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Mediator regulates root system architecture in Arabidopsis

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PFT1/MED25 regulates lateral root formation via auxin signaling in *Arabidopsis thaliana*

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ABSTRACT

Root system architecture is a major determinant of water and nutrient acquisition as well as stress tolerance in plants. The Mediator complex is a conserved multi-protein complex that acts as universal adaptor between transcription factors and the RNA polymerase II. In this report, we characterized possible roles of the MED8 and MED25 subunits of the plant Mediator complex in the regulation of root system architecture in Arabidopsis (*Arabidopsis thaliana*). We found that loss-of-function mutations in PFT1/MED25 increase primary and lateral root growth as well as lateral and adventitious root formation. In contrast, *PFT1/MED25* over-expression reduces these responses, suggesting that PFT1/MED25 is an important element of meristematic cell proliferation and cell size control in both lateral and primary roots. PFT1/MED25 negatively regulates auxin-transport and response gene expression in most parts of the plant, as evidenced by increased and decreased expression of auxin-related reporters *PIN1::PIN1::GFP*, *DR5:GFP*, *DR5:uidA* and *BA3:uidA* in *pft1-2* mutants and in *35S:PFT1* seedlings, respectively. No alterations in endogenous auxin levels could be found in *pft1-2* mutants or in *35S:PFT1* over-expressing seedlings. However, detailed analyses of *DR5:GFP* and *DR5:uidA* activity in wild-type, *pft1-2* and *35S:PFT1* seedlings in response to indole-3-acetic acid (IAA), naphthaleneacetic acid (NAA), and the polar auxin-transport inhibitor 1-*N*-naphthylphthalamic acid (NPA), indicated that PFT1/MED25 principally regulates auxin transport and response. These results provide compelling evidence for a new role for PFT1/MED25 as an important transcriptional regulator of root system architecture through auxin-related mechanisms in Arabidopsis.

INTRODUCTION

The indeterminate growth of the plant root system through continuous cell division and elongation processes can be profoundly affected by nutrient and water availability as well as various stress conditions such as extreme temperatures, drought, and/or salt stress (López-Bucio et al., 2003; Malamy, 2005). Therefore, the plasticity of root system is of critical importance for the plant to compete for resources and adapt to constantly changing growth conditions. The Arabidopsis root system architecture, which consists of a primary root, lateral roots, and root hairs, determines the exploratory ability of roots in the soil. Lateral roots initiate from a few pericycle cells that acquire the attributes of founder cells, then divide asymmetrically to give rise to lateral root primordia (LRP), which continue to grow and eventually emerge from the primary root (Dubrovsky et al., 2000; Dubrovsky et al., 2008). Finally, the new apical meristem is established and takes over the control of growth of mature lateral roots (Malamy and Benfey, 1997).

The phytohormone auxin (indole-3-acetic acid, IAA) plays an important role during all stages of lateral root formation (Casimiro et al., 2003; De Smet et al., 2006; Fukaki et al., 2007). Application of IAA or synthetic auxins such as naphthaleneacetic acid (NAA) stimulates lateral root formation (Celenza et al., 1995), whereas treatment with polar auxin transport inhibitors prevents lateral root initiation (Casimiro et al., 2001; Himanen et al., 2002). Consistently, Arabidopsis mutants with increased auxin levels, such as *rooty* (*rty*) and its alleles *aberrant lateral root formation1* (*alf1*) and *superroot1* (*sur1*), have increased number of lateral roots (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995), while mutants such as *aux1*, *axr1*, *doc1*, *slr1* and *arf7arf1*9 with defective auxin transport, perception, and/or signaling, show reduced lateral root formation (Lincoln et al., 1990; Gil et al., 2001; Swarup et al., 2001; Fukaki et al., 2002). Auxin is unique among plant hormones in being actively and directionally transported from the place of synthesis in young apical parts to distant tissues. The auxin-efflux regulators PIN-FORMED (PIN) are crucial for auxin distribution throughout the plant. PIN proteins have been shown to mediate various developmental processes. For instance, vascular tissue and flower development are regulated by PIN1 (Gälweiler et al., 1998), while tropisms by PIN2 and PIN3 (Friml et al., 2002b) and the patterning of the root by PIN4 (Friml et al., 2002a).

Auxin can interact with other plant hormones to orchestrate root development. Recently, it has been found that COI1, the jasmonic acid (JA) receptor, is required to mediate lateral root formation in response to JA, a canonical defense signal, thus indicating the participation of downstream signaling components integrating the responses of two or more plant hormones into plant organogenesis (Raya-González et al., 2012).

The Mediator complex is a large multiprotein complex conserved in all eukaryotes, from yeast to human. Mediator acts as a bridge between the RNA polymerase II complex and the myriad of transcription factors present within the cell (Kim et al., 1994; Koleske and Young, 1994). Mediator fine-tunes diverse regulatory inputs and presents a balanced output to the RNA polymerase II to initiate transcription by binding to distal activators/repressors as well as general transcription factors at the promoter site (Malik and Roeder, 2005). In *Saccharomyces cerevisiae*, Mediator is composed of 25 subunits, of which 22 subunits are at least partially conserved among eukaryotes (Boube et al., 2002; Bourbon et al., 2004). Specific Mediator subunits are required for growth and development in organisms such as *Drosophila*, zebrafish, mice and *Caenorhabditis elegans* (Loncle et al., 2007; Lehner et al., 2006; Wang et al., 2006; Jiang et al., 2010).

Plant growth is regulated by both cell number and cell size, which in turn are controlled by coordinated cell proliferation and expansion events during organogenesis (Mizukami, 2001; Sugimoto-Shirasu and Roberts, 2003). Recently, the participation of the Mediator complex in plant organ size control and cell differentiation was evidenced through the identification of 21 conserved and six putative plant-specific Mediator subunits in Arabidopsis and the analysis of Arabidopsis mutants defective on MED14 and MED25 (Autran et al., 2002; Xu and Li, 2011; Sundaravelpandian et al., 2012). Prior to its identification as Mediator subunit 14, STRUWWELPETER (SWP) was found to regulate cell number and shoot meristem development (Autran et al., 2002). Arabidopsis MED25 was originally identified as PHYTOCHROME AND FLOWERING TIME 1 (PFT1), a nuclear protein acting in a photoreceptor pathway that induces flowering in response to suboptimal light conditions (Cerdan and Chory, 2003). In addition, Arabidopsis mutants compromised in the kinase module of the Mediator complex, MED12, MED13 and CDK8, all show developmental phenotypes due to altered cell differentiation (Wang and Chen, 2004; Ito et al., 2011; Gillmor et al., 2010). The *med12* and *med13* mutants are affected in the transition of

embryos from globular to heart stage due to a delay in the expression of *KANADI1* and *KANADI2* transcription factors during early development (Gillmor et al., 2010). The effect of the *med13* mutation on cell differentiation has also been explained by a defective response to the hormone auxin (Ito et al., 2011). Even though MED8 and MED25 play important roles in jasmonic acid signaling, stress responses and plant development such as root hair formation and flowering time, currently, possible roles of these and other Mediator complex subunits on root system architecture and auxin signaling are unknown.

In this study, we tested the possible participation of MED8 and PFT1/MED25 subunits of Mediator in root development and auxin signaling in Arabidopsis. While *med8* mutants did not show any evident alteration of root architecture, the *pft1* mutants had increased primary root growth and root branching. Analysis of cell cycle marker *CycB1:uidA*, cell size measurements, auxin transport and auxin-responsive reporter gene expression in *pft1-2* and *35S:PFT1* seedlings before and after treatments with IAA, NAA and the auxin transport inhibitor NPA further indicated that the Mediator subunit PFT1/MED25 acts as negative regulator of cell proliferation, lateral root formation, auxin transport and auxin-responsive gene expression in Arabidopsis.

RESULTS

PFT1/MED25 subunit of Mediator regulates root architecture in Arabidopsis

To determine the participation of the two subunits of Mediator, MED8 and PFT1/MED25, as a regulators of Arabidopsis root architecture, we compared root growth phenotypes of wild-type (WT, Col-0), *pft1-2*, and *med8* single, and *pft1 med8* double mutants, as well as *pft1-1* mutants that were transformed with a genomic copy of *PFT1* (G1 complementation line; *gPFT1*; Cerdan and Chory, 2003) and *PFT1/MED25* over-expression seedlings (*35S:PFT1*). The seedlings were grown on agar-solidified 0.2X Murashige and Skoog (MS) medium and primary root lengths, lateral root numbers and lengths were quantified 8 days after germination. We found that *pft1-2* and *pft1 med8* seedlings had longer primary roots than WT seedlings, whereas *med8* and *gPFT1* plants were not affected in primary root growth (Fig. 1A). In contrast, *35S:PFT1* plants had shorter primary roots than WT plants

(Fig. 1A). Interestingly, *pft1-2* and *pft1 med8* seedlings showed nearly two-fold increase in lateral root numbers and lateral root lengths, when compared to WT, *gPFT1* and *med8* seedlings (Fig. 1, B-D). In contrast, *35S:PFT1* seedlings showed fewer and shorter lateral roots than WT and *gPFT1* seedlings (Fig. 1, B-D). An increase in primary root growth and lateral root formation in *pft1-1*, *pft1-2* and *pft1-3* mutants relative to WT was confirmed in additional experiments (Supplemental Fig. S1). Together, these results indicate that PFT1/MED25 regulates root system architecture in Arabidopsis.

Adventitious roots originate from stem tissue and provide an increased ability for the plant to explore soil for water and nutrients. To evaluate the participation of PFT1/MED25 and MED8 in adventitious root formation, we obtained stem explants from WT, *pft1-2*, *35S:PFT1*, *med8*, and *pft1 med8* seedlings grown in dark conditions. These explants were cultured for 5 d over the surface of Petri plates containing agar-solidified 0.2X MS medium and both first- and second-order adventitious roots were quantified. In these experiments, *pft1-2* and *pft1 med8* seedlings had a two- and five-fold increase in first- and second-order adventitious root numbers, respectively, when compared to WT seedlings while the formation of second-order adventitious roots was drastically reduced in *35S:PFT1* seedlings (Supplemental Fig. S2). These results suggest that PFT1/MED25 also regulates processes associated with adventitious root development in Arabidopsis.

PFT1/MED25 controls cell division and elongation

Primary root growth depends on two basic processes: cell division in the root apical meristem and elongation of divided cells that subsequently leave the root meristem (Blilou et al., 2002). Because root architecture was altered in *pft1* mutants, we next explored the role of PFT1/MED25 on cell division and elongation of primary and lateral roots. For this aim, we crossed *pft1-2* and *35S:PFT1* plants with Arabidopsis plants expressing the cell cycle marker *CycB1:uidA* (Colón-Carmona et al., 1999), which monitors cell cycle progression in the root meristem. We found that *pft1-2/CycB1:uidA* root meristems contain 40% more dividing cells than WT meristems, as revealed by increased number of blue spots in the GUS-expression domain of *pft1-2/CycB1:uidA* primary root meristems (Fig. 2, A and D), which were also larger than *CycB1:uidA* and *35S:PFT1/CycB1:uidA* root

meristems (Fig. 2B). In contrast, *35S:PFT1/CycB1:uidA* seedlings had fewer dividing cells and smaller root meristems than *CycB1:uidA* seedlings (Fig. 2, A, B and D). Contrasting expression of *CycB1:uidA* was also observed in emerged lateral roots of *pft1-2/ CycB1:uidA* and *35S:PFT1/CycB1:uidA* seedlings (Supplemental Fig. S3). To determine the involvement of PFT1/MED25 in cell elongation, we measured fully developed cortical cells of the primary and lateral roots of *CycB1:uidA*, *pft1-2/CycB1:uidA* and *35S:PFT1/CycB1:uidA* seedlings and found that cortical cells of *pft1-2/CycB1:uidA* seedlings were in average 15% longer than those of *CycB1:uidA* seedlings (Fig. 2C, Supplemental Fig. S3). In contrast, cortical cells of *35S:PFT1*/*CycB1:uidA* seedlings were significantly shorter than those of *pft1-2/CycB1:uidA* seedlings (Fig. 2C). Taken together, these data indicate that PFT1/MED25 acts as a repressor of cell division and elongation during primary and lateral root growth in Arabidopsis.

PFT1/MED25 regulates lateral root primordia development through auxin signaling

To understand the role played by PFT1/MED25 during lateral root formation and its possible relationship with auxin signaling, which regulates this organogenesis process, we analyzed lateral root primordia (LRP) originating from the primary roots of WT, *pft1-2* and *35S:PFT1* seedlings, according to Malamy and Benfey (1997). In 7-d-old *pft1-2* roots, the number of Stage I and II LRP were two-fold higher than WT roots (Fig. 3, A and B), suggesting that *pft1-2* primary roots are more branched because they produce more *de novo* LRP from pericycle cells. Given that lateral root formation is a process regulated by auxin signaling, we then evaluated the expression of the auxin responsive marker *DR5:uidA* during LRP development in *DR5:uidA*, *pft1-2/DR5:uidA*, and *35S:PFT1/DR5:uidA* seedlings. *DR5:uidA* expression in *pft1-2/DR5:uidA* seedlings was stronger at all LRP developmental stages and in mature lateral roots than in *DR5:uidA* and *35S:PFT1*/ *DR5:uidA* seedlings (Supplemental Figs. S4 and S5). These data suggest that PFT1/MED25 modulates lateral root formation by regulating auxin signaling during LRP development.

PFT1/MED25 regulates auxin-responsive reporter gene expression in roots and shoots

Auxin signaling has been implicated in many development processes in both root and shoot systems. To determine whether PFT1/MED25-mediated alterations in auxin responses could also occur in other plant tissues, we evaluated reporter gene activity in WT as well as in *pft1-2* and *35S:PFT1* seedlings harboring the *DR5:GFP*, *DR5:uidA* or *BA3:uidA* gene constructs. Firstly, seedlings harboring the *DR5:GFP* construct were grown for 7 d on agar-solidified 0.2X MS medium with or without 60 nM IAA and different parts of the plants including root tips, vascular tissues and root/shoot transition zones analyzed by confocal microscopy. GFP expression was higher in *pft1-2/DR5:GFP* but lower in *35S:PFT1/DR5:GFP* in all three regions analyzed (Fig. 4). Exogenous auxin further induced *GFP* expression in *pft1-2/DR5:GFP* and *DR5:GFP* while no such induction was evident in *35S:PFT1/DR5:GFP* seedling roots (Fig. 4). We next analyzed the *DR5:uidA*–driven GUS activity in cotyledons, young leaves, shoot meristems, the stem/root transition zone and the primary root tip. GUS activity was present in the primary root tip region and in leaves of untreated *DR5:uidA* seedlings (Supplemental Fig. S6). However, an increase in GUS activity was evident in most tissues of *pft1-2/DR5:GUS* seedlings, including cotyledons, petioles, the stem/root transition zone, lateral roots, and primary root tips. In contrast, the GUS activity was much lower in *35S:PFT1* seedlings than in *DR5:GUS* and particularly in *pft1-2/DR5:GUS* in all tissues analyzed (Supplemental Fig. S6).

PFT1/MED25 regulates auxin response

The observation of contrasting root growth phenotypes resulting from the loss- and gain-of-PFT1/MED25 function, together with contrasting expression of auxin reporter genes in *pft1-2* and *35S:PFT1* seedlings, suggests a role for PFT1/MED25 in the auxin signaling pathway. As IAA is a major regulator of root architecture, PFT1/MED25-mediated effects in root development could be due to potential alterations in auxin biosynthesis, auxin transport and/or auxin response. To determine whether *pft1* mutants could over-accumulate auxin, we first measured free IAA levels in WT, *pft1-2* and *35S:PFT1* whole seedlings by gas chromatography-mass spectrometry (GC-MS). No significant differences in auxin accumulation were observed between WT, *pft1-2* and *35S:PFT1* seedlings (Supplemental Fig. S7), suggesting that PFT1/MED25 is not a regulator of auxin biosynthesis.

To assess whether the observed root phenotypes could be due to changes in auxin-responsive gene expression, we performed experiments with WT, *pft1-2* and *35S:PFT1* seedlings harboring the *DR5:uidA* or *BA3:uidA* gene constructs after treatments with IAA. Arabidopsis seedlings were grown for 7 d on 0.2X MS medium solidified with agar and then transferred to liquid 0.2X MS medium supplemented with either the solvent only or different concentrations of IAA, and incubated for 8 h at 22 ^{0}C. In solvent-treated *DR5:uidA* seedlings, GUS expression was present in leaves and primary roots (Fig. 5). As expected, *DR5:uidA* seedlings treated with IAA showed a dose-dependent increase in GUS activity (Fig. 5). In contrast, the GUS activity in response to IAA was higher and lower in *pft1-2/DR5:uidA* and *35S:PFT1/DR5:uidA* seedlings, respectively than in *DR5:uidA* (Fig. 5).

We further evaluated the IAA response in WT, *pft1-2* and *35S:PFT1* harboring the *BA3:uidA* marker. We found that this marker is strongly expressed in petioles and vascular tissues of *pft1-2/BA3:uidA* seedlings under standard growth conditions (Supplemental Fig. S8). In response to IAA, *pft1-2/BA3:uidA* showed even a stronger GUS activity than WT in petioles, vascular tissues and primary root elongation zones (Supplemental Fig. S8). In contrast, *35S:PFT1*/*BA3:uidA* seedlings showed a weaker GUS activity than *BA3:uidA* in petioles and primary roots both in the absence of any treatment and in response to IAA (Supplemental Fig. S8). These results indicate that PFT1/MED25 modulates auxin response in Arabidopsis.

PFT1/MED25 affects lateral root formation in response to auxin

Auxin has been shown to inhibit the elongation of the primary root and to stimulate lateral root formation, whereas auxin-transport inhibitors (e.g. 1-*N*-naphthylphthalamic acid, NPA) antagonize lateral root formation (Blakely et al., 1988; Muday and Haworth, 1994; Casimiro et al., 2001). To determine whether auxin transport is an important determinant of the root developmental changes mediated by PFT1/MED25, we evaluated root architectural responses of WT, *pft1-2* and *35S:PFT1* seedlings grown in Petri plates containing 0.2X MS medium supplied with low concentrations of naphthalene acetic acid (NAA), a synthetic auxin that enters the root cells via diffusion. Primary root growth was similarly inhibited in

WT and *pft1-2* seedlings in response to 5 to 30 nM NAA (Fig. 6A). Interestingly, *pft1-2* seedlings showed an increased response to NAA as evidenced by two-to-three-fold increases in the number and density of lateral roots in all evaluated NAA concentrations (Fig. 6B-D). In contrast, in response to NAA, *35S:PFT1* seedlings produced lower number and density of lateral roots than WT and *pft1-2* seedlings (Fig. 6B-D). When the lateral root data are normalized to the value obtained in the untreated control for each genotype, the fold-increases in lateral root numbers appear clearly different in all three genotypes, with a nearly two-fold higher relative lateral root formation in *pft1-2* seedlings than WT (Fig. 6C). Together, these observations suggest that PFT1/MED25 regulates pericycle cells to divide in response to auxin.

PFT1/MED25 regulates expression and distribution of auxin transporter PIN1

Auxin positively influences the PIN family of auxin transporters in a tissue-specific manner through an AUX/IAA-dependent signaling pathway (Vieten et al., 2005). PIN1 and PIN2 play important roles in lateral root formation and auxin-mediated gravitropism, respectively (Benková et al., 2003). To test whether PFT1/MED25 could regulate primary root growth and/or lateral root formation through differential expression of PIN1 or PIN2, we analyzed the spatial pattern of PIN1 and PIN2 localization in WT, *pft1-2* and *35S:PFT1* seedlings. In primary roots of seedlings expressing *PIN1::PIN1:GFP* (Vieten et al., 2005), the GFP fluorescence was detected in the stele and endodermis cells (Fig. 7A). In *pft1-2* primary roots, the GFP fluorescence was stronger than *PIN1::PIN1:GFP* and extended towards the root differentiation zone (Fig. 7B), while in *35S:PFT1* seedlings GFP fluorescence was weaker than *PIN1::PIN1:GFP* and *pft1-2/PIN1::PIN1:GFP* and remained somewhat restricted (Fig. 7C). An analysis of PIN1 localization during lateral root initiation showed that in WT plants, Stage V and VII primordia displayed the typical localization of PIN1 in most external cell layers while an increased and decreased PIN1 expression was evident in Stage V and VII primordia of *pft1-2/PIN1::PIN1:GFP* and *35S:PFT1/PIN1::PIN1:GFP* seedlings, respectively (Fig. 7, D-I). In contrast to differential expression of PIN1 in *pft1*, PIN2 expression was similarly detected in cortex and epidermal cells of WT, *pft1-2* and *35S:PFT1* primary roots (Supplemental Fig. S9). These findings suggest that PFT1/MED25

specifically regulates the expression and distribution of the PIN1 auxin transporter.

PFT1/MED25 modulates response of root architecture to NPA

To further analyze the participation of PFT1/MED25 in auxin transport, we evaluated the effects of the polar auxin transport inhibitor NPA on root architecture in WT, *pft1-2* and *35S:PFT1* seedlings. Arabidopsis seedlings were grown side by side for 8 d in agar plates containing MS 0.2X medium supplied either with 0.25-4 µM NPA or without NPA.
NPA (4 µM) inhibited approximately 60 % primary root growth in WT seedlings when the data for root lengths were normalized to the values obtained in their untreated counterparts. Relative to the response we observed in WT seedlings, *pft1-2* seedlings showed reduced response to 2 and 4 µM NPA (Fig. 8A), while *35S:PFT1* seedlings had a WT-like response (Fig. 8A). As expected, NPA dramatically inhibited lateral root formation in WT seedlings (Fig. 8, B-D). However, lateral root formation in *pft1-2* seedlings was less while in *35S:PFT1* seedlings more sensitive to NPA than WT. Together, these contrasting responses of *pft1-2* and *35S:PFT1* seedlings to an IAA efflux inhibitor in terms of both primary root growth and lateral root formation suggest a role for PFT1/MED25 in modulating auxin transport and response.

Root architectural responses of *pft1* and *coi1* mutants to jasmonic acid

Recent reports have shown that PFT1/MED25 is required for JA-mediated defense gene expression (Kidd et al., 2009). Because JA affects both auxin signaling and root architecture (Raya-González et al., 2012), it was important to test the function of PFT1/MED25 in JA-mediated root architecture regulation. The primary root growth and lateral root formation was analyzed in WT, *pft1-2* and *coi1-1* seedlings grown side by side. In these experiments, *pft1-2* showed a WT-like phenotype in its relative response to JA inhibition of primary root growth (Fig. 9, A and B). Also, a similar increase in lateral root numbers as WT seedlings in response to 4 µM JA was evident in *pft1-2* seedlings (Fig. 9, C-E). In contrast, as expected, *coi1-1* seedlings were highly resistant to primary root inhibition and lateral root promotion by JA (Fig. 9, B and E). Thus, these data show that

PFT1/MED25 likely acts independently of the jasmonate receptor COI1 and jasmonic acid to regulate lateral root formation in Arabidopsis.

DISCUSSION

PFT1/MED25 plays a role in root development

The root system, which plays an important role in anchoring the plant to the soil and in water and nutrient acquisition, exhibits an amazing architectural diversity manifested through changes in root hair, lateral and adventitious root formation and primary root growth (López-Bucio et al., 2003; Nibau et al., 2008). Engineering of root architecture of crop plants can be of value for increasing plant stress tolerance but this requires a thorough understanding of complex and interacting endogenous and exogenous factors that control individual aspects of root system configuration.

In this study, we investigated possible roles of MED8 and PFT1/MED25 Mediator subunits as regulators of root system architecture of Arabidopsis seedlings. To the best of our knowledge, so far the Mediator complex has not been implicated in lateral root development and auxin signaling, despite its requirement as an essential component of gene transcription in all eukaryotes. Our results show that while *med8* single mutants had root architecture similar to WT seedlings, the loss- and gain-of-function of PFT1/MED25 showed opposite effects on lateral and adventitious root development and on primary root growth, indicating that PFT1/MED25 functions as a key modulator of cellular processes that control root architecture configuration.

The root phenotypes observed in *pft1-2* and *35S:PFT1* seedlings correlated with changes in *CycB1:uidA* expression and cell expansion in primary and lateral roots (Fig. 2, Supplemental Fig. S3). Therefore, it is plausible that PFT1/MED25 regulates root development by inhibiting cell division and expansion in the roots. Through microarray analyses conducted on WT and *pft1* roots, Sundaravelpandian et al. (2012) found that the genes implicated in growth- and cell cycle-associated processes were differentially expressed between *pft1-2* and WT seedlings. For instance, the genes associated with the "indole butyric acid-related processes" were found to be differentially expressed in *pft1*

roots. In the light of our results, this is of particular interest, as genetic evidence in Arabidopsis suggests that indole-3-butyric acid (IBA) converted into IAA by peroxisomal beta-oxidation plays an important role in root hair formation and other developmental events by regulating cell expansion (Strader et al., 2010). Recently, Xu and Li (2011), through the use of transgenic Arabidopsis plants harboring the *MED25* promoter::*GUS* fusion (*pMED25::GUS*) construct, showed that *MED25* is expressed throughout the plant. The expression of *PFT1/MED25* in roots is particularly relevant to our findings, which suggest that both cell proliferation and elongation are affected by PFT1/MED25 in the Arabidopsis root system. Also, given that PFT1/MED25 was first described as a positive regulator of shade avoidance and later as a regulator of basal defense and abiotic stress responses (Cerdan and Chory, 2003; Bäckström et al., 2007; Wollenberg et al., 2008; Kidd et al., 2009; Elfving et al., 2011; Chen et al., 2012), it seems likely that PFT1/MED25 represents a molecular node for the integration of distinct environmental and developmental cues.

The PFT1/MED25 protein is highly conserved across diverse eukaryotes. Remarkably, MED25 in other eukaryotes is also associated with several developmental processes. For example, the RNAi-mediated suppression of *MED25* expression in *Drosophila* results in the failure of extension of some axons that affect the embryonic nervous system (Koizumi et al., 2007). In zebrafish, morpholino-mediated knockdown of *MED25* induces palatal malformation, suggesting an important role for MED25 in cartilage development (Nakamura et al., 2011). Remarkably, through characterization of mutants, complemented and over-expressing lines of PFT1/MED25, we obtained evidence in this study that PFT1/MED25 functions in development of organs and tissues ubiquitous to plants such as lateral and adventitious roots.

PFT1/MED25 is a negative regulator of lateral root primordium development

Lateral root formation is initiated when the pericycle cells respond to auxin and acquire the status of founder cells and through subsequent asymmetric cell divisions, give rise to a new LRP (Boerjan et al., 1995; Malamy and Benfey, 1997; Dubrovsky et al., 2008). The loss of PFT1/MED25 function leads to the formation of increased numbers of lateral root

primordia, particularly in Stage I and II. In contrast, the over-expression of *PFT1/MED25* resulted in decreased primordium formation (Fig. 3). These results indicate that PFT1/MED25 modulates root branching in Arabidopsis by inducing *de novo* formation of lateral root primordia from pericycle cells. Kidd et al. (2009) showed that *pft1* seedlings form more rosette leaves than WT plants. In plants, lateral root formation and leaf development are both regulated by auxin. Increased and reduced lateral root formation in *pft1-2* and *35S:PFT1* seedlings, respectively, were correlated with alterations in *DR5:uidA*-driven reporter gene activity detected in all stages of lateral root primordia and emerged lateral roots (Supplemental Fig. S4). Benková et al. (2003) showed that auxin accumulates at developing primordia. Subsequently, an auxin gradient is established with its maximum at the tip of the forming lateral root, in which the auxin transporter PIN1 plays an important role (Benková et al., 2003). Therefore, both increased distribution of auxin from producing cells and efficient transport by PIN1 may explain why *pft1-2* mutant shows an accelerated lateral root formation and greater proliferative capacity in pericycle cells than WT seedlings. Our results indicate that PFT1/MED25 negatively regulates lateral root initiation and development, probably by modulating an initial step required for the establishment of an auxin response maximum in lateral root founder cells.

PFT1 modulates auxin-inducible gene expression

The plant growth hormone auxin has been implicated in regulating many developmental and cellular processes by altering basic patterns of gene expression (Kende and Zeevaart, 1997). The availability of well established auxin markers such as *DR5:GFP*, *DR5:uidA* and *BA3:uidA* provides important genetic tools to study the involvement of auxin signaling in plant development as different markers show different sensitivity to endogenous and applied auxins. Importantly, we found differential expression of all three markers, *DR5:GFP*, *DR5:uidA* and *BA3:uidA*, in *pft1-2* and *35S:PFT1* seedlings (Fig. 4; Supplemental Figs. S5 and S6). These results indicate that PFT1/MED25 might regulate either auxin distribution and/or response. PFT1/MED25 was initially identified as a nuclear protein that acts in the phytochrome B (phyB) pathway that induces flowering in response to suboptimal light conditions. *pft1* mutants display defects in hypocotyl elongation under

both red and far-red light conditions (Cerdán and Chory, 2003). The involvement of auxin in photomorphogenesis, including shade avoidance response, has long been known (Steindler et al., 1999; Shinkle et al., 1998; Gil et al., 2001). It is noteworthy that *axr1*, *doc1/tir3* and other auxin-related mutants with altered responses to light and shade avoidance manifest important alterations in root system architecture, including root hair and lateral root formation (Lincoln et al., 1990; Pitts et al., 1998; López-Bucio et al., 2005). The expression of *AXR1* is localized to zones of active cell division and elongation and in epidermal cells that differentiate the root hairs. This expression pattern is correlated with a defect in root hair elongation observed in *axr1* mutant seedlings (Pitts et al., 1998; del Pozo et al., 2002). It is therefore possible that altered auxin responses observed in the *pft1* mutant could contribute to the developmental phenotypes such as altered flowering time, response to light quality and root development.

Through the analysis of the microarray data reported by Kidd et al. (2009), we identified several auxin-associated genes, such as *ANTHRANILATE SYNTHASE1* (*ASA1*), *TRYPTOPHAN SYNTHASE1* (*TSB2*), *CHORISMATE MUTASE3* (*CM3*), several auxin responsive *GH3* family genes, *IAA17, AUX/IAA*, an amino acid permease and a gene similar to *AUX1*, were differentially expressed between WT and *pft1* seedlings. Although our analyses did not show any alteration in free auxin levels in the *pft1-2* mutants (Supplemental Fig. S7), the gene expression data are overall in agreement with the detailed experiments reported here with the plants expressing well-established auxin reporters in *pft1* and *35S:PFT1* backgrounds.

The phytohormone jasmonic acid (JA) is a crucial component of the plant defense signaling system. JA and its metabolites, collectively called jasmonates, are lipid-derived signals produced during defense responses against insects and pathogens, but also under exposure to ozone, UV light, wounding, and other abiotic stresses (Wasternack, 2007). Reduction in root growth and carbon allocation patterns in several plant species upon mechanical wounding or by herbivory was ascribed to JA. In Arabidopsis, treatment with jasmonates strongly inhibits primary root growth and promotes lateral root formation (Sun et al., 2009; Raya-González et al., 2012). JA promoted lateral root formation through auxin biosynthesis and transport by directly inducing the auxin biosynthesis gene *ASA1* (Sun et al., 2009). Emerging evidence suggests that jasmonate and auxin signaling contains many common

components (reviewed by Cuéllar Pérez and Goossens, 2013). However, the exact cellular/tissue responses to jasmonates during Arabidopsis root system remodeling are currently not understood. Given the involvement of PFT1/MED25 in regulation of both JA (Kidd et al., 2009) and auxin responses (this study), it was important to test the function of this protein in JA-mediated root architecture. A comparison of root architecture of WT, *pft1-2* and *coi1-1* seedlings indicates that the increased lateral root formation in *pft1-1* is likely independent of COI1 and JA signaling, in which a loss-of-function mutation in COI1 renders the plants highly resistant to JA in both primary root growth inhibition and lateral root formation (Fig. 9).

It is worth noting that auxin itself positively feeds back on *PIN* gene expression in a tissue-specific manner through an AUX/IAA-dependent signaling pathway. Vieten et al. (2005) suggested a positive effect of IAA on *PIN1* expression. Our data suggest that both auxin response and transport rather than auxin accumulation might be important factors involved in root system remodeling in *pft1* mutants. Indeed, an analysis of the spatial pattern and abundance of PIN1 localization in WT, *pft1-2* and *35S:PFT1* revealed an increased GFP fluorescence in the stele and endodermal cells of *pft1-2* primary roots (Fig. 7A-C) and in lateral root primordia (Fig. 7 D-I). In contrast, PIN2 was detected only in the cortex and epidermal cells in a similar manner in WT, *pft1-2* and *35S:PFT1* primary roots (Supplemental Fig. S9). These findings suggest that PFT1/MED25 regulates the expression and distribution of PIN1 auxin transporter, and may explain why *pft1* seedlings show an amplified response to exogenous auxin based on enhanced auxin responsive gene expression and associated root developmental phenotypes. These results are also informative in explaining why *pft1* seedlings are more resistant than WT and *35S:PFT1* to NPA-mediated inhibition of lateral root formation.

PFT1 regulates lateral root and root hair formation through different mechanisms

Auxin is important for a multitude of physiological processes and regulates plant development through its biosynthesis and transport. The ability of plant cells to respond to this phytohormone in an appropriate manner is also critical for auxin-mediated plant development (Okushima et al., 2007). Our analysis by gas chromatography-mass

spectrometry (GC-MS) revealed that PFT1/MED25 is unlikely to be involved in general auxin biosynthesis (Supplemental Fig. S7). The activity of the *DR5:GFP* and *DR5:uidA* markers may not necessarily reflect global auxin levels but the sensitivity of tissues to IAA or other auxins (Benková et al., 2003). Our results suggest that auxin distribution and/or response could be involved in the activation of auxin-inducible genes involved in lateral root formation modulated by PFT1/MED25. Detailed analysis of *DR5:uidA* and *BA3:uidA* in WT, *pft1-2* and *35S:PFT1* in response to IAA revealed that *pft1-2* and *35S:PFT1* were more sensitive and resistant, respectively, to auxin (Fig. 5; Supplemental Fig. S8). This indicates that PFT1/MED25 modulates auxin response rather than auxin biosynthesis, and auxin transport through the regulation of PIN1 auxin transporter. This hypothesis is supported by the finding that *pft1-2* and *35S:PFT1* seedlings had opposite responses to NAA during lateral formation (Fig. 6). The increased and reduced NAA response shown by *pft1-2* and *35S:PFT1* seedlings, respectively, suggest that PFT1/MED25 is a key element that controls pericycle cell activation during lateral root formation, which is modulated by the auxin signaling pathway. Similarly, *35S:PFT1* seedlings showed developmental alterations and auxin-responsive gene expression consistent with a decreased auxin transport that correlates with their increased sensitivity to NPA (Figs. 4, 5, 6 and 8). This suggests that PFT1/MED25 might be involved in auxin transport, possibly by mediating the transcriptional regulation and/or distribution of PIN1 (Fig. 7).

In a recent work, Sundaravelpandian et al. (2012) reported that PFT1/MED25 controls root hair differentiation through reactive oxygen species (ROS) distribution. Both *pft1-2* and *pft1-3* Arabidopsis mutants showed a short root hair phenotype that was correlated with perturbations in hydrogen peroxide (H_2O_2) and superoxide distribution. Supply of H_2O_2, or KCN rescued the *pft1* mutant phenotype, indicating that PFT1/MED25 regulates root hair differentiation through ROS. The short root hair phenotype of *pft1* mutants could be reproduced in our research. Furthermore, we also found that *35S:PFT1* shows the opposite phenotype with root hairs longer than WT and *pft1* seedlings (Supplemental Fig. S10). Considering the positive effect of auxin on root hair growth (Pitts et al., 1998), the short root hair phenotype of *pft1* mutants indicates that the role of PFT1/MED25 in epidermal cell differentiation most likely occurs through an auxin-independent mechanism.

In conclusion, our results have shown that: (i) gain- and loss-of PFT1/MED25 function lead to opposite responses in primary root growth, lateral and adventitious root development. (ii) PFT1/MED25 negatively regulates cell division and elongation processes that are important in modulating the configuration of the root system; (iii) PFT1/MED25 regulates auxin responsive gene expression during LRP development, (iv) PFT1/MED25 modulates auxin responses to endogenous and supplied auxin and in lateral root formation, which seems to be independent from jasmonic acid signaling. Emerging evidence indicates that PFT1/MED25 plays multiple roles in a number of essential plant processes including light signaling and flowering time (Cerdán and Chory, 2003; Wollenberg et al., 2008; Inigo et al., 2012; Klose et al 2012), JA-mediated pathogen defense (Kidd et al., 2009), organ growth (Xu and Li, 2011), abiotic stress (Elfving et al., 2011), and JA and abscisic acid (ABA) signaling (Cevik et al., 2012; Chen et al., 2012). This is consistent with the expectation that individual Mediator subunits recognize and respond to a subset of the ~1500 transcription factors present in the Arabidopsis genome. Therefore, determining which transcription factor(s) interacts with PFT1/MED25 to coordinate auxin responses in pericycle cells should provide important information about the role of PFT1/MED25 and the Mediator complex in root morphogenesis.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana* Col-0), the transgenic Arabidopsis lines *35S:PFT1* (Cerdán and Chory, 2003), *CycB1:uidA* (Colón-Carmona et al., 1999), *DR5:uidA* (Ulmasov et al., 1997), *DR5:GFP* (Ottenschläger et al., 2002) *BA3:uidA* (Oono et al., 1998), *PIN1::PIN1::GFP* (Benkova et al., 2003), *PIN2::PIN2::GFP* (Blilou et al., 2005) and the mutant lines, *pft1-1* (Cerdan and Chory, 2003), *pft1-2* (SALK_129555), *pft1-3* (SALK_059316), *med8* (SALK_092406) *pft1 med8* (Kidd et al., 2009) and *coi1-1* (Feys et al., 1994) were used for the experiments reported here. To generate WT, *pft1-2* and *35S:PFT1* lines expressing auxin reporter gene constructs, crosses were made between the

respective lines and the lines homozygous for both loci were used in subsequent experiments. Seeds were surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach for 7 min. After five washes in distilled water, seeds were germinated and grown on agar plates containing 0.2X MS medium. The MS medium (Murashige and Skoog Basal Salts Mixture, catalog no. M5524) was purchased from Sigma. Phytagar (commercial grade) was purchased from Gibco-BRL. Plates were placed vertically at an angle of 65º to allow root growth along the agar surface and to allow unimpeded aerial growth of the hypocotyls. Plants were placed in a plant growth chamber (Percival AR-95L) with a photoperiod of 16 h of light/8 h darkness, light intensity of 300 μmol/m^{-2}/s^{-1}, and temperature of 22 ºC.

Chemicals

IAA (indole-3-acetic acid), NAA (1-Naphthaleneacetic acid), and NPA (1-*N*-naphthylphthalamidic acid) were purchased from Sigma and dissolved in dimethyl sulfoxide (DMSO). In control treatments, the solvents were used in equal amounts as present in the greatest concentration of each compound tested.

Analysis of growth

Arabidopsis root system and primary root meristem integrity were analyzed with a stereoscopic microscope (Leica, MZ6). All lateral roots emerged from the primary root and observed with the 3X objective were taken into account for lateral root number data. Images were captured with a Samsung SCC 131-A digital color camera adapted to the microscope. Primary root length was determined for each root using a ruler. Lateral root number was determined by counting the lateral roots per seedling, and lateral root density was determined by dividing the lateral root number value by the primary root length values for each analyzed seedling. For all experiments with WT and mutant lines, the overall data were statistically analyzed using the SPSS 10 program. Univariate and multivariate analyses with Tukey´s post-hoc test were used for testing the differences in growth and root development responses. Different letters were used to indicate means that differ

significantly (P<0.05).

Determination of developmental stages of LRP

Lateral root primordia (LRP) were quantified 7 d after germination. Seedling roots were first cleared to enable primordia at early stages of development to be visualized and counted. Each LRP was classified according to its stage of development as reported by Malamy and Benfey (1997). The developmental stages are as follows; Stage I: LRP initiation. In the longitudinal plane, approximately 8-10 'short' pericycle cells are formed. Stage II: the LRP is divided into two layers by a periclinal division. Stage III: the outer layer of the primordium divides periclinally, generating a three-layer primordium. Stage IV: an LRP with four cell layers. Stage V: the LRP is midway through the parent cortex. Stage VI: the LRP has passed through the parent cortex layer and has penetrated the epidermis. It begins to resemble the mature root tip. Stage VII: the LRP appears to be just about to emerge from the parent root.

Histochemical analysis

For histochemical analysis of GUS activity, Arabidopsis seedlings were incubated overnight at 37 ºC in a GUS reaction buffer (0.5 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide in 100 mM sodium phosphate, pH 7). The stained plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62 ºC. The solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Plants were dehydrated with ethanol treatments at 40, 20 and 10% (v/v) for a 24h period each, and fixed in 50% glycerol (v/v). The processed roots were placed on glass slides and sealed with commercial nail varnish. For each marker line and for each treatment at least 15 transgenic plants were analyzed.

Propidium iodide staining and *GFP* detection

For fluorescent staining with propidium iodide (PI), plants were transferred from the

growth medium to 10 mg ml^{-1} of PI solution for 1 min. Seedlings were rinsed in water and mounted in 50 % glycerol on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with an 568-nm excitation line and emission window of 585–610 nm, and *GFP* emission with 500- to 523-nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1000) after which the two images were merged to produce the final image.

Free IAA determination

The determination of IAA was from whole plants grown on agar-solidified 0.2X Murashige and Skoog (MS) medium for 10 days and IAA free was quantified as described by Edlund et al. (1995).

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FIGURE LEGENDS

Figure 1. PFT1/MED25 regulates root system architecture in Arabidopsis. WT (Col-0), *pft1-2*, *gPFT1*, *35S:PFT1*, *med8* and *pft1 med8* Arabidopsis seedlings were germinated and grown for 8 days on agar solidified 0.2X MS medium. (A) Primary root length. (B) Lateral root number. (C) Lateral root length. Root traits were scored as indicated in Materials and Methods. Error bars represent standard errors from 30 seedlings. Different letters indicate statistical differences at $P<0.05$. (D) Photographs of representative WT (Col-0), *pft1-2*, *gPFT1*, *35S:PFT1*, *med8* and *pft1 med8* seedlings are shown. Note that an opposite response is seen in lateral root formation and lengths between the *pft1-2* mutant and *PFT1* over-expressing seedlings. The experiment was repeated twice with similar results. Scale bar = 1 cm.

Figure 2. PFT1/MED25 represses cell division and elongation in primary root apical meristems. WT (Col-0), *pft1-2* and *35S:PFT1* Arabidopsis seedlings harboring the *CycB1:uidA* gene construct were germinated and grown for 7 days on agar solidified 0.2X MS medium. (A) Number of GUS positive spots/root meristem. (B) Meristem lengths and (C) Cortical cell lengths were scored as indicated in Materials and Methods. (D) Primary roots of young seedlings were stained for GUS activity and cleared to show the expression of *CycB1:uidA*. Photographs show representative individuals from 15 GUS-stained seedlings. Error bars represent standard errors from 15 GUS-stained seedlings analyzed. Different letters indicate means statistically different ($P<0.05$). The experiment was repeated two times with similar results. Scale bar =100 µm.

Figure 3. PFT1/MED25 modulates lateral root primordium formation. WT (Col-0), *pft1-2* and *35S:PFT1* Arabidopsis seedlings were germinated and grown for 7 days on agar solidified 0.2X MS medium and development of root primordia evaluated. (A) Lateral root primordium (LRP) per plant. (B) Lateral root primordia density (LRP per cm). LRP stages were recorded according to Malamy and Benfey (1997). Error bars represent standard errors from 15 GUS-stained seedlings analyzed. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated two times with similar results.

Figure 4. PFT1 modulates auxin-responsive gene expression. WT, *pft1-2* and *35S:PFT1* seedlings harboring the *DR5:GFP* gene construct were germinated and grown for 7 days in agar solidified 0.2X MS medium and then transferred for 8 h to liquid medium supplemented with or without IAA. Photographs show representative individuals of at least 15 seedlings analyzed by confocal microscopy. Note that *pft1-2* and *35S:PFT1* seedlings show stronger and weaker *DR5:GFP* reporter expression, respectively, than WT seedlings in all regions analyzed. Scale bar = 100 µm.

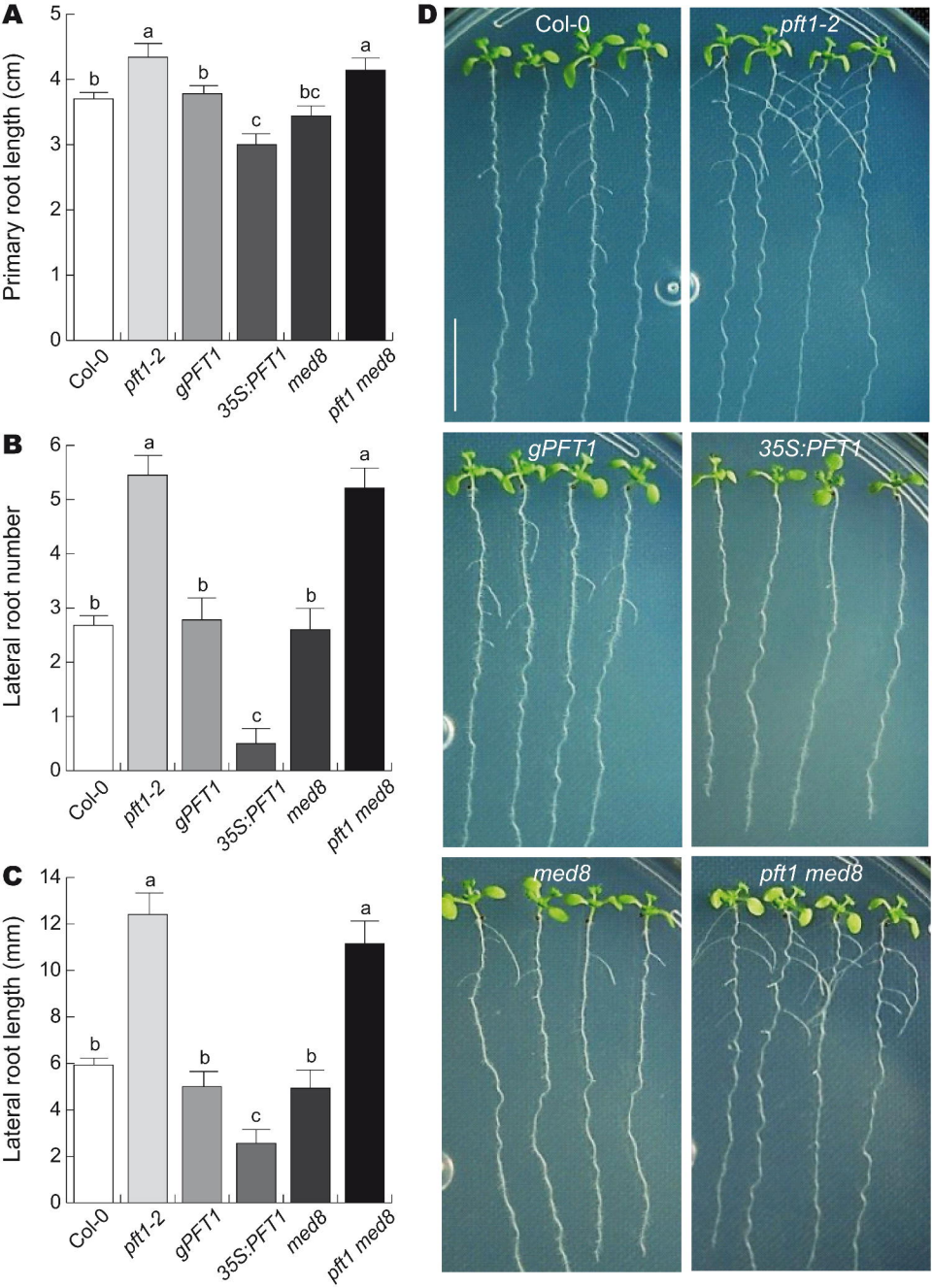
Figure 5. Effects of IAA on auxin-responsive reporter gene expression in WT, *pft1-2* and *35S:PFT1* seedlings. WT, *pft1-2* and *35S:PFT1* seedlings harboring the *DR5:uidA* gene construct were germinated and grown for 7 days in agar solidified 0.2X MS medium and then transferred for 8 h to liquid medium supplemented with or without IAA. Photographs show representative individuals of at least 15 GUS-stained seedlings analyzed. Note that *pft1-2* and *35S:PFT1* seedlings show stronger and weaker GUS activity, respectively, than WT seedlings. Scale bar = 200 µm.

Figure 6. Effects of NAA on root system architecture of WT (Col-0), *pft1-2* and *35S:PFT1* seedlings. Arabidopsis plants were germinated and grown for 7 days with or without NAA. (A) Relative primary root growth. (B) Lateral root number. (C) Relative lateral root formation (fold-induction). (D) Lateral root density. Primary root lengths at 0 µM NAA were 26 mm for Col-0, 30 mm for *pft1-2* and 19 mm for *35S:PFT1*. Error bars represent standard errors from 15 seedlings. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated two times with similar results.

Figure 7. *PIN1* expression in WT, *pft1-2* and *35S:PFT1* seedlings. *PIN1::PIN1::GFP*, *pft1-2/PIN1::PIN1::GFP* and *35S:PFT1/PIN1::PIN1::GFP* were germinated and grown on agar solidified 0.2X MS medium. Seven days after germination the seedlings were stained with propidium iodide and analyzed by confocal microscopy. (A-C) Primary root apical meristems. (D-F) Stage V LRP, (G-I) Stage VII LRP. Representative photographs of primary roots and lateral root primordia (n=10). Note the increase and decrease of *PIN1::PIN1::GFP* in *pft1-2* and *35S:PFT1* backgrounds, respectively. Scale bar = 100 μm.

Figure 8. Effect of NPA on root architecture in WT, *pft1-2* and *35S:PFT1* seedlings. Arabidopsis plants were germinated and grown for 8 days with or without NPA. (A) Relative primary root growth. (B) Lateral root number. (C) Relative lateral root formation. (D) Lateral root density. Primary root lengths at 0 μM NPA were 38 mm for Col-0, 40 mm for *pft1-2* and 27 mm for *35S:PFT1*. Error bars represent standard errors from 15 seedlings. Different letters indicate statistical differences at $P<0.05$. The experiment was repeated three times with similar results.

Figure 9. Effect of jasmonic acid on root development of WT, *pft1-2* and *coi1-1* seedlings. WT and *pft1-2* seedlings were germinated and grown for 4 d on 0.2X MS medium and homozygous *coi1-1* seedlings were selected from a *coi1-1/COI1*segregating population in medium supplemented with 4 μM JA. Four-day-old seedlings were transferred and grown side by side over the surface of 0.2X MS agar plates supplied or not with 4 μM JA, and primary root growth was measured every two days (A). (B) Relative primary root growth in response to 4 μM JA. (C) Lateral root numbers. (D) Relative lateral root formation. Error bars represent standard errors from 15 seedlings. Different letters indicate statistical differences at $P<0.05$. (E) Photographs of representative WT, *pft1-2* and *coi1-1* seedlings illustrating the phenotype in response to JA. Relative primary root growth and lateral root formation were analyzed 8 days after transfer to JA. The experiment was repeated three times with similar results. Scale bar = 1cm.



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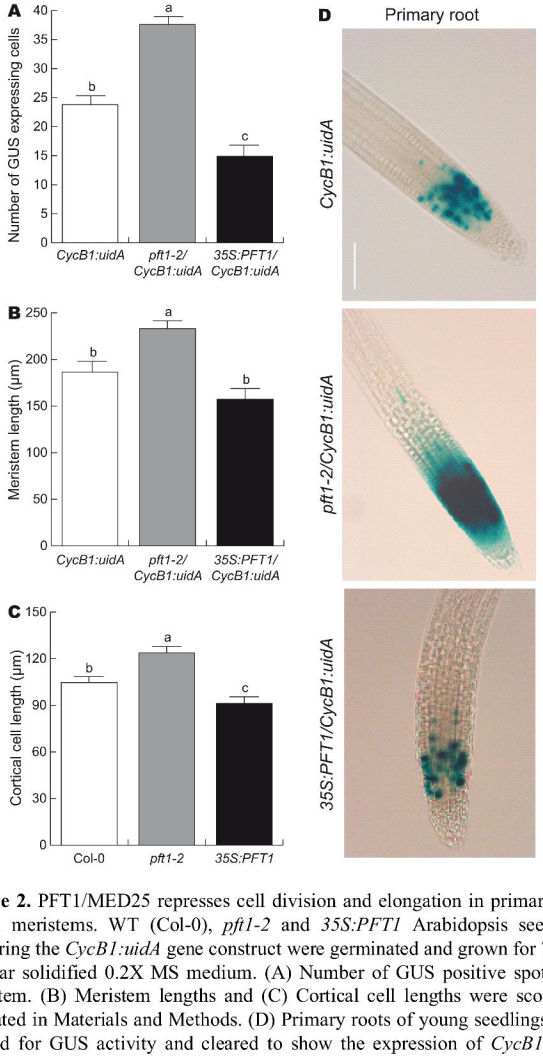

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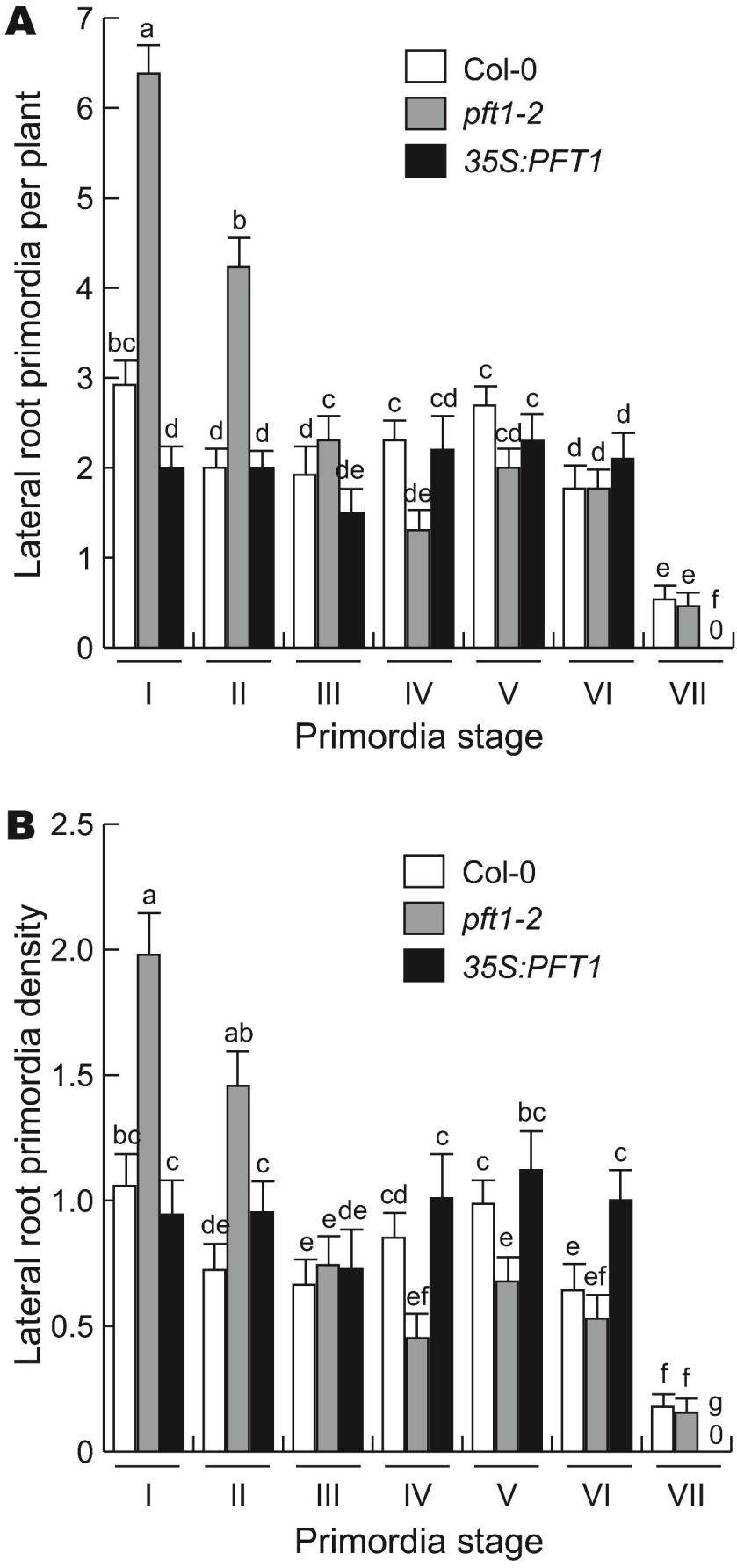

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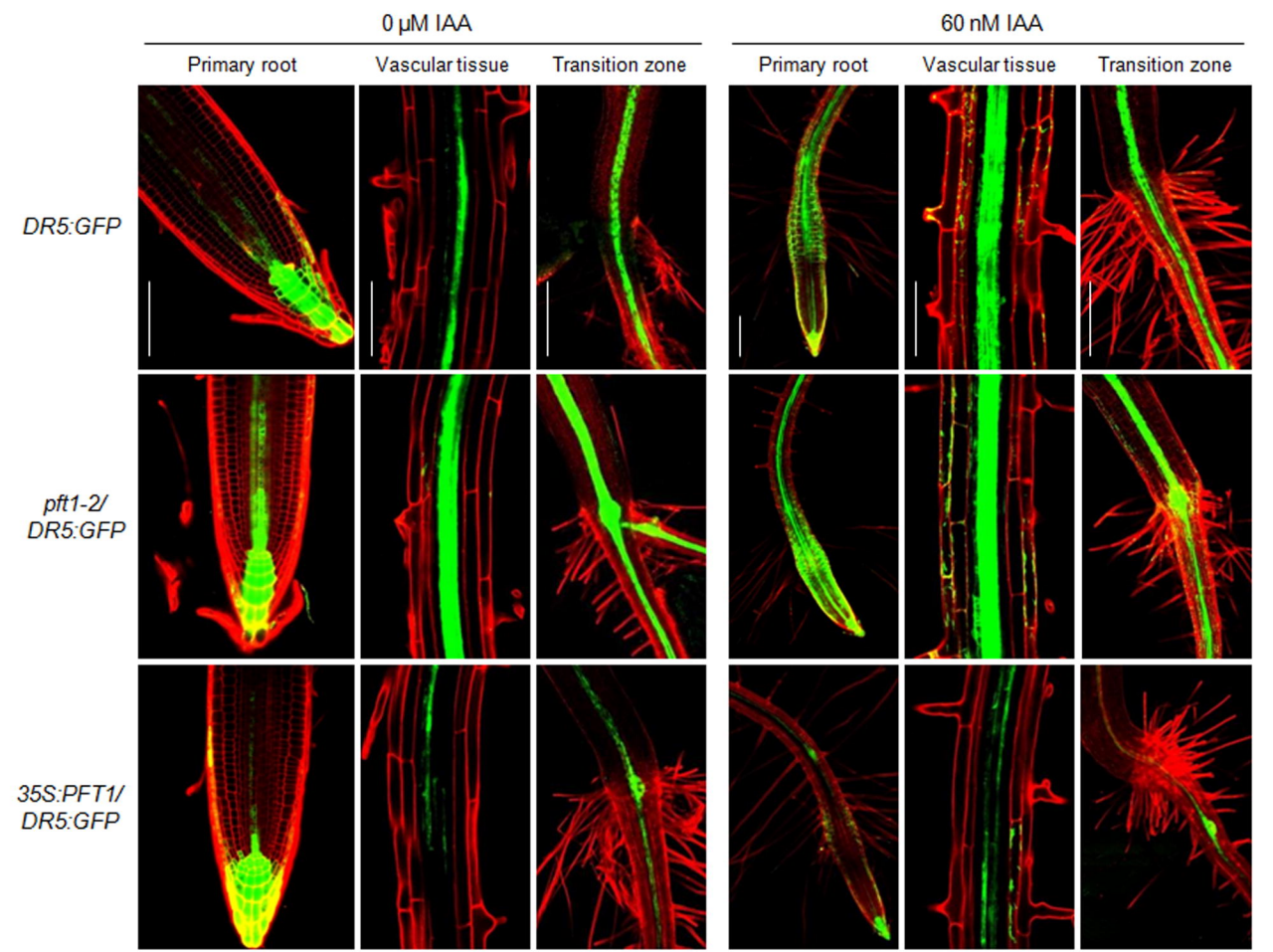

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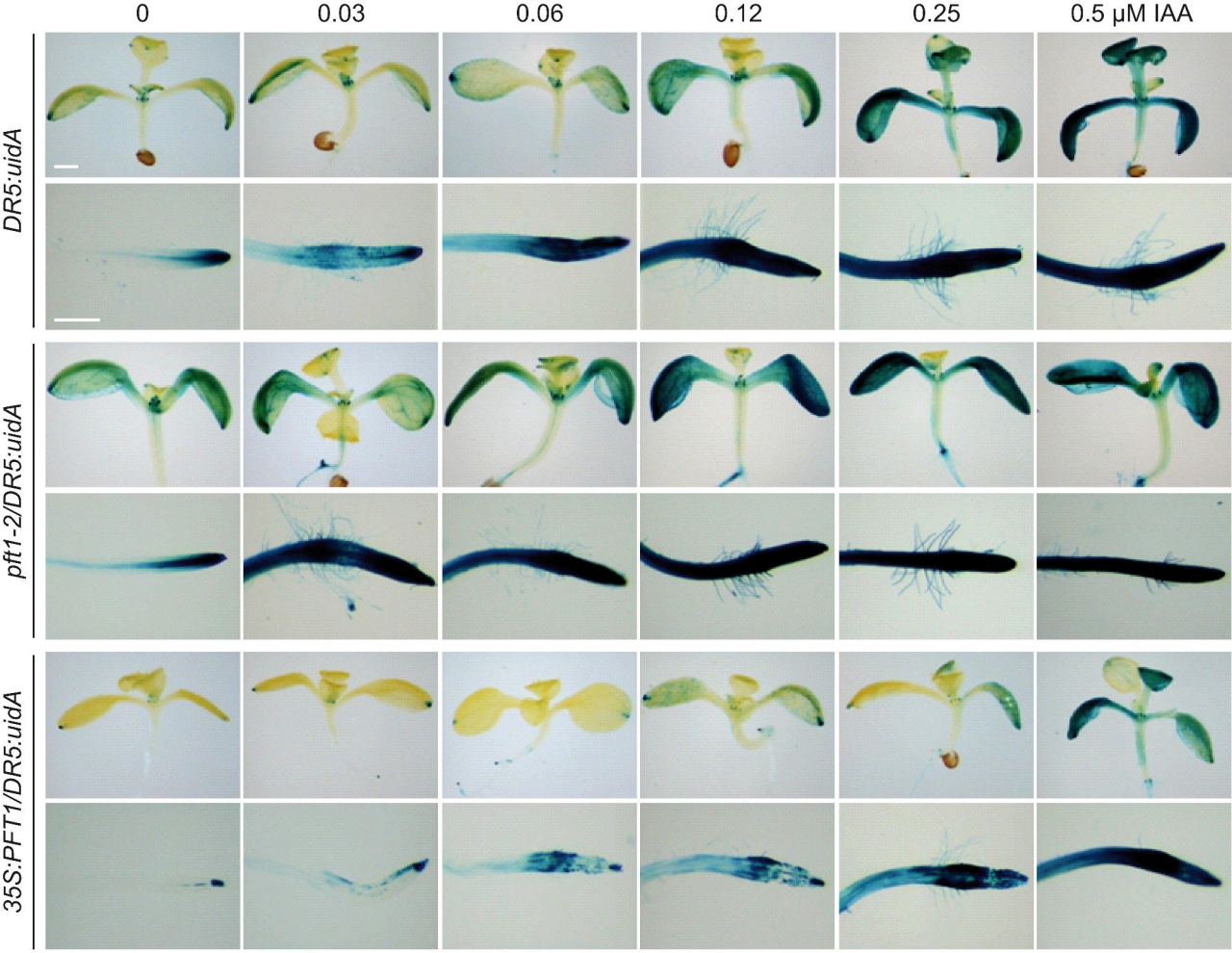

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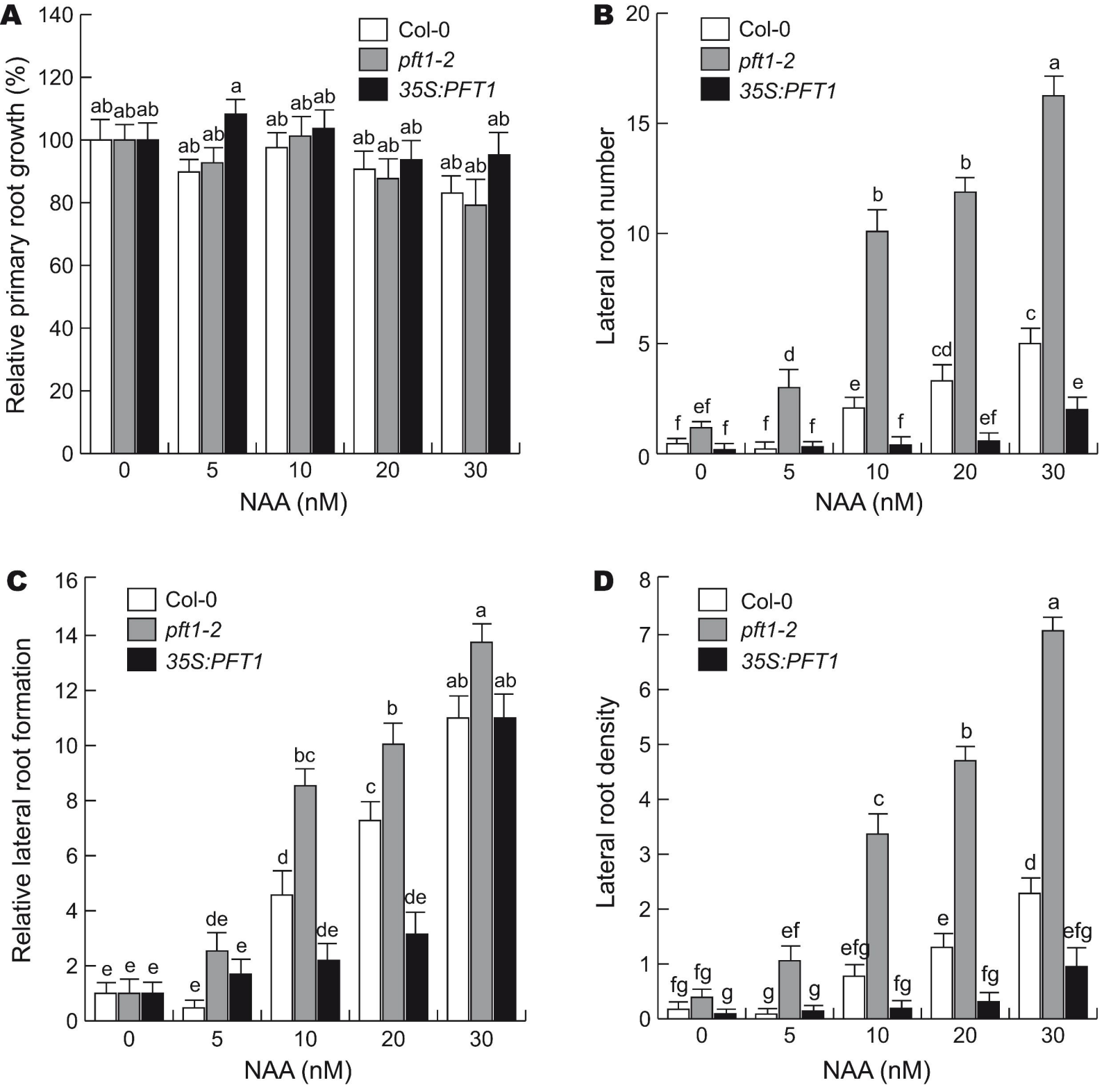

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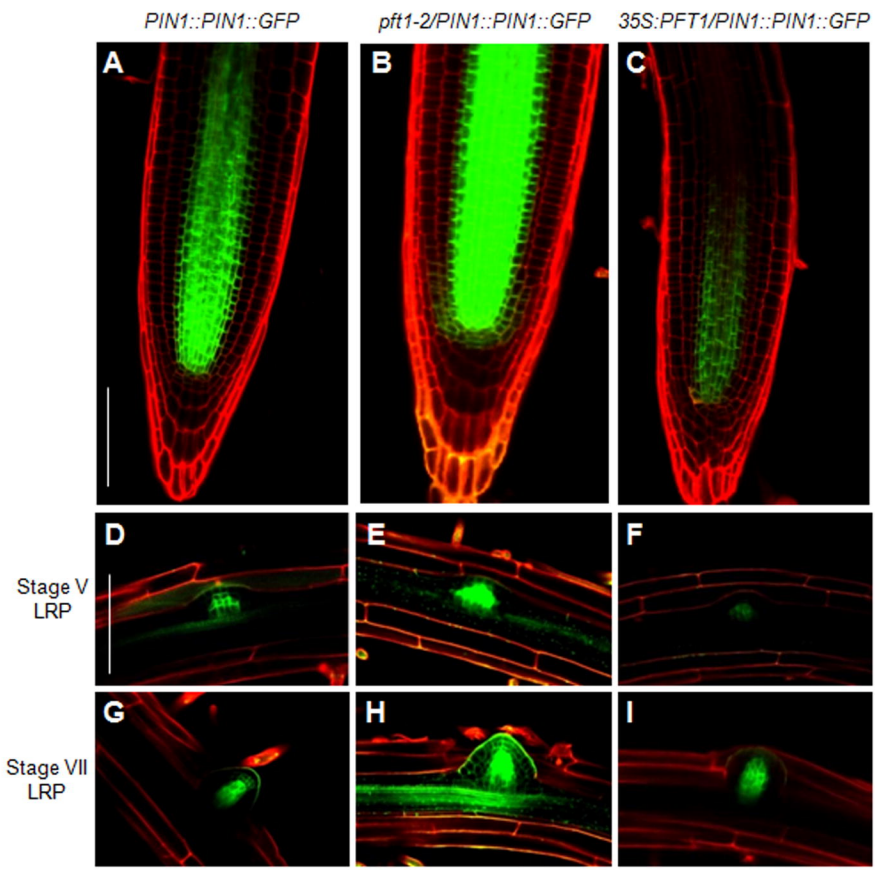

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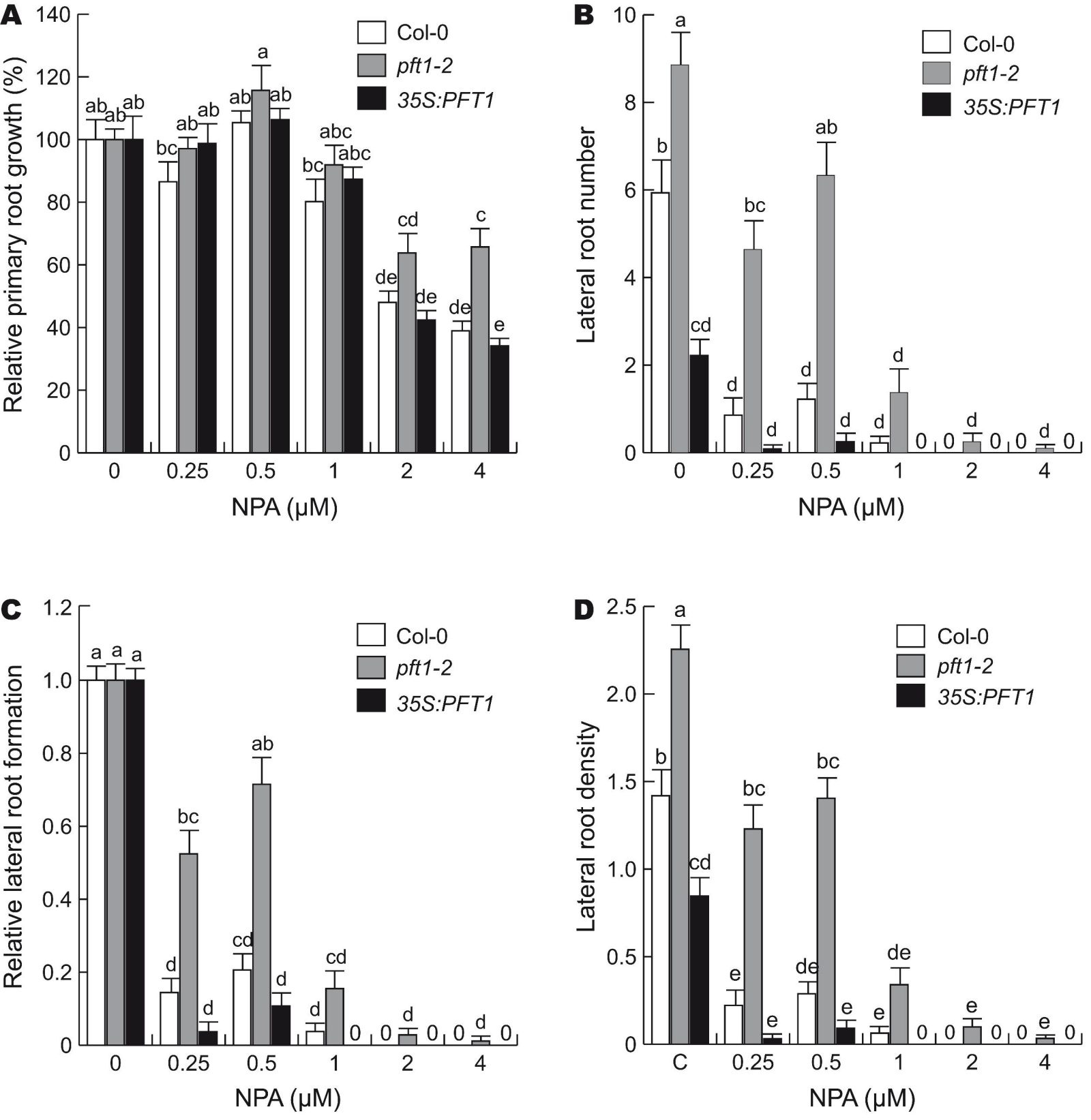

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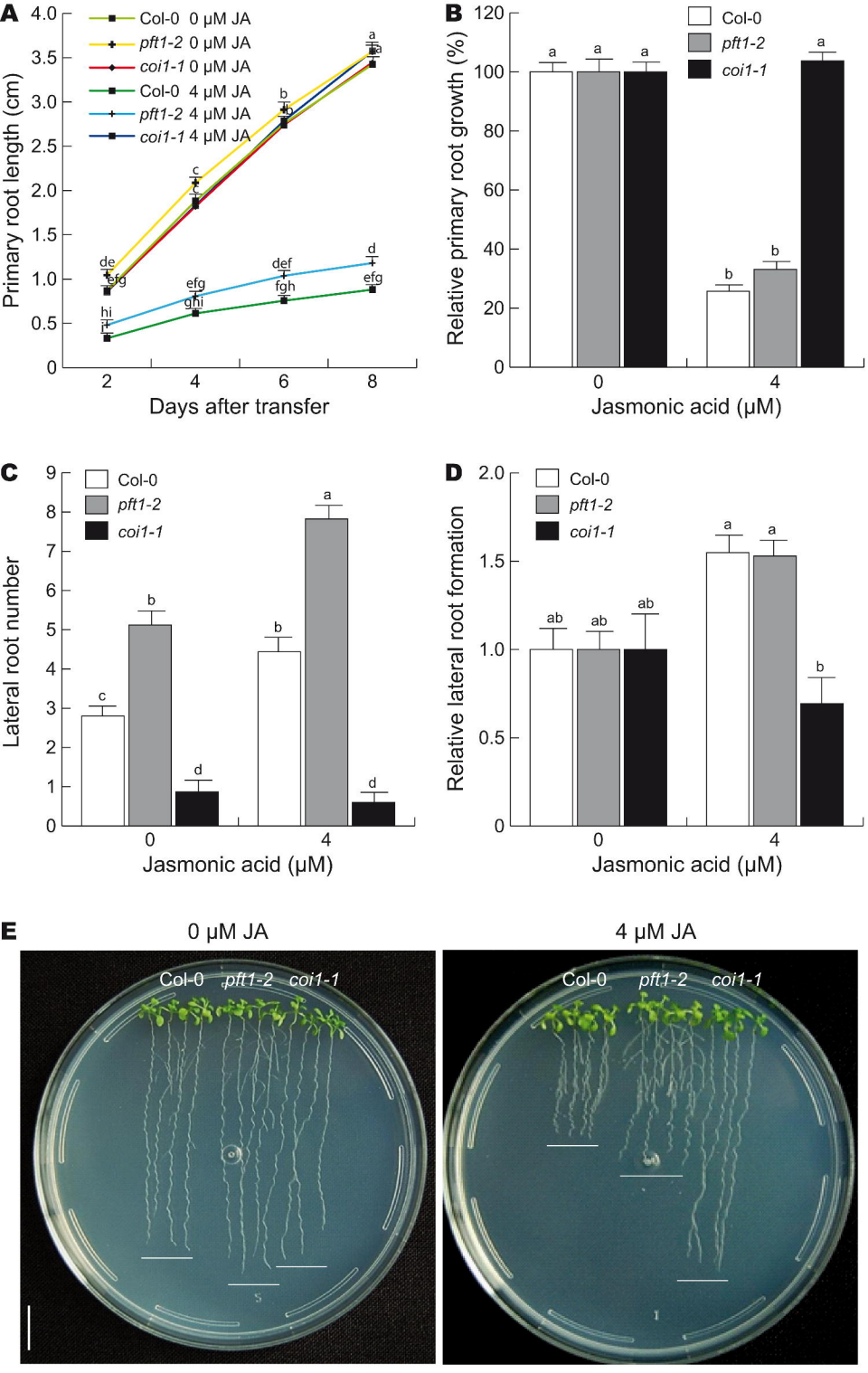

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