Amino acid metabolism in systemic acquired resistance of *Arabidopsis thaliana*; identification of pipecolic acid as a critical component of innate and inducible immunity

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1 SUMMARY

Plants are continuously exposed to various pathogens towards which they have evolved a sophisticated network of defence mechanisms. One of these defence mechanisms is systemic acquired resistance (SAR) which activates defence responses spreading from the site of pathogen attack through the whole plant. The resistance responses are mostly dependent on the hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). The aim of this thesis was to provide a better understanding of the molecular characteristics of SAR establishment. Therefore, I studied metabolic changes that occur in *Arabidopsis thaliana* after *Pseudomonas syringae* pv. *maculicola* (*Psm*) attack, at the site of inoculation and also in systemic (not inoculated) leaves.

Following pathogen recognition, several amino acids increased their levels, such as Trp, Phe, Leu, Lys, however, particularly pipecolic acid (Pip) and α-aminoadipic acid (Aad) highly accumulated in inoculated leaves. Pip and Aad are products of Lys catabolism. It was found that Aad production is dependent on LKR/SHD (known enzyme of Lys degradation via saccharopine pathway). In contrast, Pip level in *lkr* mutants was comparable to wild type. It was established that Pip is synthesized by AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1)-aminotransferase. The *ald1* mutant plants displayed defects in SAR as well as in basal and specific resistance. Moreover, Pip was found to highly accumulate also in leaves distal from the site of initial inoculation, and, compared to many other amino acids, in petiole exudate collected from pathogen-inoculated leaves. Exogenous application of Pip rescued the defects of *ald1* in resistance responses and increased resistance in the wild type. Thus Pip accumulation is critical for SAR and local resistance to bacterial pathogen attack.

Exogenous β-aminobutyric acid (BABA) treatment increased Pip level in wild type plants. The BABA-induced resistance was fully blocked in the *ald1* mutant, indicating that induced biosynthesis of Pip following BABA application is causative for the enhanced plant resistance. In addition, biologically-induced SAR was associated with enhanced levels of SA, phytoalexin camalexin and Pip. Exogenous Pip also positively affected SA and camalexin biosynthesis, as well as enhanced expression of defence related genes. Moreover, biologically induced defence priming was absent in the *ald1* mutant and this effect was partly restored by exogenous Pip. These data indicate that Pip promotes plants to a primed, SAR-like state.

Exogenous Pip potentiated *ALD1* expression also. This observation, combined with the fact that Pip accumulation is boosted during SAR indicates a positive defence amplification loop within SAR. This amplification seems to include SAR important player

FMO1 (*FLAVIN-DEPENDENT MONOOXYGENASE 1*), since *fmo1* mutant plants were fully blocked in Pip induced resistance, did not possess *ALD1* expression systemically, and expression of *FMO1* was primed upon Pip treatment. Characterized FMOs from plants or animals oxygenize either nitrogen- or sulphur-containing substrates. It is thus tempting to speculate that FMO1 would convert Pip or a Pip derivative into an oxygenized form to transduce the Pip signal. This hypothesis would also explain the observation that *fmo1* over-accumulates Pip in pathogen-inoculated leaves.

This thesis provides evidence that non-protein amino acid, pipecolic acid, a common lysine catabolite in plants and animal, functions as a novel critical regulator of inducible plant immunity.

1 ZUSAMMENFASSUNG

Pflanzen sind kontinuierlich verschiedenen Schädlingen ausgesetzt, gegen die sie ein hochentwickeltes Netzwerk von Verteidigungsmechanismen entwickelt haben. Einer dieser Mechanismen ist die systemisch erworbene Resistenz (Systemic Acquired Resistance, SAR), welche Verteidigungsreaktionen aktiviert, welche sich vom Ort des Pathogenangriffs auf die ganze Pflanze ausbreiten. Die Resistenzreaktionen sind hauptsächlich von Salicylsäure (salicylic acid, SA), Jasmonsäure (jasmonic acid, JA) und Ethylen (ethylene, ET) abhängig. Ziel dieser Dissertation ist, ein besseres Verständnis der molekularen Charakteristika der Entstehung von SAR zu ermöglichen. Hierfür untersuchte ich die metabolischen Veränderungen, welche nach einem Angriff durch *Pseudomonas syringae* pv. *maculicola* (*Psm)* in *Arabidopsis thaliana* auftreten, sowohl am Ort der Inokulation als auch in systemischen (nicht inokulierten) Blättern.

Als Folge der Pathogenerkennung erhöhte sich bei mehreren Aminosäuren, u.a. Trp, Phe, Leu und Lys der Spiegel. Insbesondere Pipecolinsäure (Pip) und α-aminoadipische Säure (Aad) reicherten sich stark in inokulierten Blättern an. Es wurde festgestellt, dass Pip durch AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1)-aminotransferase synthetisiert wird. *ald1-Mutanten* zeigten sowohl Defekte in SAR als auch in der basalen und spezifischen Resistenz. Weiterhin wurde festgestellt, dass Pip auch in vom Ort der Inokulation entfernten Blättern und im Gegensatz zu anderen Aminosäuren auch im Blattstiel-Exsudat, welches von mit Pathogen inokulierten Blättern gesammelt wurde, stark akkumulierte. Zuführen von Pip über die Wurzeln der Pflanzen kompensierte die Defekte von *ald1* in Resistenzreaktionen und erhöhte die Resistenz im Wildtyp. Daher ist Pip-Anreicherung von kritischer Bedeutung für SAR und lokaler Resistenz gegen Angriff durch bakterielle Pathogene.

Exogene Behandlung mit β-aminobutyric Säure (BABA) erhöhte den Pip-Spiegel im Wildtyp. BABA-induzierte Resistenz war im a*ld1*-Mutanten vollkommen geblockt, was zeigt, dass die induzierte Biosynthese von Pip nach Anwendung von BABA erhöhte Resistenz der Pflanze verursacht. Zusätzlich stand biologisch induzierte SAR in Zusammenhang mit erhöhten Mengen an SA, Phytoalexin, Camalexin und Pip. Exogenes Pip beeinflusste sowohl die Biosynthese von SA und Camalexin positiv und verstärkte die Expression von Abwehrgenen. Außerdem fehlte biologisch induziertes „defence priming“ im *ald1*-Mutanten. Dieser Effekt wurde durch exogenes appliziertes Pip teilweise wiederhergestellt. Diese Daten zeigen, dass Pip Pflanzen in einen ‚geprimeten‘, SAR-ähnlichen Zustand versetzt.

Exogenes Pip potenzierte auch die Expression von ALD1. Diese Beobachtung, kombiniert mit der Tatsache, dass die Akkumulation von Pip während SAR verstärkt ist zeugt von einem positiven Verteidigungs-Amplifikations-Loop innerhalb SARs. Diese Amplifikation scheint den wichtigen in SAR mitwirkenden Stoff *FMO1* (*FLAVIN-DEPENDENT MONOOXYGENASE 1*) zu beinhalten, da *fmo1*-Mutanten in Pip-induzierte Resistent vollkommen geblockt waren, systemisch keine *ALD1*- Expression zeigten und die Expression von *FMO1* nach Behandlung mit Pip ‚geprimt' war. Charakterisierte pflanzliche oder tierische FMOs oxidieren entweder Stickstoff oder Schwefelhaltige Substrate. Insofern liegt es nahe zu spekulieren, dass FMO1 Pip oder eines seiner Derivate eine oxidierte Form verwandelt, um das Pip-Signal zu übertragen. Diese Hypothese würde auch die Beobachtung erklären, dass Pip in pathogen-inokulierten Blättern überakkumuliert.

Diese Arbeit liefert den Beweis, dass Pipecolinsäure, eine nicht proteinische Aminosäure und ein Pflanzen und Tieren gemeiner Lysin-Katabolit, als neuer kritischer Regulator induzierbarer Pflanzenimmunität fungiert.

2 INTRODUCTION

2.1 PLANT RESISTANCE

Plants live in complex environments in which they intimately interact with a broad range of other plants, animals, and microbial pathogens. Evolution has provided plants with a highly sophisticated defence system which is triggered after pathogen attack.

The initial (constitutive) obstacles that phytopathogens encounter are physical and biochemical impediments, such as a waxy leaf cuticule, the barrier provided by the plant cell wall, which can be reinforced by the deposition of callose (glucan polymers). Moreover, the presence of many apoplastic antimicrobial compounds represents initial obstacles to infection.

Despite the diversity of pre-existing defensive barriers, many microbes succeed in breaking through this first layer of defence (Figure 1). The first active line of defence recognizes and responds to molecules common to many classes of microbes, including non-pathogens. It is based on extracellular recognition of highly conserved elicitor molecules called pathogen-associated molecular patterns (PAMPs) [more accurately called microbe-associated molecular patterns (MAMPs)] - such as lipopolysaccharides, peptidoglycans, and bacterial flagellin - through plasma membrane receptors known as pattern recognition receptors (PRRs). Activation of PRRs by PAMPs induces a range of cellular responses resulting in functional immunity called PAMP-triggered immunity (PTI).

To circumvent PTI, adapted pathogens can deliver molecules directly into the plant cell. These so called virulence effector proteins are transported into the plant cell via a specialised type-III secretion system (TTSS). The second branch of plant immunity is triggered when intracellular receptor complexes based on nucleotide-binding-leucine rich repeats proteins recognise effectors as elicitors, leading to effector-triggered immunity (ETI) (Gimenez-Ibanez and Rathjen, 2010; Spoel and Dong, 2012).

Next to this innate immunity, plants can activate yet another line of defence that is referred to as ‘induced resistance’. Over the past three decades, distinct forms of induced resistance have been identified, mainly defined by differences in the signal transduction pathways. Systemic acquired resistance (SAR) (Durrant and Dong, 2004), which is triggered by pathogen causing limited infection; rhizobacteria-induced systemic resistance (ISR) (van Loon *et al.*, 1998), which is activated upon colonisation of root by selected strains of non-pathogenic rhizobacteria; wound-induces resistance (WIR) (Howe, 2004), which is typically elicited upon tissue damage, such as caused by insect feeding or β-aminobutyric acid

(BABA) induced resistance (BABA-IR), induced by treating plant with this non-protein amino acid (Zimmerli *et al.*, 2000).

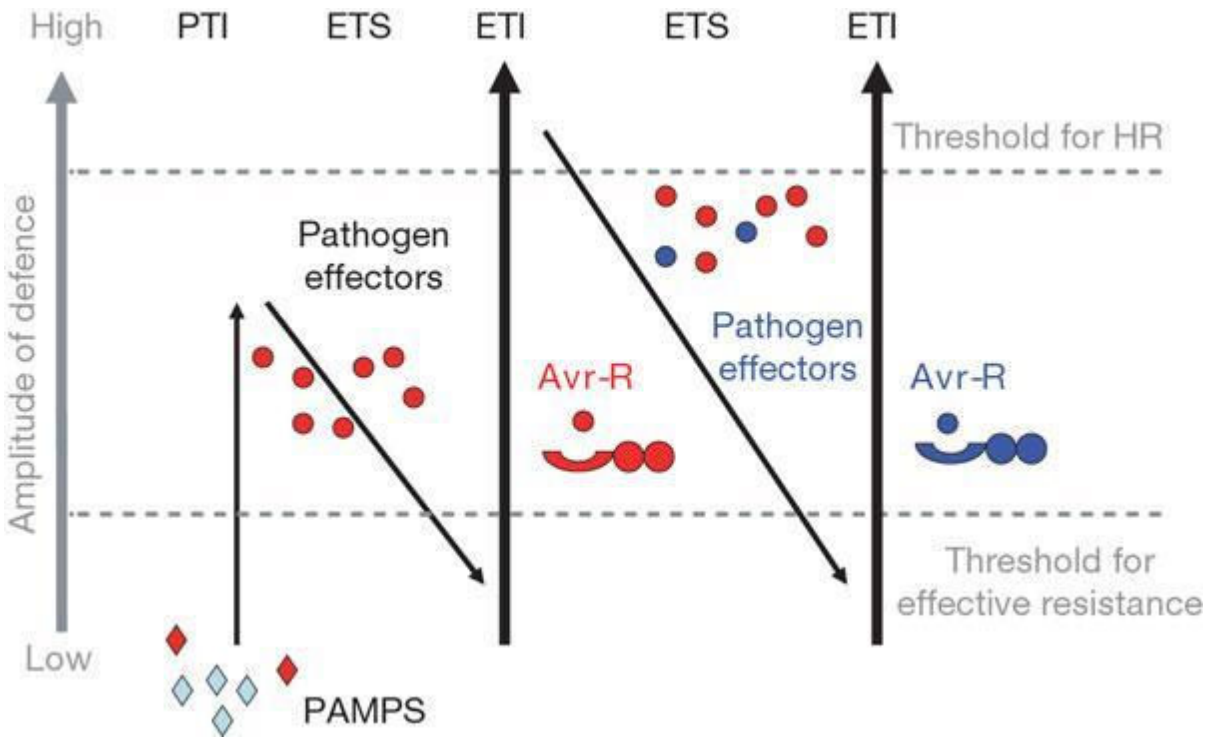


Figure 1. A zigzag model illustrates the quantitative output of the plant immune system. In this scheme, the ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI]. In phase 1, plants detect microbial/pathogen-associated molecular patterns (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). In phase 2, successful pathogens deliver effectors that interfere with PTI, or otherwise enable pathogen nutrition and dispersal, resulting in effector-triggered susceptibility (ETS). In phase 3, one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR). In phase 4, pathogen isolates are selected that have lost the red effector, and perhaps gained new effectors through horizontal gene flow (in blue)-these can help pathogens to suppress ETI. Selection favours new plant NB-LRR alleles that can recognize one of the newly acquired effectors, resulting again in ETI (Jones and Dangl, 2006).

2.1.1 PAMP-triggered immunity (PTI)

The MAMP elicitors constitute highly conserved molecular structures typical of whole classes of pathogens that are indispensable for the microbial lifestyle and cannot be easily modified to evade recognition. Similar signals, called damage-associated molecular patterns (DAMPs), are molecules released by plant cells undergoing microbe infection (Lotze *et al.*, 2007). Plants can recognise a wide number of bacterial MAMPs including flagellin, elongation factor Tu (EF-Tu), cold shock protein, peptidoglycan (PGN), lipopolysaccharides (LPS), necrosis-inducing protein, and superoxide dismutase. Oomycete MAMPs include transglutaminase, and lipid-transfer proteins (elicitins) and fungi MAMPs include xylanase, β-glucans, chitin, ergosterol, and cerebrosides (Nürnberger *et al.*, 2004).

The critical sensory function for MAMPs is conferred by PRRs which are typically localized in the plasma membrane. Two types of plant PRR have been described. These include receptor kinases (RKs) and receptor-like proteins (RLPs) (Shiu and Bleecker, 2001). The RK class of receptors is defined structurally by the presence of a ligand-binding extracellular domain, a single transmembrane-spanning domain, and a cytoplasmic kinase signalling domain. In contrast, RLPs are transmembrane proteins with a ligand-binding extracellular domain, a short cytoplasmic tail, but lacking an intracellular signalling moiety (Fritz-Laylin *et al.*, 2005).

In *Arabidopsis thaliana* (Arabidopsis), the two best-characterized MAMP/PRR pairs, flagellin/FLS2 and EF-Tu/EFR, are discussed in detail and put into a phylogenetic perspective. Both FLS2 and EFR are leucine-rich repeat receptor kinases (LRR-RKs) and use the receptor kinase BAK1 as a co-factor.

Flagellin is the principal component of bacterial flagella, the organ of motility for many bacterial species. The conserved 22 amino acid epitope present in its N-terminus is sufficient for induction of defence responses (*flg22*). *Flg22* is a ligand of the RK FLAGELLIN SENSING 2 (FLS2). FLS2 contains an extracellular leucine-rich repeat (LRR) domain for ligand binding, a transmembrane domain and a serine/threonine kinase domain. Upon treatment with flg22, FLS2 forms a heteromeric complex with brassinosteroid insensitive 1-associated kinase 1 (BAK1), an LRR-RK that also acts as co-receptor for the brassinolide receptor BRASSINOSTEROID-INSENSITIVE 1 (BRI1). This rapid oligomerization of BAK1 with FLS2 activates innate immune signalling (Gómez-Gómez and Boller, 2000). The signal-specific activation of plant PRRs by different MAMPs leads to seemingly generic responses.

EF-Tu is an essential protein involved in translation and is one of the most conserved and abundant cytoplasmic proteins in bacteria. Intact EF-Tu protein or a derived 18 amino acid epitope present in its N-terminus known as elf18 is sensed by the Arabidopsis RK ELONGATION FACTOR Tu RECEPTOR (EFR). Interestingly, EF-Tu perception is restricted to Arabidopsis and other members of the *Brassicaceae* family, suggesting that this perception system arose relatively recently compared to flagellin recognition. Moreover, BAK1 is required for full signalling upon perception of the elf18 (Kunze *et al.*, 2004). These data suggest a model in which BAK1 is a central regulator of plant signalling by acting as a general adaptor for multiple PRRs (Gimenez-Ibanez and Rathjen, 2010).

2.1.2 Effector-triggered immunity (ETI)

Pathogens have evolved the ability to suppress PTI by secreting virulent effectors directly inside the plant cell cytosol by TTSS. The intended function of these effector proteins is to promote bacterial virulence. However, specific plant cultivars have developed the ability to recognize particular effector proteins by matching resistance (R) proteins. The effectors are in this case named avirulence (*avr*)-proteins. Plant R proteins monitor the actions of isolate specific pathogen effectors, and can trigger programmed cell death, a defence reaction known as the hypersensitive response (HR).

R proteins typically consist of a variable amino terminus followed by a nucleotide-binding site (NB) domain in the middle and an LRR domain at the carboxyl terminus. NB-LRR proteins form two groups distinguished by the presence of an N-terminal coiled-coil (CC) or TIR (toll, interleukin 1R and resistance) domain (da Cunha *et al.*, 2006). Based on homology modelling to the well-studied potato R protein Rx, a general mechanistic model for R protein activation has been proposed. In the absence of a ligand, intramolecular interactions occur between the variable N terminus, the NB and the LRR domain of an R protein. This limits nucleotide exchange and hydrolysis in the central NB, thereby inhibiting the activity of the receptor. Following ligand binding, this intramolecular inhibition is thought to be alleviated, resulting in receptor activation, which is associated with nucleotide exchange and hydrolysis. In addition, receptor activation leads to possible conformational changes (Takken *et al.*, 2006).

In the *Pseudomonas syringae* – Arabidopsis interaction, essential CC-NB-LRR proteins are RPM1 which recognises AvrRpm1 and AvrB, RPS5 which responds to AvrPphB, and RPS2 which recognises AvrRpt2. The TIR-NB-LRR class includes RPS4 which recognises the effector AvrRps4. With only a few exceptions, most of the R proteins studied so far do not interact with their cognate pathogen effectors directly, but bind to 'guard' pathogen-targeted self proteins. R protein activation is triggered when self proteins are perturbed or modified by pathogen effectors. The best-studied R protein-guarded cellular target is the *A. thaliana* protein RPM1-INTERACTING PROTEIN 4 (RIN4). In the unchallenged state, R proteins that have a CC-NB-LRR domain structure detect unmodified RIN4 and this interaction maintains these R proteins (RPM1 and RPS2) in an inactive state. (1) Injection of the effector molecules AvrB and AvrRpm1 into the plant cell, results in RPM1-induces protein kinase-mediated phosphorylation of RIN4. Phosphorylated RIN4 is detected by RPM1, resulting in its activation and induction of downstream signalling (Liu *et al.*, 2011a). (2) Injection of the effector molecule AvrRpt2 (bacterial cysteine protease) into

the plant cell results in the cleavage of RIN4, which leads to the activation of RPS2 and effector-triggered immunity (Axtell and Staskawicz, 2003). RIN4 is a negative regulator not only ETI, but also PTI, where it regulates stomatal apertures via the H^+-ATPases AHA1 and AHA2 (Liu *et al.*, 2009), thereby controlling the entry of bacteria into the plant leaf.

While CC-NB-LRR and TIR-NB-LRR proteins elicit very similar reactions during ETI, they can be distinguished by their genetic requirements for function. In Arabidopsis, CC-NB-LRR proteins generally require the *NON-RACE SPECIFIC DISEASE RESISTANCE-1* (*NDR1*) gene for activity, the putative glycosylphosphatidylinositol-anchored plasma membrane protein that interacts with RIN4. NDR1 is required for immunity mediated by RPS2, RPM1 and RPS5 against *P. syringae* pv. *tomato* DC3000, and overexpression of *NDR1* in Arabidopsis leads to enhanced bacterial resistance (Coppinger *et al.*, 2004). A recent study revealed that NPR1 plays a role in preventing fluid loss and maintaining cell integrity through plasma membrane-cell wall adhesions (Chaturvedi *et al.*, 2012). The TIR-NB-LRR proteins require the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), which together with its interacting homologs PHYTOALEXIN DEFICIENT 4 (PAD4) and SENESCENCE-ASSOCIATED GENE 101, constitutes a regulatory node in immunity (Aarts *et al.*, 1998; Coppinger *et al.*, 2004).

2.1.3 Intracellular signal transduction in plant innate immunity

Most of the downstream defence events associated with PTI and ETI are shared. However, individual recognition events appear to dictate specific signalling routes that employ a distinct set of secondary messengers and activate a characteristic portion of the complex defence machinery. These responses include ion fluxes, the oxidative burst, activation of a downstream mitogen-activated protein kinase cascade, transcriptional changes and the production of antimicrobial compounds, such as pathogenesis-related (PR) proteins and phytoalexins.

In the first minutes after pathogen infection the increased influx of H^+ and Ca^{2+} and a concomitant efflux of K^+ is observed; an efflux of anions, in particular of nitrate, has also been reported. The ion fluxes lead to membrane depolarization. Rapid increase in cytoplasmic Ca^{2+} concentrations might serve as second messenger to promote the opening of other membrane channels or to activate calcium-dependent protein kinases (Lecourieux *et al.*, 2002; Ludwig *et al.*, 2005).

A characteristic marker of the defence response is the rapid production of a burst of highly reactive and toxic reactive oxygen species (ROS) such as superoxide anions (O_2^-),

hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and closely related active species of nitric oxide (NO), among others. The major ROS-producing enzymes for plant immunity are the plasma membrane NADPH oxidases which are also called respiratory burst oxidase homologs (RBOHs) (Lamb and Dixon, 1997). The H_2O_2 originating from the oxidative burst induces a number of plant genes involved in cellular protection and defence such as glutathione S-transferase (GST, EC 2.5.1.18) and is necessary for the initiation of host cell death following the HR (Levine *et al.*, 1994). ROS can act as antibiotic agents directly, or they may contribute indirectly to defence by causing cell wall cross-linking. In addition, ROS may act as secondary stress signals to induce various defence responses (Lamb and Dixon, 1997). NO has been described as a critical signal in plant-pathogen interactions. NO functions in combination with ROS to potentiate the hypersensitive cell death against *P. syringae* in both soybean cells and Arabidopsis leaves. NO increase seems to play a role in induction of the PR proteins and accumulation of salicylic acid (SA), a key resistance hormone, as well in other physiological processes, including guard cell abscisic acid signalling (Desikan *et al.*, 2002; Durner *et al.*, 1998). NO possibly functions in incompatible plant-pathogen interactions by inhibiting the plant antioxidative machinery, and thereby ensuring locally prolonged H_2O_2 levels (Zeier *et al.*, 2004). NO production promotes the S-nitrosylation of cysteine thiols, leading to the formation of protein *S*-nitrosothiols (known for regulating the activity of a wide variety of proteins involved in signalling and metabolism in mammals), which may serve protein stabilization, or functions as a mobile reservoir of NO bioactivity (Feechan *et al.*, 2005). However, NO involvement is still unclear because there was no enzyme with NO synthase activity found in Arabidopsis (Zemojtel *et al.*, 2006) and the widely-used method for measure NO may not be specific enough to discriminate it from other ROS products (Planchet and Kaiser, 2006).

Mitogen-activated protein (MAP) kinases (MAPK) constitute central points of cross-talk in stress signalling in plants including the protection against microbial invasion. MAPK pathways are ubiquitous signal transduction components in eukaryotes and transfer signals from extracellular receptors to cellular responses. A MAPK cascade typically consists of a modular complex consisting of a MAPK kinase kinase (MAPKKK), which phosphorylates a MAPK kinase (MAPKK), which phosphorylates a MAPK. These pathways regulate the activity of various substrates, such as transcription factors and protein kinases (Asai *et al.*, 2002). The Arabidopsis MAPK cascade appears to be (non-exclusively) responsive to PAMP (*flg22*, elf18) treatment or infection. In particular, the MAPKKs MKK4[5] upstream of the MAPKs MPK3[6], lead to the activation of WRKY-type transcription factors and culminate

in the expression of defence genes. Moreover, MPK6 activates ethylene biosynthesis through phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase (ACS6; EC 4.4.1.14) upon *flg22* perception (Liu and Zhang, 2004).

Taken together, calcium levels, ROS, NO, and MAPK cascades are basic elements of signalling responses; their perception orchestrates an extensive reprogramming of gene transcription in favour of defence and of regulation of hormone signalling after pathogen attack.

2.1.4 Plant defence hormones

Plant hormones play important roles in signalling networks involved in plant responses to a wide range of biotic and abiotic stresses. Significant progress has been made in identifying the key components and understanding the role of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) in plant responses to biotic stresses. SA plays a crucial role in plant defence and is generally involved in the activation of defence responses against biotrophic and hemi-biotrophic pathogens as well as the establishment of systemic acquired resistance. By contrast, JA and ET are usually associated with defence against necrotrophic pathogens and herbivorous insects. These two pathways were described as acting antagonistically to some extent (Glazebrook, 2005). A recent study found complex interactions between SA and JA-ET signalling in a detailed combinatorial study using multiple mutants blocked in different pathways. The SA and JA-ET pathways seemed to act synergistically in PTI to amplify the response. This may explain why many pathogen effectors are able to suppress PTI by interacting with different targets; because the signal itself is relatively weak, blocking just one component is sufficient to substantially perturb the response. However, the ETI response is stronger and involves redundant activities of SA and JA-ET pathways. Thus, even in the absence of SA signalling, the JA-ET response contributes to maintaining a substantial level of pathogen resistance. These compensatory interactions may simply result from the higher signal flux in ETI, and probably make this response more robust against pathogen interference (Tsuda *et al.*, 2009).

Two different biosynthetic pathways are known for the production of SA in plants (Figure 2), both starting with chorismate as a precursor. Chorismate-derived phenylalanine can be converted into SA via either benzoate intermediates or coumaric acid via a series of enzymatic reactions initially catalysed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5). Although, PAL is induced by pathogen inoculation, it is not the major pathway for SA synthesis after infection (Lee *et al.*, 1995). Chorismate can also be converted into SA via

isochorismate in a two-step process involving chloroplast-localized isochorismate synthase 1 (ICS1, EC 5.4.99.6), and putative isochorismate pyruvate lyase (EC 4.1.99.-).

Two SA induction-deficient Arabidopsis mutants, *sid1* and *sid2*, were found (Nawrath and Metraux, 1999). A breakthrough in our understanding of SA biosynthesis came when *SID2* was cloned and shown to encode a chloroplast-localized ICS1. Transcripts for *ICS1* accumulated in inoculated leaves following infection with the fungal biotroph *Golovinomyces orontii* (formerly called *Erysiphe orontii*) or a virulent strain of the bacterial hemi-biotroph, *P. syringae* pv. *maculicola*. *ICS1* expression correlated with SA accumulation and expression of the SA-associated *PR* genes. Mutation of the *ICS1* gene reduces SA accumulation after infection to only 5–10% of wild-type levels and compromise both basal and systemic resistance. This demonstrates that the isochorismate pathway in plants is the main source of SA synthesis during SAR. Since basal SA synthesis is not completely abolished in *sid2* plants, some SA must be produced either through the activity of another ICS-like protein, such as ICS2, or through the phenylpropanoid pathway. Interestingly, *ENHANCED DISEASE SUSEPTIBILITY5* (*EDS5/SID1*) encodes another protein required for SA accumulation, which has sequence similarity to the multidrug and toxin extrusion (MATE) family of transporter proteins. This suggests that EDS5 might be involved in moving SA or a phenolic precursor out of the plastid after synthesis (Nawrath and Metraux, 1999; Wildermuth *et al.*, 2001).

Once synthesized, SA may undergo a number of biologically relevant chemical modifications including glucosylation, methylation, and amino acid conjugation (Figure 2). Most of the SA produced *in planta* is converted into SA O-β-glucoside (SAG) by a pathogen-inducible SA glucosyltransferase (SAGT, EC 2.4.135). SAG may exist in an inactive storage form that can be converted back to SA. Glucosylation is also probably associated with SA detoxification. Methylation inactivates SA while increasing its membrane permeability, as well as its volatility, thus possibly allowing more effective long distance transport of this defence signal (Dean *et al.*, 2005). Moreover, a hydroxylated form of SA, 2,5-dihydroxybenzoic acid (gentisic acid), which also accumulates in plants, induces a specific set of *PR* genes in tomato that are not induced by SA (Bellés *et al.*, 1999). Amino acid (AA) conjugation of SA is less well characterized, but may be involved in SA catabolism and/or affects disease resistance. Salicyloyl-L-aspartate might be the dominant stable SA-AA conjugate, but this conjugation has been proposed to play a role in SA catabolism. The acyl-adenylate/thioester-forming enzyme (GH3.5), which is involved in the conjugation of amino acids to SA and to auxin, affects disease resistance. *GH3.5* positively regulates the SA signalling pathway in plant defence and modulates the auxin pathway to enhance host

susceptibility, whereas during an incompatible interaction SA responses are enhanced, increasing resistance (Zhang *et al.*, 2007c). In contrast to *gh3.5* mutants, null mutants in *GH3.12* (*PBS3*) exhibited substantively reduced pathogen-induced SAG accumulation, *PR* expression, and resistance to *P. syringae*. Because SA was able to rescue the compromised resistance phenotypes of GH3.12 null mutant *pbs3* (*AvrPphB susceptible3*), it likely acts upstream of SA in defence signalling. How it compromises resistance is unclear, but it was proposed that GH3.12's product, 4-hydroxybenzoate-glutamic acid, might induce or prime SA biosynthesis, with SA feedback inhibiting GH3.12's activity and thereby modulating its own synthesis (Okrent *et al.*, 2009).

Other plant hormones, including abscisic acid (ABA) (Mauch-Mani and Mauch, 2005), brassinosteroids (Nakashita *et al.*, 2003), and auxin (Wang *et al.*, 2007) have also been implicated in plant defence, but their significance is less well understood.

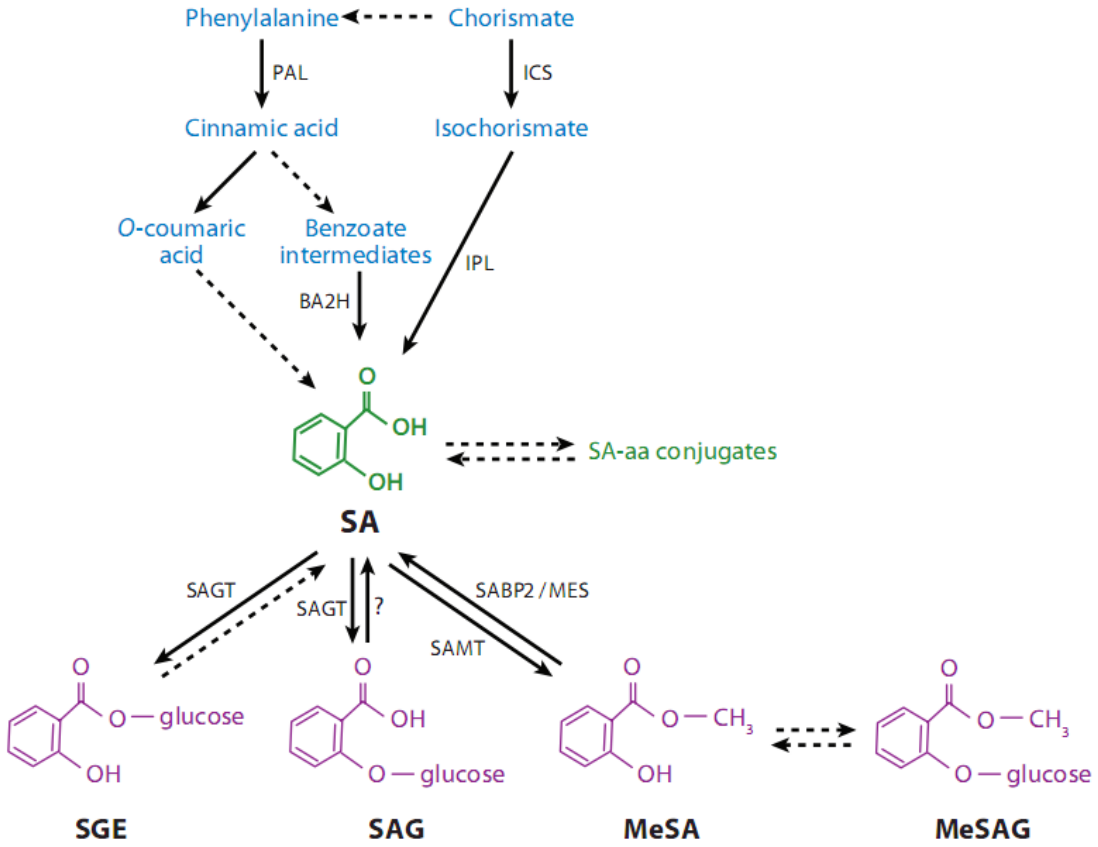


Figure 2. Simplified schema of pathways for SA biosynthesis and metabolism. Abbreviations: PAL, phenylalanine ammonia lyase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; BA2H, benzoic acid-2-hydroxylase; SA, salicylic acid; SAGT, SA glucosyltransferase; aa, amino acid; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase; SGE, salicyloyl glucose ester; SAG, SA O-β-glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate O-β-glucoside (Vlot *et al.*, 2009).

2.1.5 Systemic acquired resistance (SAR)

In the 1960s, Ross showed that tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed increased resistance to secondary infection in distal tissues. This spread of resistance throughout the plant tissues was termed systemic acquired resistance. The resistance conferred by SAR is long-lasting and effective against a broad pathogen spectrum, including viruses, bacteria, oomycetes and fungi (Durrant and Dong, 2004). SAR develops not only in response to a pathogen that causes a necrotic lesion as a consequence of a hypersensitive response (HR) but also as a result of disease symptom development in the course of a compatible interaction (Hammerschmidt, 1999a). Moreover, non-adapted (non-host) pathogens and MAMP treatment can induce SAR (Mishina and Zeier, 2007), showing that the event initiating SAR still remains elusive. Establishment of SAR is dependent on a functional SA signalling pathway. Accumulation of SA in local and systemic tissues of infected plants during SAR, as well as exogenous SA treatment, induce a signalling pathway leading to expression of *PR* genes and production of defence proteins.

2.1.5.1 SA signalling

Research over the past 25 years has revealed many defence-associated signalling and regulatory processes mediated by SA. A variety of studies have demonstrated that pathogen-induced SA accumulation is dependent on NDR1 and/or on EDS1 and its interaction partners (see 2.1.2). The ability of SA treatment to rescue defence gene induction in *eds1* and *pad4* mutant plants, as well as the SAR-deficient phenotype of *ndr1*, indicates that corresponding genes act upstream of SA in the defence signalling cascade. EDS1 and PAD4 expression are induced by SA suggests that together they may form a positive feedback loop that amplifies defence signalling (Wiermer *et al.*, 2005).

SA treatment triggers MAPK associated protein phosphorylation cascades and especially MPK3 and MPK6 are well established SA-mediated defence regulators. For instance, MAP kinase kinase 7 (MKK7), a negative regulator of polar auxin transport, is involved in basal resistance and SAR. Moreover, the MAP kinase MPK4 has been hypothesized to be a negative regulator of SAR. Recent genetic analyses suggest that MPK4 regulates both SA signalling and JA-ET defence pathways via EDS1 and PAD4 (Brodersen *et al.*, 2006; Zhang *et al.*, 2007b).

SA is capable of modulating pools of major cellular antioxidants such as glutathione, thioredoxin, and ascorbate. The redox modulating and antioxidant capacity of SA may be its major link with other stress responses in which it is involved, because ROS (see 2.1.3) occur

during many stress situations. The relationship between SA and ROS is complicated. To reconcile a considerable body of conflicting results regarding whether SA is upstream of ROS or *vice versa*, several researchers proposed that SA and H_2O_2 form a self-amplifying feedback loop (Vlot *et al.*, 2009).

A central regulatory protein of the SA pathway is NPR1/NIM1 (NON-EXPRESSOR OF PR GENES1/ NONINDUCIBLE IMMUNITY1) (Cao *et al.*, 1994). After SA accumulation in the cytoplasm, the redox state changes leading to reduction of disulphide bridges holding NPR1 in oligomeric form. NPR1 monomers are subsequently translocated from the cytosol into the nucleus, where they activate defence gene transcription (Mou *et al.*, 2003). NPR1 is an ankyrin-repeat containing protein with a BTB/POZ (*broad-complex, tramtrack, and bric-à-brac/poxvirus, zinc finger*) domain. Once inside the nucleus, NPR1 binds to TGA (TGACG motif binding) transcription factors of basic leucine zipper (bZIP) transcription factors, enhancing their binding to SA-responsive promoters. In Arabidopsis, NPR1 interacts especially with TGAs 2, 5 and 6 (this interaction is enhanced by SA), which are required for activation of *PR1* (coding an unknown protein with antifungal activity) expression (Zhang *et al.*, 2003). By contrast, expression of *PR2* (*BGL2*, coding β-1,3-endoglucanase) and *PR5* (coding thaumatin-like protein) does not necessarily depend on SA (Nawrath and Metraux, 1999). But all three PR proteins are accumulated during SAR. In addition to TGAs, several members of the WKRY (containing W-box) family of transcription factors differentially regulate SA signalling, which interacts mostly with NPR1, either positively or negatively. A recent study demonstrated that the NPR1 paralogues, NPR3 and NPR4, directly bind SA, and that this binding modulates their interaction with NPR1. NPR3 and NPR4 function as adaptors of the Cullin 3 ubiquitin E3 ligase to mediate NPR1 degradation in the nucleus (Fu *et al.*, 2012). The ability of SA to induce gene expression via an NPR1-independent pathway may require members of the WHIRLY (WHY) transcription factor family. These proteins bind single-stranded DNA in an NPR1-independent fashion.

Because SA is a defence signal, maintaining the right concentration of it in the cell is important for proper equilibrium between plant development and immune responses. SA metabolism is primarily regulated at the transcriptional level. In the absence of an inducing stress or hormone, the genes involved in SA synthesis and modification are expressed at low levels. Following an (a)biotic stress, activation of *ICS1* expression likely requires both derepression of negative transcriptional regulators, such as ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) (Chen *et al.*, 2009), and activation by positive regulators, including CALMODULIN-BINDING PROTEIN 60-LIKE G (CBP60G), SAR-DEFICIENT

1 (SARD1) (Zhang *et al.*, 2010), and/or WRKY28 (van Verk *et al.*, 2011). SA-mediated activation of NPR1 leads to feedback inhibition of *ICS1* expression, thereby preventing runaway SA accumulation. Transcriptional regulation of SA modifying genes such as *BA/SA CARBOXYL METHYLTRANSFERASE 1* (*BSMT1*) and *GH3.5* by the hormones indole-3-acetic acid (IAA, auxin), JA and ET (but not SA) also may play a role in controlling cellular SA levels. In this manner, IAA, JA, and/or ET can limit free SA accumulation, which in turn supresses the activation of SA-induced defence responses. For example, *Pseudomonas* spp. produces coronatine, which mimics JA-Ile (see 2.3.3), activating JA signalling that is able to suppress SA-mediated host defence responses. In addition, ET and JA promote the expression/stabilization of EIN3, which negatively regulates *ICS1* expression and thereby supresses SA levels. SA metabolism can be rapidly fine-tuned at the biochemical level, largely by modulating the activities of SA modifying enzymes (see 2.1.4) (Dempsey *et al.*, 2011).

2.1.5.2 SAR elusive signal(s)

SA rises dramatically in the phloem of SAR-induced tobacco and cucumber plants which led to the hypothesis, that SA itself can be the mobile signal. First experiments argue against SA being the systemic signal. Detachment of Pseudomonas syringae-infected cucumber leaves before SA levels had increased in the petiole did not block the development of SAR (Rasmussen *et al.*, 1991). Consistently, grafting experiments in tobacco between wild-type scions and NahG-expressing (*nahG* encodes salicylate hydroxylase and removes SA by conversion to catechol) plants provided evidence that SA is not the mobile SAR signal, but that an accumulation of SA in the distal leaves is necessary to establish a SAR reaction (Vernooij *et al.*, 1994).

Over the decades, scientists world-wide have tried to identify the mobile immune signal. Such a signal should be generated in the infected tissue and be rapidly transported to uninfected parts of the plant. It might also be able to encode detailed information about the primary pathogen infection. If the last criterion holds true, then more than one kind of mobile signal, functioning in a synergistic manner, might be needed to relay such complex information to systemic tissues.

After exclusion of SA, the scientist focused on methylated SA derivative (MeSA) as a candidate. Data describing the function of MeSA are controversial. Park *et al.* (2007) investigated SAR in the interaction of tobacco with TMV, and based on their results, they proposed the following model for MeSA as the mobile signal: in infected leaves,

accumulating SA is converted to MeSA, which travels through the phloem to distant leaves. There, the esterase activity of the SA-BINDING PROTEIN 2 (SABP2) reconverts the arriving MeSA into SA, causing the establishment of SAR (Park *et al.*, 2007). Also in Arabidopsis, SABP2 orthologues were found (Vlot *et al.*, 2008). Investigation of T-DNA knock out lines of some of these methyl esterases resulted in an impaired SAR activation prompting the authors to suggest that MeSA is the unique and conserved mobile signal for SAR in many plants species. By contrast, the study of Arabidopsis mutants impaired in MeSA production, which is caused by the pathogen-inducible SA methyl transferase BSMT1, disproved the above-mentioned model, because *bsmt1* knockout mutants completely lacking pathogen inducible MeSA production were still fully capable of establishing SAR (Attaran *et al.*, 2009). Furthermore, the same study demonstrates that inoculated Arabidopsis leaves emit 97% of volatile and pathogen-induced MeSA into the air, doubting a role for MeSA as a good and efficient phloem-mobile signal. However, a recent publication demonstrated that MeSA was required when primary infection occurred late in the day, when infected plants received very little light exposure before entering the dark period and that MeSA was generally required for optimal SAR development (Liu *et al.*, 2011b).

Another group of candidates for mobile SAR signals are lipid-based molecules. The *dir1* (*defective in induced resistance1*) mutant exhibits wild-type-like local defence responses, functional SA metabolism and normal response to SA, but is not able to develop SAR. Vascular exudates from pathogen-inoculated wild-type plants induced immune-related genes, whereas those from mutant *dir1* plants did not. As *DIR1* codes for a putative lipid transfer protein, the DIR1 protein might act as a chaperone for a lipid-derived SAR signal generated in inoculated leaves (Maldonado *et al.*, 2002). Support for lipid-derived long distance signals also comes from studies of mutants *pad4* and *eds1*, which are both defective in lipase-like proteins and are impaired in the establishment of SAR (Durrant and Dong, 2004). Additionally, SAR is compromised in a further mutant defective in lipid metabolism, *sfd1* (*suppressor of fatty acid desaturase 1*), with deficiency in a glycerol-3-phosphate (G3P) dehydrogenase (Nandi *et al.*, 2004). Importantly, another mutant, *gli1*, impaired in biosynthesis of G3P, fails to develop SAR, where *GLI1* encodes glycerol kinase. Resistance in distal tissues was rescued in these mutant plants by the local application of exogenous G3P or SAR-induced vascular exudates from wild-type plants. Radioactive tracer experiments showed that a G3P derivative is translocated to distal tissues, and this requires the lipid transfer protein, DIR1 (Chanda *et al.*, 2011). Mutations in three other genes involved in chloroplast galactolipid metabolism, fatty acid desaturase 7 (FAD7), monogalactosyldiacyl-

glycerol synthase 1 (MGD1), and SFD2, abolished SAR without affecting basal resistance or the accumulation of SA in leaves infected with avirulent *P. syringae* (Chaturvedi *et al.,* 2008). However, a recent publication showed that this lipid-based signalling does not play a major role in SAR responses based on fact that *dir1* and *sfd1* mutants can establish SAR under certain condition (Liu *et al.*, 2011b). In addition, C9-dicarboxylic acid, azelaic acid, was shown to accumulate in response to pathogens in the phloem, to prime plants for SA accumulation and to activate the immune-related genes. Moreover, it induced the expression of AZELAIC ACID INDUCED 1 (AZI1), which is predicted to encode a secreted lipid-transfer protein putatively involved in the production and/or translocation of a mobile immune signal (Jung *et al.*, 2009).

Another potential SAR activator is from the class of diterpenoids, which are major constituents of conifer resins. When locally applied to leaves, dehydroabietinal (DA), an abietane diterpenoid, is translocated through the plant, systemically inducing the accumulation of SA and leading to enhanced resistance against subsequent infections. In response to the biological induction of SAR, DA in vascular sap is redistributed into a SAR-inducing 'signalling DA' pool that is associated with a trypsin-sensitive high molecular weight fraction, thus suggesting that DA orchestrated SAR involves a vascular sap protein(s) (Chaturvedi *et al.*, 2012).

Peptides also have been implicated in SAR activation. In response to pathogen infection, the apoplastic aspartate protease CDR1 (CONSTITUTIVE DISEASE RESISTANCE 1) is thought to generate a mobile peptide required for SAR (Xia *et al.*, 2004).

In distant leaves, the SAR signal must be perceived and translated into responses leading to SA accumulation and *PR* genes expression. However, the mechanism by which this is accomplished is largely unknown. The ACYL CARRIER PROTEIN 4 (ACP4) was proposed to be involved in the perception of the SAR signal. Although *acp4* plants generated the mobile signal, they failed to induce the systemic immunity response (Xia *et al.*, 2009).

Plants mutated in FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) are capable of normal resistance responses at the site of infection with *P. syringae* pv. *maculicola*, but are totally compromised in systemic responses; such as SA accumulation, systemic expression of SA-dependent and -independent *PR* genes and ROS generation (Mishina and Zeier, 2006). These results show the importance of FMO1 in systemic tissue for translation of the SAR signal. Koch *et al.* (2006) described an *fmo1* mutant more locally susceptible to *H. parasitica* and *P. syringae* pv. *tomato* in comparison to the wild-type control. Moreover, *fmo1* mutants were impaired in defence reactions mediated by TIR-NB-LRRs, whereas CC-NB-

LRR-mediated defence remained effective. Expression of *FMO1* in inoculated leaves was dependent on EDS1- and PAD4-mediated pathways (Bartsch *et al.*, 2006). In contrast, these results indicate that FMO1 has a function also at the site of infection.

Flavin-containing monooxygenases (FMOs, EC1.14.13.8) in plants were discovered only recently. In humans, only five FMOs have been identified, whereas *Arabidopsis* has 29 putative FMO-like genes. FMOs catalyse the oxygenation of heteroatoms, particularly the soft nucleophilic atoms including nitrogen and sulphur in small organic molecules. In contrast to the animal FMOs, whose main function appears to be the metabolization and detoxification of xenobiotics, plants use FMOs to synthesize signalling molecules that play essential roles in many aspects of plant growth and resistance (Schlaich, 2007). Arabidopsis FMO-like protein, termed YUCCA1, was identified as a key enzyme that catalyses a rate-limiting step in a tryptophan-dependent auxin biosynthesis pathway (Zhao *et al.*, 2001). A further example of FMO, $FMO_{GS\text{-}OX}$, was found to catalyse the conversion of methylthioalkyl glucosinolate into methylsulfinylalkyl glucosinolate (Hansen *et al.*, 2007).

2.1.4.3 Long-lasting immunity

Primary pathogen exposure can induce life-long protection against secondary infection, indicating an ‘immune memory’. Immune memory in plants and invertebrate animals has been associated with cell priming, which results in a sensitized state that allows cells to respond faster and with greater amplitude to secondary pathogen attack, thereby rapidly limiting pathogen proliferation and spread. However, the molecular mechanisms of cell priming are not well understood. Priming is widely speculated to result from the cellular accumulation of signalling components that are activated only following exposure to a secondary pathogen attack. It is plausible that chromatin remodelling by proteins involved in DNA repair and homologous recombination might underpin gene priming. Changes in the methylation and acetylation status of DNA and histones have been associated with the activation of immune-related genes in plants. Moreover, it has been suggested that plants can establish transgenerational immune memory through epigenetic changes and by increasing the rate of DNA rearrangement to generate new R genes. But whether this is a widespread phenomenon requires further investigation (Spoel and Dong, 2012).

2.2 USED PATHOSYSTEM *ARABIDOPSIS THALIANA – PSEUDOMONAS SYRINGAE*

In the 1980s, *Pseudomonas syringae* was the first pathogen demonstrated to infect the model crucifer *Arabidopsis thaliana* (Arabidopsis) and cause disease symptoms in the laboratory (Dong *et al.*, 1991). Depending on the *P. syringae* strain or Arabidopsis accession, three principal outcomes of a plant-pathogen interaction can be studied: non-host resistance, incompatible interaction based on gene-for-gene resistance, and compatible interactions underlying basal resistance resulting in susceptibility.

Arabidopsis thaliana L. Heynh is a small annual flowering plant belonging to the mustard family (*Brassicaceae*), which includes cultivated species such as cabbage and rape. Its advantages as a model organism are numerous. The life cycle is short, about 6-8 weeks from germination to seed maturation, which offers easy, fast and frequent cultivation in the laboratories. Approximately 115 Mb of the 125 Mb genome was sequenced and annotated in 2000. It provides easy use for transformation with *Agrobacterium tumefaciens*, and allows the application of forward and reverse genetic approaches to investigate the approximately 30000 genes, located on 5 chromosomes. Therefore, a large number of mutants are available. *Arabidopsis thaliana* has more than 750 natural accessions. The most widely used Arabidopsis ecotypes are Columbia (Col-0), Landsberg (Ler-0), Wassilewskija (Ws) and C24. The Col-0 genome has been sequenced and the majority of available mutants have Columbia as background.

Pseudomonas syringae is a gram-negative and strictly aerobe rod-shaped bacterium with polar flagella. Specific strains are assigned to one of the over 50 known pathovars (pv.) based on their ability to infect different plant species. *P. syringae* is a phytopathogen with a biotrophic or hemibiotrophic lifestyle. The bacterium infects the host through wounds and stomata and multiplies in the intercellular spaces. In the early stages of compatible infections, host cell death does not occur, but later stages of infection are associated with host tissue chlorosis and necrosis. Many strains infecting Arabidopsis produce toxins that contribute to pathogenicity. *P. syringae* actively transports dozens of proteins into host cells through a specialized system known as type III secretion (Glazebrook, 2005). The compatible interaction which occurs after infection with virulent pathogens that overcome basal resistance, can be investigated with *P. syringae* pv. *maculicola* ES4326 (*Psm*). The discovery of bacterial avirulence genes like *avrRpm1* and the plant resistance genes *RPM1* facilitated the study of gene-for-gene resistance which is an incompatible interaction (Dangl *et al.*, 1992; Dong *et al.*, 1991). The SAR responses can be studied by local inoculation of avirulent strains

that causes a necrotic lesion as a consequence of an HR. But robust SAR is developed also upon virulent *P. syringae* inoculation (Mishina and Zeier, 2007). Some *P. syringae* strains (pv. *tomato* DC3000, pv. *syringae* B728a, pv. *phaseolicola* 1448A) are completely sequenced and therefore amenable to genetic manipulation supporting their use as a model organism.

2.3 AMINO ACIDS IN PLANT STRESS

Amino acids are more than just structural components of proteins. They serve as precursors for a large array of metabolites with multiple functions in plant growth and response to various stresses. In this part of the thesis, amino acids will be discussed in correlation to biotic or abiotic stresses.

2.3.1 Aspartate-derived amino acids

The aspartate-family network (Figure 3) consists of three major branches. The first branch leads to lysine biosynthesis. The second branch leads to the biosynthesis of methionine and the third to the biosynthesis of threonine. The entire aspartate-family pathway, except the last step of methionine synthesis (methionine synthase), occurs in the plastid and is regulated by several feedback inhibition loops (Less *et al.*, 2010).

Aspartate kinase (AK, EC 2.7.2.4) and aspartate semialdehyde dehydrogenase (ASDH, EC 1.2.1.11) catalyse the first two steps of the aspartate-derived amino acid pathway, prior to the branch-point at L-aspartate-4-semialdehyde. In *A. thaliana*, five genes have been confirmed to encode aspartate kinase activity. As a group, the aspartate kinase enzymes are subject to complex allosteric regulation (Figure 3, Figure 4). Expression of AK is also dependent on the light intensity; it is strongly enhanced by sucrose and repressed by phosphate. This regulation suggests that aspartate kinase is an important checkpoint for balancing the relative flux of different plant amino acid biosynthesis pathways (Jander and Joshi, 2010).

Bioinformatics analysis of genes coding catabolic enzymes of the aspartate-family network showed that they were generally positively correlated with stress-associated genes, while negatively correlated with growth-associated genes (Less and Galili, 2009).

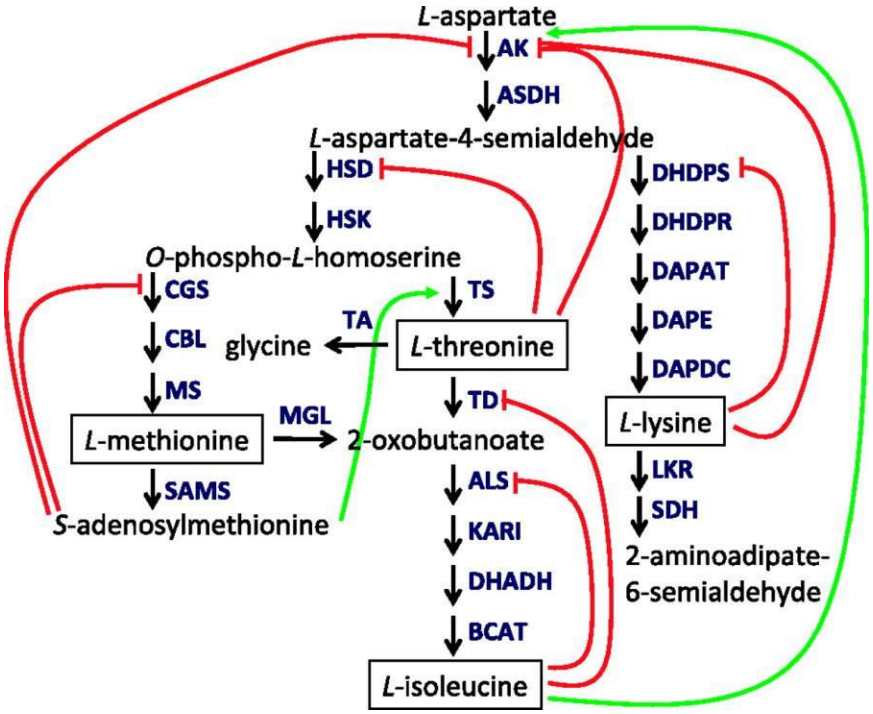


Figure 3. Biosynthesis of methionine, threonine, isoleucine, and lysine via the aspartate-derived amino acid biosynthesis pathway in plants. Known mechanisms of feedback inhibition (red lines) and activation (green lines) are indicated. AK, aspartate kinase; ALS, acetolactate synthase; ASDH, aspartate semialdahyde dehydrogenase; BCAT, branched chain amino acid aminotransferase; CBL, cystathionine beta-lyase; CGS, cystathionine gamma-synthase; DAPAT, diaminopimelate aminotransferase; DAPDC, diaminopimelate decarboxylase; DAPE, diaminopimelate epimerase; DHADH, dihydroxy-acid dehydratase; DHDPR, dihydrodipicolinate reductase; DHDPS, dihydrodipicolinate synthase; HSD, homoserine dehydrogenase; HSK, homoserine kinase; KARI, ketol-acid reductoisomerase; LKR, lysine ketoglutarate reductase; MGL, methionine gamma-lyase; MS, methionine synthase; SAMS, *S*-adenosylmethionine synthase; TA, threonine aldolase; TD, threonine deaminase; TS, threonine synthase (Jander and Joshi, 2010).

2.3.1.1 Lysine

Lysine (Lys) belongs to the group of amino acids with a positively charged side chain. Because Lys is an essential amino acid for humans, its metabolic pathway is extensively studied, with the aim to increase the levels of Lys in cereals (Pfefferle *et al.*, 2003).

Lys biosynthesis starts by degradation of a common substrate L-aspartate-4-semialdehyde with dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52), to synthetize L-2,3-dihydrodipicolinate (Figure 4). DHDPS in *A. thaliana* and other plants is feedback-inhibited by Lys and is the major regulatory checkpoint for Lys production (Jander and Joshi, 2009). The second reaction is mediated by dihydrodipicolinate reductase, synthesizing tetrahydrodipicolinate. Compared to bacteria, Lys synthesis in Arabidopsis needs just one catalytic step to convert tetrahydrodipicolinate into L,L-diaminopimelate. It is done by L,L-diaminopimelate aminotransferase (LL-DAP-AT, EC 2.6.1.83) (Hudson *et al.*, 2006). At4g33680, the locus encoding LL-DAP-AT, was previously identified based on the

phenotype of a point mutant that caused aberrant growth defects and cell death named *agd2*, where the protein AGD2 was mis-classified as a lysine transaminase (Song *et al.*, 2004a).

The saccharopine pathway is generally regarded as the major metabolic route for Lys degradation in plants. Lys is catabolized in plants via saccharopine into glutamate, pipecolic acid and acetyl-CoA (Figure 5). Two enzymes linked on a single bifunctional polypeptide catalyse the first two steps of this pathway, lysine ketoglutarate reductase (LKR, EC 1.5.1.8) and saccharopine dehydrogenase (SDH, EC 1.5.1.9). LKR first combines Lys and α-ketoglutarate into saccharopine and SDH then converts saccharopine into α-aminoadipic-δ-semialdehyde and glutamate. These two enzymatic steps can be viewed as an atypical transamination reaction in which the ε-amino group of Lys is transferred to α-ketoglutaric acid to form glutamic acid. Additional enzymatic reactions convert α-aminoadipic-δ-semialdehyde, via α-aminoadipic acid, into acetyl-CoA and several additional glutamate molecules (Galili *et al.*, 2001).

At least two other pathways for Lys degradation likely exist in higher plants, but have not yet been studied very extensively. Lysine decarboxylase (EC 4.1.1.18) activity, which catalyses the formation of cadaverine from Lys, has been reported in pea seedlings. However, although there are two genes with similarity to lysine decarboxylases encoded in the Arabidopsis genome, their potential role in Lys catabolism has not yet been investigated. A possible lysine aminotransferase in Arabidopsis (At2g13810; AGD2-LIKE DEFENSE RESPONSE PROTEIN 1, ALD1) may represent another pathway for Lys catabolism. In this case, the product of the reaction would be either piperidine-2-carboxylate or piperidine-3-carboxylate (Figure 5). However, there is yet no *in vivo* evidence for this degradation pathway in Arabidopsis or other plants (Jander and Joshi, 2010).

Lys catabolism in plants is a super-regulated metabolic pathway that has multiple developmental and stress-associated functions. Although the α-aminoadipic acid pathway consists of many enzymatic steps, its flux is apparently regulated mainly by the first bifunctional enzyme, LKR/SDH. Expression of this gene in plants is subject to complex transcriptional (and/or posttranscriptional) regulation as well as posttranslational control (Galili, 2002). Enzymatic studies suggest that LKR/SDH is a highly regulated enzyme in which LKR activity can be negatively regulated by the SDH domain by a mechanism that is controlled by Lys and operates via phosphorylation/dephosphorylation of LKR by casein kinase-II and a putative protein phosphatase. In contrast, the monofunctional LKR is a highly efficient enzyme (Galili *et al.*, 2001).

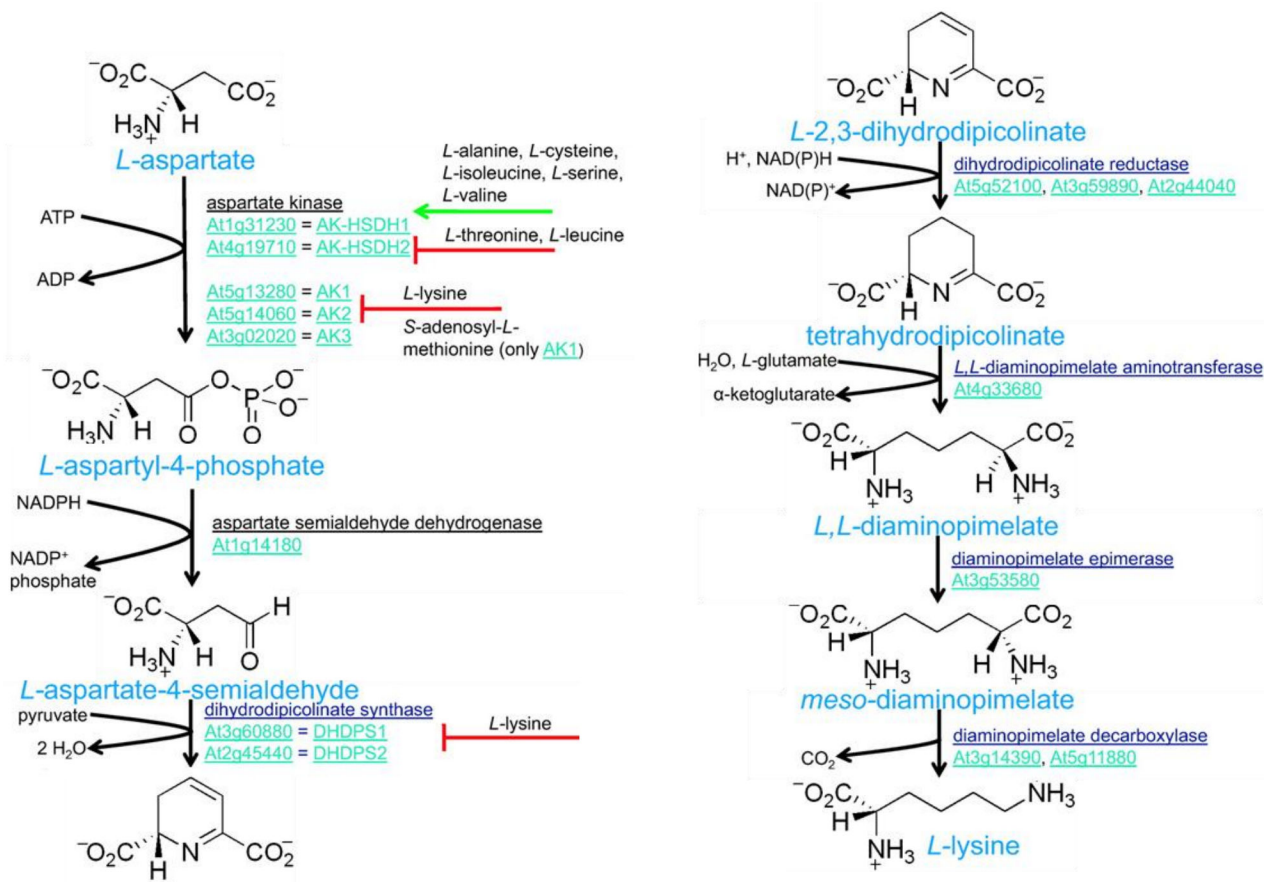


Figure 4. Lysine biosynthesis in *A. thaliana*. Enzymes involved in the biosynthesis of L-lysine from L-aspartate. Known allosteric regulation is shown, activation with a green arrow and inhibition with a red bar adopted by (Jander and Joshi, 2009).

The LKR and SDH activities in rapeseed leaves are changed in response to osmotic stress. Before the stress response, LKR and SDH activities in rapeseed leaves are relatively low and approximately equal; they are apparently performed by basal levels of bifunctional LKR/SDH. The initial response to osmotic stress includes increased SDH but not LKR activity, which is apparently due to excess production of monofunctional SDH. This increase in SDH activity may allow a slight increase in the flux of Lys catabolism. When the osmotic stress becomes more severe, there is a significant differential increase in LKR activity. This increase is apparently due to differential production of monofunctional LKR and/or stimulation of LKR activity by phosphorylation. Upon recovery from the stress, LKR activity decreases much faster than that of SDH (Moulin *et al.*, 2000).

The Arabidopsis LKR/SDH gene is strongly up-regulated by the two hormones ABA and jasmonate. The Arabidopsis LKR/SDH gene was also stimulated by carbon starvation but repressed by nitrogen starvation. These findings are in agreement with the expected regulatory function of an amino acid catabolic enzyme in carbon/nitrogen partitioning. Under sugar

starvation, plant cells generally convert amino acids to sugars to balance the sugar levels, whereas under nitrogen starvation such conversion must be blocked. One of the major catabolic products of the Lys catabolism is acetyl-CoA, which is readily converted to various sugars (Stepansky and Galili, 2003). Thus, the complex regulatory pattern of LKR/SDH gene expression in plants suggests that Lys catabolism participates in multiple metabolic processes in plants.

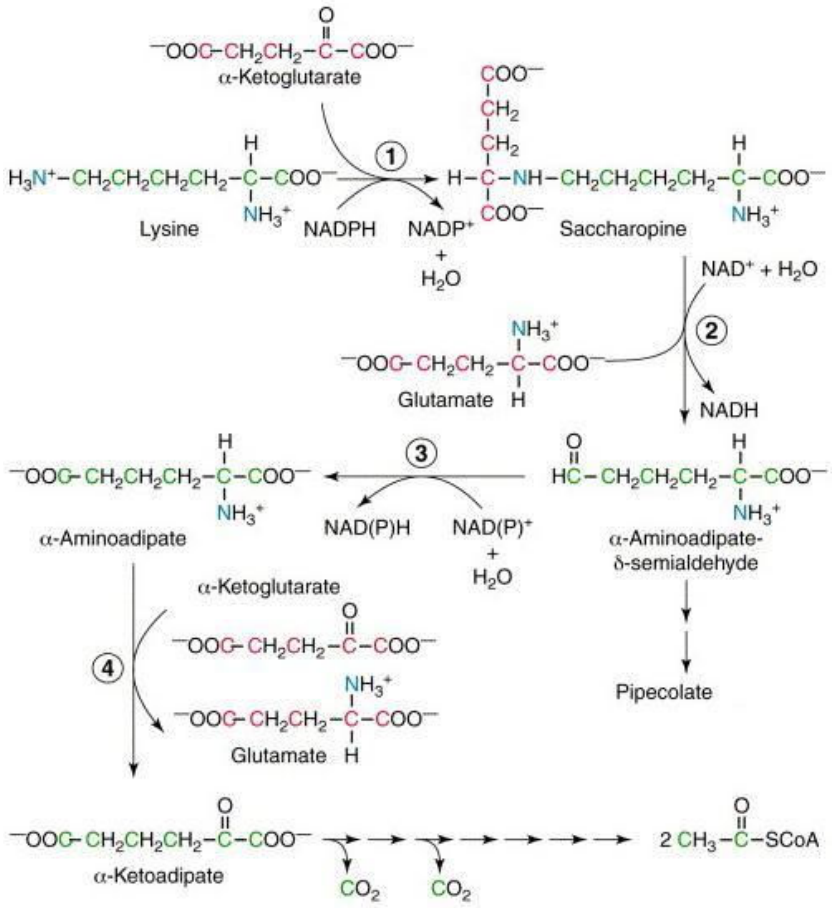


Figure 5. The pathway of lysine degradation in plants. The enzymes indicated are: (1) lysine–keto-glutaric acid reductase (LKR); (2) saccharopine dehydrogenase (SDH); (3) aminoadipic acid semialdehyde dehydrogenase (AADH); (4) aminoadipic acid aminotransferase (AAALKR–SDH and AAA activities incorporate lysine nitrogen atoms (blue) into two molecules of glutamic acid. Lys and α-ketoglutaric acid carbon atoms are green and red, respectively, adopted from (Arruda *et al.*, 2000).

2.3.1.2 Methionine, Threonine, Isoleucine

L-aspartate-4-semialdehyde, the last common intermediate in the aspartate-derived amino acid pathway, is a substrate also for homoserine dehydrogenase (HSD, EC 1.1.1.3), the committing enzyme for threonine, methionine, and isoleucine biosynthesis, which competes with DHDPS (Figure 3). Homoserine kinase (HSK, EC 2.7.1.39), which catalyses the formation of O-phosphohomoserine from homoserine, is the next step in the pathway leading

to the formation of methionine and threonine. Like L-aspartate-4-semialdehyde, O-phosphohomoserine is a branch point metabolite in aspartate-derived amino acid biosynthesis in plants. Both, threonine synthase (TS, EC 4.2.3.1), the committing enzyme for threonine biosynthesis, and cystathionine γ-synthase (CGS, EC 2.5.1.48), the committing enzyme for the methionine branch of the pathway, use O-phosphohomoserine as a substrate, and are therefore also a key regulatory point for the biosynthesis of threonine and methionine.

The great majority of methionine in plants is not utilized for protein biosynthesis, but is instead converted to S-adenosylmethionine (SAM) by S-adenosylmethionine synthase (SAMS, EC 2.5.1.6) (Jander and Joshi, 2010). SAM serves as an essential cofactor in numerous methylation reactions, for example DNA methylation (and therefore replication), chlorophyll and lignin (cell wall component) biosynthesis. SAM is also the precursor of plant metabolites such as hormone ethylene, polyamines, biotin, vitamin B1 and the iron chelator mugineic acid. Moreover, SAMS is inhibited by S-nitrosylation. Since SAM is a precursor for ethylene biosynthesis, inhibition of SAMS by NO may mediate cross-talk between ethylene and NO signalling pathways in plants. Moreover, the gene coding SAM decarboxylase was repressed under phosphate starvation. In addition, methionine is the precursor of S-methylmethionine, which plays a major role in phloem sulphur transport and is itself a precursor of the osmoprotectant 3-dimethylsulfoniopropionate and of dimethylsulfide, a biogenic source of atmospheric sulphur. Methionine also serve for isoleucine biosynthesis which its accumulation putatively serve as a response to drought stress (Amir *et al.*, 2002; Anderson, 1990; Hernández *et al.*, 2007; Ma *et al.*, 1995; Nambara *et al.*, 1998; Sun, 1998).

Methionine is further converted into aliphatic glucosinolates that occur almost exclusively in members of the *Brassicaceae*. When tissue is damaged, for example by herbivory, glucosinolates are degraded in a reaction catalysed by thioglucosidases, denoted myrosinases, also present in these species. Thereby, toxic compounds such as nitriles, isothiocyanates, epithionitriles and thiocyanates are released. The glucosinolate-myrosinase system is generally believed to be part of the plant's defence against insects and pathogens, like *Hyaloperonospora arabidopsidis* (downy mildew), *Leptosphaeria maculans* (black leg), and *Alternaria* spp. (pod spot). Methyl jasmonate (MeJA) and SA each induce also a different and limited range of glucosinolates and neither response fully mimics pathogen attack (Rask *et al.*, 2000).

Threonine can be further conversed to glycine, that is important for seed development (Joshi *et al.*, 2006) or to isoleucine which plays a role in osmotic stress (Nambara *et al.*, 1998).

2.3.2 Aromatic amino acids

The aromatic amino acids (AAA), phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) are central molecules in plant metabolism. The biosynthetic pathways of aromatic amino acids proceed via central metabolite chorismate. Chorismate is produced with seven reactions catalysed by six enzymes initiates from phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E-4P), the shikimate pathway (Figure 6). The first enzyme of shikimate pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS; EC 2.5.1.54), catalyses the formation of 3-dehydroquaianate from PEP and E4-P. In Arabidopsis plants, the DAHPS1 gene is induced by physical wounding and MeJA (Devoto *et al.*, 2005; Yan *et al.*, 2007), by infiltration with pathogenic *P. syringae* strains (Keith *et al.*, 1991), by redox state (Entus *et al.*, 2002), and ABA (Catala *et al.*, 2007). 5-Enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), which catalyses the formation of enolpyruvylshikimate-3-phosphate (EPSP), is the sixth enzymatic reaction (Tzin and Galili, 2010). The expression of the EPSPS gene is induced in response to infection by the necrotrophic fungal pathogen *Botrytis cinerea* (Ferrari *et al.*, 2007) and by sulphate starvation (Nikiforova *et al.*, 2003).

Beyond chorismate, the shikimate pathway branches into a pathway leading to Phe and Tyr and into another one leading to Trp. The first committed step of Phe and Tyr biosynthesis is catalysed by chorismate mutase (CM, EC 5.4.99.5), which converts chorismate to prephenate. Arabidopsis has two plastidic CMs, CM1 and CM3. CM1 is generally inhibited by Phe and Tyr and activated by Trp. The expression of *CM1* and, to a lesser extent, *CM3* is inducible in response to elicitors and pathogen treatments (Mobley *et al.*, 1999). It was found that *Ustilago maydis* actively secretes CM (Cmu1) into the plant cell as a virulence factor. The common precursor, chorismate, is then preferably converted to prephenate, and therefore less salicylic acid is synthesized. Such a host-manipulated plant has suppressed resistance responses (Djamei *et al.*, 2011). Prephenate can be converted by two different enzymatic pathways to Phe. The first pathway is via arogenate, catalysed by the enzymes prephenate aminotransferase (PAT, EC 2.6.1.79) and arogenate dehydratase (ADT, EC 4.2.1.49) respectively. Recently identified *A. thaliana* genes, encoding PATs, drive carbon flux from prephenate toward arogenate, making the arogenate pathway predominant in plant Phe biosynthesis (Maeda *et al.*, 2011). The second pathway includes conversion of prephenate via phenylpyruvate (PPY) to Phe, using enzymes with prephenate dehydratase (PDT) and AAA aminotransferase activities (Tzin and Galili, 2010). The AAA aminotransferase activity was recently confirmed by Tzin *et al.* (2009), when transgenic Arabidopsis plants with bacterial enzyme constitutively converting prephenate to PPY did not accumulate PPY but Phe.

Arogenate is then also converted into Tyr by arogenate dehydrogenase (ADS, EC 1.3.1.43). An alternative route of Tyr biosynthesis has also been suggested, which includes the conversion of prephenate to p-hydroxyphenylpyruvate and subsequently is converted into Tyr by a broad range AAA aminotransferases, but this route was not really confirmed *in planta* (Tzin and Galili, 2010).

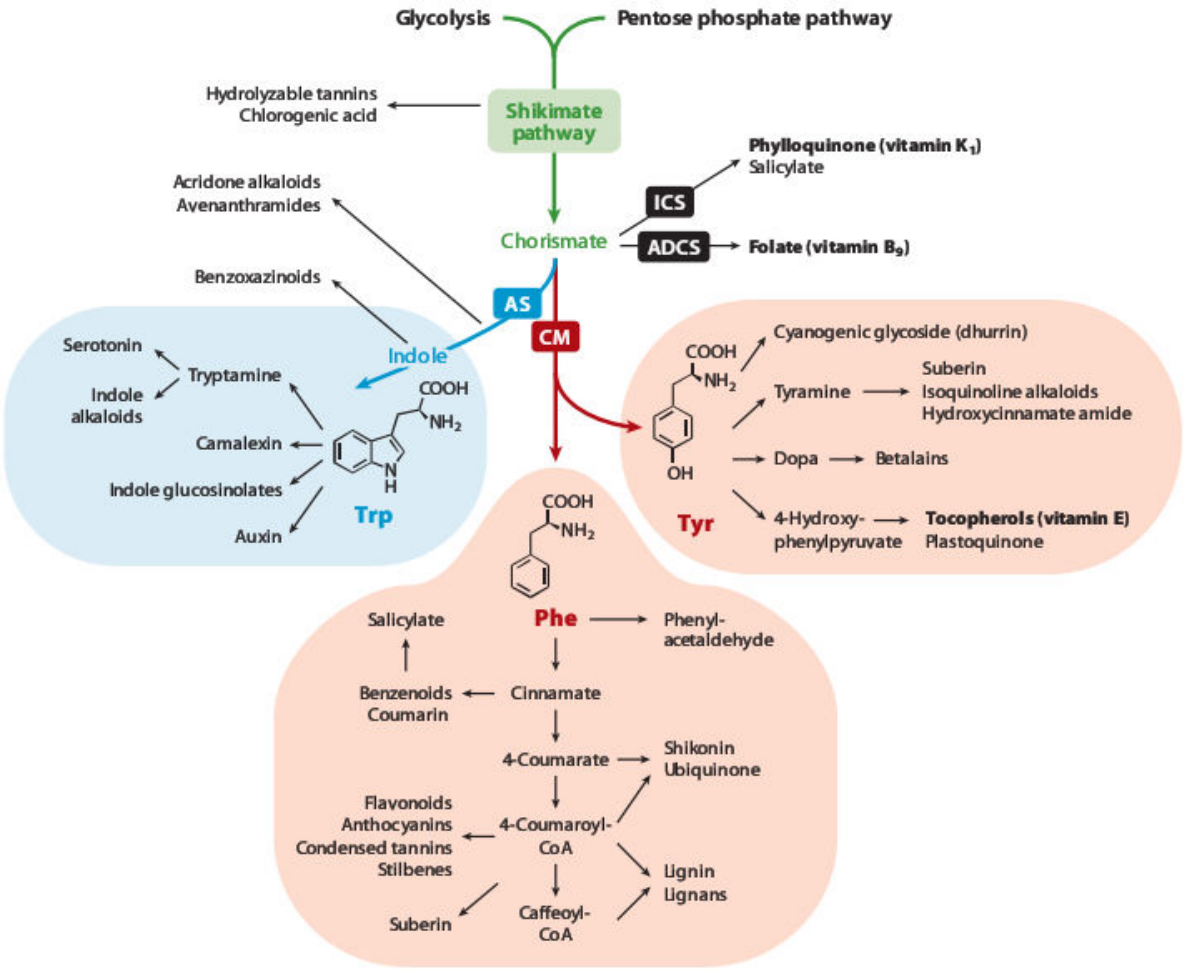


Figure 6. The aromatic amino acid pathways support the formation of numerous natural products in plants. The shikimate pathway (shown in green) produces chorismate, a common precursor for the tryptophan (Trp) pathway (blue), the phenylalanine/tyrosine (Phe/Tyr) pathways (red), and the pathways leading to folate, phylloquinone, and salicylate. Trp, Phe, and Tyr are further converted to a diversearray of plant natural products that play crucial roles in plant physiology, some of which are essential nutrients in human diets (bold).Other abbreviations: ADCS, aminodeoxychorismate synthase; AS, anthranilate synthase; CM, chorismate mutase; CoA, coenzyme A; ICS, isochorismate synthase (Maeda and Dudareva, 2012).

The first committed step of Trp biosynthesis includes a transfer of an amino group of Gln to chorismate to generate anthranilate and pyruvate, catalysed by anthranilate synthase (AS; EC 4.1.3.27). The fourth enzyme of the Trp biosynthesis pathway is indole-3-glycerol phosphate synthase (IGPS; EC 4.1.1.48), which catalyses the formation of indole-3-glycerol phosphate from 1-(O- carboxyphenylamino)-1-deoxyribulose-5-phosphate. IGPS provides an important enzymatic step in the biosynthesis of Trp and the hormone auxin because it is the

only known enzyme that catalyses the formation of the indole ring. The Arabidopsis gene encoding IGPS is regulated by the hormones jasmonate (Dombrecht *et al.*, 2007) and salicylate (Chibani *et al.*, 2006). The last two steps of Trp biosynthesis are catalysed by Trp synthase (TS, EC 4.2.1.20), which contains both alfa (TSα) and beta (TSβ) subunits. Indole-3-glycerol phosphate is cleaved by TSα to indole and glyceraldehyde-3-phosphate (α-reaction). Then, indole is transported to TSβ, which catalyses its condensation with serine (β-reaction) to produce Trp. TSα and TSβ Arabidopsis proteins (as well as AS) are induced after *Pseudomonas syringae* pv. *tomato*, *P. s.* pv. *maculicola* or *Xanthomonas campestris* pv. *campestris* infection, after abiotic elicitors silver nitrate and α-amino butyric acid, under amino acid starvation and under oxidative stress (Zhao *et al.*, 1998; Zhao and Last, 1996).

In bacteria, this AAA pathway is almost exclusively used for protein synthesis, but in plants, AAA also serve as precursors for a variety of plant hormones as well as for a very wide range of aromatic secondary metabolites with multiple biological functions.

Phe serves as a precursor for a large array of multiple functional secondary metabolites. Most plant phenolic natural compounds are derived from trans-cinnamic acid, formed by deamination of Phe by PAL. Phenylpropanoids, include flavonoids that protect the plant against UV-B radiation (Casati and Walbot, 2005). Phenylpropanoid-based polymers, like lignin, suberin, or condensed tannins, contribute substantially to the stability and robustness of herbivore gymnosperms and angiosperms and towards mechanical or environmental damage, like drought or wounding. Nearly 1% of the secondary metabolites derived from Phe are volatile compounds involved (Vogt, 2010). Another class of sulphur-rich Phe-derived secondary metabolites includes the Phe-glucosinolates but they are generally not widespread in Arabidopsis (Tzin and Galili, 2010).

Tyr serves as a precursor of several families of secondary metabolites, including tocochromanols (vitamin E), plastoquinones, isoquinoline alkaloids, several non-protein amino acids, and perhaps also some phenylpropanoids. In Arabidopsis, putative Tyr/L–dopa decarboxylase (TYDC, EC 4.1.1.25) increased its mRNA level after treatment with fungal elicitors (Trezzini *et al.*, 1993). In plants, TYDC catabolizes Tyr into tyramine on the route of producing of cell wall-bound hydroxycinnamic acid amides. The deposition of hydroxycinnamic acid amides, and other phenolics, in the cell wall, is believed to create a barrier against pathogens by reducing cell wall digestibility and/or by directly inhibiting the growth of fungal hyphae (Facchini *et al.*, 2000).

Trp is catabolized into many indole-containing secondary metabolites, such as IAA (Östin *et al.*, 1998), terpenoid indole alkaloids (Facchini *et al.*, 2004), and tryptamine

derivatives (Facchini *et al.*, 2000). The indole ring is also used in the biosynthesis of certain phytoalexins, low molecular weight antimicrobial compounds, ranging from simple aliphatics to complex terpenoids that are synthesized by plants in response to infection (Arabidopsis -*P. syringae*, tomato - *Cladosporium fulvum*, potato -*Phytophthora infestans*) (Hammerschmidt, 1999b).

Camalexin (3-thiazolylindole), the major indolic phytoalexin in Arabidopsis, accumulates upon leaf infection with both biotrophic and necrotrophic plant pathogens. These pathogens include bacteria (*P. syringae* pv. *tomato* or pv. *maculicola* or pv. *syringae*, *Xanthomonas campestri)* (Glazebrook and Ausubel, 1994), viruses (cauliflower mosaic virus, turnip crinkle virus) (Callaway *et al.*, 1996; Dempsey *et al.*, 1997), fungi (*Hyaloperonospora arabidopsidis*, *Alternaria brassicicola*) (Glazebrook *et al.*, 1997; Thomma *et al.*, 1999), and oomycetes (*Phytophthora* species) (Roetschi *et al.*, 2001). Camalexin originates from cysteine and tryptophan, which is converted to indole-3-acetaldoxime and subsequently dehydrated to indole-3-acetonitrile (Rauhut and Glawischnig, 2009). The camalexin biosynthetic capacity is not restricted to leaves, as also roots infected with the oomycete *Pythium sylvaticum* accumulated camalexin (Bednarek *et al.*, 2005). In addition, a number of abiotic treatments, presumably triggering the formation of reactive oxygen species (ROS), induced camalexin biosynthesis. These include application of heavy metals ions (Tsuji *et al.*, 1993), ROS-inducing chemicals, such as acifluorfen (Zhao *et al.*, 1998), UV-B radiation (Mert-Türk *et al.*, 2003), the toxin fusaric acid (Bouizgarne *et al.*, 2006), silver nitrate (Zhao and Last, 1996) and the volatile allo-ocimene (Kishimoto *et al.*, 2006). This compound was shown to inhibit pathogen growth *in vitro* and to accumulate in plants in a pattern negatively correlated with *in vivo* pathogen growth. Camalexin deficient *pad3* (mutation in CYP71B15, the last enzyme in camalexin synthesis) mutant was more susceptible to *H. arabidopsidis* and *B. cinerea* in comparison to wild type. The *pad4* mutation was shown to be a recessive allele of a single gene, and causes reduced camalexin synthesis in response to *P. syringae* infection, and strongly enhanced susceptibility to *P. syringae* (Glazebrook *et al.*, 1996). Other mutants were affected in camalexin induction include *pad1, pad2, pad5* (Glazebrook *et al.*, 1997), *bos2* (Veronese *et al.*, 2004) and *ald1*, which is defective in an aminotransferase (Song *et al.*, 2004b). Therefore camalexin plays a role in defence against (hemi)biotrophic pathogens.

Moreover, transcripts associated with several metabolic pathways, particularly plastid-based primary carbon metabolism, pigment biosynthesis, and AAA metabolism, are significantly modified by the bacterial challenge within the first 12 h post inoculation (Truman *et al.*, 2006).

2.3.3 Branched chain amino acids

Valine (Val), leucine (Leu) and isoleucine (Ile) contain short branched carbohydrate residues responsible for their classification as branched-chain amino acids (BCAA). In plants it seems clear that these amino acids are synthesized in plastids, Val and Leu originate from pyruvate, while Ile is synthetized from Thr where the first reaction is catalysed by threonine deaminase (TD, EC 4.2.1.16) to form α-ketobutyrate. TD has been very recently found among several jasmonate inducible plant enzymes that accumulate in the midgut of herbivorous insect larvae, where it is responsible for the degradation of Thr and in effect lowering the availability of this essential amino acid for the herbivore (Chen *et al.*, 2005). Val and Ile are synthesized in two parallel pathways each with a set of three identical enzymes catalysing the reactions with different substrates, resulting in the generation of 3-methyl-2-oxopentanoate (3MOP) that is transaminated to Ile, or of 3-methyl-2-oxobutanoate (3MOB) that is either transaminated to Val or that serves as substrate for the biosynthesis of Leu. The latter requires four additional reactions including a final transamination reaction converting 4-methyl-2-oxopentanoate (4MOP) to Leu (Knill *et al.*, 2008). All of the final transamination steps are catalysed by the pyridoxal-5'-phosphate-dependent branched-chain aminotransferases (BCATs, EC 2.6.1.42). In Arabidopsis, these enzymes are encoded by a small gene family of six transcribed members. These are targeted to different sub-cellular locations in the plant, one in the mitochondria, two in the cytosol, and three in the plastids (Diebold *et al.*, 2002). Urano *et al*. (2009) reported that the dehydration-inducible accumulation of BCAAs was correlated with induced expression of the BCAT2 gene, which is regulated by endogenous ABA (Urano *et al.*, 2009).

Many-fold higher accumulation of BCAAs during transient osmotic stress followed by rapid depletion during the recovery process clearly implies tight regulation of the BCAA catabolism by environmental perturbation. High level of BCAA was also observed after high temperature stress. It has been suggested that, to avoid excessive accumulation, BCAAs promote their own degradation during seed germination, senescence, or under sugar starvation, and thereby provide alternative carbon sources for plants during stress conditions (Taylor *et al.*, 2004).

Ile is an important player of JA defence signalling pathway, its conjugation to JA create the active hormone JA-Ile that binds to the SCF^{COI1} protein (COI1 is an F-box component of SKIP-CULLIN-F-box complex) and so assists in its binding to a jasmonate ZIM domain (JAZ) protein. The complex is then degraded by the ubiquitin ligase-dependent 26S proteasome pathway. This interrupts the repression of JA-responsive genes (by the

release of MYC2 and possibly other transcription factors) and eventually leads to local and systemic defensive response, such as the emission of predator-attractive volatiles (Yan *et al.*, 2009).

Isoleucine-related molecule, isoleucic acid (2-hydroxy-3-methyl-pentanoic acid) was recently shown to enhance resistance towards *P. syringae*, positively regulates SA pathway and *PR1* expression. Isoleucic acid, but also valic acid (2-hydroxy-3-methylbutyric acid) inhibited root growth, indicating also resistance related properties (von Saint Paul *et al.*, 2011).

2.3.4 Proline

In plants, proline (Pro) is synthesized mainly from glutamate, which is converted by two successive reductions catalysed by pyrroline-5-carboxylate synthase (P5CS, EC 2.7.2.11) and pyrroline-5-carboxylate reductase (P5CR, EC 1.5.1.2), respectively. P5CS is a bifunctional enzyme catalysing first the activation of glutamate by phosphorylation and second the reduction of the labile intermediate γ-glutamyl phosphate into glutamate semialdehyde (GSA), which is in equilibrium with the Δ^1-pyrroline-5-carboxylate (P5C) form. An alternative precursor for Pro biosynthesis is ornithine (Orn, that is created from arginine by arginase), which can be transaminated to P5C by Orn-δ-aminotransferase (δ-OAT, EC 2.6.1.13), a mitochondrial located enzyme. Pro biosynthesis occurs in the cytosol and in the plastids, like chloroplasts in green tissues (Kishor *et al.*, 2005; Verbruggen and Hermans, 2008).

Pro degradation is the reverse process of Pro biosynthesis and catalysed by Pro dehydrogenase (PDH, EC 1.5.99.8) and P5C dehydrogenase (P5CDH, EC 1.5.1.12) and takes place in mitochondria.

Pro accumulation has been reported to occur after high salinity, drought (Yoshiba *et al.*, 1995), high light and UV radiation (Saradhi *et al.*, 1995), heavy metals (Schat *et al.*, 1997), oxidative stress or nutrient deficiency (Hare and Cress, 1997). Pro was considered as an inert compatible osmolyte that protects subcellular structures and macromolecules under osmotic stress. However, Pro accumulation can influence stress tolerance in multiple ways: (1) it has been shown to function as a molecular chaperone able to protect protein integrity and enhance the activities of different enzymes, (2) as an antioxidant, suggesting ROS scavenging activity and Pro acting as a singlet oxygen quencher, (3) it is feasible that Pro metabolism can stabilize cellular homeostasis during stress conditions (Pro biosynthesis in chloroplasts during stress can maintain the low $NADPH/NADP^+$ ratio, contribute to

sustaining the electron flow between photosynthetic excitation centres, stabilize the redox balance, and reduce photoinhibition and damage of the photosynthetic apparatus) (Szabados and Savouré, 2010).

ABA has strong and moderate enhancement on the expression of P5CS and P5CR during abiotic stress, respectively (Ábrahám *et al.*, 2003). Local Pro accumulation was observed in Arabidopsis challenged with avirulent *P. syringae* strains, though a direct causal relationship has not been demonstrated (Fabro *et al.*, 2004). Exogenous application of 0.5mM SA increased Pro levels in lentil (*Lens esculenta*) (Misra and Saxena, 2009), but plants with low SA levels due to mutation of *EDS5* or overexpression of a bacterial salicylate hydroxylase (NahG) did not show enhanced P5CS expression or Pro accumulation in response to pathogen attack (Fabro *et al.*, 2004).

It was recently shown that Pro catabolic products can play role in biotic stress. After MeJA treatment, Pro (as well as Arg, and Glu) was converted to Orn, which was acetylated by NATA1 to produce the uncommon non-protein amino acid N^{δ}-acetylornithine. Both insect feeding and *P. syringae* infection increase NATA1 expression and N^{δ}-acetylornithine accumulation (Adio *et al.*, 2011). Pro degradation enzyme, PDH, was also recently determined as a defence component contributing to HR caused by *P. syringae* pv. *tomato avrRpm1* and disease resistance. Expression of *PDH* was found to be dependent on salicylic acid, and an increase in PDH activity was detected in cells destined to die. *PDH*-silenced plants displayed reduced ROS and cell death levels as well as enhanced susceptibility in response to avirulent pathogens (Cecchini *et al.*, 2011).

2.3.5 Histidine

Histidine (His) biosynthesis is linked to purine metabolism through its precursors 5-phosphoribosyl-1-pyrophosphate and ATP, and the release of an intermediate (5′-phosphoribosyl-4-carboxamide-5-aminoimidazole) at the branch point step catalysed by imidazole-glycerol phosphate synthase (IGPS, EC 2.4.2.-), which enters the *de novo* purine synthesis pathway. The first compound of the His biosynthesis branch is imidazole glycerol phosphate; an α-keto compound is obtained by dehydration of the glycerol phosphate moiety. This compound undergoes a transamination reaction to yield a compound that upon dephosphorylation is still charged, so that it can serve as a biosynthetic intermediate. Dephosphorylation yields histidinol, which is the only histidine biosynthetic intermediate that can enter the cell at an appreciable rate. Histidinol is oxidized in a two-step reaction to form

His. While the route of His biosynthesis has now been established, the pathway of His catabolism in plants has not been elucidated (Ingle, 2011).

His is not extensively studied amino acid in plants. But it was shown that increased endogenous pool of free His resulted in enhanced tolerance of Arabidopsis to Ni^{2+} ions (Wycisk *et al.*, 2004). His was also found to act as chelator of Zn^{2+} (Salt *et al.*, 1999) and Cu^{2+} ions in plants (Liao *et al.*, 2000).

2.3.6 Other amino acids

Amino acid metabolism may play an important role in plant stress tolerance through the accumulation of compatible osmolytes (Campalans *et al.*, 1999), by intracellular pH regulation, and by detoxification of reactive oxygen species, xenobiotics, and heavy metals (Alia *et al.*, 2001). The pool of free amino acids would be available for the synthesis of new proteins in response to stress.

It was shown that during oxidative stress (60 μM menadione) the levels of alanine, β-alanine, asparagine, glycine, glutamate, glutamine, serine, aspartate and glycine were decreased compare to control (Baxter *et al.*, 2006). Several amino acids (β-alanine, asparagine, alanine, γ-aminobutyric acid) were observed to accumulate under heat stress and on the other hand, arginine, cysteine, glycine and serine were increased in pool size under cold stress (Kaplan *et al.*, 2004).

The non-protein amino acid β-aminobutyric acid (BABA) is extensively studied. In 1994 BABA was determined as a protective compound to *Peronospore tabacina* in Tabacco (Cohen, 1994). The broad spectrum protective effect of BABA against numerous plant diseases has been well documented (Cohen, 2002). In the case of Arabidopsis, BABA treatment increased resistance against *H. arabidopsidis* and *P. syringae* pv. *tomato* (Zimmerli *et al.*, 2000), *Plectosphaerella cucumerina* and *Alternania brassisicola* (Ton and Mauch-Mani, 2004), and *Botrytis cinerea* (Zimmerli *et al.*, 2001). BABA was shown to be effective not only to necrotrophic and biotrophic pathogens, but as well to abiotic stresses like drought and salinity (Jakab *et al.*, 2005). One of the cellular mechanisms behind BABA-induced resistance is callose deposition at sites of penetration, along walls of the penetrated host cells and in the pistil which causes female sterility. The cellular mechanisms behind BABA-IR are diverse. Although BABA-IR against *P. syringae* pv. *tomato* depends solely on SA and NPR1 (Zimmerli *et al.*, 2000), the BABA-IR against pathogenic fungi and oomycetes is controlled by a different defence pathway involves ABA- and phosphoinositide (PI)-dependent signalling (Ton and Mauch-Mani, 2004). Recently, Tsai *et al.* (2011) found that BABA

specifically represses plant responses to bacterial effector coronatine. BABA treatment blocked a coronatine-mediated stomata opening and suppression of SA signalling. BABA resistance, therefore, seems to involve SA-dependent, SA-independent and ABA-dependent defence mechanisms, and the importance of these defences varies according to the nature of the challenging pathogen (Ton *et al.*, 2005).

3 MATERIAL AND METHODS

3.1 PLANT MATERIAL

Seeds of wild-type *Arabidopsis thaliana* (L. Heynth., Arabidopsis) ecotype Col-0, mutants in Col-0 background *ald1* (SALK_007673), *lkr-1* (SALK_068769), *lkr-2* (SALK_127160) from the SALK Institute collection (Alonso *et al.*, 2003) and *fmo1* (Mishina and Zeier, 2006), *npr1-2* (*npr1*, NASC ID: N3801), *pad4-1* (*pad4*, Glazebrook *et al.*, 1997), *cpr5* (Bowling *et al.*, 1997), *sid2-1* (*ics1*, Nawrath and Métraux, 1999) were sown in soil. The 14-days-old seedlings were transferred into 120-ml pots containing a mixture of soil (Klasmann-Deilmann, Substrat BP3) vermiculite and sand (8:1:1). Plants were cultivated in a growth chamber at photoperiod 9 h day (110 µE/m^2/sec, 21°C) and 15 h night (18°C) cycle at 70% relative humidity. Plants were watered as needed. All experiments were done with 5-6-weeks old, unstressed plants exhibiting a uniform appearance.

Nicotiana benthamiana plants were grown in a green house and kindly provided by F. K. H. Breuers (A. P. M. Weber laboratory, Heinrich Heine Universität).

3.2 CULTIVATION OF PSEUDOMONAS SYRINGAE

The virulent pathogen *Pseudomonas syringae* pv. *maculicola* strain ES4326 (*Psm*), strain *Pseudomonas syringae* pv. *maculicola* ES4326 with plasmid carrying the avirulence gene pLAFR3::*avrRpm1* [*Psm(avrRpm1)*] were grown on King's medium B (King *et al.*, 1954) containing agar plates supplemented with appropriative antibiotics: *Psm* with 50 µg/l rifampicine, and *Psm(avrRpm1)* with 50 µg/l rifampicine (A2220, AppliChem) and 15 µg/l tetracycline (A2228, AppliChem), for 2 days at 28°C.

3.3 INOCULATION OF PSEUDOMONAS SYRINGAE

Prior inoculation, bacteria were cultured overnight at 28°C (240 rpm) in 2.5 ml liquid King's medium B with appropriate antibiotics. The log phase cultures were washed three times with 10 mM $MgCl_2$ (Acros Organics) and diluted to the desired optical density at 600 nm (OD_{600}). For determination of local metabolite levels, local gene expression, SAR induction and petiole exudates collection, *Psm* diluted to OD_{600} 0.005 was used. For bacteria grows assays, *Psm* was diluted to OD_{600} 0.001 and *Psm(avrRpm1)* to OD_{600} 0.002.

The bacterial solution was infiltrated by 1ml syringe without a needle into the abaxial side of a leaf area covering about 80% of total leaf area. Control (mock) inoculations were performed with 10 mM $MgCl_2$ only.

For characterization of systemic resistance responses, three lower 1° leaves of a given plant were first infiltrated with a suspension of virulent *Psm* (OD_{600} 0.005) between 10-12am to induce SAR, or with 10 mM $MgCl_2$ as a control. Two days later (except for time course analyses), non-treated, upper 2° leaves were either harvested for metabolite determination and gene expression analysis, or plants were inoculated on three upper leaves with *Psm* (OD_{600} 0.001) for growth determination (as described below).

For assessment of defence priming during SAR, 2° leaves were infiltrated with either 10mM $MgCl_2$ or *Psm* (OD_{600} 0.005) two days after the 1° leaves treatment. 2° leaves were collected 10 hours after they had been treated.

For growth assays plants were inoculated with virulent *Psm* (OD_{600} 0.001) or *Psm(avrRpm1)* (OD_{600} 0.002) and levels of bacteria were assessed 3 days later. The 3 discs originating from infiltrated areas of different leaves were homogenized in 500 µl of 10 mM $MgCl_2$, plated on appropriate dilutions on King's B medium containing 50 µg/l rifampicin, and colony numbers were quantified after 2 to 3 days.

3.4 EXOGENOUS CHEMICAL TREATMENT

One day prior to local inoculation or 1° treatments in case of a SAR experiment, 10 ml of 1mM (10 µmol) D,L-pipecolic acid solution (S47167; Sigma-Aldrich), 10ml of 0.5mM (5µmol) L-pipecolic acid solution (P1404; TCI Europe), 10ml of 0.5mM (5µmol) D-pipecolic acid solution (P1830; TCI Europe) or 10 ml of 1mM (10 µmol) β-aminobutyric acid (A44207; Sigma-Aldrich) solution was pipeted onto the soil substrate of individually cultivated plants. Control plants were supplemented in the same manner with 10 ml of water only.

For different complementation strategies, the leaves were sprayed with 500µM D,L-pipecolic acid solution supplemented with 0.02% Silwet at 6 and 24 h post inoculation with *Psm* (OD 0.005). Leaves were co-inoculated with *Psm* suspension supplemented with 1mM D,L-pipecolic acid, as the third complementation method.

3.5 CHARACTERIZATION OF MUTANTS

The T-DNA insertion in each line was confirmed by three PCRs with gene-specific and T-DNA-specific primers as follows. Genomic DNA (gDNA) was extracted from leaf of 4-week-old plants. Leaf material was ground by a tissue homogeniser (Qiagen, TissueLyser II) in Eppendorf tube, and resuspended in 800 µl of extraction buffer (1.5M Tris-HCl pH 8.0; 1M NaCl; 0.5M EDTA; 10% w/v SDS). The sample was heated for 5 min at 65°C and then 200 µl of chloroform was added. The plant material was pelleted by 5 min centrifugation at

14000 rpm. The supernatant (500 µl) was transferred to a new Eppendorf tube and precipitated with the same volume of 2-propanol for 10 min, -20°C. The gDNA was pelleted by centrifugation 5 min, at 14000 rpm and the pellet was rinsed by 70% v/v ethanol, centrifuged and air-dried for 10 min. The resulting pellet was dissolved in 30 µl of water (MolBio grade, AppliChem). An aliquot (2 µl) of the resulting DNA solution was amplified in the first PCR with only gene-specific primers (RP+LP; Table I) and another two aliquots were amplified with gene-specific primer RP or LP and LBa primer specific for the T-DNA insertion (Table I). PCR was performed with 0.25 µM of each primer, 60 µM of each nucleotide, 0.25 U of Taq DNA polymerase (M0273, New England Biolabs) and 5x ThermoPol buffer in the final volume of 20 µL. PCR annealing temperature was 55 °C, polymerising for 1 min and 35-times amplified. The PCR product was separated on a 1% w/v agarose gel with 0.5x TAE buffer [20 mM Tris; 10 mM boric acid; 0.5 mM EDTA] and 0.05 µg/ml ethidium bromide and visualized under UV light (GelDoc, INTAS). Based on the result of the three PCRs, seeds of plants homozygous in the T-DNA insertion were harvested and used for further experiments.

Table I. List of primers used for characterization of T-DNA insertion lines.

Locus	Gene name	T-DNA line	Primer sequence	
At2g13810	*ALD1*	SALK_007673	LP	5′ TTACGATGCATTTGCTATGACC 3′
			RP	5′ TTTTAAATGGAACGCAAGGAG 3′
At4g33150	*LKR*	SALK_068769	LP	5′ TCATTCTGCCTTCTCCATCAG 3′
			RP	5′ AGCAACAACGATATTTCGTGG 3′
At4g33150	*LKR*	SALK_127160	LP	5′ CGCTTCGATCATATCAAGAGC 3′
			RP	5′ CCCCTATGACTTTCTGTGCAG 3′
At1g29930	*CAB1*	abundant gene	Fw	5′ GCCGCCGTGTACCCTTCGCTTCTC 3′
			Rv	5′ ATGCGCCTCCCGGGCACTTCTTCT 3′
			LBa	5′ TGGTTCACGTAGTGGGCCATC 3′

3.6 GAS CHROMATOGRAPHIC DETERMINATION OF DEFENCE METABOLITES

The determination of metabolites levels in leaves was performed by a modified vapour-phase extraction method (Schmelz *et al.*, 2004). Briefly, ca 150 mg frozen leaf tissue was homogenized with 600 µl of extraction buffer (H_2O : 1-propanol : HCl, 1:2:0.005). After addition of internal standards (D_4-salicylic acid, dihydrojasmonic acid, indole-3-propionic acid; 100 ng each) and 1 ml of dichloromethane (KK47, GC ultra-grade, Roth), the mixture was shaken thoroughly and centrifuged at 14 000 rpm, 1 min for phase separation. The lower,

organic phase was then removed, dried over Na_2SO_4 and treated with 2 µl of 2M trimethylsilyldiazomethane (36,283-2, Sigma-Aldrich) in hexane for 5 min at room temperature to convert carboxylic acid groups into their corresponding methyl esters. After stopping the methylation reaction with 2 M acetic acid in hexane, the sample was subjected to a vapour phase extraction procedure (Schmelz *et al*., 2004) using a volatile collector trap packed with Porapak-Q absorbent (VCT-1/4X3-POR-Q; Analytical Research Systems). The final evaporation temperature was set to 200°C, and samples were eluted from the collector trap with 1 ml dichloromethane. Finally, the sample volume was reduced to 15 µl in a stream of nitrogen, and then subjected to GC/MS analysis.

For determination of bound SA levels, the upper, aqueous phase resulting from the centrifugation step previously described was supplemented with 100 ng D_4-salicylic acid for internal standardization and 1 ml of 0.1M hydrochloric acid and was heated to 100 °C for 30 min to convert the bounded SA to free SA. After cooling, the aqueous solution was extracted three times with 2 ml dichloromethane, and the combined organic extracts were dried over Na_2SO_4. After removal of the organic solvent under a stream of nitrogen, the residue was dissolved in 400 ml of dichlormethane/methanol (3:1), methylated and subjected to vapor phase extraction as described previously.

The sample mixture (4 µl) was separated on a gas chromatograph (GC 7890 A; Agilent Technologies) equipped with a fused silica capillary column (ZB-5MS 30m x 0.25mm, Zebron, Phenomenex) and combined with a 5975C (EI) mass spectrometric detector (Agilent Technologies). The initiation injection was at 250°C and then the metabolites were separated by a temperature program: 50°C/3min with 8°C/min to 240°C, with 20°C/min to 320°C/3 min, under constant flow of helium, 1.2 ml/min. For quantitative determination of metabolites, peaks originating from selected ion chromatograms were integrated. The area of a substance peak was related to the peak area of the corresponding internal standard [SA (m/z 120) –D_4-salicylic acid (m/z 124); jasmonic acid (m/z 151)–dihydrojasmonic acid (m/z 156), camalexin (m/z 200)–indolepropionic acid (m/z 130), azelaic acid (m/z 152) - dihydrojasmonic acid (m/z 156)], and experimentally determined correction factors for each substance/standard pair were considered.

3.7 GAS CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS

The amino acid levels were determined by the EZ:faast free amino acid analysis kit for GC-MS (Phenomenex), which is based on the separation and mass spectrometric identification of propyl chloroformate-derivatized amino acids (Figure 7) (Kugler *et al.*,

2006). Homogenized leaf material (50mg) was extracted with 200µl of buffer (25% acetonitrile in 0,01N HCl). The sample was shaken thoroughly for 15 min at room temperature and centrifuged at 14 000 rpm, for 4min. Aliquot (100µl) of supernatant was extracted following the EZ:faast user's manual (Phenomenex). The dry residue was then dissolved in 70µl of dichloromethane and subjected to GC/MS analysis.

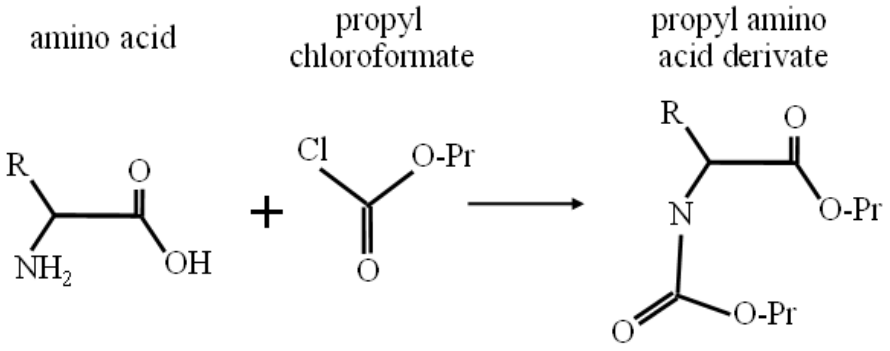


Figure 7. Schema of propyl chloroformate derivatization of amino acids. Conversion of amino groups into propyl carbamate and carboxyl groups into propyl ester derivatives.

The sample mixture (3 µl) was separated on a silica capillary column (ZB-AAA 10m x 0.25mm, Zebron, Phenomenex). The initiation injection was at 250°C and then the metabolites were separated by a temperature program: 70°C/3min with 8°C/min to 240°C, with 20°C/min to 320°C/2 min, under constant flow of helium, 1.2 ml/min. For quantitative determination of individual amino acids, peaks originating from selected ion chromatograms were integrated: Gly (m/z 116), Ala (m/z 130), Val (m/z 158), β-Ala (m/z 116), Leu (m/z 172), Ile (m/z 172), GABA (m/z 130), Ser (m/z 146), Thr (m/z 101), Pro (m/z 156), Pip (m/z 170), Aad (m/z 244), Asp (m/z 216), Glu (m/z 84), Asn (m/z 69), Gln (m/z 84), Cys (m/z 248), Orn (m/z 156), Lys (m/z 170), His (m/z 282), Phe (m/z 148), Tyr (m/z 107), and Trp (m/z 130). The area of substance peak was related to the peak area of norvaline (m/z 158), which served as an internal standard. Experimentally determined correction factors for each amino acid were considered. Arg and Met could not be analysed with the applied method.

3.8 QUANTITATIVE REAL-TIME PCR ANALYSIS

Total RNA was isolated from frozen leaves using peqGOLD RNAPure reagent (PeqLab, Erlangen) following the manufacturer's instructions. RNA concentration was determined by absorption at 260 nm (BioPhotometer plus, Eppendorf) and 1 µg was treated with DNase I (EN0521, Fermentas) for 30 min at 37°C to remove genomic DNA. The DNase reaction was inactivated by incubation at 70°C for 10 min in the presence of 2.5 mM EDTA.

The mRNA was converted to cDNA with the OligodT primers and the reverse transcriptase (205113, Omniscript RT Kit, Qiagen) according to the manufacturer's instructions. An equivalent of 11.3 ng of total RNA was amplified in the total 10 µl of reaction volume with 5µl of SenziMix SYBR Green (SMP5-111C, Bioline) and 0.75 µM gene-specific primers (Table II). The qPCR reaction was performed in triplicate in a Rotor-Gene Q apparatus (Qiagen) using the following cycling program: 95°C for 7 min, followed by 45 cycles at 95°C for 10s, 60°C for 30s, and finally 72°C for 3 min.

The gene encoding a polypyrimidine tract-binding (PTB) protein 1 (At3g01150) was used as a housekeeping gene. The data were analyzed using the Rotor-Gene Q 2.0.2 software, setting the threshold of the normalized fluorescence to 0.1, which corresponded to the exponential phase of the fluorescence signal. The resulting C_T and E values were used to calculate the relative mRNA abundance according to the $\Delta\Delta C_T$ method. The values were normalized to those of the reference gene and expressed relative to the $MgCl_2$-treated wild-type control sample.

Table II. List of primers used for quantitative PCR.

Locus	Gene name	Primer sequence	
At3g01150	*PTB*	Fw	5′ GATCTGAATGTTAAGGCTTTTAGCG 3′
		Rv	5′ GGCTTAGATCAGGAAGTGTATAGTCTCTG 3′
At2g13810	*ALD1*	Fw	5′ GTGCAAGATCCTACCTTCCCGGC 3′
		Rv	5′ CGGTCCTTGGGGTCATAGCCAGA 3′
At4g33150	*LKR*	Fw	5′ CATGTTGATGGGAAGAATCT 3′
		Rv	5′ AATATCGTTGTTGCTTCGCT 3′
At2g14610	*PR1*	Fw	5′ GTGCTCTTGTTCTTCCCTCG 3′
		Rv	5′ GCCTGGTTGTGAACCCTTAG 3′
At1g74710	*ICS1*	Fw	5′ TTCTGGGCTCAAACACTAAAAC 3′
		Rv	5′ GGCGTCTTGAAATCTCCATC 3′
At1g19250	*FMO1*	Fw	5′ TCTTCTGCGTGCCGTAGTTTC 3′
		Rv	5′ CGCCATTTGACAAGAAGCATAG 3′

3.9 PETIOLE EXUDATE COLLECTION AND ANALYSIS

Plant leaves were either infiltrated with a suspension of *Psm* (OD 0.005) or 10mM $MgCl_2$ as a control treatment. Six hours after infiltration, leaves were cut at the base of their petioles and the cut surface sterilizes by successive dipping in 50% v/v ethanol and 0,0005% bleach for 10 s. After rinsing with sterile 1mM EDTA, pH 8.0 (AppliChem), the petioles were

submerged in fresh EDTA-solution for exudate collection. 12-well tissue culture plates were used for this purpose, whereas each well was filled with 2.5 ml of collection solution and equipped with 10 harvested leaves. Exudates were continuously collected in the period from 6 to 48 hpi.

For the determination of amino acid (defence metabolite) content, 100 µl (1 ml) of collected exudates were analysed as describe above.

3.10 FLAGELLIN AND LIPOPOLYSACCHARIDES TREATMENT

The *flg22* peptide and purified LPS from *E. coli* were diluted in 10 mM $MgCl_2$ to a final concentrations of 200 nM (*flg22*) and 100 µg ml^{-1} (LPS), and infiltrated into leaves. The *flg22* peptide, representing the elicitor active domain of bacterial flagellin was kindly provided by Vera Göhre. Chromatographically purified lipopolysaccharides (LPS) preparations were acquired from Sigma-Aldrich (L3024). Control infiltrations were performed with 10 mM $MgCl_2$.

3.11 CHROMATIN IMMUNOPRECIPITATION

The systemic leaves (~3.5g) from plants infected in 1° leaves with *Psm* (OD_{600} 0.005) or 10mM $MgCl_2$ were determined for histone modification, following the chromatin immunoprecipitation (ChIP) protocol according to Saleh *et al.* (2008). For immunoprecipitation, 8 µg of antibodies against H3K4m2 (dimethylated Lys 4 at histone H3), H3K4m3, H3K9m2, H3K27m2 and H3K27m3 (Millipore) were added to a 1.5 ml solution containing diluted chromatin extracts and incubated overnight at 4°C with gentle rotation. Immunoprecipitated chromatin-DNA (IP-DNA) and 50 µL of input chromatin-DNA (without IP) were subjected to reverse cross-link and purified for PCR analysis of *ISC1* promoter (At1g74710). PCR was performed with 0.3 µM of each primer (Table III), 50 µM of each nucleotide, 0.25 U of Taq DNA polymerase (M0273, New England Biolabs) and 5x ThermoPol buffer in the final volume of 20 µL. PCR annealing temperature was 55 °C, polymerising for 1 min and 35-times amplified. The PCR product was separated on a 1% w/v agarose gel with 0.5x TAE buffer and 0.05 µg/ml ethidium bromide and visualized under UV light.

Table III. List of primers used for chromatin immunoprecipitation assay of *isochorismate synthase 1* (*ICS1*) promoter. Location confers to nucleotide number, where starting adenine of *ICS1* gene was set up as 1.

Primer name	Location	Primer sequence	
A	-2605 -2140	LP	5′ TCTGACCAGTGACCAGCGAT 3′
		RP	5′ CGTCTTCTGGAGTCTGAGTTCC 3′
B	-1932 -1564	LP	5′ TGTTAGCGTCAGTGTGAAAGCA 3′
		RP	5′ AATGTTGGACGCGGTCTCAG 3′
C	-1655 -1259	LP	5′ AGAAATTCGTAGCATCCACA 3′
		RP	5′ TATGGTGATTAAGACGGCTC 3′
H	-765 -481	LP	5′ GCGTTTGCAATTTGTAGTGAC 3′
		RP	5′ CCGGATTCGTTTGGTTGA 3′

3.12 MAPK IN GEL KINASE ASSAY

The in-gel kinase assay was performed as previously described by Kameshita and Fujisawa (1989), with minor modifications.

Systemic leaves (~150mg) of Col-0 and *fmo1* were harvested at 48 hpi of local leaves with mock or *Psm* (OD_{600} 0.005), grinded and extracted in 300µl of buffer E (50mM HEPES. pH 7.5, 5mM EGTA, 5mM EDTA, 1mM Na_3VO_4, 50mM glycerolphosphate, 10mM NaF, 5µg/ml leupeptin, 1mM PMSF, 2mM DTT). Protein concentration was determined using Bradford method (Bio-Rad).

Extracts containing 20 µg of total protein per lane were separated on a 10% SDS–polyacrylamide gel embedded with 0.25 mg/ml myelin basic protein (MBP; Millipore) as a kinase substrate. After electrophoresis, the SDS was removed by two washings (30 min each) in buffer A (50 mM Tris-HCl, pH 8.0, 20% v/v isopropanol) followed by two washings in buffer B (50 mM Tris-HCl, pH 8.0, and 5 mM ß-mercaptoethanol). The proteins in the gel were denatured in buffer C (50 mM Tris-HCl, pH 8.0, 5 mM ß-mercaptoethanol, and 6 M guanidinium chloride) for 1 h at room temperature and renatured overnight at 4°C in buffer D (50 mM Tris-HCl, pH 8.0, 5 mM ß-mercaptoethanol, and 0.04% v/v Tween 40). After equilibration in reaction buffer (40 mM HEPES, pH 7.5, 2 mM DT T, 20 mM $MgCl_2$, and 0.1 mM EGTA) for 1 h at room temperature, the gel was incubated in 20 ml of reaction buffer including 25 µM ATP plus 15 µCi γ-^{32}P-ATP (3000 Ci/mmol) for 1 h at room temperature. The reaction was stopped by transferring the gel into 5% w/v trichloroacetic acid and 1% w/v potassium pyrophosphate. Unincorporated γ-^{32}P-ATP was removed by an intensive washing for 3 h with six changes of the same solution. The gels were dried on Whatman paper and

exposed to X-Omat AR film (Sigma). MAP kinase activity was quantified using a PhosphoIm-ager (Molecular Dynamics Inc). Prestained molecular marker (ColorPlus, NEB) was used to estimate the apparent molecular mass of the MAP kinases after in-gel kinase assays.

3.13 CLONING OF 35S::FMO1::GFP AND AGROBACTERIUM PREPARATION

The gene of *FMO1* (At1g19250) was amplified from the cDNA of Col-0 using the primer pair: 5'-CACCATGGCTTCTAACTATGATAAGCTTACT-3' and 5'- AGCAGT-CATATCTTCTTTTTCTTC-3' and cloned to pENTR™/D-TOPO (Invitrogen). This subclone was used for LR reaction with pB7FWG2 (Karimi *et al.*, 2002) for C-terminal GFP-fusion under 35S promoter. For the 35S::GFP control construct, the pB7FWG2 was cut with restriction enzyme *EcoRV* to remove the suicide cassette. *Agrobacterium tumefaciens* [C58C1 (pGV2260)] (Agrobacterium) was transformed with created plasmids using the freeze-thaw method (Holsters *et al.*, 1978) and grown on LB medium (5g yeast extract, 10g tryptone, 5g NaCl in 1l H_2O, pH 7) containing rifampicin (50µg/ml), ampicilin (50µg/ml) and spectomycin (50µg/ml). Colonies were checked for *FMO1* gene using PCR and positive colonies were grown for 32h at 28°C for transformation of *Nicotiana benthamiana*.

3.14 FLUORESCENCE MICROSCOPY

The Agrobacterium carrying the construct 35::FMO1::GFP, control vector 35S::GFP, and mCherry construct reporting the Golgi apparatus was used. Prior inoculation, Agrobacterium was cultured overnight at 28°C (240 rpm) in 5 ml liquid Luria Broth medium supplemented with 50µg/ml ampicilin, 50µg/ml rifampicin, and 50µg/ml spectinomycin was prepared. The bacteria were washed one times with infiltration buffer (2mM sodium phosphate, pH 6.5; 450 µM acetosyringone and 50mM MES, pH 5.7, 0.5% w/v glucose). Bacteria were then diluted to OD_{600} 0.1 and inoculated to the leaves of four- to six-weeks old *Nicotiana benthamiana*.

One day later the leaf tissue were mounted in water and viewed using a Laser Scanning Micsroscope LSM 780 (Carl Zeiss Microscopy, Germany). GFP (mCherry) was visualized using an excitation wavelength of 499 (582) nm and a bandpass 551 (641) nm emission filter.

3.15 REPRODUCIBILITY OF EXPERIMENTS AND STATISTICAL ANALYSES

The data shown in the figures generally resulted from a single biological experiment. Unless otherwise stated, the results were similar in three biologically independent experiments. For metabolic analysis, the data represents mean ±SD of at least three replicates in $\mu g.g^{-1}$ fresh weight (FW). Bacterial numbers values represent mean values ±SD of colony forming units (cfu) per square centimetre from at least seven replicate samples, each consisting of three leaf disks. No significant differences in initial bacterial numbers (1 hpi) were detected. Statistical analyses were performed using two tailed Student's *t* test. Asterisks denote statistically significant differences between *Psm*- (OD 0.005) and $MgCl_2$-samples (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

4 AIM OF THE THESIS

After pathogen attack, plants trigger a sophisticated complex network of resistance responses at the site of infection. Moreover, resistance signals are spread through the whole plant to distal tissues. Although several components of plant defence against bacterial pathogens have been identified, many of the molecular events involved in plant resistance to bacterial pathogens need to be elucidated.

Since the discovery of SAR, much research has been devoted to identify the principles of induced resistance at the whole plant level. Questions about the mobile signal responsible for this phenomenon, its translation in systemic leaves and the main factors influencing the translation of the signal arose. This thesis aims to contribute to a better understanding of the SAR signalling pathway, focusing on metabolic changes that occur during SAR. For this purpose, the Arabidopsis-*Pseudomonas syringae* interaction model has been utilised as an experimental system.

One focus of this research has been to examine free amino acid levels in plant-bacteria interaction using GC/MS analysis. With this approach, a new component of inducible plant immunity was discovered and determined as pipecolic acid (Pip), a non-protein amino acid. Subsequently, local and systemic defence responses of Arabidopsis mutants putatively impaired in Pip production were investigated upon infection. Furthermore, based on the observation that Pip-deficient mutant is more susceptible to bacterial infection, the chemical complementation of plants was performed. Moreover, the regulatory events leading to Pip production and analysis of the biological responses in which Pip is integrated were unravelled.

Another aim of this thesis was to identify the biochemical function of the previously described critical SAR player FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) (Mishina and Zeier, 2006). Its function in chromatine remodelling and MAP kinase signalling was observed. After Pip discovery, the Pip-FMO1 relation in signal transduction was examined.

5 RESULTS

5.1 PIPECOLIC ACID AS A CRITICAL PLAYER IN SAR

5.1.1 Changes of free amino acid levels after pathogen infection

In order to gain insights into metabolic changes that occur in the course of SAR establishment, a comparative analysis of free amino acids was performed. Arabidopsis leaves infiltrated with *Psm* or 10mM $MgCl_2$ were named as local (1°) leaves and upper, not inoculated leaves as systemic (2°) leaves. Both types of leaves were sampled 48 hours post inoculation (hpi) and amino acid analysis was performed after derivatization with propyl chloroformate via GC/MS.

Massive changes of amino acids levels were observed in pathogen infected leaves (Table IV, left column). A strong increase by a factor of 5 to 20 was observed for Lys, the aliphatic amino acids Val, Leu, Ile and β-Ala, and the aromatic amino acids Phe, Tyr, Trp, and His (highlighted in orange). Moderate, but still significant, pathogen-induced accumulations were detected for γ-aminobutyric acid (GABA), Cys, Asn, Ala, Gly, Ser, and Orn. Outstandingly, two substances increased their levels by about 70-fold times in *Psm* treated leaves compared to mock treatment: α-aminoadipic acid (Aad) and an initially unknown compound with a major fragment ion of m/z 170 (highlighted in red). Conclusion by analogy, taking into account the mass spectra of Lys, Orn, and Pro derivatives, strongly suggested the identity of the unknown compound to be pipecolic acid (piperidine-2-carboxylic acid, homoproline, Pip), the methylene homologue to Pro (Figure 8). This assumption was confirmed when the authentic Pip derivative gave rise to the same mass spectra as the derivatized extract peak (Figure 8 A, E). Moreover, co-injection of the leaf extract with authentic Pip led to a specific increase of the extract peak (ion of m/z 170) while the internal standard norvaline (NorVal) and other peaks of extracted amino acids did not increase (Figure 8 F). Furthermore, amino acid analysis of local leaves revealed that only Asp, from 23 determined amino acids, significantly decreased after pathogen infection.

Changes of free amino acid levels in systemic (2°) leaves were less pronounced than at the inoculation site. The only amino acid with a strong 10-fold increase, compared to mock treatment at 48 h after local pathogen inoculation, was Pip (Table IV, central column). The levels of Aad, Phe, Val and Tyr also rose significantly, albeit to lower amounts (about 2-fold). Systemic content of remaining amino acids did not differ between *Psm*- and mock-treated leaves.

Table IV. Changes of the levels of free amino acids in Arabidopsis Col-0 plants upon leaf inoculation with *P. syringae* pv. *maculicola* (*Psm*). Left column: inoculated, lower leaves at 48 hpi. Central column: non-treated, upper (systemic) leaves at 48 hpi. Right column: petiole exudates of inoculated leaves collected between 6-48 hpi. Mean values are given in µg.g^{-1} fresh weight (FW) ± SD from at least four replicate samples. Mock-treatment was performed by infiltration of leaves with 10mM $MgCl_2$ solution. Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-samples (two tailed *t* test).

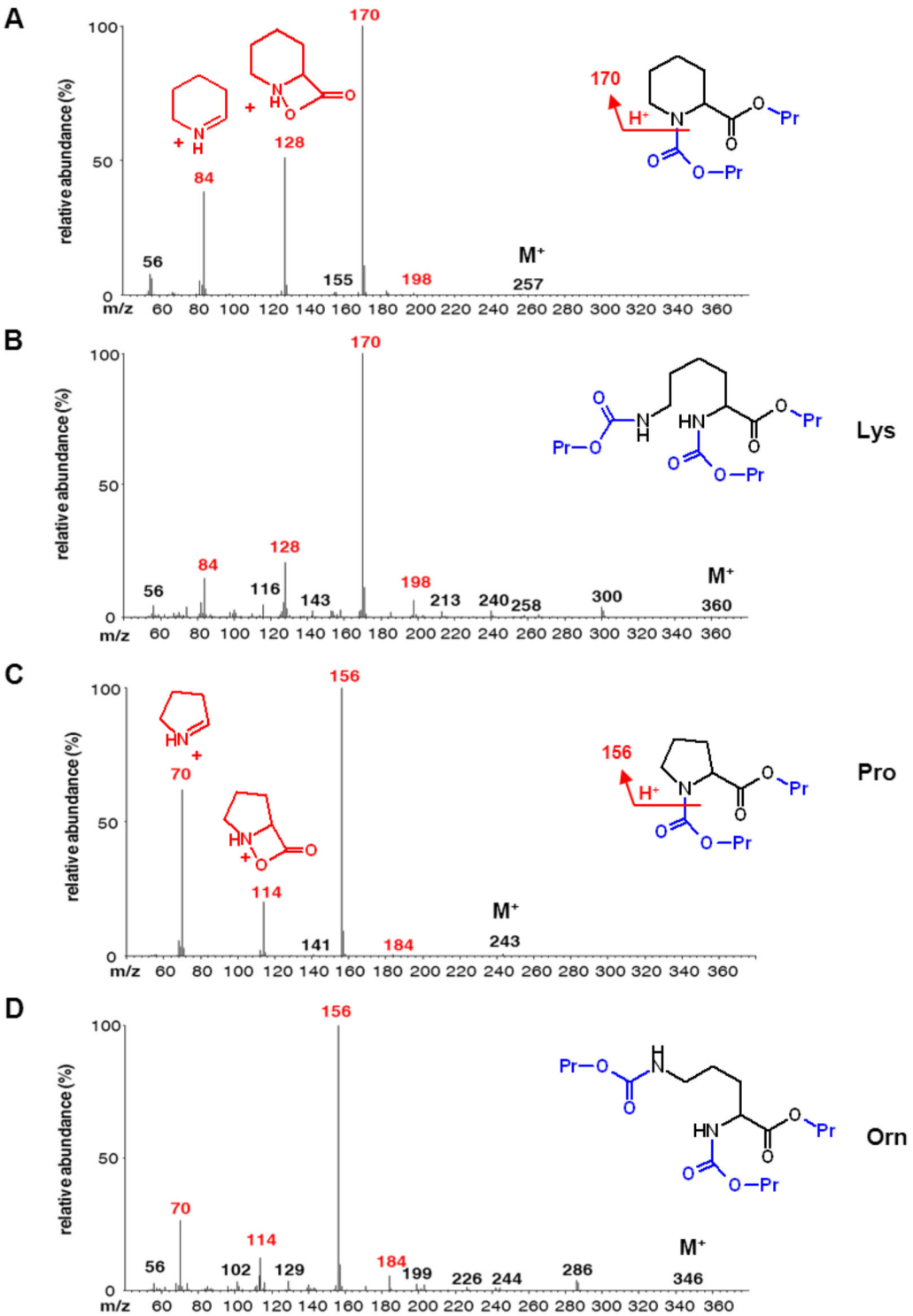
µg g^{-1} FW	inoculated leaves (48 hpi)			systemic leaves (48 hpi)			petiole exudates (6-48 h)		
amino acid	µg g^{-1} FW		ratio	µg g^{-1} FW		ratio	µg g^{-1} FW		ratio
	$MgCl_2$	*Psm*	P / M	$MgCl_2$	*Psm*	P / M	$MgCl_2$	*Psm*	P / M
Gly	12.8 ± 1.0	21.7 ± 5.4	1.7 *	16.5 ± 1.2	18.0 ± 3.7	1.1	12.6 ± 1.4	17.9 ± 0.4	1.4 ***
Ala	78.4 ± 7.3	141.0 ± 32.5	1.8 **	64.0 ± 7.0	87.9 ± 18.2	1.4	41.0 ± 4.2	52.1 ± 4.6	1.3 *
Val	7.9 ± 1.3	54.4 ± 27.1	6.9 **	6.7 ± 0.4	9.8 ± 1.9	1.5 *	15.7 ± 0.8	18.0 ± 1.5	1.1
β-Ala	2.5 ± 1.2	15.5 ± 7.3	6.2 **	2.5 ± 0.8	3.7 ± 0.7	1.5	0.43 ± 0.05	0.51 ± 0.05	1.2
Leu	3.3 ± 0.6	49.2 ± 26.7	14.8 **	2.5 ± 0.5	4.6 ± 1.6	1.8	16.2 ± 1.9	18.3 ± 1.0	1.1
Ile	2.6 ± 0.4	31.4 ± 16.0	12.0 **	2.6 ± 0.4	3.8 ± 1.3	1.5	17.4 ± 0.6	14.1 ± 1.0	0.8 **
GABA	6.5 ± 0.5	25.1 ± 6.5	3.8 **	5.6 ± 1.5	6.1 ± 0.9	1.1	19.7 ± 2.3	22.3 ± 3.8	1.1
Ser	127.5 ± 17.8	230.5 ± 46.4	1.8 *	110.7 ± 25.5	146.3 ± 41.0	1.3	31.9 ± 3.4	37.7 ± 8.9	1.0
Thr	58.9 ± 13.0	98.5 ± 33.7	1.7	85.8 ± 18.4	122.6 ± 27.1	1.4	33.8 ± 3.6	22.7 ± 4.9	0.7 *
Pro	11.1 ± 1.5	10.0 ± 3.0	0.9	13.5 ± 2.7	12.4 ± 2.4	0.9	2.8 ± 0.5	2.9 ± 0.4	1.0
Pip	0.6 ± 0.2	42.2 ± 15.8	65.3 ***	0.3 ± 0.1	3.1 ± 1.3	10.1 **	0.5 ± 0.1	3.3 ± 0.6	7.0 ***
Aad	0.4 ± 0.1	25.6 ± 6.6	71.2 ***	0.19 ± 0.03	0.42 ± 0.12	2.2 *	0.24 ± 0.05	0.11 ± 0.03	0.5 *
Asp	160.0 ± 46.6	91.7 ± 30.7	0.6 *	145.0 ± 30.1	194.0 ± 57.7	1.3	98.7 ± 3.2	91.7 ± 23.5	0.9
Glu	277.0 ± 63.9	282.5 ± 95.1	1.0	282.0 ± 44.5	347.1 ± 63.5	1.2	91.0 ± 7.2	85.0 ± 28.6	0.9
Asn	28.1 ± 4.3	78.9 ± 22.3	2.8 **	39.6 ± 10.0	41.0 ± 15.2	1.0	15.3 ± 3.6	17.1 ± 5.5	1.1
Gln	254.1 ± 65.2	374.5 ± 72.0	1.5	358.2 ± 84.2	372.3 ± 78.5	1.0	98.9 ± 28.7	110.4 ± 28.6	1.1
Cys	0.09 ± 0.01	0.25 ± 0.03	2.9 *	0.05 ± 0.02	0.08 ± 0.03	1.5	0.02 ± 0.00	0.02 ± 0.00	1.0
Orn	0.7 ± 0.1	1.1 ± 0.2	1.6 *	1.4 ± 0.3	1.0 ± 0.1	0.7	0.49 ± 0.10	0.73 ± 0.31	1.5
Lys	5.1 ± 1.1	28.8 ± 11.7	5.7 **	5.1 ± 0.6	6.1 ± 1.1	1.2	12.9 ± 1.5	3.4 ± 0.5	0.3 ***
His	0.3 ± 0.2	2.9 ± 2.1	9.4 *	0.42 ± 0.21	0.64 ± 0.25	1.5	0.28 ± 0.06	0.28 ± 0.09	1.0
Phe	3.7 ± 0.2	20.3 ± 4.9	5.5 *	4.2 ± 0.6	9.1 ± 3.3	2.2 *	8.4 ± 0.9	13.9 ± 1.5	1.7 **
Tyr	3.3 ± 1.0	23.9 ± 4.5	7.3 ***	3.3 ± 0.3	5.4 ± 0.8	1.6 *	0.65 ± 0.06	0.69 ± 0.19	1.1
Trp	2.7 ± 0.7	24.7 ± 11.7	9.2 **	2.9 ± 0.3	4.3 ± 0.9	1.5	0.31 ± 0.06	0.30 ± 0.05	1.0

fold change P/M

> 20
5 - 20
3 - 5
1.6 - 3
0.5 – 0.8
< 0.5

t-test P vs. M

* P < 0.05
** P < 0.01
*** P < 0.001


A
relative abundance (%)
m/z
170
128
84
56
155
198
M+
257
B
relative abundance (%)
m/z
170
128
84
56
116
143
198
213
240
258
300
M+
360
Lys
C
relative abundance (%)
m/z
156
70
114
141
184
M+
243
Pro
D
relative abundance (%)
m/z
156
70
114
56
102
129
184
199
226
244
286
M+
346
Orn

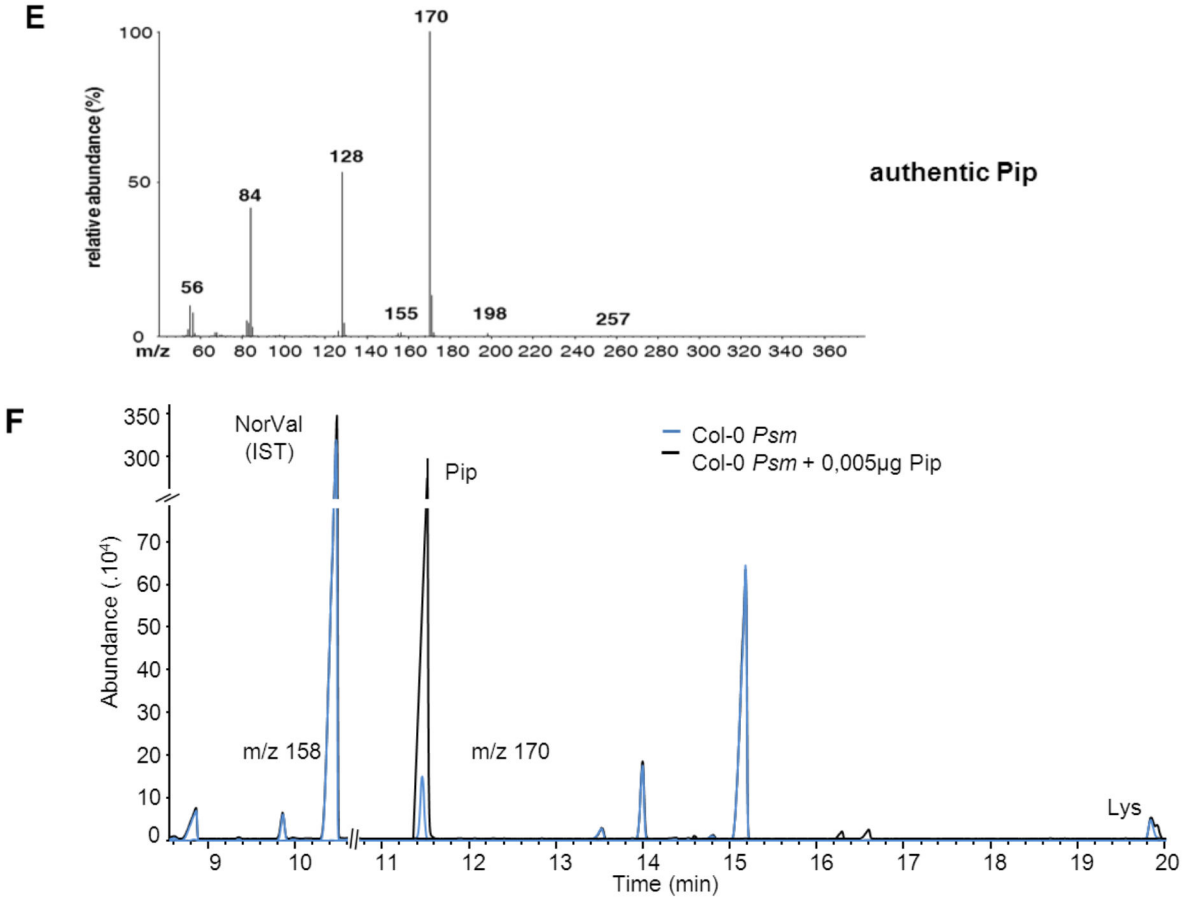


Figure 8. Mass spectral identification of the initially unknown substance detected in extracts of *P. syringae*-inoculated leaves as pipecolic acid. Spectra were determinated and interpreted by Jürgen Zeier. (**A**) Mass spectrum of the unknown substance after derivatization with propyl chloroformate converting amino groups into propyl carbamate and carboxyl groups into propyl ester derivatives. The mass spectrum showed similarities to the spectrum of the lysine derivative (**B**). Moreover, the m/z 257 ion appeared to be the molecular ion (M^+) of the unknown substance. A homologous relationship existed to the spectrum of the proline derivative (**C**) in a way that each of the fragments m/z 257 (M^+), 198, 170, 128, and 84 of unknown substance spectrum (A) was replaced by a fragment reduced by 14 mass unites: m/z 243 (M^+), 184, 156, 114, and 72, respectively (C). The spectra of the derivatives of lysine ("homoornithine") (B) and ornithine (**D**) exhibited a similar 14 mass unit fragment shift, which is consistent with a presence of an additional methylene group in lysine compared to ornithine. These observations and deduced structures of fragment ions (A, C) were consistent with the assumption that the unknown compound is pipecolic acid (homoproline), the methylene homologue of proline. (**E**) The mass spectrum of authentic pipecolic acid with agreement of "unknown compound" (A). (**F**) The chromatogram of pathogen-inoculated leaf extract ± 0.005µg pipecolic acid. Mass unit m/z 158 at retention time of 10.4 min defines internal standard norvaline (NorVal) and ion m/z 170 at retention time of 11.5 min determines Pip.

5.1.2 Changes of free amino acid levels after PAMP treatment

The accumulation of amino acids might initially be triggered by perception of conserved bacterial structures, such as PAMPs. Therefore, exogenous application of two well-characterized bacterial PAMPs, flagellin (the proteinaceous building unit of the bacterial flagellum) and lipopolysaccharide (LPS, a major component of the outer membrane of Gram-negative bacteria) was investigated. Arabidopsis Col-0 plants were infiltrated with *flg22*,

a peptide corresponding to the elicitor active epitope of flagellin (Gómez-Gómez *et al.*, 1999), or LPS to trigger PAMP-induced resistance. Infiltrated leaves were analysed for amino acid levels 48 h after treatment. Control treatment was performed with 10mM $MgCl_2$ solution only.

Table V. Changes of the levels of free amino acids in Arabidopsis Col-0 plants upon leaf inoculation with PAMPs, flagellin (*flg22*) and lipopolysaccharides (LPS) at 48 h post treatment. Left column: *flg22* treatment. Right column: LPS treatment. Mean values are given in $\mu g.g^{-1}$ fresh weight (FW) ± SD from three replicate samples. Mock-treatment was performed by infiltration of leaves with 10mM $MgCl_2$ solution. Asterisks denote statistically significant differences between PAMP- and $MgCl_2$-samples (two tailed *t* test).

amino acid	µg g^{-1} FW $MgCl_2$	µg g^{-1} FW *flg22*	ratio *f* / M	µg g^{-1} FW $MgCl_2$	µg g^{-1} FW LPS	ratio L / M
Gly	18.9 ± 2.0	65.5 ± 15.1	3.5 **	18.9 ± 2.0	30.5 ± 4.1	1.6 *
Ala	54.8 ± 7.3	73.8 ± 20.5	1.3	54.8 ± 7.3	70.7 ± 18.3	1.3
Val	4.9 ± 0.4	13.7 ± 1.3	2.8 ***	4.9 ± 0.4	5.8 ± 0.7	1.2
β-Ala	0.4 ± 0.05	2.4 ± 0.2	6.5 ***	0.4 ± 0.05	0.7 ± 0.08	1.9 **
Leu	1.0 ± 0.05	8.4 ± 1.6	8.5 **	1.0 ± 0.05	1.5 ± 0.1	1.5 **
Ile	1.3 ± 0.07	6.0 ± 1.0	4.8 **	1.3 ± 0.07	1.6 ± 0.08	1.3 **
GABA	1.7 ± 0.5	4.0 ± 0.4	2.4 **	1.7 ± 0.5	2.8 ± 0.6	1.6
Ser	94.2 ± 36.9	225.8 ± 14.9	2.4 **	94.2 ± 36.9	151.9 ± 41.5	1.6
Thr	63.1 ± 1.1	67.7 ± 6.4	1.1	63.1 ± 1.1	90.4 ± 18.2	1.4
Pro	18.0 ± 4.1	31.8 ± 1.0	1.8 **	18.0 ± 4.1	26.3 ± 3.2	1.5
Pip	0.1 ± 0.02	7.0 ± 0.9	54.7 ***	0.1 ± 0.02	2.0 ± 0.3	15.7 ***
Aad	0.1 ± 0.01	4.2 ± 0.8	31.1 **	0.1 ± 0.01	0.7 ± 0.2	5.3 **
Asp	412.6 ± 26.0	523.0 ± 19.4	1.3 **	412.6 ± 26.0	644.9 ± 143	1.6 *
Glu	125.4 ± 6.7	266.0 ± 5.2	1.8 ***	125.4 ± 6.7	213.9 ± 41.3	1.7 *
Asn	15.5 ± 0.8	22.7 ± 1.4	1.5 **	15.5 ± 0.8	23.8 ± 5.6	1.5
Gln	62.8 ± 39.2	108.2 ± 55.5	1.7	62.8 ± 39.2	86.0 ± 47.0	1.4
Cys	0.1 ± 0.01	0.3 ± 0.07	2.5 *	0.1 ± 0.01	0.2 ± 0.04	1.4
Orn	0.4 ± 0.03	0.4 ± 0.06	1.0	0.4 ± 0.03	0.5 ± 0.08	1.1
Lys	1.2 ± 0.1	6.1 ± 0.3	5.0 ***	1.2 ± 0.1	1.9 ± 0.4	1.6 *
Phe	2.7 ± 0.2	10.6 ± 1.8	3.9 **	2.7 ± 0.2	3.8 ± 0.9	1.4
Tyr	0.3 ± 0.02	1.9 ± 0.4	6.0 **	0.3 ± 0.02	0.5 ± 0.1	1.6 *
Trp	0.2 ± 0.04	1.4 ± 0.4	7.7 **	0.2 ± 0.04	0.3 ± 0.02	2.0 **

fold change P/M

> 20

5 - 20

3 - 5

1.6 - 3

t-test P vs. M

* P < 0.05

** P < 0.01

*** P < 0.001

Infiltration of 200nM *flg22* triggered biosynthesis of several amino acids (Table V., left column). Moderate enhancement of Gly, Val, Ile, GABA, Ser, Cys, and Phe was observed after *flg22* treatment (highlighted in yellow). Strong accumulation by factor 5 to 20 was

determined for β-Ala, Leu, Lys, Tyr, and Trp (highlighted in orange). More than 30-fold higher levels of Pip and Aad were accumulated after *flg22* treatment (highlighted in red). The accumulation pattern of amino acids after *flg22* infiltration is similar to the pattern observed for pathogen-inoculated leaves. In both treatments, aliphatic, aromatic amino acids and outstandingly Pip and Aad were accumulated to higher levels compared to mock treatments. However, for most of the amino acids, *flg22*-triggered levels did not reach the values of those triggered by pathogen. Significant increases in discussed amino acids also occurred when leaves were treated with gel-purified LPS preparations from *Escherichia coli* at a concentration of 100 µg/ml, but trailed behind *flg22* or pathogen treatments (Table V., right column).

5.1.3 Pathogen-induced activation of Lys degradation pathway

Pip and Aad are both catabolites of Lys (see 2.3.1.1). In plants only one major Lys catabolic pathway has been described. The first step of this pathway is catalysed by the LKR/SDH enzyme, creating α-amino adipic semialdehyde, the Aad precursor. In some publications, α-amino adipic semialdehyde is proposed to be a substrate for Pip (Arruda *et al.*, 2000; Goyer *et al.*, 2004). This would need Δ^1-piperideine-6-carboxylic acid as an intermediate. However, a radiolabel experiment by Gupta and Spenser (1970) proposed that Pip is produced in bean plants from Δ^1-piperideine-2-carboxylic acid (Pip2C). This reaction would need an aminotransferase with the specificity to the α-amino group of Lys. Such a reaction would convert Lys into ε-amino-α-ketocaproic acid and by its spontaneous dehydratation, the Pip2C would be created. Afterwards, a reduction step would be necessary for Pip biosynthesis. An aminotransferase with the highest enzymatic activity to Lys and specificity to the α-amino group was described and named as ALD1. In addition, *ald1-T1,T2* mutants were more susceptible to *Pseudomonas* infection (Song *et al*., 2004a). These data led us to the hypothesis that ALD1 could be involved in pathogen-triggered biosynthesis of Pip in Arabidopsis (Figure 9).

To test this, initially the transcript levels of *ALD1* and *LKR* were determined after *Psm* inoculation in the local leaves at 24 hpi and in systemic leaves at 48 hpi. Both mRNA levels increased in local leaves (Figure 11 A), supporting the hypothesis that Lys degradation is the pathogen-triggered event. Increased *ALD1* expression was also observed in distal leaves upon local *Psm* inoculation (Figure 11 B).

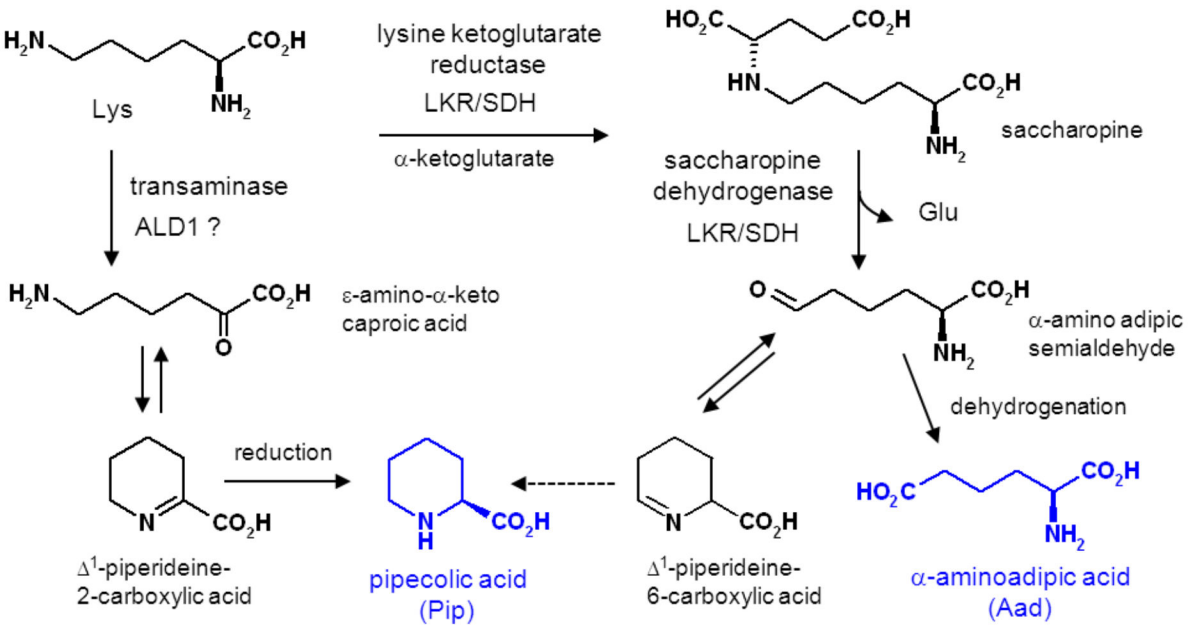


Figure 9. Proposed scheme for pathogen inducible pipecolic acid and α-aminoadipic acid biosynthesis in plants via lysine degradation.

To study the Lys catabolic pathway on the metabolic level, mutants (in Col-0 background) with the T-DNA insertion in locus *ALD1* and *LKR/SDH* were ordered from the SALK Institute collection (Alonso *et al.*, 2003).

The *ald1* mutant (SALK_007673; *ald1-T2*, Song *et al.*, 2004a) has an insertion in the exon located on the beginning of the *ALD1* gene on chromosome 2. The insertions were annotated within the intron and exon of the *LKR/SDH* gene on chromosome 4 for the *lkr-1* (SALK_068769) and *lkr-2* (SALK_127160) mutants, respectively (Figure 10 A). The homozygous mutant line was subsequently identified by multiplex PCR (Figure 10 B). Obtained homozygotes were tested for the expression of *ALD1* and *LKR/SDH* at 24h after *Psm* inoculation (Figure 10 C). The *ald1* and *lkr-2* mutants showed to be null mutants and *lkr-1* had approximately 80% reduced expression of *LKR* in comparison to wild type.

Afterwards, the *ald1* and *lkr* mutants were examined for metabolite levels by GC/MS analysis. Mutant *ald1* was fully blocked in *Psm*-induced Pip biosynthesis at the inoculation site, but both *lkr* mutants showed Pip levels similar to wild type (Figure 11 C). In contrast, *Psm*-induced Aad production in local leaves was strongly attenuated in both *lkr-1* and *lkr-2*, but rose in *ald1* to wild type-like levels (Figure 11 D). Blockage of either pathway in *ald1* or *lkr1/2* led to a hyper-accumulation of the common precursor Lys after pathogen infection (Figure 11 E).

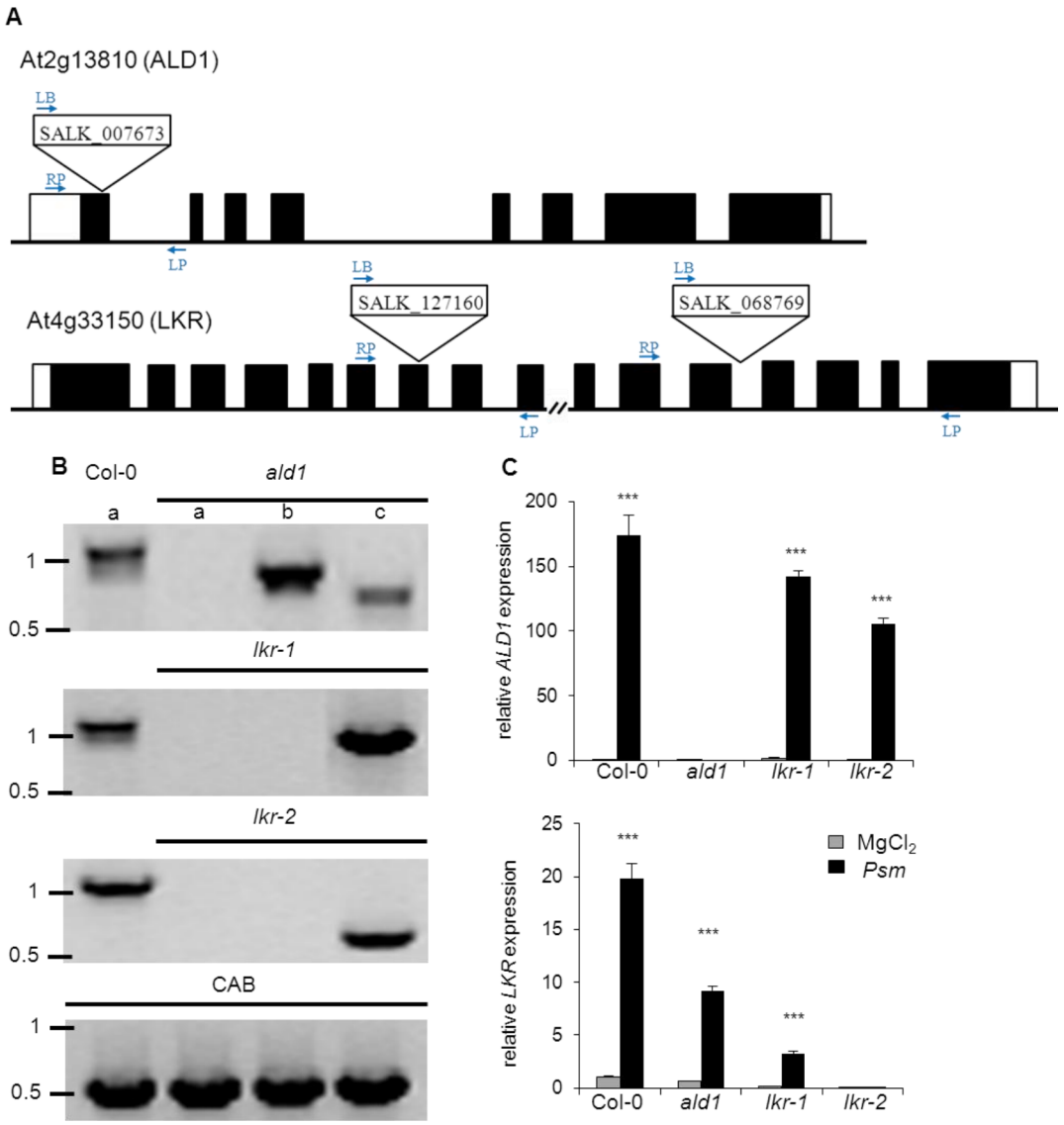


Figure 10. Molecular characterization of *ald1* and *lkr-1,2* mutants. (**A**) Diagrams showing the position of the T-DNA insertions at the *ALD1* and *LKR* locus. Black boxes represent coding sequence and the spaces spliced introns. Blue arrows indicate the position of primers used for PCR analysis. (**B**) PCR analysis of gDNA isolated from Col-0 and indicated mutants. a: LP and RP primers combination, b: LP and LB primers combination, c: RP and LB primers combination, CAB: chlorophyll a/b binding protein (positive control). (**C**) *Psm*-induced *ALD1* and *LKR* expression in inoculated leaves of Col-0, *ald1*, *lkr-1* and *lkr-2* plants at 24 hpi. Transcript levels were assessed by quantitative PCR analysis; values are given as means ± SD of three replicate samples, and are expressed relative to the respective mock-control value. Asterisks denote statistically significant differences between *Psm*- (OD 0.005) and $MgCl_2$-samples (***: $P < 0.001$; two tailed *t* test).

5.1.4 Pipecolic acid and α-aminoadipic acid accumulate at the site of bacterial inoculation

To determine the kinetics of Pip and Aad accumulation in competent and incompetent plant-pathogen interaction, the time course analyses of local leaves were performed. Leaves of Arabidopsis were inoculated with *Psm* (OD_{600} 0.001) or *Psm* carrying avirulence gene *avrRpm1* (*Psm avrRpm1*) (OD_{600} 0.002) and then analysed for Pip and Aad levels at 0, 8, 24,

32, and 48 hpi. Pip was produced faster in leaves inoculated with the incompatible, HR-inducing *Psm avrRpm1* strain.

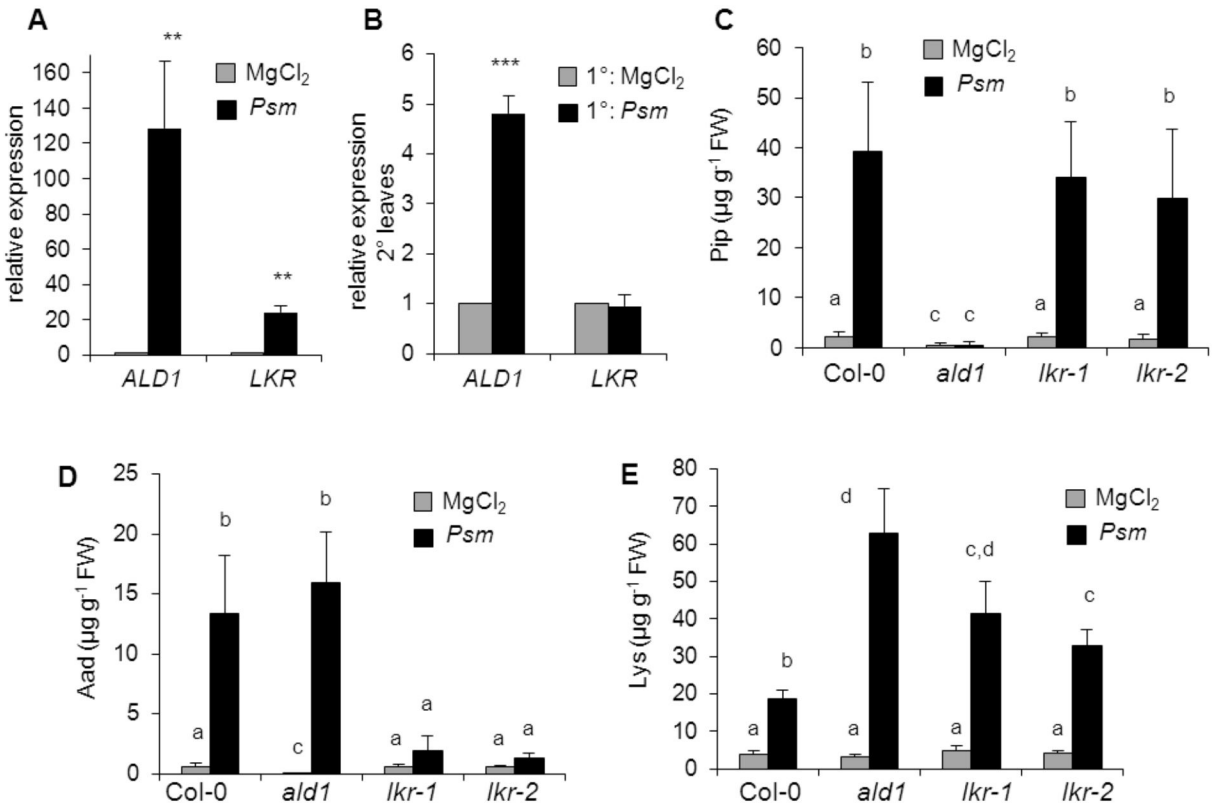


Figure 11. Characterization of plants mutated in enzymes involved in lysine degradation pathway.(**A-C**) *Psm*-induced *ALD1* and *LKR* expression in Col-0 plants. Transcript levels were assessed by quantitative PCR analysis, values are given as means ± SD of three replicate samples, and are expressed relative to the respective mock-control value. Asterisks denote statistically significant differences between *Psm*- (OD 0.005) and $MgCl_2$-samples (***: $P < 0.001$; **: $P < 0.01$; two tailed *t* test). (**A**) Relative expression in *Psm*-inoculated leaves (24 hpi). (**B**) Relative expression in upper (2°) leaves upon *Psm*-inoculation of lower (1°) leaves (48 hpi). (**C-E**) Pathogen induced biosynthesis of Pip and Aad proceed via ALD1 and LKR, respectively. Accumulation of Pip (**D**), Aad (**E**), and Lys (**F**) in *Psm*-inoculated leaves of Col-0, *ald1*, *lkr-1* and *lkr-2* plants at 48 hpi. Mean values are given in $\mu g.g^{-1}$ fresh weight (FW) ± SD from at least four replicate samples. Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

Whereas accumulation of Pip already took place at 8 hpi with avirulent *Psm avrRpm1*, substantial increase of Pip content with virulent *Psm* was observed after 24 hpi. Pip accumulation at 24 h of the incompatible interaction reached its maximum, in contrast to compatible interaction where Pip accumulated to high levels during later stages. Aad biosynthesis was also triggered upon inoculation with both compatible and incompatible bacteria but the values were similar for both strains at all analysed times (Figure 12 A, B).

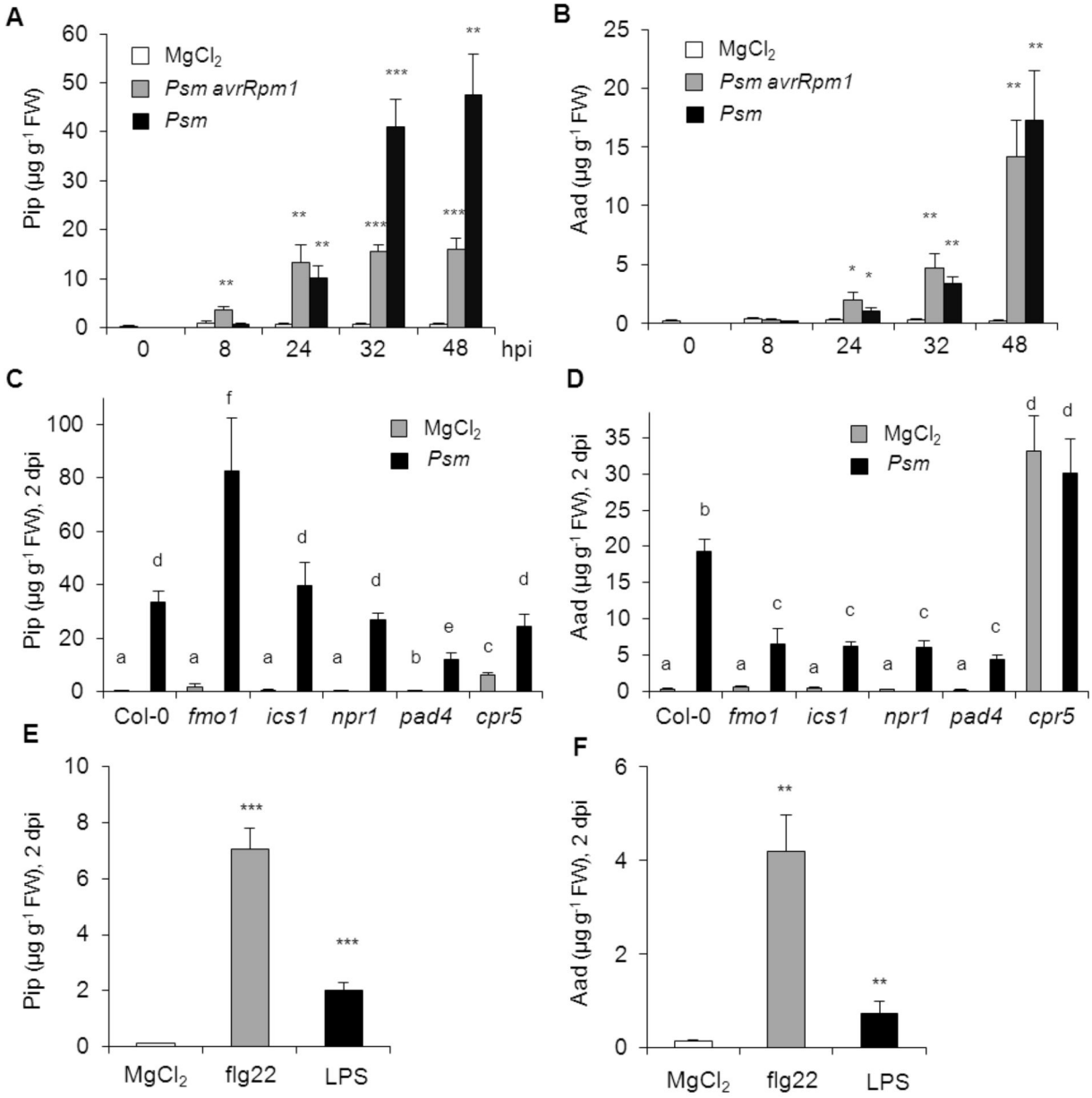


Figure 12. Metabolite accumulation upon *P. syringae* attack in inoculated Arabidopsis leaves. Mean values are given in µg.g^{-1} fresh weight (FW) ± SD from at least three replicate samples. (**A**, **B**) Time course of pipecolic acid (**A**) and α-aminoadipic acid (**B**) accumulation in leaves inoculated with compatible *Psm* (OD 0.001) and incompatible *Psm avrRpm1* (OD 0.002). (**C**, **D**) Accumulation of Pip (**C**) and Aad (**D**) in *Psm*-inoculated leaves of Col-0 and selected defence mutants at 48hpi. (**E**, **F**) Accumulation of Pip (**E**) and Aad (**F**) after 48 hpi of flagellin peptide (*flg22*, 200nM) or lipopolysaccharides (LPS, 100µg/ml) treatment. Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-samples (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; two tailed *t* test). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

In order to investigate whether induced Pip biosynthesis is regulated via classical defence signalling pathways, Pip accumulation was determined in leaves of various well-characterized Arabidopsis defence mutants after *Psm* inoculation. At inoculation site, the Pip accumulation in *ics1* (*sid2*), the mutant compromised in SA biosynthesis, rose to wild type-

like level, suggesting that Pip biosynthesis is independent of SA biosynthesis. The same result was observed for the *npr1* mutant; therefore Pip accumulation is mediated upstream of NPR1-signalling in local leaves. In contrast, the level of Pip was partially dependent on the defence regulator PAD4. The *fmo1* mutant showed consistently higher Pip levels than in Col-0 at 48 hpi (Figure 12 C).

The regulation of induced Aad and Pip biosynthesis seems to differ because Aad accumulation was partially dependent on SA-, FMO1-, NPR1- and PAD4-mediated signalling in the local leaves (Figure 12 D). Moreover, the basal levels of Pip and particularly of Aad were elevated in the constitutively resistant *cpr5* mutant (Figure 12 C, D).

Moreover, single treatments of leaves with *flg22* or LPS were sufficient to induce Pip and Aad formation, suggesting that bacterial PAMP recognition initiates Lys degradation after *P. syringae* inoculation in Arabidopsis leaves.

5.1.5 Pipecolic acid accumulates in leaves distal from pathogen inoculation

The SA-signalling pathway is essential for the establishment of SAR in Arabidopsis-*P. syringae* interaction. Pip also accumulated in systemic leaves at 48 hpi after local inoculation with *Psm* (Table IV). To compare the kinetics of systemic Pip and SA accumulation, levels of both were determined in systemic leaves after 16, 24, 30, 36, and 48 h after local *Psm* inoculation. Significantly enhanced systemic levels of Pip but not of SA were already observed at 36 hpi (Figure 13 A, B). A closer time course analysis was then performed in systemic leaves, where samples were taken every two hours from 32 to 42 hpi. Figure 13 C shows that systemic Pip and SA levels are not statistically different at 32 hpi. At 34 hpi the levels start to differ and while Pip accumulation already increased 2.5-times at 42 hpi compared to the 32 hpi value, SA content did not increased in the investigated time period. These data suggest that Pip accumulation precedes SA accumulation in distal leaves at the onset of SAR

To examine whether systemic accumulation of Pip depends on SA signalling and SA-related defence pathways, the systemic Pip levels were checked in different defence mutants upon local pathogen treatment. In the SAR deficient mutant *fmo1* and the SA-biosynthesis mutant *ics1*, the systemic accumulation of Pip exhibited about 4-fold lower levels compared to Col-0 plants. However, this was still significant increase compared to plants that were pre-treated with $MgCl_2$ solution only. Moreover, a similar result was observed with the defence-

signalling mutant *pad4*. In another SA-signalling mutant, *npr1*, the pathogen-triggered systemic accumulation of Pip was completely supressed (Figure 13 D).

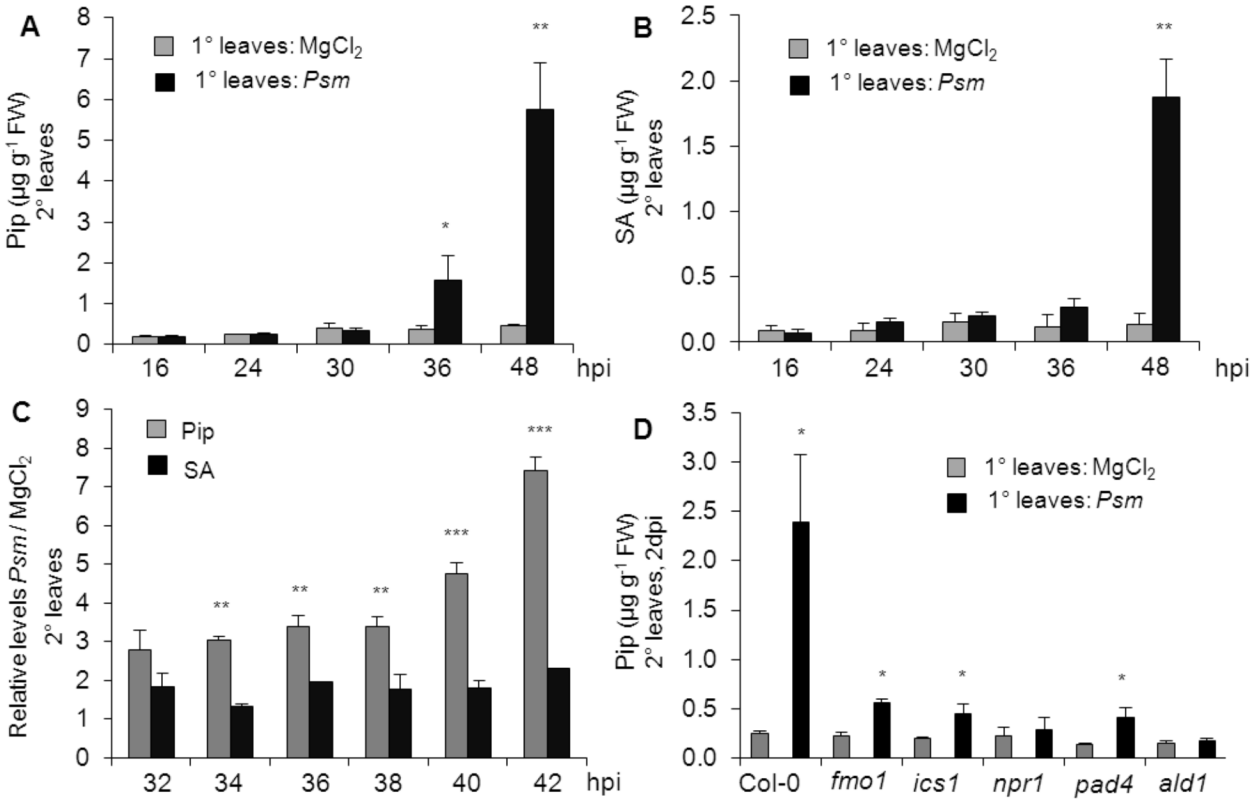


Figure 13. Metabolite accumulation upon *P. syringae* attack in systemic Arabidopsis leaves. Mean values are given in µg.g^{-1} fresh weight (FW) ± SD from at least three replicate samples. (**A**, **B**, **C**) Time course of Pip and salicylic acid accumulation in upper (2°) leaves following inoculation of lower (1°) leaves with *Psm* (OD 0.005). In (C) the values represents the levels accumulated after *Psm* compared already to $MgCl_2$ levels. (**D**) Systemic Pip accumulation at 48 hpi in Col-0 and different defence mutant plants. Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-samples, only in C the difference between Pip and SA content (***: P < 0.001; **: P < 0.01; *: P < 0.05; two tailed *t* test).

5.1.6 Pipecolic acid strongly accumulates in exudate collected from inoculated leaves

The outstanding status of Pip in the course of SAR establishment was corroborated in analyses with petiole exudates. The exudates were collected from inoculated Arabidopsis between 6 and 48h after infiltration with *Psm* (OD 0.005). Upon pathogen treatment, levels of Pip increased by a factor of 7, whereas other amino acid levels remained at control values, showed less pronounced increases (Phe, Gly, and Ala) or were reduced (Lys, Aad, Ile, and Thr) (Table IV, right column, Figure 14 A, B).

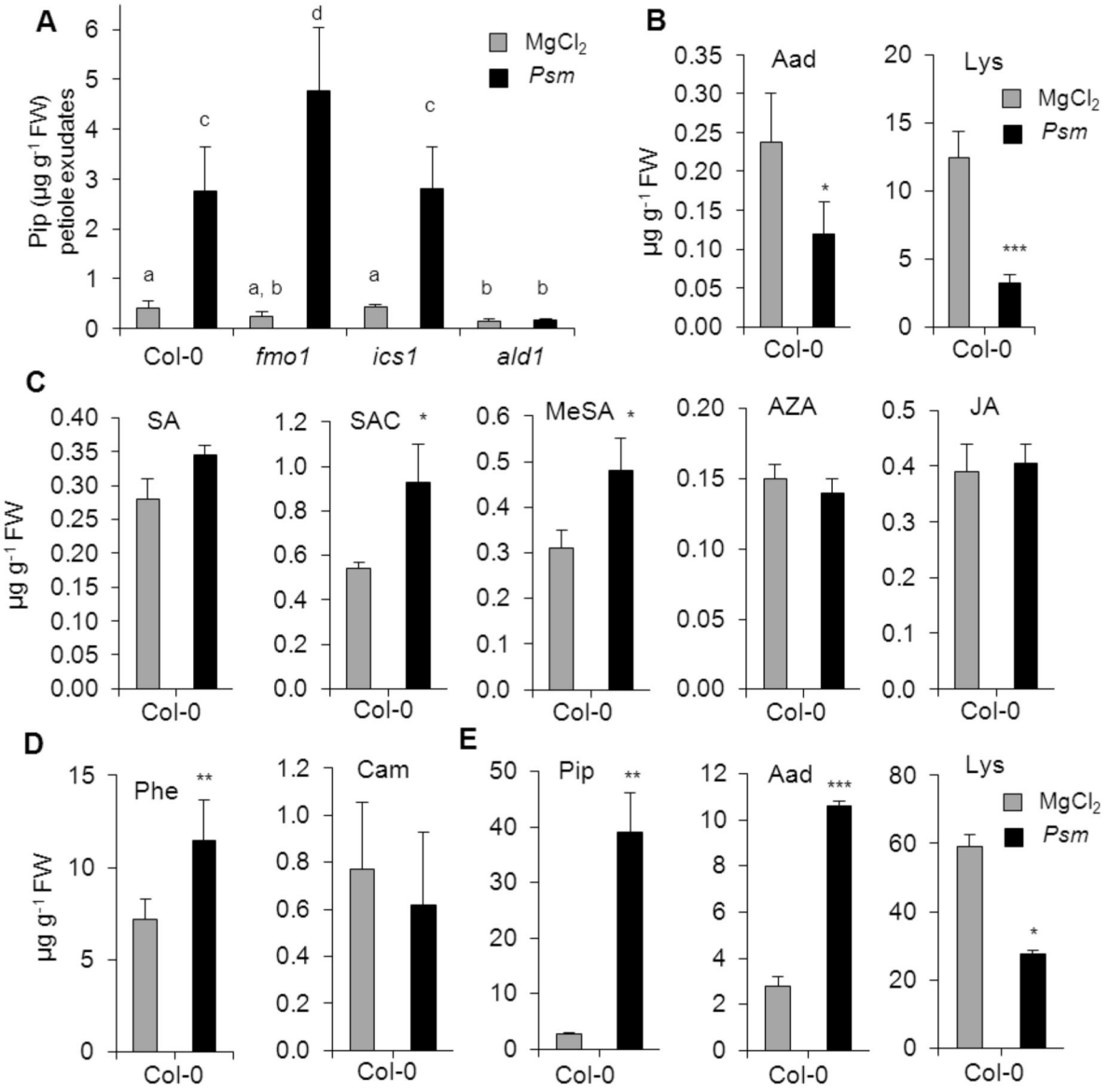


Figure 14. Metabolite levels in petiole exudates of leaves collected between 6 to 48 h post *Psm*- or $MgCl_2$-infiltration. Mean values are given in µg.g^{-1} fresh weight (FW) ± SD from at least three replicate samples. (**A**) Pipecolic acid (Pip) levels in Col-0 and different defence mutant plants. (**B-D**) Metabolite levels in petiole exudates. Aad: α-aminoadipic acid, Lys: lysine, SA: free salicylic acid, SAC: conjugated salicylic acid, MeSA: methyl salicylate (analysed by E. Attaran), AZA: azelaic acid, JA: jasmonic acid, Phe: phenylalanine, Cam: camalexin. (**E**) Metabolite levels in leaves from which the exudate was collected. Asterisks denote statistically significant differences between *Psm*- (OD 0.005) and $MgCl_2$-samples (***: $P < 0.001$; **: $P < 0.01$; *: $P < 0.05$; two tailed *t* test). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

Compared to the substantial pathogen-induced increase of Pip in exudates, the exuded levels of other defence-related metabolites, such as SA, SA conjugates (SAC), methyl salicylate (MeSA), azelaic acid (AZA), JA, or camalexin did not or did only modestly increase after *Psm* inoculation (Figure 14 C-D). However, alongside Pip, several of these metabolites and amino acids do strongly accumulate in inoculated leaves. The Pip transport out of inoculated leaves seems therefore to be favoured over the transport of many other

accumulating metabolites. Enrichment of Pip in petiole exudates of pathogen-inoculated leaves was also detected in *fmo1* and *ics1* and thus independent of FMO1- and SA-mediated signalling (Figure 14 A). The contradictory behaviour was especially found in the accumulation of Lys in local leaves (where increased) and petiole exudate (where decreased), therefore the amino acid analysis was carried out also in the leaves that were used for petiole exudate collection. The Lys levels also significantly decreased after pathogen inoculation in leaves that were detached from the plants (Figure 14 E) suggesting the consumption of Lys for synthesis of Aad and Pip.

5.1.7 L-pipecolic acid is a metabolic regulator of several forms of induced resistance in plants

It was shown that pathogen-triggered biosynthesis of Pip does not occur in the *ald1* mutant (Figure 11 C). Moreover, Pip was accumulated upon *Psm* infiltration (Table IV) and as well *ALD1* gene expression was enhanced by pathogen attack as well (Figure 11). It was previously described that *ald1-T2* (Song *et al.*, 2004a) is strongly attenuated not only in basal and specific resistance, but is also fully blocked in SAR. To test whether these changes of the defence responses of *ald1* occur in our genetically different pathosystems, the number of colony-forming units of bacteria in the apoplast was determined 3 days after leaf inoculation. To investigate the biological induction of SAR, leaves of a given plant were treated with *Psm* (OD 0.005) as a primary inoculation and 2 days later a secondary infection with virulent *Psm* (OD 0.001) was performed in systemic leaves. When primary leaves of wild-type plants were pre-inoculated with *Psm*, a significant reduction of bacterial growth in the subsequent challenge infection in systemic leaves was observed in comparison to treatment of primary leaves with a control solution of $MgCl_2$, demonstrating the establishment of SAR. In marked contrast, SAR did not develop in *ald1* mutant plants because growth of *Psm* in systemic leaves proved to be equally pronounced in plants pre-treated in primary leaves with $MgCl_2$ or *Psm* (Figure 15 A). Moreover, numbers of colonies were significantly higher in *ald1* mutant after inoculation with the compatible *Psm* (OD 0.001) and the incompatible *Psm avrRpm1* (OD 0.002) strain compared to wild-type levels, confirming the defect in basal and specific resistance, respectively (Figure 15 B). The endogenous accumulation of Pip is therefore critical for resistance establishment.

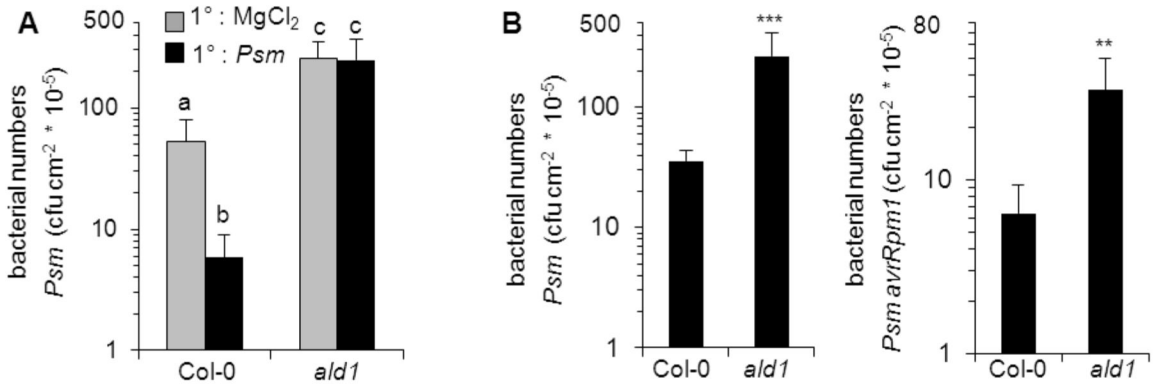


Figure 15. Comparison of SAR, basal and specific resistance in Col-0 and *ald1* mutant. (**A**) Systemic acquired resistance assay. Lower leaves (1°) were infiltrated with *Psm* (OD 0.005) or 10mM $MgCl_2$ and 2 days later the upper (2°) leaves were challenge with *Psm* (OD 0.001). (**B**) Bacterial numbers of compatible *Psm* (OD 0.001) and incompatible *Psm avrRpm1* (OD 0.002) in Arabidopsis Col-0 and *ald1* leaves at 3 dpi. Values represent mean values ±SD of colony forming units (cfu) per square centimetre from at least seven replicate samples, each consisting of three leaf disks. Asterisks denote statistically significant differences between *ald1*- and Col-0 samples (***: $P < 0.001$; **: $P < 0.01$; two tailed *t* test).). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test). No significant differences in initial bacterial numbers (1 hpi) were detected.

Next, it was tested whether exogenous application of Pip could complement the attenuated resistance in *ald1*. Different approaches to exogenous Pip application were performed. Spraying 500µM D,L-Pip supplemented with 0.02% Silwet after *Psm* inoculation did not rescue the *ald1* phenotype. The numbers of bacteria determined after inoculation with either *Psm* suspension alone or *Psm* suspension supplemented with 1mM D,L-Pip, were also not statistically different. When 10 ml of 1mM (~10µmol) Pip was supplied to the soil one day prior to inoculation, the numbers of bacteria decreased more than 10-times compared to water control treatment (Figure 16). Therefore, further experiments were performed using the watering method.

To examine whether exogenous application of Pip will change the local resistance responses in Col-0 and SAR-deficient mutant *fmo1*, the plants of Col-0, *ald1* and *fmo1* were watered with 10µmol of racemic D,L-Pip one day prior to inoculation with *Psm* or *Psm avrRpm1*. Numbers of bacteria were determined 3 days later. Exogenous Pip significantly increased the resistance in Col-0 in both compatible and incompatible interaction, and rendered basal and specific resistance of *ald1* to wild-type levels (Figure 17 A-C). The phenotype was not rescued in the case of the *fmo1* mutant, indicating that FMO1 plays a role downstream of Pip.

To investigate the substance limit where Pip confers resistance induction, different amounts of Pip were applied to the soil. Application of 1µmol but not of 100nmol Pip per plant was sufficient to significantly enhance resistance to *Psm* in Col-0 or *ald1*, with 10µmol

further augmenting the effect (Figure 17 D). To investigate whether the D- or L- enantiomer can chemically complement the phenotype of *ald1*, plants were watered with 5 µmol D- or L-Pip only, or 10µmol D,L-Pip. The L-Pip enantiomer and racemic D,L-Pip treatments decreased the numbers of bacteria 10 times compared to the D-Pip enantiomer or control H_2O application (Figure 17 E). These data suggest that L-enantiomer is the biologically active form of Pip.

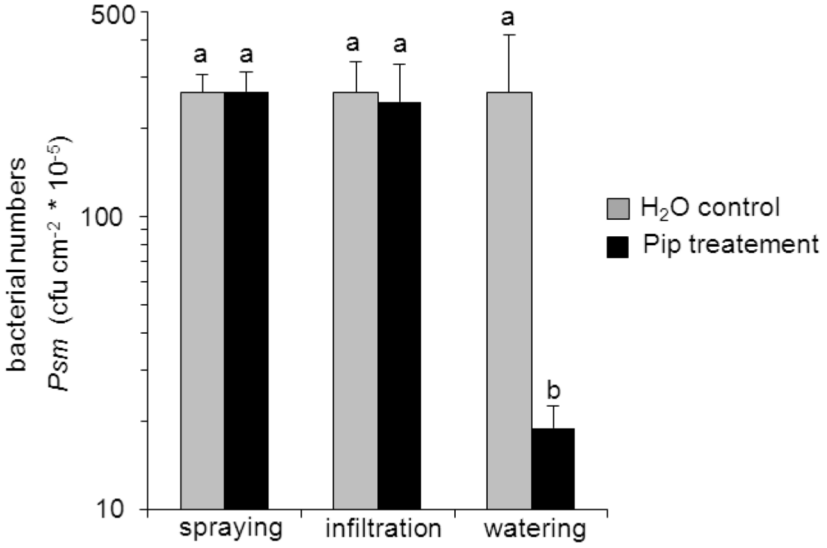


Figure 16. Comparison of different exogenous application of Pip in *ald1* mutant. Bacterial numbers after *Psm* (OD 0.001) inoculation in Arabidopsis ald1 mutant were determined 3 dpi. Spraying: plants were sprayed at 6 and 24 hpi with 500µM D,L-Pip solution supplemented with 0.02% Silwet. Infiltration: leaves were infiltrated either with *Psm* suspension only or with *Psm* suspension supplemented with 1mM D,L-Pip. Watering: 10µmol of D,L-Pip was supplemented to the soil one day prior to *Psm* inoculation. The appropriate water treatments were performed as controls. Values represent mean values ±SD of colony forming units (cfu) per square centimetre from at least seven replicate samples, each consisting of three leaf disks. Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test). No significant differences in initial bacterial numbers (1 hpi) were detected.

It was shown that exogenous application of Pip can rescue the attenuated local resistance of *ald1* plants. To test whether exogenous Pip will also rescue also its SAR deficiency, the *ald1* plants were watered with 10µmol D,L-Pip one day prior to the primary $MgCl_2$ or *Psm* treatment in lower leaves. Two days later, the upper leaves were challenged with *Psm*, and the bacterial growth in upper leaves was assessed another 3 days later. In the presence of Pip, *Psm* inoculation of *ald1* plants in lower leaves also rendered upper leaves more resistant to subsequent infection, demonstrating that SAR can be restored by exogenous application of Pip (Figure 18 A). Thus, exogenous Pip can chemically complement the defects of *ald1* in basal, specific and systemic acquired resistance.

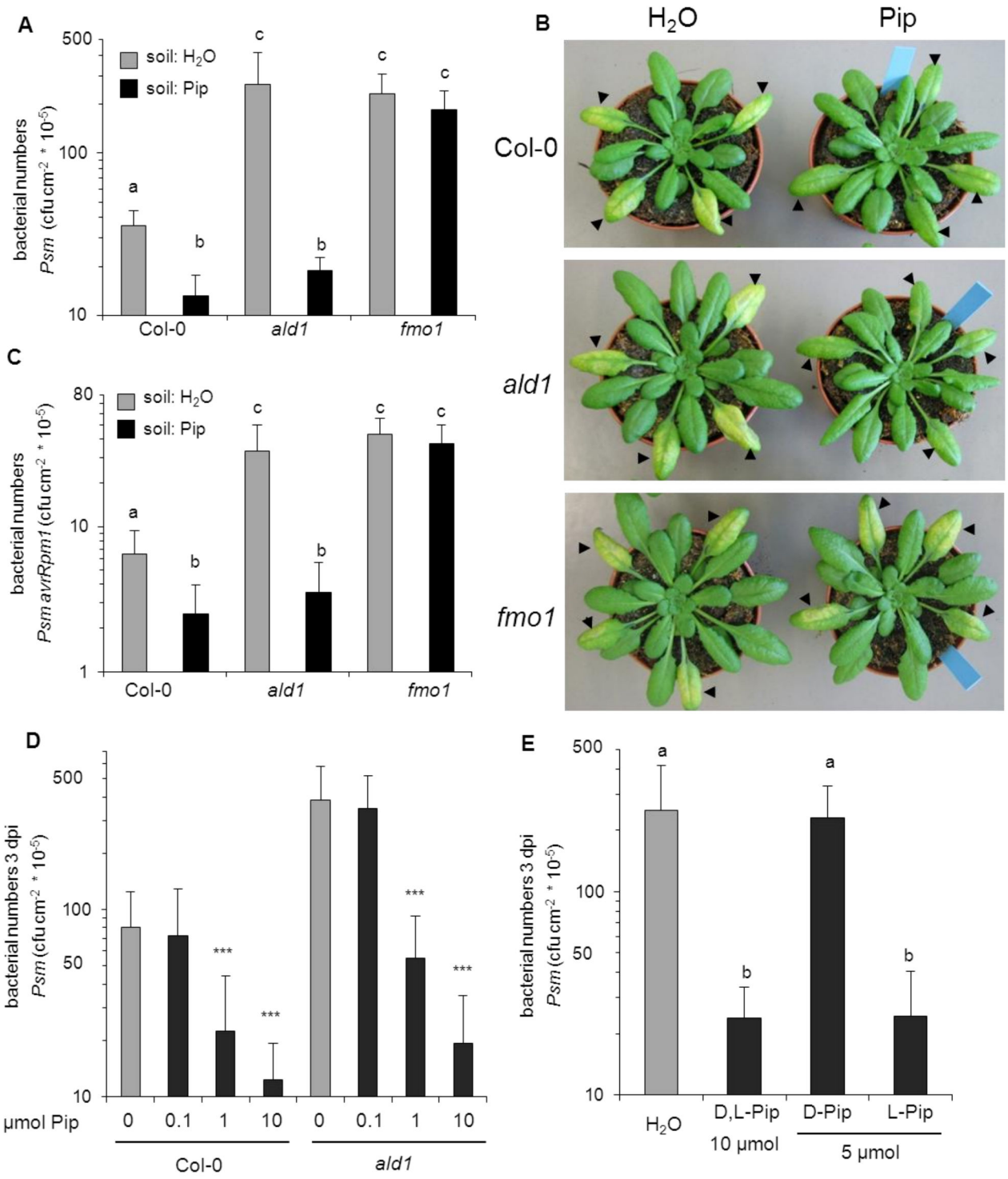


Figure 17. Basal and specific resistance changes after exogenous application of Pip. (**A**) Bacterial numbers of compatible *Psm* (OD 0.001) in Arabidopsis Col-0, *ald1* and *fmo1* leaves at 3 dpi. Pots were supplied with 10 ml of 1mM D,L-Pip (10µmol) or H_2O one day prior to inoculation. (**B**) Disease symptoms of *Psm*-infected plants in the absence and presence of exogenous D,L-Pip. Arrows denote inoculated leaves. (**C**) Bacterial numbers of incompatible *Psm avrRpm1* (OD 0.002) in Arabidopsis Col-0, *ald1* and *fmo1* leaves at 3 dpi. (**D**) Concentration dependency of resistance induction by exogenous D,L-Pip in Col-0 and *ald1* plants. (**E**) Indicated amount of racemic D,L-Pip, D-Pip, or L-Pip were fed to roots one day prior to *Psm* inoculation of *ald1* plants, and bacterial numbers were scored at 3 dpi. Values represent mean values ±SD of colony forming units (cfu) per square centimetre from at least seven replicate samples, each consisting of three leaf disks. Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-samples (***: $P < 0.001$; two tailed *t* test). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test). No significant differences in initial bacterial numbers (1 hpi) were detected.

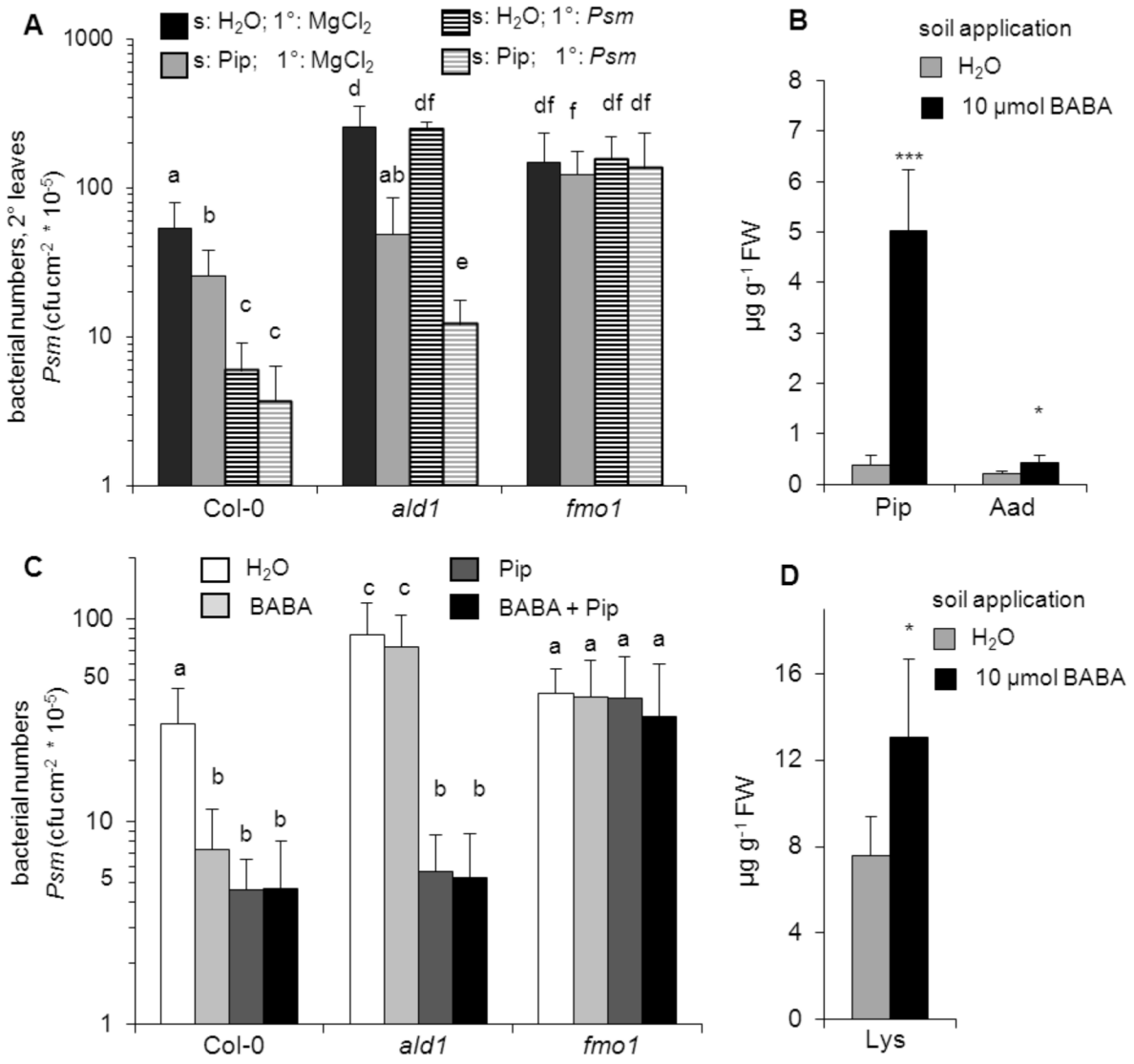


Figure 18. Induced resistance changes after exogenous application of Pip. (**A**) Systemic acquired resistance in Col-0, *ald1* and *fmo1*. One day prior to lower leaves inoculation, H_2O or 10 µmol D,L-Pip were applied through the soil (s) to each pot. Lower leaves (1°) were infiltrated with *Psm* (OD 0.005) or 10mM $MgCl_2$ and 2 days later the upper (2°) leaves were challenged with *Psm* (OD 0.001). Bacterial growth was assessed 3 days post 2° leaf-inoculation. For more see Figure 17 (**B, D**) Leaf content of Pip, Aad, and Lys after exogenous β-aminobutyric acid (BABA). Leaves were collected one day after supplying plant pots with 10 µmol BABA or H_2O. Mean values are given in $\mu g.g^{-1}$ fresh weight (FW) ± SD from at least three replicate samples. Asterisks denote statistically significant differences between samples from BABA-fed and from control plants (***: $P < 0.001$; *: $P < 0.05$; two tailed *t* test). (**C**) BABA-induced resistance assay in Col-0, *ald1* and *fmo1*. H_2O, 10 µmol D,L-Pip, 10µmol of BABA or the combination of both were applied through the soil, and resistance toward *Psm* was assessed as detailed in A. Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

BABA is an amino acid that is not produced in plants, but effectively enhances plant disease resistance when exogenously supplied through the soil via a SAR-like mechanism (Zimmerli *et al.*, 2000). Plants watered with 10µmol BABA resulted in moderate augmentation of Lys and Aad levels and a strong increase of Pip levels in the leaves (data provided by J. Zeier) which was quantitatively comparable to the systemic accumulation of

Pip during SAR (Figure 18 B, D; Figure 13 A). Therefore, the mode of action of BABA was analysed also in *ald1* and *fmo1*. Plants were fed with BABA one day prior to inoculation of *Psm* (OD 0.001) and the number of bacteria was scored 2 days later. BABA application 4-times increased the resistance of Col-0 plants, similarly to exogenous Pip treatment, and application of both BABA and Pip did not further increased the resistance. BABA did not enhance the resistance against *Psm* in the *ald1* mutant, indicating that induced biosynthesis of Pip following BABA application is causative for the enhanced plant resistance towards *P. syringae* (Figure 18 C). Similar to SAR, BABA-induced resistance was also fully blocked in *fmo1*, but exogenous Pip was neither able to restore SAR nor BABA-induced resistance in this mutant (Figure 18 A, C). This result is consistent with a possible function of FMO1 downstream of Pip in resistance induction.

5.1.8 Pipecolic acid primes defence responses

It was previously demonstrated that BABA-IR is predominantly based on a defence priming mechanism, *i.e.* BABA primes SA-inducible defences (Zimmerli *et al.*, 2000). Since exogenous BABA application induced Pip biosynthesis, we reasoned that BABA-IR can be mediated by Pip synthesis.

Although defence priming induced by resistance activating chemicals such as BABA or S-methyl-1,2,3-benzothiadiazole-7-carbothioate (BTH) is a well-studied phenomenon, evidence for priming in plants in which SAR has been biologically induced is rare (Jung *et al.*, 2009; Pick *et al.*, 2012). To examine defence priming during biological SAR, a double inoculation experiment was set up. Arabidopsis plants were first treated in lower (1°) leaves with $MgCl_2$ or *Psm* (OD 0.005), and two days later, upper (2°) leaves were infiltrated with $MgCl_2$ or *Psm*. This yielded four distinguishable cases corresponding to a control situation (1° $MgCl_2$/ 2° $MgCl_2$ = “mm”), a systemic pathogen stimulus (1° *Psm*/ 2° $MgCl_2$ = “pm”), a local pathogen stimulus (1° $MgCl_2$/ 2° *Psm* = “mp”), and a combination of both systemic and local stimuli (1° *Psm*/ 2° *Psm* = “pp”). Upper leaves were then sampled at 10 h post 2° treatment.

The defence metabolite levels were then determined. Total SA (sum of free and conjugated SA) was accumulated in both “pm” and “mp” treatments combinations in Col-0 plants. However, reduced SA contents were observed in the *ald1* mutant, which is consistent with Song *et al.* (2004b) observation. In double pathogen inoculation (“pp”), a higher increase in SA level was observed that corresponded approximately to the sum of local and systemic response. This additive effect was lost in the *ald1* mutant (Figure 19 A).

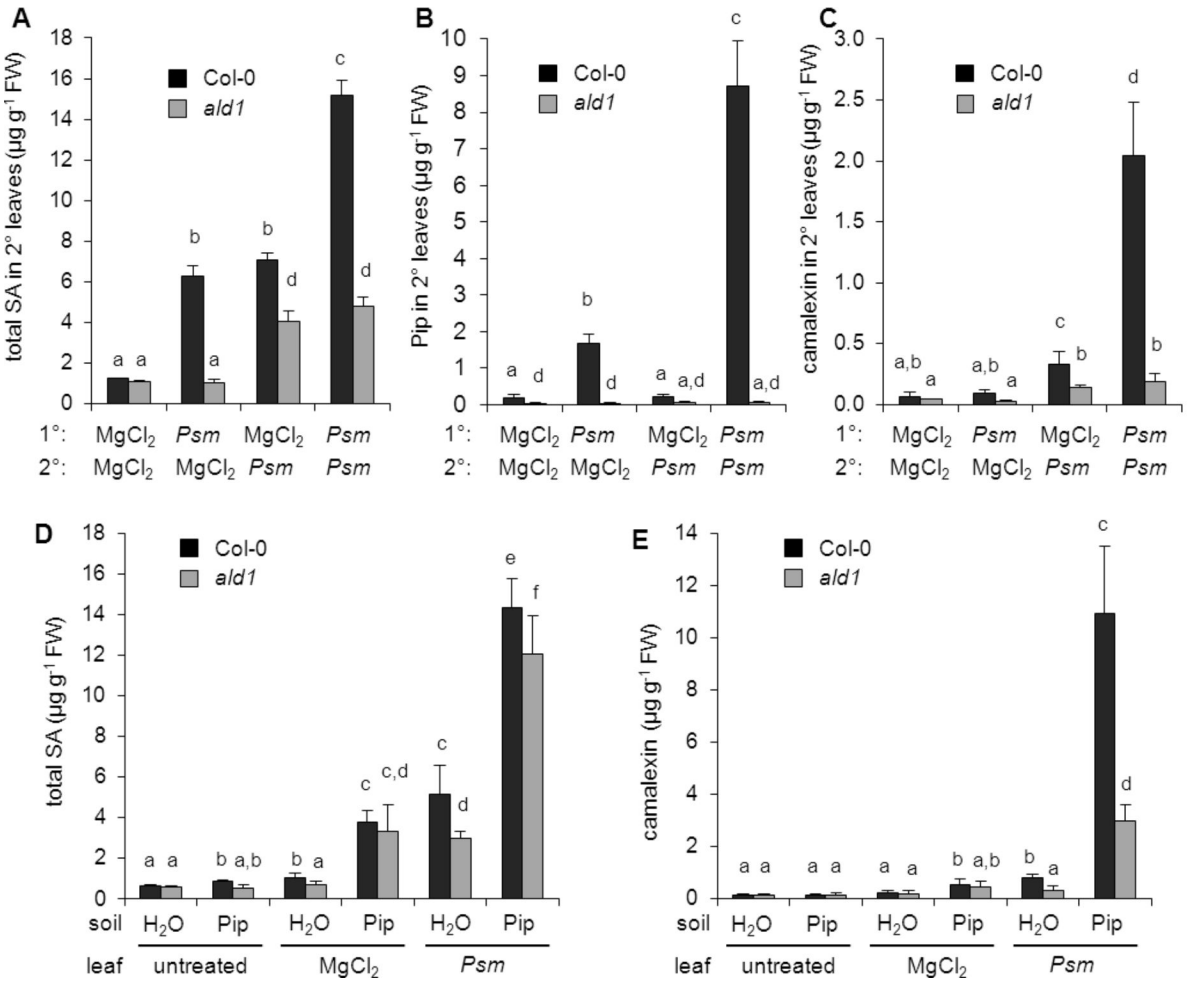


Figure 19. Priming of defence responses following biological SAR induction and exogenous Pip application. (**A-C**) Double inoculation experiment to assess defence priming during SAR in Col-0 and *ald1*. Plants were treated in lower (1°) leaves with $MgCl_2$ or *Psm* (OD 0.005), and two days later, upper (2°) leaves were infiltrated with $MgCl_2$ or *Psm*. Upper leaves were then scored for total SA (sum of free and conjugated SA) (A), Pip (B), and camalexin accumulation (C) at 10 h post 2° treatment. This yielded four distinguishable cases corresponding to a control situation (1° $MgCl_2$/ 2° $MgCl_2$), a systemic pathogen stimulus (1° *Psm*/ 2° $MgCl_2$), a local pathogen stimulus (1° $MgCl_2$/ 2° *Psm*), and a combination of both the systemic and the local stimuli (1° *Psm*/ 2° *Psm*). (**D**, **E**) Priming of local defence responses after exogenous Pip application in Col-0 and *ald1*. H_2O or 10µmol D,L-Pip were applied to plants as outlined for Figure 17. Leaves were infiltrated one day later with $MgCl_2$ or *Psm* (OD 0.005) or not infiltrated at all, and local defence responses were scored 10 h after infiltration. (D) Total SA accumulation. (E) Camalexin accumulation. Mean values are given in $\mu g.g^{-1}$ fresh weight (FW) ± SD from at least three replicate samples. Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

Pip was produced only in Col-0 plants. At 10 h after local inoculation, no production of Pip was determined and only moderate increase was observed for "pm". In contrast to SA accumulation, Pip biosynthesis increased in combined pathogen treatments more than 4-times compared to the sum of independent pathogen inoculations (Figure 19 B). This suggests that in the SAR induced state Pip accumulates to high levels earlier than in the case of the non-induced state. Camalexin accumulated in WT only as a consequence of the local, but not the systemic, pathogen trigger. Camalexin levels in the *ald1* mutant were significantly lower compared to the wild type; such observation is in agreement with Song *et al.* (2004b). The

“pp” treatment potentiated this wild-type local response more than 4-times (Figure 19 C). However, camalexin strong potentiation was completely lost in the *ald1* mutant. These data suggest that SA, Pip and camalexin are primed during the biologically induced SAR (data partly produced by A. Döring). Moreover, the *ald1* mutant failed to accumulate camalexin as well as exhibiting attenuated SA accumulation, indicating that pathogen-triggered Pip accumulation is necessary for priming.

To investigate whether exogenous Pip would render the plant into a primed state, plants were supplemented with 10µmol of D,L-Pip or H_2O to the soil. One day later, Col-0 and *ald1* plants were either left untreated or inoculated, with 10mM $MgCl_2$ or *Psm* (OD 0.005), and the leaves were then collected 10 hpi. Pip treatment alone was not sufficient for SA induction in the plants. However, exogenous Pip treatment in combination with mock inoculation induced SA biosynthesis in both Col-0 and *ald1* plants. These data suggest that Pip positively regulates local SA production after mechanical stimuli. Pick *et al.* (2012) showed that infiltration with water only primed defence gene activation after BTH pre-treatment, suggesting that Pip positively regulates defence responses in the same manner as BTH. Moreover, application of Pip prior to *Psm* inoculation potentiated SA biosynthesis up to 3-fold higher levels in Col-0 and *ald1* than those detected in *Psm*-infected leaves of unfed plants (Figure 19 D). Exogenous Pip treatment complemented the *ald1* SA biosynthesis deficiency. In addition, Pip feeding restored the capacity of the *ald1* plant to systemically enhance SA levels upon localized pathogen inoculation, indicating the importance of Pip for systemic accumulation during SAR (Figure 21 A).

Camalexin levels were not augmented at 10 h post any treatment condition except for Pip soil application combined with *Psm* inoculation. Here, camalexin levels increased 10-times over the H_2O/*Psm* combination in Col-0 (Figure 19 E). However much the Pip application increased the camalexin level in Pip/*Psm* treatment in the *ald1* mutant, the level did not reach the value of Col-0. This observation indicates that exogenous Pip cannot completely rescue the endogenous biosynthesis of Pip in plants (Figure 19 E). Taken together, similar to the priming response characteristic for biological SAR, exogenous Pip positively regulates SA biosynthesis and strongly potentiates camalexin production.

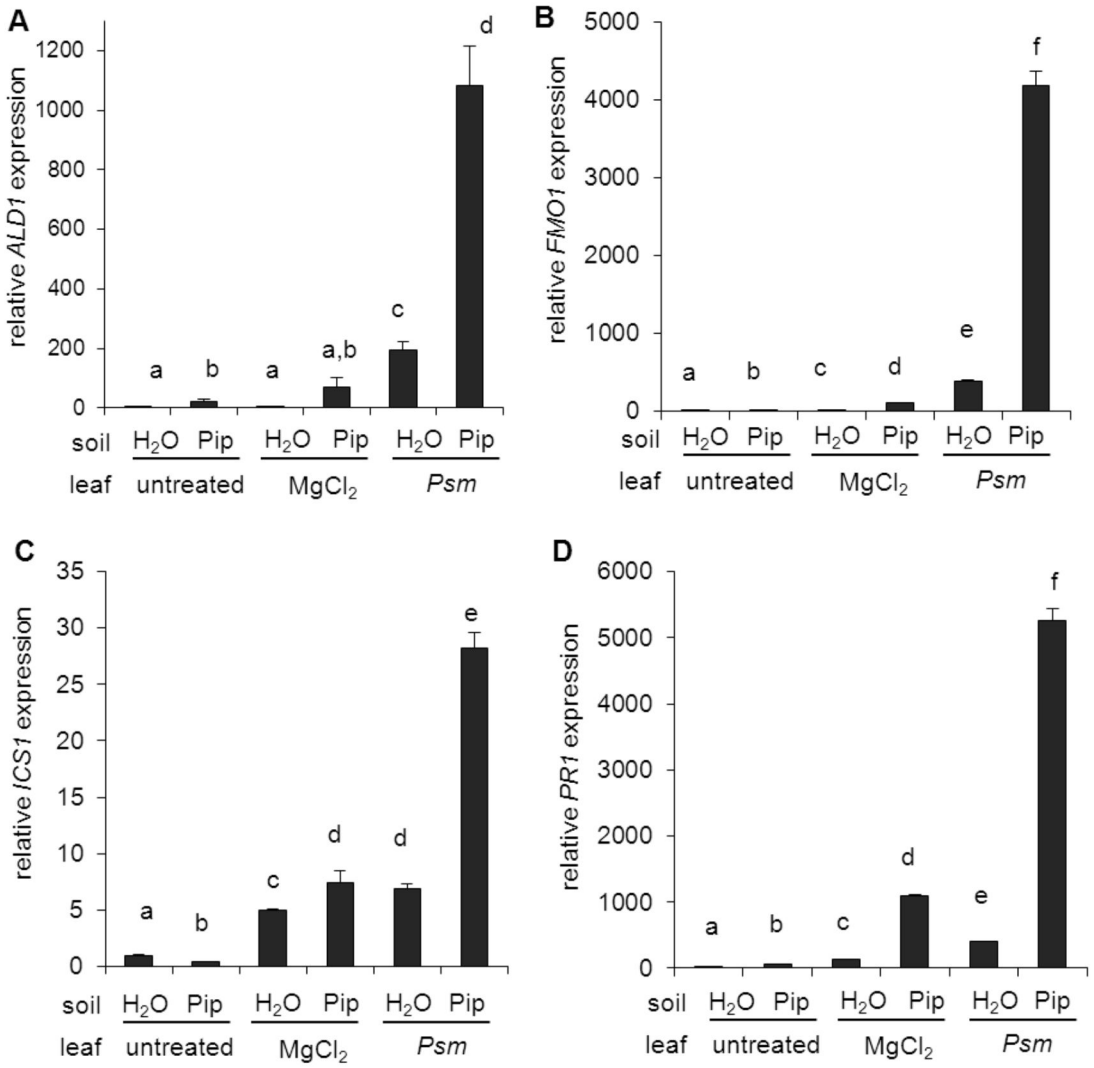


Figure 20. Gene expression following the exogenous Pip application in Col-0. H_2O or 10µmol D,L-Pip were applied to plants as outlined for Figure 17. Leaves were infiltrated one day later with $MgCl_2$ or *Psm* (OD 0.005) or not infiltrated at all, and local defence responses were scored 10 h after infiltration. Transcript levels were assessed by quantitative PCR analysis, values are given as means ± SD of three replicate samples, and are expressed relative to the respective mock-control value. Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).The expression of *ALD1* (**A**), *FMO1* (**B**), *ICS1* (**C**) and *PR1* (**D**).

Moreover, Pip feeding to Col-0 plants significantly primed *ALD1* expression after pathogen inoculation (Figure 20 A), indicating a positive regulatory role for Pip on its own biosynthesis. Exogenous Pip also primed plants for pathogen-triggered transcript levels of *FMO1*, *ICS1* and *PR1* gene (Figure 20 B-D). In addition, the expression of *ALD1* determined in systemic leaves after 48 h post 1° leaves inoculation, was triggered by pathogen in Col-0 plants but no significant rise was observed in the *fmo1* and *ics1* mutants (Figure 21 B). Therefore, there are differences in the signalling networks underlying local and systemic resistance.

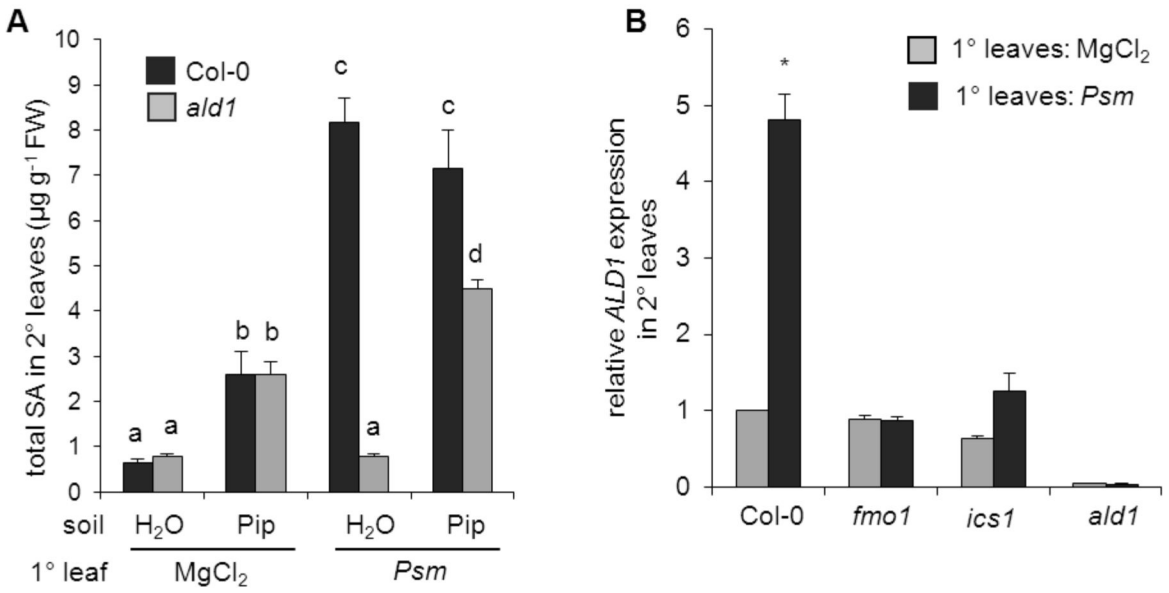


Figure 21. Responses in systemic leaves. The data in 2° leaves were assessed 2 days after 1° inoculation of either 10mM $MgCl_2$ or *Psm* (OD 0.005), (**A**) The relative expression of *ALD1* in Col-0 and several mutants, for more details see Figure 20. (**B**) Systemic accumulation of total SA upon SAR induction following exogenous Pip feeding (see Figure 19). Asterisks denote statistically significant differences between *Psm*- and $MgCl_2$-samples (*: $P < 0.05$; two tailed *t* test). Different letters above the bars denote statistically significant differences ($P < 0.05$; two tailed *t* test).

5.2 BIOCHEMICAL FUNCTION OF FMO1

5.2.1 A role in histone modification during SAR

Preliminary results from transient expression of 35S::FMO1::GFP constructs in onion epidermal cells suggested a nuclear localization of FMO1 (data produced by T. Zeier). These observations led to the hypothesis that FMO1, as a nuclear protein, could mediate the *ICS1* expression through putative histone modification. In animals, amine oxidases, which mediate similar reaction as FMOs, are involved in histone H3 demethylation (Shi *et al.*, 2004). Therefore, changes in histone methylation pattern changes in 2° leaves upon SAR-induction were tested in Col-0 and in *fmo1*. The naïve plants were inoculated with either 10mM $MgCl_2$ or *Psm* (OD 0.005) in 1° leaves and two days after the 2° leaves were harvested for chromatin immunoprecipitation assay. The cross-linked DNA to proteins was precipitated with antibodies to histones: H3K4m2 (dimethylated Histone H3 at Lys 4), H3K4m3 (trimethylated Histone H3 at Lys 4), H3K9m2 (dimethylated Histone H3 at Lys 9), H3K27m2 (dimethylated Histone H3 at Lys 27) and H3K27m3 (trimethylated Histone H3 at Lys 27).

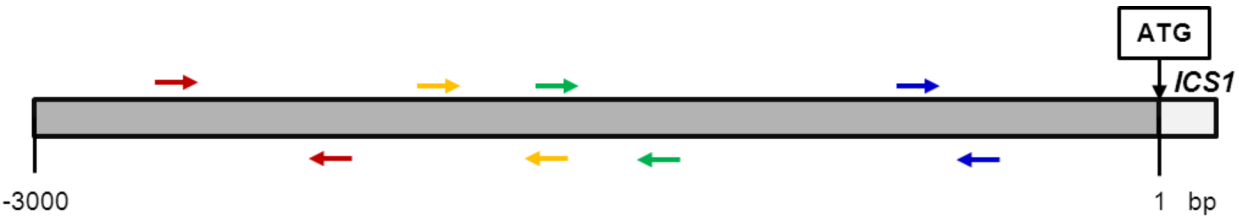


Figure 22. Diagram of ChIP primer pairs matching the promoter of *ICS1* (At1g74710). Adenine, first base of *ICS1* gene was set up as 1. Primers A are signed by red arrows, primers B by yellow arrows, primers C by green arrows and primers H by blue arrows.

To investigate whether histone modifications are involved in the transcriptional regulation of *ICS1*, different primer pairs matching its promoter were designed: primer pair **A** matches the region (-2605, -2140), **B** (-1932, -1564), **C** (-1655, -1259), and **H** (-765, -481), where the first adenine of the starting codon of *ICS1* gene was set up as 1 (Figure 22).

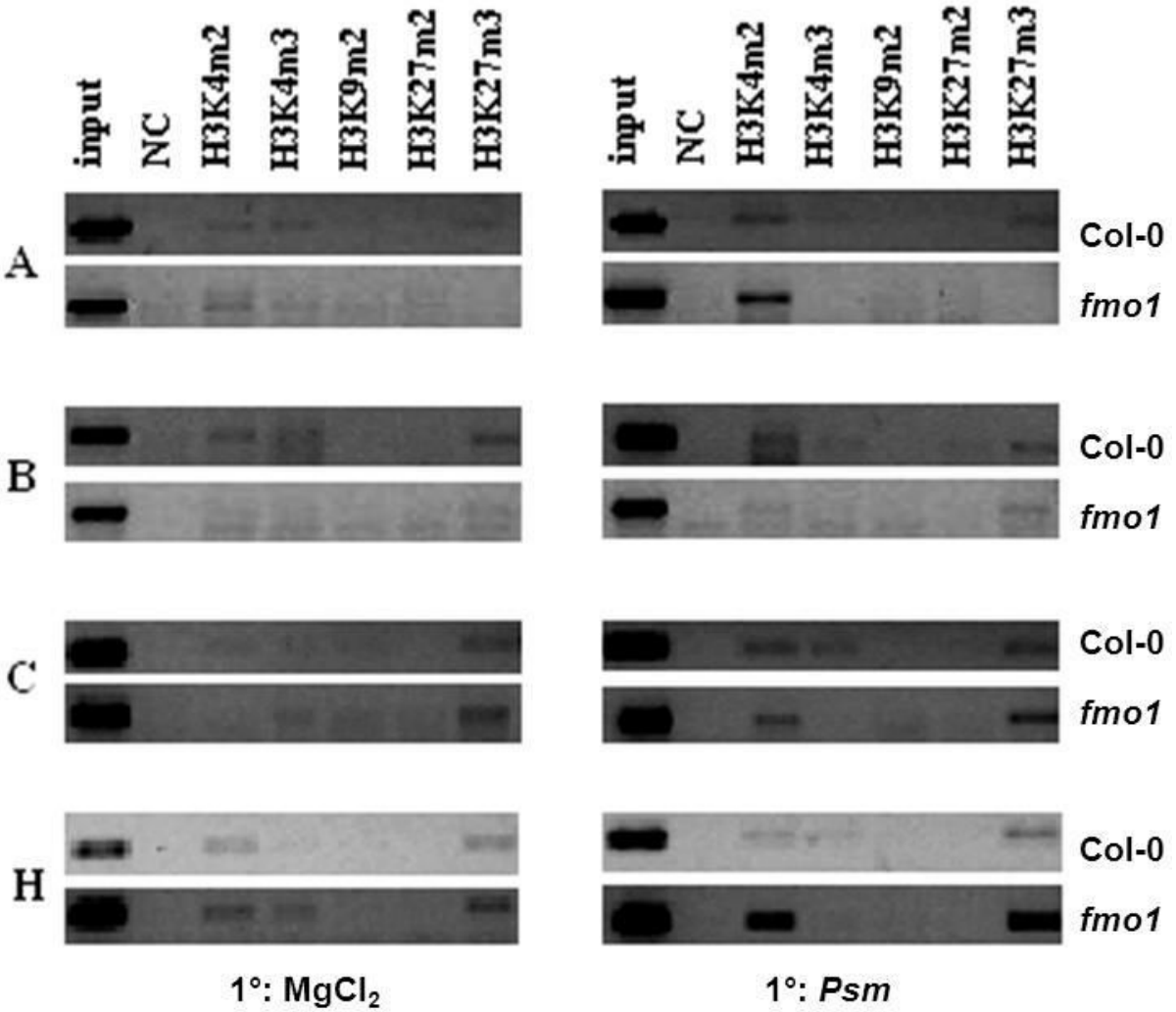


Figure 23. The agarose gel analysis of chromatin immunoprecipitation assay. PCR products of *ICS1* promoter DNA which were cross-linked to H3 histone. DNA from the 2° leaves of Col-0 or *fmo1* was obtained 2 days after 1° inoculation with either 10mM $MgCl_2$ or *Psm* (OD 0.005). Input: positive control (genomic DNA), NC: negative control (without Ab). H3K4m2: Histone H3 Lys 4 dimethylation, H3K4m3: Histone H3 Lys 4 trimethylation, H3K9m2: Histone H3 Lys 9 dimethylation, H3K27m2: Histone H3 Lys 27 dimethylation and H3K27m3: Histone H3 Lys 27 trimethylation.

No changes of H3K9m2 and H3K27m2 of the *ICS1* promoter were observed. For all primer pair combinations, the *ICS1* promoter was associated with enhances levels of H3K4m2 and H3K4m3 in systemic tissue of Col-0 upon local *Psm* inoculation (visible especially in primer pairs C). Histone methylation was found for trimethylation of Lys 4 on histone H3 on promoters and coding sequences of active genes. By contrast, the roles of dimethylation of the same residue in gene regulation are less defined. Genes associated with H3K4m2 are, in the absence of H3K4m3, expressed at very low levels. However, dimethylation, together with trimethylation of H3K4, is likely associated with active transcription (Zhang *et al.*, 2009). Therefore these methylation changes could contribute to priming of the *ICS1* gene and synthesis of SA in systemic leaves of Col-0. In contrast, *fmo1* failed to show H3K4m3 enrichment at the *ICS1* promoter, but displayed significant increase with H3K4m2. These data suggest that the *ICS1* promoter is silenced in *fmo1* plants (Figure 23).

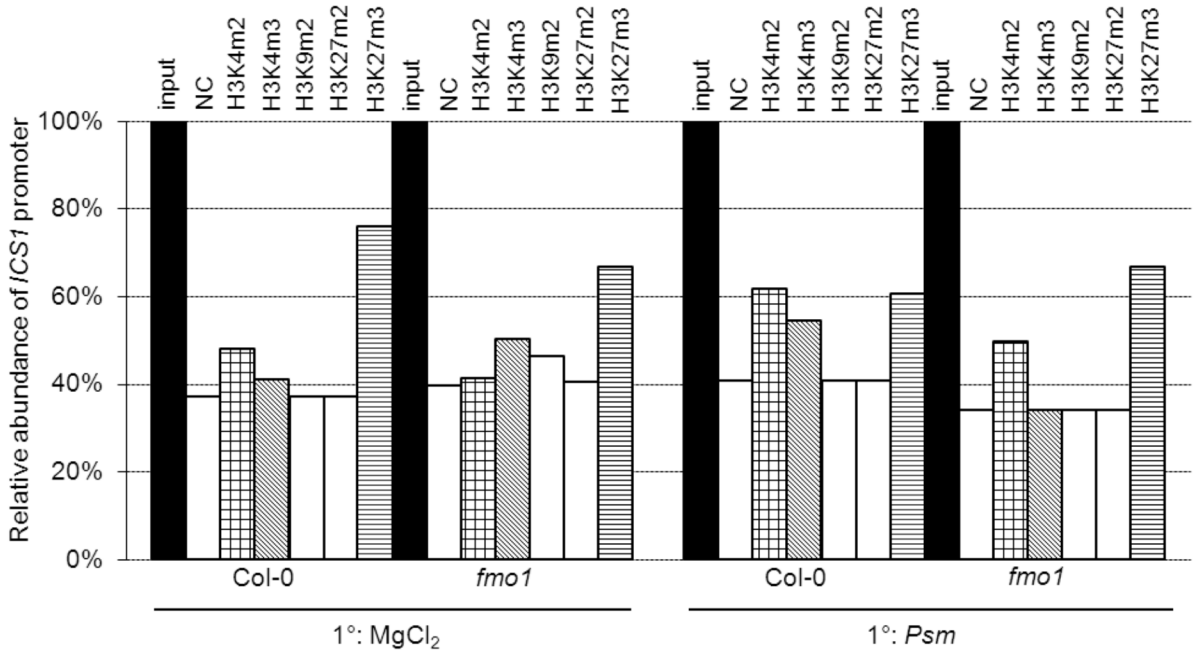


Figure 24. Quantification of *ICS1* promoter. The signal intensities of PCR products detected with C primer pair (see Figure 23) were measured using ImageJ analysis software. The relative levels of target DNA expression were determined by normalizing their individual band intensity to the input band intensity, which was set up as 100%.

Furthermore, the *ICS1* promoter was not altered in H3K27m3 levels neither in $MgCl_2$ nor *Psm* pre-treated *fmo1* plants (Figure 24). In contrast, a small decrease of *ICS1* promoter with H3K27m3 was observed in Col-0 systemic leaves after local *Psm* inoculation. Since H3K27m3 is associated with transcriptional silencing (Zhang *et al.*, 2007a), this histone modification could contribute to increased transcriptional capacity.

5.2.2 MAP kinase activity in *fmo1*

MAP kinases are involved in signal transduction in eukaryotic cells. FMO1 could influence the MAP kinase activity, the signals are then wrongly or not sufficient transferred and therefore *fmo1* fails to establish SAR. The systemic leaves were collected at 48 h post local inoculation by 10mM $MgCl_2$ or *Psm* (OD 0.005) and total proteins aliquot (20µg) was loaded for detection of MAPK activity in gel using radioactive γ-^{32}P-ATP incorporation.

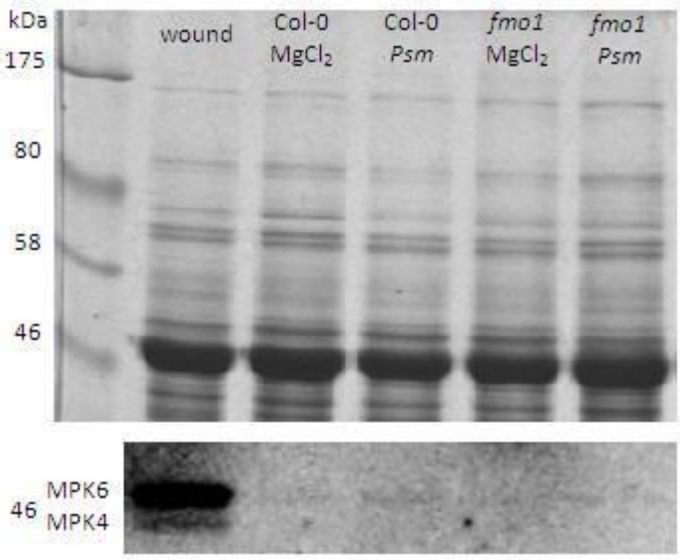


Figure 25. The MAP kinase in gel activity assay. The systemic leaves of Col-0 and *fmo1* plants sampled 48 h post local 10mM $MgCl_2$ or *Psm* inoculation were used for protein purification. The SDS-PAGE shows an equal protein distribution. Radiophotoscan of γ-^{32}P-ATP incorporated in a MAP kinase substrate (myelin basic protein) in gel, detecting the MPK6 and MPK4 kinases. Wound: positive control, sampled 5 min after wounding with forceps.

The SDS-PAGE shows an equal protein distribution. It was possible to detect the strong MPK6 activity and slight MPK4 activity in positive control (leaves sampled 5 min after wounding with forceps). The AtMPK6 and AtMPK4 are rapidly activated by wounding with maximum activity by around 5-10 min (Ichimura et al., 2000). However, only weak activity of MPK6 was observed in systemic leaves at 2 days post local *Psm* inoculation. Moreover, no difference was observed among $MgCl_2$, *Psm*, Col-0 or *fmo1* samples. These data suggest that MAP kinases are not active in systemic leaves of SAR-induced plants at 2 dpi (Figure 25). Therefore the kinetic experiment should be performed for better understanding of MAPK activity in the course of SAR establishment.

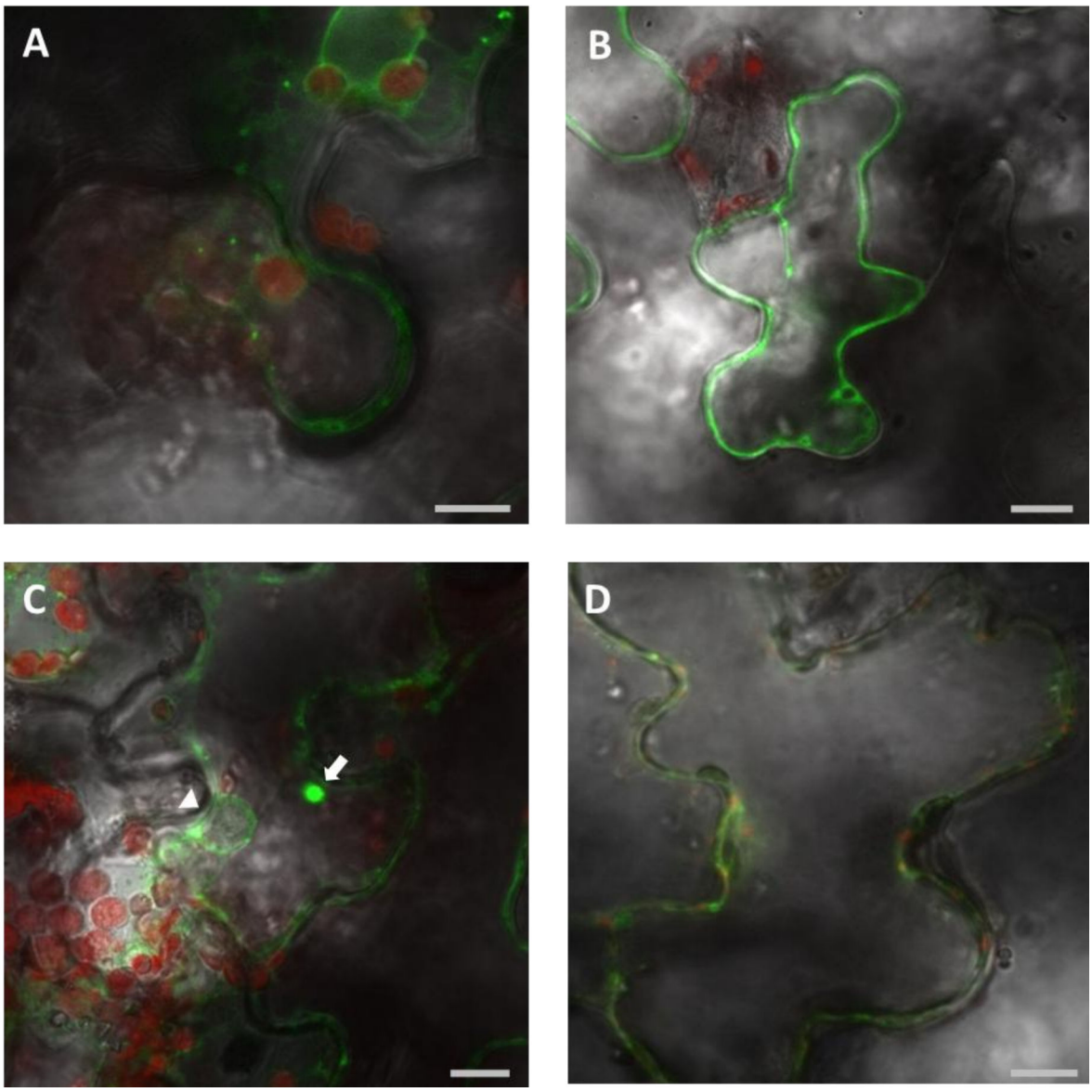


Figure 26. Expression pattern of FMO1-GFP-fusion protein in *Nicotiana benthamiana*. Fluorescence microscopic analysis in leaf tissue of *N. benthamiana*. Overlay images of bright-field microscopic graph (grey, showing epidermal cells), (**A**) 35S::FMO1::GFP green fluorescence (detecting GFP), red autofluorecsence (detecting chloroplasts) (**B**) 35S::GFP green fluorescence (detecting GFP), red autofluorecsence (detecting chloroplasts) (**C**) 35S::FMO1::GFP over expression (3 days after infiltration), green fluorescence (detecting GFP), red autofluorecsence (detecting chloroplasts), white arrow detect circular aggregate, white arrow head detect the nucleus (**D**) 35S::FMO1::GFP, GA-mCherry merge image green fluorescence (detecting GFP), red fluorescence (detecting GA-mCherry). Size bars: 10µm (microscopy performed by F. K. H. Breuers).

5.2.2 Localization of FMO1

The subcellular localization of FMO1 could provide valuable information about its function. The transient expression of the 35::FMO1::GFP construct and the control 35::GFP vector were performed in *Nicotiana benthamiana* using *Agrobacterium tumefaciens* transformation. Four to six-weeks old plants were infiltrated with the Agrobacterium C58C1 strain carrying the GFP-reporting vectors in a density of OD_{600} 0.1. One, two, and three days after infiltration, fluorescence microscopic examination was performed.

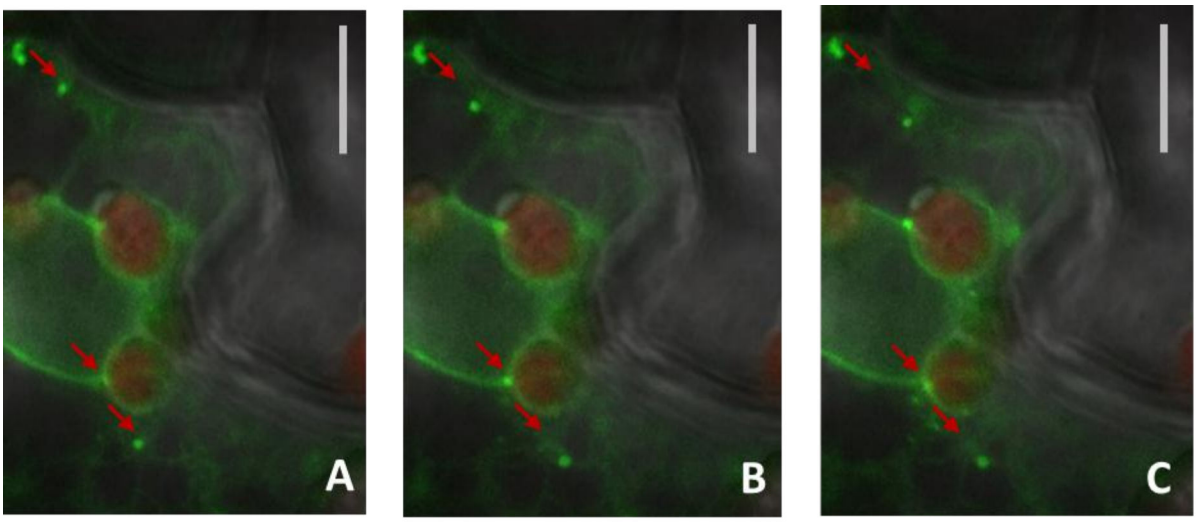


Figure 27. Time points series of expression pattern of 35S::FMO1::GFP. Overlay images of bright-field microscopic graph (grey, showing epidermal cells), green fluorescence (detecting GFP), red autofluorecsence (detecting chloroplasts). Time points at (**A**) 0 s, (**B**) 7 s, and (**C**) 34 s of 35S::FMO1::GFP in epidermal cells of *Nicotiana benthamiana*. Red arrows show moving vesicles. Size bars: 10µm (microscopy performed by F. K. H. Breuers).

In contrast to previous observations, the FMO1-GFP expression was detected in small amounts in the cytoplasm but mostly in circular fast moving vesicles (Figure 27, Figure 26 A). The 35S::GFP control construct showed cytoplasm localization only (Figure 26 B). Previously the same construct transiently expressed in onion epidermal cell showed localization in nucleus (T. Zeier, unpublished data). In the over-expressed sample (3 days after inoculation), the GFP signal was highly detected in the endoplasmatic reticulum (ER) and in big circular aggregates. Figure 26 C shows the nucleus (arrowhead) and the circular aggregate (highlighted by arrow) in over-expressed leaf tissue. So it is tempting to speculate, that the FMO1 protein is targeted to the secretory pathway, but also accumulates in the cytosol while the ER and the Golgi apparatus is overwhelmed by the protein amount. The bombardment of FMO1-reporting construct to onion epidermal cell (T. Zeier, unpublished data) could also cause the over-expression and therefore high accumulation of the FMO1 protein in ER which surrounds the core. The localization of GFP to the nucleus and not only to a compartment surrounding the nucleus was not observed in this Nicotiana based study. The Golgi apparatus (GA) mCherry-reporting construct was also detected in moving vesicles (data not shown); however, in coexpression of GA-mCherry and FMO1-GFP no colocalization was observed (Figure 26 D). Therefore further studies with other organelle-reporting constructs will be necessary for an accurate subcellular localization of the FMO1 protein.

6 DISCUSSION

Systemic acquired resistance (SAR) is an enhanced state of broad-spectrum disease resistance that develops in the whole plant in response to a locally restricted leaf inoculation with microbial pathogen. Many aspects of SAR still remain elusive, although the molecular mechanisms of plant defence have become an intensive research topic over the last years. Upon infection with a pathogen, plants induce a multitude of metabolic changes with potential impact on plant resistance. The current work is predominantly focussed on changes of amino acids contents in the model plant *Arabidopsis thaliana* in response to *Pseudomonas syringae* infection and their biological relevance.

Here, we demonstrate that several free amino acids highly accumulate after *P. syringae* attack in local (inoculated) leaves (Table IV.). They are mostly aliphatic branched-chain amino acids (BCAAs) such as Val, Leu and Ile. It was described that the breakdown products of BCAAs, which include acetyl-CoA, propionyl-CoA, and acetoacetate, are potential energy sources for plants (Taylor *et al.*, 2004). Therefore their accumulation could help support the energy-demanding fight against the pathogen. It has been also proposed that accumulation of free BCAAs may serve as a substrate for the synthesis of stress-induced proteins and that BCAAs may act as signalling molecules to regulate gene expression (Nambara *et al.*, 1998). Ile conjugation to jasmonic acid (JA) creates the functionally active derivative of JA, which then contributes to the JA defence-signalling pathway (Thines *et al.*, 2007). The Arabidopsis defence against *P. syringae* is based on salicylic acid (SA) signalling, that actually acts antagonistically to JA-dependent defence mechanisms (Glazebrook, 2005). Therefore the Ile accumulation could be induced by the pathogen to support the JA responsive pathway. Activation of JA signalling might repress the SA resistance pathway, facilitating pathogen proliferation in the plant apoplast.

However, the isoleucine-related molecule, isoleucic acid (2-hydroxy-3-methyl-pentanoic acid) was recently shown to enhance resistance towards *P. syringae*, positively regulating the SA pathway and *PR1* expression. In plants, isoleucic acid is glucosylated by UGT76B1 and its overexpression led to reduced *PR1* expression and in contrast increased expression of JA-responsive gene *VSP2*. This disturbed equilibrium between SA- and JA-pathways suggests the role for UGT76B1 in the cross-talk between these two hormones. Isoleucic acid, but also valic acid (2-hydroxy-3-methylbutyric acid) inhibited root growth, indicative also of resistance related properties (von Saint Paul *et al.*, 2011).

The aromatic amino acid (AAA; Phe, Tyr, Trp) levels also rise during pathogen invasion. AAAs are generated via the shikimate pathway. The exposure of plants to various

stresses generally induces the expression of genes encoding enzymes involved in the shikimate pathway and the AAA metabolism. For example, oligogalacturonides that are released from plant cell walls upon infection with the *Botrytis cinerea* pathogen stimulate a number of genes encoding enzymes of the shikimate and AAA biosynthesis pathways, as well as genes encoding enzymes of secondary metabolites derived from the AAA (Ferrari *et al.*, 2007). Phe secondary metabolites, like phenylpropanoid-based polymers, mostly serve as supportive compounds to increase robustness of the cell wall against mechanical damages, caused either by herbivores or drought (Vogt, 2010), but might also have the function in bacterial infection. Trp is the precursor for the synthesis of a number of indole-containing secondary metabolites, e.g. indole-glucosinolates, which are associated with plant–insect and plant–pathogen interactions (Halkier and Gershenzon, 2006). The indole ring serves for the biosynthesis of phytoalexins, from which the best described, is camalexin. Induction of camalexin biosynthesis in response to biotrophic and necrotrophic pathogens is part of an elaborated network of defence mechanisms, which involves salicylic acid, jasmonate, ethylene dependent signalling pathways, the glutathione status, and in particular the generation of reactive oxygen species (Glawischnig, 2007). Increase of Trp and therefore possible increase of camalexin levels contribute to plant resistance.

Proline which plays a critical role in abiotic stresses, like high salinity or drought (Yoshiba *et al.*, 1995) did not change its level during the infection (Table IV.), resulting in no need for this amino acid in resistance to *Psm*. However, about five-fold levels of Pro were determined in incompatible interaction of Arabidopsis with *P. syrinagae* pv. *tomato avrRpm1* (Fabro *et al.*, 2004). Moreover, the Pro catabolic pathway was found to play a role in biotic stress. Cecchini *et al.* (2011) showed that the Pro catabolic enzyme, Pro dehydrogenase (PDH), increased its activity in the hypersensitive response of plants infiltrated by *P. syrinagae* pv. *tomato avrRpm1*. From these data, it can be concluded that Pro biosynthesis but and in particular its degradation are important in incompatible, HR-inducing interaction.

Similar Pro levels in pathogen-treated and mock-treated leaves also suggest that the differences in the amino acid levels originate from activation of plant biosynthetic pathways and not from the bacteria. This is also confirmed by PAMPs treatment. Especially after *flg22* inoculation, most of the amino acids that were triggered by the pathogen were accumulated (Table IV, V). Amino acids can, in plants, be produced by either protein degradation or by *de novo* synthesis. It can be concluded that non-protein amino acids, pipecolic acid and α-aminoadipic acid, are *de novo* synthesised in plants after *Psm* inoculation. The explanation for protein amino acid accumulation in *Psm*-infected plants is more complicated. Less and

Galili (2008) studied the expression of all annotated Arabidopsis proteases in response to drought and other abiotic stress, and concluded that increased protease production cannot account for the observed increase in free amino acid accumulation. This assumption is also supported by our observation that amino acids did not accumulate to the same extent after pathogen attack as would be expected by protein degradation. Moreover, pathogen-triggered *de novo* biosynthesis can be concluded for Asp derived amino acids, such as Lys and Ile, because Asp decrease was observed after pathogen attack. Therefore, I believe that free amino acid levels also in biotic stress do not change due to the protein breakdown. Nevertheless, one cannot rule out the possibility of up-regulated protein targeting to already existing protease complexes, *e.g.* by the activity of E3 ubiquitin ligases.

Moreover, amino acids are used for conjugation to plant hormones. Amino acid (AA) conjugation to SA and the growth hormone auxin was described. SA-AA conjugation has been proposed to play a role in SA catabolism (Zhang *et al*., 2007b), resulting in decrease of SA and the subsequent more susceptible state of the plant. However, the balance of the free SA level after pathogen attack is important for proper regulation of defence responses (Dempsey *et al.*, 2011). Moreover, the *pbs3* mutant, lacking an enzyme putatively involved in SA conjugation to AA, is more susceptible to *P. syringae* infection (Nobuta *et al.*, 2007). AA conjugation to auxin inactivates auxin-responsive processes. Auxin was described as an enhancer of pathogen susceptibility (Wang *et al.*, 2007). However, a recent publication showed that fungal and bacterial plant pathogens promote the accumulation of auxin-Asp, leading to disease development (Gonzalez-Lamothe *et al.*, 2012).

In our study, no significant changes of Gln were observed after pathogen attack. However, the Gln deficiency mutant *lht1* was more resistant to *P. syringae* pv. *tomato* compared to wild type. For LYSINE HISTIDINE TRANSPORTER1 (LHT1), Gln was determined as a main physiological substrate. Moreover, the *lht1* mutant showed an enhanced oxidative burst that was dependent on endogenous SA level. Higher basal and pathogen-triggered SA levels were observed in this mutant. Collectively, these results demonstrate that LHT1 and therefore a high Gln level negatively modulates defence responses by interacting with the SA-dependent pathway (Liu *et al.*, 2010).

Taken together, generally the amino acids levels balance has a major contribution to proper development of plant resistance.

Among others, a highly accumulating amino acid upon *P. syringae* infection was Lys, an Asp-derived amino acid. Asp levels significantly decreased after *Psm* inoculation, which is concomitant with Lys biosynthesis as a pathogen-triggered event.

Glutamate, a major product of Lys catabolism, serves as a primary precursor for three important metabolites that have a function in abiotic stress. These are proline, a strong osmolyte (see above), γ-amino butyric acid (GABA), a stress-related signalling molecule; and arginine, which is the potential precursor of the stress-related compounds, polyamines and nitric oxide (Galili *et al.*, 2001). None and moderate increments upon *P. syringae* infection were observed for Pro and GABA accumulation, respectively; thus it is unlikely that Lys accumulation would serve as a precursor for these amino acids in biotic stress.

Outstandingly, two other Lys catabolic products were accumulated after pathogen attack: the non-protein amino acids, pipecolic acid (Pip) and α-aminoadipic acid (Aad). Pip was found to accumulate to high levels after pathogen attack in inoculated leaves, in systemic tissue and in leaf petiole exudates (Table IV.) as well as in leaves treated with PAMPs (Figure 12 E). However, the absolute values of Pip and Aad accumulation were lower for PAMPs than for *Psm* treatment. This could be the reason why Asp levels did not decrease after PAMP treatments (Table V).

Other non-protein amino acids were described as occurring in plants, mostly in the role of resistance against herbivores. These amino acids are stored in a form that makes it metabolically unavailable to herbivores. For instance, degradation of Arg to Orn by tomato arginase significantly reduces *Manduca sexta* weight gain (Chen *et al*., 2005), showing that availability of a single essential amino acid can be growth-limiting for insects. The next example, L-Canavanine is synthesized by leguminous plants for N-storage, but L-Canavanine containing plants were less eaten by *Manduca sexta* (Rosenthal, 2001) and caused behavioural avoidance of *Drosophila melanogaster* (Mitri *et al*., 2009). Accumulation of GABA in plants has an effect on the inhibition of neurotransmission in herbivores (Huang *et al.*, 2011). Furthermore, *p*-aminophenylalanine was found to be an important component of resistance against two important pest bruchids of *Vigna* and *Phaseolus* crops (Birch *et al.*, 1986).

Here, we provide the evidence that non-protein amino acid plays a role in resistance to bacterial infection. Since plant resistance is dependent on endogenous Pip levels, because the *ald1* mutant was more susceptible in compatible as well as incompatible interaction to the pathogen (Figure 17 A-C) and was not capable of establishing SAR (Figure 15). Moreover, characteristic local defence responses and the accumulation of SA and camalexin, were significantly altered in the *ald1* mutant (Figure 19 A, C, Song *et al*., 2004b).

However, Pip is not the only described non-protein amino acid involved in bacteria-plant interaction. A recent study showed that N^{δ}-acetylornithine also was strongly induced by

the phytohormone methyl jasmonate (MeJA) and after virulent *P. syringae* infection (Adio *et al.*, 2011).

Pip has been described as a diagnostic marker of pyridoxine-dependent epilepsy in human plasma and cerebrospinal fluid (Plecko *et al.*, 2005). However, Pip has no role in mediating resistance responses in this disease. The accumulation of Pip in human plasma is caused by the deficiency of an α-amino adipic semialdehyde dehydrogenase, converting α-amino adipic semialdehyde to Aad. This leads to a highly spontaneous cyclization of α-amino adipic semialdehyde to Δ^1-piperideine-6-carboxylic acid, which is in humans converted to Pip (Struys and Jakobs, 2010). Furthermore, Pip has been also recognized as a weak inhibitory neurotransmitter and GABA agonist (Charles, 1986), and the pipecolate pathway represents the major Lys catabolic route in rat brain (Chang, 1976). These data show an occurrence of Pip in animals.

Pip has also been described as an osmoprotectant for *E. coli* (Gouesbet *et al.*, 1994), *Brevibacterium ammoniagenes* (Gouesbet *et al.*, 1992), and *Undifilum oxytropis* (Mukherjee *et al.*, 2010), showing that Pip arises in microorganisms under stress conditions as well.

In plants, enhanced Pip levels have been measured after osmotic stress in *Brassica napus* (Moulin *et al.*, 2006) and *Citrus* genus plants (Servillo *et al.*, 2011). Pip was described in *Trifolium repens*, *Conium maculatum*, and *Luzula sylvatica* as a constitutivly occuring non-protein amino acid (Morrison, 1953) and in *Hordeum vulgare* after water stress (Singh *et al.*, 1973). Pip also accumulated in the strawberry plant following growth affecting treatments such as application of the plant growth regulator maleic hydrazine (Moulin *et al.*, 2006; Yatsu and Boynton, 1959). The physiological function of Pip in plants has remained elusive, although it was described as a flower-inducing substance in the aquatic plant *Lemna gibba* (Fujioka *et al.*, 1987) and as an indicator of abnormal protein metabolism in diseased plants (Pálfi and Dézsi, 1968). Thus pipecolic acid has a widespread occurrence in several plant species.

Mutations of enzymes involved in Asp catabolism, DIHYDRODIPICOLINATE SYNTHASE 2 (DHDPS2) and ASPARTATE KINASE 2 (AK2), caused reduced susceptibility of Arabidopsis to *H. arabidopsidis*. AK2 is the first enzyme of Asp catabolism. DHDPS2 is the first enzyme in Lys biosynthesis after the branching point of Asp catabolism. In *dhdps2* mutant plants, Asp-derived amino acids, such as Thr, Met and Ile were accumulated. Chemical treatments mimicking the mutants' metabolic state identified Thr as the amino acid suppressing pathogen growth (Stuttmann *et al.*, 2011). However, the low levels of Pip can be expected because of the lack of Lys. The resistance responses to *H.*

arabidopsidis, as a (hemi)biotrophic pathogen, are associated with SA-dependent defence (Ton *et al.*, 2002). Hence, as Pip positively regulates the SA pathway, the more susceptibility would be expected in these mutants. Therefore the perturbations in amino acid homeostasis rendering the mutant plants unsuitable as an infection substrate for *H. arabidopsidis* had a higher impact on the growth of this oomycete than hormone levels.

Interestingly, mutation in another Asp catabolic pathway enzyme, HOMOSERINE KINASE (HSK), increased the resistance of Arabidopsis to *H. arabidopsidis* infection. This mutant (*dmr1*) showed consistently increased level of homoserine, a precursor for Thr, Ile and Met synthesis. However, the levels of these three amino acids were not decreased in *dmr1* mutant. Exogenous application of homoserine increased the resistance of wild-type plants. Therefore homoserine increased level was responsible for decreased susceptibility to *H. arabidopsidis* (van Damme *et al*., 2009). These data suggest that Asp catabolic pathway plays an important role in plant resistance to different pathogens.

It was established that Pip production is in Arabidopsis dependent on ALD1 as an aminotransferase with the highest specificity to Lys, as reported by Song *et al*. (2004a). Since *lkr* mutans were able to synthesize Pip, Pip accumulation is not dependent on the well-described first catabolic enzyme LKR/SDH which converts Lys via saccharopine pathway into α-amino adipic semialdehyde (Galili, 2002). This result is in contrast with the Goyer *et al.* (2004) study, where the authors proposed that α-amino adipic semialdehyde is a substrate for Pip production. However, such a reaction is not a bad thought, because the saccharopine pathway has been described as a Pip synthesis pathway in fungi (Wickwire *et al.*, 1990). In summary, Pip biosynthesis according to our observation happens in plants through ALD1, which putatively transfers the α-amino group of Lys and produces ε-amino-α-ketocaproic acid; by its spontaneous cyclization Δ^1-piperideine-2-carboxylic acid is produced and a putative reductase then catabolizes Pip production.

The *ALD1* expression is in agreement with Pip biosynthesis, its expression also increased in local and systemic leaves upon *Psm* inoculation (Figure 11 A, B). High expression of *ALD1* was determined in the *lht1* mutant. However, the double mutant *ald1lht1* did not change phenotype in comparison to *LHT1* mutation only, suggesting that these pathways function independently in resistance responses (Liu *et al.*, 2010). However, mutations in *ALD1* suppressed all *enhanced disease resistance2* (*edr2*)-mediated phenotypes, including powdery mildew resistance, programmed cell death and ethylene-induced senescence. The suppression of *edr2*-associated ethylene senescence suggests that ALD1 is required by ethylene signalling. In the same study, the authors suggest that EIN3 (a positive

regulator of the ethylene pathway) could bind to the *ALD1* promoter directly (Nie *et al.*, 2011). Thus ALD1 could represent a link among Pip, SA and ethylene signalling.

SA is a well-described plan hormone which is important for resistance responses to *P. syringae* and in SAR establishment. The kinetics comparison of SA and Pip accumulation in local leaves after pathogen infection should be discussed. The SA and Pip accumulation patterns after both *Psm avrRpm1* and *Psm* were similar at 8, 24 and 48 hpi (Mishina *et al.*, 2008, Figure 10 A). The SA and Pip accumulation significantly increased at 8 h in the case of incompatible interaction compared to mock treatment and further rose at 24 and 48 hpi. With virulent *Psm*, the substantial increase of Pip and SA content was observed at 24 hpi. Furthermore, total SA accumulation in Col-0 increased about 7-times at 10 h after virulent *Psm* inoculation compared to $MgCl_2$ treatment (Figure 19 A). The Pip level in local leaves at 10 hpi did not increased (Figure 19 B), suggesting that SA accumulation precedes the Pip *Psm*-triggered biosynthesis in local leaves. In contrast, Pip production preceded the SA biosynthesis (Figure 13 A, B) in systemic leaves upon local *Psm*-inoculation. These data indicate different metabolic and molecular regulations of defence responses at the site of pathogen attack and in leaves distal from pathogen infection.

Pip was not only accumulated in local and systemic leaves, its strong enrichment was found in petiole exudates that were collected from inoculated leaves (Figure 14 A). Compared to other amino acids and many defence-related compounds that strongly accumulated in local leaves, only a high level of Pip was determined in these exudates. The transport of Pip out of inoculated leaves seems therefore favoured over the transport of many other accumulating metabolites. Substantial decrease of Lys level was determined in petiole exudates (Figure 14 B), leading to the hypothesis that Pip and Lys could use the same transporter out of the inoculated leaf. This hypothesis was partly rejected, when a lesser content of Lys was also determined in the leaves that were used for petiole exudates collection (Figure 14 E). The possible explanation could be that detached local leaves are not able to import N-compounds for Lys formation and therefore Pip biosynthesis led to a higher consumption of Lys in this case.

Moreover, exogenously applied Pip through soil, but not by infiltration or spraying, was able to complement *ald1* disease susceptibility phenotype (Figure 16). These data suggest that the plant is not able to assimilate Pip from the apoplast or the surface of the leaves, but that Pip can be up-taken by the plant through the root system. This was confirmed when Pip was determined in shoots of *ald1* plants after Pip application via the roots (data produced by

F. Bernsdorff). This conclusion, together with the enrichment of Pip in petiole exudates, indicates that Pip has the ability to transfer through the plant tissue.

Interestingly, soil application of BABA strongly increased Pip levels in Col-0 leaves (Figure 18 B). BABA is an amino acid that is not produced in plants, but effectively promotes plants into a primed state via a SAR-like mechanism (Zimmerli *et al.*, 2000). Exogenously supplied BABA did not enhance *ald1* resistance towards *P. syringae*. These data suggest that Pip is important for BABA-induced resistance in Arabidopsis. Neither exogenous Pip nor BABA were able to enhanced resistance of *fmo1* plants (Figure 18 C). These data, together with higher accumulation of Pip in *fmo1* mutant in local leaves (Figure 12 C) as well as in petiole exudates (Figure 14 A), suggest that FMO1 plays a role downstream of Pip.

After concluding that Pip is important for BABA-induced resistance, we tested whether Pip accumulation is potentiated during the primed state of plants. Indeed, multifold enhancement of the Pip level was determined in SAR-induced plants (Figure 19 B). Moderate and strong primed production was observed in SAR-induced plants for SA and camalexin, respectively (Figure 19 A, C). However, these priming effects were absent in the *ald1* mutant, indicating the importance of endogenous Pip production for defence priming during SAR. Moreover, plants which were fed with Pip also accumulated potentiated levels of SA and camalexin after *P. syringae* inoculation (Figure 19 D, E). Therefore, Pip promotes pathogen-triggered SA biosynthesis and consistently also promotes expression of the *ICS1* gene (Figure 20 C). Based on this, it can be concluded, that Pip positively regulates SA biosynthesis, a phenolic plant defence regulator that features structural similarity to Pip (Figure 28). The *ald1* mutant was able to produce pathogen-triggered camalexin after soil application of Pip. However, the value did not reach the wild-type level, suggesting that defence priming mediated by exogenous Pip is still reinforced by the capacity of endogenous Pip biosynthesis in wild type. Interestingly, Pip application to Col-0 plants strongly primed plants for expression of the *ALD1* after pathogen inoculation (Figure 20 A), indicating a positive regulatory role for Pip on its own biosynthesis.

Simultaneous experiments to answer the question about the biochemical function of FMO1 were performed. Preliminary results suggested a nuclear localization of FMO1 and led to the hypothesis that FMO1 could mediate expression of important SAR-defence genes by histone modification of their promoters. Histone modifications during SAR have recently become an intensively studied topic. The idea is that exposure to a priming agent could activate a set of genes. However, instead of reverting the genes to the transcriptionally silent

state once the stimulus is removed, an epigenetic mark could perhaps be left, facilitating quicker and more potent responses to subsequent attacks.

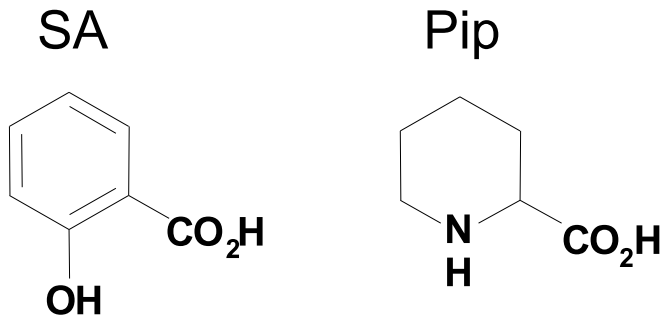


Figure 28. Structural comparison of salicylic acid (SA) and pipecolic acid (Pip).

It was found that histone modifications, such as H3 and H4 acetylation, and H3K4 methylation are systemically set during a priming event (Jaskiewicz *et al.*, 2011). In this study, chromatin modifications in distal leaves upon local *P. syringae* inoculation were induced on *WRKY6* and *WRKY29* promoters that are normally found on active genes, although the genes remain inactive. Moreover, SAR was epigenetically inherited following disease pressure by *P. syringae* pv. *tomato* to subsequent generations. These plants showed higher resistance to hemibiotrophic *H. arabidopsidis*. The new generation of plants was enriched with acetylated histone H3 at lysine 9, a chromatin mark associated with a permissive state of transcription, on SA-inducible promoters of *PR1*, *WRKY6*, and *WRKY53* (Luna *et al.*, 2012)

In the course of SAR, changes of the histone H3 methylation pattern at the promoter region of *ICS1* were found. Increase of trimethylation of Lys 4 on histone H3 and a small decrease of H3K27m3 in Col-0 suggests the active transcription of *ICS1* in systemic leaves upon local *Psm* infection. These data are consistent with increased SA synthesis during SAR (Durrant and Dong, 2004). In contrast, *fmo1* plants failed to show increased levels of H3K4m3 indicating transcriptional silencing. Since *fmo1* fails to establish SAR and does not accumulate SA in systemic leaves (Mishina and Zeier, 2006), this histone modification could contribute to suppressed resistance responses in the *fmo1* mutant. However, analysis in *Nicotiana benthamiana* transformed plants localized FMO1 in moving vesicles and not in the nucleus, thus it is difficult to judge whether FMO1 has a direct effect on histone modification of the *ICS1* promoter.

Taken together, a hypothetical model of SAR establishment can be proposed. Pip is initially produced in pathogen-inoculated leaves where it enhances resistance responses, such as accumulation of SA and antimicrobial phytoalexin. A small amount of Pip is then putatively transported to systemic leaves. The initial systemic accumulation of Pip, exemplified by the small increase of Pip in SAR-defective *fmo1* plants (Figure 13 D), might trigger its own biosynthesis by high expression of *ALD1*. The *fmo1* mutant, in contrast to the wild-type, does not elevate systemic *ALD1* expression and therefore is not able to initiate Pip biosynthesis in distal leaves (Figure 21 B). The rise of Pip level in distal leaves further initiates other resistance responses, such as increased *ICS1*, *FMO1* and *PR1* expression (Figure 20). Moreover, similarly to *fmo1*, the *ics1* mutant also fails to accumulate *ALD1* transcript systemically after pathogen attack (Figure 21 B), supporting the conclusion that increased Pip levels can ensure a primed state that allows positive feedback regulation of Pip biosynthesis and rapid defence activation following subsequent pathogen encounter (Figure 29). Functional FMO1 is required for both Pip-induced resistance and SA accumulation and thus represents a possible link between Pip and SA biosynthesis. Different FMOs were described to incorporate oxygen into nitrogen or sulphur containing compounds (Schlaich, 2007). Therefore it is tempting to speculate that Pip, an N-containing compound, could serve as a substrate for FMO1. Oxygenated Pip putatively represents the signal transduction point between Pip and SA in systemic leaves (Figure 29).

The ability of Pip to transfer through the plant and its involvement in SAR responses implicate that Pip could be a mobile signal for SAR. Since Pip was not up-taken by the plant through infiltration, proving this hypothesis is made difficult. Drafting experiments with Col-0 root stock and the *ald1* scion could give the answer. If Pip were accumulated in the grafted *ald1* scion upon *Psm* inoculation of Col-0 leaves, its systemic mobility would be proven. However, drafting experiments with Arabidopsis are difficult to perform. Another approach could use the *ALD1* expression under control of locally induced promoter. The promoter of a gene that is only induced upon pathogen inoculation in local leaves, but which shows no increase of expression in systemic leaves, would be used for control of expression of *ALD1*. Transformed *ald1* plants with this construct would be pathogen-inoculated and, if Pip were accumulated in systemic leaves, its mobility would be proven.

Higher Pip accumulation in petiole exudates compared to other locally accumulated amino acids suggests a specific transporter for Pip in the cell wall that exports Pip from the

intercellular space to the vascular system. Further studies will be necessary to clarify this hypothesis.

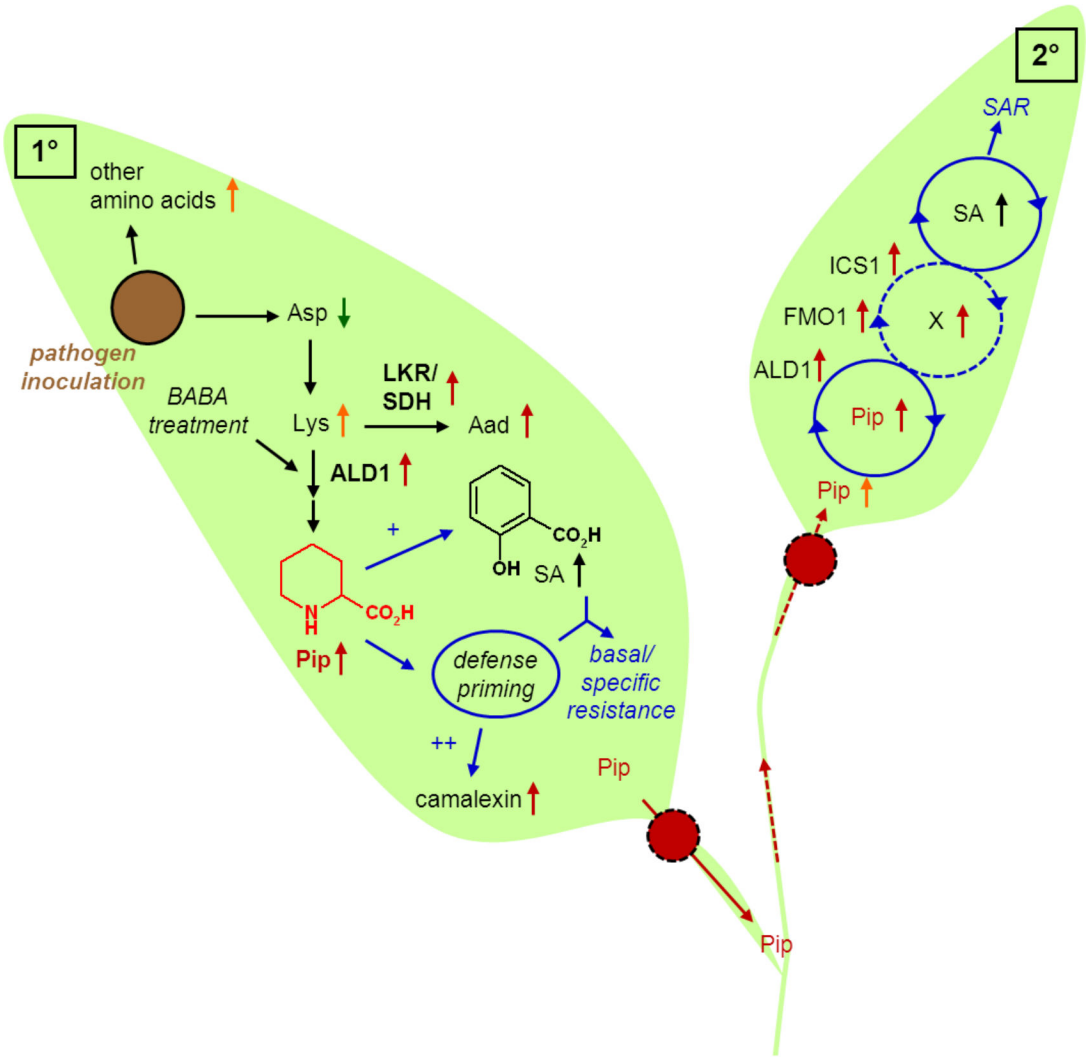


Figure 29. Schema of SAR establishment. After pathogen encounter in local leaves, the several amino acids (Phe, Trp, Tyr, Ala, Val, Leu) increase their levels. In contrast, Asp, the precursor of Lys, is decreased. The level of Lys rise as well as transcript levels of *ALD1* and *LKR/SDH*, two enzymes mediated catabolism of Lys. Through LKR/SDH, the α-aminoadipic acid (Aad) is synthesised and through ALD1 pipecolic acid (Pip) is produced. Both Pip and Aad dramatically accumulate after pathogen attack. Pip positively influences the salicylic acid (SA) biosynthesis and thus leading to subsequent resistance responses. Pip has priming effect, where *e.g.* primes camalexin levels, the antimicrobial phytoalexin. Pip is in small amount exudated from the petiole of inoculated leave and putatively transferred to systemic leaf. There Pip positively influences the expression of *ALD1* and therefore its own biosynthesis. Higher level of Pip triggers also *FMO1* and *ICS1* expression. FMO1 presumably convert Pip to active compound which induce SA biosynthesis and other defence responses. Dashed lines: hypothetical pathways. Orange arrow: modest accumulation. Red arrow: high accumulation. Green arrow: decrease of level.

The metabolic analysis of the *ald1* mutant revealed that *ALD1* is important for Pip biosynthesis. The highest enzymatic activity of recombinant ALD1 was obtained with Lys (Song *et al.*, 2004a). Further recombinant ALD1 enzyme assays could determine if intermediates on the way to Pip are synthesized with Lys as a substrate. Furthermore, in order

to prove Lys as a precursor for Pip, C- or ε-N-radiolabeled Lys applied through soil to wild type plants should reveal radiolabeled Pip after pathogen inoculation in leaves.

Loss of function of the *ALD1* suppressed *edr2* mutant phenotype and *ALD1* promoter localization in senescent leaves of Arabidopsis suggested its role in the ethylene pathway (Nie *et al.*, 2011). Further studies of *ALD1* and Pip levels in ethylene response defected mutants could answer the question how Pip influences ethylene triggered resistance. Moreover, ALD1 could be involved in SA/ET crosstalk. Further studies between PAD4 and ALD1 are also necessary to clarify their interaction in resistance responses of Arabidopsis. An important future task will be to also systematically clarify also the interrelationship between Pip and other metabolic SAR activators, such as glycerol-3-phosphate, azelaic acid, and dehydroabietinal.

In summary, the pipecolic acid-deficient mutant *ald1* fails to establish basal as well as systemic acquired resistance. Pipecolic acid application primes major defence responses to biotrophic pathogens, such as salicylic acid and camalexin accumulation, and pathogen-related gene expression. Therefore it can be concluded that pipecolate is a novel critical regulator of innate and inducible immunity of Arabidopsis to *P. syringae* infection.

7 ABBREVIATIONS

AAA	aromatic amino acids
ABA	abscisic acid
ABI	ABSCISIC ACID INSENSITIVE
Avr	avirulence
BABA	β-aminobutyric acid
BAK	BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE
BCAA	branched-chain amino acids
BRI	BRASSINOSTEROID-INSENSITIVE
bZip	basic leucine zipper
CC	coiled-coil
cDNA	complementary DNA
cfu	colony forming units
CoA	coenzym A
c_T	cycle threshold
DA	dehydroabietinal
DAMP	damage-associated molecular patterns
DIR	DEFECTIVE IN INDUCED RESISTANCE
E	efficiency
EDR	enhanced disease resistance
EDS	enhanced disease susceptibility
EDTA	ethylen-diamine tetra-acetic acid
EFR	elongation factor Tu receptor
EF-Tu	elongation factor Tu
EIN	ETHYLENE INSENSITIVE
ET	ethylene
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FLS2	FLAGELLIN SENSING 2
FMO1	FLAVIN-DEPENDENT MONOOXYGENASE 1
FW	fresh weight
G3P	glycerol-3-phosphate
GABA	γ-aminobutyric acid
gDNA	genomic DNA
GFP	green fluorescent protein
GST	gluthathione S-transferase
h	hours
hpi	hours post inoculation
HR	hypersensitive response
IAA	indole acetic acid, auxin
ICS1	isochorismate synthase 1
IR	induced resistance
ISR	induced systemic resistance
JA	jasmonic acid
JAZ	jasmonate ZIM domain
LHT	LYSINE HISTIDINE TRANSPORTER

LKR	lysine ketoglutarate reductase
LPS	lipopolysaccharides
LRR	leucine-rich repeat
M	$mol.l^{-1}$
MAMP	microorganism-associated molecular patterns
MAP	mitogen-activated protein
MAPK/MPK	mitogen activated protein kinase
MeJA	methyl jasmonate
MeSA	methyl salicylate
mRNA	messenger RNA
NahG	salicylate hydroxylase
NB	nucleotide-binding site domain
NB-LRR	nucleotide-binding – leucine rich repeats
NDR1	NON RACE SPECIFIC DISEASE RESISTANCE 1
NO	nitric oxide
NPR1	NON-EXPRESSOR OF PR GENES1
PAD	PHYTOALEXIN DEFICIENT
PAL	phenylalanine ammonia-lyase
PAMP	pathogen-associated molecular patterns
PBS3	AvrPphB SUSEPTIBLE 3
PCR	polymerase chain reaction
PDH	proline dehydrogenase
PGN	peptidoglycan
PR	pathogenesis-related
PRR	pattern recognition receptors
Psm	*Pseudomonas syringae* pv. *maculicola* ES4326
Psm(avrRpm1)	*Psm* carrying the avirulence gene pLAFR3::*avrRpm1*
PTI	PAMP-triggered immunity
qRT-PCR	quantitative RT-PCR
R	resistance
RBOH	RESPIRATORY BURST OXIDASE HOMOLOG
RIN4	RPM1-INTERACTING PROTEIN 4
RK	receptor kinase
RLP	receptor-like proteins
ROS	reactive oxygen species
SA	salicylic acid
SABP2	SA BINDING PROTEIN 2
SAM	S-adenosylmethionine
SAR	systemic acquired resistance
SD	standard deviation
SDH	saccharopine dehydrogenase
SID	SALICYLIC ACID INDUCTION DEFICIENT
TAE	tris-acetate-ethylenediamine tetraacetic acid
T-DNA	transposable DNA
TIR	toll, interleukin 1R and resistance
TMV	tobacco mosaic virus

TTSS	type-III secretion system
WIR	wound-induces resistance

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9 CURRICULUM VITAE

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EXPERIENCE

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TACHING EXPERIENCE

Teaching assistant: laboratory practice in cloning for master students, Fribourg 2010
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CONFERENCES

Sep 4-8, 2011 PR-proteins and induced resistance workshop Neuchatel	Metabolic regulation of acquired resistance in Arabidopsis
Sep 13-17, 2010 12th Conference of experimental plant biology Prague	Oral presentation: Molecular characterization of systemic acquired resistance in *Arabidopsis thaliana*
Jun 6-10, 2010 21st International Conference on Arabidopsis research Yokohama	Poster presentation: Molecular characterization of systemic acquired resistance in *Arabidopsis thaliana*
Jun 30-Jul 4, 2009 20st International Conference on Arabidopsis research Edinburgh	Poster presentation: The site of action of salicylate antagonism in the jasmonate signalling pathway
Jul 4-9, 2009 34th FEBS Congress Prague	Poster presentation: Distinct isoforms of Arabidopsis phospholipase D are involved in defence response against *Pseudomonas syringae*

PUBLICATIONS

Navarova H., Döring A., Bernsdorff F., Zeier J.: Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity (revision in The Plant Cell)

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11 ERKLÄRUNG

Erklärung gemäß § 3 Abs. 1 Ziff. 2

der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät

der Heinrich-Heine Universität Düsseldorf

Hiermit erkläre ich, die vorgelegte Arbeit mit dem Titel „Amino acid metabolism in systemic acquired resistance of Arabidopsis thaliana; identification of pipecolic acid as a critical component of innate and inducible immunity“ eigenständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Diese Dissertation hat weder in gleicher noch ähnlicher Form einer anderen Institution vorgelegen. Ich habe bisher keine erfolglosen Promotionsversuche unternommen

Düsseldorf, den 17.10.2012

(Hana Návarová)