Auxin is involved in the regulation of leaf and root development by LAF1 under short day conditions

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Abstract

LAF1 (Long after far-red radiation 1) is a R2R3 Myb transcription factor and a signal transducer of far-red radiation. To investigate the role of LAF1 in leaf and root development, the leaf growth and vein patterning in *laf1* mutants under short day conditions were examined. The length of rosette leaves was reduced and the width of the midvein was increased in *laf1* mutants compared to their wild-type (WT) counterparts. In addition, cell size and cell number were both decreased in the *laf1* mutant in comparison to the WT plant. A comparative analysis of gene expression showed that the transcript levels of *PIN* and *IAA* genes, encoding auxin carrier and response proteins, were decreased in *laf1* mutants. *LAF1* expression was also shown to be induced by 1-naphthaleneacetic acid. These results suggest that both auxin transport and auxin responses are impaired in *laf1* mutants, and that LAF1 is involved in the regulation of leaf and root development mediated by auxin signaling under short day conditions.

Additional key words: IAA, midvein, mutant, NAA, PIN, short day.

Introduction

Plant growth and reproduction are primarily influenced by radiation quantity and quality, leading to the evolution of several different photoreceptors, including phytochromes (Chen *et al.* 2004). These receptors are potent regulators of plant development, affecting a broad range of responses throughout the plant life cycle, including hypocotyl elongation, leaf expansion and apical dominance. These responses are coordinated by interactions between light signals and various hormones.

Auxins participate in every aspect of plant growth and development and indole-3-acetic acid (IAA) is synthesized by at least two separate biosynthesis pathways (Barlier *et al*. 2000, Zhao *et al.* 2001, Cheng *et al*. 2006). *De novo* synthesized IAA is distributed to specific tissues by polar transport (Rubery and Sheldrake 1974, Raven 1975) and the polarity is regulated by the expression pattern and subcellular localization of auxin efflux associated proteins belonging to the AtPIN family (Paponov *et al*. 2005, Teale *et al*. 2006). PIN proteins have been suggested to participate directly in IAA transport or to help in the assembly of other proteins with efflux activity (Benjamins and Scheres 2008).

The plant vascular system is a continuous cellular network composed of the phloem and the xylem, and these vessels play a crucial role in plant development. Many essential substances, including photosynthates, are transported through the sieve elements of the phloem, which has triggered extensive research into the mechanisms of vascular tissue patterning and development in the leaf and the root. The involvement of IAA in vascular differentiation was discovered through a screen for mutants with characteristic vascular defects. Among others, mutations in the *MONOPTEROS* (*MP*)/*AUXIN RESPONSE FACTOR 5* (*AFR5*), *AUXIN*

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Abbreviations: FR - far-red; HFCA - 9-hydroxyfluorene-9-carboxylic acid; IAA - indole-3-acetic acid; NAA - 1-naphthaleneacetic acid; NPA - *N*[1]-naphtylphtalamic acid; RT-PCR - real time polymerase chain reaction; TIBA - 2,3,5-triiodobenzoic acid.
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RESISTANT 6 (*AXR6*), and *BODENLOS* (*BDL*)/*IAA12* genes resulted in incomplete vascular system development and defects in the formation of the embryo axis and embryonic root (Przemeck *et al*. 1996, Hamann *et al*. 1999, Hobbie *et al*. 2000).

In addition, the inhibition of IAA transporters showed that normal vascular strand patterning during *Arabidopsis* organogenesis depends on IAA-transport (Mattsson *et al*. 1999, Sieburth 1999). Additionally, these studies showed that IAA transport is tightly associated with vascular patterning (Sieburth and Deyholos 2006). At the same time, phytochrome-mediated responses have been shown to be linked to IAA (Cluis *et al.* 2004, Sibout *et al.* 2006). More recently, Salisbury *et al*. (2007) showed that phytochrome plays a role as a coordinator of shoot and root development, suggesting the presence of a tight link between phytochrome and auxins (Salisbury *et al*. 2007). There are five members of the *Arabidopsis* phytochrome protein family (phyA - phyE), but only phyA can perceive far red (FR) radiation signal (Sharrock and Quail 1989, Clack *et al.* 1994). After the perception, phyA signaling is modulated through its direct or indirect interaction with various factors, including LAF1, HFR1, SPA1, and COP1 (Chen *et al*. 2004, Seo *et al.* 2003, 2004, Jang *et al.* 2005, 2007). LAF1 is a R2R3-MYB transcription factor and its mutant *laf1* displays a long hypocotyl under FR (Ballesteros *et al*. 2001), suggesting that LAF1 functions as a positive regulatory component of phyA signaling. LAF1 is localized to nuclear speckles, and its stability is regulated by the activity of the E3 ubiquitin ligase COP1 in the COP1-SPA1 protein complex (Seo *et al.* 2003). Recently, it has been reported that LAF1 and HFR1 stabilize each other through COP-1 mediated inhibition of ubiquitination of them, thereby enhancing phyA photoresponses (Jang *et al.* 2007).

To investigate the specific roles of LAF1 in auxin-mediated leaf and root development, we analyzed the *laf1* mutant under short day conditions. Defective leaf development was detected in *laf1* mutants as changes in leaf size, midvein thickness, cell number and cell area when compared to wild type plants. *LAF1* expression was induced by synthetic auxin analogs such as 1-naphthaleneacetic acid (NAA), and transcript levels of *PIN* and *IAA* genes were decreased in *laf1* mutants.

Materials and methods

The *Arabidopsis thaliana* L. ecotype Landsberg *erecta* (L*er*) and its *laf1* mutant (Ballesteros *et al*. 2001) were used. Wild type (WT) and *laf1* mutant seeds were germinated and grown on Murashige and Skoog (MS) agar media. After two weeks, seedlings were transferred into MS liquid media and kept in this media for 2 d as an adaptation period. Treatment with NAA (*Sigma-Aldrich*, St. Louis, MO, USA) was carried out using samples grown on MS media. Liquid media were replaced with 2 μM NAA-containing liquid media and incubated for another 12 or 24 h in a growth chamber. Plants were grown in growth chambers under 16-h photoperiod, irradiance of 150 μmol m^{-2} s^{-1}, day/night temperature of 22/20 °C either on 0.75 % agar media containing Murashige and Skoog (MS) basal salts (*Sigma-Aldrich*, St. Louis MO), or Haughn and Somerville (1986) nutrient solution containing 0.5 g dm^{-3} 2-(N-morpholino) ethanesulfonic acid, and 10 g dm^{-3} sucrose, or mixture of *Metro-Mix 200* and *Vermiculite* (3:1; *Scotts*, Marysville, OH, USA). Samples were taken from the shoots or roots and frozen in liquid nitrogen for later use in RT-PCR. For the analysis of vein pattern and leaf size, 4-d-old seedlings were transferred onto the soil and grown for 4 weeks until flowering. Samples were taken from the shoots or roots and frozen in liquid nitrogen for later use in RT-PCR. For the analysis of vein pattern and leaf size, 4-d-old seedlings were transferred onto the soil and grown for 4 weeks until flowering.

To check the cell size and number, 4-d-old seedlings were transferred onto the soil and grown for 3 weeks under long day (16-h photoperiod) or short day (8-h photoperiod) conditions. For the measurement of root growth rate and lateral root growth, WT and *laf1* mutant seeds were germinated and grown on MS agar media. After 4 d, the seedlings were transferred into MS agar media with or without 2 μM NAA and grown for an additional 6 d.

Total RNAs were isolated from shoots or roots of wild-type plants and *laf1* mutant using *TRIzol* (*Gibco BRL*, Grand Island, NY, USA) according to the manufacturer's instructions. Total RNA (2 μg) was reverse-transcribed with 200 units of *SuperScript*™ (*Invitrogen*, San Diego, CA, USA). The resulting cDNA:RNA hybrids were treated with 10 units of DNase I (*Roche*, Waterville, OH, USA) for 30 min at 37 °C, purified on a *Qiaquick* PCR column (*Qiagen*, Germantown, MD, USA), and used as template for RT-PCR, which was conducted by using the MyiQ RT-PCR detection system (*Bio-Rad*, Hercules, CA, USA) and *iQ Sybergreen Supermix* (*Bio-Rad*). Primer sequences were
LAF1, 5'-TCTTCATCTCCCTCACAAGAAAGC-3'
and 5'-TCATAAGCTGAGAGAGTCTCTTCG-3';
PIN1, 5'-CGGCTATGAGATTTGTCGTTGGAC-3'
and 5'-AAGAGTTATGGGCAACGCGATCAA-3';
PIN2, 5'-CAGCCACCTCAATAGCAATTGGTA-3'
and 5'-AGCCCCAAAAGAACGTAGTAGAGT-3';
PIN3, 5'-TATAGCCATCGGATTACGTGGTGA-3'
and 5'-CGAGTAGAATGTAGTAAACCAGCG-3';
PIN7, 5'-TTCTTTACTGGACCAGCGGTAATG-3'
and 5'-AGTGTAATCGGTAGTGCGATAAGC-3';
IAA1, 5'-CAGAGCTTCTCAAAGCACTAGAGA-3'

and 5'-GGAGCTTCGGATCCTTTCATGATT-3';
IAA3, 5'-GCATGAGGGTCAAGGAATCTATGT-3'
and 5'-GAGCATCCAATCACCATCTTTGTC-3';
IAA7I, 5'-CGAGAGCAAGCTAATGAATCTGCT-3'
and 5'-CTTGCAGTACTTCTCCATTGCTCT-3';
IAA14, 5'-GAGTTATGGAGCACAAGGGATGAT-3'
and 5'-CATTGCTCTTGGAGCAAGTCCAAT-3'.

Primers for tubulin amplification (5'-GTGAGCGAA CAGTTCACAGCGATG-3' and 5'-GTGGAAGTGCAG GAGGAGCAATAA-3') were added as an internal control together with gene-specific primers. Amplified cDNA fragments were analyzed on 1.2 % agarose gels with *Gel Doc 1000* software (*Bio-Rad*).

Digital images of detached 7th and 8th leaves were acquired by using a scanner. Leaf area and lengths of a longitudinal and a maximum-width axes of leaf blades, as well as petiole length, were determined with the image-analyzing program *SCIONIMAGE* (*Scion Corporation*; http://www.scioncorp.com).

Leaves were fixed with an ethanol:acetic acid (6:1) solution for 4 h, washed with 100 % ethanol three times with gentle shaking and then with 70 % ethanol once, after which all tissues were cleared in a chloral-hydrate solution (8 g of chloral hydrate, 1 cm^3 of glycerol, and 2 cm^3 of distilled water) for at least 1 h, and then mounted on a glass slide. The microscopic images of leaf surface were obtained by using a *DIC* microscope (*Zeiss Axioplan*, Thornwood, NJ, USA). Subepidermal palisade cells aligned along the leaf maximum-width region were counted. To determine cell area, 20 cells that were grouped halfway from the midvein to the leaf margin at the widest point were analyzed with the *SCIONIMAGE* software. Vein diameters were determined at three different locations along the midvein axis: distal, medial, and proximal to the leaf base. The distal measurement was done at the branching point of the midvein. All of the experiments were repeated three times.

Results

The aim of this study was to investigate the potential role of LAF1 (AtMYB18, accession number: At4G25560) in leaf and root development, including the patterning of vascular tissues regulated by auxin. To address this question, we initially performed a phenotypic analysis of the *laf1* mutant grown in soil under long day or short day conditions. There was no difference in leaf growth between wild-type (WT) plants and *laf1* mutants under long day (Fig. 1*A*). However, under short day, whole plant size, as well as leaf size, was significantly decreased in *laf1* mutants compared to WT plants (Fig. 1*B*). The length and the width of the leaf were shorter and narrower in *laf1* mutants than in WT. Petiole length was also shorter in *laf1* mutants than in WT. Leaf area and leaf index were significantly decreased in *laf1* mutants (Table 1).

In general, leaf size depends on both cell size and cell number. First, to assess whether cell size was affected, the subepidermal palisade was observed under a light microscope. Interestingly, *laf1* mutation revealed leaf cells of reduced size in comparison to WT. The everage cell area of *laf1* mutants was approximately 73 % of that of WT, and the average number of cells of *laf1* mutants was about 83 % of that of WT (Table 1, see Materials and methods). This indicated that both reductions in cell size and cell number contributed to the smaller leaf size of *laf1* mutants.

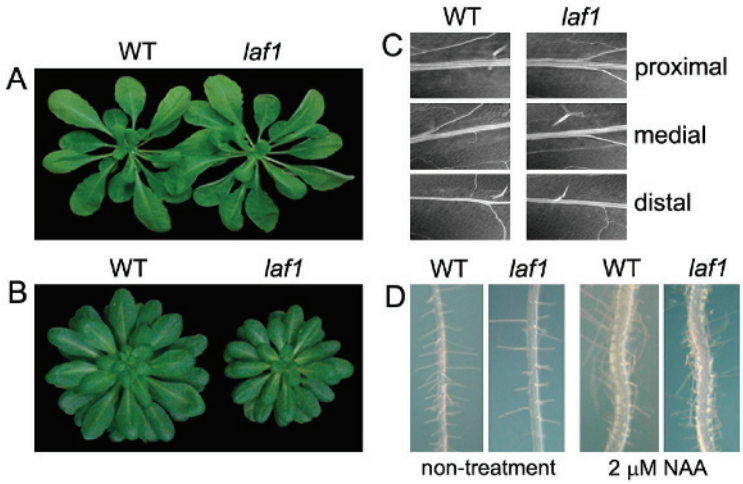

Fig. 1. Phenotypic characterization of *laf1* mutants. Phenotypes of the *laf1* mutant and WT plant grown in soil under long day conditions (*A*) or short day conditions (*B*). Proximal, medial and distal regions of leaf midveins were photographed in WT plants and *laf1* mutants grown in soil under short day conditions (*C*). The root phenotypes of WT and *laf1* mutant plants grown under short day conditions in the presence or absence of NAA (*D*).

Polar auxin transport inhibitors such as N^1-naphtylphtalamic acid (NPA), 9-hydroxyfluorene-9-carboxylic acid (HFCA) and 2,3,5-triiodobenzoic acid (TIBA) induced alterations in the midvein and in the veins adjacent to the leaf margin, and one of the most pronounced effects of these inhibitors was the thickening of the midvein. Treatment with inhibitors that interfere with auxin activity causes the same effects as polar auxin transport inhibitors. We therefore measured projected midvein width in wild type and *laf1* mutants grown in soil under short day conditions by light microscopy (Fig. 1*C*, Table 1). The midvein of the *laf1* mutant was unchanged at the proximal portion, but it was slightly increased in size at the central portion in comparison to the WT leaf. The distal region of the midvein showed a considerable increase in width in the *laf1* mutant compared to the WT (Table 1).

Table 1. Leaf characteristics of the WT and *laf1* mutant leaves. Means ± SE, $n = 10$; repeated three times with similar results.

Characteristics	WT	*laf1*
Leaf area [mm^2]	123.94 ± 6.29	54.73 ± 4.45
Leaf length [mm]	19.93 ± 0.40	13.00 ± 1.07
Leaf width [mm]	8.03 ± 0.34	5.11 ± 0.13
Leaf index	2.52 ± 0.14	2.92 ± 0.01
Petiole length [mm]	11.57 ± 0.45	7.66 ± 0.36
Cell area [μm^2]	4097.33 ± 327.1	3004.51 ± 243.5
Transverse cell number	113.83 ± 6.18	94.50 ± 3.30
Proximal midvein [μm]	99.74 ± 6.60	92.65 ± 2.35
Medial midvein [μm]	90.66 ± 2.86	95.10 ± 2.64
Distal midvein [μm]	56.77 ± 0.82	103.05 ± 3.81

Table 2. Effect of NAA (2 μM) applied for the indicated time on the root development in WT and *laf1* mutant plants. Means ± SE, $n = 20$; repeated three times with similar results.

Characteristics	Time [d]	NAA	WT	*laf1*
Primary root length [cm]	1	-	1.21 ± 0.13	0.82 ± 0.93
	1	+	0.27 ± 0.12	0.38 ± 0.11
	2	-	1.62 ± 0.14	1.22 ± 0.16
	2	+	0.38 ± 0.13	0.51 ± 0.12
	3	-	2.75 ± 0.21	2.06 ± 0.13
	3	+	0.93 ± 0.15	0.97 ± 0.25
Lateral root number	4	-	111.0 ± 6.60	53.0 ± 8.90
	4	+	139.6 ± 11.7	133.6 ± 22.4

Previous results have shown that auxin-related mutants have fewer lateral roots or display reduced root growth. We therefore examined the root growth of *laf1* mutants under short day conditions. Our results showed that the growth rate of the primary root of the *laf1* mutant was reduced in comparison to that of WT (Fig. 1*D*, Table 2). In addition, the number of lateral roots was reduced in *laf1* mutants compared to WT (Table 2). Recent results have shown that NAA induces adventitious rooting (Ďurkovič and Bukovská 2009). In addition, Peres *et al*. (2009) showed a positive correlation between ABA/IAA ratio and root length, suggesting that these two hormones interact in controlling root growth and that IAA inhibits root growth. We thus checked the root growth pattern after treatment with 2 μM NAA. The NAA-induced inhibition of primary root growth was slightly higher in WT than in *laf1* mutants (Table 2). Although this suggested that *laf1* mutants were less sensitive to NAA, there was no significant difference in lateral root formation between WT and *laf1* mutants (Fig. 1*D*, Table 2).

Table 3. Relative expression levels of *PIN* and *IAA* genes in the *laf1* mutant. Leaves and roots of the WT and *laf1* mutant grown in soil under short day conditions were harvested and total RNAs were isolated for the evaluation of transcript levels by real-time RT-PCR, using gene-specific primers. *A* - the transcript levels were normalized to a value of 1.00 for the transcript levels in the leaves or roots of the WT plants. *B* - The transcript levels were normalized to a value of 1.00 for the transcript levels of *PIN1* and *IAA1* in the leaves or roots of the WT plants. Means ± SE, $n = 3$; repeated three times with similar results.

Gene	Leaf		Root	
	WT	*laf1*	WT	*laf1*
A				
PIN1	1.00 ± 0.08	0.99 ± 0.05	1.00 ± 0.05	0.30 ± 0.01
PIN2	1.00 ± 0.13	0.53 ± 0.12	1.00 ± 0.08	0.53 ± 0.04
PIN3	1.00 ± 0.03	1.06 ± 0.11	1.00 ± 0.08	0.98 ± 0.05
PIN7	1.00 ± 0.07	0.92 ± 0.08	1.00 ± 0.09	1.14 ± 0.11
IAA1	1.00 ± 0.03	0.84 ± 0.01	1.00 ± 0.11	0.15 ± 0.01
IAA3	1.00 ± 0.03	1.08 ± 0.01	1.00 ± 0.04	0.21 ± 0.02
IAA7	1.00 ± 0.03	0.46 ± 0.02	1.00 ± 0.07	0.63 ± 0.06
IAA14	1.00 ± 0.04	0.74 ± 0.01	1.00 ± 0.11	0.23 ± 0.02
B				
PIN1	1.00 ± 0.09	0.99 ± 0.05	1.00 ± 0.06	0.30 ± 0.02
PIN2	0.46 ± 0.04	0.24 ± 0.05	2.35 ± 0.14	1.25 ± 0.08
PIN3	0.96 ± 0.06	1.00 ± 0.09	3.49 ± 0.31	3.41 ± 0.22
PIN7	1.02 ± 0.07	0.94 ± 0.07	2.76 ± 0.25	3.13 ± 0.32
IAA1	1.00 ± 0.03	0.84 ± 0.01	1.00 ± 0.06	0.15 ± 0.01
IAA3	0.32 ± 0.01	0.37 ± 0.01	4.84 ± 0.06	1.03 ± 0.06
IAA7	0.01 ± 0.00	0.00 ± 0.00	0.21 ± 0.01	0.13 ± 0.01
IAA14	0.25 ± 0.01	0.19 ± 0.00	0.29 ± 0.02	0.07 ± 0.00

We investigated the expression of auxin efflux-related *PIN* genes and auxin response *IAA* genes in *laf1* mutants grown in soil under short day conditions. Transcript levels of *PIN1*, *PIN3* and *PIN7* genes were similar in the leaves of *laf1* mutants and WT plants; however, *PIN2* expression was significantly lower in the leaf of the *laf1* mutant than in the WT. In the root, the transcript

Table 4. *LAF1* expression induced by NAA. WT plants were treated with 2 μM NAA, and the leaves and roots were harvested at the indicated time points. Total RNAs were isolated and relative transcript levels were determined by real-time RT-PCR using *LAF1* primers. The transcript levels were normalized to numerical values based on a value of 1.00 for the transcript levels present in the leaves or roots at 0 h. Means ± SE, $n = 3$; repeated three times with similar results.

Time [h]	Leaf [fold]	Root [fold]
0	1.00 ± 0.05	1.00 ± 0.06
12	3.18 ± 0.10	4.00 ± 1.11
24	3.91 ± 0.02	4.44 ± 0.27

levels of *PIN1* and *PIN2* genes were lower in the *laf1* mutant, while *PIN3* and *PIN7* expression were comparable in the *laf1* mutant and WT. We also examined the expression of auxin-responsive genes in the leaf and root of the *laf1* mutants and WT plants (Table 3). The expression of *IAA1* and *IAA14* was lower in the leaf of the *laf1* mutants, and the transcript level of IAA7 was about half of that in WT. On the other hand, *IAA3* expression was the same in the two plant types. The transcript levels of *IAA1*, *IAA3* and *IAA14* genes were lower in the roots of the *laf1* mutant. The transcript level of *IAA7* was also decreased, but by a smaller amount. *LAF1* expression was also induced by auxin, which established that LAF expression is regulated by auxin (Table 4).

Discussion

In this study, we show that LAF1 can act as a positive regulator of auxin-mediated leaf and root tissue development. Phytochromes are tightly connected with IAA signaling, and previous work has shown that phytochromes regulate a subset of IAA-responsive genes, including *IAA1* and *IAA3* (Tian *et al.* 2002, Devlin *et al.* 2003), as well as components of the IAA transport machinery, including *PIN3* and *PIN7* (Sidler *et al.* 1998, Devlin *et al.* 2003, Lin and Wang 2005). Phytochrome B interacts with IAA3, a known auxin and phyB signaling component (Tian *et al.* 2003).The shoots of the *phyA-phyB* null mutant have higher contents of *IAA1* and *IAA3* mRNA, whereas the roots have slightly lower contents of these same mRNAs. The transcription factor HY5 binds to the *AXR2* promoter *in vitro,* and *IAA14*/*IAA28* and *AXR2*/*IAA7* gene expression is reduced in *hy5* mutants (Cluis *et al.* 2004). In addition, cryptochromes regulate root growth by controlling IAA transport (Canamero *et al.* 2006). Salisbury *et al*. (2007) have shown that phytochrome regulates lateral root emergence by manipulating IAA distribution, thereby showing that the shoot-localized form of phytochrome can act over long distances through IAA to control root development. More recently, irradiance has been shown to play an important role in the intracellular localization of the IAA efflux regulator PIN2, demonstrating that root development could be regulated by changes in the intracellular localization of PIN proteins according to irradiance (Laxmi *et al*. 2008). Taken together, these results indicate that light signaling and auxin responses are tightly linked in a signaling network.

Sieburth *et al*. (1999) showed that a block in IAA transport or the inhibition of IAA activity causes a thickening of the primary vein. In this study, *laf1* mutants displayed a much wider distal midvein when grown in soil under short day conditions (Table 1), suggesting that LAF1 is involved in IAA transport, even if the phenotype is mainly restricted to the distal region of leaf vascular tissue.

Microscopy showed that the cell number was decreased in *laf1* mutants grown under short day conditions, together with a smaller total cell area (Table 1). From these results, we can conclude that the decrease in the leaf size of *laf1* mutants is a consequence of the abnormal development of vascular tissue (Fig. 1*C*, Table 1) brought about by the LAF1 mutant protein.

The expression of nearly all of the genes tested in this experiment was changed by the LAF1 mutation, although the basal expression levels of the genes were different. Among IAA efflux-related genes, *PIN2* gene expression was much lower in the leaves of the *laf1* mutant than in the WT plant (Table 3). In addition, the expression of *PIN1* and *PIN2* were dramatically decreased in the roots of the *laf1* mutant (Table 3). Laxmi *et al*. (2008) have shown that FR changed the intracellular distribution of PIN2 from the plasma membrane to vacuoles in the root. Taken together, the results indicate that phy A signaling through LAF protein possibly regulates the localization and transcription of PIN proteins, including PIN2. Most genes, except for *IAA3,* were down-regulated in the *laf1* mutant with a more pronounced effect in the root, except for *IAA7* (Table 3). These results indicate that all *IAA* genes are involved in the regulation of the auxin response by LAF1 protein; therefore auxin efflux and auxin response genes are all affected by mutation of LAF1, with variable effects depending on the individual gene. Induction of *LAF1* expression by auxin (Table 4) also supports the notion that LAF1 can regulate auxin signaling by controlling auxin efflux and auxin response-related gene expression.

The data in this paper provide insights into how LAF1, a signal transducer of phyA, coordinates shoot and root development by affecting auxin signaling under short day conditions. Our results also support previous reports that auxin signaling is connected with light signaling.

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