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Patterns and Timing in Expression of Early Auxin-Induced Genes Imply Involvement of Phospholipases A (pPLAs) in the Regulation of Auxin Responses

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ABSTRACT While it is known that patatin-related phospholipase A (pPLA) activity is rapidly activated within 3 min 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* knockdown 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 hypersensitive auxin-induced gene expression in *ppla* mutants. While, in three *ppla* mutants, which 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 posttranslational mechanism as a functional link of pPLAs to auxin signaling. The need for a receptor triggering an auxin response without employing transcription regulation is discussed.

Key words: hormonal regulation; hormone biology; signal transduction.

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

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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, 1998b; 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; Reed, 2001; Tian et al., 2003) 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 RNAs and are transcribed within 2–5 min of exogenous auxin application (Hagen and Guilfoyle, 2002). The function of proteins 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 Figure 1. In fact, only one of the *ppla* mutants, *ppla-IIIδ*, showed an auxin-related phenotype in root growth (Figure 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 Figure 2).

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 and Scherer, 2011; Effendi et al., 2011).

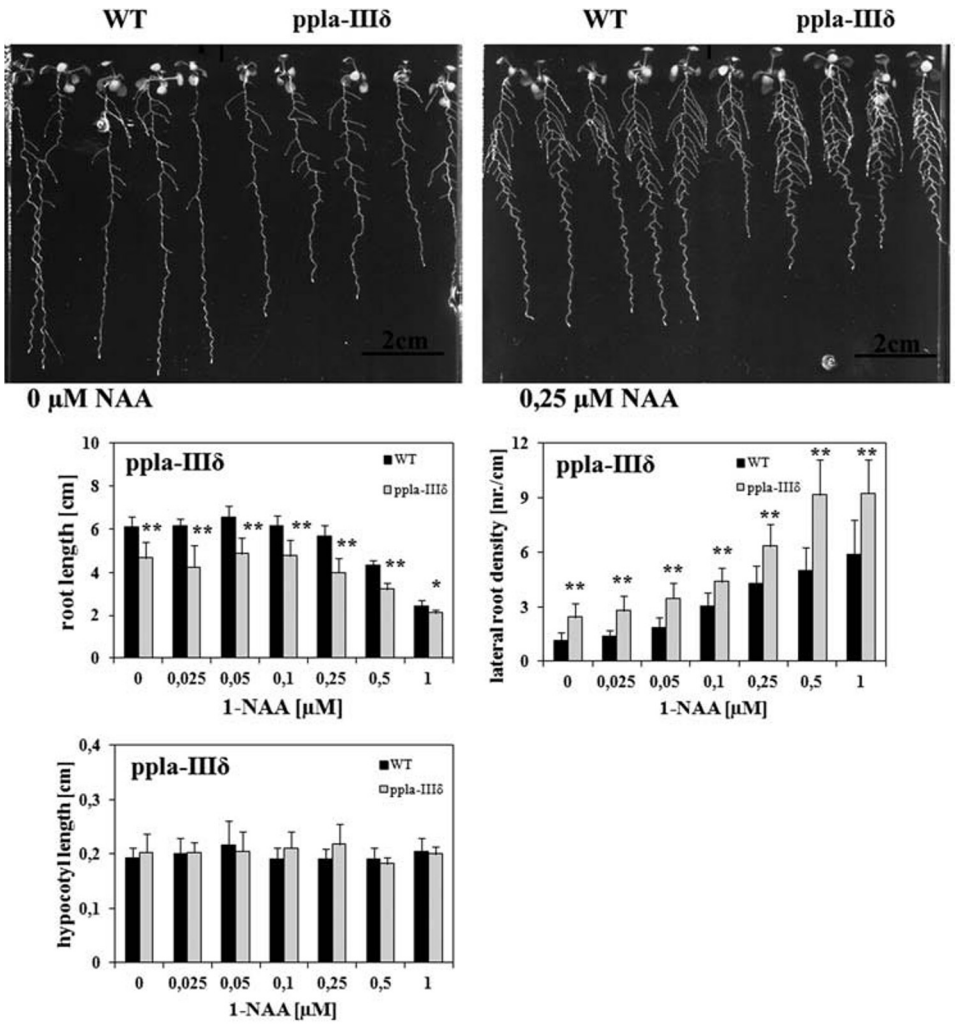

Figure 1. Growth Response of Light-Grown *ppla-IIIδ* Mutants and Wild-Type Plants in Response to Auxin.
Seedlings were grown for 7 d on 1 ATS medium with different 1-NAA concentrations.
(A) Comparison of growth patterns (bar = 2 cm).
(B) Root length.
(C) Lateral root density.
(D) Hypocotyl length. Asterisks 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).

Several groups of early auxin-induced genes were chosen for this investigation. In wild-type seedlings, most of the genes were up-regulated 2–12-fold in comparison to untreated controls after 30 min auxin treatment (Figures 2–4). Among the *IAA* genes, *IAA2* failed to be up-regulated in the *ppla* mutants most often (five times) (Figure 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 *ppla-IIα* and *ppla-IIγ* mutants, respectively.

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 (Figure 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* (five times).

Considering all nine *ppla* mutants together, always at least one of the three tested *SAUR* genes responded more weakly to the auxin stimulus. In two ppla mutants, all three *SAURs*

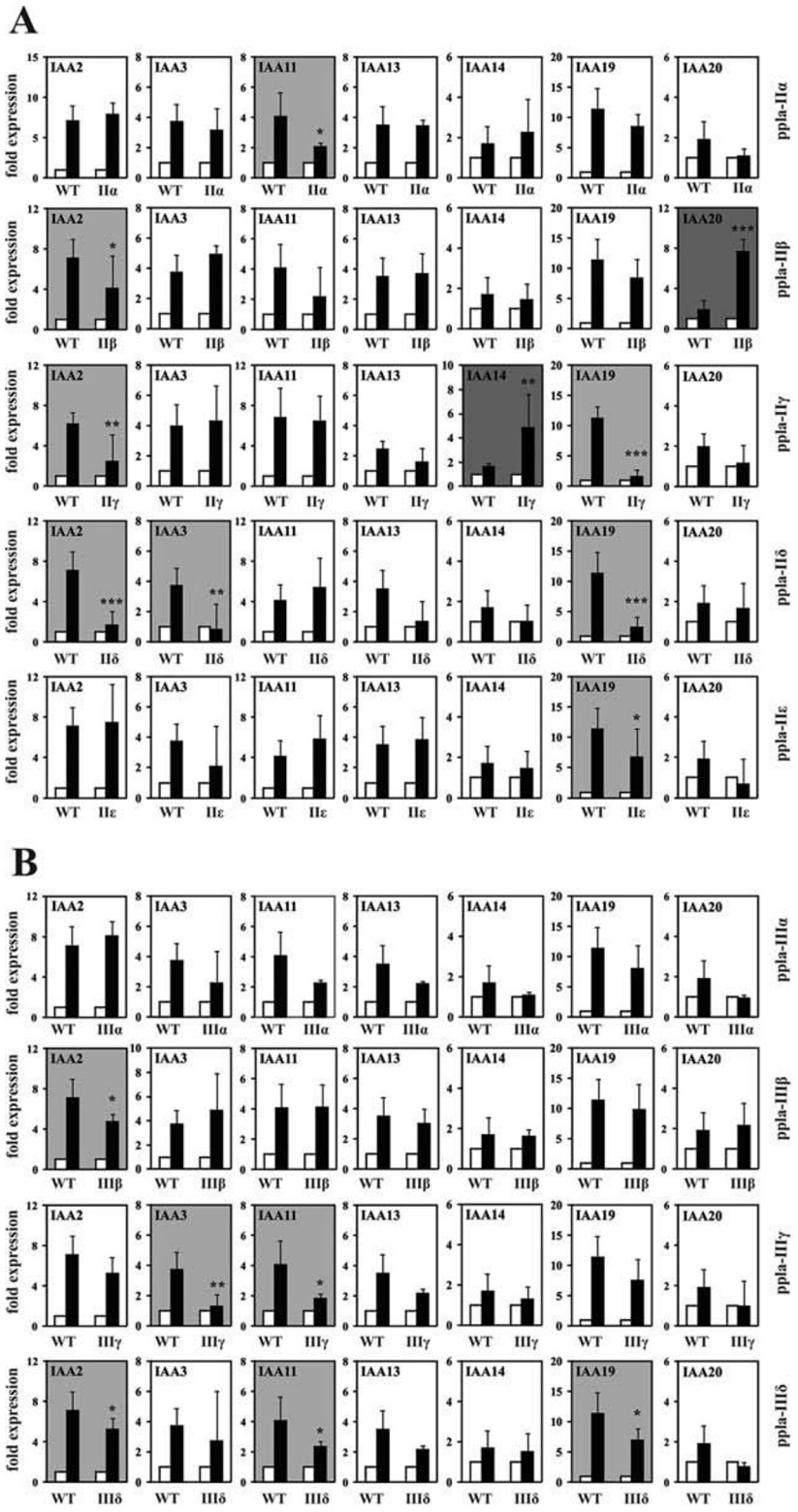

Figure 2. Expression of *IAA* Genes in Light-Grown *ppla* Mutant and Wild-Type Seedlings.

(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 $t = 30$ min IAA were calculated accordingly (black bars).

(*SAUR9*, *SAUR15*, *SAUR23*) were less up-regulated (Figure 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 misregulated in two-thirds of all cases investigated. However, we could not detect a common pattern of misregulation.

The auxin-inducible expression levels of the auxin response genes reported (Figures 2–4) were also tested in non-treated mutants and wild-type (Figure 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, 24 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 (three to six per mutant) than the five group II mutants (0–4) per mutant) compared to wild-type. However, we could not observe any recognizable pattern (Figure 5) that was reflected by their auxin-inducible gene expression (Figure 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 Figure 1.

pPLAs Interfere with Gene Expression as Early as 10 Min After Auxin Application

pPLA genes themselves could be regulated by auxin and, potentially, a lack of their transcription could be the cause of 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 onwards

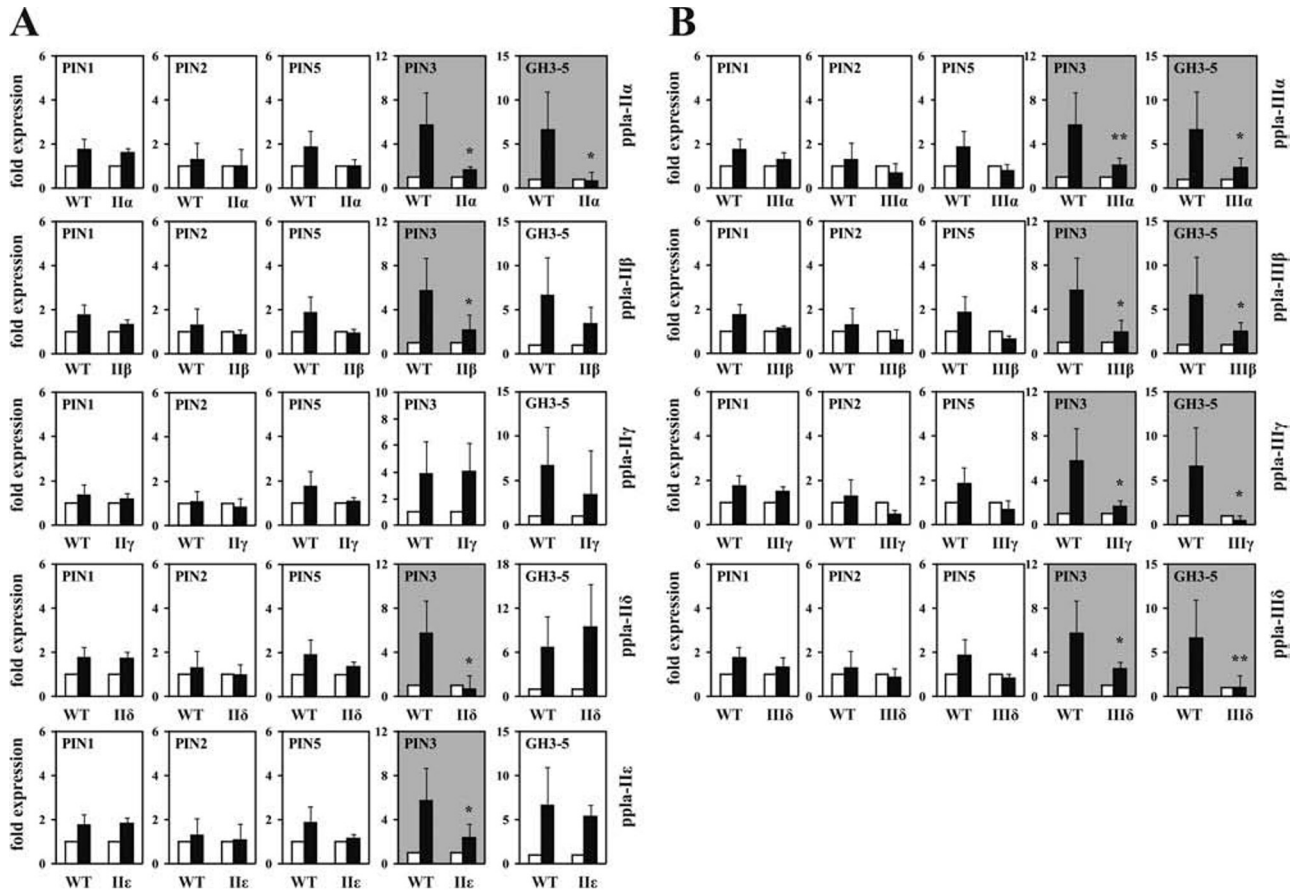

Figure 3. Expression of Several *PIN* Genes and *GH3.5* in Light-Grown *ppla* Mutant and Wild-Type Seedlings.
(A) Group II genes.
(B) Group III genes. The background of the panels is shaded whenever significant differences between wild-type and mutant were obtained. Asterisks above columns indicate significant 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 $t = 30$ min IAA were calculated accordingly (black bars).

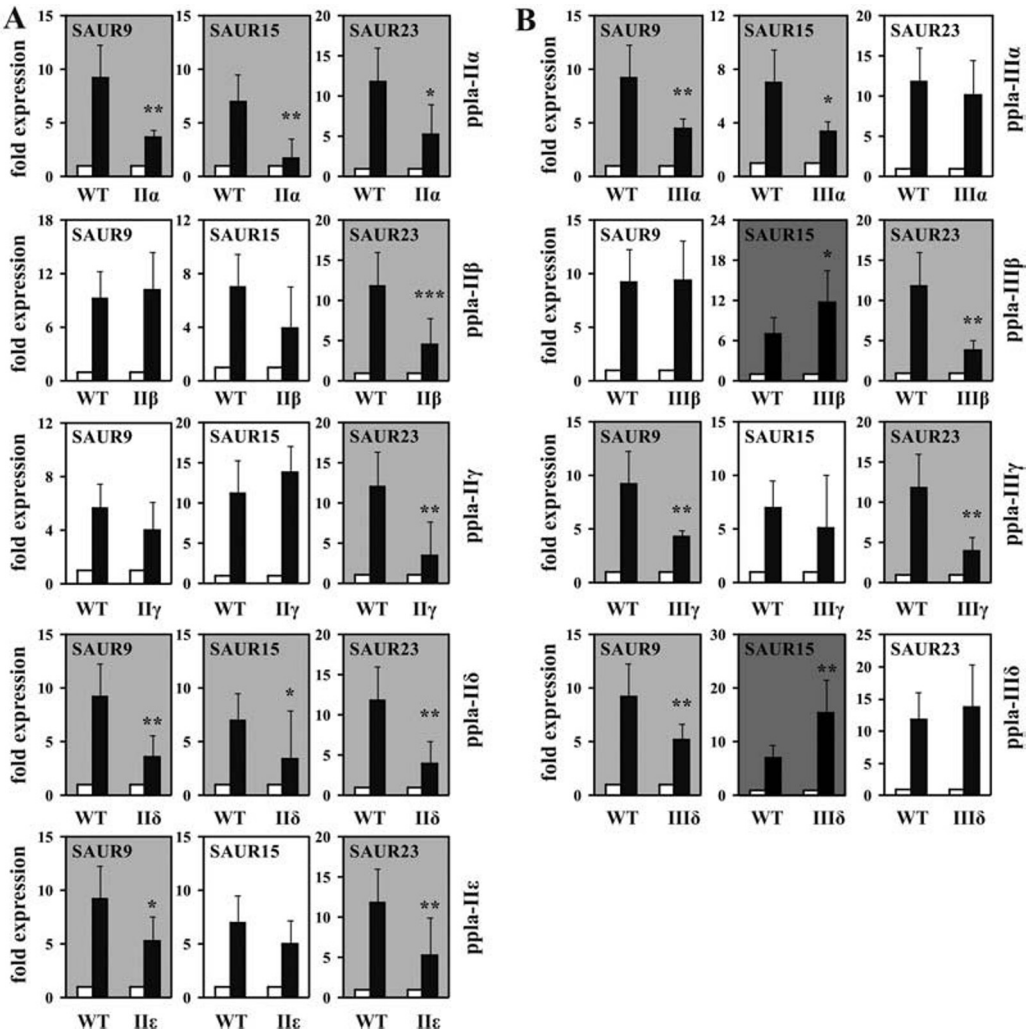

Figure 4. Expression of Several *SAUR* Genes in Light-Grown *ppla* Mutants and Wild-Type Seedlings Grown in the Light.
(A) Group II genes.
(B) Group III genes. Background of panels is shaded whenever significant differences between wild-type and mutant were obtained.
Asterisks above columns indicate 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 $t = 30$ min IAA were calculated accordingly (black bars).

(Figure 6). None of the *pPLA* genes was 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.

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 10 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 (Figure 7). 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, misregulation 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 (Figure 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

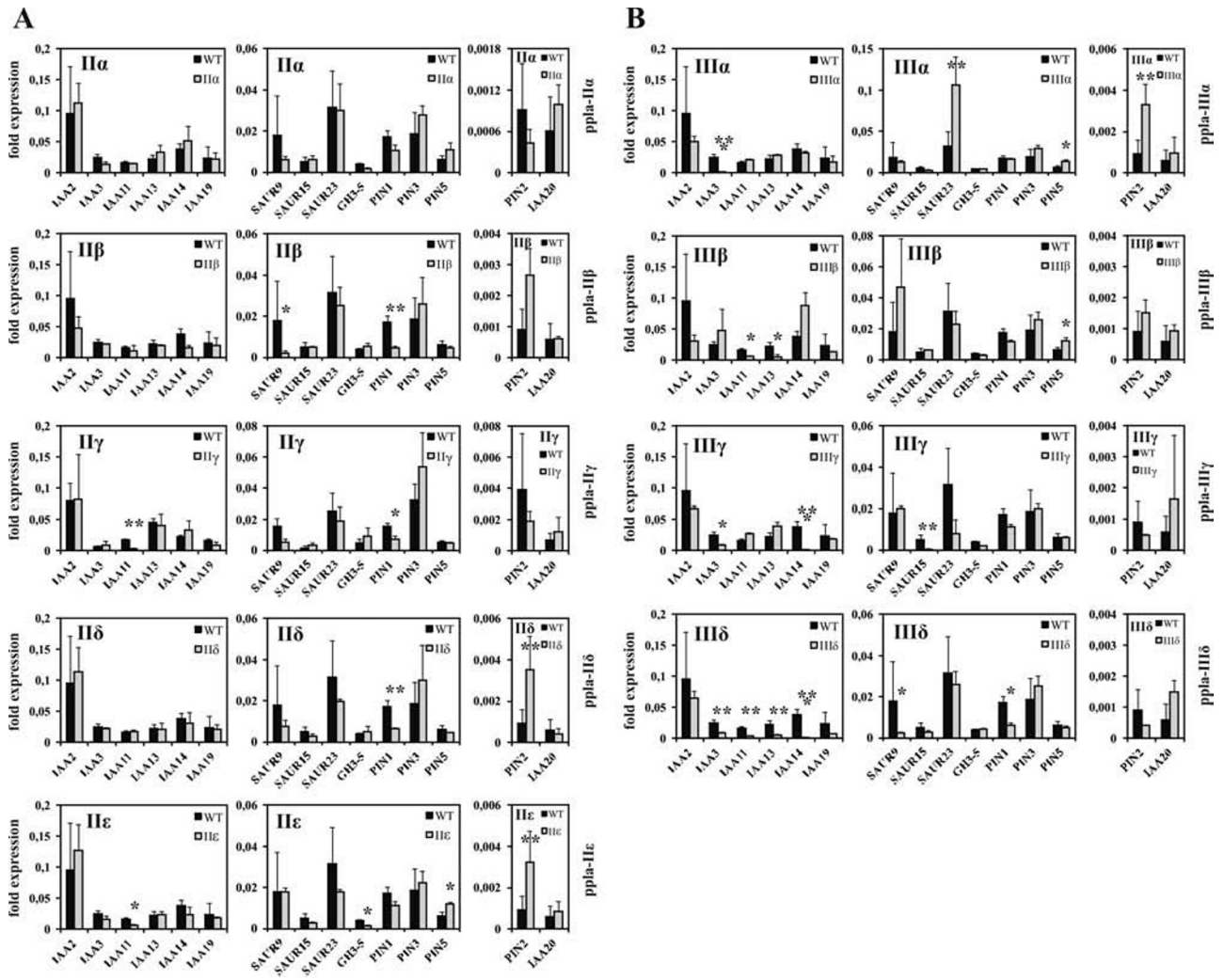

Figure 5. Comparison of Expression Profiles of All Genes Tested in Light-Grown *ppla* Mutants and Wild-Type Seedlings without Auxin Treatment ($t = 0$).

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*.

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 assignment of this activation to any single enzyme. Several previously described *ppla* mutants exhibit developmental phenotypes related to auxin physiology (Figure 1 and Supplemental Figure 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 with Supplemental Figure 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 d after germination in our growth conditions. Possible effects

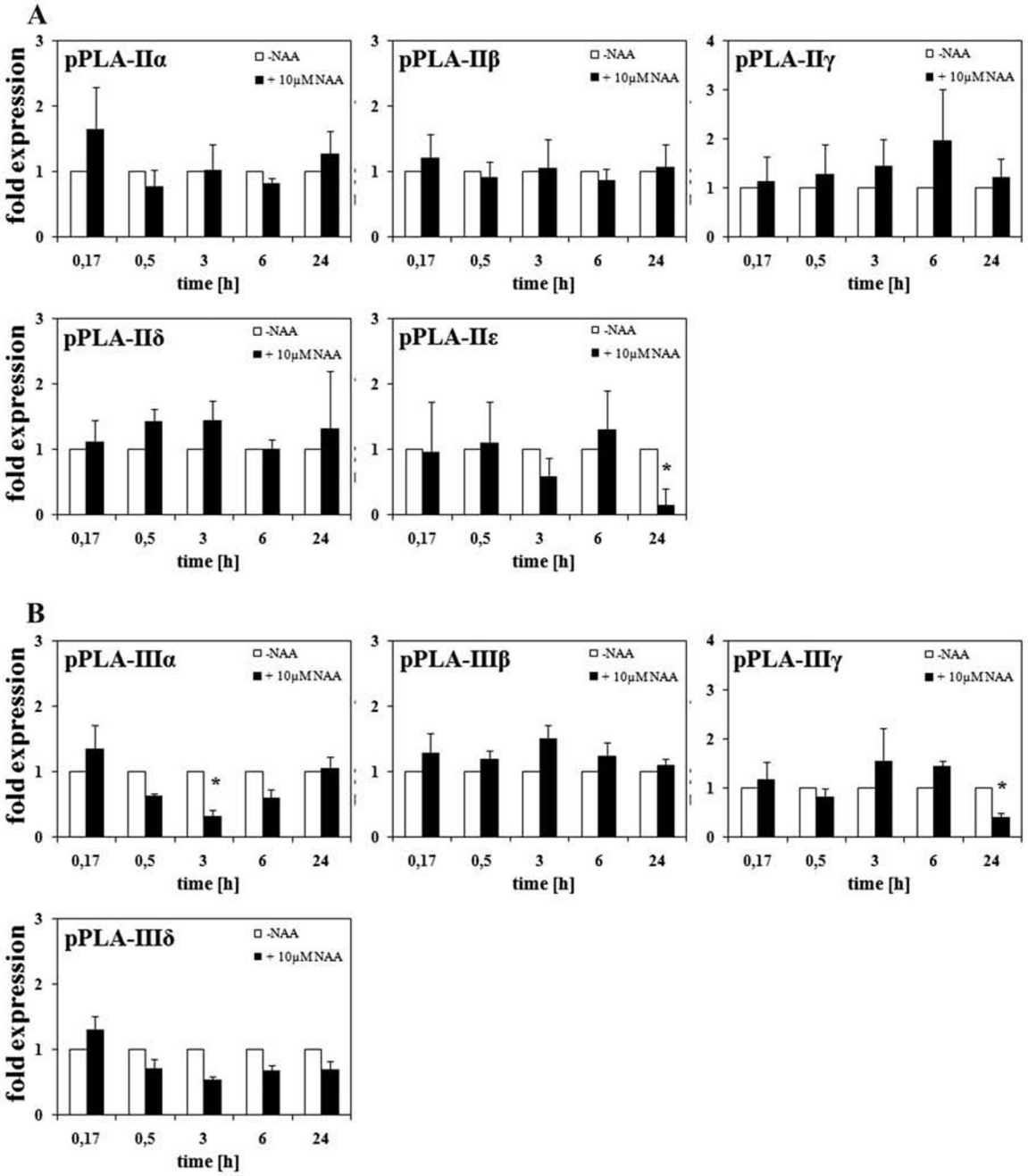

Figure 6. Time Courses of Expression of *pPLA* Genes in Wild-Type after Auxin Application.
Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; *t*-test). Relative expression levels were 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).

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 aberrant epidermal cell patterning indicate a role for auxin in this regard. In addition, while

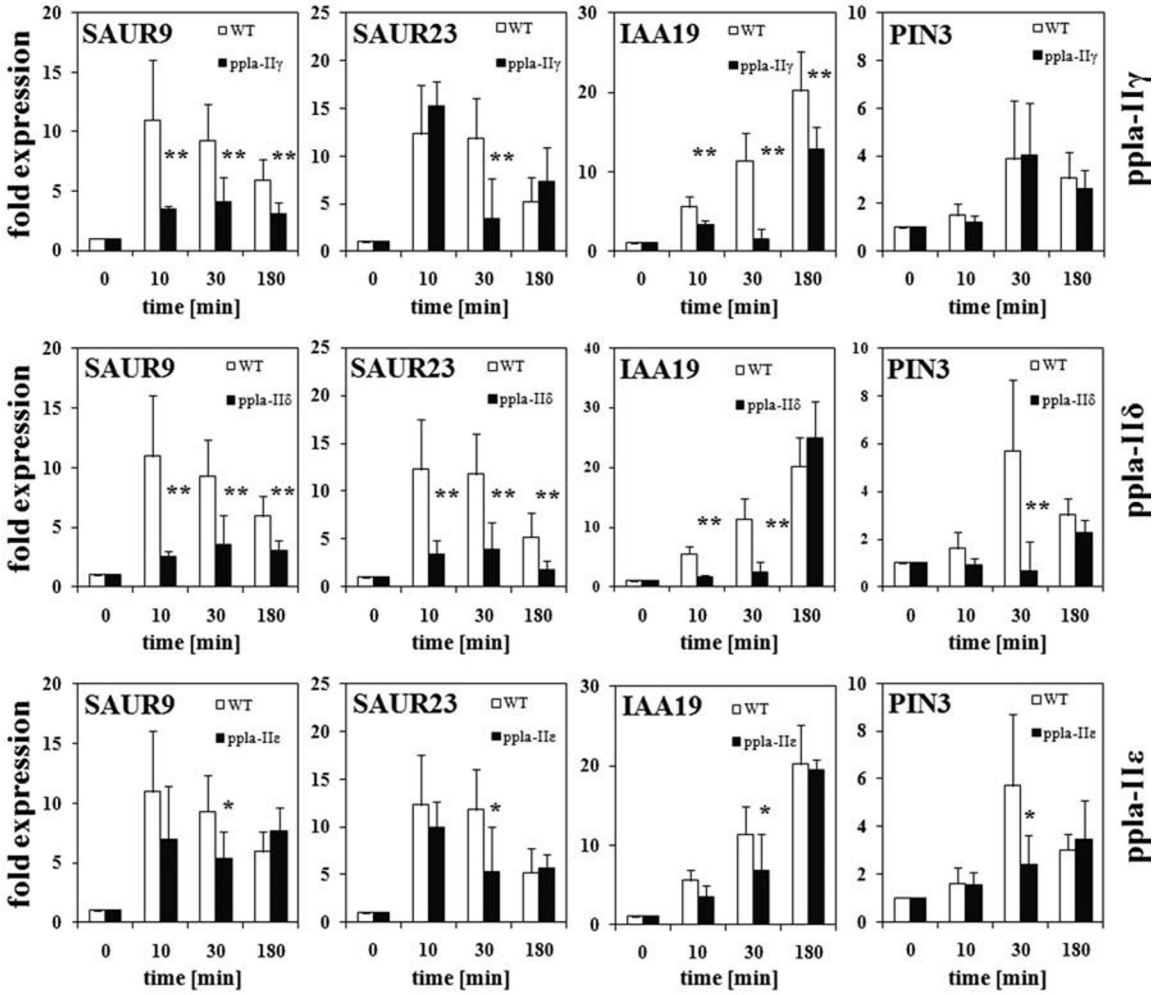

Figure 7. Time Courses of Expression of Selected Genes in Three Light-Grown *ppla* Mutant and Wild-Type Seedlings.
Asterisks above columns indicate significant differences between the mutants and the corresponding wild-type treatments at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0{,}001$ (***) levels according to *t*-test. Relative expression levels were calculated by setting values at $t = 0$ min to 1 (white bars); values at $t = 10$, 30 and 180 min IAA were calculated accordingly (black bars).

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 Figure 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 misregulated (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., 2010; 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 misregulated 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 spatiotemporal 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 all *ppla* mutants exhibited delayed expression of auxin-induced genes.

Several genes were preferentially misregulated in *ppla* mutants: *IAA2* (five times), *IAA19* (four times), *PIN3* (five times), *GH3.5* (five times), *SAUR9* (six times), *SAUR15* (four times), and *SAUR23* (six times). The likely 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 (Benková et al., 2003; Péret 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 a few were recently shown to have a function in auxin-dependent elongation in rather specific circumstances (Franklin et al., 2011; Chae et al., 2012; Spartz 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-III*δ (Figure 5) in which six genes were differently expressed and *ppla-III*δ indeed has a morphological phenotype (Figure 1).

The initial imbalance in regulation of auxin-induced gene expression is mostly attenuated after 3 h (Figure 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; Delker et al., 2008; Mockaitis and Estelle, 2008). This makes all *ppla* mutants to auxin-signaling mutants.

Any auxin-induced response must start with binding of auxin to a receptor. In the 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, namely 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 (Figure 5), and (3) after 30 min (Figure 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). 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 (Figure 6). The hypothesis we found more attractive is that pPLA activation is triggered by a different receptor, which then regulates TIR1/AFB activity by an as-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 occur within 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 posttranslational 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 (Figure 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 those 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 Figure 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 the 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, such as 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; Mano 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 min 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, such as 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 in 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).

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 Figure 1).

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

scanned and analyzed. Root and hypocotyl lengths were measured using AXIOVISIOLE version 4.6 software (Zeiss, 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 d under long-day conditions. Prior to treatment with auxin, the medium was replaced by fresh medium. After 4-h calibration in the fresh medium, seedlings were treated either with 10 μM 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 a 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 a 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 (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 Figure 3. For quantitative PCR reactions, SYBR Green Master Mix was used in a StepOnePlus™ system (Applied Biosystem). About 30 ng cDNA, 200 nM primers, 0.5 μM ROX (Invitrogen), 0.1 SYBR Green (Invitrogen), and 0.03 U 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 onefold. 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 performed with REST 2009 software (Pfaffl et al., 2002). The expression level at $t = 0$ was set to onefold for all lines or calculated relative to the reference gene *UBQ10*.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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