4	2.28	0.84	1.28	0.97	1.08	1.21	1.08	0.49	1.1	0.96	1.04
At1g28390	CR4L	-	0.8	1.77	1.24	1.31	0.87	0.91	0.78	0.6	0.53	1.28	0.85	0.88
At4g04490	DUF26	ES, M	0.69	1.96	1.05	1.85	0.67	1.51	0.99	1.21	1.64	0.69	0.84	1.87
At4g11890	DUF26	M	0.96	1.78	1.11	2.1	0.79	1.37	0.39	-	0.54	1.06	1.01	1.15
At4g23190	DUF26	M	0.59	2.05	0.87	1.64	0.81	1.11	-	-	-	0.49	0.76	2.63
At4g23210	DUF26	ES	1.16	1.87	1.41	1.47	0.76	1.04	0.9	1.02	1.57	1.82	1.42	2.91
At5g60300	L-lectin	ES, M, N	1.78	1.11	1	0.84	1.04	0.83	1.22	1.1	2	1.99	1.58	3.87
At1g51850	LRR 1	ES, M, PSII	1.45	2.23	2.11	1.52	1.04	1.04	0.42	-	0.49	0.89	0.97	1.02
At1g34210	LRR 2	ES	2.16	0.95	1.06	0.93	1.01	0.89	1.33	0.99	0.51	1.63	1.34	1.47
At2g24230	LRR 7	ES	1.06	0.9	0.53	0.71	1.03	0.79	0.52	-	0.38	0.67	0.78	0.97
At1g56140	LRR 8-2	M	0.91	1.84	1.15	1.1	0.88	1.04	0.74	1.11	0.53	0.94	1.03	1.01
At5g25930	LRR 11	ES, M, PSII	0.59	2.06	1.36	1.8	0.9	1.22	1.05	0.65	2.27	1.19	1.05	1
At2g41970	RLCK 8	-	1.83	1.09	0.79	0.91	0.97	0.98	0.86	-	0.55	0.98	0.92	1
At1g61360	SD-1	ES	1.48	1.86	1.38	1.38	0.93	1	0.92	0.3	1.55	0.73	0.7	0.98
At1g19390	WAKL	ER, ES	2	1.12	0.72	0.75	1.23	0.88	1.1	2.46	0.49	0.87	0.99	0.99

Table 4-2: *Receptor-like kinases from Arabidopsis in response to LPS treatment in both, plant and cells*.

At the indicated time points after LPS treatment mRNA from cells and plants was hybridized to microarrays from Agilent. A complete data set is presented in the Supplement (Tab. 9-4). Here, RLKs that respond to LPS in both test-systems (cells and leaves) were present. White boxes are indicative for no or to 1.5-fold activation. Genes are highlighted in light yellow (higher than 1.5-to less than 2.0-fold activation), yellow (2.0- to less than 2.5-fold activation), orange (2.5- to less than 3.0-fold activation) and red (3.0-fold or more activation). Greenish colors indicate repression. Kinase locations are supplemented by TIGR database. The genes are arranged in subfamily alphabetical order. Abbreviations; ER, extracellular region; ES, endomembrane system; M, membrane; KF, keratin filament; PSII, photosystem II reaction center.

LPS responding genes in both test-systems are shown in table 4-2. Gene-induction in plants was only found in local leaves at comparatively basely level. In cells, various genes are repressed at early time-points with an up-regulation after 24 hr of LPS

treatment. Most of LPS induced receptor kinases are located in the endomembrane system, which is a collection of membranous structures involved in transport within the cell and include endoplasmic reticulum, Golgi bodies, vesicles, cell membrane and nuclear envelope. Unfortunately, this part of my work is not completed yet. Further investigations should use knock-out mutants for severe RLKs, especially those are listed in Tab. 4-2, for searching after plants with disturbed LPS-signaling. Assays therefore could be LPS-induced NO burst and/or defense-gene activation.

4.5.5 GENE INDUCTION IS NEARLY ABOLISHED IN ATNOS1-MUTANT

We compared gene expression dynamics in LPS treated *Arabidopsis* wild-type, atnos1-mutant plants and cell suspension by using a (biased) custom-designed cDNA microarray that included about 700 defense-related genes encoding PR-proteins or proteins induced by pathogens and abiotic stresses (Huang, von Rad, and Durner, 2002).

ID	Name	WT						*atnos1*		cells					
		local				systemic		local							
		4 hr	8 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	0.5 hr	1 hr	2 hr	4 hr	8 hr	24 hr
ABC transporter family protein	At1g59870	0.59	1.68	0.47	2.15	0.45	1.31	0.87	-	2.14	2.62	1.73	2.03	2.26	2.32
ABC transporter homolog PnATH - like	At5g60790	2.17	0.92	1.44	0.93	1.09	0.8	-	-	1.46	1.05	1.34	1.68	1.86	2.81
Atpm24.1 glutathione S transferase	At4g02520	0.19	5.52	0.46	2.59	0.4	1.61	0.62	0.94	2.44	1.92	3.07	1.97	2.51	1.76
blue copper binding protein	At5g20230	0.45	2.37	0.43	2.27	0.74	1.38	0.9	1.09	1.74	2.22	1.43	0.85	0.67	0.78
cytochrome P450 71B15, putative	At3g26830	0.19	14.6	0.3	4.2	0.27	1.7	-	-	2.76	1.75	4.25	-	2.78	2.31
cytochrome P450 family protein	At4g12320	1.89	0.6	2.01	0.57	1.15	1.03	1.17	-	-	-	0.41	0.18	0.59	0.22
cytochrome P450 family protein	At4g37370	0.37	5.28	0.31	2.2	0.48	1.57	-	-	0.79	1	1.21	-	0.88	0.99
cytochrome P450 family protein	At2g45560	2.21	0.75	1.7	0.57	1.15	0.89	-	-	0.74	-	0.91	-	1.31	1.08
cytochrome P450 family protein	At2g24180	0.55	2.08	0.57	1.42	0.85	1.4	-	-	1.16	0.59	1.24	1.15	1.14	1.7
fasciclin-like arabinogalactan-protein 7	T88134	0.67	2.49	0.52	1.98	0.72	1.22	0.79	-	1.4	1.38	2.06	1.69	0.9	1.14
flavanone 3-hydroxylase (FH3);	At3g51240	0.48	1.72	0.58	1.95	0.72	1.29	-	-	1.1	0.53	1.42	0.83	1.58	1.09
germin-like protein (GLP4)	At1g09560	0.41	1.58	0.49	2.07	0.93	1.22	0.97	1.36	0.85	-	1.09	0.71	1.05	1.43
glutathione peroxidase 1	At2g25080	2.04	2.52	1.81	0.61	1.01	0.91	1.19	0.96	0.74	-	0.86	-	0.98	1.24
glutathione reductase	At3g24170	0.44	2.18	0.79	1.55	0.76	1.3	0.96	1.19	1.38	0.95	1.21	-	1.55	1.86
glutathione S-transferase	At1g02920	0.49	3.49	0.42	3.2	0.21	1.87	0.75	1.11	1.92	1.88	2.67	2.12	0.88	1.75
glutathione S-transferase (GST14)	At5g62480	0.19	4.91	0.44	2.48	0.39	1.63	-	-	-	-	-	-	-	-
glutathione S-transferase, putative	At1g78370	1.38	0.49	1.46	0.71	1.07	0.83	-	-	-	-	-	-	-	-
gluthatione s-transferase	At1g02930	0.65	2.72	0.46	2.95	0.25	1.42	0.98	1.18	1.75	1.74	2.41	1.85	3.18	1.42
HIN1 family protein	At2g35980	0.44	1.81	0.45	1.5	1.02	1.18	0.99	-	2.6	3	1.47	0.72	1.12	1.66
pathogenesis-related protein 1 (PR-1)	At2g14610	0.1	10.9	0.29	3.63	0.12	6.1	0.7	1.34	0.83	0.28	0.9	1.14	1.01	1.06
pathogenesis-related protein 2 (PR-2)	At3g57260	0.25	4.86	0.75	2.18	0.28	2.32	1.05	1.44	0.42	-	0.57	0.67	1.23	0.44
pathogenesis-related protein 3 (PR-3)	At3g12500	0.42	2.45	0.45	2.03	0.9	1.57	0.94	1.04	1.63	1.41	0.9	1.18	1.16	7.07
pathogenesis-related protein 4 (PR-4)	At3g04720	0.62	2.59	0.49	2.48	0.41	1.52	0.81	-	1.7	1.78	1.92	1.15	1.04	1.83
pathogenesis-related protein 5 (PR-5)	At1g75040	0.12	6.47	0.69	3.43	0.27	2.47	0.92	-	0.38	-	0.74	0.78	1.19	0.54
peroxidase; prxCb	At3g49120	0.41	2.46	0.35	2.3	0.71	1.63	0.78	0.96	1.56	1.62	1.4	1.37	1.23	2
RuBisCO activase	At2g39730	2.33	0.66	1.16	0.79	0.92	0.68	-	-	1.33	1.12	1.03	1.86	1.64	1.54
starch excess protein (SEX1)	At1g10760	2.42	0.67	1.55	0.99	0.95	0.92	-	-	0.46	0.65	0.7	0.71	1.25	0.58
Triose phosphate isomerase	U02949	0.36	3.13	0.48	2	0.59	1.47	-	-	-	-	-	-	-	-
UDP-glucose glucosyltransferase, putative	At1g05530	0.69	2.89	0.43	-	0.89	1.67	-	-	-	0.46	0.71	0.69	1.25	0.78
UDP-glucose glucosyltransferase, putative	At1g22400	0.38	3.44	0.45	-	0.37	2.04	-	-	0.09	-	0.91	-	0.67	-
UTP-glucose glucosyltransferase	At5g66690	-	4.74	0.17	-	-	-	-	-	0.19	-	0.36	-	0.77	0.41
violaxanthin de-epoxidase precursor	TC109750	2.03	0.98	1.33	0.78	0.91	0.82	-	-	0.91	0.86	0.96	1.33	0.93	1.04

Table 4-3: *DNA microarray analyses of transcriptional changes in Arabidopsis thaliana plants (WT and atnos1) and suspension cells in response to LPS treatment.*

At the indicated time points after LPS treatment, RNA reverse transcribed from local and systemic leave tissue or cells was hybridized to the cDNA array. A complete data set is presented in the Supplement (Tab. 9-3). Here, genes that respond to LPS in both test-systems (cells and leaves) were present. White boxes are indicative for no or to 1.5-fold activation. Genes are highlighted in light yellow (higher than 1.5-to less than 2.0-fold activation), yellow (2.0- to less than 2.5-fold activation), orange (2.5- to less than 3.0-fold activation) and red (3.0-fold or more activation). Greenish colors indicate repression. The genes are arranged in alphabetical order.

The results shown in Table 4-3 demonstrate that LPS induces an array of defense or stress-associated genes including glutathione S-transferases, cytochrome P450 and many genes encoding PR-proteins in wild-type plants and in cells (for a complete set of data see the Supplement Tab. 9-3). Interestingly, while the local response is stronger, several of the LPS-induced genes were activated in systemic leaves, too. Most importantly, gene expression was almost completely abolished in atnos1 mutant plants (blue frame). This result suggests a functional link between LPS induced NO-production and gene induction.

4.5.6 PR-GENE INDUCTION IS DEPENDENT ON NO

To verify the induction of PR-genes by LPS and for a more exactly investigation of NO-dependent gene induction, northern blots were performed. Analysis for the PR-genes 1, 2, 3, 4 and 5 were made after LPS (100 µg/ml) or SNP (500 µM) treatment in wild-type and atnos1-mutant plants.

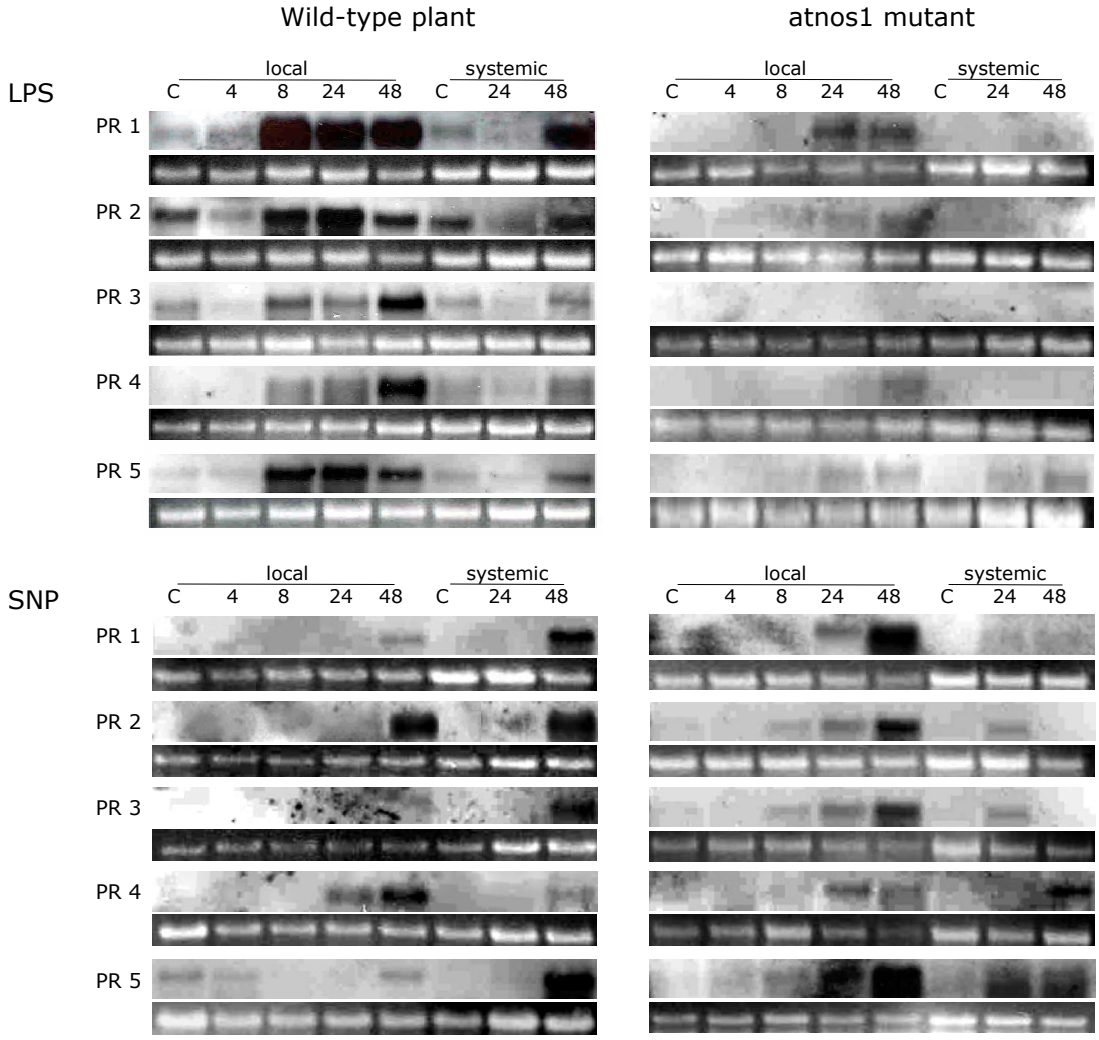

Figure 4-16: *Induction of local and systemic PR gene expression in leaves of wild-type or atnos1 mutant plants by LPS and SNP.*

Arabidopsis leaves were treated with LPS (100µg/ml) or SNP (500 µM) and collected at the times indicated for RNA preparation (4–48 h). Northern blots were probed with cDNAs for PR1, PR2, PR3, PR4 and PR5. Shown is the region between 1.8 and 1.0 kb. Ethidium bromide staining shows gel loading.

After LPS elicitation showed the wild-type a strong gene induction especially in local leaves, which also appears somewhat weaker in systemic leaves. This accumulation of transcript is nearly abolished in atnos1-mutant (Fig. 4-16, upper row). These data confirm the array analysis described above (Tab. 4-2). To test, if this gene expression is really dependent on NO, wild-type plants and atnos1-mutant were also challenged with SNP. This NO donor induces PR-transcript accumulation not only in wild-type but more interestingly also in the atnos1-mutant plant (Fig. 4-16, lower row).

4.5.7 GENE INDUCTION IN NAHG AND JIN1 PLANTS

LPS induced gene-expression is dependent on NO, but mostly transcription of resistance genes like PR 1 to 5 is also dependent on jasmonic and/or salicylic acid (JA; SA).

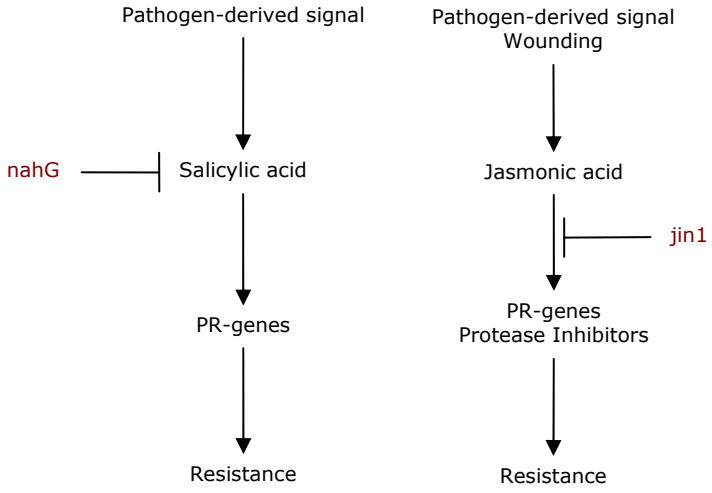

Figure 4-17: *Schematic simplified model of SA and JA defense pathways.*

Produced SA in the nahG plant will be degraded by a bacterial SA hydroxylase gene. The jin1 mutant is a JA insensitive plant.

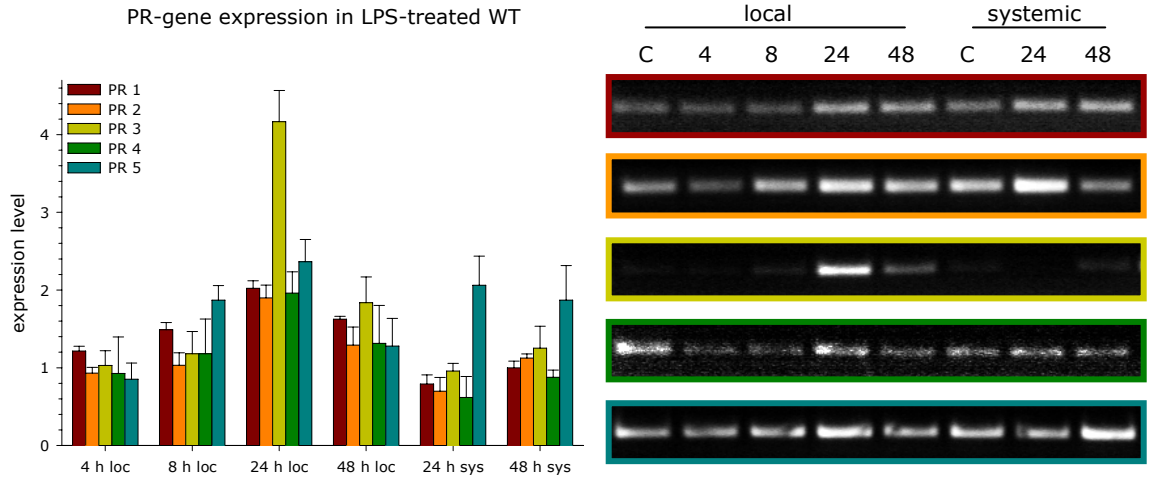

Figure 4-18: *PR-gene expression in wild-type plants as detected by real-time PCR.*

Plants were inoculated with LPS (100 µg/ml) and harvested at indicated time-points. Isolated RNA was reverse transcribed and gene induction in local and systemic leaves was determined using real-time PCR. Left bar diagram indicates gene expression levels in compare to control plants. The values represent a mean of 3 independent experiments. Right photos show representative gel pictures.

These two important plant signaling molecules play a key role in plant defense reactions (Ryals et al., 1996; Devoto and Turner, 2003; Shah, 2003). For this investigation two mutants were used, which have a disturbed jasmonic or salicylic acid pathway (Fig. 4-17). The jin1 plant is a jasmonic acid insensitive mutant (jin; (Berger, Bell, and Mullet, 1996; Berger, 2002)), whereas the nahG plant contains a bacterial salicylate hydroxylase gene (Gaffney et al., 1993). This enzyme catalyzes the conversion of SA to catechol, which is not an active defense inducer.

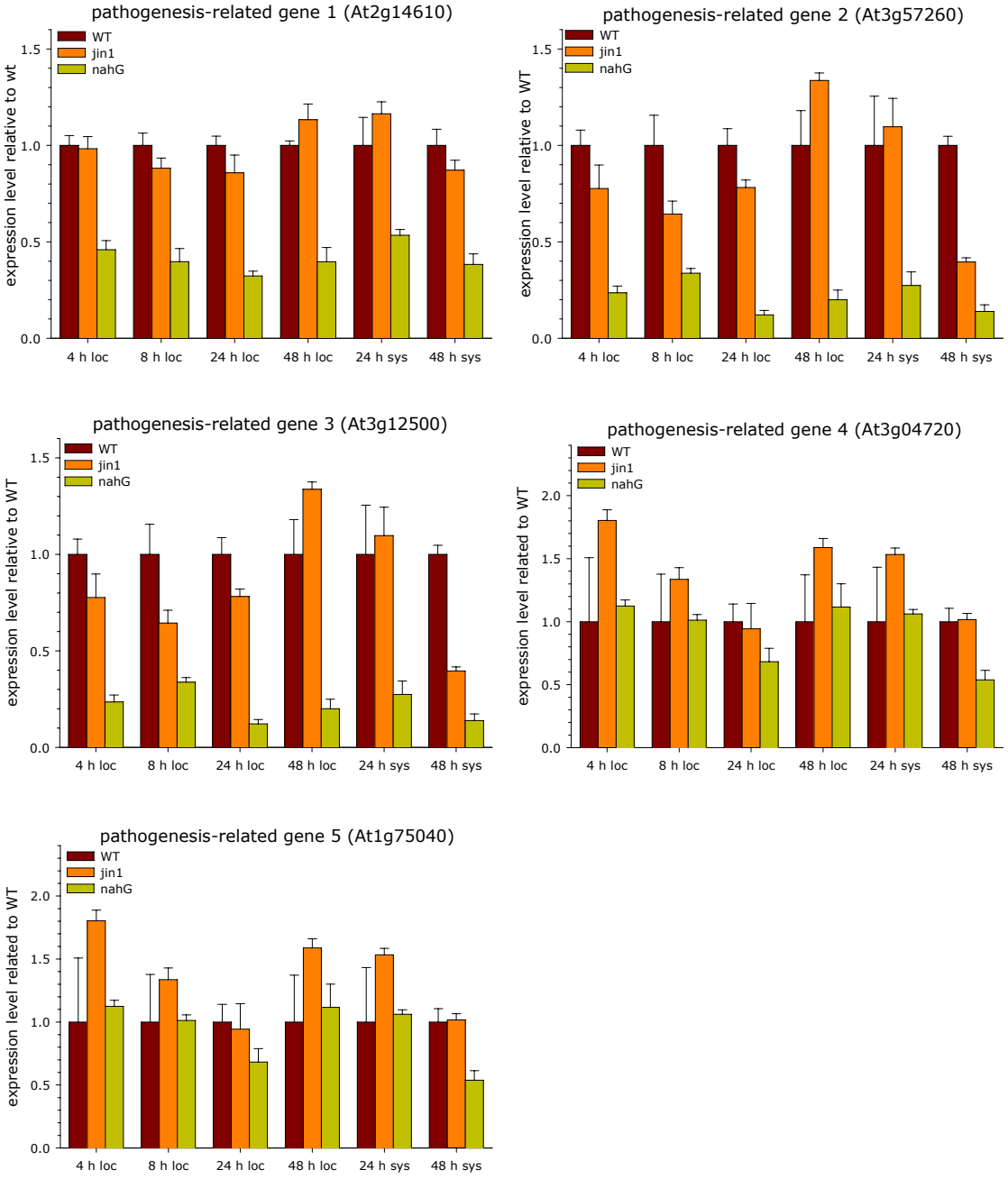

Figure 4-19: *PR-gene expression in jin1 and nahG plants in compare to wild-type as detected by real-time PCR.*

Plants were inoculated with LPS (100 µg/ml) and harvested at indicated time-points. Isolated RNA was reverse transcribed and gene induction in local and systemic leaves was determined using real-time PCR. The values represent a mean of 3 independent experiments.

The LPS mediated PR-gene expression after LPS treatment was first determined in the wild-type plant (Fig. 4-18) using real-time PCR and were then compared with expression level in the mutants (Fig. 4-19). The JA insensitive mutant jin1 showed no derogation in expression levels, whereas the gene expression of PR1, PR2, and PR3 in the nahG plants was clearly decreased in comparison to the wild-type. Expression of PR4 and PR5 was decreased only after 24 hr of LPS treatment, but this time-point represents highest gene-induction in wild-type plants (Fig. 4-18).

4.5.8 ATNOS1-GENE EXPRESSION

Because of the important role of the AtNOS1 enzyme in LPS induced NO (Fig. 4-8) and in pathogenesis (Fig. 4-9), the gene-expression levels in wild-type plants after LPS treatment was measured and compared with jin1, nahG and atnos1 mutants (Fig. 4-20). In wild-type plants the expression of the atnos1-gene was somewhat induced after 8 and 24 hr in local leaves (left). No gene induction was measured in the nahG and not surprisingly in the atnos1 mutant, whereas the jin1 plant showed an increased gene expression level after 4, 8 and 48 hr in local leaves in comparison to wild-type (right).

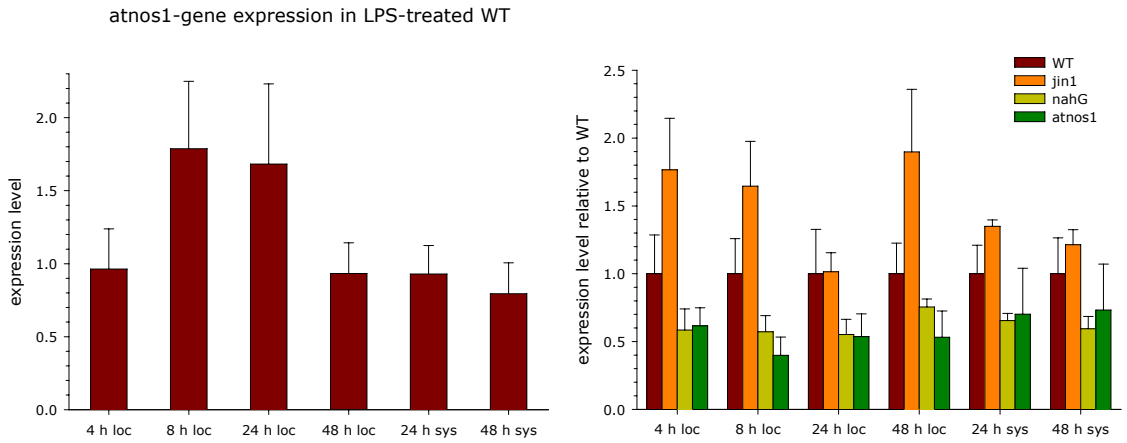

Figure 4-20: *Atnos1-gene expression in wild-type, jin1, nahG and atnos1 plants after LPS treatment as detected by real-time PCR.*

After LPS inoculation, local and systemic leaves were harvested at indicated time-points, RNA was isolated, reverse transcribed and expression levels were determine using real-time PCR. Values represented a mean of 3 independent experiments.

4.6 LPS TREATMENT RESULTED IN INCREASED SA ACCUMULATION

For activation of PR-gene expression and development of SAR elevated levels of SA are necessary (Ryals, Uknes, and Ward, 1994; Ryals et al., 1996). LPS elicited induction of PR (Tab. 4-3, Fig. 4-16) and other SAR (Fig. 4-15) related genes were good reasons for analyzing SA levels in LPS treated plants. Indeed, treatment of *Arabidopsis* leaves with LPS resulted in increased accumulation of conjugated SA in local tissue after 8 (5.72 nmol/g FW), 24 (4.42 nmol/g FW) and 48 hr (3.28 nmol/g FW). These data are also in

line with decreased expression of PR genes in nahG plants. This mutant carries a bacterial SA hydroxylase gene, which leads to SA degradation (Fig. 4-19).

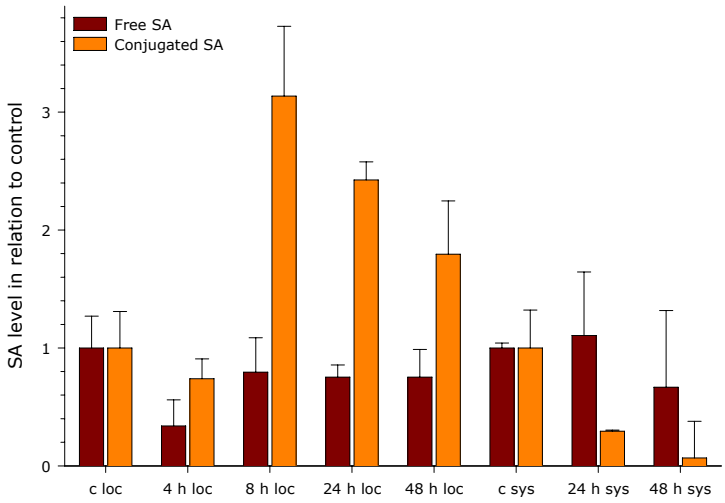

Figure 4-21: *LPS elicited SA levels in Arabidopsis plants in relation to control.*

SA accumulation in local and systemic leaves of LPS treated or untreated control plants were analyzed at indicated time-points. Values are displayed in relation to control leaves and represent a mean of duplicates.

4.7 LPS INDUCE SYSTEMIC AQUIRED RESISTANCE

Pathogenesis-related proteins are important markers for systemic acquired resistance (Ward *et al.*, 1991; Hunt *et al.*, 1996). For this reason, it was investigated, if the LPS mediated induction of PR-genes really resulted in a resistance against pathogens. *Arabidopsis* plants were pre-treated with LPS (100 µg/ml) for two days and systemic leaves were subsequently challenged with the bacterial plant-pathogen *Pseudomonas syringae* pv. *tomato* DC 3000 (*Pst* DC3000) using the virulent and the avirulent strain.

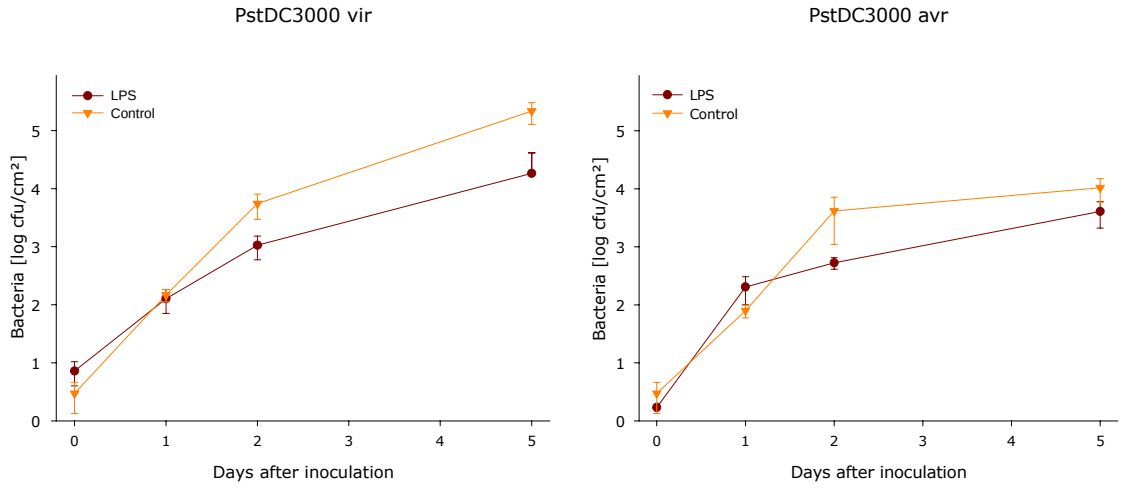

Figure 4-22: LPS induce systemic acquired resistance against *Pst DC3000.*

Wild-type were pre-treated with LPS for 2 days and systemic leaves were then inoculated with *Pst* DC3000 using the virulent and the avirulent strain. The diagrams indicate the number of *Pst* DC3000 bacteria extracted from systemic leaves 0, 1, 2 and 5 days after infection.

After 0, 1, 2 and 5 day leaves were harvested and bacterial number inside the host tissue was quantified (Fig. 4-22). The LPS pre-treated plants showed after 2 days of infection less bacterial growth then the untreated control. This different bacterial growth between control and LPS triggered plants becomes clearer after 5 days in plants inoculated with virulent strain (Fig. 4-22, left), whereas the number of bacteria was nearly the same in control and LPS elicited plants infected with the avirulent strain (Fig. 4-22, right).

4.8 LPS MOBILIZATION

4.8.1 DETECTION BY FLUORESCENCE MICROSCOPY

LPS from *B. cepacia* are able to induce a NO burst (Fig. 4-1) generated by the AtNOS1 (Fig. 4-8) and an array of defense genes in *Arabidopsis thaliana* plants and cells (Tab. 4-3).

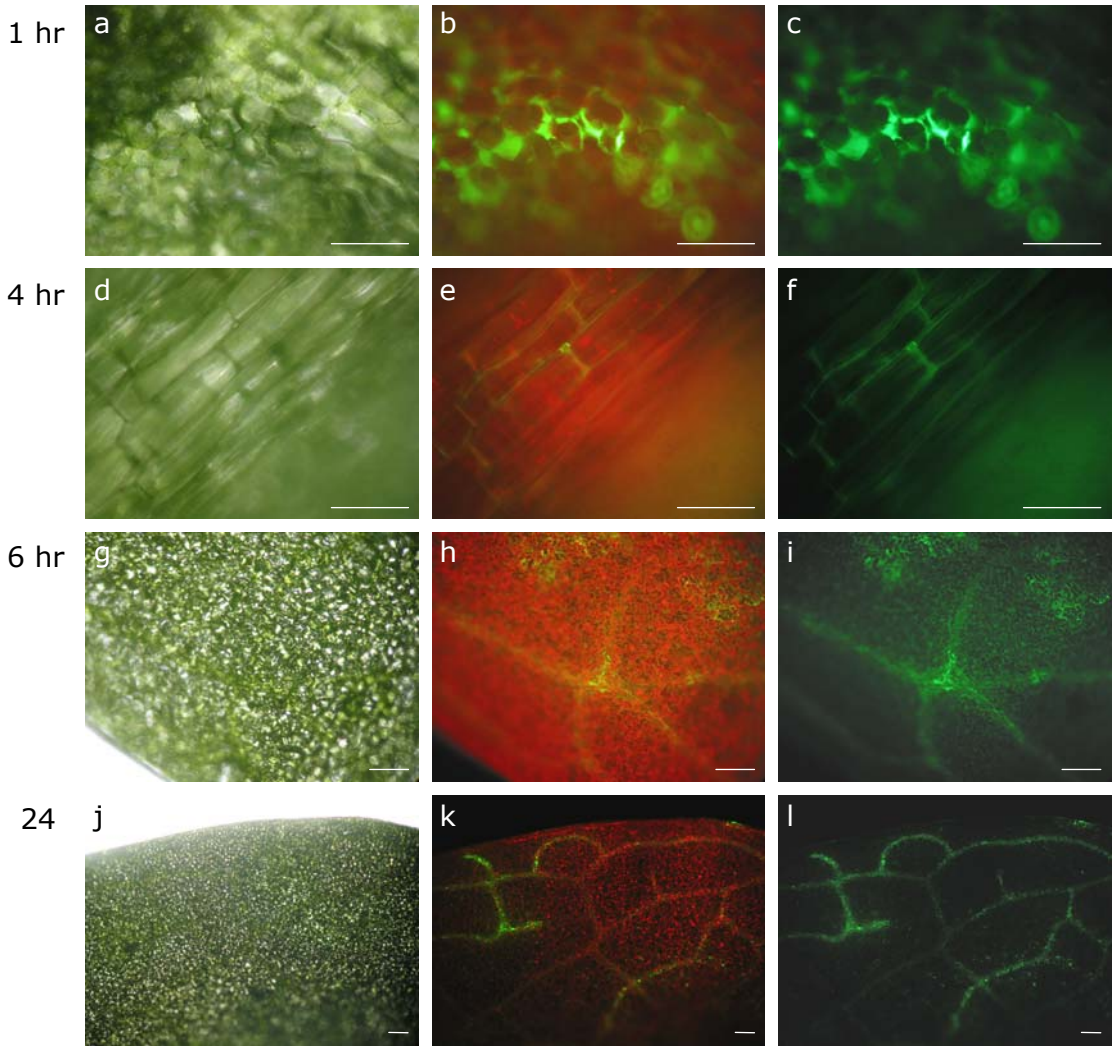

Figure 4-23: *Investigation of LPS mobilization in Arabidopsis leaves by using fluorescent-labeled LPS from S. minnesota.*

After pressure infiltration of fluorescent-labeled LPS, images were obtained from the abaxial leave side at indicated time-points under bright field (a, d, g and j) and under fluorescence light (green light filter, 505-530 nm; c, f, i and l). Chlorophyll autofluorescence was captured with a long-pass filter (585 nm; b, e, h and k).

In order to monitor the localization of LPS during and after these reactions, fluorescent-labeled LPS molecules from *S. minnesota* were used. For microscopically investigations *Arabidopsis* leaves were pressure infiltrated with the fluorescein-labeled LPS and a fluorescence microscope was used to localize the bound LPS. Observation of leaves 1 hr after supplementation with fluorescent-LPS revealed a fluorescence signal at the intercellular space (Fig.4-23a-c). After 4 hours (Fig. 4-23d-f) the LPS-fluorescence became visible in the middle-rip of *Arabidopsis* leaves. This fluorescence distributes over smaller leave veins near by the middle-rip after 6 hr (Fig. 4-23g-i) and was detectable in whole veins after 24 hr (Fig. 4-23j-l).

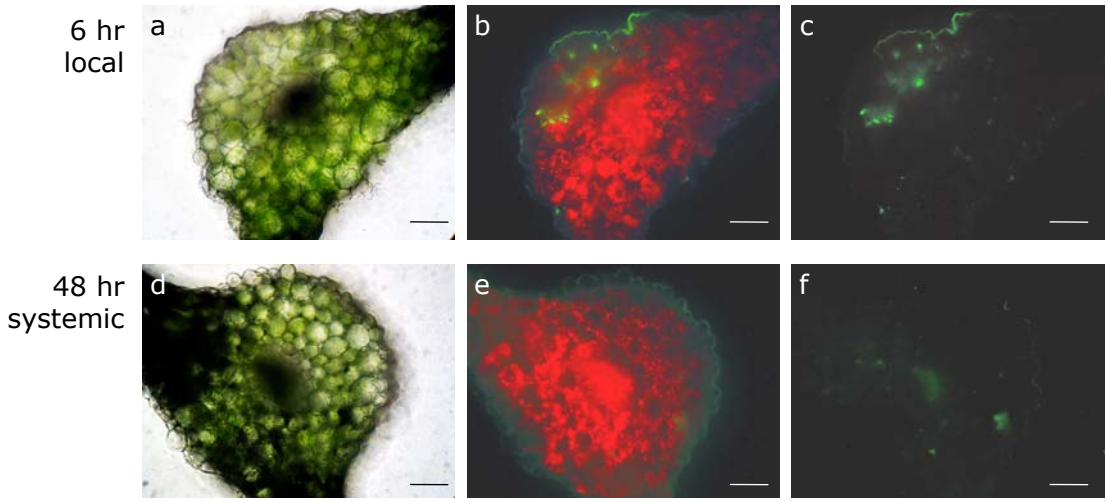

Figure 4-24: *Investigation of LPS mobilization in cross sections of Arabidopsis leaves by using fluorescent-labeled LPS from S. minnesota.*

After pressure infiltration of LPS and the cut of cross sections, images were obtained at indicated time-points under bright field (a and d) and under fluorescence light (green light filter, 505-530 nm; b and e). Chlorophyll autofluorescence was captured with a long-pass filter (585 nm; c and f).

To get a better overview, where in the veins and in which part of the vascular bundle the LPS-fluorescence appears, cross sections of the middle rips where made. The investigations with the microscope were performed as described above. Observation of cross sections 6 hr after supplementation with fluorescein-LPS revealed a clear fluorescent signal in outer vascular bundle cells (Fig. 4-24a-c). This fluorescence distributes over the plant and appears in systemic leaves after 48 hr (Fig. 4-24d-f).

4.8.2 DETECTION BY CAPILLARY ELECTROPHORESIS

To ensure, that the detected label was not due to a fluorescent group that was separated from the rest of the molecule, an independent technique was used. For this purpose, verification of the previous results was obtained by capillary zone electrophoresis. Distribution of fluorescent-labeled LPS from *S. minnesota* was observed in direct treated and in systemic leaves. For investigation of local leaves, extracts of middle-rips were made and separated by CZE.

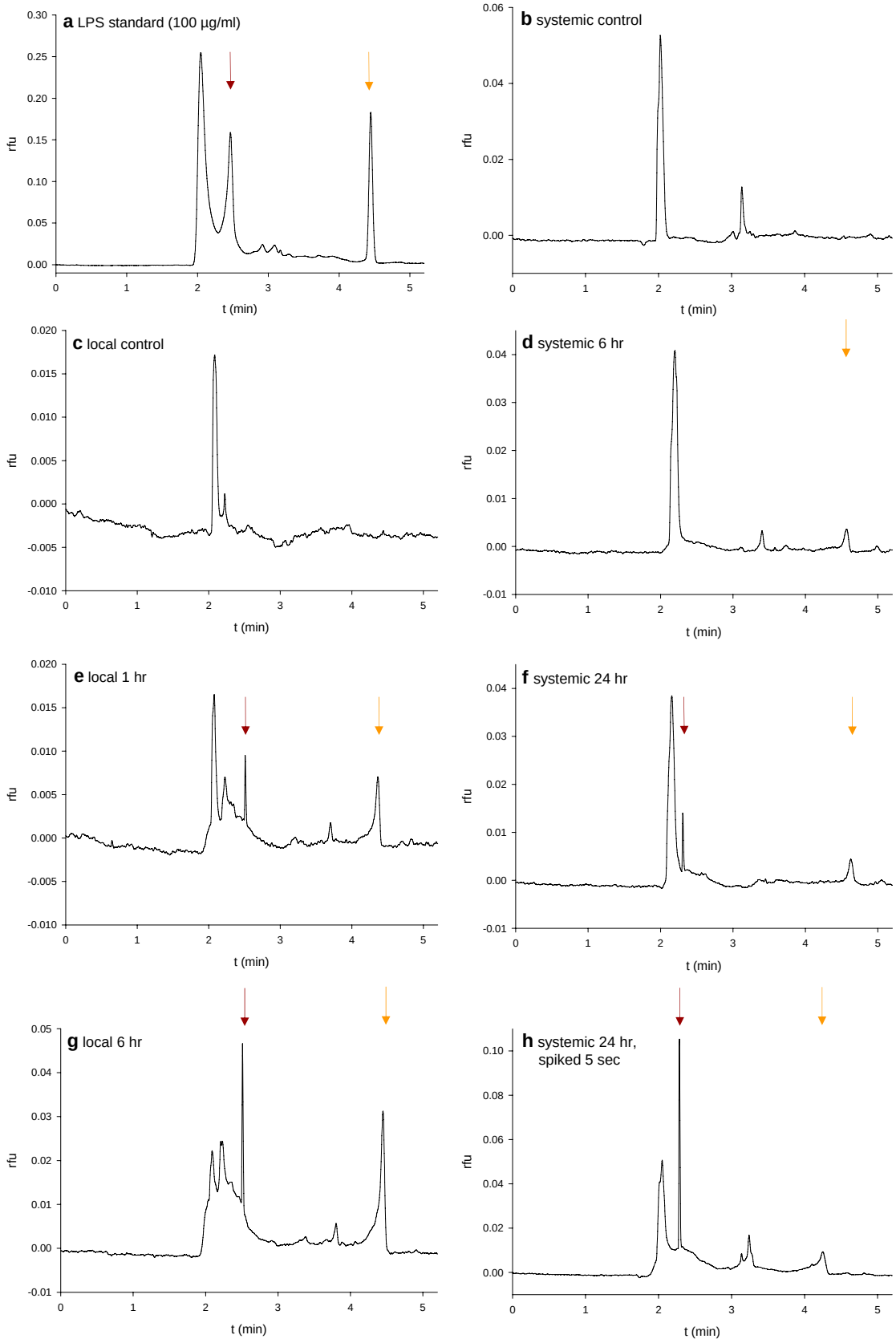

Figure 4-25: *Investigation of LPS mobilization by using Capillary zone electrophoresis.*

After the treatment of *Arabidopsis* leaves with fluorescent labeled LPS, leaves were harvested at indicated time-points and veins (local leaves) or whole leave (systemic leaves) were investigated. LPS signal (dark red arrows) became visible after 1 and 6 hr in local middle-rips and after 24 hr in systemic leaves. An additionally peak indicative for separated dye from LPS molecule was also observed (orange arrow).

To get standard separation data the first run was performed with LPS stock solution (Fig. 4-25a). For detection of LPS, peak in samples of untreated leave veins (Fig. 4-25c) were compared with treated veins after 1 hr (Fig. 4-25e) and after 6 hr (Fig. 4-25g). The LPS peak (dark red arrow) is visible after 1 hr of treatment which became higher after 6 hr. Additionally, a peak indicative for separated fluorescence group was observed (orange arrow). The same investigations were made for systemic leaves. No LPS could be detected in control leaves (Fig. 4-25b) and after 6 hr (Fig. 4-25d). But after 24 hr (Fig. 4-25f) a clear LPS signal (dark red arrow) appears which could be intensified by spiking for 5 sec with LPS standard (5 µg/ml; Fig. 4-25h).

4.8.3 DETECTION BY SDS-PAGE

To obtain further evidence of LPS mobilization, middle-rip samples of LPS treated local leaves were investigated by SDS-gel electrophoresis. After electrophoresis, gels were stained with different techniques to detect LPS and proteins. The ProQ Emerald stain is specific for LPS and visualized the characteristic LPS ladderlike pattern (dark red arrow) in middle- rips of LPS treated leaves after 1, 6 and 24 hr (Fig. 4-26, lane 5-7). Such bands reflect the number of repeating units represent in the O-chain of LPS. The protein contain in separated samples are detected with Sypro stain (Fig. 4-26B) to view the equal loading. The silver stain was used for detection of LPS and proteins together (Fig. 4-26C), whereas the LPS-pattern is also visible.

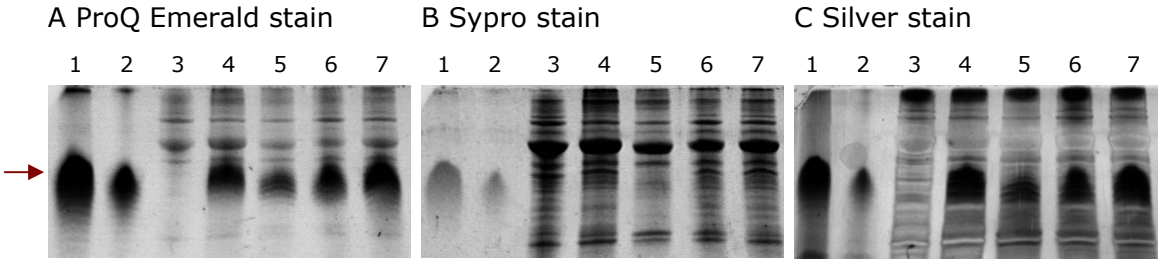

Figure 4-26: *Visualization of LPS in middle-rips of Arabidopsis leaves with SDS-gel electrophoresis.*

After gel run gels were stained with LPS specific stain [A], with protein specific stain [B] or with unspecific stain [C], respectively. Lanes: 1-LPS standard (100 µg/ml); 2-LPS standard (10 µg/ml); 3-untreated control; 4-whole treated leave direct after LPS inoculation; 5-middle-rip after 1 hr; 6-middle-rip after 6 hr and 7-midlle rip after 24 hr.

5 DISCUSSION

The aim of this study was to address the effects of LPS treatment on innate immunity of *Arabidopsis thaliana*. One aspect of plant resistance against pathogens is specific *R*-mediated innate immunity. R-genes recognize pathogen strain- or race specific factors and allow the establishment of pathogen-host specific disease resistance (Nuernberger and Lipka, 2005). Another principle represents the recognition of invariant PAMPs, which trigger innate immune responses in various vertebrate and invertebrate organisms and have long been known to act as general elicitors of defense responses in a multitude of plant species, too (Nuernberger and Brunner, 2002). In animals, one of the best studied PAMP are LPS and an important hallmark of innate immunity is the LPS-mediated induction of NO production (Nathan, 1995; Alexander and Rietschel, 2001), a molecule whose importance in plant growth and defense is just emerging (Wendehenne, Durner, and Klessig, 2004). In plants, NO and ROS play a major regulatory and/or executive role in defense responses and cell death events that are associated with microbial pathogen attack (Delledonne et al., 1998; Durner, Wendehenne, and Klessig, 1998; McDowell and Dangl, 2000).

5.1 LPS INDUCE A NO BURST AND ACTIVATE THE ATNOS1

The first finding of this work was the demonstration of LPS elicited NO production by the use of the NO-specific fluorophore DAF-FM DA in conjunction with confocal laser scanning microscopy in *Arabidopsis* cells (Fig. 4-1). This result showed that plants react like animals on LPS stimulation with production of NO (Nathan and Shiloh, 2000). Data were substantiated with different techniques (Fig. 4-2 and 4-3) and the NO burst could also be detected in *Arabidopsis* plants (Fig. 4-6, 4-7). The LPS elicited NO always appeared within a few minutes of treatment. Because of the promptness and intensity of the NO burst, the induction of NO seems to be a very early LPS response, similar to an elicitor-induced NO burst in tobacco, mechanical stress of various gymnosperms or wounding (Foissner et al., 2000; Pedroso, Magalhaes, and Durzan, 2000; Huang et al., 2004). In the human immune system NO often functions together with reactive oxygen species (ROS), for example in macrophage killing of bacteria and tumor cells, so that the capacity of LPS to elicit an oxidative burst was elucidated (Schmidt and Walter, 1994; Nathan, 1995; Delledonne et al., 1998). As shown in Fig. 4-10 and 4-11, LPS challenge resulted in rapid oxidative burst in tobacco as well as in *Arabidopsis* cells (Gerber et al., 2004). Induction of an oxidative burst was also shown for LPS from *Xanthomonas campestris* in tobacco cells (Meyer, Puehler, and Niehaus, 2001). These results emphasize the role of ROS in plant defense and LPS response.

The next investigations concentrated on the source of LPS-induced NO. In animals, NO is generated primarily by NOS, a group of evolutionarily conserved iso-enzymes that convert L-arginine to L-citrulline and NO (Nathan and Xie, 1994). Plants can produce NO

by NOS-like enzymes or by nitrate reductase (Shapiro, 2005). To obtain a first hint a pharmacological approach was performed which suggested that LPS elicited NO burst is independent of NR (Fig. 4-5), which was corroborated with the measured LPS induced NOS-activity from around 12 pmol/min x mg in *Arabidopsis* leaves (Fig. 4-7). In this experimental phase we assumed the presence of two plant NOS-like enzymes: a pathogen-inducible NOS from *Arabidopsis* and tobacco (varP; (Chandok et al., 2003)) and a hormone-activated NOS from *Arabidopsis* (AtNOS1; (Guo, Okamoto, and Crawford, 2003)). The first enzyme was described as a variant of the P protein of the glycine decarboxylase complex and was shown to produce NO in *Arabidopsis* plants that were resisting infection by turnip crinkle virus, and in tobacco plants treated with tobacco mosaic virus (Durner, Wendehenne, and Klessig, 1998). The association of varP with pathogen responses suggested this enzyme to be responsible for a LPS-induced NO burst. However, T-DNA insertion lines of varP turned out to be not affected in LPS-induced NO production (Fig. 4-8). This result was not unexpected, because the slow (transcriptional) induction of varP (Durner, Wendehenne, and Klessig, 1998; Chandok et al., 2003) does not correlate with the almost immediate NO-burst after LPS contact (Figs. 4-1 to 4-3, 4-6). In addition to varP, Guo and colleagues have cloned a NOS on the basis of its sequence similarity to a protein implicated in NO synthesis in the snail *Helix pomatia* (Guo, Okamoto, and Crawford, 2003). AtNOS1 does not share sequence identity with either mammalian NOS or the plant NOS varP, and surprisingly, displays a flavin-, heme-and tetrahydrobiopterin-independent NOS activity. AtNOS1 has been implicated in NO production in response to hormonal signals including abscisic acid and seems to be constitutively expressed. Strikingly, AtNOS1 appears to be the initial source for the LPS-mediated NO burst in *Arabidopsis* leaves, because the LPS induced NO was reduced by about 80% in the atnos1 mutant compared with the wild-type (Fig. 4-8). But the NO burst in response to LPS was not completely prevented in the mutant, suggesting that the AtNOS1 is the main but not the only NO source. Another NO generating enzyme in plants is the NR, which is a central enzyme of nitrogen assimilation in plants (Lea, 1999). NR also catalyzes the reduction of nitrite to NO, which have been demonstrated both *in vitro* and *in vivo* (Dean and Harper, 1986; Yamasaki and Sakihama, 2000; Rockel et al., 2002). NR-mediated NO synthesis plays a role in physiological processes, where it is required for ABA-induced stomatal closure in *Arabidopsis* (Desikan et al., 2002). The NR inhibitor sodium azide did not dramatically reduce the LPS elicited NO (ca. 20%); suggesting that the NR is not the main source for NO production in LPS response (Fig. 4-5). But it is possible, that the NR is involved in basal NO production. Cryptogein, an elicitor of tobacco defense responses, triggers a NO burst within few minutes in epidermal sections of tobacco leaves. This cryptogein-elicited NO was also sensitive to NOS but insensitive to NR inhibitors (Lamotte et al., 2004). Another possibility of NO formation is the non-enzymatic dismutation of nitrite to NO and nitrate. This reaction strongly depends on acidic pH and on extracellular accumulation of nitrite, which may

happen under anaerobic conditions (Stoehr and Ullrich, 2002). Apoplastic synthesis of NO occurs in barley aleuron layers when nitrite is added to the medium in which they are incubated (Bethke, Badger, and Jones, 2004). However, the biological significance of non-enzymatic NO synthesis is still unclear.

Innate immunity becomes apparent as basal resistance against pathogens. In a reverse genetics approach it was demonstrated that defective perception of the bacterial elicitor flagellin leads to enhanced susceptibility of *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Zipfel et al., 2004). Since NO is an important component of innate immunity and induced by LPS, we asked for its role in plant disease resistance. Here, we show that plants lacking NOS are more susceptible to pathogenic bacteria. Atnos1 plants showed a faster and much more severe development of disease symptoms than wild-type plants (Fig. 4-9). These results are in line with a report that demonstrated that suppression of NO through NOS inhibitors increases susceptibility to *Pst* (Delledonne et al., 1998).

What might be the molecular mechanisms underlying perception of LPS in plants? LPS is comprised of three distinct regions: lipid A, the oligosaccharide core, and commonly a long-chain polysaccharide O antigen that causes a smooth phenotype. Lipid A is the most conserved part and the endotoxic centre of LPS. It is connected to the core part, which links it to the highly polymorphic O repeating units (Beutler and Rietschel, 2003). In our hands about fifteen LPS preparations as well as LTA from Gram-positive *Staphylococcus aureus* were found to trigger rapid NO-production in suspension cultured *Arabidopsis* cells (Fig. 4-4). Lipid A was as effective as most LPS preparations and may serve as the active part of LPS, as reported for animal-microbe interactions (Alexander and Rietschel, 2001; Trent, 2004). However, interpretations of differences in LPS-mediated responses should be made with caution, since any readout (such as NO) might be related to the LPS-conformation. Biological activities of LPS are determined by the shape of their lipid A portion, and it is still unclear whether monomeric LPS molecules are able to activate cells or whether only larger aggregates or even an intact bacterial surface are active (Schromm *et al.*, 2000; Gerber *et al.*, 2004; Mueller *et al.*, 2004).

5.2 LPS INDUCE DEFENSE GENE EXPRESSION

The effects of Gram-negative bacterial LPS on mammalian and insect cells have been well documented. LPS have been shown to activate the synthesis of antimicrobial peptides in *Drosophila*, as well as the production of immunoregulatory and cytotoxic molecules in humans (Lemaitre et al., 1996; Medzhitov and Janeway, 2002). As for plants, evidence is emerging implicating bacterial LPS in enhancement of the plants response to subsequent pathogen attack by pre-treatment with LPS. While treatment of leaves with LPS from a number of bacteria did not induce the synthesis of defense-related secondary conjugates, it primed its induction upon subsequent bacterial inoculation (Newman et al., 2002). LPS

pre-treatment also potentiated the expression of PR genes upon subsequent bacterial inoculation (Dow, Newman, and von Roepenack, 2000). Currently, we can only speculate how the activation of innate immune responses in plants as a consequence of PAMP recognition works together with the more specific recognition via *avr* factors/*R* genes.
In a first step a global transcriptional profile by studying gene expression in LPS treated *Arabidopsis* cells with a microarray, consisting of ~16,000 genes was obtained. Fig. 4-12 shows that LPS regulate 13 different classes of genes distributed over the whole *Arabidopsis* genome with many components involved in defense response. To better understand LPS response and to investigate systemic processes, expression profile in *Arabidopsis* plants was examined using full genome arrays. LPS treatment resulted in the regulation of ~1,800 genes, 20% of them in systemic leaves (Fig. 4-13 and 4-14).

Concerning to the fact, that LPS has been shown to induce and to potentiate the induction of plant defense response, signaling pathways underlying this mechanism were analyzed (Erbs and Newman, 2003). One prevalent defense mechanism in plants is the development of a long-lasting resistance in response to pathogen attack (Durrant and Dong, 2004). This systemic acquired resistance (SAR) is expressed locally as well as distally from the attempted site of pathogen invasion and is connected with the expression of PR-(pathogenesis-related) genes (Uknes et al., 1992). PR-genes encode small antimicrobial proteins that are either secreted from the cell or targeted to the vacuole (Sticher, Mauch-Mani, and Metreaux, 1997). Formation of SAR is dependent on salicylic acid (SA) an important signal molecule in plant defense against pathogen attack (Gaffney et al., 1993; Shah, 2003).

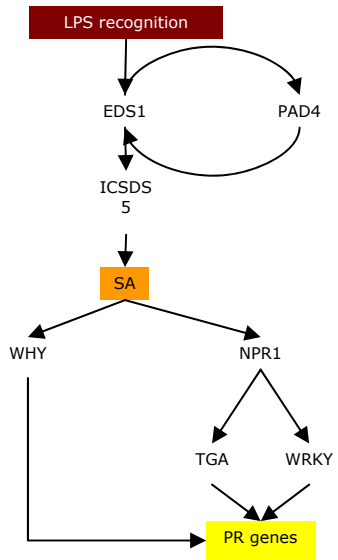

Figure 5-1: *The sequence of events from LPS recognition to defense gene induction leading to devlopment of SAR.*

Modified after (Durrant and Dong, 2004). Abbreviations; EDS, enhanced disease susebtibility; PAD, phytoalexin deficient; ICS, isochorismate synthase; SA, salycilic acid; WHY, whirly transcription factor; NPR, non-expressor of PR genes; TGA and WRKY, transcription factors; PR, pathogenesis related

SA required for SAR is synthesized from chorismate by Isochorismate synthase 1 (ICS1) just like in bacteria (Fig. 5-1; (Serino et al., 1995; Wildermuth et al., 2001)). SA accumulation is regulated by the EDS5 (enhanced disease susceptibility) gene, which may be involved in transport of phenolic compounds that are precursors of SA biosynthesis (Nawrath et al., 2002). *EDS1* in association with *PAD4* (phytoalexin deficient 4) are required for SA synthesis and regulate it upstream of *EDS5* (Feys et al., 2001). SA itself contributes to the expression of both *EDS1* and *PAD4* as part of a positive feedback loop that appears to be important in defense amplification (Wiermer, Feys, and Parker, 2005). Transduction of the SA signal to activate PR-gene expression and SAR requires the function of *NPR1* (non-expressor of pr-genes 1) (Pieterse and Van Loon, 2004). Activation of *NPR1* through SA occurs by translocation of *NPR1* into the nucleus (Kinkema, Fan, and Dong, 2000). *NPR1* acts through the members of bZIP-TGA or WRKY transcription factors, which are implicated in the activation of SA-responsive PR-genes (Zhang *et al.*, 1999; Despres *et al.*, 2000; Zhou *et al.*, 2000). The Whirly family of plant transcription factors function do not require the global SAR regulator *NPR1* for their SA-dependent activation (Durrant and Dong, 2004). They do, however, function with NPR1 to control SA-regulated gene expression (Desveaux et al., 2004). Transcripts of all named members of SAR signaling cascade were found in response to LPS treatment, partially in systemic leaves too (Fig. 4-15). This suggests the possibility of LPS to induce SAR in *Arabidopsis* plants. The resistance state is associated with local and systemic accumulation of PR-genes (Tab. 4-3 and Fig. 4-16) and has already been well documented in tobacco, cucumber and *Arabidopsis* (Uknes *et al.*, 1992; Smith, 2000).

Because LPS induced NO will be generated by the AtNOS1 (Fig. 4-8) and for a more closely examination of defense-gene expression, transcription pattern of LPS treated wild-type plants was compared with atnos1 mutant using self-made microarrays consisting of 700 defense- and stress-related genes. LPS induce an array of defense or stress-associated genes including PR-genes, glutathione S-transferases and cytochrome P450, both locally and systemically (Tab. 4-3). For example, flagellin also acted as an elicitor in whole *Arabidopsis* plants, inducing an oxidative burst and leading to the induction of defense-related genes such as *PR1*, *PR5*, *PAL1* and *GST1* (Gomez-Gomez and Boller, 2002; Zipfel et al., 2004).

Most interestingly, (defense) gene expression was almost completely abolished when atnos1 mutant plants were treated with LPS. This result suggests a functional link between LPS induced NO-production and gene induction. Performance of Northern blots for the detection of PR1-5 from LPS-treated wild-type and atnos1 plants prove this presumption and the array results. Additionally, the NO donor SNP could not only induce the PR-gene expression in wild-type plants but also rescue the mutant (Fig. 4-16). Note that these data do not implicate that gene induction by LPS is always dependent on NO. But, transcript accumulation of various defense related genes in *Arabidopsis* after NO

stimulation have been demonstrated by different scientists (Huang et al., 2002; Polverari et al., 2003).

New cognitions could be obtained by analyzing transcriptional changes in response to LPS treatment and disease susceptibility using NO or AtNOS1 overproducing plants (Li et al., 1995; Streatfield et al., 1999; Guo, Okamoto, and Crawford, 2003; He et al., 2004). If LPS mediated gene induction is really NO dependent, any NO over-producer should not be LPS inducible or show a much stronger response as wild-type plants. Because NO plays an important role in plant defense, such mutants should have enhanced disease resistance against phytopathogens than the wild-type (Fig. 4-9, (Delledonne et al., 1998; Wendehenne, Durner, and Klessig, 2004). The nox1 mutant is a NO overproducing *Arabidopsis* plant. This mutant flowered later than the wild-type, because NO represses the *Arabidopsis* floral transition (He et al., 2004). It was not time left to investigate LPS effects on this mutant, but it should be an interesting goal for the future. Another exciting test object could be an atnos1-overproducing plant. Such a mutant could be provided by Nigel Crawford and should also be analyzed in its LPS response (Guo, Okamoto, and Crawford, 2003).

Two other important signal molecules apart from NO in plant defense response are salicylic and jasmonic acid (SA and JA; (Glazebrook, 2001). It has been shown, that SA levels increase in plant tissue following pathogen infection, and exogenous application of SA results in enhanced resistance to a broad range of pathogens (Ryals et al., 1996). JA, a fatty acid derived signaling molecule, is also involved in defense against microbial pathogens. *Arabidopsis* mutants that are impaired in JA production or perception exhibit enhanced susceptibility to a variety of pathogens, including *Erwinia carotovora* (Norman-Setterblad, Vidal, and Palva, 2000; Kunkel and Brooks, 2002). The dependency of LPS induced PR-genes on SA or JA signaling was tested using nahG and jin1 plants. The first mutant carries a bacterial SA hydroxylase gene, which led to a degradation of SA to catechol (Gaffney et al., 1993). The jin1 plant is JA insensitive mutant (Berger, Bell, and Mullet, 1996) and showed no disruption in accumulation of PR transcripts (Fig. 4-19). In contrast, in the nahG plant a clearly lower expression level was measured in relation to wild-type plants (Fig. 4-19). This applies especially for the PR-genes 1-3, but also for PR 4 and 5 24 hr after LPS challenge in local leaves. At this time-point, highest induction was measured for all PR-genes in the wild-type (Fig. 4-18). Additionally, content of conjugated SA were found to be two to three fold higher as in control after 8, 24 and 48 hr of LPS treatment in local leaves (Fig. 4-21). Requirement of SA in signaling pathway leading to SAR and accumulation of SA in pathogen-infected leaves correlated with the induction of both SAR genes and resistance have already been shown for *Arabidopsis*, tobacco and cucumber (Malamy *et al.*, 1990; Métraux *et al.*, 1990; Enyedi *et al.*, 1992; Gaffney *et al.*, 1993; Uknes *et al.*, 1993). Additionally to the NO dependency of LPS-

mediated PR-gene activation these results indicate also a SA dependent PR-gene induction in response to LPS treatment.

5.3 LPS INDUCE SYSTEMIC ACQUIRED RESISTANCE (SAR)

The previously described induction of SAR signaling cascade in LPS response and the LPS mediated accumulation of PR-genes in local as well as in systemic leaves were good reasons for analyzing the ability of LPS to enhance resistance to infection by bacterial pathogens. Systemic leaves of LPS pre-treated *Arabidopsis* plants were challenged with the phytopathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 using the virulent (vir) and the avirulent (AvrRpt2) strain. LPS pre-treated *Arabidopsis* plants showed enhanced resistance against *Pst/vir* in relation to control after 2 and 5 days of infection (fig. 4-22). LPS pre-treatment of pepper leaves with LPS from *Xanthomonas campestris* pv. *campestris* (*Xcc*) lead to an analogical limiting effect on growth from *X. axonopodis* pv. *vesicatoria* (*Xav)* like here shown for *Arabidopsis* (Newman et al., 2002). Enhanced resistance against phytopathogens was also obtained by treatment of *Arabidopsis* with the chemical elicitor 1,2-bentoisothiazol-3 (2H)-one 1,1-dioxide (BIT) 4 days prior inoculation of *Pst/vir* or with the bacterial elicitors flagellin and harpin (Dong et al., 1999; Yoshioka et al., 2001; Zipfel et al., 2004). LPS-induced limitation of bacterial growth in *Arabidopsis* leaves is not as strong as after harpin or flagellin treatment. This effect could be an indirect consequence of the missing of hypersensitive response (HR) in LPS primed plants (Sequeira, 1983; Newman, Daniels, and Dow, 1997; Newman et al., 2002). HR is a programmed cell death (PCD) and its classification is based mainly on morphological criteria of the resultant cell-death lesions as well as the functional suppression of pathogen growth (Heath, 2000a, b). The HR occurs at the site of pathogen entry and involves PCD in and around the infection site. It is also accompanied by the induction of plant defense response that serves to confine the pathogen and protect the plant (Lam, Kato, and Lawton, 2001). Harpin and flagellin are strong inducers of HR in different plant species (Che et al., 2000; Tampakaki and Panopoulos, 2000; Xie and Chen, 2000; Shimizu et al., 2003; Tanaka et al., 2003; Krause and Durner, 2004). In contrast, LPS did not induce, but could prevent the HR, so that LPS pre-treatment leads to weaker plant protection against infection with phytopathogens compared with harpin or flagellin (Newman et al., 2000).

The slower growth rate of the avirulent *Pst/avrRpt2* in comparison to the virulent *Pseudomonas* strain is a normal phenomenon (Fig. 4-22; (Dong et al., 1991)). The reason therefore is, that the pathogen recognition is determined by the resistance gene RPS2 in the *Arabidopsis* plant with specificity for the bacterial avirulence gene avrRpt2 (Kunkel et al., 1993). This recognition leads to the rapid induction of plant defense mechanisms that limit multiplication and spread of the avirulent *Pst* within the *Arabidopsis* leaves (Lamb et al., 1989). The differences in bacterial growth between

control and LPS pre-treated plants which appear after 2 days of infection are no more visible after 5 days (Fig. 4-22). Accordingly, LPS priming leads merely to a somewhat faster activation of plant defense response against the avirulent strain of *Pst*. This result agrees with bacterial growth of *Xav/avrBs1* in pepper leaves after LPS treatment (Newman et al., 2002).

5.4 LPS INDUCE RECEPTOR-LIKE KINASES (RLKS)

Successful activation of gene expression by LPS through SA and NO are plant defense mechanisms which require the recognition of LPS as a pathogen derived molecule by the plant, but to date no LPS-recognition receptor is known (Montesano, Brader, and Palva, 2003). The first step in the signal perception and transduction of the LPS-induced defense responses is probably the interaction of LPS with a plant cell wall- or plasma membrane-bound receptor or binding protein. LPS have been reported to bind to the mesophyll cell wall of tobacco cells and to induce ultrastructural changes such as vesiculation (Graham, Sequeira, and Huang, 1977). In animals, LPS act as prototypic model PAMP and initial steps of LPS recognition and signaling include multiple proteins such as TLR4, CD-14 and MD-2 (Dobrovolskaia and Vogel, 2002; Miyake, 2004). However, there is no sequence with (convincing) homology to TLR4 in *Arabidopsis* genome. Furthermore, CD 14/TLR4 mediated perception of LPS operates in the pg or ng (per ml) range (Du et al., 1999), while in most plants defense responses require higher amounts of LPS (Fig. 4-3 and (Coventry and Dubery, 2001; Meyer, Puehler, and Niehaus, 2001; Newman et al., 2002; Gerber et al., 2004). On the other hand, animals possess additional, low affinity systems to detect LPS. Heat shock proteins 70 and 90, chemokine receptor 4 and growth differentiation factor 5 are main mediators of activation by bacterial LPS (Triantafilou, Triantafilou, and Dedrick, 2001). Other LPS receptors with affinity in the µg/ml range are L-selectins, which mediate production of oxygen free radicals (Baveye et al., 2000). In our opinion the putative LPS receptor in plants may be of such a low affinity type. A promising family for possible LPS receptors are receptor-like kinases (RLKs), which are transmembrane proteins with striking resemblance in domain organization to animal receptors (Shiu and Bleecker, 2001b). The diversity and the large number of *Arabidopsis* RLKs suggest that RLKs may be involved in the perception of a wide range of stimuli, including elicitor produced both during symbiosis and in plant-pathogen interactions (Endre et al., 2002; Stracke et al., 2002; Montesano et al., 2003). In *Arabidopsis*, the FLS2 protein represents the only RLK known to be involved in PAMP perception, but other members of this large family are likely to play similar functions (Gomez-Gomez and Boller, 2000). Therefore, the superfamily of RLKs with 610 members was single out from Agilent array experiments. Further, several other RLKs have been implicated in plant defense response such as the wall-associated kinase (WAK) family or the proline extensin-like receptor kinase (PERK) related genes (He, He, and Kohorn,

1998; Silva and Goring, 2002; Morris and Walker, 2003). LPS treatment resulted in the regulation in a total of 102 RLKs in plant and cell test systems, 36 of them has leucine-rich repeat domains, 6 were WAK-like and 4 PERK-like kinases (Tab. 4-1). Interestingly, most of the 16 kinases which are regulated in both model systems are localized in endomembrane system (Tab. 4-2).

Name	Subfamily	LPS												Flagellin			
		plants						cells						seedlings		cells	
		local				systemic											
		4h	8h	24h	48h	24h	48h	0.5h	1h	2h	4h	8h	24h	0.5h#	0.5h*	0.5h*	1h*
At1g70530	DUF26	-	-	-	-	-	-	0.7	0.9	0.9	1.9	1.4	1.5	1.9	-	-	-
At1g70740	DUF26	-	-	-	-	-	-	0.8	0.6	2.3	1.2	1.0	1.0	5.7	-	-	-
At4g11890	DUF26	1.0	1.8	1.1	2.1	0.8	1.4	0.4	-	0.5	1.1	1.0	1.1	-	7.5	-	-
At4g23180	DUF26	-	-	-	-	-	-	-	1.0	1.3	0.6	1.3	2.0	8.9	8.3	7.0	20.7
At4g23190	DUF26	0.6	2.0	0.9	1.6	0.8	1.1	-	-	-	0.5	0.8	2.6	9.3	9.6	3.2	8.2
At4g23210	DUF26	1.2	1.9	1.4	1.5	0.8	1.0	0.9	1.0	1.6	1.8	1.4	2.9	2.8	-	-	-
At4g23280	DUF26	0.8	1.8	1.2	1.0	0.7	0.9	-	-	-	-	-	-	-	6.5	2.7	10.5
At2g37710	L-lectin	0.8	1.7	0.7	1.9	1.0	1.2	-	-	-	-	-	-	1.5	-	-	-
At4g28350	L-lectin	-	-	-	-	-	-	0.9	-	0.6	1.3	1.2	1.2	9.1	2.5	-	5.5
At5g01540	L-lectin	0.8	2.3	1.2	2.5	0.8	1.3	-	-	-	-	-	-	10.8	-	-	-
At5g01550	L-lectin	0.7	3.1	1.3	2.7	1.1	1.1	-	-	-	-	-	-	3.9	-	-	-
At5g01560	L-lectin	2.5	1.5	1.1	0.9	1.1	1.0	-	-	-	-	-	-	3.4	-	-	-
At1g25390	LRK10L-1	-	-	-	-	-	-	0.4	1.8	0.5	1.4	0.7	1.0	2.7	-	-	-
At1g51620	LRR 1	1.0	1.8	1.3	1.4	0.9	1.1	-	-	-	-	-	-	5.7	-	-	-
At1g51790	LRR 1	1.1	1.9	1.2	1.8	0.9	1.2	-	-	-	-	-	-	2.8	-	-	-
At1g51800	LRR 1	1.0	3.2	1.4	2.7	1.4	1.4	-	-	-	-	-	-	2.9	-	-	-
At1g51850	LRR 1	1.4	2.2	2.1	1.5	1.0	1.0	0.4	-	0.5	0.9	1.0	1.0	4.1	-	-	-
At1g55610	LRR 10	0.7	1.4	1.0	2.3	1.2	1.3	-	-	-	-	-	-	-	-	-	-3.4
At1g74360	LRR 10	0.7	2.1	1.1	1.6	0.9	1.2	-	-	-	-	-	-	4.5	-	-	-
At5g48380	LRR 10	0.8	1.4	1.0	2.0	0.8	1.2	-	-	-	-	-	-	5.1	-	-	-
At2g31880	LRR 11	0.8	1.4	0.8	2.0	0.8	1.3	-	-	-	-	-	-	11.1	13.4	4.6	7.6
At5g25930	LRR 11	0.6	2.1	1.4	1.8	0.9	1.2	1.1	0.6	2.3	1.2	1.0	1.0	6.5	11.3	2.7	7.4
At4g08850	LRR 12	0.8	1.6	1.1	2.0	1.2	1.2	-	-	-	-	-	-	3.7	-	-	-
At1g53430	LRR 8-2	-	-	-	-	-	-	1.2	0.2	0.5	0.9	1.0	0.8	3.6	-	-	-
At2g33580	LysM	-	-	-	-	-	-	1.1	1.1	0.5	0.8	0.8	1.0	-	17.7	5.2	3.9
At5g46080	N. A.	0.4	2.3	0.8	1.3	1.0	1.1	1.2	1.1	0.5	1.1	1.0	1.0	2.7	-	-	-
At1g67470	RLCK 3	-	-	-	-	-	-	1.5	1.0	0.9	0.9	0.5	1.0	2.6	5.7	-	-
At5g58940	RLCK 4	0.7	1.8	1.1	1.7	0.7	1.2	-	-	-	-	-	-	15.1	-	-	-
At2g05940	RLCK 7	-	-	-	-	-	-	2.2	-	0.5	1.3	0.8	1.1	4.0	3.0	10.7	5.3
At3g59350	RLCK 8	-	-	-	-	-	-	2.2	0.8	1.3	0.2	0.5	2.7	3.2	-	-	-
At5g61560	RLCK 9	1.0	1.8	0.9	1.2	1.1	1.1	-	-	-	-	-	-	5.2	-	-	-
At1g61360	SD-1	1.5	1.9	1.4	1.4	0.9	1.0	0.9	0.3	1.6	0.7	0.7	1.0	5.9	-	-	-
At1g61370	SD-1	-	-	-	-	-	-	0.4	0.5	0.8	0.5	0.6	0.8	2.4	3.0	-	-
At1g18390	WAKL	0.9	1.8	1.2	1.3	0.9	1.1	-	-	-	-	-	-	3.6	-	-	-
At1g79680	WAKL	0.6	1.7	1.3	1.1	0.9	1.2	-	-	-	-	-	-	2.5	-	-	-

Table 5-1: *Comparison of transcriptional changes of RLKs in response to LPS and flagellin treatment.*

LPS induced RLKs in local *Arabidopsis* leaves obtained from transcript analysis with full-genome arrays were compared with flagellin induced RLKs in *Arabidopsis* seedlings and cells as shown by (Navarro et al., 2004; Zipfel et al., 2004). At the indicated time points after LPS treatment, RNA reverse transcribed from local leave tissue hybridized to the Agilent array. A complete data set is presented in the Supplement (Tab. 9-4). Transcriptional analysis from flagellin treated seedlings and cell culture was performed with full-genome Gene-Chip ATH1 from Affymetrix. Here, RLKs that respond to LPS and flagellin were present. White boxes are indicative for no or to 1.5-fold activation. Genes are highlighted in light yellow (higher than 1.5-to less than 2.0-fold activation), yellow (2.0- to less than 2.5-fold activation), orange (2.5- to less than 3.0-fold activation) and red (3.0-fold or more activation). Greenish colors indicate repression. The genes are arranged in subfamily alphabetical order. The abbreviations for the extracellular domains stand for: DUF 26, domain of unknown function 26; L-lectin, Legume lectin; LRK10L-1, wheat LRK10-like; LRR, leucine-rich repeat, the numbers refer to the number of repeats; LysM, lysine motif; RLCK, receptor-like cytoplasmic kinase; SD, S-locus glycoprotein-like domain; WAKL, wall-associated kinase like; N.A., not available. Symbols; #, data extracted from Zipfel et al., 2004; *, data extracted from Navarao et al., 2004.

Additionally, comparative analysis of LPS and flagellin induced RLKs provide a first insight into substantial overlap between LPS and flagellin recognition and highlights common defense processes.

In Table 5-1 is shown, that 35 kinases respond to both elicitors in different test-systems. This means, that 34 % of all LPS activated RLK genes are also respond to flagellin treatment. Further, in both elicitor systems was observed that more transcripts were induced than repressed. Although the FLS2 (At5g46330), a LRR-RLK responsible for flagellin recognition in *Arabidopsis* (Gomez-Gomez and Boller, 2000, 2002), was not induced by LPS, this comparison suggests that detection systems for PAMPs have similar and complementary functions in controlling pathogen invasion at different step of infection processes. The demonstration of the biological relevance of putative PAMPs and the identification of their corresponding receptors represent an exciting goal for the future.

First test-systems in identification of possible LPS recognition receptor could be knock-out mutants for severe RLKs, especially those are listed in Table 4-2 and 5-1. These mutants could be investigated for LPS insensitivity using LPS induced NO burst and activation of PR genes as analysis tools.

5.5 LPS IS MOBIL IN *ARABIDOPSIS* PLANTS

As discussed so far, LPS from the Gram-negative bacterium *Burkholderia cepacia* are able to induce a rapid and strong NO burst (Fig. 4-1 and 4-6) produced by the AtNOS1 (Fig. 4-8) and an array of defense genes in *Arabidopsis thaliana* plants and cells (Fig. 4-15; 4-16 and Tab. 4-3). In order to monitor the localization of LPS in the *Arabidopsis* plant during and after those processes, fluorescent-labeled LPS molecules from *Salmonella minnesota* were used. The possibility of plants for an uptake and the diffusion of external molecules in leaves have been shown using two fluorescent dyes as model xenobiotics in broad bean plants (Liu and Gaskin, 2004). Another studies have demonstrated the internalization of fluorescent-labeled LPS in tobacco cells (Gross et al., 2005) and the accumulation of inoculated pathogenic plant viruses in minor leave veins of *Solanaceae* and *Fabaceae* plants (Ding et al., 1998). In this study, disposal of inoculated fluorescent conjugates in *Arabidopsis* leaves was analyzed with fluorescence microscopy. After one hour of inoculation of green fluorescent LPS in *Arabidopsis* leaves the LPS appeared in the intercellular space. Three hours later LPS became visible in the middle-rip wherefrom the LPS spread through the smaller veins over the whole leave after 24 hr (Fig. 4-23). Similar results were obtained by separating middle-rip extracts with SDS gel electrophoresis and staining the gel with LPS specific dyes (Fig. 4-26). Analysis via capillary electrophoresis could be excluded that merely the fluorescent dye diffused throughout the leave. This technique was also helpful to determine labeled LPS in systemic leaves, too (Fig. 4-25). These results suggest that LPS is transported

throughout the plant in directly inoculated leaves as well as in systemic leaves and that LPS can pass plasma membranes and cell walls. The intercellular signal transduction mechanisms leading to the development of SAR are not well understood. Maybe, LPS itself is one translocated signal of SAR; it is found in systemic tissue and is able to induce SAR (Fig. 4-15; 4-23 and 4-24). However, it is also possible, that another yet unknown molecule is the responsible signal. A candidate therefore might be SA, which is an important translocated signal involved in SAR and systemic induction of PR proteins in the upper leaves of tobacco mosaic virus inoculated tobacco (Shulaev, Léon, and Raskin, 1995). Furthermore, SA synthesized in infected cotyledons of cucumber can also be translocated to the first leave and this transport occurs before SAR establishment (Moelders, Buchala, and Métraux, 1996). But the measurable levels of SA in systemic tissue after LPS treatment in *Arabidopsis* are not increased in relation to control (Fig. 4-21).

Taken together the presented results indicate that LPS triggered NO generated by the AtNOS1 in *Arabidopsis* plays a key role in LPS signaling. Furthermore, it was shown, that LPS induce alterations in gene expression in cell culture and in local as well as in systemic leaves. The LPS signaling pathway includes a NO burst, SA accumulation and SAR-gene expression leading to systemic acquired resistance against *Pseudomonas syringae* pv. *tomato* (Fig. 5-2).

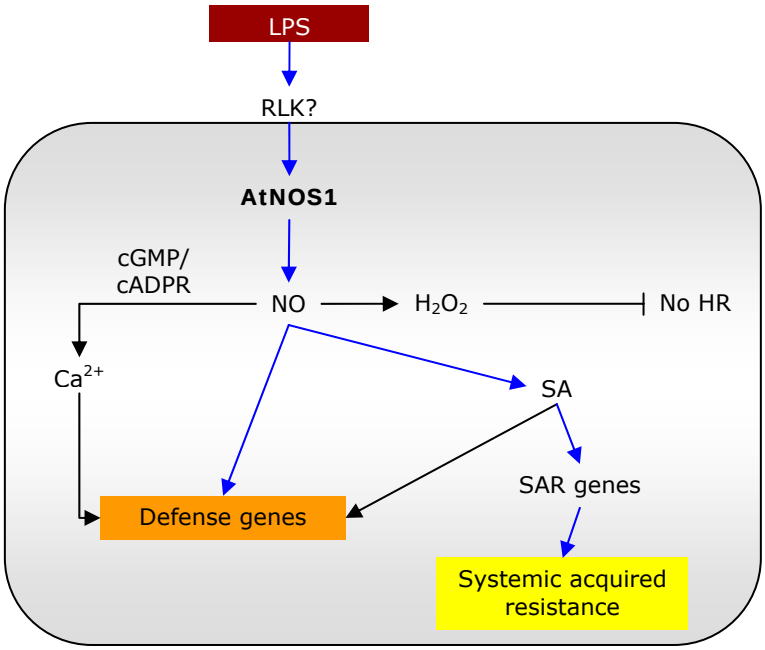

Figure 5-2: *Model for LPS signaling in Arabidopsis.*

LPS activate the AtNOS1 which resulted in a strong and rapid NO burst. NO and Salicylic acid (SA) are required for the induction of defense and systemic acquired resistance (SAR) genes. In contrast to the systemic acquired resistance mediated by defense genes, this pathway does not lead to a hypersensitive response and cell death. Blue arrows indicate LPS signaling processes presented in this work.

Thus, perception of LPS and generation of NO appear to be part of an important signaling and response system in plant–pathogen interactions involved in broad-spectrum defense mechanisms. The demonstration of the biological relevance of putative PAMPs and the identification of their corresponding receptors represent an exciting goal for the future.

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7 CURRICULUM VITALAE (LEBENSLAUF)

ZUR PERSON

Name	Dana Zeidler (geb. Georgi)
Geburtstag	02.02.1978
Geburtsort	Karl-Marx-Stadt (jetzt Chemnitz)

SCHULBILDUNG

Sep-84 to Jun-92	POS „Valeska Meinig" in Pleißa
Sep-92 to Jun-96	„Albert-Schweitzer-Gymnasium" in Limbach-Oberfrohna; mathematisch-naturwissenschaftliches Profil
Schulabschluss 1996	Allgemeine Hochschulreife

STUDIUM

Nov-96 to Feb-02	Biologiestudium an der Bayerischen Julius-Maximilians-Universität in Würzburg
Apr-99	Vordiplom
Apr-01	Diplomprüfung;
	Hauptfach: Biochemie, Nebenfächer: Genetik, Tierökologie
Mai-01 bis Jan-02	Diplomarbeit im Fach Biochemie:
	„Modifikation von tierischen Zellen zum Einsatz für die Gentherapie"
Abschluss 2002	Diplom-Biologin

PROMOTION

Seit Jul-02	Doktorarbeit im GSF-Forschungszentrum für Umwelt und Gesundheit GmbH in München am Institut für Biochemische Pflanzenpathologie:
	„Activation of nitric oxide synthase and induction of defense genes in *Arabidopsis thaliana* by bacterial lipopolysaccharides"

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9 SUPPLEMENT

Supplements will be presented as electronic dataset on CD. CD contains complete analyses from all displayed microarray experiments as pdf and excel files.

File	Table
Zeidler 9-1	LPS elicited transcriptional changes in *Arabidopsis* cell culture as analyzed with Agilent microarrays.
Zeidler 9-2	Transcriptional changes in local and systemic *Arabidopsis* leaves in response to LPS treatment as analyzed with full-genome microarrays from Agilent.
Zeidler 9-3	Transcriptional changes after LPS treatment in *Arabidopsis* plants and cells in compare to the atnos1 mutant as analyzed with in-house microarrays.
Zeidler 9-4	Receptor-like kinases from *Arabidopsis* in response to LPS treatment in both, plant and cells.