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Overexpression of 3β-Hydroxysteroid Dehydrogenases/C-4 Decarboxylases Causes Growth Defects Possibly Due to Abnormal Auxin Transport in Arabidopsis

Bokyung Kim[1], Gyusik Kim[1], Shozo Fujioka[2], Suguru Takatsuto[3], and Sunghwa Choe[1,4,*]

Sterols play crucial roles as membrane components and precursors of steroid hormones (e.g., brassinosteroids, BR). Within membranes, sterols regulate membrane permeability and fluidity by interacting with other lipids and proteins. Sterols are frequently enriched in detergent-insoluble membranes (DIMs), which organize molecules involved in specialized signaling processes, including auxin transporters. To be fully functional, the two methyl groups at the C-4 position of cycloartenol, a precursor of plant sterols, must be removed by bifunctional 3β-hydroxysteroid dehydrogenases/C-4 decarboxylases (3βHSD/D). To understand the role of *3βHSD/D* in Arabidopsis development, we analyzed the phenotypes of knock-out mutants and overexpression lines of two *3βHSD/D* genes (At1g47290 and At2g26260). Neither single nor double knock-out mutants displayed a noticeable phenotype; however, overexpression consistently resulted in plants with wrinkled leaves and short inflorescence internodes. Interestingly, the internode growth defects were opportunistic; even within a plant, some stems were more severely affected than others. Endogenous levels of BRs were not altered in the overexpression lines, suggesting that the growth defect is not primarily due to a flaw in BR biosynthesis. To determine if overexpression of the sterol biosynthetic genes affects the functions of membrane-localized auxin transporters, we subjected plants to the auxin efflux carrier inhibitor, 1-N-naphthylphthalamic acid (NPA). Whereas the gravity vectors of wild-type roots became randomly scattered in response to NPA treatment, those of the overexpression lines continued to grow in the direction of gravity. Overexpression of the two Arabidopsis *3βHSD/D* genes thus appears to affect auxin transporter activity, possibly by altering sterol composition in the membranes.

INTRODUCTION

Sterols are important components of membranes and play crucial roles in the growth, differentiation, and development of eukaryotic organisms. Yeast, higher plants, and animals have different forms of major sterols, i.e., ergosterol, sitosterol, and cholesterol, respectively (Benveniste, 2004). Plant sterols, including sitosterol, stigmasterol, 24-methylcholesterol, and campesterol (CR), regulate membrane permeability and fluidity by interacting with other lipids and proteins within the membranes (Hartmann, 1998; Kim et al., 2011). Sterols are often enriched in detergent-insoluble membranes (DIMs), which are thought to organize specialized signaling proteins at specific positions within the plasma membrane (Simon-Plas et al., 2011). DIMs are thought to play a role in signaling processes that involve Pathogen-Associated Molecular Patterns (PAMPs) (Bhat et al., 2005) and auxin transporters (Hanzal-Bayer and Hancock, 2007; Titapiwatanakun et al., 2009). In addition, sterols serve as biosynthetic precursors of growth-promoting steroid hormones, such as brassinosteroids (BRs) in plants (Kwon and Choe, 2005).

The biosynthesis of plant sterols from the initial precursor squalene involves up to 13 enzymatic steps (Schaller, 2004). Squalene is cyclized to produce the first 4-ring structure, named cycloartenol (Darnet and Rahier, 2004). To become a functional sterol, the two methyl groups at the C-4 position of cycloartenol should be removed by an enzyme complex that includes a sterol 4α-methyl oxidase (SMO) (Darnet and Rahier, 2004). In Arabidopsis, two redundant genes encoding bifunctional 3β-hydroxysteroid dehydrogenases/C-4 decarboxylases (3βHSD/D) were shown to be responsible for this step (Rahier et al., 2006).

The plant growth promoting steroid hormones, brassinosteroids (BRs), regulate cell division (Cheon et al., 2010; Ibanes et al., 2009), cell growth (Clouse and Sasse, 1998), and stress responses (Krishna, 2003). BR biosynthesis is affected by both

[1]School of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-747, Korea, [2]RIKEN Advanced Science Institute, Wako-shi, Saitama 351-0198, Japan, [3]Department of Chemistry, Joetsu University of Education, Niigata 943-8512, Japan, [4]Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-921, Korea
*Correspondence: shchoe@snu.ac.kr

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an endogenous signal, such as auxin (Chung et al., 2011; Maharjan et al., 2011), and an exogenous cue, such as temperature (Gray et al., 1998; Maharjan and Choe, 2011). In the BR biosynthetic pathways, 3βHSD/D epimerizes the hydroxyl group at the C-3 of CR to convert it to brassinolide (BL), the most bioactive BR compound (Fujioka and Yokota, 2003; Kwon and Choe, 2005); however, the gene governing this step has not been identified. Mutants that are defective in either sterol or BR pathways highlighted the importance of these compounds in plant development. Sterol mutants, such as *cyclopropylsterol isomerase* (*cpi*) (Willemsen et al., 2003), *fackel* (*fk*) (Jang et al., 2000; Schrick et al., 2000), *cytochrome P450 51A2* (*cyp51A2*) (Kim et al., 2010), and *hydra1*(*hyd1*) (Souter et al., 2002), display severe growth defects, partly due to abnormal positioning of PIN2 in response to developmental signals (Men et al., 2008). By contrast, BR-deficient mutants, such as *dwf7* (Choe et al., 1999b), *dwf1* (Choe et al., 1999a), *dwf5* (Choe et al., 2000), *det2* (Li et al., 1996), *dwf4* (Choe et al., 1998), and *cpd* (Szekeres et al., 1996), and a double *cyp85a1* and *cyp85a2* mutant (Kim et al., 2008), display characteristic dwarf phenotypes that can be rescued by exogenous treatment with BRs. However, the phenotype of sterol mutants (e.g., *fackel*, *hydra1*, and *cyp51A2*) was not restored to wild type by BRs, which indicates the essential role of sterols in plant development. Because the sterol mutants had an embryonic lethal phenotype, it is difficult to establish the effects of these mutations on later developmental processes.

Previously, Rahier et al. (2006) reported that two Arabidopsis genes encode 3β-hydroxysteroid dehydrogenases/C-4 decarboxylases (*3βHSD/D)*, i.e., *3βHSD/D1* (*At1g47290*) and *3βHSD/D2* (*At2g26260*), and found that these genes can complement a deficiency of 4α-methyl oxidase function in the yeast *erg25* mutant. However, genetic or transgenic analysis of these genes has yet to be conducted. In this study, we report the importance of these genes in Arabidopsis development by analyzing both knock-out mutants and overexpression lines. The transgenic lines exhibit phenotypes that specifically involve inflorescence internodes. The overexpression lines showed different degrees of internode shortening that depended on the degree of *3βHSD/D1* and/or *3βHSD/D2* expression. Our data provide evidence that *3βHSD/Ds* are important to maintain sterol quantity and/or composition at optimal levels which are essential for proper growth and development of Arabidopsis plants.

MATERIALS AND METHODS

Plant growth conditions and the gravitropism assay

Seeds were surface sterilized before being plated on 1/2 MS (Duchefa, Netherland) medium containing 1% (w/v) sucrose and 0.7% (w/v) plant agar. After stratification at 4°C for 3 days, seedlings were grown under long-day conditions (16 h light/8 h dark) at 22°C. Two days after germination, seedlings were transferred to hormone media and grown vertically for 4 more days. The concentration of growth regulators was 10^{-7} and 10^{-8} M for epi-BL and 5×10^{-6} M for NPA. Because NPA was dissolved in DMSO for stock solution, we added equal amount of DMSO to the control and consider it as mock treatment. To measure the orientation of the root tip, photographs of square plates were taken and analyzed using ImageJ (http://rsbweb.nih.gov/ij). The number of seedlings with the following root orientations was counted: 45°-135°, 135°-225°, 225°-315°, and 315°-45°.

Table 1.

Gene		Primer sequences (5' to 3')
For RT_PCR		
Actin	F	AGTGTGTCTTGTCTTATCTGGTTCG
(At5g09810)	R	AATAGCTGCATTGTCACCCGATACT
DWF4	F	AGATGTTCGGTACAAAGGATACGATATC
(At3g50660)	R	GTTTATCATCTTCTGCTAATTCCCAATTG
AtCYP85A2	F	TGTGGTTGGGATGATCTTGA
(At3g30180)	R	CTCCACTGCGGTAATTCGTT
At1g47290	F	GCAGCATATTTGGTCCTGGT
	R	CCAGCAGCTTTTGCACATAC
At2g26260	F	TTTTTGGTCCCGGTGATAGA
	R	ATATGCCTGTCCTGCAGCTT
At2g33630	F	TCACTGGAGGTCTTGGCTTC
	R	GCAAAACACAGTCTGCTCCA
At2g43420	F	ATCTGCAGCCCAACTTCTGT
	R	TCCAGTCTCCTCCACTGCTT
For cloning		
At1g47290	F	CACCATGGTGATGGAAGTTACAGGAGACTG
	R	GTCGATCTTCTTGCTCCCGAACA
At2g26260	F	CACCATGTCGCCGGCAGCTACG
	R	TTAGTCATGTTTCTTGCTTCCGAAC
At2g33630	F	CACC ATGCATTTGAGTGAGAATGAAG
	R	AATCTGGTGCTCTTTCGCT
At2g43420	F	CACC ATGGACGAAGATTCCGTCC
	R	CATGAACATTGGTTTAGATGAAG

Cloning of Arabidopsis *3β-HSD* cDNAs

The coding sequences of the four *3β-HSDs* genes were PCR amplified using the primers listed in Table 1 and cloned into the pENTR/SD/D-TOPO Gateway vector (Invitrogen, USA). Clones with PCR errors were eliminated by sequencing before proceeding to the next step. cDNAs in the entry vector were transferred to the pEarley101 destination vector for overexpression in Arabidopsis (Earley et al., 2006) and the pYES_DEST52 vector (Invitrogen, USA) for overexpression in yeast.

Total RNA preparation and semi-quantitative RT-PCR analysis

Seven-day-old seedlings of wild-type and overexpression lines were grown in the light and harvested for RNA preparation using TRI Reagent (Molecular Research Center, USA). Three micrograms of total RNA was reverse-transcribed using M-MuLV Reverse Transcriptase (Fermentas, USA). Equal amounts of RT product were used for the PCR reactions. Each template RNA was normalized using the Arabidopsis Actin gene (*At5g09810*) as a loading control. The oligonucleotide sequences used for RT-PCR are listed in Table 1.

Quantitative analysis of endogenous BRs and sterols by gas chromatography-mass spectrometry (GC-MS)

To determine the endogenous levels of both sterols and BRs, the aerial parts of 5-week-old Arabidopsis plants were harvested to obtain 30 g of fresh tissues, which was frozen in liquid nitrogen and immediately lyophilized at -80˚C. The tissues were

A

	At1g47290	At2g26260	HSD3B1	ERG26
At1g47290	100			
At2g26260	55.7	100		
HSD3B1	24.1	17.2	100	
ERG26	31.9	21.4	24.5	100

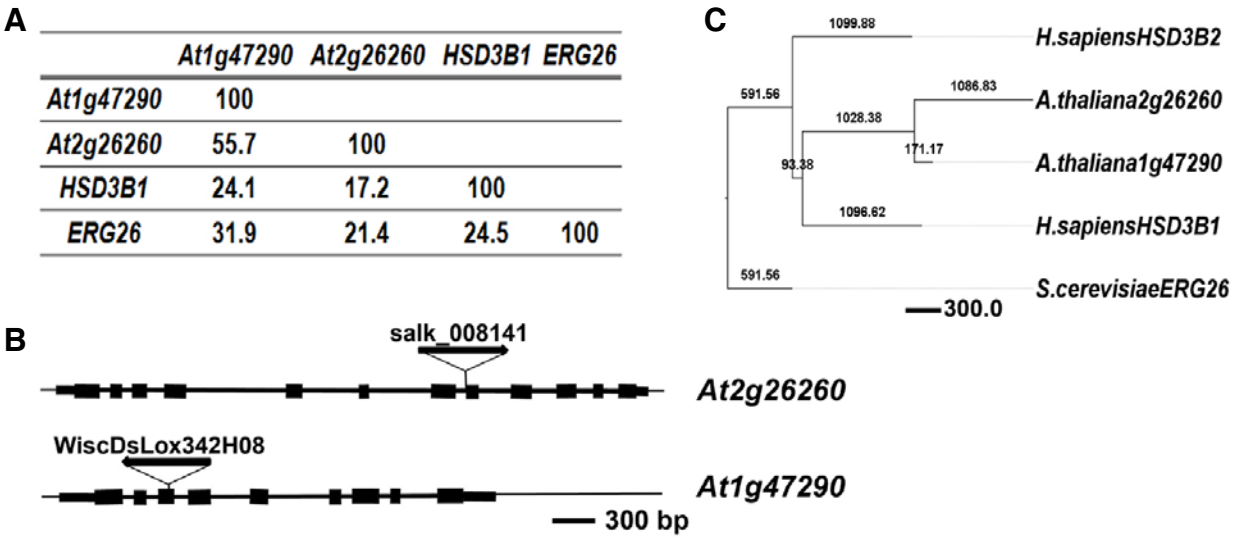

Fig. 1. Phylogenetic analysis of 3β-hydroxysteroid dehydrogenases/ C-4 decarboxylases from yeast, Arabidopsis, and humans. (A) The percent identity between Arabidopsis 3β-hydroxysteroid dehydrogenase/C-4 decarboxylases (3βHSD/D) and the homologs from human and yeast. The numbers indicate percent identity based on multiple alignments of the protein sequences using ClustalW. (B) The phylogenetic tree was generated using Jalview and Figtree 1.3.1 programs. Bootstrap values based on 100 replicates (from 10,000 trials) are shown at the branching points. Scale bar = length of bootstrap value 100. (C) Schematic representation of *At2g26260* and *At1g47290*. The positions of T-DNA inserts are shown with the line numbers. GenBank accession numbers are NP_000853 for human HSD3B1, NP_000189 for human HSD3B2, YGL001C for yeast ERG26, NM_103623 for Arabidopsis At1g47290 (3βHSD/D1), and NM_128183 for Arabidopsis At2g26260 (3βHSD/D2).

extracted twice with 300 ml of MeOH. Deuterium-labeled internal standards synthesized in our lab were added to the extracts. Purification and quantification of sterols and BRs were performed according to the method described previously (Fujioka et al., 2002; Kim et al., 2008).

RESULTS AND DISCUSSION

Two closely related Arabidopsis *3βHSD/D* genes

Based on previous findings that Arabidopsis has multiple homologs of *3βHSD/D* (Rahier et al., 2006), we performed *in silico* screening of the NCBI protein database using the sequence of human hydroxy-delta-5-steroid dehydrogenase 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase (*HSD3B1*) as a query. The protein sequences deduced from yeast *ERG26* and Arabidopsis *At1g47290, At2g26260*, *At2g43420*, and *At2g33630* emerged as the five sequences with the highest sequence similarity. Multiple sequence alignment analysis revealed that amino acid residues Aspartic acid-39, Tyrosine-159, and Lysine-163 of yeast ERG26 are well conserved in these protein sequences. Since we aimed to understand the effects of the sterol-3-hydroxylase genes in Arabidopsis, we chose to focus on the two functionally characterized genes, *At1g47290* and *At2g26260*. According to the previous report, we will call *At1g47290* as *3βHSD/D1* and *At2g26260* as *3βHSD/D2* throughout the manuscript.

The percent identity between human *HSD3B1* and the Arabidopsis and yeast protein sequences are presented in Fig. 1A. A phylogenetic tree based on this sequence comparison revealed that *At1g47290* was closer to human *HSD3B1* than *At2g26260* (Fig. 1B). To identify knock-out mutants of these genes, we searched the Arabidopsis database (http://www.arabidopsis.org). We found that the SALK_008141 and WiscDsLox342H08 lines have a T-DNA insertion in the *At2g26260* and *At1g47290* loci, respectively. We identified homozygous lines for these T-DNA insertional mutants by a combination of segregation analysis and PCR-based genotyping. Figure 1C illustrates the position of T-DNA insertion events in these two loci.

Lack of visible phenotype in the double knock-out mutant

To understand the role of these two *3βHSD/D* genes in Arabidopsis development, we examined the phenotypes of single and double mutants of these loci. A single mutant for each of these genes did not display a visible phenotype at any point in the life cycle (data not shown). Thus, we generated a double mutant by crossing the two single mutants. After identifying a putative double mutant based on segregation analysis of the antibiotic marker genes in the T-DNA, we confirmed that this plant was indeed a double mutant by PCR amplification of the sequence surrounding the insertion sites. Fig. 2A shows a schematic representation of these loci and the positions of the T-DNA insertion as well as the primer binding sites. An amplification product was only obtained from the wild-type DNA (Fig. 2A), suggesting that the isolated line was homozygous for both of these knock-out genes.

To detect any phenotypic differences between the wild type and the double mutant, we grew the seedlings on agar-solidified media. The root lengths were not significantly different ($p < 0.05$, $n > 15$) (Fig. 2B). Furthermore, the overall morphologies of the seedlings and adult plant were not noticeably different (Fig. 2C).

Short internode phenotype during late developmental stages

To examine the function of these genes using inverse approaches, we generated gain-of-function lines by overexpressing these genes. We first evaluated the morphologies of independent transformants harboring the overexpression construct of the

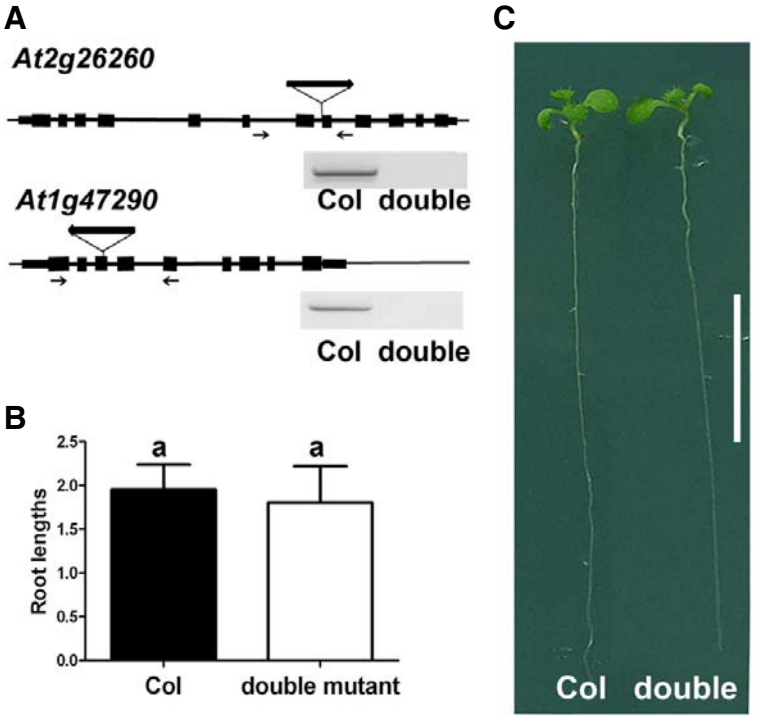

Fig. 2. Phenotypic comparison of 7-day-old seedlings of the wild type and double mutant of *At2g26260* and *At1g47290*. (A) Confirmation of the double mutant by showing failure to amplify the DNA around the T-DNA insertion sites. PCR amplifications (shown beneath the diagrams) were performed using the primer pairs shown. DNA amplification was successful only with wild-type DNA, but not with double mutant DNA (double). (B) Average root lengths of the wild type and double mutant. Letter 'a' indicates no statistical difference according to Student's *t*-test ($p < 0.05$, $n > 10$). Error bars represent standard deviation. (C) Morphology of the whole seedlings grown for 1 week under the long-day condition. Bar = 1 cm.

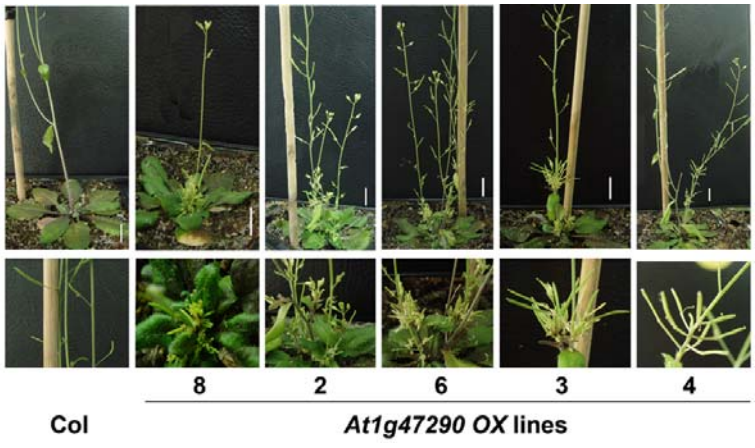

Fig. 3. Representative phenotypes of five independent *At1g47290 OX* lines and the wild type at 5 weeks post germination. The T1 generations are shown. OX lines consistently displayed characteristic phenotypes of opportunistically shortened internodes. For instance, one of the stems of inflorescences in a single line (number 8) appears normal, but the others are greatly affected and show extremely clustered internodes. Similar phenotypes are shown for other lines. The bottom row shows magnified views of the abnormal parts. Bar = 1 cm.

At1g47290 gene (*At1g47290 OX*). At the T1 stage, many of the transgenic lines consistently showed growth retardation (Fig. 3). The five independent lines showed phenotypes of opportunistic suppression of inflorescences. Even within one plant, the growth of some stems was relatively more suppressed than others (Fig. 3). The expansion of leaf blades was unequal toward the long axis, resulting in wrinkled leaves. In addition, the internodes were greatly shortened, causing siliques to aggregate (Fig. 3).

To examine the morphologies of the plants from earlier stages, we obtained homozygous lines for the transgenes at the T3 generation. These transgenic lines expressed higher levels of the transgene than of the nascent genes (Fig. 4A). This finding suggests that the transgenes did not cause co-suppression of the nascent genes. From 1 to 7 days post germination, visible phenotypes were not apparent (Figs. 4B and 4C). However, at later stages, the overexpression lines displayed obvious alterations in their leaf, flower, and inflorescence morphologies (Figs. 4D and 4E). Most strikingly, both overexpression lines displayed growth retardation in inflorescences (line 1-1 in Fig. 4D and 8-4 in Fig. 4E). As seen in the T1 generation (Fig. 3), the siliques were clustered due to suppression of internodes (1-1, Fig. 4D). In addition, the flower shapes of these lines were not normal. Compared to the wild-type control, the overexpression line 8-4 had longer petals and gynoecia, and its petals did not bend outwards (Fig. 4E inset). As a result of the malformed floral structure, the overexpression lines produced fewer seeds than did the wild type.

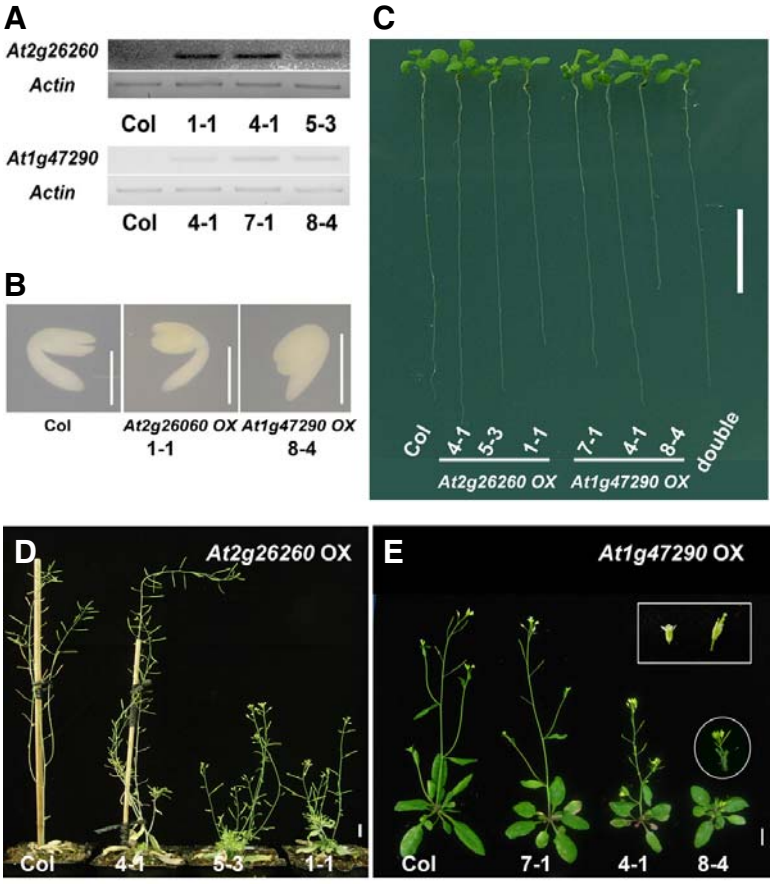

Fig. 4. Morphology of wild-type, *At2g26260 OX,* and *At1g47290 OX* plants. (A) Gel image showing cDNA amplified by RT-PCR from the wild type and individual transgenic lines. Identification numbers of independent transformants are indicated below the lanes. The levels of cDNA in transgenic lines were greater than those of the wild type. The Actin gene was used as a loading control. (B) Seedlings at 1 day after germination. Bar = 1 mm. (C) Comparison of OX lines, the wild type, and the double mutant at 7 days after germination. Hardly any phenotypic variation was detected during the seedling stages. (D, E) Phenotypes of the adult stages of the *At2g26260 OX* and *At1g47290 OX* lines at 6 and 5 weeks post germination, respectively. Magnified views of flowers from the wild type (left) and 8-4 (right) are shown in the inset of (E). The circled inset shows the floral cluster from line 8-4.

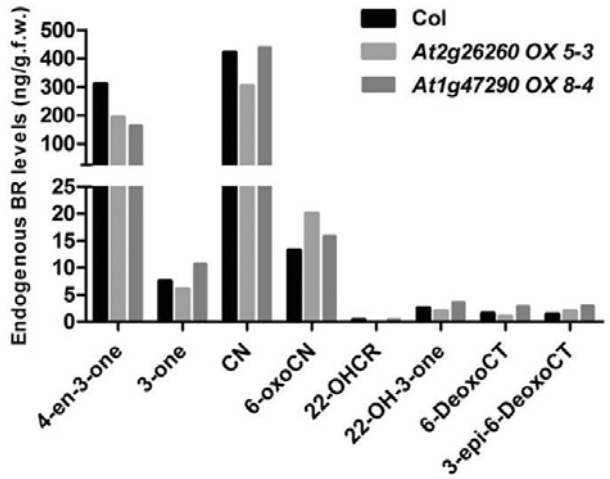

Fig. 5. Endogenous levels of BR biosynthetic intermediates in wild-type (Col-0), *At2g26260 OX*, and *At1g47290 OX* lines. BR content was determined using the aerial parts of plants at 5 weeks post germination. The concentration of BRs in the two overexpression lines is similar for all intermediates examined. Abbreviations: 4-en-3-one, 22-hydroxy-ergost-4-en-3-one; 3-one, 22-hydroxy-5β-ergostan- 3-one; CN, campestanol; 6-OxoCN, 6-oxocampestanol; 22-OH CR, 22-hydroxycampesterol; 22-OH-3-one, 22-hydroxy-5-ergostan-3-one; 6-DeoxoCT, 6-deoxocathasterone; and 3-epi-6-DeoxoCT, 3-epi-6-deoxocathasterone.

Similar levels of BRs in the overexpression lines

To examine if the morphological aberrations are due to a deficiency in endogenous BRs, we examined the levels of this steroid hormone in both wild type and overexpression lines (Fig. 5). The concentration of this hormone was essentially the same in the wild type and overexpression lines (Fig. 5). For one intermediate of the BR pathway (6-oxoCN), the level was higher in the overexpression lines, whereas the level was lower for 4-en-3-one (Fig. 5). Thus, it is not likely that the phenotypes of the overexpression lines are due to alterations in endogenous BR levels.

Different mechanisms of transcriptional regulation from BR biosynthetic genes

To further rule out the possibility that the overexpression phenotypes of the two *3βHSD/D* genes are not due to BRs, we examined the expression pattern of two genes involved in BR biosynthesis after BR treatment. When wild-type seedlings were treated with two different concentrations (10^{-8} and 10^{-6} M) of epi-BL, the expression of two genes involved in BR biosynthesis, *CYP85A2* (Kim et al., 2008) and *DWF4* (Chung et al., 2011; Kim et al., 2006; Maharjan and Choe, 2011; Maharjan et al., 2011), was downregulated in proportion to the concentration of epi-BL applied (Fig. 6). However, the transcript levels of two *3βHSD/D* genes hardly changed after epi-BL treatment. This result suggests that the transcriptional regulation mechanism of these two genes differs from feedback downregulation by BL concentrations.

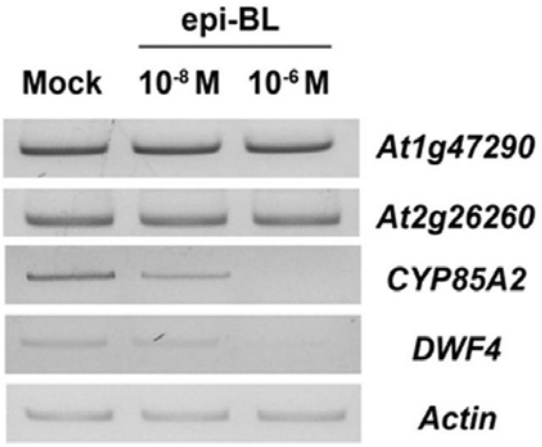

Fig. 6. The transcript levels of BR biosynthetic genes and *3β-HSD/D*s from Arabidopsis Col-0 wild-type seedlings. *DWF4*, *CYP85A2*, *At1g47290,* and *At2g26260* transcript levels were examined using total RNA prepared from seedlings treated with and without epi-BL. The levels of BR biosynthetic genes (*DWF4* and *CYP85A2*) gradually decreased proportional to the concentration of epi-BL, whereas those of *At1g47290* and *At2g26260* were unchanged by the treatments. *Actin* was used as a loading control.

Altered response of the overexpressors to an auxin efflux inhibitor

To explore the possibility that auxin is involved in the phenotype of the overexpression lines, we examined whether the overexpression lines had altered responses to treatment with the auxin efflux inhibitor, 1-N-naphthylphthalamic acid (NPA). Root gravity vectors were examined in Arabidopsis seedlings grown on agar-solidified media supplemented with mock (DMSO) or 5 μM NPA in a vertical position. As expected, mock-treated wild-type seedlings grew toward gravity and the angle between hypocotyls and roots fell within the range of 135-225° (Figs. 7A and 7B). In contrast, the root orientations of wild-type seedlings treated with NPA were distributed in all four categories of angles defined. The abnormal transport of endogenous auxin toward the root tip appeared to affect gravity-responsive growth and caused the roots to grow in random directions.

The root orientations of the NPA-treated overexpression lines differed from those of the wild type; the root angles mostly fell into the range of 135-225° regardless of the treatment administered (Figs. 7C and 7D), suggesting that overexpression of these genes hampered NPA responsiveness in these lines.

It was reported that quantitative and qualitative changes in sterol levels in plasma membranes affect the proper re-positioning of auxin efflux carriers like PIN1 (Titapiwatanakun et al., 2009). The altered response to NPA observed in our overexpression lines suggests that increased function of these genes led to biochemical changes in membrane sterols. Accordingly, this change might have made the overexpression lines resistant to NPA. Furthermore, it is likely that the opportunistic suppression of internode elongation (Figs. 3 and 4) is due to failure to support the proper re-distribution of auxin transport machinery in response to signals triggered by endogenous developmental programs. To investigate the roles of the two *3βHSD/D* genes in Arabidopsis, we examined the phenotypes of both single double mutants of these genes. The observation that the double mutant had virtually no visible phenotype (Fig. 2) suggests that the Arabidopsis genome may contain additional functionally redundant genes besides these two. Further studies of the genes that we identified as distant homologs should address this question.

In contrast, overexpression analysis led to the discovery of novel phenotypes, especially at later developmental stages. Overexpression lines of the two genes exhibited the same phe-

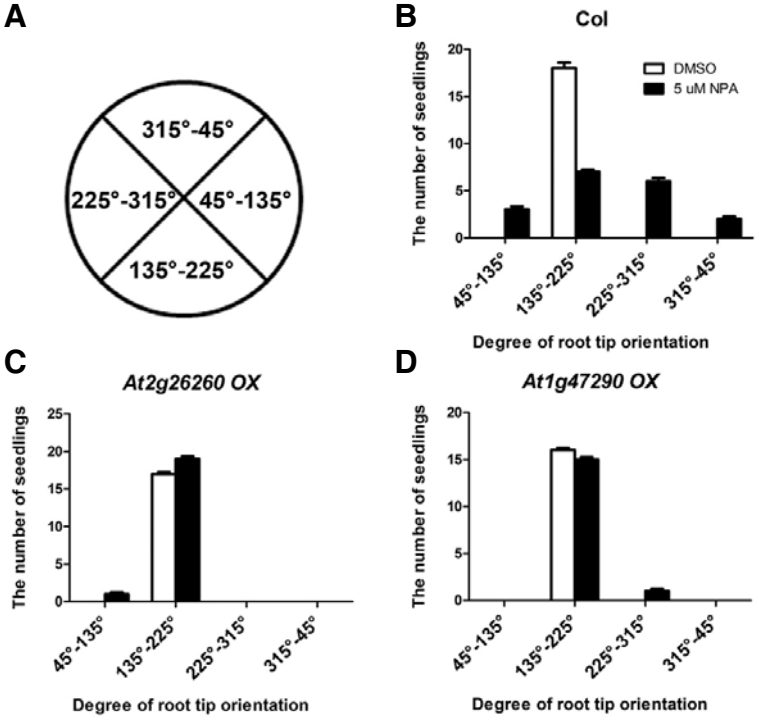

Fig. 7. Comparison of root growth vectors in wild-type and overexpression lines after treatment with the auxin efflux inhibitor, NPA. (A) Classification of the angles of root growth vectors. After treatment with mock (DMSO) or NPA, the growth vectors were determined and placed in one of the four categories. (B) The root vector of mock-treated wild-type plants pointed down in the direction of gravity (i.e., 180°); however, the vectors were scattered randomly amongst the four divisions after NPA treatment. (C) The *At2g26260 OX* line, which is insensitive to NPA, grew in the direction of gravity. (D) *At1g47290 OX* displayed a similar response as *At2g26260 OX*. Average values are shown with standard deviation (n > 5). Three biological replicates were performed.

notypes, such as wrinkled leaves, malformed flowers, and clustered siliques due to shortening of the internodes (Figs. 3 and 4). Consistency in the overexpression phenotypes of these two genes indicates that they participate in the same biochemical reactions; this was indeed shown in previous reports (Rahier et al., 2006; 2009). Since the endogenous levels of BRs were not significantly altered in the overexpression lines (Fig. 5), it is likely that the phenotypes stem from changes in the biochemical properties of membrane sterols. Elevation of *3βHSD/D* function in the overexpression lines might have caused the 3-one (22-hydroxy-5β-ergostan-3-one) type of sterol biosynthetic intermediates to accumulate, and this might hinder the formation of modified sterols via the hydroxyl function at C-3. Indeed, it was reported that free and conjugated sterols with fatty acids or sugars at C-3 can be enriched in DIMs or membrane rafts (Simon-Plas et al., 2011). Given that signaling proteins, including auxin transporters (Titapiwatanakun and Murphy, 2009) and receptors (Keinath et al., 2010), are organized in DIMs, failure to supply normal composition of sterols in the overexpression lines might have affected the function of these signaling proteins accordingly.

Using the overexpression lines presented in this study, questions regarding the *in vivo* function of these genes in sterol metabolic pathways, the biochemical properties of DIMs in plasma membranes, and the signal-triggered relocation processes of auxin transporters, can be addressed in future studies.

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