Phospholipid signalling in the salicylic acid pathway of Arabidopsis thaliana

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Présentée par

Mr Ondřej Krinke

Pour obtenir le grade de

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Sujet de la thèse :

Rôle de la signalisation phospholipidique dans la voie de réponse à l'acide salicylique chez *Arabidopsis thaliana*

soutenue le

devant le jury composé de :

Olga VALENTOVA	Professeur, ICT, Prague	Directeur de thèse
Alain ZACHOWSKI	Directeur de Recherche, CNRS, Paris	Directeur de thèse
Patrick MOREAU	Chargé de Recherche, CNRS, Bordeaux	Rapporteur
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DISSERTATION OF THE
UNIVERSITE PIERRE ET MARIE CURIE

Specialization

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Presented by

Mr Ondřej Krinke

To obtain the title of

DOCTOR of the UNIVERSITÉ PIERRE ET MARIE CURIE

Title of the thesis :

Phospholipid signalling in the salicylic acid pathway of *Arabidopsis thaliana*

defended on the

in front of the jury composed of:

Olga VALENTOVA	Professor, ICT, Prague	Supervisor
Alain ZACHOWSKI	Research Director, CNRS, Paris	Supervisor
Patrick MOREAU	Research Associate, CNRS, Bordeaux	Reviewer
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Résumé

Chez les plantes, l'acide salicylique (SA) a un rôle central dans la réponse à de nombreuses contraintes environnementales et lors du développement. Cependant les événements de signalisation précoces qu'il déclenche sont peu connus. Nous montrons, par marquage métabolique au $^{33}P_i$ sur une suspension cellulaire d'*Arabidopsis thaliana*, que le SA induit une diminution rapide et précoce d'un pool de phosphatidylinositol (PI). Celle-ci est accompagnée d'une accumulation de PI 4-phosphate et PI 4,5-bisphosphate. Ces changements sont inhibés par de la wortmannine à 30 µM mais pas à 1 µM, ce qui implique une activation de PI 4-kinase de type III. C'est pourquoi une étude des effets de la wortmannine sur les modifications de transcriptome par le SA a été menée à l'aide de la puce « Complete Arabidopsis Transcriptome MicroArray » (CATMA). Sur 773 gènes régulés par le SA, 112 sont sensibles à 30 µM de wortmannine. En parallèle, nous voyons que l'acide phosphatidique issu de la phospholipase D (PLD) est important pour la réponse génique précoce au SA. Une expérience de puces menée pour identifier les gènes régulés par la PLD en réponse au SA a révélé que parmi 1327 gènes régulés par le SA, 97 gènes sont régulés positivement, et 117 gènes négativement, par la PLD. Les régulons de la voie sensible à la wortmannine et de la voie PLD se chevauchent fortement, ce qui suggère que les deux activités agissent en synergie dans la même voie de signalisation en réponse au SA.

Mots clés :
Arabidopsis thaliana, Phosphatidylinositol 4-kinase, Phospholipase D, Signalisation phospholipidique, Acide salicylique, Transcriptome

Summary

Salicylic acid (SA) has a pivotal role in many plant stress and developmental responses but little is known about the early signalling events triggered by this molecule. Using *Arabidopsis thaliana* suspension cells, it was shown by *in vivo* metabolic phospholipid labelling with $^{33}P_i$ that SA induced rapid and early decrease in a pool of phosphatidylinositol (PI). The decrease was accompanied by an increase in PI 4-phosphate and PI 4,5-bisphosphate contents. These changes could be inhibited by 30 μM wortmannine, but not by 1 μM wortmannine, implying that a type III PI 4-kinase was activated in response to SA. Therefore, a study of wortmannin effects on SA transcriptome was undertaken. Using the Complete Arabidopsis Transcriptome MicroArray (CATMA) chip, 773 SA-regulated genes were identified. Among these, the SA response of 112 was inhibited by 30 μM wortmannine, but not by 1 μM wortmannin. In parallel, it was discovered that phospholipase D (PLD) derived phosphatidic acid was important for the early SA-regulated gene expression. Microarray experiment aimed to identify genes regulated by PLD in response to SA revealed that out of 1327 genes regulated by SA, 97 genes were positively and 117 genes were negatively regulated by PLD. The wortmannin-sensitive pathway and PLD pathway regulons share an important overlap implying that the two enzyme activities act synergistically in the same signalling pathway in response to SA.

Key words:
Arabidopsis thaliana, phosphatidylinositol 4-kinase, phospholipase D, phospholipid signalling, salicylic acid, transcriptome

La thèse a été préparée dans le Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes (UMR 7180), Université Pierre et Marie Curie-Paris 6 et Centre National de la Recherche Scientifique, Ivry-sur-Seine F-94200, France et dans le Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague 166 28, Czech Republic.

Je soussigné, déclare que j'ai préparé la thèse moi-même en mentionnant tout les sources utilisées ainsi que les co-auteurs.

A Prague, le 19 juillet 2007

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Signature

The thesis was worked out at the Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes (UMR 7180), Université Pierre et Marie Curie-Paris 6 and Centre National de la Recherche Scientifique, Ivry-sur-Seine F-94200, France and at the Department of Biochemistry and Microbiology, Institute of Chemical Technology, Prague 166 28, Czech Republic.

I hereby declare that I have developed this thesis independently while noting all resources used, as well as all co-authors.

In Prague, 19th July 2007

.....

Signature

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1 INTRODUCTION

During their development plants are submitted to various stresses, either abiotic or biotic. To cope with these stresses, plants synthesize molecules that trigger adaptive responses. Salicylic acid (SA) is one of those molecules. Its role is mainly established in response to biotic stresses where it acts both locally and systemically as a mediator of the systemic acquired resistance (SAR; Bostock, 2005). Treatment with salicylates induces synthesis of pathogenesis related (PR) proteins and enhances resistance to infections. Both these effects were abolished in *nahG* transgenic plants where the level of endogenous SA was dramatically decreased by expression of a bacterial gene encoding SA hydroxylase (Bostock, 2005). However, SA was produced in other physiological situations, such as chilling (Scott et al., 2004), exposure to ozone (Rao et al., 2002) and UV-C stress (Yalpani et al., 1994). The role of SA in those processes may be through SA-induced changes in the gene transcription or protein accumulation (Kliebenstein et al., 2006; Rajjou et al., 2006).

While final effects of SA are well documented, knowledge about the events it triggers is rather fragmentary. Some events that follow application of SA have recently been reported. Several high affinity SA-binding proteins have been identified in tobacco (Forouhar et al., 2005). SA induced an increase of reactive oxygen species (ROS), which may then serve as signalling mediators themselves (Mateo et al., 2006). SA-induced protein kinase (SIPK), belonging to the MAP kinase family, was rapidly activated by phosphorylation in tobacco, and its activation was shown to be necessary for the phosphorylation of the transcription factor WRKY1 (Menke et al., 2005). A number of studies have been focused on the regulatory sequences upstream of *PR1* and necessary for its SA induction.

Many signalling pathways are activated (often in concert) in plants in response to various stresses, including cytoplasmic Ca^{2+}-, protein kinase-, protein phosphatase- and lipid-signalling cascades. Phospholipid signalling is emerging as a major component of pathways responding to many stresses and hormones in plants (Meijer and Munnik, 2003). Several low-abundant phospholipid species that are rapidly and transiently generated in response to various stresses have been proposed to function as second messengers. These comprise some phosphoinositides and phosphatidic acid (PA) which can bind to various protein kinases, protein phosphatases or ion channels and thus modulate their activity. They constitute a minor portion of membrane lipids

under control conditions, but their levels significantly increase upon exposure to numerous stresses and may form membrane microdomains which increase local concentration of proteins with specific lipid-binding domains (Davis et al., 2007). Arabidopsis *sac9* mutant, defective in phosphoinositide phosphatase, had increased phosphatidylinositol (PI) 4,5-bisphosphate ($PI(4,5)P_2$) level and exhibited constitutive stress response accompanied by constitutive overexpression of stress-induced genes (Williams et al., 2005). In Arabidopsis cell suspensions and plants, net $PI(4,5)P_2$ synthesis was enhanced by hyperosmotic stress (Meijer and Munnik, 2003). Phospholipase D (PLD) is an enzyme generating PA from structural phospholipids. PLD transcripts increased after infiltration of Arabidopsis plants by both virulent and avirulent strains of *Pseudomonas syringae* pv. *tomato* (Bargmann and Munnik, 2006). PLD activity increased in transgenic Arabidopsis plants during elicitor treatment (Bargmann and Munnik, 2006). PLD was activated in response to abscisic acid in Arabidopsis plants (Bargmann and Munnik, 2006) or cells (Hallouin et al., 2002). It was also activated upon cold stress in Arabidopsis cells and this activation was upstream of a cluster of cold-regulated genes (Vergnolle et al., 2005). It is believed that specificity of a signalling cascade is achieved by the spatio-temporal combination of the activation/deactivation of its different components.

In this context, my goal was to investigate the involvement of phospholipid signals in response to SA. Using Arabidopsis cell suspensions, a model where SA uptake (Clarke et al., 2005) and SA-triggered responses at protein level (Rajjou et al., 2006) are well documented, it was shown for the first time that PI 4-kinase and PLD are rapidly and transiently activated in response to SA. PI 4-kinase activation led to an increase in PI 4-phosphate (PI(4)P) and, more strikingly, in $PI(4,5)P_2$ level. SA transcriptome and its changes induced by wortmannin, an inhibitor of PI 4-kinase, and by *n*-butanol, an agent which diverts the production of PLD-derived PA (PA_{PLD}) to non-active phosphatidylbutanol (PBut), were studied using a microarray chip. Doing so, 112 genes whose response to SA was inhibited by 30 μM wortmannin but not by 1 μM wortmannin (W30-sensitive) were identified as well as 97 genes positively and 117 genes negatively regulated by PLD. Potential *cis*-elements which may coordinate the changes in gene expression upon SA treatment are reported, and the overlap of a W30-sensitive pathway regulated cluster of genes with that regulated by PLD is discussed.

2 CURRENT STATE OF KNOWLEDGE

2.1 Role of salicylic acid in plants

In 1979 SA was identified as a compound capable to decrease the severity of infection when applied on plants before inoculation (the process was later called priming; Durrant and Dong, 2004). The majority of our knowledge about its mode of action comes from the studies of plant innate immunity. However, SA is involved in other physiological processes including adaptation to stress and development and thus can be seen as a plant hormone.

2.1.1 Systemic acquired resistance and SA

In the 1960s, it was shown that tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed increased resistance to secondary infection in distal tissues (Durrant and Dong, 2004). This spread of resistance throughout the plant tissues was termed SAR (Ross, 1961). Flor (1971) proposed a molecular model for plant infection called 'gene-for-gene'. In this model, pathogen bears a gene of avirulence (*Avr*) and plant bears the corresponding gene of resistance (*R*). When the R protein recognizes the corresponding Avr protein, an incompatible reaction is triggered leading to localized programmed cell death (PCD) and eventually to SAR development. The localized PCD is SA-dependent and it is called hypersensitive response (HR). During HR reactive oxygen species (ROS) are produced and this slows down or even stops the infection. Interestingly, ROS generated by Arabidopsis respiratory burst oxidase (AtRBOH) proteins can antagonize SA-dependent pro-death signals. This is a good example of how both SA and ROS control localized PCD (Torres et al., 2005). When R protein does not recognize its Avr partner, compatible interaction is triggered. Compatible reaction often leads to development of infection and eventually to death of the challenged plant. We now know that SAR can be activated in many plant species by pathogens that cause necrosis. The process of SAR development is SA-dependent and it can also be activated by SA alone. The role of SA in SAR has been discussed extensively in a number of reviews (Bostock, 2005; Dempsey et al., 1999; Dong, 2001; Durrant and Dong, 2004; Nürnberger and Scheel, 2001; Ryals et al., 1996; Shah and Klessig, 1999). The obtained resistance is long-lasting, sometimes for the lifetime of the plant, and effective against a broad spectrum of pathogens including viruses, bacteria,

fungi, and oomycetes representing various parasitic strategies (i.e. biotrophs, hemibiotrophs, and necrotrophs).

Molecularly, SAR is characterized by the increased expression of a large number of pathogenesis-related genes (*PR* genes), in both local and systemic tissues. PR proteins were first described in the 1970s when accumulation of various novel proteins after infection of tobacco with TMV was observed (Durrant and Dong, 2004). Although many PR proteins have antimicrobial properties *in vitro*, the function of some of them in the defence response has not been clearly defined (Table I). It is generally thought that SAR results from concerted interplay of many PR proteins rather than from a specific PR protein. *PR* genes serve as useful molecular markers of the onset of SAR.

Another type of systemic resistance is the jasmonic acid (JA) -mediated induced systemic resistance (ISR). ISR protects plants against a range of pathogens, but it is independent of SA and *PR* gene induction. It is usually associated with the soil bacteria, often strains of *Pseudomonas fluorescens*. It can be triggered by various volatile oxylipins including JA and methyljasmonate (MeJA).

Table I *Examples of some PR proteins and their function.*
Adopted from van Loon et al. (2006).

Family	Representative	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase, type I,II,IV,V,VI,VII
PR-4	Tobacco R	Chitinase, type I,II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase, type III
PR-9	Tobacco ‘lignin forming peroxidase‘	Peroxidase
PR-10	Parsley ‘PR1‘	Ribonuclease-like
PR-11	Tobacco ‘class V‘ chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

SAR has been studied using many approaches including analyses of concentration changes of SA and its conjugates in plant tissues prior to, during or after the resistance is expressed. Various mutants constitutively expressing resistance genes or affected in development of systemic resistance were the most useful tools. Alternative ways of inducing plant resistance were sought to facilitate its study

including treatment of plants with SA or various SA-mimicking, but less phytotoxic, compounds, such as benzothiadiazole S-methyl ester (BION; BTH; Actiguard®) and 2,6-dichloroisonicotinic acid (INA). Although these compounds are thought to be equivalent to SA treatment (they induced similar sets of *PR* genes), a more detailed study on the whole transcriptome scale revealed that many genes are regulated in a different manner, especially between SA and BTH treated plants (von Rad et al., 2005).

SAR needs a systemic signal to be emitted and perceived in distant tissues. The nature of the systemic signal is yet unknown, but according to the literature it is not species-specific and it is transmitted via phloem (Durrant and Dong, 2004). The detection of increased SA levels in systemic leaves and in the phloem led many researchers to believe that SA might be a systemic signal for SAR. Malamy et al. (1992) showed that the endogenous SA concentration raises both in local and systemic tissues after infection of tobacco with TMV and this increase correlates with *PR* gene induction. Métraux et al. (1990) found that cucumber plants infected with either *Colletotrichum lagenarium* or tobacco necrosis virus (TNV) have considerably elevated levels of SA in the phloem sap. Radiolabelling studies in TMV-infected tobacco showed that most of the SA (69%) accumulating systemically was made and exported from the inoculated leaf (Shulaev et al., 1995). Similarly, in cucumber infected with TNV, SA found in systemic leaves was both imported from the infected leaf and synthesized *de novo* (Durrant and Dong, 2004). A number of experiments argue against SA being the systemic signal. One of them shows that detachment of *Pseudomonas syringae*-infected cucumber leaves before SA levels had increased in the petiole did not block the development of SAR (Rasmussen et al., 1991). Alternative hypotheses have been raised pointing to nitric oxide (NO) or methylsalicylate (MeSA; Forouhar et al., 2005). Feed-back loop probably exists in the SA-NO relationship as NO triggered SA production (Wendehenne et al., 2004) and it was produced after SA treatment of Arabidopsis seedlings (Zottini et al., 2007). MeSA as a volatile compound could induce resistance not only in the uninfected parts of the same plant but also in neighbouring plants. In a very interesting field study MeSA emitted from soybean infested with soybean aphid (*Aphis glycines*) was identified as the major olfactory attractant for predacious seven-spotted lady beetles (*Coccinella septempunctata*; Zhu and Park, 2005). NO indeed functions in local pathogen perception where it stimulates ROS production during the oxidative burst and it induces the general phenylpropanoid pathway (Zeier et al., 2004a). Unfortunately, NO can easily be quenched in contact with

the cellular environment (Baudouin et al., 2006) so it would be difficult to imagine it as a long distance signal. Others have suggested members of the lipid transfer protein (LTP) family (Durrant and Dong, 2004). These are small proteins which specifically bind fatty acids and which can be easily transported through phloem. They have been studied in various species but their exact role is not clear. Recently LTP covalently modified by an oxylipin has been found in barley (LTP1b) thus opening interesting perspectives regarding the roles of LTPs in plant defence and development (Bakan et al., 2006).

2.1.1.1 SA signalling pathway

Locally produced SA can act both in the cell where it was produced and in neighbouring cells. A mechanism was proposed in which pathogen elicitor caused apoplast alkalization which in turn promoted pH-independent passive export of SA. Released SA moves through the plant tissue in a wave preceding the infection and it enters cells which still have acid apoplasts to trigger defence responses there (Clarke et al., 2005). SA uptake was higher at lower extracellular pH and took place probably via an H^+ symport mechanism in Arabidopsis cell suspensions. As its pKa is 2.97 a small portion remains in non-ionized state and can be retained inside the cell by ion-trap mechanism. Accordingly, SA treatment caused transient cytoplasmic acidification. However, the SA uptake occurred even at pH close to neutrality. The mobility of SA in the phloem of *Ricinus communis* seems to be facilitated by a pH-dependent carrier system translocating aromatic monocarboxylic acids (Rocher et al., 2006).

Till now, no canonical receptor molecule for SA has been identified in plants despite the numerous efforts. Nevertheless, several high affinity binding proteins have been identified in tobacco (e.g. catalase, ascorbate peroxidase, and carbonic anhydrase; Kumar and Klessig, 2003). One of them, called SA binding protein 2 (SABP2), was first shown to have a lipase activity and later its MeSA hydrolyzing esterase activity was described (Forouhar et al., 2005). Furthermore, silencing the *SABP2* gene by RNAi diminished both local resistance and SAR (Kumar et al., 2006). These observations led authors to propose that SABP2 is the receptor for SA; however, the exact position of SABP2 in the SA signalling pathway is not clear yet. Interestingly, its tomato orthologue is rather a MeJA esterase (MJE; Shah, 2005).

SA treatment triggers protein phosphorylation cascades. These involve MAP kinases (reviewed by Innes, 2001), specifically the wound-induced protein kinase

(WIPK) and SA-induced protein kinase (SIPK) both being described in tobacco. WIPK is activated by wounding and SIPK is activated by both NO and SA. Neither of them responds to JA or ethylene. Moreover SIPK is strongly activated by ROS and it appears to modulate the cellular response to ROS and the level of WIPK. Arabidopsis MPK4 negatively regulated SA-mediated defences and its direct binding partners from the family of MAPKK and MAPKKK have been identified by yeast two-hybrid screen (Innes, 2001).

Ca^{2+}-dependent protein kinases (CDPKs) are also involved in plant immune response. They serve as important sensors of Ca^{2+} fluxes. Specific isoforms of this multigene family are implicated in signalling pathways leading to abiotic and biotic stress resistance, including disease resistance. CDPKs that are up-regulated by wounding, elicitors, and/or infection, including *Avr*/*R* gene interactions have been characterized from tobacco, tomato, and maize (Bostock, 2005). However, further research is needed to determine if and how these kinases operate in connection with induced resistance pathway regulation and signal cross-talk.

Perception of the unknown systemic signal and/or perception of SA lead to induction of early transcription factors (e.g. WRKY or basic leucine zipper, bZip, family) and to a shift of cytoplasmic redox potential to more reductive state. Changes in the redox state affect the activity of SAR-related transcription factors and of enzymes regulating levels of reactive oxygen species (ROS) in the cell. SA is capable of modulating pools of major cellular antioxidants such as glutathione, thioredoxin, and ascorbate. The redox modulating and antioxidant capacity of SA may be its major link with other stress responses where it is involved because ROS occur during many stress situations. On the other hand, failure to control ROS is one of the causes leading to cell death. Potential SA targets in cellular redox management involve hydrogen peroxide scavengers, SA binding proteins, catalase and ascorbate peroxidase, and chloroplastic carbonic anhydrase. The ability of SA to interact with these proteins may be partially caused by its affinity to transition metals (e.g. heme and nonheme iron; Bostock, 2005).

Reductive state of the cytoplasm leads to monomerization of homotrimeric transcription factor ‘non-expressor of PR-1’ (NPR1; Bostock, 2005). NPR1 is considered as the main switch in the signalling network of plant induced resistance (Pieterse and van Loon, 2004). It is also well conserved between monocots and dicots (Yuan et al., 2007). NPR1 is expressed throughout the plant at low levels and its mRNA level raises two- to three-fold after pathogen infection or treatment with SA. NPR1

expression is likely mediated by WRKY transcription factors as mutation of the WRKY binding sites (W-boxes) in the NPR1 promoter inhibited it. Monomers of NPR1 can easily enter the nucleus and in combination with other transcription factors trigger the next wave of gene expression (Bostock, 2005; Yuan et al., 2007). Such self-amplification of the initial signal is typical for many signalling pathways.

The absence of any obvious DNA-binding domain and the presence of protein-protein interaction domains in NPR1 prompted several laboratories to carry out yeast two-hybrid screens for NPR1-interacting proteins. In one of the screens, three small structurally similar proteins named NIMIN1, NIMIN2, and NIMIN3 (non-inducible immunity interactor) were identified. NIMIN1 and NIMIN2 interact with the C-terminus of NPR1, while NIMIN3 interacts with the N-terminus. NIMINs contain stretches of acidic amino acids and are hypothesized to be transcription factors; however, more experiments are required to demonstrate their biological activity (Durrant and Dong, 2004). Other NPR1 interactors found in the yeast two-hybrid screens were members of the TGA family of bZip transcription factors. NPR1 interacts indeed with the Arabidopsis TGA factors, TGA2, TGA3, TGA5, TGA6, and TGA7 but only weakly or not at all with TGA1 and TGA4. Interaction with TGA1 and TGA4 was dependent on their redox status (Durrant and Dong, 2004). TGA2, TGA5 and TGA6 were functionally redundant and only their triple mutant was compromised in SAR induction. All these TGA factors were also important for SA tolerance and for the negative regulation of the basal expression of *PR1* (Zhang Y et al., 2003). 51 TGA2-binding elements in the promoters of Arabidopsis genes were identified and, indeed, SA-induced genes were significantly over-represented among the genes neighbouring putative TGA2-binding sites (Thibaud-Nissen et al., 2006). Using truncated or mutant forms of NPR1, the ankyrin-repeat domain in the middle of the protein was shown to be essential for binding TGA factors, while the N-terminal region appears to enhance the binding (Durrant and Dong, 2004). The exact mode of interaction between NPR1 and TGA2 was studied in detail. It was postulated that TGA2 and NPR1 are recruited independently to the promoter of *PR1* and upon SA stimulation they combine to form an enhanceosome which triggers *PR1* expression. TGA2 itself (without NPR1 or SA stimulus) functions as a repressor of *PR1* expression in the proposed model (Rochon et al., 2006). NPR1 also interacts with TGA factors from tobacco and rice (Durrant and Dong, 2004).

TGA factors bind to activator sequence-1 (*as-1*; TGACG) or *as-1*-like promoter elements, which have been found in several plant promoters activated during defence, including Arabidopsis *PR1*, an important SAR marker. Linker scanning mutagenesis of the *PR1* promoter identified two *as-1*-like elements, *LS7* and *LS5*. *LS7* is a positive regulatory element required for induction by INA, whereas *LS5* is a weak negative regulatory element. It was shown that both TGA2 and TGA4 could bind to *LS7*, whereas only TGA2 could bind to *LS5*. Furthermore, binding of TGA2 but not TGA4 was enhanced by the addition of NPR1, consistent with the yeast two-hybrid interaction data (Durrant and Dong, 2004).

Although NPR1 is clearly a positive regulator of *PR* genes, it may exert its function by either enhancing a transcriptional activator or inhibiting a transcriptional repressor. The presence of multiple *as-1*-like elements in the *PR1* promoter and the differential binding affinities of each TGA factor to these elements as well as to NPR1 highlight the complexity of the regulatory mechanism. The NPR1/TGA system seems to be operative even in monocots as demonstrated in the experiment where the ectopic expression of the Arabidopsis NPR1 in rice enhanced resistance to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (Bostock, 2005). NPR1 controlled the expression of the protein secretory pathway genes. Up-regulation of these genes was essential for SAR and was ensured through a previously undescribed *cis*-element activated by another transcription factor that translocated into the nucleus upon SAR induction (Wang et al., 2005). However the NPR1 is considered as the major transcriptional switch in the network of induced resistance, there is growing evidence for a SA-dependent NPR1-independent pathway(s) that contributes to defence gene induction (Durrant and Dong, 2004).

Transcription factors bind to their respective boxes or *cis*-elements in promoters of target genes. Several *cis*-elements are known to be associated with SAR. Interestingly, only 17 of 26 genes from the *PR1* regulon (a cluster of genes similarly regulated as *PR1*) have an *as-1* element in their promoters, whereas W-boxes were overrepresented, occurring an average of 4.3 times per promoter. This suggests that WRKY factors rather than TGA factors are important for coregulation of *PR1* regulon genes. The *LS4* W-box in the *PR1* promoter acts as a strong negative *cis*-element, leading some authors to propose that WRKY factors repress the expression of *PR1* regulon genes (Durrant and Dong, 2004). This issue was clarified in an elegant study where 8 WRKY transcription factors were directly regulated by NPR1 and also by TGA

transcription factors. Among these WRKY58 was the negative regulator and four others were positive regulators of *PR* gene expression (Wang D et al., 2006). WRKY25 was another identified negative regulator of SA-mediated induced resistance to bacterial pathogen (Zheng et al., 2007). It is not surprising because it directly interacts with MKS1, a substrate for MPK4, which is a repressor of SA-dependent defence responses (Andreasson et al., 2005). Some WRKY transcription factors (e.g. WRKY33) are specific for some types of pathogens (Zheng et al., 2006). WRKY transcription factors may seem specific for plant-pathogen interaction, however, they are not. For instance, one of the positive SAR regulators, WRKY70, is also a negative regulator of developmental senescence (Ulker et al., 2007). Another promoter analysis on 1058 genes that were induced by pathogen infection, SA, MeJA, or ozone revealed that *as-1* elements, W-boxes, abscisic acid (ABA) response elements (ABRE), and G-boxes were overrepresented across all treatments, whereas the Myc motif (CANNTG) was overrepresented only in the SA-induced genes (Mahalingam et al., 2003). Upon activation of SAR, NPR1-dependent derepression would occur, possibly through the inactivation of transcription factor 'suppressor of NPR1, inducible' (SNI1) which is required to maintain the low basal expression of *PR* genes (Durrant and Dong, 2004). The signalling cascade which triggers *PR1* expression is summarized in Figure 1.

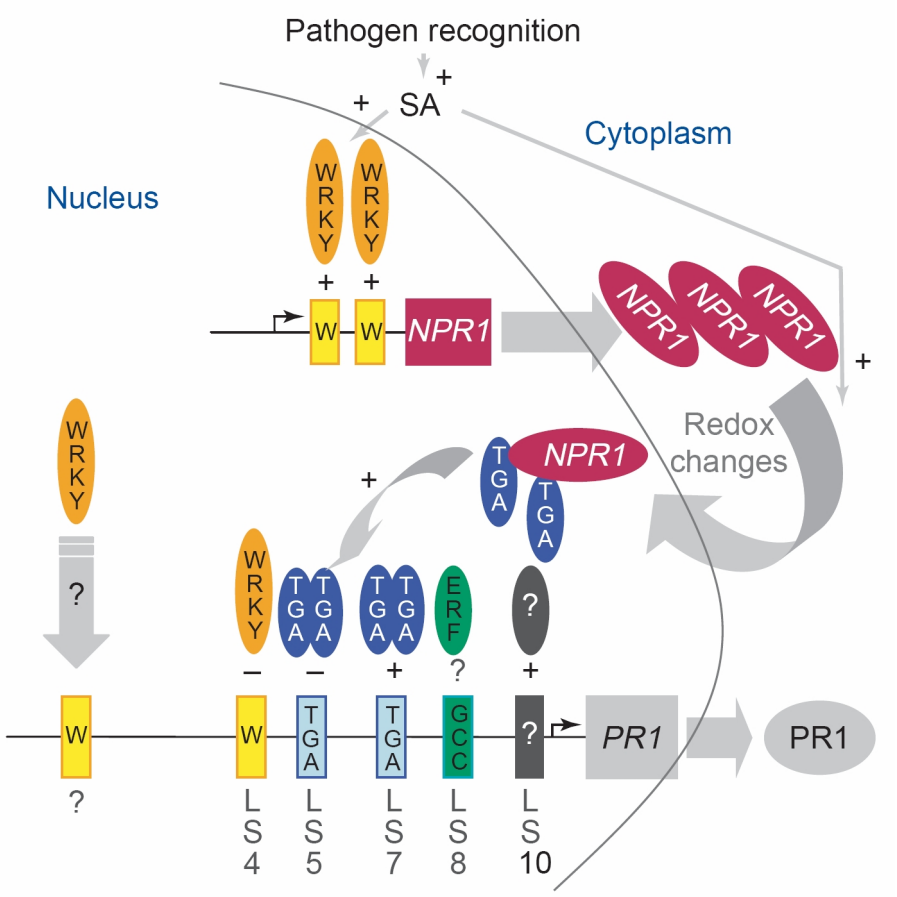


Figure 1. Molecular cascade relaying SA-dependent signals to *PR1*.
Elevated SA levels trigger increased *NPR1* transcription via WRKY factors binding to two W-boxes in the 5'-UTR of *NPR1*. In resting Arabidopsis cells, NPR1 oligomers are sequestered in the cytoplasm. Elevated SA levels trigger a biphasic change in the cellular redox environment leading to the reduction of the intermolecular disulfide bounds of NPR1 oligomers. The resulting NPR1 monomers localize to the nucleus where they activate binding of TGA factors to TGA boxes (*LS5* and *LS7*) in the *PR1* promoter. The TGA box *LS5* and *LS4*, a W-box that probably interacts with WRKY factors, are negative elements that repress *PR1* expression. The TGA box *LS7* and *LS10*, which appears to interact with unknown transcription factors, are positive elements. The GCC box, *LS8*, is likely to interact with ERF transcription factors. The effects of this element on *PR1* expression are not known. However, SAR induction stimulates binding of protein factors to this element. Additional WRKY factors might contribute to *PR1* regulation indirectly or by interacting with putative W-boxes upstream from *LS4*. Causal interactions are indicated by grey arrows. '+' means activating mechanisms, '-' means repressing mechanisms. Coding regions of genes are represented by squares, *cis*-elements by upright rectangles and transcription factors as well as NPR1 by ovals. Transcription start sites of genes are marked by black arrows. Adopted from Eulgem (2005).

Once produced, PR proteins are probably down-regulated by the proteasome complex as documented for pepper where CaRFP1, encoding the C3-H-C4 type RING-finger protein, was isolated from pepper leaves infected by *Xanthomonas campestris* pv. *vesicatoria*. CaRFP1 physically interacted with the basic PR1 protein (CaBPR1) and it acted as an E3 ligase for polyubiquitination of target PR proteins. It was induced by several stresses including SA treatment and its over-expression negatively regulated disease resistance (Hong et al., 2006). These results could be partly

explained in terms of negative regulation of SA production as documented for Arabidopsis. The *SIZ1* gene, which encodes an Arabidopsis small ubiquitin-like modifier (SUMO) E3 ligase, regulated innate immunity. Mutant *siz1* plants exhibited constitutive SAR characterized by elevated accumulation of SA, increased expression of *PR* genes and increased resistance to a bacterial pathogen. Transfer of the *nahG* gene to *siz1* plants resulted in reversal of these phenotypes back to wild type. SIZ1 interacted epistatically with PAD4, phytoalexin deficient, to regulate *PR* gene expression and disease resistance in NPR1-independent manner and it was required for SA and PAD4-mediated *R* gene signalling pathway (Lee et al., 2007). Characterization of mutants of two ubiquitin-activating enzyme genes revealed that ubiquitination pathway was crucial in the activation and downstream signalling of several R-proteins in Arabidopsis (Goritschnig et al., 2007).

2.1.1.2 Studies of SAR transcriptome

During the past seven years, differential display, DNA microarrays, and other high throughput transcriptomic approaches have been applied to gain a more comprehensive view of changes in global gene expression associated with plant responses to diverse stressors. Information obtained on SAR transcriptome, i.e. studies where wild type or mutated plants were infected with different pathogens (Glazebrook et al., 2003), are the main source of our knowledge about the differential activation of signalling pathways during the onset of plant immune response. Other studies in Arabidopsis have focused on expression profiles induced by pathogens or pathogen-related activities using multiple conditions rather than pairwise comparisons to better assess the complexity of signal networks, e.g. transcriptional profiling of plants infected with various pathogens or treated with various plant activators including SA, SA analogues and JA. Schenk et al. (2000) performed transcription profiling of infected plants and plant activator-treated plants on a custom microarray with 2375 EST-derived clones. 323 genes were differentially expressed after 24h of SA treatment and overlaps between infection-regulated and plant activator-regulated genes were discussed. Mahalingam et al. (2003) identified 732 transcripts as differentially expressed after pathogen infection or plant activator treatment (among these 68 by SA treatment) on a custom microarray constructed from stress cDNA library of 1058 gene transcripts. Von Rad et al. (2005) used a custom microarray for transcriptional profiling of about 700 genes after treatment with commercial plant activators and found that their mode of

action is rather complex and mimics neither pure JA nor pure SA action. Transient JA pathway activation was followed by a more sustained activation of the SA pathway. Pylatuik and Fobert (2005) tested several whole genome scale microarray platforms and took SA treatment as a model treatment. Several hundreds of genes differentially expressed after 2h and 8h of SA treatment were identified along with NPR1-dependent and independent gene clusters. However, the complete list of differentially expressed genes was not provided. Thibaud-Nissen et al. (2006) identified 1265 SA-induced genes after 2h of SA treatment of Arabidopsis plants and used the expression data to prove that newly-discovered TGA2-binding elements are over-represented in their promoters. However, the list of SA-induced genes was not provided either. Kliebenstein et al. (2006) also took the SA treatment as a model treatment to demonstrate gene expression diversity in Arabidopsis. They identified 439 SA-induced and only 19 SA-repressed genes in the wild type Columbia background. The SA transcriptome was not discussed at all in the study. Using various SAR-inducing and SAR-repressing conditions comparison Maleck et al. (2000) identified several regulons. Regulons share common *cis*-elements in their promoters and can be attributed to specific transcription factor families. Other studies focused on regulatory *cis*-elements in the promoters of SAR-regulated genes. Using cDNA-AFLP display Blanco et al. (2005) identified around 40 genes differentially regulated by SA treatment. They identified common *cis*-elements of NPR1-dependent and NPR1-independent genes. Interestingly, NPR1-dependent genes did not contain *as-1* element and were mostly involved in signal transduction while NPR1-independent genes had *as-1* element in their promoters and were mostly involved in cellular detoxification. Accordingly, two categories of SAR-regulated genes with distinct profiles of *cis*-regulatory elements in their promoters were identified in a bioinformatic study (Pan et al., 2004). Glucocorticoid receptor (GR) fused to a protein can control its nuclear localization in a dexamethasone-dependent manner. Using the whole genome transcriptomic approach on *npr1* plants transformed with NPR1-glucocorticoid receptor (NPR1-GR), Wang et al. (2005) identified around 150 direct transcriptional targets of NPR1 in response to SA, pointed out the importance of the protein secretory pathway for successful development of SAR and showed that its induction is NPR1-dependent. In an expression profiling of 402 transcription factors known to be involved in various stress responses including SAR, Chen et al. (2002) identified about 40 transcription factors that were specifically up-regulated during infection with various pathogens. These transcription factors may be responsive to SA

too. Dong et al. (2003) performed transcription profiling of 72 WRKY transcription factors in Arabidopsis and found that six were repressed and 43 induced by both SA treatment and incompatible reaction induced by *Pseudomonas syringae* pv. *tomato*. It confirms the central role of WRKY transcription factors during the development of plant immune response.

The timing of the systemic response is not the same for bacteria, viruses, fungi or chemical inducers like SA or INA. Pathogens need some time for germination (when they land on the host as a spore), propagation in the host and for causing a threshold damage which triggers the SAR. This causes a temporal delay ranging from about 12 hours for bacteria up to 3 days for sporulated fungi and viruses (Pieterse et al., 1996; Ward et al., 1991). When SA or INA is applied, the first wave of gene expression culminates between 2 and 4 hours (transcription factors) and the second between 6 and 12 hours (*PR* genes) after the treatment (Ward et al., 1991). Transcriptomic studies confirm that the SAR development has several waves of gene induction triggered successively. Expression profiling seems to provide a good starting point to decrypt the staggering array of changes occurring in plants under various biotic and abiotic stresses. Although it is still unclear how expression profiling will inspire field application of induced resistance, the integration of this knowledge with proteomic and metabolomic approaches should ultimately explain why unanticipated or undesirable physiological shifts occur.

2.1.2 Mutants with altered SA/SAR signalling

The modern research and progress in life sciences cannot be imagined without the use of mutants. Either loss-of-function or gain-of-function mutants both being tools of reverse genetics can be engineered by the means of molecular biology. Indeed, both categories have been very useful in unravelling the SA signalling pathway. Most of them are Arabidopsis mutants which makes it a model plant of choice for the majority of genetic studies.

Perhaps the crucial experiments for establishing an important link between SA and SAR were those utilizing transgenic plants in tobacco and Arabidopsis expressing the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that converts SA to catechol. The *nahG* transgenic plants are diminished in their ability to accumulate SA, thus essentially abolishing the SAR response, and are rendered more susceptible to pathogens. The expression of resistance in *R* gene-mediated interactions with

incompatible strains of pathogens is also abrogated in such plants, indicating an important role of SA-mediated signalling downstream from the recognition between *Avr* and *R* gene products. Furthermore, *nahG* transgenics shed light on the question whether SA is the mobile signal of SAR. Grafting experiments in tobacco between wild type scions and *nahG*-expressing rootstocks showed that, although the rootstock was unable to accumulate SA, the SAR signal was still produced and translocated to the scion. The reciprocal grafting experiment showed that the systemic tissue must accumulate SA for the SAR signal to be perceived (Durrant and Dong, 2004).

In many plants SAR is preceded by an increase in SA concentration. However, some plants such as potato and rice have high endogenous levels of SA under non-inducing conditions. It is not surprising that application of SA to potato does not protect it against *Phytophthora infestans*. However, expression of *nahG* in potato blocks resistance to *Phytophthora infestans* induced by arachidonic acid, a lipid elicitor from this pathogen (Durrant and Dong, 2004). This suggests that after treatment with arachidonic acid, instead of SA levels rising, the potato plants become more sensitive to SA already present in the cells. Thus, SA is an essential signal for SAR across a range of plants, although the mechanism by which SA induces SAR might differ.

Interpretation of the experiments utilizing SA-modifying plants (*nahG*) is not without complication, particularly in light of unexpected phenotypes in certain backgrounds (Heck et al., 2003; van Wees and Glazebrook, 2003). Although most of the evidence documents the capacity of the plant to respond rapidly to changes in cellular SA levels, the data also suggest that there are impacts on yet unclear response pathway when SA levels are severely disrupted like in the *nahG* transgenics. For example, expression of *nahG* in tomato can result in a lesion-mimic phenotype, and DNA-laddering characteristics of PCD can be observed in nuclear extracts of lesioned tissue (Bostock, 2005). The lesion-mimic created by *acd2* (accelerated cell death) in Arabidopsis is thought to result from an accumulation of free radical breakdown products of chlorophyll. *NahG* worsens the effect of *acd2* which is consistent with the participation of SA in the modulation of the balance of antioxidants and oxidants in the cell and in PCD (Bostock, 2005). Ectopic expression of the Arabidopsis *NPR1* gene in rice also results in a lesion mimic, a phenotype that is potentiated by SA (Bostock, 2005). The possibility of altered flux through the isochorismate/phenylpropanoid pathway to compensate for the SA defect in *nahG* backgrounds could create collateral perturbations in plant metabolism that compromise host resistance in a more generally

disruptive manner. Genetic evidence that *nahG* plants display additional alterations unrelated to reduced SA levels that impact defence-related responses is provided by mutants that are SA-deficient (*pad4*) or SA induction deficient (*eds5*, enhanced disease susceptibility, and *sid2*, SA induction deficient), and combinations thereof (Bostock, 2005). NahG could attack other substances in the cell besides SA and even direct effect of SA on pathogen activity should not be ruled out. The extent to which SA modification, regardless of how this is accomplished, spills over to disrupt other metabolic processes in the host and possibly in the pathogen remains a fertile area of investigation.

2.1.2.1 Mutants pointing to lipid-based signalling in SAR

Exciting new findings suggest that a lipid-based molecule may be the mobile signal for SAR. The *dir1* (defective in induced resistance) mutant has normal local resistance to pathogens but is unable to develop SAR or express *PR* genes in systemic leaves. Therefore, wild type DIR1, which has sequence homology to lipid transfer proteins (LTPs), might function in the generation or transmission of the mobile signal. Indeed, experiments using petiole exudates showed that the phloem sap from *dir1* is deficient in the mobile signal for SAR. However, the mutant plants could still respond to a signal contained in the sap from wild type plants, ruling out a role for DIR1 in signal perception. Furthermore, the *dir1* plants have wild type SA metabolism and a normal response to SA and INA (Durrant and Dong, 2004). LTPs form a multigene family in Arabidopsis with 71 predicted members. Interestingly, they share sequence similarity with protein elicitors, elicitins, from *Phytophthora* spp. The extracellular location of LTPs and elicitins is consistent with a role in signalling and implies the presence of plasma membrane (PM) receptors involved in signal transduction. Indeed, wheat LTP1 binds to the same PM receptor as the *Phytophthora* elicitin cryptogein (Durrant and Dong, 2004). However, over-expression of DIR1 was not sufficient for SAR initiation which is in favour of a yet unidentified phospholipid, which would bind to DIR1, as the true long distance signal (Suzuki et al., 2004). The nature of the lipid molecule carried by DIR1 could be assessed from the characterization of Arabidopsis mutant called *sfd1* (suppressor of fatty acid desaturase deficiency) in which the SAR is compromised but the basal resistance to a bacterial pathogen is unaffected. SAR-associated SA accumulation is also compromised in *sfd1* mutants. The SAR defect of *sfd1* is restored by the application of BTH, a functional analogue of SA. This

suggests that SFD1 is most likely required for the synthesis and/or translocation of the mobile signal from the necrotizing pathogen-inoculated organ, or for the perception of the mobile signal or subsequent signal transduction, leading to SA accumulation in the distal organ. SFD1 is a dihydroxyacetone phosphate reductase that is required for an early step in glycerolipid biosynthesis, thus pointing to the involvement of lipid or lipid-derived molecule in the SAR initiation (Shah, 2005).

Further evidence for a lipid-based signal molecule comes from the characterization of the *eds1* and *pad4* mutants, which are both defective in lipase-like proteins. The *eds1* mutant was originally identified for its compromised local resistance to *Peronospora parasitica* mediated by several *R* genes, whereas *pad4* was isolated in a screen for mutants with enhanced susceptibility to a virulent strain of *Pseudomonas syringae* pv. *maculicola*. It was subsequently discovered that *pad4* weakens local resistance mediated by the same subset of *R* genes that are blocked by *eds1*. These *R* genes encode TIR-NB-LRR-type resistance proteins. However, many other *R* genes act through an EDS1-independent signalling pathway. In *eds1* and *pad4* plants, even when a normal HR is elicited by pathogens that trigger the EDS1-independent pathway, SAR cannot be induced. Experiments using phloem exudates have shown that EDS1 is required for both production of the mobile signal in the local tissue and perception of the signal in the systemic tissue. It has to be mentioned in this context that the second activity of the tobacco SABP2 is a lipase and that its lipase activity is increased four- to five-fold upon addition of SA (Durrant and Dong, 2004). Either EDS1 or PAD4 could therefore be the Arabidopsis orthologue of tobacco SABP2.

In a revolutionary study JA was proposed as the long sought systemic signal of SAR (Truman et al., 2007). Several mutants affected in JA biosynthesis or perception were tested with respect to their ability to trigger systemic response upon incompatible interaction with bacterial pathogen. The timing of the response was studied in detail and it revealed a peak of JA synthesis which preceded the SAR development in systemic tissues. Moreover, the expression pattern of the systemic response was very similar with the pattern of wounding and JA pathway. The well-known antagonism between these two signals (see chapter 2.1.2.5 for details) is overcome by the temporary character of the JA signal. The SA vs. JA antagonism is likely to be unidirectional, with SA pathway damping the JA pathway and not vice versa. Given that the tobacco LTP1 binds JA, and LTP1-JA, but not LTP1 or JA alone, enhances resistance in tobacco to *Phytophthora parasitica* (Buhot et al., 2004), jasmonates are potential ligands for another LTP (DIR1)

in Arabidopsis. The SFD1 (plastidial dihydroxyacetone phosphate reductase involved in glycerolipid synthesis) was placed either upstream of or at the same level as the DIR1 in the SAR signalling cascade (Nandi et al., 2004). SFD1 could therefore be involved in the plastidial biosynthesis of a specific glycerolipid with an oxylipin moiety esterified to the *sn*-1 position of the glycerol backbone. Such oxylipin would be released by a plastidial lipase, much like the DAD1 (phospholipase A_1; see chapter 2.2.2.7 for details) releases linolenic acid, the JA precursor, from phosphatidylcholine. Released oxylipin could then bind to DIR1 thus forming the active systemic signal of SAR. Although many important questions still need to be addressed, presented data strongly suggest a role for lipids (including oxylipins) in SAR signalling.

2.1.2.2 Mutants affected in SA synthesis

Many mutants have also been useful in dissecting between the two possible biosynthetic pathways for SA in plants. SA can be synthesized via two different metabolic pathways: via isochorismate, starting with isochorismate synthase (ICS) or via phenylpropanoid pathway starting with phenylalanine-ammonia lyase (PAL). Relative fluxes through these pathways may vary in different plant species. It was presumed that the SAR-related SA is mainly synthesized by the phenylpropanoid pathway (Ferrari et al., 2003) but it was never proven. Expression of the bacterial enzymes catalyzing these reactions, ICS1 and isochorismate pyruvate lyase 1 (IPL1), in tobacco and Arabidopsis results in increased SA accumulation and pathogen resistance suggesting that this is the dominant pathway in the two species (Durrant and Dong, 2004).

SA induction-deficient Arabidopsis mutants, *sid1* and *sid2*, failed to accumulate SA after SAR induction. More alleles of *sid1* and *sid2*, called *eds5* and *eds16*, respectively, were identified independently by virtue of their enhanced disease susceptibility phenotype. Breakthrough in our understanding of SA biosynthesis came when SID2/EDS16 was cloned and shown to encode a chloroplast-localized ICS1 (Strawn et al., 2007). Mutations of the *ICS1* gene, in *sid2* and *eds16*, reduce SA accumulation after infection to only 5–10% of wild type levels and compromise both basal and systemic resistance. This demonstrates that the isochorismate pathway in Arabidopsis is the main source of SA in SAR. Consistent with this conclusion, *ICS1* expression is induced by infection both in local and systemic tissues. In the proposed model the phenylpropanoid pathway is responsible for the rapid production of SA

associated with local cell death, whereas the isochorismate pathway is more important for sustained SA synthesis during SAR development (Durrant and Dong, 2004). Arabidopsis ICS1 is a plastidial enzyme localized in the stroma, it is not regulated by light-dependent changes in the stromal microenvironment and it is remarkably active even at 4°C (Strawn et al., 2007). Interestingly, EDS5/SID1 encodes another protein required for SA accumulation which has sequence homology to the multidrug and toxin extrusion (MATE) family of transporter proteins. This suggests that EDS5 might be involved in the export of SA or of its phenolic precursor out of the plastid after synthesis (Durrant and Dong, 2004). Taking into account the plastid localization of SA production, it is not surprising that prolonged changes in light conditions largely affect SAR development. SAR was completely lost in dark cultivated plants, normal in ordinary light conditions and was present but not associated with SA and *PR1* accumulation in systemic leaves under high light conditions, demonstrating that under specific conditions SAR can be executed independently from these molecular markers (Zeier et al., 2004b).

2.1.2.3 SA signalling in redox mutants

Ascorbate and GSH are implicated as the major redox buffers in the cell, and operate with the enzymes that use them to regulate levels of H_2O_2 to protect cells against damage by this highly reactive form of oxygen. The GSH/GSSG and the ascorbate/dehydroascorbate redox couples are known to modulate gene expression. For instance, NPR1 monomerization depends on the redox state of the cytoplasm, especially on the GSH/GSSG ratio. The ascorbate mutants are hypersensitive to ozone, and are locked into a program of slow growth, delayed flowering, and accelerated senescence. They also have high constitutive levels of SA and certain *PR* gene transcripts. Recently, it was shown that ascorbate-deficient mutants (*vtc1* and *vtc2*) display an enhanced resistance to bacterial and fungal challenge relative to wild type plants, an effect that the investigators attribute to the premature senescence and corresponding heightened levels of SA associated with the mutant phenotype. These plants are already 'primed' to respond, i.e. they are in a state of enhanced ability to mobilize defence responses. However, in the case of *vtc1*, the connection between this priming and altered redox status is unclear because the levels of other leaf antioxidants (e.g., GSH) in the mutants are unaltered and H_2O_2 levels are similar to that of the wild type. Nonetheless, these

studies provide a link between SAR induction and the redox homeostasis (Bostock, 2005).

2.1.2.4 NPR1-dependent and –independent pathways

To identify other components of SAR signalling, several genetic screens have been conducted looking for suppressors of *npr1*. The recessive *sni1* mutant restores SA-inducible *PR* gene expression and pathogen resistance in the *npr1* background. The *sni1* mutant has wild type levels of SA and only slightly elevated expression of *PR* genes in the absence of an SAR inducer, indicating that *sni1* is likely downstream of *npr1*. The *sni1* phenotype suggests that SNI1 is a negative regulator of *PR* gene expression and SAR, whose repressive effect is overcome by NPR1 after induction. The low basal levels of *PR* gene expression and restored *PR* gene induction in the *sni1npr1* double mutant indicate that, in addition to NPR1-mediated inactivation of SNI1, a SA-dependent but NPR1-independent regulatory step is also required for SAR. Consistent with the hypothesis that SNI1 is a repressor of *PR* genes, SNI1-GFP has been observed in the nucleus when bombarded into onion epidermal cells. Moreover, when fused to the DNA-binding domain of the transcription activator of galactosidase genes in yeast (GAL4) and expressed in yeast, SNI1 repressed transcription of a reporter carrying activation sequence of GAL4 promoter upstream of its constitutive promoter (Durrant and Dong, 2004). This result suggests that SNI1 may repress a general transcriptional mechanism that is conserved between yeast and plants.

SNI1 is a novel plant-specific protein with no similarity to proteins of known function. However, putative homologues have been found in many plant species including barley, *Medicago truncatula*, potato, rice, soybean, and sugar cane, indicating that SNI1 function may be conserved. SNI1 contains no obvious DNA-binding domain, suggesting that it represses transcription through interaction with other factors rather than binding directly to the promoter. Linker scanning mutagenesis of the *PR1* promoter identified the negative regulatory *cis*-element, *LS4*, which has a W-box consensus sequence. Mutation of *LS4* resulted in elevated basal expression and stronger induction in response to INA treatment, a pattern similar to that of *PR1* in *sni1*. This suggests that SNI1 might be recruited to the *PR1* promoter through interaction with a WRKY factor (Durrant and Dong, 2004).

As an alternative approach to investigate the function of SNI1, a screen for suppressors of *sni1* (*ssn*) was performed. Three mutants were identified, *ssn1*, *ssn2*, and

ssn3, which reduce both the dwarf morphology and the background *PR* gene expression of *sni1*. In the *sni1ssn* double mutants, the pattern of *PR* gene expression is the same as in the wild type, whereas in the *sni1npr1ssn* triple mutants induction of *PR* gene expression by SA is blocked, similar to *npr1*. These results indicate that the same transcription factor(s) is probably responsible for the basal and SA-inducible NPR1-independent *PR* gene expression (Durrant and Dong, 2004). Other negative regulators of *PR* gene expression were identified by Zhang et al. (2006a). These were paralogues of NPR1 called NPR3 and NPR4. Both interacted with TGA2, TGA3, TGA5 and TGA6 transcription factors; the interaction between NPR3 and TGA2 took place in the nucleus. Thus TGA factors may enhance *PR* genes expression also by sequestering the negative regulators like NPR3 and NPR4.

Likely candidates for regulators of NPR1-independent *PR* gene expression and resistance are the Whirly (Why) family of transcription factors, named after the whirligig appearance of their crystal structure. Potato StWhy1 specifically binds the single-stranded form of a *cis*-element in the *PR10a* promoter. Arabidopsis has three genes encoding Whirly proteins, of which *AtWhy1* is the most similar to *StWhy1*. SA treatment induced AtWhy1 DNA-binding activity in both wild type and *npr1* plants, indicating that AtWhy1 activation is independent of NPR1. Knock-out mutants of *AtWhy1* were lethal. However, two lines with missense mutations in the ssDNA-binding domain (*atwhy1.1*) or the central α-helical region (*atwhy1.2*) were viable and showed reduced DNA-binding activity, reduced SA-induced *PR1* transcript accumulation, and reduced resistance to *Peronospora parasitica*. These observations suggest that AtWhy1 is important for NPR1-independent *PR* gene expression (Durrant and Dong, 2004). It would be interesting to test whether *atwhy1* mutants can block the NPR1-independent *PR* gene expression observed in the *sni1npr1* background.

Another component of the NPR1-independent SA-inducible pathway was identified thanks to the *dth9* (detachment) mutant. The *dth9* has an increased susceptibility to virulent pathogens, accumulates elevated levels of SA, and fails to develop SAR in response to pathogen infection and SA treatment. These phenotypes are reminiscent of *npr1*; however, *dth9* differs from *npr1* in that its *PR1* and *PR2* expression in response to infection or SA treatment was unaltered. Since SA treatment did not reverse the disease susceptibility observed in the mutant, DTH9 should be placed downstream of SA in a pathway parallel to NPR1 that contributes to SAR. Interestingly, *dth9* is also insensitive to auxin treatment, indicating that auxin signalling

may play a role in plant defence (Durrant and Dong, 2004). Figure 2 shows a signalling network of induced resistance where important mutants described above are placed with respect to their impact on either local or systemic resistance.

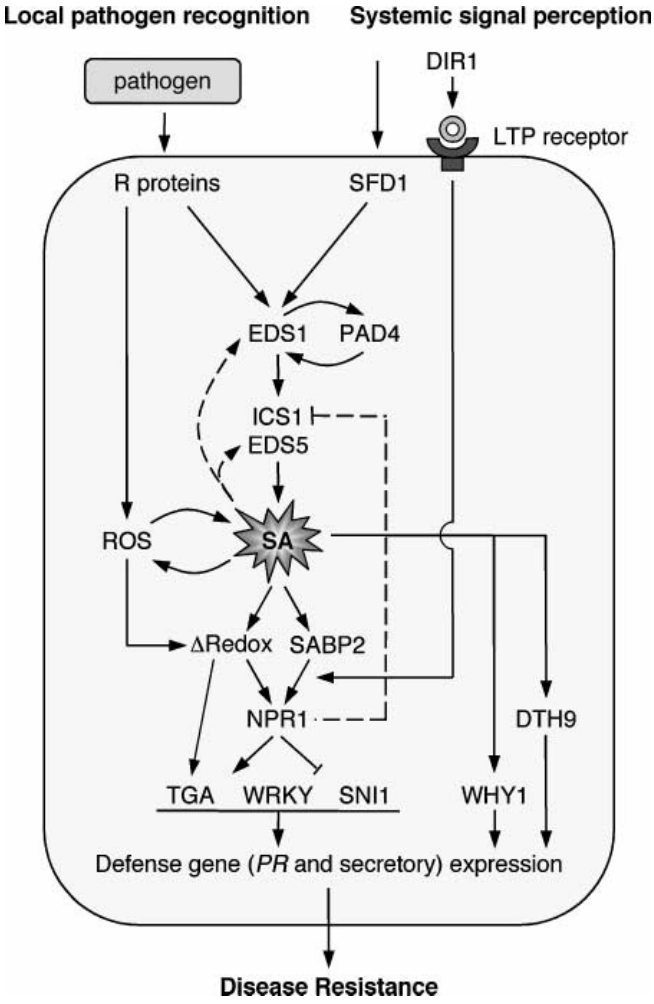


Figure 2. The sequence of events from pathogen recognition to defence gene induction.
Important mutants affected in the signalling pathway leading to induced resistance are placed in a signalling network with respect to their influence on either local or systemic resistance. Adopted from Durrant and Dong (2004).

2.1.2.5 Connections and cross-talks

It is impossible to understand SAR fully without studying its interaction with other biological processes. It is hypothesized that plant defence pathways interact synergistically or antagonistically to fine tune responses according to the challenging organism(s). Activation of one pathway may lead to cross-talk inhibition of another that is less effective against the pathogen. The genetic evidence shows points of cross-talk and convergence in the signalling pathways leading to SAR, *R*-gene-mediated resistance, and basal (i.e. non-induced or constitutive) resistance to pathogens. SAR

development is known to be somehow antagonistic to JA-mediated ISR. Experiments with transgenic plants compromised in the SAR response also provide good evidence for the trade-off between SA- and JA-mediated signalling. Tobacco plants silenced for PAL and thus containing reduced levels of phenylpropanoid compounds as well as plants expressing *nahG* both displayed a stronger ISR in response to larval feeding by the tobacco hornworm *Manduca sexta* (Bostock, 2005). Overexpression of glutaredoxin GRX480, a direct TGA2 binding partner, in Arabidopsis damped the JA gene responses (Ndamukong et al., 2007). WRKY transcription factors were also proposed as elements of such negative cross-talk between SA and JA pathways, in the direction of SA damping the action of JA (Mao et al., 2007). Only a few studies provide evidence for negative cross-talk in the direction of JA damping SA action. Arabidopsis mutants have been useful in this regard and also in illustrating possible ecotype differences with respect to degree of the cross-talk. Growth of *Pseudomonas syringae* pv. *tomato* in the Wassilewskija ecotype was reduced after SA treatment, but this effect was negated when plants were treated both with SA and JA (Bostock, 2005). Surprisingly, the same study found little evidence of a negative cross-talk between SA and JA in either direction in the Columbia ecotype. In another study with Arabidopsis, the constitutive activation of JA responses that occurs in the *cev1* (constitutive expression of VSP1) mutant resulted in inhibition of SA-dependent responses (Bostock, 2005).

Another connection can be drawn for ABA, which is considered as a negative regulator of SAR and of biotic stress in general, but in some cases it can increase the resistance of plants towards pathogens via its positive effect on callose deposition (Mauch-Mani and Mauch, 2005). Microbial entry into host tissue is the first critical step during infection both in animals and plants. Stomatal closure is a part of the plant innate immune response which helps to restrict the bacterial invasion. Stomatal guard cells of Arabidopsis perceive bacterial surface molecules and close stomata in SA-dependent manner, a process which requires the FLS2 receptor, production of NO, and the guard-cell-specific OST1 kinase. The process, as stomatal closure in general, is inevitably linked to ABA signalling. To circumvent this innate immune response, plant pathogenic bacteria have evolved specific virulence factors (like the polyketide toxin, coronatine) to effectively cause stomatal reopening as an important pathogenesis strategy (Melotto et al., 2006). Comprehensive scheme of SA/SAR signalling where important mutants are placed together with connections to other signalling pathways is given in Figure 3.

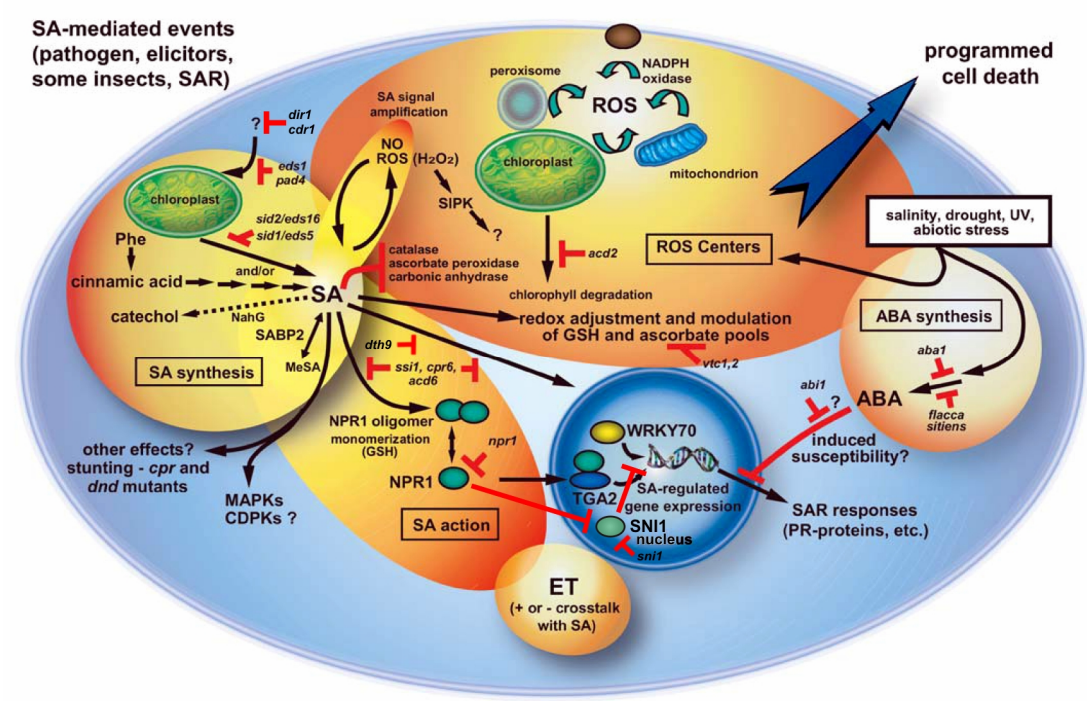


Figure 3. Synthesis and action of SA in local and systemic acquired resistance (SAR) and cross-talk with stress-induced ROS, ethylene (ET) and abscisic acid (ABA).
Key steps in SA synthesis and action and locations of some mutant blocks as discussed in the text are indicated. Mutations are primarily those in Arabidopsis, except *flacca* and *sitiens* (tomato). Possible separation of the indicated processes in different cell types is not considered in the figure. SA as a central molecule of SAR signalling is produced mainly in chloroplasts via isochorismate and partially in the phenylpropanoid pathway via cinnamic acid. It can be transformed to methylsalicylate (MeSA), removed by salicylate hydroxylase in *nahG* transgenics or it can generate reactive oxygen species (ROS) in a self-amplification loop. It induces shift of redox potential of the cytoplasm to a reductive state which promotes monomerization of transcription factor NPR1. NPR1 relieves the constitutive block on expression of *PR* genes (represented here by a negative transcription regulator SNI1) and in cooperation with TGA and WRKY transcription factors triggers the expression of *PR* genes. This expression can be negatively regulated by activated ABA signalling. ROS can be generated by other abiotic stresses and cause programmed cell death when not handled properly. SA helps to modulate ROS levels via the indicated means. *aba*, ABA-deficient; *abi*, ABA-insensitive; *acd*, accelerated cell death; *cdr*, constitutive disease resistance; *cpr*, constitutive expressor of *PR* genes; *dir*, defective in induced resistance; *dnd*, defence, no death; *dth*, detachment; *eds*, enhanced disease susceptibility; ET, ethylene; NO, nitric oxide; *npr1*, non-expressor of pathogenesis-related protein 1; *pad*, phytoalexin deficient; SABP2, SA binding protein 2; *sid*, SA induction-deficient; SIPK, SA-induced protein kinase; *sni1*, suppressor of NPR1, inducible; *ssi*, suppressor of SA insensitivity; *vtc*, ascorbate-deficient. Adopted from Bostock (2005) and modified.

2.1.2.6 Metabolic costs of induced resistance

Transgenic plants provide valuable information about the impact of induced resistance on plant fitness and reproduction. One study showed that *nahG* and *nim1* (non-inducible immunity) plants grown in a growth chamber have high seed production in the absence of SAR induction (Durrant and Dong, 2004). On the contrary, mutants with constitutively expressed resistance (*cpr*) have lower fitness than the wild type and they often display a stunted phenotype. SAR was found to be costly even when induced

with chemical inducers. When SAR was induced in wheat by BTH, there was 5-75% reduction in fitness. A high dose of SA caused a reduction in seed yield in *Arabidopsis thaliana* (Heidel et al., 2004). A fitness cost represented by the reduction of relative growth rate and seed production was reported also for BTH treated Arabidopsis plants; the observed changes were caused by the activation of NPR1-dependent defences (van Hulten et al., 2006). This supports the idea that constitutive SAR expression, no matter how it is brought about, has costs. Such results have important implications for development of crop protection strategies through SAR manipulation.

The fitness cost can be partially attributed to the large number of proteins that are induced by SAR compared to a relatively small number of the repressed (Maleck et al., 2000; Chen et al., 2002; Glazebrook et al., 2003). In tobacco, two PR proteins can reach up to 3% of soluble protein (Heidel et al., 2004). Parallel transcriptional reduction in other genes could potentially limit the immediate cost of the SAR. Indeed, reductions in photosynthesis transcripts/proteins are common in defensive responses and while these may 'save' on protein synthesis, they themselves may be symptoms of how costs are expressed (e.g., reduced photosynthetic rate). Another fitness cost was documented for *cpr1* which exhibited reduced cold tolerance and lowered overwinter survival rate (Heidel et al., 2004).

Supplemental costs are represented by the activation of secondary metabolism to produce antimicrobial compounds like phytoalexins (Durner et al., 1997) and alkaloids (Matsuo et al., 2001). Enhanced lignin synthesis (Durner et al., 1997) and callose deposition (Bostock, 2005) are other costly metabolic pathways which are activated during the onset of resistance. Strengthening of the cell wall through lignification and callose deposition is believed to prevent the pathogen from infecting the plant cell (Durner et al., 1997). Some reports point to SAR inducer-stimulated anthocyanin synthesis which may be seen as related to accelerated senescence or as a part of the mobilization of antioxidants (Iriti et al., 2004; Kong JM et al., 2003; Sudha and Ravishankar, 2003).

There has been a substantial discussion on the role of resource availability in the expression of induced defences in plants. One expectation is that metabolic costs will be enhanced under resource-limited conditions. On the other hand, it can also be argued that if a resistance is costly, then it will be downregulated under nutrient stress to minimize the cost. A study of BTH-induced SAR in wheat revealed that under sterile

conditions low nutrients caused a greater relative cost. However, under field conditions they found the reverse situation (Heidel et al., 2004).

The presented fitness costs can be summarized as a metabolic reconfiguration in favour of a shift away from growth and reproduction to other processes. Such trade-off is typical for inducible resistance. Mechanisms of induced resistance allow the plant to reallocate its limited resources for the defences only when necessary. Another benefit of the induced versus basal resistance is that the induced resistance represents a 'surprise' to the invading pathogen letting it no chance to adapt and become resistant to its defences.

2.1.3 SA in other physiological contexts

2.1.3.1 Temperature stress

Most of the knowledge about SA comes from studying SAR. However, there are other physiological situations in which SA is somehow involved. SA helps the plant to cope with the ROS generated during many different stress situations including temperature stress. There is much more literature covering the involvement of SA in temperature stress than in any other (besides SAR) and therefore it will be discussed separately. SA was produced early upon the onset of both cold and heat stress (Scott et al., 2004; Liu et al., 2006a Liu et al., 2006b). Kang and Saltveit (2002) showed that pre-treatment of rice and cucumber seedlings reduced the chilling-induced electrolyte leakage from leaves. This was accomplished by induction of GSSG reductase and guaiacol peroxidase in aerial parts. Similar results were obtained when tomato plants were pre-treated either with MeSA or MeJA (Ding et al., 2002). However, chilling did not substantially induce expression of SA marker genes. Surprisingly, Scott et al. (2004) showed that *nahG* plants were more tolerant to chilling and displayed greater growth rates at 5°C than the wild type. The partially SA-insensitive *npr1* mutant displayed growth intermediate between *nahG* and Col-0, while the SA-deficient *eds5* mutant behaved like *nahG*. In contrast, the *cpr1* (constitutive expressor of *PR* genes) mutant at 5°C accumulated very high levels of SA and its growth was much more inhibited than that of the wild type and displayed more extensive oxidative damage at normal and chilling temperatures. The observed discrepancy with previous pharmacological studies was explained by the use of higher temperatures not causing substantial damage (5°C comparing to 2.5°C in Kang and Salveit, 2002) and longer treatment times (weeks

comparing to a few days in Kang and Salveit, 2002). SA was produced and the differences between the studied genotypes were observed starting only from the second week of such mild chilling treatment.

Heat stress causes similar oxidative damage as chilling. Indeed, SA (but also ABA and ethylene) protected Arabidopsis plants against oxidative damage caused by heat stress. Moreover, mutants defective in the three mentioned signalling pathways (*nahG*, *abi1*, ABA-insensitive, and *etr1*, ethylene receptor) were more susceptible to elevated temperatures (Larkindale and Knight, 2002). In a comprehensive study Clarke et al. (2004) showed that SA promotes basal thermotolerance but it is not required for acquired thermotolerance. SA was important during the heat shock and the subsequent recovery. Recovery from heat shock involved an NPR1-dependent pathway but thermotolerance during heat shock did not. SA induced membrane thermoprotection and expression of some heat shock proteins. Accordingly, heat shock protein Hsp17.6 appeared more slowly in *nahG* plants. All genotypes (including *nahG* and *npr1*) when heat acclimated, expressed heat shock proteins and acquired heat shock tolerance. Based on the characterization of many mutants (including *nahG*) during heat stress Larkindale et al. (2005) showed that signalling pathways involving SA and ROS are critical for events both during basal and acquired thermotolerance. The role of SA in acquired thermotolerance was also demonstrated in pea (Liu et al., 2006a; Liu et al., 2006b) suggesting that such mechanism of temperature adaptation is common for higher plants.

2.1.3.2 Oxidative stress

Role of SA in other stress responses can be summarized as mobilization of antioxidant capacity of the cell. SA induces a more reductive state of the cytoplasm which helps the plant to cope with ROS generated during various stresses. Rice has high basal levels of SA. Expression of *nahG* in rice demonstrated that SA modulates redox balance and protects rice plants from oxidative stress by mobilizing the plant's capacity to detoxify ROS (Yang et al., 2004). Examples of protective role of SA during stress are numerous: SA was produced during exposure to ROS (Danon et al., 2005), UV-B (Surplus et al., 1998) and UV-C stress (Yalpani et al., 1994). It was produced during osmotic stress (Borsani et al., 2001), specifically to cope with the oxidative damage. Senaratna et al. (2000) found that SA promoted drought tolerance in bean and tomato (dicots). Interestingly, SA decreased drought tolerance in maize and wheat (monocots; Nemeth et al., 2002). Accordingly, SA promoted tolerance to high light stress (Mateo et

al., 2006) and paraquat treatment (Kim HS et al., 2003), both being situations where ROS are produced. Despite its protective role against ROS, SA itself was proposed to induce ROS via uncoupling and inhibition of the mitochondrial electron transport. Such localized and controlled ROS production triggered a specific gene response (Norman et al., 2004).

Another role of SA is its capability to promote PCD (Overmyer et al., 2003). Ethylene is the substance which induces PCD during ozone treatment and SA is produced upon exposure to ozone to promote ethylene production (Rao et al., 2002). Ogawa et al. (2005) elaborated this observation by finding that SA is produced upon exposure to ozone both in Arabidopsis and tobacco, yet by different biosynthetic pathways. In this case SA production was under the control of ethylene. These two seemingly opposite findings suggest a self-amplifying feed-back mechanism between SA and ethylene production. Similar self-amplifying feed-back mechanism was documented also during singlet oxygen generation (Danon et al., 2005) suggesting a common mechanism by which PCD is triggered in higher plants.

2.1.3.3 Plant development

SA production *in planta* was shown to have some developmental consequences, especially in stress-influenced developmental transitions. Both exogenous and endogenous SA inhibited root growth in legumes and also inhibited nodule formation on both determinate and indeterminate nodule-forming hosts (Stacey et al., 2006). It induced earlier flowering (Martinez et al., 2004), promoted tuberization (Lopez-Delgado and Scott, 1997) and senescence (Morris et al., 2000). Its role in senescence can be directly linked to its capability to trigger PCD (Overmyer et al., 2003). Its role in developmental senescence but not in dark-induced senescence was also established by the means of whole genome transcriptional profiling (Buchanan-Wollaston et al., 2005). SA reduced seed yield in Arabidopsis (Heidel et al., 2004; chapter 2.1.2.6). Growth of tobacco seedlings on 100 μM SA reduced shoot biomass and leaf epidermal cell size (Dat et al., 2000) and endogenous SA content correlated with the growth retardation during chilling (Scott et al., 2004; chapter 2.1.3.1).

2.2 Phospholipid signalling in plants

Current challenge in plant biology is to unravel intracellular communication and elucidate the pathways involved in signalling. Transduction starts with receptors, often in the plasma membrane, that perceive the change and relay the information into the cell. It is clear that lipid-based signalling is also involved in plant immune response (some evidence was already discussed in chapter 2.1.2.1). Most reports on plant defence-related lipid signalling point to phospholipids and phospholipid-modifying enzymes. Therefore a more detailed description of these will be given in this chapter. Figure 4 shows selected phospholipid signalling pathways. The signal perceived on the cell surface by a membrane receptor is often transmitted via an effector enzyme that converts lipids into signalling molecules inside the cell.

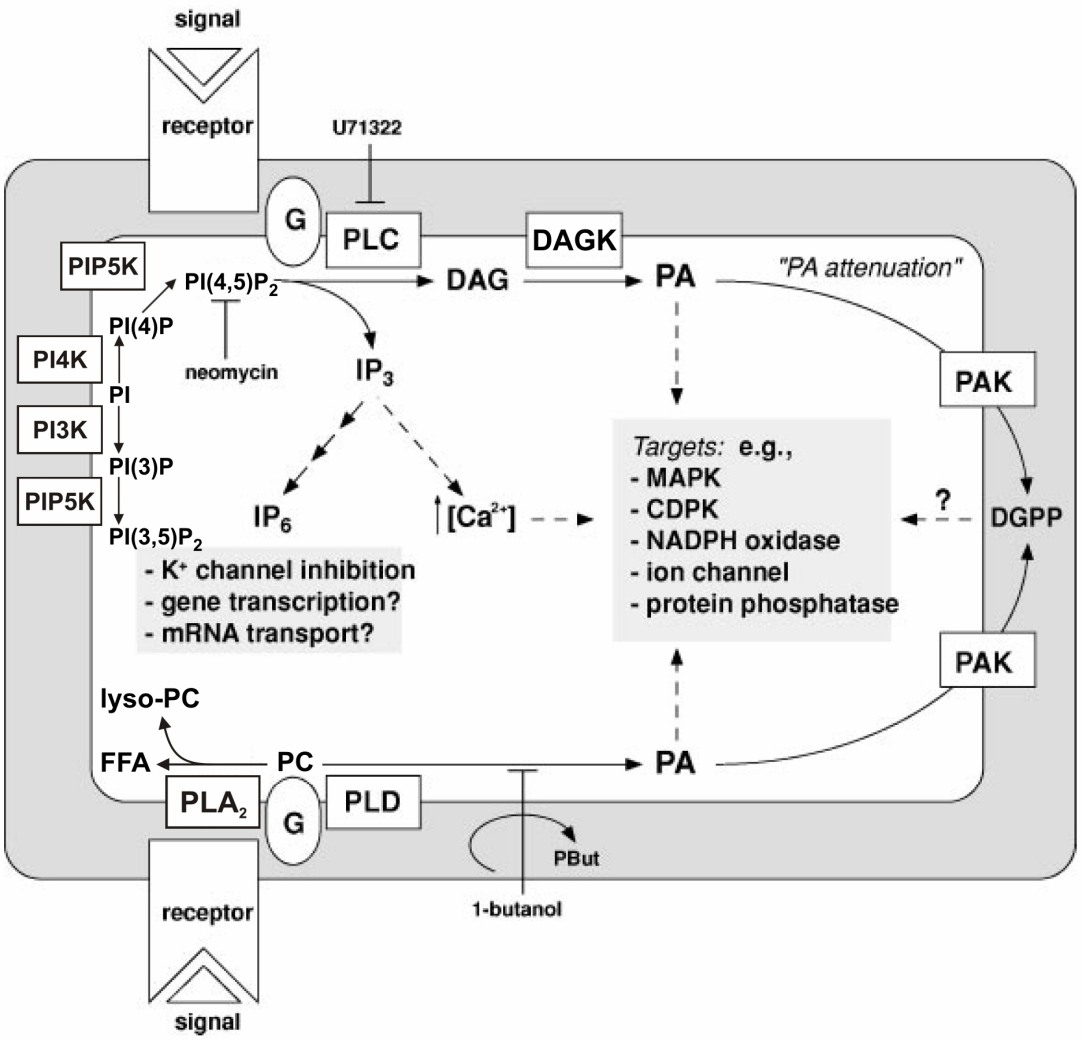
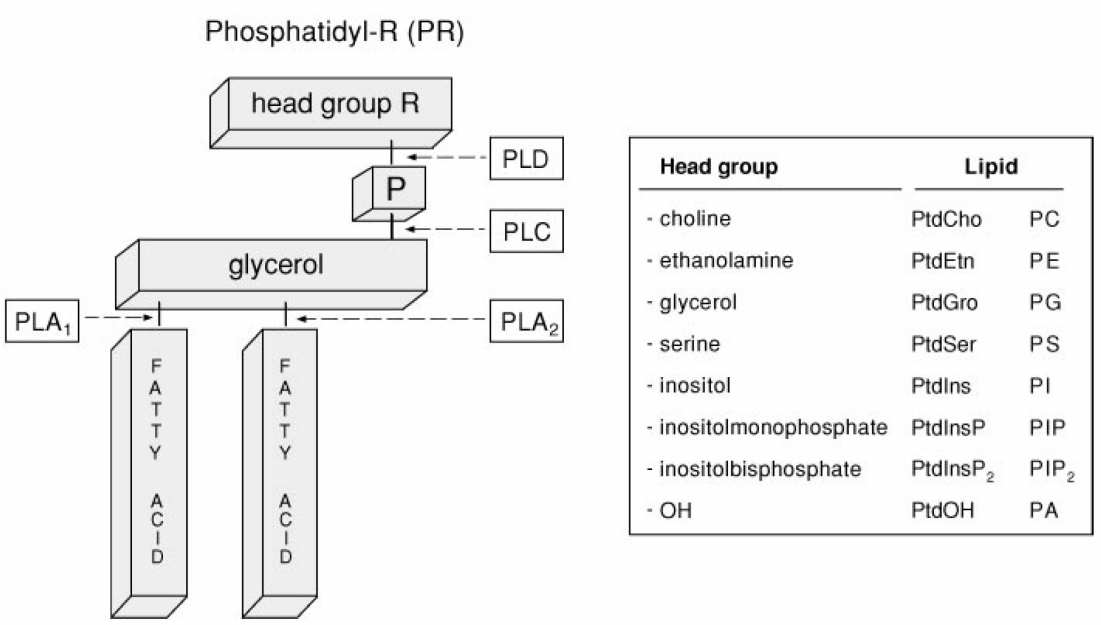


Figure 4. Model of phospholipid signalling in plants.
PI can be phosphorylated either by PI 3-kinase (PI3K) or by PI 4-kinase (PI4K) to form PI(3)P or PI(4)P respectively. These can be further phosphorylated by PIP 5-kinase (PIP5K) to give $PI(3,5)P_2$ or $PI(4,5)P_2$. PLC hydrolyzes $PI(4,5)P_2$ into IP_3 and diacylglycerol (DAG). IP_3 diffuses into the cytosol where it releases Ca^{2+} from intracellular stores or is converted to IP_6, triggering both Ca^{2+} and nuclear responses. DAG remains in the membrane where it is phosphorylated to PA by DAG kinase (DAGK). PLD generates PA directly by hydrolyzing structural lipids such as phosphatidylcholine (PC). PC can be hydrolyzed to lyso-PC and free fatty acid (FFA) by phospholipase A_2 (PLA_2). Increased PA levels affect several plant processes via various intracellular targets. Signalling is attenuated when PA is phosphorylated to DGPP by PA kinase (PAK) or hydrolyzed to lyso-PA by PLA. Inhibitors and the putative involvement of G-protein (G)–coupled receptors are indicated. For explanation of other abbreviations see text. Adapted from Meijer and Munnik (2003) and modified.

2.2.1 Key phospholipid signalling molecules

Common phospholipids are membrane components represented by the structure shown in Figure 5, which also includes the abbreviations used throughout the text. Structure and metabolism of the subgroup of phospholipids derived from phosphatidylinositol (phosphoinositides) is depicted in Figure 6.



Head group	Lipid	
- choline	PtdCho	PC
- ethanolamine	PtdEtn	PE
- glycerol	PtdGro	PG
- serine	PtdSer	PS
- inositol	PtdIns	PI
- inositolmonophosphate	PtdInsP	PIP
- inositolbisphosphate	$PtdInsP_2$	PIP_2
- OH	PtdOH	PA

Figure 5. Structure, hydrolysis, names, and abbreviations of the common phospholipids.
On the left, the general structure of a phospholipid is shown, consisting of two fatty acyl chains esterified to a glycerol backbone at the *sn*-1 and *sn*-2 positions, a phosphate at the *sn*-3 position (creating the 'phosphatidyl' moiety), to which a variable headgroup (R) is attached. The positions that are subject to phospholipase activity are also indicated. On the right side, the possible headgroups are indicated together with the abbreviations that are used in the text. The exception to this nomenclature, and therefore not included, is diacylglycerol pyrophosphate (DGPP), which has an extra phosphate group on the phosphatidyl moiety. Adapted from Meijer and Munnik (2003).

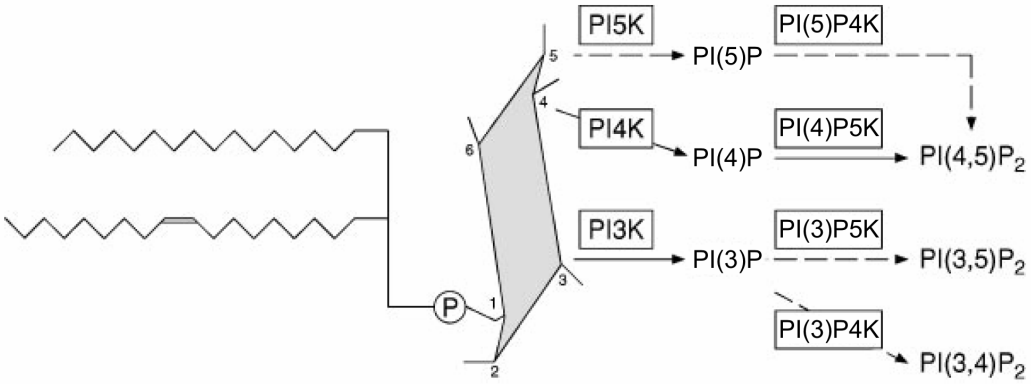


Figure 6. Possible pathways of phosphoinositide synthesis in plants.
Solid arrows indicate established pathways; dashed arrows remain to be established. Adapted from Meijer and Munnik (2003).

2.2.1.1 Phosphoinositides

Plants contain three PIP isomers (PI(3)P, PI(4)P, and PI(5)P) and three PIP_2 isomers ($PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(4,5)P_2$) but no PIP_3. This is significant because $PI(3,4,5)P_3$ is a prevalent signal in animal cells (Meijer and Munnik, 2003). Phosphorylation of the inositol ring on the D-3-, D-4-, or D-5-position is carried out by

specific phosphoinositide kinases, including PI 3-kinase, PI 4-kinase, PI 5-kinase, the PI(3)P 5-kinase, PI(4)P 5-kinase, and the PI(5)P 4-kinases (Figure 6).

PI(3)P was found in various plant species. In *Chlamydomonas* its turnover rate is much faster than that of structural phospholipids; this is typical for signalling lipids (Meijer and Munnik, 2003). PI(3)P was important for ABA-induced ROS generation and stomatal closure in Arabidopsis (Park et al., 2003) and its levels raised during salt stress (Meijer and Munnik, 2003). PI(3)P forms a docking site for proteins with specific domains, e.g. FYVE or PX domains. A chimera of FYVE and GFP first labelled the *trans*-Golgi network (TGN) when expressed in Arabidopsis, suggesting that PI(3)P could be synthesized there. In time course studies, the GFP label was transported to the prevacuolar body and then to the tonoplast and finally it was internalized as vacuolar vesicles. When PI(3)P docking sites were saturated, protein transport to the vacuole was inhibited. These results are reminiscent of the phenotype seen when the yeast PI 3-kinase (Vps34p) was knocked out, emphasizing that the role of polyphosphoinositides (PPIs) in membrane trafficking and vacuole turnover in yeast could be similar to that in plants (Meijer and Munnik, 2003). In an imaging study of *in vivo* distribution PI(3)P colocalized with highly motile structures undergoing fusion and fission, presumably pre-vacuolar compartments. In addition, it was present in the vacuolar membrane of Arabidopsis guard cells and in the newly formed cell plate during cytokinesis of BY-2 cells (Vermeer et al., 2006). PI(3)P also binds to dynamin-related protein 2A from Arabidopsis (AtDRP2A) which is involved in protein trafficking from TGN to the central vacuole (Lee et al., 2006).

PI(4)P decreased during hypoosmotic treatment of tobacco pollen tubes (Zonia and Munnik, 2004), both PI(4)P and PI(4,5)P_2 levels increased upon spermidine and spermine treatment of *Coffea arabica* suspension cells (Echevarria-Machado et al., 2005). PI(4)P binds to the PH domain of some proteins but these domains can bind other phospholipids too, thus the specificity of the given PH domain must be assayed experimentally (Meijer and Munnik, 2003).

PI(5)P is the most recently identified PPI. It was initially overlooked because it is not easily separated from PI(4)P, which is present in much higher concentrations. Kinase assays and HPLC techniques can be used to distinguish PI(5)P from PI(4)P and PI(3)P. The amount of PI(5)P in animal systems is relatively low, representing around 2% of the PIP pool, compared with 3% to 18% in plants. It is still not clear how cellular PI(5)P is generated. *In vitro*, both PI(4)P 5-kinase and PI(3)P 5-kinase can be

phosphorylated PI to PI(5)P (Figure 6). Recently, an inositol lipid 3-phosphatase (MTMR3) was described that converted PI(3,5)P_2 to PI(5)P with high specificity. PI(5)P can then be converted to PI(4,5)P_2 by PI(5)P 4-kinases. About 8% of the *Chlamydomonas* PIP pool was PI(5)P, compared with about 12% PI(3)P, whereas 80% was PI(4)P. It does not seem to be converted to PI(4,5)P_2 because when turnover of the latter was stimulated, PI(4)P but not PI(5)P levels changed accordingly. During hyperosmotic stress, the PI(5)P level increases together with PI(3,5)P_2. This indicates that PI(5)P is involved in osmotic stress and suggests that it may be a precursor or a metabolic product of PI(3,5)P_2 (Meijer and Munnik, 2003). PI(5)P regulated organogenesis related transcription factor ATX1 in Arabidopsis (Alvarez-Venegas et al., 2006).

PI(4,5)P_2 is prevalently synthesized via phosphorylation of PI(4)P, although it can also be formed by phosphorylation of PI(5)P on the 4-position (Figure 6). In higher plants, PI(4,5)P_2 levels are about 10 times lower than in animals or in lower plants such as *Chlamydomonas*. This means that its turnover upon PLC activation must be exceptionally high to generate D-*myo*-inositol 1,4,5-trisphosphate (IP_3) concentrations equivalent to those in animals. In fact, the need for IP_3 in plants is even higher because the plant IP_3-induced Ca^{2+} release requires higher concentration of IP_3 than the animal one (Krinke et al., 2007a). Apart from being a substrate or a newly formed docking site for signalling proteins, hydrolysis of PI(4,5)P_2 could also mean a loss of such docking sites. This is the basis of a plasma membrane-to-nucleus signalling system involving 'Tubby' proteins in animals. These are transcription factors that dock to PI(4,5)P_2 in the plasma membrane via their carboxy terminal Tubby domains. When PLC hydrolyzes the docking sites, the Tubby proteins are released to migrate to the nucleus and influence gene expression. Tubby-like sequences are present in plant databases. Besides PLC, down-regulation of PI(4,5)P_2 increase in Arabidopsis took place via a phosphoinositide 5-phosphatase (At5PTase14; Zhong and Ye, 2004). Another enzyme exhibiting such activity was identified thanks to *fra3* (fragile fiber 3) mutant with defective secondary wall in fiber cells and xylem vessels (Zhong et al., 2004). Confirmation that PI(4,5)P_2 is a stress response regulator in plants comes from the characterization of Arabidopsis *sac9* mutant which is defective in phosphoinositide phosphatase and exhibits constitutive stress response accompanied by constitutive overexpression of stress-induced genes (Williams et al., 2005). The presence of different PI(4,5)P_2 pools in the cell was illustrated for the alga *Galdieria sulphuraria*.

The $PI(4,5)P_2$ levels in plasma membranes decreased during the stationary phase of growth, whereas that in microsomes increased (Meijer and Munnik, 2003).

When *Chlamydomonas* was subjected to hyperosmotic stress or G-protein activation, PLC was activated and $PI(4,5)P_2$ levels initially decreased, followed after 30 sec by up-regulation of its synthesis. By pre-labelling cells with $^{32}P_i$ for only 30 sec, which favours the detection of synthesis rather than hydrolysis, mastoparan, a G-protein activator, immediately stimulated its synthesis. As labelling times became longer, the concomitant activation of PLC dominated the labelling pattern, and the synthesis of $PI(4,5)P_2$ was not observed, even though present. In Arabidopsis cell suspensions and plants, net $PI(4,5)P_2$ synthesis was enhanced by hyperosmotic stress. Concentrations reached 8 to 25 times the control values within 10 to 20 min and were associated with similar increases in IP_3 production (Meijer and Munnik, 2003). Both $PI(3,5)P_2$ and $PI(4,5)P_2$ levels increased during hyperosmotic stress in tobacco pollen tubes (Zonia and Munnik, 2004).

$PI(4,5)P_2$ is important for vacuole function in yeast. The vacuole is a multivesicular organelle that can fuse to form a single vesicle or fragment into smaller ones. In *Schizosaccharomyces pombe*, vacuole fusion and fragmentation are triggered by hypo- and hyperosmotic stress, respectively. In *Saccharomyces*, $PI(4,5)P_2$ is needed for vacuole fusion, whereas $PI(3,5)P_2$ seems to play a role in vacuole fragmentation (see below). Much less is known about plants, but some evidence suggests that $PI(4,5)P_2$ plays a role in vesicular trafficking (Monteiro et al., 2005). Profilin, a G-actin binding protein thought to be regulated by $PI(4,5)P_2$, is a potent controller of actin dynamics and was recently found to be localized in the bulges of outgrowing root hairs and pollen tubes (Meijer and Munnik, 2003). $PI(4,5)P_2$ regulated pollen tube growth in *Petunia inflata* where it was preferentially localized at sites undergoing rapid expansion (Dowd et al., 2006). $PI(4,5)P_2$ and profilin could together determine the site for vesicle delivery and so regulate exocytosis. In support, disrupting actin filaments or hydrolyzing $PI(4,5)P_2$ disrupted tip growth (Meijer and Munnik, 2003). $PI(4,5)P_2$ modulated the activity of actin capping protein from Arabidopsis (AtCP; Huang et al., 2003).

$PI(3,5)P_2$ is a recently discovered phosphoinositide that is synthesized from PI(3)P by PI(3)P 5-kinase. This enzyme is present in plants, bears FYVE domain and it is homologous to yeast Fab1p. This means that PI(3)P is needed not only for membrane trafficking and sorting to the vacuole but also for the formation of the subsequent $PI(3,5)P_2$ signal. $PI(3,5)P_2$ levels may be down-regulated by another phosphoinositide

5-phosphatase (At5PTase11) identified in Arabidopsis. Its expression was regulated by ABA, JA and auxin (Ercetin and Gillaspy, 2004). Another enzyme exhibiting such activity (AtSAC1) was identified in *fra7* (fragile fiber 7) mutant. It was important for normal cell morphogenesis of vascular tissues, cell wall synthesis, and actin organization and it colocalized with Golgi apparatus marker (Zhong et al., 2005).

$PI(3,5)P_2$ was identified in the yeast *S. cerevisiae* and in various plant species, where its levels increased 2- to 20-fold during hyperosmotic stress. Yeast mutants completely lacking $PI(3,5)P_2$ were viable but they failed to internalize and recycle the tonoplast. This underlines the importance of $PI(3,5)P_2$ in vacuolar turnover. $PI(3,5)P_2$ may help maintain vacuolar integrity during hyperosmotic conditions. As the vacuole shrinks because of water loss, the surface area of the tonoplast can be maintained by fragmentizing the vacuole, a process in yeast that is promoted by $PI(3,5)P_2$. The lipid also promotes vacuolar H^+-ATPase activity, therefore yeast mutants depleted in $PI(3,5)P_2$ have neutral rather than acidic pH in their vacuoles. During osmotic stress, yeast and plants synthesize osmolytes to compensate dehydration. They are transported into the vacuole by proton exchangers. Therefore, $PI(3,5)P_2$ synthesis could help the transport by stimulating the proton pump to maintain proton gradient over the tonoplast. Hyperosmotic stress in Arabidopsis increased the formation of $PI(4,5)P_2$ rather than $PI(3,5)P_2$. Nonetheless, $PI(3,5)P_2$ increased in several other higher plants. The PH domain of centaurin β2 specifically binds $PI(3,5)P_2$. Centaurin activates the GTPase activity of ARF, which is involved in membrane trafficking. It could therefore be the first true target for $PI(3,5)P_2$ that has been discovered (Meijer and Munnik, 2003).

2.2.1.2 PA and DGPP

PA can be generated either by direct hydrolysis of structural lipids (by the action of PLD) or in a two-step process through phosphorylation of DAG generated by PLC (Figure 4) either acting on $PI(4,5)P_2$ or on PI(4)P (PI-PLC) or on PC (PC-PLC). Abbreviation PLC will designate the canonical $PI(4,5)P_2$-hydrolyzing PLC in the following text. PA is a special phospholipid with conically shaped molecule (Bargmann and Munnik, 2006). When it is enriched in some membrane domain, it can modify the structure of cellular membranes by perturbing the lateral diffusion of phospholipids and by forming hexagonal structures causing membrane curvature. This property of PA is enhanced by micromolar concentrations of Ca^{2+} (Faraudo and Travesset, 2007). The origin of PA can be determined thanks to the transphosphatidylation reaction of PLD or

by analyzing the fatty acyl composition of PA (see chapter 2.2.2.6 for details). The involvement of PLC in a given stress response can be tested by application of various inhibitors (U73122, edelfosine and PI(4,5)P_2-chelating agent neomycin). Whereas DAG is a second messenger in animals, this has not been established for plants or fungi. Plant DAGK rapidly converts the DAG produced by PLC into PA. In contrast, plant PA is now accepted as a signalling molecule, the point of convergence in the network of plant phospholipid signalling. Its central role in plant stress response was reviewed by Testerink and Munnik (2005) and by Wang (2005). Nice comprehensive review on physiological role of PA both in animals and plants has recently been published (Wang X et al., 2006). Examples of its involvement in diverse stress situations are given in Table II.

Table II. *Examples of involvement of PA and DGPP in plant stress responses.*

Stress	Plant species	Enzyme involved	PA level[a]	DGPP level[a,b]	Reference
elicitors (chitotetraose, flg22)	*Lycopersicon esculentum*	PLC	+	?	Meijer and Munnik, 2003
elicitors (xylanase)	*Lycopersicon esculentum*	PLC/PLD	+	?	Meijer and Munnik, 2003
incompatible reaction triggered by fungal elicitor AVR4 from *Cladosporium fulvum*	*Nicotiana tabacum* (expressing tomato resistance gene *Cf-4*+)	PLC	+	+	Meijer and Munnik, 2003
Rhizobium Nod factor	*Vicia sativa*	PLC/PLD	+	+	Meijer and Munnik, 2003
wounding	*Arabidopsis thaliana* *Glycine max*	PLD	+	?	Meijer and Munnik, 2003
mastoparan	*Chlamydomonas moewusii*	PLC/PLD	+	+	Meijer and Munnik, 2003
hypoosmotic stress	*Nicotiana tabacum* (pollen tube)	PLD	+	?	Zonia and Munnik, 2004
low concentration of salt	*Chlamydomonas moewusii*	PLD	+	?	Meijer and Munnik, 2003
hyperosmotic stress	*Nicotiana tabacum* (pollen tube)	PLD	-	?	Zonia and Munnik, 2004
salt stress	*Zea mays*	PLD	+	?	Zhang et al., 2006b
ABA	*Arabidopsis thaliana*	PLD	+	+	Zalejski et al., 2005
cold	*Arabidopsis thaliana*	PLC/PLD	+	?	Ruelland et al., 2002
Al^{3+}	*Coffea arabica*	PLC/PLD	-	?	Ramos-Diaz et al., 2007
Cu^{2+}	*Triticum durum*	PLD	+	?	Navari-Izzo et al., 2006

[a]plus sign means that the level increased, minus sign means that the level decreased

[b]question mark denotes that the DGPP level was not determined in the study

PA accumulation from the PLC/DAGK pathway (PA_{PLC}) was placed upstream of the elicitor-induced oxidative burst caused by NADPH oxidase activation (Meijer

and Munnik, 2003). On the other hand, PLD-produced PA (PA_{PLD}) was important for down-regulation of oxidative burst-induced cell death in Arabidopsis (Shah, 2005). A complex interplay probably exists between PLC and PLD pathways. Tobacco cells treated with xylanase elicitor responded by synthesizing *N*-acylphosphatidyl-ethanolamine (NAPE). Upon treatment, *N*-acylethanolamine (NAE) is rapidly accumulated 10 to 50 times of the normal concentration, which is thought to be generated from NAPE by PLD. NAE in nanomolar concentrations activates PAL, a key regulatory enzyme in phenylpropanoid metabolism ultimately leading to SA synthesis and lignin accumulation. The fact that PLD can also give rise to two second messengers (PA and NAE) is exciting, but whether it occurs *in vivo* remains to be established (Meijer and Munnik, 2003). Nevertheless, these results establish PA as an early signalling component in plant-microbe interactions.

PA_{PLD} can mimic abscisic acid (ABA) responses by closing inward-rectifying K^+-channels and stomatal aperture (Meijer and Munnik, 2003). It inhibited a protein phosphatase 2C (ABI1) which is a negative regulator of ABA responses. PA bound directly to ABI1 thus positively influence the ABA signal transduction (Zhang et al., 2004). This was summarized in a bifurcating model where AtPLDα1-produced PA binds to the ABI1 to signal ABA-promoted stomatal closure, whereas AtPLDα1 and PA interact with the Gα-subunit of heterotrimeric G-protein to mediate ABA inhibition of stomatal opening (Mishra et al., 2006). PLD-produced PA is also suggested to promote ethylene responses. PA probably binds to CTR1, a negative regulator of ethylene responses, which heads a MAP kinase cascade. PA_{PLD} could create a membrane-docking site for CTR1, so that its relocalization relieves the block on ethylene signalling (Meijer and Munnik, 2003).

Interestingly, more and more proteins are found to bind PA. So far the best characterized are the serine/threonine kinase Raf-1 and a cAMP-specific phosphodiesterase in animal cells. A wound-responsive CDPK was directly activated by PA in maize (ZmCPK11; Szczegielniak et al., 2005). PA bound directly to protein phosphatase ABI1 during ABA signalling (Zhang et al., 2004). PA also activated animal and some plant PIP 5-kinases *in vitro* (Perera et al., 2005). PA activates these enzymes directly, but it will indirectly activate others by increasing the local enzyme concentrations thus promoting direct protein-protein interactions.

PA formation in plants is sometimes accompanied by apparition of a novel molecule, **DGPP**. DGPP activated an inflammatory-like response in macrophages

including enhanced prostaglandin production via activation of a cytosolic PLA_2. DGPP formation is antagonized by lyso-PA signalling by its competition for PA. However, DGPP has not been found in higher animals. DGPP, generated from PA by PA kinase (PAK), can be seen as a product of attenuation of the PA signal but it may have a signalling role in plants itself. There are enzymes that down-regulate DGPP in plants. DGPP phosphatase activity has been identified in *Catharanthus roseus*. Two genes cloned from Arabidopsis (AtLPP1 and 2) use PA and DGPP as substrate. Two more genes from the same gene family are predicted in the Arabidopsis genome. AtLPP1 was up-regulated by various stresses known to stimulate DGPP synthesis (van Schooten et al., 2006). DGPP is involved in plant stress responses too (Table II).

2.2.1.3 Free fatty acids and lyso-phospholipids

Free fatty acids (**FFAs**) are mostly generated by PLA_2 which hydrolyzes the acyl ester bond of glycerophospholipids at the *sn*-2 position yielding a FFA and a lyso-phospholipid (Figure 5). Both products have many downstream targets. The FFA can function as a second messenger or as a precursor of various oxylipins, e.g. linolenic acid (18:3) serves as precursor for jasmonic acid (JA), whereas the lyso-phospholipid can have a second messenger or membrane perturbing function (Meijer and Munnik, 2003). Oxylipins are potent signalling molecules in the defence response in animals and plants. The starting point in the synthesis of oxylipins is catalyzed by lipoxygenases (LOXs), which add molecular oxygen to polyunsaturated fatty acids (PUFAs) to yield the corresponding fatty acid hydroperoxides that are substrates for other enzymes in the octadecanoic pathway, synthesis of antifungal compounds such as aldehydes and divinyl ethers, and a variety of plant specific volatiles. There are other parallel pathways besides the *de novo* oxylipin synthesis. Oxylipins are often esterified to the *sn*-2 position of phospholipids or *sn*-1 position of galactolipids suggesting that these constitute a preformed pool of esterified oxylipins. Besides, fatty acids regulate activities of enzymes involved in the generation of other signal molecules in plant defence. Increase in FFA, PUFA or lyso-phospholipid levels was reported in various stress responses, examples are given in Table III.

Arabidopsis PLDδ stimulated by an unsaturated fatty acid, oleic acid (18:1), protects cells from oxidative burst-induced cell death. Interestingly, in animal cells, 18:1 reduced apoptosis (Shah, 2005). The mechanism underlying this phenomenon of protection was unravelled in a recent study where administration of oleic acid to cell

suspensions of *Taxus cuspidata* resulted in activation of NADPH oxidase and both intra- and extracellular oxidative burst which later lead to mobilization of ROS scavenging machinery (Xu et al., 2005). Activation of NADPH oxidase may be due to the PLDδ-produced PA. Interestingly, reduction of oleic acid (18:1) levels in the *ssi2* (suppressor of SA insensitivity) Arabidopsis mutant plants lead to the constitutive activation of NPR1-dependent and -independent defence responses. This explains why *ssi2* plants are compromised in the induction of JA-responsive gene PDF1.2 and in the resistance to necrotrophic pathogen *Botrytis cinerea* (Kachroo et al., 2003).

Both arachidonic acid (20:4) and linolenic acid (18:3) activated oxidative burst in potato and when applied to the plasma membrane fraction from tobacco suspension cells, respectively (Shah, 2005). Arachidonic acid triggers PCD in various plant species (Weyman et al., 2006). Various oxylipins generated from fatty acids by peroxidation were shown to execute the hypersensitive cell death in tobacco leaves (Montillet et al., 2005).

Table III. *Examples of involvement of FFA and lyso-phospholipids in plant stress responses.* Linolenic acid can be further transformed to JA via octadecanoid pathway.

Stress	Plant species	Molecule produced	Reference
Pseudomonas syringae (incompatible interaction)	*Arabidopsis thaliana*	hexadecatrienoic acid (16:3) linolenic acid (18:3)	Shah, 2005
TMV-induced HR	*Nicotiana tabacum*	JA	Meijer and Munnik, 2003
elicitor treatment	*Eschscholtzia californica*	linolenic acid (18:3) lyso-PC	Shah, 2005
Wounding	higher plants	linolenic acid (18:3) lyso-phospholipids	Meijer and Munnik, 2003

Lyso-PC caused temporary acidification of cytoplasm upon elicitor-triggered Gα-subunit stimulation in California poppy independently of the hypersensitive response (Viehweger et al., 2006). **Lyso-PE** inhibited PLD activity *in vivo* and thus promoted enhanced freezing tolerance in Arabidopsis since PLD is involved in membrane breakdown upon freezing (Rajashekar et al., 2006). In mammalian systems, extracellular **lyso-PA** is an important signalling molecule that provokes cell division and cytoskeleton rearrangements and activates resting platelets. Hyperosmotic stress activated PA- and PC-hydrolysis by PLA_2 in green algae. Channelling PA to the DGPP production diminished lyso-PA formation suggesting that PLA_2 and PAK compete for the same PA pool. Interestingly, lyso-PA was not generated during other treatments that induced formation of DGPP and PA. Hyperosmotic stress induced vacuole

fragmentation both in yeast and plant systems, a process requiring membrane curvature. Lyso-PA could stimulate the membrane curvature along with PA, however, in plants the role of lyso-PA in the process has not been demonstrated yet (Meijer and Munnik, 2003).

2.2.2 Key enzymes in phospholipid signalling

Phospholipids and lipids prove to be important signals in plants and especially in plant stress responses. Structural phospholipid (PI) can be phosphorylated either to PI(3)P or to PI(4)P by PI 3-kinase or PI 4-kinase, respectively. PI(3)P and PI(4)P can be further transformed to $PI(3,5)P_2$ and $PI(4,5)P_2$ by PIP 5-kinase. Phosphoinositide kinases are further classified into subclasses or subtypes according to their biochemical properties and sequence homology. A complicating factor is that *in vitro* some enzymes phosphorylate PI indiscriminately, whereas under physiological conditions, they prefer a specific hydroxyl group (Meijer and Munnik, 2003). $PI(4,5)P_2$ is hydrolyzed by PLC to give DAG and IP_3. DAG can be further phosphorylated by DAGK and PAK to produce PA and DGPP, respectively. PA can also be produced via PLD-catalyzed hydrolysis of structural phospholipids (e.g. PC, PE). See Figure 4 for overall scheme. Only enzymes involved in phospholipid signalling are described in detail, purely metabolic enzymes synthesizing structural phospholipids are omitted for clarity.

2.2.2.1 PI 3-kinase

PI 3-kinase (EC 2.7.1.137) phosphorylates inositol on the D-3-position (Figure 6). Three different classes are known (classes I–III) depending on their regulation and substrate specificity. Plants contain only class III PI 3-kinases, which phosphorylate PI (but not PPIs) to produce PI(3)P. PI 3-kinases have been cloned from several plant species and they are presumably localized in the nucleus or in its close proximity. Various PI-kinases in animals and yeast have been reported to be active in the nucleus, and plant nuclei also contain PI- and PIP kinase activities, although it is not clear which PPIs they produce. A chimera of FYVE and GFP first labelled the *trans*-Golgi network (TGN) when expressed in Arabidopsis, supposing that PI 3-kinase might be located there (Meijer and Munnik, 2003).

Several roles for PI 3-kinase in stress responses can be outlined. Autophagy has a protective role during HR by restricting the PCD (Liu et al., 2005). PI 3-kinase was essential for autophagy in tobacco cell suspensions (Takatsuka et al., 2004). PI 3-kinase

was also important for ABA-mediated ROS generation during stomatal closure (Park et al., 2003). Accordingly, expression of *Brassica napus* homologue of yeast Vps34 was strongly induced during salt stress and drought (Das et al., 2005). Similarly, PI 3-kinase was involved in auxin-mediated ROS generation during gravitropic signalling (Joo et al., 2005).

PI 3-kinases are involved in vacuolar trafficking, cell proliferation, and cytoskeleton organization. They are all homologues of the yeast Vps34p, which is required for vesicular-protein-sorting (Meijer and Munnik, 2003). Accordingly, expression pattern of PI 3-kinase in *Medicago truncatula* revealed that it is over-represented in tissues with high degree of vesicle trafficking and cell elongation (Hernandez et al., 2004). There is evidence of direct interaction between PI 3-kinase and profilin, a protein involved in the dynamics of the actin cytoskeleton and vesicular trafficking, in *Phaseolus vulgaris* (Aparicio-Fabre et al., 2006).

2.2.2.2 PI 4-kinase

PI 4-kinase (EC 2.7.1.67) phosphorylates inositol on the D-4-position (Figure 6). In mammals, type II and type III enzymes have been identified (later on type I turned out to be a PI 3-kinase). Genes from the type II family encode 45–55 kDa membrane proteins that appear to be membrane associated via a palmitoylated Cys-rich central region. Homologues in Arabidopsis exist. Type III PI 4-kinases are much larger (110–210 kDa) and often soluble enzymes. In yeast, three PI 4-kinases (Pik1, Stt4 and Lsb6) resemble mammalian type III (Meijer and Munnik, 2003). Stt4p is required for the maintenance of vacuolar morphology, cell wall integrity, and cytoskeleton organization, particularly during osmotic stress, while Pik1p plays an essential role in secretion, dynamics of Golgi and vacuolar membrane, and endocytosis (Müller-Röber and Pical, 2002).

In plants, PI 4-kinase activity was purified from several species and it was present in different cellular compartments. Arabidopsis genome contains 8 type II (γ variants) and 4 type III PI 4-kinases (α and β variants). Two representatives of the type III have been cloned (α1 and β1). The PI 4-kinase α contains a PH domain that is absent from the β variant. When this PH domain was expressed in bacteria, it bound PA, PI(4)P, and PI(4,5)P_2 (Meijer and Munnik, 2003). It regulated the activity of AtPI4Kα1 and it was responsible for its localization in the perinuclear region (Stevenson-Paulik et al., 2003). All Arabidopsis type II PI 4-kinases are γ variants and form a novel class of

PI 4-kinases, however, none of them has yet been shown to encode a functional PI 4-kinase. These are biochemically distinguishable from the type III as they cannot be inhibited by wortmannin but they are inhibited by adenosine and high Ca^{2+} concentration. On the contrary, type III, represented by two α and two β variants, can be inhibited by wortmannin and cannot be inhibited by adenosine and high Ca^{2+} concentration. The inhibitory concentrations of wortmannin are much higher than those reported for PI 3-kinases (Müller-Röber and Pical, 2002).

Hyperosmotic stress activates protein kinases in *Glycine max* which indirectly activate both PI 3-kinase and PI 4-kinase via phosphorylation of their interaction partner Ssh1p, a phosphatidylinositol transfer protein (Monks et al., 2001). Among the tested stress conditions expression of an orthologue of AtPI4Kβ1 in *Brassica napus* was induced only by drought (Das et al., 2005). Another orthologue of AtPI4Kβ1 was cloned from rice (OsPI4K1) and its expression was found to be specifically induced by SA treatment (Kong XF et al., 2003). PI 4-kinase activity increased during Al^{3+} stress in *Coffea arabica* cell suspensions (Martinez-Estevez et al., 2003).

When AtPI4Kβ1 gene was knocked out in Arabidopsis protoplasts, vesicular trafficking was inhibited up to 50% illustrating that PPI species play a role in the turnover of intracellular membrane compartments (Meijer and Munnik, 2003). The AtPI4Kβ1 lacking any PH domain was shown to colocalize with small vesicles in the cytoplasm and it was stimulated by PI(4)P (Stevenson-Paulik et al., 2003). This was later explained by identification of a highly charged repetitive segment designated PPC (plant PI 4-kinase charged) region specific to β-isoforms of PI 4-kinase. PPC bound PI, PI(4)P and PA and was responsible for membrane targeting of the protein (Lou et al., 2006).

2.2.2.3 PIP 5-kinases

Two distinct enzyme activities phosphorylating PI monophosphates on D-5-position fall in this category: PI(3)P 5-kinase (EC 2.7.1.150) and PI(4)P 5-kinase (EC 2.7.1.68). Both are present in plants, although the PI(3)P 5-kinase has not been cloned yet. Yeast **PI(3)P 5-kinase**, called Fab1p, is a large protein with an N-terminal PIP kinase domain, a central chaperonin-like domain, and a C-terminal FYVE domain. Arabidopsis genome contains four putative *Fab1* homologues, although they are smaller and two of them lack the N-terminal FYVE domain. One of the FYVE domains was cloned and shown to bind PI(3)P. All *Fab1*-like genes contain a chaperonin-like region

and a highly conserved C-terminal PIP kinase domain that is different from that of PI(4)P 5-kinases. *Fab1*-homologues are also present in other plant genomes according to EST databases (Meijer and Munnik, 2003).

Yeast *Fab1* deletion mutants failed to internalize and recycle the tonoplast. Interestingly, PIKfyve lipid kinase, the mammalian orthologue of Fab1p can synthesize PI(5)P and PI(3,5)P_2 *in vitro* from PI and PI(3)P, respectively, and it was critical for endomembrane homeostasis in mammalian cells (Meijer and Munnik, 2003).

Several **PI(4)P 5-kinase** isoforms have been characterized in animal systems but only one in yeast (Mss4p). The Arabidopsis genome is predicted to contain nine genes, of which only two cDNAs (AtPIP5K1 and AtPIP5K10) have been cloned. AtPIP5K1 interacts with F-actin and targets AtPI4Kβ1 to the actin cytoskeleton because AtPI4Kβ1 cannot bind to the cytoskeleton directly. Such an interaction is believed to gather the kinases together to create a multienzyme complex which is able to form membrane microdomains enriched in PI(4,5)P_2 (Davis et al., 2007). Arabidopsis PI(4)P 5-kinase contains a lipid kinase domain, an N-terminal extension, and eight MORN (membrane occupation and recognition nexus) motifs, which are novel protein-folding modules that bind proteins to membranes. They are responsible for modulation of PIP 5-kinase activity by both PA and PI(4,5)P_2 and for the sub-cellular localization of the protein (Im et al., 2007a). Animal PI(4)P 5-kinases, but surprisingly not all Arabidopsis PI(4)P 5-kinases are activated by PA. Comparative analysis of recombinant AtPIP5K10 and AtPIP5K1 with recombinant HsPIP5KIα revealed that the Arabidopsis enzymes have roughly 200- and 20-fold lower V_{max}/K_m, respectively. This is one possible explanation for the longstanding mystery of the relatively low PI(4,5)P_2:PI(4)P ratio in terrestrial plants (Perera et al., 2005). Accordingly, PIP 5-kinase was identified as the bottleneck of PI(4,5)P_2 synthesis in tobacco (Im et al., 2007b).

A PI(4)P 5-kinase from *Oryza sativa* (OsPIP5K1) was a negative regulator of floral initiation with delayed leaf development (Ma et al., 2004). PI(4)P 5-kinase expression in Arabidopsis was induced by drought, salt, and ABA treatments, all of which are thought to activate PLC and reduce PI(4,5)P_2 pools. Enhanced expression could therefore supply the consumed PI(4,5)P_2 (Meijer and Munnik, 2003). PI(4)P 5-kinase activity increased also during Al^{3+} treatment of *Coffea arabica* cell suspensions (Martinez-Estevez et al., 2003).

2.2.2.4 Phospholipase C

Phosphoinositide-specific phospholipase C (PLC; EC 3.1.4.11) usually produces two second messengers DAG, which is usually rapidly converted to PA, and IP_3. IP_3 mobilizes intracellular Ca^{2+} via IP_3-receptor which has not been identified in plants yet (Krinke et al., 2007a). Expression pattern of nine PLC isoforms present in Arabidopsis genome was studied in different tissues and stress conditions. Three of them (AtPLC7, AtPLC8 and AtPLC9) seem to be inactive because of substitutions in their active sites (Hunt et al., 2004). PLC activity was associated with plasma membrane in hypocotyls of seedlings of *Brassica napus* (Novotná et al., 2003a) and its plasma membrane localization was confirmed for AtPLC4 (Cao et al., 2007).

PLC activation occurs as a general early stress response in plants. ABA is an important signal in drought stress response. Accordingly, both hyperosmotic stress and drought activated PLC signalling. AtPLC1 was negatively regulated by SOS2, a protein kinase involved in plant salt tolerance (Meijer and Munnik, 2003). A PLC transcript was induced in maize roots in response to both hyper- and hypoosmotic stress (Zhai et al., 2005). Involvement of PLC in other stress responses is summarized in Table IV.

The ABA signal was transduced via PLC because ABA did not inhibit germination or growth in plants expressing antisense-*AtPLC1* but did so in wild type plants. Overexpression of AtPLC1, however neither resulted in germination or growth inhibition nor in the expression of ABA-responsive genes, and therefore it could be required for secondary responses. The primary ABA-induced responses were attributed to the production of cyclic ADP-ribose (cADPR) followed by a Ca^{2+} influx (Meijer and Munnik, 2003). Stomatal closure is another phenomenon regulated by ABA. Arabidopsis mutants defective in GPA1 (a heterotrimeric GTP-binding Gα-subunit gene) did not close their stomata after ABA treatment. The normal ABA-induced increase in Ca^{2+} in guard cells seems to be due to the combined actions of IP_3 and cADPR (Meijer and Munnik, 2003). A Ca^{2+}-mobilizing role during stomatal closure was proposed also for IP_6 (Krinke et al., 2007a). Interestingly, in yeast, PLC-generated IP_3 is phosphorylated to IP_6, which affects gene transcription and mRNA transport. Perhaps plants, fungi, and slime molds evolved a form of PLC signalling different from that in animals, generating rather IP_6 and PA as messengers instead of IP_3 and DAG (Meijer and Munnik, 2003).

PLC activation occurred during cell cycle in tobacco cell suspensions (Apone et al., 2003). PLC activity was indispensable for tip growth of pollen tubes of *Agapanthus*

umbellatus (Monteiro et al., 2005) and *Petunia inflata* where its relation to actin cytoskeleton was demonstrated (Dowd et al., 2006). Activity of pollen PLC was stimulated by calmodulin (CaM) in *Lilium daviddi* (Pan et al., 2005).

Table IV. *Examples of involvement of PLC in plant stress responses.*

Stress	Plant species	Role of PLC	Reference
infection by fungus *Alternaria alternate*	*Citrus limon*	*in vivo* activation	Ortega et al., 2005
incompatible reaction triggered by *Pseudomonas syringae*	*Arabidopsis thaliana*	*in vivo* activation	Andersson et al., 2006
incompatible reaction triggered by fungal elicitor AVR4 from *Cladosporium fulvum*	*Nicotiana tabacum* (expressing tomato resistance gene *Cf-4*+)	*in vivo* activation	de Jong et al., 2004
xylanase, chitotetraose	*Medicago sativa*	*in vivo* activation	den Hartog et al., 2003
N-acetylchitooligosaccharide	*Oryza sativa*	*in vivo* activation	Yamaguchi et al., 2005
Cryptogein	*Nicotiana tabacum*	*in vivo* activation	Kašparovský et al., 2004
SA, BTH, MeJA	*Brassica napus*	*in vitro* activity increased	Profotová et al., 2006
Ethylene	*Citrullus lanatus*	induced expression and *in vitro* activity	Karakurt and Huber, 2004
Nod factor	*Medicago sativa*	*in vivo* activation	den Hartog et al., 2003
cytokinin and gravistimulation	*Physcomitrella patens*	*in vivo* activity (demonstrated by gene knock-out)	Repp et al., 2004
auxin and gravistimulation of shoots	*Avena sativa*	*in vivo* activation	Yun et al., 2006
GA_3	*Pisum sativum*	*in vivo* activation	Kolla et al., 2004
Putrescine	*Coffea arabica*	*in vitro* activity decreased	Echevarria-Machado et al., 2005
Al^{3+}	*Coffea arabica*	*in vivo* activity decreased	Ramos-Diaz et al., 2007
cold stress	*Brassica napus*	induced expression	Das et al., 2005
	Arabidopsis thaliana	*in vivo* activation	Vergnolle et al., 2005
heat stress	*Arabidopsis thaliana*	*in vivo* activation	Liu et al., 2006c
	Pissum sativum	*in vivo* activation	Liu et al., 2006b
blue light	*Arabidopsis thaliana*	*in vivo* activation	Harada et al., 2003
extremely low frequency electromagnetic fields	*Peganum harmala*	*in vivo* activation	Piacentini et al., 2004

A special type of PLC, **PC-specific PLC** (PC-PLC; EC 3.1.4.3), is present both in bacteria and animal cells where it is involved in signal transduction. In plants the first report showed that the activity was inhibited in response to elicitor treatment in *Petrosilenum crispum* and *Nicotiana benthiana* cell suspensions (Scherer et al., 2002). The same activity was greatly induced during phosphate starvation in Arabidopsis where six genes putatively encoding PC-PLC were found. One of them (NPC4) was a functional PC-PLC. It was strongly expressed during phosphate starvation, Ca^{2+}-independent and predominantly localized on the plasma membrane in roots (Nakamura et al., 2005).

2.2.2.5 DAG- and PA-kinase

Plant DAGK (EC 2.7.1.107) is very active and ensures *in vivo* rapid conversion of DAG to PA. PA kinase (PAK; EC 2.7.4.-) is a novel lipid kinase originally discovered *in vitro*. It phosphorylates PA to DGPP, a lipid that was discovered to accumulate *in vivo* when PLC or PLD signalling was activated.

DAGK activity has been purified and characterized from various plant materials. It is largely present at the plasma membrane, with some activity associated with the cytoskeleton and nucleus. Interestingly, chloroplasts incubated with ^{32}P-ATP produced several labelled phospholipids including PA (Meijer and Munnik, 2003). AtDAGK2 was cloned, biochemically characterized and shown to be induced by wounding and cold treatment (Gomez-Merino et al., 2004; Gomez-Merino et al., 2005). DAGK activation laid down-stream of cold-induced membrane rigidification therefore it may translate the physical stimulus into a chemical signal (Vaultier et al., 2006). Expression of a DAGK orthologue in *Brassica juncea* was induced by Cd^{2+} treatment (Minglin et al., 2005).

DAGK7 from Arabidopsis was expressed in flowers and young seedlings (Gomez-Merino et al., 2005). Interestingly, DAGK is one of the components involved in the pathway leading to monoterpene biosynthesis which is responsible for the floral scent in *Phalaenopsis* species (Hsiao et al., 2006).

PAK activity was present in many plant species and tissues and it was predominantly localized on the plasma membrane. The activity was also described in yeast, oomycetes, and trypanosomes but not yet in mammalian cells (Meijer and Munnik, 2003). Until now, the gene encoding PAK has not been cloned. Most of the knowledge about PAK is derived from the biochemical characterization of the partially purified enzyme from *Catharanthus roseus* cell suspensions. PAK is a small integral membrane protein expressed in all tissues. It can use both ATP and GTP as phosphate donors (van Schooten et al., 2006).

2.2.2.6 Phospholipase D

Biochemical properties of plant phospholipase D

When phospholipase D (PLD; EC 3.1.4.4) hydrolyzes a structural phospholipid, it transfers the phosphatidyl group to water, producing PA, but in the presence of primary alcohols, it preferentially transfers the phosphatidyl group to this acceptor,

forming a phosphatidylalcohol. Since this is a unique property of PLDs, it means that activity can be unambiguously assessed *in vivo*, by using low concentrations of alcohol to measure the production of phosphatidylalcohol. A note of caution must be added since some PLDs are claimed to have no transphosphatidylation activity, but these constitute some extraordinary PLDs, like PI-specific PLD. Conventional plant PLDs harbouring two catalytic H*K****D motifs (e.g. all known Arabidopsis PLDs) are all capable of transphosphatidylation reaction (Liscovitch et al., 2000). The architecture of the HKD motif and its close neighbourhood is responsible for the transphosphatidylation capacity of the PLD (Lerchner et al., 2005). The most favourite tracer of PLD activity is *n*-butanol. Production of phosphatidylbutanol (PBut) is a credible proof of PLD activity. The formation of phosphatidylalcohols provides a relative, rather than absolute, measure of activity. Finally, when using $^{32}P_i$-labelling to monitor lipid synthesis, PLD's lipid substrate (structural phospholipids) must be well labelled, which requires relatively long prelabelling times.

Several PLDs have been cloned from Arabidopsis, rice, and tomato. The Arabidopsis genome is predicted to contain 12 PLDs, out of which 10 C2-PLDs containing a C2 (CalB; Ca^{2+}-lipid binding) domain. The C2-PLDs have been arranged into classes based on their sequences and their Ca^{2+} and $PI(4,5)P_2$ dependency and are referred to as PLDα1-4, β1-2, γ1-3 and δ. PLDζ1-2 are PXPH-PLDs, containing a PX (or PHOX) and PH (pleckstrin homology) domain. First insights into the tertiary structure of the plant protein have been gained only recently for PLDα2 from white cabbage (Stumpe et al., 2007). Strikingly, mammals and yeast contain only PXPH-PLDs and no C2-PLDs (Meijer and Munnik, 2003).

In vitro and *in vivo* assays showed that different PLD isoforms activated under various stresses preferred diverse structural phospholipids, such as PC, PG, and PE, as substrates (Arisz et al., 2003; Devaiah et al., 2006; Li et al., 2006b). PLDα1 from Arabidopsis preferentially hydrolyzed PC. This was concluded from the comparison of the fatty acyl composition of PA with possible PLD substrates in wild type and antisense PLDα1 plants. A drawback of this approach was that it did not discriminate between PA_{PLD} and PA derived from other pathways. Fatty acyl fingerprint of stress-induced PBut during stress-activated signalling in *Chlamydomonas* revealed that PE is the PLD substrate there. It has to be noted that there is no PC in *Chlamydomonas* (Arisz et al., 2003). *In vitro*, NAPE was hydrolyzed by Arabidopsis PLDβ1 and PLDγ1

but not by PLDα1 when expressed in *E. coli* (Meijer and Munnik, 2003). Both PLDζs were PC-specific (Li et al., 2006b).

Most PLDs were activated by Ca^{2+} both *in vitro* and *in vivo*. Activation required micromolar (PLDβ, PLDγ, and PLDδ) to millimolar (PLDα) concentrations. The Ca^{2+}-dependency of C2-PLDs was largely determined by its C2 domain, which was also responsible for phospholipid binding. In particular, PLDβ1 and γ1, but not α at millimolar Ca^{2+}, required $PI(4,5)P_2$ for activity (Meijer and Munnik, 2003). Surprisingly, even two closely related PLD isoforms like PLDγ1 and PLDγ2 can have distinguishable biochemical properties and expression patterns. The *in vitro* activity of PLDγ2 was inhibited by Triton X-100 while that of PLDγ1 was stimulated. This was explained by interference of Triton X-100 with $PI(4,5)P_2$ binding in the case of PLDγ2. Two splice variants were identified for PLDγ2 (Qin et al., 2006). Both Ca^{2+} and $PI(4,5)P_2$ bound to C2 domain of plant PLDα and PLDβ in a competitive manner. However, PLDα is considered as $PI(4,5)P_2$-independent because at high Ca^{2+} concentration its $PI(4,5)P_2$ dependence is lost. C2 domain of PLDβ bound PC at lower Ca^{2+} concentration than PLDα which suggests its role in membrane anchorage (Zheng et al., 2000). A novel $PI(4,5)P_2$-binding domain called PBR1 was identified in PLDβ and it was conserved also in PLDγ, but not in PLDα. $PI(4,5)P_2$-bound PBR1 promoted substrate binding by PLDβ (Zheng et al., 2002). Ca^{2+} bound to the catalytic domain of PLDβ stimulated $PI(4,5)P_2$ binding of the protein (Pappan et al., 2004). Increases in cytosolic Ca^{2+} concentration may well evoke conformational changes in the PLD protein that will result in its translocation to $PI(4,5)P_2$-rich membranes, where it is activated to hydrolyze substrate lipids. Such mechanism of PLD activation could therefore be one of the downstream components in stress-responsive signalling pathways, where local increase in Ca^{2+} concentration is the primary response. The fact that some Ca^{2+}-dependent PLDs are also dependent on $PI(4,5)P_2$ emphasizes the complexity of lipid signalling. In contrast to other PLDs, the newly described Arabidopsis PLDζ1 activity was not Ca^{2+}-dependent, required $PI(4,5)P_2$ for activity, but PE was not needed in the substrate vesicles (unlike PLDβ or γ). Both the PX domain and the PH domain are probably involved in targeting PLDζ1 to the appropriate membrane by binding PPIs whose identity is as yet unknown (Meijer and Munnik, 2003).

Several reports show that plant PLDs can be stimulated by G-protein activators like mastoparan, alcohols, and cholera toxin. A PLDα from tobacco directly interacted

with the Gα-subunit of a trimeric G-protein (Meijer and Munnik, 2003). GDP-bound Gα-subunit bound to PLDα1 via its DRY motif and caused its inhibition in Arabidopsis. At the same time PLDα1-Gα-subunit complex enhanced the GTPase activity of the Gα-subunit (Zhao and Wang, 2004). PLD from *Brassica campestris* was positively regulated by small GTPases from the Ras superfamily; the interaction required protein kinase C (Kim et al., 2004). Another mode of PLD regulation is phosphorylation. Dephosphorylation of Ser/Thr of PLDγ from *Brassica oleracea* caused its inactivation (Novotná et al., 2003b).

PLD in plant stress responses

Most reports on PLD activation in plants are related to stress signalling. Expression of a PLD gene was induced upon exposure to *Xanthomonas oryzae* pv. *oryzae* in rice leaves. Furthermore, in the rice cells a PLD protein was recruited to sites on the plasma membrane where the pathogen was present, suggesting a role in plant response to the pathogen (Meijer and Munnik, 2003). Application of incompatible reaction-inducing elicitor activated first PLC and then PLD in Arabidopsis (Andersson et al., 2006). Treatment of tomato cell suspensions with fungal elicitor xylanase activated LePLDβ1 and enhanced its expression which was accompanied by its translocation within the cytosol. LePLDβ1-silenced cells were impaired in the HR and their normal response to elicitor (Bargmann et al., 2006) perhaps due to impaired NAE production (see chapter 2.2.1.2 for details). PLDδ was involved in phytoalexin synthesis following *N*-acetylchitooligosaccharide elicitor treatment of rice cell suspensions and its activity was stimulated by H_2O_2 (Yamaguchi et al., 2004). Stimulation by H_2O_2 occurred also in Arabidopsis cell suspensions and PLDδ-ablated Arabidopsis plants exhibited increased susceptibility to various stresses (Zhang W et al., 2003) implicating that PLDδ is a sensor of ROS generated during many stresses. PTI1-2, recently identified Ser/Thr protein kinase, represents a point of convergence between elicitor-stimulated PLD-derived PA and NADPH oxidase-generated ROS during plant-pathogen interaction. It is placed downstream of PA-activated protein kinase PDK1 (3-phosphoinositide-dependent protein kinase 1) and upstream of MAPK module (Anthony et al., 2006). The highest specific PLD activity was found in the plasma membrane fraction and it was activated after treatments of *Brassica napus* plants with inductors of plant systemic resistance (SA, BTH and MeJA; Novotná et al., 2003a; Profotová et al., 2006).

PLDs, especially PLDα, were long time considered as enzymes involved mainly in catabolism (e.g., senescence or wounding). In PLDα1 antisense Arabidopsis plants, 97% of all *in vitro* activity was lost (PLDα1 contributed to phospholipid metabolism in all organs except leaves and siliques). PLDα1 plays an important role in seed deterioration and aging in Arabidopsis. A high level of PLDα1 was detrimental to seed quality and attenuation of PLDα1 expression improved oil stability, seed quality and seed longevity (Devaiah et al., 2007). However, its signalling role cannot be overlooked. Despite its abundance in non-stimulated cells, its expression could be induced during stress treatment (Devaiah et al., 2006). Wounding induced PLDα1, β1, γ1 and γ2. The differential regulation of the PLD isoforms suggests that they may be involved in different aspects of the wound response. Formation of JA was slightly delayed and expression of JA-dependent genes was reduced in wounded PLDα1-antisense Arabidopsis plants placing JA synthesis downstream of PLD signalling (Meijer and Munnik, 2003). A rice orthologue of PLDα1 (RPLD1) was also induced by wounding (McGee et al., 2003).

Silencing the PLDα1 in Arabidopsis resulted in the loss of ABA sensitivity as well as in the decrease of stomatal closure (Mishra et al., 2006), and therefore these plants wilted earlier owing to increased transpirational water loss. Over-expression of castor bean PLDα1 in tobacco cells resulted in more rapid and more sensitive response to ABA (Meijer and Munnik, 2003). Therefore it is not surprising that PLD has a role in drought and osmotic stress. The extent of PLD activation correlated with different drought sensitivity in peanut cultivars (Guo et al., 2006). PLD activity was important for hyperosmotically induced macrotubule formation in *Triticum turgidum* root cells (Komis et al., 2006). Hyperosmotic stress activated PLD in several other species (*Chlamydomonas moewusii*, *Lycopersicum esculentum*, *Medicago sativa* and *Craterostigma plantagineum*; Meijer and Munnik, 2003). PA_{PLD} activated vacuolar H^+-ATPase during salt stress in maize (Zhang et al., 2006b). Hyperosmotic stress specifically induced expression of PLDδ in Arabidopsis, whereas other isoforms were not affected. However, PLDδ-antisense plants had no phenotypic defects under normal or dehydrated conditions, even though PA and PBut production was reduced by 20% to 50% (Meijer and Munnik, 2003). Over-expression of PLDδ in Arabidopsis increased its freezing tolerance (Li et al., 2004). Freezing decreases water availability in cells and PLDδ may enhance osmolyte synthesis and thus establish enhanced freezing tolerance. A cluster of PLD-regulated cold-responsive genes was identified in Arabidopsis cell

suspensions (Vergnolle et al., 2005). On the contrary, PLDα1 was a negative regulator of freezing tolerance in Arabidopsis. This was especially due to increased accumulation of osmolytes with cryoprotective role in PLDα1-antisense plants (Rajashekar et al., 2006). The latter observation correlates well with the finding that PLD negatively regulates proline synthesis, a major hyperosmotic stress response, in Arabidopsis (Thiery et al., 2004). Moreover, silencing PLDα1 in tomato cells also results in a partial (40%) reduction in the osmotic stress-induced PLD activation (Meijer and Munnik, 2003). A transcriptomic study revealed no changes between wild type and PLDα1 antisense plants in the absence of stress. On the contrary, a group of differentially regulated genes between the two genotypes was identified after a few days of drought stress. Expression of PLDδ was highly induced in drought stressed PLDα1 antisense plants (Mane et al., 2007). The implication is that PLDα1 and PLDδ play distinct roles in water-stress signalling. Thus, PLDδ may be a target of PLA-derived free fatty acids, and the activation of various phospholipases may act in concert, PLDα → PLA → PLDδ, to regulate the production of lipid messengers as part of plant responses to stress (Wang X et al., 2006). On the contrary, hypoosmotic stress activated PLD and hyperosmotic stress inhibited PLD in tobacco pollen tubes (Zonia and Munnik, 2004). PLDδ is the most expressed PLD isoform in tobacco pollen tubes (Potocký, unpublished data). This apparent contradiction could be attributed to the specificity of pollen tube as a cell type.

PLD during plant morphogenesis

Besides these major roles in plant stress responses, some minor roles for PLD were reported. In yeast and mammalian cells, PLD is involved in various fundamental processes such as meiosis, secretion, vesicular trafficking, and actin assembly (Meijer and Munnik, 2003). PLD along with PA phosphatase activity increased during phosphate starvation of oat where structural phospholipids were progressively replaced by non-phosphorous-containing galactolipids (Andersson et al., 2005). Even though Arabidopsis *pldζ1pldζ2* double mutant did not exhibit alterations of root hair formation (supposedly because of phenomenon of compensation by other PLD isoforms) both AtPLDζs (but especially AtPLDζ2) were important for the regulation of root development during phosphate starvation. One hypothesis was that PA generated by AtPLDζs promotes root elongation by activating a protein kinase cascade that is regulated by AtPDK1, which has previously been linked to PA and root growth (Li et

al., 2006a). Second hypothesis linked AtPLDζ2 (accompanied by increased PA phosphatase activity) with increased phospholipid breakdown and their progressive replacement by galactolipids (Cruz-Ramirez et al., 2006; Li et al., 2006b). PLD also antagonized gibberellic acid (GA_3) stimulated germination and α-amylase production in barley aleurone layer (Meijer and Munnik, 2003). Cu^{2+} treatment increased PLD transcript abundance and activity in *Triticum durum* seedlings. PLD activity was necessary for NADPH oxidase activation leading to superoxide production (Navari-Izzo et al., 2006).

Plant PLD was activated by ethylene, which is a hormone controlling developmental processes like senescence and fruit ripening. When plants were sprayed with lyso-PE, which inhibits PLD *in vitro*, senescence and ripening were retarded. Expression of PLDα1 in Arabidopsis leaves was stimulated by ethylene, whereas suppression of PLDα1 reduced ethylene-induced senescence (Meijer and Munnik, 2003). PLD expression was also induced after ethylene treatment of watermelon (Karakurt and Huber, 2004). Treatment with polyamine putrescine increased PLD activity in *Coffea arabica* cell suspensions; putrescine naturally accumulates at the end of stationary phase and thus such activation may also be seen as senescence-related (Echevarria-Machado et al., 2005). AtPLDζ2 positively mediated auxin responses, including root gravitropism and hypocotyl elongation under high temperature, and was important for normal PIN2 cycling (Li and Xue, 2007). PLD was activated by Nod factor in *Medicago sativa* (den Hartog et al., 2003), PLD activity was necessary for nodulation in *Medicago truncatula* (Charron et al., 2004) and a PLD protein was induced during nodulation in soybean (Wan et al., 2005). AtPLDζ1 was an effector of transcription factor GL2 which is important for root hair development (Ohashi et al., 2003). Inhibition of PLD blocked root and root hair development along with disruption of microtubule organisation in Arabidopsis (Gardiner et al., 2003). PA_{PLD} was important for pollen tube growth in tobacco (Potocký et al., 2003) and in *Agapanthus umbellatus* (Monteiro et al., 2005). Inhibition of PLD by *n*-butanol slowed down cell growth and division in BY-2 cells because PLD was an important component of the cortical microtubule tethering complex (Dhonukshe et al., 2003). It has to be noted that *n*-butanol itself can act as microtubule depolymerizing agent as documented both *in vivo* in BY-2 cells and *in vitro* (Hirase et al., 2006). PA induced actin polymerization in soybean (Lee et al., 2003) via a PA-dependent protein kinase. In reverse, plant PLD was

activated by polymerized actin (Kusner et al., 2003) suggesting a positive feed-back mechanism in the regulation of actin dynamics by PLD.

2.2.2.7 Phospholipase A

Two enzymatic activities fall in the phospholipase A (PLA) category: PLA_1 (EC 3.1.1.32) hydrolyzing the acyl chain on the *sn*-1-position and PLA_2 (EC 3.1.1.4) hydrolyzing the acyl chain on the *sn*-2-position of the glycerol backbone of phospholipids and lysophospholipids. PLA activity generates two second messengers, a lysophospholipid and a FFA.

In the **PLA_1** family, 12 PC-specific isoforms have been identified in the Arabidopsis genome database. Based on the presence of particular N-terminal stretches and sequence similarities in the catalytic region, these proteins have been grouped into three classes (Ryu, 2004). JA biosynthesis can depend on PLA_1 as documented for *Acer pseudoplatanus* and Arabidopsis. An Arabidopsis mutant *dad1* (defective in anther dehiscence 1) impaired in the synthesis of developmental JA was mutated in a class I PLA_1 protein. DAD1-GFP was localized in chloroplasts as were other enzymes of the JA pathway (Meijer and Munnik, 2003). Arabidopsis mutant impaired in shoot gravitropism (*sgr2*) is defective in a putative PA-PLA_1 gene (At1g31480). Vacuolar membranes are involved in the early step of gravity sensing and, as expected, the cells of some *sgr2* embryos had abnormal structures in their vacuolar membranes (Ryu, 2004). A novel PLA_1 with a homology to animal lecithin:cholesterol acyltransferases was recently cloned from Arabidopsis and biochemically characterized (AtLCAT3; Noiriel et al., 2004).

Simplified classification of all **PLA_2s** results into four groups: cytosolic Ca^{2+}-dependent ($cPLA_2$); secretory, low-molecular-weight ($sPLA_2$); intracellular Ca^{2+}-independent PLA_2; and platelet activating factor-specific acylhydrolases (PAF-PLA_2). PLA_2s have been purified from many plant species. They are mostly related to $sPLA_2$s. This is illustrated by the purification and characterization of a PLA_2 from developing seeds of elm which was small (14 kDa), Ca^{2+}-dependent and active at alkaline pH. It specifically hydrolyzed the *sn*-2 fatty acyl. Two small PLA_2s were found in rice both containing 12 conserved Cys residues in their active site, a Ca^{2+}-binding loop and a putative secretion signal. Another $sPLA_2$ was found in carnation flower (CFMI-3; Meijer and Munnik, 2003). Four isoforms of $sPLA_2$s denoted AtsPLA$_2$-α (At2g06925), AtsPLA$_2$-β (At2g19690), AtsPLA$_2$-γ (At4g29460) and AtsPLA$_2$-δ

(At4g29470) were identified in Arabidopsis (Ryu, 2004). AtsPLA$_2$-α was cloned and biochemically characterized. It preferentially cleaved linoleic acid over palmitic acid from PE and PC in a Ca^{2+}-dependent manner (Ryu et al., 2005). A detailed review focused on plant sPLA$_2$s was published by Lee et al. (2005).

Activation of plant PLA$_2$ was reported during seed germination when the phospholipid monolayers of lipid bodies were degraded. PLA$_2$ is also activated in response to auxin, during wounding, pathogen attack, and hyperosmotic stress (Meijer and Munnik, 2003). PLA$_2$ mediated the oxidative burst induced by ergosterol in tobacco cells (Kašparovský et al., 2004). How auxin stimulates cell elongation is still unclear but there is much evidence that PLA$_2$ is an important intermediate of auxin-induced cell elongation. Moreover, treatments with sPLA$_2$ inhibitors, aristolochic acid and manoalide, blocked auxin-induced cell wall acidification of corn coleoptiles. AtsPLA$_2$-β probably participated in this process because AtsPLA$_2$-β-over-expressing or -silenced transgenic plants showed altered gravitropism in their inflorescence stems and hypocotyls (Ryu, 2004). Significantly higher specific activity of PLA$_2$ over other organs and tissues was found in tobacco flowers (Fujikawa et al., 2005).

In plants, PLA$_2$ activity is also exhibited by **patatin-like enzymes**, which are structurally unrelated to the PLA$_2$s mentioned above. Patatin is a storage protein with lipid acyl hydrolase activity, cleaving fatty acyls from various glycerolipids. Patatin isolated from cowpea preferentially hydrolyzed galactolipids, whereas phospholipids were only hydrolyzed at a very slow rate. This emphasizes that not all patatin-like proteins are PLA$_2$s. All patatin genes encoded a protein of around 45 kDa, showed a combined PLA$_1$ and PLA$_2$ activity, and some of them were stress-induced. Patatin-like proteins were induced in tobacco prior to oxylipin accumulation and were also transiently induced during drought and seed germination. Arabidopsis patatin-like PLA genes (PAT-PLA) were divided into three classes based on their domain structure. AtPAT-PLA-IIα was induced by various abiotic and biotic stresses including pathogen inoculation, SA and MeJA. Patatin-like PLA$_2$ activation was reported in elicitor-treated and TMV-infected tobacco plants. Arabidopsis mutant *sturdy*, exhibiting defects that might result from low auxin and cytokinin levels, turned out to be mutated in a patatin-like gene (AtPAT-PLA-IIIδ; Ryu, 2004).

Endogenous lyso-PC levels increased 2–3 min after elicitor treatment of intact cells of California poppy due to Gα-subunit activated PLA$_2$. *In situ* lyso-PC treatment caused transient cytoplasmic acidification by activation of tonoplast H^+–Na^+ antiporter.

The cytoplasmic acidification was involved in phytoalexin biosynthesis independent of HR (Viehweger et al., 2006). This example documents how the products of PLA activity were involved in plant response to pathogen elicitors.

Several connections of PLA signalling with other signalling pathways can be outlined. For example, PLDα was inhibited by the lyso-PE that was generated by PLA. Inversely, PLDδ was activated by unsaturated fatty acids that were generated by PLA, thus PLDδ might be a downstream target of PLA. PA-PLA_1 hydrolysed PA that was generated by PLD into lyso-PA, which is another bioactive molecule, suggesting that the pathway downstream of PLD can be regulated by PA-PLA_1. It is conceivable that both PLD-mediated activation of PLA and feedback inhibition of PLD by PLA can occur in plants (Ryu, 2004).

2.2.3 Possible cellular targets of phospholipid signals

Lipid second messengers can act as membrane-docking molecules for proteins containing the appropriate lipid-binding domain. Downstream protein targets can translocate from cytosol to membrane sites rich in signal. For instance, phospholipases could hydrolyze membrane lipids to produce another round of lipid signalling in a self-amplifying feed-back loop. This represents a passive way of stimulation of signal transmission, but active ways can be envisaged, e.g. lipid-induced conformational changes that enhance enzyme activity. Such interactions are promoted by lipid-binding domains. A number of domains have been characterized in animals and these domains are also present in plants, even though their lipid-binding properties have seldom been determined.

2.2.3.1 FYVE domain

FYVE is an acronym from the names of four proteins in which the domain was first identified (Fab1p, YOTB, VAC1p, and EEA1). It specifically binds PI(3)P. A double FYVE-GFP construct has been used to visualize PI(3)P in internal membranes of mammalian cells. Labelling occurred on endosomes, Golgi and multivesicular bodies, in agreement with PI(3)P's role in endocytosis and membrane trafficking. In a comparable study (Vermeer et al., 2006), a GFP-FYVE construct was transiently expressed in Arabidopsis protoplasts. It labelled the TGN, pre-vacuolar compartment, and vacuolar membranes, indicating that PI(3)P occurred in similar intracellular compartments in plants, animals, and yeast. The FYVE domain cloned from the

Arabidopsis Fab1 homologue bound PI(3)P better than other polyphosphoinositides, while the (incomplete) FYVE-domain from the Arabidopsis PRAF1 gene bound PI(3,4,5)P_3 and PI(3,4)P_2 better than PI(3)P. The Arabidopsis genome contains 17 proteins with FYVE domains (Meijer and Munnik, 2003).

2.2.3.2 PH domain

Pleckstrin homology (PH) domains bind PPIs but sometimes so weakly and so indiscriminately that one can question whether that is their function. However, a few bind with high affinity and specificity: for instance the PH domain of PLCδ1 that binds PI(4,5)P_2. Some animal PH domains have high affinity for PI(3,4,5)P_3. PH domain from centaurin β2 bound specifically to PI(3,5)P_2. GFP chimeras showed that PH domains can target proteins to specific cell membranes. PH domains are sometimes located next to FYVE or PX domain (see chapter 2.2.3.4), all of which might be important for the properties of the effector protein. Several PH domains have been described in plants, and ~50 PH-containing proteins are coded in the Arabidopsis genome, yet only two have had their lipid-binding properties characterized. One is from a PI 4-kinase that binds PI(4)P and PI(4,5)P_2 but also, surprisingly, to PA, locating the enzyme to membranes involved in PPI-based signalling (Meijer and Munnik, 2003). The PH domain of PI 4-kinase modulates its activity (Stevenson-Paulik et al., 2003). Recently, a dynamin-like gene with a PH domain was identified in Arabidopsis (ADL6 or AtDRP2A). It is a GTPase which is involved in clathrin-coated vesicle formation during endocytosis, a process that is regulated by PPIs (Meijer and Munnik, 2003). PI(3)P and a Sec13 homologue 1 (AtSeh1) competed for the PH domain of AtDRP2A thus regulating the cycling of AtDRP2A between membrane-bound and soluble forms (Lee et al., 2006). Interestingly, PH domain of sucrose synthase from maize is also responsible for its membrane/cytosol partitioning (Hardin et al., 2006). An ADP-ribosylation factor-GTPase activating protein (ARF-GAP) identified in *van3* mutant contained a PH domain and specifically bound PPIs. VAN3 was located in a subpopulation of the TGN. Expression of this gene was induced by auxin and positively regulated by VAN3 itself. It functioned in vein pattern formation by regulating auxin signalling via a TGN-mediated vesicle transport system (Koizumi et al., 2005). Likewise, both AtPLDζ1 and AtPLDζ2 contain a PH domain, although its lipid binding and specificity remain to be determined (Meijer and Munnik, 2003). Arabidopsis mutant displaying enhanced disease resistance (*edr2*) to biotrophic powdery mildew pathogen

Erysiphe cichoracearum, responded normally to *Pseudomonas syringae* pv. *tomato* strain DC3000. Crossing with various mutants proved that *edr2*-mediated disease resistance was SA-dependent. EDR2 contained N-terminal mitochondrial targeting sequence, PH and START domains both implicated in lipid binding. EDR2 might provide a link between lipid signalling and activation of mitochondria-mediated PCD (Tang et al., 2005).

2.2.3.3 C2 domain

C2 domain, also known as CalB (calcium and lipid binding), is a conserved membrane-docking module with affinity for Ca^{2+} and lipids. C2 domains were found in many proteins involved in signal transduction and membrane trafficking. The module usually bound acidic or neutral phospholipids in a Ca^{2+}-dependent manner, but some had a clear preference for PPIs. In plants, a number of genes encoding proteins with C2 modules were identified. Some of them , such as PI 3-kinase, PLC, and PLD, are involved in lipid signalling (Meijer and Munnik, 2003). Arabidopsis genome is predicted to contain 84 genes coding for proteins with a C2 domain (Krinke et al., 2007b). Plant PLDs illustrate that Ca^{2+}-sensitivity of the C2 domain can largely vary (PLDα needs millimolar concentrations, whereas PLDβ and γ are activated in the micromolar range; Meijer and Munnik, 2003). Both Ca^{2+} and $PI(4,5)P_2$ bind to C2 domain of PLDα and PLDβ in a competitive manner (Zheng et al., 2000). They could therefore translocate to signal-rich locations via their C2 domains. C2 domain of plant PLD may also serve for recognition of other proteins and mediate the protein-protein interaction as shown for an aspartic proteinase (Simoes et al., 2005) and for annexins, a class of Ca^{2+}-binding proteins (Morgan et al., 2006). A rice protein responsible for pollen development and germination (Delegen14) bears also a C2 domain (Jiang et al., 2005). A heavy metal-responsive C2 domain-containing protein from barley (HvC2d1) translocated to the nucleus in a Ca^{2+}-dependent manner and was induced during heavy metal treatment, oxidative stress and leaf senescence (Ouelhadj et al., 2006). Two small proteins with C2 domain were isolated from elicitor-treated rice. Both bound to lipid vesicles in a Ca^{2+}-dependent manner and their transcription was strongly up-regulated following elicitor treatment (Kim CY et al., 2003). Another small C2 domain-containing protein from Arabidopsis (BAP1) negatively regulated basal defences and PCD via SA-dependent pathway (Yang et al., 2006).

2.2.3.4 Other phospholipid-binding domains

PX or PHOX (phagocyte oxidase) is another phospholipid-binding domain. Most of the characterized PX domains bound PI(3)P with high specificity, although some bound other PPIs. Proteins containing this domain were involved in membrane trafficking, organizing the actin cytoskeleton, and protein sorting. In Arabidopsis, 11 genes contain a PX domain. At least two of them, PLDζ1 and PLDζ2, are involved in lipid signalling (Meijer and Munnik, 2003). Interestingly, another subfamily of PLD (PXTM-PLD) was characterized in *Phytophthora infestans*. Besides the typical catalytic domain with two HKD motifs, these PLDs contain only the PX domain and a membrane-spanning region (Meijer et al., 2005).

A zinc finger PPIs-binding domain called **PHD** (plant homeodomain) was identified in animals. Animal nuclear stress receptor ING2 was regulated by PI(5)P via its PHD domain (Gozani et al., 2003; Jones et al., 2006). PHD domain of animal protein Pf1 specifically bound PI(3)P (Kaadige and Ayer, 2006). A plant transcriptional activator (EDM2), bearing the PHD domain, was associated with disease resistance against *Hyaloperonospora parasitica* isolate Hiks1 in *Arabidopsis thaliana*. It was placed downstream of a specific resistance protein RPP7 (Eulgem et al., 2007).

A PI(3)P-binding domain was described in Arabidopsis **ZAC** protein (zinc and Ca^{2+} binding). PI(3)P binding was dependent on its zinc finger motif, but the motif was not sufficient in itself, indicating that other residues were involved (Meijer and Munnik, 2003).

Another phospholipid-binding domain identified in plant PIP 5-kinases was called **MORN** (membrane occupation and recognition nexus). MORN motifs bound strongly to PA and relatively slightly to PI(4)P and $PI(4,5)P_2$ (Ma et al., 2006) and it was responsible for the activation of PIP 5-kinase from Arabidopsis by both PA and $PI(4,5)P_2$ (Im et al., 2007a). MORN motif was a part of the ARC3 protein functioning during chloroplast division much like the prokaryotic FtsZ protein (Shimada et al., 2004).

PA, PI and PI(4)P were all specifically bound by **PPC** domain (plant PI 4-kinase charged) originally identified in the PI 4-kinase β from Arabidopsis and *Oryza sativa*. PPC domain was responsible for the plasma membrane localization of the enzymes (Lou et al., 2006).

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and kits

Special chemicals and kits used in this research are listed in Supplemental Table I. Other ordinary chemicals were of analytical grade.

3.1.2 Plant materials

Cell suspension of Arabidopsis (*Arabidopsis thaliana*), ecotype Columbia-0, derived from line T87 originally initiated from young cotyledon leaves by Dr. Michèle Axelos (Centre National de la Recherche Scientifique, Toulouse, France; Axelos et al., 1992). Cells were grown in the Gamborg B5 basal medium supplemented with vitamins, 15 $g.L^{-1}$ sucrose and 0.1 $mg.L^{-1}$ 2,4-D; pH was adjusted to 5.7 with 50 µM Na^{+}-phosphate buffer and KOH. Every 7^{th} day, 20-mL aliquots of cell suspension were transferred into 500-mL Erlenmeyer flasks containing 200 mL of fresh medium. Cells were grown under continuous light (100 $\mu mol.m^{-2}.s^{-1}$) at 22 °C, with orbital agitation (130 rpm). Experiments were performed on 5-day-old cultures, which corresponds to the end of exponential phase.

Arabidopsis plants ecotype Columbia-0 and the T-DNA insertion mutants were grown for 4 weeks in a growth chamber in sterilized substrate at temperature ranging from 22 °C to 25 °C in 70% relative humidity and were watered as needed. The light intensity varied between 150 and 200 $\mu mol.m^{-2}.s^{-1}$. The photoperiod was that of a short day (8 h light/16 h dark).

3.1.3 Characterization of T-DNA insertion mutants

The *pi4kβ1*, *pi4kβ2* and *pi4kβ1pi4kβ2* plants were kindly provided by Dr. Erik Nielsen and characterized as described in Preuss et al. (2006). The T-DNA insertion mutants for PLD isoforms were obtained from the SALK T-DNA collection (SALK_053785, SALK_079133, SALK_113607, SALK_066687, SALK_089965, SALK_084335, SALK_083090 and SALK_094369; Arabidopsis Biological Resource Center; Alonso et al., 2003). The T-DNA insertion in each line was confirmed by two PCRs with gene-specific and T-DNA-specific primers as follows. DNA was extracted from leaf discs of 4-week-old plants. All operations were performed at room

temperature. Leaf discs were ground by a small pestle in an Eppendorf tube, and resuspended in 400 µL of extraction buffer (200 mM Tris-HCl pH 8.0; 250 mM NaCl; 25 mM EDTA; 0.5% w/v SDS). Plant material was pelleted by 1 min centrifugation at 14,000xg. Supernatant (300 µL) was transferred to a new Eppendorf tube and precipitated by the same volume of 2-propanol for 2 min. DNA was pelleted by 5 min centrifugation at 14,000xg and the pellet was dissolved in 100 µL of buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 7.6). Aliquot (1 µL) of the resulting DNA solution was amplified in the first PCR with only gene-specific primers (RP+LP; Supplemental Table II) and another aliquot was amplified in the second PCR with gene-specific primer RP and either LBa1 or LBb1 primer specific for the T-DNA insertion (Supplemental Table II). PCR was performed with 1.5 µM of each primer, 200 µM of each nucleotide, 1 U of Taq polymerase and reaction buffer supplied by manufacturer in the final volume of 20 µL. Annealing temperature was 55 °C, polymerising time was 30 s, amplification was done with 40 cycles. Based on the result of the two PCRs seeds of plants homozygous in the T-DNA insertion (with no product in the first PCR and with one product of the expected size in the second one) were harvested and used for further experiments.

3.2 Characteristics of cell suspensions

3.2.1 Growth curve

Cell suspensions (200 mL) were cultivated in 500 mL Erlenmeyer flasks from which 5 mL aliquots were withdrawn (utmost five times from the same flask) during the 8 days of culture. Cells were vacuum filtered and weighted to determine the fresh weight, then dried 2 h at 110 °C, cooled down in desiccator next 15 min and weighted again to determine the dry weight.

3.2.2 Viability staining

Cells (7 mL of cell suspension; 1 g of fresh weight) were cultivated in the presence of various SA concentrations or water and 50 µL aliquots were withdrawn at indicated times. SA (sodium salt) did not show any buffering or pH modifying capacity up to 5 mM. Aliquots were mixed with 5 µL of 0.4% w/v Trypan blue on a glass slide. Cells were incubated 10 min with the dye at room temperature and then the fraction of

dead (blue) cells (averaged from five independent counting fields) was estimated for each aliquot at 200x magnification under the light microscope (Olympus).

3.3 Phospholipid analysis

3.3.1 SA treatment and phospholipid analysis

Cells (7 mL of cell suspension; 1 g of fresh weight) were treated by 250 μM SA unless stated otherwise. Cells were labelled by $^{33}P_i$ (53 kBq.mL^{-1}) for the time indicated.

Lipid extraction was performed by adding 2.14 volumes of ice-cold chloroform:methanol:HCl (37% v/v) (50:100:1.5 v/v) into the flasks. The mixture was transferred into tubes, and a two-phase system was induced by the addition of 0.7 volume of chloroform and 0.7 volume of NaCl solution (9% w/v). The tubes were vigorously shaken and phases were allowed to form at 4 °C. The upper phase and interphase pellet were discarded and the organic phase was evaporated under nitrogen stream.

Total lipids were separated by TLC. Structural phospholipids and phosphatidic acid were separated in the acid solvent system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1 v/v/v/v) according to Lepage (1967). Phosphoinositides were separated in the alkaline solvent system composed of chloroform:methanol:ammonia solution (5% w/v) (9:7:2 v/v/v) where the TLC plates were soaked in potassium oxalate solution before heat activation according to Munnik et al. (1994). PI(3)P and PI(4)P were separated in the borate solvent system composed of methanol:chloroform:pyridine:boric acid:water:formic acid (88% v/v):2,6-di-*tert*-butyl-4-methylphenol:ethoxyquin (75:60:45:12:7.5:3:0.375:0.075 v/v/v/w/v/v/w/v) where the TLC plates were soaked in *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid solution before heat activation according to Walsh et al. (1991). PBut was separated in a modified ethyl acetate solvent system (organic upper phase of a mixture of ethyl acetate:isooctane:acetic acid:water, 12:2:3:10 v/v/v/v) according to de Vrije and Munnik (1997). Radioactive spots were quantified by autoradiography using a Storm phosphorimager (Amersham Biosciences, UK). Separated phospholipids were identified by comigration with authentic non-labelled standards visualised by primuline staining (under UV light) or by phosphate staining.

3.3.2 Analysis of PI species by RP-HPLC

Cells (7 mL of cell suspension; 1 g of fresh weight) were labelled by $^{33}P_i$ (1.1 MBq.mL^{-1} when labelling for 5 min or 0.26 MBq.mL^{-1} when labelling for 24 h). Lipids were extracted and separated in the acid solvent system described above, the lipid spots were visualised by primuline staining (under UV light) and the spot comigrating with the PI standard from soybean was scraped from the TLC plate. Lipids were extracted by methanol acidified with 1% v/v acetic acid and separated on μBondapak^{TM} C18 RP-HPLC column (Waters-Millipore) in isocratic solvent system methanol:water:acetonitrile:choline chloride (905:40:25:2.8 v/v/v/w). Liquid scintillation was measured in parallel to UV absorbance at 205 nm and the area of radiolabelled peaks corresponding to molecular species of PI was calculated.

3.4 Gene expression analyses

3.4.1 RNA extraction for semiquantitative RT-PCR and microarray experiments

Cells (2 mL of cell suspension; 280 mg of fresh weight) were filtered and immediately frozen in liquid nitrogen or four fully developed rosette leaves were cut and immediately frozen in liquid nitrogen. RNA was isolated using the phenol/chloroform extraction. All aqueous solutions contained 0.1% v/v DEPC to prevent RNA degradation by RNases. Cells or leaves were ground in liquid nitrogen and the powder was transferred to 500 μL of extraction buffer (200 mM Tris-HCl pH 8.0; 250 mM NaCl; 25 mM EDTA; 0.5% w/v SDS; 1% w/v PVP-40) and vigorously vortexed. Organic phase was created by addition of 500 μL of phenol-chloroform mixture (phenol saturated with 0.1 M citrate buffer pH 4.3:chloroform:isoamylalcohol 25:24:1 v/v/v). Mixture was vortexed and short centrifuged 1 min at room temperature, 12,000xg. Upper aqueous phase (600 μL) was extracted by 600 μL of phenol-chloroform mixture using the same procedure. Upper aqueous phase (550 μL) was extracted by 550 μL of phenol-chloroform mixture using the same procedure. Upper aqueous phase (500 μL) was precipitated by addition of 500 μL of 4 M LiCl over night. The RNA was pelleted by centrifugation 15 min at 4 °C, 12,900xg and the pellet was washed by 70% v/v ethanol, dried 5 min in water bath at 37 °C. RNA pellet was dissolved in 150 μL of water and reprecipitated by addition of 150 μL of 4 M LiCl for next 6 h. The RNA was pelleted by centrifugation 15 min at 4 °C, 12,900xg and the pellet was washed by 70% v/v ethanol, dried 5 min in water bath at 37 °C. The resulting

pellet was dissolved in 50-100 µL of RNase-free water. Quantity of total RNA was estimated after 100x dilution from the difference A_{260}-A_{320} using the specific extinction coefficient ε=0.025 $ng^{-1}.\mu L.cm^{-1}$. RNA quality was checked by horizontal electrophoresis in 1% w/v agarose gel with 0.5x TBE buffer (65 mM Tris; 22.5 mM boric acid; 1.25 mM EDTA) and 0.5 $\mu g.mL^{-1}$ ethidium bromide.

3.4.2 RNA extraction for QRT-PCR

Cells (0.6 mL of cell suspension; 85 mg of fresh weight) were filtered and immediately frozen in liquid nitrogen or four fully developed rosette leaves were cut and immediately frozen in liquid nitrogen. RNA was extracted using the Trizol extraction (TRI Reagent). Cells or leaves were ground in liquid nitrogen and the powder was transferred to 500 µL of TRI Reagent. After 5 min incubation at room temperature, the suspension was centrifuged 10 min at 4 °C, 12,000xg. Supernatant was transferred to a new Eppendorf tube, extracted with 100 µL of chloroform and the solution was centrifuged 15 min at 4 °C, 12,000xg, then 30 µL of 2-propanol were added to the upper aqueous phase and the solution was centrifuged again 10 min at 4 °C, 12,000xg. Upper aqueous phase was transferred to a new Eppendorf tube and after addition of 220 µL of 2-propanol the RNA was precipitated 15 min at 4 °C. RNA was pelleted by centrifugation 10 min at 4 °C, 12,000xg and supernatant was removed. RNA pellet was washed by 75% v/v ethanol in sterile water, centrifuged 5 min at 4 °C, 7,500xg and supernatant was removed. Dried RNA pellet was dissolved in 100 µL of sterile water for samples from cell suspensions or in 60 µL for samples from plant leaves. RNA concentration was determined by absorbance measurement at 260 nm in spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, USA). RNA quality was checked by horizontal electrophoresis in 1% w/v agarose gel with 0.5x TBE buffer (65 mM Tris; 22.5 mM boric acid; 1.25 mM EDTA) and 0.5 $\mu g.mL^{-1}$ ethidium bromide.

3.4.3 Semiquantitative RT-PCR analysis

1 µg of total RNA was treated by DNase I and reverse transcribed using the Omniscript reverse transcriptase kit and oligo$(dT)_{15}$ primers according to manufacturer's instructions. Resulting cDNA corresponding to an equivalent of 40 ng of total RNA was amplified in the successive 20 µL PCR with 0.6 µM gene-specific primers. The gene encoding a 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. Annealing temperature was 53 °C for all primer pairs. Primer

sequences are listed in Supplemental Table III. A suitable number of PCR cycles were used for each primer pair. PCR was separated on horizontal electrophoresis in 1% w/v agarose gel with 0.5x TBE buffer (65 mM Tris; 22.5 mM boric acid; 1.25 mM EDTA) and 0.5 $\mu g.mL^{-1}$ ethidium bromide.

3.4.4 QRT-PCR analysis

1 µg of total RNA was treated by DNase I and reverse transcribed using the iScript cDNA Synthesis kit according to the manufacturer's instructions. An equivalent of 25 ng of total RNA was amplified in the successive 15 µL QPCR with 0.3 µM gene-specific primers designed by Vector NTI software (version 10.3.0). The gene encoding a 40S ribosomal protein S24 (At3g04920) was used as a housekeeping gene. 10- and 100-fold dillutions of one reference sample were prepared to determine the efficiency of QPCR. Amplification was done on Bio-Rad MJ Mini Opticon thermal cycler with iQ SYBR Green Supermix according to manufacturer's instructions. Annealing temperature was 57 °C for all primer pairs. Primer sequences are listed in Supplemental Table IV. Threshold cycles (c_T) for each sample were determined with Opticon Monitor software (version 3.1). QPCR efficiency was determined from the slope of the plot of c_T against log of dillution of the reference sample. Gene expression in each sample was normalized to the expression of the housekeeping gene.

3.4.5 Transcriptome studies

The microarray analysis was carried out at the Unité de Recherche en Génomique Végétale (INRA UMR1165, CNRS UMR8114), Evry, France, using the CATMA array (Crowe et al., 2003; Hilson et al., 2004), containing 24,576 gene-specific tags (GSTs) from *Arabidopsis*. RNA samples from two independent biological replicates were used. For each biological repetition, RNA samples for a condition were obtained by pooling RNAs from three independent extractions (1 mg of fresh weight per extraction). For each comparison, one technical replication with fluorochrome reversal was performed for each biological replicate (i.e. four hybridizations per comparison). The reverse transcription of RNA in the presence of Cy3-dUTP, or Cy5-dUTP (PerkinElmer-NEN Life Science Products, Courtaboeuf, France), the hybridization of labelled samples to the slides and the scanning of the slides were performed as described in Lurin et al. (2004).

3.4.6 Statistical analysis of microarray data

Experiments were designed with the aid of Marie-Laure Martin-Magniette from the statistics group of the Unité de Recherche en Génomique Végétale. The statistical analysis was based on two dye-swaps (i.e. four arrays, each containing 24,576 GSTs and 384 controls) as described in Lurin et al. (2004). The controls were used for assessing the quality of the hybridizations but were not included in the statistical tests or the graphical representation of the results. For each array, the raw data comprised the logarithm of median feature pixel intensity at wavelengths 635 nm (red) and 532 nm (green). No background was subtracted. In the following description, log ratio refers to the differential expression between two conditions. It is either $\log_2$(red/green) or $\log_2$(green/red) according to the experimental design. Array-by-array normalization was performed to remove systematic biases. First, we excluded spots that were considered badly formed features. Then, we performed a global intensity-dependent normalization using the LOESS procedure to correct the dye bias. Finally, for each block, the log ratio median calculated over the values for the entire block was subtracted from each individual log ratio value to correct print tip effects on each metablock. To determine differentially expressed genes, we performed a paired *t* test on the log ratios, assuming that the variance of the log ratios was the same for all genes. Spots displaying extreme variance (too small or too large) were excluded. The raw p-values were adjusted by the Bonferroni method, which controls the familywise error rate (FWER). We considered as being differentially expressed the genes with an FWER<5%.

3.5 Data deposition

Microarray data from the experiment with wortmannin were deposited at Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/; accession no GSE7495) and at Complete Arabidopsis Transcriptome Database (CATdb; http://urgv.evry.inra.fr/CATdb/; Project: RS05-04_salicylic-acid) according to the Minimum Information About a Microarray Experiment (MIAME) standards.

Microarray data from the experiment with *n*-butanol and *tert*-butanol are not published yet therefore these were not deposited at public databases.

4 RESULTS

4.1 Introductory experiments leading to characterization of the model system

4.1.1 Non-lethal and effective SA concentration

The presented research was aimed to study changes in the phospholipid metabolism and putative involvement of phospholipid signalling system in the SA signalling pathway of *Arabidopsis thaliana* (Arabidopsis). The major part of the experiments was performed with cell suspensions derived from the wild type of Arabidopsis. Arabidopsis plants are known to trigger SA signalling pathway upon spraying their leaves with SA. However, the SA responsiveness of the cell suspension had to be experimentally determined. Growth curve of the cell suspension is shown in Figure 7. The cell suspension was multiplied every 7th day but it reached the stationary phase on the 5th day after passage. In order to avoid senescence related processes and to work with sufficient amount of biomass, all the experiments were performed on the 5th day of cultivation.

SA can be phytotoxic when applied at high concentration. Arabidopsis plants are usually treated with SA at concentration around 1 mM, but cells in suspension culture can be more sensitive as they do not possess the waxy cuticle on their surface. Thus it was important to estimate the maximal non-lethal concentration of SA. This was done by Trypan blue staining for two periods of SA treatment (3 h and 24 h; Figure 8A). The concentration not causing substantial damage even after 24 h of SA treatment was indeed 1 mM SA, as the fraction of dead cells in the water treated samples after 24 h was about 5%.

The SA responsiveness of the cell suspension was further characterized for three non-toxic concentrations (i.e. 250 µM, 500 µM and 1 mM SA) by semiquantitative RT-PCR analysis of expression of the principal marker gene of the SA pathway (*PR1*; At2g14610). Expression of *PR1* was comparable for all three concentrations after 24 h of SA treatment (Figure 8B). Based on this advantage, the lowest concentration (250 µM) was chosen for further experiments to minimize the possible undesired side-effects of higher concentration.

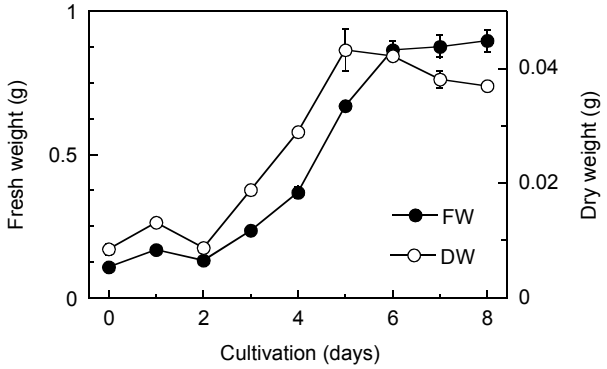


Figure 7. Growth curve of Arabidopsis cell suspensions.
The data are means of two independent experiments. DW, dry weight; FW, fresh weight.

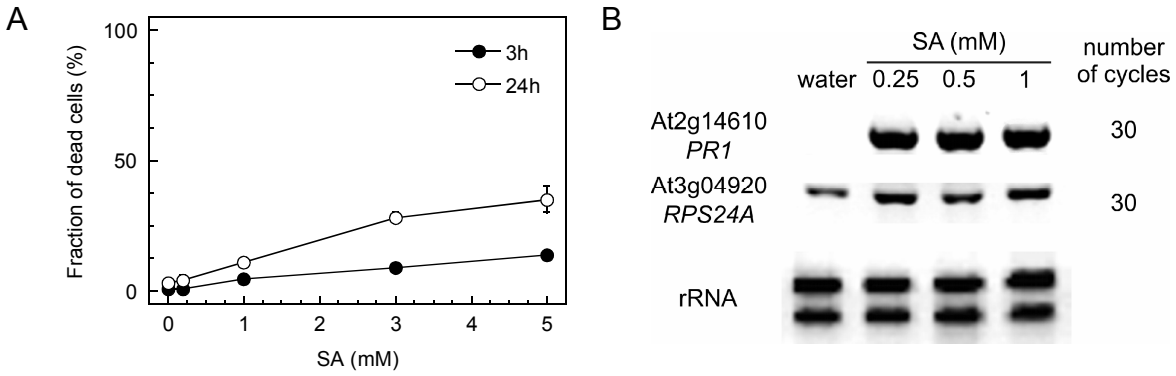


Figure 8. Non-lethal but effective SA concentration.
(A) Viability of cells determined by Trypan blue staining after 3 and 24 h of SA treatment. The data are means of two independent experiments. (B) Expression of *PR1* after 24 h treatment with different SA concentrations or water analyzed by semiquantitative RT-PCR. 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result of two independent experiments. (A, B) SA treatment started on the 5th day of sub-cultivation in both experiments. rRNA, ribosomal RNA.

4.1.2 Marker genes of the SA pathway

Expression of marker genes is a very useful tool to estimate if and to what extent the studied pathway has been induced. Many marker genes of the SA pathway are known but their expression profile was studied only in whole plants. Therefore their induction and the time course of their induction had to be proved experimentally for cell suspensions. Expression of a small selection of genes (*GST6*, *NPR1* and *PR1*) was studied by RT-PCR (Figure 9). All three genes were induced by 250 µM SA and had their maxima at 2 h, 4 h and 6 h, respectively. Such result is in good correlation with the sequence of events in the whole plants (Bostock, 2005; Wagner et al., 2002), thus confirming the correct triggering of the SA pathway in the studied cell suspensions.

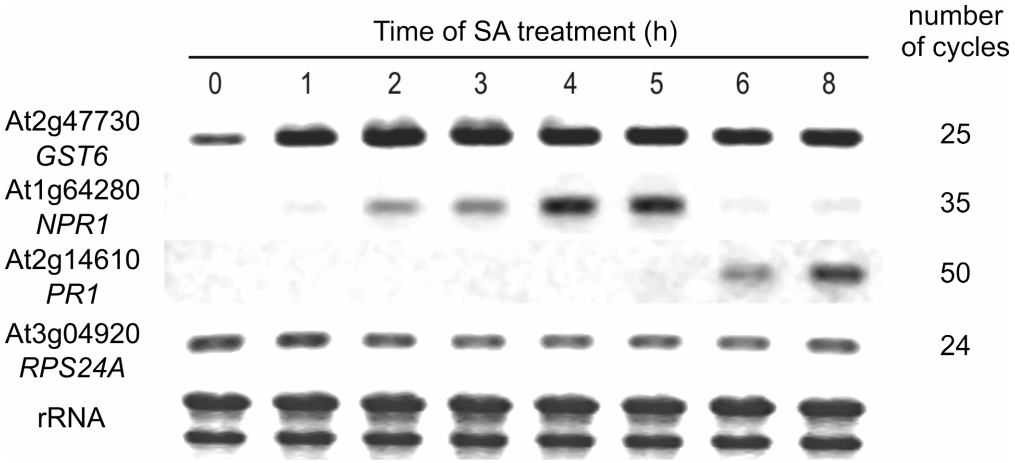


Figure 9. Expression of a selection of SA marker genes in Arabidopsis cell suspensions.
Cells were treated with SA at time 0 and RNA was isolated from aliquots at indicated times. Gene expression was analyzed by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result from five independent experiments. rRNA, ribosomal RNA.

4.1.3 Radiolabelling of phospholipids in vivo

In order to be able to evaluate changes in the phospholipid pattern after SA treatment, it was necessary to have an estimation of the turnover of phospholipids in the cell suspensions as well as of the time course of their labelling. In one experiment, $^{33}P_i$ was added to the cell suspension at the time 0 to initiate metabolic labelling of phospholipids. Aliquots were then extracted at different times (Figures 10A and 10B). The time course of the metabolic labelling of each phospholipid was followed during the first 3 h. Another experiment was a pulse-chase study where the labelling was allowed to proceed during the first 20 min and then it was quenched by dilution of the radioactive tracer with cold P_i (Figures 10C and 10D). Two classes of phospholipids could be distinguished. Phospholipids bearing monoesterified phosphate (PIP, PI(4,5)P_2 and PA) were rapidly labelled. Their labelling reached a plateau, and then it slowly decreased. On the other hand, phospholipids with only diesterified phosphate (PI, PC, PE, PG) were labelled more slowly, and their labelling reached a plateau probably beyond the end of the experiment. These two classes behaved differently even in the pulse-chase experiment. PIP, PI(4,5)P_2 and PA lost the radioactive label rapidly upon cold P_i addition. In case of PIP and PI(4,5)P_2 the resting radioactivity could probably come from the diesterified phosphate which is harder to replace. In case of PA it could correspond to a pool which comes from the inter-conversion of structural lipids rather than from direct phosphorylation of DAG. It could also come from the hydrolysis of PE or PC by PLD *in vivo* but it is known that PA can be formed through PLD action even

during lipid extraction. Labelling of structural phospholipids simply 'stopped' at the moment of addition of cold P_i. Comparison of normal labelling time course with a pulse chase study for each phospholipid is given in Figure 11.

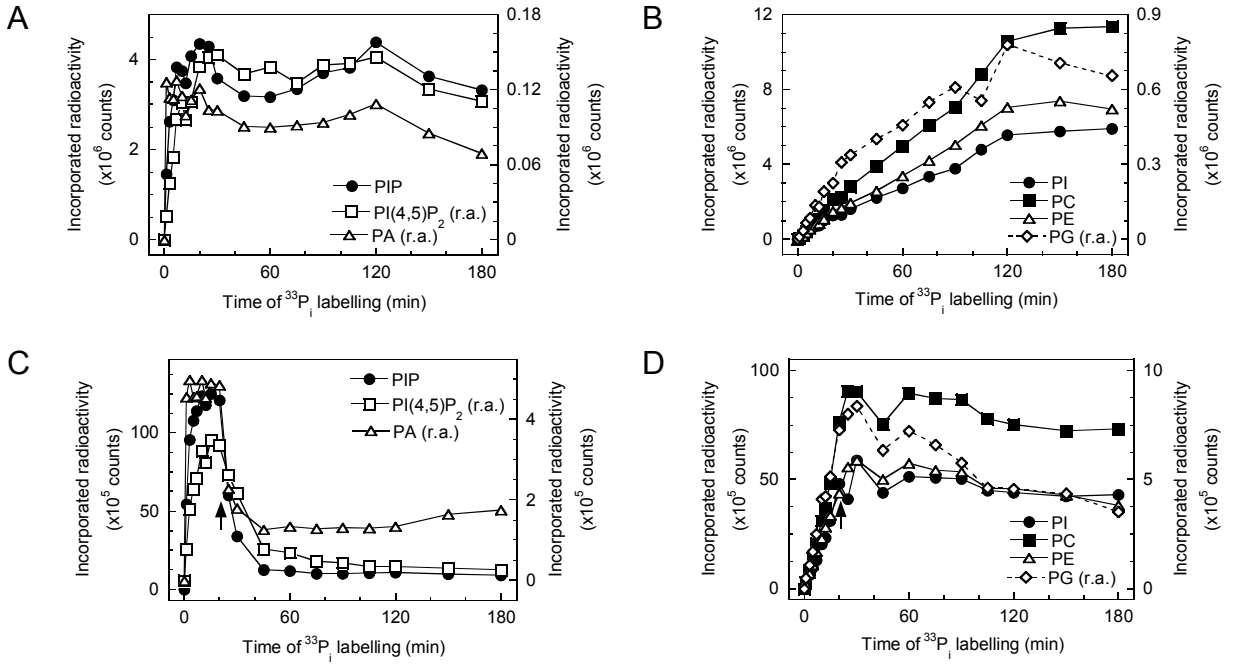


Figure 10. Pulse-chase study of metabolic labelling of phospholipids.
(A, B) Cells were labelled at the time 0 with $^{33}P_i$ and lipids were extracted at the times indicated. (C, D) After 20 min of metabolic labelling the radioactive tracer was diluted with a surplus of $^{31}P_i$ (indicated by an arrow). The data represent a typical result from three independent experiments. PG, phosphatidylglycerol; r.a., right axis scale.

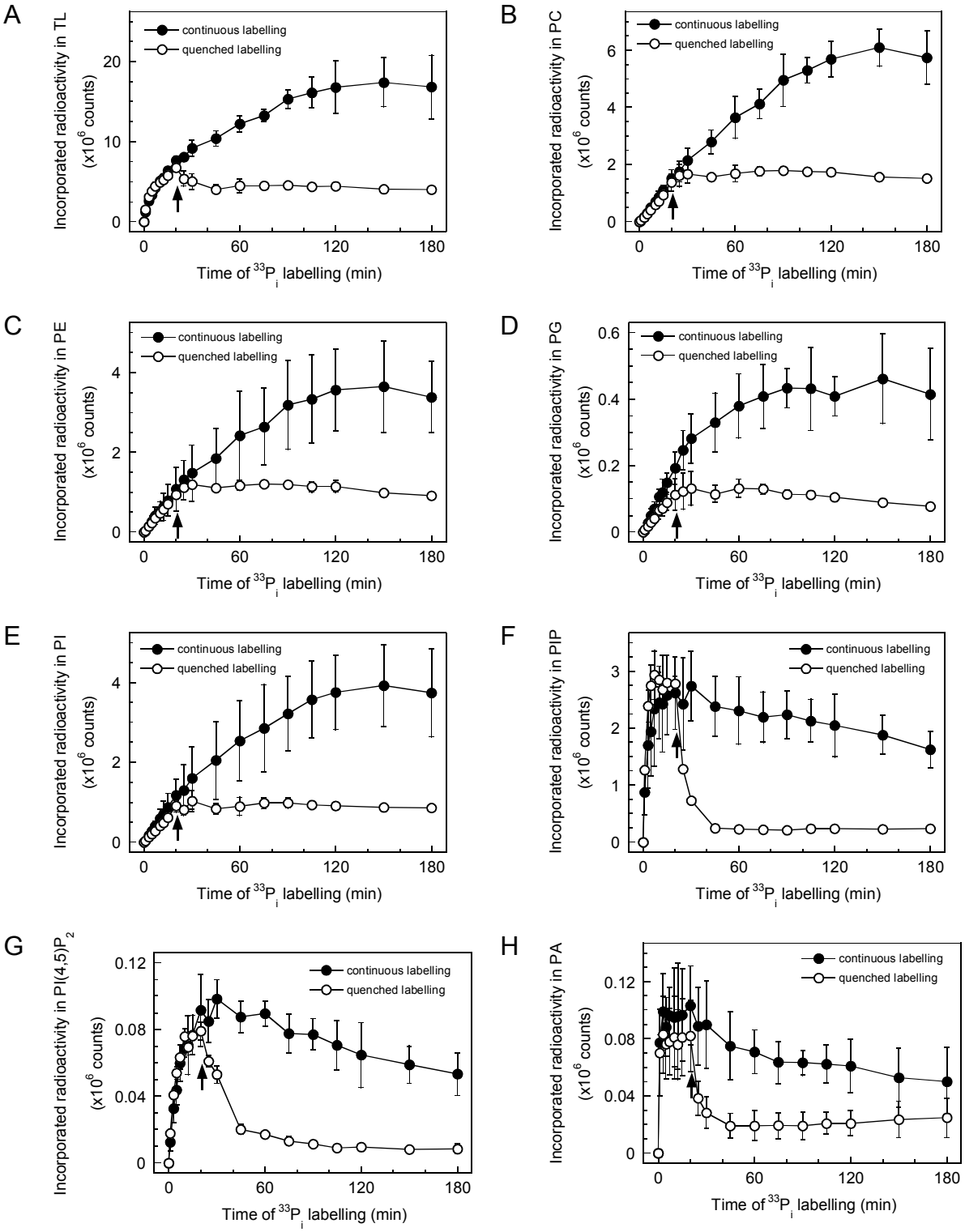


Figure 11. Pulse-chase study of metabolic labelling for each studied phospholipid.
Cells were labelled at the time 0 with $^{33}P_i$ and lipids were extracted at the times indicated (continuous labelling). After 20 min of metabolic labelling the radioactive tracer was diluted with a surplus of $^{31}P_i$ (quenched labelling; indicated by an arrow). The data are means of three independent experiments for undisturbed labelling and of two independent experiments for quenched labelling. PG, phosphatidylglycerol; TL, total labelled lipids.

4.2 SA activates PI 4-kinase *in vivo*

4.2.1 Radiolabelling of phospholipids during SA treatment

Changes in the pattern of radiolabelled phospholipids were investigated in the first three hours of SA treatment. Three different times of phospholipid labelling preceding SA application were chosen for the preliminary screening (5 min, 15 min and 24 h). The procedure of metabolic labelling followed by autoradiography of TLC-separated phospholipids bears several critical steps which may introduce a bias into the final signal (unequal mass of cells in aliquots of cell suspension, unequal amount of radioactive tracer due to pipetting error, unequal phospholipid extraction efficiency or deposit of extracted phospholipids on the TLC plate due to rapid evaporation of the solvent). The best solution how to circumvent these errors is to perform a normalisation step where the amount of the studied phospholipid is made relative to a phospholipid which is present in sufficient amount and which does not vary in the treated cells and the controls. PC and PE fulfilled the prerequisite for each time of labelling (data not shown). PC was more labelled and its TLC spot was more compact (thus enabling more accurate quantification) than that of PE. PC was therefore used as the internal standard for all the following normalisation steps unless stated otherwise. The proportion of radiolabelled phosphoinositides differed between SA and water treatment only when cells were labelled for a short time (5 or 15 min). The biggest difference in the PI/PC ratio occurred after 5 min of labelling (Figure 12A) and thus this time was chosen for further experiments.

No changes in radiolabelled phosphoinositides were observed after 24 h of labelling. The dissimilar results obtained with different times of labelling (i.e. 5 or 15 min of labelling compared to 24 h labelling) could originate in the fact that the radioactive label is present in distinct pools of cellular PI. PI is synthesized on the ER and then transported to other cellular membranes (Justin et al., 2003). These pools may differ in their fatty acyl composition. RP-HPLC analysis of the PI labelled for 5 min and for 24 h revealed slight differences in their fatty acyl composition (Figure 12B). This could be in favour of the existence of distinct pools of PI which are differently affected by SA treatment.

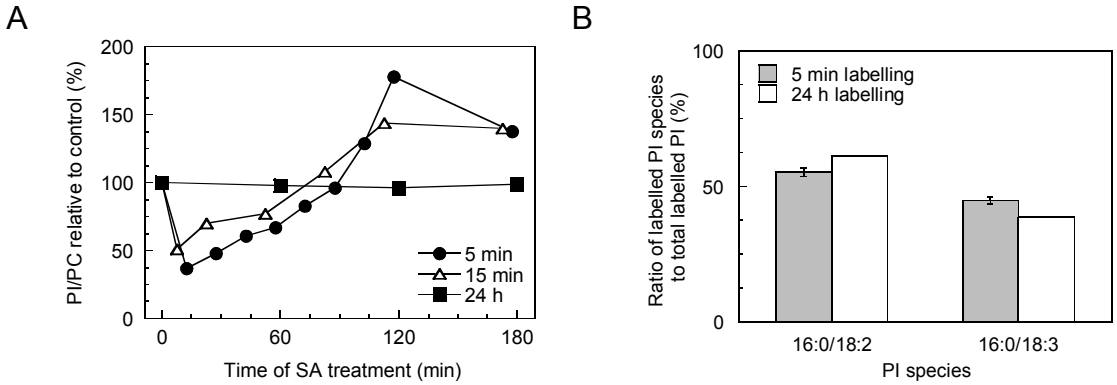


Figure 12. Different periods of radiolabelling mark different PI pools in the plant cell.
(A) Cells were treated with SA at the time 0 and labelled for different periods of time before lipid extraction. The data represent a typical result from two independent experiments. (B) Cells were labelled for either 5 min or 24 h before lipid extraction and the radiolabelled PI species were quantified by RP-HPLC. The data are means of two independent experiments.

4.2.2 Characteristics of the SA-induced changes in labelled phospholipids

To get a closer insight into the phosphoinositide changes, the time course of changes in the pattern of labelled phospholipids was studied during the first 3 h of SA treatment. A decrease of PI/PC was accompanied by an increase in PIP/PC and PI(4,5)P_2/PC within 45 min of SA treatment (Figure 13). After that, the situation was inversed, PI/PC increased above the control level and PIP/PC decreased below the control level. The decrease of PI/PC was the most pronounced change in the first minutes of SA treatment (Figure 13B). No changes were observed in the PE/PC ratio during the first 3 h of SA treatment. The early changes in phosphoinositides could not be inhibited by cycloheximide, an inhibitor of protein synthesis, while the later turn of the trend was inhibited (Figure 14) which suggests that the late decrease depends on protein synthesis.

The observed changes in radiolabelled phosphoinositides were dose-dependent reaching a maximum at 1 mM SA (Figure 15A). However, concentrations above 250 μM substantially perturbed the labelling of structural phospholipids (especially that of PE; Figure 15B).

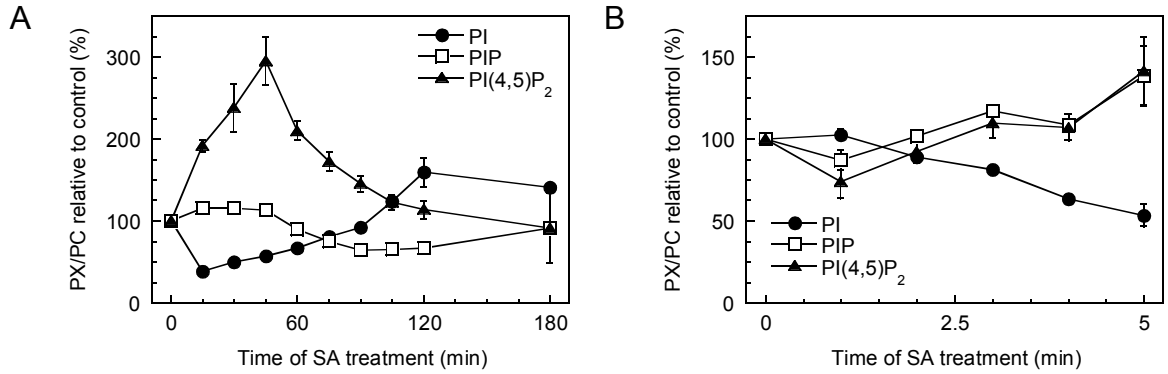


Figure 13. Time course of phosphoinositide labelling in SA-treated Arabidopsis cells.
(A) Changes in the phosphoinositide labelling rate during the first 3 h of SA treatment. (B) Changes in labelled phosphoinositides appear during the first minutes of SA treatment. (A, B) Cells were labelled with $^{33}P_i$ 5 min before lipid extraction. PIP and PI(4,5)P_2 were separated in the alkaline solvent system, PI was separated in the acid solvent system. Values for water-treated cells were taken as controls (100%). The data are means of two independent experiments. PX, phospholipid.

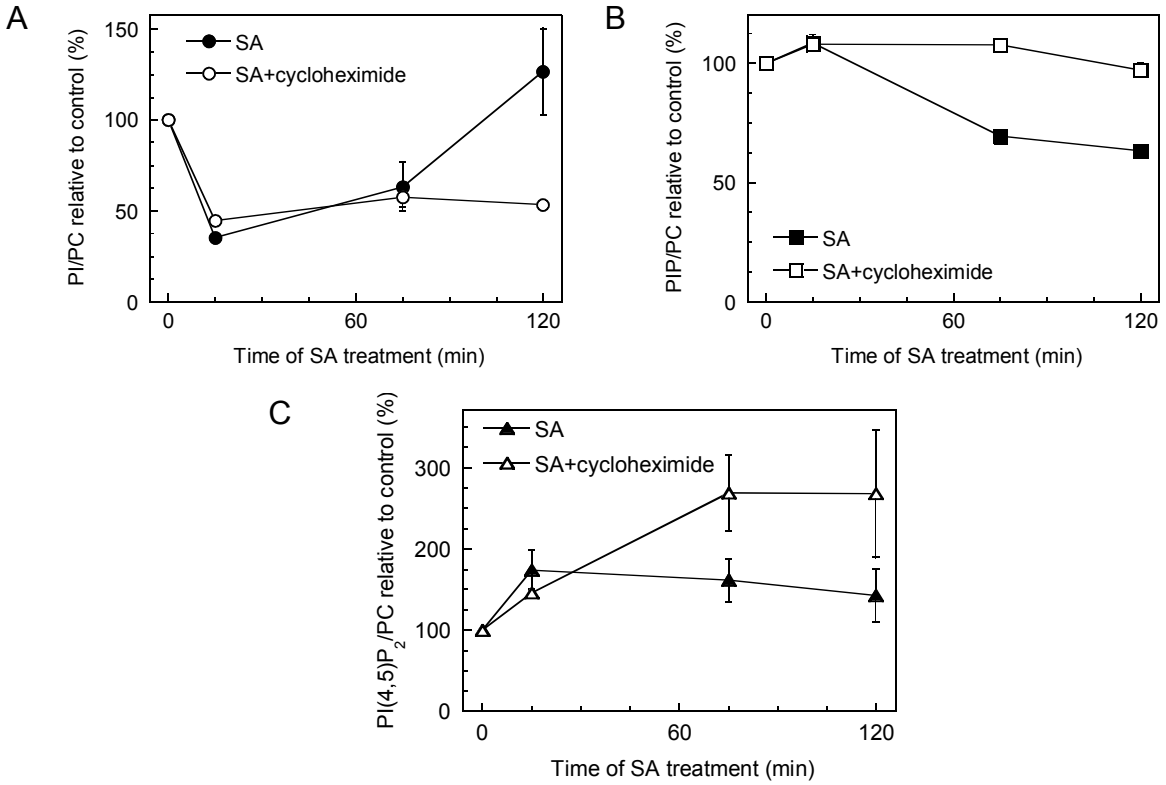


Figure 14. The early changes in labelled phosphoinositides during SA treatment are not inhibited by cycloheximide.
Cycloheximide was dissolved in water to final concentration of 20 μM. Cells were labelled with $^{33}P_i$ 5 min before lipid extraction. Values for water-treated cells are taken as controls (100%). The data are means of two independent experiments.

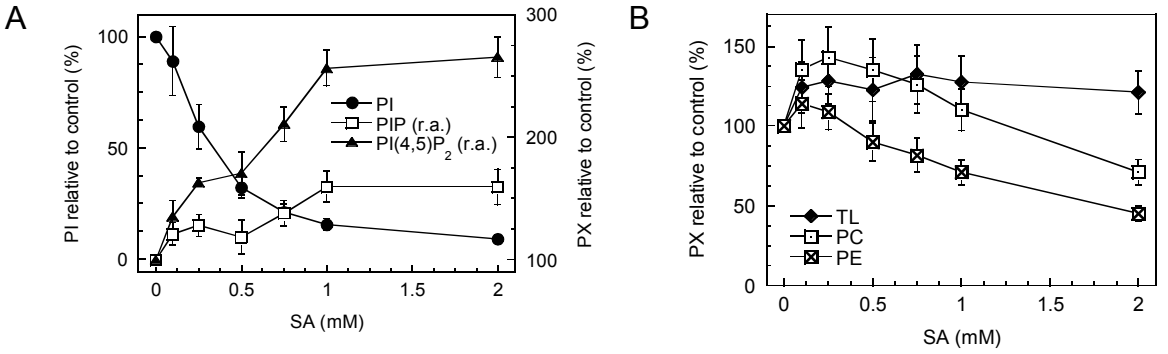


Figure 15. Dose dependence of changes in labelled phospholipids.
(A) Dose dependence of changes in labelled phosphoinositides. (B) Dose dependence of changes in phospholipid labelling and in labelled structural lipids. (A, B) Cells were treated with the corresponding concentration of SA for 8 min before lipid extraction. Cells were labelled with $^{33}P_i$ for 5 min before lipid extraction. PIP and PI(4,5)P_2 were separated in the alkaline solvent system, PI, PC and PE were separated in the acid solvent system. Values for water-treated cells were taken as controls (100%). The data are means of two independent experiments. PX, phospholipid; r.a., right axis scale; TL, total labelled lipids.

4.2.3 SA-induced PIP is PI(4)P

Several enzyme activities could be responsible for the observed changes (PI 3-kinase, PI 4-kinase, PIP 5-kinase, PLC or a combination thereof). All of the enzyme activities are present in Arabidopsis. First step was to determine whether the induced PIP is PI(3)P or PI(4)P. These two isomers cannot be separated in the alkaline solvent system routinely used for separation of phosphoinositides in the experiments. However, they can be separated in a borate solvent system. It is based on stronger binding of BO_3^{3-} by the phosphoinositol ring of PI(4)P than by that of the PI(3)P (Walsh et al., 1991). SA and water treated samples were run in parallel in the alkaline and borate solvent systems (Figure 16). The published retention factors (R_f) are 0.66 for PC, 0.46 for PI(4)P and 0.51 for PI(3)P in the borate system. R_f values of 0.60 for PC and of 0.39 for PI(4)P were found; the R_f value for the PI(3)P spot would be expected to be close to 0.45, but no spot with this mobility was detected (Figure 16B). Quantification of the PIP spots in both separation systems confirmed that all the radioactivity in the PIP spot in the alkaline solvent system was present in the spot corresponding to the PI(4)P in the borate system. Almost no radioactivity was detected in the PI(3)P spot and, more important, this amount did not vary with SA treatment. Thus it can be concluded that the PIP changes are equivalent to PI(4)P changes and therefore the PIP will be called PI(4)P in the following text.

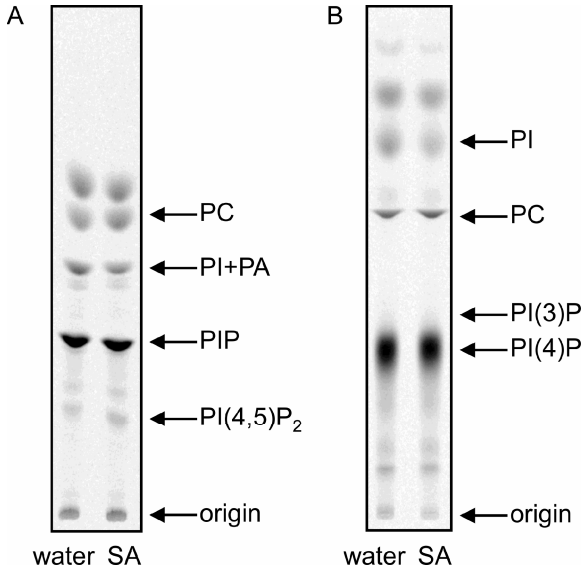


Figure 16. SA-induced PIP is PI(4)P.
Cells were treated with SA or with water 8 min before lipid extraction and labelled by $^{33}P_i$ 5 min before lipid extraction. Lipids were extracted and separated in parallel using an alkaline solvent system (A) and a borate solvent system (B). The expected position of PI(3)P is also indicated (R_f 0.45). The images represent a typical result from two independent experiments.

4.2.4 PI decrease can be impaired by inhibitors of type III PI 4-kinase

Inhibitors are valuable tools in dissecting among various enzyme activities involved in the studied response. Inhibitors were used for identification of the enzymatic activity responsible for the observed changes in labelled phosphoinositides. Unfortunately, the application of inhibitors is usually limited by their side effects. They may influence the rate of metabolic labelling or either activate or deactivate enzymes metabolizing the structural lipids. Some substances used in the following experiments increased PLD activity which led to a loss of labelled PC and an increase of labelled PA. This was further confirmed by increased production of PBut in the presence of the tested inhibitor (when tracing the PLD activity with *n*-butanol; data not shown). In such cases the PI/PC ratio was replaced by the PI/PE ratio. This is also indicated in the corresponding figures and their legends. All inhibitors were applied in DMSO 15 min before SA treatment unless stated otherwise.

The decrease of PI/PC ratio accompanied by an increase of PI(4)P/PC ratio suggested that the involved activity was that of a PI 4-kinase. There are two types of PI 4-kinases (type II and type III) which differ in their sensitivity to micromolar concentrations of wortmannin and in their Ca^{2+} dependence. No plant type II PI 4-kinase has been biochemically characterized yet. Based on animal and yeast studies, type II PI 4-kinases are inhibited by high Ca^{2+} concentrations but cannot be

inhibited by micromolar concentrations of wortmannin, contrary to type III PI 4-kinases that are inhibited by micromolar concentrations of wortmannin (Müller-Röber and Pical, 2002), by phenylarsine oxide (PAO; Rajebhosale et al., 2003) and are not Ca^{2+} dependent (Müller-Röber and Pical, 2002). PAO and high concentrations of wortmannin (30 μM) inhibited the SA response while low concentration of wortmannin (1 μM) had no effect (Figure 17). These data were in favour of the PI decrease being due to the activity of a PI 4-kinase of the type III.

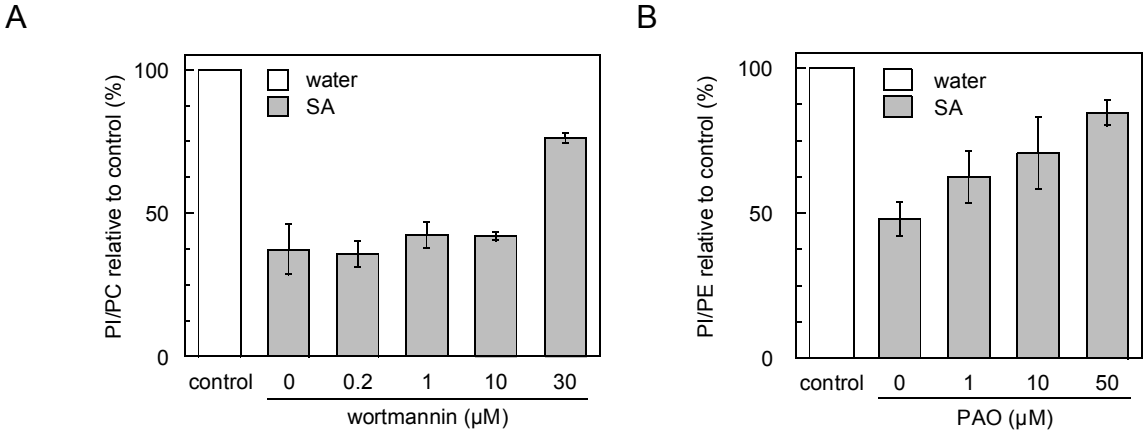


Figure 17. Inhibitory effect of wortmannin and phenylarsine oxide (PAO) on the SA-induced decrease in labelled PI.
Cells were incubated 45 min with wortmannin (A) or 15 min with PAO (B) prior to SA application and then treated with SA for 8 min before lipid extraction. Cells were labelled by $^{33}P_i$ for 5 min before lipid extraction. Inhibitors were applied in DMSO (final concentration 0.5% v/v). Controls (100%) represent water-treated cells. PI/PE ratio was chosen to express the results of experiment with PAO (B) due to activation of PC-specific PLD during PAO treatment. The data are means of three independent experiments.

A yeast mutant, *stt4*, deficient in yeast orthologue of *AtPI4Kα1*, had diminished formation of PE due to perturbed transport of PS from ER to Golgi (Trotter et al., 1998). Thus the level of PE labelling should to certain extent mirror the activity of PI4Kα1. 30 μM wortmannin decreased PE labelling in water-treated cells (Figure 18A) suggesting that the PI 4-kinase activity inhibited by 30 μM wortmannin was that of PI4Kα1. If the SA activated PI 4-kinase isoform was PI4Kα1, the PE/PC ratio in SA treated cells relative to PE/PC ratio in water treated cells should have decreased in the presence of 30 μM wortmannin but in fact it increased (Figure 18B) which is in favour of other PI 4-kinase isoform being activated in response to SA. Unfortunately, it was not possible to estimate the labelling of PE in the presence of PAO because the level of labelled PC changed due to activation of PC-specific PLD.

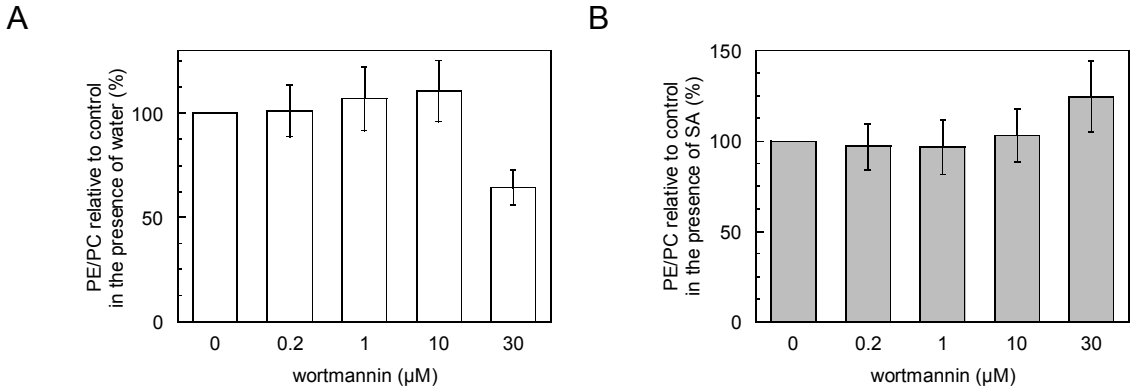


Figure 18. Effect of wortmannin on PE labelling.
The effect of wortmannin on PE labelling was studied after treatment with water (A) or SA (B). Cells were incubated 45 min with wortmannin prior to water or SA application and then treated with water or SA for 8 min before lipid extraction. Cells were labelled by $^{33}P_i$ for 5 min before lipid extraction. Wortmannin was applied in DMSO (final concentration 0.5% v/v). The PE/PC ratios in figure B are made relative to that of water treated cells. Controls (100%) represent DMSO-treated cells. The data are means of three independent experiments.

To exclude that the observed changes were not due to the activity of other plant PI-kinases more inhibitors were tested. Plant PI 3-kinases are inhibited by sub-micromolar concentrations of wortmannin and by LY294002 (Turck et al., 2004). Low concentrations of wortmannin (up to 10 µM; Figure 17A) and 50 µM LY294002 (data not shown) did not affect the response to SA, confirming that the PI 3-kinase was not responsible for the observed changes. Additional point of regulation can take place at the level of PIP 5-kinase. This activity in plants can be inhibited by 30 µM wortmannin too (Jung et al., 2002). Thus the concomitant activation of a PIP 5-kinase (in addition to that of the PI 4-kinase) cannot be excluded.

The increase in PI(4)P and PI(4,5)P_2 levels could be seen as a way of supplying substrates for the action of a phosphoinositide-specific phospholipase C (PI-PLC). PI-PLC inhibitors U73122 (60 µM) and edelfosine (150 µM) did not affect the increase of labelled PI(4,5)P_2 after 8 min of SA treatment (data not shown). Both these compounds were previously shown to inhibit PI-PLC *in vivo* in Arabidopsis cells (Vergnolle et al., 2005). Moreover, no production of $InsP_3$, the second product of PLC activity, was observed from 1 to 150 min of SA treatment (data not shown). Therefore the involvement of PI-PLC in the early SA response seems unlikely.

4.2.5 Phosphorylation events are involved in the activation of PI 4-kinase

In plants, regulation of PI 4-kinase by preceding phosphorylation events was described for the PI 4-kinase interacting protein PIKA49 (later identified as elongation

factor EF-1α with F-actin binding capacity) in carrot cell suspensions (Yang and Boss, 1994). PIKA49 was phosphorylated on Ser by CDPK in a Ca^{2+}-dependent manner. Another study reported on phosphatidylinositol transfer protein from soybean (Ssh1p) which was phosphorylated on Ser/Thr during hyperosmotic stress and which was *in vitro* capable to activate both PI 3-kinase and PI 4-kinase in Arabidopsis (Monks et al., 2001). Therefore it was interesting to investigate whether the PI 4-kinase activation in response to SA is preceded by a phosphorylation event.

Several inhibitors can be used to test the involvement of protein kinases/phosphatases in a response. None of them is really specific but the use of their combination may give an idea of the involved phosphorylation events (Davies et al., 2000). The concentrations of inhibitors used are those published to be active in plants. Figure 19A summarizes the effects of inhibitors of Ser/Thr protein kinases/phosphatases. Okadaic acid (1 μM) is an inhibitor of protein phosphatases 2A and calyculin A (1 μM) is an inhibitor of both protein phosphatases 1 and 2A. K252-a (5 μM) preferentially inhibits CaM-dependent protein kinase (CaMK). Staurosporine (1 μM) is less selective and inhibits several types of protein kinases. K252-a, staurosporine and okadaic acid had no effect (the difference from the control was not statistically significant) and only calyculin A inhibited the SA-induced PI decrease. Based on this it can be concluded that a dephosphorylation on Ser/Thr has to take place before the SA-induced PI 4-kinase activation and that this dephosphorylation is mediated by type 1 protein phosphatases.

The effects of inhibitors of Tyr protein kinases/phosphatases are summarized in Figure 19B. 3,4-dephostatin (25 μM) inhibits a large spectrum of Tyr phosphatases and genistein (100 μM) inhibits a large spectrum of Tyr kinases. Daidzein (100 μM) is the inactive analogue of genistein. It can be seen that 3,4-dephostatin as well as daidzein had no effect. On the other hand there was a clear inhibitory effect of genistein on the SA-induced PI decrease. In conclusion, a phosphorylation step on Tyr also precedes the PI 4-kinase activation in response to SA. Therefore, the phosphorylation events are at least two (one on Ser/Thr and one on Tyr). Based on these data, one cannot conclude in which order they take place or whether they affect directly the PI 4-kinase protein or its interaction partner(s).

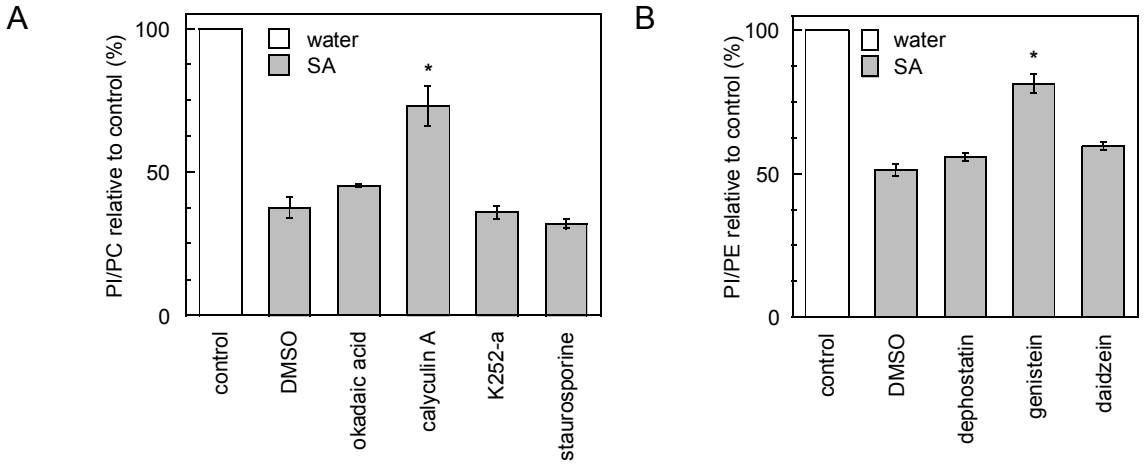


Figure 19. Effect of inhibitors of protein phosphorylation and dephosphorylation on the SA-induced decrease in labelled PI.
Cells were incubated 15 min with the corresponding inhibitor of Ser/Thr kinases/phosphatases (A) or of Tyr kinases/phosphatases (B) prior to SA application and then treated with SA for 8 min before lipid extraction. Cells were labelled by $^{33}P_i$ for 5 min before lipid extraction. Inhibitors were given in DMSO (final concentration 0.5% v/v). Okadaic acid was 1 μM, calyculin A was 1 μM, K252-a was 5 μM, staurosporine was 1 μM, 3,4-dephostatin was 25 μM, genistein was 100 μM and daidzein was 100 μM. Controls (100%) represent water-treated cells. PI/PE ratio was chosen to express the results of experiment with inhibitors of Tyr kinases/phosphatases (B) due to activation of PC-specific PLD during genistein treatment. The data are means of two independent experiments.

4.2.6 Ca^{2+} influx in response to SA

Ca^{2+} influx is a general mechanism which is involved in many plant stress responses. An elevation of cytosolic Ca^{2+} concentration upon SA treatment was reported in BY-2 cells expressing aequorin (Lin et al., 2005). Therefore it was worthwhile to check whether the changes in cytosolic Ca^{2+} concentration also play a role in the SA response of Arabidopsis cell suspensions. Direct measurements of bioluminescence in Arabidopsis cell suspensions expressing aequorin were performed. Two concentrations of SA (250 μM and 1 mM) were tested and compared to the application of the same volume of distilled water (negative control) and to the generation of hypoosmotic stress conditions by two-fold dilution of the cell suspensions (positive control). The whole system was then calibrated by disintegration of the cells to obtain the total amount of releasable Ca^{2+}. The change in bioluminescence during 10 min of SA treatment was not significantly different from the negative control (data not shown), which is in favour of the absence of any Ca^{2+} influx during the first ten minutes of SA treatment. Moreover, no changes of the SA-induced PI decrease were observed after 8 min of SA treatment in the presence of 1 mM La^{3+}, a plasma membrane Ca^{2+} ion channel blocker (data not shown). Both results suggest that Ca^{2+} signalling is not involved at the very beginning of the PI 4-kinase activation (during the first 10 min of SA treatment).

4.2.7 Expression of genes involved in the phosphoinositide metabolism during SA treatment

The PI 4-kinase activation is too rapid to be a result of altered gene expression or protein synthesis. However, the later changes in the phosphoinositide pattern could originate from altered expression of genes involved in phosphoinositide metabolism. There are four genes corresponding to the type III PI 4-kinase (Müller-Röber and Pical, 2002) and two genes for PI synthase in the genome of Arabidopsis (Justin et al., 2003). Expression of all the six genes was followed during the first 4 h of SA treatment by QRT-PCR with gene-specific primers (Figure 20). Expression levels of both PI synthases, *PI4Kα1* and *PI4Kβ1* did not vary significantly during the experiment, while that of *PI4Kα2* decreased about two-fold around 2 h of SA treatment and that of *PI4Kβ2* increased about two-fold starting from 2 h of SA treatment. *PI4Kα2* is probably a very lowly expressed isoform based on its high threshold cycle (around 35) during the QRT-PCR analysis. Therefore the slight increase of labelled PI(4)P between the second and the third hour of SA treatment (Figure 13A) could result from the increased expression of *PI4Kβ2*.

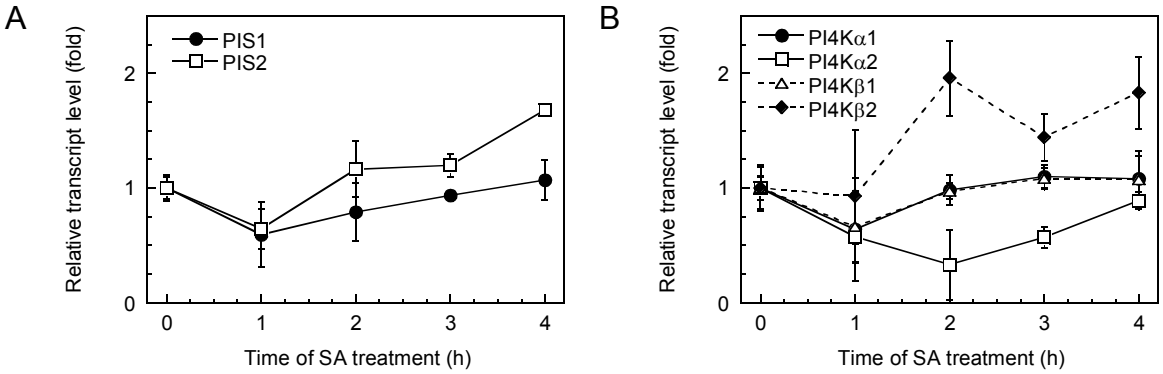


Figure 20. Expression of genes involved in the phosphoinositide metabolism during SA treatment.
Cells were treated with SA at the time 0 and RNA was isolated from aliquots at indicated times. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of water-treated cells was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results. PIS, PI synthase.

4.3 SA transcriptome is partially regulated by a W30-sensitive pathway

4.3.1 SA induces changes in the transcriptome of Arabidopsis

A few studies on SA transcriptome have been published (see chapter 2.1.1.2) but only in the whole plants of Arabidopsis. Therefore it was interesting to investigate it in

the cell suspensions and compare the results with the known facts. Preliminary results showed that SA-triggered transcript level changes were hardly detectable before 4 h, but became visible after 4 h (Figures 9 and 32). Thus this time was chosen to study the changes of Arabidopsis transcriptome. RNA samples from two independent biological repetitions were analyzed on the Complete Arabidopsis Transcriptome MicroArray (CATMA) chips as described in Lurin et al. (2004). RNAs were extracted from 1 mg of fresh weight of 5 days old cells that had been treated for 4 h with water or with SA. For each biological repetition, RNA samples for each condition were obtained by pooling RNAs from three independent extractions. One dye−swap (technical replicate with fluorochrome reversal) was made per biological repetition, i.e. four hybridizations per comparison. The differentially expressed genes were identified by a paired *t* test on the $\log_2$ ratios based on the four hybridizations. The raw p-values were adjusted by Bonferroni methods, which control the familywise error rate (FWER). With an FWER set at 5%, most of the probes, i.e. 19,040 probes (96.0% of total monitored probes), showed no changes in their transcript levels. Nevertheless, 474 genes (2.4% of total monitored genes) could be considered as up-regulated, while 317 (1.6%) were down-regulated. The list of the 791 differentially regulated genes is available as Supplemental Table V (electronic supplement only).

The 25 most induced genes and the 25 most repressed genes are described in Tables V and VI, respectively. Genes with unambiguous probe-to-gene assignment were extracted from the list of SA-regulated genes. They were then classified according to the Munich Information Center for Protein Sequences (MIPS) functional catalogue categories (Ruepp et al., 2004), using the MIPS interface (Figure 21). In the whole genome each functional category is represented by a certain portion of annotated genes. The MIPS interface determines the probability that in a given set of genes the functional category is over-represented relatively to its proportion in the whole genome. Only over-representation is considered in the analysis because under-representation of the other categories is its simple consequence. It means that random choice of a subset of genes from the whole genome would produce p-values$<<$0.05 for no functional category. It was found that categories ‘Cell rescue and defence’, ‘Metabolism’ and ‘Cellular transport’ were significantly over-represented among the SA-induced genes and only the category ‘Cell fate and differentiation’ was significantly over-represented among the SA-repressed genes.

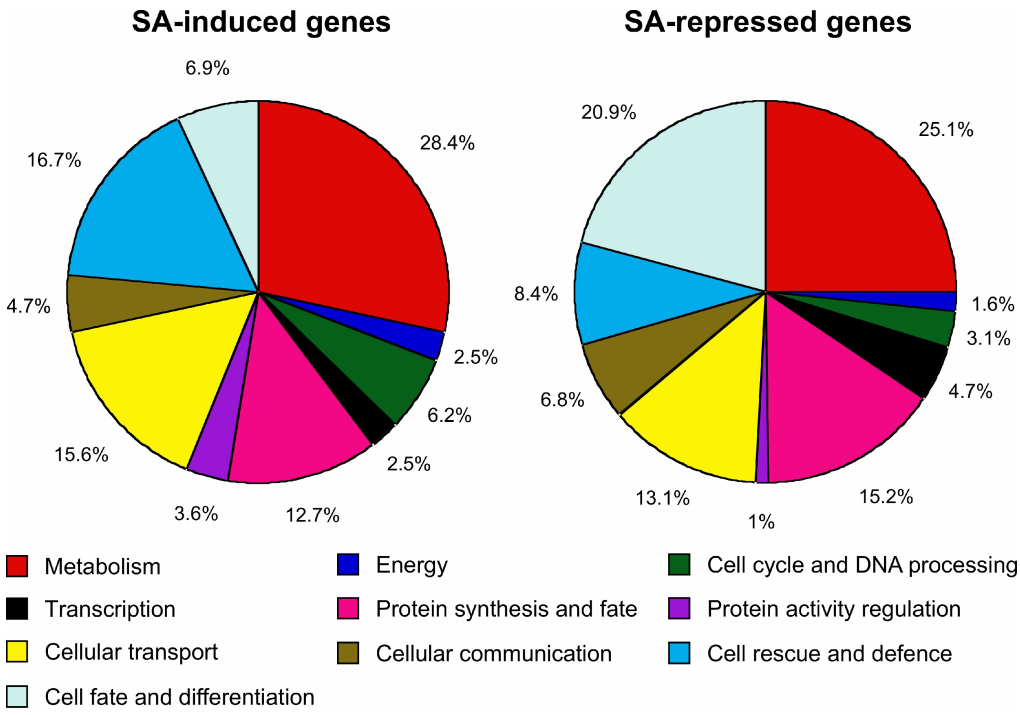


Figure 21. Functional categories of SA-induced and SA-repressed genes after 4 h of SA treatment in Arabidopsis cell suspensions.
Genes with unambiguous probe-to-gene assignment that were either up-regulated or down-regulated according to the microarray analysis (751 genes) were classified into functional categories using the MIPS functional catalogue interface (Ruepp et al., 2004). Proportions of functional categories within the sum of genes for which a function could be assigned (275 SA-induced and 191 SA-repressed) are displayed in the graph.

SA treatment activated protein secretory pathway which is other name for unfolded protein response (UPR) in Arabidopsis plants via NPR1-dependent mechanism. The pathway was essential for secretion of PR proteins and SAR development (Wang et al, 2005). Therefore it was interesting to check whether SA initiated UPR in Arabidopsis cells. Three microarray experiments on whole plants of Arabidopsis identified marker genes of the UPR (Kamauchi et al., 2005). A list of UPR marker genes was compiled and crossed with the set of genes differentially expressed upon SA treatment. Of the 90 induced UPR markers, 19 were induced by SA and of the 131 repressed UPR markers, 15 were repressed by SA treatment in the microarray experiment (Supplemental Table VI; electronic supplement only).

Additional information was obtained from the analysis based on MapMan software (Thimm et al., 2004) where genes can be mapped to user-defined graphic schemes. All relevant figures generated with this software are supplied as (Supplemental Figure 1; electronic supplement only).

Expression data for the genes that are known to be involved in phospholipid signalling are summarized in Supplemental Table VII (electronic supplement only).

Among the genes with a corresponding CATMA probe only *PI4Kγ4*, gene encoding a type II PI 4-kinase, was significantly induced by SA treatment.

Table V. *List of the 25 most induced genes after SA treatment, ranked by decreasing degree of induction.*

AGI, Arabidopsis Genome Initiative gene index; $\log_2$ I, $\log_2$ average signal intensity (6-7 corresponds to a low level of expression, 14-15 corresponds to a high level of expression); $\log_2$ ratio = $\log_2 I_{SA} - \log_2 I_{water}$. The difference in transcript level was significant in all cases (α=0.05).

Protein	Function	AGI	$\log_2 I_{water}$	$\log_2 I_{SA}$	$\log_2$ ratio
Legume lectin family protein	Unknown	At5g03350	7.72	13.03	5.31
Amino acid transporter family protein	Amino acid transport	At4g35180	7.49	12.76	5.28
Expressed protein	Defence response	At1g31580	7.86	13.02	5.16
NPR1/NIM1-interacting protein 2 (NIMIN-2)	Unknown	At3g25882	7.50	11.79	4.29
WRKY38	Regulation of transcription, DNA dependent	At5g22570	7.56	11.78	4.22
Expressed protein	Unknown	At1g56580	9.20	13.16	3.96
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Metabolism	At3g11340	7.32	11.08	3.75
WRKY66	Regulation of transcription, DNA dependent	At1g80590	7.55	11.05	3.50
Glycerophosphoryl diester phosphodiesterase family protein	Glycerol metabolism	At1g66970	8.92	12.38	3.46
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Metabolism	At2g43820	7.45	10.88	3.43
Glutaredoxin family protein	Electron transport	At1g03850	7.41	10.80	3.39
Expressed protein	Unknown	At3g29240	9.77	13.13	3.36
Expressed protein	Unknown	At1g19960	6.86	10.22	3.36
NPR1/NIM1-interacting protein 1 (NIMIN-1)	Unknown	At1g02450	6.82	10.12	3.31
Oxidoreductase, 2-oxoglutarate and Fe(II)-dependent oxygenase family protein	Flavonoid biosynthesis	At5g24530	7.59	10.77	3.18
DC1 domain-containing protein	Intracellular signalling cascade	At2g44370	8.48	11.60	3.12
GTP-binding protein, putative	Small GTPase mediated signal transduction	At1g09180	8.16	11.10	2.94
Leu-rich repeat family protein	G-protein coupled receptor protein signalling pathway	At1g49750	9.12	12.06	2.94
ADP-ribosylation factor, putative	N-terminal protein myristoylation	At1g70490	11.06	13.97	2.92
Glutaredoxin family protein	Electron transport	At1g28480	7.32	10.20	2.88
WRKY54	Regulation of transcription, DNA dependent	At2g40750	6.80	9.55	2.76
Toll-interleukin-resistance (TIR) domain-containing protein	Defence response signalling pathway, resistance-gene dependent	At1g72930	7.51	10.23	2.72
D-3-phosphoglycerate dehydrogenase (3-PGDH)	L-Ser biosynthesis	At1g17745	8.74	11.46	2.72
Expressed protein	Unknown	At4g34630	11.13	13.78	2.65
Glutathione S-transferase 6 (GST6)	Defence response	At2g47730	9.86	12.43	2.57

Table VI. *List of the 25 most repressed genes after SA treatment, ranked by decreasing degree of repression.*
AGI, Arabidopsis Genome Initiative gene index; $\log_2$ I, $\log_2$ average signal intensity (6-7 corresponds to a low level of expression, 14-15 corresponds to a high level of expression); $\log_2$ ratio = $\log_2 I_{SA} - \log_2 I_{water}$. The difference in transcript level was significant in all cases (α=0.05).

Protein	Function	AGI	$\log_2 I_{water}$	$\log_2 I_{SA}$	$\log_2$ ratio
Pectate lyase family protein	Unknown	At4g24780	13.83	10.82	-3.02
Multi-copper oxidase type I family protein	Pollen germination	At1g76160	13.09	10.54	-2.55
Multi-copper oxidase type I family protein	Pollen germination	At4g22010	10.79	8.42	-2.37
Pectate lyase family protein	Unknown	At1g04680	11.99	9.86	-2.12
Xyloglucan:xyloglucosyl transferase, putative/xyloglucan endotransglycosylase, putative/endo-xyloglucan transferase, putative	Carbohydrate metabolism	At4g30290	10.42	8.32	-2.10
Multi-copper oxidase, putative (SKU5)	Tip growth	At4g12420	11.08	8.99	-2.08
Endo-1,4-β-glucanase, putative/cellulase, putative	Carbohydrate metabolism	At1g64390	10.68	8.60	-2.08
Nodulin family protein	Unknown	At4g34950	12.72	10.71	-2.01
Expressed protein	Unknown	At1g29980	10.93	8.94	-2.00
Asp protease family protein	Proteolysis and peptidolysis	At3g61820	11.68	9.75	-1.93
Leu-rich repeat transmembrane protein kinase, putative	Transmembrane receptor protein Tyr kinase signalling pathway	At2g41820	9.83	7.93	-1.90
Expressed protein	Transport	At4g27720	11.61	9.74	-1.87
Phosphate-responsive 1 family protein	Unknown	At5g51550	11.86	10.04	-1.82
β-expansin, putative (EXPB3)	Cell elongation	At4g28250	13.66	11.84	-1.82
Tubulin β-8 chain (TUB8)	Response to cold	At5g23860	11.30	9.49	-1.81
Myosin heavy chain-related	Unknown	At4g03620	11.37	9.56	-1.81
Fasciclin-like arabinogalactan-protein (FLA2)	Cell adhesion	At4g12730	11.31	9.52	-1.79
Expansin, putative (EXP6)	Cell wall modification during cell expansion (sensu *Magnoliophyta*)	At2g28950	11.28	9.50	-1.78
Ovule development protein aintegumenta (ANT)	Regulation of transcription, DNA dependent	At4g37750	9.50	7.72	-1.78
Phosphate-responsive 1 family protein	Unknown	At2g17230	9.61	7.84	-1.77
Plastocyanin-like domain-containing protein	Electron transport	At5g15350	11.35	9.58	-1.77
Osmotin-like protein, putative	Response to pathogen	At2g28790	9.90	8.14	-1.76
Sugar transporter family protein	Carbohydrate transport	At1g73220	11.93	10.19	-1.74
Pentatricopeptide repeat-containing protein	Unknown	At2g30780	8.79	7.06	-1.73
Expressed protein	Unknown	At4g33625	9.82	8.12	-1.71

4.3.2 Effect of wortmannin on the SA-regulated transcriptome

As described in chapter 4.2.4, 30 µM wortmannin inhibited the SA-triggered PI 4-kinase activation while 1 µM wortmannin did not (Figure 17A). Thus it was meaningful to analyze the effect of wortmannin at both concentrations on the SA-responsive transcriptome. RNA was isolated from 5-day-old cells that had been submitted to either: a treatment with SA in the presence of 30 µM wortmannin (hereafter, SAW30), a treatment with SA in the presence of 1 µM wortmannin (hereafter, SAW1), a treatment with 30 µM wortmannin (hereafter, W30), a treatment with 1 µM wortmannin (hereafter, W1), or a treatment with 0.5% v/v methanol (hereafter, M). Methanol at 0.5% v/v final concentration was used as the solvent for wortmannin. Cells were pre-treated with inhibitors for 15 min and then treated with SA for four more hours where indicated. RNAs extracted from three independent repetitions were pooled and reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP to perform two-colour hybridization with the CATMA chip. One dye-swap was made for three combinations: SAW1 vs. SA, SAW30 vs. SAW1 and W30 vs. W1. This has been repeated with another pool of three independent RNA extracts for each condition, leading to biological replicates. W30 or SAW30 is always compared to W1 or SAW1, respectively, to ensure that the effect of 30 µM wortmannin is not due to an effect already present at 1 µM concentration. Hereafter, 'gene regulated by a W30-sensitive pathway' will imply that its regulation is disturbed by 30 µM wortmannin, and that the effect cannot be attributed to 1 µM wortmannin.

In order to identify the genes whose response to SA is affected by W30 and not by W1, the following cluster analysis was performed. Among the 791 genes which were differentially regulated in response to SA, 773 produced good hybridization in all tested dye-swaps. Eight genes whose transcript levels were different between SAW1 and SA were not considered, as they may be regulated by methanol or W1-sensitive pathway (Figure 22). Among the remaining 765 genes, 310 showed a transcript level difference between SAW30 and SAW1. Among the 310 genes which were specifically regulated by a W30-sensitive pathway in response to SA, 208 genes showed lower transcript levels in SAW30 vs. SAW1. Among these 208 genes, 101 had also lower transcript levels in W30 vs. W1, arguing for an effect on the regulatory events controlling the basal levels of those transcripts. For these genes, the effect of SAW30 vs. SAW1 could therefore be a consequence of a perturbation of basal transcript level regulation and not on the SA response (Figure 23). The regulation mode of genes in such cluster could not

be unambiguously determined based only on the microarray data (the cluster contains false positive genes with different modes of regulation). QRT-PCR analysis of expression profile of each gene would be necessary to ultimately classify it. Therefore the whole cluster was excluded from the following analyses that are aimed to decrypt the W30-sensitive pathway dependent regulation of SA transcriptome. However, many of the excluded genes may indeed be regulated by a W30-sensitive pathway even in their SA response. Among the 310 genes which were specifically regulated by a W30-sensitive pathway, 102 genes had higher transcript levels in SAW30 vs. SAW1. Again, 80 of them showed the same changes in W30 vs. W1 and thus were excluded too. In the remaining group of 129 genes, which were specifically regulated by a W30-sensitive pathway during the SA response, 108 genes were SA-induced and 21 were SA-repressed. Among the 108 genes induced by SA, 99 showed lower transcript levels in SAW30 than in SAW1, showing an inhibitory effect on the SA induction. Among the 21 SA-repressed genes, 13 showed higher transcript levels in SAW30 than in SAW1, showing an inhibitory effect on the SA repression. Taken together 112 out of 129 genes (i.e. 87%) showed a repressing effect of 30 μM wortmannin on their response to SA treatment. Possibly underestimated, the number of genes induced by a W30-sensitive pathway, and not by a W1-sensitive one, represents 21% of the SA-induced genes. The overall number of genes regulated by a W30-sensitive pathway represents 14% of all SA-regulated (i.e. repressed or induced) genes.

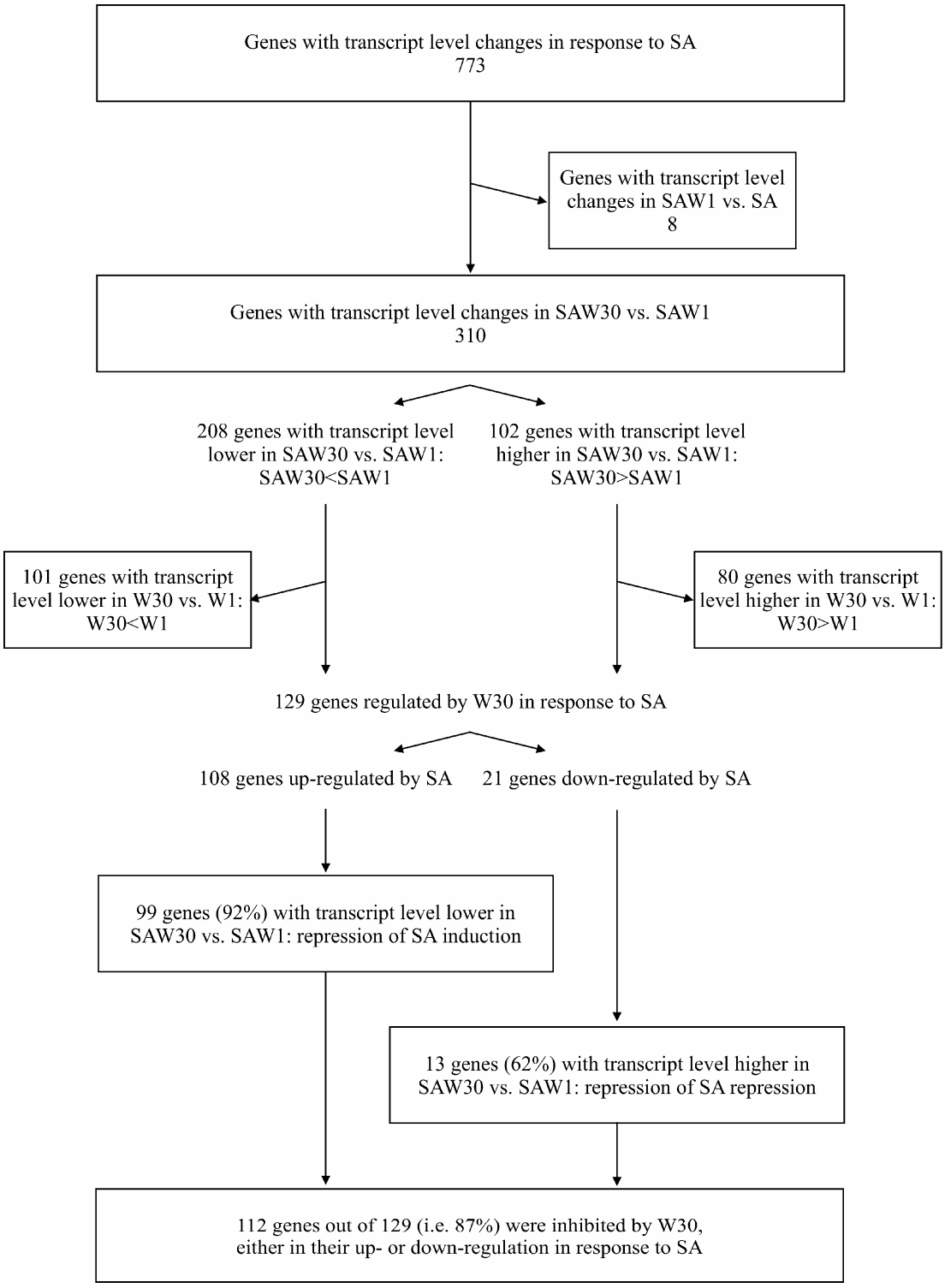


Figure 22. Determination of SA-regulated genes downstream of the PI 4-kinase pathway.
Among the 3,266 genes which showed differential gene expression in one of the four tested comparisons, 129 showed transcript level changes both in SA vs. water and in SAW30 vs. SAW1 comparisons. For these genes the transcript levels did not change in the same direction in W30 vs. W1 as in SAW30 vs. SAW1. Hence these genes are potentially SA-regulated via PI 4-kinase pathway. Of these genes, 108 were up-regulated and 21 were down-regulated by SA treatment. 112 genes out of 129 showed positive action of the W30-sensitive pathway on their SA response.

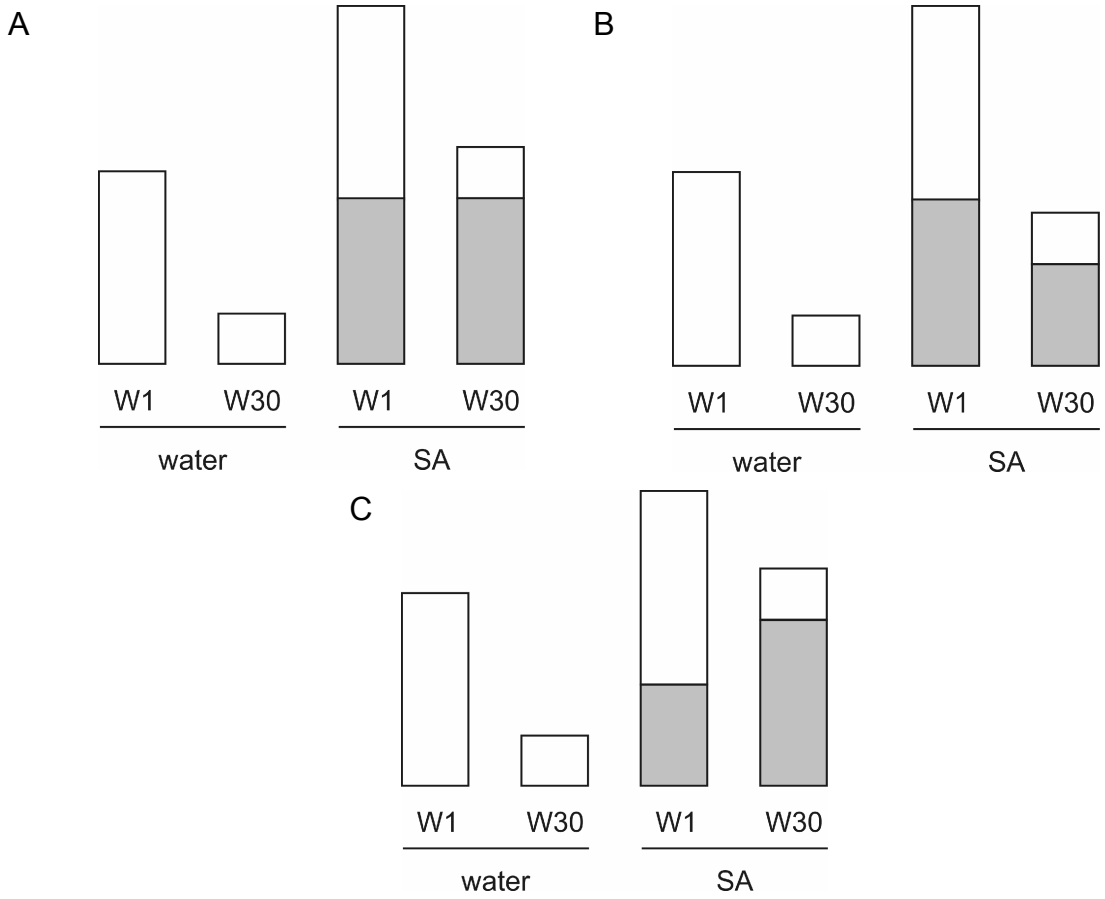


Figure 23. Scheme of two possible cases of regulation of gene expression within the cluster of genes regulated in the same way both in the SA vs. water and SAW30 vs. SAW1 comparisons.
(A) The gene is regulated by a W30-sensitive pathway already in the basal state. SA induces expression of the gene via a W30-insensitive pathway. (B) The gene is regulated by a W30-sensitive pathway in the same direction both in the basal and SA-stimulated state (i.e. its expression is enhanced by a W30-sensitive pathway in both cases). (C) The gene is regulated by a W30-sensitive pathway both in the basal and SA-stimulated state but in the opposite direction. (A, B, C) Overall bar height mirrors the gene transcript level (higher bar corresponds to higher transcript level). Grey bars represent SA-regulated portion of the gene transcript level.

To make sure that the observed numbers are not a product of random events a test of colocalization of the two phenomena of regulation was performed, i.e. colocalization of the SA-regulated gene expression (SA vs. water comparison) with the regulation of SA response by W30 (SAW30 vs. SAW1 comparison). The genes regulated non-specifically by W1 or methanol in their response to SA (i.e. genes with transcript level change in SAW1 vs. SA comparison) were removed from the analysis as well as the genes showing the same mode of regulation even in the non-stimulated (water treated) cells (i.e. in W30 vs. W1 comparison) as these may artificially increase the observed overlap of regulation modes (they represent false positives). Each gene among the remaining ones could be unambiguously classified into one of the four groups: genes with transcript level change both in SA vs. water and SAW30 vs. SAW1

comparisons, genes with transcript level change only in one comparison and genes with no transcript level change in any of the two comparisons. Probabilities that the observed distributions are due to random events (calculated by Fisher's test) are given in Table VII. All four possible colocalizations were not products of coincidence (α=0.05) showing that the regulation of gene expression by SA and regulation of the same response by a W30-sensitive pathway colocalize in the transcriptome more than would be expected in case of independence of the two phenomena.

Table VII. *Colocalization analysis of regulation by SA with regulation by a W30-sensitive pathway in response to SA on the whole transcriptome scale.*

Genes regulated in SAW1 vs. SA comparison and genes regulated in the same way in SAW30 vs. SAW1 as in W30 vs. W1 comparison were removed from the analysis. The remaining 17,983 genes were classified with respect to their regulation in SA vs. water and SAW30 vs. SAW1 comparisons. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. P-values are those of Fisher's test. Categories printed in bold are over-represented in the experimental data. d_{obs}, observed distribution; d_{theor}, theoretical distribution.

SA regulation	SAW30 vs. SAW1	Number of genes		p (d_{obs}=d_{theor})
		Observed	Theoretical	
induced	repressed	**99**	**6**	**7.7e-94**
	induced	**9**	**3**	**8.3e-3**
repressed	induced	**13**	**3**	**1.3e-7**
	repressed	**8**	**4**	**2.9e-2**

Although the positive mode of regulation was prevalent among the group of genes regulated via a W30-sensitive pathway in response to SA, it was interesting to evaluate the relative importance of the two modes of regulation using a statistical analysis. Cluster of 129 genes specifically regulated by W30 in response to SA (Figure 22) was classified into four sub-groups with respect to the induction or repression of their expression by SA and inhibition or not of this response in the presence of W30 compared to W1. For each cluster it was possible to calculate a theoretical number of genes based on the hypothesis that the transcript level difference between SAW30 and SAW1 was independent of the transcript level difference in the presence or absence of SA. The observed number of genes in each cluster was then compared to this theoretical number (Table VIII). It is clear that there was an over-representation of genes showing a positive action of the W30-sensitive pathway on the response to SA (i.e. an inhibiting effect of W30 on the induction of SA response) combined with the under-representation of genes not showing such a positive action. Fisher's test (α=0.05) indicated that these differences in distribution were not a product

of random events. Thus the positive mode of regulation of gene expression by a W30-sensitive pathway specific to SA response is really more important than the negative one both for SA-induced and SA-repressed genes.

Table VIII. *Analysis of expected and observed clusters of genes classified by their response to SA and the effect of wortmannin on this response.*

The expected number of genes for each cluster was calculated and compared to the actual number observed in the microarray analysis. With respect to the expected numbers, the categories printed in bold are over-represented in the experimental data, while the categories written in italics are under-represented. SA-induced and SA-repressed genes were considered separately.

Number of genes	SA>H_2O		subtotal	SA<H_2O		subtotal
	SAW30>SAW1	SAW30<SAW1		SAW30>SAW1	SAW30<SAW1	
Theoretical	*18*	**90**	108	**4**	*17*	21
Observed	*9*	**99**	108	**13**	*8*	21

The list of 112 (99+13) genes positively regulated via a W30-sensitive pathway in response to SA is provided as Supplemental Table VIII (electronic supplement only). The first ten SA-induced genes ranked by decreasing degree of their repression by W30 are listed in Table IX.

Table IX. *List of the 10 SA-induced genes most repressed by W30 treatment, ranked by decreasing degree of repression by W30.*

The difference in transcript level was significant in all cases (α=0.05). AGI, Arabidopsis Genome Initiative gene index; I, average signal intensity.

Protein	**Function**	**AGI**	**$\log_2 I_{SAW30}/I_{SAW1}$**	**$\log_2 I_{SA}/I_{water}$**
Glycerophosphoryl diester phosphodiesterase family protein	Kinase activity	At1g66970	-3.87	3.46
Legume lectin family protein	Carbohydrate binding	At5g03350	-3.48	5.31
Expressed protein	Unknown	At1g31580	-3.36	5.16
33 kDa secretory protein-related	Unknown	At5g48540	-3.01	2.19
WRKY38	Transcription factor activity	At5g22570	-2.76	4.22
Cytochrome P450 83B1 (CYP83B1)	Cytochrome P45 activity	At4g31500	-2.74	1.30
AAA-type ATPase family protein	ATPase activity	At3g28540	-2.57	2.48
Toll-Interleukin-Resistance (TIR) domain-containing protein	Defence/immunity protein activity	At1g72930	-2.29	2.72
β-galactosidase, putative/lactase, putative	β-galactosidase activity	At5g56870	-2.25	2.26
DC1 domain-containing protein	DNA binding	At2g44370	-2.22	3.12

The results obtained by DNA microarray analysis were confirmed on a selection of seven genes showing high induction by SA and important inhibition of this response in the presence of 30 μM wortmannin. RT-PCR analysis of an independent non-pooled

set of RNAs revealed a clear and specific inhibitory effect of 30 µM wortmannin on the SA induction for all tested genes (Figure 24).

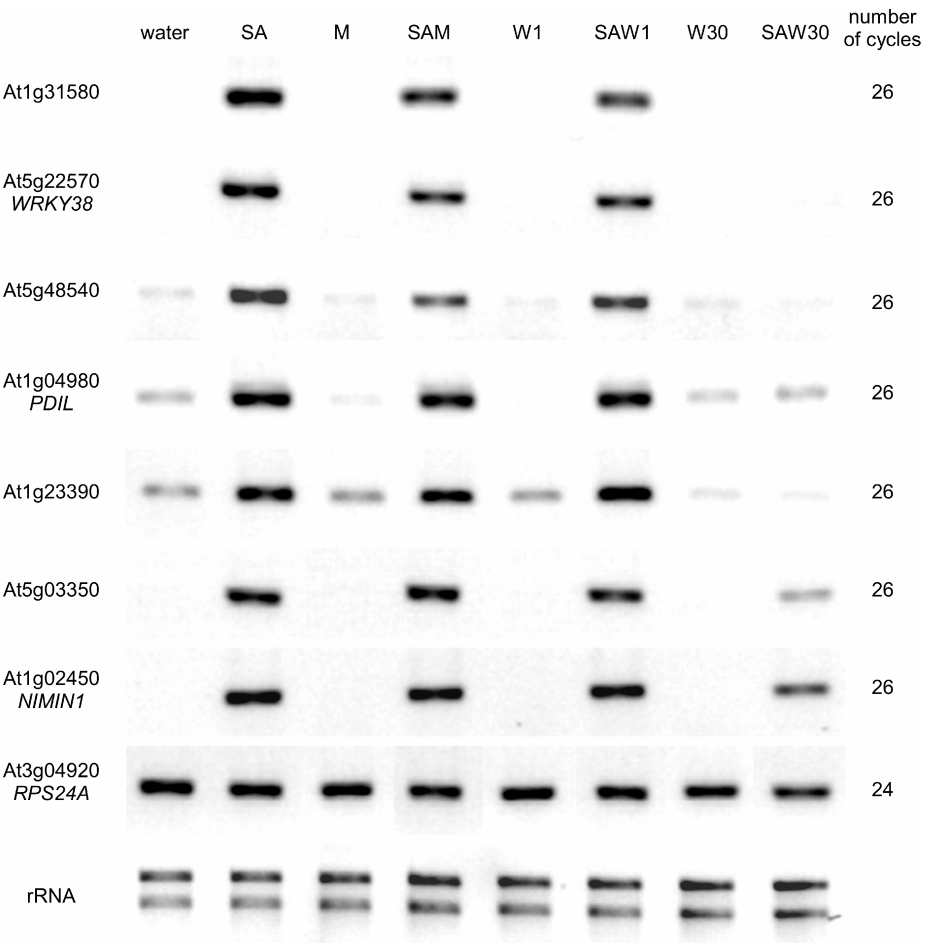


Figure 24. Confirmation of microarray results on an independent set of non-pooled RNAs.
RNA was isolated from 5-day-old cell suspensions; cells were pre-treated with wortmannin or methanol for 15 min and then treated with SA for 4 h. The level of expression was verified by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. M, 0.5% v/v methanol as solvent control for wortmannin; rRNA, ribosomal RNA; W1, 1 µM wortmannin in 0.5% v/v methanol; W30, 30 µM wortmannin in 0.5% v/v methanol.

4.3.3 Common cis-elements in the promoters of SA-regulated genes

Signalling cascades often lead to activation of a set of transcription factors which ensure the changes in gene expression. To have an idea of the transcription factors involved in the SA response, common *cis*-elements in the promoters of SA regulated genes were identified. A subset of probes with unambiguous probe-to-gene assignment was extracted from the list of SA-regulated probes. Genes were divided into two groups: SA-induced genes and SA-repressed genes. Public repository of promoter sequences at The Arabidopsis Information Resource (TAIR) (Rhee et al., 2003) was mined for common motifs in promoters (up to -1000 bp) of each of these groups using

the Motif Analysis tool. In parallel these groups were analyzed using the SIFT software (Hudson and Quail, 2003), where the promoter region spans by default up to -2000 bp. Common motifs 4 to 8 bp long were chosen for each group and separately statistically analyzed using the R package software (version 2.4.1). Their frequency in promoters up to -1000 bp on the whole genome scale was determined by the Patmatch tool at TAIR and was compared to the observed number of appearances in the tested gene groups using a general bootstrapping method with 200 random trials. The probability that any random trial will produce the observed or higher/lower frequency of the given motif was assigned to each motif. Motifs were compared to the RIKEN Arabidopsis Genome Encyclopedia (RARGE) database using the build-in Promoter Search tool (Sakurai et al., 2005) or to the *Arabidopsis thaliana cis*-regulatory database (AtcisDB) on the Arabidopsis Gene Regulatory Information Server (AGRIS; Palaniswamy et al., 2006) and its name or the name of its closest homologue was assigned to it. Several significantly over-represented and under-represented motifs specific both for SA-induced (Table X) and SA-repressed genes (Table XI) were identified.

Table X. *Cis-elements identified in the promoters of SA-induced genes.*
The analysis was performed on a subset of 448 genes with unambiguous probe-to-gene assignment. The number of appearances of each motif in promoters (-1000 bp) of SA-induced genes (f_{obs}) was compared to its frequency in promoters of genes of the whole genome (f_{theor}) by general bootstrapping technique. Only motifs significantly over- or under-represented ($p<0.05$) are listed.

Motif description	Searched motif	Found motifs	f_{obs}	f_{theor}	Alternative	p ($f_{obs}=f_{theor}$)
TATCCAY, GATA-box	TATCCA	368	0.82	0.59	over-represented	1.1e-11
I-box, MYB-like family	GATAAG	334	0.75	0.54	over-represented	4.7e-11
TAG1/RTBP1-binding, MYB-like family	AAACCC	270	0.60	0.80	under-represented	6.5e-5
ASF-1, TGA family	TGACG	520	1.16	0.89	over-represented	3.8e-10
ASF-1-like, TGA family	TGACGTG	72	0.16	0.09	over-represented	1.0e-6
W-box, WRKY family	TTGACY	735	1.64	1.21	over-represented	3.0e-14
W-box-like, WRKY family	TTGACTT	228	0.51	0.32	over-represented	2.8e-13
bZip	ACGT	2468	5.51	4.53	over-represented	1.2e-8
A-box bZip variants	BACGTA	444	0.99	0.78	over-represented	1.5e-5
C-box bZip variants	YACGTC	239	0.53	0.37	over-represented	8.4e-8
G-box bZip variants	YACGTG	372	0.83	0.66	over-represented	1.7e-3
DRE/CRT core, DREB1A	ACCGACA	35	0.08	0.06	over-represented	2.9e-2

Table XI. *Cis-elements identified in the promoters of SA-repressed genes.*
The analysis was performed on a subset of 303 genes with unambiguous probe-to-gene assignment. The number of appearances of each motif in promoters (-1000 bp) of SA-repressed genes (f_{obs}) was compared to its frequency in promoters of genes of the whole genome (f_{theor}) by general bootstrapping technique. Only motifs significantly over- or under-represented ($p<0.05$) are listed.

Motif description	Searched motif	Found motifs	f_{obs}	f_{theor}	Alternative	p (f_{obs}=f_{theor})
TATCCAY, GATA-box	TATCCA	146	0.48	0.59	under-represented	8.2e-3
I-box, MYB-like family	GATAAG	120	0.40	0.54	under-represented	1.1e-3
TAG1/RTBP1-binding, MYB-like family	AAACCC	213	0.70	0.80	under-represented	4.1e-2
W-box, WRKY family	TTGACY	317	1.05	1.21	under-represented	1.3e-2
A-box bZip variants	BACGTA	279	0.92	0.78	over-represented	1.9e-2
G-region like (ethylene responsive)	AGCCGTT	41	0.14	0.07	over-represented	4.2e-6
DRE/CRT core, DREB1A	ACCGACA	40	0.13	0.06	over-represented	4.9e-8
CBF1 binding site	RGGCCY	176	0.58	0.74	under-represented	2.3e-2
GT repeat	GTGTGTAT	27	0.09	0.07	over-represented	4.9e-2

It was interesting to determine whether any of the motifs specific for the SA-induced genes is over-represented in the group of genes regulated by SA via a W30-sensitive pathway. A subset of genes with unambiguous probe-to-gene assignment was extracted from the list of genes induced by SA via a W30-sensitive pathway. For each motif its frequency in the group of 95 genes regulated by a W30-sensitive pathway was compared to its frequency in the subset of 439 SA-induced genes using the same bootstrapping method on the subset of SA-induced genes. Only one motif, W-box-like (TTGACTT), was found to be significantly over-represented (f_{obs}=0.73, p=1.9e-4) in the group of genes induced by SA via a W30-sensitive pathway. Thus WRKY transcription factors may be situated downstream of such W30-sensitive pathway in response to SA. Such an analysis was not possible for SA-repressed genes or for genes negatively regulated by a W30-sensitive pathway because these subgroups were too small (containing utmost 13 genes) making any statistics meaningless. On the contrary, analysis of the group of genes positively regulated by a W30-sensitive pathway both in their SA induction and in water-treated condition revealed under-representation of the W-box motif (TTGACY; data not shown) confirming that leaving those genes in the promoter analysis would bias its result.

4.3.4 Expression of selected marker genes of the W30-sensitive pathway in the T-DNA mutants of PI 4-kinase β

One way to identify the PI 4-kinase isoform involved in the SA response is to study expression of marker genes of the W30-sensitive pathway in the T-DNA mutant lines for the respective genes. The idea was to find a T-DNA line which would show lowered induction of these marker genes in response to SA. Among the 4 isoforms of PI 4-kinase in Arabidopsis, no T-DNA insertion line is available for the *PI4Kα1* gene suggesting that the complete knock-out is impaired in reproduction. Based on the high QRT-PCR threshold cycle for *PI4Kα2* (around 35; data not shown) and on the fact that there are currently no ESTs assigned to it in plant databases, it is probable that the *PI4Kα2* gene is very poorly expressed and therefore its T-DNA knock-out mutant is likely to have no phenotype. It was thus possible and interesting to test only the single and double knock-outs of both β isoforms. The lines had homozygous T-DNA insertions only in the studied genes.

Genes regulated by a W30-sensitive pathway whose expression profile was confirmed by semiquantitative RT-PCR (Figure 24) were tested with respect to their induction by SA in the whole plants of Arabidopsis. Four of them were induced to various extents after 4 h of SA treatment (*WRKY38*, *NIMIN1*, At5g48540 and At2g44370). Their induction by SA was then followed both in the wild type and in the *pi4kβ1*, *pi4kβ2*, *pi4kβ1pi4kβ2* mutant plants (Figure 25). Interestingly, *pi4kβ1* and *pi4kβ1pi4kβ2* plants had higher basal level of these transcripts while their induction by SA was maintained. This finding led to the question whether the increased basal expression of these genes leads to induction of *PR1*, the principal marker gene of the SA pathway. Indeed, it was induced both in *pi4kβ1* and *pi4kβ1pi4kβ2* plants (Figure 26A).

The W30-sensitive activity (presumably the PI 4-kinase activity) was identified as a positive regulator of SA response in the previous microarray experiment. Here its marker genes are in fact induced in the absence of one PI 4-kinase isoform suggesting that the missing PI 4-kinase is a negative regulator of the response. One explanation of such apparent contradiction could be that the knock-out mutants may carry out an adaptive response of compensation for the lost isoform (Kaiser et al., 2002). To find out whether such compensation could explain the observed phenotypes, the basal level of expression of *PI4Kα1*, *PI4Kβ1* and *PI4Kβ2* isoforms was checked in the three mutant lines (Figures 26B, C and D). Transcript levels of the *PI4Kβ1* isoform were not checked

in the mutants where the gene was knocked-out (same for the *PI4Kβ2* isoform). Basal level of no tested PI 4-kinase gene was significantly induced in any of the mutant lines.

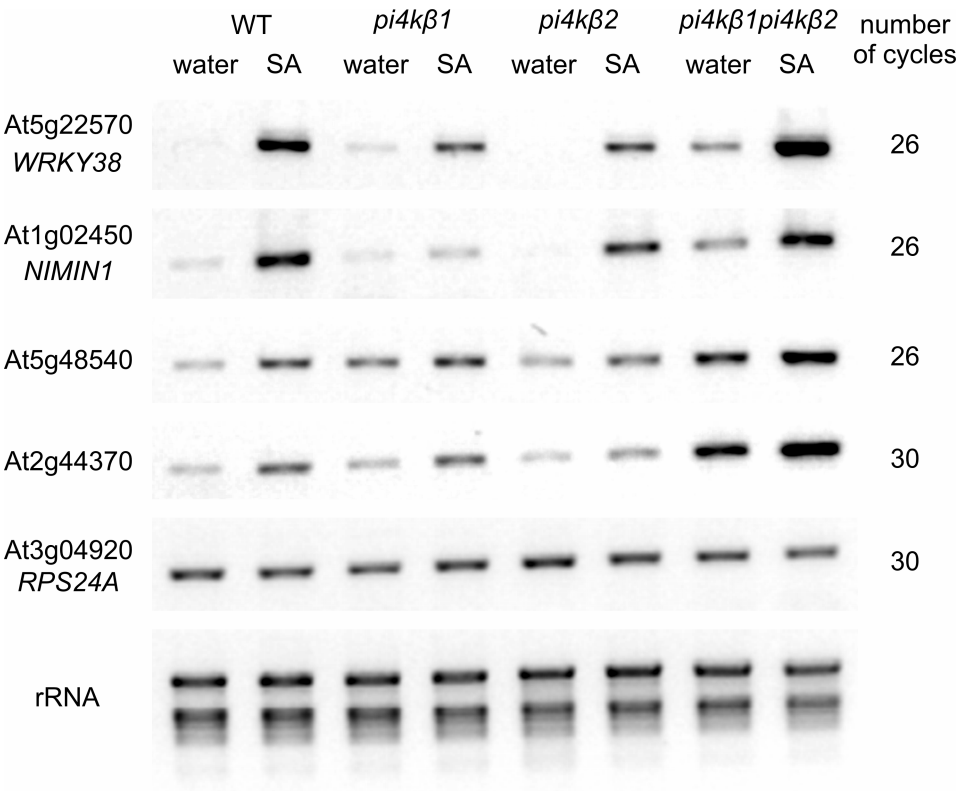


Figure 25. Expression of genes regulated via a W30-sensitive pathway in their SA response in Arabidopsis wild type plants and in T-DNA knock-out lines of PI 4-kinase β.
Plants were sprayed with 1 mM SA or water and RNA was isolated from their leaves after 4 h of treatment. Leaves from six plants were pooled for each condition. Transcript levels were determined by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result of two independent experiments. rRNA, ribosomal RNA; WT, wild type.

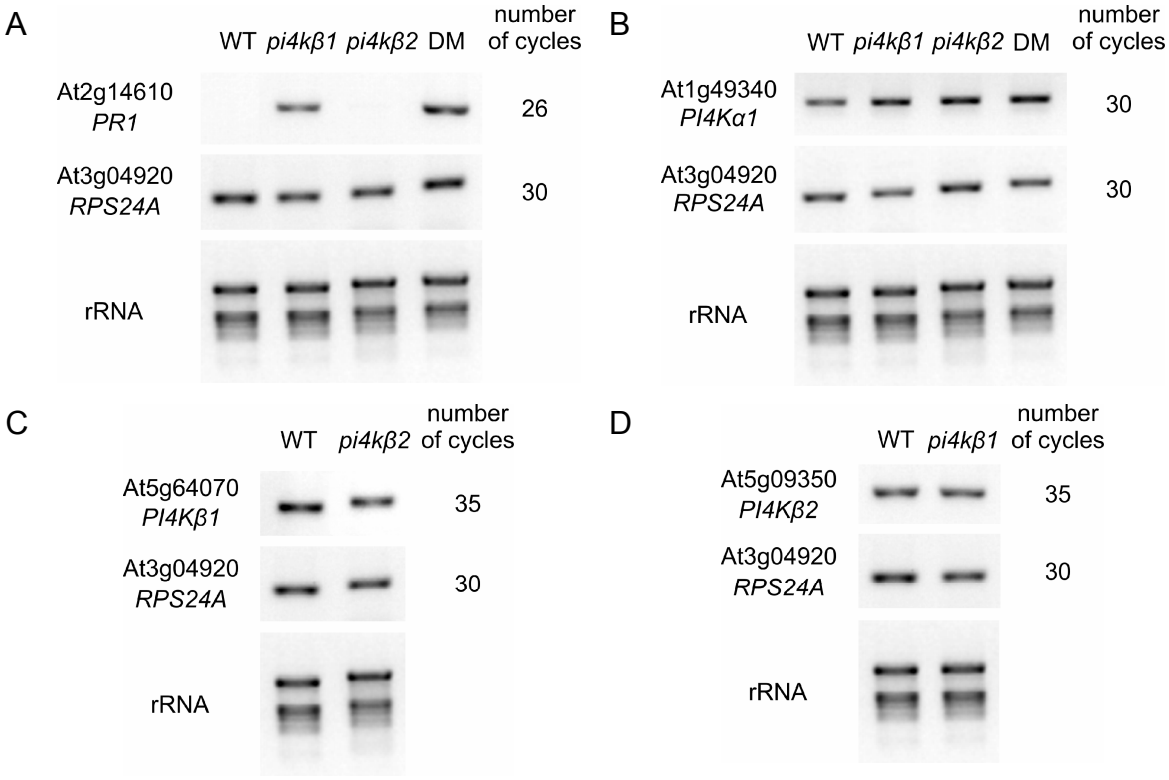


Figure 26. Expression of *PR1* and of genes encoding type III PI 4-kinases in non-treated Arabidopsis wild type plants and in T-DNA knock-out lines of PI 4-kinase β.
Leaves from six plants were pooled for each genotype. Transcript levels were determined by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result of two independent experiments. DM, *pi4kβ1pi4kβ2* double mutant; rRNA, ribosomal RNA; WT, wild type.

4.4 SA activates PLD *in vivo*

4.4.1 Expression of several SA-regulated genes is regulated by PLD

The early SA response is mediated by PI 4-kinase activation (Krinke et al., 2007b). Nevertheless, other phospholipid signalling pathways may be involved in the response. PLD is now considered to be one of the key players in plant phospholipid signalling. PLD pathway is involved in many stress responses including pathogen attack and elicitor treatment (Bargmann and Munnik, 2006). SA induces an increase of $PI(4,5)P_2$, which in this case does not activate PLC (see chapter 4.2.4) but it is a well-known PLD activator. It suggests that PLD signalling pathway might be involved in the SA response. Involvement of the PA_{PLD} in a studied response can be easily verified by application of different alcohols with distinct ability to inhibit its production. The application of alcohols could be troublesome as they may induce various side effects (e.g. G-protein activation; Munnik et al., 1995). To find the alcohol concentration which would not substantially affect other signalling pathways besides

that of PLD, *n*-butanol was chosen as a representative because it is believed to be the best inhibitor of PA_{PLD} production and the strongest G-protein activator among the used alcohols. An experiment was designed in which the production of radioactive PA, a marker of G-protein activation, and of PBut, a marker of PLD activity, was followed every 5th min during the first 15 min of *n*-butanol treatment (Figure 27). The highest concentration of *n*-butanol (0.7% v/v) caused an increased synthesis of labelled PA and moreover the synthesis of labelled PBut was not linear with time. Therefore, 0.1% v/v concentration of *n*-butanol and of other alcohols was chosen for further experiments.

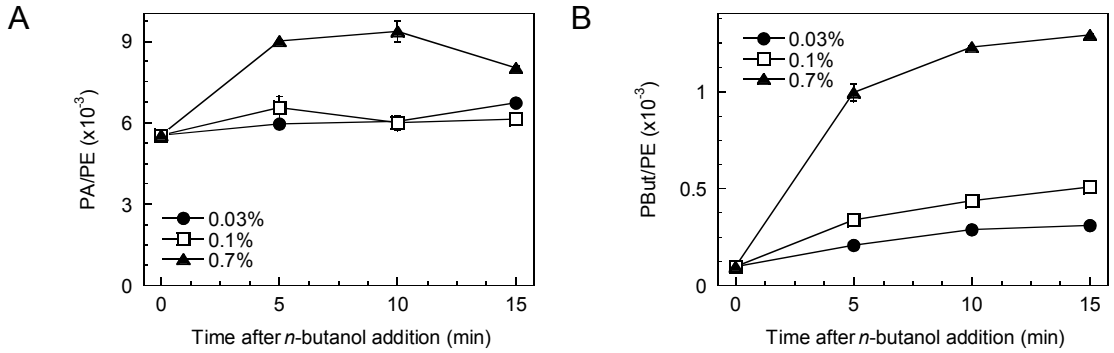


Figure 27. Suitable *n*-butanol concentration for tracing PLD activity *in vivo*.
Cells were first labelled for 24 h with $^{33}P_i$ and then treated with various concentrations of *n*-butanol at the time 0. Lipids were extracted at indicated time points and the amounts of labelled PA, PBut and PE were determined by TLC in a modified ethyl acetate solvent system. The data are means of two independent experiments.

PR1 is the principal marker gene of the SA pathway. Many signalling pathways related to SA response converge to trigger its expression. Therefore in a preliminary experiment, the expression of *PR1* was measured after SA treatment of cell suspensions in the presence of several alcohols (Figure 28). Different alcohols affected the PLD activity to various extents (see chapter 2.2.2.6 for details). The inhibitory effect on the SA induction of *PR1* increased with the chain length of the primary alcohol and was not affected by their secondary and tertiary analogues. Moreover, the inhibitory effect of ethanol and *n*-butanol on the SA induction of *PR1* was dose dependent (Figure 29). Such regulation of gene expression corresponded well to that expected for a PLD-regulated gene.

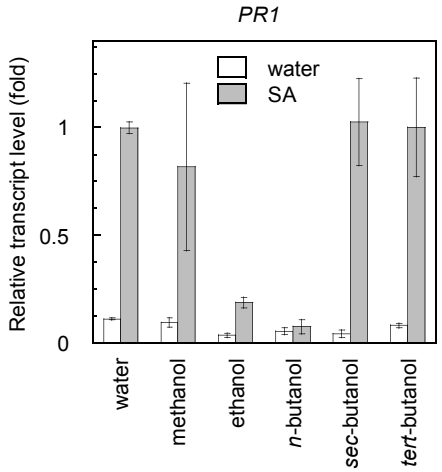


Figure 28. Alcohols differentially affect SA induction of *PR1*.
Cells were incubated 15 min with different alcohols (final concentration of all alcohols was 0.1% v/v) prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of each gene in cells treated with SA and not treated with any alcohol was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

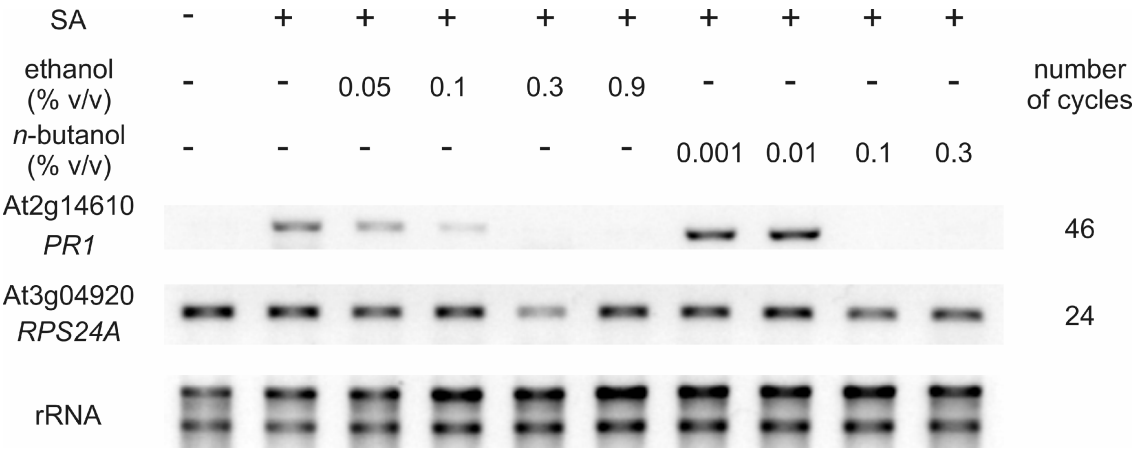


Figure 29. Dose dependence of the inhibitory effect of ethanol and *n*-butanol on the SA induction of *PR1*.
Cells were incubated 15 min with water or with different concentrations of ethanol or *n*-butanol prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was analyzed by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result of two independent experiments. rRNA, ribosomal RNA.

If the SA-induced PI(4,5)P_2 really serve to activate the PLD, expression of genes influenced by wortmannin in response to SA should also be affected by *n*-butanol. To verify whether the PLD activation is located in the W30-sensitive pathway previously identified in chapter 4.3.2, the inhibitory effect of *n*-butanol was checked on a selection of genes induced by SA via a W30-sensitive pathway. Several genes chosen for RT-PCR confirmation of the microarray experiment with wortmannin (Figure 24) were

much less induced by SA after *n*-butanol pre-treatment (Figure 30). Among these, *WRKY38* showed the highest induction by SA and the highest repression in the presence of *n*-butanol. Like in the case of *PR1*, the expression profile of *WRKY38* in the presence of SA and of different alcohols corresponded to that of a gene regulated by PLD in its SA response (Figure 31).

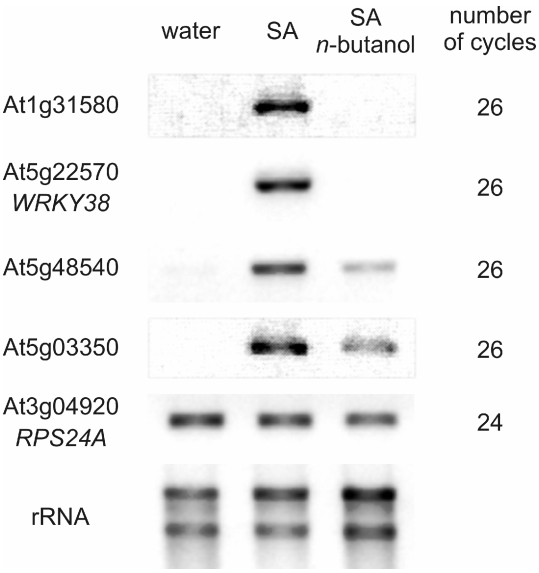


Figure 30. SA induction of several genes regulated by a W30-sensitive pathway is inhibited by *n*-butanol. Cells were incubated 15 min with water or with 0.1% v/v *n*-butanol prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was analyzed by semiquantitative RT-PCR. Number of cycles was optimized for each primer pair (indicated on the right). 40S ribosomal protein S24 (At3g04920) was used as housekeeping gene. The data represent a typical result of two independent experiments. rRNA, ribosomal RNA.

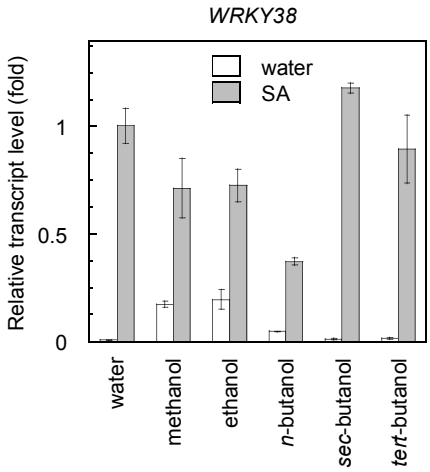


Figure 31. Alcohols differentially affect SA induction of *WRKY38*.
Cells were incubated 15 min with different alcohols (final concentration of all alcohols was 0.1% v/v) prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of each gene in cells treated with SA and not treated with any alcohol was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

Contrary to *PR1*, *WRKY38* was induced already in the first hour of SA treatment (Figure 32). To have a better idea of the timing of the PLD action, and not to base conclusions only on one particular gene, a similar set of following experiments was carried out on both genes.

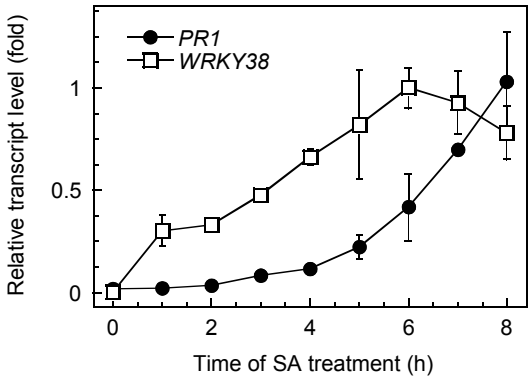


Figure 32. Time course of SA induction of *PR1* and *WRKY38*.
Cells were treated with SA at the time 0 and RNA was isolated from aliquots at indicated times. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The maximal normalized expression level of each gene was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

Hirase et al. (2006) published that *n*-butanol depolymerises microtubules in tobacco cells even *in vitro*. Such an effect could alter gene expression and be the true cause of the observed inhibition of SA response. In order to check whether the depolymerisation of microtubules is not the cause of the observed altered gene expression, cells were treated with 5 μM oryzalin, a microtubule depolymerising drug effective in plants (Binet et al., 2001) and the expression of *PR1* and *WRKY38* was measured after 6 h of SA treatment (Figure 33). The expression of *WRKY38* was not affected at all. Slightly lowered expression of *PR1* could not explain the observed inhibition of SA induction by *n*-butanol.

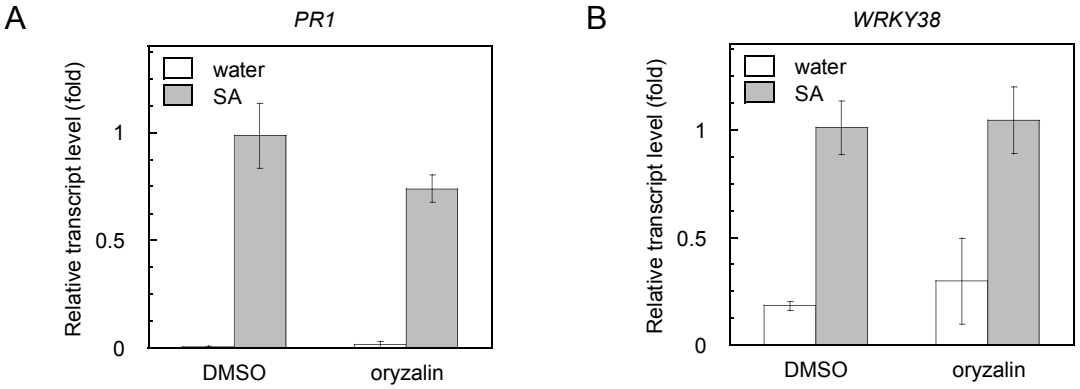


Figure 33. Effect of oryzalin on the SA induction of *PR1* and *WRKY38*.
Cells were incubated 15 min with 5 µM oryzalin in 0.0125% v/v DMSO prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of each gene in cells treated both with SA and DMSO was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results. DMSO, dimethyl sulfoxide.

4.4.2 PLD intervenes in the early stages of SA treatment

A way of getting insight in the timing of PLD intervention in the SA response is to inhibit the enzyme at various time points of SA treatment and to look for the time from which the inhibition does not interfere with gene expression. This was done for SA induction of both *PR1* and *WRKY38*; *n*-butanol was added from 0 to 5 h in one hour intervals and all samples were analyzed after 6 h of SA treatment (Figure 34). PA_{PLD} was important during the whole interval of SA treatment for *PR1* induction while the expression of *WRKY38* was lowered only within the first three hours. The PA_{PLD} is therefore important at least till the third hour of SA treatment.

The advantage of *WRKY38* over *PR1* was that its expression with and without *n*-butanol treatment could be compared as early as in the first hour of SA treatment (no need to wait till the 6th hour to see any important SA induction). Such a comparison was done during the first 4 h of SA treatment (Figure 35). It can be seen that *n*-butanol application almost abolished *WRKY38* induction which means that the PA_{PLD} is indeed important from the very beginning of SA response.

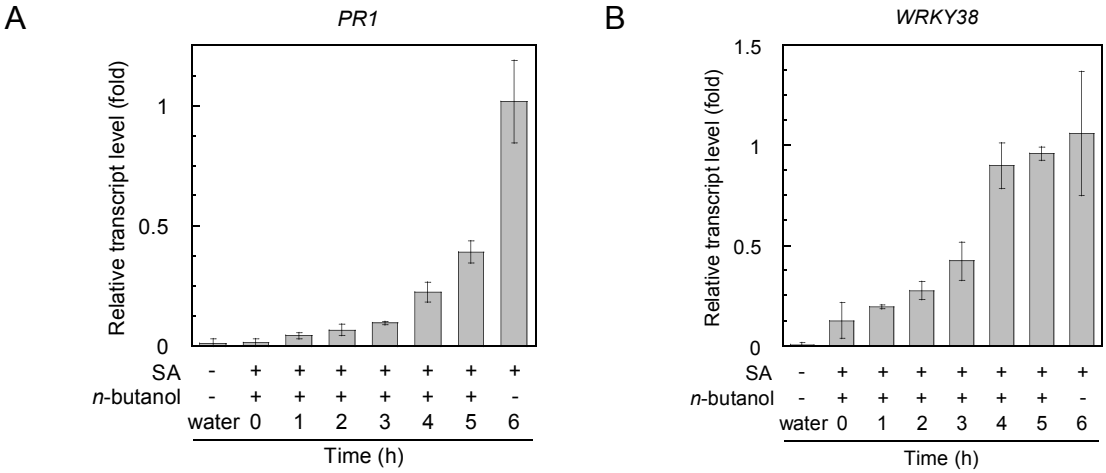


Figure 34. Delimitation of the period of time necessary for the correct SA induction of *PR1* and *WRKY38*.
Cells were treated with SA at time 0 where indicated and 0.1% v/v *n*-butanol was added at indicated times. 'Time' denotes the period of time for which the cells were incubated without *n*-butanol. RNA was isolated from all samples 6 h after addition of SA or water. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). For each gene the normalized expression level of simple SA treatment was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

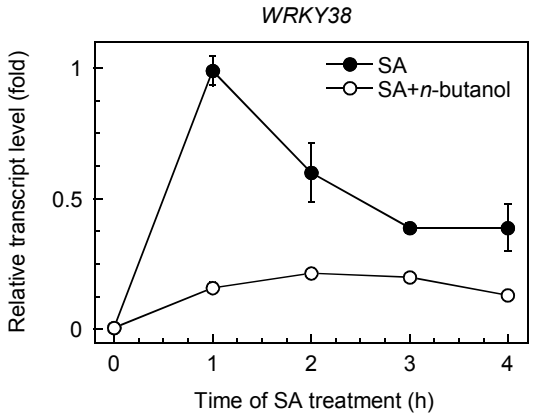


Figure 35. *WRKY38* induction is compromised by *n*-butanol treatment already in the first hour of SA treatment.
Cells were incubated 15 min with 0.1% v/v *n*-butanol prior to SA application where indicated and then treated with SA at the time 0. RNA was isolated from aliquots at indicated times. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The maximal normalized expression level of simple SA treatment was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

If the increase of PLD activity resulted from the synthesis of the enzyme, another hint for delimiting both, the time when PLD was turned on in SA response and the involved PLD isoform(s) would be the induction of any PLD isoform during the SA treatment. Gene specific primers were designed for the 12 PLD isoforms currently

annotated in the Arabidopsis genome and their expression in the cell suspensions during the first 4 h of SA treatment was checked by QRT-PCR. Expression of *PLDα3* and *PLDα4* could not be quantified (c_T beyond 40) suggesting that these two isoforms are very lowly expressed. Among the other PLDs, no isoform was significantly induced at the gene level during the monitored time course and only the *PLDβ2* was slightly repressed starting from the first hour of SA treatment (Figure 36). Thus the apparition of PLD activity cannot be satisfactorily explained by altered gene expression of PLD isoforms.

The ultimate way of delimiting the PLD activation would be monitoring its *in vivo* activity. This can be done by measuring PBut production following the *n*-butanol treatment. Indeed a peak of PBut production (approximately 2.5-fold increase of the basal level) was found around 45 min after SA addition (E. Ruelland, personal communication).

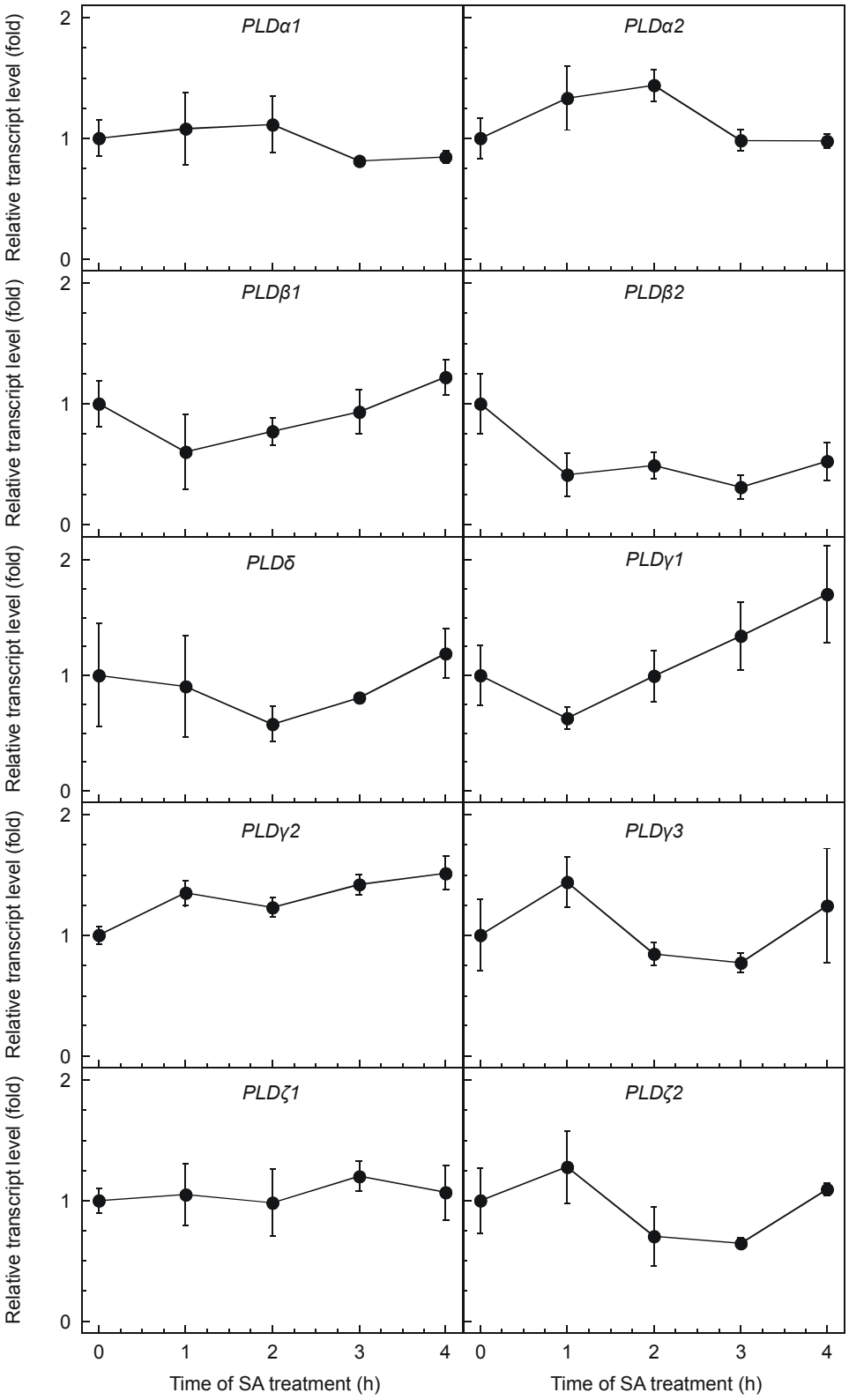


Figure 36. Expression of PLD isoforms during SA treatment.
Cells were treated with SA at the time 0 and RNA was isolated from aliquots at indicated times. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). The normalized expression level of water-treated cells was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

4.4.3 Exogenous PA application did not reverse the n-butanol inhibition of SA response

In a few studies the lack of PA_{PLD} caused by *n*-butanol treatment was overcome by treatment with exogenous PA (Bi et al., 1997; Potocký et al., 2003; Sang et al., 2001; Zhang W et al., 2003). Such inhibition reversal experiments were done with PA bearing both long-chain and short-chain fatty acid residues as these exhibit different properties both in terms of their water solubility and affinity for their potential cellular protein targets. Short-chain fatty acyl PA (8:0/8:0) was usually somewhat less effective although capable of the *n*-butanol inhibition reversal too. Long-chain fatty acyl PA is more biological but it must be applied in the form of liposomes, the preparation of which is not always reproducible, and moreover one can hardly determine the available concentration. Therefore only dioctanoyl-PA, a water soluble sodium salt, was used in the following experiments aimed to reverse the *n*-butanol inhibition of gene expression. Cells were pre-treated with water, PA, *n*-butanol or with both PA and *n*-butanol, and then treated with either water or SA for 6 h. In the end of the experiment the expression of *PR1* and *WRKY38* genes was determined by QRT-PCR (Figure 37). Not only the exogenous PA did not release the *n*-butanol block on SA induction of *PR1* or of *WRKY38* genes, but even PA alone inhibited their SA induction.

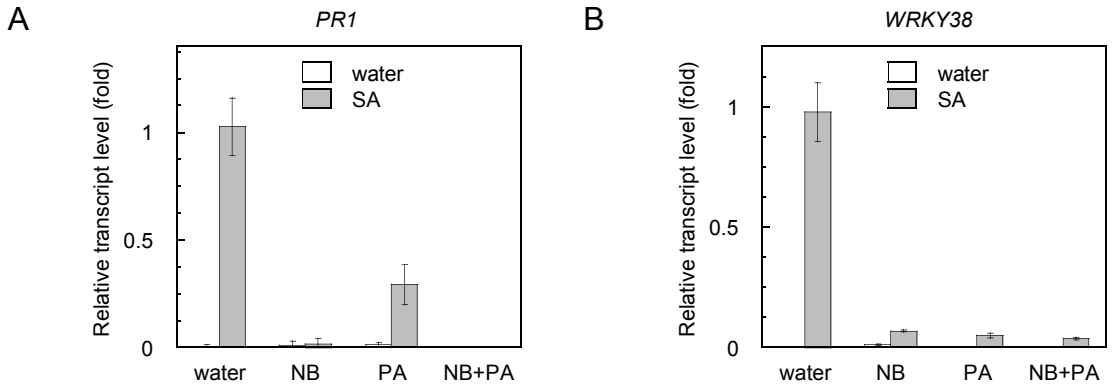


Figure 37. PA did not reverse the *n*-butanol block on SA induction of *PR1* and *WRKY38* genes.
Where indicated cells were incubated 15 min with 300 μM PA (8:0/8:0) or 0.1% v/v *n*-butanol prior to SA application and then treated with SA for six more hours before RNA isolation. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). For each gene the normalized expression level of simple SA treatment was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results.

4.5 SA transcriptome is partially regulated by the PLD pathway

4.5.1 Identification of PLD-regulated genes in the SA transcriptome

Previous experiments have shown that PA_{PLD} affects the SA-regulated gene expression (see chapter 4.4.1). Moreover, the principal marker gene of SA action, *PR1*, exhibits also properties of a gene regulated by PLD. Therefore, it was interesting to evaluate the impact of PLD-derived PA on the whole SA transcriptome. To be able to compare the cluster of PLD-regulated genes in response to SA with the W30-sensitive cluster in the same response (see chapter 4.3.2), the same time of SA treatment (4 h) was chosen. RNAs were isolated from 5-day-old cells that had been submitted to either: a treatment with SA in the presence of 0.1% v/v *n*-butanol (hereafter, SANB), a treatment with SA in the presence of 0.1% v/v *tert*-butanol (hereafter, SATB), a treatment with 0.1% v/v *n*-butanol (hereafter, NB), or a treatment with 0.1% v/v *tert*-butanol (hereafter, TB). As the gene response to SA may slightly change from one experiment to another, RNAs were also isolated from cells treated only with SA or water. Cells were pre-treated with alcohols for 15 min and then treated with SA for four more hours when indicated. RNAs were extracted from two independent biological repetitions (no pooling was performed), reverse transcribed in the presence of Cy3-dUTP or Cy5-dUTP to perform two-colour hybridization with the CATMA chip. One dye-swap was made per biological repetition (i.e. four hybridizations per comparison) for four combinations: SANB vs. SATB, SANB vs. SA, SATB vs. SA and NB vs. TB. Again, the differentially expressed genes were identified by a paired *t* test on the $\log_2$ ratios based on the four hybridizations. The raw p-values were adjusted by the Bonferroni methods, which control the FWER. NB or SANB is always compared to TB or SATB, respectively, to ensure that the effect of NB is not due to a general effect of alcohols. The additional comparison SANB vs. SA was done to ensure exclusion of any non-specific response to alcohols. Only genes differentially expressed and with the transcript level change in the same direction in SANB vs. SATB like in SANB vs. SA (i.e. induced in both comparisons or repressed in both comparisons) were claimed as PLD-regulated. Hereafter, 'PLD-regulated gene' will imply that its regulation is disturbed by NB, and that the effect cannot be attributed to general effect of alcohols.

In order to identify the genes whose response to SA is affected by NB and not by TB, the following cluster analysis was performed. Among the 1380 genes which were differentially regulated in response to SA, 1327 produced good hybridization in all

tested dye-swaps. Thirteen genes whose transcript levels were different between SATB and SA were not considered, as they may be regulated by alcohols (Figure 38). Among the remaining 1314 genes, 380 could be considered as PLD-regulated. Among the 380 PLD-regulated genes in response to SA, 238 genes showed lower transcript levels in SANB vs. SATB. Among these 238 genes, 86 had also lower transcript levels in NB vs. TB, arguing for an effect on the regulatory events controlling the basal levels of those transcripts. For these genes, the effect of SANB vs. SATB could therefore be a consequence of a perturbation of basal transcript level regulation and not specifically on the SA response. Reasoning analogical to that of Figure 23 could be used to explain the ambiguity. These genes were excluded because they may represent a considerable bias for the cluster analysis (the cluster contains false positive genes with different modes of regulation). Among the 380 PLD-regulated genes, 142 genes had higher transcript levels in SANB vs. SATB. Again, 80 of them showed the same changes in NB vs. TB and thus were excluded too. In the remaining group of 214 genes, which were specifically regulated by PLD during the SA response, 147 genes were SA-induced and 67 were SA-repressed. Among the 147 genes induced by SA, 91 showed lower transcript levels in SANB than in SATB, showing an inhibitory effect on the SA induction. Among the 67 SA-repressed genes, only six showed higher transcript levels in SANB than in SATB, showing an inhibitory effect on the SA repression. Taken together 97 out of 214 genes (i.e. 45%) showed a positive action of PLD on their response to SA treatment. Possibly underestimated, the number of genes induced by the PLD pathway represents 12% of the SA-induced genes. The overall number of genes regulated by the PLD pathway represents 7% of all SA-regulated (i.e. repressed or induced) genes.

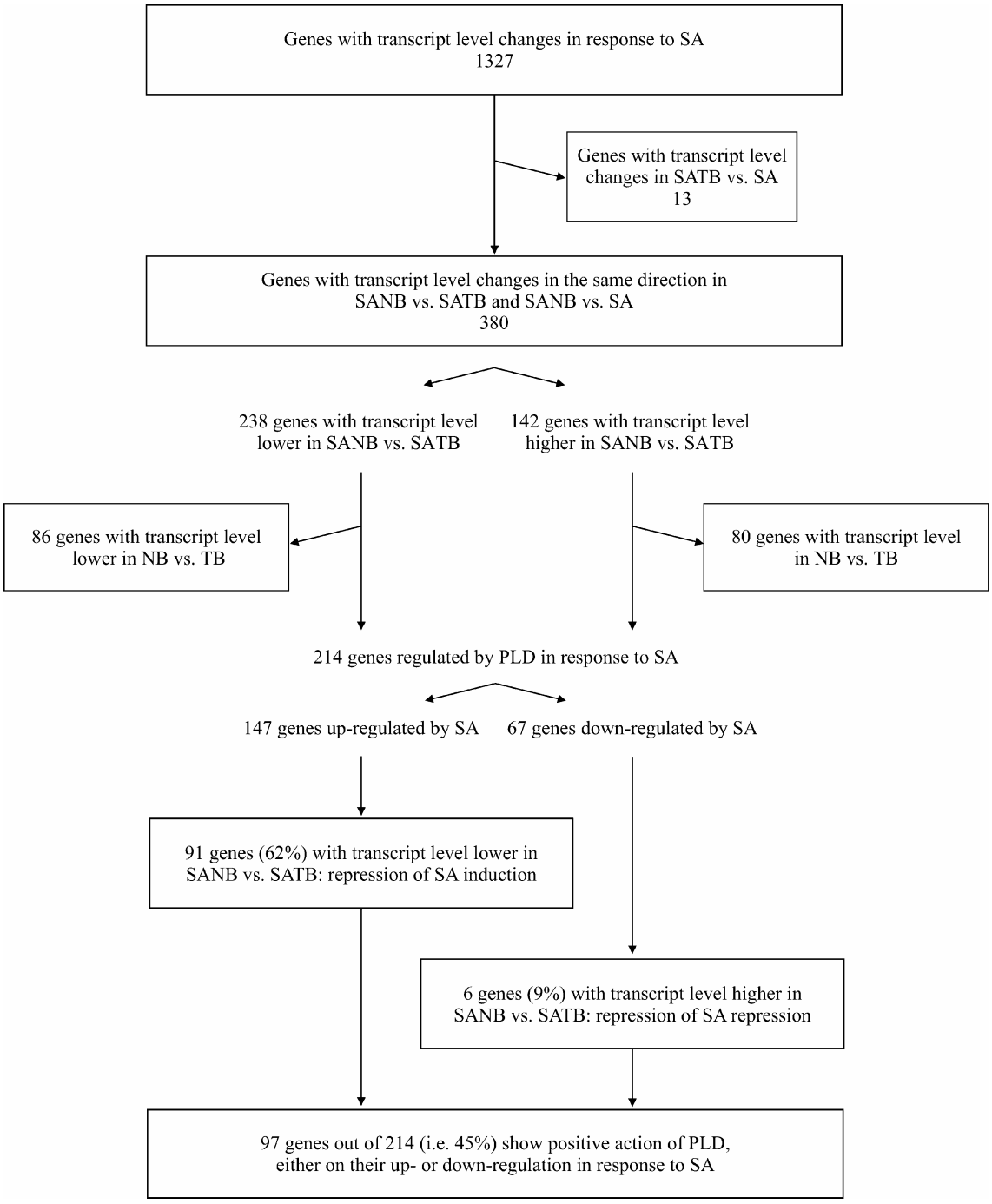


Figure 38. Determination of SA-regulated genes downstream of the PLD pathway.
Among the 4,022 genes which showed differential gene expression in one of the five tested comparisons, 214 showed transcript level changes both in SA vs. water comparison and in the same direction in SANB vs. SATB comparison as in SANB vs. SA comparison. For these genes the transcript levels did not change in the same direction in NB vs. TB as in SANB vs. SATB. Hence these genes are SA-regulated via PLD pathway. Of these genes, 147 were up-regulated and 67 were down-regulated by SA treatment. 97 genes out of 214 showed positive action of PLD on their SA response.

Strikingly, in this microarray analysis the positive action of PLD could be attributed to a relatively small portion of PLD-regulated genes. It was thus interesting to identify the group of genes negatively regulated by PLD in response to SA (Figure 39). Among the 147 genes induced by SA, 56 showed higher transcript levels in SANB than

in SATB, showing an inhibitory effect on the repression of SA induction. Among the 67 SA-repressed genes, 61 showed lower transcript levels in SANB than in SATB, showing an inhibitory effect on the repression of SA repression. Taken together 117 out of 214 genes (i.e. 55%) showed a negative action of PLD on their response to SA treatment. Possibly underestimated, the number of genes negatively regulated by the PLD pathway represents 10% of the SA-repressed genes. The overall number of genes regulated by the PLD pathway represents 9% of all SA-regulated (i.e. repressed or induced) genes.

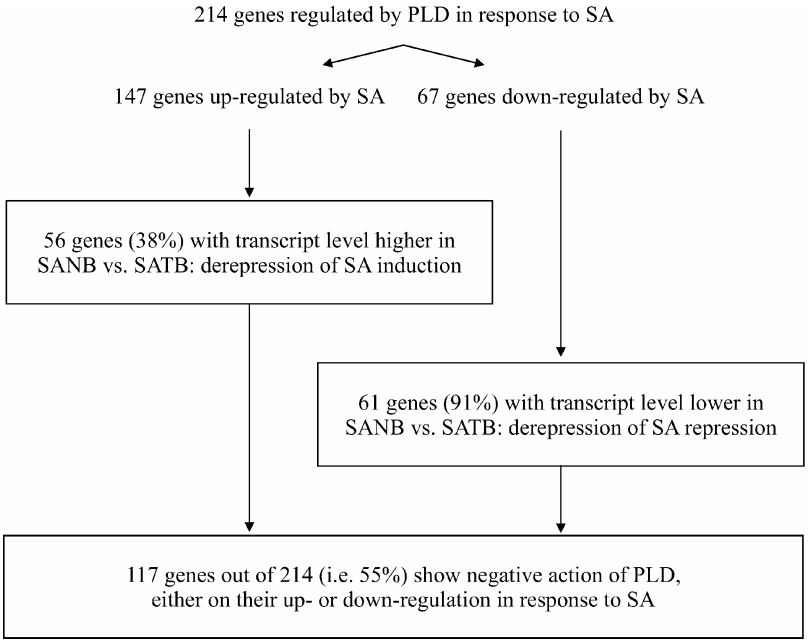


Figure 39. Focus on the negative action of PLD on gene regulation in response to SA.
Of 214 genes regulated by PLD in response to SA, 147 were up-regulated and 67 were down-regulated by SA treatment. 117 genes out of 214 showed negative action of PLD on their SA response.

To make sure that the observed numbers are not a product of random events a test of colocalization of the two phenomena of regulation was performed, i.e. colocalization of the SA-regulated gene expression (SA vs. water comparison) with the regulation of SA response by NB (SANB vs. SATB comparison). The genes regulated non-specifically by alcohols in their response to SA (i.e. genes with transcript level change in SATB vs. SA comparison or with different behaviour in SANB vs. SATB and in SANB vs. SA comparisons) were removed from the analysis as well as the genes showing the same mode of regulation even in the non-stimulated (water treated) cells (i.e. in NB vs. TB comparison) as these may artificially increase the observed overlap of regulation modes (they represent false positives). Each gene among the remaining ones

could be unambiguously classified into one of the four groups: genes with transcript level change both in SA vs. water and SANB vs. SATB comparisons, genes with transcript level change only in one comparison and genes with no transcript level change in any of the two comparisons. Probabilities that the observed distributions are due to random events (calculated by the Fisher's test) are given in Table XII. Three of the four possible colocalizations were not a product of coincidence (α=0.05) showing that the regulation of gene expression by SA and regulation of the same response by PLD colocalize in the transcriptome much more than would be expected in case of independence of the two phenomena. Only the group of positively PLD-regulated SA-repressed genes may result from random overlap of the two modes of regulation of gene expression.

Table XII. *Colocalization analysis of regulation by SA with regulation by PLD in response to SA on the whole transcriptome scale.*

Genes regulated in SATB vs. SA comparison or with different behaviour in SANB vs. SATB and in SANB vs. SA comparisons and genes regulated in the same way in SANB vs. SATB as in NB vs. TB comparison were removed from the analysis. The remaining 17,343 genes were classified with respect to their regulation in SA vs. water and SANB vs. SATB comparisons. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. P-values are those of Fisher's test. Categories printed in bold are over-represented in the experimental data. d_{obs}, observed distribution; d_{theor}, theoretical distribution.

SA regulation	SANB vs. SATB	Number of genes		p (d_{obs}=d_{theor})
		Observed	Theoretical	
induced	repressed	**91**	**11**	**7.4e-58**
	induced	**56**	**9**	**8.6e-29**
repressed	induced	6	7	1.0
	repressed	**61**	**8**	**1.0e-34**

To estimate the relative importance of the two modes of regulation a statistical analysis was performed. Cluster of 214 genes specifically regulated by NB in response to SA (Figures 38 and 39) was classified into four sub-groups with respect to the induction or repression of their expression by SA and inhibition or not of this response in the presence of NB compared to TB. For each cluster it was possible to calculate a theoretical number of genes based on the hypothesis that the transcript level difference between SANB and SATB was independent of the transcript level difference in the presence or absence of SA. The observed number of genes in each cluster was then compared to this theoretical number (Table XIII). It is clear that there was an over-representation of genes showing a negative action of the PLD pathway on the response to SA (i.e. an inhibiting effect of NB on the repression of SA response)

combined with the under-representation of genes not showing such a negative action. Fisher's test (α=0.05) indicated that these differences in distribution were not a product of random events. Thus the negative mode of regulation of gene expression by PLD specific to SA response is indeed more important than the positive one both for SA-induced and SA-repressed genes.

Table XIII. *Analysis of expected and observed clusters of genes classified by their response to SA and the effect of NB on this response.*

The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. With respect to the expected numbers, the categories printed in bold are over-represented in the experimental data, while the categories written in italics are under-represented. SA-induced and SA-repressed genes were considered separately.

Number of genes	SA>H_2O		subtotal	SA<H_2O		subtotal
	SANB>SATB	SANB<SATB		SANB>SATB	SANB<SATB	
Theoretical	**43**	*104*	147	*19*	**48**	67
Observed	**56**	*91*	147	*6*	**61**	67

The list of 97 genes positively and 117 genes negatively regulated by the PLD pathway in response to SA is provided as Supplemental Table IX (electronic supplement only). Unfortunately, there is no CATMA probe for *PR1*, thus its PLD-dependence could not be confirmed in the microarray experiment. The first ten genes from the category of SA-induced genes positively regulated by PLD and from categories of SA regulated genes negatively regulated by PLD ranked by decreasing degree of their repression or induction by NB are listed in Table XIV.

Expression data for the genes that are known to be involved in phospholipid signalling are summarized in Supplemental Table X (electronic supplement only). Among the genes with a corresponding CATMA probe *PI4Kγ7* (gene encoding a type II PI 4-kinase), *PLC7*, *PLDα1* and *PLDζ2* were significantly repressed by SA treatment. The repression of *PLDα1* and *PLDζ2* represents an artefact of the microarray experiment because it was not confirmed by QRT-PCR (Figure 36) and therefore will not be considered. *PLC7* was positively regulated by PLD while *PI4Kγ7* was negatively regulated by PLD in its SA response.

Table XIV. *List of SA regulated genes most influenced by NB treatment, ranked by decreasing degree of repression or induction by NB.*

(A) Top 10 genes positively regulated by PLD in their SA induction. (B) Top 10 genes negatively regulated by PLD in their SA induction. (C) Top 10 genes negatively regulated by PLD in their SA repression. The difference in transcript level was significant in all cases (α=0.05). AGI, Arabidopsis Genome Initiative gene index; I, average signal intensity.

A)

Protein	Function	AGI	$\log_2 I_{SANB}/I_{SATB}$	$\log_2 I_{SA}/I_{water}$
Expressed protein	Unknown	At1g31580	-3.85	3.66
Glycerophosphoryl diester phosphodiesterase family protein	Kinase activity	At1g66970	-3.54	2.91
WRKY66	Transcription factor activity	At1g80590	-2.91	4.02
WRKY38	Transcription factor activity	At5g22570	-2.77	4.59
Heavy-metal-associated domain-containing protein	Metal ion binding	At5g26690	-2.56	2.88
AAA-type ATPase family protein	ATPase activity	At3g28540	-2.44	2.62
D-3-phosphoglycerate dehydrogenase (3-PGDH)	Phosphoglycerate dehydrogenase activity	At1g17745	-2.26	2.96
Cytochrome P450 83B1 (CYP83B1)	Cytochrome P45 activity	At4g31500	-2.09	1.63
Expressed protein	Unknown	At1g19960	-2.04	2.81
Protein phosphatase 2C, putative	Protein phosphatase type 2C activity	At1g34750	-2.03	2.15

B)

Protein	Function	AGI	$\log_2 I_{SANB}/I_{SATB}$	$\log_2 I_{SA}/I_{water}$
60S ribosomal protein L10 (RPL10C)	Structural constituent of ribosome	At1g66580	2.07	0.92
UDP-glucoronosyl/UDP-glucosyl transferase family protein	Transferase activity, transferring glycosyl groups	At2g15490	1.94	2.42
Dehydrin xero2 (XERO2)/low-temperature-induced protein LTI30 (LTI30)	Unknown	At3g50970	1.84	1.87
Hydrophobic protein, putative/low temperature and salt responsive protein, putative	Unknown	At4g30660	1.69	0.81
Transcription initiation factor IIA γ chain/TFIIA-γ (TFIIA-S)	RNA polymerase II transcription factor activity	At4g24440	1.61	0.67
Complex 1 family protein/LVR family protein	Catalytic activity	At2g39725	1.61	0.73
Pseudogene, glycine-rich protein	Structural molecule activity	At2g36120	1.53	0.84
Expressed protein	ATP binding	At1g15230	1.50	1.06
Drought-responsive family protein	Unknown	At3g06760	1.45	0.84
Transcription factor, putative	Transcription factor activity	At1g56170	1.34	1.15

C)

Protein	Function	AGI	$\log_2 I_{SANB}/I_{SATB}$	$\log_2 I_{SA}/I_{water}$
Expressed protein	Unknown	At5g42090	-1.96	-1.35
SEC14 cytosolic factor family protein/phosphoglyceride transfer family protein	Transporter activity	At1g72150	-1.86	-1.16
Pyrophosphate-fructose-6-phosphate 1-phosphotransferase β subunit, putative/pyrophosphate-dependent 6-phosphofructose-1-kinase, putative	6-phosphofructokinase activity	At4g04040	-1.83	-1.35
Cellulose synthase, catalytic subunit (Ath-B)	Cellulose synthase activity	At5g05170	-1.59	-2.04
RNA polymerase α chain	Unknown	AtCg00740	-1.55	-1.90
Amino acid transporter family protein	Amino acid permease activity	At3g30390	-1.46	-0.88
Ribosomal protein L2	Unknown	AtCg00830	-1.37	-1.14
Expressed protein	Unknown	At3g49990	-1.36	-0.82
Endomembrane protein 70, putative	Transporter activity	At4g12650	-1.31	-1.57
ATP synthase CF1 β chain	Unknown	AtCg00480	-1.29	-0.78

The results obtained by DNA microarray analysis were confirmed on a selection of eight genes showing high induction by SA and important inhibition of this response in the presence of *n*-butanol. QRT-PCR analysis of an independent non-pooled set of RNAs revealed a clear and specific inhibitory effect of *n*-butanol on the SA induction for all of the tested genes (Figure 40).

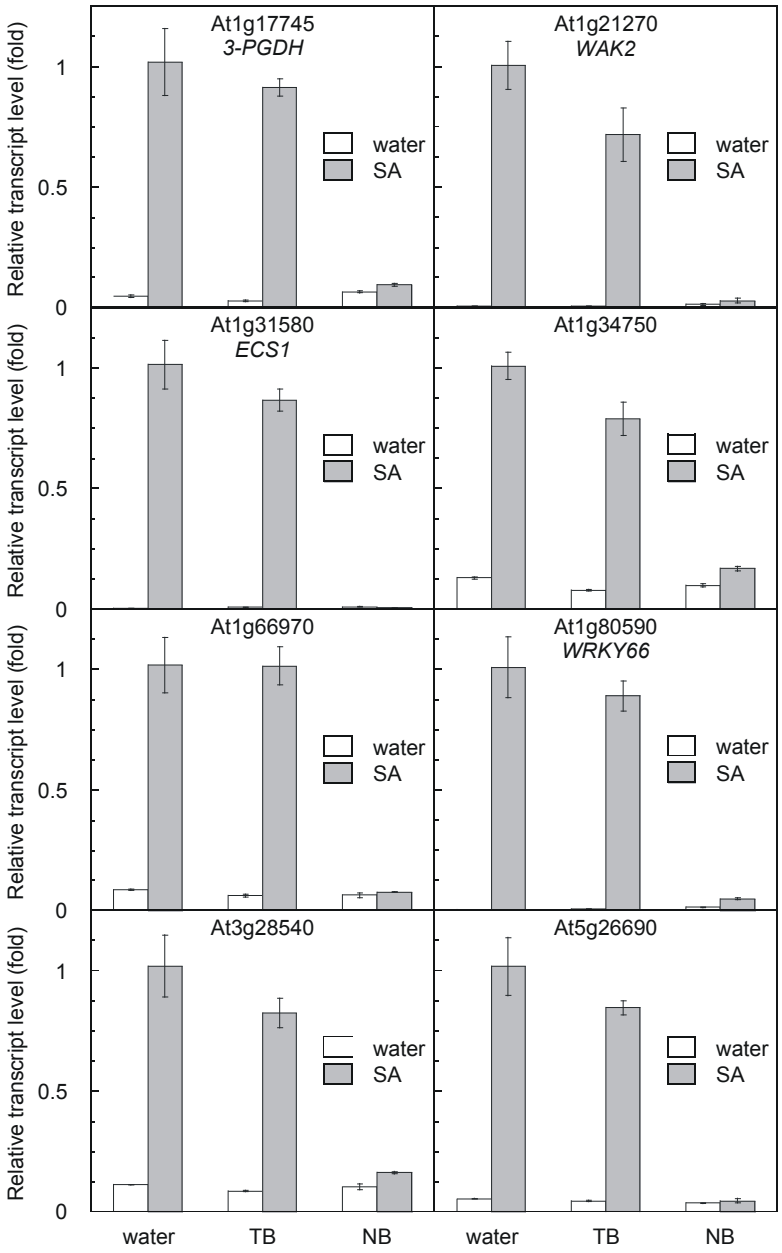


Figure 40. Confirmation of microarray results on an independent set of non-pooled RNAs.
RNA was isolated from 5-day-old cell suspensions; cells were pre-treated with 0.1% v/v *n*-butanol (NB) or 0.1% v/v *tert*-butanol (TB) for 15 min and then treated with SA for 4 h. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). For each gene the normalized expression level of simple SA treatment was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs. 3-PGDH, D-3-phosphoglycerate dehydrogenase; WAK2, wall-associated kinase 2; ECS1, ecotype specific 1.

4.5.2 Common cis-elements in the promoters of PLD-regulated genes in response to SA

It was interesting to determine whether any of the motifs specific for the SA-regulated genes are over-represented in the group of genes regulated by SA via the PLD pathway. Only clusters of genes regulated by PLD specifically in their SA response with sufficient number of genes were considered for this analysis. A subset of

genes with unambiguous probe-to-gene assignment was extracted from the list of PLD-regulated genes in response to SA. For each SA-specific motif identified in chapter 4.3.3, its frequency in the group of genes regulated by the PLD pathway was compared to its frequency in the subset of SA-regulated genes (either induced or repressed depending on the tested PLD regulon) using the same bootstrapping method on the subset of SA-regulated genes. No motifs were differently represented in the cluster of SA-repressed genes negatively regulated by PLD. Significantly over- and under-represented motifs are listed in Table XV. One motif, the W-box (TTGACTT/TTGACY) motif, was significantly over-represented in the group of genes induced by SA via the positively acting PLD pathway. These data suggest that WRKY transcription factors may be situated downstream of the positive action of PLD. Another motif, the CBF1 binding site (RGGCCY), was under-represented in the group of genes positively regulated by PLD and over-represented in the group of genes negatively regulated by PLD in their SA induction suggesting its role downstream of the negative PLD action. Analysis of groups of genes regulated by PLD both in their SA response and in water-treated condition revealed different motifs (data not shown) confirming that leaving those genes in the promoter analysis would bias its result.

Table XV. *Cis-elements identified in the promoters of the sub-group of genes induced by SA via the PLD pathway.*

The analysis was performed on a subset of genes with unambiguous probe-to-gene assignments. The number of appearances of each motif in promoters (-1000 bp) of the cluster of 84 genes induced by SA via the positive action of PLD ($f_{obs,p}$) and in promoters of the cluster of 55 genes induced by SA via the negative action of PLD ($f_{obs,n}$) was compared to its frequency in promoters of 681 genes induced by SA (f_{theor}) by general bootstrapping technique. n.s.d., observed frequency is not significantly different from the theoretical one ($p \geq 0.05$).

Motif description	Searched motif	f_{theor}	$f_{obs,p}$	p ($f_{obs,p}=f_{theor}$)	$f_{obs,n}$	p ($f_{obs,n}=f_{theor}$)
I-box, MYB-like family	GATAAG	0.64	n.s.d.	-	0.42	2.2e-2
ASF-1-like, TGA family	TGACGTG	0.12	0.05	2.0e-2	n.s.d.	-
W-box, WRKY family	TTGACY	1.51	2.02	8.4e-5	n.s.d.	-
W-box-like, WRKY family	TTGACTT	0.43	0.77	2.9e-7	n.s.d.	-
bZip	ACGT	5.13	4.19	9.1e-3	n.s.d.	-
G-box bZip variants	YACGTG	0.77	0.33	8.8e-4	n.s.d.	-
CBF1 binding site	RGGCCY	0.88	0.60	4.0e-2	1.31	1.6e-2
SORLIP2, light responsive	TGGGCC	0.49	0.33	4.2e-2	n.s.d.	-

4.5.3 Expression of PR1 and WRKY38 in the T-DNA mutants of several PLDs

Changes in the SA induction of PLD marker genes in T–DNA lines for PLD isoforms could indicate isoform(s) involved in the SA response. Arabidopsis genome

contains 12 PLD isoforms, and until now 7 T-DNA knock-out lines homozygous in the insertion have been prepared in our laboratory. *PR1* and *WRKY38* genes, both exhibit behaviour typical of genes regulated by PLD in response to SA (see chapter 4.4.1). Both are known to be induced by SA in the whole plants of Arabidopsis. Therefore these two genes were tested with respect to the time course of their induction by SA in the whole plants. Both were sufficiently induced 6 h after spraying the plants with 1 mM SA. Their induction by SA was then checked in the wild type and in the *pldα1*, *pldβ1*, *pldγ1*, *pldγ2*, *pldγ3*, *pldζ1* and *pldζ2* plants (Figure 41). The degree of SA induction of these two genes was lower in the mutant lines *pldα1*, *pldβ1* and *pldγ3* than in the wild type. In the line *pldγ1* the degree of SA induction of these two genes was higher than in the wild type. Therefore these are the PLD isoforms which should be further characterized. Moreover, the expression pattern of the two genes across the tested isoforms was very similar. The only difference was observed between the basal levels of expression of the two genes in *pldγ1* and *pldζ2* mutants. The basal gene expression was increased for *PR1* but not for *WRKY38* in these two lines. Such similarity of their expression patterns further confirms a role for PLD in their SA induction.

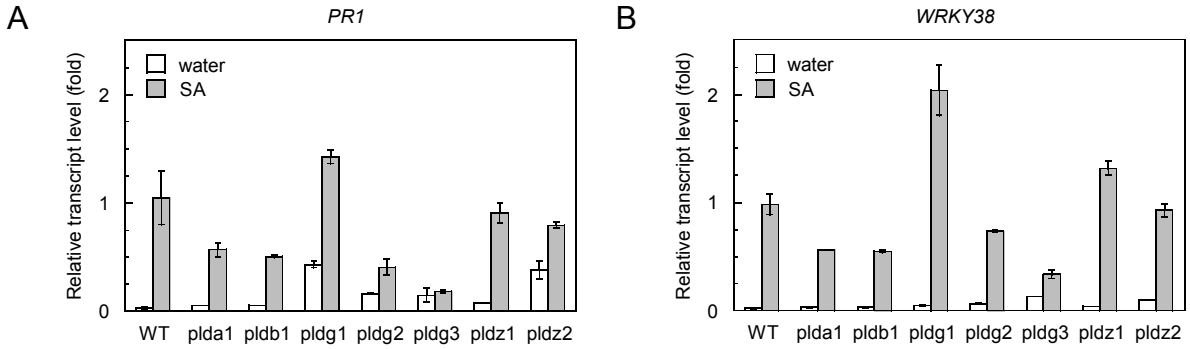


Figure 41. Expression of *PR1* and *WRKY38* in Arabidopsis wild type plants and in T-DNA knock-out lines of several PLD isoforms after SA treatment.
Plants were sprayed with 1 mM SA or water and RNA was isolated from their leaves after 6 h of treatment. Five leaves from one plant were pooled for each condition. Gene expression was measured by QRT-PCR with relative calibration. Expression levels were normalized for the cDNA content by the expression level of a housekeeping gene (At3g04920; 40S ribosomal protein S24). For each gene the normalized expression level of simple SA treatment of wild type plants was arbitrarily set to 1 and that of other samples was made relative to it. The data are means of three PCRs from one of two independent experiments with similar results. WT, wild type.

4.6 Overlap of W30-sensitive and PLD-regulated SA transcriptomes

Two elements upstream of the SA regulated transcriptome, i.e. a W30-sensitive pathway and PLD, have been identified in the two microarray experiments (chapters 4.3

and 4.5). To be able to place these elements in the SA signalling network it was important to check whether the two distinct groups of genes share a common overlap and whether the overlap is more than a simple coincidence. There were quite large groups of genes in both experiments for which the regulation either by W30 or by PLD was already present in water treated cells but lost in SA treated cells (data not shown) which means that maintaining the regulation in the presence of SA has a physiological relevance. Therefore it was not necessary to remove genes sharing common regulation in water and SA treated cells because, even if it was non-specific of SA, the regulation was still present after SA treatment and therefore important for the correct development of the SA response.

A common base for the overlap analysis was constructed according to the following rules: the genes had to be regulated in the same way in both microarray experiments (i.e. SA-induced in both or SA-repressed in both). Moreover, genes removed from either analysis for their non-specific regulation (i.e. regulated by methanol or W1 in the SA response in the microarray analysis of the W30-sensitive pathway or regulated by TB in the SA response in the microarray analysis of the PLD-regulated pathway) were omitted from the overlap analysis. This led to the construction of a set of 273 SA-induced genes and a set of 153 SA-repressed genes where the mode of regulation of gene expression in response to SA could be unambiguously attributed to each gene (i.e. positive or negative regulation of SA-induction or SA-repression). In this way eight colocalization analyses (four for SA-induced genes and four for SA-repressed genes) could be designed (Table XVI). Based on Fisher's test (α=0.05) in most of them the observed distribution was not a product of random events. Strikingly, the overlaps of both positive regulations and both negative regulations were over-represented while overlaps of one negative mode of regulation with one positive mode of regulation were under-represented. It means that the two enzyme activities are placed in the same signalling pathway acting synergistically on the SA transcriptome both in its positive and negative regulation.

To ascertain this, another analysis was done only for the genes present in the eight constructed overlaps (Table XVII). The overlaps corresponding to the same mode of regulation (i.e. positive or negative) were over-represented while the overlaps corresponding to mixed modes of regulation were under-represented both for SA-induced and SA-repressed genes. It means that the two differently regulated groups of genes (i.e. positively or negatively regulated) are quite distant in the SA

transcriptome and that their cross-overlapping is rather rare. Genes belonging to the four over-represented clusters from Table XVII are listed in Supplemental Table XI (electronic supplement only).

Table XVI. *Analyses of overlaps of different modes of regulation of the SA transcriptome in the microarray experiment which identified genes regulated via a W30-sensitive pathway and in the microarray experiment which identified PLD-regulated genes.*

Genes regulated in the same way by SA in both microarray experiments were taken as a base for the overlap analyses. Genes differentially expressed in SAW1 vs. SA and SATB vs. SA comparisons and genes with different behaviour in SANB vs. SATB and in SANB vs. SA comparisons were removed from the analyses. The remaining genes (273 SA-induced and 153 SA-repressed genes) were classified with respect to their regulation in SAW30 vs. SAW1 (a W30-sensitive pathway) and SANB vs. SATB (PLD pathway) comparisons. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. P-values are those of Fisher's test. Categories printed in bold are over-represented while those printed in italics are under-represented in the experimental data. d_{obs}, observed distribution; d_{theor}, theoretical distribution.

SA regulation	SAW30 vs. SAW1	SANB vs. SATB	Number of genes		p (d_{obs}=d_{theor})
			Observed	Theoretical	
induced	repressed	repressed	**48**	**25**	**1.4e-10**
		induced	*5*	*13*	*3.7e-3*
	induced	repressed	*1*	*16*	*1.8e-8*
		induced	**24**	**8**	**5.1e-9**
repressed	repressed	repressed	**17**	**8**	**2.0e-4**
		induced	*2*	*8*	*7.7e-3*
	induced	repressed	2	3	7.4e-1
		induced	4	3	3.1e-1

Table XVII. *Analyses of overlaps of modes of regulation of the SA transcriptome in microarray experiments which identified genes regulated by SA via a W30-sensitive pathway and via PLD.*

Genes in the overlaps listed in Table XVI were classified with respect to their positive or negative regulation by a W30-sensitive pathway or PLD. The expected number of genes for each cluster was calculated and compared to the actual number observed in the analysis. P-values are those of Fisher's test. Categories printed in bold are over-represented while those printed in italics are under-represented. SA-induced and SA-repressed genes were considered separately. d_{obs}, observed distribution; d_{theor}, theoretical distribution.

SA regulation	Number of genes	Mode of regulation of the SA transcriptome				p (d_{obs}=d_{theor})
		W30-sensitive pathway positive		W30-sensitive pathway negative		
		PLD positive	PLD negative	PLD positive	PLD negative	
induced	theoretical	**33**	*20*	*16*	**9**	3.4e-14
	observed	**48**	*5*	*1*	**24**	
repressed	theoretical	**1**	*5*	*5*	**14**	1.5e-2
	observed	**4**	*2*	*2*	**17**	

Clusters of the W30-sensitive pathway dependent and PLD dependent SA regulated genes for which the regulation was not already present in water treated conditions were often too small to perform any statistics. The only possible test of

overlap (i.e. that of genes positively regulated by a W30-sensitive pathway and positively regulated by PLD in their SA induction) also showed an over-representation of a group of genes regulated by both pathways (data not shown). It confirms that leaving the genes with regulation already present in water treated conditions did not introduce a substantial bias in the presented overlap analysis.

5 DISCUSSION

5.1 Arabidopsis cell suspensions are an admissible model to study SA signalling

The fact that cells have direct access to the medium assures synchronous uptake of radioactive label and applied agonist leading to high reproducibility of results (Meijer and Munnik, 2003). This makes cell suspensions the material of choice for studying plant lipid signalling. SA can be taken up by Arabidopsis cells (Clarke et al., 2005) and it induces changes in their proteome (Rajjou et al., 2006). Genes known to be SA-induced in Arabidopsis plants (Blanco et al., 2005; Kliebenstein et al., 2006; Thibaud-Nissen et al., 2006; Wagner et al., 2002) were also induced in Arabidopsis cells making this system a suitable model for studying SA signalling.

5.2 Activation of a PI 4-kinase as an early response to SA

In Arabidopsis cell suspensions, changes of phosphoinositide levels were studied after metabolic labelling with $^{33}P_i$. When cells are labelled for a very short time (5 or 15 min before lipid extraction), application of SA induces a rapid decrease in the pool of newly labelled PI, concomitantly with an increase of labelled PI(4)P and PI(4,5)P_2. This makes a compelling argument for a PI 4-kinase being activated by SA. There are two types of PI 4-kinases (type II and type III) which differ in their sensitivity to different concentrations of wortmannin. No plant type II PI 4-kinase has been biochemically characterized yet. Based on animal and yeast studies, type II PI 4-kinases cannot be inhibited by micromolar concentrations of wortmannin, contrary to type III PI 4-kinases that are inhibited by micromolar concentrations of wortmannin (Müller-Röber and Pical, 2002) and by PAO (Rajebhosale et al., 2003). The involvement of PI 4-kinase in the SA response could thus be confirmed by the use of inhibitors: the decrease in labelled PI level is inhibited by PAO and high concentrations of wortmannin, but not by low concentration of wortmannin or by LY294002, indicating that the changes in labelled phosphoinositides are due to the activation of a type III PI 4-kinase, which is the prevalent PI 4-kinase activity *in planta* (Müller-Röber and Pical, 2002).

Biochemically, the reaction catalyzed by PI kinases is close to that catalyzed by protein kinases. Their close relationship is documented by the fact that some protein kinases are members of the PI 3-kinase superfamily (Davies et al., 2000). It is thus not surprising that some protein kinases and PI kinases can be inhibited by the same

inhibitors. Consequently, LY294002 can also inhibit animal phosphorylase kinase and casein kinase 2 (Davies et al., 2000) but as it does not inhibit the SA-induced PI decrease it was not necessary to prove that it acted solely on PI 3-kinase. Wortmannin inhibits also animal myosin light chain kinase (MLCK; Davies et al., 2000). This kinase can also be inhibited by staurosporine. However, 1 µM staurosporine did not inhibit the SA-induced PI decrease. PAO is known to inhibit protein Tyr phosphatases (PTPs; Reyes et al., 2006). As 25 µM 3,4-dephostatin, another inhibitor of PTP (MacRobbie, 2002), has no influence on the SA-induced PI decrease, the effect of PAO on the SA response can be attributed to direct inhibition of a PI 4-kinase.

The activation of PI 4-kinase occurs shortly after SA addition and lasts about one hour after which its activity returns to initial level. Labelled PI(4,5)P_2 follows the trends of labelled PI(4)P both in SA dose dependence and time course. This suggests that PI(4,5)P_2 accumulation is only a simple consequence of PI(4)P accumulation and that no supplemental control at the level of PIP 5-kinase has to take place during the SA response. This is also the case in yeast, where regulation of PI(4,5)P_2 synthesis rate takes place at the PI 4-kinase level (Godi et al., 1999). Besides the wortmannin-sensitive PI 4-kinase activation, the increase in PI(4,5)P_2 could also result from inactivation of a phosphoinositide phosphatase. In Arabidopsis the mutation of *SAC9* (supressor of actin mutation domain phosphoinositide phosphatase-like 9) and *FRA3* (fragile fiber 3, a gene encoding a type II inositol polyphosphate 5-phosphatase) has indeed led to accumulation of PI(4,5)P_2 in non-stressed plants (Williams et al., 2005; Zhong et al., 2004). Therefore deactivation of a phosphoinositide phosphatase may contribute to the observed changes of labelled phosphoinositides.

An increased synthesis of PI(4,5)P_2 could be seen as an enhanced supply of substrate for a PI-PLC activity which would then produce $InsP_3$ and DAG. No increased production of $InsP_3$ was observed during the first 150 min of SA treatment. This suggests a direct role of PI(4)P and/or PI(4,5)P_2 in the SA response. Based on the sequence of the Arabidopsis genome, van Leeuwen et al. (2004) identified 53 different proteins with PH domains, which can bind both PI(4)P and PI(4,5)P_2, and I have identified 84 proteins with a C2 domain (data not shown), which can bind PI(4,5)P_2 under low Ca^{2+} concentrations (Meijer and Munnik, 2003). All these proteins represent possible cellular targets of elevated PI(4)P and/or PI(4,5)P_2 levels.

5.3 Phosphorylation events precede the PI 4-kinase activation in response to SA

Even though the activation of PI 4-kinase takes place in the first minutes of SA treatment, it is probably not the very first event in the SA signalling pathway. Both calyculin A, an inhibitor of Ser/Thr protein phosphatases 1 and 2A, and genistein, an inhibitor of Tyr protein kinase, inhibited the SA-induced PI decrease. Such results are in favour of some events upstream of the PI 4-kinase activation. There is at least one phosphorylation on Ser/Thr which is a negative regulator (it has to be removed for the SA response to proceed correctly) and one phosphorylation on Tyr which is a positive regulator (it has to occur for the SA response to proceed correctly). This implies some signalling elements upstream of and possibly also in parallel to the PI 4–kinase activation. Such hypothesis is in good correlation with the size of identified W30-sensitive cluster, which does not comprise majority of the SA transcriptome (utmost 40% of SA-regulated genes) indicating that not all genes are regulated via the PI 4–kinase pathway and that other parallel pathways are probably operating during the development of the SA response.

5.4 Ca^{2+} signalling is not involved in the early SA response

An elevation of cytosolic Ca^{2+} concentration upon SA treatment was previously reported in BY-2 cells expressing aequorin (Lin et al., 2005). Contrary to this observation, SA did not trigger Ca^{2+} influx in the studied Arabidopsis cells transformed with aequorin at least in the first ten minutes of treatment. Such discrepancy could be explained by differences between the two species. Various plant species may differ slightly in the same signalling pathways. For instance potato has constitutively elevated SA level and it becomes more sensitive to the endogenous level upon stimulation (Durrant and Dong, 2004) contrary to most other plant species which increase SA synthesis upon stimulation. Another example may be the PLC activation which occurred after SA treatment in cucumber (Li et al., 1998) but it was not observed in Arabidopsis cells.

5.5 Protein synthesis differentially affects the SA response

As the decrease of PI corresponds to an increase in PI(4)P, and the decrease is inhibited by high concentration of wortmannin (30 μM) but not by its low concentration (up to 10 μM), the activation of PI 4-kinase surely takes place in the SA response. The early PI decrease was not influenced by treatment with cycloheximide, an inhibitor of

protein synthesis, suggesting that the PI 4-kinase activation is not due to an increased level of enzyme but rather due to the activation of the pre-existing protein. The only question is to what extent the PI decrease reflects the PI 4-kinase activation. Expression of PI synthase and type III PI 4-kinase isoforms did not vary during the first hour of SA treatment. Moreover, the decrease of labelled PI during SA treatment starts too early to be attributed to altered expression of either PI synthase or type III PI 4-kinase isoforms. However, rigorously, it cannot be excluded that the decrease in labelled PI is partially due to decreased activity of PI synthase. Regulation of plant PI synthase is not yet well understood and inhibitors effective on the animal enzyme are not effective on its Arabidopsis counterpart (S. Collin, unpublished data) therefore such possibility could not be verified.

On the other hand, the decrease in PI(4)P and PI(4,5)P_2 after 45 min of SA treatment depended on protein synthesis because application of cycloheximide blocked it and arrested the cells in the stimulated state (based on the levels of labelled phosphoinositides). The down-regulation of altered phosphoinositide levels in cells with unaffected protein synthesis after 45 min of SA treatment cannot be attributed to altered gene expression of either PI synthase or type III PI 4-kinase isoforms. Based on the high threshold cycle in the QRT-PCR analysis of *PI4Kα2* expression and on the fact that there are currently no ESTs assigned to it in plant databases, it can be concluded that its transcript level change is likely not to be important for the observed changes of PI(4)P levels. On the other hand, the slight increase in PI(4)P level between two and three hours of SA treatment could be assigned to the induction of *PI4Kβ2*. Characterization of the corresponding knock-out or knock-down mutant would be required to confirm such hypothesis. Accordingly, one of the PI 4-kinase isoforms in rice (*OsPI4K1c*, an orthologue of *AtPI4Kβ1*) was also induced by SA treatment (Kong XF et al., 2003). A simpler explanation of such slight increase of PI(4)P would be the progressive deactivation of PI(4)P 5-kinase.

5.6 SA-regulated transcriptome

Two microarray experiments aimed to identify the SA-regulated transcriptome in Arabidopsis cell suspensions were performed in the frame of my thesis. However, their design was different. The first one aimed to identify W30-sensitive cluster of genes comprised two biological repetitions collected with a one year interval. RNAs for each repetition were pooled from three independent extractions. There were also two

biological repetitions in the second experiment aimed to identify PLD regulated genes but these were collected with a one month interval and the RNAs for each repetition come from single extractions. Therefore it is not surprising that the first experiment identified less SA-regulated genes than the second one. On the other hand, the genes identified in the first experiment can be considered as more reliable since the study was dealing with more samples thus decreasing the biological variation. Moreover, the fact that the same expression pattern was obtained with two distinct pools collected in a one year interval adds a lot of confidence to the obtained data. Thus only these data will be discussed in the following chapter.

With more than 790 genes identified as SA-regulated, this experiment represents a very large collection of early SA-regulated plant genes. Transcriptomic response to SA and its physiological significance in Arabidopsis plants has already been discussed (Bostock, 2005; Thibaud-Nissen et al., 2006). Many of the previously described changes in gene expression related to SA treatment were indeed found among SA-regulated genes in the microarray experiment (based on the MapMan analysis). These pointed to activation of heterotrophic metabolism (Clifton et al., 2006), perturbation of photosynthesis (Mateo et al., 2006), repression of development (van Hulten et al., 2006), induction of PR proteins (Durrant and Dong, 2004), activation of the phenylpropanoid pathway leading to increased lignin synthesis (Durner et al., 1997; He and Wolyn, 2005) and increased anthocyanin synthesis (Gruhler et al., 2005; Taguchi et al., 2001). Nevertheless, the data reveal several new aspects of the mode of action of SA: triggering of the UPR, modes of cellular redox regulation, and interaction with hormone responsive pathways.

SA treatment activated protein secretory pathway (another name for UPR) in Arabidopsis plants via NPR1-dependent mechanism. The pathway was essential for secretion of PR proteins and SAR development (Wang et al, 2005). An important overlap between SA and UPR transcriptomes was also observed in Arabidopsis cells. UPR is triggered when a stress, including biotic ones, causes the protein folding in the ER to be slowed down, resulting in the temporary presence of a lot of unfolded proteins in the ER (Vitale and Ceriotti, 2004). It is characterized by the induction of chaperones, and of proteins involved in disulfide bond formation and in protein degradation. Genes encoding these proteins were indeed induced by SA treatment in the microarray experiment in Arabidopsis cells. For instance, SA induced chaperones from the DnaJ family, several components of the proteasome complex, especially F-box proteins,

RING proteins and members of the ubiquitin-dependent protein catabolism. The observed transcriptome overlap included protein disulfide isomerases and a luminal binding protein (BiP-3) which is an important marker of the UPR in plants (Kamauchi et al., 2005; Wang et al., 2005). This further documents a strong correlation between SA and UPR transcriptomes in Arabidopsis cells. It has to be noted that an elevation of $PI(4,5)P_2$ level observed in response to SA in cells was also reported during UPR in plants (Shank et al., 2001).

After 4 h of SA treatment, the ratio of reduced to oxidized glutathione was increased in the studied cell suspensions (M. Flemr, unpublished data). A more reducing environment, together with induction of thioredoxins and glutaredoxins, would lead to structural changes of several transcription factors (e.g. NPR1 and TGA1) which are known to be active in their reduced form (Bostock, 2005). Besides, the induction of a number of glutathione S-transferases points to mobilization of the detoxification machinery against oxidized molecules (Gruhler et al., 2005). SA acts in a number of physiological situations and one of its modes of action could indeed be mobilization of antioxidant cell capacity against ROS produced during various stresses (Borsani et al., 2001; Rao et al., 2002; Danon et al., 2005; Mateo et al., 2006).

The biosynthetic pathway of ethylene was induced (e.g. a homologue of the tomato ethylene synthesis regulatory protein E8 and ACC oxidase) along with developmental genes related to ethylene signalling (ethylene response factor 1, ERF1, and members of the AP2/EREBP transcription factor family). The ethylene pathway was shown to be important for PR protein accumulation (Bostock, 2005). An overlap of SA and auxin response is also suggested: two genes coding for auxin efflux proteins were induced while a gene coding for auxin influx protein (AUX1) was suppressed. This should result in reduced auxin uptake by the cells.

5.7 Effects of wortmannin on the SA-responsive transcriptome

Because wortmannin at 30 μM, but not at 1 μM, inhibited SA-triggered PI 4-kinase activation, the effects of wortmannin on the SA transcriptome were studied at both concentrations. Treatments with 30 μM wortmannin (W30) were always compared to treatments with 1 μM wortmannin (W1) to exclude the effect already present at 1 μM. In the microarray analysis, genes for which the differential response in the presence of 30 μM wortmannin was the same either with or without SA represent an ambiguity with respect to their mode of regulation in response to SA and therefore these

were not considered for analyses where false positives might introduce a bias in the statistical tests. Doing so, it was possible to identify 112 genes (mostly SA-induced) whose response to SA was altered by 30 μM, but not by 1 μM wortmannin. For these genes, there was a good correlation between the effect of wortmannin on SA-triggered PI 4-kinase activation and on the SA transcriptome. The W30-sensitive pathway acts mainly positively on the SA response showing that it is involved in the generation of the SA response and not in turning it off. The cluster of W30-sensitive genes comprises transcription factors and pathogenesis-related genes important for SAR development (e.g., *NIMIN1*, *WRKY38*, several ERF/AP2 family transcription factors, TIR-class and NBS-LRR-class disease resistance proteins, various lectins and class IV chitinase) and redox potential regulating and regulated enzymes.

It was shown that 30 μM wortmannin inhibited SA-triggered PI 4-kinase activity while 1 μM wortmannin did not. It is therefore tempting to conclude that the genes, whose response to SA is inhibited by 30 μM, but not by 1 μM wortmannin, are responding via a PI 4-kinase dependent pathway. However, rigorously, it cannot be excluded that the effect of 30 μM wortmannin on the SA transcriptome is due to another mechanism. For instance, wortmannin has been reported to inhibit PIP 5-kinase (Jung et al., 2002) or some protein kinases, such as Ataxia-telangiectasia mutated (ATM) protein (Sarkaria et al., 1998). And thus wortmannin-sensitive protein kinases could act upstream of some of the identified genes in response to SA.

5.8 PI4Kβ1 may be involved in the SA response

The rate of PE synthesis in yeast mirrors to certain extent the activity of PI4Kα1 (Trotter et al., 1998). Among the effects caused by addition of 30 μM wortmannin, a reduced metabolic labelling of PE was observed in water treated Arabidopsis cells. However, when comparing SA treated and water treated cells the PE labelling was higher in SA treated cells in the presence of 30 μM wortmannin which contradicts with PI4Kα1 being involved in the SA response. The PI4K β-isoform is prevalent relative to the α-isoform in studies of whole plant extracts and AtPI4Kβ1 isoform is less sensitive to wortmannin than the AtPI4Kα1 when assayed *in vitro* (Stevenson-Paulik et al., 2003). This is in agreement with a relatively high wortmannin concentration (30 μM) needed for inhibition of the SA-induced PI decrease. The AtPI4Kβ1 was also involved in tip growth of root hairs via an interaction with Rab GTPase, RabA4b, and a Ca^{2+}

sensor, AtCBL1, leading to Ca^{2+}-dependent production of PI(4)P and PI(4,5)P_2 on the RabA4b-labelled membranes (Preuss et al., 2006).

It has to be underlined that the decrease of labelled PI upon SA treatment was only detected when cells were labelled for a very short time (5 or 15 min). During the short labelling time, only newly synthesized PI are preferentially labelled, and the radiolabelled PI are still near their site of synthesis. This suggests that SA-triggered PI 4-kinase is located close to the site of PI synthesis, most probably on the ER (Sandelius and Morré, 1987). GFP-tagged AtPI4Kβ1 was primarily located in the cytoplasm with increased fluorescence around dispersed, possibly Golgi, vesicles, when expressed in insect cells (Stevenson-Paulik et al., 2003). ER and Golgi are quite close in the cellular secretory pathway and thus the PI4Kβ1 isoform could be responsible for the observed SA-induced PI decrease.

Additional data come from experiments with *PI4Kβ1* knock-out mutant plants (i.e. *pi4kβ1* and *pi4kβ1pi4kβ2* lines). It is clear that whole plants cannot be completely substituted by a dedifferentiated cell suspension. Cell suspensions do not posses tissues and organs; there are no metabolite fluxes between distal parts, no stomatal movements related to transpiration and no sexual reproduction mechanisms. Each physiological mechanism discovered in cell suspensions is only a hint of what goes on in the whole plants and vice versa. Arabidopsis cells used in this research were derived from young cotyledon leaves thus it is possible that mechanisms working in the cells will be operative at least in leaves. However, simple transfer of knowledge from one system to another is not possible without additional verifications. Experiments with *PI4Kβ1* knock-out mutant plants revealed that the basal level of several SA marker genes was elevated even in non-treated plants. No compensation by other isoforms took place in the tested mutant lines. However confusing, there was a SA-related alteration of gene expression in *PI4Kβ1* knock-out mutant plants which was absent from *PI4Kβ2* knock-out mutants. This would make a compelling argument for the negative regulatory role of the PI4Kβ1 isoform on the SA response in plants. It is quite surprising when taking into account the fact that the W30-sensitive pathway acted mainly positively on the SA transcriptome in the cells. This may be a good example of the difficulties with the transfer of knowledge between cells and plants. Only the demonstration that marker genes of the W30-sensitive pathway are not induced in a knock-down or knock-out mutant cell line would ultimately identify the PI 4-kinase isoform responsible for the

positive modulation of SA response in cells. The presented data suggest that *PI4Kβ1* knock-out or knock-down mutant would be a good candidate for such experiments.

5.9 PLD influences SA-regulated gene expression

In the presence of primary alcohols PLD is capable of transphosphatidylation reaction in which the PA_{PLD} production is diverted to the production of the corresponding phosphatidylalcohol thus attenuating the PLD signal. The induction of *WRKY38* and *PR1* genes was inhibited in the presence of aliphatic alcohols and the inhibitory effect increased with the length of alkyl chain. The inhibition by *n*-butanol was not due to its non-specific effect on the polymer state of microtubules (Hirase et al., 2006) or on G-protein activation (Munnik et al., 1995) and thus can be attributed to direct inhibition of PA_{PLD} production (Meijer and Munnik, 2003). Expression of the SA marker genes in the presence of different alcohols revealed the importance of PA_{PLD} for correct development of the SA response.

With the intention to place the PLD activation to a specific time point of SA treatment an experiment was done where *n*-butanol was added in various times of SA treatment. The SA induction of *WRKY38* and *PR1* was inhibited by *n*-butanol even when added as late as three hours after SA treatment but the results were slightly different for the two genes. The discrepancy may be explained by the late time of RNA extraction (after 6 h of SA treatment) when later regulatory phenomena may interfere with the initial PLD signal and bias the result. For example, there may be two peaks of PLD activation and the *PR1* may require both or only the later one while *WRKY38* requires only the early one. However, PLD activity (either increased or basal) is necessary at least till the fourth hour of SA treatment. The early SA response could be studied only on *WRKY38* for which the inhibitory effect of *n*-butanol on its SA induction was detected already in the first hour of SA treatment. The fact that *WRKY38* is induced already in the first hour of SA treatment in cells disproves its NPR1 dependence published by Wang D et al. (2006) since NPR1 is induced and monomerized not earlier than in the second hour of INA treatment in plants (Mou et al., 2003).

The PLD isoform involved in the SA response is probably activated at the protein level (i.e. by increased translation or by activation of the protein already present) as the expression of no PLD isoform was significantly induced during the first 4 h of SA treatment. Plant PLD can be stimulated by both phosphorylation and activator

binding (Bargmann and Munnik, 2006). Therefore these two possibilities should be further investigated when looking for the cause of the observed activation.

Reversal of the *n*-butanol inhibition by application of exogenous PA could ultimately confirm the involvement of PLD in the studied response as documented for pollen tube growth (Potocký et al., 2003) and for the transport of glycoproteins from the ER to the Golgi complex in Chinese hamster ovary cells (Bi et al., 1997). Surprisingly, even PA itself inhibited the SA induction of *PR1* and *WRKY38*. The phenomenon could be explained by the fact that exogenous PA induced ABA-related processes (Zhang W et al., 2004). The antagonistic effect of ABA on plant response to pathogen was observed in several studies (reviewed in Mauch-Mani and Mauch, 2005). Apparition of PA_{PLD} is a very early event in ABA signalling (Zalejski et al., 2005) and thus it can be easily replaced by pre-treatment with exogenous PA while PA_{PLD} appears later in the SA response and may require some prerequisites to be correctly perceived as SA-related. ABA related PA is generated by the activity of prevalent PLD isoform in Arabidopsis (PLDα1; Zhang W et al., 2004). It is therefore possible that the SA response is mediated by another minor PLD isoform with different subcellular localization and with different effector proteins in its vicinity. The hypothesis of negative ABA cross-talk is further supported by the increasing inhibitory effect of PA with increasing time of PA pre-treatment (M. Flemr, unpublished data) allowing for development of the negatively intervening ABA pathway.

Another possible explanation would be the activation of PLC/DAGK pathway and negative influence of PA_{PLC} on the PLD pathway. Such activation of PLC/DAGK pathway by exogenous PA was never documented in the literature and moreover when PLC/DAGK pathway is activated in addition to PLD the two pathways act synergistically in most stress responses (Meijer and Munnik, 2003; Ramos-Diaz et al., 2007; Vergnolle et al., 2005). However, negative impact of other signalling pathways is only one possible cause. Alternatively, the exogenous PA might have remained in the plasma membrane and did not reach the right intracellular membrane compartment where it is normally produced during PLD activation.

5.10 Effects of PLD on the SA-responsive transcriptome

Because *n*-butanol, but not *tert*-butanol, inhibited SA-triggered expression of both *PR1* and *WRKY38*, the effects of both alcohols on the SA transcriptome were studied. Treatments with *n*-butanol were always compared to treatments with

tert-butanol to exclude the non-specific effect of alcohols. In the microarray analysis, genes for which the differential response in the presence of *n*-butanol was the same either with or without SA represent an ambiguity with respect to their mode of regulation in response to SA and therefore these were not considered for analyses where false positives might introduce a bias in the statistical tests. Doing so, it was possible to identify 97 genes (mostly SA-induced) whose response to SA was prevented by *n*-butanol, but not by *tert*-butanol. These were positively regulated by PLD in response to SA. Surprisingly, 117 genes were negatively regulated by PLD in response to SA. Contrary to the W30-sensitive pathway, PLD acts both positively and negatively on the SA transcriptome showing that PLD pathway is involved both in stimulation and down-regulation of the SA response. The two modes of regulation may be ensured by two distinct PLD isoforms which differ in their subcellular localization and thus modulate activity of different effector enzymes or transcription factors. Over-representation of the CBF1 binding site (RGGCCY) in promoters of negatively PLD regulated SA-induced genes and its under-representation in promoters of positively PLD regulated SA-induced genes suggest that CBF pathway may promote the negative action of PLD on the SA-induced transcriptome. Interestingly, CBF pathway was also placed downstream of PLD action in the cold response of Arabidopsis cells (Vergnolle et al., 2005).

The cluster of genes positively regulated by PLD contains transcription factors and pathogenesis-related genes important for SAR development (e.g., *NPR1*, *NIMIN1*, *NIMIN2*, *WRKY38*, *WRKY66*, *TGA1*, NBS-LRR-class disease resistance proteins, *PAD4*, and a small ubiquitin-like modifier 3). The presence of *NPR1* in this cluster of genes is in good agreement with the PLD regulation of the *PR1* because *PR1* expression is NPR1-dependent. Therefore the PLD regulation of *PR1* is probably indirect via the NPR1 or possibly via other element upstream of NPR1. Contrary to the W30-sensitive pathway, redox potential regulating and regulated enzymes are almost absent from the cluster positively regulated by PLD.

Clusters of genes negatively regulated by PLD, both SA-induced and SA-repressed, comprised many genes involved in transcription (a helicase, transcription factors, transcription initiation factor, phosphoribulokinase/uridine kinase-related protein, RNA polymerase and a Ran-binding protein 1 domain-containing protein which is a component of the nuclear transport mechanism) but even more of those involved in translation (amino acid transporter family protein, a tRNA synthetase, various

ribosomal proteins both nuclear and cytoplasmic, translation initiation factor, elongation factor EF-1α, a chaperonin, ubiquitin interaction motif-containing protein and a protease inhibitor). Besides these two groups, there was a relatively large group of genes specific for stress signalling pathways unrelated to the primary role of SA (e.g. dehydration, cold, salt stress, avirulence and hypoxia related genes, an ethylene responsive factor and an auxin efflux carrier protein). These may be negatively regulated, not to trigger some undesired stress responses. Negatively PLD regulated SA-induced genes contain *WRKY51*, negatively PLD regulated SA-repressed genes include several genes encoding lipid metabolizing enzymes (e.g. *PI4Kγ7*, monogalactosyldiacylglycerol synthase and alkaline phytoceramidase).

5.11 PI(4,5)P_2-dependent PLDs modulate the SA response

Alcohols differentially affect the gene response to SA and this response is not due to perturbation of microtubule cytoskeleton. It is a convincing argument for a role of PLD in the SA response in Arabidopsis cells. However, the ultimate confirmation may come only from characterization of the SA response in PLD mutant lines. The expression profile of *WRKY38* and *PR1* in the seven T-DNA knock-out mutant plants did not identify one particular PLD isoform but several lines had lower transcript levels of the two genes. Such result is in favour of functional redundancy within the plant PLD family. The question is, whether each isoform contributes partially to the observed phenotype or whether the knock-out of one isoform up-regulates the others. Such compensation would explain the difference observed between *pldγ1* and *pldγ3*. The two isoforms are the most homologous within the PLD family and even their promoter sequences are closer to one another than to that of PLDγ2 (data not shown). If PLDγ3 is the isoform mainly involved in the SA response, knock-out of PLDγ1 could activate the PLDγ1 promoter by a negative feed-back mechanism and thus also trans-activate the PLDγ3 promoter ultimately leading to over-expression of the SA-responsive isoform PLDγ3 and to the increased SA sensitivity of the *pldγ1* plants. Such hypothesis could be checked by measuring the transcript levels of PLDγ1 and PLDγ3 both in wild type plants and in the two mutant lines. Again these results obtained on plants are only indicative of what is going on in the cell suspensions. Only cells transformed with RNAi constructs against these PLD isoforms together with overexpressing mutants would identify the isoform involved in the SA response in cell suspensions.

PLD β, γ, ζ and even α isoforms (but only under low Ca^{2+} concentration) require $PI(4,5)P_2$ as activator (Bargmann and Munnik, 2006). Mutant plants *pldα1*, *pldβ1* and *pldγ3* had lowered expression of SA marker genes. In SA-treated cells the peak of PBut production correlated with the peak of $PI(4,5)P_2$ production. Based on these data, although obtained on different materials (i.e. plants and cells), a hypothesis can be drawn in which the PI 4-kinase activation leads to increased $PI(4,5)P_2$ production which in turn activates $PI(4,5)P_2$-dependent PLDs. After 45 min of SA treatment, concomitantly with the PLD activation, the PI 4-kinase activation is down-regulated in the cells. Moreover, the deactivation of PI 4-kinase depends on protein synthesis and thus possibly also on the gene expression. Such negatively acting gene expression could be PLD-driven because PLD regulates the SA transcriptome both positively and negatively.

5.12 PI 4-kinase and PLD act synergistically in the SA signalling pathway

An important synergism was observed when evaluating transcriptome overlaps between the W30-sensitive pathway-regulated and PLD-regulated clusters. Genes positively regulated by both pathways and negatively regulated by both pathways were significantly over-represented both in the cluster of SA-induced and SA-repressed genes. On the other hand mixed overlaps between one positive and one negative mode of regulation were under-represented. Moreover, promoter analysis of genes positively regulated both by a W30-sensitive pathway and PLD pathway in their SA induction revealed over-representation of W-boxes, *cis*-elements binding WRKY transcription factors. Therefore these are putative elements which could ensure the observed transcriptome overlap of genes induced by SA via the two pathways at the level of direct regulation of gene transcription.

PI 4-kinase is activated in the first minutes of SA treatment while PLD is activated later concomitantly with the peak of $PI(4,5)P_2$. Such observations are in favour of the hypothesis which places the two enzyme activities in a sequence of the same signalling pathway where PI 4-kinase is activated first and in turn activates PLD by elevating the $PI(4,5)P_2$ level (Figure 42). Besides the direct activation of PLD by $PI(4,5)P_2$, an indirect modulation of PLD activity can take place via the impact of $PI(4,5)P_2$ on actin cytoskeleton (Meijer and Munnik, 2003) or via the $PI(4,5)P_2$ binding to various lipid binding domains (PH, C2, MORN, PBR1; see chapters 2.2.2.6 and 2.2.3 for details) of interaction partners of PLD. Membrane microdomains enriched in

$PI(4,5)P_2$ can attract regulatory proteins bearing such domains and thus indirectly activate PLD. However, independent parallel activation of the two enzyme activities cannot be excluded.

There is always a reason for adding additional element to a signalling pathway, so one cannot expect that the two activities will regulate exactly the same set of genes. PLD could serve for fine tuning of the SA response but it could also enhance the initial signal or enlarge its impact on other genes not affected by the primary phosphoinositide signal. One such interaction can be outlined for the redox modifying enzymes that are SA-induced via the positive action of the W30-sensitive pathway and their up-regulation is further modulated by the negative PLD action.

Based on the presented data one cannot conclude whether the increase of $PI(4,5)P_2$ level is sufficient or even necessary for the PLD activation or whether other elements intervene to potentiate the phosphoinositide signal. However, the proposed model is worth further investigation. My findings add an additional tile to the fascinating mosaic of plant SA signalling and outline possibilities for future exploration of the research domain.

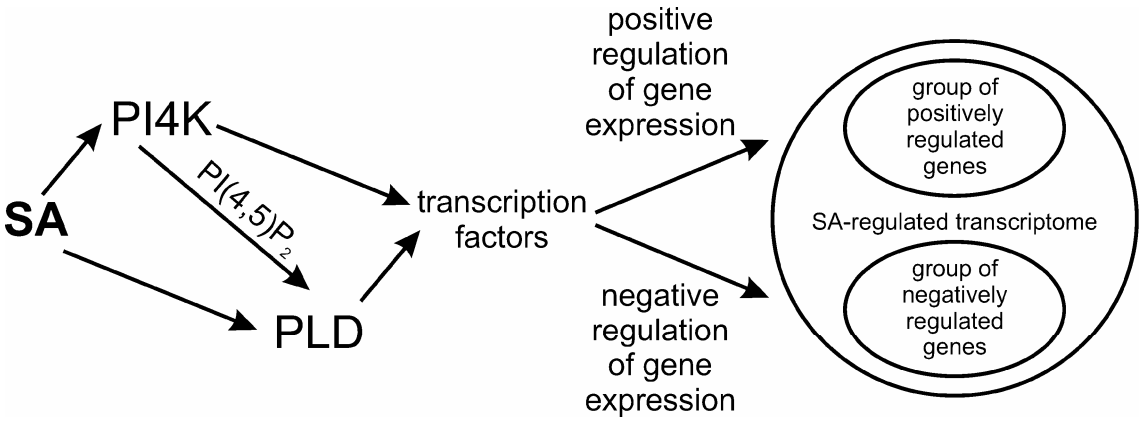


Figure 42. A model positioning PI 4-kinase and PLD in the SA signalling pathway and outlining the modes of regulation of SA transcriptome by the two enzyme activities.

6 CONCLUSIONS

- It was shown by metabolic radiolabelling of phospholipids that SA treatment induced rapid and early changes in labelled phosphoinositides. The level of labelled PI decreased while that of PI(4)P and especially that of PI(4,5)P_2 increased during the first hour of SA treatment. These changes could be inhibited by inhibitors of a type III PI 4-kinase.
- The early PI 4-kinase activation was not caused by changes in protein synthesis while the down-regulation of the response which came after 45 min of SA treatment depended on protein synthesis but was not due to changes in transcription of either PI synthase or type III PI 4-kinase isoforms.
- The early PI decrease could be inhibited by calyculin A, an inhibitor of Ser/Thr protein phosphatases 1, and by genistein, an inhibitor of Tyr protein kinases, which suggests at least two phosphorylation events upstream of the PI 4-kinase activation.
- No Ca^{2+} influx was detected in cells transformed with aequorin during the first ten minutes of SA treatment.
- A large collection of 474 SA-induced and 317 SA-repressed genes was identified in a microarray experiment with CATMA chips. Potential *cis*-regulatory elements differentially represented in the promoters of genes in the two clusters are reported.
- A cluster of 112 SA responsive genes positively regulated by a 30 μM wortmannin-sensitive pathway was identified; it comprises genes regulated by a type III PI 4-kinase. WRKY transcription factors may be upstream of the positively regulated cluster of SA-induced genes. The cluster contains genes coding for transcription factors, disease resistance related proteins and redox potential regulating and regulated enzymes.
- Expression of several genes regulated by a 30 μM wortmannin-sensitive pathway in cells was disturbed in T-DNA knock-out plants with T-DNA insertion in *PI4Kβ1*.
- Treatment with ethanol and *n*-butanol, but not with *sec*- and *tert*-butanol, inhibited SA induction of *PR1* and *WRKY38*, marker genes of the SA pathway, as soon as the first hour of SA treatment. The inhibition was dose-dependent.

Based on their expression profile under various conditions, the two genes could be claimed as PLD-regulated in response to SA.

- No PLD isoform was substantially induced during the first 4 h of SA treatment, suggesting that the PLD activation is due to either increased protein translation or activation of the protein already present.
- Clusters of 97 genes positively and 117 genes negatively regulated by PLD in response to SA were identified. WRKY transcription factors may be upstream of the positively PLD regulated cluster and CBF pathway may be upstream of the negatively PLD regulated cluster of SA-induced genes. Positively PLD regulated genes include those coding for transcription factors, including *NPR1*, and disease resistance related proteins. Negatively PLD regulated genes comprise those involved in transcription, translation and in stress responses different from SAR.
- Mutant plants *pldα1*, *pldβ1* and *pldγ3* had a lowered expression of SA marker genes *PR1* and *WRKY38,* suggesting that these isoforms may be activated in response to SA.
- Overlap analysis of gene clusters regulated by a 30 μM wortmannin-sensitive pathway and by PLD revealed that these enzyme activities act synergistically in the SA signalling pathway. The possibility that the observed synergism is ensured by $PI(4,5)P_2$ is discussed.

7 ABBREVIATIONS

ABA	abscisic acid
ABI	abscisic acid-insensitive
ABRE	abscisic acid response element
ACD	accelerated cell death
AFLP	amplified fragment length polymorphism
AGRIS	Arabidopsis gene regulatory information server
ARF-GAP	ADP-ribosylation factor-GTPase activating protein
AtcisDB	*Arabidopsis thaliana cis*-regulatory database
ATM	Ataxia-telangiectasia mutated
AUX	auxin influx protein
Avr	avirulence protein
BiP	luminal binding protein
BTH	benzothiadiazole S-methyl ester
bZip	basic leucine zipper
C2	calcium and lipid binding
cADPR	cyclic ADP-ribose
CaM	calmodulin
CaMK	calmodulin-dependent protein kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CATdb	complete Arabidopsis transcriptome database
CATMA	complete Arabidopsis transcriptome microarray
cDNA	complementary DNA
CDPK	Ca^{2+}-dependent protein kinase
CEV	constitutive expression of vegetative storage protein 1
CP	capping protein
cPLA	cytosolic phospholipase A
CPR	constitutively expressed resistance
c_T	threshold cycle
CTR	constitutive expression of ethylene-regulated genes
DAD	defective in anther dehiscence
DAG	diacylglycerol

DAGK	diacylglycerol kinase
DEPC	diethylpyrocarbonate
DGPP	diacylglycerol pyrophosphate
DIR	defective in induced resistance
DMSO	dimethyl sulfoxide
DRP	dynamin-related protein
DTH	detachment
EDR	enhanced disease resistance
EDS	enhanced disease susceptibility
EDTA	ethylen-diamine tetra-acetic acid
EF	elongation factor
ER	endoplasmic reticulum
ERF	ethylene response factor
EST	expressed sequence tag
ETR	ethylene receptor
FFA	free fatty acid
FRA	fragile fiber
FWER	familywise error rate
FYVE	phosphatidylinositol 3-phosphate binding domain
G	G-protein
GA_3	gibberellic acid
GEO	gene expression omnibus
GFP	green fluorescent protein
GR	glucocorticoid receptor
GSH	glutathione, reduced
GSSG	glutathione, oxidized
GST	gene sequence tag
HR	hypersensitive response
ICS	isochorismate synthase
INA	2,6-dichloroisonicotinic acid
IP_3	D-*myo*-inositol 1,4,5-trisphosphate
IP_3-R	receptor for D-*myo*-inositol 1,4,5-trisphosphate
IP_6	D-*myo*-inositol 1,2,3,4,5,6-hexakisphosphate

IPL	isochorismate pyruvate lyase
ISR	induced systemic resistance
JA	jasmonic acid
LCAT	lecithin:cholesterol acyltransferase
LOESS	locally weighted scatter plot smooth
LOX	lipoxygenase
LPP	diacylglycerol pyrophosphate phosphatase
LTP	lipid transfer protein
MAPK	mitogen activated protein kinase
MATE	multidrug and toxin extrusion
MeJA	methyljasmonate
MeSA	methylsalicylate
MIAME	minimum information about a microarray experiment
MIPS	Munich information center for protein sequences
MJE	methyljasmonate esterase
MLCK	myosin light chain kinase
MORN	membrane occupation and recognition nexus
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NAE	*N*-acylethanolamine
NahG	salicylate hydroxylase
NAPE	*N*-acylphosphatidylethanolamine
NB	*n*-butanol
NIM	non-inducible immunity
NIMIN	non-inducible immunity interactor
NO	nitric oxide
NPR1	non-expressor of pathogenesis related 1
PA	phosphatidic acid
PAD	phytoalexin deficient
PAF-PLA_2	platelet activating factor-specific acylhydrolase
PAK	phosphatidic acid kinase
PAL	phenylalanine-ammonia lyase
PAO	phenylarsine oxide

PA_{PLC}	phosphatidic acid generated via the phospholipase C/diacylglycerol kinase pathway
PA_{PLD}	phosphatidic acid generated by phospholipase D activity
PAT-PLA	patatin-like phospholipase A
PBR1	phosphatidylinositol 4,5-bisphosphate-binding domain in phospholipase D
PBut	phosphatidylbutanol
PC	phosphatidylcholine
PCD	programmed cell death
PC-PLC	phosphatidylcholine-specific phospholipase C
PDF	plant defensin
PDK	3-phosphoinositide-dependent protein kinase
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PH	pleckstrin homology
PHD	plant homeodomain
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol 3-phosphate
$PI(3,4)P_2$	phosphatidylinositol 3,4-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-trisphosphate
$PI(3,5)P_2$	phosphatidylinositol 3,5-bisphosphate
PI(4)P	phosphatidylinositol 4-phosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI(5)P	phosphatidylinositol 5-phosphate
PI3K	phosphatidylinositol 3-kinase
PI4K	phosphatidylinositol 4-kinase
PI5K	phosphatidylinositol 5-kinase
PIP	phosphatidylinositolmonophosphate
PIP_2	phosphatidylinositolbisphosphate
PIP_3	phosphatidylinositoltrisphosphate
PIP5K	phosphatidylinositolmonophosphate 5-kinase
PI-PLC	phosphoinositide-specific phospholipase C
PLA	phospholipase A

PLC	phospholipase C
PLD	phospholipase D
PM	plasma membrane
PPC	plant phosphatidylinositol 4-kinase charged
PPI	polyphosphoinositide
PR	pathogenesis related
PS	phosphatidylserine
PTP	protein Tyr phosphatase
PUFA	polyunsaturated fatty acid
PVP	polyvinylpyrrolidon
PX	phagocyte oxidase
QPCR	quantitative PCR
QRT-PCR	quantitative RT-PCR
R	resistance protein
RARGE	RIKEN Arabidopsis genome encyclopedia
RBOH	respiratory burst oxidase
RNAi	RNA interference
ROS	reactive oxygen species
RP-HPLC	reverse phase HPLC
SA	salicylic acid
SABP2	salicylic acid binding protein 2
SAC	supressor of actin mutation domain phosphoinositide phosphatase-like
SAR	systemic acquired resistance
SDS	dodecyl sulfate, sodium salt
SFD	suppressor of fatty acid desaturase deficiency
SGR	shoot gravitropism
SID	salicylic acid induction deficient
SIPK	salicylic acid-induced protein kinase
SNI1	suppressor of non-expressor of pathogenesis related 1, inducible
sPLA	secretory phospholipase A

SSN	suppressors of ‘suppressor of ‘non-expressor of pathogenesis related 1, inducible’
SUMO	small ubiquitin-like modifier
TAIR	the Arabidopsis information resource
TB	*tert*-butanol
T-DNA	transposable DNA
TGN	*trans*-Golgi network
TMV	tobacco mosaic virus
TNV	tobacco necrosis virus
tRNA	transfer RNA
UPR	unfolded protein response
VTC	ascorbate-deficient
W1	1 μM wortmannin
W30	30 μM wortmannin
Why	Whirly transcription factor
WIPK	wound-induced protein kinase
ZAC	zinc and Ca^{2+} binding

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9 SUPPLEMENTAL DATA

Supplemental Table I. *List of chemicals and kits used in this research.*
All chemicals were of analytical grade unless stated otherwise.

Chemical or kit	Manufacturer
Acetic acid	VWR
Aceton	VWR
Acetonitrile (HPLC grade)	VWR
Agarose	Sigma-Aldrich
Boric acid	Sigma-Aldrich
Calyculin A	Sigma-Aldrich
Chloroform	VWR
Choline chloride	Sigma-Aldrich
Cycloheximide	ICN Biomedicals
Daidzein	Sigma-Aldrich
Deoxyribonuclease I	Sigma-Aldrich
3,4-dephostatin	Sigma-Aldrich
2,4-dichlorphenoxyacetic acid (2,4-D)	Sigma-Aldrich
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
2,6-di-*tert*-butyl-4-methylphenol	Sigma-Aldrich
DNAse I, RNAse free	Sigma-Aldrich
dNTP mix	Finnzymes
Dodecyl sulfate, sodium salt (SDS)	Sigma-Aldrich
Ethanol	VWR
Ethidium bromide	ICN Biomedicals
Ethoxyquin	Sigma-Aldrich
Ethylenediaminetetraacetic acid, disodium salt (EDTA)	ICN Biomedicals
Formic acid	VWR
Gamborg B5 basal medium with vitamins	Duchefa
Genistein	Sigma-Aldrich
iQ SYBR Green Supermix	Bio-Rad
iScript cDNA Synthesis kit	Bio-Rad
Isoamylalcohol	VWR
Isooctane	VWR
K252-a	Sigma-Aldrich
LiCl	Sigma-Aldrich
Methanol	VWR
Methanol (HPLC grade)	VWR
n-butanol	VWR
Okadaic acid	Sigma-Aldrich
Oligo$(dT)_{15}$ primer	Eurogentec
Omniscript RT kit	Qiagen
Oryzalin	Duchefa
$^{33}P_i$	GE Healthcare
Phenol saturated with 0.1 M citrate buffer pH 4.3	Sigma-Aldrich
Phenylarsine oxide (PAO)	Sigma-Aldrich
Phosphatidic acid (dioctanoyl), sodium salt	Sigma-Aldrich
Phospholipid standards (non-labelled)	Sigma-Aldrich
Polyvinylpyrrolidon 40,000 (PVP-40)	Sigma-Aldrich
Primuline	Sigma-Aldrich
2-propanol	VWR
Pyridine	Sigma-Aldrich
QPCR primers	Sigma-Aldrich
Salicylate, sodium salt	Sigma-Aldrich
sec-butanol	VWR
Semiquantitative PCR primers	Eurogentec
Staurosporine	Sigma-Aldrich

Chemical or kit	**Manufacturer**
Sucrose	Lachema
Taq DNA polymerase	New England Biolabs
tert-butanol	VWR
TLC plates coated with Silica gel 60 (20 cmx20 cm)	Merck
trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid	Sigma-Aldrich
TRI reagent	Sigma-Aldrich
Trizma base (Tris)	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Wortmannin	Sigma-Aldrich

Supplemental Table II. *List of primers used for characterization of T-DNA insertion lines.* All primers were desalted by manufacturer.

Gene	AGI locus	T-DNA line	Primer	Sequence (5'→3')
PLDα1	At3g15730	SALK_053785	RP	TGGAGGGAAGAACTATGAGCC
			LP	GCAAAAGAACTCCCAAGGAAG
PLDβ1	At2g42010	SALK_079133	RP	GCACATGTCCCAGAAGGGATG
			LP	CAAGCCCACCGACAAAAGCTA
PLDβ2	At4g00240	SALK_113607	RP	CCCTAGACTCTCTGGCTCCAC
			LP	CAATGGTCTTGACAACCTTGG
PLDγ1	At4g11850	SALK_066687	RP	GGATTCAAACAAGGACTTGGG
			LP	AAGAAATTGGGACAAACCAGG
PLDγ2	At4g11830	SALK_089965	RP	GCTTCAGAAAGTCCCATGATG
			LP	ATTGTTCATGGAGCTTATCGC
PLDγ3	At4g11840	SALK_084335	RP	CATCATTTGCATGGTTTTGTG
			LP	ATCCGCTATTCTTGATCCATG
PLDζ1	At3g16785	SALK_083090	RP	CGTCGCATAATAGATGCTTGG
			LP	TGAAAAGCATGGAAATTTTCG
PLDζ2	At3g05630	SALK_094369	RP	TCGCACCACGAGAAAATAGTG
			LP	CCGGAGATGAAGAATTGGTTC
-	-	-	LBa1	TGGTTCACGTAGTGGGCCATCG
-	-	-	LBb1	GCGTGGACCGCTTGCTGCAACT

Supplemental Table III. *List of primers used for semiquantitative RT-PCR.*
All primers were desalted by manufacturer.

AGI locus	Gene name	Primer	Sequence (5'→3')	Product size (bp)
At1g02450	NIMIN1	sense	TGTGGAGAGCAAAGAGACGAT	345
		antisense	AAAGCCTTGTCTTCGTTTCG	
At1g04980	PDIL	sense	AGAATCCAATGCTGGTCCTG	383
		antisense	GCAAATTCCCTTTTCCTCCT	
At1g23390	-	sense	CTTTCCGTAGCGGTGAGTTC	327
		antisense	CGTTAACGGCCAATCAGAAT	
At1g31580	-	sense	ACGAATGGAACTCCGTGAAC	313
		antisense	ATGACAAACACGTGACATGC	
At1g49340	PI4Kα1	sense	AACCACAGGCTTGCATTTTC	481
		antisense	TGAGTCATTTCGTGGCTCAG	
At1g64280	NPR1	sense	TGTTCCTCCCTCTTTTGCAG	480
		antisense	TCTGTCAGGGACGAATTTCC	
At2g14610	PR1	sense	TTCTTCCCTCGAAAGCTCAA	411
		antisense	CGTTCACATAATTCCCACGA	
At2g44370	-	sense	TCCCCACCTTACGAGTCAAC	423
		antisense	TTGAGCTTCCAACATTGCAG	
At2g47730	GST6	sense	CATCCCCGTCGATATGAGAG	400
		antisense	GAAGGAATCACCAGCCAAGA	
At3g04920	RPS24A	sense	TCCAGGAAGCAGTTCGTTATTGAT	345
		antisense	TCACTTCTTCTTGGCATCACCAG	
At5g03350	-	sense	TTCTCTCACGGCCAAAGTCT	376
		antisense	TCCCAATTCCTGTCTGAACC	
At5g09350	PI4Kβ2	sense	AGCATGGCTGGATATTCTTTG	562
		antisense	CCTGTTTAGTCTCTGATCAGGTTG	
At5g22570	WRKY38	sense	CCGGATCCGATTTACTACGA	485
		antisense	CTTTCACTGCCAGATGACGA	
At5g48540	-	sense	GAACCAATCCGACTCAAGGA	475
		antisense	CGGAGAGAGATGAACGGAAG	
At5g64070	PI4Kβ1	sense	GAATTCTCCTGGTGGCGTAA	406
		antisense	CCCTTATTAGGTTGGTTCTTTGG	

Supplemental Table IV. *List of primers used for QRT-PCR.*
All primers were purified on reverse phase cartridge by manufacturer (Sigma-Aldrich).

AGI locus	Gene name	Primer	Sequence (5'→3')
At1g17745	PGDH	sense	GGCAGCATTAGATGTGTTCTG
		antisense	CTCAATGGCTACACCTTCCTG
At1g21270	WAK2	sense	CCAAACGGTTGTCAAGACATC
		antisense	TTAAGGGAATCTTTGCGGTAA
At1g31580	ECS1	sense	ATGGAACTCCGTGAACTTGGA
		antisense	GCACCTTCTGGGACGATATG
At1g34750	-	sense	TAGCGGTTTCTCGTGCTTTT
		antisense	GGTTAGCCATCACCTTCCAT
At1g49340	PI4Kα1	sense	TGGGCTGCTTGTTCAATTAC
		antisense	AACTCGTTCAGGAGGGTAGGA
At1g51040	PI4Kα2	sense	CGACACGACCGATGAAAAC
		antisense	GCAATAAAACGCCGAAATC
At1g52570	PLDα2	sense	TATCCGAGCACAAGAAGCAC
		antisense	GCACCATCCATTGACCTCTG
At1g55180	PLDε	sense	AGATGGGGAGTTTGAAGCAG
		antisense	ATGTATGTGTCGTCCACTATCATG
At1g66970	-	sense	AAACGCTACAAAACAAGTGGAT
		antisense	AAGGGTACACCGTCGCTAGA
At1g68000	PIS1	sense	GCAAAGAACCAATCTGAAAATC
		antisense	CAATGTCAAAGCCAGGAGAA
At1g80590	WRKY66	sense	TCTCGTTTGCTCTGTCTTCTCA
		antisense	GGTCTTTCGCATAGGCACAT
At2g14610	PR1	sense	TTGTAGGTGCTCTTGTTCTTCC
		antisense	CAACCCTCTCGTCCCACT
At2g42010	PLDβ1	sense	GCATTCTCCCCACAAGATTA
		antisense	CTGCTTTTCCGACTCAACG
At3g04920	RPS24A	sense	AGCGGACGAAAACACTCA
		antisense	GAAGAACATCAATAACGAACT
At3g05630	PLDζ2	sense	GAACGGAATGAAGTGGATGG
		antisense	CGGGATGCACGAGAAGAC
At3g15730	PLDα1	sense	AGAAACCAGACCCCGACACT
		antisense	TGATGTTAGCAGACCCAATGA
At3g16785	PLDζ1	sense	GTCTTAGGCTCTCTTTGTGGT
		antisense	TGTGTTTGTCTTTGCGGTTG
At3g28540	-	sense	GCAGAGGAAGAACACAAAAAGAA
		antisense	GACAACACCATACCAAAACACAA
At4g00240	PLDβ2	sense	AACCCATGACGAGGAGACAC
		antisense	GATGGTTCCAACTTCCCTCT
At4g11830	PLDγ2	sense	TCGTGGAATAGGGAAAGGAA
		antisense	TCAATAGAACGGAAAACCTGAAC
At4g11840	PLDγ3	sense	TTGAGGAACGCTGGATGAAG
		antisense	GCTTCGGAAAGTCCCATTAT
At4g11850	PLDγ1	sense	TCTGGGATTCAAAACACAAGG
		antisense	TGATGCGTGTAGATTGTTCCA
At4g35790	PLDδ	sense	GCACCAAAGATACTGAAATCG
		antisense	GCCTAAATGCTCTGCCCATA
At4g38570	PIS2	sense	CTTCTCATAGCAACGAACCAAAC
		antisense	GGACCAACCAAATATGCTCAAAG
At5g09350	PI4Kβ2	sense	TCTCGGTGGAGACGCATAGT
		antisense	CACTTTCCAAACCAGTCCTCA
At5g22570	WRKY38	sense	GCCCCTCCAAGAAAAGAAAG
		antisense	CCTCCAAAGATACCCGTCGT
At5g25370	PLDα3	sense	ATGACTTTCACCAGCCGAAC
		antisense	CCTTCTACCACTACCTTGTTTCA
At5g26690	-	sense	ACCGTGGTGGGTACTATGGA
		antisense	GGGTCCTTCTTAGGGTCTGG
At5g64070	PI4Kβ1	sense	AAGTCAGAGGTGAAGGTGAAAC
		antisense	ATCATTGTTCAGCCCAGTCC

List of electronic supplements

Supplemental Figure 1. Summary of interesting outcomes from the MapMan analysis.

Supplemental Table V. List of genes differentially expressed upon SA treatment in the microarray experiment with wortmannin.

Supplemental Table VI. Overlap between unfolded protein response-regulated transcriptome and transcriptome regulated by SA in the microarray experiment with wortmannin.

Supplemental Table VII. Expression profiles of genes involved in phospholipid signalling in the microarray experiment with wortmannin.

Supplemental Table VIII. Expression profiles of genes regulated by a W30-sensitive pathway in response to SA.

Supplemental Table IX. Expression profiles of genes regulated either positively or negatively by PLD in response to SA.

Supplemental Table X. Expression profiles of genes involved in phospholipid signalling in the microarray experiment with alcohols.

Supplemental Table XI. Expression profiles of genes from overlaps of clusters regulated via a W30-sensitive pathway and via PLD in response to SA.