Patatin-Related Phospholipases (pPLA) and Other Cytosolic Components in AUXIN BINDING PROTEIN1 (ABP1)-Mediated Auxin Signaling are Revealed by Using Early Auxin-Induced Gene Expression as a Biotest in *Arabidopsis thaliana*

Von der Naturwissenschaftlichen Fakultät

der Gottfried Wilhelm Leibniz Universität Hannover

Zur Erlangung des Grades

Doktorin der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

Dipl.-Biol. Corinna Labusch

geboren am 14.06.1984 in Herne

2013

Referent : Prof. Dr. Günther Scherer
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Tag der Promotion: 27.03.2013

Für Dennis

ABSTRACT

Patatin-related phospholipases (pPLA) are supposed to have a function in auxin signal transduction. The pPLA activity is induced 2-5 min after auxin treatment (Paul et al., 1998) and expression of some auxin-induced genes was decreased 2 hours after treatment with a pPLA-inhibitor (Scherer, 2007). This rapid pPLA activation by auxin excludes TIR1 as the relevant receptor. A study on an *abp1/ABP1* heterozygous mutant showing mis-regulated early auxin-induced gene expression 30 min after auxin treatment (Effendi et al., 2011) led to the hypothesis of an involvement of pPLAs in ABP1-mediated auxin signal transduction. A biotest was designed to investigate early auxin response independent from morphological phenotype expermiments. Several early auxin-induced genes from the *IAA*-, *SAUR*- and *GH3*-family were chosen and gene expression was measured 10 and 30 min after auxin application in all ten *ppla* T-DNA insertional mutants. The results show a high number of early auxin-induced genes with delayed or increased gene expression 10 and 30 min after auxin treatment. This and the fact that none of the *pPLA* genes themselves were auxin-induced definietely excludes TIR1/AFBs as the responsible receptors. These rapid responses connect pPLAs to ABP1-signal transduction because of similar results in mis-regulation of early auxin-induced gene expression in *abp1* mutants (Effendi et al., 2011; Effendi and Scherer, 2011). Next to pPLA activity in ABP1-mediated auxin signaling more cytosolic components are needed for this network. Thus mutants of different kinds of potential components were chosen to be tested with this biotest. The mutant group consists of several membrane lipid-deficient mutants (*fad2-1*, *fad6-1*, *ssi2*, *lacs4*, *lacs9*, *lacs4 las9*), protein kinases (*cpk3*, *cpk3 ox*, *d6pk-1*, *d6pk-1 d6pk1-1 d6pk2-2*) and protein phosphatases (*ibr5*, *pp2a*). Some of them were already described to be involved in auxin responses (Michniewicz et al., 2007; Strader et al., 2008; Zourelidou et al., 2009) and therefore selected. Membrane lipid-deficient mutants were chosen because of hydrolysis of phospholipids. As shown for *pplas* the tested mutants of this group also showed delayed or increased early auxin-induced gene expression. Altogether this work demonstrates an involvement of pPLAs and other cytosolic components in ABP1-mediated auxin signal transduction. With the used biotest several mutants were identified to be auxin mutants although only for a few an auxin-related developmental phenotype could be shown.

Keywords: Auxin-Signaltransduction, Phospholipases, ABP1

ZUSAMMENFASSUNG

Patatin-ähnlichen Phospholipasen (pPLA) wird eine Funktion in der Auxin Signaltransduktion zugesprochen. Die pPLA-Aktivität wird innerhalb von 2 bis 5 min nach Auxin Behandlung induziert (Paul et al., 1998) und nach einer zweistündigen Behandlung mit einem pPLA-Inhibitor war die Expression einiger auxin-induzierte Gene reduziert (Scherer 2007). Diese schnelle auxininduzierte pPLA-Aktivierung schließt TIR1 als den verantwortlichen Auxin-Rezeptor aus. Dagegen zeigt eine Studie mit einer *abp1/ABP1* heterozygoten Mutante eine defekte Regulierung früher auxininduzierter Gene 30 min nach Auxin Behandlung (Effendi et al., 2011). Dies führt zu der Hypothese, dass die pPLAs in der ABP1-vermittelten Auxin Signaltransduktion beteiligt sind. Um diese Hypothese zu testen wurde ein Biotest entwickelt, mit dem frühe Auxin-Antworten unabhängig von Wachstumsversuchen untersucht werden können. Dazu wurden einige frühe auxininduzierte Gene der *IAA*-, *SAUR*- and *GH3*-Familie ausgewählt und die Genexpression 10 und 30 min nach Auxin Behandlung in allen zehn ppla T-DNA Insertionsmutanten gemessen. Die Ergebnisse zeigen, dass eine große Anzahl der getesteten frühen auxin induzierten Gene eine verspätete oder erhöhte Expression 10 und 30 min nach Auxin Zugabe aufwiesen. Zusammen mit dem Ergebnis, das die *pPLA* Gene selbst nicht durch Auxin induziert sind, kann eine Rezeptor Beteiligung von TIR1/AFBs hier ausgeschlossen werden. Aufrgund derselben defekten Genregulation früher auxininduzierter Gene in *abp1* Mutanten (Effendi et al., 2011; Effendi und Scherer, 2011) können die pPLAs zur ABP1-vermittelten Signaltransduktion zugeordnet werden. Neben der pPLA Aktivität im ABP1-vermittelten Signalweg werden mehr cytosolische Komponenten für dieses Netzwerk benötigt. Daher wurden Mutanten von verschiedenen potentiellen Komponenten ausgewählt und mithilfe des Biotests untersucht. Diese Gruppe besteht aus mehreren Mutanten mit veränderter Fettsäurekonzentration in den Membranlipiden (*fad2-1*, *fad6-1*, *ssi2*, *lacs4*, *lacs9*, *lacs4 las9*), Protein Kinasen (*cpk3*, *cpk3 ox*, *d6pk-1*, *d6pk-1 d6pk1-1 d6pk2-2*) und Protein Phosphatasen (*ibr5*, *pp2a*). Einigen aus der Gruppe wurde bereits eine Funktion in Auxinantworten nachgewiesen (Michniewicz et al., 2007; Strader et al., 2008; Zourelidou et al., 2009) und wurden daher ausgewählt. Die Aktivität der pPLAs ist die Hydrolyse von Phospholipiden, weshalb die Mutanten mit veränderter Fettsäurekonzentration in den Membranlipiden ausgewählt wurden. Wie für die pPLA Mutanten gezeigt werden konnte, haben auch die weiteren

getesteten Mutanten Defekte in der Regulation früher auxininduzierter Gene. Zusammenfassend zeigt diese Arbeit eine Beteiligung von pPLAs und anderen cytosolischen Komponenten an der ABP1-vermittelten Auxin Signaltransduktion. Mit dem hier verwendeten Biotest konnten mehreren Mutanten als Auxin-Mutanten identifiziert werden, obwohl nur wenige einen wachstumsbedingten Auxin-Phänotyp aufwiesen.

Schlagworte: Auxin-Signaltransduktion, Phospholipasen, ABP1

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ABBREVIATIONS

ABP1	AUXIN BINDING PROTEIN1
Col-0	Columbia-0
CPK3	Calcium-dependent protein kinase 2
FAD	Fatty acid desaturase
IAA	Indoleacetic acid
IBR5	INDOLE-3-BUTYRIC ACID RESPONSIBLE 5
LACS	Long-chain acyl-CoA synthetase
1-NAA	1-naphthaleneacetic acid
pPLA	Patatin-related phospholipase
PP2A	Protein phosphatase 2A
qRT-PCR	Qualitative Real Time Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SSI2	Stearoyl acid carrier protein desaturase
TIR1	TRANSPORT INHIBITOR RESISTANT1
Ws	Wassilewskija

CHAPTER 1:

GENERAL INTRODUCTION

GENERAL INTRODUCTION

An important part of plant research is the investigation of mechanisms, which allow the plant to deal with and react to various internal signals and environmental influences. Because of their sessile way of life, plants need to adapt their growth, development and reproduction to different environmental signals, like varying light conditions or changes in temperature among the seasons. Plant hormones, which are small signaling molecules, play an essential role in regulating and coordinating plant growth and are involved in all developmental processes. The phytohormone auxin acts as a versatile signal for many developmental responses like cell differentiation, cell division, cell elongation, embryogenesis, morphogenesis, organogenesis, and reproduction. Furthermore auxin plays a role in mediating responses to different environmental signals like gravity and light (Davies, 1995; Leyser, 2006; Benjamins and Scheres, 2008; Mockaitis and Estelle, 2008; Chapman and Estelle, 2009).

Auxin signal transduction and the two-receptor concept

To give a response to an auxin signal a detection mechanism, a transfer of the signal and an influence on gene expression via a signal transduction cascade is required. Although much is known about auxin function in plant development the molecular mechanism of auxin signaling pathways is still poorly understood (Leyser, 2001; Scherer, 2002; Dharmasiri and Estelle, 2004; Mockaitis and Estelle, 2008). In plant signal transduction the intercellular transmission of signals starts at a receptor. Two potential auxin receptors are known so far: AUXIN BINDING PROTEIN1 (ABP1) and TRANSPORT-INHIBITOR-RESISTANT1 (TIR1). ABP1 was at first described as a protein having a high specificity of binding and affinity to auxin (Hertel et al., 1972; Löbler and Klämbt, 1985, Inohara et al., 1989; Hesse et al., 1989; Jones and Venis, 1989) and several mutants of ABP1 are known to have various impacts on auxin functions (Chen et al., 2001b; David et al., 2007; Braun et al., 2008; Effendi et al., 2011; Robert et al., 2010; Xu et al., 2010). ABP1 is localized on the plasma membrane where it is secreted to some extent to the extracellular space and to the endoplasmatic reticulum (ER) (Jones and Herman, 1993; Tian et al., 1995; Henderson et al., 1997). ABP1 is essential for embryogenesis (Chen et al., 2001), postembryonic shoot and root development (Braun et al., 2008; Tromas et al., 2009).

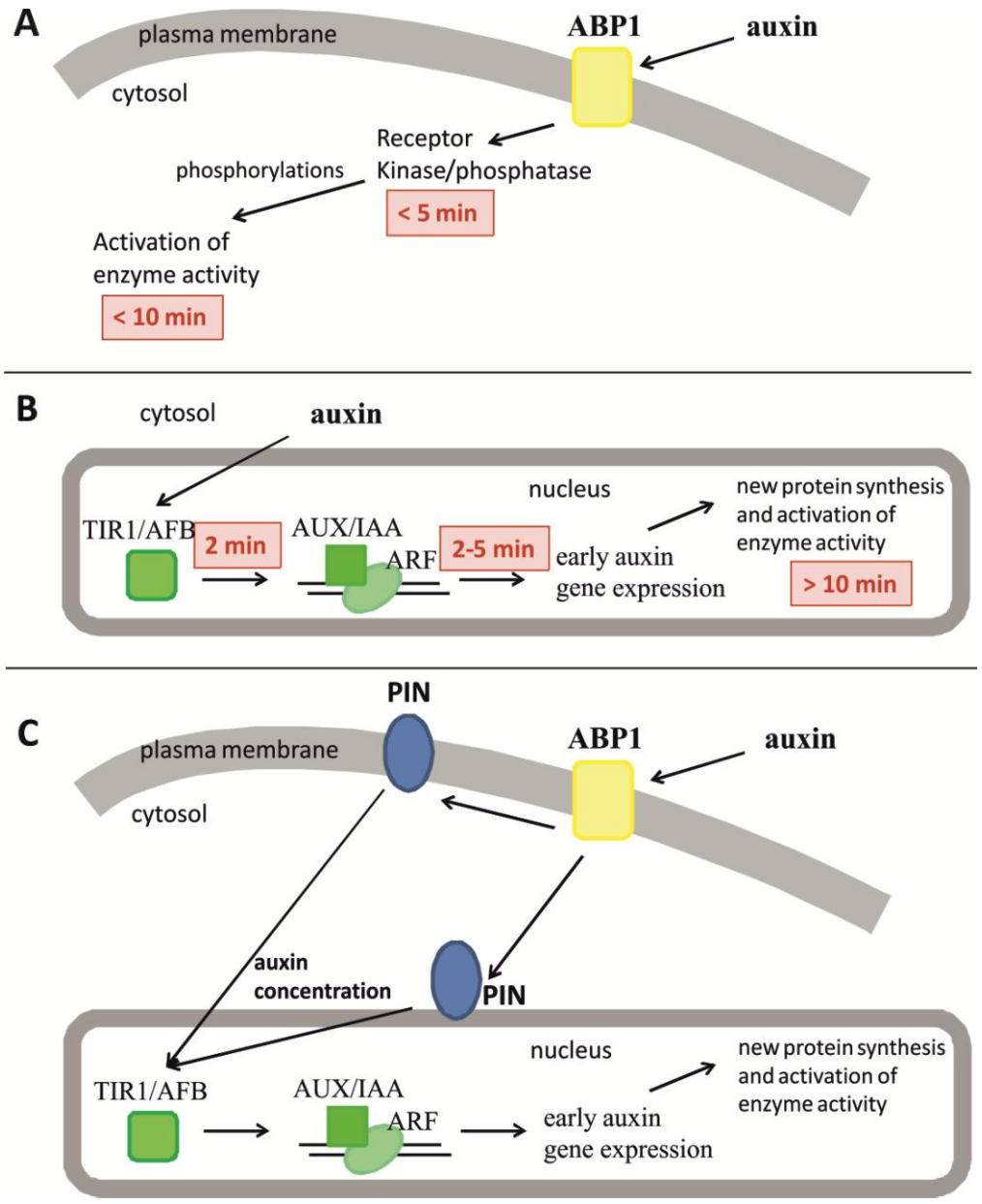

Figure 1.1: Model of ABP1-mediated auxin signal transduction and interaction of ABP1 and TIR1.

A: Auxin perception of ABP1 leads to a cytosolic signal cascade. Activation of kinases and phosphatases within minutes leads to an activation of further enzyme activity by phosphorylation within or faster 10 min. **B**: TIR1 receptor activity leads to IAA protein degradation within 2 min which induces expression of early auxin-induced genes in 2-5 min the earliest and then to transcription-dependent auxin responses later than 10 min. **C**: Cytosolic signaling pathways of ABP1 may include the regulation of the phosphorylation status of PIN proteins and indirectly control this way auxin concentration in the cell by extracytosolic auxin transport. PIN proteins may also control auxin concentration in the nucleus where auxin is sensed by the receptor TIR1. (combined from several sources)

Furthermore ABP1 plays a role in auxin transport by influencing PIN proteins (Robert et al., 2010; Effendi et al., 2011; Effendi and Scherer, 2011). Figure 1.1 A shows a simplified model of ABP1 activity and how ABP1-mediated auxin signal transduction pathway across the plasma membrane may happen. TIR1 action in the nucleus and its influence on gene expression is described in Fig. 1.1 B. The possibility of taking an influence on TIR1 via PIN protein-regulated auxin concentration is depicted in Fig. 1.1C. The plasma membrane located ABP1 binds auxin and the transmission of the signal may happen in activation of an unknown transmembrane receptor kinase or some other unknown transmembrane protein. This leads to phosphorylations which could lead to activation of enzyme activity in the cytosol. Recent studies suggest an interaction between ABP1 and TIR1 in auxin signal transduction (reviewed by Scherer, 2011). TIR1 is located in the nuclear cytosol and auxin receptor activity could be demonstrated (Dharmasiri et al., 2005; Kepinsi und Leyser 2005; Tan et al, 2007). TIR1 is an F-Box protein and an auxin activated E3 ubiquitin ligase (Dharmasiri et al., 2005). It binds auxin in a sandwich complex consisting of TIR1, auxin and the substrate proteins AUX/IAA (Tan et al., 2007). Auxin binding stimulates ubiquitination which leads to a degradation of the AUX/IAA proteins and thereby to a regulation of early auxin-induced genes (Dharmasiri and Estelle, 2004). Thus TIR1 determines early auxin-induced gene expression (Fig.1.1 B). The important detail of the two-receptor concept in auxin signal transduction is the timing of the early auxin responses. As a first mechanism a receptor can induce a response to auxin without any newly made proteins in regulating post-translationally enzymatic and other protein activities. An example for such a mechanism could recently be shown for PIN proteins by Robert et al. (2010). PIN proteins are important plasma membrane and ER located proteins with functions in auxin transport (Kleine-Vehn and Friml, 2008; Mravec et al., 2009). Auxin-induced stimulation of efflux in auxin transport occur within 3 min (Paciorek et al., 2005). This probably happens by inhibiting endocytosis of the plasma membrane-bound PIN proteins. TIR1-induced gene expression and synthesis of new proteins is to slow to be a mechanism for these rapid changes in auxin transport and a receptor different from TIR1 has to be responsible for this auxin response. Robert et al. (2010) could show that ABP1 is the responsible receptor in this case. Thus ABP1 seem to be responsible for rapid auxin responses faster than 10 to 30 min that do not depend on newly made proteins while TIR1 function is to induce the biosynthesis of new proteins in auxin signaling (Scherer, 2011). It is very probable that ABP1 and TIR1 act together in auxin signal transduction. It is

supposed that TIR1 is influenced by ABP1 via PIN protein regulation and therefore via auxin concentration (Fig.1.1 C) (Effendi and Scherer, 2011).

Auxin signal transduction is probably a network of many proteins and triggered by two receptors. In general, receptor activation first triggers the generation of second messengers by the activation of specific enzymes within seconds or minutes. There are a number of second messengers in plants like reactive oxygen species (ROS), nitric oxide (NO), cGMP, cADPR and lipid breakdown products like free fatty acids (FFA) or phosphatidic acid (Laxalt and Munnik, 2002; Scherer, 2002; Kwak et al., 2003; Bastian et al., 2010). Early auxin activation within minutes could be shown for phospholipases A which release free fatty acids and lysophospholipids from phospholipids as potential second messengers (Paul et al., 1998). For several more phopholipases an involvement in auxin signaling could be shown: PLD (Lanteri et al., 2008), $sPLA_2$ (Lee et al., 2003) and pPLA (Paul et al., 1998; Scherer et al., 2007). The first part of this work concentrates on the involvement of patatin-related phospholipases in early auxin signal transduction.

Patatin-related phospholipases in auxin signal transduction

Evidence for the involvement of pPLAs in auxin signal transduction was already found 1989, when auxin treatment of isolated membrane vesicles of soybean, hypocotyls of zucchini and sunflower led to an activation of pPLA *in vitro* (Scherer und André, 1989; 1993; Scherer, 1995). This pPLA activation happened already a few minutes after auxin treatment and was measured by the increase of free fatty acid and lysoloipid levels (Scherer und André, 1989; 1993; Paul et al., 1998). Some years later with the help of $pPLA_2$ inhibitors more evidence for a function of pPLA in auxin signal transduction could be gathered. These inhibitors decreased the auxin-mediated pPLA activation (Paul et al., 1998), the hypocotyl elongation (Holk et al., 2002; Yi et al., 1996; Scherer and Arnold 1997) and the activation of some early auxin-induced genes (Scherer et al., 2007). A 2 hour treatment of seedlings with 2,4-D and the inhibitor resulted in an inhibition of auxin-induced gene expression. The fact that pPLAs are activated by auxin within minutes and that auxin-induced gene expression was inhibited after inhibitor treatment led to the hypothesis that pPLAs are involved in early auxin signal transduction with ABP1 as the responsible receptor (Fig.1.2).

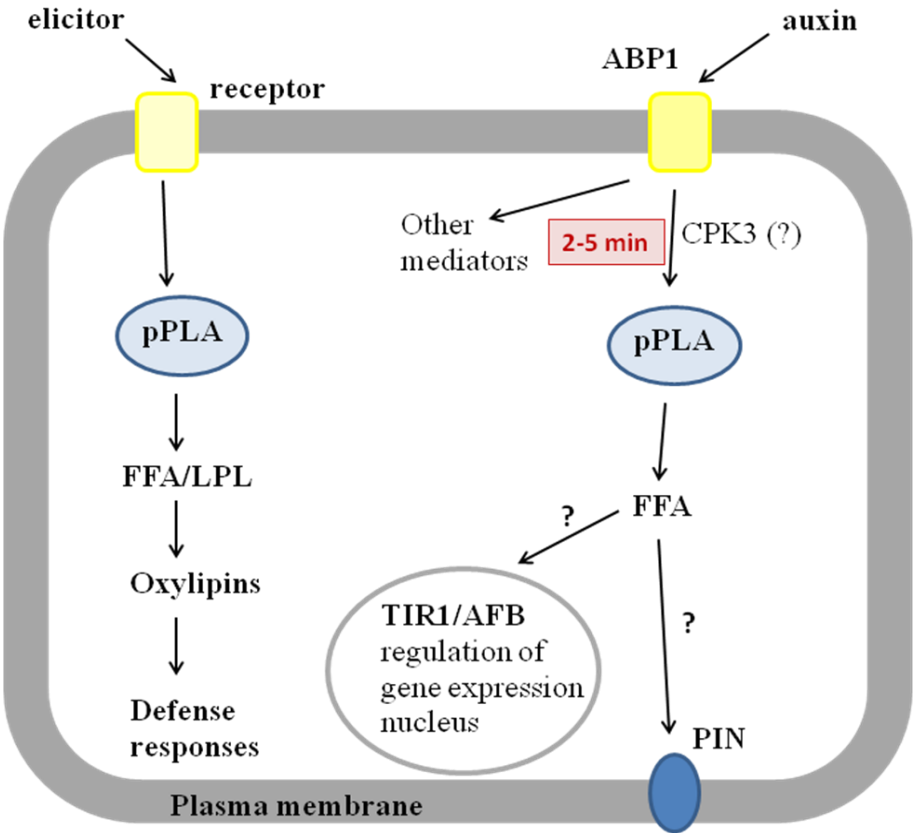

Fig. 1.2: Potential functions of patatin-related phospholipases in *Arabidopsis*.

Simplified Scheme of possible pPLA functions in pathogen and stress responses as well as in auxin signal transduction of the plant cell (modified from Scherer et al., 2010). Description in the text. CPK3: calcium-dependent protein kinase3; FFA: free fatty acid; LPL: lysophospholipid; PIN: PIN-formed protein; R: receptor

Quantification of gene expression after 2h, however, leaves room for mechanisms participating that also might involve translational mechanisms, i.e., TIR1. Only rapid pPLA auxin activation within 2-5 min (Rietz et al., 2010) excludes TIR1 as the receptor. Auxin responses occurring later than 10-30 min, require a receptor other than TIR1, for this is the time span which is at least necessary for a de novo synthesis of proteins from a fast auxin activated promoter (Calderon-Villalobos et al., 2006; review in Scherer 2011). Thus a time span of a 2 hour treatment and measurement of auxin-induced gene expression to disprove participation of TIR1 was to long. To show an involvement of pPLAs in early auxin signal transduction a much shorter auxin treatment for quantification of the response would be useful.

Furthermore, to find out which of the ten pPLA genes are involved in this auxin pathway the use of knock down mutants instead of pPLA inhibitor was obviously more precise. A

recent study could show a defect in expression of early auxin-induced genes in the heterozygous *abp1/ABP1* mutant (Effendi et al., 2011). Many early auxin-induced genes from the early gene families *IAA*, *SAUR* and *GH3* were less or slower up-regulated after a 30 min auxin treatment. Including all these facts a biotest was designed using a short-time auxin treatment of 10 and 30 min of pPLA mutants and measure the expression of the same early auxin-induced genes as in Effendi et al. (2011) in comparison to the wild type.

Functions of pPLAs in plant defense signaling

Besides to the involvement of pPLAs in auxin signal transduction several studies demonstrate a potential function in pathogen defence signal transduction (Yang et al., 2007; Ackermann et al., 1994; Lee at al., 1997; Narváez-Vasquez et al., 1999; Roos et al., 1999; Scherer et al., 2000; Viehweger et al., 2002; Narusaka et al., 2003; Rietz et al., 2004). Plants of a *ppla-I* knock down mutant showed a lower level of basal jasmonic acid (JA) (Yang et al., 2007). Furthermore overexpression of *pPLA-IIα* demonstrated increased cell death and a higher resistance against the mosaic virus of cucumber. These overexpression plants were further less resistant to *B.cinerea* and *P.syringae* (Ackermann et al., 1994). An involvement of pPLA-IIα in mobilization of fatty acid precursors for the biosynthesis of specific oxilipins was supposed (La Camera et al., 2009).

Patatin-related phospholipases: Classification

Patatin-related phospholipases (pPLA) belong to the Phospholipases A (PLA) group. PLAs are acyl hydrolases that hydrolyse phospholipids either at the hydroxyl group of the C_1- (Phospholipase A_1; PLA_1) or of the C_2-atom (Phospholipase A_2; PLA_2) (Fig.1.3). Enzymes which show this activity differ in their structure from each other (Scherer, 2010). To these group of PLAs belong the lipase related PLA_1, the secreted PLA_2 ($sPLA_2$) and the patatin related PLA_2 ($pPLA_2$).

The plant $sPLA_2$ are phospholipases which, in contrast to the most other PLA_2, only catalyze the hydrolysis at the C_2-atom and not at the C_1-atom (Lee et al., 2003). They have a size of about 14 kDa, they are calcium dependent and have a structure homologous to animal sequences, although the sequence homology in other parts except the catalytic centre and the calcium binding position is not very high (Ståhl et al., 1998; Mansfeld et al.,

2006; Bahn et al., 2003). The function of $sPLA_2$ in plant is still widely unknown. The investigation of a $sPLA_2$ *Arabidopsis* mutant as well as the corresponding overexpression line could show differences in cell elongation and defects in gravitropic responses (Lee et al., 2003). Furthermore the transcript of this $sPLA_2$ was upregulated by auxin after one hour. A role of $sPLA_2$ in gravitropism, possibly by influencing auxin induced cell elongation it supposed.

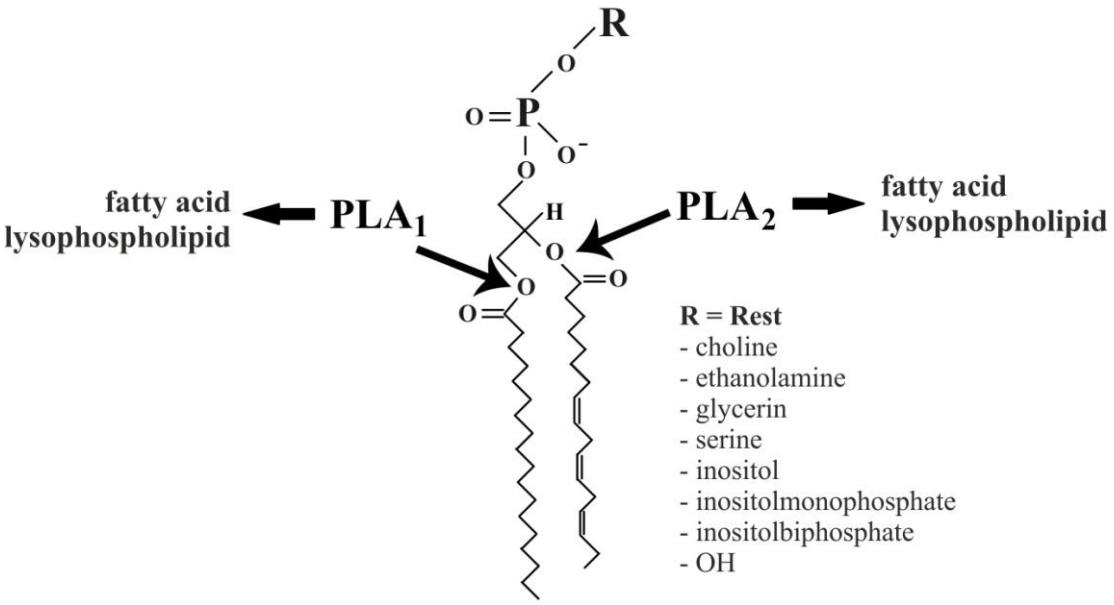

Figure 1.3: Schematic picture of phospholipid hydrolysis by phospholipase A

A schematic, exemplary picture of a phospholipid is visible. Possible headgroups (rest) and reaction products of the PLA mediated reaction are listed. R: Rest/headgroup, PLA_1: Phospholipase A_1, PLA_2: Phospholipase A_2

The patatin related phospholipases were named after their close relation to storage proteins in the potato tuber, which have acyl hydrolase activity (Hirschberg et al., 2001; Schewry 2003; Scherer et al., 2010). The enzymes of this group have a molecular size of about 50 kDa but some have additional domains. They are characterized by their catalytic centre, which consists of the esterase box GTSTG und of the phosphate or anion binding element DGGGXRG (Holk et al., 2002). Based on sequence comparisons the pPLA family is ordered into three groups (Figure 1.4, Holk et al., 2002; Ryu 2004, Matos et al., 2009; Scherer et al., 2010). Group I consists of the single gene/protein pPLA-I. It has similarities to animal iPLAs and is characterized by a leucin rich domain at the C-terminus, an

enzymatic domain and a N-terminal domain. Because of these structural properties pPLA-I is supposed to be the evolutionary oldest pPLA.

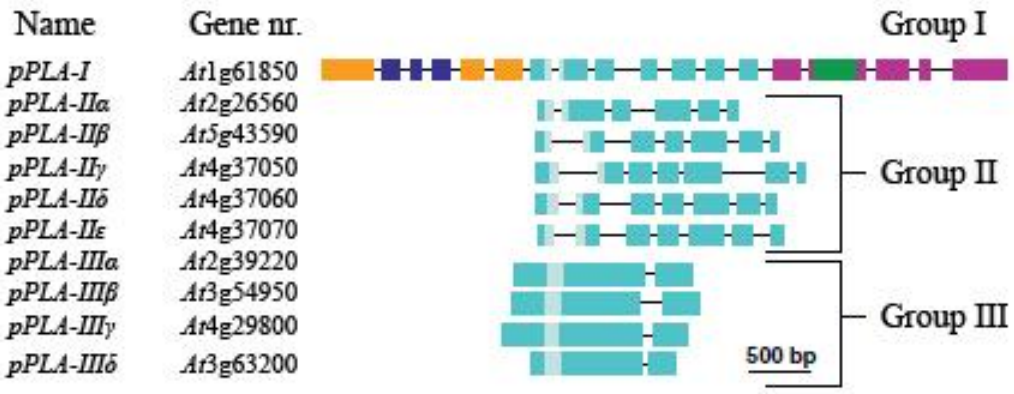

Fig. 1.4: **Exon-Intronstructure of patatin related phospholipases A in *A.thaliana.***
Exons are colored; Introns are black lines between exons. Enzymes are colored in blue, the catalytic centers light blue. The N-terminal domain of pPLA-I is marked with yellow color and contains a leucin rich repeat domain (dark blue). The C-terminal domain of pPLA-I is pink and the position of the ankyrin-like element is colored in green. Scheme from Scherer et al. (2010).

Group II in *Arabidopsis* consists of *pPLA-IIα- pPLA-IIε* (Fig.1.4). In comparison to group III (*pPLA-IIIα-pPLA-IIIδ*), the members of group II are more closely related to the potato tuber storage proteins. The structure of the genes/proteins of group III clearly differs from the ones of group I and II. They have only one intron and their catalytic centre slightly differs from those of the other pPLA groups. Until now these characteristics were not found in any other subfamily of other organisms which have a patatin domain. Thus it is supposed that group III is a plant specific subgroup of pPLA genes (Scherer et al., 2010).

Activity tests of proteins from group I and II could confirm they posess acyl hydrolase activity. They showed a broad enzymatic specificity and accept galactolipids as well as phospholipids as substrates *in vitro* (Matos, 2001; Dhondt et al., 2002; Holk et al., 2002; La Camera et al., 2005; Yang et al., 2007; Matos et al., 2008; Rietz et al., 2010) and *in vivo* (Reina-Pinto et al., 2009). Furthermore it could be shown that pPLA-I prefers oxidated galactolipids over non-oxidated (Yang et al., 2007). pPLA-IIα, pPLA-IIγ and pPLA-IIε also have a substrate preference for galactolipids *in vitro* (Matos et al., 2001, La Camera et al., 2005; Yang et al., 2007, Holk et al., 2002). Galactolipids are mainly present in

chloroplastid membranes. For none of the three pPLAs (pPLA-IIα, pPLA-IIγ and pPLA-IIε) localization to plastids could be demonstrated and pPLA-I only shows a partial localization to plastids (Holk et al., 2002).

How could pPLA be involved in auxin signal transduction?

Although many facts about pPLAs are known which pinpoint to a role in auxin signal transduction, many questions are still open. If pPLA is so rapidly activated by auxin within 2 min, does this really exclude TIR1 to exert influence on pPLA gene expression? If TIR1 indeed could not be the relevant receptor can one show more conclusively that ABP1 is the relevant receptor? Can we find a more precise hypothesis what the consequences of membrane lipid hydrolysis could be in the plant cell?

To get an answer we used at first pPLA T-DNA insertion mutants in a special biotest measuring short-time early auxin-induced gene expression that was already successfully carried out for an *abp1/ABP1* heterozygous mutant by Effendi et al. (2011) to get a link between pPLAs and ABP1. Furthermore membrane lipid-deficient mutants were chosen to support the hypothesis of pPLA action and therefore membrane lipid involvement in auxin signal transduction as described below.

ABP1-mediated auxin signaling probably includes more cytosolic components

It is assumed that auxin signaling mediated by ABP1 located in the plasma membrane and ER (Jones and Herman, 1993; Tian et al., 1995; Henderson et al., 1997) occurs in the cytosol of the cell (Robert et al., 2010; Scherer, 2011). Besides cytosolic pPLAs there probably are more components included in the auxin signaling pathway. In general signal transduction includes a number of characteristic enzymes like protein kinases and protein phosphatases (reviewed in Scherer et al., 2012). Furthermore other signaling components like second messengers are involved. Several mutants of protein kinases and protein phosphatases were suggested in the literature to be part of auxin signal transduction (Monroe-Augustus et al., 2003; Michniewicz et al., 2007; Zourelidou et al., 2009). Membrane lipids are the substrates for phospholipases but phospholipase activities could be influenced by membrane lipid composition. Therefore, enzymes mediating fatty acid concentration were selected to be tested in this work to be involved in auxin signaling.

Potential involvement of fatty acids in auxin signal transduction

Fatty acids are components of membrane lipids and they act as second messengers in signal transduction (Lee et al., 1997; Ryu and Wang, 1998; Shanklin and Cahoon, 1998; Kachroo et al., 2001, 2003; Laxalt and Munnik, 2002; Li et al., 2003). As a direct product of the pPLA hydrolysis they or their derivates could act as second messengers in early auxin signal transduction in an as yet unknown way. Furthermore, the fatty acid composition in lipids and membranes could have an influence on membrane attached proteins or intrinsic membrane proteins. Thus several mutants which are deficient in fatty acid biosynthesis and therefore have changed fatty acid compositions in phospholipids were used in this work. Selected were *ssi2*, *fad2-1*, *fad6-1*, *lacs4*, *lacs9* and *lacs4 lacs9*. *SSI2* codes for a stearoyl acyl carrier protein desaturase (S-ACP-DES), which is a typical member of the soluble fatty acid desaturase family (Kachroo et al., 2003). These enzymes play an important role in the regulation of unsaturated fatty acid levels in the cell. The activity of the enzyme S-ACP-DES was 10 fold decreased which led to an increase of stearic acid (18:0) concentration (Kachroo et al., 2001) and furthermore resulted in an induction or inhibition of several defense mechanisms (Kachroo et al., 2003). The *Arabidopsis* gene *FAD*2 encodes an enzyme that is localized at the endoplasmatic reticulum (ER). A mutation of this gene leads to an increase of oleic acid (18:1) concentration. The *fad6* mutant has a defect in the activity of a chloroplastid localized desaturase, which converts palmitoleic acid (16:1) and 18:1 to delta-7-cis,10-cis-hexadecadienic acid (16:2) and linoleic acid (18:2). Thus the mutation in the *fad6* mutant leads to an increased concentration of 16:1 and 18:1 in the membrane lipids. The *Arabidopsis LACS* genes belong to the large superfamily of CoA ligase-related encoding genes called acyl-activating enzymes (AAEs) and the *LACS* gene family consists of nine genes (Shockey et al., 2002; Shockey and Browse, 2011). They encode long-chain acyl-CoA synthestases which catalyze the activation of free fatty acids to acyl-CoAs. Some functions for LACS are for example in providing of fatty acids for cuticular lipid synthesis (Schnurr et al., 2004; Bessire et al., 2007; Lü et al., 2009), the activation of fatty acids for β-oxidation in the peroxisome (Fulda et al., 2002) and involvement in the biosynthesis of triacylglycerols (TAGs) (Zhao et al., 2010). In bacteria and mammalian cells an important function of LACS in long-chain fatty acid transport could be shown (Eaton et al., 1996; Hettema and Tabak, 2000). A role for all these membrane lipid-deficient mutants in auxin

signaling is not shown yet but undoubtly membranes with a changed lipid composition provide a different substrate for phospholipases which are activated by auxin.

CPK3 as a potential activator of pPLA activity

Because of the rapid activation of pPLAs by auxin within a few minutes (Paul et al., 1998), a de novo synthesis of these enzymes to achieve increased activity can be excluded. A possible pPLA activation mechanism can be phosphorylation. For pPLA-IIε a C-terminal phosphorylation and activation by a calcium dependent kinase (CPK3) could be demonstrated (Rietz et al., 2010). CPK3 belongs to the large family of calcium dependent protein kinases (CDPK). Theses CDPKs have a characteristic structure in which an N-terminal Serine/Threonine domain is coupled to a carboxyterminal calmodulin related domain (Cheng et al., 2002; Harmon et al., 2001; Klimecka and Muszyńska, 2007; Liese and Romeis, 2012). Therefore the activation of CDPKs does not depend on the interaction with exogenous calmodulin but happens directly by calcium binding. They have functions in plant signal transduction by either influencing directly enzymatic activity by phosphorylation or by changing indirect gene expression patterns (Sathyanarayanan und Poovaiah, 2004). CDPKs also act as abscisic acid (ABA) signal components and are involved in ABA mediated gene expression, germination, growth of seedlings and stomata movement (Choi et al., 2005; Mor et al., 2006; Zhu et al., 2007). A recent study demonstrated that CPK3 activity is influenced by salt and other stress elicitors (Mehlmer et al., 2010). The *cpk3* knockout mutant showed a salt sensitive phenotype, the overexpression line showed the corresponding increased salt tolerance. Another study demonstrated that CPK3 is involved in an herbivore induced signaling pathway by posttranslational regulation of the defense mechanism of the plant (Kanchiswamy et al., 2010). In addition the phosphorylation of pPLA-IIε by CPK3 (Rietz et al., 2010) could show a potential function of CPK3 in activation of pPLAs. Thus the *cpk3* knock down mutant and the corresponding overexpression line were included to be tested in this work.

Protein phosphatases and protein kinases in auxin signal transduction

Typical components of signal transduction networks are protein phosphatases and protein kinases which activate or inactivate further enzymes and proteins by phosphorylation or dephosphorylation (reviewed in Scherer, 2011). One group of candidates are protein

kinases like mitogen-activated protein kinases (MAPKs). MAPKs describe a conserved family of enzymes that regulate physiological processes and are a component of signaling molecules, the so-called MAPK cascades (Ulm et al., 2001; Bartels et al., 2010). These MAPK cascades may lead to the phosphorylation of transcription factors and it could be shown that a MAPK is activated by auxin within 5 min (Mockaitis and Howell, 2000). In *Arabidopsis* it could be shown for five MAPK phosphatases, the DUAL-SPECIFICITAY PROTEIN TYROSINE PHOSPHATASE 1 (DSPTP1), MAP KINASE PHOSPHATASE2 (MPK2), INDOLE-3-BUTYRIC ACID RESPONSE 5 (IBR5); PROPYZYAMIDE HYPERSENSITIVE 1 (PHS1) AND MAP KINASE PHOSPHATASE1 (MPK1), to interact with MAPK (Lee and Ellis, 2007; Ulm et al., 2002; Gupta et al., 1998; Walia et al., 2009; Lee et al., 2009; Andreasson and Ellis, 2010). One of these five dual-specificity MAPs, IBR5, is a positive regulator of auxin and abscisic acid (ABA) responses (Monroe-Augustus et al., 2003). An *Arabidopsis ibr5* null mutant is less responsive to IBA, an endogenous auxin, and to auxin transport inhibitors (Monroe-Augustus et al., 2003). Typical auxin-response mutant-phenotypes like fewer lateral roots, longer roots and increased leaf serration were found in the *ibr5* plants (Monroe-Augustus et al., 2003; Strader et al., 2008). In a study to ascertain a possible link between IBR5 action and TIR1 auxin receptor, a *tir1* background enhanced the *ibr5* auxin-related physiological phenotypes as well as the *ibr5* ABA resistance (Strader et al., 2008, BMC). Therefore it has been suggested that IBR5 and TIR1 promote auxin and ABA responsiveness independently of one another (Strader et al., 2008). Thus, IBR5 could be a candidate in early auxin signaling using ABP1 as the receptor to further regulate the expression of early auxin-induced genes.

Other substrates for the phosphorylation by protein phosphatases in ABP1-mediated auxin signal transduction are PIN proteins (Huang et al., 2010; Michniewicz et al., 2007). The phosphorylation status of PIN proteins is important for auxin transport and thus auxin concentration in the cell compartments. Several mutants were chosen whose proteins have a function in phosphorylation of PIN proteins. Protein phosphatase 2A (PP2A) directly modulates the phosphorylation status of plasma membrane localized PINs in roots and shoot apex (Michniewicz et al., 2007). It could be shown that PP2A and a PIN-regulating protein kinase (PINOID) which is involved in regulation of polar delivery of PIN proteins

(Christensen et al., 2000; Benjamins et al., 2001), act antagonistically in mediating polar targeting of PINs (Michniewicz et al., 2007; Li et al., 2011).

Other kinases involved in PIN regulation in auxin transport are the other members of the AGC kinase family (Zourelidou et al., 2009). For members of the AGCVIIIa kinase subfamily, D6PKs, it could be shown that PIN proteins are their phosphorylation substrates and that PINs and D6PKs are colocalized at the basal membrane of root cells (Zourelidou et al., 2009). They belong to the same AGC kinase subfamily as PINOID and the *d6pk* mutants showed reduced auxin transport and have defects in lateral root formation and gravitropism. Thus the *ibr5, pp2a* and *d6pk* mutants were included in the mutant selection of this work because of their probable involvement in ABP1-mediated regulation of PIN proteins.

Objetives of the thesis

The aim of this work was the investigation of ABP1-mediated auxin signal transduction in defining further candidates for this TIR1-independent auxin signaling network. Several mutants were chosen, especially *ppla* knockout mutants of all 10 family members in *Arabidopsis* (Chapter 2 and 3), as well as mutants which have defects in their fatty acid composition in membrane lipids (*fad2-1*, *fad6-1*, *ssi2*, *lacs4*, *lacs9*, *lacs4 lacs9*) and mutants of protein kinases and protein phosphatases with potential functions in auxin signaling (*cpk3*, *cpk3-ox*, *ibr5*, *pp2a*, *d6pk-1*, *d6pk-1 d6pk1-1 d6pk2-2*) (Chapter 4). A special biotest was used like successfully done with the *abp1/ABP1* heterozygous mutant by Effendi et al. (2011). Several early auxin-induced genes were chosen and their expression measured 10 and 30 min after auxin treatment. Especially the 10 min value of transcription would indicate that a receptor other than TIR1 (Fig.1.1) is necessary to explain a decrease of function induced by the particular mutant. The 30 min allows to better characterize the different mutants.

OUTLINE THESIS

Chapter 1 gives a general introduction and an overview about the hypothesis of this work.

In **Chapter 2** the involvement of the nine pPLA mutants *ppla-IIα, ppla-IIβ, ppla-IIγ, ppla-IIδ, ppla-IIε, ppla-IIIα, ppla-IIIβ, ppla-IIIγ, ppla-IIIδ* in early auxin signal transduction is demonstrated. Many early auxin-induced genes were defect in their auxin response after 10 and 30 min auxin treatment in comparison to the wild type. Interestingly only one of the mutants, *ppla-IIIδ*, showed an auxin-related phenotype.

Chapter 3 focuses on pPLA-I, which has a function in light responses and gravitropism. Also the two *ppla-I* knockout mutants have defects in early auxin-induced gene expression after auxin treatment. Figure 3 and the corresponding text show the part of the paper which was done in this thesis.

Based on the results of the pPLA investigations, several other mutants were tested for the regulation of early auxin-induced genes as described in **Chapter 4**. Mutants with changed fatty acid concentration (*fad2, fad6*, *ssi2*, *lacs4*, *lacs9* and *lacs4 lacs9*), mutants of protein kinases (*cpk3*, *cpk3-ox*, *d6pk-1* and *d6pk-1 d6pk-1-1 d6pk2-2*) and protein phosphatase mutants (*ibr5* and *pp2a*) were selected. Again, many early auxin-induced genes showed defects in auxin-induced expression 10 and 30 min after auxin treatment. In addition, *tir1-3* was also used for this test and did not show this strong misregulation of early auxin-induced genes.

Chapter 5 contains a general discussion and summary of the previous chapters.

CHAPTER 2:

PATTERNS AND TIMING IN EARLY AUXIN-INDUCED GENES IN PHOSPHOLIPASE A (pPLA) T-DNA INSERTION MUTANTS REVEAL THEIR FUNCTION IN AUXIN SIGNALING.

Patterns and Timing in Expression of Early Auxin-Induced Genes in Phospholipase A (pPLA) T-DNA Insertion Mutants Reveal Function in Auxin Signaling

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ABSTRACT

While it is known that patatin-related phospholipase A (pPLA) activity is rapidly activated within three minutes by auxin, hardly anything is known about how this signal influences downstream responses like transcription of early auxin-induced genes or other physiological responses. We screened mutants with T-DNA insertions in members of the *pPLA* gene family for molecular and physiological phenotypes related to auxin. Only one in nine *Arabidopsis thaliana ppla* knock down mutants displayed an obvious constitutive auxin-related phenotype. Compared to wild type, *ppla-IIIδ* mutant seedlings had decreased main root lengths and increased lateral root numbers. We tested auxin-induced gene expression as a molecular readout for primary molecular auxin responses in nine *ppla* mutants and found delayed up-regulation of auxin responsive gene expression in all of them. Thirty minutes after auxin treatment, up-regulation of up to 40% of auxin-induced genes was delayed in mutant seedlings. We observed only a few cases with hypersentitive auxin-induced gene expression in *ppla* mutants. While in three *ppla* mutants, that were investigated in detail, rapid up-regulation (as early as 10 min after auxin stimulus) of auxin-regulated genes was impaired, late transcriptional responses were wild type like.

This regulatory or dynamic phenotype was consistently observed in different *ppla* mutants with delayed up-regulation that frequently affected the same genes. This defect was not affected by *pPLA* transcript levels which remained constant. This indicates a post-translational mechanism as functional link of pPLAs to auxin signaling. The need for a receptor triggering an auxin response without employing transcription regulation is discussed.

Keywords : ABP1, auxin signal transduction, patatin-related phospholipases, early auxin-induced genes

INTRODUCTION

The generation of lipid messengers like free fatty acids and lysolipids by phospholipase A_2 (PLA_2) enzymes is an important step in early plant signal transduction because they regulate distinct proteins or downstream processes. In plants, two main PLA_2 gene families function in signal transduction, patatin-related phospholipase A and secreted PLA_2 (Scherer et al., 2010). In *Arabidopsis thaliana*, the *pPLA* family is represented by 10 genes that can be classified into three sub-groups (I-III) based on sequence comparisons (Holk et al., 2002; Ryu, 2004; Scherer et al., 2010).

Group I consists of the single gene *pPLA-I* that has clear homology to animal Ca^{2+}-independent PLA_2 (iPLA) enzymes (Balsinde and Balboa, 2005). The remaining nine group II and III pPLAs have only rudimentary sequence homology to animal sequences, indicating that group I *pPLA-I* is evolutionarily ancient (Holk et al., 2002). *pPLA-I* T-DNA insertion mutants exhibit reduced basal levels of jasmonic acid. However, pathogen or wounding induced jasmonic acid levels are indistinguishable from wild type (Yang et al., 2007). In addition, we found that auxin-inducible gene expression and shade-induced elongation growth are affected in *ppla-I* insertion mutants (Effendi et al., unpublished).

Group II consists of five genes (*pPLA-IIα* – *pPLA-IIε*). *pPLA-IIα* transcription is up-regulated by abiotic and biotic stresses (Matos et al. 2001; Rietz et al., 2004; La Camera et al., 2005). The phenotypic analysis of *ppla-IIδ* and *ppla-IIε* suggested a function in root architecture regulated by auxin and ABA (Rietz et al., 2010).

The members of group III (*pPLA-IIIα – pPLA-IIIδ*) are plant specific and differ in their intron/exon structures and catalytic centers from the other subfamilies (Scherer et al., 2010).. Interestingly, only one of the insertion mutants (*ppla-IIIβ*) displayed a subtle morphological auxin response phenotype (Li et al., 2011).

The above described phenotypes together with rapid activation of pPLAs 2-5 min after auxin treatments (Scherer and André, 1989; Paul et al., 1998) indicate a possible function of pPLA enzymes in auxin signaling. However, in recent years additional evidence in support of this line of argumentation has accumulated. Pharmacological approaches, for example, revealed that pPLA inhibitors block auxin-induced elongation growth (Scherer and Arnold, 1997; Holk et al., 2002) and auxin-induced gene expression (Scherer et al., 2007). On the biochemical level, we could recently show that pPLA-IIδ and pPLAε are activated by CPK3 (Rietz et al., 2010).

To substantiate a possible role of pPLAs in early auxin signaling, we here aimed to systematically analyze the auxin inducibility of classic auxin response genes in pPLA mutant backgrounds. In contrast to classic physiological assays, such a rapid response biotest is able to show the direct effect of pPLAs on the primary molecular auxin response clearer than in developmental tests taking days. The classical example for this principle is the hypocotyl and coleoptile elongation test with etiolated tissues where a response can be detected after about 10 min. We included various auxin response genes in this analysis, some of which are induced within minutes independent of *de novo* protein synthesis (e.g. the *IAA, GH3* and *SAUR* families) (Hagen and Guilfoyle, 2002; Abel et al., 1994; 1995; Abel and Theologis 1996; Guilfoyle et al., 1998a; b; Paponov et al., 2008). Thus, these genes are referred to as early or primary auxin response genes. IAA proteins are unstable transcriptional repressors and developmental responses to auxin are sensitive to the levels of these proteins (Dreher et al., 2006; Mockaitis and Estelle, 2008). Mutations of several *IAA* genes like *MASSAGU2* (*MSG2/IAA19*) (Tatematsu et al., 2004), *SUPPRESSOR OF HY2* (*SHY2/IAA3*) (Abel et al., 1995; Kim et al., 1996; Soh et al., 1999; Tian and Reed 1999; 2003; Reed, 2001) or *SOLITARY ROOT* (*SLR/IAA14*) (Abel et al., 1995; Fukaki et al., 2002; 2005; Vanneste et al., 2005) reduce multiple auxin responses. Especially, they have defects in auxin-induced lateral root formation and reduced cell cycle activity (Fukaki et al., 2006; Mockaitis and Estelle, 2008). The *GH3* gene family in *Arabidopsis* consists of three subfamilies and several of the members encode IAA-amido synthetases (Hagen and

Guilfoyle, 2002; Staswick et al., 2005). A rapid transcriptional activation leads to a higher amount of IAA-amido synthetase, which then converts auxin to amino acid conjugates that are either inactive or become degraded. Thus, a function of *GH3* is to maintain IAA homeostasis (Staswick et al., 2005). From *SAUR* genes are small, auxin-induced RNA's and are transcribed within 2-5 min of exogenous auxin application (Hagen and Guilfoyle, 2002). The function of proteins is coded by them is still unknown but it was suggested that they play a role in auxin signal transduction involving calcium and calmodulin (Yang and Poovaiah, 2000; Hagen and Guilfoyle, 2002; Jain et al., 2006; Wang et al., 2009). Recently, it has been shown that transcription of several *PIN* genes that encode auxin efflux transporters is also responsive to auxin (Vanneste et al., 2005; Vieten et al., 2005; Effendi and Scherer, 2011). They were therefore included in our analyses although not all of them are rapidly regulated by auxin (Vanneste et al., 2005 Vieten et al., 2005; Effendi and Scherer, 2011).

To complement the transcriptional analyses, which emphasize rapid auxin responses, we also addressed physiological responses such as primary root growth and lateral root formation in the pPLA mutant backgrounds. In brief, we show that the transcription of several early auxin response genes is transiently delayed in *ppla* mutants in response to auxin, corroborating a role of pPLAs in early auxin signaling.

RESULTS

ppla-IIIδ shows an auxin-sensitivity phenotype in the classical root response

Isolation of several of the *ppla* insertion mutants used here (*ppla-I, ppla-IIα; ppla-IIγ, ppla-IIδ, ppla-IIε, ppla-IIIβ*) was published (La Camera et al., 2005; Yang et al., 2007; Rietz et al., 2010; Li et al., 2011). Experimental evidence about the transcript null status of the other T-DNA insertion mutants is presented in Supplemental Fig. 1. In fact, only one of the *ppla* mutants, *ppla-IIIδ*, showed an auxin-related phenotype in root growth (Fig. 1). This finding suggests a dominant negative function for *ppla-IIIδ* although auxin sensitivity was not affected. None of the other *ppla* mutants showed an auxin phenotype in this particular test (Supplemental Fig. 2).

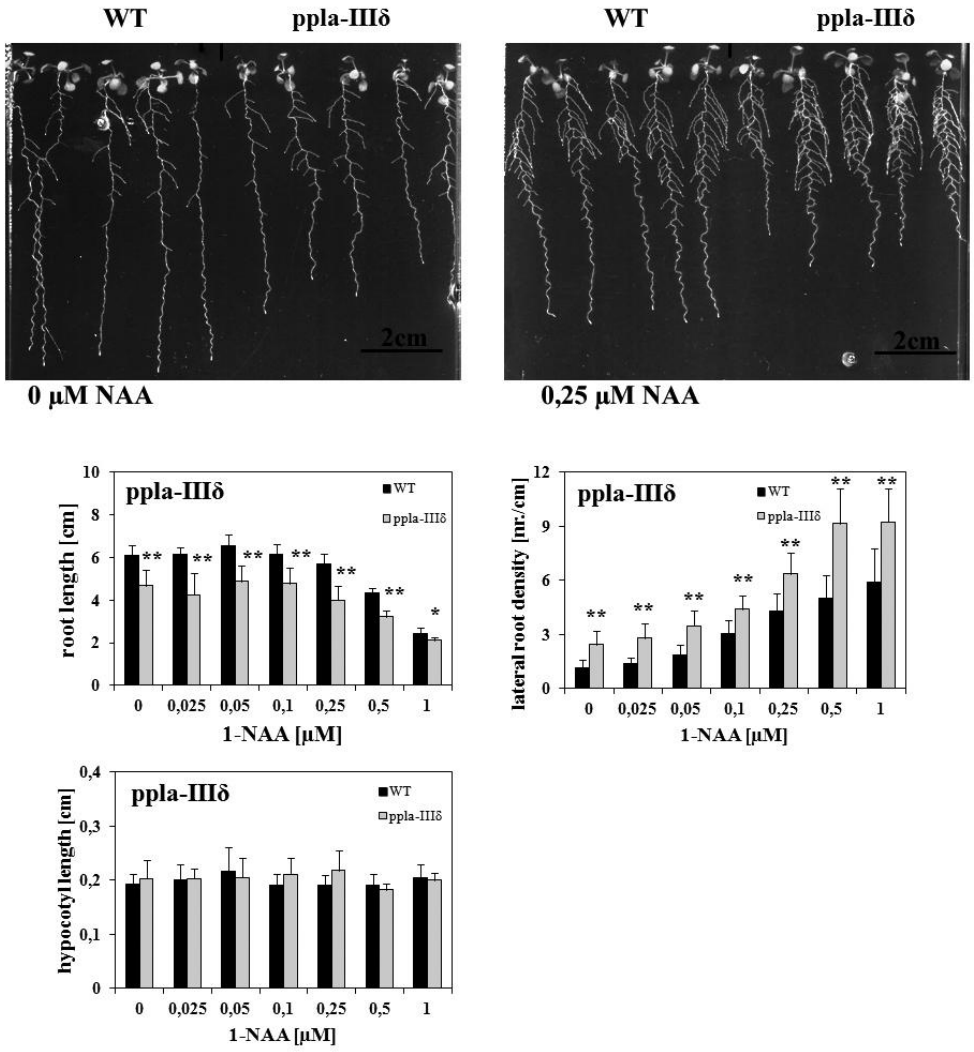

Figure 1. Growth response of light-grown *ppla-IIIδ* mutants and wild type plants in response to auxin.
Seedlings were grown for 7 days on 1xATS medium with different 1-NAA concentrations. (A) Comparison of growth patterns (bar = 1cm). (B) Root length. (C) Lateral root density. (D) Hypocotyl length. Asterisks above columns indicate significant differences between treatments of mutant and the corresponding wild-type (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0,001$; t-test).

pPLA-group II mutants are defective in early auxin induced gene expression

Developmental responses to auxin need several days to become manifested. Therefore, we wanted to employ a rapid response to auxin and chose transcription of early auxin-induced genes as a test system as we recently did for the *abp1/+* and *pin2* mutant (Effendi et al., 2011; Effendi and Scherer, 2011).

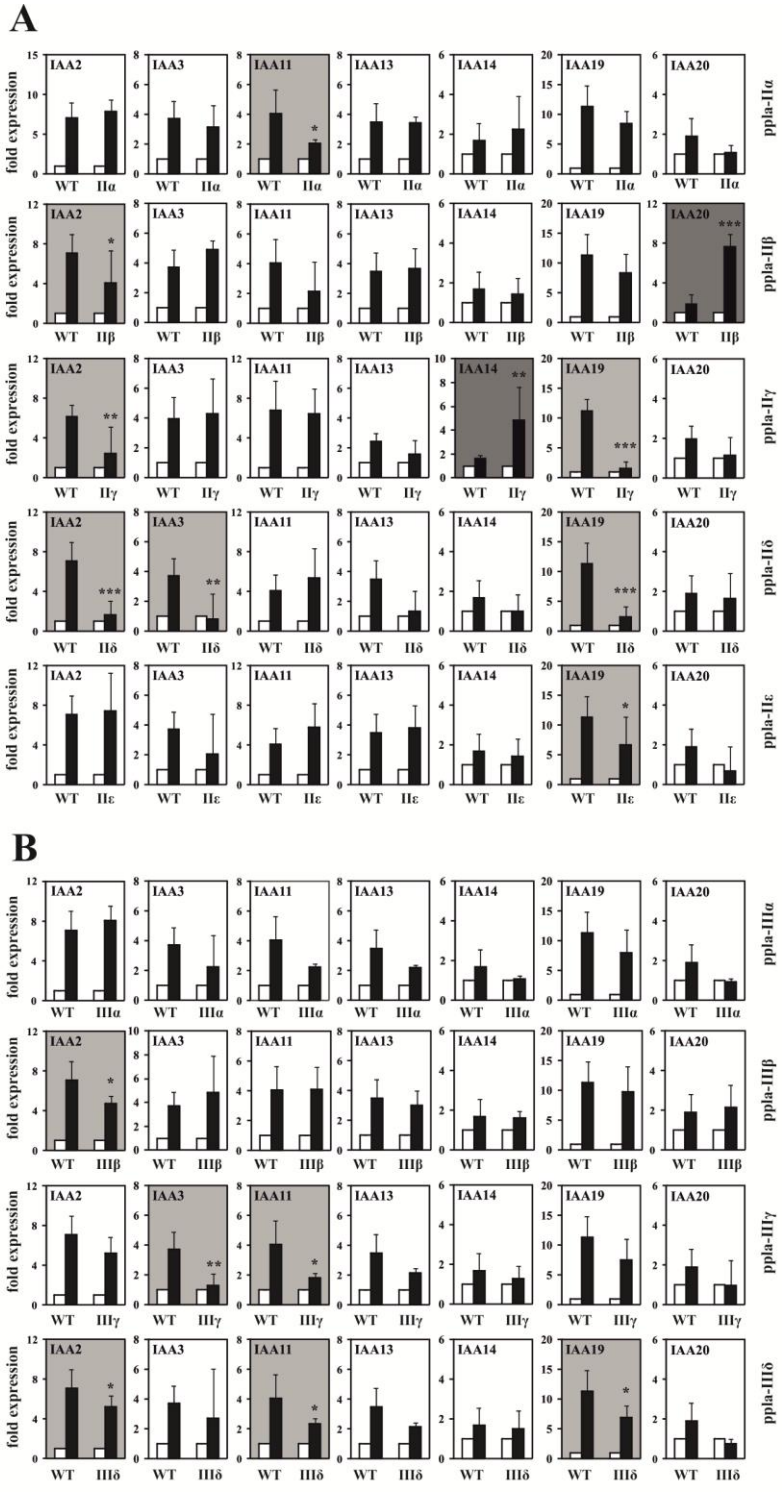

Figure 2. Expression of *IAA* genes in light-grown *ppla* mutant and wild-type seedlings. (A) Group II genes. (B) Group III genes. The background of the panels is shaded whenever significant differences between wild type and mutant were obtained. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments type (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0{,}001$; t-test). Relative expression levels were calculated by setting values at t=0 min to 1 (white bars); values at t=30min IAA were calculated accordingly (black bars).

Several groups of early auxin-induced genes were chosen for this investigation. In wild type seedlings most of the genes were up-regulated two- to twelve-fold in comparison to untreated controls after 30 min auxin treatment (Figs. 2-4). Among the *IAA* genes *IAA2* failed to be up-regulated in the *ppla* mutants most often (5x) (Fig. 2). Similarly, several *ppla* mutants were unable to induce *IAA19*. In all remaining *ppla* mutants, as a tendency, *IAA19* induction was weaker when compared to wild type. *IAA11* failed to be regulated only in three mutants (*ppla-IIα, ppla-IIIβ,* and *ppla-IIIδ*) which was similar for *IAA13* (*ppla-IIIα, ppla-IIIδ*). *IAA3* was less up-regulated in two *ppla* mutants (*ppla-IIδ* and *ppla-IIIγ*). Interestingly, *IAA20* and *IAA14* induction was faster in in *ppla-IIα* and *ppla-IIγ* mutants, respectively.

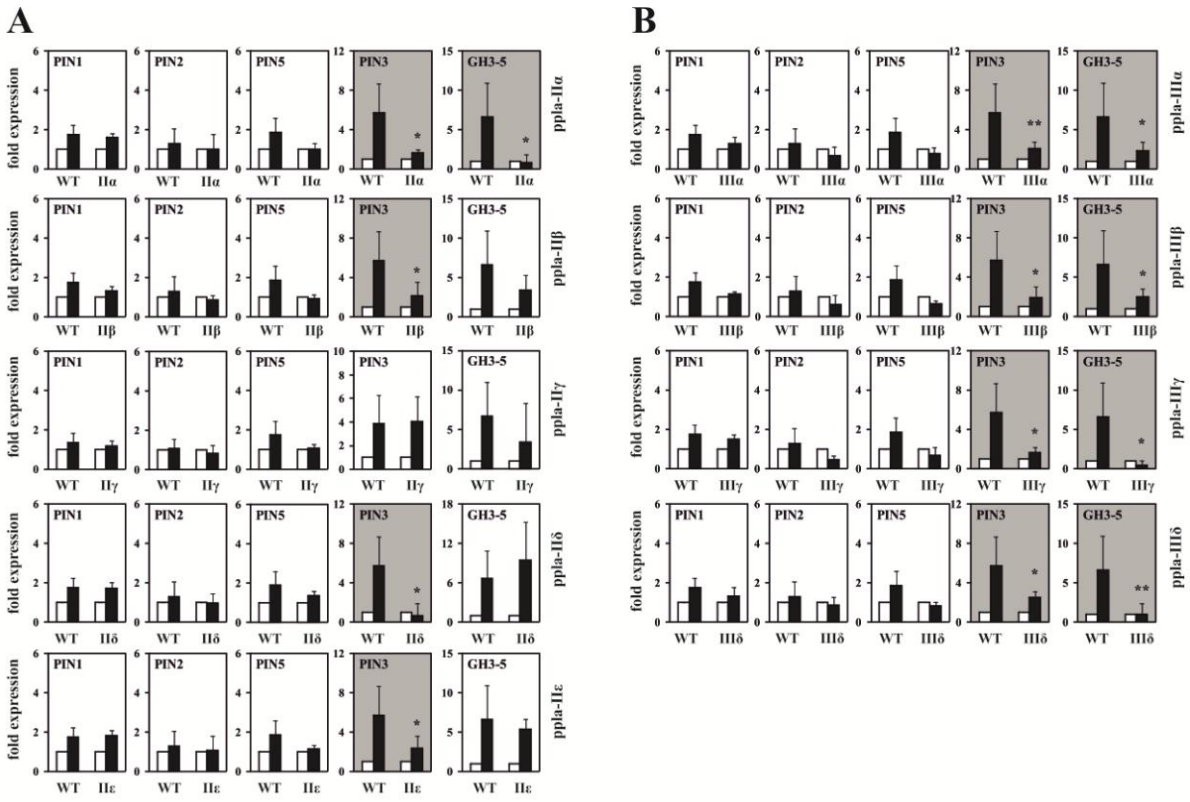

Figure 3. Expression of several *PIN* genes and *GH3.5* in light-grown *ppla* mutant and wild-type seedlings.
(A) Group II genes. (B) Group III genes. The background of the panels is shaded whenever significant differences between wild type and mutant were obtained. Asterisks above columns indicate significant differences between the mutants and the corresponding wild type treatments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0{,}001$; t-test). Relative expression levels were calculated by setting values at t=0 min to 1 (white bars); values at t=30min IAA were calculated accordingly (black bars).

As they might potentially regulate cellular auxin concentrations, we also analyzed four *PIN* genes (*PIN1, PIN2, PIN3,* and *PIN5)*. *PIN2, PIN3,* and *PIN5* were shown previously to be regulated by auxin (Vieten et al., 2005; Effendi and Scherer, 2011). *GH3.5* was added to this group as another gene that may affect auxin concentration by conjugating auxin with amino acids (Staswick et al., 2005). Within 30 min, *PIN1, PIN2*, and *PIN5* were not significantly up-regulated by auxin (Fig. 3). *PIN3* expression, however, was delayed in eight out of the nine *ppla* mutants. Similarly, *GH3.5* up-regulation was significantly weaker in all *ppla-III* mutants and in *ppla-IIα* (5x).

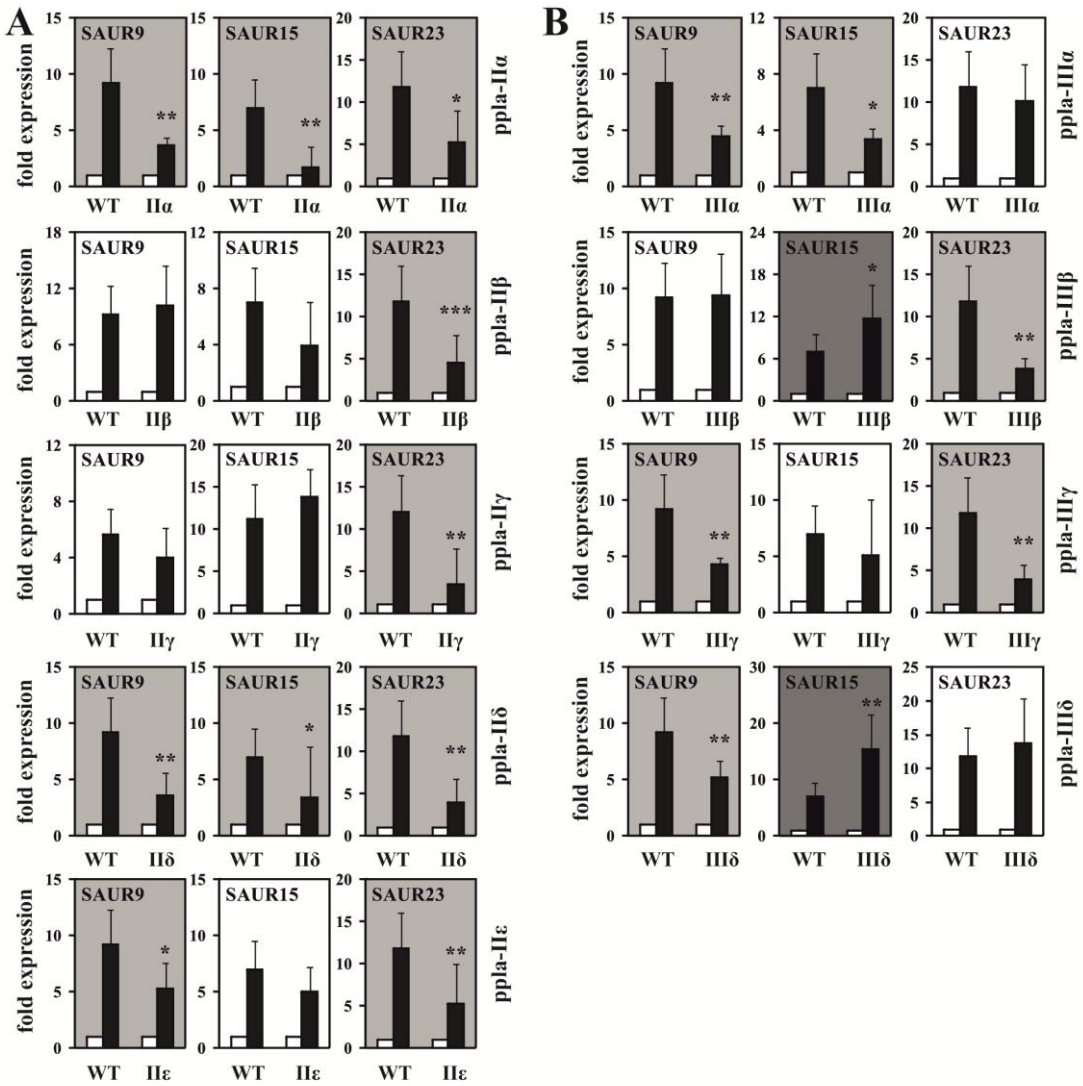

Figure 4. Expression of several *SAUR* genes in light-grown *ppla* mutants and wild-type seedlings grown in the light.

(A) Group II genes. (B) Group III genes. Background of panels is shaded whenever significant differences between wild type and mutant was obtained. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0{,}001$; t-test). Relative expression levels were calculated by setting values at t=0 min to 1 (white bars); values at t=30min IAA were calculated accordingly (black bars).

Considering all nine *ppla* mutants together, always at least one of the three tested *SAUR* genes responded weaker to the auxin stimulus. In two ppla mutants all three *SAUR*s (*SAUR9, SAUR15, SAUR23*) were less up-regulated (Fig. 4). *ppla-IIIβ* and *ppla-IIIδ* were unusual in that here *SAUR15* responded more strongly when compared to the wild type. The *SAUR* genes selected by us were mis-regulated in two-thirds of all cases investigated. However, we could not detect a common pattern of mis-regulation.

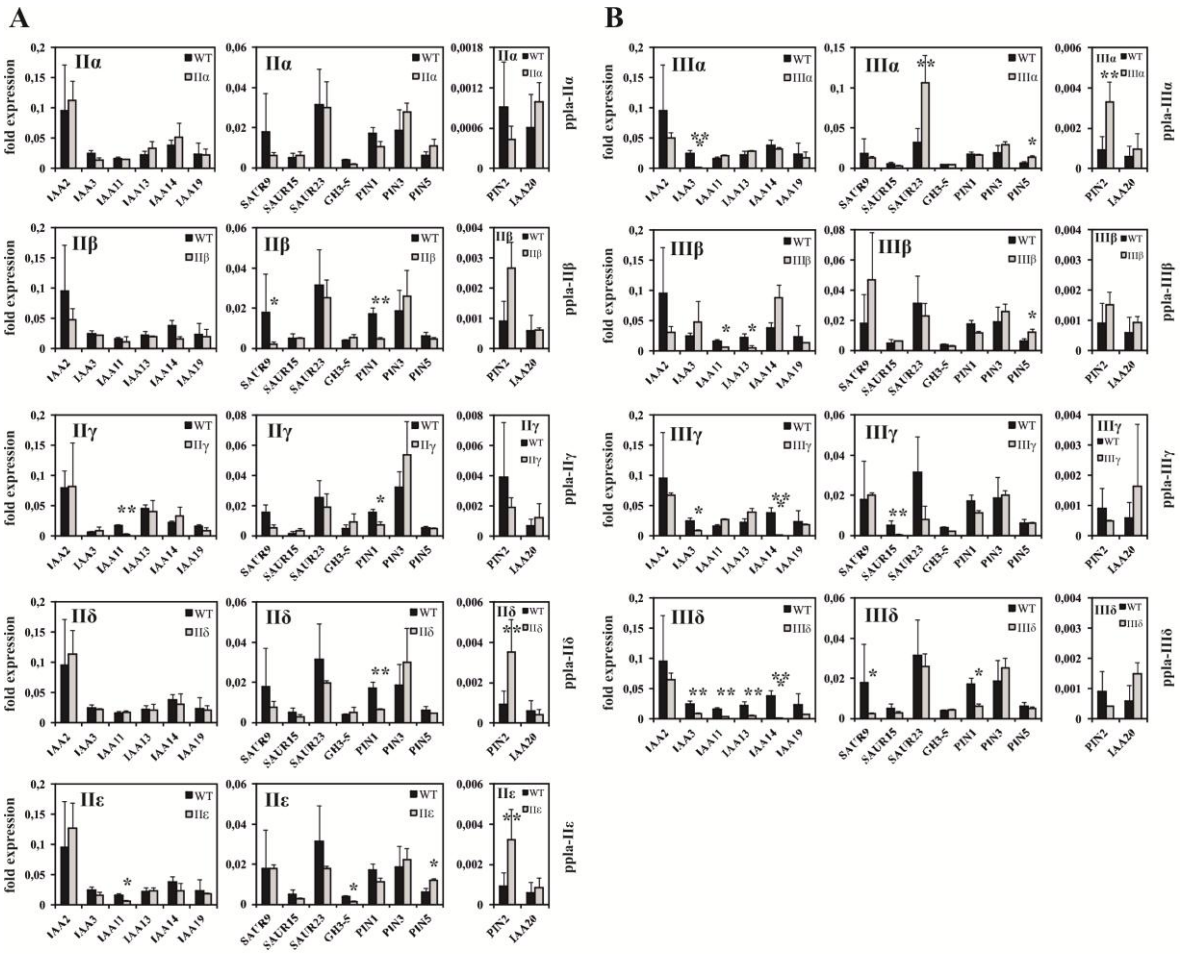

Figure 5. Comparison of expression profiles of all genes tested in light-grown *ppla* mutants and wild type seedlings without auxin treatment (t=0).

Quantified genes are grouped according to expression levels in all mutants and wild type. (A) Group II genes. (B) Group III genes Black bars: wild types; gray bars: mutants. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0{,}001$; t-test). Relative expression rates were calculated relative to the reference gene *UBQ10*.

The auxin inducible expression levels of the auxin response genes reported (Figs. 2 - 4) were also tested in non-treated mutants and wild type (Fig. 5). To address this, we quantified expression of the same reference gene in all samples as a basis for the comparison by qPCR. In the non-treated seedlings, twenty-four genes out of a total of 135 measured (~18%) were differently expressed in all mutants when compared to the wild type. We found no correlation between highly or lowly expressed genes and the number of differentially expressed genes in mutants and wild type. Rather, the four group III mutants showed more differences (3-6 per mutant) than the five group II mutants (0-2 (-4) per mutant) compared to wild type. However, we could not observe any recognizable pattern (Fig. 5) that was reflected by their auxin inducible gene expression (Fig. 2-4). In other words, a logical context between initial expression levels and auxin-induced expression responses could not be found; auxin induction seemed not to be coupled to basal levels of expression. Similarly, the number or type of differentially regulated genes in the non-treated state gave no simple prediction basis for morphologically aberrant phenotypes in mutants with one notable exception: the mutant with the highest number of six aberrantly expressed genes was *pplaIIIδ* and also displayed the root phenotype shown in Fig. 1.

pPLA's interfere with gene expression as early as 10 min after of auxin application

pPLA genes themselves could be regulated by auxin and, potentially, a lack of their transcription could be the cause for any lack of proper up-regulation of early auxin-induced gene expression. Therefore, expression of all *pPLA* genes was quantified after auxin application from 10 min on (Fig. 6). None of the *pPLA* genes were regulated as quickly as within 10 min. *pPLA-IIα* was transiently down-regulated, but statistically significant first after 3 h, and *pPLA-IIε* and *pPLA-IIIγ* were down-regulated at 24 h. Early effects of auxin on transcription of *pPLA* genes, thus, cannot explain the transcriptional effects in *ppla* mutants on auxin-induced genes.

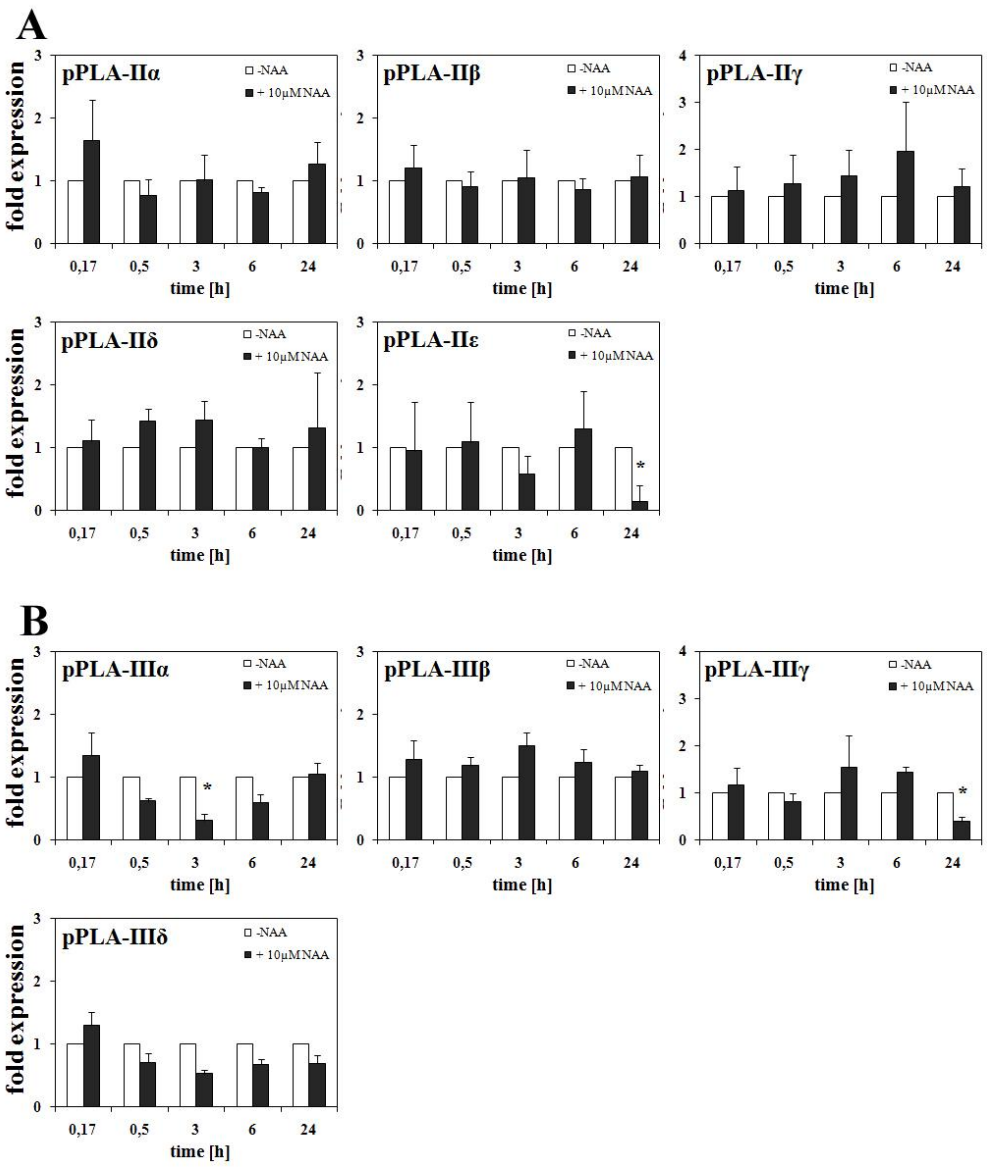

Figure 6. Time courses of expression of *pPLA* genes in wild type after auxin application. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0{,}001$; t-test). Relative expression levels were calculated by setting values at t=0 min to 1 and at other time points values were calculated accordingly for each genotype (controls: white bars; 10 µM auxin: black bars).

pPLA enzymes are predicted to be cytosolic enzymes, which could be shown for some (Holk et al., 2002; La Camera et al., 2005). Therefore, it was important to test how fast a cytosolic component could influence nuclear events in auxin gene expression regulated by TIR1. We narrowed down the time span from stimulus application to quantification of transcription of auxin-induced genes to ten min in a few selected examples. *SAUR9*,

SAUR23, *IAA19*, and *PIN3* were chosen and transcription quantified at 10 min, 30 min, 60 min, and 180 min in three *ppla* mutants (Fig. 7).

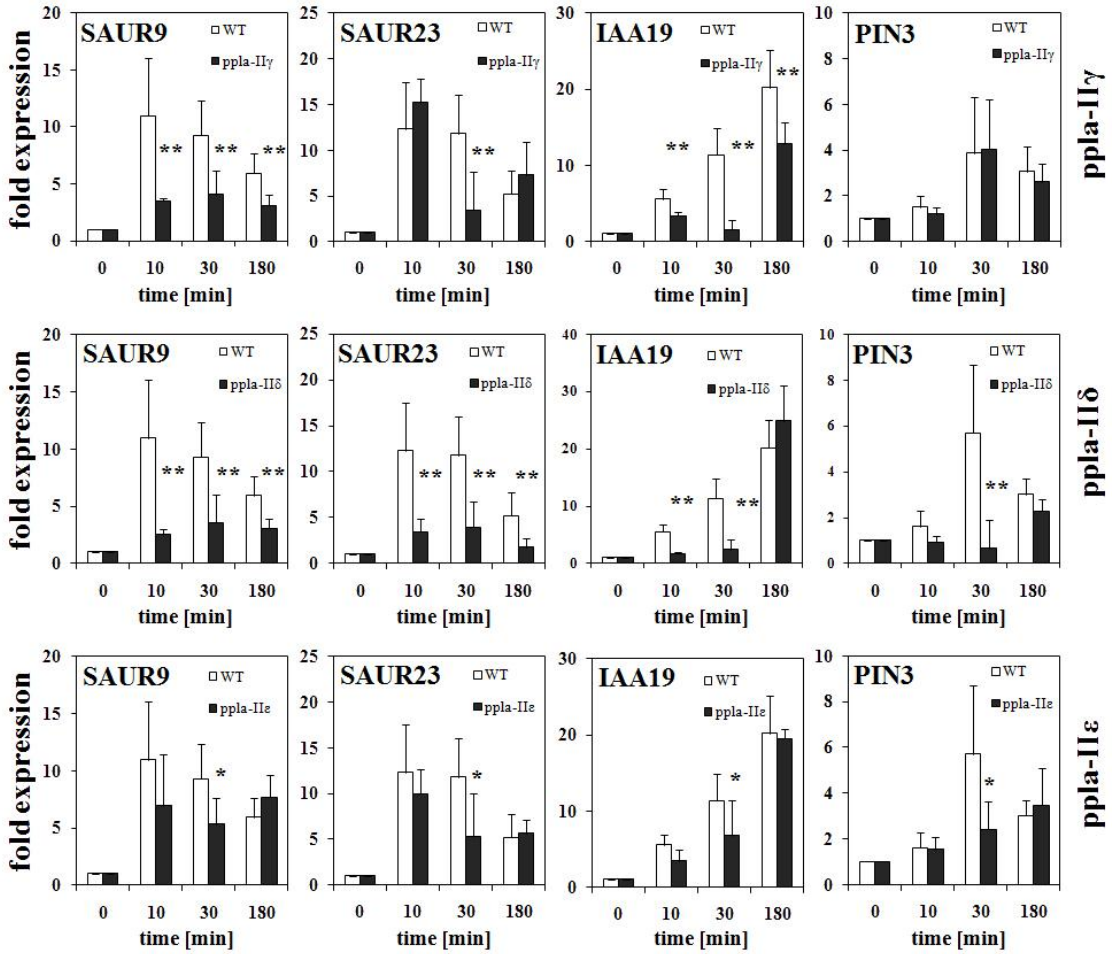

Figure 7. Time courses of expression of selected genes in three light-grown *ppla* mutant and wild type seedlings.
Asterisks above columns indicate significant differences between the mutants and the corresponding wild type treatments at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0{,}001$ (***) level according to t-test. Relative expression levels were calculated by setting values at t=0 min to 1 (white bars); values at t=30min IAA were calculated accordingly (black bars).

In the *ppla* mutants, especially *SAUR23* and *IAA19* were less up-regulated already after 10 min when compared to the wild type. *PIN3* and *SAUR9* reacted only after 30 min. In the genes selected, mis-regulation was transient and at 180 min expression of all genes in mutants was statistically indistinguishable from that in wild type even though persistent small differences may have gone undetected. In some other genes (Fig. 5) persistent differences could be found.

DISCUSSION

Half of ppla mutants have a developmental phenotype related to auxin functions

In this study we aimed to understand whether some or all *pPLA* genes or proteins have a function related to auxin by assessing classical root developmental responses in combination with auxin responsive gene expression. In auxin signaling, activation of pPLA was described (Scherer and André, 1989; Paul et al., 1998) but did not allow to assign this activation to any single enzyme. Several previously described *ppla* mutants exhibit developmental phenotypes related to auxin physiology (Fig. 1; Suppl. Fig. 1; Huang et al., 2001; Rietz et al. 2010; Li et al., 2011). In response to auxin, *ppla-IIε* developed fewer lateral roots than wild type seedlings only under nutrient stress (Rietz et al., 2010). *ppla-IIγ* showed changes in root architecture in response to phosphate deficiency and ABA (Rietz et al., 2010), but not in response to auxin alone (compare suppl. Fig.1). Root responses to phosphate deficiency involve several hormones but increased lateral root formation and inhibition of main root growth is a hallmark of auxinic compounds so that auxin is regarded as the main growth-regulating hormone involved in phosphate deficiency (Lopez-Bucio et al., 2002; Pérez-Torres et al., 2008). A careful investigation of the *ppla-IIIβ* mutant and *pPLA-IIIβ* overexpressing plants showed some – relatively weak – characteristics, which could be ascribed to an auxin-related phenotype: slightly longer roots and hypocotyls in *ppla-IIIβ* mutant and shorter roots and hypocotyls, as well as smaller leaves in the overexpressors in young seedlings (Li et al., 2011). These subtle differences in the *ppla-IIIβ* mutant might have gone undetected when seedlings were measured at 7 days after germination in our growth conditions. Possible effects of metabolites generated by pPLA-IIIβ on root growth were compared between *ppla-IIIβ* and wild type. The free fatty acids 18:2 and 18:3 inhibited root growth in both *ppla-IIIβ* mutant and wild type plants; however, root growth inhibition was more pronounced in mutant seedlings (Li et al., 2011). Strikingly, reduced lobe formation in the interdigitating pattern of leaf epidermis cells resembled those observed in *abp1* auxin receptor mutants (Xu et al., 2011). Together, these defects and abberant epidermal cell patterning indicate a role for auxin in this regard. In addition, while *pPLA-IIIβ* overexpressing plants showed enhanced stem fragility, stems of insertional mutants were slightly more elastic than in the wild type plants, which was interpreted as a lack of cellulose biosynthesis in the mutants associated

with this trait (Li et al., 2011). A fragility phenotype was also found in plants overexpressing *pPLA-IIIδ* that were isolated by activation tagging and named *STURDY* (Huang et al., 2001). Further characteristics of *pPLA-IIIδ* overexpressing *STURDY* plants were thicker stems and siliques and some proliferation of the xylem which was similar to *pPLA-IIIβ* overexpressors described by Li et al. (2011).

As shown in Fig. 1, only *ppla-IIIδ* seedlings exhibited an obvious auxin-related developmental phenotype (root growth). Compared to the wild type, mutant seedlings developed more lateral roots and shorter primary roots. The total number of genes tested here which were up-regulated by exogenous auxin in *ppla-IIIβ* or in *ppla-IIIδ* was neither so different between these two mutants nor different when compared to other *ppla* mutants. Thus, there is no indication that rapid regulation of auxin-induced genes is "translated" into this auxin-related phenotype in *ppla-IIIβ* and in *ppla-IIIδ*. In summary, in half of the *ppla* mutants a developmental phenotype was found. It is obvious that it is more difficult to find a developmental phenotype than finding the regulatory phenotype described here (see below).

ppla mutants exhibit delayed auxin-induced gene expression

Using basically the same transcriptional approach as described here, we identified a clear regulatory phenotype in the *abp1/+* auxin receptor mutant in which all the genes tested here were likewise mis-regulated (Effendi et al., 2011). Application of 1 µM or 10 µM auxin yielded similar results in *abp1/ABP1* plants (Effendi et al., 2011) and several *ppla* mutants (Scherer et al., 2012). 50% of the *ppla* mutants exhibit subtle auxin-related developmental phenotypes (Rietz et al., 201; Li et al., 2011; Effendi et al., unpublished). In addition, all mutants investigated display defects in the transcriptional regulation of early auxin-induced genes suggesting functional redundancy. The number of mis-regulated genes does not correlate with the penetrance of developmental phenotypes. Possibly, the number of genes tested was too small. Alternatively, at the level investigated here, required spatio-temporal co-expression patterns of pPLAs and auxin response genes may not be optimal. Furthermore, the majority of the early auxin response genes such as *GH3*s and *IAA*s function in the regulation of the auxin signaling loop itself. Thus, direct connections to specific developmental phenotypes are unclear. Likewise, the *PIN* genes were also

selected for their function in regulating auxin gradients. It should be stressed, however, that iall *ppla* mutants exhibited delayed expression of auxin-induced genes.

Several genes were preferentially mis-regulated in *ppla* mutants: *IAA2* (5x), *IAA19* (4x), *PIN3* (5X), *GH3.5* (5x), *SAUR9* (6x), *SAUR15* (4x) and *SAUR23* (6x). The likely the reason for this finding is functional redundancy of *pPLA* genes. For some of the investigated response genes (*IAA3, IAA19, PIN3*) a function in lateral root formation is known (Benkova et al., 2003; Peret et al., 2009). Possibly, this relates to several of the observed root phenotypes in some *ppla* mutants. *GH3.5* codes for one of the auxin conjugating enzymes (Staswick et al., 2005), but the link to developmental phenotypes is unclear. How *SAURs* exert their function is still unknown (Jain et al., 2006) Only few were recently shown to have a function in auxin-dependent elongation in rather specific circumstances (Franklin et al., 2011; Spartz et al., 2012; Chae et al., 2012) so that searching for a phenotype based on the results of our gene expression study alone is not promising. Analyzing transcriptional differences between wild type and mutants in non-treated seedlings also provided no safe basis to predict morphological phenotypes except for *ppla-IIIδ* (Fig. 5) in which six genes were differently eypressed and *ppla-IIIδ* indeed has a morphological phenotype (Fig. 1)

The initial imbalance in regulation of auxin-induced gene expression is mostly attenuated after 3 h (Fig. 7). The response genes chosen for this experiment were rapidly auxin inducible, but unaffected under non-induced conditions. Together, this part of our investigation indicates that, despite the absence of obvious morphological phenotypes in some *ppla* mutants, *pPLAs* generally may exhibit functions in auxin physiology at least by defining a gene regulatory phenotype.

Slowing of auxin-induced gene expression in *ppla* mutants within 10 min and 30 min indicate a link of pPLAs to auxin signaling

In each of the *ppla* mutants we found a delay in up-regulation of auxin-induced gene expression, the primary molecular response to auxin (Quint and Gray 2006; Mockaitis and Estelle, 2008; Delker et al., 2008). This makes all *ppla* mutants to auxin signaling mutants.

Any auxin-induced response must start with binding of auxin to a receptor. In case of the TIR1/AFB auxin receptors, the auxin response will indeed start with regulation of gene

expression. These regulated genes have binding sites for ARF transcription factors. In the presence of high cellular auxin levels, TIR1 will have increased activity as E3 ligase and, in turn, trigger the degradation of the IAAs, which act as repressors of the ARF transcription factors (Delker et al., 2008). This will lead to derepression of these genes i.e. activation.

Next, we compare three auxin-induced actions (1) auxin-induced activation after 2 min (Paul et al., 1998), (2) delayed activation of auxin-induced genes in *ppla* mutants after 10 min (Fig. 5), and (3) after 30 min (Fig. 6), and test the idea that TIR1/AFBs can or cannot be responsible for these responses. Obviously, change of transcriptional activity after 2 min excludes transcription as a mechanism and, thus, the TIR1/AFBs as receptors for this auxin-induced response. This situation is similar to several other rapid auxin-induced responses. It has been amply discussed that ABP1 may be a more likely receptor for such rapid responses (Napier et al., 2002; Badescu et al., 2006). The lower time limit for physiological responses (as opposed to transcription of early auxin-induced genes without direct physiological function of mRNA) regulated by TIR1/AFBs was found to be 10 min (Scherer, 2011).

What might cause the delayed up-regulation of auxin-induced genes 10 or 30 min after auxin treatment in *ppla* mutants? Possible explanations would be either a decrease in TIR1/AFB protein levels or modulation of TIR1/AFB activity. The latter possibility might be equivalent to receptor activity modulation by pPLA activity. If TIR1/AFBs would be the receptor for this specific rapid response it would have to make use of the only known function of TIR1, regulation of auxin-dependent transcription. In fact, none of the *pPLA* genes showed increased auxin-induced expression. We found only decreased auxin-induced expression after 3 h at the earliest (Fig. 6). The hypothesis we found more attractive is that pPLA activation is triggered by a different receptor, which then regulates TIR1/AFB activity by a yet unknown mechanism. ABP1 apparently exhibits the property to modulate TIR1 activity, but activation of pPLA activity by ABP1 remains to be shown (Effendi et al., 2011).

An alternative hypothesis for the modulation of TIR1/AFB activity is TIR1/AFB induced expression of one or several proteins that then stimulate pPLA activity. This would allow for back-coupling to TIR1/AFB activity by an initially solely transcriptional mechanism.

But can it happen within 10 min? Although degradation of IAA proteins can occurwithin minutes (Zenser et al., 2001), this event still needs to exert an influence on the biosynthesis of the postulated unknown protein, which again will take at least several minutes. Likely, it is inconceivable that such a mechanism is rapid enough to allow *detectable* back-coupling to TIR1/AFB activity within 10 min. Hence, delayed activation of auxin responsive genes 10 min after auxin treatment in *ppla* mutants is very unlikely to be caused by transcriptional regulation by TIR1/AFBs. More likely, a different auxin receptor is required, which then exerts an unknown but rapid effect on TIR1/AFBs. This is compatible with a post-translational mechanism of pPLA activity by auxin.

After 30 min the same trends in expression of the most rapidly regulated genes were observed as after 10 min. However, the response was much stronger than after 10 min (Fig. 5). Considering the timing for a 30 min response, a reaction chain for back-coupling to TIR1/AFB triggered regulation of newly induced expression of an unknown protein to regulate TIR1/AFB activity, this seems quite possible. It should be asked, however, why a *different* second mechanism should be evoked if there is already one operating without evoking this additional component? We rather assume that the same mechanism should be assumed for pPLA action in auxin signal transduction which operates with ABP1 as a receptor at 10 min or at 30 min (for similar data see: Effendi et al., 2011).

There are more hypothetical mechanisms possible than the ones described, but one question remains eminent: how compatible are they with cellular timing? The unknown component postulated, for instance, could be a protein which regulates auxin concentration either by decrease/degradation or by biosynthesis. Again, if fitting to our experiment in Fig. 5, the kinetics must be rapid. A gene having such a property could be *GH3.5*, which decreases free auxin levels by conjugating IAA to amino acids (Staswick et al., 2005). The problems with the timing are the same as above: first, a gene's expression is regulated, then the enzyme must regulate to an effective amount the auxin concentration to which then TIR1/AFBs can react. The necessary kinetic data are not known and the success of such a reaction chain seems barely possible for a time span of 30 min, but not at all for 10 min. Similarly, we can apply this kinetics concept to auxin biosynthesis, e.g. increasing auxin concentration as a necessary component to execute a response needing pPLAs. Although we know even less about auxin biosynthesis we arrive at the same conclusions regarding the required velocity of these responses. Currently, there are not enough data on

the *rapidity* of changes in auxin biosynthesis rates (Quint et al., 2009; Mashiguchi et al., 2011;; Mana and Nemoto, 2012; Ruiz Rosquete et al., 2012) to draw a final conclusion.

In conclusion, we hypothesize that auxin activation of pPLA activity within 2 minutes must start with binding of auxin to a receptor other than TIR1/AFBs and likely a direct or indirect influence is exerted by pPLA activation on TIR1/AFBs resulting in TIR1/AFB dependent auxin responsive gene expression within 10 min after an auxin stimulus. As of yet, we are unable to pinpoint by what mechanism TIR1/AFB activity may be modulated in *ppla* and/or *abp1* mutants (Effendi et al., 2011). Although we favor the hypothesis that the regulation of auxin concentrations by PIN proteins is the "regulatory glue" between ABP1 and TIR1/AFBs, this needs to be substantiated (Effendi et al., 2011; Scherer et al., 2012). A biochemical modification of TIR1, e.g. by NO, seems to be an additional possibility. However, this would once more evoke the question about the auxin receptor triggering the increase in NO (Terrile et al., 2012). pPLA enzymes, by sequence analysis, are likely cytosolic enzymes, even though they can be associated with the membranes (Holk et al., 2002; La Camera et al., 2005; Li et al., 2011). We found that two pPLA enzymes, pPLA-IIδ and pPLA-IIε, are activated by protein kinases (Rietz et al., 2010). Several more potential phosphorylation sites i pPLA C-termini were also suggested. These sites differ between group II and group III enzymes, offering the possibility of co-existence of different signal input pathways regulated by different protein kinases (Scherer et al., 2012). Such pathways could also be, for instance, related to the defense functions of *pPLA* genes (Scherer et al., 2010). The pPLA activation response and many other rapid responses to auxin require enzymatic reactions in the cytosol and a receptor other than TIR1/AFBs (Scherer et al., 2007; Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011). Whether the enigmatic receptor ABP1 bridges the missing gaps in the current models remains open (Sauer and Kleine-Vehn, 2011; Shi and Yang, (2011); Scherer et al., 2012).

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana T-DNA insertion lines were obtained from the SALK collection (Columbia ecotype) (Alonso et al., 2003) and from the Wisconsin collection (Wassilewskija ecotype) (Sussman et al., 2000). We used the T-DNA insertion lines of

pPLA-IIα (SALK_0591195.53.75.x), *pPLA-IIβ* (SALK_130122.24.55.x), *pPLA-IIγ* (= *ppla-IIγ-1*; Wisconsin collection; Sussman et al., 2000), *pPLA-IIδ* (SALK_090933.54.20.x = *ppla-IIδ-2*), *pPLA-IIε* (SALK_027625.39.00.x = *ppla-IIε-2*), *pPLA-IIIα* (SAIL830G12), *pPLA-IIIβ* (SALK_057212.52.95.x), *pPLA-IIIγ* (SALK_088404) and *pPLA-IIIδ* (SALK_029470). All SAIL and SALK lines are in the Columbia background, only *pPLA-IIγ* is in Wassiliewskija. Some of the mutants were described (*ppla-IIγ-1*, *ppla-IIδ-2*, *ppla-IIε-2*, Rietz et al., 2010). The homozygous status of the remaining mutants was determined by PCR and (Supplemental Fig. 1).

Seedlings were grown under long day conditions (16h white light, 8h dark, 40 µE). For physiological auxin experiments seedlings were pre-grown for 3 days on ATS agar with 1% sucrose and then transferred to ATS-medium (Estelle and Somerville, 1987) containing appropriate concentrations of 1-NAA. After 12 days on vertical agar plates the seedlings were scanned and analyzed. Root and hypocotyl lengths were measured using AXIOVISIOLE version 4.6 software (Zeiss, http://www.zeiss.com/). Lateral root density was determined based on Dubrovsky and Forde (2012).

To investigate early auxin gene expression in *ppla* mutants, seedlings were grown in MS/2 liquid medium for 7 days under long day conditions. Prior to treatment with auxin the medium was replaced by fresh medium. After 4 hour calibration in the fresh medium, seedlings were treated either with 10µM IAA or only with MS/2 liquid medium for 10 min, 30 min, or longer. Plant material was quickly blotted on filter paper and frozen in liquid nitrogen.

Nucleic acid analysis

For quantitative RT-PCR, total RNA from auxin treated seedlings was prepared using TRIzol® reagent according to the manufacturer´s instructions (Invitrogen) and converted to cDNA with RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas). Total RNA was treated with DNase I (Invitrogen, manufacturer's instructions) and converted to cDNA with RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas). Primers were selected from previous works (Li et al., 2009; Rietz et al., 2010; Effendi et al., 2011) or designed by using Primer 3 software (http://frodo.wi.mit.edu/primer3/) and Netprimer software (http://www.premierbiosoft.com/netprimer/index.html). Primer efficiency was

checked by using different cDNA concentrations and only primer with mathematical efficiency between 95 and 105% were used. Primers are listed in Supplemental Fig. 3. For quantitative PCR reactions SYBR-Green Master Mix was used in a StepOnePlus™ system (Applied Biosystem). About 30ng cDNA, 200 nM primers, 0,5 µM ROX (Invitrogen), 0.1x SYBR Green (Invitrogen) and 0,03U Hot Start Polymerase (DNA cloning service) were utilized in one PCR reaction. The specificity of PCR amplification was examined by monitoring the presence of a single peak in the melting curves for quantitative PCR. In each experiment four to six biological repeats and for each biological treatment three technical repeats were performed for the subsequent qPCR reaction. The expression level for the control treatment was set to 1-fold. PCR conditions were: activation of the polymerase at 95°C for 10 min; 40 cycles of DNA melting at 95°C for 15 s, and DNA annealing at 62°C for 60 s. Relative expression calculation and statistical analysis were done with REST 2009 software (Pfaffl et al., 2002). The expression level at t = 0 was set to 1-fold for all lines or calculated relative to the reference gene *UBQ10*.

ACKNOWLEDGEMENTS

Support from the Deutsches Zentrum für Luft- und Raumfahrt (contract number 50WB0627) and from the Deutsche Forschungsgemeinschaft (Sche207/24-1) is gratefully acknowledged. Work in XW laboratory was supported by a grant from the National Science Foundation (MCB-0922879). We thank M. Quint (Halle) for help with language edition and many helpful discussions.

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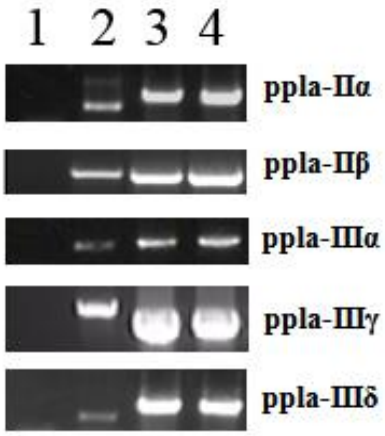
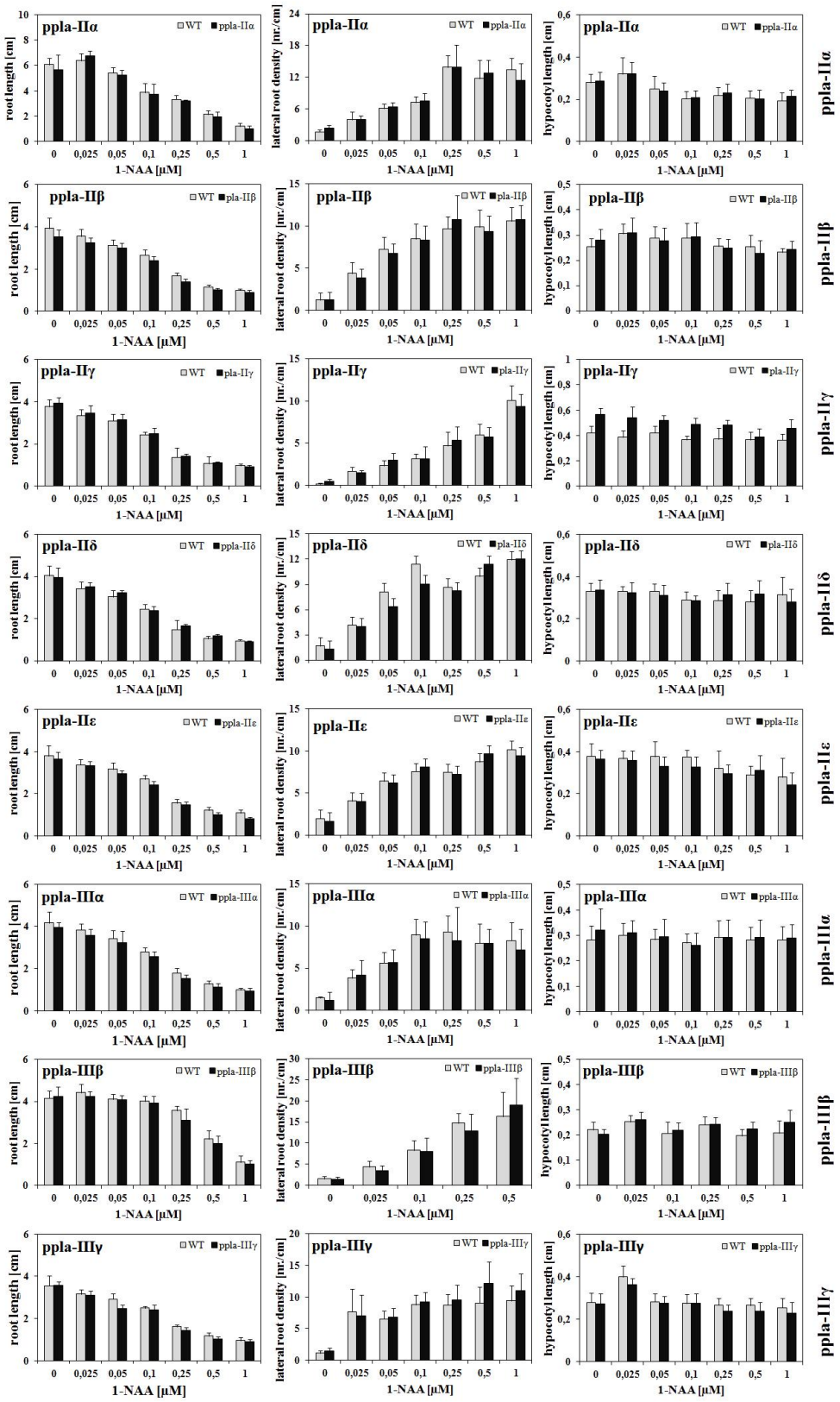
SUPPORTING INFORMATION



Supplemental Figure 1. Transcriptional analysis of relevant *pPLA* in *ppla* knock out mutants

RT-PCR analysis to show the deficiency of *ppla-IIα, ppla-IIβ, ppla-IIIα, ppla-IIIγ* and *ppla-IIIδ* transcripts in homozygous mutant plants. *Actin* was used as an internal control. 1: mutant cDNA and gene primer, 2: Wildtype cDNA and gene primer 3: mutant cDNA and *Actin* primer, 4: Wild type and *Actin* primer



Supplemental Figure 2. Physiological responses to auxin of T-DNA insertion mutants. Plants were grown in white light for 7 d on upright agar plates in the presence of increasing concentrations of 1-NAA.

Supplemental Table 1. Primer list

Oligoname	Gene	Sequence (5`- 3`)
UBQ10_for	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10_rev	AT4G05320	AAAGAGATAACAGGAACGGAAACATAGT
IAA2_for	AT3G23030	GGTTGGCCACCAGTGAGATC
IAA2_rev	AT3G23030	AGCTCCGTCCATACTCACTTTCA
IAA3_for	AT1G04240	AACTGAAACATCCCCTCCTC
IAA3_rev	AT1G04240	CCATCTCTCTCAAAGTACTCTCC
IAA11_for	AT4G28640	CCTCCCTTCCCTCACAATCA
IAA11_rev	AT4G28640	AACCGCCTTCCATTTTCGA
IAA13_for	AT2G33310	CACGAAATCAAGAACCAAACGA
IAA13_rev	AT2G33310	CACCGTAACGTCGAAAAGAGATC
IAA14_for	AT4G14550	CCTTCTAAGCCTCCTGCTAAAGCAC
IAA14_rev	AT4G14550	CCATCCATGGAAACCTTCAC
IAA19_for	AT3G15540	GGTGACAACTGCGAATACGTTACC
IAA19_rev	AT3G15540	CCCGGTAGCATCCGATCTTTTCA
IAA20_for	AT2G46990	CAATATTTCAACGGTGGCTATGG
IAA20_rev	AT2G46990	GCCACATATTCCGCATCCTCT
SAUR9_for	AT4G36110	GACGTGCCAAAAGGTCACTT
SAUR9_rev	AT4G36110	AGTGAGACCCATCTCGTGCT
SAUR15_for	AT4G38850	ATGGCTTTTTTGAGGAGTTTCTTGGG
SAUR15_rev	AT4G38850	TCATTGTATCTGAGATGTGACTGTG
SAUR23_for	AT5G18060	ATGGCTTTGGTGAGAAGTCTATTGGT
SAUR23_rev	AT5G18060	TCAATGGAGCCGAGAAGTCACATTGA
GH3.5_for	AT4G27260	AGCCCTAACGAGACCATCCT
GH3.5_rev	AT4G27260	AAGCCATGGATGGTATGAGC
PIN1_for	AT1G73590	ATGGCTTCTGGTGGTGGTCGGAA
PIN1_rev	AT1G73590	AGCAGGACCACCGTCTTCTTCGT
PIN2_for	AT5G57090	TATCAACACTGCCTAACACG
PIN2_rev	AT5G57090	GAAGAGATCATTGATGAGGC
PIN3_for	AT1G70940	TGGTCCAAATCGTCGTCCTCCA
PIN3_rev	AT1G70940	TGGAAGCAGCCGTCTCAGGGA

PIN5_for	AT5G16530	CCATCGGCTCTATTGTCCTTG
PIN5_rev	AT5G16530	GCGACGAGCACAGGTAGAGA
pPLA-IIα_for	AT2G26560	TCCTCTCCAGCCCCCGACCTAT
pPLA-IIα_for	AT2G26560	AGCCCTCTAATGCCACCACCGT
ppLA-IIβ_rev	AT5G43590	TGTCGAAGCAAGGTATGACAACG
ppLA-IIβ_for	AT5G43590	AGGAGGACCCAAGTTCAACGG
ppla-IIγ_for	AT4G37050	CCTTGATTGAGATTGTAAAGGAGAAGAG
ppla-IIγ_rev	AT4G37050	AGATAGAACAGTTGCTTGTCTTCC
ppla-IIδ_for	AT4G37060	CCGAGGAAAGGAAATTAAGGAGAA
ppla-IIδ_rev	AT4G37060	TACAGCCGGAAAATCACTCTCG
ppla-IIε_for	AT4G37070	GGAAATCATTCGTGCTTTTGTGTGAA
ppla-IIε_rev	AT4G37070	ACGTCCATTACTTTATATGCTGTGAG
ppla-IIIα_for	AT2G39220	ACAAGAACAAAAGCATCGTTTGCGA
ppla-IIIα_rev	AT2G39220	AGGCATTATCGCCAAGACAAGACA
ppla-IIIβ_for	AT3G54950	ACATGATGAGAAAAGGGGAAACACCT
ppla-IIIβ_rev	AT3G54950	TGGGCAGCATGGATTAAGGAGC
ppla-IIIγ_for	AT4G29800	GAGCTTGTGGACCGAACGTGGAC
ppla-IIIγ_rev	AT4G29800	TGCTTCCGAACAAAACCGACTCCA
ppla-IIIδ_for	AT3G63200	CGAGCTTCGACTTCGAGCTGTGG
ppla-IIIδ_rev	AT3G63200	AGGTTTTCCCGTCCACCGACACT

CHAPTER 3

AUXIN-RELATED AND PHYTOCHROME-RELATED RESPONSES ARE COMPROMISED IN PATATIN-RELATED PHOSPHOLIPASE-A-I (PPLA-I) KNOCKOUTS OF *ARABIDOPSIS THALIANA*.

This Paper contains results from experiments that were done in this thesis. The expression of early auxin-induced genes was tested in the *ppla-I* mutants. The results in Chapter 2 show the expression of early auxin-induced genes in 9 of the 10 mutants of pPLA genes in Arabidopsis. The missing *ppla-I* is going to be published seperateley as addition to a broad investigation of pPLA-I in Arabidopsis. Figure 3 shows the results of this thesis.

Auxin-Related and Phytochrome-Related Responses are Compromised in Patatin-Related Phospholipase-A-I (pPLA-I) knockouts of Arabidopsis thaliana

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SUMMARY

pPLA-I is the evolutionarily oldest patatin-related phospholipase A (pPLA) in plants. Molecular and physiological analysis of two allelic null mutants (*ppla-I-1* in Wassilewskija (Ws), *ppla-I-3* in Columbia (Col) revealed *pPLA-I* has functions in auxin and light signaling. In both alleles, a number of auxin-induced genes are up-regulated at a slower rate after 10 min and after 30 min and both alleles showed a slower gravitropic response of hypocotyls, indicating compromised auxin functions. We found impaired phyA-modulated functions like slower phototropic bending in *ppla-I-1* and gravitropically less disoriented hypocotyls in both alleles. phyB-dependent elongation in shade was hypersensitive in both

alleles whereas phyB-modulated properties like early flowering and formation of root coils were found only in *ppla-I-1*. The data are consistent with an explanation that *ppla-I-1* behaves like a *phyBphyD* mutant because Ws and *ppla-I-1* are devoid of *phyD* and Col and *ppla-I-3* are not.

Keywords: gravitropism, patatin-related phospholipase A pPLA-I, phototropism, phytochrome, root coiling, shade avoidance, Arabidopsis

INTRODUCTION

Phospholipase A (PLA) hydrolyses phospholipids either at the hydroxyl group of the C_1 (phospholipase A_1; PLA_1) or of the C_2 atom (phospholipase A_2; PLA_2) and liberates free fatty acids and lysophospholipids as products. Several structurally different enzymes can show this activity in plants (Scherer, 2010; Scherer et al., 2010). These enzymes are the small (14 kDa) secreted phospholipases A_2 ($sPLA_2$) (Ståhl et al., 1999), also found in fungi and animals, and the larger soluble patatin-related phospholipases A (pPLA), which include the homologous soluble calcium-independent phosholipases A_2 ($iPLA_2$) in animals (Six and Dennis, 2000; Balsinde and Balboa, 2005) and patatin-related phospholipases A (pPLA) (Scherer et al., 2010). pPLAs hydrolyse fatty acids of both phospho- and galactolipids (Matos et al., 2001; Yang et al., 2007), whereas the lipase-like phospholipase A_1 hydrolyses only at the C_1 hydroxyl group and prefers galactolipids over phospholipids (Aoki et al., 2007). Lipases do not hydrolyse phospholipids or glycolipids. The enzymes of the pPLA group with no additional domains have a molecular weight of around 50 ±5 kDa. The plant *pPLA* gene family is divided into three subfamilies (Holk et al., 2002; Scherer et al., 2010). Proteins of subfamilies II and III do not have domains besides the enzymatic domain itself and can be distinguished by overall homology and their exon-intron structure. Group I in *Arabidopsis* comprises only one gene (*At-pPLA-I*) having an additional N-terminal LRR domain with a G protein-binding motif within this LRR domain, and a C-terminal domain with unknown function. Gene *pPLA-I* is the one gene most similar to the homologous so-called calcium-independent PLAs (iPLA) described for

animals (Winsteadt et al., 2000; Holk et al., 2002) and, therefore, probably the evolutionarily oldest *pPLA* gene in plants.

Our laboratory investigated the function of auxin-activated and elicitor-activated patatin-related phospholipase A (pPLA). Activation of pPLA by auxin is detectable after 2-5 min, depending on the method of measurement (Scherer and André 1989; Paul et al., 1998). pPLA blockers inhibit purified recombinant patatin-related pPLA, auxin activation of pPLA activity *in vivo*, elongation growth (Scherer and Arnold, 1997; Holk et al., 2002) and auxin-induced proton secretion (Yi et al., 1996). Moreover, auxin regulation of the *DR5* promoter and of several *IAA* genes is sensitive to pPLA inhibitors (Scherer et al., 2007). Three group II pPLAs can be phosphorylated at a serine in the C-terminus; this phosphorylation enhances activity. Moreover, *pPLA-IIε* knockout mutants have fewer lateral roots, and a *pPLA-IIγ* null mutant does not respond to phosphate deficiency (Rietz et al., 2010) by forming lateral roots and decrease of main root length (Pérez-Torres et al., 2008). Several laboratories have demonstrated rapid activation of pPLAs, also in defence-related processes. Plant defence elicitors were shown to induce the production of free fatty acids and lysophospholipids within minutes in tomato leaves (Narváez-Vásquez et al., 1999), *Eschscholtzia californica* cells (Viehweger et al., 2002 and 2006), and *Petroselinum crispum* cells (Scherer et al., 2000; 2002). Viehweger et al. (2006) were the first to show that elicitors induce a transient rise of lysophosphatidylcholine (LPC), which is followed by activation of a Na^+/H^+ exchange transporter which thus acidifies the cytosol. LPC is also a second messenger in mycorrhiza formation (Drissner et al., 2007). Taken together, this supports a function of pPLAs in auxin and defence signaling.

Due to the presence of ten *pPLA* genes in the *Arabidopsis* genome, a functional analysis of individual genes is needed. The first knockout plants of the *pPLA-I* gene of this gene family, *pplaI-1* and *pplaI-2*, were described by Yang et al. (2007). The T-DNA insertion mutants of *pPLA-I* investigated here, *pplaI-1* (in Ws) and *pplaI-3* (in Col), do not exhibit an obvious growth defect when grown in soil or on agar plates. However, we found a complex phenotype of the mutants when we investigated the early regulation of auxin-responsive genes, gravitropism and phototropism. Moreover, the mutants showed a hypersensitive growth response in shade avoidance conditions and decreased inhibition of hypocotyl gravitropism by far red (FR) light pointing to compromised phyB signaling.

Here we report that *pPLA-I* knockout plants have a complex phenotype in auxin and, unexpectedly, also in light signaling.

RESULTS

Small differences in phenotypes of *ppla-I-1* and *ppla-I-3* are not explained by structural difference in gene or mRNAs

We isolated two T-DNA insertion mutants for the *pPLAI* gene, *pplaI-1* (Ws background) and *pplaI-1* (Col background). For *pPLA-I* an early annotation lists a sequence with 17 exons (http://www.ncbi.nlm.nih.gov/ nuccore/NM_104867.3), whereas the newest annotation predicts 18 exons in the gene and two splicing sites different from the earlier version (http://www.ncbi.nlm.nih.gov/ nuccore/NM_104867).

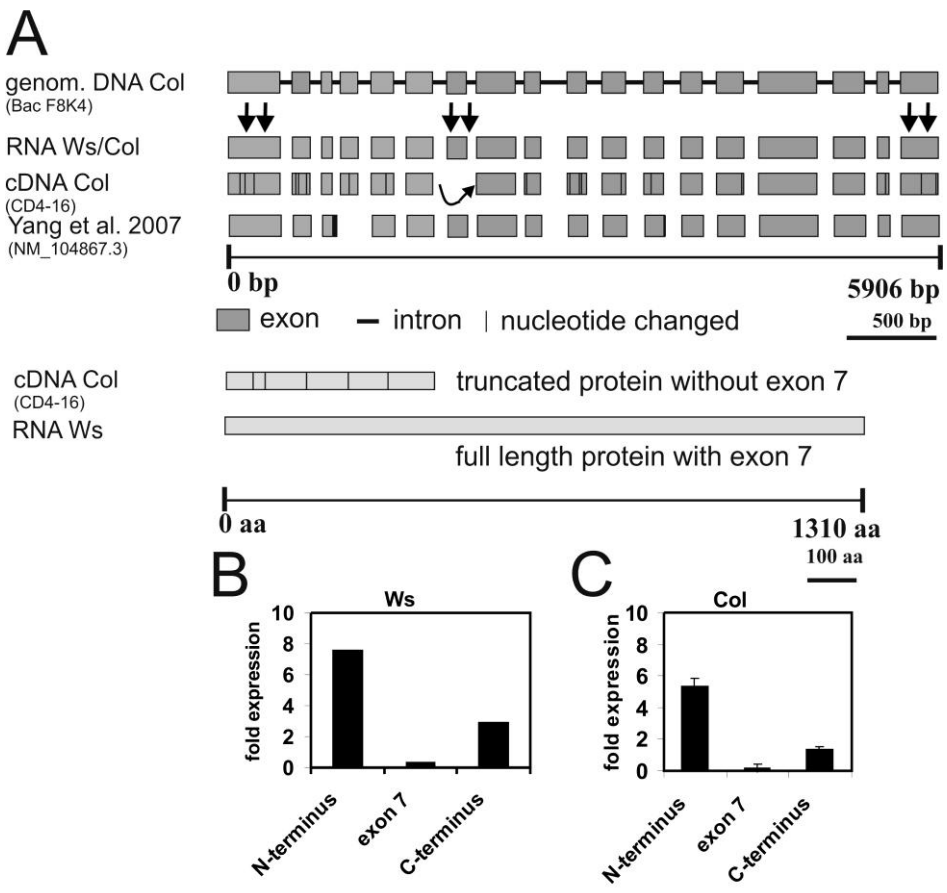


Figure 1. Genomic and exon-intron structure, mRNA sequences and splicing and transcription of *pPLA-I*.
(A) Genomic sequence, known and postulated RNA sequences and protein sequence. (B) Schematic presentation of potential protein sequences translated from the above mRNAs. (C, D) Relative amplicon frequencies as derived from the RNAs from Ws and from Col.

During the course of our work, we noted some differences between the two mutant lines which might have been due to sequence or splicing differences. This prompted us to sequence the genomic loci and cDNAs of both lines, which proved to be identical to the latest annotation (18 exons each; Fig. 1 A). Yang et al. (2007) described the sequence of a BAC clone CD4-16 encompassing the *pPLA-I* cDNA with exon4 missing, and used this clone for expression of the pPLA-I protein. We found, however, that this clone lacked exon7, not exon4, and contained 19 single base errors when compared to the genomic sequence, apparently introduced by the reverse transcriptase used to create the clone. According to our sequence data of CD4-16, it can code for a protein of about one-third of the complete protein only and contains several amino acid changes in that fragment. After that, a stop codon would terminate this potential protein (Fig. 1 B). The missing exon7 in CD4-16 could be due to a splicing error. We tested this by choosing primers for quantitative RT-PCR in the upstream 5'-terminal part of the RNA, for the exon7 alone, and for the downstream 3'-terminal part of the RNA. In both Ws and Col wild type the exon7 was present in mRNA at levels 25 times lower than the 5'-terminal part and about eight times less than the 3'-terminal part (Fig. 1 C). From this we conclude that mis-splicing, resulting in the excision of exon7, occurs 25 times more often than formation of the complete mRNA productive for protein biosynthesis. The reason for not coding a full protein is that the potential translation of mRNA without exon7 is also terminated prematurely in a stop codon. We cannot exclude that the full-length mRNA transcribed from the *pPLA-I* gene was present in low abundance in the CD4-16 clone but, despite much effort, the missing exon7 was not found there and CD4-16 was not useful for protein expression. In conclusion, differences in *pPLA-I* gene or mRNA sequence, or in RNA splicing, are not a cause for the differences observed in the mutant lines *ppal-I-1* or *ppla-I-3*.

Expression pattern of pPLA-I

Both mutant lines are null mutants (Fig. 2 a, b). We isolated plants transformed by a p_{AtPLAI}*::uidA* construct linking the *uidA* indicator gene to the promoter of the *AtPLA-I* gene. When staining was done over night, we found p_{AtPLAI}-directed GUS activity in the vascular bundles of mature leaves and in pollen and trichomes only (Fig. 2 e, h, i, j). With extended staining up to 48 h, the stem vascular bundles and the root stele were also stained (Fig. 2 c,

d, f, g), as was the columella, albeit weakly (Fig. 2 i). At the site of lateral root emergence we found stronger GUS activity in the stele of the newly formed lateral roots (Fig. 2 g).

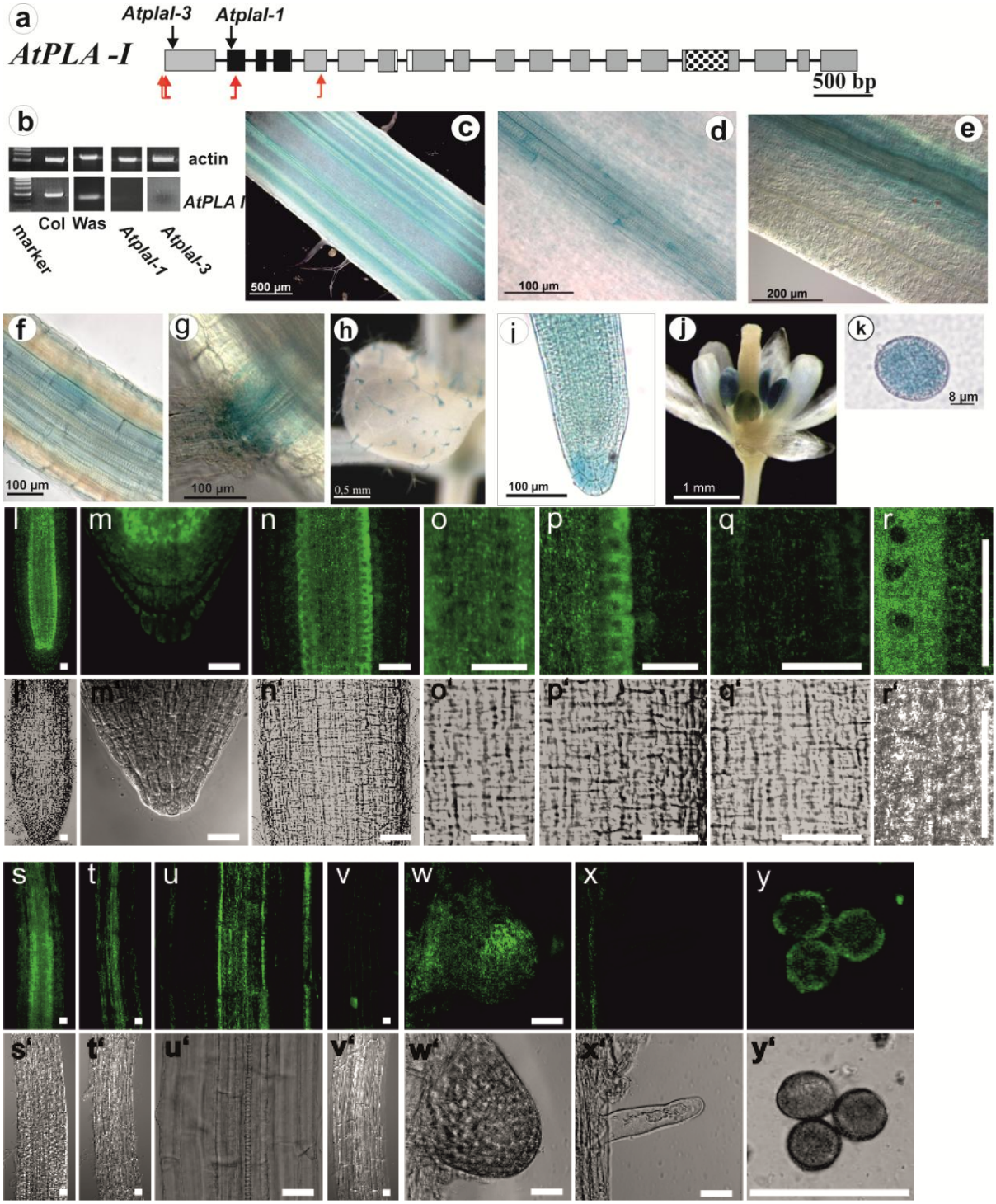


Figure 2. Expression of *pPLA-I*.
(a) Exon-intron structure of the *pPLA-I* gene. Black boxes highlight the LRR structure; the small white boxes the catalytic center. Black arrows for insertions, red arrows for RT-PCR as shown in (b). (b) RT-PCR on one-week-old seedlings of *pPLA-I* expression in Col, Ws, *pplaI-1*, and *pplaI-3*. GUS expression overview from P_{PLAI}*::uidA* (c -k). (c) P_{PLAI}*::uidA*

expression in the vascular of the inflorescence stem, (d) in a single the bundle of an inflorescence stem, (e) in a bundle of a petiole, (f) in the stele of the root, (g) in emerging lateral root, (h) in trichomes, (i) in the root tip. (j) Localisation of P_{PLAI}*::uidA* in the flower, (k) in the pollen. (l-y) pPLA-I-GFP expression in *ppla-I-1* background. Plants were grown in white light for 7 days. (l) root tip; (m) quiescent center with root cap; (n) mitotis root zone; (o) stele with endodermis, (p) stele, endodermis and cortex and rhizodermis, (q) rhizodermis; (r) endodermis plus cortex to the right; (s) transition zone elongation towards maturation; (t) elongation zone (u) elongation zone at higher magnification; (v) mature zome; (w) lateral root tip; (x) root hair; (y pollen. Bars: 20 µm. (l'-y') light microscopy of otherwise identical settings.

Tissue and subcellular localization was investigated also in plants expressing $_{PpPLA\text{-}I}$*:pPLA-I* in *ppla-I-1* (Fig. 2) and *ppla-I-3* background (suppl. Fig. 1) showing essentially the same tissue patterns. pPLA-I-GFP was expressed in the root tip (Fig. 2 l-n), and there less in the rhizodermis and cortex but strongly in the stele and endodermis (Fig. 2 n-q). Expression in the columella was weak but visible (Fig. 2 m). Expression decreased towards the root base (Fig. 2 l, s-v). Young lateral root tips showed clear expression in the tip meristem (Fig. 2 w). In root hairs no expression was apparent (Fig. 2 x) but strongly in pollen (Fig. 2 y).

At higher magnification (Fig. 2 p, r), a perinuclear localization, probably at ER and nuclear membrane became visible. This could be observed clearly in endodermis cells but less intense in cortex cells exhibiting weaker expression. In addition, we found pPLA-I localization in the cytosol and near the plasma membrane. In more mature parts of the root, in the transition from meristematic part to the elongation zone and in elongation zone, pPLA-I-GFP was found near the plasma membrane and not perinulear. In the more elongated and vacuolated cells cytoplasmic localisation and/or localisation near the plasma membrane could not be clearly distinguished (Fig. 2 p,q,r). Expression in the *ppla-I-3* background was nearly identical (suppl. Fig. 1). Tissue localization of GUS activity and pPLA-I-GFP were in good agreement to each other.

ppla-I mutants have a dynamic auxin phenoytpe

When seedlings were grown in white light or darkness, no obvious differences between the respective wild types and *ppla-I-1* and *ppla-I-3* mutant lines were observed. When we tested the response to auxin, similarly, no obvious differences in main root length or lateral root formation were found in mutants or wild types.

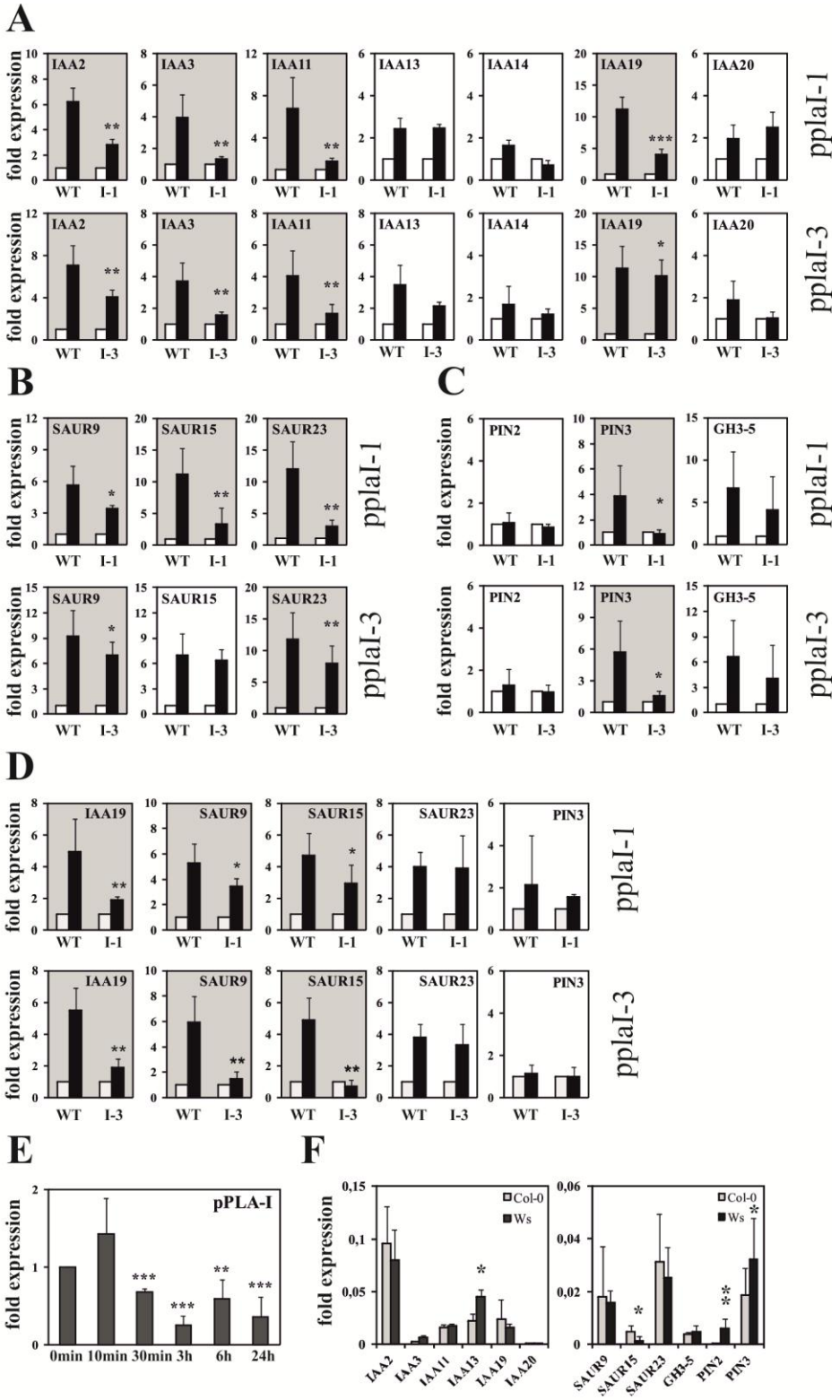


Figure 3. Regulation of early auxin-inducible genes in light-grown *ppla-I-1* and *ppla-I-3* knockout lines and respective wild type seedlings.

The results are arranged into different groups of early auxin-inducible genes and other genes. (A) Regulation of selected *IAA* genes. (B) Transcription of *SAUR* genes (C) Expression levels of *PIN* genes and *GH3-5*. Black bars: auxin-treated 30 min; white: non-treated 0 min. (D) Responses after 10 min in selected genes. Grey background in panels

highlight significant differences wild types versus mutant lines. (E) Decrease of transcription of *pPLA-I* after extended treatment with 10 µM IAA (measured in WS background). (F) Comparison of wild types Col (grey bars) and Ws (black bars) of all tested genes in non-treated samples. *PIN2* was about 30fold higher in Ws. The results are from four to six biological treatments with three technical repeats for each measurement. Relative expression calculation and statistical analysis were carried out with REST 2009 software (Pfaffl et al., 2002). Asterixes above columns indicate significant differences between the mutants and the corresponding wild-type or treatments at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0{,}001$ (***) level according to t-test.

Further, we tested regulation of early auxin-activated genes in both mutant lines and wild types as an auxin response (Fig. 3). Rapidity of regulation by exogenous auxin of the test genes was chosen as a criterion (Effendi et al., 2011), and selected test genes could be grouped into three groups: *IAA* genes some of which are involved in the formation of lateral roots (Fig. 3 A), *SAUR* genes (Fig. 3 B) and PIN2, PIN3, *GH3.5* (Fig. 3 C). In *ppla-I-1*, eight of thirteen genes tested responded weaker than in wild type (Ws) 30 min after application of 10 µM IAA, in *ppla-I-3* seven genes.

In order to narrow down the time span during in which pPLA-I could exert an influence on TIR1-directed transcription of early auxin genes we investigated the expression of selected genes also at 10 min in the knockouts (Fig. 3 D). Again, in both alleles delayed up-regulation of *IAA19, SAUR9* and *SAUR15* was observed even at 10 min. *pPLA-I* expression itself is not influenced by auxin in 10 min (Fig. 3 E) so that transcription regulation of *pPLA-I* is not the cause of the auxin-induced effect on expression of early auxin genes. When basal expression of Col and Ws wild types were compared only *PIN2* was clearly differently expressed (Fig. 3 F). Together, despite the absence of a growth phenotype, both mutant lines showed a clear auxin-induced regulatory phenotype.

Phototropism and gravitropism are impeded and root coiling increased in *ppla-I* mutants

If both mutant lines were grown in the dark for 3 days, only the *pplaI-1* mutant showed a delayed phototropic response, but *pplaI-3* did not, when compared to the respective wild types (Fig. 4 A, B). After excluding sequence differences as potential cause for different phenotypes in the two alleles we speculated that the lack of phytochrome D (Aukermann et al., 1997) could be the cause for such dissimilarities. Phototropism can be increased by far red and this reaction depends on phyA (Laringuet and Fankhauser, 2004) so that we

compared the phototropic response in *phyA* and *phyB* mutants. Indeed, the *phyA* mutant showed a reduced phototropic reaction, similar to *ppla-I-1*, whereas *phyB* reacted indistinguishable from wild type (Fig. 4 C, E).

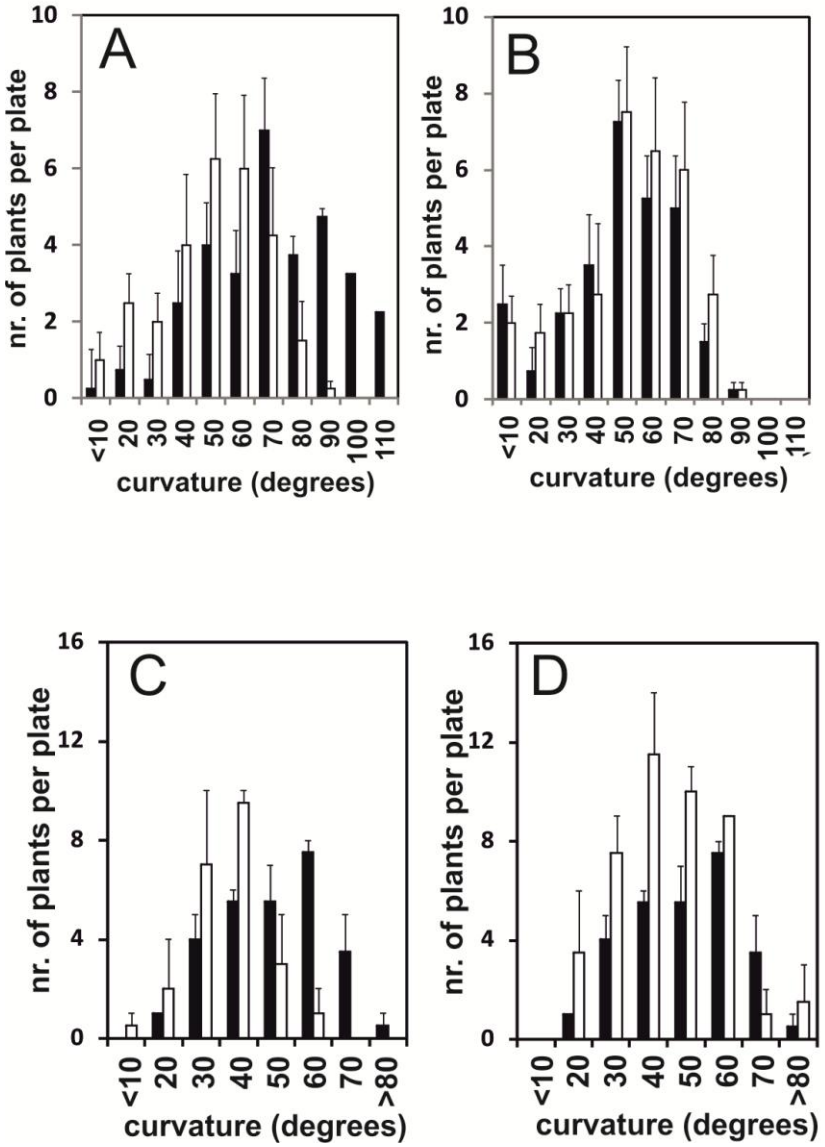


Figure 4. Phototropic bending of hypocotyls of 3-day-old dark-grown seedlings after 12 h lateral blue light (1 µE).
Black bars: wild types, white bars: mutants. (A) Ws: 67.1°, *ppla-I-1*: 45.6° ($p<0.001$). (B) Col: 46.6°, *ppla-I-3*: 47.2°. (C) Col: 51.2°, *phyA:* 33.1° ($p<0.001$). (D) Col: 51.2°, *phyB*: 41.2° ($p<0.001$). (means indicated, n=66-129).

The hypocotyls of *pplaI-1* and *ppla-I-3* responded to a gravitropic stimulus considerably slower after 24 h than the respective wild types (Fig. 5 A, B).

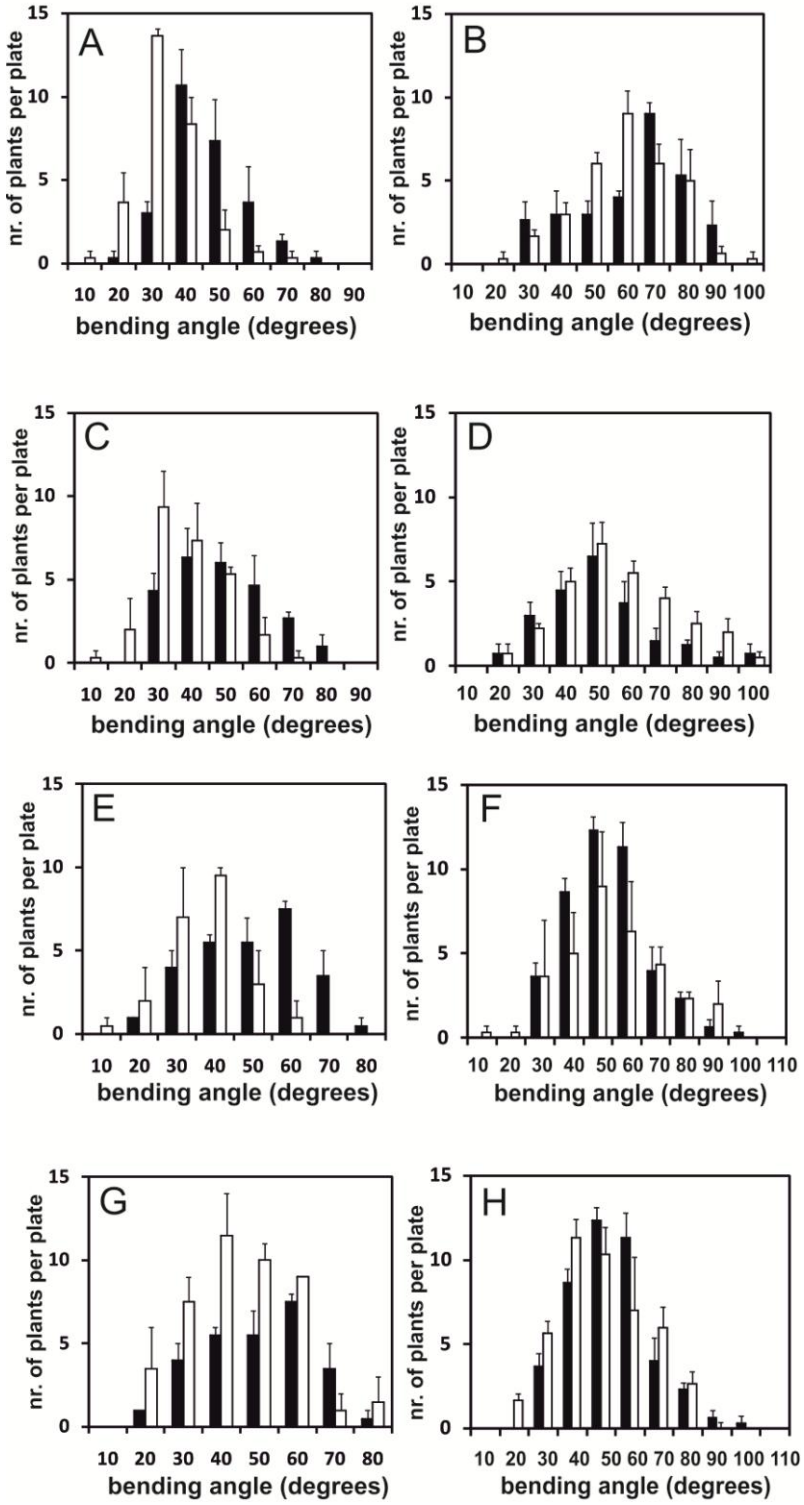


Figure 5. Gravitropic bending of hypocotyls or roots of 3-day-old dark-grown seedlings after 24 h tilting by 90°.

(A, C, E, G) Hypocotyl bending angle frequencies. (B, D, F, H) Root bending angle frequencies. Black bars: wild types. White bars: mutants. (A) Ws: 43.1°; *ppla-I-1*: 30.0° ($p<0.001$). (C) Col: 45°; *ppla-I-3*: 33.8° ($p<0.001$). (E) Col: 51.6°; *phyA*: 48.9°. (G) Col: 51.6°; *phyB*: 40.4° ($p<0.001$). (B) Ws: 58.5°; *ppla-I-1*: 54.7°. (D) Col: 46.4°; *ppla-I-3*:

52.4° ($p<0.014$). (F) Col: 54°; *phyA*: 54.9°. (H) Col: 54° *phyB*: 51.1°. (Means indicated, n=73-120).

The average bending angle of the roots of dark-grown *ppla-I-3* seedlings was statistically indistinguishable after 24h gravitropic reaction whereas the one of *ppla-I-1* was slightly smaller than in wt (Fig. 5 C, D). Gravitropism also can be decreased by red light (Robson and Smith, 1996). Therefore, we tested gravitropism in *phyA* and *phyB* seedlings under the same conditions as for *ppla-I* alleles. Only *phyB* hypocotyls showed a clearly reduced gravitropic response and only *phyB* roots responded slower to gravitropic stimulus whereas *phyA* roots and hypocotyls were indistinguishable from wild type in their responses (Fig. 5 E-H). Furthermore, we investigated the inhibition of hypocotyl gravitropism by far red light, which is greatest in *phyB* mutants and small in *phyA* mutants (Fankhauser and Casal, 2004). In *phyB* mutants, FR light abrogated gravitropism *via* phyA therefore hypoctyls appears random (Fig. 6 B). In fact, both mutant lines showed a similar, though weaker abrogation of hypocotyl gravitropism than respective wild types, while the *phyA* mutant, since blind to FR, grew almost upright (Fig. 6 B). In this test, both *ppla-I* knockouts displayed phenotypes resembling a weak defect in phyA signaling, by being more similar to *phyA* than to *phyB* in their responses.

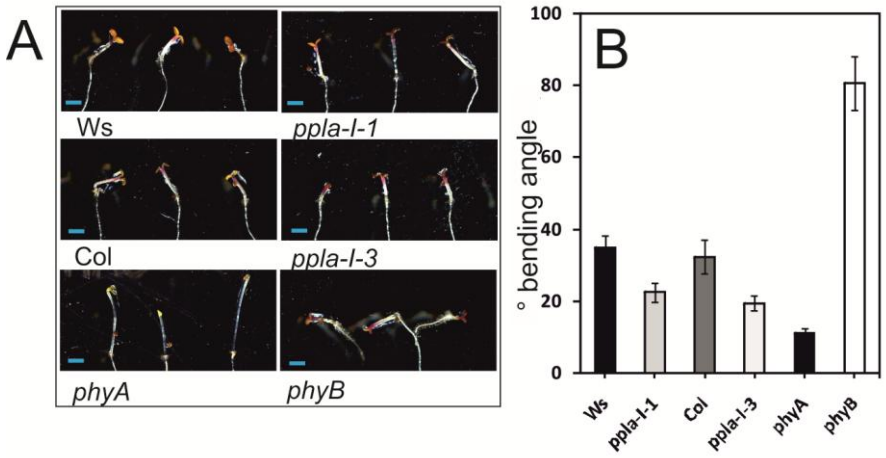


Figure 6. Interaction of far red light with hypocotyl gravitropism. Growth of seedlings in continuous far red light (10 µE) for 3 days and inhibition of gravitropism.
(A) Representative seedlings of the different genotypes used. (B) The bending angle is the angle deviating from the plumb line in absolute values. In all treatments, the differences between respective wild types and *ppla-I* mutants were highly significant ($p<0.001$; n=50-80).

A rather obvious feature of the mutant *pplaI-1* was its much higher tendency of root coil formation on 45° tilted hard agar plates (Simmons et al., 1995) than the Ws wild type's tendency (Fig. 7 A, B, G). Here the *ppla-I-3* line was indistinguishable from the Col wild type (Fig. 7 D, E, G), corresponding to results on root gravitropism. Coil formation was increased by increasing osmotic strength of the medium (Fig. 7 H).

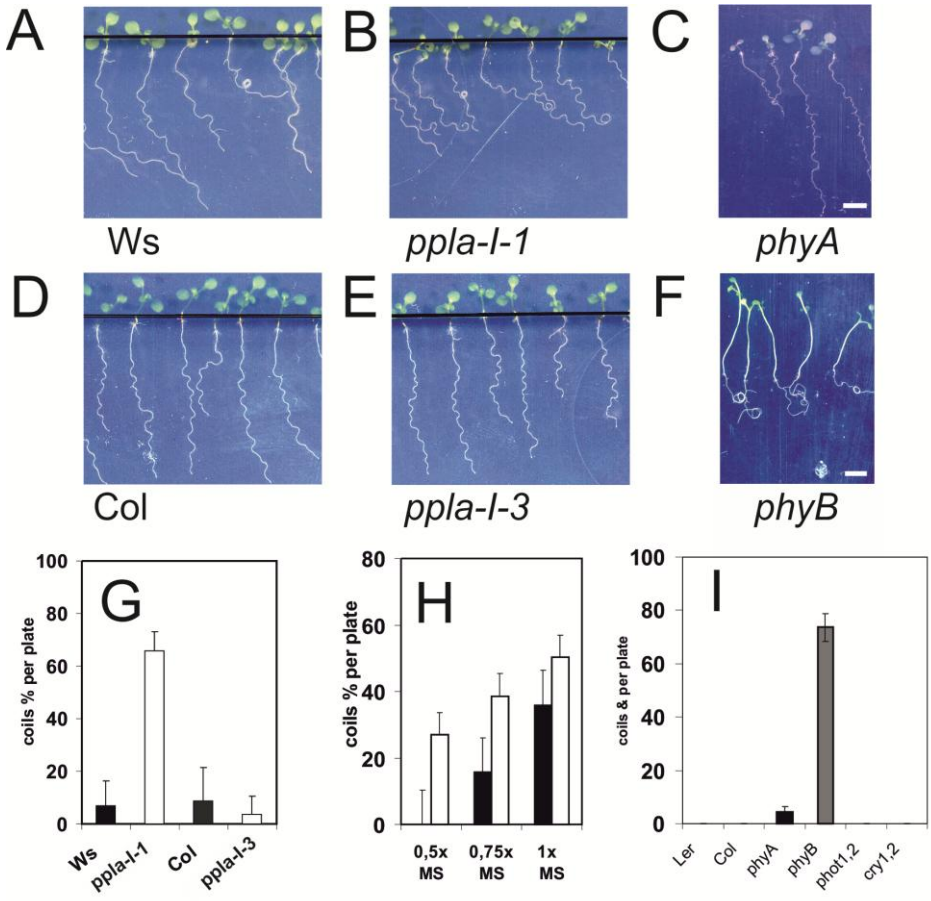


Figure 7. Formation root coils in seedlings grown on hard agar at a 45 ° tilted angle in (A, B, D, E) on 1/1 MS agar and in (C, F) on ½ MS agar. A plate with Ws wild type (A), *pplaI-1* (B), *phyA* (C), Col (D), *ppla-I-3* (E), and *phyB* in (F) is shown. (G) Comparison of coil frequency on 1/1 MS medium of the two mutant *ppla-I* lines. (H) Dependence of coiling response on the osmolarity of the medium in *ppla-I-1* (white bars) and Ws wild type (black bars). (I) Comparison of coiling in of several photoreceptor mutants on ½ MS agar. Each experiment was conducted with 4-6 agar plates harbouring 15 or 30 seedlings each (S.E.).

This has been noted before (Buer et al., 2000). When we tested *phyA* and *phyB* seedlings for root coil formation, *phyB* roots on 45° tilted agar produced about 80% coils whereas *phyA* roots only produced 5-10% (Fig. 7 C, F, I). To test contributions from blue light

receptors we also investigated *cry1,2* and *phot1,2* double mutants. Both produced no coils (Fig. 7 I).

ppla-I mutants have a red light phenoytpe

We noticed that in short days, *ppla-I-1* flowered earlier. We verified this in a growth chamber under controlled conditions but *ppla-I-3* did not flower earlier (Fig. 8), Early flowering is consistent with reduced phyB signaling (Devlin et al., 1999). Therefore, on our quest for a light phenotype, we quantified the responses of the *ppla-I* mutant lines to continuous R, FR, and B (blue light) (supplemental Fig. 2). We could not find convincing differences between mutants and corresponding wild types when grown in a single light color continuously (R, FR. B of 1 or 5 µmol m^{-2} s^{-1}). Only when we combined FR and B (1 µmol m^{-2} s^{-1} each) were the mutants significantly taller (Fig. 9 A).

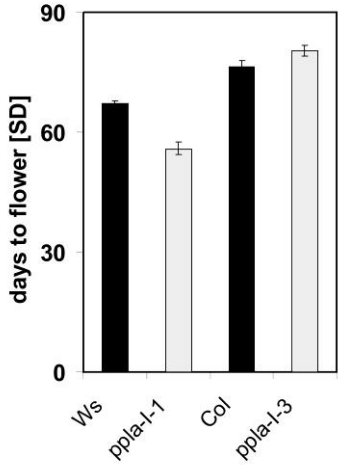


Figure. 8. Flowering dates of wild types and *ppla-I* mutants in short days (22° C; 16L/8D).
The day of the appearance of the first flower was recorded. Differences between corresponding wild types/ mutants were highly significant (both $p < 0.001$; n=36-39; S.E.).

The combination of FR and B light could indicate a different response to shade just as early flowering. Therefore, we also tested the elongation response to low R:FR and high R:FR in white light in hypocotyls of *ppla-I-1* and *ppla-I-3* and the respective wild types and compared it to *phyA* and *phyB* mutants (Fig. 9). In low R:FR conditions, both *ppla-I* mutant lines were much taller than the respective wild types (Fig. 9 B, C). Both wild types were taller in low R:FR than in high R:FR, but the differences between *ppla-I-1* and Ws or *ppla-I-3* and Col in high R:FR were much greater so that the mutants were hypersensitive

in their shade avoidance responses in low R:FR light. In low R:FR conditions *phyB* could not perform the hypersensitive elongation and were, as expected, red-insensitive in the high ratio R:FR light (Fig. 9 F, G).

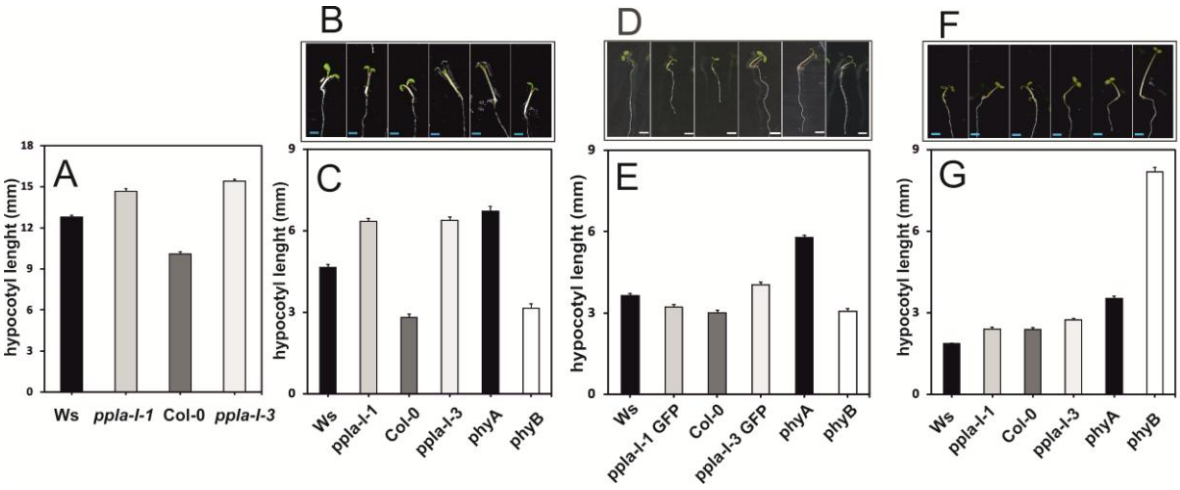


Figure 9. Light and shade avoidance response phenotype of *ppla-I* mutants.
(A) Seedlings were grown for 3 d in the dark and then treated for 12 h with continuous far red and blue light (1 µE each). (B, C) Growth of seedlings in white light supplemented with a low ratio of R:FR (red/ far red). Bar=2mm. In (C) the differences between respective wild types and *ppla-I* mutants were highly significant ((p<0.001). (D, E) Wt and complemented seedlings grown in white light supplemented with a low ratio R:FR light. Bar=4 mm. (F, G) Growth of seedlings in white light supplemented with a high ratio of R:FR (red/ far red). Bar=2mm; n=50-80.

When we used the *ppla-I* lines complemented by transformation of $P_{pPLA\text{-}I}$*:pPLA-I-GFP* the response to low ratio R/FR light was not hypersensitive in the complemented plants (Fig. 9 D, E). The shade avoidance response to low R:FR is mediated by low signaling output of phyB (Lau and Deng, 2010; Stamm and Kumar, 2010). Comparison to a *phyA* and *phyB* mutant in each assay showed that tall *ppla-I* hypocotyls likely corresponded to this low signaling output from *phyB*, as was obvious in the *phyB* mutant tested for comparison.

DISCUSSION

ppla-I-1 in Ws background and *ppla-I-3* in Col background show identical phenotypes with some exceptions

In regulation of early auxin genes, gravitropism of dark-grown seedlings, inhibition of hypocotyl gravitropism by FR light, and in the shade avoidance response of seedlings, *ppla-I-1* and *ppla-I-3* are very similar. On the other hand, they differ in other responses (phototropism, early flowering, root coils). Several of these responses are known to involve phytochrome signaling or to potentially involve phytochrome signaling. In fact, Ws is a natural deletion mutant for *phyD* (Aukerman et al., 1997) and Col is not. We excluded differences in genomic and mRNA sequence and in RNA splicing as a basis for the phenotypic differences of the two lines (Fig. 1). Moreover, the shade avoidance hypersensitive phenotype common to both alleles was complemented by expressing the pPLA-I-GFP protein in both alleles (Fig. 8 I). Comparison of tissue distribution of *pPLA-I*-promoter-controled GUS expression with the pPLA-I-GFP expression pattern also indicates tht the pPLA-I protein is expressed in the same tissues, probably reconstituting function in the null background. Other gene differences besides *phyD* between Ws and Col are known (Ulm et al., 2001; Yang and Hua, 2004; Bartels et al., 2009). Whereas the lack of *phyD* in *ppla-I-1* and Ws clearly seems to have an impact on the mutant phenotype, it remains elusive whether other genetic differences could have an impact on responses in *ppla-I* mutants.

ppla-I mutants have a dynamic auxin phenotype similar to the receptor mutant *abp1/ABP1*

Despite a lack of obvious morphological responses to exogenous auxin in the knockout lines compared to corresponding wild types, we found clear defects in both knockout lines in auxin-induced gene regulation using a set of selected genes as a physiological test (Fig. 3). This is reminiscent of our findings on the heterozygous *abp1/ABP1* and *eir1/pin2* mutants (Effendi et al., 2011; Effendi and Scherer, 2011). In *abp1/ABP1* seedlings, all auxin-regulated genes were mis-regulated; in *eir1/pin2* 9 of 12 were. Auxin-induced inhibition of *pPLA-I* transcription was only slight after 30 min (Fig. 3 E), so that transcriptional regulation of *pPLA-I* itself as a component of this signaling appears rather

unlikely (Fig. 3 D). In contrast to the *pPLA-I* gene itself whose expression was not changed within 10 min we found already after 10 min delayed transcription of early auxin-induced genes in *ppla-I* alleles (Fig. 3D). A transcriptional regulation by auxin of *pPLA-I* could only be induced by TIR1 yet it did not happen but an auxin-induced change of TIR1 activity was observed. This auxin-induced event needs another auxin receptor. We think it rather indicates an auxin triggered post-transcriptional activation/inactivation event on pPLA-I, being more rapid than 10 min. Although here we cannot provide positive proof for such a mechanism nor for the identitiy of the second receptor ABP1 is the most likely candidate receptor. The strikingly similar phenotypes of *abp1/ABP1* (Effendi et al., 2010) and the *ppla-I* alleles here and the proven capacity of ABP1 to trigger rapid auxin responses (Scherer 2011, Scherer et al., 2012) make ABP1 the likely candidate. Moreover, the similarity in subcellular localisationn of pPLA-I (Fig. 2 q-r) and ABP1 (Klode et al., 2010) in the perinuclear ER and nuclear membrane provides another hint of their functional association.

Compromised gravitropism, phototropism, and root coiling in *ppla-I* mutants could have reduced mobility of PIN proteins and phytochrome signaling output as a common denominator

Gravitropism

Gravitropic and phototropic bending involve differential growth regulation by auxin redistribution. Redristribution of auxin transport is mainly dependent on mobile PIN proteins, more specifically PIN2 and PIN3 and gravitropic is compromised in pin2 and pin3 mutants. In darkness, defects in gravitropism must have their origin in gravisensing by statoliths and subsequent regulation of PIN2 and PIN3 subcellular localisation and auxin transport activity (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Friml et al., 2002; Abas et al., 2006; Kleine-Vehn and Friml, 2008; Ding et al., 2011). Mis-regulation of early auxin-induced genes during the gravitropic response could be another reason to explain the gravitropic defects. Up-regulation of *PIN3* transcription by auxin was delayed in both mutant lines and may contribute to delayed gravitropism in our experiments. Other auxin-dependent genes *ARF7*, *IAA19*, and *SHY2/IAA3* are candidates whose products are involved in hypocotyl gravitropism (Kim et al., 1998; Soh et al., 1999;

Harper et al., 2000; Liscum and Reed, 2002; Tatematsu et al., 2004). *PIN3, IAA19, IAA3, and SAUR15* were mis-regulated in *ppla-I-1* and *ppla-I-3*, though *IAA19* and *SAUR15* only slightly in *ppla-I-3*. These components are probably needed to regulate growth asymmetry in response to lateral auxin transport carried out by PIN2 and PIN3 (Chen et al., 1998; Luschnig et al., 1998; Müller et al., 1998; Friml et al., 2002).

Gravitropism is abrogated by FR and R (Poppe et al., 1996; Robson and Smith, 1996; Correll and Kiss, 2005; Shin et al., 2009; Kim et al., 2011). A delayed gravitropic response of hypocotyls grown in darkness was found in both *ppla-I* lines and in *phyB* but to a much lower extent in *phyA*, meaning that in this experiment, gravitropism was not influenced by light and yet *ppla-I* alleles responded in a manner similar to a *phyB* mutant. We point out, however, that in a *phyB* signaling mutant possessing no PIF1 or PIF3 transcription factors, gravitropic orientation is disturbed also in the dark (Shin et al., 2009). This phenotype is much stronger in the quadruple loss-of-function mutant *pif1pif3pif4pif5* in both darkness and light (Kim et al., 2011). Further components downstream from phyB which act in the regulation of hypocotyl orientation are GIL1 (Allen et al., 2006) and PKS4 (Schepens et al., 2006). It is tempting to speculate that signaling by mainly phyB to these transcription factors is affected negatively in *ppla-I* mutants because the link to phytochromes signaling is obvious in other aspects of the phenotype (see below).

Phototropism

Phototropin (PHOT) is the blue-light receptor mediating phototropism in higher plants (Christie et al., 1998; Christie, 2007). Few intermediates downstream from PHOT1 are known. Interactors with PHOT1 are NPH3 and PKS1. NPH3 binds to PHOT1 (Motchoulsky and Liscum, 1999; Pedmale and Liscum, 2007), and PKS1 is one of four homologous proteins which act as intermediates between phototropin and phytochromes (Lariguet et al., 2006; Molas and Kiss, 2008). Long known is the positive influence of red light on phototropism and phyA was identified as the responsible phytochrome (Liu and Iino, 1996a/b; Parks et al., 1996; Stowe-Evans et al., 2001; Lariguet and Fankhauser, 2004; Whippo and Hangarter, 2004). Further downstream, PIN2 and PIN3 relocalisation most likely mediates the auxin flux to the shaded flank in order to execute the asymmetrical growth response (Müller et al., 1998; Friml et al., 2002; Ding et al., 2011). Quite surprisingly, the phototropic defect in *ppla-I-1* was not found in *ppla-I-3*. As stated above,

one major difference between the Ws-derived mutant *ppla-I-1* and the Col-derived mutant *ppla-I-3* is the lack of *phyD* in *ppla-I-1* and Ws (Aukerman et al., 1997). Conceivably, this could change interaction of intermediates common to the PHOT-induced and phytochrome-induced pathways. How pPLA-I fits into these interactors remains unclear. Also, a membrane association of PHOT1 with the help of PKS1 has been proposed, therefore a proximity of the PHOT-signaling complex to the membrane and the pPLA-I localization near the plasma membrane would allow interaction with those (Fig. 2 n).

Coiling

Root coiling in the *ppla-I-1* mutant is proposed here to be caused by decreased gravisensing in the root and several auxin signaling mutants show this phenomenon (Simmons et al., 1995; Sedbrook and Kaloriti, 2008). Further factors contributing to root coiling are circumnutation and negative thigmotropism (Mullen et al., 1998; Migliaccio and Piconese, 2001). Lack of *phyA* or *phyB* is known to decrease hypocotyl gravitropism (Robson and Smith, 1996) and root gravitropism (Kunihiro et al., 2011) which can explain that the *phyB* mutant strongly and the *phyA* mutant to a weaker degree produced root coils (Fig. 6). Again, the additional *phyD* deletion in *ppla-I-1* may make it a stronger *phyB*-like mutant, explaining the higher numbers of coils in *ppla-I-1* as compared to *ppla-I-3*.. Measurements on the influence of light on auxin transport showed that in tomato phyB2 exerts the strongest positive influence (Liu et al., 2011), fitting our notion that root coils in *ppla-I-1* could be a consequence of reduced auxin transport due to compromised phyB signaling in the roots leading to reduced performance in gravitropism (Fig. 7).

ppla-I mutants have a shade avoidance phenotype

Both lines show hypersensitivity of the elongation response in shade conditions - here white light with a low R:FR ratio (Fig. 9 B, C). Elongation of hypocotyls in shade is considered to be due to down-regulation of phyB signal output as the major receptor for this response (Lau and Deng, 2010; Stamm and Kumar, 2010). Shade avoidance is somewhat enhanced in a *phyBphyD* double mutant but also enhanced in a *phyD* single mutant (Devlin et al., 1999), showing that the two genes are redundant in regulation of this response. phyA has been shown to regulate hypocotyl extension negatively in light-grown plants and thereby reacts and contributes to changes in the R:FR ratio (Johnson et al.,

1994). In low R:FR *phyA* mutants had a long hypocotyl similar to that of *phyB* seedlings in high R:FR (Fig. 9). This reaction on the part of *phyA* seedlings makes a lack of suppression of elongation by phyA apparent in low R:FR shade (i.e. high FR) conditions which was indeed less in wild-type Ws than in the Col wild type (Fig. 9 B, C).

A significant FR-insensitive phenotype typical for *phyA* mutants was not observed, or only at very low FR intensities. Furthermore, the blue-light insensitive phenotype was rather minimal (suppl. Fig. 2). Only in a combination of FR plus blue light, similar to shade, could a clear hypocotyl growth inhibition be observed (Fig. 9 A). The early-flowering phenotype can also be explained as a stronger decrease of phyB regulatory output in Ws as a "functional *phyBphyD*" mutant (Fig. 8). As there are more genetic differences than the *phyD* deletion known between Ws and Col (Ulm et al., 2001; Yang and Hua, 2004; Bartels et al., 2009), we cannot completely exclude other reasons for the differences between the two *ppla-I* alleles in phyB-related responses. Nonetheless, the lack of phyD as a regulatory photoreceptor homologous and redundant to phyB already provides a sound explanation for the differences between *ppla-I-1* and *ppla-I-3*.

Hypersensitivity to shade conditions may explain why a low JA content in *ppla-I-1* and *ppla-I-2* has been found (Yang et al., 2007). Both their *ppla-I-1* and *ppla-I-2* alleles are in the Ws background. Moreover, the accumulation of jasmonate in response to *Botrytis* was indistinguishable in Ws and mutants so that pPLA-I was concluded not to be the enzyme to release linolenic acid for jasmonate biosynthesis (Yang et al., 2007). Jasmonate is an inhibitor of elongation so that, depending on the quality and quantity of light, the jasmonate content of plants defective in phyB signaling could be low (Robson et al., 2010). Consistent with this notion, because the jasmonate receptor mutant *coi1-16* showed exaggerated sensitivity of hypocotyl elongation in the shade avoidance response (Robson et al., 2010), we conclude facts reported here on *ppla-I-1* mutant in Ws background are consistent with findings on JA content in *ppla-I-1* (Yang et al., 2007).

The *ppla-I* mutant identifies a function at the intersection of auxin and light signaling. There is overall similarity of phenotypes of the *abp1/+* mutant and the *ppla-I* mutants, which consist of the similar regulatory defects in auxin-dependent responses like gene regulation, phototropism, gravitropism, also found in *abp1/+* mutants (Effendi et al., 2011; Effendi and Scherer, 2011). Additionally, both *ppla-I -1* and *abp1/ABP1* flower early.

This, together, indicates that ABP1 and pPLA-I could share the same signaling pathway (Scherer, 2011; Scherer et al., 2012). Due to the lack of *phyD* in Ws and *ppla-I-1*, the differences between *ppla-I-1* and *ppla-I-3* are consistent with the notion that lack of *phyD* enhances the effects of apparent reduced signaling output by phyB in both *ppla-I* alleles. Our comparison of *phyA* and *phyB* with *ppla-I* mutants identified phytochromes as influential on the phenotypes as the *ppla-I* alleles resemble partial phenocopies of phytochrome mutants. The phyB-modulated responses were associated with shade avoidance (hypocotyl elongation and early flowering), root gravitropism, and root coiling. The phyA-modulated responses in *ppla-I* mutants were enhancement of phototropism by phyA and inhibition of hypocotyl gravitropism. We suggest that pPLA-I functions downstream from ABP1 and from phytochromes using downstream factors common to both ABP1 and mainly phyB to integrate light and auxin signalling.

MATERIALS AND METHODS

Growth conditions and physiological experiments

For gravitropism and phototropism experiments, seeds were stratified for 4d, treated for 4h with white light and grown for 3 days on upright 0.5 x MS agar plates in the dark at 22.5°C. For testing gravitropism, plants were turned by 90° for 24h and then scanned; for phototropism 1 µE lateral blue light was applied and scanned after 8 hours (CanonScan 8800F; resolution 600 dot per inch). For testing shade avoidance, after stratification seeds were treated with white light for 4 h, and then kept in the dark for 24 h. For 3 subsequent days, white light (24.5 µmoles $m^{-2}s^{-1}$) was applied, after that red and far red either with a high R:FR ratio (2.1) or a low R:FR ratio (0.098) was applied in an LED box at 22.5 °C (CLF, Plant Climatics) for another 3 days at 22.5 °C. Light-growth experiments with continuous light were carried out in a similar fashion. Lengths or angles were measured using AxioVision LE Ver.4.6 software (Zeiss Germany). For flowering experiments, plants grew in a growth chamber at 22.5 °C in 8/16 (L/D). For root coiling assays, seedlings were grown in the light on 2% hard agar containing half or full concentration of MS medium plus 1% or 2% sucrose, respectively (Murashige and Skoog, 1951). Plates stood upright for 3 days to orient the roots according to gravity, then tilted to 45° to induce coiling (Simmons et al., 1995).

Identification of homozygous knockout lines

Homozygous *AtplaI-1* knockout plants in the Wassilewskia background were isolated as described (Krysan et al., 1996) (see primer list). The site of T-DNA integration for *Atpla-1-1* was localised by sequencing of amplified PCR product in the second exon at nucleotide 22856893, corresponding to nucleotide 483 counted from the A in the AUG codon in the coding sequence and thus inserted into the second intron. Yang et al. (2007) isolated two knockout alleles in the Ws background from the same collection (Krysan et al., 1996) and localised one insertion in exon2, but reported the insertion at nucleotide 438 after the AUG codon which, however, corresponds to the end of the first exon (assuming the AUG is included into counting). However, in their Fig. 1 the insertion is placed into in the second exon. Therefore, we conclude that their T-DNA insertion mutant, which gives rise to their mutant *ppla-I-1,* is highly likely to be identical to the one we isolated (nucleotide 483 instead of 438), so we also named it also *ppla-I-1*. Homozygous knockout *pplaI-3* plants (SALK_061667) in the Columbia background were isolated by using the same gene-specific primers described above plus the Lba1 primer and the T-DNA position for *pplaI-3* was 22856359 in the first intron. The lack of mRNA expression in the homozygous lines was shown by RT-PCR (see primer list).

P_{PLAI}::uidA construct

The promoter sequence of *AtPLAI* (P_{PLAI}) comprises 1,940 bp in the 5´-region of *AtPLAI* (At1g61850) and ends at nucleotide +1 prior to the translation initiation site, including the unknown transcriptional start site. The primers (see list) included *Sal*I and *Xba*I restriction sites at their 5´ends, respectively. The promoter fragment was digested with *Sal*I and *Xba*I restriction enzymes and ligated in front of the *uidA* gene of the binary plant transformation vector pGPTV and introduced into the *Agrobacterium tumefaciens* strain GV3101. *Arabidopsis thaliana* (Col) were used for *in planta* transformation (Bechtold et al., 1993).

Histochemical GUS assay

Plants were grown in soil in the greenhouse to stain shoots and petioles, and grown in vitro on MS agar for staining roots, leaves and flowers. To stain shoots, petioles and roots, the plant tissue was soaked with with 2 mM 5-bromo-4-chloro-3-indoyl-b-D-glucuronic acid

(X-Gluc) in 50 mM NaH_2PO_4 (pH 7.0), 0.5% Triton-X, and 0.5 mM potassium ferri- and ferrocyanide for 24-48 h at 37 °C. To stain leaves and flowers, this substrate buffer containing 1 mM X-Gluc was used for 16-22 h at 37 °C (Jefferson et al. 1987). Three different lines showed the same pattern of GUS staining.

Expression of $P_{pPLA\text{-}I}$*:pPLA-I-GFP* in *ppla-I* lines and CLSM

A 7.8-kb genomic fragment of DNA containing the coding region of pPLA-I (*At1g61850*) and 1.42 kb 5´- and 0.55 kb 3´-untranslated regions was amplified by PCR from BAC clone F8K4 and the cDNA sequence was verified. The construct was introduced into Agrobacterium tumefaciens strain GV3101 with the vector pGWB4 (Nakagawa et al. 2007) by heat shock transformation. Plant transformation was done into *pPLA-I* knockout mutant *ppla-I-1* using the vacuum infiltration method (Bechthold et al. 1993) to produce *PpPLA-I::pPLA-I-GFP*. Seed selection was done by plant growth on 1/2 MS media containing hygromycin. Selected seedlings were cultivated in the greenhouse and seeds were harvested.

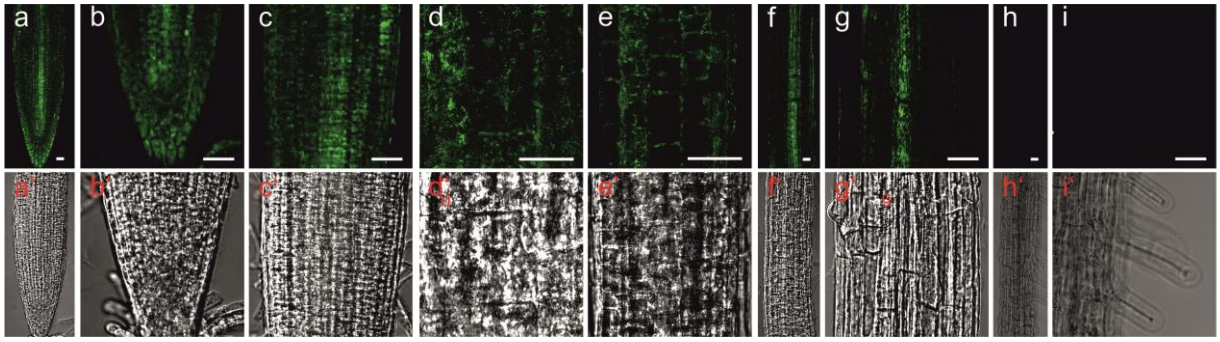
Seedlings of the transgenic line *PpPLA-I::pPLA-I-GFP* with native promotor were grown for 7-28 days on 1/2 MS media, 1%Agar on vertically plates and exposed to white light (16/8h). Seedlings were mounted in water, covered with glass slides and sealed using nail polish. Samples were visualized using an inverted confocal laser scanning Carl Zeiss LSM 510 Axiovert 200M Zeiss microscope with standard filter set. Laser monochromatic excitation light = 488nm was obtained from Argon/Krypton gas mixture. Emission light was collected using a long-pass 505nm filter for GFP signal. Image analysis was done with the Zeiss LSM software and ImageJ.

Transcriptional measurements

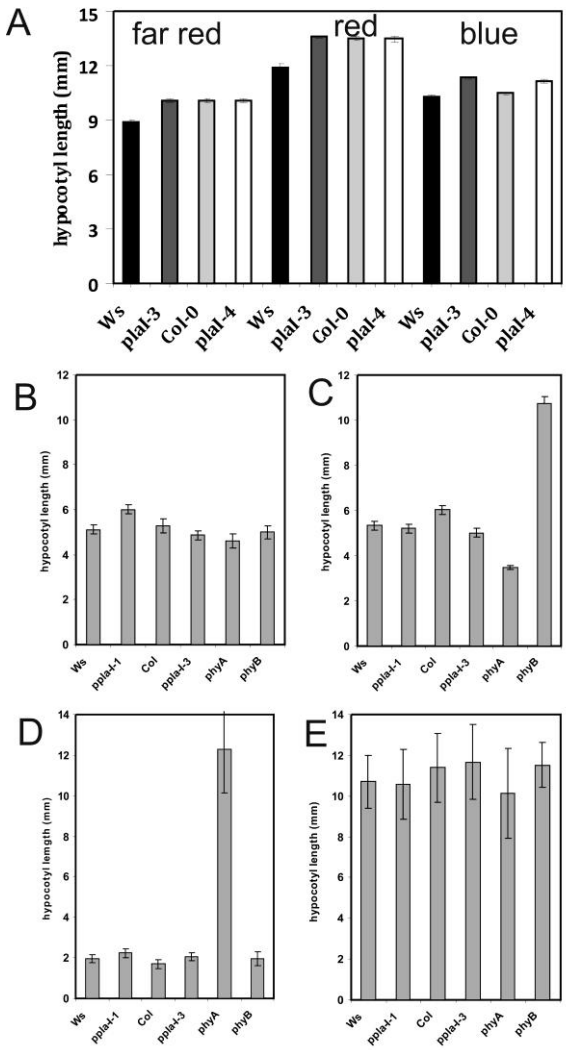
For transcription measurements of early auxin genes, seedlings were grown in MS/2 liquid medium for 7 days under long day conditions. For auxin treatment, the medium was removed and replaced with fresh medium. After 4 hours of calibration in the fresh medium, seedlings were treated either with 10µM IAA or only with MS/2 liquid medium for 30min or as indicated. Seedlings were blotted on filter paper and frozen in liquid nitrogen for further use.

For quantitative RT-PCR, total RNA from auxin-treated seedlings was prepared using TRIzol® reagent according to the manufacturer´s instructions (Invitrogen). RNA was converted to cDNA with RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas). Primers were selected from previous works (Li et al., 2009; Effendi et al., 2011) (see primer list). Relative expression calculation and statistical analysis were carried out with REST 2009 software (Pfaffl et al., 2002). The expression level for the control treatment was set as 1-fold. PCR conditions were: activation of the polymerase at 95°C for 10 min; 40 cycles of DNA melting at 95°C for 15 s and DNA annealing at 62°C for 60 s. Each data point is the mean value of 4-6 biological treatments and 3 technical repeats for each of those.

SUPPLEMENTAL DATA:



Supplemental Figure 1. pPLA-I-GFP expression in *ppla-I-3* background.
Plants were grown in white light for 7 days. (a) root tip; (b) quiescent center with root cap; (c) mitotic root zone; (d) stele with endodermis enlarged, (e) stele, rhizodermis and cortex in meristematic zone enlarged (f) beginning elongation zone (g) elongation zone (h) mature elongation (i) root hair. Bars: 20 µm. (a'-i') light microscopy of otherwise identical settings.



Supplemental Fig. 2. Light-response phenotype of *ppla-I* mutants.

(A) Seedlings were grown for 3 d in the dark and then treated for 12 h with continuous far red, red and blue light (1 µE each). (far red: Ws/*ppla-I-1*: difference highly significant $p<0.001$; n=67, n=111 (S.E.). Col/*ppla-I-3*: difference not significant n=113; n=100 (S.E.). Red: Ws/*ppla-I-1*: difference highly significant $p<0.001$; n=70, n=108 (S.E.). Col/*ppla-I-3*: difference not significant n=106; n=107 (S.E.). Blue: Ws/*ppla-I-1* difference highly significant $p<0.001$; n=159, n=225. Col/*ppla-I-3*: difference highly significant $p<0.001$ n=215; n=204 (S.E.)). *ppla-I-1* showed significant differences in hypocotyl lengths in all light colours, *ppla-I-3* only in blue light. (B, C, D) Treatment of seedlings with continuous far red (B), red (C), and blue light (D) (5 µE each) for 3 days. (S.E.; n=21-26 for each average; differences not significant or at $p<0.05$).

Primer List

Knockout isolation Ws

The primer sequences were
AtPLAI forward 5´-GTC GAT GTC TTC TAC ATG TTC TTC TCC AT-3´,
AtPLAI reverse 5´-TTT AAC AGT CTC TCA AAC TCG TTT GCA CT-3
JL202 5´-CAT TTT ATA ATA ACG CTG CGG ACA TCT AC-3´ located in the T-DNA sequence.

Additional primer for knockout isolation in Col background:
Lba1 primer. (5´-TGG TTC ACG TAG TGG GCC ATC G-3´)

Transcript null test :
ppla I-1 the forward primer 5´-ATG TCT TCT ACA TGT TCT TCT CCA T-3´ and
the reverse primer 5´-TAT CAT ACT TAT AAG CTG CCT CAC C-3´,
for *pplaI-3*, the forward primer 5´-GCT CTA AGC ACA TTG CTC CC-3´ and
the reverse primer 5´-GGA AGC ATT TGT TGC TAC GC-3´.
Actin genes *ACT2* and *ACT7* were used as a standard to normalize RT-PCR amplification,
primers sense 5´- AGG ATA TTC AGC CAC TTG TCT GTG-3´
antisense 5´-AGA AAC ATT TCC TGT GAA CAA TCG-3'.

Promoter isolation
P_{PLAI} (forward) 5´-TTG TCG ACA TAT AGT TTT GTG ATG TAC C-3´ and
P_{PLAI} (reverse) 5´-TCT AGA CCG ACG TTG GAG ATC CGA ATG G-3´,

Real time PCR

Primers were:
UBQ10 forward (5´-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3´),
UBQ10 reverse (5´-AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3´),
IAA2 forward (5´-GGT TGG CCA CCA GTG AGA TC-3´),
IAA2 reverse (5´-AGC TCC GTC CAT ACT CAC TTT CA-3´),
IAA11 forward (5`-CCT CCC TTC CCT CAC AAT CA-3´),
IAA11 reverse (5´-AAC CGC CTT CCA TTT TCG A-3´),
IAA13 forward (5´-CAC GAA ATC AAG AAC CAA ACG A-3´),
IAA13 reverse (5´-CAC CGT AAC GTC GAA AAG AGA TC-3´),
IAA14 forward (5´-CCT TCT AAG CCT CCT GCT AAA GCA C-3´),
IAA14 reverse (5´-CCA TCC ATG GAA ACC TTC AC-3´),
IAA19 forward (5´-GGT GAC AAC TGC GAA TAC GTT ACC-3´),
IAA19 reverse (5´-CCC GGT AGC ATC CGA TCT TTT CA-3´),
IAA20 forward (5´-CAA TAT TTC AAC GGT GGC TAT GG-3´),
IAA20 reverse (5´-GCC ACA TAT TCC GCA TCC TCT-3´),
SAUR9 forward (5´-GAC GTG CCA AAA GGT CAC TT-3´),
SAUR9 reverse (5´-AGT GAG ACC CAT CTC GTGCT-3´),

SAUR15 forward (5´-ATG GCT TTT TTG AGG AGT TTC TTG GG-3´),
SAUR15 reverse (5´-TCA TTG TAT CTG AGA TGT GACTGTG-3´),
SAUR23 forward (5´-ATG GCT TTG GTG AGA AGT CTA TTG GT-3´),
SAUR23 reverse (5´-TCA ATG GAG CCG AGA AGT CAC ATT GA-3´),
GH3-5 forward (5´-AGC CCT AAC GAG ACC ATC CT-3´),
GH3-5 reverse (5´-AAG CCA TGG ATG GTA TGA GC-3´),
PIN2 forward (5´-TAT CAA CAC TGC CTA ACA CG-3´),
PIN2 reverse (5´-GAA GAG ATC ATT GAT GAG GC-3´),
PIN3 forward (5´-TGG TCC AAA TCG TCG TCC TCC A-3´),
PIN3 reverse (5´-TGG AAG CAG CCG TCT CAG GGA-3´).
pPLA-I N-terminus forward: TGT CTT CTA CAT GTT CTT CTC,
pPLA-I N-terminus reverse: GCC GTC CAA TCT AAA TCA AT,
pPLA-I exon7 forward: CTT TAA AAT CCT TGT GTG CCC,
pPLA-I exon7 reverse: TCC CAA CTG CTA ATA AGG CT,
pPLA-I C-terminus forward: CAC TAA TCA CAT CCC AAG GC,
pPLA-I C-terminus reverse: CGT TCT GAT CTC CAA TGT TG.

ACKNOWLEDGEMENTS

This work was supported by DLR-BMBF (grant 50 WB 50WB0627 and 50WB0633), DFG (Sche 207/9-1 and Sche207/24-1) and a grant from the Ministerium für Wissenschaft und Kultur "Biotechnologie Niedersachsen". Technical assistance by Martin Pähler, Peter Pietrzyk, Christa Ruppelt, Marianne Langer and Melanie Bingel is gratefully acknowledged. Kate Hunter helped with language corrections.

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CHAPTER 4

Timing and pattern of auxin-induced expression of early auxin genes in known and unknown auxin mutants reveal cytosolic components having an influence on auxin signal transduction.

Timing and pattern of auxin-induced expression of early auxin genes in known and unknown auxin mutants reveal cytosolic components having an influence on auxin signal transduction

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ABSTRACT

ABP1-mediated early auxin signal transduction has to be a network of many components consisting for example of second messengers, protein kinases and protein phosphatases. Regulation of early auxin-induced genes in mutants gives evidence about a function of potential signaling components in ABP1-mediated auxin signaling. We screened mutants of different potential components involved in this pathway for molecular phenotypes related to auxin. Selected were the membrane lipid-deficient mutants *fad2-1*, *fad6-1*, *ssi2*, *lacs4*, *lacs9* and *lacs4 lacs9*; a knock down mutant of the calcium-dependent protein kinase CPK3, *cpk3*, and the corresponding overexpresson line; mutants of several protein phosphatases: *pp2a*, *ibr5*, *d6pk-1* and *d6pk1 d6pk1-1 d6pk2-2*. We tested early auxin-induced gene expression as a molecular readout for primary molecular auxin responses in the selected mutants and found delayed or increased up-regulation of auxin-induced gene expression in all of them. Thirty minutes after auxin treatment, regulation of up to 45% of auxin-induced genes was delayed or increased in mutant seedlings in comparison to wild type. The measurement of regulation of selected early auxin-induced genes as early as 10 min after auxin stimulus also resulted in mis-regulation of expression of some of these genes. The genes of the tested potential signaling components themselves were not regulated by auxin within 30 minutes. The mutants furthermore did not show a direct auxin-induced physiological phenotype. The results indicate an involvement of fatty acid compositions in membranes, protein kinases and protein phosphatases in ABP1-mediated auxin signaling.

Keywords: auxin signal transduction, ABP1, early auxin-induced genes

INTRODUCTION

Auxin plays an essential role in plant development, growth and in the response to several signals like light and gravitropic signals. It has for example functions in embryogenesis, lateral root development, vascular differentiation, apical dominance, tropic responses and flower development (Aloni et al., 2006; Cecchetti et al., 2008; Friml et al., 2003; Esmon et al., 2005). Although much is known about auxin function, the auxin signaling pathways are still poorly understood. Auxin signal transduction pathways begin with a receptor. Until now two auxin receptors are known: AUXIN BINDING PROTEIN 1 (ABP1) (Hertel et al., 1972; Löbler and Klämbt, 1985) and TRANSPORT-INHIBTOR-RESISTANT 1 (TIR1) (Dharmasiri et al., 2005; Kepinski and Leyser 2005; Tan et al., 2007). ABP1 is a small glycoprotein localized on the extracytosolic side of the plasma membrane and in the ER (Napier et al., 2002). ABP1 is important for development and many rapid cellular changes (Jones et al., 1998, Chen et al., 2001). Several mutants of ABP1 show defects in auxin function (Chen et al., 2001; David et al., 2007; Braun et al., 2008; Effendi et al., 2011; Robert et al., 2010; Xu et al., 2010) and regulation of early auxin-induced genes could be shown (Effendi et al., 2011). TIR1 is an F-Box protein and therefore an auxin activated E3 ubiquitin ligase which binds auxin in a sandwich complex consisting of TIR1, auxin and the substrate proteins AUX/IAA (Tan et al., 2007). The *Arabidopsis* genome encodes five F-Box proteins having 50-70% sequence identity with TIR1, the auxin signaling F-Box proteins 1 to 5 (AFB1-AFB5) (Dharmasiri et al., 2005b). Unlike ABP1, where a complete knockout of the gene results in embryo lethality, a single loss-of-function of TIR1 or AFBs does not cause dramatic developmental defects. However, triple or quadruple mutants like *tir1afb2afb3* and *tir1afb1afb2afb3* show a strongly reduced auxin response and varieties of auxin-related developmental defects (Dharmasiri et al., 2005b).

In the heterozygous ABP1 mutant *abp1/ABP1* 12 early auxin-induced genes out of 15 were up-regulated less efficiently in the mutant than in wild type 30 min after auxin application (Effendi et al., 2011; Effendi and Scherer 2011). In addition also in *Arabidopsis* mutants of patatin-related phospholipases A (pPLA) 30-50% of the tested early auxin induced genes were mis-regulated within 30 min and several as fast as 10 min after auxin application (Labusch et al., 2013). This leads to the suggestion of an early auxin signal transduction pathway including ABP1 as a receptor and pPLAs as signaling intermediates (Scherer et

al., 2012; Labusch et al., 2013). There have to be many more components in this pathway to transmit the signal and to lead to early auxin responses. Signal transduction starts with a receptor and then leads to intermediate reactions and the release of second messengers. Furthermore, signal transduction includes a number of characteristic enzymes like protein kinases and protein phsophatases (reviewed in Scherer er al., 2012). To find some signaling intermediates for ABP1-mediated auxin signal transduction we have chosen mutants of different enzymes, like protein kinases, protein phosphatases or fatty acid desaturases to get a more detailed insight of auxin signal transduction.

The reaction products of pPLAs are free fatty acids (FFA) and lysophospholipids. In a research to test pPLA activity, an increase of FFA and lysophospholipid levels could be shown 2 to 5 min after auxin treatment of parsley and soybean cell cultures (Scherer and André, 1989; Paul et al., 1998). Paul et al. (1998) demonstrated that phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are substrates for pPLAs. Free fatty acids or their derivates may act as second messengers and their composition in membrane lipids could have a function in early auxin signal transduction. Membrane lipid-deficient mutants with changes in fatty acid concentrations in phospholipids were chosen: *fad2-1, fad6-1, ssi2, lacs4, lacs9* and *lacs4lacs9*. *SSI2* encodes a stearoyl acid carrier protein desaturase (S-ACP-DES) which plays an important role in the regulation of unsaturated fatty acid levels in the cell (Kachroo et al., 2003). The *ssi2* mutant has an increase of stearic acid (18:0) concentration in phospholipids and a decreased oleic acid (18:1) level (Kachroo et al., 2001). SSI2 is supposed to play a role in plant defense, because the SSI2-catalyzed synthesis of 18:1 appears to influence defense responses by regulating special resistance (*R*) genes (Chandra-Shekara et al., 2007). Fatty acid desaturase-2 (FAD2) of the endoplasmatic reticulum (ER) and fatty acid desaturase-6 (FAD6) of the plastids encode two ω-6 desaturases that convert oleic acid (18:1) to linoleic acid (18:2) (Zhang et al., 2011). It is suggested that FAD2 plays an important role in regulating the fatty acid composition of intracellular membrane lipids (Zhang et al., 2012). Similar results could be demonstrated for FAD6 (Zhang et al., 2009). The *Arabidopsis LACS* genes belong to the large superfamily of CoA ligase-related encoding genes called acyl-activating enzymes (AAEs) and the *LACS* gene family consists of nine genes (Shockey et al., 2002; Shockey and Browse, 2011). They encode long-chain acyl-CoA synthestases which catalyze the activation of free fatty acids to acyl-CoAs. Some functions for LACS are for example the

involvement in providing fatty acids for cuticular lipid synthesis (Schnurr et al., 2004; Bessire et al., 2007; Lü et al., 2009; Wang et al., 2009), the activation of fatty acids for β-oxidation in the peroxisome (Fulda et al., 2002) and involvement in the biosynthesis of triacylglycerols (TAGs) (Zhao et al., 2010). In bacteria and mammalian cells an important function of LACS in long-chain fatty acid transport could be shown (Eaton et al., 1996; Hettema and Tabak, 2000). A role of LACS in auxin signaling is not shown yet.

One question about the role of phospholipases in signal transduction is the activation mechanism of pPLAs. Because of their rapid activation by auxin within minutes (Paul et al., 1998), a *de novo* synthesis can be excluded. A recent study demonstrated a potential molecular mechanism to activate two group II pPLA enzymes by phosphorylation (pPLA-IIδ and pPLA-IIε; Rietz et al., 2010). It could be shown that a calcium dependent protein kinase, CPK3, was able to phosphorylate and activate the two pPLAs at their C-Termini *in vitro*. Auxin induced calcium influx could activate CPK3 (Monshausen et al., 2011). Thus CPK3 or other members of the CDPK family could probably be responsible for pPLA activation and *cpk3* knock down overexpression line were investigated here as potential auxin mutants.

Other possible candidates to act in early auxin signal transduction are protein phosphatases. A MAPK phosphatase, the INDOLE-3-BUTYRIC ACID RESPONSIBLE 5 (IBR5), is a positive regulator of auxin and abscisic acid (ABA) responses (Monroe-Augustus et al., 2003). The *ibr5* null mutant shows typical auxin-mutant phenotypes like fewer lateral roots, longer roots and decreased leaf serration (Monroe-Augustus et al., 2003; Strader et al., 2008). Also another protein phosphatase, PP2A, is also involved in early auxin signal transduction (Michniewicz et al., 2007). For the ABP1 receptor an involvement in auxin transport including PIN proteins could be shown (Effendi et al., 2010; Robert et al., 2010). It is known that PP2A modulates the phosphorylation status of plasma membrane localized PINs in root and shoot apex in *Arabidopsis* (Michniewicz et al., 2007). It could be shown that PP2A and a PIN-regulating protein kinase (PINOID) which is involved in regulation of polar delivery of PIN proteins (Christensen et al., 2000; Benjamins et al., 2001), act antagonistically in mediating polar targeting of PINs (Michniewicz et al., 2007; Li et al., 2011).

Kinases involved in auxin transport are the members of the AGC kinase family (Zourelidou et al., 2009). For members of the AGCVIIIa kinase subfamily, D6PKs, it could be shown that PIN proteins are their phosphorylation substrates and that PINs and D6PKs are colocalized at the basal membrane of root cells (Zourelidou et al., 2009). The *d6pk* mutants show reduced auxin transport and have defects in lateral root formation and gravitropism.

In our hypothetical model we postulated that besides pPLAs also other cytosolic components might contribute to ABP1 triggered auxin signal transduction. As a final target of ABP1-triggered signaling we postulated PIN protein activity so that cytosolic proteins known to be important for auxin and/or PIN action (Effendi and Scherer 2011) could be part of a cytosol-based signaling system. We further postulated that a PIN-dependent regulation of auxin transport will lead to regulation of auxin transport which, in turn, will influence cytosolic and nuclear auxin concentration and thus, expression of early auxin-induced genes. The receptor to regulate auxin-induced gene expression is TIR1 so that crosstalk of ABP1 and TIR1 was postulated (Effendi et al., 2011).

To test this hypothesis we used the short-time measurement of early auxin-induced genes 10 and 30 min after auxin application as a biotest (Effendi et al., 2011; Labusch et al., 2013). This test should show an involvement in ABP1-mediated signaling, because of similar experiments with an *abp1* mutant (Effendi et al., 2011). Furthermore the short-time measurements of gene expression should give evidence for a TIR1-independent auxin response, because of an auxin response independent of new protein synthesis.

Several early auxin-induced genes were chosen because of their rapidity in response to auxin. This group of early auxin-induced genes tested in this work includes three gene families called *AUX/IAA*, *GH3* and *SAUR* (small auxin-up RNA) (Hagen and Guilfoyle, 2002; Abel et al., 1994; 1995; Abel and Theologis, 1996; Guilfoyle et al., 1998a, b; Paponov et al., 2008). Many of these genes are up-regulated within minutes of exposure to active auxin, independent of *de novo* synthesis (Abel and Theologis 1996). AUX/IAA proteins are short lived and they play a crucial role in auxin-mediated signaling (Dharmasiri and Estelle, 2004; Dreher et al., 2006). Mutations of several *IAA* genes have defects on multiple auxin responses, especially on auxin-induced lateral root formation and auxin-mediated cell cycle activity (Fukaki et al., 2006; Mockaitis and Estelle, 2008). The

GH3 gene family in *Arabidopsis* encodes IAA-amido synthetases which have function to maintain IAA homeostasis in converting auxin to inactive amino acid conjugates (Staswick et al., 2005). *GH3-5* is another candidate for the regulation of auxin concentration, because it encodes an IAA-amido-synthetase which conjugates auxin with amino acids (Staswick et al., 2005). Expression of *SAUR* mRNAs is induced by auxin within 2 to 5 min (McClure et al., 1989; Hagen and Guilfoyle, 2002). The protein function is still unknown but they are maybe involved in auxin signal transduction including calcium and calmodulin (Yang and Poodaiah, 2000; Hagen and Guilfoyle, 2002; Jain et al., 2006; Wang et al., 2009).

This work demonstrates the potential involvement of membrane phospholipids, protein kinases and protein phosphatases in ABP1-mediated early auxin signal transduction. We used the mutants *fad2-1*, *fad6-1*, *ssi2*, *cpk3*, *cpk ox*, *ibr5*, *ppa2* and *tir1-3* to test the expression of early auxin-induced genes (*IAA2*, *IAA3*, *IAA11*, *IAA13*, *IAA14*, *IAA19*, *IAA20*, *SAUR9*, *SAUR15*, *SAUR23*, *GH3-5*, *PIN1*, *PIN2*, *PIN3*, *PIN5*) after 30 min auxin treatment in comparison to wild type. Selected genes were also tested 10 min after auxin application and responded with delayed up-regulation in comparison to the wild type.

RESULTS

Several mutants show a growth phenotype but no detectable auxin phenotype except *ibr5*

Phenotypes on medium without addition or under stress conditions of several mutants here were already described (Kachroo et al., 2001; 2003; Monroe-Augustus et al., 2003; Zhang et al., 2009; 2011; Li et al., 2011). The membrane lipid-deficient mutants *fad2-1*, *fad6-1* and *ssi2* showed shorter main roots on medium without auxin as described by Kachroo et al. (2001; 2003) and Zhang et al. (2009; 2011) (Suppl-.Fig.1 A-C). On auxin containing medium they did not show any different response to auxin in comparison to the phenotypes on non auxin containing medium. As expected, the auxin insensitive mutant *ibr5* had longer main roots, less lateral roots and shorter hypocotyls (Monroe-Augustus et al., 2003; compare to Suppl.-Fig.1 D). Neither the knockout mutant *cpk3* nor the corresponding overexpression line *cpk3-ox* showed a phenotype on auxin containing medium or medium without auxin (Suppl.-Fig.1 E-F). *pp2a* had shorter main roots and showed an impaired

gravity response as described by Li et al. (2011) and did not show any growth differences on auxin containing medium in comparison to non auxin containing medium (Fig.1 G).

Expression of early auxin-induced genes in fatty acid deficient mutants is often mis-regulated

Since only those mutants where a phenotype was already described ("known auxin mutants") showed an auxin response phenotype and other mutants ("new auxin mutants") did not we chose transcription of early auxin-induced genes as a test system as we successfully did for the *abp1/ABP1*, *eir1* and *ppla* mutants (Effendi et al., 2011; Effendi and Scherer, 2011; Effendi et al, 2013; Labusch et al., 2013). The same members of the known groups of early auxin-induced genes (*IAA, GH3, SAUR)* were selected. In addition, *PIN* genes were chosen which potentially could have a function to auxin (Labusch et al., 2013). In wild type seedlings expression of most of the genes was up-regulated two- to twelve-fold in comparison to untreated controls after 30 min auxin treatment (Fig.1-4) except *PIN1*, *PIN2* and *PIN5*.

In the membrane lipid-deficient mutants *fad2-1*, *fad6-1* and *ssi2* of the 15 tested early auxin-induced genes 5-7 showed delayed expression after 30 min (Fig.1). The expression of *PIN3* was delayed in all three mutants. Interestingly, in the membrane lipid-deficient mutants the *SAUR* gene expression was often mis-regulated (Fig.1C). *SAUR9* and *SAUR23* were significantly defect in their regulation by auxin in all three mutants, *SAUR15* only showed a mis-regulation in *fad6-1*. Surprisingly, expression of all three *SAUR* genes was higher up-regulated in the *fad6-1* mutant than in wild type seedlings.

In addition to auxin-induced expression basal expression levels of all genes tested here were compared in the mutants to the wild type (Fig.1 D-F). We took expression of the same reference gene in untreated wild type samples as a basis for the comparison to mutant of expression of early auxin-induced genes by qPCR. For *fad2-1* no significant differences in the basal levels between wild type and mutant could be detected (Fig.1D). In contrast, four genes (*IAA14*, *SAUR23*, *PIN2*, *PIN3*) showed different basal levels in the *fad6-1* mutant in comparison to the wild type (Fig.2E). Expression of two of them, *SAUR23* and *PIN3*, was also mis-regulated in the mutant after auxin treatment (Fig.1B; C). Also in the *ssi2* mutant three genes showed significant differences in basal level expression (*IAA14*,

IAA20, *SAUR23*) (Fig.2F). Expression of one of these genes, *SAUR23*, was also delayed in the mutant (Fig.1C).

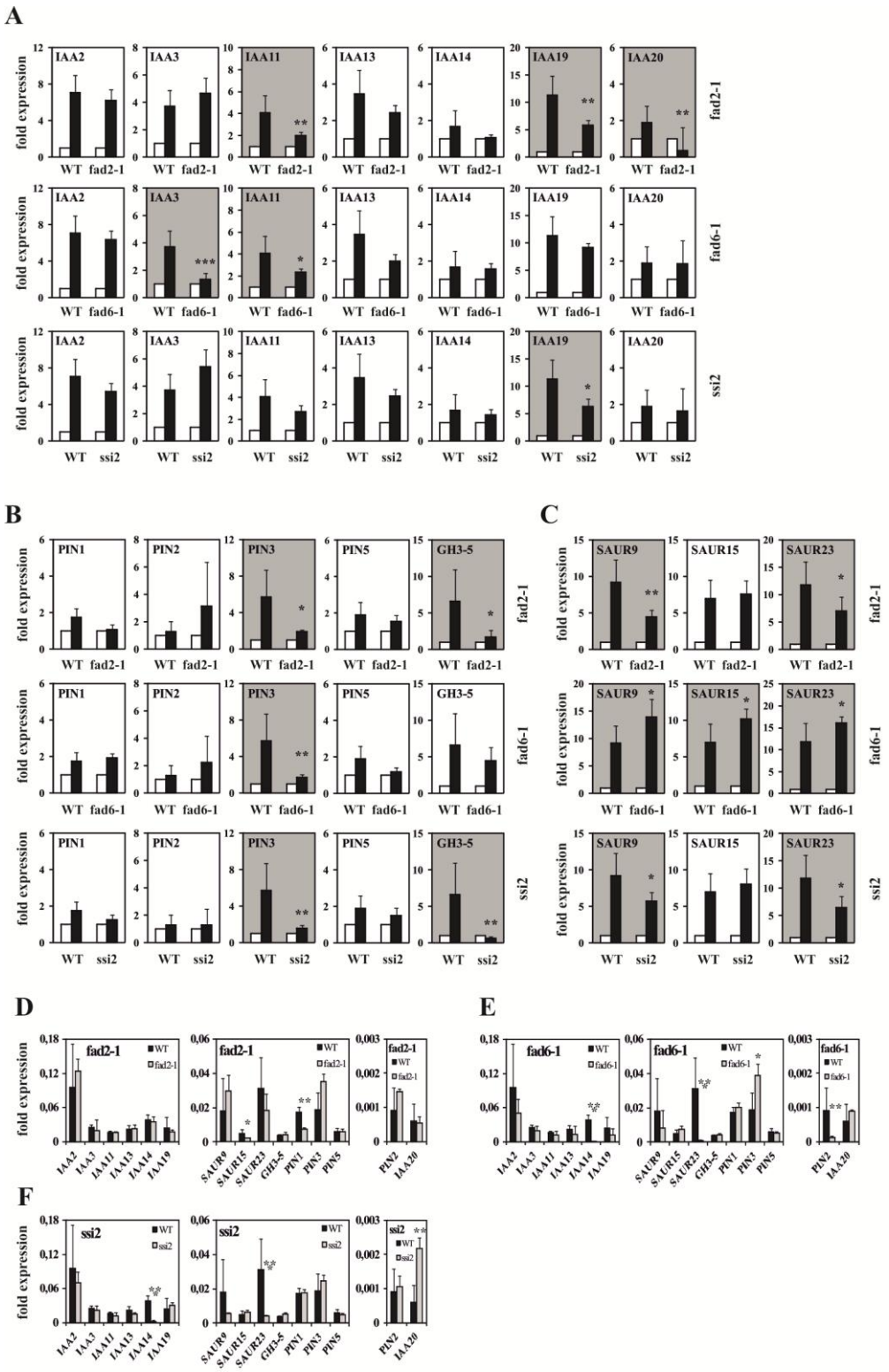


Figure 1. Expression of early auxin-induced genes from the *IAA*, *SAUR* and *GH3* family as well as several *PIN* genes in light-grown *fad2-1*, *fad6-1* and *ssi2* mutants and wild type seedlings.
(A) IAA genes. (B) *PIN* genes and *GH3-5*. (C) *SAUR* genes. (D) Basal levels of all selected genes in *fad2-1* mutant. (E) Basal levels of all selected genes in *fad6-1* mutant. (F) Basal levels of all selected genes in *ssi2* mutant. Background of panels in A-C is shaded whenever significant differences between wild type and mutant was obtained. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments ((*): $p < 0.05$, (**): $p < 0.01$, (***): $p < 0{,}001$; t-test). Relative expression calculation was done setting untreated controls as 1 (white bars) and values at t=30min IAA were calculated accordingly (black bars).

Expression of early auxin-induced genes in mutants with changed very long chain fatty acids in lipids

In the *lacs4lacs9* double mutant seven of the 15 tested genes were significantly delayed oder increased in their expression after 30 min auxin treatment (Fig.2). In *lacs4lacs9* mainly *IAA* genes were defect in their expression and only one *SAUR* gene. Again, *PIN3* showed a significant less up-regulation in comparison to the wild type. The single mutants *lacs4* and *lacs9* did not show many changes in early auxin-induced gene expression except of *IAA19* in *lacs4* and of *SAUR9* in *lacs9*.

In the two single mutants *lacs4* and *lacs9* 5 of the 15 tested genes showed significant decreased basal levels (Fig. 2 D-E). Interestingly in the *lacs4 lacs9* double mutant 9 of the 15 genes showed different basal levels in comparison to the wild type. All *IAA* genes except *IAA13* and all tested SAUR genes had significantly decreased or, in one case (*IAA20*), increased basal levels (Fig .2 F).

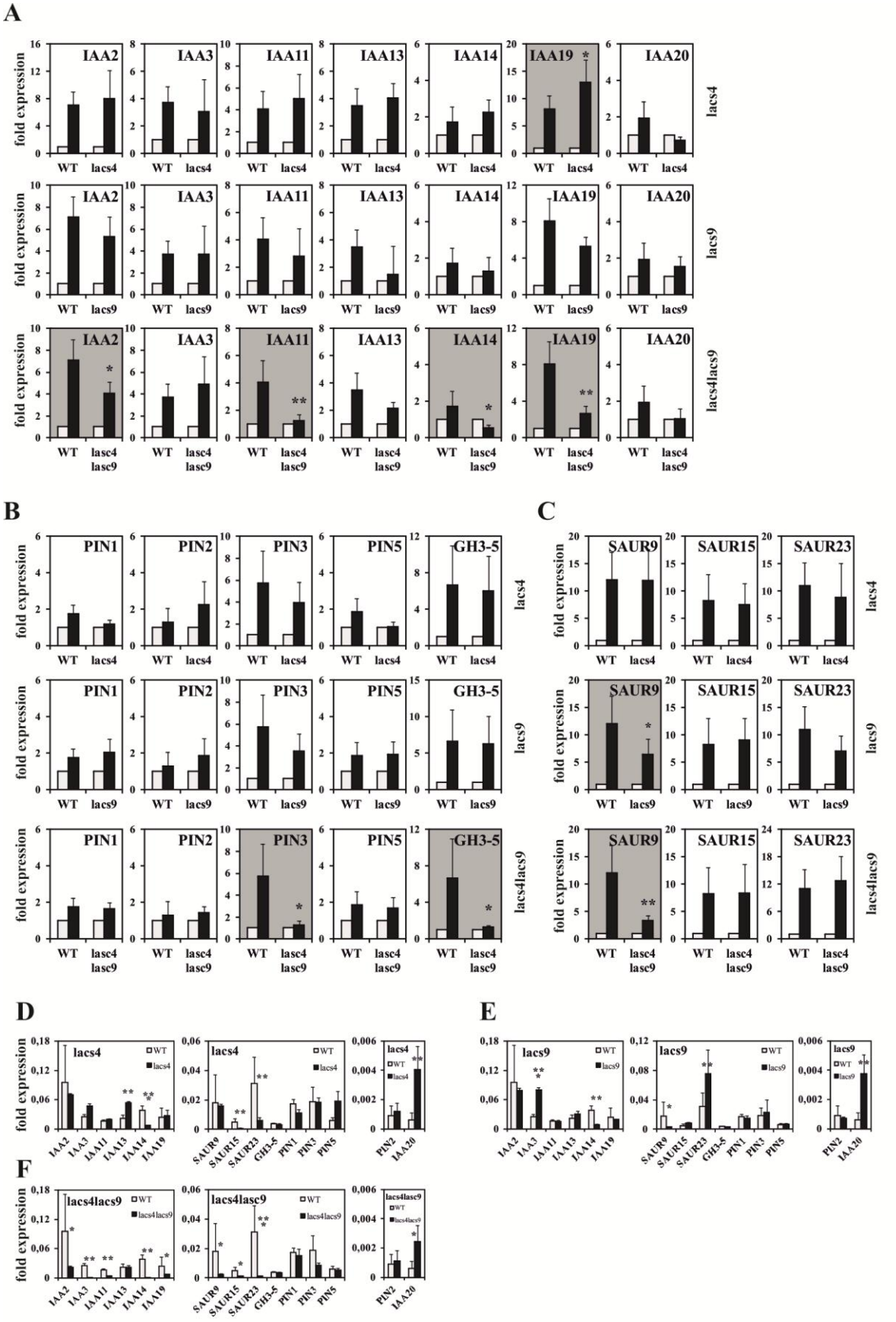
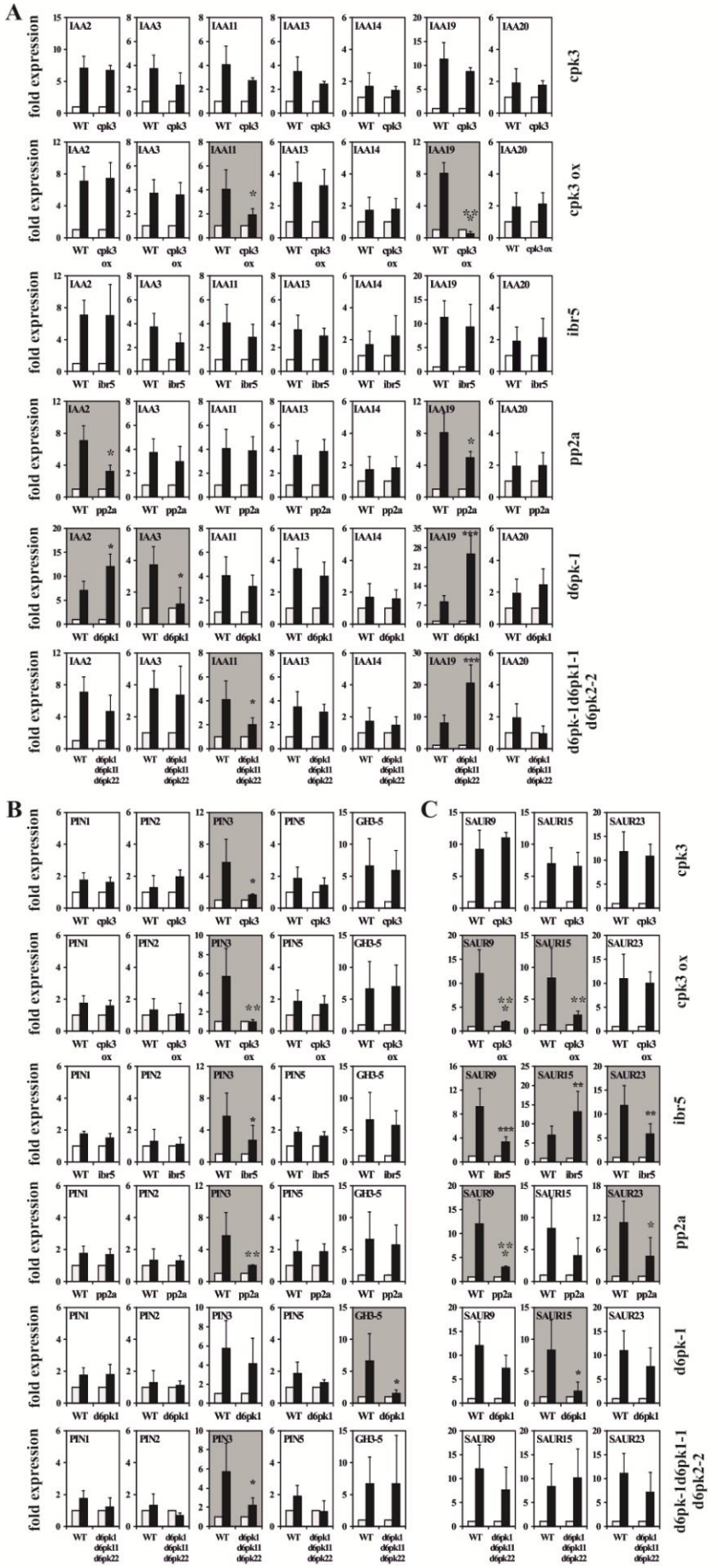

A
IAA2
IAA3
IAA11
IAA13
IAA14
IAA19
IAA20
fold expression
WT
lacs4
lacs9
lasc4 lasc9
lacs4lacs9
B
PIN1
PIN2
PIN3
PIN5
GH3-5
C
SAUR9
SAUR15
SAUR23
D
E
F
lacs4
lacs9
lacs4lacs9
lacs4lasc9
SAUR9
SAUR15
SAUR23
GH3-5
PIN1
PIN2
PIN3
PIN5
IAA2
IAA3
IAA11
IAA13
IAA14
IAA19
IAA20

Figure 2. Expression of early auxin-induced genes from the *IAA*, *SAUR* and *GH3* family as well as several *PIN* genes in light-grown *lacs4, lacs9* and *lacs4lacs9* mutants and wild type seedlings.
(A) *IAA* genes. (B) *PIN* genes and *GH3-5*. (C) *SAUR* genes. (D) Basal levels of all selected genes in *lacs4* mutant. (E) Basal levels of all selected genes in *lacs9* mutant. (F) Basal levels of all selected genes in *lacs4lacs9* mutant. Background of panels in A-C is shaded whenever significant differences between wild type and mutant was obtained. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments ((*): $p < 0.05$, (**): $p < 0.01$, (***): $p < 0{,}001$; t-test). Relative expression calculation was done setting untreated controls as 1 (white bars) and values at t=30min IAA were calculated accordingly (black bars).

Expression of early auxin-induced genes is often defect in protein kinase or phosphatase mutants

In this category 6 mutants (*cpk3*, *cpk3ox*, *ibr5*, *pp2a, d6pk-1* and *d6pk-1 d6pk1-1 d6pk2-2*) were investigated. Espression of several *IAA* genes, *IAA2, IAA3, IAA11* and *IAA19* was less up-regulated in the mutants tested here (Fig.3A). *IAA19* was significantly mis-regulated in *cpk3ox, pp2a* and the *d6pk* mutants, expression of *IAA11* was less up-regulated in *cpk3ox* and the *d6pk* triple mutant and *IAA2* was defectively up-regulated in *pp2a* and *d6pk-1*. Among the *PIN* genes expression of *PIN3* was mis-regulated in all mutants except *d6pk-1*. None of the other tested *PINs* or *GH3-5* showed a difference in their auxin response in comparison to wild type. The *SAUR* gene expression was strongly mis-regulated in *cpk3-ox, ibr5* and *pp2a*. Expression of all of them was less up-regulated than the corresponding wild type, except *SAUR15* in *ibr5*, which was more up-regulated in comparison to wild type. Interestingly, no significant defects in the regulation of auxin-induced genes, except *PIN3*, could be detected in the *cpk3* mutant. The corresponding overexpression line *cpk3ox* showed mis-regulation of expression of 5 genes out of 15.

In all four mutants alltogether the basal levels of the genes were 24 times out of 90 (27%) different in comparison to wild type (Fig.3). Nine times the basal level of genes that were also mis-regulated in the mutant was changed. Especially many of the tested genes showed significant lower basal levels in the *cpk3* and *d6pk-1* mutants although in *cpk3*, except one gene, no signifacant defects in auxin response of the genes could be detected.


A
B
C
fold expression
IAA2
IAA3
IAA11
IAA13
IAA14
IAA19
IAA20
PIN1
PIN2
PIN3
PIN5
GH3-5
SAUR9
SAUR15
SAUR23
WT
cpk3
cpk3 ox
ibr5
pp2a
d6pk1
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d6pk-1d6pk1-1 d6pk2-2

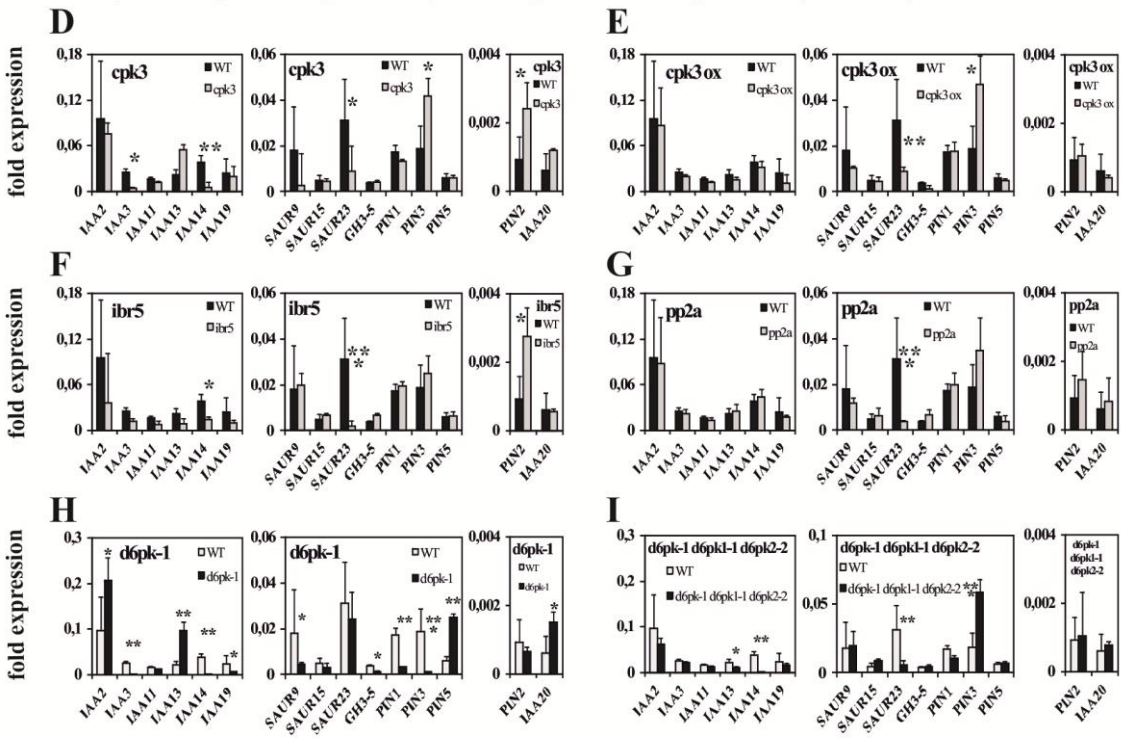

Figure 3. Expression of early auxin-induced genes from the *IAA*, *SAUR* and *GH3* family as well as several *PIN* genes in light-grown *cpk3*, *cpk30x*, *ibr5*, *pp2a*, *d6pk-1* and *d6pk-1d6pk1-1d6pk2-2* mutants and wild type seedlings.
(A) *IAA* genes. (B) *PIN* genes and *GH3-5*. (C) *SAUR* genes. (D) Basal levels of all selected genes in *cpk3* mutant. (E) Basal levels of all selected genes in *cpk3ox* mutant. (F) Basal levels of all selected genes in *ibr5* mutant. (G) Basal levels of all selected genes in *pp2a* mutant. (H) Basal levels of all selected genes in *d6pk-1* mutant. (I) Basal levels of all selected genes in *d6pk-1 d6pk1-1 d6pk2-2* mutant. Background of panels in A-C is shaded whenever significant differences between wild type and mutant was obtained. D-I: Quantified genes are grouped according to expression strength in all mutants and wild types. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments ((*): $p < 0.05$, (**): $p < 0.01$, (***): $p < 0{,}001$; t-test). Relative expression calculation was done setting untreated controls as 1 (white bars) and values at t=30min IAA were calculated accordingly (black bars).

Only some early auxin genes were mis-regulated in *tir1*

Another important mutant to test in this work was *tir1*. It was interesting to know if the early auxin-induced gene expression was also mis-regulated in *tir1*. In fact, only twice (*IAA19* and *PIN3*) the expression of the 15 tested early auxin genes was mis-regulated (Fig.4). Expression of *IAA19* was significantly stronger up-regulated than in the corresponding wild type while *PIN3* expression was less up-regulated. The basal levels of the genes tested here were not significantly different from the wild type.

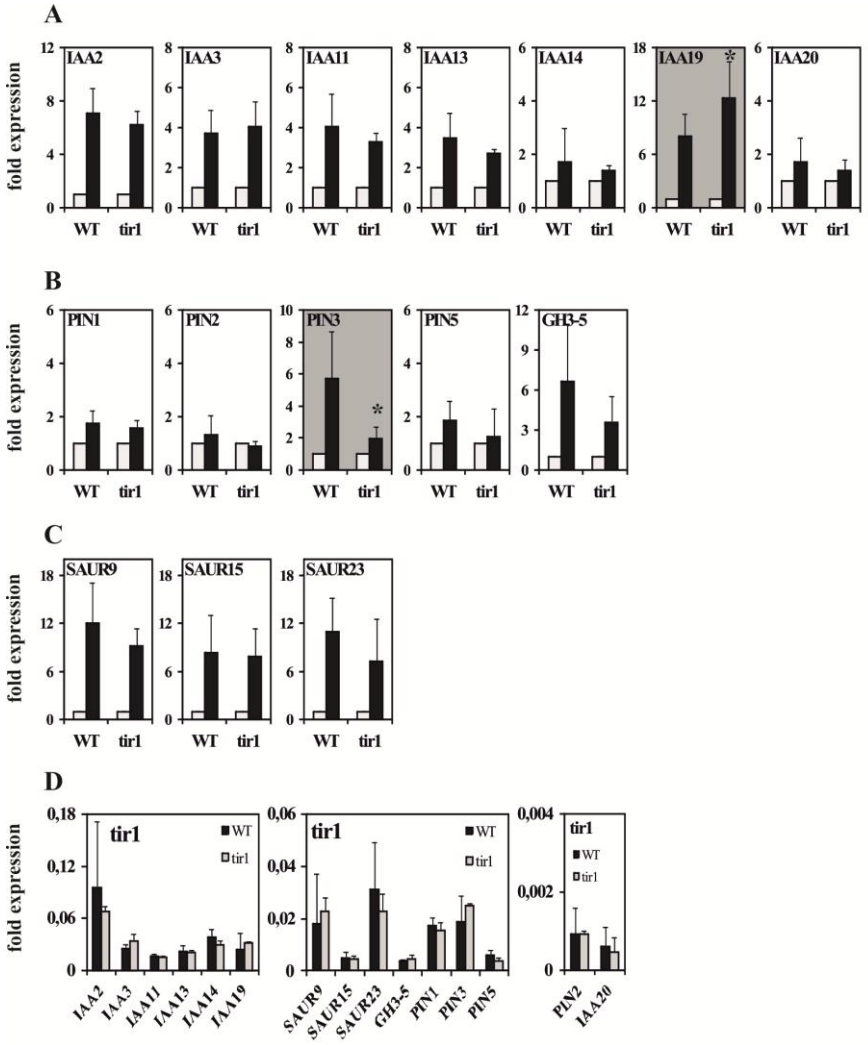

Figure 4. Expression of early auxin-induced genes from the *IAA*, *SAUR* and *GH3* family as well as several *PIN* genes in light-grown *tir1* mutant and wild type seedlings.
(A) IAA genes. (B) *PIN* genes and *GH3-5*. (C) *SAUR* genes. (D) Basal levels of all selected genes in *tir1* mutant. Background of panels in A-C is shaded whenever significant differences between wild type and mutant was obtained. D: Quantified genes are grouped according to expression strength in all mutants and wild types. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments ((*): $p < 0.05$, (**): $p < 0.01$, (***): $p < 0{,}001$; t-test). Relative expression calculation was done setting untreated controls as 1 (white bars) and values at t=30min IAA were calculated accordingly (black bars).

Early auxin-induced gene expression of selected genes was delayed in nearly all tested mutants 10 min after auxin treatment

The expression of selected early auxin-induced genes was also tested 10 min after auxin treatment in all mutants tested here (Fig.5). *IAA19*, *PIN3*, *SAUR9*, *SAUR15* and *SAUR23*

were chosen because of the high occurrence of mis-regulation after 30 min auxin treatment in the tested mutants.

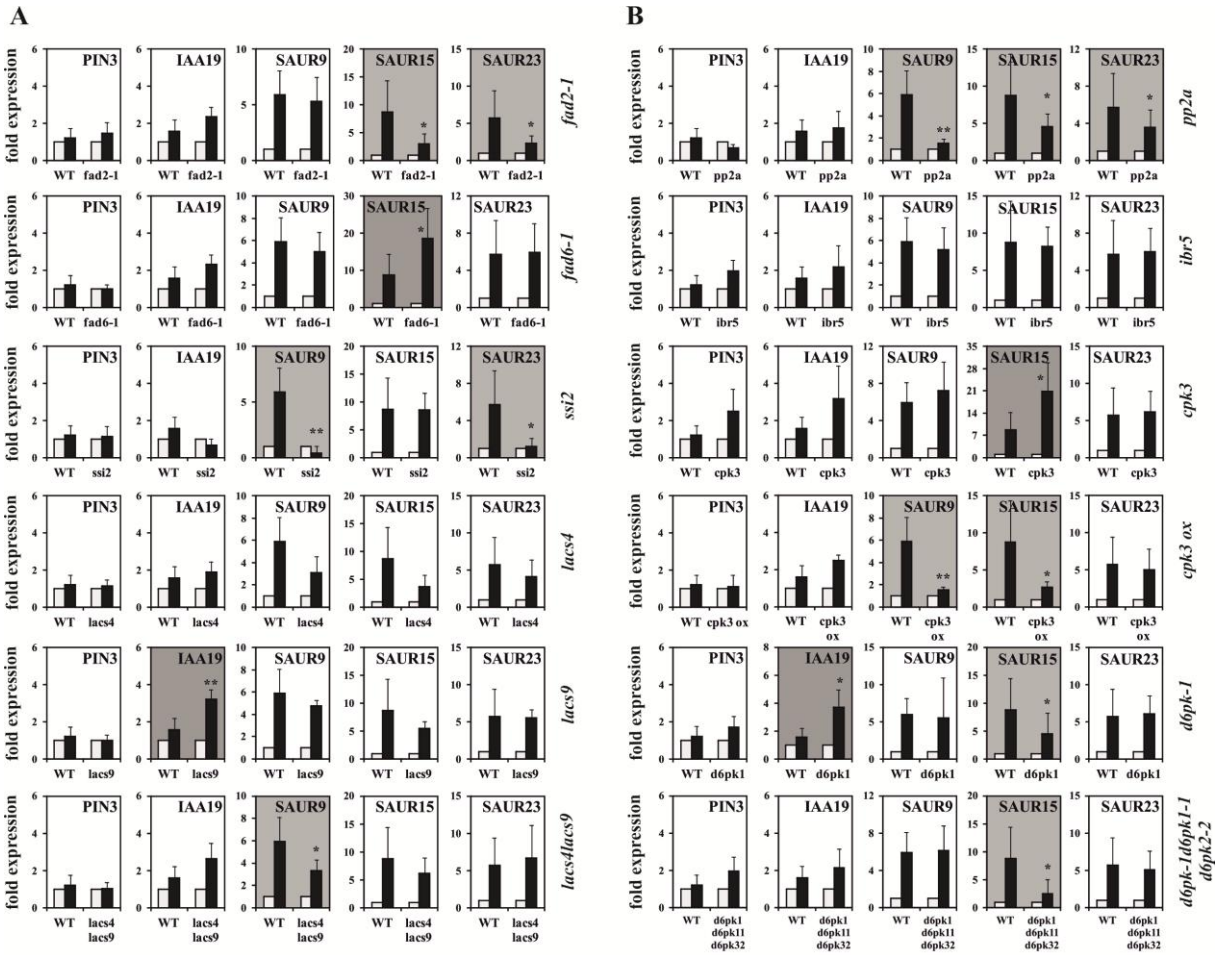

Figure 5. Expression of selected early auxin-induced genes in light-grown *tir1* mutant and wild type seedlings after 10 min auxin treatment.
(A) Early auxin gene expression in fatty acid-deficient mutants. (B) Expression of early auxin genes in protein kinase and phosphatase mutants. Background of panels is shaded whenever significant differences between wild type and mutant was obtained. The intensity of the grey color depends on a decrease or increase of gene expression in comparison to the wild type. Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments ((*): $p < 0.05$, (**): $p < 0.01$, (***): $p < 0,001$; t-test). Relative expression calculation was done setting untreated controls as 1 (white bars) and values at t=10min IAA were calculated accordingly (black bars).

Expression of some of the selected genes was significantly mis-regulated, especially in *fad2-1*, *ssi2*, *pp2a*, *d6pk-1* and *cpk3 ox*. Except in two instances (*IAA19* in *lacs9* and *d6pk-1*) all mis-regulated gene expression belong to the *SAUR* group.

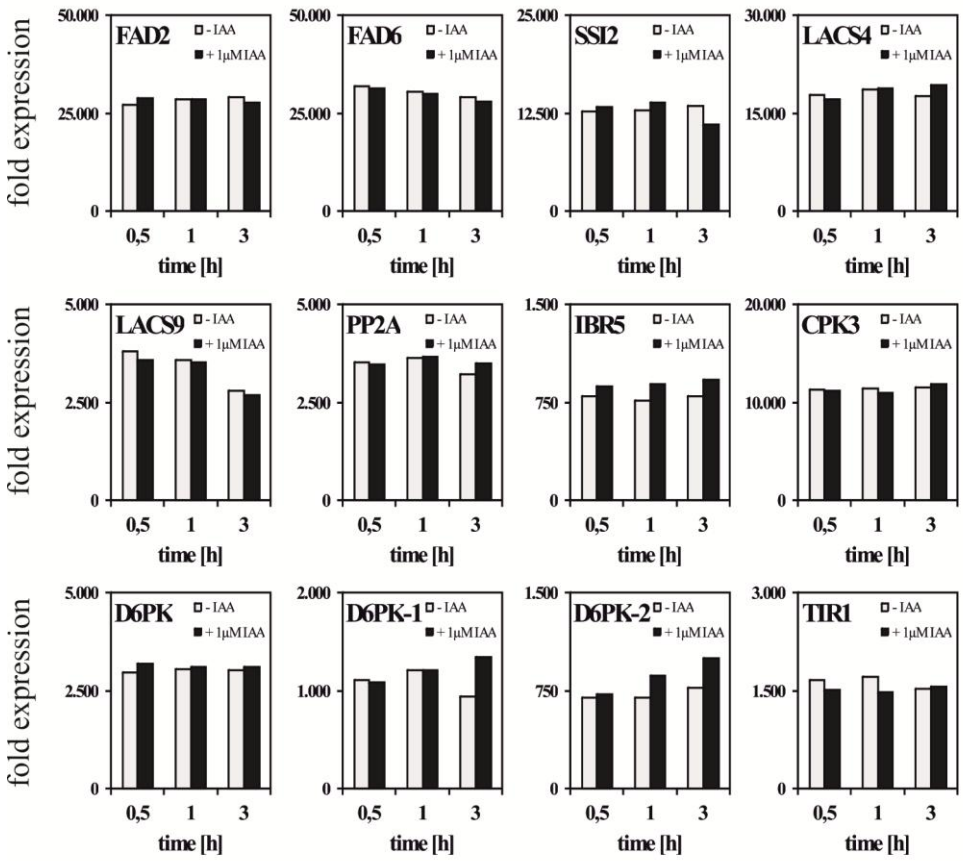

Figure 6. Time courses of expression of the genes in wild type after auxin application. Data was used from AtGenExpress Visualisation Tool (http://jsp.weigelworld.org/expviz/expviz.jsp) in experiments done by Nemhauser et al., 2006. (controls: white bars; 1 µM auxin: black bars).

The genes defined by the mutants themselves are not influenced in their expression by auxin within 3 hours (Fig. 6). We compiled data provided by the AtGenExpress Visualisation Tool (http://jsp.weigelworld.org/expviz/expviz.jsp) in experiments done by Nemhauser et al. (2006). None of the genes showed a change in gene expression after treatment with 1 µM IAA in a time course starting at 30 min and the latest 3 hours. Only *IBR5*, *D6PK-1* and *D6PK-2* seem to be slightly up-regulated after 3 hours.

DISCUSSION

In this work we wanted to investigate the function of potential cytosolic components in early auxin signal transduction. Previous studies showed an involvement of patatin-related phospholipases (pPLA) in ABP1-mediated signaling (Labusch et al., 2013). Furthermore a function of ABP1 in regulating PIN proteins and auxin transport was demonstrated (Effendi et al., 2011; Effendi and Scherer, 2011) which led to the hypothesis of a signaling network of ABP1, PIN proteins and pPLAs (Scherer, 2011). To get a better insight of this network we wanted to find more components of the cytosolic part of this signal transduction. For this mutants of proteins where chosen which are known to have functions in PIN phosphorylation (Michniewicz et al., 2007; Zourelidou et al., 2009), in auxin responses (Strader et al. 2008) and pPLA activation (Rietz et al., 2010). Furthermore membrane lipid-deficient mutants were used (Kachroo et al., 2003; Shockey et al., 2002) because the pPLA hydrolysis products are fatty acids and lysophospholipids with potential functions in auxin signal transduction.

A biotest was used to investigate early auxin function (Effendi et al., 2011; Labusch et al., 2013). Expression of early auxin-induced genes within 10 or 30 min was tested in the selected mutants (Fig.1-4). The genes to be tested were selected for their rapidity only. In addition, *PIN* genes were tested because of functional reasons. For completion we also tested the phenotypes of the most mutants on auxin-containing medium (Suppl.-Fig.1). Only *ibr5* showed a clear auxin insensitive phenotype with longer main roots, less lateral roots and shorter hypocotyls as it was described already by Monroe-Augustus et al. (2003). Some of the other mutants have other phenotypes not directly assigned to auxin and that were already described in previous studies (Kachroo et al., 2001; 2003; Zhang et al., 2009; 2011 Li et al., 2011; Zourelidou et al., 2009). The missing direct auxin-induced developmental phenotypes when grown on auxin-containing medium showed that rapid auxin-induced responses cannot be investigated with long-term methods.

Delayed early auxin-induced gene expression in the mutants indicate a function in auxin signaling

All mutants tested in this work show a more or less strong mis-regulation of expression of early auxin-induced genes upon auxin addition. Of the mis-regulated genes 16% were

higher up-regulated in comparison to the wild type. Some mutants with similar functions like *fad2-1* and *fad6-1* show opposite results for the mis-regulation of *SAUR* gene expression. Also in 3 mutants expression of *IAA19* is higher up-regulated. It remains unclear how a significantly different delayed or overshooting regulation in comparison to the wild type can be explained and integrated into our model of early auxin signal transduction.

Interestingly the basal levels of the tested early auxin-induced genes were often significantly changed in the mutants in comparison to the wild type with one exception, namely *tir1*. A comparison of the changed basal levels (Suppl.-Fig. 2) and the mis-regulation of gene expression (Fig.7) did not show a correlation of both results. Thus, the differences in basal transcription in mutants do not decide whether the expression of these genes is mis-regulated.

To get a clear overview and to answer the question if the number of mis-regulated expression of genes in the mutants is important, in Figure 7 the results of all mutants are shown and sorted according to the number of mis-regulated genes. In addition the results of the similar experiments with *abp1/ABP1*, *abp1-5*, *eir1* and all *ppla* mutants (Effendi et al., 2011; Effendi and Scherer 2011; Effendi et al., 2013; Labusch et al., 2013) are included in this picture. The mutants can be devided into several groups. At first there is the group of mutants in which the expression of the tested early auxin-induced genes is strongly mis-regulated. In the first three mutants in Fig 7, *abp1/ABP1*, *eir1* and *ppla-I* 50-86% of the tested early auxin-induced genes expression was less up-regulated after 30 min auxin treatment in comparison to the wild type. This group is followed by mutants that show a relative strong mis-regulation of early auxin genes. About 40-45% of the tested genes were mis-regulated in many *ppla* mutants as well as in several membrane lipid-deficient mutants, *abp1-5* and *pp2a*. In half of the *ppla* mutants, *ibr5*, and *d6pk* up to 30% of the tested genes showed defects in their regulation of expression by auxin. The last group of mutants with a weak mis-regulation of expression of early auxin-induced genes consists of some single membrane lipid-deficient mutants, *cpk3* and *tir1*. Not only after 30 min, but also 10 min after auxin treatment a delayed expression of selected early auxin-induced genes occurred in the mutants tested here (Fig. 5). What do these results presented in Fig 7 mean for the involvement and function of all tested proteins in auxin signal transduction?

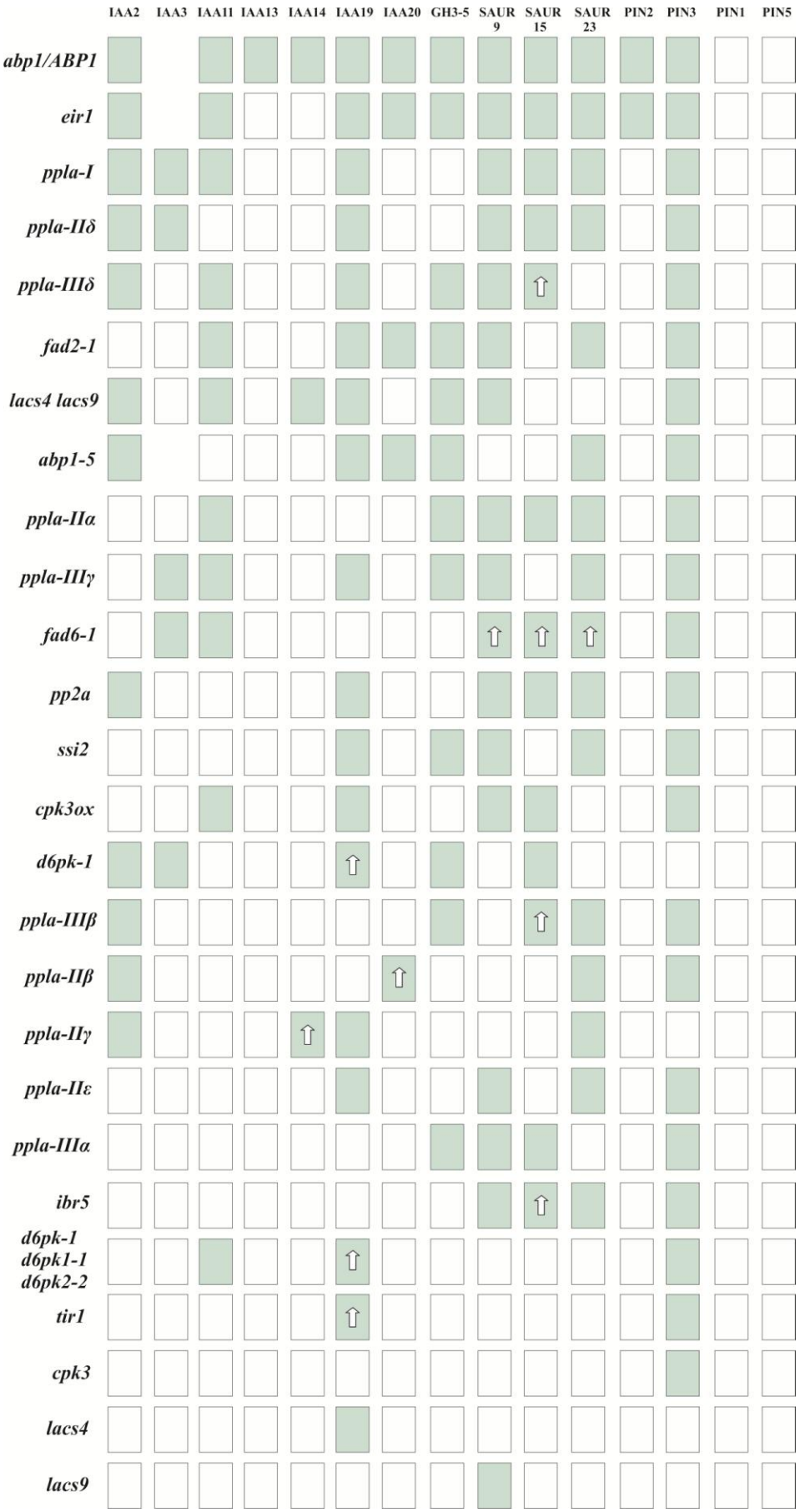
IAA2
IAA3
IAA11
IAA13
IAA14
IAA19
IAA20
GH3-5
SAUR 9
SAUR 15
SAUR 23
PIN2
PIN3
PIN1
PIN5
abp1/ABP1
eir1
ppla-I
ppla-IIδ
ppla-IIIδ
fad2-1
lacs4 lacs9
abp1-5
ppla-IIα
ppla-IIIγ
fad6-1
pp2a
ssi2
cpk3ox
d6pk-1
ppla-IIIβ
ppla-IIβ
ppla-IIγ
ppla-IIε
ppla-IIIα
ibr5
d6pk-1
d6pk1-1
d6pk2-2
tir1
cpk3
lacs4
lacs9

Figure 7. Summary of mutants and the number of defects in early auxin gene expression
Mutants were sorted by the number of expression of mis-regulated genes. Grey boxes show defect gene expression white boxes show no differences to the wild type. Arrows represent a significant higher up-regulation in comparison to the wild type.

The results reveal cytosolic components in ABP1-mediated early auxin signal transduction

There are two important auxin receptors: ABP1 and TIR1/AFBs. TIR1 act as an E3 ligase and decreases the numbers of negative IAA transcriptional cofactors by ubiquitination which (in general) leads to a derepression of ARF genes (Mockaitis and Estelle, 2008). Because the auxin response including TIR1 as a receptor starts with regulation of transcription and *de novo* synthesis of proteins, it probably can be excluded as a receptor for responses faster than 10 min. For *abp1/ABP1* heterozygous mutant defects in early auxin-induced genes could be shown (Effendi et al., 2011). 86% of the expression of the early auxin-induced genes was mis-regulated including the genes which were also tested here. Interestingly, in *tir1* only two of the 15 tested genes were significantly delayed or overshooting in comparison to the wild type. TIR1 is a redundant gene related to five more AFB genes which could be an explanation for these results. But it seems to be clear that ABP1 exerts influence on TIR1 output activity.

ABP1 probably uses multiple components in auxin signal transduction forming a signaling network including PINs, pPLAs, fatty acids, protein kinases like D6PKs and CPK3 and protein phosphatase PP2A. Some of the components, like PIN2 and some pPLAs, are probably upstream to ABP1 in the signaling pathway and the mutants therefore also show this strong mis-regulation of early auxin induced gene expression. Recent studies showed a connection between PIN activity and ABP1 (Robert et al., 2010; Effendi et al., 2011; Effendi and Scherer 2011) by showing that endocytosis inhibition by auxin depend on intact ABP1 and that early auxin-induced gene expression was mis-regulated in the PIN2 mutant *eir1*. Thus the influence of ABP1 on TIR1 probably acts including PIN proteins and therefore over auxin concentration in the cell.

Fatty acid composition of membrane lipids has function in auxin signaling

Furthermore the early mis-regulation of early auxin-induced gene expression in *ppla* mutants (Labusch et al., 2013) indicates an involvement of this enzymatic phospholipid hydrolyzing activity in ABP1-mediated auxin signaling. By testing several mutants with changed concentrations in fatty acid composition, especially 18:0, 18:1, 18:2 and very long chain fatty acids (VLCFAs) we could support the hypothesis that phospholipids containing these FAs are involved in auxin signal transduction. A recent study showed the involvement of VLCFAs in PIN1 polarity (Roudier et al., 2010). The investigation of a mutant (*pas1*) having reduced levels of VLCFAs resulted in defect PIN1 polarity in the apical region of *pas1* embryos which altered polar auxin transport and led to a lack of local redistribution of auxin accumulation. Thus fatty acid composition of phospholipids could influence the attachment to membrane surfaces of proteins like PP2A or D6PKs (Michniewicz et al., 2007; Zourelidou et al., 2009) and have an influence of the membrane fluidity including PIN proteins (Roudier et al., 2010).

CPK3 is involved in auxin signal transduction probably in activating pPLA activity

A recent study could show that CPK3 is able to phosphorylate two pPLA proteins *in vitro* (Rietz et al., 2010). Interestingly, only *PIN3* was less up-regulated in the *cpk3* knock down mutant (Fig.3). In contrast, 5 of the 15 tested genes were mis-regulated in the corresponding overexpression line *cpk3-ox*. *CPK3* is a redundant gene and there are many other CPKs that could rescue the function of the missing CPK3 in the mutant. The CPK3 overexpression could result in a lower specificity for the substrate pPLA and therefore lead to less activation of pPLAs by auxin and to a mis-regulation of early auxin-induced gene expression. These results and from Rietz et al. (2010) indicate a function of CPK3 in auxin signal transduction, probably in activating pPLA activity.

Protein kinases and protein phosphatases are involved in the early auxin signaling network

Protein phosphatases and kinases play an important role in signal transduction pathways (reviewed in Scherer et al., 2012). A tested protein phosphatase here, PP2A, is involved in auxin signaling by regulating PIN apical-basal targeting and auxin distribution

(Michniewicz et al., 2007). It could be shown that PP2A and PINOID both partially colocalize with PINs and therefore mediate PIN apical-basal polar targeting. The mis-regulation of early auxin-induced genes 30 min after auxin treatment therefore supports the findings of other studies that PP2A is involved in auxin signaling. Other possible candidates for the cytosolic part of ABP1-mediated auxin signaling are D6PKs, which are also involved in regulating the phosphorylation status of PIN proteins (Zourelidou et al., 2009). Also the two tested mutants *d6pk-1* and *d6pk-1 d6pk1-1 d6pk2-2* showed mis-regulated expression of early auxin-induced genes that clearly shows a link between ABP1-mediated auxin signaling involving PIN proteins and the D6PK proteins.

A broad investigation of IBR5 demonstrated that IBR5 phosphatase activity is necessary for full auxin and ABA responsiveness (Strader et al., 2008). Two Aux/IAA family members, IAA17 and IAA28, were not stabilized in *ibr5*, suggesting that IBR5 acts as downstream of auxin recognition by the $SCF^{TIR1/AFB}$ –AUX/IAA complexes. A mis-regulation of *IAA* genes could not be shown for *ibr5* here and *IAA17* and *IAA28* were not included in the group of tested genes, but the fact that some early auxin-induced genes were defect in their expression in the mutant after 30 min auxin treatment supports the knowledge that IBR5 has a function in auxin signaling.

In summary we could identify cytosolic components in ABP1-mediated early auxin signal transduction by using a biotest that shows that early auxin-induced gene expression is regulated by ABP1 (Effendi et al., 2001), pPLAs (Labusch et al., 2013), PIN proteins (Effendi et al., 2011), composition of FAs in membrane lipids, protein kinases like D6PKs and CPK3 and protein phosphatases like PP2A and IBR5. All these findings give an idea of a complex ABP1-mediated network occurring minutes after auxin signal perception.

ACKNOWLEDGEMENTS

Support from the Deutsches Zentrum für Luft- und Raumfahrt (contract number 50WB0627) and from the Deutsche Forschungsgemeinschaft (Sche207/24-1) is gratefully acknowledged.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana mutant lines *fad2-1*, *fad6* and *ssi2* were obtained from the department of Plant Pathology at the University of Kentucky (Kachroo et al, 2003). The mutant lines *lacs4*, *lacs9* and *lacs4 lacs9* were provided by the department of Plant Biochemistry at the Georg-August University in Göttingen. We used the *ibr5* mutant line from the Department of Biochemistry and Cell Biology at the Rice University in Houston (Monroe-Augustus et al., 2003; Strader et al., 2008; 2010). The *d6pk* lines were provided by the department of Plant Systems Biology at the Technische Universität München (Zourelidou et al., 2009). The *cpk3-2* T-DNA insertion line (SALK_022862) was obtained from the SALK collection (Columbia ecotype) (Alonso et al., 2003). The *cpk3* overexpresser was obtained from Steffen Rietz from the University of Kiel.

Seedlings were grown under long day conditions (16h white light, 8h dark, 30-40 µE). For auxin phenotype experiments seedlings were pre-grown for 3 days on ATS agar with 2% sucrose and then transferred to ATS-medium (Estelle and Somerville, 1987) containing appropriate 1-NAA concentrations. After 12 days growth on vertical agar plates the seedlings were scanned.

To investigate early auxin gene expression in the used mutants, seedlings were grown in MS/2 liquid medium for 7 days under long day conditions. Prior to treatment with auxin the medium was replaced by fresh medium. After 4 hour calibration in the fresh medium, seedlings were treated either with 10µM IAA or only with MS/2 liquid medium for 10min or 30min. Plant material was quickly blotted on filter paper and frozen in liquid nitrogen.

Nucleic acid analysis

For quantitative RT-PCR, total RNA from auxin treated seedlings was prepared using TRIzol® reagent according to the manufacturer´s instructions (Invitrogen), treated with DNaseI (Invitrogen) and converted to cDNA with RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas). Primers were selected from previous works (Li et al., 2009; Rietz et al., 2010; Effendi et al., 2011, Labusch et al., 2013). Primer efficiency was checked by using different cDNA concentrations and only primer with mathematical

efficiency between 95 and 105% were used. Primers are listed in supplemental material (Supplemental table 1). For quantitative PCR reactions SYBR-Green Master Mix was used in a StepOnePlus^{TM} system (Applied Biosystem). About 30ng cDNA, 200 nM primers, 0,5 µM ROX (Invitrogen), 0,1x SYBR Green (Invitrogen) and 0,03U Hot Start Polymerase (DNA cloning service) were utilized in one PCR reaction. The specificity of PCR amplification was examined by monitoring the presence of a single peak in the melting curves for quantitative PCR. In each experiment four to six biological repeats, and for each biological treatment three technical repeats were performed for the subsequent qPCR reaction. Relative expression calculation and statistical analysis were done with REST 2009 software (Pfaffl et al., 2002). The expression level of the untreated controls was set as 1-fold for all lines.

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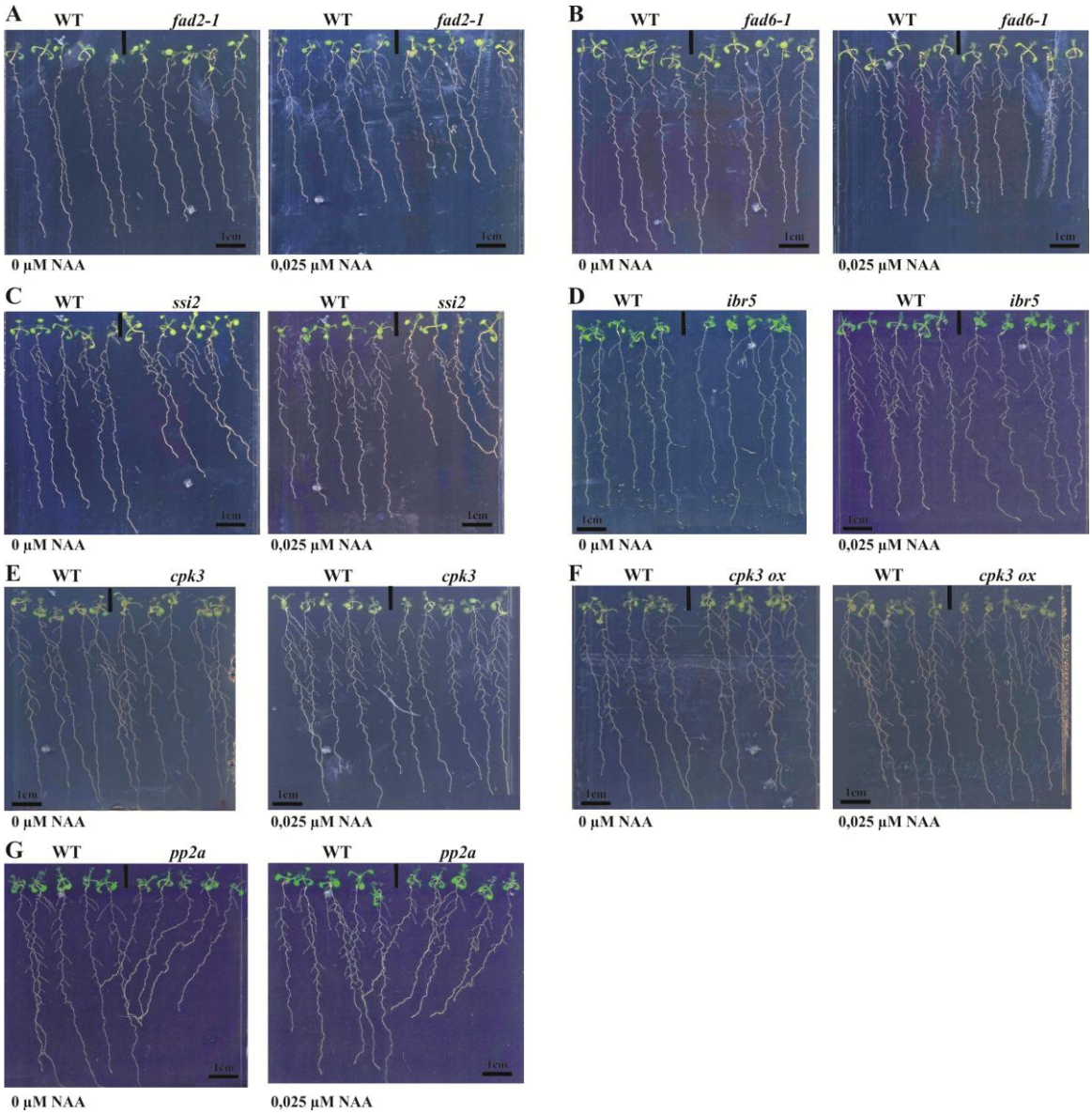
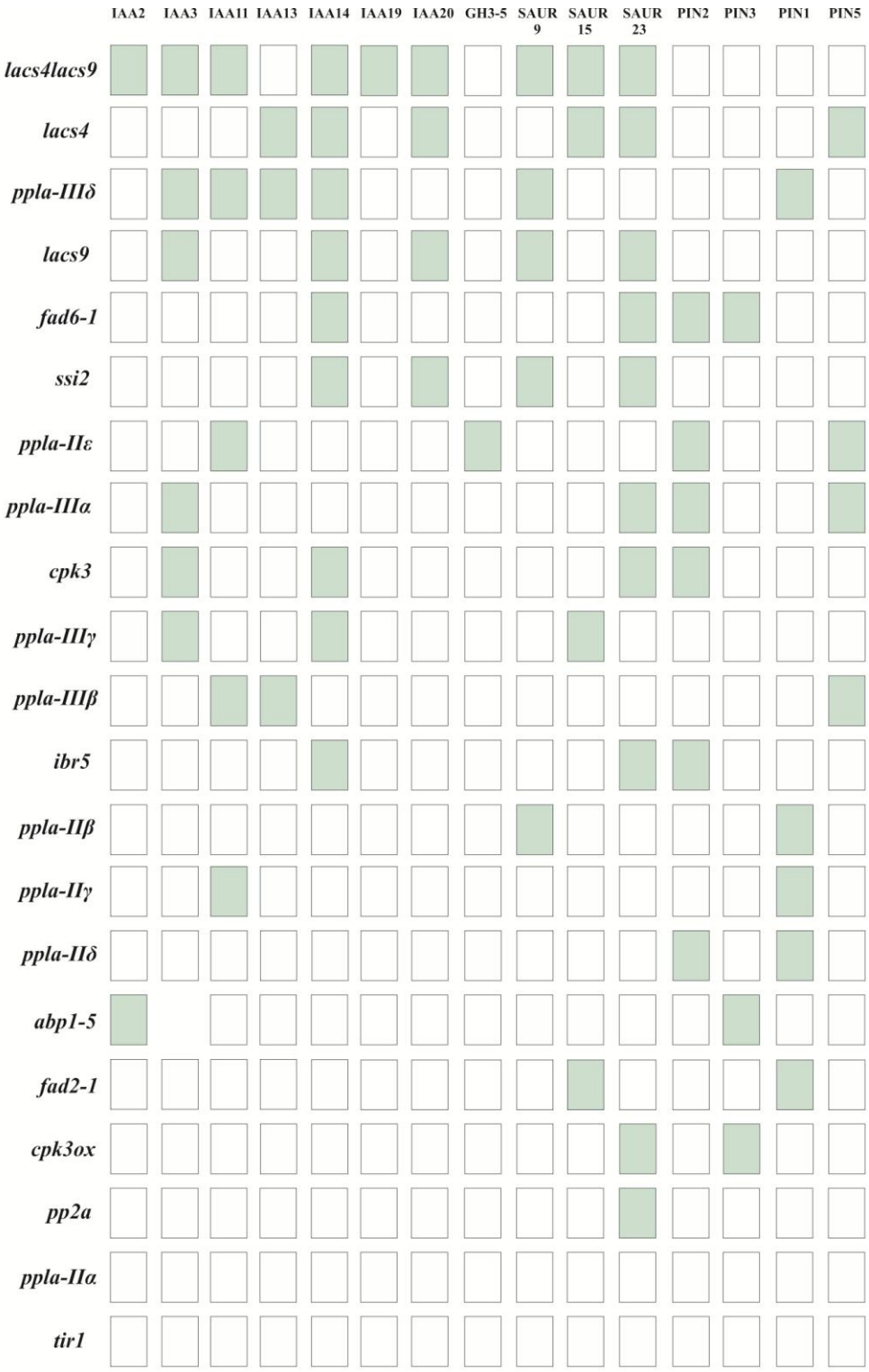
SUPPLEMENTAL DATA



Suppl.-Figure 1. Growth response of light-grown mutants and wild type plants to auxin. Seedlings were pregrown on 1xATS for 3 days and transferred to 1xATS medium in the presence of different 1-NAA concentrations. Comparison of wild type and mutant seedlings on 1xATS medium without auxin and with 0,025µM auxin is shown. Some auxin mutants were described in the literature (Zourelidou et al., 2009) and were not included because of lack of sufficient number of seeds. (A) *fad2-1* (B) *fad6-1* (C) *ssi2* (D) *ibr5* (E) *cpk3* (F) *cpk3ox* (G) *pp2a*



Suppl.-Fig. 2. Summary of mutants and the number of changed basal transcription of early auxin genes.

Mutants were sorted by the number of changed basal transcription of early auxin responsive genes. Grey boxes show changed gene expression white boxes show no differences to the wild type.

Supplemental Table 1. Primer list

Oligoname	Gene	Sequence (5`- 3`)
UBQ10_for	AT4G05320	GGCCTTGTATAATCCCTGATGAATAAG
UBQ10_rev	AT4G05320	AAAGAGATAACAGGAACGGAAACATAGT
IAA2_for	AT3G23030	GGTTGGCCACCAGTGAGATC
IAA2_rev	AT3G23030	AGCTCCGTCCATACTCACTTTCA
IAA3_for	AT1G04240	AACTGAAACATCCCCTCCTC
IAA3_rev	AT1G04240	CCATCTCTCTCAAAGTACTCTCC
IAA11_for	AT4G28640	CCTCCCTTCCCTCACAATCA
IAA11_rev	AT4G28640	AACCGCCTTCCATTTTCGA
IAA13_for	AT2G33310	CACGAAATCAAGAACCAAACGA
IAA13_rev	AT2G33310	CACCGTAACGTCGAAAAGAGATC
IAA14_for	AT4G14550	CCTTCTAAGCCTCCTGCTAAAGCAC
IAA14_rev	AT4G14550	CCATCCATGGAAACCTTCAC
IAA19_for	AT3G15540	GGTGACAACTGCGAATACGTTACC
IAA19_rev	AT3G15540	CCCGGTAGCATCCGATCTTTTCA
IAA20_for	AT2G46990	CAATATTTCAACGGTGGCTATGG
IAA20_rev	AT2G46990	GCCACATATTCCGCATCCTCT
SAUR9_for	AT4G36110	GACGTGCCAAAAGGTCACTT
SAUR9_rev	AT4G36110	AGTGAGACCCATCTCGTGCT
SAUR15_for	AT4G38850	ATGGCTTTTTTGAGGAGTTTCTTGGG
SAUR15_rev	AT4G38850	TCATTGTATCTGAGATGTGACTGTG
SAUR23_for	AT5G18060	ATGGCTTTGGTGAGAAGTCTATTGGT
SAUR23_rev	AT5G18060	TCAATGGAGCCGAGAAGTCACATTGA
GH3.5_for	AT4G27260	AGCCCTAACGAGACCATCCT
GH3.5_rev	AT4G27260	AAGCCATGGATGGTATGAGC
PIN1_for	AT1G73590	ATGGCTTCTGGTGGTGGTCGGAA
PIN1_rev	AT1G73590	AGCAGGACCACCGTCTTCTTCGT
PIN2_for	AT5G57090	TATCAACACTGCCTAACACG
PIN2_rev	AT5G57090	GAAGAGATCATTGATGAGGC
PIN3_for	AT1G70940	TGGTCCAAATCGTCGTCCTCCA
PIN3_rev	AT1G70940	TGGAAGCAGCCGTCTCAGGGA
PIN5_for	AT5G16530	CCATCGGCTCTATTGTCCTTG
PIN5_rev	AT5G16530	GCGACGAGCACAGGTAGAGA

CHAPTER 5

GENERAL DISCUSSION

GENERAL DISCUSSION

Among all the known functions of the plant hormone auxin, its early signal transduction pathway is still poorly understood. The general model of signal transduction describes that beginning with the receptor the physiological responses occur hours or days after signal perception (reviewed in Scherer et al., 2012). At first second messengers and specific proteins, like protein kinases and phosphatases, are activated within minutes which leads then to a change of activity of transcription factors and to new protein synthesis within minutes and hours (reviewed in Scherer et al., 2012). The aim of this work was to identify signaling components involved in ABP1-mediated early auxin signal transduction. Based on the knowledge of previous studies several mutants of phospholipases, fatty acid desaturases, protein kinases and protein phosphatases were chosen to be tested as candidates for this auxin signalling pathway.

How to devise a system to test an auxin function in a mutant?

Auxin functions are well known (Davies, 1995) so that one way to test whether or not a mutant is disturbed in a typical auxin function is to compare its phenotype to the respective wild type. This was done for all ten *ppla* mutants (Chapter 2 and 3) and a number of other candidate mutants (Chapter 4) with only limited success. Only few mutants had recognizable auxin-related phenotypes (Fig. 1, Chapter 2; Huang et al., 2001; Rietz et al. 2010; Li et al., 2011; Monroe-Augustus et al., 2003).

As a second test of auxin function in mutants we used a short-term test of expression of early auxin-induced genes that could be performed within 10 or 30 min (Effendi et al., 2011). The genes to be tested in this expression test were selected for their rapidity of regulation only. In addition we checked for all genes defined by the mutants whether expression of these genes themselves (in wild types) was regulated by auxin and none of them were (Fig. 6, Chapter 2; Fig. 3, Chapter 3; Fig. 6, Chapter 4).

While there is complete agreement that expression of early auxin-induced genes is regulated by TIR1/AFBs (Mockaitis and Estelle, 2008) it came as surprise that in *abp1/ABP1* mutant all tested early auxin-induced genes were delayed in induction by auxin (Effendi et al., 2011). Because it can safely be assumed that an output like more active

protein for a physiological response takes at least 10 min (Fig.1.1; see also Scherer, 2011 and Scherer et al., 2012) we added tests of expression already at 10 min which were not included in previous work. If, in a mutant other than *tir1*, early auxin-induced expression is influenced than this mutated protein functions in a process that influences TIR1 activity (Fig. 1.1, Chapter 1; Fig.7, Chapter 2; Fig. 3, Chapter 3; Fig.5, Chapter 4). The 30 min expression values allow differentiating better between different mutants because expression of more genes was tested.

All mutants tested have defects in their early auxin-induced gene expression

All of the mutants tested show a delayed (most of the genes) or increased (few genes) up-regulation of early auxin-induced gene expression 30 min after auxin treatment. Figure 7 of Chapter 4 shows a summary of the results of all mutants tested. The mutants were sorted according to the numbers of mis-regulation of expression of genes. The results of similar experiments with the ABP1 mutants *abp1/ABP1* and *abp1-5* as well as the PIN2 mutant *eir1* were included to visualize the connection of the receptor with the other potential signaling components (Effendi et al. 2011, Effendi and Scherer 2011). Fig. 7 shows clearly that the highest number of defects in early auxin-induced gene expression can be found in the mutants *abp1/ABP1*, *eir1* and *ppla-I* closely followed by *ppla-IIδ*, *ppla-IIIδ*, *fad2-1* and *lacs4 lacs9*. These mutants show a high number of early auxin-induced genes with delayed gene expression 30 min after auxin treatment (50-85%). Many of the other mutants, like *abp1-5*, the membrane lipid-deficient mutants *fad6-1* and *ssi2*, the other *ppla* mutants, *d6pk-1* and *pp2a* still had a remarkable number of mis-regulated expression of genes (30-40%). Interestingly, the mutants with the least number of defective genes are *tir1* and *cpk3*. TIR1 is a redundant gene and six AFB homologues exist. Probably the AFB homologues rescue the function of the missing TIR1 in the *tir1* mutant.

For selected early auxin-induced genes also a mis-regulation in most of the tested mutants could be shown 10 min after auxin treatment. This supports the idea of a rapid auxin response which needs all these proteins (defined by the mutants) as components of a network. Even though TIR1 regulates expression of early auxin-induced genes and the glycoprotein ABP1 cannot directly interfere with TIR1, the strongest influence is exerted by proteins other than TIR1. As outlined below for this network, only ABP1 can be the receptor, as indicated by its strongest influence on early auxin-induced gene expression.

Does testing expression of test genes at 30 min in mutants allow a predicition of morphological phenotypes? Probably not, because long-term expression patterns are the basis of morphological phenotypes. Thus expression of the test genes was compared in all mutants to the respective wild types with no auxin treatment (Fig. 5, Chapter 2; Fig. 1-4, Chapter 4). Remarkably, the *ppla-IIIδ* mutant that showed an auxin-related phenotype also had a high number of differentially expressed early auxin-induced genes (Fig. 5; Chapter 2) but most mutants did not. Moreover expression of 25% of the genes mis-regulated in their basal level transcription were also mis-regulated 30 min after auxin treatment, so that these two groups auxin-regulated expression vs. permanently different expression did only partial correlate (Fig. 7 and Suppl.-Fig. 2, Chapter 4).

Involvement of the tested potential signalling components already 10 min after auxin treatment does therefore not depend on auxin-induced gene regulation but posttranslational activation. This shows again that TIR1/AFBs cannot be the responsible receptor for the signalling network involving all tested components but ABP1 which is responsible for early auxin events that do not depend on newly synthesized proteins.

Defects in early auxin-induced gene expression in the tested mutants give a new insight of the cytosolic part of ABP1-mediated early auxin signal transduction

Together with the known functions of some tested proteins in auxin responses the results of this work demonstrate new cytosolic components in ABP1-mediated early auxin signal transduction. Figure 5.1 shows a summarized model of the ABP1-mediated early auxin signal transduction including the potential signaling components identified in this work. Auxin perception in auxin signaling occurs at the two receptors ABP1 and TIR1. ABP1 is the relevant receptor for reactions that occur faster than within 10 min, like the activation of the pPLAs and the corresponding release of free fatty acids as potential second messengers and as potential regulators of membrane attached proteins (Rudier et al. 2010). Further the results of this work show other potential cytosolic components, like PP2A and D6PKs that are probably involved in this signaling pathway by exerting an influence on PIN proteins as previously shown (Michniewicz et al., 2007; Zourelidou et al., 2009). It was shown that pPLA activation can be catalyzed by protein kinases like CPK3 (Rietz et al., 2010) or CK2 and in the case of pPLA-I by small G proteins (Scherer et al., 2012). As a mechanism of how ABP1 mediates signal transduction it was hypothesized that ABP1

regulates PIN proteins (auxin transport) and thus changes auxin concentration in the cell so that this is perceived by TIR1 which executes auxin-induced gene expression accordingly. The role of the proteins of this cytosolic network is discussed in the following paragraphs.

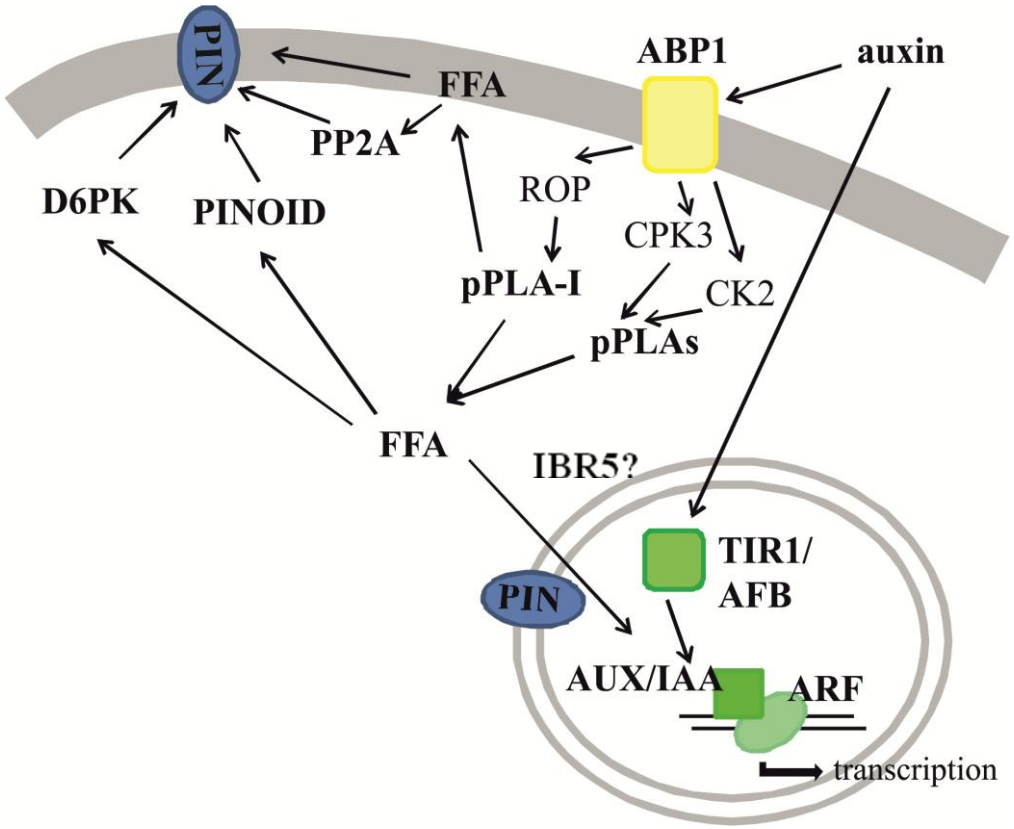


Fig. 5.1. Model of ABP1-mediated early auxin signal transduction
Simplified model of the auxin signal transduction pathway with ABP1 as the receptor. For Explanations see text. ABP1: AUXIN-BINDING PROTEIN1; ARF: AUXIN RESPONSE FACTOR; Aux/IAA: auxin/indoleacetic acid proteins; CK2: casein kinase 2; CPK3: calcium-dependent kinase 3; D6PK: protein kinases of the AGCVIIIa kinase subfamily; FFA: free fatty acid; IBR5: INDOLE-3-BUTYRIC ACID RESPONSE 5; PIN: pin-formed, auxin efflux transporter; PINOID: protein kinase; PP2A: protein phosphatase 2A; pPLA: patatin-related phospholipase A; pPLA-I: patatin-related phospholipase A-I; ROP: Rho GTPase; TIR1/AFB: TRANSPORT-INHIBITOR-RESISTANT1 and homologous AUXIN UP-REGULATED F-BOX PROTEIN. Based on Scherer et al. (2012).

Patatin-related phospholipases are involved in auxin signalling

Activation of pPLAs by auxin within 2-5 min (Paul et al., 1998), auxin-related phenotypes of half of the *ppla* mutants (Huang et al., 2001; Rietz et al. 2010; Li et al., 2011; Effendi et al., 2013; this work) and mis-regulated expression of early auxin-induced genes makes

clear that *ppla* mutants are auxin mutants. How can pPLAs be integrated into the ABP1-mediated auxin signaling with an influence on TIR1? At first, pPLA activation by auxin was described by measuring an increase of pPLA reaction within minutes (Scherer and André 1989; Paul et al., 1998). Furthermore some *ppla* mutants showed developmental phenotypes that can be assigned to auxin functions, although only one mutant (*ppla-IIIδ*) showed a phenotype when grown on auxin medium (Chapter 2). Nevertheless, *ppla-IIε* developed fewer lateral roots than wild type seedlings under nutrient stress and *ppla-IIγ* had changes in root architecture in response to phosphate deficiency and ABA (Rietz et al., 2010). Such responses involve several hormones, but auxin is regarded to be the main growth-regulating hormone involved in phosphate deficiency (Lopez-Bucio et al., 2002; Pérez-Torres et al., 2008). Li et al. (2011) also found a weak auxin-related phenotype for *ppla-IIIβ* and the corresponding overexpresser plants. The auxin phenotype test could demonstrate a dominant-negative auxin-related phenotype only for *ppla-IIIδ* (Chapter 2).. Thus, for half of the mutants developmental phenotypes could be detected which are auxin-related.

Chapter 3 focuses on the investigation of pPLA-I function in *Arabidopsis*. Two *ppla-I* knockout mutants were tested under several physiological conditions and secondly early auxin-induced gene expression was measured which is part of this work. Next to the similar gene regulation defects as it was shown for *abp1/ABP1*, *ppla-I* mutants also had clear phenotypes in phototropism, gravitropism and root coiling. The hypocotyls of dark-grown mutant seedlings responded slower to a gravitropic stimulus than the wild type. Furthermore one of the alleles had a higher tendency of root coil formation and the effect was increased by raised osmotic strength of the medium. In addition, mutant seedlings were much taller than the wild type when grown in a shade avoidance condition, which means light conditions in a low red and far red ration. Thus, these results indicate that pPLA-I has a function in the intersection of auxin- and light signalling.

But the main evidence for an involvement of all pPLAs in auxin signalling was the regulatory phenotype that was shown for all ten mutants. All had defects in early auxin-induced gene expression. Expression of 4-11 out of 15 tested genes was mis-regulated 30 min after auxin treatment of mutant seedlings. For selected genes and mutants this defect could be also shown at 10 min after auxin application (Chapter 2, Fig. 7; Chapter 3, Fig. 3). Interestingly, this mis-regulation of auxin induced gene expression was mostly not

detectable any more after 3 hours (Chapter 2, Fig. 7). This shows a transient effect in the early stages of auxin signaling. After 3 hours of auxin treatment there may be other pPLA-independent signals regulating early auxin-induced gene expression. The mutant with the highest number of mis-regulated genes is *ppla-I*, followed by *ppla-IIδ* and *ppla-IIIδ*. The number of early auxin-induced genes with expressional defects in *ppla-I* is close to the number shown for *abp1/ABP1* and *eir1* (Effendi et al., 2011; Effendi and Scherer 2011). The similar regulatory phenotypes of both, *ppla-I* and *abp1/ABP1*, consisting of defects in auxin-induced gene regulation, phototropism, gravitropism and early flowering (Effendi et al., 2011; Effendi and Scherer 2011) demonstrates for the gene *pPLA-I* that this phospholipase A is most likely to be linked to ABP1 as the responsible receptor.

ppla-I, *abp1/ABP1* and *eir1* showed a 55-80% mis-regulated expression of all tested early auxin-induced genes (see Fig. 8 in Chapter 4). Four other *ppla* mutants showed a noticeable defect of 40-50% of the tested genes: *ppla-IIα*, *ppla-IIδ*, *ppla-IIIγ*, and *ppla-IIIδ*. The remaning five mutants *ppla-IIβ*, *ppla-IIγ*, *ppla-IIε*, *ppla-IIIα* and *ppla-IIIβ* showed 25-30% misregulation of the tested genes.

The little information about the pPLAs and the results of this work give evidence for an involvement in auxin signaling. Whether they are also involved in the link of auxin- and light signalling or in other signaling pathways was not further investigated.

Expression of several genes was preferentially mis-regulated in *ppla* mutants: *IAA2*, *IAA19*, *PIN3*, *GH3.5* and the tested *SAUR* genes. A reason for this could be the redundancy of pPLAs in *Arabidopsis*. For some of the investigated early auxin-induced genes (*IAA3*, *IAA19*, *PIN3*) a function in lateral root formation is known (Benkova et al., 2003; Peret et al., 2009). Some *ppla* mutants exhibit root phenotypes that could be related to this results. *GH3.5* codes for one of the auxin conjugating enzymes (Staswick et al., 2005) with a function of regulation of auxin concentration in the cell. How *SAURs* exert their function is still unknown (Jain et al., 2006) Only few were recently shown to have a function in auxin-dependent elongation but always in requiring additional conditions to reveal their auxin functions (Franklin et al., 2011; Spartz et al., 2012; Chae et al., 2012). It is not possible to explain why especially these early auxin-induced genes are defect in practically all tested mutants but the fact that some were preferentially mis-regulated not only in *ppla* but also

other tested mutants show a common involvement of the genes/proteins defined by the mutants in ABP1-mediated auxin signaling.

Change of fatty acid composition in membrane lipids lead to mis-regulated early auxin-induced gene expression

The demonstration of function of pPLAs in auxin signaling led to the idea to test more mutants with this specific biotest of testing early auxin-induced gene expression after 10 and 30 min auxin treatment. It seemed to be possible to identify more cytosolic signaling components besides pPLAs in ABP1-mediated auxin signaling.

Mutants with changes in concentrations of specific fatty acids in phospholipids of membranes should provide a different substrate to phosphlipases so that, for instance, pPLA activation would create differences in membrane surfaces. To test this general hypothesis, fatty acid biosynthesis mutants were chosen that have either an increased or decreased amount of specific fatty acids like 18:0, 18:1 and 18:2 (Kachroo et al., 2001, 2003). Furthermore mutants with either decreased levels of very long chain fatty acids (VLCFAs) (*lacs9*) or increased levels of VLCFA (*lacs4*) were selected.

The tested mutants *fad2-1*, *fad6-1*, *ssi2* and *lacs4 lacs9* showed indeed mis-regulated early auxin-induced gene expression similar to *ppla* mutants. The two single mutants *lacs4* and *lacs9* did not show remarkable defects in auxin-induced gene expression as it could be shown for the double mutant (Fig. 2, Chapter 4). The defect of fatty acid composition in the single mutants is probably not strong enough to interfere auxin signaling. None of the mutants showed a direct developmental auxin-induced phenotype, although *fad2-1*, *fad6-1* and *ssi2* already had a phenotype when grown without any treatment. They had shorter main roots and it is known that retarded growth was increased under abiotic stress conditions (Zhang et al., 2009; 2012). However other typical auxin-phenotypes like more lateral roots on auxin-containing medium were missing. Nevertheless the clear regulatory phenotype of the tested membrane lipid-deficient mutants makes them to auxin mutants and shows importance of membrane lipid composition and, thus, of fatty acids in auxin signaling.

So, how can fatty acids influence auxin signaling in the ABP1-mediated auxin network? One possibility is that free fatty acids (FFA) provided by pPLAs act as second messengers.

In some signaling pathways an involvement of FFA could be shown. In plant defense plastidial fatty acids regulate resistance gene-dependent defense signaling (Chandra-Shekara et al., 2007). In animal systems the ratio of saturated and unsaturated fatty acids is thought to control many cellular functions, including cell growth, differentiation, and apoptosis (Kates et al., 1984; Ntambi, 1995). Studies in mammalian systems have shown that 18:1 can induce the translocation of protein kinase C from the cytosol toward the membranes via direct or indirect processes (Diaz-Guerra et al., 1991) but a protein kinase C was not found in plants (Bögre et al., 2003). Thus FFA could influence the activation of not yet identified enzymes which then leads to regulation of early auxin-induced gene expression in an unknown way (Scherer, 2010).

Another possibility is that FAs or lipids derived from them have an influence on membrane bound proteins. A recent study showed the involvement of very long chain fatty acids in PIN1 polarity (Roudier et al., 2010). They investigated a mutant (*pas1*) having reduced levels of VLCFAs. In the apical region of *pas1* embryos PIN1 polarity was defect and this altered polar auxin transport and led to a lack of local redistribution of auxin accumulation. Thus the fluidity of the membranes could be important for auxin transport in altering relocalization of PIN-proteins. Furthermore there are several enzymes like protein phosphatases which are important for the phosphorylation status of PINs. PINOID, PP2A and D6PKs (Michniewicz et al., 2007; Zourelidou et al., 2009) are some examples for this and could be influenced by fatty acid composition of membranes because of their attachment to the plasma membrane (Fig. 5.1).

Mis-regulation of early auxin-induced gene expression identified protein kinases and protein phosphatases as cytosolic components in ABP1-mediated auxin signal transduction

ABP1-mediated auxin signal transduction including PIN activity needs protein kinases and protein phosphatases to regulate the phosphorylation status of PINs and also activate further enzymes like pPLAs via phosphorylation. Thus several mutants were selected with known functions either in pPLA activation (Rietz et al., 2010) or PIN phosphorylation (Michniewicz et al., 2007; Zourelidou et al., 2009). In addition a MAPK phosphatase mutant (*ibr5*) was selected (Strader et al., 2008).

Two mutants, *cpk3* and the corresponding overexpression line, were selected because of the auxin-induced activation mechanism of pPLAs. A de novo synthesis of pPLAs can be excluded because of the rapid activation by auxin within 2-5 minutes (Paul et al., 1998). A recent study identified potential phosphorylation sites in the pPLA protein structure and could show the activation of group II pPLA enzymes via phosphorylation by a calcium dependent protein kinase (CPK3) (Rietz et al., 2010). Auxin-induced calcium influx could , for instance, activate CPK3 (Monshausen et al., 2011). In the *cpk3* knock down mutant only one of the 15 tested early auxin-induced genes was mis-regulated. The *CPK3* overexpression line otherwise showed a remarkable mis-regulation of 30% of the tested genes after 30 min auxin treatment. CPK3 belongs to a large family of calcium dependent protein kinases and some other members of the family with similar functions could replace the function of the missing CPK3 in the knockout mutant. The defect expression of early auxin-induced genes in the overexpression line could be perhaps explained by a loss of substrate specificity of the enzymes in a high concentration so that increased phosphorylation could have a disturbing effect. Nevertheless phosphorylation of pPLAs as an activation mechanism could be a function where CPK3 is integrated in auxin signaling.

For nine of the ten pPLAs potential phosphorylation sites in the C-termini could be identified (Scherer et al., 2012). The single group I enzyme pPLA-I does not posess such a phosphorylation site (Scherer et al., 2012). For pPLA-I activation by G proteins was supposed. Small G proteins are involved in auxin signal transduction (Tao et al., 2002; 2005; Wu et al., 2011) and were shown to be involved in ABP1 mediated auxin signaling (Xu et al., 2010). As a recent study by Effendi et al. (2013) and this work associated pPLA-I with ABP1-mediated auxin signaling, an activation of pPLA-I by G proteins seems a possibility. Thus, several activation mechanisms for the three pPLA groups are possible and CPK3 and other protein kinases as well as small G proteins could present more potential components in early auxin signaling (Fig.5.1).

IBR5 phosphatase activity is necessary for full auxin and ABA responsiveness (Strader et al., 2008). In *ibr5* two Aux/IAA family members, IAA17 and IAA28, were not degraded as rapidly as in wild type, suggesting that IBR5 acts downstream of auxin recognition by the $SCF^{TIR1/AFB}$ –AUX/IAA complexes. A mis-regulation of *IAA* gene expression could not be shown for *ibr5* in this work and *IAA17* and *IAA28* were not tested here. Furthermore only 20% of the tested early auxin-induced genes were mis-regulated. IBR5 is known to be

involved in auxin responses and the results could indicate an involvement in early auxin signaling. However, IBR5 is probably less tightly linked to the early steps of ABP1/PIN/pPLA-mediated auxin signaling which lead to regulation of TIR1 activity.

PP2A and D6PKs are involved in auxin signaling by influencing PIN proteins employing the kinase PINOID (Michniewicz et al., 2007; Zourelidou et al., 2009). PP2A and PINOID partially colocalize with PINs and regulate PIN apical-basal targeting by phosphorylation/dephosphorylation (Michniewicz et al., 2007). D6PKs, which belong to the same AGCVIIIa kinase subfamily as PINOID, were shown to phosphorylate PIN proteins and furthermore PINs and D6PKs are colocalized at the basal membrane of root cells (Zourelidou et al., 2009). Of the tested early auxin-induced gene expressions up to 40% were mis-regulated in the *d6pk* mutants and *pp2a* (Fig. 3; Chapter 4). The defects in gene expression in these mutants as well as in ABP1 mutants *abp1/ABP1* and *abp1-5* and also in the PIN2 mutant *eir1* supports the idea of an ABP1-mediated auxin signal transduction including several signaling components like PP2A and D6PKs taking influence on PIN protein phosphorylation status and therefore on auxin transport (Fig.5.1).

The ABP1 receptor activity and the identified cytosolic components of the ABP1 network describe an early auxin signal transduction pathway with influence on TIR1 activity

Together with previous results on the *abp1/ABP1*, *abp1-5* and *eir1* mutants (Effendi et al., 2011; Effendi and Scherer 2011) the results of this work show a complex network of early auxin signal transduction (Fig. 5.1). It was demonstrated that not only TIR1/AFBs but also many other components regulate (very) early auxin-induced gene expression. All these results support the idea of a two-receptor concept in auxin signalling. TIR1/AFBs function in auxin signal transduction is to initiate regulation of gene expression and the synthesis of new proteins which leads then to typical physiological auxin responses. It is clear that TIR1/AFBs cannot be the responsible receptor for rapid auxin responses like for example relocalization of PIN proteins within minutes after auxin signal perception or the activation of pPLA activity by auxin within minutes. If TIR1/AFBs would be the receptor for this specific rapid response it would have to make use of the only known function of TIR1/AFBs, regulation of auxin-dependent transcription. In fact, none of the tested genes providing the mutants showed increased auxin-induced expression (Chapter 2, Fig. 6;

Chapter 3, Fig. 3; Chapter 4, Fig.6). Thus two receptors, ABP1 and TIR1/AFBs, are responsible for different auxin signal transduction pathways. ABP1 is associated with the regulation of rapid cytosol-based signaling mechanisms while TIR1/AFBs regulate negative transcriptional co-regulators in the nucleus (Fig.5.1).

An interesting idea is a co-operation of ABP1 and TIR1/AFBs in an auxin signal transduction network (Scherer et al., 2012). The final purpose of the network is postulated to be regulation of the auxin concentration in the cell. ABP1 can perceive apoplastic auxin concentration in regulating PIN proteins and auxin transport and therefore indirectly regulates auxin concentration in the cell (Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011). The PIN2 and PIN3 proteins are located at the plasma membrane and therefore regulate extracytosolic and, thereby, cytosolic auxin concentration while PIN5 which is located at the ER may regulate nuclear auxin concentration or inside the ER. Thus a link of ABP1 and TIR1/AFBs may happen via auxin concentration using regulation of PIN protein activity as a short term mechanism and regulation of transcription of PIN proteins as a long term mechanism (Robert et al., 2010; Xu et al., 2010; Effendi et al., 2011; Scherer, 2011).

Outlook

In Fig.7 in Chapter 4 all mutants were ordered according to the number of defects in gene expression. Although this may still be an incomplete matrix one can tentatively derive the conclusion that ABP1 and proteins postulated to be involved in auxin transport/concentration regulation showed the highest number of defects. TIR1 itself has little capacity to regulate its own activity within 30 min. Gene families with many members exert a low influence, e.g. CPK3 or LACS. All single mutants of pPLA have a more or less strong influence.

To get more detailed or more conclusive knowledge about the mechanisms of ABP1-mediated auxin signal transduction the identification of more cytosolic components would be helpful. More mutants of potential candidates could be tested with the developed biotest. Candidates for this could be mutants of PINOID, more PIN protein mutants, further protein kinase and protein phosphatase mutants. Also multiple mutants of TIR1 and its AFB homologues would complement the knowledge about potential defects of early auxin-induced gene expression after a short time auxin treatment like 10 or 30 minutes.

Also multiple mutants of members of the pPLA family could exclude functional redundancy and result in morphological phenotypes. Also double mutants of *abp1* and the identified components could result in either morphological or regulatory phenotypes.

Another important experiment would be a closer look on the activation mechanism of pPLAs. To confirm an activation mechanism via phosphorylation a site directed mutangenesis of the potential phosphorylation sites in the pPLA structures (Scherer et al., 2012) would be helpful. The exchange of amino acids in the protein structure and the following activity tests or again using the biotest of measuring early auxin-induced gene expression could show if pPLAs are activated via phosphorylation.

Furthermore a closer look on the involvement of the components identified to be involved in ABP1-mediated auxin transport would help to understand how ABP1 and the other cytosolic components of the early auxin signal transduction may interact. For example if the lipid composition of the membranes is important for fluidity of the membranes and thus localization of PIN proteins, early localization studies of PIN proteins with PIN antibodies in membrane lipid-deficient mutants could show if PINs are defect in relocalization after auxin signal perception. These expermiments could also be done for the other mutants tested here, especially pPLA mutants.

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APPENDICES

DANKSAGUNG

An erster Stelle möchte ich mich bei meinem Doktorvater Herrn Prof. Dr. Scherer bedanken. Nicht nur für die Vergabe des interessanten Themas, sondern besonders für sein Vertrauen in meine Fähigkeiten und für Zuspruch und Kritik zu genau der richtigen Zeit.

Herrn Prof. Dr. Peterhänsel und Frau Prof. Dr. Piechulla danke ich für die Übernahme des Zweit- und Drittgutachtens.

Ein besonderer Dank geht an die Mitarbeiter der AG Scherer, im speziellen an Peter P., der mir in den Jahren immer wieder sowohl im Labor, als auch freundschaftlich zur Seite stand.

Felix Tebartz durfte ich während meiner Doktroarbeit kennen lernen und er war mir in den Höhen und Tiefen der letzten Jahre immer ein guter Freund. Ohne seine Freundschaft wäre die Fertigstellung dieser Arbeit um einiges schwieriger gewesen. Sabrina Spengler möchte ich ebenfalls für ihre Freundschaft, Unterstützung und Durchsicht dieser Arbeit danken.

Darüber hinaus möchte ich mich sehr bei meinen Eltern und im besonderem bei meinem Bruder Matthias für die immerwärende Unterstützung, Geduld und das immer offene Ohr bedanken. Vor allen anderen aber danke ich Dennis, dem diese Arbeit gewidmet ist, dafür immer für mich da zu sein, für sein unerschütterliches Vertrauen und bedingungslose Freundschaft.

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LIST OF PUBLICATIONS

Labusch C. and Scherer G.F.E. (2013). Timing and pattern of auxin-induced expression of early auxin genes in known and unknown auxin mutants reveal cytosolic components having an influence on auxin signal transduction. (in progress)

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Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

Für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, das ich meine Dissertation mit dem Titel

Patatin-related phospholipases (pPLA) and other cytosolic components in AUXIN BINDING PROTEIN1 (ABP1)-mediated auxin signaling are revealed by using early auxin-induced gene expression as a biotest in *Arabidopsis thaliana.*

selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, den. 20.12.2012

Corinna Labusch