Consequences of a Deficit in Vitamin B₆ Biosynthesis de Novo for Hormone Homeostasis and Root Development in Arabidopsis¹[OPEN]

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Vitamin B₆ (pyridoxal 5'-phosphate) is an essential cofactor of many metabolic enzymes. Plants biosynthesize the vitamin de novo employing two enzymes, pyridoxine synthase1 (PDX1) and PDX2. In Arabidopsis (Arabidopsis thaliana), there are two catalytically active paralogs of PDX1 (PDX1.1 and PDX1.3) producing the vitamin at comparable rates. Since single mutants are viable but the pdx1.1 pdx1.3 double mutant is lethal, the corresponding enzymes seem redundant. However, the single mutants exhibit substantial phenotypic differences, particularly at the level of root development, with pdx1.3 being more impaired than pdx1.1. Here, we investigate the differential regulation of PDX1.1 and PDX1.3 by identifying factors involved in their disparate phenotypes. Swapped-promoter experiments clarify the presence of distinct regulatory elements in the upstream regions of both genes. Exogenous sucrose (Suc) triggers impaired ethylene production in both mutants but is more severe in pdx1.3 than in pdx1.1. Interestingly, Suc specifically represses PDX1.1 expression, accounting for the stronger vitamin B₆ deficit in pdx1.3 compared with pdx1.1. Surprisingly, Suc enhances auxin levels in pdx1.1, whereas the levels are diminished in pdx1.3. In the case of pdx1.3, the previously reported reduced meristem activity combined with the impaired ethylene and auxin levels manifest the specific root developmental defects. Moreover, it is the deficit in ethylene production and/or signaling that triggers this outcome. On the other hand, we hypothesize that it is the increased auxin content of pdx1.1 that is responsible for the root developmental defects observed therein. We conclude that PDX1.1 and PDX1.3 play partially nonredundant roles and are differentially regulated as manifested in disparate root growth impairment morphologies.

Vitamin B₆ is the collective term used to refer to a group of related water-soluble compounds, namely pyridoxine, pyridoxal, pyridoxamine, and their phosphorylated derivatives, also known as vitamers. The importance of vitamin B₆ is determined mainly by the involvement of one of its forms, pyridoxal 5'-phosphate (PLP), in amino acid, lipid, and carbohydrate metabolism as an essential cofactor in over 140 enzymatic reactions (Percudani and Peracchi, 2003; Hellmann and Mooney, 2010). Animals and humans cannot biosynthesize vitamin B₆ de novo, so they rely on dietary sources for the acquisition of the necessary amounts. Deficiency has been implicated in cardiovascular disease, diabetes, neurological disorders, carpal tunnel syndrome, premenstrual syndrome, andpellagra skin disease (Fitzpatrick et al., 2012). In contrast, plants, fungi, and microorganisms are able to biosynthesize their own vitamin B₆ employing one of two mutually exclusive pathways. The deoxyxylulose 5-phosphate (DXP)-dependent pathway, discovered in Escherichia coli, involves seven enzymes (Lam and Winkler, 1992; Zhao and Winkler, 1996; Cane et al., 1998, 1999; Laber et al., 1999) and is only present in a small subset of bacteria (Ehrenshaft et al., 1999; Mittenhuber, 2001). In all plants and fungi, as well as the rest of bacteria, a DXP-independent pathway operates (Tambasco-Studart et al., 2005), employing only two enzymes (pyridoxine synthase1 [PDX1] and PDX2; Ehrenshaft et al., 1999; Ehrenshaft and Daub, 2001; Burns et al., 2005; Raschle et al., 2005).

In Arabidopsis (Arabidopsis thaliana), there are three homologs of PDX1 (PDX1.1, PDX1.2, and PDX1.3) and a single PDX2 homolog. PDX1.1 and PDX1.3 are catalytically active in the biosynthesis of vitamin B₆ (Tambasco-Studart et al., 2005; Titz et al., 2006), whereas PDX1.2 was recently assigned a function of pseudoenzyme involved in enhancing the activity of the catalytic homologs under stress conditions (Moccand et al., 2014). The DXP-independent pathway uses Gln, ribose 5-phosphate, and glyceraldehyde 3-phosphate as precursors to produce PLP (Burns et al., 2005; Raschle et al., 2005; Strohmeier et al., 2006; Zein et al., 2006).
Mutants impaired in vitamin B₆ biosynthesis de novo have been described in Arabidopsis. Knocking out either the single PDX2 or both PDX1.1 and PDX1.3 homologs was found to be lethal, resulting in an arrest of embryo development at the globular stage (Tambasco-Studart et al., 2005; Titiz et al., 2006). On the other hand, the two single pdx1 mutants are viable (Chen and Xiong, 2005; Titiz et al., 2006; Wagner et al., 2006) but display distinctive phenotypes. Both mutants have been reported to have a short-root phenotype, which is considerably more pronounced in pdx1.3 (Titiz et al., 2006). The pdx1.3 mutant was also shown to be more sensitive to salt, osmotic, and photooxidative stresses compared with pdx1.1 (Titiz et al., 2006). Additionally, pdx1.3 mutants hemizygous for PDX1.1 display more drastic phenotypic differences compared with pdx1.1 mutants hemizygous for PDX1.3, both morphologically and developmentally (Titiz et al., 2006). This despite the fact that both proteins are 87% identical and are able to biosynthesize the vitamin at comparable rates (Tambasco-Studart et al., 2005).

While pdx1.3 was shown to be more deficient in vitamin B₆ compared with pdx1.1, a mechanistic explanation for these differences has not been provided. A study of pdx1.3 alone concluded that the stunted root growth of this mutant results from an impairment in local auxin production (Chen and Xiong, 2009a, 2009b). However, direct evidence for this conclusion was lacking. Moreover, the factors behind the root growth impairment of its paralogous knockout mutant, pdx1.1, have not been addressed. Reasons to investigate the homologs more closely derive from recent work that shows that PDX1.1 can be overexpressed while PDX1.3 cannot (Raschke et al., 2011). Indeed, a more strict regulation of the latter has been suggested, as it is a ubiquitination target (Manzano et al., 2008). In addition, data from the Arabidopsis microarray database, Genevestigator, suggest differential expression of PDX1.1 and PDX1.3 under several conditions (Hruz et al., 2008). Notably, all plants for which sequences are available carry at least two copies of PDX1 (Moccand et al., 2014). Taken together, the data suggest that the catalytic PDX1s are not completely redundant in planta and warrant further investigation.

In this study, we provide detailed comparative analyses of pdx1.1 and pdx1.3. The differential sensitivity of both mutants to Suc provides a tool to unravel the consequences of a deficit in vitamin B₆. First, an assessment of the promoter regions provides evidence for nonredundant roles of PDX1.1 and PDX1.3. In pdx1.3, impairment in ethylene production plays a critical role in stunted root growth propagated by a deficit in auxin accumulation as well as the SHORT-ROOT (SHR) transcription factor. It has been shown previously that root apical meristem activity is disturbed in pdx1.3 (Chen and Xiong, 2005), and here we also show impaired lateral root formation. PDX1.1 expression, on the other hand, is rapidly down-regulated by Suc. Interestingly, and in stark contrast to pdx1.3, auxin levels are substantially increased in pdx1.1 but confined to the root. Therefore, we conclude that PDX1.1 and PDX1.3 play nonredundant roles and that the phenotypes of the corresponding mutants are a result of differential regulation of the genes.

RESULTS

Differential Impairment of Root Growth and Development in pdx1 Mutants

This study was initiated because of the severe root growth defect of the pdx1.3 mutant and the surprising fact that root tissue demonstrates a relatively low level of expression of PDX1.3 in comparison with other tissues, according to two independent quantitative PCR (qPCR) studies (Titiz et al., 2006; Wagner et al., 2006). Furthermore, previous analyses of expression by fusion of the promoter to GUS showed comparatively high activity in leaves with much weaker activity detected in roots (Wagner et al., 2006). In this study, we have reanalyzed the expression of PDX1.3 in more detail in an attempt to explain its disparate phenotype compared with its closest paralog, PDX1.1, assumed to be redundant. Our own analysis with promoter-GUS fusions of the PDX1.3 gene in the wild-type Columbia-0 (Col-0) background corroborates the previous conclusions, although we noted that GUS expression driven by the promoter of PDX1.3 (pPDX1.3:GUS) is much stronger than that of PDX1.1 (pPDX1.1:GUS; Fig. 1A, top row). Upon weaker staining using a lower temperature, we noticed a concentration of GUS activity in the root tip and lateral root emergence areas with pPDX1.3:GUS in particular, which is not observed in pPDX1.1:GUS (Fig. 1A, bottom row). Interestingly, root growth impairment becomes more severe in pdx1.3 in the presence of Suc (Fig. 1B), while it is of benefit to the growth of pdx1.1, similar to that observed in wild-type seedlings. Nonetheless, the growth of both pdx1.1 and pdx1.3 is retarded compared with the wild type in the presence of Suc. However, growth of the seedlings in the presence of mannitol was not significantly different from the control samples, indicating that the phenotype is not a result of osmotic stress. The differential impairments in root growth are clearly discernible in a plot of primary root growth of the mutants and the wild type in the presence and absence of Suc (Fig. 1C). In addition, both pdx1.1 and pdx1.3 have reductions in root hairs compared with the wild type (Fig. 1B). Of note also is that pdx1.3 displays altered root architecture, with anchor roots (adventitious roots emerging from the hypophysis) appearing 5 d after germination (DAG; Fig. 1D), while there are none in pdx1.1 or the wild type under these conditions. In the presence of Suc, all pdx1.3 seedlings developed anchor roots, whereas in its absence, less than 20% formed such structures (Fig. 1E). The pdx1.3 mutant is also significantly impaired in the development of lateral roots, with only 25% to 30% of the seedlings developing such organs at 10 DAG, while all wild-type plants have well-developed lateral roots by this time.
Notably, the number of lateral roots per plant was normalized against the average length of the differentiation zone of the primary root, according to the recommendations of Dubrovsky and Forde (2012). In the presence of Suc, there is also lateral root growth impairment in pdx1.1, but it is less pronounced than that observed with pdx1.3 (Fig. 1E).

Evidence for Differential Regulation of PDX1.1 and PDX1.3

While pdx1.1 and pdx1.3 can be phenotypically distinguished in culture medium by their differential retardation in root growth, they both display a deficiency in total vitamin B6 content, which is especially pronounced in pdx1.3 (Titiz et al., 2006). Root growth is restored for both mutants upon supplementation with the B6 vitamer, pyridoxine (Titiz et al., 2006). In our previous work, combining qPCR and protein expression studies, we demonstrated that PDX1.3 is generally expressed at a higher level than PDX1.1 but is particularly more abundant in roots (Titiz et al., 2006). As judged from the promoter-GUS fusion lines, pPDX1.3:GUS and pPDX1.1:GUS, it becomes apparent that the higher expression level of PDX1.3 compared with PDX1.1 seems to be inherent to the promoter.
sequence (Fig. 1A). An in silico examination of the upstream regions reveals putative cis-regulatory elements, some of which are disparate between PDX1.1 and PDX1.3 (Fig. 2A). In particular, we found sequences corresponding to the ethylene-responsive element (ERE; AGCCGCC; Shinshi et al., 1995) and a partial auxin response element (AuxRE; TGTCTc; Ulmasov et al., 1997a) in the upstream region of PDX1.3, which are absent in PDX1.1. On the other hand, we noted that the upstream region of PDX1.1 harbors several abscisic acid-responsive elements (CACGT; Iwasaki et al., 1995) as well as a sugar response element (SRE; TTATCC; Tatematsu et al., 2005), which are absent from the corresponding region in PDX1.3. Potential binding sites for members of the MYB (WAACCA and CNGTTR), MYC (CANNTG; Abe et al., 2003) and WRKY (TTGAC; Yu et al., 2001) transcription factor families are observed in the upstream regions of both genes. The latter are involved in gene regulation in response to various stresses, including drought, cold, and pathogen attack. With a particular focus on the short-root phenotype and in order to study the functional relevance of the potential differential regulation of expression, we performed promoter-swap experiments. We transformed pdx1.3 with chimeras of the region (approximately 1,400 nucleotides) that lies upstream of the transcriptional start site of either PDX1.1 or PDX1.3 (pPDX1.1 and pPDX1.3, respectively) and fused to either the PDX1.3 or the PDX1.1 coding region. The corresponding lines are named pPDX1.1:PDX1.3 and pPDX1.3:PDX1.1 as well as the control line pPDX1.3:PDX1.3. We observed that while the integration of pPDX1.3 could
complement the pdx1.3 short-root phenotype to the same extent as the control line pPDX1.3:PDX1.3, pPDX1.1: PDX1.3 could not (Fig. 2B). This clearly indicates that the upstream region of PDX1.3 contains specific elements not present in the corresponding region of PDX1.1 and that these elements are responsible for coordinating the proper expression of PDX1.3.

Since both auxin and ethylene are important determinants of root growth and vitamin B₆ is required for their biosynthesis (Mooney and Hellmann, 2010; Fitzpatrick, 2011), we next focused on the involvement of these two hormones in the manifestation of the short-root phenotype. In the first instance, we noted that application of indole acetic acid (IAA) increased the expression in whole seedlings of both PDX1.1 and PDX1.3 in plants grown without Suc but only the levels of PDX1.1 in the presence of Suc (Fig. 2C). Application of the ethylene precursor, ACC, only induced the expression of PDX1.3 in the absence of Suc, albeit to a lesser extent than IAA, while both genes were induced by ACC in the presence of Suc (Fig. 2C). This prompted us to investigate the levels of these hormones in the mutant lines pdx1.1 and pdx1.3 compared with the wild type.

Auxin Levels in Roots of pdx1.1 and pdx1.3

The pdx1.3 mutant has already been investigated with regard to auxin content by Chen and Xiong (2009a, 2009b). In the latter studies, a defect in shoot-to-root transport of auxin was ruled out as an explanation for the short-root phenotype in pdx1.3, because grafting of a wild-type scion on a pdx1.3 rootstock did not rescue the short-root phenotype. Furthermore, in the same studies, the treatment of pdx1.3 harboring DR5-GUS (the artificial AuxRE derived from the natural GH3 element in soybean that reports endogenous IAA sensing/distribution; Ulmasov et al., 1997b) with IAA indicated a functional auxin response (Chen and Xiong, 2009b). Yet, impairment in local auxin biosynthesis in the root and/or transport could not be excluded. The pdx1.1 mutant was not investigated in the latter studies. Therefore, in order to compare the pdx1 mutants and probe their levels and response to auxin, a transgenic line carrying the DR5-GUS fusion gene (Col-0 background) was crossed with both pdx1.1 and pdx1.3, and homozygous mutant lines carrying the transgene were selected. While the GUS staining around the leaf perimeter (as is typically observed with the expression of this construct) was similar for lines carrying the transgene in the wild-type, pdx1.1, and pdx1.3 backgrounds, the staining in the root tip was significantly weaker for pdx1.3 compared with the wild type (Fig. 3A) and was similar to what has been reported previously (Chen and Xiong, 2009b). Surprisingly, on the other hand, the staining was considerably stronger for pdx1.1 in the root tip compared with the wild type (Fig. 3A). Quantitative GUS analysis of the entire seedling substantiated the observations for pdx1.1 in particular (Fig. 3B, right). Interestingly, quantifying the GUS activity driven by DR5 showed that the latter was significantly higher in the pdx1.1 mutant compared with the wild type only in the presence of Suc (Fig. 3B, compare left and right). To corroborate the differential observations in shoot versus root in the presence of Suc, we measured the endogenous auxin levels in the whole root versus shoot of pdx1.1 and pdx1.3 compared with the wild type using gas chromatography-mass spectrometry. Indeed, while the overall levels of IAA and its conjugates were not significantly different from the wild type in the shoot of pdx1.1 or pdx1.3, the levels of free IAA, in particular in the root of pdx1.1 and pdx1.3, were significantly stronger and weaker, respectively, when calculated on a per root basis (Fig. 3C). The latter was used because of the difference in development and size of this tissue.

Addressing pdx1.1 first, several mutants with elevated auxin levels have been studied (e.g. YUC flavin monoxygenase-like enzyme [yucca], superroot1 [sur1], and sur2) and have characteristic phenotypes comprising elongated petioles, narrow epinastic leaves and cotyledons, short roots, but more and longer root hairs (Zhao et al., 2001). The absence of a similar shoot phenotype in pdx1.1 but impaired root growth is consistent with the restriction of auxin accumulation to the root under similar conditions (Fig. 3C) but implies a different mechanism to the reported classical auxin-accumulating mutants. In the case of pdx1.3, our data would appear at the outset to support the conclusion of Chen and Xiong (2009a, 2009b) that local auxin biosynthesis in the root must be impaired. However, expression of the bacterial auxin biosynthetic gene indole acetamide hydrolase under the control of the quiescent center-specific promoter WUSCHEL-related homeobox5 did not alter the root growth of pdx1.3 (Chen and Xiong, 2009a). Therefore, an alternative explanation must be sought.

Ethylene Response Is Impaired in pdx1.1 and pdx1.3

Interactions between ethylene and sugar signaling pathways are well documented. Specifically, several studies have demonstrated that an increased ethylene response or levels can result in increased resistance to Suc and Glc (Zhou et al., 1998; Gibson et al., 2001) and that a decreased ethylene response can lead to Suc and Glc hypersensitivity during early seedling development (Zhou et al., 1998; Gibson et al., 2001). Therefore, the observation of a putative ERE in the promoter region of PDX1.3 as well as its Suc hypersensitivity is interesting. In this context, we sought to test the ethylene biosynthesis and/or responsiveness in relation to the PDX genes. In the first instance, we compared the ability of wild-type plants and pdx1.1 and pdx1.3 mutants to produce ethylene in response to the peptide derived from bacterial flagellin, flg22 (Felix et al., 1999; Zipfel et al., 2004). While both pdx1.3 and pdx1.1 produced lower amounts of ethylene compared with the wild type in the presence of Suc, that of pdx1.3 was
more impaired than that of *pdx1.1* (Fig. 4). On the other hand, in the absence of Suc, inhibition of ethylene production was less pronounced in *pdx1.3* but stronger for *pdx1.1* (Fig. 4). A repetition of the experiment with seedlings that had been grown in the presence of pyridoxine supplementation restored *flg22*-induced ethylene production/responsiveness toward wild-type levels in both *pdx1* mutants (Fig. 4). Interestingly, the same experiment with the swapped promoter lines *pPDX1.1*:*PDX1.3* and *pPDX1.3*:*PDX1.1* in the *pdx1.3* background demonstrated that the impairment in *flg22*-induced ethylene production/responsiveness by *pdx1.3* can be restored by reintroducing its own promoter sequence to control the expression of either *PDX1.1* or *PDX1.3* but not the promoter sequence of *PDX1.1* (Fig. 4). Therefore, while the *PDX1* genes are clearly required for the production and/or response to ethylene under these conditions, the deficits observed must have contributions from the *PDX1.3* promoter sequence.

**Suppression of PDX1.1 Expression by Suc**

As stated above, vitamin B6 in its form as PLP is required as a cofactor for both auxin and ethylene biosynthesis (Mooney and Hellmann, 2010; Fitzpatrick, 2011). Therefore, the lower abundance and impaired ability to produce these hormones, respectively, in *pdx1.3* in particular, may be simply linked to a stronger deficiency in the vitamin related to the fact that *PDX1.3* is more abundant than *PDX1.1* (Titiz et al., 2006). However, during the course of the studies described here, we observed that the expression of *PDX1.1* is considerably down-regulated (approximately 4- to 5-fold) when cultured in medium containing Suc compared with its absence (Fig. 5A). Interestingly, *PDX1.1* is up-regulated (approximately 2-fold) in *pdx1.3* cultured in medium that does not contain Suc (Fig. 5A). While the expression of *PDX1.3* is also slightly down-regulated in the presence of Suc, it is much less pronounced than that observed with *PDX1.1* (Fig. 5A). Given that the respective phenotypes can be rescued (albeit not completely; see below) by supplementation with pyridoxine, it would appear that the level of impairment observed in *pdx1.3* and *pdx1.1* is related to the respective deficiencies in vitamin B6 content (Titiz et al., 2006; Supplemental Fig. S1). In other words, the down-regulation of *PDX1.1* expression in the presence of Suc contributes to the strong *pdx1.3* phenotype under these conditions. However, the fact that *PDX1.1* cannot compensate for the loss of *PDX1.3*, as it does not rescue the *pdx1.3* phenotype even in the absence of Suc (Fig. 1, B and E), still reveals important differences between the two genes and lends support to divergence in their regulation.

In light of the above data and our own studies described so far, we probed the impact of the ethylene precursor, ACC, on the *pdx1* mutants. Supplementation with ACC concentrations in the 100 nm range and above inhibits wild-type root growth as reported previously (Rahman et al., 2001; Swarup et al., 2002; Fig. 5C).
However, supplementation with ACC at concentrations of 5 nM significantly increased root growth in pdx1.3 and pdx1.1 (Fig. 5B). Indeed, concentrations as low as 0.1 pm enhanced root length in pdx1.3 (Fig. 5C). The promotion of root growth by such low concentrations of ethylene is not without precedence and has been reported previously, where concentrations of 20 nM were observed to promote root elongation (Konings and Jackson, 1979). The increase in root growth was more pronounced for pdx1.3 compared with pdx1.1 or the wild type (Fig. 5C). This suggests that a deficit in ethylene production contributes to the short-root phenotype of pdx1.3. Notably, when ACC was applied in similar concentrations (i.e. 5 nM) to plants grown without Suc, it also appeared to stimulate the root growth of pdx1.3, although the effect was weaker than in the presence of Suc (Supplemental Fig. S2A).

Expression Levels of SHR Are Altered during the Early Developmental Stages of pdx1.3

It has been reported previously that pdx1.3 has reduced root apical meristem activity (Chen and Xiong, 2005). The observed differences in root morphology and root apical meristem activity of pdx1.3 in the early stages of development draw some parallels with the shr mutant (Lucas et al., 2011). SHR is a Gibberellic acid insensitive, Repressor of GA1, and SCARECROW family transcription factor involved in root patterning during the early stages of development and is essential for the function of the root apical meristem (Benfey et al., 1993; van den Berg et al., 1995; Billiou et al., 2005). This mutant has a severely shortened main root throughout its life cycle and is impaired in lateral root development, instead forming a large number of anchor roots. Anchor root development was thus suggested to be a compensatory mechanism related to the lack of primary root growth. The latter mechanism was validated in a recent study with wild-type seedlings of Arabidopsis, where removal of the bottom 2 to 3 mm of root apical meristem at 3 DAG dramatically increased anchor root development (Lucas et al., 2011).

In this context, we examined the expression level of SHR in the pdx1 mutants. Indeed, in the presence of Suc, we observed that the expression level of SHR is reduced approximately 8-fold in pdx1.3 compared with the wild type at 5 DAG, whereas it is reduced only 1.3-fold in pdx1.1 (Fig. 6A). Expression levels of SHR are also reduced in the mutant lines in the absence of Suc but are less pronounced (Supplemental Fig. S2B). However, we observed that SHR levels are increased in pdx1.1 at a later development stage (i.e. 10 DAG), while expression in pdx1.3 also increases 3-fold toward wild-type levels (Fig. 6A). It is noteworthy that this reflects a developmental time point in pdx1.3 when the rate of root growth accelerates to approach wild-type levels (Fig. 1C). We also examined the effect of 5 nM ACC on the expression of SHR in root tips. While we saw a small induction of SHR in pdx1.1 and the wild type, it was much stronger in pdx1.3 (Fig. 6B). This implies that ethylene is associated with the decrease in SHR expression that is particularly pronounced in pdx1.3 in the presence of Suc. Interestingly, in the absence of Suc, SHR expression levels in pdx1.1 and pdx1.3 are restored to wild-type levels when supplemented with ACC (Supplemental Fig. S2B).

As SHR is associated with specific influx and efflux carriers of auxin (Teale et al., 2006) and as the latter is an important mitotic signal, we monitored the expression of selected auxin transporters in the pdx1 mutants compared with the wild type. In particular, we analyzed the auxin efflux carriers PIN-FORMED3 (PIN3; At1g70940) and PIN7 (At1g23080) and the
Auxin influx carrier Auxin transporter protein1 (AUX1; At2g38120) in pooled 10- to 12-mm root tips (meristem and elongation zone) at 10 DAG. This part of the root was chosen for expression analysis because it coincided with the area where staining was observed for the DR5-GUS lines at the same age. Both PIN3 and PIN7 are undetectable in shr at this stage of development, while AUX1 is maintained (Lucas et al., 2011). In pdx1.3 (and pdx1.1, albeit to a lesser extent), the transcript level of PIN3 is significantly decreased in the presence of Suc, while that of PIN7 is not significantly changed compared with the wild type under these conditions and at this developmental stage (Fig. 6, C and D). AUX1 expression is also decreased in both pdx1.3 and pdx1.1 (Fig. 6E). Notably, application of ACC down-regulated the expression of AUX1 and PIN3 in the wild type; in contrast, the expression of both increased in pdx1.3 to approach wild-type levels (Fig. 6, C and E). On the other hand, ACC application had no significant effect on the expression of these transporters in pdx1.1 (Fig. 6, C–E). In the absence of Suc, there was no significant induction of PIN3, PIN7, or AUX1 expression by ACC in the three plant lines (Supplemental Fig. S2, C, D, and E, respectively). We compared the above expression data with data on PIN3, PIN7, and AUX1 transcript abundance derived from whole seedlings as well as entire roots of the same plants (Supplemental Fig. S3). A decrease in AUX1 expression can be observed in entire roots in the presence of Suc as well as whole seedlings, but the decrease is negligible in the latter (Supplemental Fig. S3, A and B). The decrease in AUX1 expression in pdx1.1 appears to be independent of Suc. In the case of PIN3, changes can also be observed in these tissues and are affected by Suc but are much less pronounced than those observed in root tips. There are no significant changes in PIN7 expression in all tissues examined. Therefore, we conclude that the expression of these transporters is principally affected in the root tips of pdx1.1 and pdx1.3.

Addressing Anthocyanin Accumulation in pdx1.3

Ethylene inhibits anthocyanin accumulation induced by Suc and light (Jeong et al., 2010). This occurs through the suppression of expression of the transcription factors that positively regulate anthocyanin biosynthesis, such as GLABRA3 (GL3), TRANSPARENT TESTA8 (TT8), and PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) and PAP2, while stimulating the expression of the negative R3-MYB regulator MYBL2 (Jeong et al., 2010). We observed hyperaccumulation of anthocyanins in pdx1.3 in the presence of Suc compared with either the wild type or pdx1.1 (Fig. 7A). The impairment in ethylene biosynthesis/signaling observed in pdx1.3 in this study could thereby explain the enhanced accumulation of anthocyanins, as the negative regulation of anthocyanin biosynthesis is likely to be affected in this mutant. Indeed, upon examination of the global transcriptome effect of knocking out pdx1.3, through whole-genome RNA expression analyses performed using the Affymetrix ATH1 DNA microarray (see below), we observed that PAPI is up-regulated.
5.49-fold in shoots of *pdx1.3* (Supplemental Table S1). PAP2 is up-regulated 13.51-fold in shoots of *pdx1.3* but did not pass our statistical analysis for filtering differential expression. However, differential expression of *PAP1* and *PAP2* was confirmed by qPCR analysis (Fig. 7B). The gene encoding dihydroflavonol reductase (*At5g42800*) was also up-regulated in *pdx1.3* leaves compared with the wild type (4.52-fold). The latter is considered to be a rate-limiting step (Das et al., 2012) late in anthocyanin biosynthesis and is a target of PAP1 and PAP2 (Yan et al., 2005). On the other hand, no significant changes in the expression of *GL3*, *TT8*, or *MYBL2* were observed compared with the wild type in the microarray. Ethylene-mediated suppression of anthocyanin accumulation is dependent upon ethylene signaling components responsible for the triple response (Jeong et al., 2010). In this context, we noted that expression of the ethylene receptor *ETR2* (*At3g23150*) is down-regulated 3.3-fold in *pdx1.3* shoots compared with the wild type (Supplemental Table S1). Therefore, *pdx1.3* is not only impaired in ethylene biosynthesis but most likely also in the propagation of its signaling. The latter statement is corroborated by the fact that pyridoxine supplementation does not completely rescue the short-root phenotype, as would be expected if the phenotype is solely a consequence of a deficit in cofactor requirements (i.e. that compromises ethylene biosynthesis). Moreover, anthocyanin accumulation is observed both in the presence and absence of pyridoxine supplementation, implying a signaling event in addition to a cofactor deficit (Fig. 7A).

**Figure 6.** The altered expression levels of *SHR* and selected auxin transporters is partially rescued by application of ACC. A, *SHR* transcript abundance in roots of wild-type (WT) Col-0, *pdx1.1*, and *pdx1.3* seedlings grown in the presence of 1% (w/v) Suc at either 5 or 10 DAG. The data represent means from at least three biological replicates. Error bars represent se. Statistically significant differences (*P* < 0.05) between plants at different ages compared with the wild type are indicated with asterisks (*, 5 DAG; **, 10 DAG). B, Relative expression of *SHR* in root tips (10–12 mm) of seedlings grown in the presence of Suc for 10 DAG and in the absence or presence of 5 mM ACC. The data represent means from at least three biological replicates. Error bars represent se. Statistically significant differences (*P* < 0.05) of treatment groups compared with nontreated controls are indicated by asterisks. C to E, Transcript abundance of *PIN3* (C), *PIN7* (D), and *AUX1* (E) in root tips (10–12 mm) grown on medium containing 1% (w/v) Suc and in the presence or absence of 5 mM ACC. The expression data for each gene were normalized against GAPDH (*At1g13440*). The data represent means from at least three biological replicates. Error bars represent se. Statistically significant differences (*P* < 0.05) between wild-type and mutant lines with (**) and without (*) ACC treatment are indicated.

**Global Transcriptome Effect of *pdx1.3***

As *pdx1.3* is more severely affected than *pdx1.1* in Arabidopsis, a whole-genome RNA expression analyses was performed on *pdx1.3* using the Affymetrix ATH1 DNA microarray in order to ascertain the global transcriptome effect. Total RNAs were extracted from the separated roots and shoots of 10-d-old seedlings of all lines grown in the presence of 1% (w/v) Suc. Significantly differentially regulated genes were clustered according to their transcriptional behavior after statistical analyses (see “Materials and Methods”). A group of 538 and 317 genes were found to be either up-regulated or down-regulated, respectively, in leaves of *pdx1.3* compared with the wild type (Supplemental Table S1). The number of genes being significantly altered in the root was substantially lower, with 156 and 162 genes being either up-regulated or down-regulated, respectively (Supplemental Table S1). A comparison of the functional distribution of altered genes according to the Gene Ontology biological process annotation tool at
The Arabidopsis Information Resource (http://www.arabidopsis.org/tools/bulk/go/) indicates a significant enrichment of genes involved in metabolism and stress response in both leaves and roots (Supplemental Fig. S4). In the context of this study, it is notable that several genes involved in ethylene biosynthesis (amino-cyclopropane carboxylate synthase [ACS7; At4g26200], ACS8 [At4g37770], ACS11 [At4g08040], and ACC oxidase [At1g62380]) and signaling (ETR2 mentioned above and Ethylene response factor73 [ERF73; At1g72360]) were found to be down-regulated in shoots, while ERF B-2 (At5g61590) was found to be down-regulated in roots. Genes implicated in auxin signaling are also down-regulated, among which are the auxin-regulated gene involved in organ size (At3g59900), Small auxin-upregulated72 [SAUR72; At3g12830], and YUCCA (At5g43890) in leaves as well as SAUR76 (At5g20820) in roots. Many of the differentially expressed genes are of unknown function and may be noteworthy for future studies. In this context, it is interesting that the triterpene synthase, marneral synthase, is the most up-regulated transcript in pdx1.3 roots (118-fold) and that the related thalianol synthase is up-regulated as well (3-fold). Over-expression of these genes causes a dwarf phenotype (Field and Osbourn, 2008; Field et al., 2011). However, the exact role of these molecules in Arabidopsis has not yet been elucidated.

**DISCUSSION**

In this study, we report on the incomplete redundancy of two genes involved in vitamin B6 biosynthesis de novo in Arabidopsis. The study provides important information on the individual role these genes play and their significance toward maintaining sufficient levels of the phytohormones, ethylene and auxin, and the downstream effects that this has on plant development with a particular focus on the root. The possibility for differential regulation of PDX1.1 and PDX1.3 genes addressed in this work was initially suggested by two earlier studies. The first study, by Titiz et al. (2006), showed that, in spite of the ability of both enzymes to biosynthesize vitamin B6 at comparable rates (at least in vitro), the two respective knockout mutants displayed distinct phenotypes. Moreover, mutants carrying a single functional copy of either PDX1.1 or PDX1.3 were drastically different, with pdx1.3 mutant plant lines hemizygous for PDX1.1 more severely impaired in development than pdx1.1 mutant plant lines hemizygous for PDX1.3 (Titiz et al., 2006). A second study, describing the overproduction of vitamin B6 in Arabidopsis (Raschke et al., 2011), demonstrated that while both PDX1.1 and PDX1.3 could be overexpressed at the transcript level, only the PDX1.1 paralog could be increased at the protein level. The fact that PDX1.3 had been reported to be a ubiquitination target (Manzano et al., 2008) led to the postulation of a more stringent regulation of this paralog and suggested incomplete redundancy of the PDX1 genes.

Suc can serve as a carbon source, cause osmotic stress (depending on its concentration), or act as a signaling molecule. According to a recent article (Kircher and Schoepfer, 2012), sugar produced by newly established photosynthesis during early seedling development is sufficient for the regulation of root elongation in light. Therefore, Suc supplied through the growth medium is beneficial for the root and subsequently for the growth of the entire seedling, since a larger root provides better...
access to nutrients from the medium. While this proves to be true for wild-type and *pdx1.1* seedlings in this study, the growth of *pdx1.3* mutant seedlings is severely impaired by the presence of Suc in the culture medium. The stunted growth of the main root was combined with altered morphology, fewer lateral roots, and the appearance of adventitious (anchor) roots, known to serve as a compensatory mechanism when the main root is damaged or missing (Lucas et al., 2011). Features revealed in this study can explain the rather pleiotropic phenotype observed. First, we noted that *PDX1.1* expression was down-regulated in the presence of Suc within 30 min (Supplemental Fig. S5). This may be explained by the presence of a sequence (TAACAAA) assigned as a sugar response element (Morita et al., 1998) immediately upstream of *PDX1.1* (Fig. 2A). Therefore, the *pdx1.3* phenotype in the presence of Suc is exaggerated by the additional depletion of *PDX1.1*. The consequential deficiency of vitamin B_{6} leads to reduced amounts of two phytohormones, auxin and ethylene, both of which require the vitamin as a cofactor for their biosynthesis. Several pathways are implicated in auxin biosynthesis (for review, see Korasick et al., 2013), comprising many aminotransferases and decarboxylases, all of which require PLP (the cofactor form of vitamin B_{6}) for catalysis. Likewise, ACC synthase, which is necessary for ethylene biosynthesis, is dependent on PLP for activity (Capitani et al., 1999). The severe depletion in auxin levels in the *pdx1.3* mutant could be shown by direct measurements using mass spectrometry as well as indirectly through the use of the synthetic DR5 reporter (Fig. 3).

The role of auxin as a morphogen and the associated generation of local auxin maxima to promote organogenesis have been studied comprehensively (Benková et al., 2003; Heisler et al., 2005; Cederholm et al., 2012). This is exemplified in the regulation of the elaborate network of efflux and influx carriers. As examples, the efflux carriers PIN3 and PIN7 and the influx carrier AUX1 are generally down- and up-regulated, respectively, at the site of lateral root primordia, promoting local auxin accumulation that drives lateral root formation (Lewis et al., 2011). In this study, we observed a down-regulation of *AUX1* and *PIN3* expression in root tips, which, in the case of *pdx1.3*, is likely due to deficits in the gradient of auxin accumulation. It is noteworthy that auxin transport is also modulated by anthocyanins (Besseau et al., 2007), which are elevated in *pdx1.3* in the presence of Suc and also negatively affect auxin distribution. As a consequence, both primary root and lateral root formation are impaired. The observation that *PDX1.3* is highly expressed in the root tip stele region that undergoes rapid elongation and division as well as at the site of lateral root emergence (Fig. 1A) is consistent with this hypothesis. The importance of a gradient of auxin distribution for the establishment and maintenance of the root apical meristem, which is also impaired in *pdx1.3*, has been discussed previously (Chen and Xiong, 2009a, 2009b). We also noted here that root growth impairment in *pdx1.3* draws some parallels with the *shr* mutant (Lucas et al., 2011). As *SHR* plays a role in maintaining the root apical meristem (Blilou et al., 2005), we investigated its expression in *pdx1.3* and could show that there is a severe deficit in *SHR* expression during the early stages of root development.

However, it is known that a reduction in ethylene biosynthesis leads to a reduction in auxin biosynthesis, particularly at the tip of the primary root (Stepanova et al., 2005). More explicitly, the effects of ethylene on root tip growth, or its modulation, are thought to lie upstream of the observed effects in auxin biosynthesis and transport (Ruzicka et al., 2007). The process depends on the ethylene-signaling pathway, because impaired ethylene perception prevents the accumulation of auxin in the root tip (Ruzicka et al., 2007). Flagellin-induced ethylene production is strongly repressed in *pdx1.3* (Fig. 4). Significantly for this study, application of the ethylene precursor ACC increases the level and redistribution of auxin, as judged from *pdx1.3* harboring the DR5-GUS fusion (Supplemental Fig. S6). Moreover, it increases *SHR* expression and the deficit in auxin transport expression (Fig. 6, B, C, and E) and, at least partially, rescues root growth (Fig. 5, B and C). In contrast, previous studies on *pdx1.3* have shown that the application of auxin or the specific induction of auxin biosynthesis genes in the root meristem did not rescue root growth (Chen and Xiong, 2009a, 2009b). Therefore, we conclude that the principal defect in postembryonic root growth of *pdx1.3* as a consequence of vitamin B_{6} deficiency is impairment in ethylene metabolism. It can be reiterated here that ACC synthase is not only dependent on the B_{6} vitamin PLP as a cofactor but is a rate-limiting enzyme for ethylene biosynthesis. As a consequence of ethylene misregulation, the downstream effects are manifested in decreased auxin biosynthesis and impairment in its distribution as well as reduced levels of *SHR*. Notably, *SHR* expression levels also can be rescued in *pdx1.3* by the application of vitamin B_{6} (Supplemental Fig. S7A). In addition, expression of *PDX1.3* is not altered significantly in the *shr2* mutant (Levesque et al., 2006) compared with the wild type (Supplemental Fig. S7B), and none of the PDX genes have been identified as direct or indirect targets of *SHR* in recent studies (Levesque et al., 2006; Cui et al., 2014). Therefore, impairment in *SHR* expression levels in this case is a consequence of the mutation in *pdx1.3*. Interestingly, levels of *SHR* expression are restored in the noncomplemented *pdx1.3* line carrying *pPDX1.1::PDX1.3* by application of vitamin B_{6} (Supplemental Fig. S7A). The latter reemphasizes the importance of the promoter region of *PDX1.3* to the regulation of these vitamin B_{6}-related parameters. Of note is that we mutated the putative ERE (AGCCGCC to ATCCCTC; Fujimoto et al., 2000) and partial AuxRE (TGACTc to TGAATc; Ulmasov et al., 1997b) in the promoter region of *PDX1.3* fused to the *PDX1.3* coding sequence by site-directed mutagenesis and retransformed *pdx1.3* to assess for complementation. Lines homozygous for these mutations were analyzed for the rescue of root growth. In almost all cases (six independent lines mutated in the ERE and four in the AuxRE), root growth was still increased compared with the *pdx1.3* mutant. However, a comparison
of pPDX1.3:pDX1.3 in the pdx1.3 mutant background with the mutated promoter lines indicates that similar root growth is only observed upon enhanced expression of PDX1.3 over that observed in pPDX1.3:pDX1.3 (Supplemental Fig. S8). Therefore, the ERE and AuxRE may contribute (albeit weakly) to the regulation of PDX1.3 expression. The other promoter regions involved in the regulation of PDX1.3 expression remain to be dissected in future experiments.

In pdx1.1, it is intriguing that the elevated auxin content in the presence of Suc is restricted to the root and, similar to other auxin overproduction mutants (Zhao et al., 2001), root growth is impaired. However, in contrast to other auxin overproduction mutants, we do not see a hairy-root phenotype (Fig. 1B). We also do not see epinastic cotyledons, increased apical dominance, or curled leaves, consistent with the restriction of the elevation in auxin content to root tissue. Nonetheless, it is not clear what is triggering elevated auxin production in pdx1.1. Some recent studies have highlighted the influence of Suc on auxin accumulation during seedling development, particularly in the root (Lilley et al., 2012; Sairanen et al., 2012). Specifically, Suc triggers an endogenous carbon-sensing pathway that results in higher free auxin levels and increased rootward auxin transport, dependent, at least in part, on the PHYTOCHROME-INTERACTING FACTOR (PIF) proteins (Lilley et al., 2012). This relay may be misregulated in pdx1.1, such that there is hyperaccumulation of auxin. It is also noteworthy in this context that PDX1.1, but not PDX1.3, expression is induced by IAA (Fig. 2C). Moreover, we have noted that PIF4 expression is up-regulated in pdx1.3 (Supplemental Table S1) in the presence of Suc (conditions where pdx1.1 is also down-regulated), providing support that there may be cross-talk between the PDX1 and PIF genes. We hypothesize that PDX1.1 must be required to prevent the overaccumulation of auxin in the presence of Suc. Whether this is a direct or indirect effect related to the presence of a sugar response element in the promoter region of PDX1.1 remains to be determined and provides a fertile area for future studies.

In summary, the PDX1.1 and PDX1.3 genes are required for postembryonic root development in Arabidopsis. Disruption of either of these genes results in a differential cascade of events as a consequence of vitamin B₆ deficiency, which, in the case of pdx1.3, is propagated by a deficit in ethylene production, followed by a dissipation of the auxin gradient in the root tip leading to reduced cell division and expansion. Root growth can be partially rescued in pdx1.3 by the application of the ethylene precursor ACC correcting the cascade of events. PDX1.1 expression is repressed by Suc, and we hypothesize that it plays a role in auxin homeostasis. We conclude that while the biochemical roles of PDX1.1 and PDX1.3 overlap, they are regulated differently and, in turn, influence phytohormone homeostasis. A model summarizing the events as unraveled in this study is depicted in Figure 8. It remains to be explored in future studies what influence the availability of PLP as a cofactor and the vitamin B₆ salvage pathway have on the overall homeostasis of this essential nutrient. A short-root phenotype and Suc sensitivity have been reported for mutants of the salvage pathway enzymes PDX3 and salt overly sensitive4 (Shi and Zhu, 2012). This relay may be misregulated in pdx1.1, such that there is hyperaccumulation of auxin. It is also noteworthy in this context that PDX1.1, but not PDX1.3, expression is induced by IAA (Fig. 2C). Moreover, we have noted that PIF4 expression is up-regulated in pdx1.3 (Supplemental Table S1) in the presence of Suc (conditions where pdx1.1 is also down-regulated), providing support that there may be cross-talk between the PDX1 and PIF genes. We hypothesize that PDX1.1 must be required to prevent the overaccumulation of auxin in the presence of Suc. Whether this is a direct or indirect effect related to the presence of a sugar response element in the promoter region of PDX1.1 remains to be determined and provides a fertile area for future studies.

In summary, the PDX1.1 and PDX1.3 genes are required for postembryonic root development in Arabidopsis. Disruption of either of these genes results in a differential cascade of events as a consequence of vitamin B₆ deficiency, which, in the case of pdx1.3, is propagated by a deficit in ethylene production, followed by a dissipation of the auxin gradient in the root tip leading to reduced cell division and expansion. Root growth can be partially rescued in pdx1.3 by the application of the ethylene precursor ACC correcting the cascade of events. PDX1.1 expression is repressed by Suc, and we hypothesize that it plays a role in auxin homeostasis. We conclude that while the biochemical roles of PDX1.1 and PDX1.3 overlap, they are regulated differently and, in turn, influence phytohormone homeostasis. A model summarizing the events as unraveled in this study is depicted in Figure 8. It remains to be explored in future studies what influence the availability of PLP as a cofactor and the vitamin B₆ salvage pathway have on the overall homeostasis of this essential nutrient. A short-root phenotype and Suc sensitivity have been reported for mutants of the salvage pathway enzymes PDX3 and salt overly sensitive4 (Shi and Zhu,
MATERIALS AND METHODS

Plant Material

All Arabidopsis (Arabidopsis thaliana) lines (wild-type Col-0, pdx1.1, and pdx1.3 [Titiz et al., 2006], schl-2 [Beney et al., 1995], Levesque et al., 2006)] as well as the constructed transgenic lines [see below] used for the described experiments were grown either on soil or in culture (as indicated) under long-day conditions (i.e. a 16-h photoperiod at 100–120 μmol photons m⁻² s⁻¹ at 22°C and 8 h in the dark at 18°C). Seedlings grown in culture were cultivated on one-half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 0.8% (w/v) agar in the presence or absence of 1% (w/v) Suc either with or without pyridoxine supplementation (5 μM) as indicated. Other treatments were performed with manitol (100 μM), IAA (1 μM), or ACC (5 μM or 1 μM), where specified, were present in the culture medium from the start of the respective experiments.

Root Growth Measurements

Arabidopsis seedlings grown on sterile medium on vertically oriented plates under the conditions described above were used to determine the root length, the root growth curves, and the number of lateral roots in the presence or absence of Suc or after ACC supplementation. Plants were photographed starting at day 1 after germination until 10 DAG, and the root length was subsequently measured using ImageJ software (http://image.jh.gov.nih.gov/). The root length and lateral root number data are from at least two independent experiments with at least 20 technical repetitions, and error bars represent SE. Statistical analyses were performed using the ANOVA with Tukey’s test.

Construction of Transgenic Lines

For the construction of the swapped promoter lines, the coding sequences of PDX1.1 and PDX1.3 were amplified from Arabidopsis genomic DNA using the following primer pairs: 5’-CTTCTTCTTACAGAAGGTCCCTTGCGGTC-3’ and 3’-GAAATTC-CTCTCTCTCTCACTTATTAC-5’. After amplification, the primers were used for site-directed mutagenesis with the following primers: 5’-CTTCTTCTTACAGAAGGTCCCTTGCGGTC-3’ and 3’-GAAATTC-CTCTCTCTCTCACTTATTAC-5’ for mutating the ERE (AGCCCCGC-6’ to ATCTCCCT; Fujimoto et al., 2000) and 5’-GCTAACATTCTTCTTACCTTATTATTTGAGAAGGTCG-3’ and 3’-CCCGAATTTTGTGACAAATATTGCGG-5’ for mutating the AuxRE (TGCTTc to TGATAc Ulmasov et al., 1997b), using pdX1.3: PDX1.3 as a template. For the promoter-GUS fusion lines, the regions immediately upstream of the start codon of either PDX1.1 or PDX1.3, amplified using the primer pairs described above, were cloned into the pCAMBIA 1201Z vector using the EcoRI and NotI restriction sites (http://www.cambia.org). In all cases, the obtained constructs were transferred into Agrobacterium tumefaciens strain C58, which was used to transform Arabidopsis (Col-0) by the floral dip method (Clough and Bent, 1998). Transformants were selected on the appropriate antibiotic, and only those lines that were homozygous and carrying a single insertion of the respective transgene were used for further analyses. Seeds harboring the DRS-GUS fusion construct originally described by Ulmasov et al. (1997b) were obtained from Jiri Friml, and progeny were crossed with the homozygous knockout mutant lines pdx1.1 and pdx1.3 (Titiz et al., 2006).

Histochemical GUS Expression Analyses

Arabidopsis seedlings grown on one-half-strength MS medium (Murashige and Skoog, 1962) containing 1% (w/v) Suc with or without pyridoxine supplementation for 10 to 12 DAG were harvested into 90% (v/v) acetone on ice and then incubated for 20 min at room temperature. The samples were rinsed three times with 10 mM NaH₂PO₄/Na₂HPO₄, pH 7, containing 0.5 mM K₃[Fe(CN)₄], 0.5 mM K₃[Fe(CN)₆], 0.1% (w/v) Triton X-100, and 10 mM EDTA·Na₂ and then incubated at 37°C in the same solution containing 0.5 mg mL⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. The incubation was done either for 2 or 3 h or overnight depending on the intensity. The excess stain and chlorophyll from the samples were removed by several rinses with 70% (v/v) ethanol. Samples were kept in 70% ethanol until examination.

Quantitative GUS Expression Analyses

Seedlings (approximately 100 mg of tissue) grown as for the histochemical analyses above either in the absence or presence of 1% (w/v) Suc were harvested and immediately frozen in liquid nitrogen. The harvested samples were ground in 50 mM NaH₂PO₄/Na₂HPO₄, pH 7, containing 50 mM EDTA·Na₂, 0.1% (w/v) Triton X-100, 0.1% (w/v) SDS, 0.025 mg mL⁻¹ phenylmethanesulfonyl fluoride, and 10 mM β-mercaptoethanol (extraction buffer). The extract was centrifuged for 10 min at 13,200 × g, and the supernatant was collected and used for further analyses. The extraction buffer containing 1 mM 4-methylumbelliferonyl-p-D-glucuronide was heated to 37°C (reaction buffer). For each sample to be analyzed, 20 μL of plant extract was added to 1 mL of reaction buffer. After 10 min of incubation, 100 μL of 1% (v/v) acetonitrile volume was added to a fresh tube containing 900 μL of stop solution (0.2 mM Na₂CO₃), and the fluorescence of the product of the reaction 4-methylumbelliferone was followed at excitation and emission wavelengths of 365 and 455 nm, respectively. The results were derived from a standard curve prepared with serial dilutions of the product. Statistical analyses were performed using the ANOVA with Tukey’s test. The results are means of three independent experiments, and error bars represent SE.

Free and Conjugated Auxin Analyses

The method used for auxin analysis was described by Penick et al. (2009). Plant material frozen with liquid nitrogen was ground with a pestle and mortar, and samples of 25 mg average weight were extracted with phosphate buffer (pH 7). The extracts were subjected to a C8-based solid-phase extraction, methylated with ethereal diazomethane, and subsequently purified by immunofluorimmunoassay. The final analysis was done by ultra-HPLC coupled to tandem mass detection.

RNA Extraction, Complementary DNA Synthesis, and qPCR Analysis

Arabidopsis seedlings were grown in culture for 10 DAG on one-half-strength MS medium with or without 1% (w/v) Suc and in the presence or absence of supplementation as indicated. Total RNA was extracted from seedling samples or separated shoots and roots using the NucleoSpin RNA kit.
from Macherey-Nagel and was reverse transcribed with SuperScript II RNase H−, using oligo(dT) primers 12 to 18 according to the manufacturer’s recommendations (Invitrogen), using 2 μg of total RNA and diluted five times. qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using 3 μL of the diluted complementary DNA samples and SYBR Green as a fluorescence detector (Power SYBR Green PCR master mix; Applied Biosystems) in accordance with the manufacturer’s instructions. Gene-specific primer pairs are listed in Supplemental Table S2. The GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH) gene was used as a reference to calculate the relative expression levels of the genes under investigation, as its transcript levels did not vary by more than one cycle threshold between treatments. All qPCR values represent three biological replicates with three technical repeats. The ANOVA Tukey or Bonferroni test was used for statistical analysis of the data together with pairwise comparisons of the mean values. The results are means of at least three independent experiments with error bars representing SE.

Microarray Analyses

Arabidopsis seedlings were grown in culture on one-half-strength MS medium (Murashige and Skoog, 1962) containing 1% (w/v) Suc without pyridoxine supplementation. Plants were cultivated under a 16-h photoperiod at 100 μmol photon s−1·m−2 at a temperature of 18°C in the dark. Roots and shoots from the wild type and pdx1.3 (three biological replicates for each line and for each type of tissue) were harvested 10 DAG, and total RNA was extracted using the Macherey-Nagel Nucleic Acid and Protein Purification kit, following the manufacturer’s instructions. The quality of the samples was checked using chip electrophoresis (Bioanalyzer 2100; Agilent). Subsequent biotin-labeled copy RNA synthesis (using 500 ng of total RNA) was done using Ambion MessageAmp II according to the manufacturer’s protocol. Hybridization of the fragmented copy RNA target to GeneChip ATH1 (22,810 probe sets), washing, labeling, and scanning were done according to Affymetrix instructions. The MAS 5.0 algorithm was used for signal expression computation and normalization. To identify differentially expressed transcripts, pairwise comparisons analyses were carried out. Each experimental sample was compared with each reference sample, resulting in nine pairwise comparisons. Transcripts were considered differentially expressed if their levels changed in the same direction in seven of nine comparisons. Further data filtering and analyses were performed with the GeneSpring software (Agilent). Only genes displaying at least 2-fold up- or down-regulation in the pdx1.3 mutant versus the wild type were considered further. Additional statistical analyses were performed using the Welch test and Student’s t test.

Ethylene Measurements

The flg22 peptide (corresponding to the sequence of Xanthomonas axonopodis citri, which was fully active to stimulate the pattern-triggered immunity response in Arabidopsis) was obtained from Peptron. Prior to use, the peptide was dissolved in water (stock solutions of 1–10 μM) and diluted to a final concentration of 10 μM in a solution containing 1% (v/v) bovine serum albumin and 0.1 M NaCl. For assaying ethylene production, entire seedlings grown for 10 d in sterile culture with and without 1 μM flg22 were collected (totaling 20 mg of fresh weight per assay) and transferred to 6-mL glass tubes containing 1 mL of water and the elicitor preparation to be tested. The tubes were closed with rubber septa, and ethylene accumulating in the free air space was measured by gas chromatography after 4 h of incubation. The average of 15 different samples was determined. Samples prepared in an identical fashion were treated with a solution containing only 0.1% (w/v) bovine serum albumin and 0.1 M NaCl (mock treatment) to serve as a control, and ethylene production was assayed in parallel. Statistical analyses were performed using the ANOVA with Tukey’s test. The results are means of three independent experiments, and error bars represent SE.

Sequence data from this study can be found in the GenBank/EMBL databases under the following accession numbers: PDX1.1, At2g38230; PDX1.3, At5g01410; AUX1, At2g38120; PIN3, At1g70940; PIN7, At1g23080; GAPDH, At1g13440; PAP1, At1g56650; PAP2, At1g66390; and SHR, At1g37650.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Total B6 vitamer levels in Arabidopsis shoots and roots.

Supplemental Figure S2. Effect of ACC application in the absence of Suc.

Supplemental Figure S3. Relative expression level of polar auxin transporters.

Supplemental Figure S4. Functional categorization of altered expression in pdx1.3.

Supplemental Figure S5. Suc-induced changes in PDX1.1 and PDX1.3 expression.

Supplemental Figure S6. Effect of ACC on DR5 expression.

Supplemental Figure S7. SHR expression in pdx1 lines and PDX1.3 expression in shr-2.

Supplemental Figure S8. Analysis of mutated PDX1.3 promoter lines.

Supplemental Table S1. Microarray analysis of pdx1.3.

Supplemental Table S2. qPCR primers used.

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