Requirement of phosphoinositol-derived signals in the wounding response of *Arabidopsis thaliana*

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Alina Mosblech

aus Paderborn

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Mitglied des Betreuungsausschusses (1. Referent):
Prof. Dr. Ingo Heilmann, bisher: Abteilung Biochemie der Pflanze, Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Georg-August-Universität Göttingen; ab 01.08.2010: Abteilung Zelluläre Biochemie, Institut für Biochemie und Biotechnologie, Martin-Luther-Universität Halle-Wittenberg

Mitglied des Betreuungsausschusses (2. Referentin):
Prof. Dr. Andrea Polle, Abteilung Forstbotanik und Baumphysiologie, Institut für Forstbotanik, Georg-August-Universität Göttingen

Mitglied des Betreuungsausschusses:
Prof. Dr. Gerhard Braus, Abteilung Molekulare Mikrobiologie und Genetik, Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen

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Herewith I affirm that I wrote this thesis independently and with no other sources and aids than quoted.

Alina Mosblech

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1 INTRODUCTION

1.1 Plant defences against herbivory

In natural ecosystems as well as in agricultural settings, plants are exposed to frequent attacks by herbivorous insects. Crop shortfalls caused by herbivory are of enormous economical relevance and infestation with larvae and beetles may cause 10-35 % of yield reduction. When rapeseed is attacked by the pollen beetle, up to 50 % of loss is possible (Kirch, 2006). To avoid yield losses caused by herbivores, preventive cultivation measures are adopted as well as technical barriers, such as nets or foil. Farmers also spend substantial sums of money for chemical treatment of their crops. In Germany, the annual expenses for insecticides reach 100-150 million EUR and for fungicides more than 500 million EUR (source: http://www.agrar-presseportal.de/Nachrichten/Jahrespressekonferenz-2010_article5618.html). Clearly, even in 2010 it is a major goal to minimize crop damage caused by herbivorous insects.

An alternative starting point to increase plants´ resistance against herbivory and pathogens is genetic modification. So far, so-called Bt maize is the only transgenic crop plant commercially cultivated in the EU today (source: http://www.bmelv.de/cln_163/SharedDocs/Standardartikel/Landwirtschaft/Pflanze/GrueneGentechnik/StandderGentechnik.html). These plants express a toxin from the bacterium *Bacillus thuringiensis* (B. t.), which is poisonous to insect pests. The commercial use of transgenic plants in agriculture suffers, however, from low public acceptance, and severe legal restrictions are supposed to control possible risks of genetically modified plants concerning the environment and ecosystem as well as the consumer of the ensuing product.

Before human intervention, plants themselves have evolved to have strategies to defend against a variety of attacks by insects and microbial pathogens. By gaining a better understanding of natural plant defence reactions, it may be possible to find new starting points of herbivorous pest control, either by genetic modifications or by other external manipulation.

The stimulus a plant receives when it is attacked by herbivorous insects is a highly complex event and is composed of different aspects, such as the loss of cellular integrity, chemical substances present in the insects´ saliva (Musser et al, 2006), or structural features of the insects´ or pathogens´ surface (Howe & Jander, 2008; Jones & Dangl, 2006). In the laboratory, the naturally occurring wounding event can be experimentally mimicked by the directed

application of an herbivorous insect, but the choice of a representative species is critical in such an experiment. To keep the experimental setup simple, mechanical wounding may serve to mimic the herbivore attack. Mechanical wounding performed by squeezing with forceps is also used in this thesis as a simplified model system to investigate general aspects of plant wound reaction. Clearly, when interpreting results obtained with such experiments it has to be taken into account that such a wounding event differs from the natural situation of herbivore attack in particular by lacking the chemical aspect (Howe & Jander, 2008).

Active wound responses of plants include those involved in healing of damaged tissue and also multiple chemical defence mechanisms. Wound responses may also serve to prevent further damage, for example by altered growth and increased trichome formation, or to limit pathogen spread by the release of antimicrobial substances. A common feature of wound responses is an alteration of gene expression patterns, which is orchestrated by a precisely adjusted signalling network, in which different phytohormones and second messengers act to sense the wounding stress and transduce this signal to manifest an appropriate response pattern. Signalling molecules important for wound responses include jasmonic acid (JA) and oxylipins, salicylic acid (SA), auxin (β-indolyl acetic acid, IAA), ethylene (ET) and Ca^{2+} (Johnson & Ecker, 1998; Kernan & Thornburg, 1989; Leon et al, 2001; O'Donnell et al, 2003). Basic research on plant-endogenous defence mechanisms against insect herbivory might provide starting points for future approaches to fight insect pests in applied settings.

1.2 Jasmonic acid signalling

1.2.1 Physiological functions of JA

The phytohormone JA is involved in the regulation of numerous cellular processes in plants, including responses to environmental as well as developmental cues (Balbi & Devoto, 2008; Turner et al, 2002; Wasternack, 2007). JA is present in all higher land plants examined, and the concentration of JA in plant tissue varies depending on the type of tissue, developmental stage and external stimuli (Creelman & Mullet, 1997). High levels of JA were reported in reproductive tissues, and JA plays a crucial role in reproduction, as JA-deficient mutants exhibit a sterile phenotype (Creelman & Mullet, 1997). Interestingly, this JA-dependent fertility is differently affected in different plant species. While JA-deficient *Arabidopsis thaliana* (Arabidopsis) mutants display male sterility (Feys et al, 1994; von Malek et al, 2002), JA-deficient tomato mutants display female sterility (Li et al, 2001). Besides its role in fertility, JA

is also involved in fruit ripening, together with the ripening hormone ET, and it is speculated to act as a scent of flowering blossoms attracting pollinating insects (Creelman & Mullet, 1997; Pichersky & Gershenzon, 2002). Furthermore, JA affects plant growth with respect to root elongation (Staswick et al, 1992) and tendril coiling (Devoto & Turner, 2003).

JA represents one of the best-studied players in wound signalling and defence. JA levels increase within a few minutes upon wounding (Glauser et al, 2008), and JA-deficient mutants fail to express a variety of wound-inducible genes (Creelman & Mullet, 1997; Park et al, 2002; Reymond et al, 2000). Moreover, JA plays a crucial role in defence against herbivorous insects (McConn et al, 1997) and against necrotrophic pathogens (Browse & Howe, 2008; Li et al, 2005; Thomma et al, 1999). Most relevant for data presented in this thesis with regard to the manifested wounding response is the fact that solanaceous plants express wound-induced genes encoding protease inhibitors active in the gut of insects to interfere with herbivore digestion (Pearce et al, 1991).

Clearly, JA is involved in numerous aspects of plant life. In this thesis, the role of JA during Arabidopsis wound response in crosstalk with other wound-induced signals, in particular to phosphoinositide (PI) signals described further down, has been investigated.

1.2.2 JA biosynthesis

JA biosynthesis via the octadecanoid pathway starts in the chloroplast (Fig. 1; Delker et al, 2006), where lipases release the triple-unsaturated C-18 fatty acid α-linolenic acid (α-LeA) from membranes of the inner plastidial envelope (Creelman & Mullet, 1997; Delker et al, 2006; Narvaez-Vasquez et al, 1999). By the lipoxygenase (LOX)-mediated introduction of molecular oxygen to the C-13 atom of α-LeA (Feussner et al, 1995), a hydroperoxy α-linolenic acid (HPOT) is produced, and allene oxide synthase (AOS), a key enzyme of JA production, forms an unstable intermediate, epoxy octadecatrienoic acid (EOT). This epoxy intermediate is transformed by allene oxide cyclase (AOC) to the cyclic compound, oxo phytodienoic acid (oPDA; Hamberg & Fahlstadius, 1990). After export from the plastid and transfer to the peroxisome, oPDA is activated and subsequently reduced by oPDA reductase 3 (OPR3) to oxo cyclopentane octanoic acid (OPC 8:0), which is converted to JA by three rounds of β-oxidation (Creelman & Mullet, 1995; Vick & Zimmerman, 1984). Alternatively, JA can be formed via the hexadecanoid pathway, which starts with the triple-unsaturated C-16 fatty acid hexadecatrienoic acid and produces JA via dinor-oPDA (dn-oDPA) and oxo pentenyl cyclopentane hexanoic acid (OPC 6:0) intermediates. JA formed by either pathway is transported from the peroxisome to the cytosol, where the JA amino acid synthetase

JASMONIC ACID RESISTANT 1 (JAR1) forms the bioactive jasmonoyl-isoleucin conjugate (JA-Ile; Fonseca et al, 2009; Staswick et al, 2002). Biosynthetic pathways involved in JA biosynthesis are, thus, in part localized in plastids, in peroxisomes and in the cytosol. JA, its precursors oPDA and dn-oPDA and the bioactive form JA-Ile are often and also in this thesis collectively referred to as jasmonates.

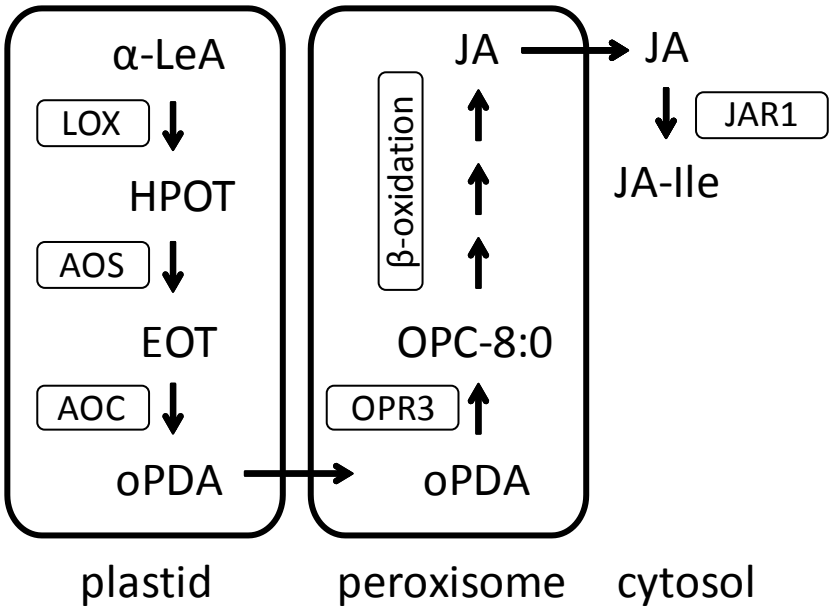

Fig. 1: Subcellular compartmentation of JA biosynthesis. The formation of jasmonic acid (JA) starts with the lipase-mediated release of α-linolenic acid (α-LeA) from membrane lipids. Lipoxygenases (LOX) transform the fatty acid into a hydroperoxy α-linolenic acid (HPOT). Allene oxide synthase (AOS) converts HPOT to an instable intermediate, epoxy octadecatrienoic acid (EOT), which is the substrate for allene oxide cyclase (AOC), yielding oxo phytodienoic acid (oPDA). oPDA is exported from the plastid and transferred to the peroxisome. There, oPDA is activated by and subsequently reduced by oPDA-reductase 3 (OPR3) to oxo pentenyl cyclopentane octanoic acid (OPC 8:0), which is transformed to JA by three rounds of β-oxidation. JA is exported to the cytosol by an unknown mechanism where it can be conjugated to isoleucine (Ile) by jasmonate amino acid transferase, JAR1, forming the biologically active JA–Ile. Illustration according to Mosblech et al (2009).

1.2.3 Perception of JA-Ile by the SCF^{COI1} complex

JA-Ile is perceived by binding to the F-box protein CORONATINE INSENSITIVE 1 (COI1). F-box proteins contain at least one F-box motif, which was first identified in the protein cyclin F. The F-box motif consists of approximately 50 amino acids and functions as a site of protein-protein interaction (Kipreos & Pagano, 2000). Together with other proteins, such as ARABIDOPSIS SKP1-LIKE 1 or 2 (ASK1 or ASK2), CULLIN 1 (CUL1) and RING-BOX PROTEIN 1 (RBX1), COI1 forms an SCF^{COI1} E3 ubiquitin ligase complex (Xu et al, 2002). In this complex, the F-box motif of COI1

interacts with ASK proteins. E3 ubiquitin ligase complexes are well known in plants to be involved in protein degradation via the ubiquitin-proteasome system (Moon et al, 2004; Santner & Estelle, 2010). The ubiquitin monomer is a highly conserved protein consisting of 76 amino acids, which is attached to target proteins by the sequential action of three enzymes: The ubiquitin activating enzyme E1 activates ubiquitin in an ATP-dependent manner and the ubiquitin conjugating enzyme E2 conjugates it to a lysine residue of a target protein, which is recruited and correctly positioned by the ubiquitin ligase complex E3 (Pickart, 2001). COI1 is, thus, part of an E3 ubiquitin ligase complex responsible for presenting specific target proteins for ubiquitination. While attachment of a single ubiquitin monomer modifies protein activity or localization of a target protein (Mukhopadhyay & Riezman, 2007), poly-ubiquitination initiates protein degradation by the 26S proteasome (Santner & Estelle, 2010). The 26S proteasome is a large ATP-dependent proteolytic complex that unfolds and degrades poly-ubiquitinated proteins (Voges et al, 1999). The ubiquitin-proteasome system is involved in numerous aspects of plant growth, including cell cycle regulation, embryogenesis, senescence, defence and hormone signalling via JA and auxin (Vierstra, 2009). While in Arabidopsis only two CUL proteins have been shown to assemble in SCF complexes and only two RBX and 21 ASK proteins are encoded in the Arabidopsis genome, over 700 genes encoding putative F-box proteins have been annotated for Arabidopsis. The large diversity enables a possibly modular assembly of specific F-box proteins with E3 ubiquitin ligase complexes (Moon et al, 2004), which denote specific protein targets for degradation. Thus, a key role in hormone perception can be attributed to F-box proteins such as COI1. Specifically, it has been demonstrated that COI1 defines the binding of target proteins determined for ubiquitination and subsequent degradation that have roles in JA signalling (Xie et al, 1998; Xu et al, 2002; Yan et al, 2009). Target proteins of COI1 are jasmonate ZIM-domain (JAZ) proteins, which are transcriptional repressors of the MYC2 transcription factor required for JA-inducible gene expression (MYC transcription factors are named after myelocytomatosis, caused by the virus in which they were first discovered; Chini et al, 2007; Katsir et al, 2008; Thines et al, 2007). In the absence of JA-Ile, JAZ proteins repress MYC2 from activating gene expression (Fig. 2 A). Upon stimulation, JA-Ile is formed and promotes the interaction between COI1 and JAZ proteins, thereby initiating JAZ degradation via the ubiquitin-proteasome system (Fig. 2 B; Thines et al, 2007). The now released MYC2 is able to activate JA-responsive genes (Fig. 2 C).

The Arabidopsis JAZ protein family consists of 12 members, all of which contain the ZIM domain in the central part of the protein and the Jas motif at the C terminus (Staswick, 2008). The ZIM motif is comprised of 28 conserved amino acids and confers the ability for homo- or heteromeric interactions among JAZ proteins independent of the presence of JA-Ile (Chini et al,

2009a; Chung & Howe, 2009). Furthermore, the ZIM domain recruits transcriptional co-repressors (Pauwels et al, 2010). The 26 amino acid comprising Jas motif displays the JA-Ile dependent COI1 binding site, presenting the "degron" of JAZ proteins, a protein sequence that acts as a starting point for degradation (Dohmen et al, 1994). Furthermore, the Jas motif mediates JAZ interaction with MYC2 in a JA-Ile independent manner (Fonseca et al, 2009). It has been proposed that both COI1 and MYC2 compete for interaction with the Jas motif of JAZ proteins, and the presence or absence of JA-Ile determines the outcome of this competition (Chini et al, 2009b; Chini et al, 2007).

Cellular processes relying on JA signalling are diverse. Likewise, different JAZ proteins display diverse tissue- and stage-specific expression patterns (Chini et al, 2009b) and furthermore are

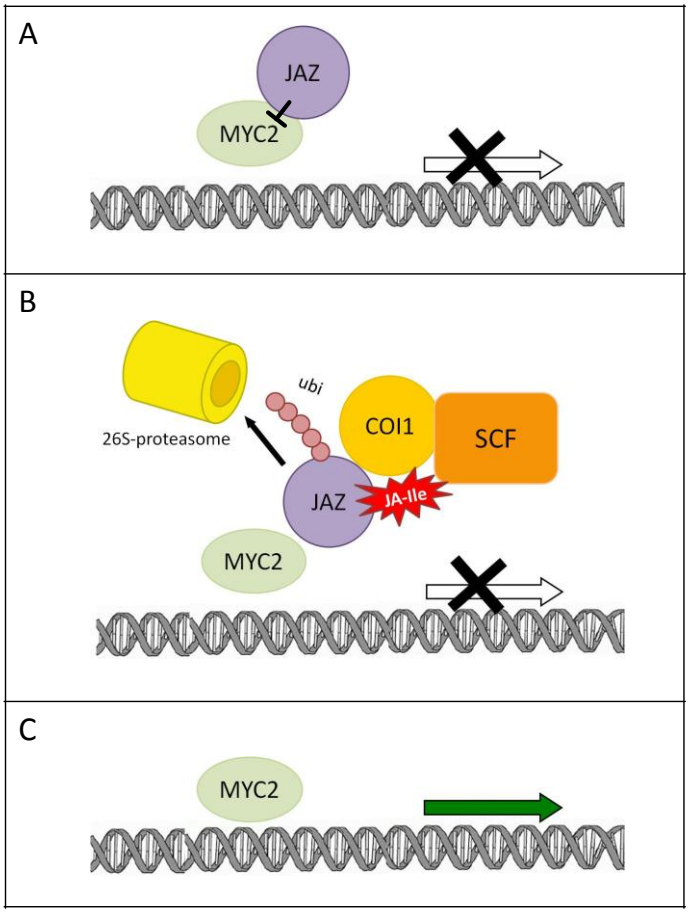

Fig. 2: JA-Ile perception via SCF^{COI1}-mediated JAZ degradation. (A) In the uninduced state, the transcription factor MYC2 is repressed by JAZ. JA-inducible genes are not transcribed, indicated by the crossed-out white arrow. (B) Upon stimulation, JA-Ile promotes the interaction of JAZ and SCF^{COI1}, JAZ is poly-ubiquitinated by the E3 ubiquitin ligase complex SCF^{COI1} and subsequently degraded by the 26S-proteasome. (C) MYC2 is now capable of activating JA-responsive gene expression, indicated by the green arrow. Illustration according to Mosblech et al (2010).

differently induced by insect feeding and wounding (Chung et al, 2008). However, individual *jaz* knockout mutants (except *jaz10*) lack JA-related phenotypes (Thines et al, 2007; Yan et al, 2007), indicating functional redundancy among JAZ family proteins (Chini et al, 2009b). The proposed diversity of JAZ actions can further be expanded by homo- and heteromeric interactions among JAZ proteins as mentioned above (Chini et al, 2009a).

The basic helix-loop-helix transcriptional activator MYC2 (Boter et al, 2004) mentioned above is the so far only identified target of JAZ repressors. Nevertheless, JAZ proteins are expected to target also other transcription factors, since MYC2 does not regulate all JA-responsive genes. Different families of transcription factors are involved in JA signalling, including ET response factors (ERFs; Lorenzo et al, 2003; McGrath et al, 2005; Pauwels et al, 2008; Pre et al, 2008), WRKYs (named after their WRKY domain, defined by the conserved amino acid sequence WRKYGQK at its N-terminal end; Li et al, 2004; Rushton et al, 1995; Xu et al, 2006) and MYBs (MYB transcription factors are named after myeloblastosis, caused by the virus in which they were first discovered; Mandaokar & Browse, 2009). Recently, JA-Ile-dependent interaction of JAZ1 with MYC3, a close relative of MYC2, was also shown (Pauwels et al, 2010), indicating an alternative candidate of JAZ targets.

As mentioned above, JA-Ile-dependent JAZ degradation is mediated by SCF complexes, which are themselves modified by addition or removal of the small ubiquitin-like peptide NEURAL PRECURSOR CELL EXPRESSED DEVELOPMENTALLY DOWNREGULATED PROTEIN 8, NEDD8 (de-/neddylation). Active cycles of neddylation and de-neddylation are required to sustain SCF activity towards its protein substrates (Cope et al, 2002; Schwechheimer & Deng, 2001). Deneddylation takes place via the COP9 signalosome (CSN), a multi protein complex that hydrolyses NEDD8 from SCFs (Schwechheimer & Isono). Interaction of CSN and SCF^{COI1} was shown by (Feng et al, 2003), and it was shown that both complexes are required for JA-responsive gene expression.

1.2.4 Manipulating JA signalling in plants

Knockout mutants are important tools to experimentally elucidate functions of single proteins or whole pathways. Two different JA-related Arabidopsis mutants were used in this thesis. The Arabidopsis *delayed dehiscence 2-2* (*dde2-2*) mutant is defective in *AOS* gene expression and exhibits reduced levels of all oxylipin metabolites downstream of AOS (Fig. 1; Park et al, 2002). As a consequence, these plants are defective in wound-induced JA accumulation as well as in JA-dependent wound-induced gene expression. Furthermore, *dde2-2* plants are male sterile,

since stamen elongation and anther development stall early and pollen fail to dehisce (Park et al, 2002). This phenotype is rescued by exogenous application of methyl-JA (MeJA).

The Arabidopsis *coronatine insensitive 1-1* (*coi1-1*) mutant was isolated in a screen for mutants displaying insensitivity towards the *Pseudomonas syringae*-derived toxin coronatine and towards MeJA (Feys et al, 1994). *coi1-1* plants lack the JA receptor COI1 and exhibit defects in JA-mediated processes, including reduced expression of JA-responsive defence genes and male sterility (Feys et al, 1994; Xie et al, 1998), similar to *dde2-2* plants. The *coi1-1* mutant also displays a flower phenotype similar to that of the *dde2-2* mutants, with shortened stamen and retarded pollen development. In contrast to *dde2-2* plants, sterility of *coi1-1* mutants cannot be rescued by treatment with exogenous MeJA. Therefore, only heterozygous *coi1-1* plants produce offspring and *coi1-1* mutant populations are maintained in a heterozygous state. When wild type seedlings are grown on agar plates containing MeJA, they react with reduced growth and especially shortened root growth. As *coi1-1* plants are impaired in JA perception, they display decreased root growth inhibition on MeJA and develop long roots (Feys et al, 1994). This response can be utilized to select for homozygous *coi1-1* individuals in a heterozygous population and is also employed in this thesis. Heterozygous *coi1-1* individuals are required for seed production and exhibit an intermediate root growth phenotype on MeJA. Both the *dde2-2* and the *coi1-1* mutants carry point mutations and are not easily genotyped. Therefore, in this thesis another allele of *coi1* was established, in which the *coi1* gene is disrupted by a T-DNA insertion, allowing genotyping by PCR.

1.3 The auxin receptor TIR1 contains an inositol polyphosphate cofactor

The principle of hormone perception via SCF ubiquitin ligases outlined above is prevalent in plant signalling and has been demonstrated to mediate the effects of JA, but also of auxins (Ruegger et al, 1998), gibberellins (McGinnis et al, 2003), ET (Gagne et al, 2004; Guo & Ecker, 2003; Potuschak et al, 2003), abscisic acid (ABA; Zhang et al, 2008) and blue light (Demarsy & Fankhauser, 2009). A large and well-studied familiy of F-box proteins are those involved in auxin signalling. The crystal structure of the auxin receptor F-box protein, TRANSPORT INHIBITOR RESPONSE 1 (TIR1), revealed the unexpected presence of an inositol hexakisphosphate ($InsP_6$) cofactor (Fig. 3), the functional relevance of which so far has not been fully assessed (Tan et al, 2007). The TIR1 structure consists of a large leucin-rich repeat (LRR) domain, responsible for auxin perception and substrate recruitment, and an F-box motif.

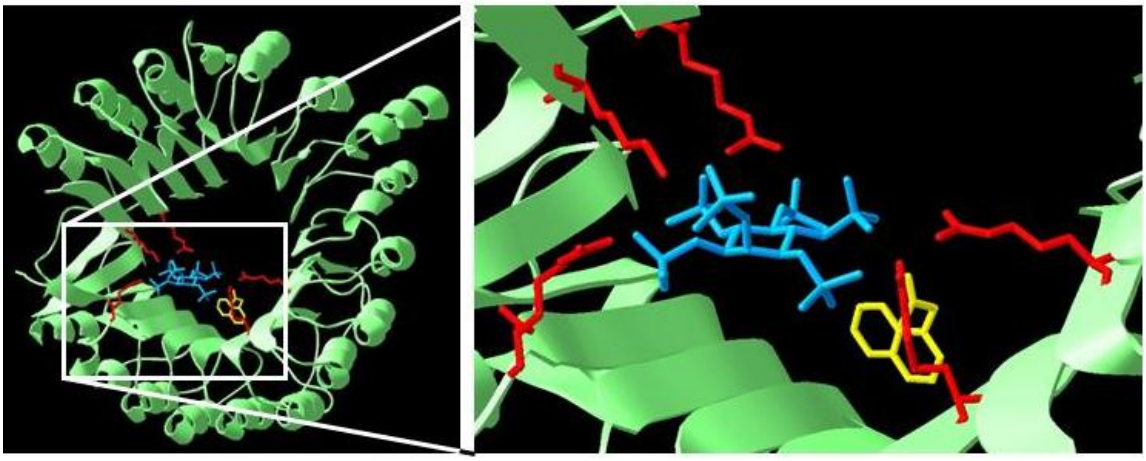

Fig. 3: Partial structure model of TIR1 LRR domain. Auxin is indicated in yellow, inositol hexakisphosphate ($InsP_6$) in blue, $InsP_6$ coordinating amino acids also conserved in COI1 are highlighted in red. Left side, overview; right side, close up of $InsP_6$ binding site. Illustration generated by Prof. Dr. Ingo Heilmann.

At the bottom of a surface pocket within the LRR domain, auxin is recognized, and $InsP_6$ supports a key arginine residue pivotally involved in auxin binding (Tan et al, 2007). The physiological role of $InsP_6$ within TIR1 is not clear. However, recent molecular dynamics simulations suggest that $InsP_6$ functions as a structural cofactor, stabilizing the local conformation of the LRR domain by a complicated hydrogen-bonding network between $InsP_6$ and the surrounding residues of the receptor (Hao and Yang, 2010).

The COI1 protein involved in JA perception is related in sequence to F-box proteins involved in auxin perception, and COI1 and TIR1 show ~ 33 % sequence identity (Yan et al, 2009). Like TIR1, COI1 contains an LRR domain and an F-box motif. The comparison of the primary structures (see also Fig. 12), revealed that the amino acid residues coordinating $InsP_6$ are not only conserved between TIR1 and other auxin signalling F-box proteins, but also largely in the JA receptor F-box protein, COI1. A cavity in the horseshoe-like structure of the LRR domain has been identified as the JA-Ile binding pocket (Yan et al, 2009). This binding pocket can be divided into four distinct areas with specific surface properties, each interacting with a different functional group of JA-Ile. One of these areas, called P1 (Yan et al, 2009), interacts with the keto group of the JA cyclopentanone ring by forming hydrogen bonds; the amino acid arginine 409 (R409), involved in forming P1, is one of the conserved amino acid residues that coordinate the $InsP_6$ within TIR1. Based on the similarity in sequence and structure between TIR1 and COI1, it can be hypothesized that an inositol polyphosphate plays a role in JA perception.

1.4 Phosphoinositide signalling

1.4.1 Signalling events involving inositol-containing factors

The PI pathway is involved in numerous cellular processes in all eukaryotes (Michell, 2008). The membrane lipid phosphatidylinositol (PtdIns) is sequentially phosphorylated by specific kinases (Fig. 4). PtdIns 4-kinases (PI4-kinases) form phosphatidylinositol 4-monophosphate (PtdIns4P) which can then be further phosphorylated by PtdIns4P 5-kinases (PIP5-kinases) to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2; Drobak et al, 1999). PI lipids act as ligands for partner proteins and display signalling function themselves. PtdIns(4,5)P_2 can also be cleaved by phospholipase C (PLC) into diacyl glycerol (DAG), which remains in the membrane, and the soluble inositol 1,4,5-trisphosphate ($InsP_3$; Fig. 4; Lee et al, 1996; Munnik et al, 1998). $InsP_3$ has been proposed to induce the release of Ca^{2+} from intracellular stores.

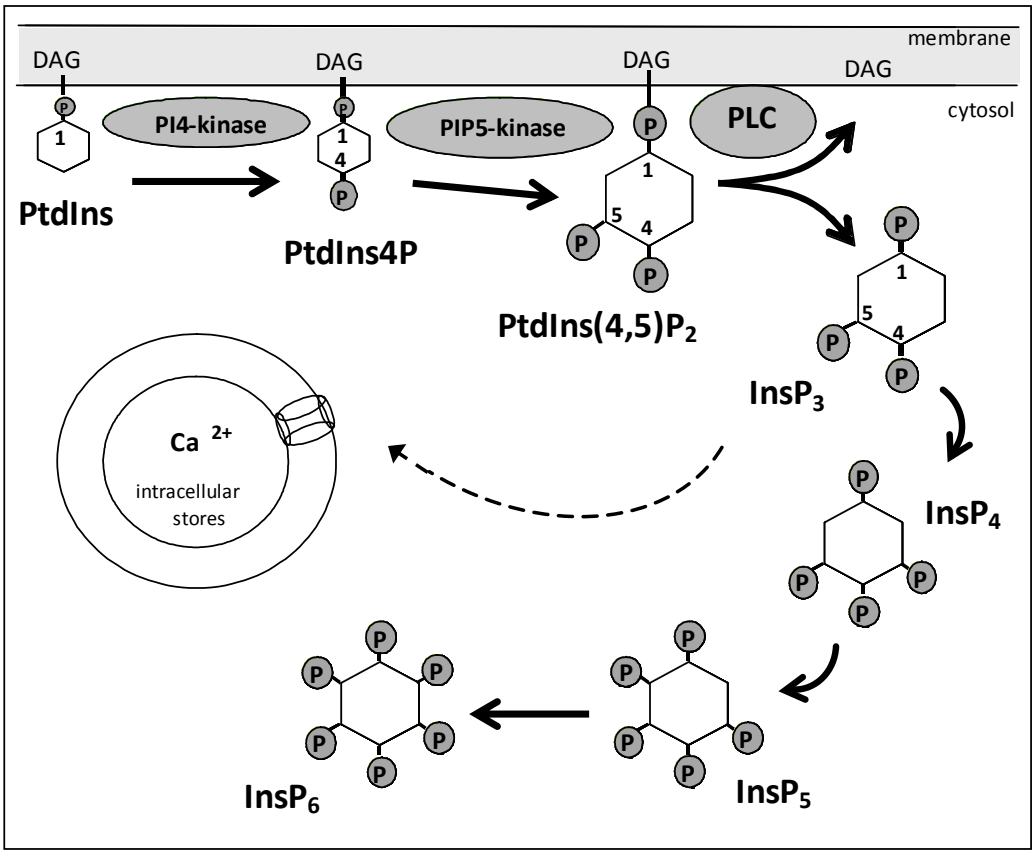

Fig. 4: The PI signalling pathway. Phosphatidylinositol (PtdIns) is phosphorylated to phosphatidylinositol 4-monophosphate (PtdIns4P) by PtdIns 4-kinases (PI4-kinase). Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), formed by further phosphorylation via PtdIns4P 5-kinases (PIP5-kinase), is cleaved by phospholipase C (PLC) to form diacyl glycerol (DAG) and the soluble messenger inositol 1,4,5-trisphosphate ($InsP_3$). $InsP_3$ can induce Ca^{2+} release from intercellular stores and $InsP_3$ can be further phosphorylated to form additional soluble inositol polyphosphates, such as inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) or inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$).

Alternatively, $InsP_3$ can be further phosphorylated to form different inositol polyphosphates, such as inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$) and inositol 1,2,3,4,5,6-hexakisphosphate ($InsP_6$).

In previous experiments, the involvement of PIs and inositol polyphosphate signals in the Arabidopsis wounding response was shown (Mosblech et al, 2008), indicated by a 6-fold increase of $InsP_3$ levels over a period of at least six hours after wounding. Based on the information available for the COI1 homologue TIR1 and those earlier results, it was the working hypothesis of this thesis that wound-induced formation of inositol polyphosphates is important for JA perception by COI1. To test this hypothesis, it is important to understand the metabolism of PIs and inositol polyphosphates in plants and their acknowledged roles in signalling and development.

1.4.2 Roles of PtdIns4P and PtdIns(4,5)P_2

As intact lipids, PtdIns4P and PtdIns(4,5)P_2 exhibit diverse signalling functions, partly due to their high density of negative charges in their head groups that are extending from the hydrophobic bilayer into the cytoplasm (Sansom et al, 2005). These special features allow the recruitment of proteins to the membrane, for example via pleckstrin homology (PH) domains. PH domains were shown to interact with a variety of different PI head groups, in some cases with high specificity towards particular PI isomers, but in most cases not discriminating between different PIs (Lemmon & Ferguson, 2000). By various indirect mechanisms, PtdIns(4,5)P_2 was shown to be involved in the regulation of the actin cytoskeleton, for example via actin binding proteins, such as profilin (Braun et al, 1999; Dong et al, 2001; Gungabissoon et al, 1998; Witke, 2004). Furthermore, PtdIns(4,5)P_2 is involved in ion channel regulation, and for instance K^+ channels are activated upon binding to PtdIns(4,5)P_2 (Hilgemann & Ball, 1996; Wigoda et al, 2010). It has been postulated that PtdIns(4,5)P_2 is involved in the fusion of secretory vesicles with the plasma membrane of yeast (Strahl & Thorner, 2007), and in neuronal cells PtdIns4P and PtdIns(4,5)P_2 are suggested to stimulate exocytosis (Berwin et al, 1998; Hay et al, 1995; Hay & Martin, 1993). Additionally, PtdIns(4,5)P_2 could be important in endocytosis, as many proteins of the endocytotic machinery are targeted to the plasma membrane by PtdIns(4,5)P_2 in yeast and mammalian cells (Ford et al, 2001; Itoh & Howe, 2001; Jost et al, 1998). Dynamin proteins, crucially involved in endocytosis, interact with PIs via their PH domain (Lemmon & Ferguson, 2000). In plants, association of PtdIns(4,5)P_2 with clathrin-coated vesicles during stress-induced endocytosis has been shown by (König et al, 2008). Moreover, vesicle transport and pectin secretion in growing pollen tubes was suggested to be

influenced by PtdIns4P and PtdIns(4,5)P_2, regulated by the action of specific lipid kinases (Ischebeck et al, 2008; Ischebeck et al, 2010). While the list of regulatory roles of intact PIs in eukaryotic cells could be expanded in much more detail, for the purpose of this thesis a focus shall be placed on the cleavage of PIs by PLC and the generation of soluble inositol polyphosphates.

1.4.3 Differences in PLC-mediated PI metabolism between animals and plants

All PI-specific PLCs (PI-PLCs) identified in plants hydrolyze PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner, and G-protein-coupled PLCs, which are present in animals, were not described (Meijer & Munnik, 2003; Mueller-Roeber & Pical, 2002). While transcription of some Arabidopsis PI-PLCs can be induced by drought, low temperature or salt stress (Hirayama et al, 1997; Parre et al, 2007), others are constitutively expressed in different plant organs (Yamamoto et al, 1995). Overexpression of PI-PLC2 in *Brassica napus* resulted in increased InsP_6 levels and also in altered phytohormone patterns, including those of ABA, auxins and cytokinins, supporting an important role for the PI system in phytohormone signalling (Georges et al, 2009).

PLC-derived DAG displays different functions in animals and plants. While DAG activates protein kinase C (PKC) in mammalian cells and therefore displays signalling function by itself, no homolog of PKC has been reported in plants (Meijer & Munnik, 2003). In contrast, in plants DAG is rapidly phosphorylated by DAG kinases to phosphatidic acid (PtdOH). PtdOH has been shown to display signalling function in plants, and increased levels of PtdOH have been reported after osmotic stress, ABA treatment, drought stress, pathogen attack and wounding (Bargmann et al, 2009; Lee et al, 2001; Munnik, 2001; Wang et al, 2000).

An InsP_3 receptor, as it was found in animals to mediate Ca^{2+} release upon stimulation, is absent in all so far sequenced genomes of land plants (Krinke et al, 2007a), and in general, the regulation of the plant PI signalling pathway appears to be different from that in animals. For instance, in animals production of InsP_3 and DAG is driven by increased activation of PLC (Berridge & Irvine, 1984), whereas in plants enhanced availability of the PLC substrate PtdIns(4,5)P_2 seems to promote this process, provided by increased activity of PIP5-kinases (Stevenson et al, 2000). Compared to mammalian cells, higher plants exhibit only low levels of PtdIns(4,5)P_2. Upon stimulation, PtdIns(4,5)P_2 levels increase locally and/or transiently, highlighting the important regulatory role of the respective lipid kinases involved in stress adaptation (Heilmann et al, 1999; Heilmann et al, 2001; Perera et al, 1999).

1.4.4 Inositol polyphosphates

$InsP_3$ signals have been reported to occur upon gravistimulation in maize and oat, accompanied by an increase of specific PIP5-kinase activity (Perera et al, 1999; Perera et al, 2001). A similar effect was reported during responses to salt and osmotic stress in plants, algae and plant cell cultures (Drobak & Watkins, 2000; Heilmann et al, 1999; Heilmann et al, 2001; König et al, 2008; König et al, 2007; Pical et al, 1999), displaying increased synthesis of $PtdIns(4,5)P_2$ accompanied by $InsP_3$ accumulation.

An increasing number of reports indicates that signalling functions can also be attributed to inositol polyphosphates other than $InsP_3$, which are predominantly derived from the PI pathway via an $InsP_3$ intermediate. In yeast, $InsP_6$ is required for mRNA export from the nucleus, and involved in the recruitment of protein complexes from the cytosol to the nuclear pore (Alcazar-Roman et al, 2006; Seeds & York, 2007; Weirich et al, 2006; York et al, 1999). $InsP_4$, $InsP_5$ and $InsP_6$ have been shown to be involved in chromatin remodelling in yeast, thereby regulating transcription (Shen et al, 2003). Additionally, inositol polyphosphates and inositol pyrophosphates control telomer length and cell death in yeast (Saiardi et al, 2005).

In plants, several reports indicate further roles for inositol polyphosphates other than $InsP_3$. Besides its acknowledged role as an important phosphate store in plant seeds (Raboy, 2003), $InsP_6$ has been shown to play a role in maintaining resistance to plant pathogens. For instance, potato plants with altered inositol polyphosphate metabolism and deficient in $InsP_6$ formation exhibit reduced resistance to viruses, bacteria and fungal pathogens (Murphy et al, 2008). Furthermore, the involvement of $InsP_6$ in ABA-mediated guard cell signalling was demonstrated in Arabidopsis, and has been attributed either to complexing of divalent cations, such as Mg^{2+} or Ca^{2+}, or to the triggering of a continuous Ca^{2+} flux into the cytosol via binding to ion channels, affecting K^+ currents (Nagy et al, 2009). Considering its presence in F-box proteins with roles in plant hormone perception, clearly roles of $InsP_6$ and other inositol polyphosphates in signalling must be further explored.

Three pathways of $InsP_6$ biosynthesis have been proposed in plants, with two of them emerging from PI lipids and the third being lipid-independent through sequential phosphorylation of *myo*-inositol or inositol-3-phosphate (Stevenson-Paulik et al, 2005). The lipid dependent pathways originate from PLC-mediated hydrolysis of $PtdIns(4,5)P_2$, generating $InsP_3$, which can be sequentially phosphorylated by the inositol polyphosphate kinases, IPK1 and IPK2. While the last step of $InsP_6$ synthesis in the lipid-dependent pathways is mediated by IPK1, the lipid-independent pathway may alternatively use IPK2 in this step.

1.4.5 Manipulating PI signals in plants

Two plant lines altered in different steps of PI metabolism were used in this thesis. Due to the expression of a human type I inositol polyphosphate 5-phosphatase (InsP 5-ptase), in transgenic *InsP 5-ptase* plants $InsP_3$ accumulation is suppressed and, as a consequence of the "pull" on the PI pathway, upstream lipid precursors are also down regulated (König et al, 2007; Perera et al, 2006; Perera et al, 2002). These plants were used in this thesis as a tool to investigate how plants with a globally downregulated PI signalling pathway would respond to wounding and herbivore attack.

To study the PI pathway with a more precise modification, *ipk1-1* plants were used. These plants lack the enzyme IPK1 and are impaired in the last step of $InsP_6$ biosynthesis and, thus, exhibit strongly reduced levels of $InsP_6$, accompanied by accumulation of metabolic precursors, such as $InsP_5$ and $InsP_4$ (Stevenson-Paulik et al, 2005).

For the purpose of this thesis, it is important to note that *InsP 5-ptase* plants display a reduction of all PI signalling components, whereas *ipk1-1* plants are only diminished in $InsP_6$ levels while accumulating inositol polyphosphate precursors.

1.5 Diverse other signals involved in plant defence

Wounding not only harms the plant by reducing tissue functionality, but a plant´s wounding site is also likely to be attacked by opportunistic pathogenic microorganisms. Therefore, it is necessary for the plant to distinguish between different attackers and to defend itself against different species of feeding insects as well as different species of pathogens. This complex task is orchestrated by a tight regulatory network of various phytohormones, with JA, SA and ET being the best studied examples (del Pozo et al, 2004; Howe & Jander, 2008; Koornneef & Pieterse, 2008; Lorenzo & Solano, 2005; von Dahl et al, 2007). The specific blend of hormones, varying in quantity, composition, and timing, results in a specific signal signature, the integration of which allows for a specific response effective against partially distinct classes of attackers (Koornneef & Pieterse, 2008). In general, SA-mediated defences are often effective against pathogens with a biotrophic lifestyle, whereas JA-mediated defences are mostly effective against necrotrophic pathogens and herbivorous insects (Glazebrook, 2005; Kessler & Baldwin, 2002; Thomma et al, 2001).

1.5.1 Salicylic acid signalling

SA is one of the best-known defence hormones involved in responses to pathogen attack and in mediating disease resistance. SA biosynthesis starts with the Shikimate pathway, in which chorismate is converted to isochorismate via isochorismate synthase (ICS; Wildermuth et al, 2001). Arabidopsis *SA induction-defective 2* (*sid2*) mutants are defective in ICS and fail to accumulate SA or to induce SA-mediated gene expression upon pathogen attack (Dewdney et al, 2000; Wildermuth et al, 2001). An alternative way to produce SA requires phenylalanine and phenylalanine ammonia lyase (PAL) activity. While the ICS-dependent pathway provides the bulk synthesis of SA, the PAL-dependent pathway only produces minor amounts (Chen et al, 2009). It has been speculated that SA derived from the PAL-dependent pathway is required to induce cell death in response to particular pathogens or fungal elicitors (Wildermuth et al, 2001). Besides *sid2* mutants, transgenic *NahG* plants expressing the bacterial salicylate hydroxylase (*nahG*) gene that encodes an enzyme degrading SA to catechol also exhibit reduced SA accumulation and increased susceptibility to viral, fungal, or bacterial pathogens (Delaney et al, 1994; Vernooij et al, 1994).

The transcriptional activator, NONEXPRESSOR OF PR GENES 1 (NPR1), represents a key node of SA signal transduction. Upon SA induction, a redox change of the cellular milieu leads to the reduction of NPR1 disulfide-bound oligomers into active monomers, which are translocated from the cytosol to the nucleus (Dong, 2004; Lu et al, 2009; Mou et al, 2003; Tada et al, 2008; Wang et al, 2006). There, NPR1 activates SA-induced gene expression by interacting with transcription factors of the TGA family (TGA transcription factors are named after their ability to bind TGACG motifs; Rochon et al, 2006). It was shown that also WRKY transcription factors are controlled by NPR1 (Niggeweg et al, 2000; Wang et al, 2006). *npr1* mutants, although accumulating elevated levels of SA after pathogen infection, display decreased disease resistance and abolished SA-responses (Cao et al, 1997; Dong, 2004), whereas NPR1 overexpressors exhibit broad resistance against diverse bacterial and fungal pathogens (Fitzgerald et al, 2004; Lin et al, 2004a; Lu et al, 2009; Makandar et al, 2006).

1.5.2 Crosstalk of JA and SA signals within plant defence

Crosstalk between different signalling pathways can be either mutually antagonistic or synergistic. For instance, SA is capable of suppressing the expression of various JA-inducible genes, thereby working as a negative regulator of JA-dependent defence pathways and representing the antagonistic aspect of the JA-SA relation (Bostock, 2005; Cui et al, 2005; Doares et al, 1995; Kloek et al, 2001; Pieterse & van Loon, 1999; Stout et al, 2006). The

expression of JA biosynthesis genes is suppressed by SA treatment, indicating that JA biosynthesis is a target of SA action in the suppression of JA signals (Doares et al, 1995; Spoel et al, 2003). SA seems to also target the JA pathway downstream of JA biosynthesis by interfering with the function of the SCF^{COI1} complex (Beckers & Spoel, 2006).

It was shown that infection with biotrophic pathogens, inducing elevation of SA levels, enhances the susceptibility of plants to necrotrophic pathogens by suppressing the JA signalling pathway (Spoel et al, 2007). In line with this observation, caterpillar induced JA-mediated defences were strongly suppressed by infection with a biotrophic pathogen (Leon-Reyes and Pieterse, unpublished data, mentioned in (Koornneef & Pieterse, 2008). Furthermore, JA treatment increases number and density of trichomes, whereas SA treatment results in decreased trichome number (Traw & Bergelson, 2003). The antagonistic relation between SA and JA is also reciprocally indicated by JA repressing SA signals. For example, JA signals in *P. syringae*-infected Arabidopsis leaves abolish SA accumulation and SA-mediated downstream signalling through NPR1. Furthermore, the suppression of JA-inducible gene expression by SA signals is blocked in Arabidopsis *npr1* mutants, indicating the crucial role of NPR1 in the crosstalk between SA and JA (Kloek et al, 2001; Spoel et al, 2003).

Mitogen-activated protein (MAP) kinases (MPKs) are important signal transducers in all eukaryotes, and they are also involved in plant defence responses (Menke et al, 2004; Nakagami et al, 2005). In Arabidopsis, MPK4 was found to negatively regulate SA signals while positively regulating JA signals (Petersen et al., 2000), indicated by increased SA levels and enhanced SA-responsive gene expression in Arabidopsis *mpk4* mutants. At the same time, *mpk4* mutants exhibit decreased JA-responsive gene expression. Interestingly, this suppression was independent of SA accumulation (Brodersen et al, 2006; Petersen et al, 2000).

WRKY transcription factors are important regulators of SA-dependent defence responses (Maleck et al, 2000; Wang et al, 2006), and WRKY70 acts as a positive regulator of SA-mediated defences while repressing JA-mediated responses (Li et al, 2004). Depending on the kind of attack, the specific signal signature enables these transcription factors to modulate the primary JA signal and to differentially activate either JA-responsive defence against necrotrophic pathogens or wound responses against insect herbivores (Koornneef & Pieterse, 2008). It has been speculated that the SA antagonism of JA signalling could be achieved by interfering with JA-mediated JAZ degradation or SCF E3 ligase components (Loake & Grant, 2007; Robert-Seilaniantz et al, 2007). However, convincing proof for this concept has not been presented to date.

Simultaneous application of SA and JA in high concentrations seems to cause antagonistic effects, which may lead to the production of reactive oxygen and cell death (Loake & Grant,

2007; Mur et al, 2006). However, application of SA and JA at low concentrations results in a synergistic effect, indicated by the expression of PR1, a typical SA-regulated gene, and simultaneous expression of classical JA-mediated defence markers (Loake & Grant, 2007; Mur et al, 2006). Positive crosstalk between JA and SA signals was reported, when JA signals were required ahead of SA and ET accumulation in disease response of tomato plants to bacterial pathogen infection (O'Donnell et al, 2003).

Clearly, the hormonal network during defence reactions is complex and highly interconnected. Transcription factors, such as MYC2 and ERF1, represent important regulatory nodes and are influenced by a variety of signalling components. Without giving any detail, at this point it should be mentioned that also JA and ABA synergistically induce MYC2-dependent gene expression after wounding, and ERF1-dependent gene induction is controlled by a combination of JA and ET in response to pathogen attack (Anderson et al, 2004; Dombrecht et al, 2007; Lorenzo et al, 2004; Lorenzo & Solano, 2005). To keep the system assessable, the focus of this thesis is on crosstalk between JA and PI signals with an outlook on the three-way interactions between JA, PI and SA signals.

1.6 A pathogen´s view of plant defence

During evolution, not only plants developed a tight signalling network of hormones to defend themselves against herbivores and pathogens; insects and microorganisms also co-evolved mechanisms to exploit these signalling cascades for their own benefit (Koornneef & Pieterse, 2008; Pieterse & Dicke, 2007). One of the best known examples is the virulent bacterium *P. syringae*, which produces coronatine, a potent mimic of JA-Ile already mentioned above (Nomura et al, 2005). Coronatine activates JA-mediated responses, which alter the initially activated defence by suppressing SA-responses and, thus, allow enhanced growth of the pathogen (Brooks et al, 2005; Cui et al, 2005; Laurie-Berry et al, 2006; Zhao et al, 2003). By actively interfering with the plant´s hormonal network, the pathogen overcomes the plant´s defence mechanisms and enables its own spreading. In JA-related research, coronatine is frequently used as JA-Ile analogue.

1.7 Goals

In previous experiments, JA and $InsP_3$ signals were shown to occur in parallel upon wounding in Arabidopsis. As JA-deficient *dde2-2* plants did not accumulate $InsP_3$ upon wounding, JA was indicated as upstream factor of PI signals.

It was the aim of this thesis to elucidate the impact of PI signals during Arabidopsis wound responses and defence reactions, and to test the hypothesis that there is crosstalk of JA and PI signals, including an inositol polyphosphate required for COI1 function in JA perception. Therefore, it was one goal to test *InsP 5-ptase* plants with globally reduced PI signalling pathway for wound-induced gene expression and their resistance to herbivore attack. Another goal was to further investigate the partial insensitivity of *InsP 5-ptase*-plants towards JA-Ile in comparison with JA-insensitive *coi1* mutants. Based on these results, it was the goal to test whether an inositol polyphosphate, such as $InsP_6$ or $InsP_5$, has a role in COI1-mediated JA responses. The effects of manipulating the putative inositol polyphosphate binding site in COI1 were to be tested in yeast two-hybrid assays as well as in complementation assays of *coi1* plants. Furthermore, the function of wild type COI1 was to be assessed in yeast and plant backgrounds altered in their inositol polyphosphate composition. Finally, it was the goal of this thesis to investigate the implementation of PI signals in a broader context of plant defence signals. Therefore, crosstalk between SA and PI signals was to be investigated using SA-deficient Arabidopsis mutants *sid2* and *NahG*.

The results of this thesis highlight the PI pathway as an important regulatory node in the defensive phytohormone signalling network.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Agar	Invitrogen, Karlsruhe, Germany
Agarose	Duchefa Biochemie, Haarlem, The Netherlands
Carbenicillin	Duchefa Biochemie, Haarlem, The Netherlands
Diisopropylamin	Aldrich, Steinheim, Germany
Desoxynucleotide triphosphate (dNTPs)	Roche Molecular Biochemicals, Mannheim, Germany
Fluorescein	Bio-Rad, Hercules, CA, USA
Kanamycin	Duchefa Biochemie, Haarlem, The Netherlands
Murashige & Skoog medium	Duchefa Biochemie, Haarlem, The Netherlands
Peptone	Invitrogen, Karlsruhe, Germany
Phospholipid standards	Avanti Polar Lipids, Inc., Alabaster, AL, USA
Rifampicin	Duchefa Biochemie, Haarlem, The Netherlands
Scintillation fluid	Zinsser Analytics, Frankfurt, Germany
Silwet-Copolymer	OSi Specialties Inc., South Charleston, WI, USA
SYBR Green I solution	Cambrex, Wiesbaden, Germany

All other chemicals were obtained from the companies Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Steinheim, Germany) or Sigma (Deisenhofen, Germany).

2.1.2 Enzymes and size markers

BIOTaq DNA Polymerase	Bioline, Luckenwalde, Germany
GeneRuler™ 1kb DNA-Ladder	MBI Fermantas, St. Leon Rot, Germany
MasterAmp *Tfl*-DNA-Polymerase	EPICENTRE Biotechnologies, Madison, WI, USA
Pfu-Polymerase™	MBI Fermantas, St. Leon Rot, Germany
Phusion High Fidelity DNA-Polymerase	Finnzymes, Espoo, Finland
Restriction endonucleases	MBI Fermantas, St. Leon Rot, Germany

RevertAid H Minus M-MuLV Reverse Transcriptase	MBI Fermantas, St. Leon Rot, Germany
T4-DNA-Ligase	MBI Fermantas, St. Leon Rot, Germany
Takara *Ex Taq*™ DNA Polymerase	Takara Bio Inc, Madison, WI, USA

2.1.3 Kits

Big Dye Terminator v1.1 Cycle Sequencing-Kit	Applied Biosystems, Darmstadt, Germany
[^{3}H] Biotrak Assay System for D-myo-inositol 1,4,5-trisphosphat	GE Healthcare/Amersham, Düren, Germany
Nucleospin Plasmid Kit	Machery & Nagel, Düren, Germany
Nucleospin Extract II Kit	Machery & Nagel, Düren, Germany
pGEM-T Easy Ligation Kit	Promega, Heidelberg, Germany

2.1.4 Equipment

Agilent 1100 HPLC system	Agilent, Waldbronn, Germany
Agilent 1100 HPLC system	Agilent, Waldbronn, Germany
Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer	MDS Sciex, Ontario, Canada
C_{18} column (EC 250/2 Nucleosil 120-5 C_{18})	Macherey & Nagel, Düren, Germany
Capillary Rtx-5MS column	Resteck, Bad Homburg, Germany
Chip ion source TriVersa NanoMate	Advion BioSciences, Ithaca, NY, USA
DB-23 capillary column	J&W, Agilent, Waldbronn, Germany
EC 50/2 Nucleodure C_{18} gravity column	Macherey and Nagel, Düren, Germany
Fluorescence Stereo Microscope Leica MZ16 FA	Leica, Wetzlar, Germany
Leica DFC480 camera	Leica, Wetzlar, Germany
GC6890 Gas chromatograph with Flame ionization detection	Agilent, Waldbronn, Germany
iCycler System	BioRad, Hercules, CA, USA
Polaris Q mass selective detector	ThermoFinnigan, Austin, Texas, USA
Shaking mill MM200	Retsch, Haan, Germany

Stereo microscope SZX12	Olympus, Hamburg, Germany
Trace gas chromatograph	ThermoFinnigan, Austin, Texas, USA
Ultra Turrax	Ika, Staufen, Germany
UV imager raytest IDA	Herolab, Wiesloch, Germany

2.1.5 Single-use materials

Bakerbond spe™ Silica Gel columns	J.T. Baker, Deventer, The Netherlands
Glass beads 2.85-3.3 mm	Roth, Karlsruhe, Germany
Silica thin layer chromatography plates Si-60, 20 x 20 cm	Merck, Darmstadt, Germany
SuperQ column SDB-L Strata	Phenomenex, Aschaffenburg, Germany

2.1.6 Software

analySIS Docu 3.2	Soft Imaging Systems GmbH, Münster, Germany
Applied Biosystems Analyst software	Foster City, California, USA
Chromas Lite v 2.0	Technelysium, Tewantin, Australia
GC ChemStation software	Agilent, Waldbronn, Germany
ImageJ	freely available at http://rsbweb.nih.gov/ij/
LSM 510 software v 4.0	Carl Zeiss Inc., Jena, Germany
Photoshop v 7.0	Adobe Systems, Munich, Germany
Xcalibur software v1.4	ThermoFinnigan, Austin, Texas, USA

2.1.7 Plant lines

Name	Species	Transgenes	Obtained from
Arabidopsis wild type Col-0	*Arabidopsis thaliana* Ecotype Columbia 0	-	Dr. Imara Perera, North Carolina State University, Raleigh, NC, USA
coi1-1	*Arabidopsis thaliana* Ecotype Columbia 0	EMS line, carrying a stop codon in the gene encoding for the JA receptor coronatin insensitive 1 (COI1; Xie et al, 1998)	Dr. Jane Glazebrook, St. Paul, MN, USA
coi1-t	*Arabidopsis thaliana* Ecotype Columbia 0	T-DNA insertion into the gene encoding for the JA receptor coronatin insensitive 1 (COI1); SALK No. 035548	SALK Institute Genomic Analysis Laboratory
dde2-2	*Arabidopsis thaliana* Ecotype Columbia 0	*delayed-dehiscence2-2 (dde2-2)*; *En1/Spm1*-transposon mutagenesis of gene encoding for allene oxide synthase (AOS), frame shift (von Malek et al, 2002)	Dr. Michael Stumpe, Georg August University, Göttingen, Germany
InsP 5-ptase	*Arabidopsis thaliana* Ecotype Columbia 0	*HsInP 5-ptase*, human type I InsP 5-ptase transgene line 2-8; Kan^R (Perera et al, 2006)	Dr. Imara Perera, North Carolina State University, Raleigh, NC, USA
ipk1-1	*Arabidopsis thaliana* Ecotype Columbia 0	T-DNA insertion into the gene encoding for inositol polyphosphate kinase 1 (IPK1; Stevenson-Paulik et al, 2005)	Dr. John York, Duke University, Durham, NC
NahG	*Arabidopsis thaliana* Ecotype Columbia 0	*Pseudomonas putida nahG* gene, encoding an SA hydroxylase, which degrades SA to catechol (Delaney et al, 1994)	Dr. Christiane Gatz, Georg August University, Göttingen, Germany
sid2	*Arabidopsis thaliana* Ecotype Columbia 0	EMS line, causing a stop codon in the gene encoding for isochorismate synthase (ICS1), involved in SA biosynthesis. SA induction–deficient 2 (*sid 2*; Nawrath & Metraux, 1999).	Dr. Christiane Gatz, Georg August University, Göttingen, Germany

2.1.8 Microorganisms

Organism	Strain	Genotype	Reference	Obtained from
E. coli	XL1-Blue	recA1endA1gyrA96 thi-1hsdR17 supE44 relA1lac[F`proAB lac9zM15 Tn19(Tetr)]	(Bullock et al, 1987)	Stratagene, Heidelberg, Germany
A. tumefaciens	pEHA105	pTiBo542ΔT-DNA RifR	(Hood et al, 1993)	-
S. cerevisiae	PJ69-4A	*MATa trpl-901 leu2-3,112 ura3-52 his3-200 ga14A ga18OA LYSZ::GALl-HIS3 GAL2-ADE2 metZ::GAL7-lacZ*	(James et al, 1996)	Dr. Corinna Thurow, Georg August University, Göttingen, Germany

2.1.9 Plasmids

Vector	Selection marker	Obtained from	Plasmid structure
pGEM-T Easy	Amp^R	Promega, Mannheim, Germany	
pCAMBIA3300-0GC	Kan^R	Dr. Ellen Hornung, Göttingen, Germany	Plasmid contains a BASTA resistence under a 35S promoter
pAG25	Amp^R; nourseothricin	Dr. Martin Fulda and Dr. Michael Scharnewski, Göttingen, Germany	nourseothricin resistance cloNAT-MX4 cassette
pGBKT7	Kan^R, TRP1 nutritional marker for selection in yeast	Dr. Corinna Thurow, Göttingen, Germany	Plasmid expresses proteins fused to amino acids 1-147 of the GAL4 DNA binding domain under the constitutive ADH1 promoter.

2.1.10 Oligonucleotides

All oligonucleotides were obtained from Invitrogen, Karlsruhe, Germany. A complete list and the respective sequences are comprised in appendix.

2.2 Methods

2.2.1 Plant growth and cultivation

For wounding experiments, caterpillar performances or treatment with MeJA, Arabidopsis plants were sown on soil (Frühstorfer Erde type: T25 Str. 1, Fein, Industrie Erdwerk Archut, Lauterbach-Wallenrod, Germany), vernalized over night at 4 °C in the dark and grown under short day conditions with 8 h of light (~130-150 µmol photons m^{-2} s^{-1}, 4 x 1,5 m fluorescent lamps, 58 W) and 16 h of darkness in climate chambers (YORK Refrigeration, YORK Industriekälte GmbH & Co. KG, Mannheim, Germany) with about 60 % humidity. Single plants were grown in pots of 6 cm in diameter for 7-8 weeks. For seed propagation, plants were grown in the greenhouse under 16 h of light (~130-150 µmol photons m^{-2} s^{-1}).

For sorbitol treatment, Arabidopsis plants were precultured under sterile conditions. Seeds were surface sterilized by exposing them to chloric gas for 4–7 h generated by adding 5 % (v/v) of 10 M HCl to a 12 % (v/v) NaClO solution in a closed exsiccator. Vacuum of about 850 mbar was applied. After 3-4 weeks of growth on sterile poplar root media (see below) in closed glass containers in Percival climate chambers under a regime of 14 h of light (~130-150 µmol photons m^{-2} s^{-1}, 4 x 1,5 m fluorescent lamps, 58 W) and 10 h of darkness, plants were transferred to 12 l hydroponic cultures, first in ddH_2O for 2-3 days, afterwards in nutrient solution (see below), purged with air of ~ 0.1 bar. If necessary, cultures were fertilised with 1-2 ml Fe-Na-EDTA und KNO_3. Growth conditions correspond to those described for soil cultures.

For vertical root growth assays, plants were cultured under sterile conditions in rectangular Petri dishes, oriented vertically, containing MS growth medium (0.22 % (w/v) Murashige & Skoog medium, 1.5 % (w/v) sucrose, 1 % (w/v) agar) under 8 h of light (~130-150 µmol photons m^{-2} s^{-1}) and 16 h of darkness in Percival climate chambers.

To select JA-insensitive mutants, plants were cultured under sterile conditions in round Petri dishes containing media as described above with additional 50 µM MeJA, dissolved in ethanol. After 2-3 weeks of growth, homozygous JA-insensitive individuals were identified by normal growth, JA-sensitive wild type individuals were identified by strongly reduced growth and heterozygous individuals by an intermediate phenotype. Desired individuals were transferred to soil and grown as described above. For wound experiments, homozygous *coi1-1* and *coi1-t* plants were grown on soil for another six weeks after selection. Wild type control plants were cultured the same way on agar plates without MeJA.

2.2.1.1 Poplar root media

Macro elements (KNO_3 0.25 M; $CaCl_2$ x $2H_2O$ 3.6 mM; $MgSO_4$ x $7H_2O$ 16.2 mM; $(NH_4)_2HPO_4$ 26 mM), micro elements (H_3BO_3 9.7 mM; Na_2MoO_4 x $2H_2O$ 0.21 mM; $MnSO_4$ x H_2O 0.9 mM; $ZnSO_4$ x $7H_2O$ 11.8 mM; $CoCl_2$ x $6H_2O$ 2.1 mM; $CuSO_4$ x $5H_2O$ 0.2 mM), vitamin solution (nicotinic acid 0.4 mM; pyridoxine-HCl 0.24 mM; thiamin-HCl 29.6 µM; glycine 2.6 mM) myo-inositol (22.2 mM), $C_{10}H_{12}FeN_2NaO_8$ (4 mM) and saccharose (73 mM) were sterile filtered (pore size 0.2 µm) and mixed as follows: macro elements 100 ml l^{-1}; micro elements 1 ml l^{-1}; vitamin solution 1 ml l^{-1}; myo-inositol 5 ml l^{-1}; $C_{10}H_{12}FeN_2NaO_8$ 5 ml l^{-1}. pH was adjusted to 5.8 using NaOH. For solid media 2.8 % (w/v) Gelrite was added.

2.2.1.2 Hydroponic culture media

Stock solutions were prepared according to Randall & Bouma (1973). Nutrient solutions: KH_2PO_4/K_2HPO_4 1 M, pH 7.2; $MgCl_2$ x $6H_2O$ 1 M; $MgSO_4$ x $7H_2O$ 1 M; $Ca(NO_3)_2$ x $4H_2O$ 1 M; KNO_3 1 M; $CaCl_2$ 1 M; KCl 1 M; trace element solution H_3BO_3 46.2 mM, $MnCl_2$ x $2H_2O$ 9 mM, $ZnSO_4$ x $7H_2O$ 750 µM, $CuSO_4$ x $5H_2O$ 318 µM, Na_2MoO_4 x $2H_2O$ 120 µM; Fe-Na-EDTA solution $FeCl_3$ x $6H_2O$ 80 mM, Na_2EDTA x $2H_2O$ 45.64 mM. Solutions were mixed according to the following scheme:

Stock solution	Volume [ml]	Final concentration
KH_2PO_4/K_2HPO_4	30	0.5 mM
$MgCl_2$ x $6H_2O$	120	2 mM
$MgSO_4$ x $7H_2O$	60	1 mM
$Ca(NO_3)_2$ x $4H_2O$	210	3.5 mM
KNO_3	180	3 mM
Trace elements	50	
H_3BO_3		38.5 µM
$MnCl_2$ x $2H_2O$		7.5 µM
$ZnSO_4$ x $7H_2O$		0.625 µM
$CuSO_4$ x $5H_2O$		0.265 µM
Na_2MoO_4 x $2H_2O$		0.1 µM
Fe-Na-EDTA solution	36	
$FeCl_3$		48 µM
Na_2EDTA		27.4 µM
filled with ddH_2O to final volume	60 l	

2.2.2 Plant treatments

2.2.2.1 Wounding

7-8-week-old Arabidopsis plants were wounded by squeezing each rosette leave 3-8 times with forceps, crossing the mid vain (Stenzel et al, 2003a). Whole rosettes were harvested at different time points and immediately frozen in liquid N_2. To account for biological variation, rosettes of 6-10 plants were pooled for each time point. Wound treatment was started in the early light phase and, if possible, completed before shift to dark phase. Frozen plant material was ground to fine powder and samples were stored at -80 °C.

2.2.2.2 MeJA application

External application of MeJA was carried out by carefully cutting whole rosettes of 6-week-old plants and placing them on 100 ml of H_2O or H_2O containing 50 µM MeJA (Sigma; solved in ethanol) in glass Petri dishes, as described (Stenzel et al, 2003a). After various times, rosettes were quickly dried on tissue and immediately frozen in liquid N_2. To account for biological variation, rosettes of four to five plants were pooled for each time point.

2.2.2.3 Sorbitol treatment

8-10-week-old plants grown in hydroponic cultures (as described above) were treated by adding a final concentration of 0.8 M sorbitol to the nutrient media. After indicated time, rosettes were cut and immediately frozen in liquid N_2. To account for biological variation, rosettes of 4-5 plants were pooled for each time point.

2.2.2.4 Root growth assay

Petri dishes of vertically grown seedlings were scanned with a flatbed scanner (CanoScan 8000F, Canon, Krefeld, Germany) and root lengths were determined using the freely available software ImageJ (http://rsbweb.nih.gov/ij/).

For root length determination of complemented *coi1-t* seedlings, Petri dishes were first scanned, then seedlings were transferred on soil, *coi1-t* homozygous individuals were identified by PCR-based genotyping, and root lengths of only those homozygous seedlings were determined.

2.2.2.5 Caterpillar feeding performance tests

Diamondback moth (*Plutella xylostella*) caterpillars of approximately 1 mg each were collected 3 d after oviposition, and 20–40 individuals were placed on the leaf surface of 5-week-old Arabidopsis plants. For the duration of the experiment, 5-6 plants were separated in closed containers, allowing gas exchange and exposition to 80–100 µmol photons m^{-1} s^{-1} in a regime of 12 h light and 12 h darkness. After various times, caterpillars were collected and weighed. After weighing, caterpillars were allowed to continue feeding on the respective plants, in order to monitor a continuous defensive response. Care was taken not to harm caterpillars during the weighing process; individuals damaged during collection were eliminated from subsequent evaluation.

2.2.3 Crossing

For easier handling, the receptor inflorescence was carefully fixed beneath the stereo microscope using linen tape. Sepals and petals of young buds, not showing any white petals yet, were carefully removed. Immature anthers, which had not released any pollen yet, were removed as well, exposing the ovary with the stigma. Mature flowers of the donor plant were slightly squeezed with forceps until pollen-carrying anthers were showing, which then were rubbed on the uncovered stigma of the mother plant. After pollen transfer, the stigma was covered with a loose bag of plastic wrap, which was removed 3-5 days after crossing procedure. All other non-treated siliques, flowers and buds were removed in order to avoid confusion.

2.2.4 Molecular biological techniques

2.2.4.1 Amplification of specific sequences by PCR

For DNA amplification from cDNA or genomic DNA for application in cloning procedures *Phusion* High-Fidelity PCR Master Mix was used following manufacturer's recommendations. For the reaction the following temperature program was applied: 30 s at 98 °C, 35 cycles of 15 s at 98 °C, 30 s at 60 °C and 1 min per 1000 bp gene length at 72 °C; 5 min at 72 °C.

For PCR-based genotyping, *TaKaRa Ex Taq*™ polymerase was used according to manufacturer's recommendations. For the reaction the following temperature program was used: 3 min at 95 °C; 28 cycles of 15 s at 95 °C, 30 s at 60 °C, 1 min per 1000 bp length at 72 °C; 4 min at 72 °C. Alternatively, MasterAmp *Tfl* DNA Polymerase, was used following manufacturer's

recommendations and the following temperature program was used: 1 min at 95 °C; 28 cycles of 30 s at 95 °C, 45 s at 60 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72 °C.

2.2.4.2 PCR-based genotyping

coi1-t T-DNA insertion mutants were genotyped by PCR using genomic DNA (isolated as described in section 2.2.4.13) as a template. PCR was performed using either *TaKaRa Ex Taq*™ Polymerase or MasterAmp *Tfl* DNA Polymerase according to manufacturer's recommendations. The PCR program described above was used. To identify mutant and wild type *coi1* alleles present in the plants, the primer combination P1 and P2 (5'-tggaccatataaattcatgcagtc-3' / 5'-ctgcagtgtgtaacgatgctc-3') was used. For identification of mutant or wild type *ipk1* alleles present in yeast, the primer combination P3 and P4 (5'-tcatcatcaatgtggctgct-3' / 5'-gatacatcgggcaaagcaag-3') was used.

2.2.4.3 Electrophoretic DNA separation

DNA was separated after addition of 1/5 volume of sample buffer (250 mM EDTA, 0.58 M sucrose, 50 % (w/v) glycerol and 0.4 % (w/v) Orange G) on TAE agarose gels (40 mM TRIS/HCl, pH 7.0, 20 mM acetic acid, 1 mM EDTA solidified with 1 % (w/v) agarose) using GeneRuler 1 kb DNA Ladder as a size marker and subsequently stained for 10 min in a 2 µg/ml ethidium bromide bath. DNA bands were then visualized using a UV-imager.

2.2.4.4 Assembly of DNA constructs

For conventional DNA construct assembly, plasmids were restricted and ligated according to the recommendations of the enzyme manufacturer. For cloning of PCR products into the pGEM-T Easy vector, an adenosine nucleotide was added to the 3'-end of the purified PCR products by using 0.5 µl of MasterAmp *Tfl* DNA Polymerase and dATP at a final concentration of 100 pM in a total volume of 50 µl buffered solution. Ligation was carried out using the pGEM-T Easy vector system Kit according to manufacturer's recommendations.

2.2.4.5 Site-directed mutagenesis

Base pair exchanges were introduced into cDNA clones using QuickChange technology (Stratagene, La Jolla, CA, USA). In this method the template plasmid is completely replicated by a DNA polymerase starting from the primers that contain the desired base pair exchanges. In one reaction, 50 ng of plasmid DNA were used in a total volume of 25 µl containing 10 pmol of

each primer, 0.75 µl DMSO and 12.5 µl of 2 x *Phusion* High-Fidelity PCR Master Mix. For the reaction the following temperature cycle was used: 30 s at 95 °C; 21 cycles of 30 s at 95 °C, 1 min at 55 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72 °C. Alternatively *Pfu* DNA Polymerase was used in a total volume of 25 µl containing 10 x *Pfu* DNA Polymerase buffer including $MgCl_2$, 0,2 mM dNTPs, 10 pmol 3'-primer, 10 pmol 5'-primer, 2,5 U μl^{-1} *Pfu* DNA polymerase und 5-50 ng dsDNA. The mixture of product and template was subsequently restricted over night with 10 U of *DpnI* that recognizes and cleaves the methylated restriction site Gm6ATC. This led to the elimination of most of the methylated template leaving the unmethylated product intact which was transformed into *E. coli* as described.

2.2.4.6 DNA sequencing

DNA was sequenced by the didesoxy termination method using 50 ng of plasmid in a total volume of 10 µl containing 1.5 µl sequencing buffer, 1.5 µl reaction mix and 10 pmol of primer. For the reaction the following temperature cycle was used: 2 min at 95 °C; 25 cycles of 30 s at 95 °C, 15 s at 55 °C, 4 min at 60 °C. The reaction product was separated by Andreas Nolte (Georg August University Göttingen, Germany) using capillary electrophoresis. Sequence analysis was performed using Chromas Lite v 2.0 software (Technelysium, Tewantin, Australia).

2.2.4.7 Preparation of chemically competent *E. coli* cells

In preparation of transformation experiments, *Escherichia coli* XL1-Blue cells were made chemically competent (Inoue et al, 1990). Cells were picked from plates and precultured in 2 ml SOB medium (2 % peptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl) overnight. Cultures were diluted 1:100, 1 ml was transferred to 250 ml of SOB medium and cells were grown at 37 °C, until an OD_{600} of 0.45–0.75 was reached. Cells were collected via centrifugation at 1000 x g and 4 °C for 10 min and resuspended in 80 ml of TFP buffer (10 mM pipes, pH 6.7, 15 mM $CaCl_2$, 250 nM KCl, 55 mM $MnCl_2$) and incubated on ice for 10 min. Afterwards, centrifugation was repeated and cells were resuspended in 21.5 ml TFB buffer containing 7 % (v/v) DMSO and incubated again for 10 min on ice prior to aliquoting, shock freezing in liquid nitrogen and storage at -80 °C.

2.2.4.8 Transformation of *E. coli* cells

For transformation according to Inoue et al (1990), 100 µl of *E. coli* cells were thawed on ice and added to the plasmid DNA in a 2 ml reaction tube. After incubation on ice for 20 min, the

cells were heat shocked at 42 °C for 45 s and immediately incubated on ice for 5 min. After addition of 300 µl SOC medium (1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, 20 mM sucrose, 20 mM $MgCl_2$) cells were incubated at 37 °C for 1 h under constant shaking and plated on solid LB medium (1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl solidified with 1.5 % (w/v) agar) containing the adequate selection antibiotic (carbenicillin 100 µg ml^{-1}, kanamycin 50 µg ml^{-1}). In liquid culture, *E. coli* cells were grown in LB medium (as above but without agarose; antibiotics were used in the same concentrations as in agar plates).

2.2.4.9 Preparation of chemically competent *A. tumefaciens* cells

The *Agrobacterium tumefaciens* strain EH 105 (Hood et al, 1993) was made competent as follows: Precultures containing 50 µg ml^{-1} rifampicin were grown in a 2 ml YEB (0.5 % (w/v) beef extract, 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.5 % (w/v) saccharose, 5 mM $MgSO_4$) culture at 28 °C over night. This culture was transferred to a flask containing 50 ml YEB and grown at 28 °C for approximately 4 h until an OD_{600} of 0.5 was reached. Cells were collected by centrifugation at 5000 x g and 4 °C for 5 min and resuspended in 10 ml of ice cold 0.15 M NaCl solution. After another identical centrifugation step, cells were resuspended in 1 ml of ice cold 75 mM $CaCl_2$ solution, aliquoted, shock frozen in liquid nitrogen and stored at -80 °C.

2.2.4.10 Transformation of *A. tumefaciens* cells

For transformation according to Hofgen & Willmitzer (1988), 100 µl of competent *A. tumefaciens* cells were thawed on ice and 3 µg of plasmid DNA were added. After incubation for 5 min, the cells were shock frozen in liquid nitrogen for 5 min and immediately heat shocked for 5 min in a 37 °C water bath. After addition of 900 µl of YEB medium, cells were incubated at 28 °C for 4 h under constant shaking and plated on solid YEB medium (solidified with 1.5 % (w/v) agarose) containing 50 µg ml^{-1} rifampicin and 50 µg ml^{-1} kanamycin.

2.2.4.11 Arabidopsis transformation

Recombinant constructs were introduced into Arabidopsis plants through *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Agrobacteria were precultured over night (30 °C and shaking at 200 rpm) in 5 ml liquid YEB medium with 100 µg ml^{-1} carbenicillin or 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} rifampicin. Precultures were

used to inoculate 400 ml of the same medium and grown over night at identical conditions. Cells were collected by centrifugation at 10000 g for 20 min and resuspended in a total volume of 600 ml 5 % (w/v) sucrose and 0.025 % (v/v) Silwet-Copolymer. Arabidopsis flowers were dipped in the bacterial suspension and plants hooded over night. The procedure was repeated one week later. Positive transformants of T1 generation were identified by DsRed fluorescence using a fluorescence stereomicroscope (Leica MZ16 FA) and a Leica DFC480 camera. Fluorescence intensities were determined using the freely available software ImageJ (http://rsbweb.nih.gov/ij/).

2.2.4.12 Isolation of plasmid DNA from bacterial cultures

Plasmids were isolated from small scale (4 ml) liquid *E. coli* cultures using the NucleoSpin Plasmid Kit as recommended by the manufacturer. For purification of DNA from solutions or agarose gel pieces, the NucleoSpin Extract II Kit was used following manufacturer's recommendations.

2.2.4.13 Isolation of DNA from Arabidopsis leaves

DNA was extracted from Arabidopsis material as follows: One medium-sized leaf per sample was frozen in liquid nitrogen and pulverized in a 2 ml reaction tube containing three 3 mm glass beads using a type MM200 shaking mill. Material was kept frozen during the whole procedure until 500 µl of CTAB extraction solution (2 % (w/v) CTAB (Cetyltrimethylammoniumbromid), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl) were added, followed by immediate agitation. The samples were heated to 65 °C for 15 min and cooled before an equal volume of $CHCl_3$:isoamyl alcohol (24:1, v/v) was added. Samples were mixed followed by a centrifugation step of 20000 x g for 2 min. 400 µl of the upper (aqueous) phase were transferred to a new reaction tube containing 40 µl of an aqueous solution of 10 % (w/v) CTAB and 0.7 % (w/v) NaCl and mixed. After a 2-minute-incubation, 500 µl of isopropanol were added and the samples were mixed. To precipitate DNA, samples were centrifuged at 20000 x g for 15 min and the supernatant was carefully removed. After a washing step with 70 % ethanol, the pellets were dried briefly and dissolved over night at 4 °C in 100 µl of ddH_2O.

2.2.5 Cloning strategies

COI1 coding region was amplified using Arabidopsis cDNA and primers P5 and P6 (5'-gattccatggaggatcctgatatcaagaggtgtaaattg-3' and 5'-gattcccgggtcagtggtggtggtggtggtgtattggctcc

ttcaggactcta-3‘), and ligated into the pGEM-T Easy vector (Promega). QuickChange PCR was used to mutate the *COI1* sequence revealing $coi1_{mut}$ variants. $coi1_{K81A}$ was generated with primers P7 and P8 (5‘-ggtcgctcaaacttgcaggcaagcctagag-3‘ and 5‘-ctctaggcttgcctgcaagtttgag cgacc-3‘), $coi1_{R348A}$ with primers P9 and P10 (5‘-cagttgaagcggctggcgattgaacgcggtgc-3‘ and 5‘-gcaccgcgttcaatcgccagccgcttcaactg-3‘), $coi1_{R516A}$ with primers P11 and P12 (5‘-gccttcactggcatacttgtgggtacaagg-3‘ and 5‘-ccttgtacccacaagtatgccagtgaaggc-3‘), $coi1_{R121A}$ with primers P13 and P14 (5‘-cggtgcacttccgagcgatgattgtcagtg-3‘ and 5‘-cactgacaatcatcgctcggaagtgca ccg-3‘), $coi1_{R409A}$ with primers P15 and P16 (5‘-cctctgtgacttcgccttgtcttactcg-3‘ and 5‘-cgagtaagacaagggcgaagtcacagagg-3‘). Multiple exchange variants were successively generated with the same primer pairs. *COI1* and mutated *coi1* variant sequences were transferred to pCAMBIA vector (kindly provided by Dr. Ellen Hornung, Georg August University Göttingen, Germany) via *NcoI* and *XmaI* sites, integrated between a p35S Cauliflower mosaic virus (CaMV) promoter and a 35S polyA terminator.

DsRed sequence including cassava vein mosaic virus (CVMV) promoter and a terminator was amplified from pPHAS-DsRed (kindly provided by Dr. John Shanklin, Brookhaven Natl. Lab., Upton, NY, USA) using primers P17 and P18 (5‘-gatcaagcttggccgcaacagaggtggatg-3‘ and 5‘-gatcaagcttagaaggtaattatccaagat-3‘), and ligated into the pGEM-T Easy vector (Promega). For reasons of cloning strategy, two *NcoI* sites were destroyed within the DsRed sequence using primer pairs P19 and P20 (5‘-gaaagtggatcgaaacgatggcctcctccgag-3‘ and 5‘-ctcggaggaggccatcgtttcgatccactttc-3‘) for the first, and primers P21 and P22 (5‘-gatgcagaagaagacgatgggctgggaggcc-3‘ and 5‘-ggcctcccagcccatcgtctt cttctgcatc-3‘) for the second. The resulting sequence was introduced to the pCAMBIA vector via *HindIII* restriction sites. All final sequences were verified by 2 rounds of sequencing with consistent results. The pCAMBIA vector carrying the DsRed as well as the COI1 sequence was first introduced to *Agrobacterium tumefaciens*, which was then used for Arabidopsis transformation.

COI1 and $coi1_{mut}$ coding regions for yeast interaction tests were amplified from pCAMBIA constructs using primers P5 and P23 (5‘-gattccatggaggatcctgatatcaagaggtgtaaattg-3‘ and 5‘-gatcgtcgactcagtggtggtggtggtggtgtattggc-3‘) and ligated to the *NcoI* and *SalI* site of pGBKT7 to obtain in frame fusion proteins with GBD. *JAZ9* coding region was ligated to pDEST-GAD-HA (Dr. Barry Causier and Dr. Brandan Davies, University of Leeds, UK) via gateway cloning (Invitrogen) to obtain in frame fusion proteins with GAD, performed by Dr. Corinna Thurow and Anna Hermann (Georg August University, Göttingen, Germany)

For yeast mutagenesis, the cloNAT-MX4 cassette (kindly provided by Dr. Martin Fulda, Georg August University, Göttingen, Germany) was used as template for introduction of the selection marker to the flanking sequences. *IPK1* flanking sequences for knock-out primer design (P24 and P25, 5'-aaaattgtcagagataagttccttttttgaaaagaaagatcgatgcgtacgctgcaggtcgac-3' and 5'-gtgcatctgccagtaccaaaggtggaaagaaaagtatacagtttaatcgatgaattcgagctcg-3') were obtained from the yeast deletion web page (http://www-sequence.stanford.edu/group/yeast_deletion_project/downloads.html).

2.2.6 Determination of specific transcript levels

2.2.6.1 RNA extraction

Total RNA was extracted from 150 to 300 mg of rosette leaf material using the TRIZOL method (Chomczynski & Mackey, 1995). Frozen plant powder was thawed by adding 1 ml TRIZOL (38 % (v/v) Roti-Phenol, 0.8 M guanidinium thiocyanate, 0.4 M ammonium thiocynanate, 133.6 mM sodium acetate pH 5.0, 5 % (v/v) glycerol) and vigorously shaking. After incubation for 5 min at room temperature and centrifugation for 10 min at 4 °C and 20000 x g, the supernatant was transferred to a new tube and 200 µl of chloroform were added. Samples were shaken by hand for 15-20 sec, and after 2-3 min of incubation at room temperature centrifuged for 15 min at 4 °C and 20000 x g. The upper phase was transferred to a new tube and half a volume of isopropanol and half a volume of high salt precipitation buffer (0.8 M sodium citrate, 1.2 M NaCl) were added. Mixture by inverting was followed by 10 min incubation at room temperature. After spinning the samples for 10 min at 4 °C and 20000 x g, the supernatant was removed and the pellet was washed twice with 900 µl of 75 % ethanol (centrifugation for 5 min at 4 °C and 10000 x g). Pellet was dried briefly at room temperature and resuspended in 20 µl of RNAse-free ddH_2O by heating to 65 °C for 5 min. During the whole procedure, care was taken to exclusively use RNAse-free equipment. Precipitation buffer was treated with diethylpyrocarbonate (DEPC) in 0.1 % (v/v) concentration, stirred over night and autoclaved.

2.2.6.2 cDNA synthesis

Reverse transcription was performed using 1 µg of total RNA, 20 pmol of oligo(dT) (18 dT), and 200 pmol of random nonamer oligonucleotides. Water was added to a final reaction volume of 12.5 µl. The mixture was heated to 70 °C for 10 min, 20 nmol deoxynucleotide triphosphate, 4 µl 5 x reaction buffer, and 30 units of ribonuclease inhibitor were added (final volume 20 µl), and the mixture was incubated at 37 °C for 10 min. 100 U of RevertAid H Minus M-MuLV

reverse transcriptase were added to a final volume of 20 µl and the mixture was incubated at 42 °C for 70 min and then heated to 70 °C for 10 min. The resulting cDNA was diluted 1:10 and 1 µl was used as a PCR template in a 25 µl reaction for Real Time RT-PCR analysis. 1 µl of undiluted cDNA was used for semi-quantitative RT-PCR.

2.2.6.3 Real-time RT-PCR analysis

Real-time RT-PCR analysis was performed as described by Fode et al (2008) using the iCycler System (BioRad). The amplification mix contained 1 x NH_4 reaction buffer (Bioline), 2 mM $MgCl_2$, 100 mM deoxynucleotide triphosphate, 0.4 µM primers, 0.25 U BIOTaq DNA Polymerase (Bioline), 10 nM fluorescein (Bio-Rad), 100000 times diluted SYBR Green I solution (Cambrex), 1 µl of 1:10 diluted cDNA as template, and ddH_2O filled to a total volume of 25 µl. For the reaction, the following temperatur programm was used: 3 min of 95 °C, 40 cycles of 20 s at 95 °C, 20 s at 55 °C, 40 s at 72 °C. Quantitect Primers for *AOS* (At5g42650), *OPR1* (At1g76680), *VSP1* (At5g24780), *RNS1* (At2g02990), *T18K17.7* (At1g73260) and *WRKY70* (At3g56400) were obtained from Qiagen. Calculations were made according to the $2_T^{-\Delta C}$ method (Livak and Schmittgen, 2001). *Protein phosphatase type 2* (*PP2A*, At1g13320, Czechowski et al., 2005) served as internal reference when transcript levels of *InsP 5-ptase* plants were determined. *Ubiqutin5* (*UBI5*, At3g62250) served as internal reference (Kesarwani et al., 2007) when transcript levels of *ipk1-1* plants were determined.

2.2.6.4 Semi-quantitative RT-PCR

To determine *coi1* transcript levels in complemented *coi1-t* plants via semi-quantitative RT-PCR, 1 µl of cDNA was used as template in *TaKaRa Ex Taq*™ PCR reactions of 27, 30 or 34 cycles with primers P26 and P27 (5'-gtcgcgacggttgatgat-3' and 5'-ggtggtggtggtggtgtatt-3').

To determine *coi1* transcript levels in *coi1-t* plants via semi-quantitative RT-PCR, 1 µl of cDNA was used as template in *TaKaRa Ex Taq*™ PCR reactions of 32 cycles with the primers P28 and P29 (5'-gattatggaggatcctgatatcaagaggt-3' and 5'-gatttcatattggctccttcaggactcta-3').

2.2.7 Sequence alignment

Sequence alignments were performed using EXPASY Proteomics Server (www.expasy.org). Conserved residues were visualized using Swiss pdb Viewer, Deep View v 3.7 (Guex & Peitsch, 1997) http://www.expasy.org/spdbv/).

2.2.8 Targeted gene disruption in *S. cerevisiae*

For gene deletion in the yeast *Saccharomyces cerevisiae*, the PCR-based method described by Wach et al (1994) was used, exploiting the fact that yeast efficiently performs homologous recombination when exposed to linear DNA fragments. Linear fragment are supposed to contain 5'-upstream regions including start codon and 3'-downstream regions including stop codon of the gene of interest, embracing the resistance cassette. Using such fragments for yeast transformation, the entire open reading frame of the gene of interest will be deleted (Fig. 5). DNA fragments for *IPK1* deletion were designed as follows: *IPK1* flanking sequences for knock-out primer design were obtained from the yeast deletion web page (http://www-sequence.stanford.edu/group/yeast_deletion_project/downloads.html). Knock-out primers contained 18 bases complementary to the resistance cassette and 45 bases complementary to IPK1 flanking sequences of the yeast genome (5'-aaaattgtcagagataagttccttttttgaaaagaaagatcgatgcgtacgctgcaggtcgac-3' and 5'-gtgcatctgccagtaccaaaggtggaaagaaaagtatacagtttaatcgatgaattcgagctcg-3'). Using these primers together with plasmid DNA (pAG25) carrying the nourseothricin resistance cloNAT-MX4 cassette (kindly provided by Dr. Martin Fulda and Dr. Michael Scharnewski, Georg August University, Göttingen, Germany) as template, the yielded PCR product was purified (Nucleospin Extract II Kit) and utilized for yeast transformation resulting in an *ipk1* deficient yeast strain resistant to nourseothricin, named *ipk1Δ*.

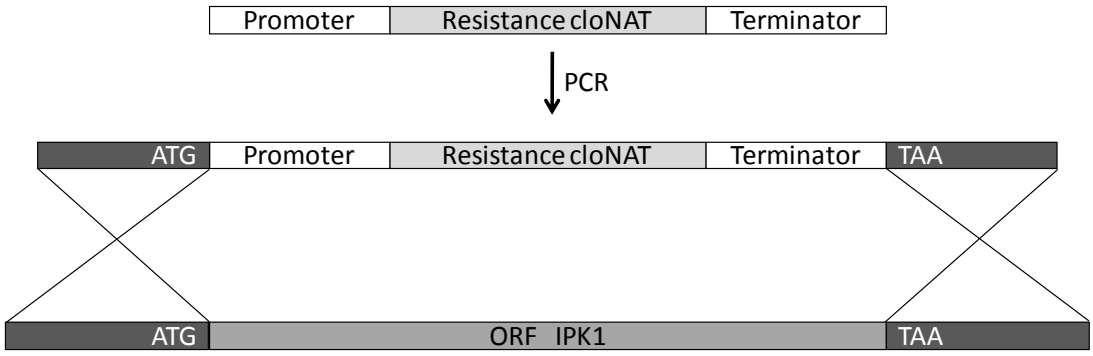

Fig. 5: Scheme of gene deletion in yeast via homologous recombination. A linear fragment containing the resistance cassette flanked by the 5'-upstream regions including start codon (ATG) and the 3'-downstream regions including stop codon (TAA) of the gene of interest is generated by PCR. By chromosomal intergration via homologous recombination the gene of interest is replaced and knocked out. Illustration according to Dr. Michael Scharnewski.

2.2.8.1 Transformation of *S. cerevisiae*

To obtain yeast cells competent for DNA uptake, an overnight culture (in liquid YPD media of 1 % yeast extract, 2 % peptone, 2 % glucose, 0.01 % adenine hemisulfate, shaking at 30 °C at 200 rpm) was diluted in 10 ml YPD to an OD_{600} of 0.3 and grown to an OD_{600} of 0.8 to 1.0. Cells were pelletized for 3 min at room temperature with 1500 x g and washed with sterile ddH_2O. The obtained cell pellet was resuspended in 400 µl of 0.1 M lithium acetate and divided into two 200 µl aliquots of competent cells.

For transformation, 25 µl of carrier DNA were preheated for 5 min to 65 °C and together with 50-80 µl of purified PCR product added to competent cells. ddH_2O was added to a control sample. 800 µl PEG solution (40 % PEG4000, 100 mM lithium acetate, 10 mM TRIS HCl, pH 7.5; sterile) were added, the samples were inverted and pivoted for 30 min at 30 °C. After heat shock of 42 °C for 25 min, cells were sedimented for 1 min at 4500 x g, resuspended in YPD and pivoted at 30 °C for 1-2 h. Subsequently, cells were centrifuged for 1 min at 1500 x g, resuspended in 200 µl of YPD and plated on nourseothricin containing YPD dishes, which were incubated at 30 °C for 3-4 days.

2.2.9 Yeast two-hybrid assay

The principle of a yeast two-hybrid test is based on the activity of a transcription factor, split into a DNA binding domain and an activation domain, which is only active upon interaction between two proteins of interest, each fused to one of the two domains. If the proteins of interest interact, the two domains are brought to close proximity and the transcription factor activates expression of a reporter gene. In this study, the Gal4 transcription factor, activating the *lacZ* gene which encodes for β-galactosidase, was used. Therefore, the JAZ9 sequence was fused to the Gal4 DNA binding domain (GBD), and the COI1 sequence was fused to the Gal4 activation domain (GAD). Upon JAZ9/COI1 interaction, β-galactosidase activity was followed by the conversion of o-nitrophenyl-β-D-galactopyranoside (ONPG), structurally similar to lactose, into galactose and o-nitrophenol, a yellow compound. β-galactosidase assays were performed in yeast strain PJ69-4A (James et al, 1996) as well as in the corresponding deletion strain *ipk1Δ*. The interaction of JAZ9 and COI1 was tested in the presence of 30 µM coronatin, which served as JA-Ile analogue. Measurement of β-galactosidase activity was performed as described by Niggeweg et al (2000).

Yeast two-hybrid tests were kindly conducted by Dr. Corinna Thurow and Anna Hermann (Georg August University, Göttingen, Germany).

2.2.10 $InsP_3$ determination

Plant material was ground with liquid nitrogen to a fine powder. $InsP_3$ levels were determined from ground powder using the $[^3H]InsP^3$ receptor binding assay system (GE Healthcare/Amersham, Düren, Germany), as previously described (Perera et al, 1999). 100-150 mg of frozen plant powder was thawed by adding 400 µl of 20 % perchloric acid and mixing vigorously. All following steps were conducted on ice. After incubation for 20 min on ice, samples were spun for 10 min at 4 °C and 20000 x g and the supernatant was transferred to a new tube. pH was adjusted to 6.5–7.5 using 1.5 M KOH/60 mM HEPES including pH indicator dye (0.5 ml Fisherdye/10 ml buffer). Following centrifugation for 10 min at 4 °C and 20000 x g, the supernatant was transferred to a new tube and the volume was determined for later calculations. $InsP_3$ determination was performed with 25 µl of binding protein and appropriate down scaled reaction volumes instead of 100 µl as recommended in the manual. All other processes were conducted according to the manual. Processed samples were incubated over night and measured in a scintillation counter (Liquid Scintillation Analyzer Tricarb 1900 TR, Canberra Packard, Dreieich, Germany).

2.2.11 Lipid analysis

Phosphoinositides were extracted from powdered plant material by using an acidic extraction protocol (Cho et al, 1992). 1.5 ml $CHCl_3$:CH_3OH (1:2), 250 µl 0.5 M EDTA, 500 µl 2.4 M HCl and 500 µl of chloroform were added to frozen plant powder, the samples were mixed after addition of each compound and subsequently shaken for 3-4 h at 4 °C. The lower organic phase was collected and the upper aqueous phase reextracted twice with 1 ml of chloroform. The combined organic phases were washed with 1.5 ml 0.5 M HCl in CH_3OH:H_2O (1:1), dried under streaming nitrogen and solubilised in 500 µl of chloroform. The samples were separated by thin layer chromatography (TLC) using silica S60 plates (Merck, Darmstadt, Germany) and $CHCl_3$:CH_3OH:NH_4OH:H_2O (57:50:4:11 v/v/v/v) as a developing solvent for separation of PtdIns4P, $PtdInsP_2$ and PtdOH (Perera et al, 2005), and methyl acetate:isopropanol:$CHCl_3$:CH_3OH:0.25 % aqueous KCl solution (25:25:25:10:9 v/v/v/v/v) as a developing solvent for separation of PtdIns (Christie, 2003). Lipids were identified by comigration of authentic standards (5 µg; Avanti Polar Lipids, Alabaster, AL, USA). Lanes with lipid standards that were run next to the plant samples were cut and visualized by the application of aqueous 10 % (w/w) $CuSO_4$ containing 8 % (w/v) H_3PO_4 followed by heating to 180 °C (König et al, 2007). Lipid bands on the unstained remaining part of the TLC plates were

scraped according to the comigrated standards, and quantified via their fatty acid content, which was determined by gas chromatography (GC) analysis.

Therefore, the lipids were re-dissolved from the scraped powder to their respective developing solvents, dried under a stream of nitrogen and transmethylated according to Hornung et al (2002). 333 µl MeOH/toluol (2:1) and 167 µl 0.5 M sodium methoxide were added to the dried lipid coat with sodium methoxide acting as nucleophile, attacking the ester bound between fatty acid and lipid backbone. Furthermore, 5 µg of tripentadecanoin as internal fatty acid standard were added to each sample and co-transmethylated. The mixture was shaken for 30 min at room temperature and afterwards stopped by adding 0.5 ml 5 M NaCl and 50 µl of 32 % HCl. Obtained fatty acid methyl esters were extracted with 2 ml hexane, and the hexane phase was washed twice with 2 ml ddH_2O and dried under a stream of nitrogen. The remaining lipid coat was resuspended in 100 µl acetonitrile and transferred to plastic inlays of GC vials, in which the volume was narrowed down to 10 µl. Samples were overlayered with argon and stored at -20 °C until GC analysis.

Compound separation via GC is based on the molecules' different chemical characteristics, resulting in different binding properties to the GC column, which in this case contained polyimide covered silica glass as stationary phase. A continuous temperature gradient applied to the GC column changes the binding conditions and results in sequential release of polar substances in the first place and nonpolar substances afterwards. For detection, molecules are thermically ionized resulting in an electric signal.

GC analysis was performed using a GC6890 gas chromatograph with flame ionization detection (Agilent, Waldbronn, Germany) fitted with a DB-23 capillary column (30 m x 250 µm, 0.25 µm coating thickness; J&W, Agilent, Waldbronn, Germany). Helium flowed as a carrier gas at 1 ml min^{-1}. Samples were injected at 220 °C. The temperature gradient was 150 °C for 1 min, 150-200 °C at 8 °C min^{-1}, 200-250 °C at 25 °C min^{-1} and 250 °C for 6 min (König et al, 2007). Fatty acids were identified according to authentic standards, and quantified according to internal tripentadecanoic acid standards of known concentration using GC ChemStation software (Agilent, Waldbronn, Germany).

2.2.12 Phytohormone analysis

2.2.12.1 Phytohormone analysis via GC/MS

Leaf material of *InsP 5-ptase* and *dde2-2* plants and corresponding wild type was extracted as described by Schmelz et al (2004) with some modifications. Frozen ground plant material (50 mg) was mixed with 1 ml diisopropylamine containing 100 ng of D6-JA, 100 ng of D6-oPDA

(kindly provided by Dr. Otto Miersch, Martin Luther University, Halle, Germany), and 50 ng of D5-SA (Icon Genetics) as internal standards. The mixture was sonified for 15 min, 1 ml of chloroform was added followed by additional sonification for 15 min. For detection, compounds were converted to their pentafluorobenzyl esters according to Mueller & Brodschelm (1994) by adding 17 mg pentafluorbenzylbromide and incubating for 1 h at 60 °C. After evaporation under streaming nitrogen, residues were dissolved in 1 ml diethyl ether and filtrated through filter paper. For complete recovery of the pentafluorobenzyl esters, the sample tube was washed with 1 ml of hexane, which was also filtered and combined with the diethyl ether filtrate. The filtered solution was evaporated under a stream of nitrogen. Vapor phase extraction was carried out at 270 °C for 5 min, with argon as a carrier gas. Vaporized substances absorbed by the SuperQ column (100 x 4.6 mm SDB-L Strata, pore size 260 A°; Phenomenex) were eluted from the SuperQ columns subsequently with 3 ml hexane and 3 ml ethyl acetate. The solution was concentrated with a rotating evaporator. The remainder was dissolved in 40 µl dichlormethane and subjected to GC coupled to mass spectrometry (GC/MS; see section 2.2.12.1.4).

2.2.12.1.1 HIP extraction method

For phytohormone analysis of *opr3* plants and corresponding wild type, 0.5 g of frozen plant powder were homogenized in 10 ml of hexane/isopropanol (HIP) solution (hexane:isopropanol (3:2), 0.0025 % 2-butyl-6-hydroxytoluen) and 300 µl glacial acetic acid together with 100 ng of D6-JA, 100 ng of D6-oPDA (kindly provided by Dr. Otto Miersch, Martin Luther University, Halle, Germany), and 50 ng of D5-SA (Icon Genetics) as internal standards, using an Ultra Turrax (13000 rpm, Ika, Staufen, Germany) under argon atmosphere for 45 s. The extract was shaken at 4 °C for 10 min and centrifuged for 10 min at 4 °C and 450 x g. The supernatant was added to 8.1 ml of 0.38 M potassium sulphate solution, shaken for 10 min at 4 °C and centrifuged for 10 min at 4 °C and 450 x g. The upper hexane-rich phase was dried under streaming nitrogen, dissolved in 200 µl MeOH and separated by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC).

2.2.12.1.2 RP-HPLC

This HPLC technique allows the separation of compound mixtures with high efficiency, forcing analytes through a stationary phase (column) by the liquid mobile phase under high pressure. Separation using RP-HPLC depends on the hydrophobic binding interactions between the dissolved compounds in the polar mobile phase and the nonpolar stationary phase. Interacting

compounds elute from the column based on their polarity and the applied gradient, resulting in separation according to individual hydrophobicities. Eluted compounds are detected by recording their UV spectra.

Here, RP-HPLC was employed for the separation of phytohormones depending on their carbon chain length and their functional groups. An Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to a diode array detector, equipped with a nonpolar C_{18} column (EC 250/2 Nucleosil 120-5 C_{18}, 250 x 2.1 mm, 5 µm particle size; Macherey & Nagel, Düren, Germany) was used, applying a binary gradient of solvent A (CH_3OH:H_2O:acetate, 75:25:0.1, v/v/v) and solvent B (CH_3OH:acetate, 100:0.1, v/v) with the following gradient parameter: 80 % solvent A, 20 % solvent B for 10 min, followed by a linear increase of solvent B to 100 % within 5 min. The flow rate increased at this point from 0.18 to 0.36 ml/min. After a run of 100 % solvent B for 10 min, the gradient changed within 2 min to 80 % solvent A and 20 % solvent B and ran with this properties for another 3 min. The absorbance at 224 nm was recorded, and the fraction from starting at 3 min till behind the oPDA peak was collected (Fammartino et al, 2007). Prior to injection, the lipid extract was dissolved in 80 µl of solvent A.

Separated phytohormones were converted to their pentafluorobenzyl esters for GC/MS analysis to allow for better ionization.

2.2.12.1.3 Derivatisation to pentafluoroesters

RP-HPLC fractions were dried under streaming nitrogen and dissolved in 200 µl of $CHCl_3$:diisopropylamin (1:1) and 10 µl of pentafluorbenzylbromide. Samples were heated to 50 °C in a water bath for 1 h and subsequently dried under a stream of nitrogen. Bakerbond spe™ Silica Gel columns (J.T. Baker, Deventer, Netherlands) were pre-rinsed twice with 2.5 ml of hexane. Afterward the dried samples were loaded onto the column, solved in 5 ml of hexane, and eluted by rinsing the column with 7 ml of hexane:diethylether (2:1). Eluate was dried under streaming nitrogen, dissolved in 10 µl acetonitrile and subjected to GC/MS analysis.

2.2.12.1.4 GC/MS analysis

The combination of GC and MS enables the separation of compound mixtures according to their chemical characteristics, resulting in different binding properties to the GC column, and subsequent characterization and quantification of individual molecules by MS, converting each molecule into its ionized form due to electron impact (EI) ionization or chemical ionization (CI) and detecting these ions using their mass to charge ratio (*m/z*). Transferred to the MS, the

molecules get ionized either directly by electrons emitted from a thermionic cathode (EI) or by electrons emitted from a thermionic cathode ionized ammonia gas, which detaches a proton from the sample molecule, resulting in an anion (CI). Using CI, the energy transfer to the molecule of interest is lower; therefore the molecules remain intact allowing for analysis of whole molecules. This technique is well suitable for quantification of known substances and was used in this study in GC-coupled MS analysis. Ionized molecules are subsequently send through an electromagnetic field, in this case the ion trap, and separated via their (m/z) ratio. Due to an electric current, generated by the ions, the molecules are detected.

GC/MS analysis was carried out using a ThermoFinnigan (Austin, Texas, USA) Polaris Q mass selective detector connected to ThermoFinnigan Trace gas chromatograph equipped with a capillary Rtx-5MS column (15 m x 0.25 mm, 0.25 µm coating thickness; Resteck, Bad Homburg, Germany). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The temperature gradient was 100 °C for 1 min, 100–300 °C at 8 °C min^{-1} and 300 °C for 5 min. The phytohormone derivates were detected by negative chemical ionization, with ammonia as ionization gas. For quantification, the diagnostic ions m/z 215 (D6-JA; Rf = 14.09, 14.45 min), 209 (JA; Rf = 14.15, 14.52 min), 141 (D5-SA; Rf = 9.16 min), and 137 (SA; Rf = 9.20 min), 296 (D_5-oPDA, R_f = 20.79, 21.17, 21.51), 291 (oPDA, R_f = 20.81, 21.21, 21.57), 263 (dn-oPDA, R_f = 18.94, 19.36, 19.70), 325 (D_3-JA-Leu, R_f = 20.87) and 322 (JA-Ile, R_f =21.06) were used. Peaks were smoothened and data were assessed using Xcalibur software (ThermoFinnigan, Austin, Texas, USA).

2.2.12.2 Phytohormone analysis via LC/MS

Extraction of *coi1-1*, *coi1-t* and corresponding wild type material for LC/MS-based analysis (RP-HPLC coupled to MS) of phytohormones was conducted according to Matyash et al (2008) as follows: 200 mg of frozen plant powder were thawed in 0.75 ml of methanol containing 10 ng D_6-SA (CDN Isotopes, Quebec, Canada), 10 ng D_6-JA, 30 ng D_5-oPDA, 10 ng D_4-jasmonic acid-leucine (D_4-JA-Leu) (all three kindly provided by Dr. Otto Miersch, Martin Luther University, Halle, Germany), 10 ng D_5-IAA (Eurisotop, Freising, Germany) each as internal standard. After vigorous mixing, 2.5 ml of methyl-*tert*-butyl ether (MTBE) were added and the extract was shaken for 1 h at room temperature. For phase separation, 0.625 ml water was added. The mixture was incubated for 10 min at room temperature and centrifuged for 15 min with 450 x g. The upper phase was collected and the lower phase was reextracted with 0.7 ml methanol and 1.3 ml MTBE as described above. The combined upper phases were dried under

streaming nitrogen and resuspended in 100 µl of acetonitrile/ddH_2O/acetic acid (20:80:0.1, v/v/v).

Phytohormone analysis via LC/MS, allows the separation followed by identification and quantification of molecules, similar to GC/MS analysis, with the difference of separation in gaseous phase via GC/MS and separation in liquid phase via LC/MS.

In contrast to MS used in GC-coupled MS analysis, the HPLC-separated compounds using LC/MS exist already as ions in the liquid surrounding of HPLC. Atmospheric pressure ionisation technique is used to eliminate the solvent and to generate ions in the gaseous phase (Agilent Technologies, 2001). Electrospray ionisation (Fenn et al, 1989) generates different precursor ions, so-called parent ions, that may form different adducts like protonated ions ($[M+H]^+$), deprotonated ions ($[M-H]^-$) and ammonium adducts ($[M+NH_4]^+$), sodium adducts ($[M+Na]^+$), chloride adducts ($[M+Cl]^-$) and acetate adducts ($[M+CH_3COO]^-$), depending on the solvent used. These parent ions are selected using either positive or negative ionization mode.

2.2.12.2.1 HPLC/ QLIT-MS analysis

Quadrupole-linear ion trap (QLIT)-MS consists of a quadrupole and a linear ion trap separated by a collision cell. Therefore, the analyte ions move from the source to the detector passing through first quadrupole, second collision cell and later ion trap, where they can be analyzed by different types of MS/MS experiments (Agilent Technologies, 2001). The quadrupole is employed as a mass filter and composed of four metallic rods arranged in a square. The two opposite rods are set at positive potential and the other two opposite rods at negative potential. The analyte ions generated in the source are directed to the centre of the four rods, where a combination of direct voltage and radio frequency controls the trajectory of the analyte ions through the quadrupole and just ions with certain values of m/z are able to pass through the quadrupole for detection, while all other ions are thrown out of the original path. The passed ions are fragmented in the collision cell by collision-induced dissociation using argon or helium as a collision gas to produce the particular fragments, which are then separated by a linear ion trap and detected due to the electric current they cause.

An Agilent 1100 HPLC system (Agilent, Waldbronn, Germany) coupled to an Applied Biosystems 3200 hybrid triple quadrupole/linear ion trap mass spectrometer (MDS Sciex, Ontario, Canada) was used. Nanoelectrospray (nanoESI) analysis was achieved using a chip ion source (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA). RP-HPLC separation was performed on an EC 50/2 Nucleodure C_{18} gravity 1.8 µm column (50 x 2.1 mm, 1.8 µm particle size; Macherey and Nagel, Düren, Germany). The binary gradient system consisted of solvent A, acetonitrile/water/acetic acid (20:80:0.1, v/v/v) and solvent B, acetonitrile/acetic

acid (100:0.1, v/v) with the following gradient program: 10 % solvent B for 2 min, followed by a linear increase of solvent B up to 90 % within 6 min and an isocratic run at 90 % solvent B for 2 min. The flow rate was 0.3 ml min^{-1}. For stable nanoESI, 50 µl min^{-1} of isopropanol/acetonitrile/water/formic acid (70:20:10:0.1, v/v/v/v) delivered by a 2150 HPLC pump (LKB, Bromma, Sweden) were added just after the column via a mixing tee valve. By using another post column splitter 740 nl min^{-1} of the eluent were directed to the nanoESI chip. Ionization voltage was set to -1.7 kV. Phytohormones were ionized in a negative mode and determined in multiple reaction monitoring mode. Mass transitions were as follows: 141/97 [declustering potential (DP) -45 V, entrance potential (EP) -7 V, collision energy (CE) -22 V] for D_6-SA, 137/93 (DP -45 V, EP -7 V, CE -22 V) for SA, 215/59 (DP -45 V, EP -9.5 V, CE -22 V) for D_6-JA, 209/59 (DP -45 V, EP -9.5 V, CE -22 V) for JA, 296/170 (DP -70 V, EP -8.5 V, CE -28 V) for D_5-oPDA, 291/165 (DP -70 V, EP -8.5 V, CE -28 V) for oPDA, 263/59 (DP -70 V, EP -8.5 V, CE -28 V) for dinor-oPDA, 325/133 (DP -80 V, EP -4 V, CE -30 V) for D_4-JA-Leu and 322/130 (DP -80 V, EP -4 V, CE -30 V) for JA-Ile. The mass analyzers were adjusted to a resolution of 0.7 amu full width at half-height. The ion source temperature was 40 °C, and the curtain gas was set at 10 (given in arbitrary units). The data were smoothed and peak areas were integrated using Applied Biosystems Analyst software. The quantification was carried out by comparison to internal standards using a calibration curve (unlabeled/deuterium-labeled) *vs.* molar amounts of unlabeled (0.3-1000 pmol).

3 RESULTS

In previous experiments, it had been shown that both JA and $InsP_3$ signals occur in parallel during the Arabidopsis wound response. *dde2-2* mutants, deficient in JA biosynthesis, were not able to accumulate $InsP_3$ upon wounding, indicating JA as an upstream factor of $InsP_3$. It was the aim of this study to elucidate the impact of PI signals during the Arabidopsis wound response and defence reactions, and to further characterize the relation and dependency between JA and PI signalling.

3.1 JA acts upstream of PI signals

3.1.1 Sorbitol treatment induces $InsP_3$ formation

To test whether JA treatment is sufficient to induce $InsP_3$ signals, whole Arabidopsis rosettes were floated on a 50 µM MeJA solution for different time spans up to 4 h and $InsP_3$ levels were determined. Control plants were floated on water. As depicted in Fig. 6 A, an increase of $InsP_3$ levels was neither detectable upon floatation on H_2O nor after MeJA application. Since technical problems of MeJA application, preventing MeJA from reaching its target within the plant leaves, cannot be excluded, endogenous JA release was triggered by exposing hydroponically cultured plants to 0.8 M sorbitol (Fig. 6 B; Reinbothe et al, 1994; Stenzel et al, 2003b; Weichert et al, 2000). While water treated plants displayed $InsP_3$ levels of 3.6 ± 1.9 to 7.8 ± 4.0 nmol g^{-1} fresh weight, $InsP_3$ levels in sorbitol treated plants increased to 39.6 ± 7.9 nmol g^{-1} fresh weight already 15 min after application and found their maximum 30 min after application with 49.3 ± 24.8 nmol g^{-1} fresh weight. After one hour of treatment, $InsP_3$ levels declined to 34.8 ± 16.0 nmol g^{-1} fresh weight and still strongly exceeded water treated controls, in which only amounts of 4.5 ± 3.4 nmol $InsP_3$ g^{-1} fresh weight were detectable. While exogenous application of MeJA did not induce substantial $InsP_3$ formation, sorbitol treatment caused strong $InsP_3$ accumulation even exceeding that previously observed with 1 h of wound treatment, which had resulted in $InsP_3$ amounts of 19.6 ± 6.4 nmol g^{-1} (Mosblech et al, 2008).

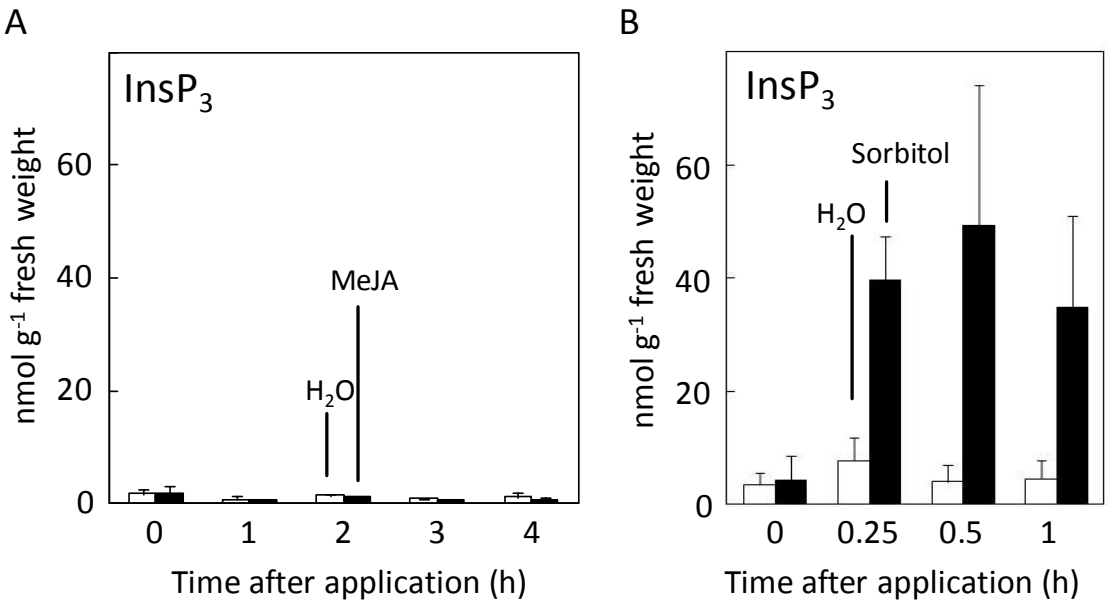

Fig. 6: $InsP_3$ induction with application of MeJA or sorbitol. (A) 5-week-old plants were floated on a 50 µM MeJA solution (black bars) and harvested after times indicated. Controls were floated on water (white bars). (B) Hydroponically grown plants were treated by addition of sorbitol to the nutrient solvent (black bars). Controls were treated with water (white bars). Data are the means of two independent experiments, each assayed in duplicates, ± SD.

3.2 PI signals are required for full induction of wound-inducible genes

It was shown in experiments performed during my diploma thesis that PI signals are involved in the Arabidopsis wound response, indicated by the accumulation of $InsP_3$. To investigate the impact of PI signals, *InsP 5-ptase* plants with global reduction of PI signalling components (König et al, 2007; Perera et al, 2006) were now wounded and analysed for downstream responses.

A number of wound-inducible genes was selected for analysis of their specific transcript levels by real-time RT-PCR (Fig. 7): *AOS* (Park et al, 2002) and *VSP1*, encoding the vegetative storage protein1 (Benedetti et al, 1995; Liu et al, 2005), both require JA for wound induction. In contrast, *OPR1* and *RNS1*, encoding oPDA reductase and ribonuclease1, respectively, are both induced by wounding independently of JA (Reymond et al, 2000). Also investigated were the transcription factor *WRKY70* (Li et al, 2006) and the Kunitz-family (Bauw et al, 2006) protease inhibitor *T18K17.7* encoded by the gene locus At1g73260.

Real-time RT-PCR analysis of wild type plants confirmed that transcript levels for *AOS*, *OPR1*, *RNS1*, *VSP1*, *WRKY70*, and *T18K17.7* were all wound-inducible (Fig. 7, white bars). In *InsP 5-ptase* plants, wound induction of the genes tested was either transiently or overall reduced

(Fig. 7, black bars) in comparison to that observed in wild type plants. Altered gene expression patterns in *InsP 5-ptase* plants included attenuated wound induction early after wounding (*AOS*), delayed wound induction (*OPR1*), or attenuated wound induction at later time points (*RNS1*, *WRKY70*, *T18K17.7*).

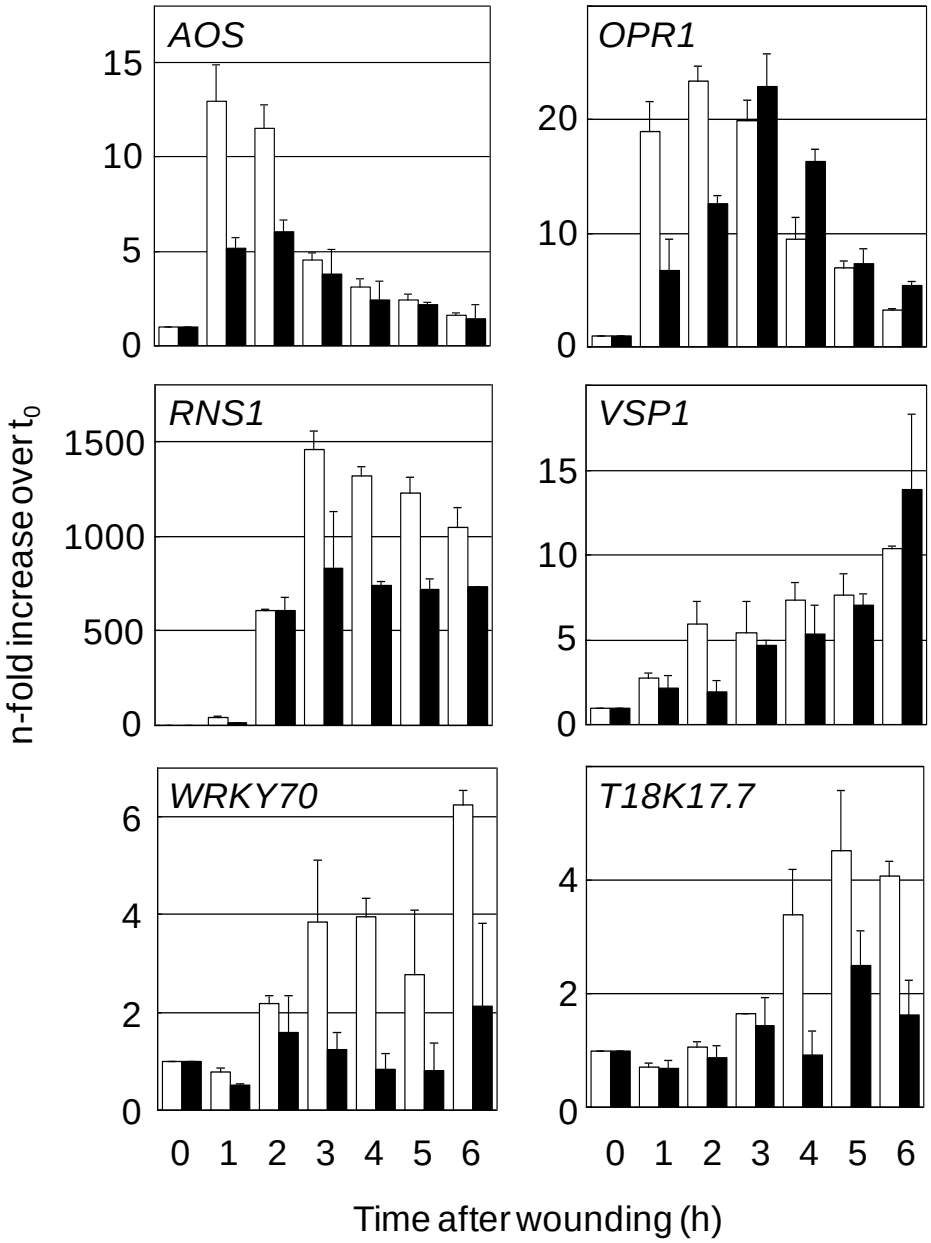

Fig. 7: Reduced induction of wound-inducible genes in *InsP 5-ptase* plants. Wild type (white bars) and *InsP 5-ptase* (black bars) plants were mechanically wounded and transcript levels were determined vie real-time RT-PCR over 6 h at time points indicated. Data are the mean of two biological replicates and given as the fold increase over transcript levels in non-wounded control plants, ± SD. *AOS*, allene oxide synthase; *OPR1*, OPDA reductase; *RNS1*, ribonuclease 1; *VSP1*, vegetative storage protein1; *WRKY70*, WRKY-family transcription factor; *T18K17.7*, Kunitz-family trypsin and protease inhibitor.

3.3 PI signals are required for functional defence against caterpillars

In addition to changes in transcript levels of wound-inducible genes, active defence of Arabidopsis plants against insect herbivory was assessed by monitoring caterpillar growth performance during feeding on wild type plants, *InsP 5-ptase* plants, or *dde2-2* plants (Fig. 8). *P. xylostella* caterpillars of approximately equal developmental stages and weight were placed on leaves of 6-week-old plants and allowed to feed. The increase in caterpillar weight was then monitored over several days. Fig. 8 illustrates the relative caterpillar weight increase over 56 h; absolute values are summarized in Table 1. Caterpillars feeding on *InsP 5-ptase* plants or on *dde2-2* plants exhibited significantly increased mean growth rates of 53 ± 8 µg h^{-1} and 68 ± 38 µg h^{-1}, respectively, and a weight increase to 328 % and 334 % of initial weight,

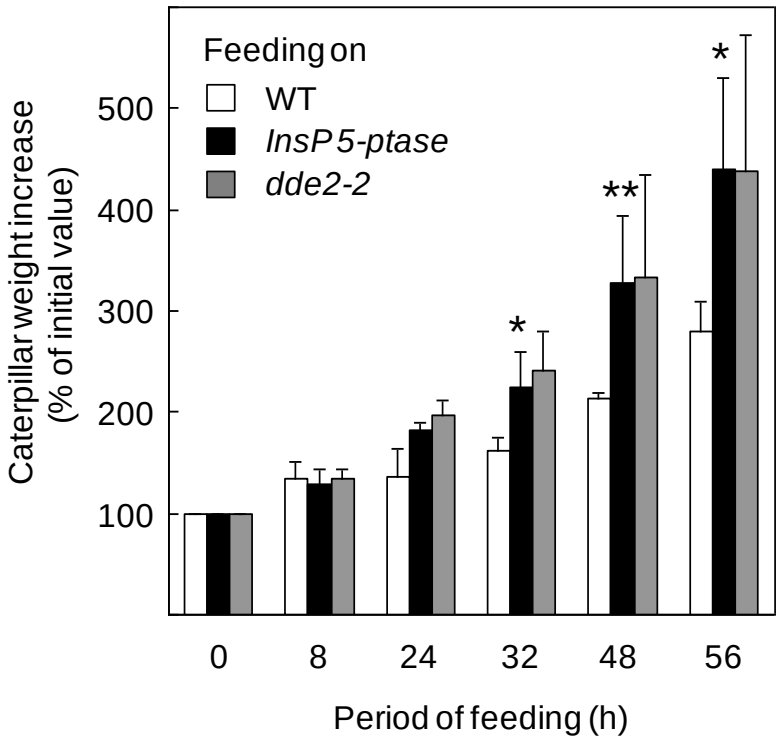

Fig. 8: Caterpillar growth performance with feeding on wild type, *InsP 5-ptase* or *dde2-2* plants. 10-12 diamondback moth caterpillars (*Plutella xylostella*) of initially ~ 1 mg were allowed to feed on wild type plants (white bars), on *InsP 5-ptase* plants (black bars) or on *dde2-2* plants (grey bars) for time periods indicated. Caterpillar weight was determined and they were allowed to continue feeding afterwards. Data were normalized against caterpillar weights at time zero and are given as percent increase over the time zero value. Data are presented from three independent biological experiments, ± SD, with 30-36 caterpillars per experiment and plant line. Asterisks indicate significant increases in caterpillar growth with feeding on transgenic plants compared with feeding on wild type plants, according to a student's *t*-test (*, p , 0.05; **, p , 0.01).

Table 1. Weight increase of *Plutella xylostella* caterpillars feeding on different Arabidopsis lines. Ten to twelve caterpillars of initially ~1 mg were allowed to feed on wild type*, InsP 5-ptase*, *or dde2-2* plants for various time periods. Caterpillar weight was determined at the times indicated, and caterpillars were allowed to continue feeding. Data are the mean of three independent biological experiments, each conducted with 30-36 caterpillars per plant line. Numbers represent mean weight per caterpillar in mg ± SD. Rates of weight increase calculated from caterpillar net weight increases are given as µg h^{-1}.

Period of feeding	0	8 h	24 h	32 h	48 h	56 h	Rate of increase [µg h^{-1}]
wild type	0.95 ± 0.13	1.30 ± 0.35	1.26 ± 0.16	1.53 ± 0.18	2.03 ± 0.35	2.69 ± 0.67	31 ± 10
InsP 5-ptase	0.96 ± 0.26	1.25 ± 0.39	1.76 ± 0.53	1.97 ± 0.49	2.98 ± 0.33	3.95 ± 0.37	53 ± 8
dde2-2	1.30 ± 0.42	1.76 ± 0.62	2.60 ± 1.00	3.24 ± 1.27	4.48 ± 1.99	5.09 ± 2.40	68 ± 38

respectively, within 48 h. In comparison, caterpillars feeding on wild type plants reached only 213 % of initial weight during this time span, which corresponds to a growth rate of only 31 ± 10 µg h^{-1}. Caterpillar weight increase with feeding on *InsP 5-ptase* plants was, thus, similar to that observed on *dde2-2* plants, and both these data sets differed significantly from that observed with feeding on wild type plants (Fig. 8).

3.4 Oxylipins accumulate in *InsP 5-ptase* plants

The first obvious difference between caterpillars feeding on wild type plants and feeding on *InsP 5-ptase* plants was observed after 24 h of feeding (Fig. 8). To investigate how oxylipins were affected in *InsP 5-ptase* plants during this extended period, JA precursors, JA and JA-Ile were determined by GC/MS, covering the time span of 24 h after wounding (Fig. 9).

All oxylipins measured displayed stronger wound-induced accumulation in *InsP 5-ptase* plants compared to wild type controls. oPDA levels of *InsP 5-ptase* plants exceeded those from wild type plants on average by about one third up to 2.5-fold, while dn-oPDA levels of *InsP 5-ptase* plants exceeded wild type levels much stronger by about two thirds till 6-7-fold. For both compounds, the oxylipin levels of wild type plants displayed a pronounced increase 4 h after wounding from 0.17 ± 0.08 to 3.63 ± 1.06 in oPDA and from 0.03 ± 0.01 to 1.44 ± 0.97 nmol g^{-1}

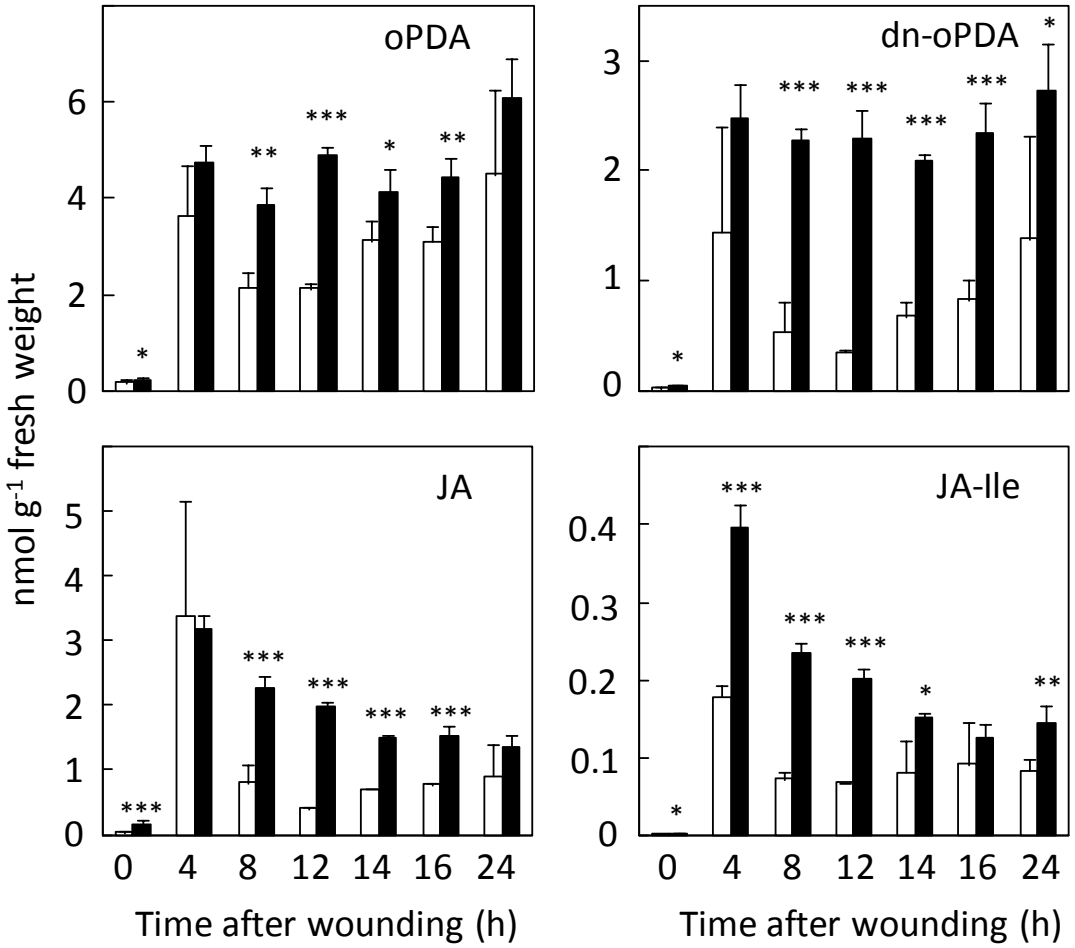

Fig. 9: Wound-induced oxylipins in *InsP 5-ptase* plants, impaired in PI signalling. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after the indicated times. Oxylipins were determined via GC/MS in wild type plants (white columns) and *InsP 5-pta*se (black columns) over 24 h. The mean values of two independent wounding experiments are shown, each assayed in duplicates, and each duplicate measured twice, ± SD. Missing error bars are too small to appear. Asterisks indicate significant differences between level detected in wild type and in *InsP 5-ptase* plants at the respective time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

fresh weight in dn-oPDA, which declined until 12 h after wounding to 2.13 ± 0.09 nmol g^{-1} fresh weight of oPDA and to 0.35 ± 0.02 nmol g^{-1} fresh weight of dn-oPDA. Afterwards, the levels rose again to reach their maximum of 4.50 ± 1.75 nmol g^{-1} fresh weight of oPDA and 1.38 ± 0.93 nmol g^{-1} fresh weight of dn-oPDA 24 h after wounding. In contrast, *InsP 5-ptase* plants displayed a rather constant pattern after wound induction of in average 4.69 ± 0.71 of oPDA and 2.37 ± 0.20 nmol g^{-1} fresh weight of dn-oPDA (Fig. 9).

As reported before in my diploma thesis, JA levels were similar between wild type and *InsP 5-ptase* plants at early time points also in the experiments of this thesis (here: 4 h after wounding, 3.39 ± 0.02 nmol g^{-1} fresh weight in wild type, 3.18 ± 0.22 nmol g^{-1} fresh weight in

InsP 5-ptase). By 8 h after wounding, wild type JA levels dropped by about three quarters to 0.81 ± 0.27 nmol g^{-1} fresh weight, whereas JA levels in *InsP 5-ptase* plants only dropped by less than one third to 2.26 ± 0.20 nmol g^{-1} fresh weight, thereby exceeding wild type levels for all later time points monitored (Fig. 9). Wound-induced JA-Ile levels increased in *InsP 5-ptase* plants over wild type levels by about one third to 3.5 times, with the strongest differences in early time points (Fig. 9). 4 h after wounding, wild type JA-Ile levels were 0.18 ± 0.02 nmol g^{-1} fresh weight, while *InsP 5-ptase* plants exhibited JA-Ile levels of 0.40 ± 0.03 nmol g^{-1} fresh weight. JA and JA precursors showed only minor differences to wild type levels in *InsP 5-ptase* plants during 6 h of wound stimulus (Mosblech et al, 2008), in contrast the levels of these oxylipins and importantly the bioactive form JA-Ile substantially exceeded wild time levels in the time period of 24 h after wounding.

The data obtained from *InsP 5-ptase* plants indicate a combination of lowered wound-induced gene expression and reduced defence against caterpillars accompanied by increased accumulation of JA-Ile over wild type levels. This pattern suggests partial JA insensitivity of the *InsP 5-ptase* plants. To test this concept, control experiments were conducted with *coi1* mutants, which represent established JA-insensitive plants.

3.5 A new T-DNA insertion line, *coi1-t*

The commonly used EMS line *coi1-1* (Feys et al, 1994) carries a point mutation within the last exon of the *COI1* gene, resulting in a premature stop codon. *coi1-1* mutants are male sterile and are propagated heterozygously. Homozygous, heterozygous and wild type individuals can be distinguished by phenotypes manifesting when grown on media containing MeJA. Whereas wild type plants respond to MeJA treatment with severely reduced growth in both roots and green parts (Feys et al, 1994; Xie et al, 1998), individuals homozygous for *coi1* display normal growth and heterozygous plants exhibit an intermediate phenotype under these conditions. Since the point mutation of *coi1-1* mutants is difficult to genotype, an Arabidopsis T-DNA insertion line was established and named *coi1-t* (Fig. 10). This line carries a T-DNA insertion in the first exon of the *coi1* gene at base pair 67 (Fig. 10 A). RT-PCR analysis revealed no detectable *coi1* transcript in these plants (Fig. 10 B).

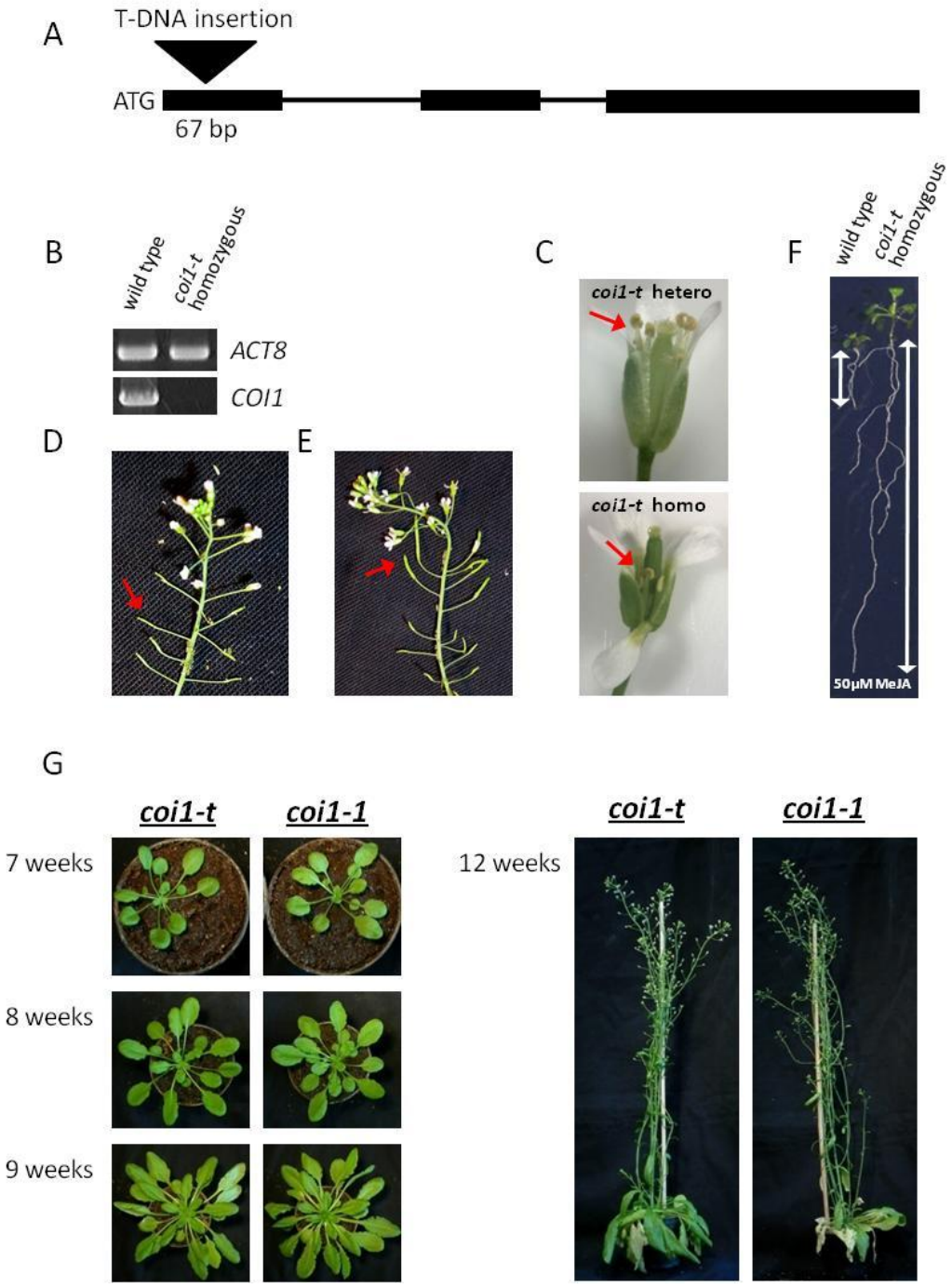

Fig. 10: *coi1-t*, a T-DNA insertion line lacking the JA receptor COI1. (A) COI1 gene structure with location of T-DNA insertion at base pair (bp) 67 within the first exon. The exact location of the insertion was determined by sequencing of the genomic locus. (B) *COI1* transcript is present in wild type but absent in *coi1-t* plants. Transcript levels were determined by performing PCR with specific primers using cDNA as template. Control PCR was performed with specific primers for actin (*ACT8*). (C) Flower phenotype of *coi1-t* stamina. Stamina of heterozygous *coi1-t* plants (*coi1-t* hetero) exhibiting normal length and stamina of homozygous *coi1-t* plants (*coi1-t* homo) exhibiting reduced length are highlighted by red arrows. (D + E) Sterility of *coi1-t* is restricted to male part. (D) *coi1-t* plants do not produce offspring, as their siliques do not carry seeds, see red arrow. (E) *coi1-t* plants produce offspring when pollinated with wild type pollen, see red arrow. (F) Impaired JA sensitivity of *coi1-t* when growing on agar plates containing 50 µM MeJA. 4-week-old wild type seedlings exhibit reduced root growth (left side), while *coi1-t* seedlings exhibit normal root length (right side), see white arrow. (G) *coi1-t* plants (left side) exhibit similar growth compared to the established *coi1-1* line (right side).

To ensure that *coi1-t* plants can be used as a tool equivalent to the *coi1-1* mutant described in the literature, JA-related phenotypes of *coi1-t* were first characterized. As described for *coi1-1* (Feys et al, 1994; Xie et al, 1998), *coi1-t* plants displayed male sterility, indicated by shortened stamina as depicted in Fig. 10 C, and abolished silique development, as depicted in Fig. 10 D. Female fertility was not affected, as flowers pollinated with wild type pollen developed normal siliques (Fig. 10 E). *coi1-t* seedlings vertically grown on media containing 50 µM MeJA displayed normal growth (Fig. 10 F). In contrast, wild type seedlings grown under these conditions exhibited reduced growth and especially shortened roots, as indicated by white arrows in Fig. 10 F. When further growth of *coi1-1* and *coi1-t* on soil was monitored over a period of 12 weeks, no obvious growth differences were recorded (Fig. 10 G).
The obtained data indicate *coi1-t* as a new COI1-deficient line, representing a valuable tool for JA-related studies.

3.6 Accumulation of JA-Ile in *coi1* mutants

The data obtained using *InsP 5-ptase* plants indicated a combination of lowered wound-induced gene expression and reduced defence against caterpillars accompanied by substantially increased accumulation of JA-Ile over wild type levels. This pattern suggests partial JA insensitivity of the *InsP 5-ptase* plants.
In order to test this concept, and because no data on oxylipin levels are currently available for JA-insensitive plants, two independent Arabidopsis *coi1* mutants lacking a functional JA receptor were tested for wound-induced JA-Ile levels: the widely used EMS line *coi1-1* (Feys et al, 1994) and the new T-DNA insertion line, *coi1-t*. In both COI1-deficient Arabidopsis lines, wound-induced levels of JA and its precursors oPDA and dn-oPDA showed induction upon wounding, but were reduced compared to wild type levels (Fig. 11). Wild type oPDA levels increased from 0.50 ± 0.15 to 3.77 ± 0.51 nmol g^{-1} fresh weight 2 h after wounding and to 4.36 ± 0.48 nmol g^{-1} fresh weight 4 h after wounding, whereas *coi1-1* plants exhibited an increase from 0.29 ± 0.02 to 1.58 ± 0.18 and 1.27 ± 0.28 nmol g^{-1} fresh weight, 2 h and 4 h

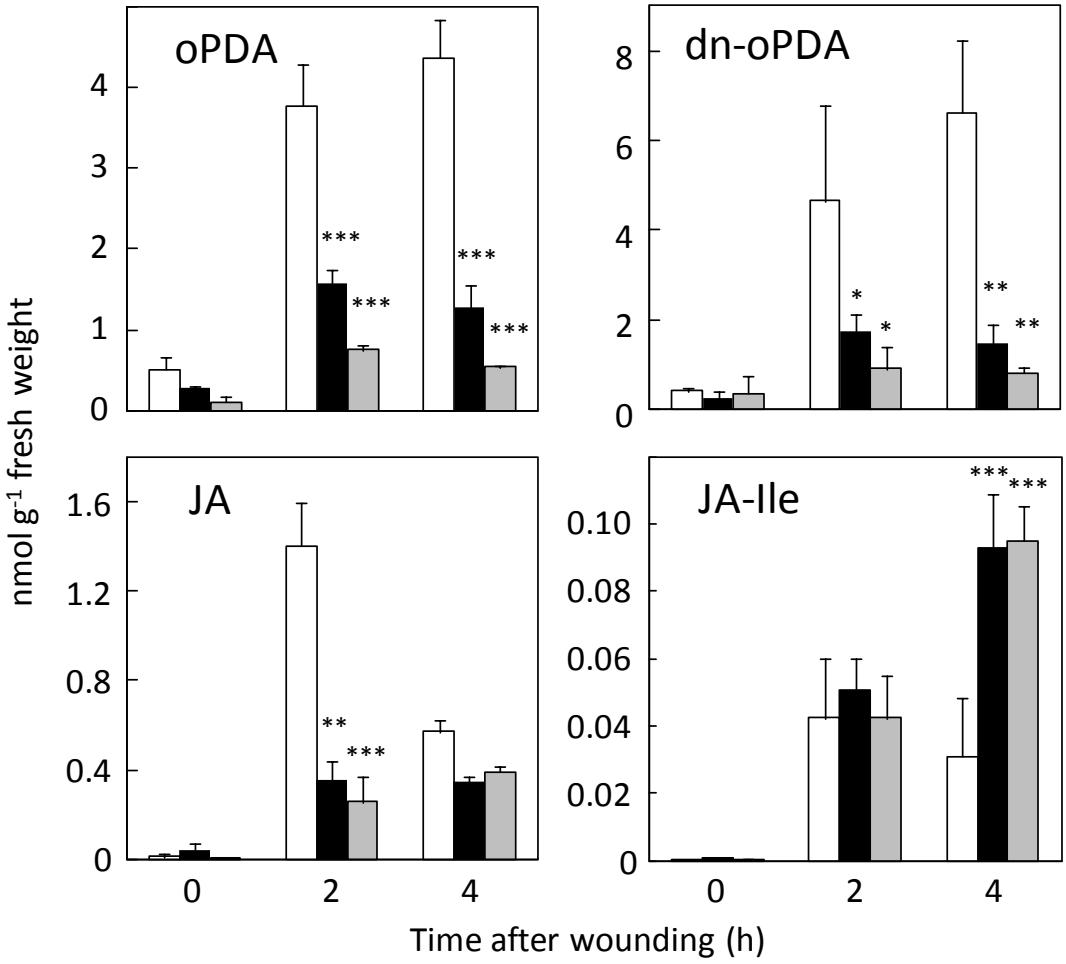

Fig. 11: Wound-induced oxylipin levels of JA-insensitive *coi1* mutants. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after indicated time spans. Oxylipins were determined via LC/MS in wild type plants (white columns), *coi1-1* (black columns) and *coi1-t* (grey columns) over 24 h. The mean values of two independent wounding experiments are shown assayed in duplicates, and each duplicate measured twice, ± SD. Missing error bars are too small to appear. Asterisks indicate significant differences between levels detected in wild type and in *coi1-1* or *coi1-t* plants, respectively, at the indicated time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

after wounding, respectively. The oPDA rise in *coi1-t* was even slower, starting with 0.11 ± 0.08 nmol g^{-1} fresh weight, increasing to 0.75 ± 0.05 and 0.54 ± 0.02 nmol g^{-1} fresh weight. The described pattern closely resembled the induction of dn-oPDA, and also the JA induction pattern was similar, but instead of still rising from 2 h to 4 h, JA levels declined in wild type plants from 1.40 ± 0.19 to 0.57 ± 0.05 nmol g^{-1} fresh weight. Both *coi1* mutant lines displayed a JA induction with wounding from levels close to detection limit (*coi1-t,* 0.04 ± 0.03, *coi1-1,* 0.00 ± 0.00 nmol g^{-1} fresh weight) to nearly constant levels of 0.34 ± 0.05 nmol g^{-1} fresh weight. Whereas levels of oPDA, dn-oPDA and JA were reduced in both *coi1* mutant lines, JA-Ile levels were clearly elevated over wild type levels with wounding. Without stimulus, JA-Ile levels were close to detection limit in wild type, *coi1-1* and *coi1-t* plants. After showing JA-Ile

levels similar to wild type 2 h after wounding (0.042 ± 0.018 nmol g^{-1} fresh weight in wild type, 0.051 ± 0.093 nmol g^{-1} fresh weight in *coi1-1* and 0.042 ± 0.095 nmol g^{-1} fresh weight in *coi1-t*), both *coi1* mutants displayed a strong and prolonged further increase of JA-Ile to a maximum of 0.093 ± 0.016 nmol g^{-1} fresh weight in *coi1-1* and 0.095 ± 0.010 nmol g^{-1} fresh weight in *coi1-t*, whereas wild type levels dropped to 0.031 ± 0.017 nmol g^{-1} fresh weight. Thereby *coi1* mutants exceeded wild type JA-Ile levels about 3-fold (Fig. 11).

To summarize this aspect of the data, in both *InsP 5-ptase* and *coi1* mutant lines the levels of the bioactive JA-Ile were highly abundant, but JA-mediated responses were reduced (Feys et al, 1994; Mosblech et al, 2008; Xie et al, 1998). The increased JA-Ile levels in the JA-Ile-insensitive *coi1* mutants resemble the JA-Ile pattern observed for *InsP 5-ptase* plants, thus supporting the notion of partial JA-Ile insensitivity of these plants. However, whereas in *InsP 5-ptase* plants oxylipin precursors of JA-Ile were also elevated over wild type levels, these oxylipins displayed reduced wound induction in *coi1* mutant plants. In *coi1* plants the JA amino acid synthetase JAR1 seems to be activated while the other enzymes of JA biosynthesis seem to be suppressed, whereas all these enzymes seem to be similarly activated in *InsP 5-ptase* plants. To elucidate the link between JA metabolism and inositol-containing signalling factors, further experiments were performed.

3.7 Amino acid residues coordinating an inositol polyphosphate cofactor in TIR1 are conserved in COI1

Based on the notion that the crystal structure of the auxin receptor F-box protein, TIR1, contains $InsP_6$ as a cofactor (Tan et al, 2007), structural information of different F-box proteins was evaluated to delineate how inositol metabolites might be bound. An alignment of the amino acid sequences of TIR1, various AUXIN SIGNALLING F-BOX (AFB) proteins homologous to TIR1, and COI1 illustrates the sequence similarity between F-box proteins involved in auxin and JA perception (Fig. 12 A). The amino acid residues coordinating the $InsP_6$ cofactor in TIR1 are strictly conserved in all proteins involved in auxin perception. Importantly, five of these residues are also conserved in COI1. These findings led to the hypothesis that an inositol polyphosphate is also bound in COI1, and that altered JA sensitivity in *InsP 5-ptase* plants is mediated by an effect of an inositol polyphosphate cofactor on COI1 function.

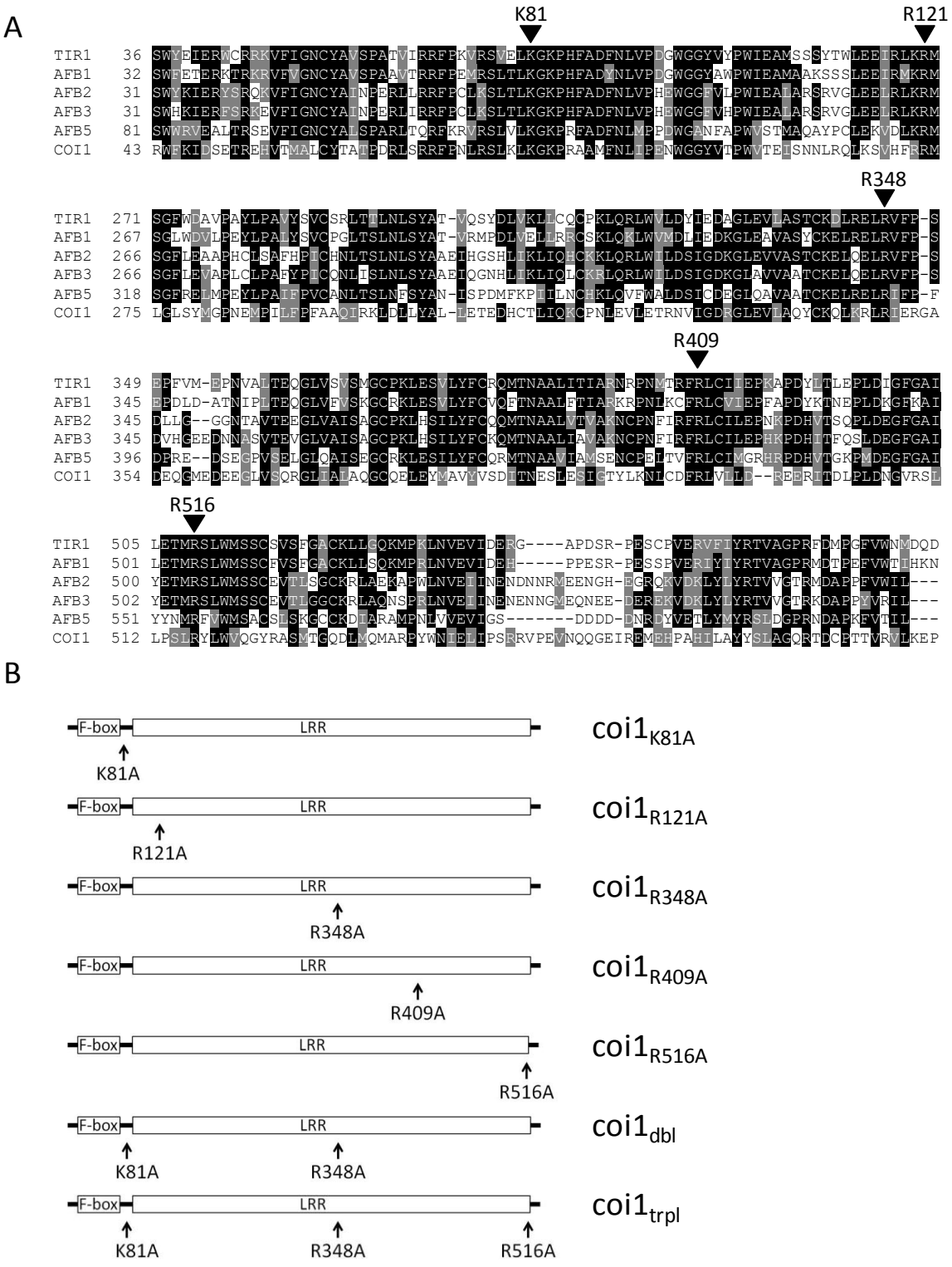

Fig. 12. Amino acid residues coordinating an inositol polyphosphate cofactor in TIR1 are conserved in COI1. (A) Partial sequence alignment of auxin binding E3 ubiquitin ligases TIR1, TIR1 homologues AFB1, 2, 3 and 5, and JA binding E3 ubiquitin ligase COI1. Putative inositol polyphosphate binding residues are marked with arrowheads. (B) Amino acid exchanges introduced into the COI1 protein. Graphical representation of COI1 with F-box domain and leucin-rich repeat (LRR) domain. Arrow heads indicate the individual positions altered in the different $coi1_{mut}$ variants, as indicated.

3.8 Putative inositol polyphosphate coordinating residues are required for COI1/JAZ9 interaction

To test this hypothesis, cDNAs encoding various mutated variants of COI1 ($coi1_{mut}$) were created, in which single or multiple putative inositol polyphosphate coordinating amino acid residues of COI1 were exchanged to alanine residues (Fig. 12 B). These variants were first tested in yeast two-hybrid assays for their capability to interact with the COI1 target, JAZ9. The interaction was tested in the presence of coronatine, a potent JA-Ile analogue (Fig 13). The

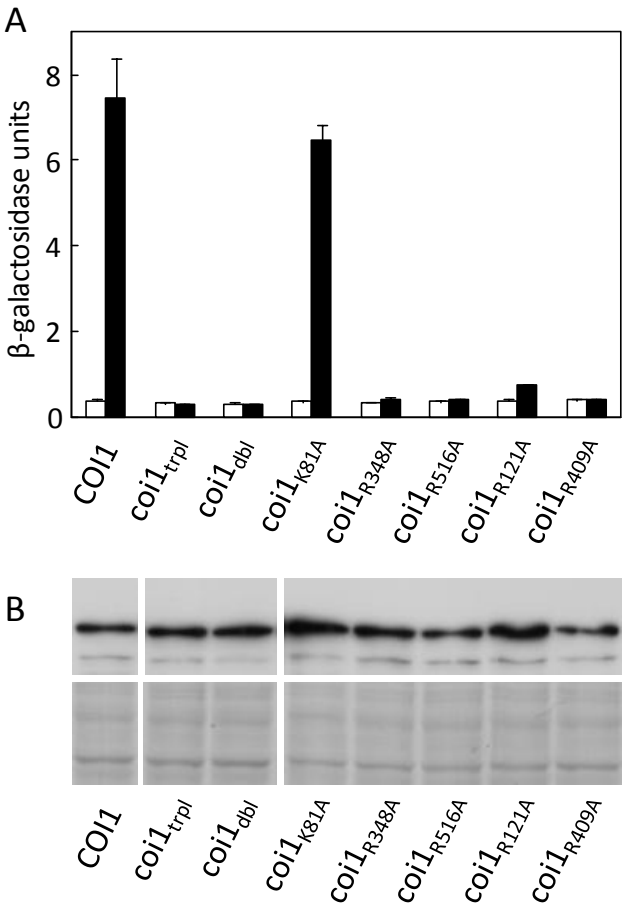

Fig 13: Putative inositol polyphosphate coordinating residues are required for the COI1/JAZ9 interaction. Constructs encoding COI1 variants with single and multiple exchanges in amino acid positions possibly involved in inositol polyphosphate binding ($coi1_{mut}$) were created and used in yeast two-hybrid tests for interaction with the COI1 target, JAZ9. (A) β-galactosidase activities indicating the COI1/JAZ9 or $coi1_{mut}$/JAZ9 interactions. Interactions were tested in the absence (white columns) or presence of 30 µM coronatin (black columns). Data represent mean values of four replicates ± SD. (B) Western blot analysis of the MYC-tagged COI1- or $coi1_{mut}$-GBD fusions. 2 µg of total protein of 4 individual clones induced with coronatin were pooled and loaded per lane. Only the specific immunodetection band of 89 kDa is shown. Top, detection of MYC-tags; bottom, coomassie-stained gel. Experiments shown in A and B were repeated once in quadruples with similar results. Yeast two-hybrid experiments and Western blot analysis were kindly performed by Dr. Corinna Thurow and Anna Hermann.

data indicate that the exchange of putative inositol polyphosphate binding residues profoundly affected the COI1/JAZ9 interaction (Fig. 13). β-galactosidase activity resulting from the interaction of the Gal4 DNA binding domain (GBD) and Gal4 activation domain (GAD) fusions was slightly lowered for the GBD-JAZ9/GAD-coi1$_{K81A}$ interaction (87 % of the activity obtained with GBD-JAZ9 and GAD-COI1; Fig. 13 A). The GBD-JAZ9/GAD-coi1$_{R121A}$ interaction was severely reduced and showed only 10 % of the β-galactosidase activity achieved with wild type COI1. The interaction of JAZ9 with all other coi1$_{mut}$ variants tested, including that of variants carrying other single as well as multiple exchanges, was fully abolished and displayed β-galactosidase activities as low as the negative controls without coronatine (Fig. 13 A). To confirm that the effects observed were not due to uneven levels of the altered coi1$_{mut}$ proteins, Western blot analysis was performed (Fig. 13 B). Similar expression levels of the GAD-COI1 and GAD-coi1$_{mut}$ fusion proteins were detected in all samples and no variations in expression levels were observed.

3.9 Reduced *in planta* functionality of COI1 variants lacking putative inositol polyphosphate binding residues

After the exchange of putative inositol polyphosphate coordinating amino acid residues in COI1 led to reduced COI1/JAZ9 interaction in yeast, the same mutated COI1 variants were used to test their capability to complement the Arabidopsis *coi1-t* mutant phenotypes. Heterozygous *coi1-t* mutant plants were transformed with either wild type COI1 or the different coi1$_{mut}$ variants and JA sensitivity was determined first on 3-week-old T1 seedlings by a vertical root growth assay. Transformants were selected by the fluorescence of a seed-expressed DsRed marker and the root lengths of seedlings growing on media containing 50 µM MeJA were determined (Fig. 14 A). Homozygous *coi1-t* backgrounds were subsequently identified by PCR-based genotyping and just these plants were considered. 10-66 plants were analyzed for each transformation construct. As positive and negative controls the root lengths of untransformed wild type and *coi1-t* plants were determined. Wild type roots reached an average length of 14.3 ± 3.4 mm, whereas *coi1-t* roots were substantially longer with 63.2 ± 17.9 mm (Fig. 14 A), indicating the discrimination of plants carrying functional or non-functional *COI1* alleles worked well. Furthermore, expression of wild type COI1 complemented fully (root lengths of 14.9 ± 4.0 mm), whereas an empty vector control did not (root length of 57.2 ± 21.8 mm). The complementation with single exchange coi1$_{mut}$ variants

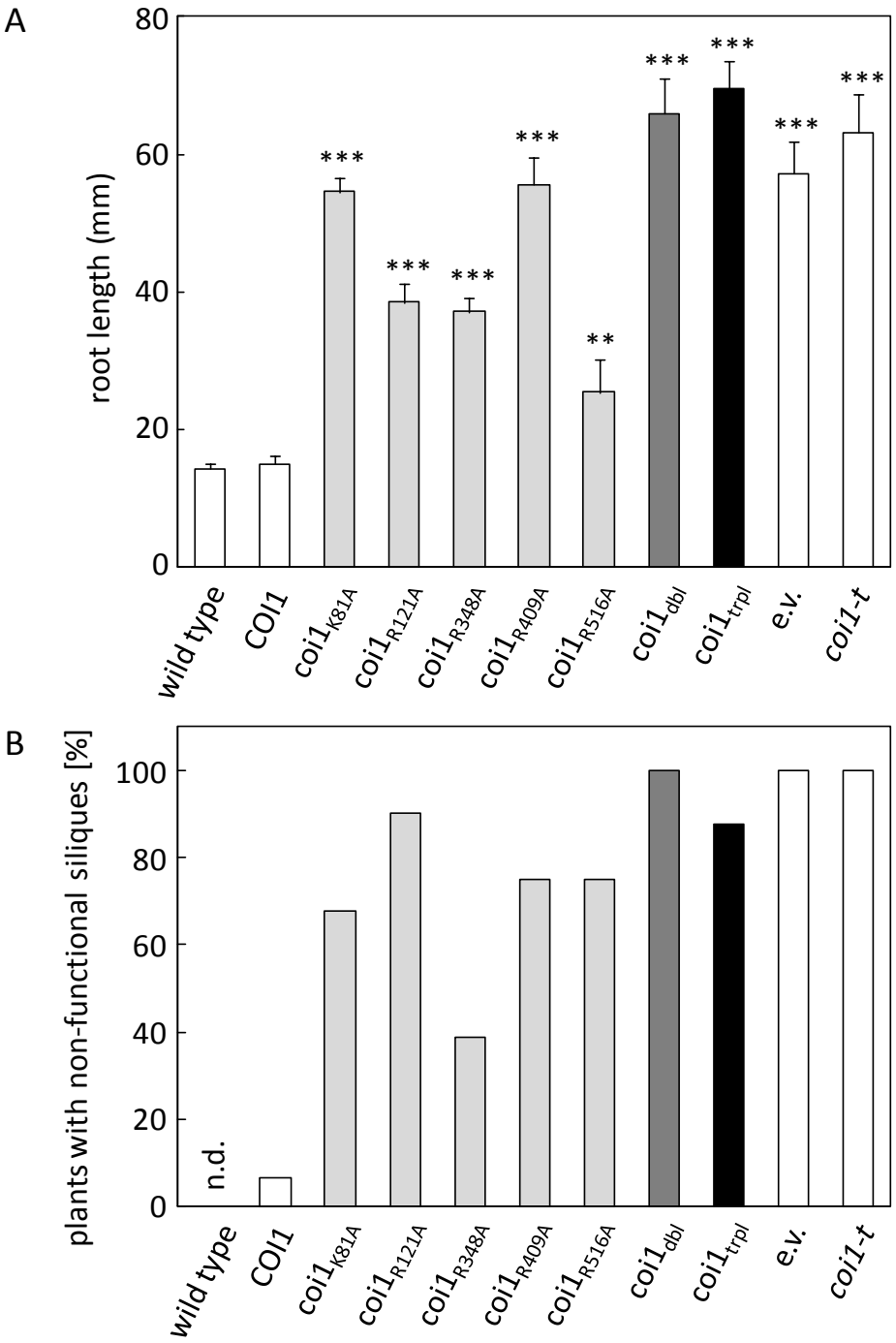

Fig. 14. Reduced *in planta* functionality of COI1 variants lacking putative inositol polyphosphate binding residues. The coi1$_{mut}$ variants carrying single or multiple exchanges were tested for their capability to rescue Arabidopsis *coi1-t* mutant phenotypes. (A) Root growth sensitivity against 50 µM MeJA was tested using 3-week-old vertically grown plants expressing different rescue constructs, as indicated. The empty vector (e.v.) control carried the DsRed sequence only; 10-66 plants per transformation construct were analyzed. Root lengths are shown in mm ± SE. (B) Male fertility was assessed by growing the same individuals assayed for root length on soil until seed set and categorizing plants producing functional or non-functional siliques. The percentage of plants generating non-functional siliques is shown; 8–60 plants were taken into account per transformation construct. White bars, controls; grey and black bars, *coi1-t* plants expressing coi1$_{mut}$ variants with single (light grey), double (dark grey) or triple exchanges (black). Asterisks indicate significant differences of root length compared to untransformed wild type, according to a student's *t*-test (**, $p \leq 0.01$; ***, $p \leq 0.001$).

resulted in varying root lengths, indicating different degrees of complementation when different amino acid positions were altered (Fig. 14 A). While plants complemented with the $coi1_{R516A}$ variant showed the shortest roots similar to complementation with wild type COI1, variants $coi1_{R348A}$ and $coi1_{R121A}$ displayed intermediate root lengths with 37.1 ± 12.7 mm and 38.5 ± 16.1 mm, respectively (Fig. 14 A). Complementation with variants $coi1_{K81A}$ and $coi1_{R409A}$ revealed the longest roots among the single exchange mutants with mean values of 54.6 ± 16.4 mm and 55.6 ± 12.8 mm, respectively (Fig. 14 A). While the single exchange variants displayed reduced complementation capability, effects observed with variants carrying multiple exchanges were even more pronounced, resulting in complete loss of complementation potential, indicated by root lengths of 66.0 ± 17.8 mm and 69.6 ± 25.9 mm, which even slightly exceeded root lengths recorded for the *coi1-t* control, possibly due to dominant negative effects. The data indicate that the exchanged amino acids are important for COI1 function and affect JA sensitivity pertaining to root growth.

To test, if other JA-mediated response were also affected, silique development was monitored in the same plants previously assessed in the root growth assay. Plants were categorized as either producing functional siliques similar to wild type controls, or as producing non-functional siliques similar to *coi1-t* plants (Fig. 14 B). In this tests 0 % of wild type plants, but 100 % of *coi1-t* plants generated non-functional siliques. Complementation of the *coi1-t* mutant with wild type COI1 revealed 6.7 % of plants with non-functional siliques, indicating close to full complementation. The single exchange variants showed reduced complementation capability indicated by an average of 69 % of non-functional siliques, with $coi1_{R348A}$ showing the fewest (39 %) and $coi1_{K81A}$, $coi1_{R409A}$ and $coi1_{R516A}$ showing intermediate proportions of non-functional siliques (68 %, 75 % and 75 %, respectively). Complementation with $coi1_{R121A}$ resulted in the lowest observed fertility rescue among the single exchange variants, with 90 % of plants showing non-functional siliques. As also seen in the root growth assay, the complementation capability was reduced even stronger with $coi1_{mut}$ variants carrying multiple exchanges, exhibiting 100 % ($coi1_{dbl}$) and 88 % ($coi1_{trpl}$) of non-functional siliques. Together the root growth assay and the determination of fertility rescue show that complementation with wild type COI1 resulted in rescue of JA sensitivity in two independent aspects of the JA response, and the rescue with $coi1_{mut}$ variants displayed similar patterns of complementation capacity between both experiments.

As before in yeast (Fig. 13 B), it was attempted to detect the C-terminal polyHis-tagged COI1 variants in plant extracts, but expression levels were too low for detection (data not shown). To test whether or not differences in rescue capability between constructs were due to positional effects of the genomic insertion loci and resulting variable expression levels,

fluorescence intensities of the co-integrated DsRed selection marker were determined in transformed seeds before experiments described in Fig. 14 were set up (Fig. 15). Data are shown for plant individuals that exhibited average values in the root length assay. Transformed seeds were grouped into categories of increasing fluorescence intensity. Seeds complemented with wild type COI1 showed DsRed fluorescence in the first (low) intensity categories and mainly in category two (Fig. 15). Single exchange variants showed fluorescence mainly in the intermediate intensity categories three and four, whereas multiple exchange variants were

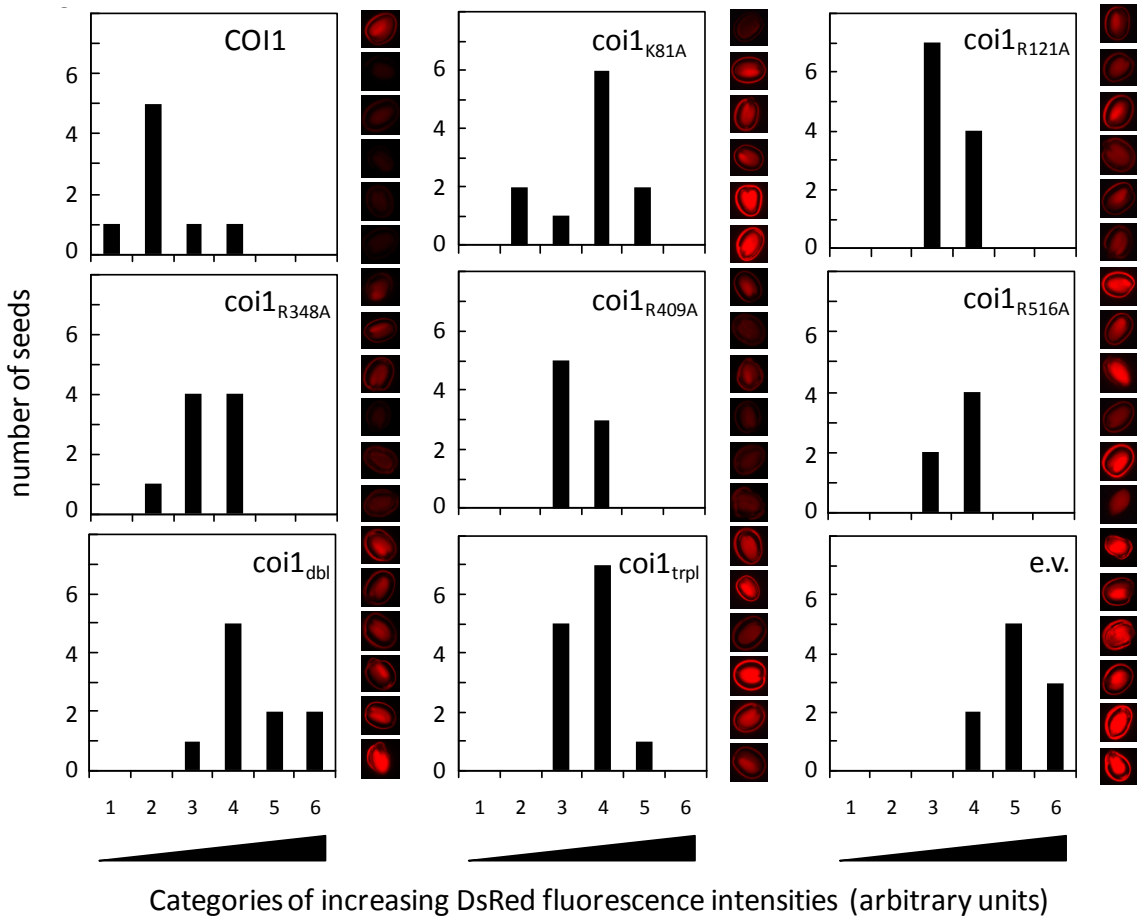

Fig. 15: Distribution of DsRed fluorescence intensities of transformed *coi1-t* seeds does not correlate with differences in complementation efficiency. Positive transformants were identified by DsRed fluorescence using the fluorescence stereomicroscope Leica MZ16 FA and a Leica DFC480 camera. Fluorescence intensities were monitored and allocated to six categories of increasing fluorescence. Seeds resulting in seedlings with average root length were taken into account; number of seeds in each category was counted and distributions of fluorescence intensities are depicted for each transformation construct. e.v.: empty vector control carrying DsRed. Representative examples of fluorescing seeds are depicted on the right of each graph.

found to exhibit fluorescence of higher intensities in categories three, four and five (Fig. 15). The empty vector control showed strongest fluorescence with intensities mainly in categories five and six (Fig. 15).

In addition to the fluorescence intensity plots, mRNA levels of the introduced COI1 and coi1$_{mut}$ variants were determined on four plants of each transformation construct via semi-quantitative RT-PCR with 27, 30 or 34 cycles, using cDNA as a template (Fig. 16). Variants with low complementation capability, such as the multiple exchange variants, displayed expression levels similar to that of wild type COI1, which complemented fully. In contrast, variants with high complementation capability, such as some of the single exchange variants, displayed lower expression levels.

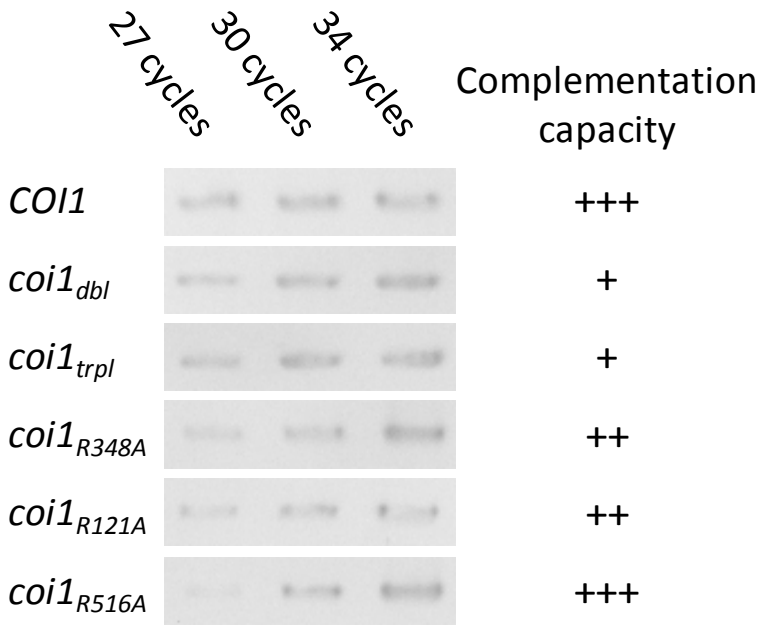

Fig. 16: Specific transcript levels for expressed *coi1$_{mut}$* variants do not correlate with patterns of complementation efficiency in *coi1-t* plants. Transcript levels for COI1 or coi1$_{mut}$ variants expressed in *coi1-t* mutants were determined by semi-quantitative RT-PCR. Transcripts were amplified using 27, 30 and 34 PCR cycles, as indicated. Complementation capacity of individual constructs was assessed from the combined results of the root growth assays and the determination of functional siliques (right). +, none to weak complementation; ++, intermediate complementation; +++, very good or full complementation. Variants with low complementation capability, as the multiple exchange variants, displayed expression levels similar to that of wild type COI1, which complemented fully. In contrast, variants with high complementation capability, such as some of the single exchange variants, displayed lower expression levels. Data are shown for a representative set of individual plants. The experiment was performed four times with similar results.

Even though functional complementation with the C-terminal polyHis-tagged COI1 protein and to a lesser extent with the C-terminal polyHis tagged $coi1_{mut}$ proteins with single amino acid exchanges was detected, immunodetection of the respective COI1 variants in *Arabidopsis* extracts failed. Semi-quantitative RT-PCR indicated similar RNA levels for all proteins, suggesting that differences observed in the complementation capabilities of $coi1_{mut}$ variants were not caused by varying expression levels due to positional effects of genomic insertion loci. The data indicate that the introduced mutations in the putative inositol polyphosphate binding sites rendered the protein either unstable or non-functional. The higher abundance of seeds expressing the fluorescent marker gene in transformants carrying the less functional $coi1_{mut}$ variants suggests that these were tolerated by the plants at higher expression levels than fully functional variants.

3.10 Modulation of inositol polyphosphate biosynthesis alters COI1/JAZ9 interactions in yeast

Mutation of putative inositol polyphosphate coordinating residues resulted in reduced interaction of COI1 and JAZ9 (Fig. 13) as well as in reduced complementation capability of COI1 in *coi1-t* plants (Fig. 14), supporting the hypothesis that an inositol polyphosphate acts as a cofactor in COI1 and is important for COI1 function. As an independent line of evidence, the effects of altered inositol polyphosphate content on the interaction of wild type COI1 with JAZ9 were investigated. Deletion of the *ipk1* gene in yeast results in a strong reduction of $InsP_6$ levels and concomitant accumulation of its precursor $InsP_5$ (Ponnusamy et al, 2008). Therefore, *IPK1* was eliminated by targeted gene disruption in the yeast strain PJ69-4a used for the two-hybrid assays. The COI1/JAZ9 interaction was then tested in this background in the presence of coronatine (Fig. 17). Samples without coronatine served as negative controls. It was surprising to find that β-galactosidase activity resembling the GBD-JAZ9/GAD-COI1 interaction was enhanced in the *ipk1Δ* strain, resulting in activities 2-3 times higher than those in the PJ69-4a background (Fig. 17 A). Western blot analysis indicates close to even levels of COI1 protein present in PJ69-4a and in the *ipk1Δ* strain, respectively (Fig. 17 B).

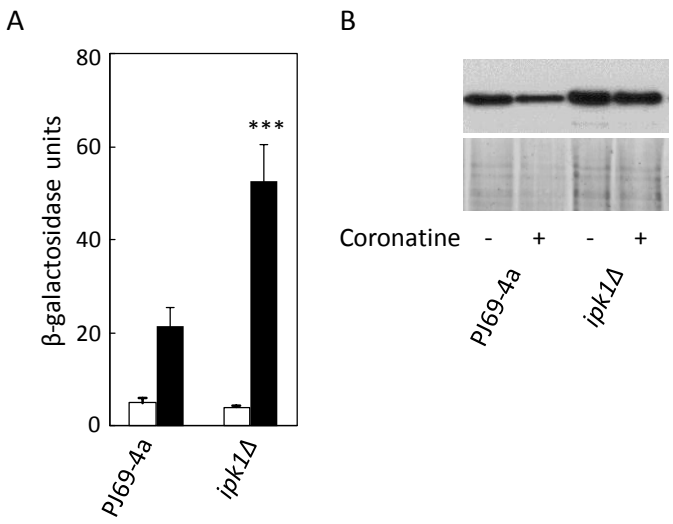

Fig. 17. Modulation of inositol polyphosphate biosynthesis alters COI1/JAZ9 interactions in yeast. The interaction of wild type COI1 and JAZ9 was assessed in yeast two-hybrid tests using an *ipk1Δ* yeast strain, in which the *IPK1* gene was deleted. (A) The COI1/JAZ9 interaction was tested in the absence (white bars) or presence of 30 µM coronatine (black bars). β-galactosidase activities indicate the COI1/JAZ9 interaction in controls or *ipk1Δ*. Mean values of four replicates ± SD are shown. Asterisks indicate significant differences of β-galactosidase activity between the parental strain PJ69-4A and *ipk1Δ*, according to a student's *t*-test (***, $p \leq 0.001$). (B) Immunodetection of COI1 in the respective yeast strains, as indicated. Top, detection of MYC tags; bottom, coomassie-stained gel. Protein from four cultures was combined for immunodetection. Experiments were repeated in quadruples once with similar results. Yeast two-hybrid experiments and Western blot analysis were kindly performed by Dr. Corinna Thurow and Anna Hermann.

3.11 Modulation of inositol polyphosphate biosynthesis alters defence responses in *ipk1-1* plants

3.11.1 Biochemical characterization of *ipk1-1* plants

3.11.1.1 Wound-induced PI signalling components in *ipk1-1* plants

To investigate COI1 function in the corresponding plant system with reduced $InsP_6$ and elevated $InsP_5$ levels, the Arabidopsis *ikp1-1* mutant (Stevenson-Paulik et al, 2005) was analysed. The Arabidopsis *ipk1-1* mutant was originally isolated for feed industrial reasons, because high abundance of $InsP_6$ in feed has detrimental influence on the uptake of phosphate, inositol, and essential minerals by monogastric animals, such as pig and poultry, and furthermore contributes to environmental phosphorus pollution (Brinch-Pedersen et al, 2002; Hamada et al, 2006; Stevenson-Paulik et al, 2005).

Disruption of the IPK1 gene locus in Arabidopsis results in decreased levels of $InsP_6$ with concomitant accumulation of $InsP_4$ and $InsP_5$ (Stevenson-Paulik et al, 2005), similar to the

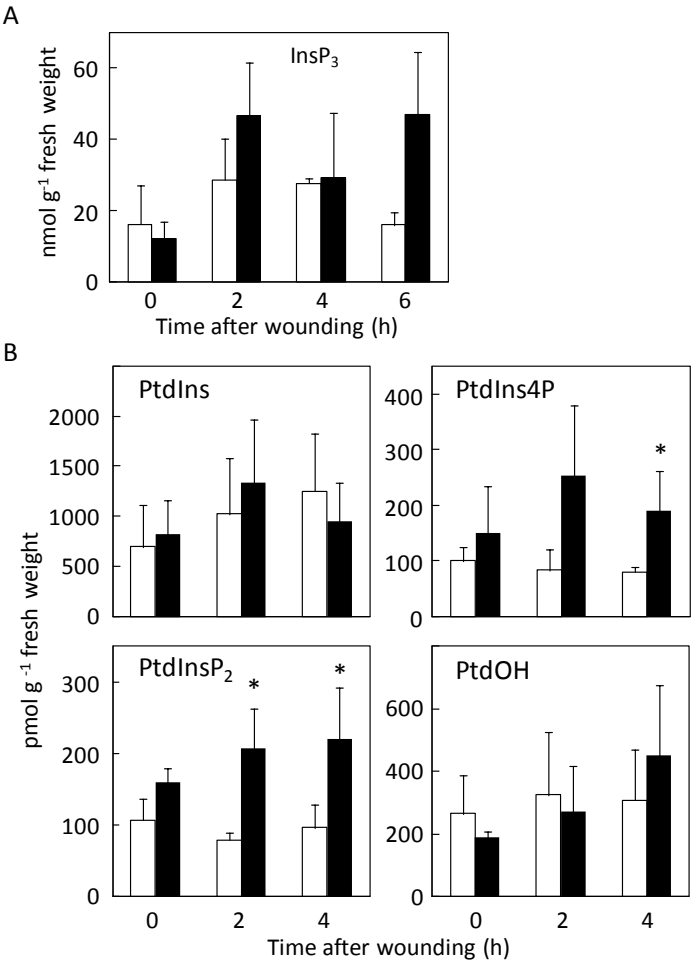

Fig. 18: Wound-induced PI signalling components in *ipk1-1* plants. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after indicated time spans. (A) $InsP_3$ levels were determined by a competitive protein binding assay in wild type (white columns) and *ipk1-1* plants (black columns). Data are mean values of one independent wounding experiments assayed in duplicates ± SD. (B) PI lipids were determined via GC/MS after TLC separation, in wild type (white columns) and *ipk1-1* plants (black columns). Lipid data are mean values of three independent wounding experiments assayed in duplicates ± SD. Asterisks indicate significant differences between level detected in wild type and in *ipk1-1* plants at the respective time point, according to a student's *t*-test (*, $p \leq 0.1$).

pattern described for yeast *ipk1Δ* (Ponnusamy et al, 2008). For a deeper biochemical characterization of *ipk1-1* plants, wound-induced $InsP_3$ levels were determined (Fig 18 A). As this experiment was only performed once, these data still need to be confirmed. Interestingly, wound induced accumulation of $InsP_3$ was also elevated over wild type controls. By 2 h after wounding, wild type plants reached maximal $InsP_3$ levels of 28.6 ± 11.7 nmol g^{-1} fresh weight, which were clearly exceeded by *ipk1-1* plants, increasing to 46.6. ± 15.1 nmol g^{-1} fresh weight (Fig. 18 A). *ipk1-1* plants reached their maximum with 47.0 ± 17.3 nmol g^{-1} fresh weight 6 h after wounding, by which time wild type controls had declined to $InsP_3$ levels of 16.0 ± 3.6 nmol g^{-1} fresh weight.

Lipid analysis of wound-induced PIs (Fig. 18 B) showed that also PtdIns4P and PtdIns(4,5)P_2 increased over wild type levels. PtdIns showed only minor differences between wild type and *ipk1-1* plants, with both reaching similar maxima (wild type 1253.5 ± 575.5 nmol g^{-1} fresh weight 4 h after wounding, *ipk1-1* plants 1332.4 ± 629.6 pmol g^{-1} fresh weight 2 h after wounding). PtdIns4P levels slightly declined in wild type plants from 100.3 ± 24.2 to 79.6 ± 9.7 pmol g^{-1} fresh weight within 4 h of wounding, whereas *ipk1-1* plants displayed increasing PtdIns4P levels from 149.3 ± 84.3 to 252.3 ± 126.1 pmol g^{-1} fresh weight already 2 h after wound stimulus. A similar pattern was recorded for PtdIns(4,5)P_2 levels, which declined from 107.4 ± 29.1 to 79.5 ± 10.0 pmol g^{-1} fresh weight in wild type controls, whereas *ipk1-1* plants showed an increase from 159.9 ± 20.1 to 219.5 ± 73.2 pmol g^{-1} fresh weight. The levels of PtdOH displayed only a minor rise in wild type plants from 264.4 ± 124.6 to 324.9 ± 199.8 pmol g^{-1} fresh weight. In *ipk1-1* plants, PtdOH levels were similar in unwounded plants (188.7 ± 18.2 pmol g^{-1} fresh weight), but rose rather strongly up to 451.0 ± 224.2 pmol g^{-1} fresh weight.

Taken together, the biochemical characterization of PI metabolites confirmed that *ipk1-1* plants displayed elevated levels of $InsP_3$ compared to wild type controls and furthermore also accumulate PI lipids over wild type levels, indicating that interruption of IPK1 influences the whole PI signalling pathway.

3.11.1.2 Altered oxylipin patterns in *ipk1-1* plants

As *InsP 5-ptase* plants with global reduction of the PI pathway displayed increased wound induction of jasmonates, the impact of PI accumulation and the loss of $InsP_6$ in *ipk1-1* plants on wound induction of oPDA, dn-oPDA, JA and JA-Ile was tested. Levels of wound-induced oxylipins were monitored via LC/MS over a period of 6 h (Fig. 19). oPDA levels of *ipk1-1* plants did not show pronounced differences to wild type levels. While early after wounding *ipk1-1* plants slightly exceeded wild type levels (0.5 h after wounding, wild type 5.45 ± 1.10, *ipk1-1* 8.92 ± 1.72 nmol g^{-1} fresh weight), the opposite pattern could be observed at most later time points (6 h after wounding, wild type 11.71 ± 7.89, *ipk1-1* 7.67 ± 3.23 nmol g^{-1} fresh weight). A similar but more pronounced pattern was depicted in dn-oPDA levels, which were about twice as high in *ipk1-1* plants early after wounding (0.5 h after wounding, wild type 10.71 ± 3.50, *ipk1-1* 23.15 ± 8.12 nmol g^{-1} fresh weight) and only decreased below wild type levels beyond 5 h of wounding (wild type 9.18 ± 7.24, *ipk1-1* 6.48 ± 3.57 nmol g^{-1} fresh weight). The time course of JA levels was very similar between wild type and *ipk1-1* plants, but *ipk1-1* plants

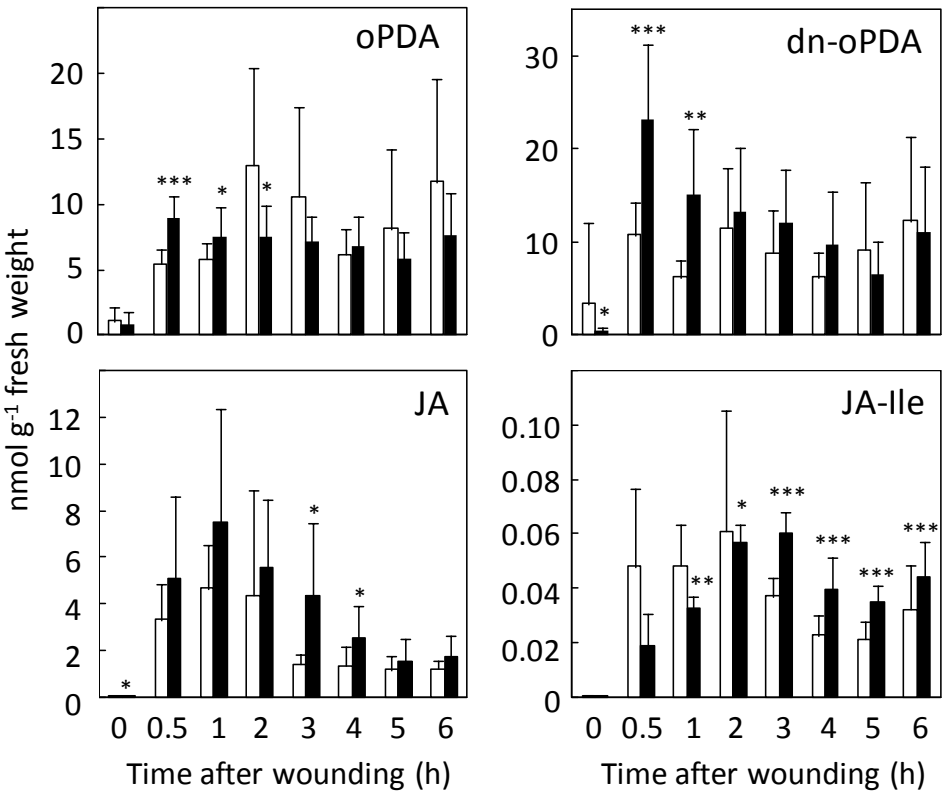

Fig. 19: Wound-induced oxylipins in wild type and *ipk1-1* plants with high $InsP_5$ and low $InsP_6$ levels. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after the indicated times. Oxylipins were determined via LC/MS in wild type plants (white columns) and *InsP 5-ptase* (black columns) over 24 h. The mean values of two independent wounding experiments are shown, each assayed in duplicates, and each duplicate measured twice, ± SD. Asterisks indicate significant differences between level detected in wild type and in *ipk1-1* plants at the respective time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

displayed elevated absolute levels compared to wild type at all time points monitored (Fig. 19). Both reached a maximum 1 h after wounding (wild type 4.67 ± 1.86, *ipk1-1* 7.50 ± 4.83 nmol g^{-1} fresh weight) and declined constantly afterwards. Whereas oPDA and dn-oPDA values were somewhat higher than wild type in early time points and lower than wild type at later time points, the opposite pattern was observed in JA-Ile time courses (Fig. 19). Wound induction of JA-Ile seemed to be delayed in *ipk1-1* plants, indicated by a postponed increase (wild type maximum 2 h after wounding of 0.061 ± 0.044 nmol g^{-1} fresh weight, *ipk1-1* maximum 3 h after wounding of 0.060 ± 0.008 nmol g^{-1} fresh weight) as well as a postponed decrease afterwards (wild type decreased to 0.037 ± 0.007 nmol g^{-1} fresh weight 3 h after wounding, *ipk1-1* decreased to comparable 0.039 ± 0.012 nmol g^{-1} fresh weight only 4 h after wounding).

In general, oxylipin patterns of *ipk1-1* plants were less different from wild type patterns than those previously observed for *InsP 5-ptase* plants.

3.11.2 Arabidopsis *ipk1-1* mutants are hypersensitive to JA

3.11.2.1 Enhanced wound-induced gene expression in *ipk1-1* mutants

It was already shown further up that *InsP 5-ptase* plants exhibit attenuated wound-induced transcript levels of *T18K17.7*, *AOS* and *WRKY70* (Mosblech et al, 2008). These genes were now also tested by quantitative real-time RT-PCR for wound induction in *ipk1-1* plants (Fig. 20). In contrast to the situation in *InsP 5-ptase* plants, wound-induced gene expression was not abolished in *ipk1-1* plants, but was clearly increased over wild type levels. Whereas all genes tested were induced by wounding in both wild type controls and in the *ipk1-1* mutant, the level of induction was substantially higher in the *ipk1-1* mutant. Relative expression levels of *T18K17.7* transcript were 2-4 times higher in *ipk1-1* plants for most time points monitored than in the corresponding wild type controls (Fig. 20).

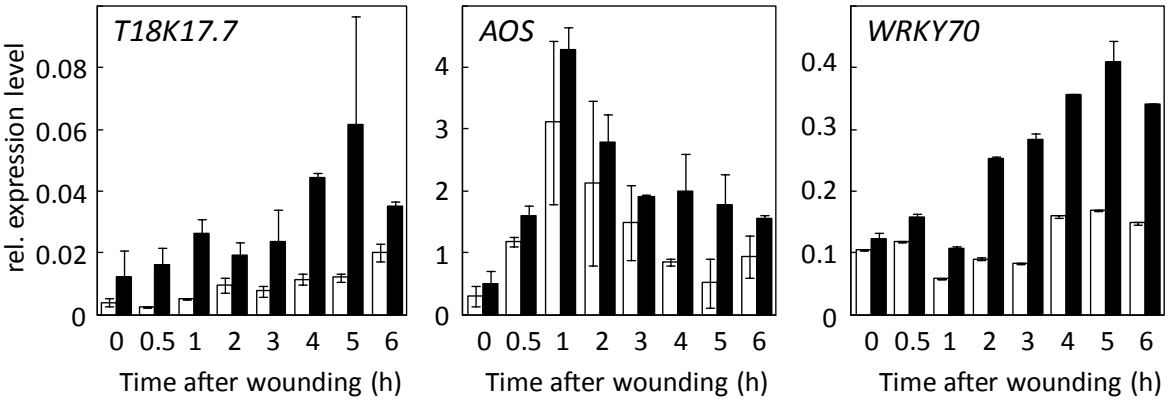

Fig. 20: Wound-induced transcript levelsof *T18K17.7*, *AOS* and *WRKY70* in wild type and *ipk1-1* plants. 7-week-old plants were wounded with forceps and harvested after the indicated times. For expression analysis of wound-inducible genes, quantitative real-time RT-PCR analysis of allene oxide synthase (*AOS*), the WRKY-family transcription factor *WRKY70* and the Kunitz-family trypsin and protease inhibitor *T18K17.7* was performed using material from wild type (white bars) or *ipk1-1* plants (black bars). The mean values of two independent wounding experiments each analyzed in duplicates ± SD are shown. Missing error bars are too small to appear.

Similarly, *AOS* transcript levels increased with a maximum 1 h after wounding and declined afterwards in both wild type and *ipk1-1* plants, with *ipk1-1* plants showing higher transcript levels compared to wild type at all time points tested. *WRKY70* transcript levels were up to three times higher in *ipk1-1* plants (Fig. 20). The data indicate that the induction of wound-induced gene expression was more pronounced in *ipk1-1* than in wild type plants.

3.11.2.2 Increased defence against caterpillars of *ipk1-1* plants

As another downstream effect of wound signalling, resistance to insect feeding was tested in the same experimental set up as was used for investigation of *InsP 5-ptase* plants (Fig. 8). *P. xylostella* caterpillars of approximately equal developmental stage and weight were placed either on wild type or on *ipk1-1* plants and were allowed to feed. Caterpillar weight gain was then monitored over several days. Fig. 21 illustrates the relative caterpillar weight increase over 56 h; absolute values are summarized in Table 2.

Importantly, the rate of weight gain of caterpillars feeding on *ipk1-1* plants was lower than that of caterpillars feeding on wild type controls, exhibiting 51 ± 7 and 61 ± 7 µg weight increase per hour, respectively. A significant difference was observed after 56 h of feeding, when caterpillars feeding on wild type plants reached a weight increase of 404.6 ± 22.7 % of initial weight, while caterpillars feeding on *ipk1-1* plants attained a weight increase of 320.3± 29.1 % of initial weight.

The data indicate that both the induction of defence gene expression and the defensive capabilities of the Arabidopsis *ipk1-1* mutant against caterpillars were increased over that of wild type plants.

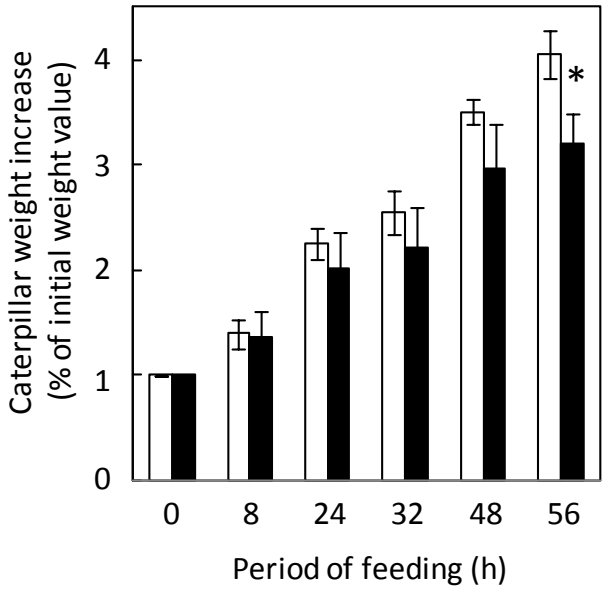

Fig. 21: Caterpillar growth performance feeding on wild type or *ipk1-1* plants. *P. xylostella* larvae of ~ 1 mg were allowed to feed on wild type plants (white bars) or on *ipk1-1* plants (black bars). Caterpillar weight was determined at the times indicated, and caterpillars were allowed to continue feeding. Data were normalized against caterpillar weights at time zero and are given as percent increase over the time zero value ± SD. Data are from three independent biological experiments wherein each experiment corresponds to the examination of 27 – 40 caterpillars for each plant line. Asterisk indicates significant increase in caterpillar growth feeding on *ipk1-1* plants compared with feeding on wild type plants according to a student's *t*-test (*, $p \leq 0.05$).

Table 2. Weight increase of *Plutella xylostella* caterpillars feeding on Arabidopsis wild type or *ipk1-1* plants. *Plutella xylostella* larvae of ~ 1 mg were allowed to feed on wild type or *ipk1-1* plants. Caterpillar weight was determined at the times indicated, and caterpillars were allowed to continue feeding. Data are from three independent biological experiments, wherein each experiment corresponds to the examination of 27 – 40 caterpillars for each plant line. Numbers represent mean weight in mg ± SD, rates of weight increase calculated from caterpillar net weight increases are given as µg h^{-1}.

Period of feeding	0	8 h	24 h	32 h	48 h	56 h	Rate of increase [µg h^{-1}]
wild type	1.12 ± 0.05	1.57 ± 0.17	2.52 ± 0.21	2.85 ± 0.27	3.93 ± 0.28	4.54 ± 0.42	61 ± 7
ipk1-1	1.32 ± 0.26	1.74 ± 0.12	2.59 ± 0.19	2.83 ± 0.21	3.82 ± 0.25	4.19 ± 0.60	51 ± 7

3.11.2.3 Enhanced root length reduction on MeJA in *ipk1-1* plants

To test whether also JA-mediated processes other than defence responses were affected in *ipk1-1* plants, root length reduction was monitored between 4-week-old seedlings vertically grown either on normal MS media or on MS media containing 50 µM MeJA (Fig. 22). Root length reduction was more pronounced in *ipk1-1* plants, exhibiting a reduction of 32.3 mm. This equals an approximately 30 % stronger reduction than that observed with wild type seedlings, which displayed only 24.9 mm of reduction. The enhanced sensitivity of roots to MeJA is consistent with results previously mentioned by Murphy et al (2008).

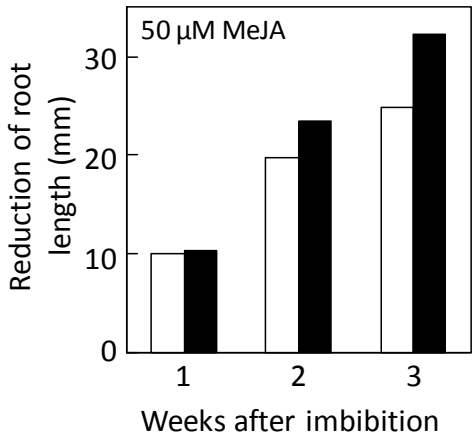

Fig. 22: Root sensitivity of wild type and *ipk1-1* seedlings against 50 µM MeJA. Reductions in root length of 4-week-old wild type (white bars) or *ipk1-1* (black bars) seedlings are shown as differences between root lengths of seedlings grown on vertically oriented MS agar plates with and without 50 µM MeJA. The difference was calculated from mean values of 85-130 root lengths with standard errors of 0.02 – 0.2.

Taken together the data of wound induced gene expression, caterpillar feeding performance and root growth on MeJA suggest that perturbed metabolism of inositol polyphosphates in *ipk1-1* plants results in hypersensitivity to JA either because of a lack of $InsP_6$ or due to an accumulation of $InsP_5$ and upstream PI metabolites. The comparison between *ipk1-1* plants and *InsP 5-ptase* plants suggests that the increase in $InsP_5$ rather than a decrease in $InsP_6$ is the cause for the JA hypersensitivity of the Arabidopsis *ipk1-1* mutant.

3.12 SA is relevant for JA as well as for PI signalling

While the focus of this study was on crosstalk between oxylipin and inositol signalling metabolites, the relevance of SA in wound responses in relation to PI signals was also investigated. JA and SA, both play important roles in plant defence against pathogens, and an antagonistic relation was previously reported in studies concerning responses to biotrophic and necrotrophic pathogens (Glazebrook, 2005; Kessler & Baldwin, 2002; Thomma et al, 2001). To investigate the role of SA in the observed crosstalk between JA and PI signals during the Arabidopsis wound response, two independent SA-deficient lines, *sid2* and *NahG*, were tested for wound induced levels of JAs and $InsP_3$. Furthermore, *InsP 5-ptase* plants, globally reduced in PI signalling, and *ipk1-1* plants, with high $InsP_5$ and low $InsP_6$ levels, were tested for their wound induced SA content. To validate that both the Arabidopsis *sid2* mutant and the *NahG* transgenic line exhibited their respective biochemical phenotypes, SA levels were determined with and without wounding (Fig. 23). In both cases, SA levels were strongly reduced compared to those observed in wild type controls, as was expected. While SA levels increased in wild type plants with wounding from 0.44 ± 0.06 to 0.97 ± 0.08 nmol g^{-1} fresh weight, there was no clear increase in both SA related mutants. *sid2* mutants displayed SA levels between 0.20 ± 0.05 and 0.25 ± 0.02 nmol g^{-1} fresh weight, without or with wounding. SA levels in *NahG* plant were even more strongly reduced, varying between 0.06 ± 0.01 and 0.10 ± 0.03 nmol g^{-1} fresh weight without or with wounding. Wound induction of oPDA was delayed in both SA related mutants (Fig. 23). While unwounded oPDA levels showed only minor differences in SA related mutants to wild type levels (wild type 0.39 ± 0.12, *sid2* 0.98 ± 0.58 and *NahG* 0.41 ± 0.22 nmol g^{-1} fresh weight), the wound-induced increase observed in wild type plants 2 h after wounding (to 6.02 ± 1.04 nmol g^{-1} fresh weight) was considerably lower in both mutants (*sid2* 2.88 ± 0.18, *NahG* 2.04 ± 0.13 nmol g^{-1} fresh weight). Nevertheless, oPDA levels of *sid2* and *NahG* even exceeded wild type levels 4 h after wounding by

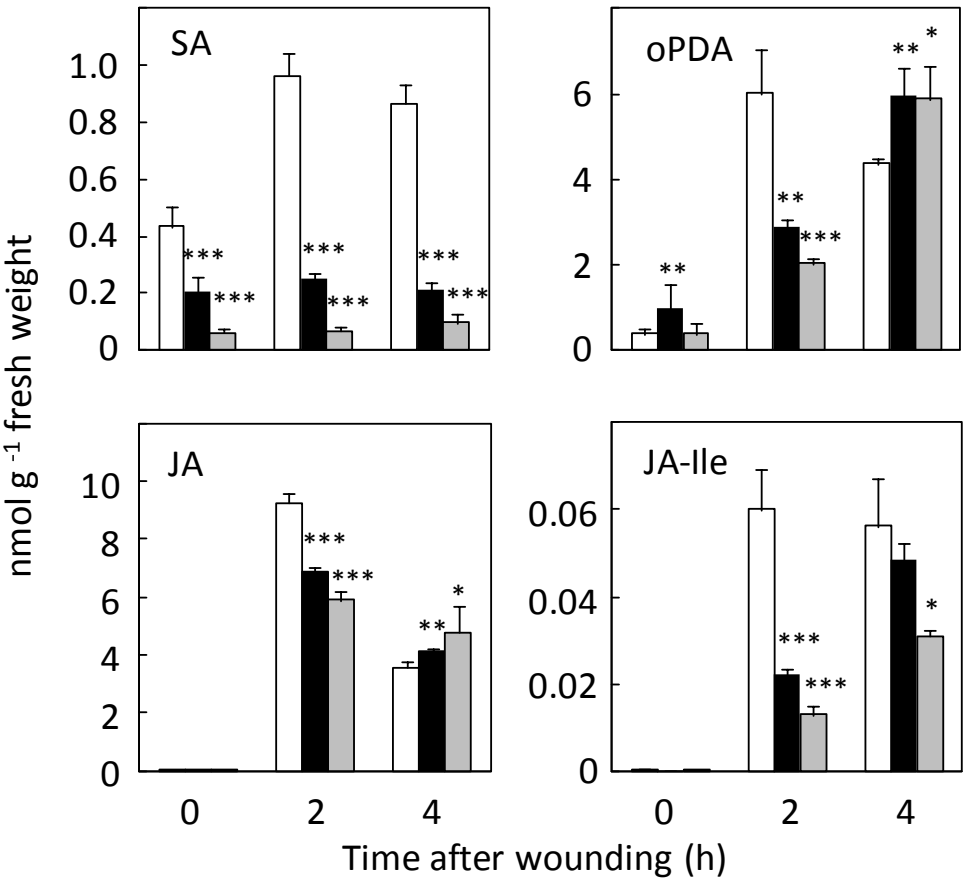

Fig.23: Wound-induced levels of SA and oxylipins in Arabidopsis mutants abolished in SA signalling. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after the indicated times. Phytohormones were determined via LC/MS in wild type plants (white columns) and *sid2* (black columns) and *NahG* (grey columns). Data of one wounding experiment are shown, each assayed in duplicates, and each duplicate measured twice, ± SD. Missing error bars are too small to appear. The experiment was repeated once using GC/MS, obtaining similar results. Asterisks indicate significant differences between levels detected in wild type and *sid2* plants or *NahG* plants, respectively, at the indicated time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

more than 25 %. Wound-induced JA levels were only slightly affected by SA alteration (Fig. 23) with lowered wound induction by about one fourth in *sid2* (6.85 ± 0.21 versus 9.25 ± 0.32 nmol g^{-1} fresh weight in wild type) and about one third in *NahG* (5.89 ±0.33 nmol g^{-1} fresh weight) 2 h after wounding, and similar levels to wild type 4 h after wounding. However, levels of JA-Ile, representing the bioactive form of JA, were strongly reduced in both mutants 2 h after wounding (Fig. 23) with wild type showing 0.06 ± 0.01, *sid2* 0.02 ± 0.01 and *NahG* 0.05 ± 0.01 nmol g^{-1} fresh weight. This difference was less pronounced 4 h after wounding, with *sid2* showing only a minor reduction and *NahG* a reduction of about 45 %.

Because SA-deficient mutants exhibited reduced wound-induced levels of JA-Ile as well as of its precursors, $InsP_3$ levels were determined after wounding in these plants (Fig. 24). Whereas wild type plants exhibited an increase in $InsP_3$ levels from 3.9 ± 2.3 to 17.4 ± 8.5 nmol g^{-1} fresh

weight, *sid2* plants showed no increase but rather constant levels of 3.4 ± 1.7 and 4.3 ± 2.9 nmol g^{-1} fresh weight (Fig. 24). *NahG* plants displayed an increase from 3.5 ± 3.4 to 11.3 ± 5.8 nmol g^{-1} fresh weight 2 h after wounding, but deceeded wild type levels by about one third. 4 h after wounding the increase in *NahG* plants was declined below untreated levels (2.7 ± 0.3 nmol g^{-1} fresh weight), whereas wild type levels were still elevated clearly over control levels (10.7 ± 1.5 nmol g^{-1} fresh weight).

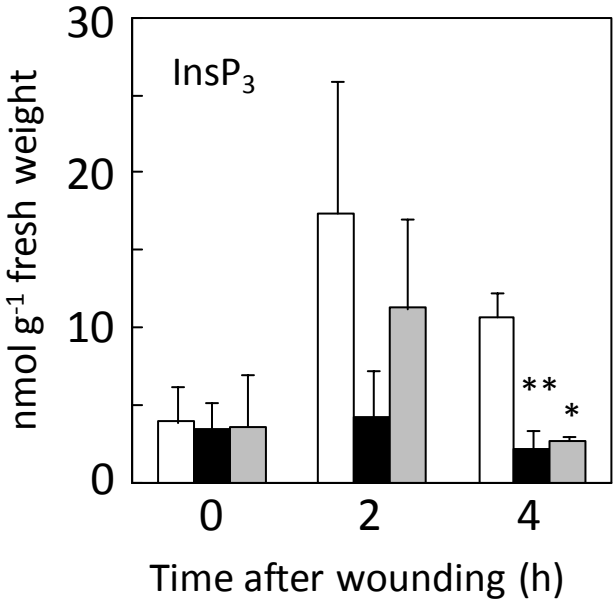

Fig. 24: Wound-induced $InsP_3$ levels in SA-deficient mutants *sid2* and *NahG*. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after the indicated times. $InsP_3$ levels were determined via a competitive protein binding assay in wild type plants (white columns) and *sid2* (black columns) and *NahG* (grey columns). Mean values of two wounding experiment are shown, assayed in duplicates, ± SD. Asterisks indicate significant differences between levels detected in wild type and in *sid2* plants or in *NahG* plants, respectively, at the indicated time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$).

Taken together, both SA-deficient plant lines exhibited reduced or even abolished $InsP_3$ induction with wounding. Whether this is a direct consequence of interference with SA content or rather has an indirect cause, possibly including lowered JA-Ile levels, remains unclear at this point.

After indicating SA signals to be relevant for proper $InsP_3$ accumulation with wounding, reciprocally the requirement of PI signals for SA formation with wounding was tested. SA levels were determined in unwounded and wounded *InsP 5-ptase* and *ipk1-1* plants, both differently impaired in PI signalling (Fig. 25). The measurement of SA content in *InsP 5-ptase* and wild type

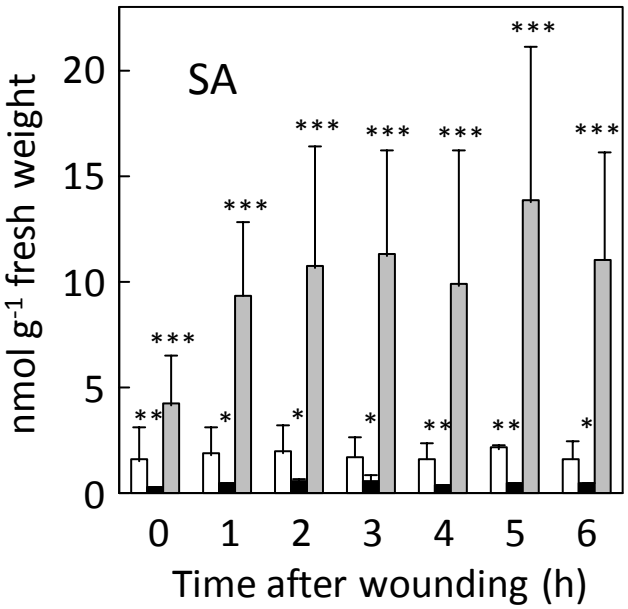

Fig. 25: Wound-induced SA levels in plants disturb in PI signalling. Rosette leaves of 7-week-old plants were wounded with forceps and harvested after the indicated times. SA levels were determined in wild type plants (white columns) and *InsP 5-ptase* (black columns) and *ipk1-1* (grey columns). Mean values of two wounding experiment are shown, assayed in duplicates, and each duplicate measured twice, ± SD. Missing error bars are too small to appear. Asterisks indicate significant differences between levels detected in wild type and in *sid2* plants or in *NahG* plants, respectively, at the indicated time point, according to a student's *t*-test (*, $p \leq 0.1$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

plants was conducted by GC/MS and in *ipk1-1* and wild type plants via LC/MS. As wild type SA levels matched between the two different methods, both results were joined in one figure (Fig. 25).

While global reduction of PI signalling components in *InsP 5-ptase* plants resulted in reduced levels of SA compared to wild type (wild type average levels of 1.8 ± 0.2 nmol g^{-1} fresh weight, *InsP 5-ptase* average levels of about 0.5 ± 0.1 nmol g^{-1} fresh weight), interruption of $InsP_5$ to $InsP_6$ conversion in *ipk1-1* plants resulted in a pronounced accumulation of SA with its maximum of 17.4 ± 8.5 nmol g^{-1} fresh weight 5 h after wounding, strongly exceeding wild type levels. Furthermore, SA levels were wound-inducible in *ipk1-1* plants, whereas SA levels proceeded rather constant in wild type and *InsP 5-ptase* plants. The effects observed in *ipk1-1* plants may be either caused by the absence of $InsP_6$, or by accumulation of precursors as $InsP_5$ or $InsP_4$.

4 DISCUSSION

Previous experiments have demonstrated that PI signals are implicated in the Arabidopsis wounding response, indicated by an increase in $InsP_3$ levels over a period of 6 h after wounding. Furthermore, wound-induced JA was indicated as an upstream factor of PI signals, as JA deficient *dde2-2* plants failed to accumulate wound-induced $InsP_3$ (Mosblech et al, 2008).

4.1 JA as an upstream factor of PI signals

To test, if JA is sufficient for $InsP_3$ induction, Arabidopsis rosette leaves were floated on MeJA solution (Fig. 6 A). Whereas this exogenous MeJA application did not induce $InsP_3$ accumulation, sorbitol treatment known to trigger endogenous JA formation caused a substantial $InsP_3$ induction (Fig. 6 B). Assuming that exogenous sorbitol treatment triggers the endogenous production of JA in plants, as has been suggested before (Reinbothe et al, 1994; Stenzel et al, 2003b; Weichert et al, 2000), these data are consistent with a role of JA upstream of $InsP_3$ in a linear signalling pathway. However, it cannot be differentiated, if sorbitol itself or JA or another factor induced by sorbitol treatment caused the accumulation of $InsP_3$. Importantly, the mechanism by which sorbitol treatment influences JA signals has not been delineated, and therefore these data must be interpreted with caution.

DNA chip-based analysis of gene expression in Arabidopsis by Lin et al (2004b) revealed that PI signalling-related genes were induced by a variety of stimuli. Among those upregulated by MeJA treatment were inositol 1,4,5-trisphosphate 3-kinase and PLC, both involved in the formation of $InsP_4$ and $InsP_5$, supporting the hypothesis that JA is an upstream factor of inositol polyphosphate signals.

4.2 PI signals required for wound responses and defence

Alteration of gene expression patterns is the key to the manifestation of specific stress responses. In order to elucidate the impact of PI signals on Arabidopsis wound responses, wound-inducible gene expression was monitored in wild type and *InsP 5-ptase* plants (Fig. 7). Transcript accumulation upon wounding was lowered or delayed for various genes in *InsP 5-*

ptase plants, including COI1-dependent (*AOS* or *VSP1*) as well as COI1-independent genes (e.g. *RNS1* or *OPR1*), suggesting that PI signals influence wound-induced gene expression in a JA-dependent as well as in a JA-independent manner. Moreover, the different expression patterns in *InsP 5-ptase* plants, displaying attenuation of early (*AOS*) or late expression (*RNS1*) or an overall delay in expression (*OPR1*), imply a complex network of PI-dependent mechanisms for the regulation of gene expression. This interpretation is supported by biochemical results, indicating that PI signals not only play a role in JA signalling but are also involved in auxin signalling (Mosblech et al, 2008) as well as in SA signalling (Fig. 24 and 25). The strongly reduced expression of WRKY70 in *InsP 5-ptase* plants furthermore enhances the effect of PI signals in SA signalling or possibly within the crosstalk between JA and SA signals, since WRKY70 represents a regulatory node between both, as it positively regulates SA-mediated defences while repressing JA-mediated responses (Li et al, 2006; Li et al, 2004). In addition, altered WRKY70 expression suggests effects of PIs on the induction of *cis*-regulatory elements. So far, there are few examples for effects of PIs on transcription factors. An example for directly PI-dependent regulation of gene expression is presented by the mammalian transcription factor TUBBY, which binds to the plasma membrane in the presence of PtdIns(4,5)P_2 and translocates to the nucleus upon PtdIns(4,5)P_2 hydrolysis (Santagata et al, 2001).

To further delineate the impact of PI signals on plant defence responses, resistance to insect feeding was assessed by caterpillar growth performance tests (Fig. 8). Caterpillar growth with feeding on *InsP 5-ptase* plants was significantly faster than that on wild type plants and resembled that observed with feeding on *dde2-2* plants (Fig. 8), suggesting that PI signals are of similar importance for Arabidopsis defence against herbivores as JA. Wound-induced production of defensins, which affect caterpillars´ digestion of plant material, has been shown before to be JA-dependent, as caterpillars feeding on JA-deficient plants grew larger compared to those feeding on wild type plants (Chen et al, 2005; Kessler et al, 2004; Liu et al, 2005). The outcome of the feeding performance test on *dde2-2* plants in this thesis (Fig. 8) closely resembled these earlier studies. The observations of this experiment support the notion that PI and JA signals act in the same signalling cascade. Moreover, the enhanced caterpillar performance on *InsP 5-ptase* plants implicates ecophysiological relevance of PI signals in mediating wound-induced plant defence reactions.

4.3 Are PI signals required for sensitivity towards JA-Ile?

Interestingly, JA as well as its precursors and also the bioactive form JA-Ile were elevated over wild type levels upon wounding in *InsP 5-ptase* plants over a 24 h period (Fig. 9), a time span within which the first differences of caterpillar growth performance on different plant lines became obvious (Fig. 8). This observation strengthens the impact of PI signals in plant defence responses, as wound-inducible gene expression and also defence against caterpillars was weakened in *InsP 5-ptase* plants, despite of elevated levels of JAs and JA-Ile. Furthermore, the combination of reduced wound response together with high levels of JA-Ile points towards the hypothesis that *InsP 5-ptase* plants impaired in PI signalling exhibit a partial insensitivity towards JA signals.

To compare *InsP 5-ptase* plants with established JA-insensitive plants, *coi1* mutants were tested for their wound-induced oxylipin pattern (Fig. 11). The here newly established T-DNA insertion line *coi1-t* exhibited JA-insensitive features known for the commonly used EMS line *coi1-1* (Fig. 10). When tested for wound-induced oxylipins, both *coi1-1* and *coi1-t* displayed reduced induction of JA and precursors, but elevated levels of JA-Ile, clearly exceeding wild type levels (Fig. 11). However, while JA-Ile levels of *InsP 5-ptase* plants displayed a similar pattern (Fig. 9), the wound induction of JA and JA precursors showed opposite patterns between *coi1* mutants (Fig. 11) and *InsP 5-ptase* plants (Fig. 9), as the latter accumulated these jasmonates as well over wild type levels. This may indicate that the conversion of JA to JA-Ile is either negatively regulated by COI1 or independently of COI1 by another factor. As several enzymes of JA biosynthesis are known to be COI1-dependently expressed (Reymond et al, 2000), it is comprehensible that levels of JA and JA precursors are reduced in *coi1* plants. However, the elevated levels of these compounds in *InsP 5-ptase* plants may be caused by a COI1-independent but PI-regulated mechanism.

The notion of partial insensitivity of *InsP 5-ptase* plants towards JA-Ile raised the question why a reduction of inositol-containing signalling factors would affect JA sensitivity. Based on the information that an $InsP_6$ is bound as a cofactor in the auxin receptor TIR1, it was a key working hypothesis of this thesis that an inositol polyphosphate is required as a cofactor also in the JA receptor COI1.

4.4 Exchange of putative inositol polyphosphate coordinating amino acids reduces COI1 functionality

To test the hypothesis of a direct influence of an inositol polyphosphate on COI1 function, different $coi1_{mut}$ variants were created (Fig. 12 B). The results of the yeast two-hybrid experiments (Fig. 13) specified the mutated amino acid residues as being important for COI1 interaction with JAZ9, and even the exchange of single amino acids already impaired the $coi1_{mut}$/JAZ9 interaction. Manipulation of amino acid residues may result in protein misfolding and subsequent protein degradation. Western blot analysis (Fig. 13 B) showed that all $coi1_{mut}$ variants were expressed to similar levels and not degraded, suggesting that reduced protein-protein interactions of $coi1_{mut}$ and JAZ9 were due to reduced inositol polyphosphate binding rather than lower expression levels of the transgenes.

Since yeast is an artificial system to investigate the interaction of plant proteins, it was tested whether the reduced interaction of $coi1_{mut}$ variants with JAZ9 as determined in the yeast system translated into a reduced functionality of the COI1 protein *in planta*. Therefore, the $coi1_{mut}$ variants were tested for altered functionality in *Arabidopsis* mutant complementation tests of the *coi1-t* mutant. Data presented in Fig. 14 demonstrate that the exchange of single amino acids resulted in reduced complementation ability, depending on the exchanged amino acid position. As was the case for the $coi1_{mut}$/JAZ9 interaction in yeast (Fig. 13), multiple exchanges led to severe reduction or complete loss of complementation ability also in Arabidopsis. It is important to note that with the exception of $coi1_{K81A}$ and $coi1_{R121A}$ single exchange variants completely lost their interaction potential with JAZ9 in yeast, but some single exchange variants still showed complementation potential in Arabidopsis, for example $coi1_{R516A}$ and $coi1_{R348A}$. This observation may be explained by the higher complexity of the plant model and in particular by the existence of other JAZ proteins, which can possibly still interact with some of the $coi1_{mut}$ variants. Furthermore, the JA-Ile analogue coronatine used in the yeast two-hybrid tests may influence the interaction of COI1 with its target proteins differently than the plant endogenous JA-Ile. Although JA-Ile is the proposed bioactive form of JA (Fonseca et al, 2009), other JA-amino acid conjugates, such as JA-leucin and JA-valin, can also promote the COI1/JAZ interaction (Katsir et al, 2008), and different JA-amino acid conjugates may, therefore, influence COI1/JAZ binding differently in different aspects of plant life.

To confirm that the function of the $coi1_{mut}$ variants was not only abolished in the artificial situation of exposing the seedlings to exogenous MeJA (Fig. 14 A), the development of functional siliques was determined (Fig. 14 B). There were slight differences upon complementation of the *coi1-t* mutant with $coi1_{mut}$ variants carrying single exchanges between

complementation of the root growth phenotype and that restoring fertility. For instance, $coi1_{R121A}$ partially complemented the root growth phenotype, but only poorly restored fertility. The opposite effect was observed for $coi1_{R516A}$, which resulted in only partial complementation of seed development but exhibited close to full complementation of the root growth phenotype (Fig. 14 A). With regard to root growth and reproduction, different JAZ proteins as well as different downstream signalling elements, such as MAP kinases (MPK6) or transcription factors may be involved (Memelink, 2009). Besides slight differences in the two single amino acid exchange variants mentioned, the overall outcome of the silique analysis closely resembled the results from the root growth assays. Furthermore, with only minor differences in the details between the yeast two-hybrid tests and the Arabidopsis mutant complementation experiments, the results display similar patterns in these two independent model systems, thus, strongly supporting the notion that interfering with inositol polyphosphate binding in COI1 impairs COI1 function.

Whereas variable expression levels could be ruled out for the yeast experiments (Fig. 13), none of the COI1-derived protein variants were directly detectable in Western blots using Arabidopsis extracts. The analysis of mRNA levels by semi-quantitative RT-PCR revealed no correlation between specific transcript levels and complementation efficiency (Fig. 16). Furthermore, the analysis of fluorescence intensities of the marker protein DsRed suggests that differences in expression levels arising from positional effects of the genomic insertion loci did not contribute to the complementation patterns observed (Fig. 15). Importantly, DsRed intensities were strongest with constructs displaying the lowest complementation capabilities, an observation in line with the results from mRNA analysis. The observation that weak functional alleles of COI1 were expressed to high levels indicates that high COI1 expression is not readily tolerated by the plants. The control experiments performed indicate that differences observed in complementation capability between different $coi1_{mut}$ variants were not a result of varying expression levels but rather originated from the manipulation of the COI1 protein.

4.5 Reduced function of COI1 in backgrounds with high $InsP_5$ and low $InsP_6$

As an alternative approach to the analysis of $coi1_{mut}$ variants, and to rule out the possibility of incorrect folding or uneven expression of the altered COI1 variants, wild type COI1 was tested for its functionality in yeast and plant backgrounds with altered inositol polyphosphate composition. In both yeast and Arabidopsis the enzyme IPK1 catalyzes a key step in the

production of $InsP_6$. Yeast and Arabidopsis mutants deficient in *IPK1* have reduced levels of $InsP_6$ and instead accumulate precursors as $InsP_5$ (Ponnusamy et al, 2008; Stevenson-Paulik et al, 2005). This situation is markedly different from that reported for *InsP 5-ptase* plants, which have reduced levels of both $InsP_6$ and $InsP_5$ (Perera et al, 2008), as was already stressed in the introduction.

4.5.1 COI1/JAZ9 interaction in *ipk1Δ* yeast

Data from yeast two-hybrid tests show that the COI1/JAZ9 interaction was stronger in the *ipk1Δ* yeast strain than in the corresponding parental strain PJ69-4a (Fig. 17). Importantly, the Arabidopsis *ipk1-1* mutant displayed enhanced responses to JA-mediated processes, including wound responses, defensive capabilities against caterpillar feeding and growth (Figs. 18-22). Preliminary microarray data indicate that COI1 expression levels were not elevated in Arabidopsis *ipk1-1* plants (data not shown). As immunodetection of COI1 or $coi1_{mut}$ variants in plants failed, it cannot be conclusively decided whether the enhanced defence response in *ipk1-1* plants was due to altered COI1 functionality or, possibly, protein stability.

4.5.2 Biochemical characterization of *ipk1-1* plants

4.5.2.1 PI signals in *ipk1-1* plants

When *ipk1-1* plants were investigated in more detail for their wound-induced levels of $InsP_3$ and PI lipids, elevated levels of $InsP_3$ as well as of PtdIns4P and PtdIns(4,5)P_2 over wild type levels were detected (Fig. 18). Without wounding, only minor differences between *ipk1-1* and wild type were monitored, indicating that especially upon wounding these signalling compounds accumulated in *ipk1-1* plants. These data indicate that the interruption of $InsP_6$ biosynthesis not only results in accumulation of $InsP_5$ and $InsP_4$ (Stevenson-Paulik et al, 2005), but that intermediates build up, at least under stress conditions. A feature shared by both plant lines *ipk1-1* and *InsP 5-ptase* is the reduction of $InsP_6$ levels. The Arabidopsis *ipk1-1* mutant is not only affected in the composition of soluble inositol polyphosphates, but displays elevated wound-induced accumulation of all PI pathway compounds, and therefore can be viewed as a tool with opposite conditions to *InsP 5-ptase* plants, which are reduced in the whole PI signalling pathway.

4.5.2.2 JA signals in *ipk1-1* plants

Wound-induced oxylipin levels were in general slightly enhanced in *ipk1-1* plants compared to wild type controls (Fig. 19), except for oPDA levels, which were reduced. JA-Ile levels in *ipk1-1* plants displayed delayed wound induction. The course of early accumulation of oPDA and dn-oPDA accompanied by increased JA levels and delayed JA-Ile formation may reflect an early block of JA conversion to JA-Ile, which results in accumulation of precursors. At later time points, JA-Ile formation seems to proceed, decreasing oPDA and dn-oPDA levels and also lowering JA accumulation. If JA-Ile perception is more pronounced in *ipk1-1* plants due to elevated $InsP_5$ levels, this may cause an intensified feedback inhibition of JA-Ile synthesis. Why JA-Ile exceeds wild type levels at later time points is still unclear.

When oxylipin levels of *InsP 5-ptase* plants with a globally reduced PI pathway and *ipk1-1* with accumulation of PI components upstream of $InsP_6$ are compared (Fig. 9 and 19), clearly a reduction of PI signals results in accumulation of oxylipins, especially JA-Ile, whereas accumulation of most PI signals in *ipk1-1* plants has only minor effects on oxylipin levels and results only in a slight elevation. Taking into account that JA-Ile is the relevant bioactive form of JA, accumulation of PI components upstream of $InsP_6$ in *ipk1-1* plants caused a delay in JA signals, whereas the global reduction of the PI pathway in *InsP 5-ptase* plants resulted in accumulation of JA signals. Considering that in both plant lines $InsP_6$ levels are reduced, whereas other PI components are manipulated in opposite patterns, the different effects monitored in both plant lines are likely due to the up or down regulation of the remaining PI signalling components, rather than a consequence of altered $InsP_6$ levels.

Thus, it may be assumed that reduction of PI signals results in accumulation of wound-induced oxylipins while increased PI signals only slightly enhanced biosynthesis of jasmonates and rather delayed JA-Ile signals.

4.5.2.3 COI1-mediated responses in *ipk1-1* plants

As *ipk1-1* plants displayed enhanced wound-inducible gene expression and increased resistance against caterpillar feeding (Fig. 20 and 21), these plants were suggested to be hypersensitive towards jasmonates.

The increased JA sensitivity in *ipk1-1* plants may be a result of the slight elevation of jasmonates with wounding in these plants (Fig. 19). However, *InsP 5-ptase* plants with reduced JA sensitivity displayed pronounced increases in wound-induced jasmonates over wild type levels (Fig. 9), suggesting that increases in JA-Ile levels do not *per se* correlate with increased JA sensitivity. Furthermore, the JA-insensitive *coi1* mutants accumulate JA-Ile over wild type

levels, indicating that levels of jasmonates and JA sensitivity are not linked. The impact of PI signals on JA sensitivity is stressed by the fact that the levels of JA-Ile in *ipk1-1* plants were rather delayed than enhanced.

The enhanced JA sensitivity in *ipk1-1* plants may either result from reduced $InsP_6$ levels or from accumulation of $InsP_5$ or other upstream components of the PI pathway. Differences between *ipk1-1* and wild type plants were most pronounced in the levels of $InsP_5$ (about 30 times higher without stimulus; Stevenson-Paulik et al, 2005) and lower phosphorylated inositols, while differences in PI lipid levels were less distinct (with wound stimulus not exceeding three times increases over wild type levels; Fig. 18 B). Thus, it is more likely that the observed increased wound response and defence in *ipk1-1* plants results from increased inositol polyphosphates rather than from increases in PI lipids. Alteration of putative inositol polyphosphate binding residues resulted in clearly lowered COI1 functionality (Figs. 13 and 14), rather than increasing it, and it appears unlikely that reduced $InsP_6$ production would enhance responses to JA-mediated processes. Furthermore, the comparison with data from experiments on *InsP 5-ptase* plants, in which both $InsP_6$ and $InsP_5$ are reduced and which showed attenuated wound responses (Fig. 7 and 8; Mosblech et al, 2008), suggests that accumulation of $InsP_5$, not a lack of $InsP_6$, might be the relevant factor. The patterns of complementation of the Arabidopsis *coi1-t* mutant by different $coi1_{mut}$ variants is also consistent with the hypothesis that the increased JA sensitivity observed in *ipk1-1* plants is a direct effect of alterations in inositol polyphosphates influencing COI1 functionality rather than having an indirect cause. In conclusion, the data indicate that the inositol polyphosphate required for COI1 function is likely $InsP_5$.

4.6 $InsP_5$ as a cofactor of COI1

The hypothesis of $InsP_5$ supporting COI1 function is additionally supported by studies performed on the CSN of mammalian cells. The inositol 1,3,4-trisphosphate 5/6 kinase (5/6 kinase) phosphorylates $InsP_3$ to form $InsP_4$, the rate limiting step of $InsP_5$ and $InsP_6$ biosynthesis in mammals (Wilson et al, 2001). Interestingly, the 5/6 kinase co-purifies with the CSN from cow brain (Wilson et al, 2001) and is conserved in sequence between kingdoms from plants to humans. It is conceivable that the association of such an enzyme with the CSN, which assembles with SCF^{COI1} also in Arabidopsis (Feng et al, 2003), provides the catalytic activity for synthesis of an inositol polyphosphate in close proximity to the COI1 protein.

Wounding-induced biosynthesis of inositol polyphosphates raises the question for the benefit of a transiently produced cofactor. In wild type yeast as well as in wild type plants the reported basal $InsP_5$ levels are very low (Stevenson-Paulik et al, 2005; York et al, 1999). However, the published data only report inositol polyphosphate levels for non-stimulated cells. While there is currently no information about $InsP_5$ levels after wounding or other JA-inducing stimuli, we have previously shown that $InsP_3$ levels strongly increase in Arabidopsis upon wounding (Mosblech et al, 2008), which may provide intermediates for further phosphorylation and production of $InsP_5$. This $InsP_3$ accumulation was even enhanced in *ipk1-1* plants (Fig. 18 A). Considering the effects of $InsP_5$ on COI1 function described here, increased production of $InsP_5$ might be part of an activating mechanism for COI1, which might enhance its affinity to JA-Ile or its target proteins.

While all data presented can be explained by focusing on known information on COI1, it is clear that plant signalling is highly complex and COI1 will not be the only player influenced by perturbed inositol polyphosphate metabolism. In this context, it should be noted that the increased complexity of the plant systems is not present in the yeast model, which served as an independent control to support reported findings made in Arabidopsis. In any case, indirect effects of altered inositol polyphosphate levels must also be considered when explaining the observations made. Since *ipk1-1* plants display low levels of $InsP_6$ (Stevenson-Paulik et al, 2005) it is likely that, for instance, auxin signalling is affected, because $InsP_6$ is bound as a cofactor to TIR1 (Tan et al, 2007). Manipulations in $InsP_6$ content can possibly lead to altered auxin signals, which could then directly or indirectly influence JA signals. Furthermore, cation balance may be influenced by $InsP_6$ reduction, as Ca^{2+} release was reported upon $InsP_6$ formation (Lemtiri-Chlieh et al, 2003), which might also be relevant for wound responses (Howe & Jander, 2008). Additionally, $InsP_6$ may build complexes with divalent cations and also affect K^+ ion channels (Nagy et al, 2009), and cation imbalances may have multiple consequences for various cellular processes including stress responses. However, such scenarios are not supported by current data and must remain speculative at this point.

The data obtained on $InsP_5$ function in COI1 in this thesis are strongly supported by a concurrent study from Sheared et al (John Browse, personal communication; data submitted to nature). Besides demonstrating that COI1 together with JAZ proteins build the receptor for JA-Ile, their data on the COI1 crystal structure together with MS data and additional pharmacological results also indicate $InsP_5$ as a cofactor of COI1. When $InsP_5$ was removed from purified COI1 protein, only trace activity of JA-Ile binding in the presence of JAZ1 protein was revealed, but this activity was restored by the addition of exogenous $InsP_5$. Interestingly, only the isomer $Ins(1,2,4,5,6)P_5$ was found to copurify with COI1. The $InsP_5$ isomer

accumulating in *ipk1-1* mutants is Ins(1,3,4,5,6)P_5, whereas the isomer detectable in wild type Arabidopsis is Ins(1,2,4,5,6)P_5 (Stevenson-Paulik et al, 2005). Possibly, COI1 also interacts with non-favoured isomers of $InsP_5$ if they are supplied in high concentrations, as it is the case in *ipk1* mutants.

Sheared et al. also identified positions K81, R121, R348 and R409 but not R516 as being likely involved in inositol phosphate binding, and additionally they identified 14 other residues to have contact to phosphate groups. This on one hand confirms four of the five residues that were tested in this thesis for their role in $InsP_5$ binding, and on the other hand highlights that $InsP_5$ binding of COI1 is far more complex than expected. Nonetheless, the study by Sheared at al. strongly supports the findings of this thesis. While their protein biochemical data represent proof that $InsP_5$ is bound to COI1, the experiments conducted in this thesis depict the physiological importance of this cofactor for plant defence as well as plant development.

In conclusion, the combined data from yeast and plant models as well as the comparison of relevant transgenic and mutant lines of Arabidopsis presented in this thesis give a well-defined picture of how $InsP_5$ is important for the regulation of COI1 functionality. The emerging view suggests that upon wounding the formation of $InsP_5$ commences, which might act as a cofactor in COI1 to mediate increased affinity to COI1 substrates. This notion is in line with previously proposed concepts (Heilmann et al, 2001; Perera et al, 2001) suggesting that upon stress an initial “wake up call” activates a whole set of internal signals, which are afterwards further integrated to establish a specific signal signature initiating an appropriate response for each stimulus perceived.

4.7 Integrating PI signals into a greater signalling network: a switch between JA and SA signalling?

Having addressed the detailed mode of action of inositol polyphosphates in JA-Ile perception by COI1, the question remains how this information must be interpreted in a greater view of plant signalling.

Analysis of jasmonates in SA-deficient Arabidopsis lines revealed that wound-induced oxylipin formation was reduced or delayed, with JA-Ile levels showing the most pronounced reduction compared to wild type induction (Fig. 23). This may indicate that JA and SA signals act synergistically during wound responses, a concept in contrast to results observed upon pathogen infection. Spoel et al (2003) reported strongly elevated JA accumulation in *NahG*

plants after inoculation with *P. syringae*, while in wild type plants only minor increases of JA were detectable. Whereas JA and SA seem to antagonize one another during responses to pathogen infection, responses to wounding may require both JA and SA, with SA acting upstream of JA signals. Synergistic effects were reported upon JA and SA application at low concentrations (Loake & Grant, 2007; Mur et al, 2006).

To elucidate the relation between PI and SA signals during wound responses, wound-induced $InsP_3$ levels were determined in *sid2* and *NahG* plants (Fig. 24) and, reciprocally, wound-induced SA-levels were determined in *InsP 5-ptase* and *ipk1-1* plants (Fig. 25). $InsP_3$ accumulation was reduced in both *sid2* and *NahG*, indicating that SA signals are relevant for PI signal induction upon wounding. This observation is in line with reports on Arabidopsis cell suspension cultures, in which PtdInsP(4,5)P_2 formation driven by increased PI4-kinase activity was inducible by SA treatment (Krinke et al, 2007b). However, $InsP_3$ accumulation was not SA-inducible in the cell culture, which may specify PI lipids as a factor being induced by SA alone. The wound stimulus may then induce further metabolism of PI lipids to form $InsP_3$ and other soluble inositol polyphosphates. As $InsP_3$ accumulation also failed in JA-deficient *dde2-2* plants (Mosblech et al, 2008), it is conceivable that both SA and JA are required in combination for PI induction upon wounding.

In a reciprocal experiment, when *InsP 5-ptase* and *ipk1-1* plants were tested for their wound-induced SA contents (Fig. 25), PI signals seemed to be required for SA formation. *InsP 5-ptase* plants with reduced PI signals displayed strongly reduced SA levels, whereas *ipk1-1* plants exhibited SA amounts strongly elevated over those of wild type controls (Fig. 25). This pattern indicates that PI signalling components other than $InsP_6$, which accumulate in *ipk1-1* plants, may boost SA accumulation upon wounding.

The results allow speculation on a self-reinforcing loop of JA, SA and PI signals in the Arabidopsis wound response. A possible network is depicted as a simplified model in Fig. 26. Upon wounding, in the first place JA-Ile is rapidly formed (Fig. 26, point 1; Glauser et al, 2008) and required for induction of PI signals, as indicated by JA-deficient *dde2-2* plants, which do not accumulate $InsP_3$ upon wounding (Fig. 26, point 2; Mosblech et al, 2008). PI signals in turn promote SA formation (Fig. 26, point 3). This notion is supported by the finding that *InsP 5-ptase* plants exhibited reduced SA-levels, whereas *ipk1-1* plants exhibit elevated SA levels (Fig. 25). SA accumulation then further induces JA-Ile signals or promotes the maintenance of the JA-Ile signal (Fig. 26, point 4), as SA-deficient mutants contained reduced levels of JA-Ile upon wounding (Fig. 23). Furthermore, the reduced wound induction of $InsP_3$ signals in SA-deficient mutants (Fig. 24) suggests that SA is required for the induction of the PI pathway or

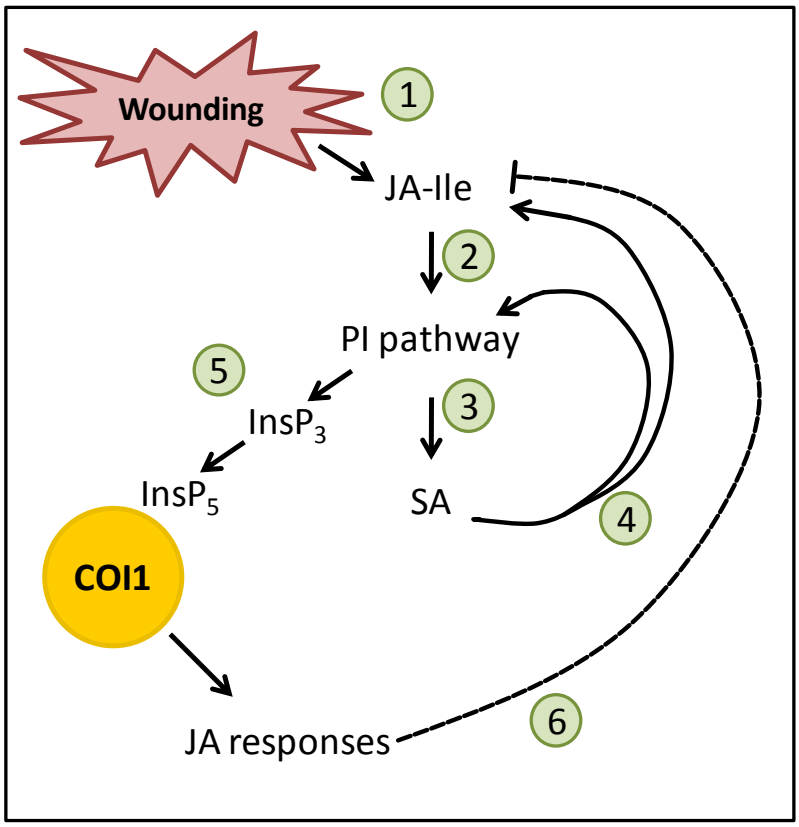

Fig. 26: Simplified model of JA/SA/PI action in Arabidopsis wound response. Black arrows indicate synergistic effects, dashed line indicates antagonistic effect. Explanation of numbers 1-6 see text.

to maintain PI signalling (Fig. 26, point 4). Data represented in this thesis suggest that JA-Ile can best be perceived with $InsP_5$ present in the COI1 protein. To allocate sufficient amounts of $InsP_5$, SA may activate a PI4-kinase and the PI pathway is put into operation (Fig. 26, point 4; Krinke et al, 2007b). With $InsP_5$ bound in the COI1 protein (Fig. 26, point 5), JA responses may proceed and induce appropriate gene expression as well as a repression of JA-Ile signals (Fig. 26, point 6), as *coi1* mutants and also *InsP 5-ptase* plants exhibit increased wound-induced accumulation of JA-Ile. In this model, the role of SA would be to potentiate the wound-induced JA signal in close interaction with PI signals. A process like this would offer various points to fine tune the signal composition in order to achieve a specific and targeted defence response.

The model presented in Fig. 26 is based on a limited volume of data and must remain largely speculative. As potato plants with altered inositol polyphosphate metabolism and deficient in $InsP_6$ formation exhibited reduced resistance to viruses, bacteria and fungal pathogens (Murphy et al, 2008), it may be speculated that $InsP_6$ is also involved in SA-mediated defence, whereas $InsP_5$ supports JA-mediated defence. The balance between $InsP_5$ and $InsP_6$ might display a switch to push the defence reaction in one or the other direction, and possibly there is a link to auxin signalling via TIR1. JA and auxin signals are both perceived via similar

machineries, including F-box protein receptors that are part of SCF E3 ubiquitin ligase complexes. Importantly, the auxin receptor TIR1 carries an $InsP_6$ as cofactor, whereas the JA receptor COI1 requires an $InsP_5$ cofactor, as demonstrated in this thesis. Truman et al (2010) have proposed a model, in which JA signals induced by pathogen infection are followed by a phase of auxin signals, which in turn initiate SA signalling. In such a model, the transition from JA to auxin signals could be supported by a change in $InsP_5$/$InsP_6$ balance, which would influence the sensitivity of either the JA or the auxin receptor. In fact, this suggestion does not interfere with the proposed scheme presented in Fig. 26, but would rather complete the suggested model.

Future analysis may fill some of the informational gaps and reveal even more links between signalling pathways that should be viewed as an integrated and interdependent signalling network of exceeding complexity.

4.8 Conclusions

The data presented in this thesis indicate that the production of inositol polyphosphates in Arabidopsis is important for sensitivity of JA perception. The proposed mechanism for this effect involves binding of $InsP_5$ as a cofactor to COI1, which is altered by this interaction either in its affinity to JA-Ile itself, its target protein, JAZ, or in its inherent stability. Stress-induced increases in inositol polyphosphates might be part of a fine-tuning mechanism for phytohormone receptors, including COI1 and TIR1. By redeploying the production of $InsP_5$ or $InsP_6$, different stress consitions may influence the plant´s signalling network towards effects mediated by JA or auxin. At this point it is clear that other mediators, such as SA, are also involved in this regulation; the precise contribution of these factors, however, remains open for future investigations.

5 SUMMARY

Plant responses to wounding are of central importance during defence against herbivores and therefore tightly regulated. Hereby the isoleucin conjugate of jasmonic acid (JA-Ile) is a major regulatory molecule. In this thesis, phosphoinositide (PI) signals not previously implicated in plant wound responses have been studied and are introduced as new components active in the signalling network regulating plant defence responses.

Transgenic Arabidopsis *InsP 5-ptase* plants that are attenuated in PI signalling exhibited decreased wound-induced gene expression as well as reduced defence against caterpillar feeding. These results indicate that wound-induced accumulation of PI signals is required for full wounding and defence responses. Besides their attenuated defence, *InsP 5-ptase* plants accumulated JA-Ile in a pattern also found for JA-insensitive Arabidopsis *coi1* mutants. These data suggest that *InsP 5-ptase* plants are partially insensitive towards JA-Ile. To explain the interdependency of PI-derived signals and JA-Ile perception, the Arabidopsis JA-Ile receptor, COI1, was compared with the auxin receptor, TIR1, which contains an inositol hexakisphosphate ($InsP_6$) cofactor. Based on the structural similarity of COI1 and TIR1 the possibility was explored that an inositol polyphosphate cofactor is required for COI1 function. COI1 variants, in which putative inositol polyphosphate coordinating amino acids were exchanged for alanines, exhibited reduced interaction with the COI1 target, JAZ9, in yeast two-hybrid tests. Consistent with the yeast two-hybrid data, the same COI1 variants displayed reduced capability to rescue jasmonate-mediated root growth inhibition or silique development in *Arabidopsis coi1* mutants. Further yeast two-hybrid tests using wild type COI1 in an *ipk1Δ* yeast strain with increased levels of inositol pentakisphosphate ($InsP_5$) and reduced levels of $InsP_6$ indicated an enhanced COI1/JAZ9 interaction. Consistent with these findings, Arabidopsis *ipk1-1* mutants, also with increased $InsP_5$ and reduced $InsP_6$ levels, showed increased defensive capabilities via COI1-mediated processes, including wound-induced gene expression, defence against caterpillars, or root growth inhibition by jasmonate. The combined data from yeast and Arabidopsis experiments indicate that an inositol polyphosphate and likely $InsP_5$ is required for COI1 function in Arabidopsis. Experiments on salicylic acid (SA)-deficient Arabidopsis mutants suggest a further interrelation between SA and PI signals in wounding responses, establishing PI-derived signals as a central component of the regulatory network controlling Arabidopsis responses to wounding.

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7 APPENDIX

7.1 List of oligonucleotides

No.	Oligonucleotide sequence	Purpose
P1	5'-tggaccatataaattcatgcagtc-3'	Genotyping Arabidopsis: *coi1*, for
P2	5'-ctgcagtgtgtaacgatgctc-3'	Genotyping Arabidopsis: *coi1*, rev
P3	5'-tcatcatcaatgtggctgct-3'	Genotyping yeast: *ipk1*, for
P4	5'-gatacatcgggcaaagcaag-3'	Genotyping yeast: *ipk1*, rev
P5	5'-gattccatggaggatcctgatatcaagaggtgtaaattg-3'	*coi1* for, introducing NcoI
P6	5'-gattcccgggtcagtggtggtggtggtggtgtattggctccttc aggactcta-3'	*coi1* rev, introducing XmaI and 6xHis tag
P7	5'-ggtcgctcaaacttgcaggcaagcctagag-3'	QuickChange site-directed mutagenesis, *coi1* K81A_for
P8	5'-ctctaggcttgcctgcaagtttgagcgacc-3'	QuickChange site-directed mutagenesis, *coi1* K81A _rev
P9	5'-cagttgaagcggctggcgattgaacgcggtgc-3'	QuickChange site-directed mutagenesis, *coi1* R348A_for
P10	5'-gcaccgcgttcaatcgccagccgcttcaactg-3'	QuickChange site-directed mutagenesis, *coi1* R348A _rev
P11	5'-gccttcactggcatacttgtgggtacaagg-3'	QuickChange site-directed mutagenesis, *coi1* R516A _for
P12	5'-ccttgtacccacaagtatgccagtgaaggc-3'	QuickChange site-directed mutagenesis, *coi1* R516A _rev
P13	5'-cggtgcacttccgagcgatgattgtcagtg-3'	QuickChange site-directed mutagenesis, *coi1* R121A _for
P14	5'-cactgacaatcatcgctcggaagtgcaccg-3'	QuickChange site-directed mutagenesis, *coi1* R121A _rev
P15	5'-cctctgtgacttcgcccttgtcttactcg-3'	QuickChange site-directed mutagenesis, *coi1* R409A _for
P16	5'-cgagtaagacaagggcgaagtcacagagg-3'	QuickChange site-directed mutagenesis, *coi1* R409A _rev
P17	5'-gatcaagcttggccgcaacagaggtggatg-3'	*DsRed* for, introducing HindIII
P18	5'-gatcaagcttagaaggtaattatccaagat-3'	*DsRed* rev, introducing HindIII
P19	5'-gaaagtggatcgaaacgatggcctcctccgag-3'	Delet NcoI in *DsRed* 1, for
P20	5'-ctcggaggaggccatcgtttcgatccactttc-3'	Delet NcoI in *DsRed* 1, rev
P21	5'-gatgcagaagaagacgatgggctgggaggcc-3'	Delet NcoI in *DsRed* 2, for
P22	5'-ggcctcccagcccatcgtcttcttctgcatc-3'	Delet NcoI in *DsRed* 2, rev
P23	5'-gatcgtcgactcagtggtggtggtggtggtgtattggc-3'	*Coi1* rev, introducing SalI
P24	5'-aaaattgtcagagataagttccttttttgaaaagaaagatcga tgcgtacgctgcaggtcgac-3'	Yeast ko, *ipk1*, for

7.1 List of oligonucleotides (continued)

No.	Oligonucleotide sequence	Purpose
P25	5‘-gtgcatctgccagtaccaaaggtggaaagaaaagtatacag tttaatcgatgaattcgagctcg-3‘	Yeast ko, *ipk1*, rev
P26	5’-gtcgcgacggttgatgat-3’	Detection of *coi1*$_{mut}$ in *coi1-t* plants, semi-quantitative RT-PCR, for
P27	5’-ggtggtggtggtggtgtatt-3’	Detection of *coi1* $_{mut}$ in *coi1-t* plants, semi-quantitative RT-PCR, rev
P28	5’-gattatggaggatcctgatatcaagaggt-3’	Detection of *coi1* in *coi1-t* plants, semi-quantitative RT-PCR, for
P29	5’-gatttcatattggctccttcaggactcta-3’	Detection of *coi1* in *coi1-t* plants, semi-quantitative RT-PCR, for

7.2 List of abbreviations

5/6 kinase	Inositol 1,3,4-trisphosphate 5/6 kinase
A. tumefaciens	*Agrobacterium tumefaciens*
ABA	Abscisic acid
Amp^R	Ampicillin
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ASK	ARABIDOPSIS SKP1-LIKE
ATP	Adenosine trisphosphate
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
cDNA	Complementary desoxyribonucleic acid
CH_3OH	Methanol
$CHCl_3$	Chloroform
$CoCl_2$	Cobalt chlorite
COI1	CORONATINE INSENSITIVE 1
coi1-1	*Coronatine insensitive 1-1*
coi1-t	*Coronatine insensitive 1 T-DNA insertion line*

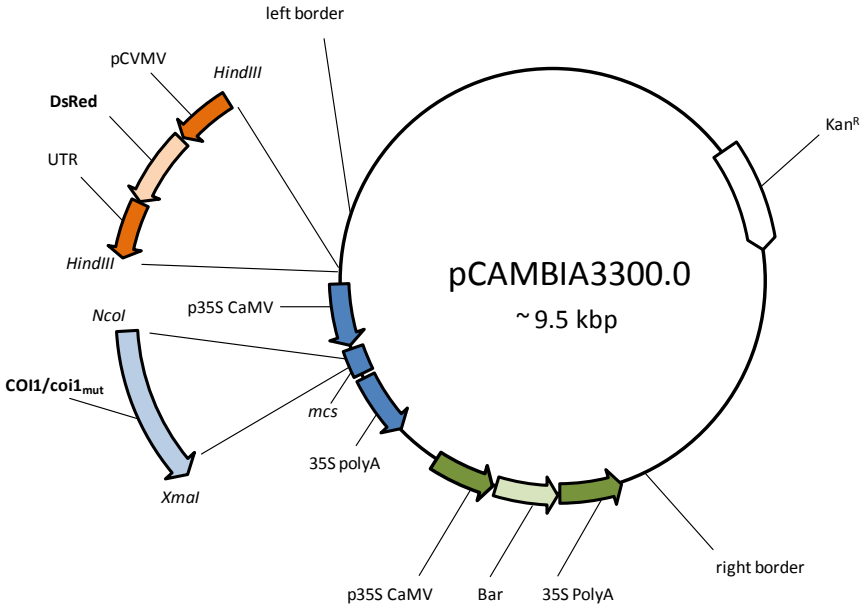
CSN	COP9 signalosome
CSN	COP9 signalosome
CTAB	Cetyltrimethylammoniumbromid
CUL1	CULLIN 1
$CuSO_4$	Copper sulfate
DAG	Diacyl glycerol
dde2-2	*Delayed dehiscence 2-2*
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DNA	Desoxyribonucleic acid
dn-oDPA	Dinor-oPDA
dNTPs	Desoxyribonucleotide
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetate
EOT	epoxy octadecatrienoic acid
ERF	Ethylene response factor
ET	Ethylene
$FeCl_3$	Iron chloride
GAD	Gal4 activation domain
GBD	Gal4 DNA binding domain
GC	Gas chromatography
GC/MS	Gas chromatography coupled to mass spectrometry
H_3BO_3	Boric acid
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIP	Hexane/isopropanol
HPOT	hydroperoxy α-linolenic acid
IAA	β-indolyl acetic acid
ICS	Isochorismate synthase
InsP 5-ptase	Inositol polyphosphate 5-phosphatase
$InsP_3$	Inositol 1,4,5-trisphosphate
$InsP_4$	Inositol 1,3,4,5-tetrakisphosphate
$InsP_5$	Inositol 1,3,4,5,6-pentakisphosphate
$InsP_6$	Inositol 1,2,3,4,5,6-hexakisphosphate

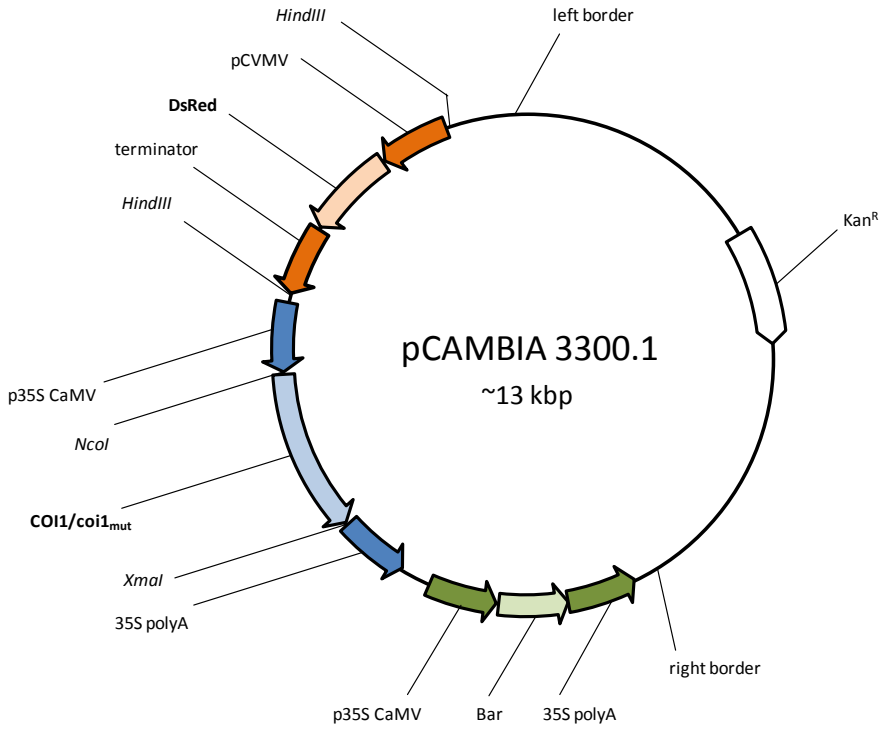
IPK	Inositol polyphosphate kinases
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucin
JAR1	JASMONIC ACID RESISTANT 1
JAZ	Jasmonate ZIM-domain
K_2HPO_4	Dipotassium phosphate
Kan^R	Kanamycin
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
LC/MS	Reversed-Phase High Performance Liquid Chromatography coupled to mass spectrometry
LOX	Lipoxygenase
LRR	Leucin-rich repeat
MAP	Mitogen-activated protein
MeJA	Methyl-JA
MeOH	Methanol
$MgCl_2$	Magnesium chlorite
$MgSO_4$	Magnesium sulfate
$MnSO_4$	Manganese sulfate
MPK	Mitogen-activated protein (MAP) kinase
mRNA	messenger RNA
MS	Mass spectrometry
MTBE	Methyl-*tert*-butyl ether
MYB	Transcription factors are named after the symptoms the virus of their first discovery causes: myeloblastosis
MYC2	JA-responsive transcription factor named after the symptoms the virus of their first discovery causes: myelocytomatosis
Na_2MoO_4	Sodium molybdate
NaCl	Sodium chlorite
NaClO	Sodium hypochlorite
NahG	Salicylate hydroxylase
NaOH	Sodium hydroxide

NEDD	Neural precursor cell expressed developmentally downregulated protein
$(NH_4)_2HPO_4$	Diammonium phosphate
NH_4OH	Ammonium hydroxide
NPR1	NONEXPRESSOR OF PR GENES 1
ONPG	o-nitrophenyl-β-D-galactopyranoside
OPC 6:0	oxo pentenyl cyclopentane hexanoic acid
OPC 8:0	oxo pentenyl cyclopentane octanoic acid
oPDA	oxo phytodienoic acid
OPR	oPDA reductase
P. syringae	*Pseudomonas syringae*
PAL	Phenylalanine lyase
PCR	Polymerase chain reaction
PH	Pleckstrin homology
PI	Phosphoinositide
PI4-kinases	PtdIns 4-kinases
PIP5-kinases	PtdIns4P 5-kinases
PI-PLCs	PI-specific PLCs
PKC	Protein kinase C
PLC	Phospholipase C
PR	Pathogenesis related
PtdIns	Phosphatidylinositol
PtdIns4,5P_2	Phosphatidylinositol 4,5-bisphosphate
PtdIns4P	Phosphatidylinositol 4-phosphate
PtdOH	Phosphatidic acid
QLIT	Quadrupole-linear ion trap
RBX	RING-BOX PROTEIN
RNS	Ribonuclease
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic acid
SCF	Skip, Cullin, F-box
sid2	*SA induction-defective 2*
TRIS	Tris(hydroxymethyl)aminomethane
VSP	Vegetative storage protein

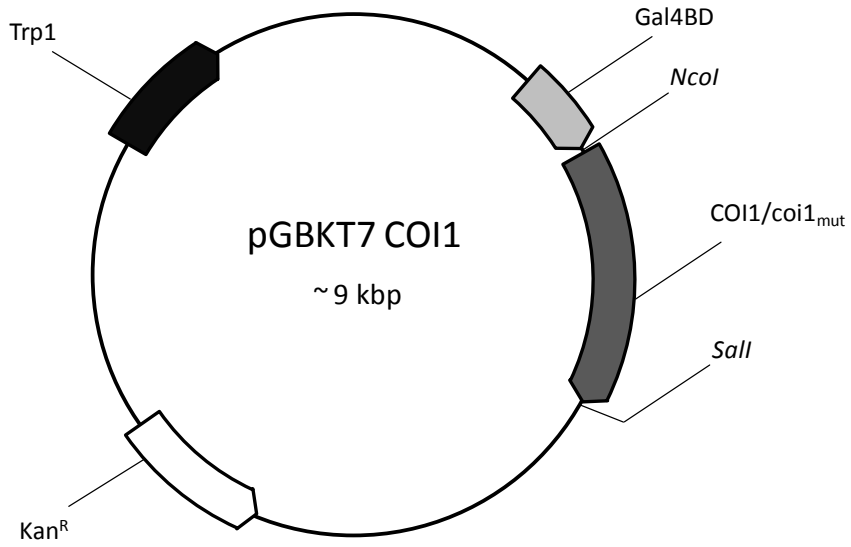
WRKY	Transcription factor, named after their WRKY domain, defined by the conserved amino acid sequence WRKYGQK
ZIM	Zinc-finger protein expressed in Inflorescence Meristem
$ZnSO_4$	Zinc sulfate
α -LeA	α-linolenic acid

7.3 Plasmid maps





Representation of the pCAMBIA plasmid, used for Arabidopsis transformation. The DsRed cassette consisting of a CVMV promoter, the DsRed coding region and a standard terminator was introduced to pCAMBIA3300.0 via *HindIII* restriction sites. The *COI1* and *coi1*$_{mut}$ coding regions were introduced into the multiple cloning site via *NcoI* and *XmaI* restriction sites, behind the 35S CaMV promoter and ahead of the 35S polyA terminator. The pCAMBIA vector containing the DsRed cassette and the *COI1* or *coi1*$_{mut}$ coding regions was named pCAMBIA3300.1



Representation of the pGBKT7 plasmid used for yeast transformation for yeast two-hybrid assays. The *COI1* and $coi1_{mut}$ coding regions were introduced via *NcoI* and *SalI* restriction sites, in frame with the Gal4BD coding region.

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9 CURRICULUM VITAE

Name	Alina Mosblech
Address	Department of Plant Biochemistry
	Albrecht-von-Haller-Institute of Plant Sciences
	Georg August University Göttingen
	Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany
Phone	[redacted]
Home address	[redacted]
Email	[redacted]

Nationality	German
Status	[redacted]
Date and place of birth	July 7th, 1980, in Paderborn, Germany

Scientific Education and Qualifications

Studies

2007-2010	**Doctoral thesis** at the Albrecht-von-Haller-Institute for Plant Sciences (with Prof. Dr. Ingo Heilmann) **Thesis title:** "Requirement of phosphoinositide-derived signals in the wounding response of *Arabidopsis thaliana*"
since 2008	Accepted to the Göttingen Graduate School for Neurosciences and Molecular Biosciences (GGNB), an interdisciplinary training platform for excellent doctoral students in neurosciences and molecular biosciences
2000 – 2007	Studies of **Biology** at Georg August University Göttingen; **Degree:** Diploma (grade "very good with distinction" / 1.0) **Major subject:** Biochemistry **Minor subjects:** Botany and Developmental Biology
2006	**Diploma thesis** at the Albrecht-von-Haller-Institute for Plant Sciences (with Prof. Dr. Ivo Feussner/Prof. Dr. Ingo Heilmann)

	Thesis title: "Inositol 1,4,5-trisphosphate and jasmonic acid signal transduction during the wound response of *Arabidopsis thaliana*" (in German)
2002 – 2003	ERASMUS exchange, University of Oulu, Finland
1999 – 2000	German language and literature studies and education science at Georg August University Göttingen

Presentations at International Conferences

2010	Oral presentation at the 23rd Conference of Plant Molecular Biology (Dabringhausen, Germany)
2009	Oral presentation at the 4th European Symposium on Plant Lipids (Göttingen, Germany)
	Poster at the International Botanical Congress (Leipzig, Germany)
2008	Oral presentation at the 18th International Symposium on Plant Lipids (Bordeaux, France)
2007	Oral presentation at the 3rd European Symposium on Plant Lipids (York, UK)
	Poster at the International Botanical Congress (Hamburg, Germany)

Scholarships

2010	GGNB Bridging Fund to finalize Ph.D. studies
2008 – 2009	GGNB Scholarship for Ph.D. studies
2002 – 2003	ERASMUS grant for a one-year stay in Oulu, Finland

Publications

Journal articles

Bargmann BO, Laxalt AM, Riet B, Testerink C, Merquiol E, **Mosblech A**, Reyes AL, Pieterse CM, Haring MA, Heilmann I, Bartels D, Munnik T (2009) Reassessing the role of phospholipase D in the *Arabidopsis* wounding response. *Plant Cell Environ* **32**: 837-850

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In Preparation

Mosblech A, Thurow C, Gatz C, Feussner I, Heilmann I "Jasmonic acid perception by COI1 requires an inositol polyphosphate cofactor in *Arabidopsis thaliana*" (currently under review at Proc Natl Acad Sci USA)