Consequences of a Deficit in Vitamin B_6 Biosynthesis de Novo for Hormone Homeostasis and Root Development in Arabidopsis[1][OPEN]

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Vitamin B_6 (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 B_6 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 B_6 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 B_6 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 B_6 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, and pellagra skin disease (Fitzpatrick et al., 2012). In contrast, plants, fungi, and microorganisms are able to biosynthesize their own vitamin B_6 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 B_6 (Tambasco-Studart et al., 2005; Titiz 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).

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Mutants impaired in vitamin B_6 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 B_6 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 B_6. 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 SHORTROOT (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

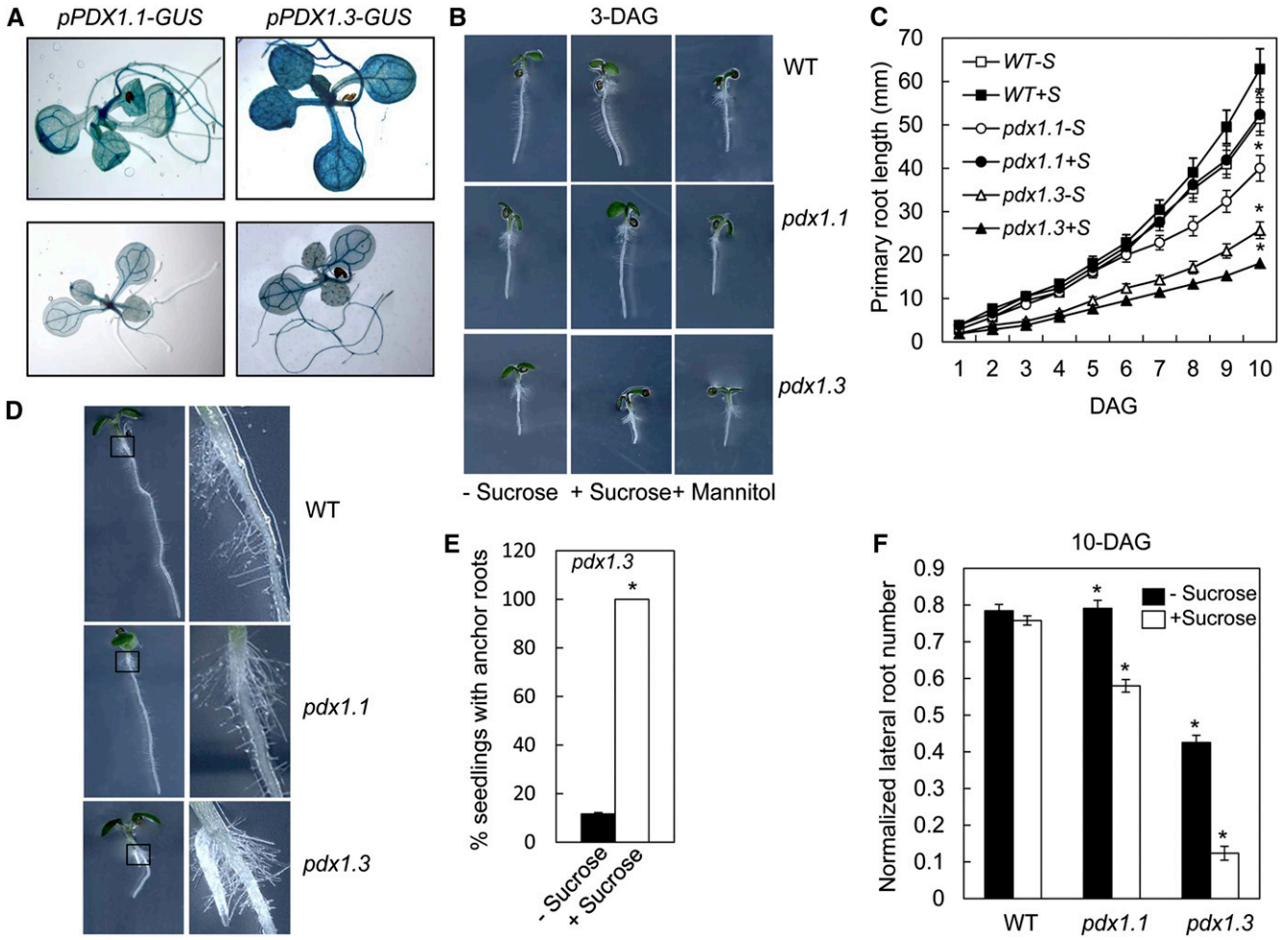

Figure 1. Gross divergence in the morphology of *pdx1.1* and *pdx1.3*. A, Staining of the promoter-GUS fusion lines *pPDX1.1*:*GUS* and *pPDX1.3*:*GUS* at 37°C (top row) and 25°C (bottom row) in the Col-0 background. B, Root growth at 3 DAG of *pdx1.1* and *pdx1.3* plants grown in the presence of 1% (w/v) Suc compared with wild-type (WT) Col-0; *pdx1.3* seedlings grown in the presence of the osmoticum mannitol (1%) are also shown. C, Kinetics of root growth of *pdx1.1* and *pdx1.3* seedlings in the presence or absence of Suc (S) compared with the wild type. The data are from three biological repetitions. Error bars indicate SE. Asterisks indicate statistically significant differences ($P < 0.001$) in comparison with the wild type grown on Suc. D, Morphology of *pdx1.1* and *pdx1.3* compared with the wild type at 5 DAG. Adventitious roots can be seen in *pdx1.3*. The black squares outline the areas magnified on the right. E, Number of *pdx1.3* seedlings that develop anchor roots in the presence or absence of Suc. The data are derived from three independent experiments. Values display statistically significant differences for $P < 0.001$. F, Number of lateral roots normalized against the length of the branching zone for each plant. Plants were grown until 10 DAG in the presence or absence of Suc. The data are from three biological repetitions. Error bars indicate SE. Asterisks indicate statistically significant differences ($P < 0.001$) when compared with the wild type.

(Fig. 1E). 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 B_6 content, which is especially pronounced in *pdx1.3* (Titiz et al., 2006). Root growth is restored for both mutants upon supplementation with the B_6 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 (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*:*PDX1.1* could

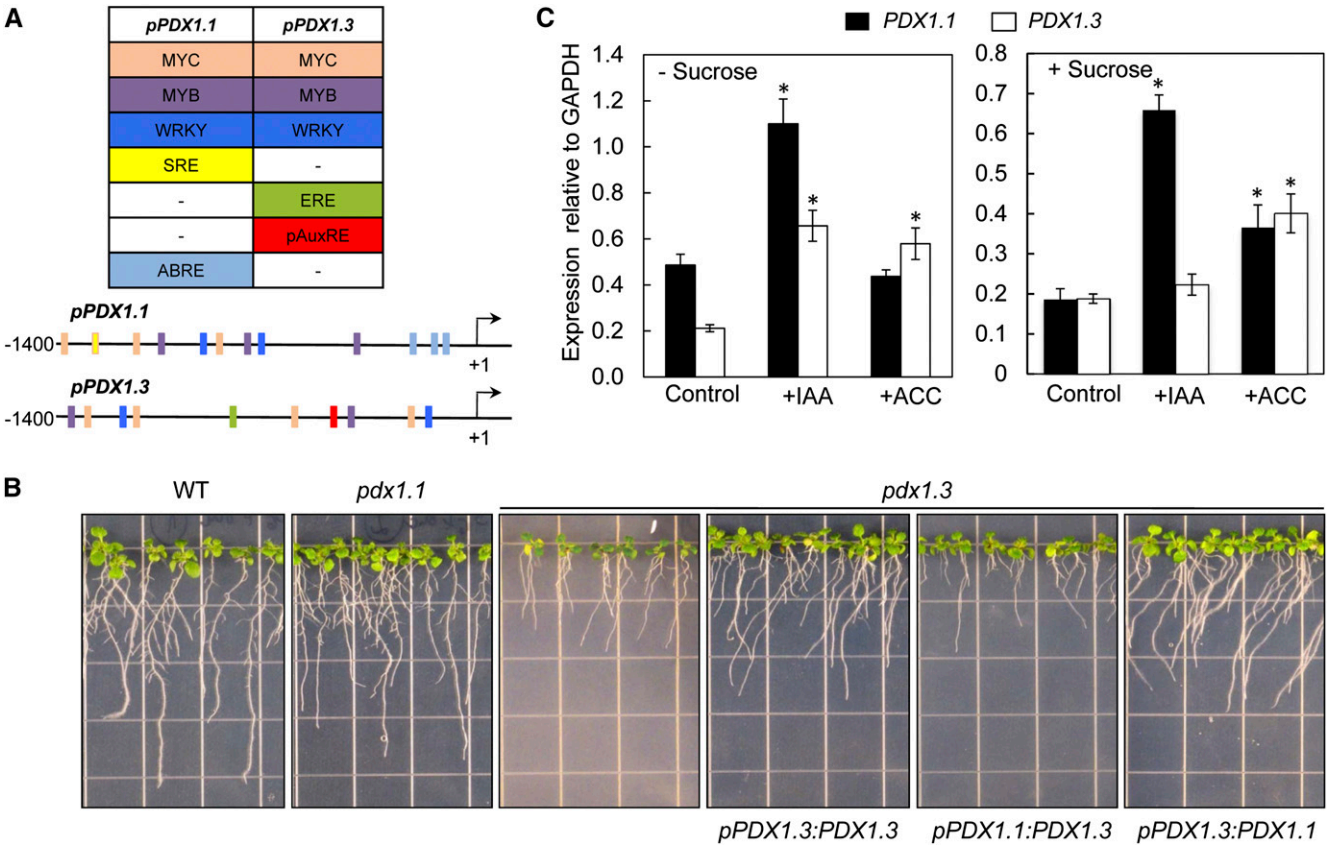

Figure 2. Evidence for differential regulation of *PDX1.1* compared with *PDX1.3*. A, The scheme depicts elements found in the upstream regions of *PDX1.1* (*pPDX1.1*) and *PDX1.3* (*pPDX1.3*). WRKY, MYB, and MYC correspond to elements recognized by the respective transcription factors. ABRE, Abscisic acid response element; pAuxRE, partial AuxRE; SRE, sugar response element; +1, transcriptional start site. B, Control of the expression of *PDX1.3* by the upstream region of *PDX1.1* cannot complement the impaired root growth of *pdx1.3*. Seedlings of wild-type (WT) Col-0, *pdx1.1*, and *pdx1.3* are compared with the *pdx1.3* line carrying swapped promoters: the upstream region of *PDX1.1* fused to *PDX1.3* (*pPDX1.1*:*PDX1.3*), the upstream region of *PDX1.3* fused to *PDX1.1* (*pPDX1.3*:*PDX1.1*), and the upstream region of *PDX1.3* fused to *PDX1.3* (*pPDX1.3*:*PDX1.3*) as a control. Seedlings were grown vertically in the presence of 1% (w/v) Suc. Images were captured at 10 DAG. C, Relative expression levels of *PDX1.1* and *PDX1.3* in Col-0 control plants (whole seedlings) after treatment with IAA and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence or absence of Suc in the growth medium. Values are relative to GAPDH (At1g13440). The data are from at least three biological repetitions. Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.05$) when treatments are compared with the respective controls.

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 B_6 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

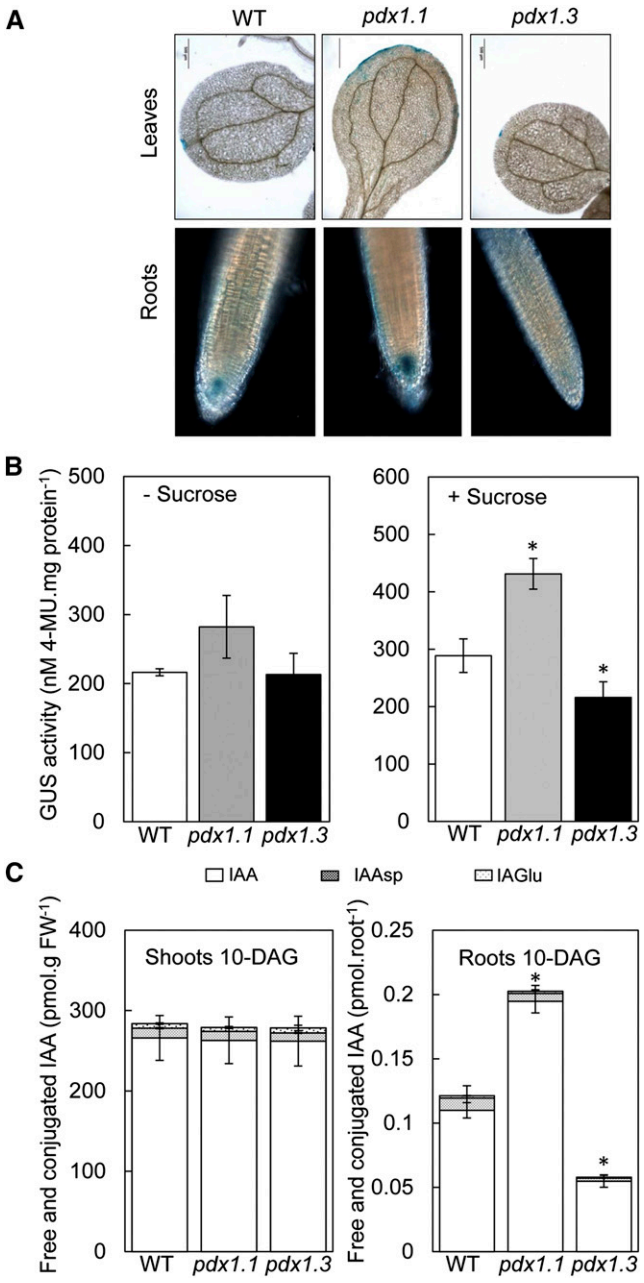

Figure 3. Levels of auxin in roots of *pdx1.1* and *pdx1.3*. A, GUS staining of leaves and roots of *pdx1.1* and *pdx1.3* compared with wild-type (WT) Col-0 carrying the auxin-responsive promoter DR5 fused to *GUS*. Seedlings were grown in the presence of 1% (w/v) Suc, and images were captured at 10 DAG. B, Quantitative GUS analysis performed with 4-methylumbelliferyl glucuronide (4-MU) in the absence (left) or presence (right) of Suc. Plant lines are at the same age as in A. The data are from at least three biological repetitions. Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.05$) when compared with the wild type. C, Quantification of free and conjugated auxin in shoots and roots of *pdx1.1* and *pdx1.3* compared with the wild type. Seedlings were grown on medium in the presence of 1% (w/v) Suc and were analyzed at 10 DAG. The data are from at least three biological repetitions. Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.05$) when compared with the wild type. FW, Fresh weight.

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 B_6 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 B_6 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).

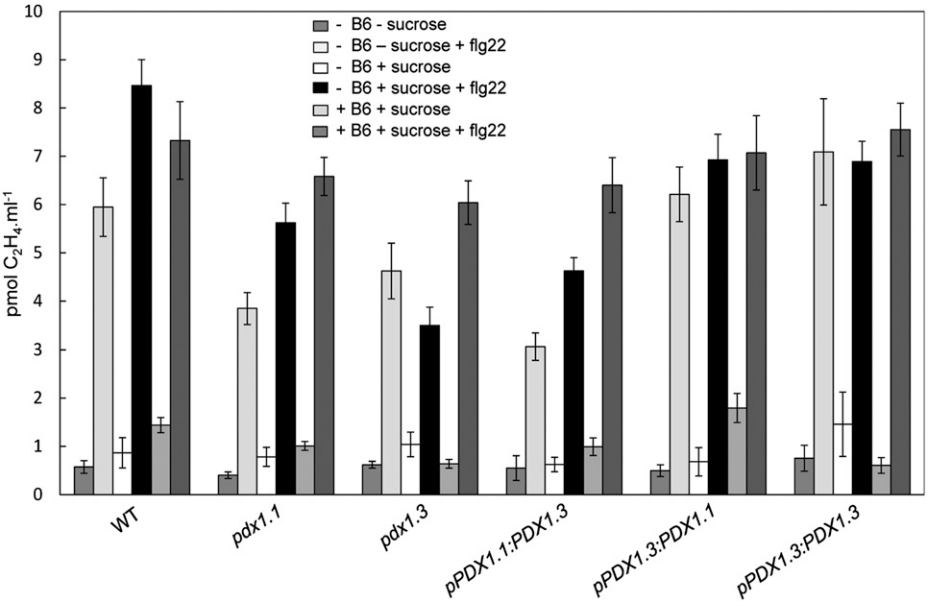

Figure 4. Ethylene production is impaired in *pdx1* mutants. Production of ethylene (C_2H_4) was induced in the presence of the flg22 peptide elicitor. Seedlings used were grown in the presence or absence of Suc and pyridoxine (B6) as indicated until 10 DAG. The lines used are the wild type (WT), *pdx1.1*, *pdx1.3*, the upstream region of *PDX1.1* fused to *PDX1.3* (*pPDX1.1*:*PDX1.3*), the upstream region of *PDX1.3* fused to *PDX1.1* (*pPDX1.3*:*PDX1.1*), and the upstream region of *PDX1.3* fused to *PDX1.3* (*pPDX1.3*:*PDX1.3*). The data are from at least five biological repetitions. Error bars represent SE.

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; Blilou 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

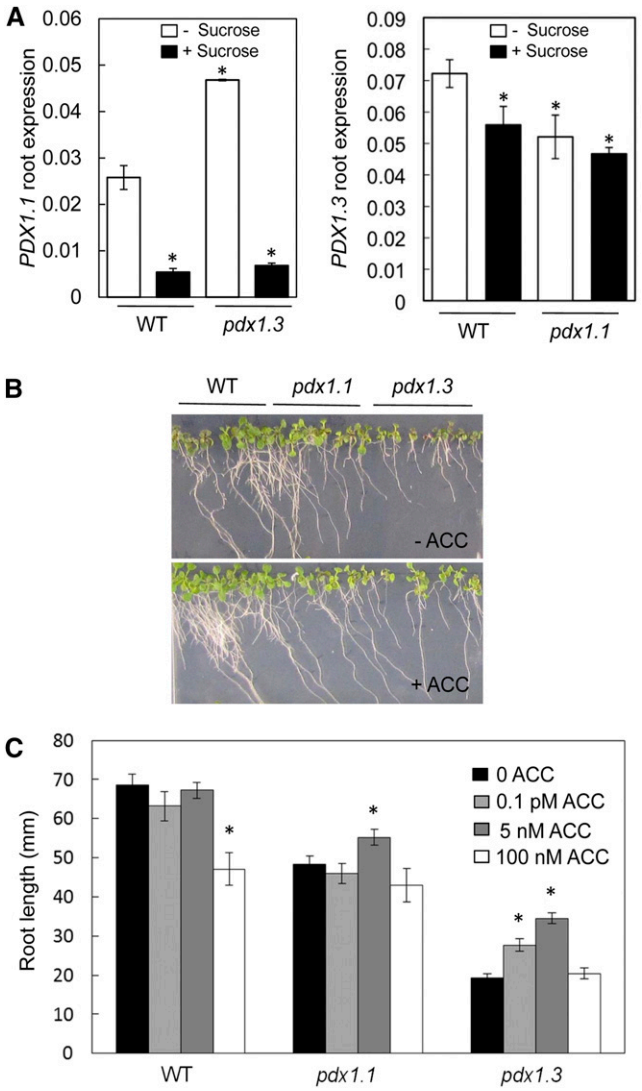

Figure 5. Exogenous Suc represses *PDX1.1* expression, but application of ACC can partially rescue the *pdx1* root phenotype. A, Expression of *PDX1.1* (left) and *PDX1.3* (right) in roots of wild-type (WT) Col-0 and *pdx1.3* or *pdx1.1* in the absence or presence of 1% (w/v) Suc. Seedlings were allowed to grow until 10 DAG. The data are from at least three biological repetitions. Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.05$) when treatments are compared with the wild type grown in the absence of Suc. B, Growth of the wild type, *pdx1.1*, and *pdx1.3* in the presence of 1% (w/v) Suc as well as in the absence (top) or presence (bottom) of 5 nM ACC. The experiment was done three times, yielding similar results; images were captured at 10 DAG. C, Root length of wild-type, *pdx1.1*, and *pdx1.3* seedlings at 10 DAG grown on 1% (w/v) Suc in the presence or absence of ACC (0–100 nM as indicated). The data are averages of three biological replicates; measurements were performed using ImageJ software (http://imagej.nih.gov/ij/). Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.05$) when compared with the wild type in the absence of ACC.

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 *PAP1* is up-regulated

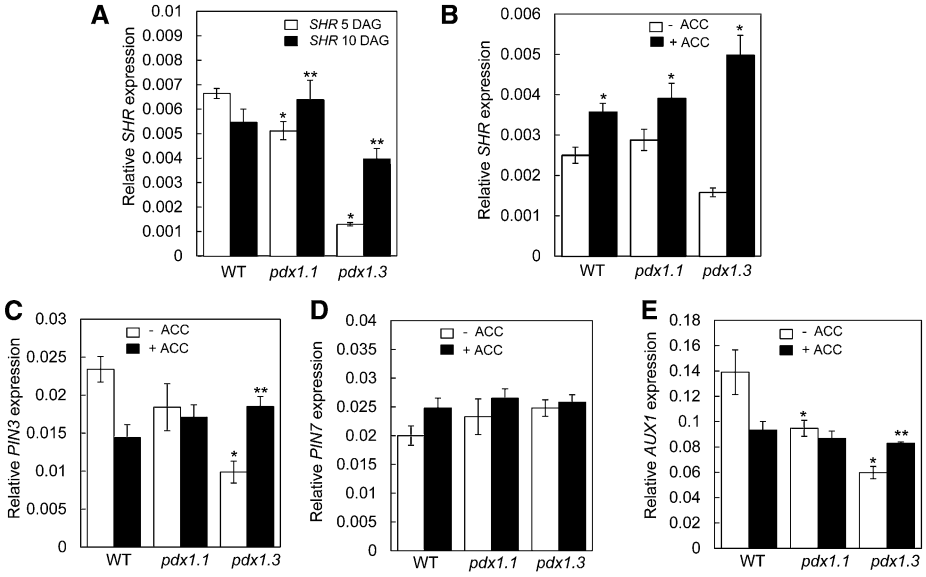

Figure 6. The altered expression levels of *SHR* and selected auxin transporters is partially rescued by application of ACC. A, *SHR* transcript abundance in roots of wild-type (WT) Col-0, *pdx1.1*, and *pdx1.3* seedlings grown in the presence of 1% (w/v) Suc at either 5 or 10 DAG. The data represent 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 presence or absence of 5 nM 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 nM 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.

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).

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

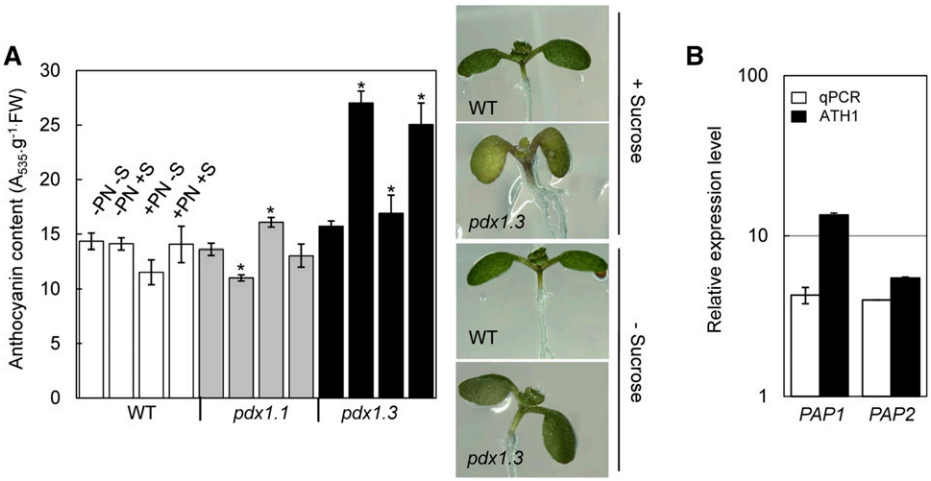

Figure 7. Anthocyanin accumulation in *pdx1.3*. A, Anthocyanin levels in *pdx1.1* and *pdx1.3* compared with wild-type (WT) Col-0 in the presence or absence of pyridoxine (PN) and/or Suc (S). Seedlings were grown until 10 DAG. The data represent means from at least three biological replicates. Error bars represent SE. Asterisks indicate statistically significant differences ($P < 0.001$) of mutant lines compared with the corresponding control wild-type line. The morphology of *pdx1.3* in the presence or absence of Suc at this stage of growth is shown on the right compared with the wild type. B, Expression levels of *PAP1* and *PAP2* transcription factors involved in anthocyanin biosynthesis in *pdx1.3* compared with wild-type Col-0. Transcript abundance was determined by both an ATH1 microarray (see text) and qPCR. In the case of the qPCR, the data are results of three biological replicates. Error bars represent SE.

The Arabidopsis Information Resource (http://www.arabidopsis.org/tools/bulk/go/) indicates a significant enrichment of genes involved in metabolism and stress responses in both leaves and roots (Supplemental Fig. S4). In the context of this study, it is notable that several genes involved in ethylene biosynthesis (aminocyclopropane 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). Overexpression 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 B_6 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 B_6 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 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 B_6 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 Schopfer, 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 (Růzicka et al., 2007). The process depends on the ethylene-signaling pathway, because impaired ethylene perception prevents the accumulation of auxin in the root tip (Růzicka 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 vitamer 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 ATCCTCC; Fujimoto et al., 2000) and partial AuxRE (TGTCTc 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 PHYTOCHOME-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 B_6 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 B_6 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,

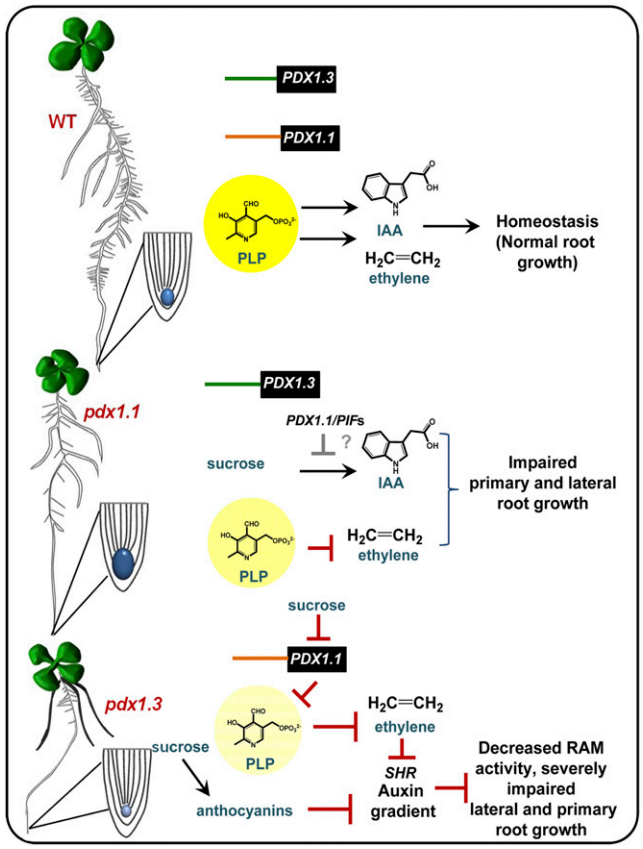

Figure 8. Consequences of a deficit in PLP for hormone homeostasis and root development in Arabidopsis. The top scenario indicates that, in the presence of sufficient PLP levels (yellow), as supplied by the catalytic action of PDX1.1 and PDX1.3, homeostasis of the two phytohormones auxin and ethylene is maintained for normal root development. The region around the root tip is amplified, part of which is shaded in blue to reflect arbitrary auxin levels at this stage of growth. The promoter regions of *PDX1.1* and *PDX1.3* are depicted by differently colored lines, to reflect their differential expression, while coding regions are shown as black boxes. The middle scenario illustrates observations in the *pdx1.1* mutant. In this scenario, there is a mild deficit in the production of PLP (paler yellow; note that *PDX1.3* is still expressed), which in turn affects ethylene production. However, auxin levels are much higher (shaded dark blue) than in the wild type, induced by the presence of Suc. The loss of *PDX1.1* contributes to the inability to repress the accumulation of auxin as a function of Suc and may be related to the misregulation of PIF proteins. Overall, this results in impaired primary and lateral root growth. The bottom scenario depicts the situation in *pdx1.3*. In the presence of Suc, the expression of *PDX1.1* is down-regulated and anthocyanins accumulate. The decrease in expression of both *PDX1.3* and *PDX1.1* leads to a major deficit in PLP production (pale yellow), which represses ethylene production. The combined deficit in ethylene production and the accumulation of anthocyanins negatively affects auxin biosynthesis (shaded pale blue) and its distribution. The expression of *SHR* is also decreased. Root apical meristem (RAM) activity is impaired, as reported (Chen and Xiong, 2005), leading to primary and lateral root growth defects and the appearance of anchor roots (dark gray).

2002; González et al., 2007), and the short-root phenotype of the *rus* (for root ultraviolet B-sensitive) mutants (Tong et al., 2008; Leasure et al., 2009) appears to be suppressed by mutations in the PLP-binding site of Asp aminotransferase as well as by pyridoxine supplementation (Leasure et al., 2011). The *rus* mutants have defects in polar auxin transport (Ge et al., 2010; Yu et al., 2013) and may provide an interesting link to this study.

MATERIALS AND METHODS

Plant Material

All Arabidopsis (*Arabidopsis thaliana*) lines (wild-type Col-0, *pdx1.1*, and *pdx1.3* [Titiz et al., 2006], *shr-2* [Benfey et al., 1993; 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 with mannitol (100 mM), IAA (1 μM), or ACC (5 nM 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://imagej.nih.gov/ij/). The root length and lateral root number data are means 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′-CATG*CCATGG*CAGGAACCGGAGTTGTGGCGGTG-TACGGCG-3′ and 3′-GG*ACTAGT*CTCAGAACGACTAGCGAACCTCTC-5′ for the *PDX1.1* gene and 5′-CATG*CCATGG*AAGGAACCGGCGTTGTGGCGGTG-TACGGTAACGG-3′ and 3′-GG*ACTAGT*CTCGGAGCGATTAGCGAACCTCTC-5′ for the *PDX1.3* gene (the region in underlined italics in each primer pair represents the incorporation of either an *Nco*I or a *Spe*I restriction site, respectively). The amplified genes were cloned into the binary vector pCAMBIA 1302 (http://www.cambia.org) using the *Nco*I and *Spe*I restriction sites. Subsequently, the 35S cauliflower mosaic virus promoter in the vector was replaced with the PCR-amplified region immediately upstream of the start codon of either *PDX1.1* (1,424 bp; primer pair 5′-CATG*CCATGG*TTTTTTCTAGGGTTTTGAGA-GAGTGTG-3′ and 3′-CCG*GAATTC*GGATTTGAGCATTTGTTAGTTTCTCTG-5′) or *PDX1.3* (1,384 bp; primer pair 5′-CATG*CCATGG*TTATCGGAGATTGAAGA-GAAATTTGTG-3′ and 3′-CCG*GAATTC*TACCATTTTTTTTGTTTGGTC-TATATA-5′) using the *Eco*RI and *Nco*I restriction sites (underlined and in italics in the primer pairs). In addition to fusing the respective upstream region with the corresponding genes *pPDX1.1*:*PDX1.1* and *pPDX1.3*:*PDX1.3*, the *PDX1.1* upstream region was fused to the *PDX1.3* gene and the *PDX1.3* upstream region was fused to the *PDX1.1* gene to generate chimeric constructs *pPDX1.1*:*PDX1.3* and *pPDX1.3*:*PDX1.1*, respectively. To prevent a cotranslational fusion with GFP in pCAMBIA 1302, either a TAA or a TGA codon was introduced immediately after the coding regions of *PDX1.1* and *PDX1.3*, respectively, using site-directed mutagenesis and the following primer pairs: 5′-GCTAGTCGTTCTGAG*TAA*ACTAGTAAAGGAGAAG-3′ and 3′-CTTCTCCTTTACTAGT*TTA*CTCAGAACGACTAGC-5′ for *PDX1.1* and 5′-CGCTAATCGCTCCGAG*TGA*ACTAGTAAAGGAG-3′ and 3′-CTCCTTTAC-TAGT*TCA*CTCGGAGCGATTAGCG-5′ for *PDX1.3*. Mutations in the putative ERE and AuxRE of *pPDX1.3*:*PDX1.3* were performed by site-directed mutagenesis with the following primers: 5′-CCTTCCTTTTCAACCACA*T*CC*T*CCTATTTCTCTATTTAC-3′ and 3′-GTAAATAGAGAAAT*A*GG*A*GGATGTGGTTGAAAAGGAAGG-5′ for mutating the ERE (AGCCGCC to ATCCTCC; Fujimoto et al., 2000) and 5′-GCTAACATTTTTTCTCCTTG*AA*TTTTATGCAACAATTAATGCGG-3′ and 3′-CCGCATTAATTGTTGCATAAAA*TT*CAAGGAGAAAAAATGTTAGC-5′ for the mutation of the AuxRE (TGTCTc to TGAATc; Ulmasov et al., 1997b), using *pPDX1.3*:*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 1291Z vector using the *Eco*RI and *Nco*I 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 *DR5-GUS* fusion construct originally described by Ulmasov et al. (1997b) were obtained from Jirí 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 NaH_2PO_4/Na_2HPO_4, pH 7, containing 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 0.1% (w/v) Triton X-100, and 10 mM EDTA-Na_2 and then incubated at 37°C in the same solution containing 0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-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 NaH_2PO_4/Na_2HPO_4, pH 7, containing 50 mM EDTA-Na_2, 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-methylumbelliferyl-β-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 the reaction volume was transferred to a fresh tube containing 900 μL of stop solution (0.2 M Na_2CO_3), 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 mean values of three independent experiments, and error bars represent SE.

Free and Conjugated Auxin Analyses

The method used for auxin analysis was described by Pencík 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 immunoaffinity extraction. 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 either 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 photons m^{-2} s^{-1} at a temperature of 22°C but were maintained at 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 comparison 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 mM) and diluted to a final concentration of 10 μM in a solution containing 0.1% (w/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 5 μM pyridoxine were used. For sampling, five separate seedlings 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*, At4g37650.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Total B_6 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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