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An auxin-inducible F-box protein CEGENDUO negatively regulates auxin-mediated lateral root formation in *Arabidopsis*

Li Dong[1,3,4], Lei Wang[1,3], Yu'e Zhang[1], Yansheng Zhang[1], Xingwang Deng[2] and Yongbiao Xue[1,*]

[1]*Laboratory of Molecular and Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences and National Centre for Plant Gene Research, Beijing 100080, China (*author for correspondence; e-mail ybxue@genetics.ac.cn);* [2]*Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520-8104, USA?* [3]*Graduate School of Chinese Academy of Sciences, Beijing 100039, China;* [4]*National Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences and National Centre for Plant Gene Research, Beijing 100080, China*

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Abstract

Previously, we characterized 92 *Arabidopsis* genes (*AtSFLs*) similar to the *S*-locus F-box genes involved in *S*-RNase-based self-incompatibility and found that they likely play diverse roles in *Arabidopsis*. In this study, we investigated the role of one of these genes, *CEGENDUO* (*CEG*, *AtSFL61*), in the lateral root formation. A T-DNA insertion in *CEG* led to an increased lateral root production, which was complemented by transformation of the wild-type gene. Its downregulation by RNAi also produced more lateral roots in transformed *Arabidopsis* plants whereas its overexpression generated less lateral roots compared to wild-type, indicating that CEG acts as a negative regulator for the lateral root formation. It was found that *CEG* was expressed abundantly in vascular tissues of the primary root, but not in newly formed lateral root primordia and the root meristem, and induced by exogenous auxin NAA (α-naphthalene acetic acid). In addition, the *ceg* mutant was hyposensitive to NAA, IAA (indole-3-acetic acid) and 2,4-D (2,4-dichlorophenoxyacetic acid), as well as the auxin transport inhibitor TIBA (3,3,5-triiodobenzoic acid), showing that *CEG* is an auxin-inducible gene. Taken together, our results show that CEG is a novel F-box protein negatively regulating the auxin-mediated lateral root formation in *Arabidopsis*.

Introduction

Lateral root formation is essential for the plant development and contributes to establish the root architecture system. The *Arabidopsis* root comprises single layers of epidermal, cortical and endodermal cells surrounding the stele (composed mainly of vascular tissue). Pericycle is the outer boundary of the stele (Dolan *et al*., 1993). Lateral roots are initiated by local activation of pericycle cells at the xylem poles (Dolan *et al*., 1993; Himanen *et al*., 2002). The subsequent development may be divided into two phases: an initial phase which lasts until the lateral root primordium is 3–5 cell layers in size, and an emergence phase during which primordium develops into a functional meristem and outgrow from the main root (Laskowski *et al*., 1995).

In recent years, a complex molecular network of lateral root formation involving the plant

hormone auxin has emerged in *Arabidopsis*. Auxin has been demonstrated to be a key regulator to promote the lateral root development. Treatment of roots with exogenous IAA (indole-3-acetic acid) induced the lateral root production (Laskowski *et al*., 1995). *Arabidopsis* mutants with increased endogenous IAA levels exhibit increased lateral root proliferation. For example, *alf1* (*aberrant lateral root formation 1*) mutant had an elevated level of endogenous auxin and produced many lateral roots, consistent with the observation that auxin promoted the lateral root formation (Celenza *et al*., 1995). In contrast, several mutants defective in auxin transport or auxin sensitivity had reduced numbers of lateral roots (Hobbie and Estelle, 1995). Moreover, auxin-resistant mutants *axr4* (*auxin resistant 4*) and *tir1* (*transport inhibitor resistant 1*) also had reduced numbers of lateral roots (Hobbie and Estelle, 1995; Ruegger *et al*., 1998; Gray *et al*., 1999). Bhalerao *et al*. (2002) reported that two separate sources of auxin influence specific stages of lateral root development. Root tip-localized auxin is important for lateral root primordium initiation, while leaf-derived auxin is critical for the primordial outgrowth. Leaf-derived IAA flux reaches the root between 5 and 7 DAG (days after germination) in *Arabidopsis*, coinciding with the emergence of lateral roots.

Auxin induces many developmental effects by regulating gene expression. Transcription factors called auxin response factors (ARFs) can bind to auxin response elements (AuxREs) in promoters of auxin-inducible genes, and mediate auxin-induced gene responses (Ulmasov *et al*., 1997a, b). *Arabidopsis* has 22 *ARF* genes encoding members of this family (Remington *et al*., 2004). *NPH4*/*ARF7* and *ARF19* have been identified to function redundantly in the auxin-induced lateral root formation (Okushima *et al*., 2005; Wilmoth *et al*., 2005). Global gene expression analysis suggested that ARF7 and ARF19 proteins play essential roles in auxin-mediated plant development by regulating both unique and partially overlapping sets of target genes (Okushima *et al*., 2005).

The ubiquitin/26S proteasome-mediated proteolysis has been implicated in the auxin signaling of the lateral root formation (Moon *et al*., 2004). In this pathway, the ubiquitin is activated by the ubiquitin-activating enzyme (E1), then transferred to the ubiquitin conjugating enzyme (E2), and finally bound to the substrate protein by the ubiquitin ligase (E3). Polyubiquitinated proteins are recruited to the 26S proteasome for degradation (Ciechanover, 1998; Ben-Neriah, 2002). Four major types of E3s, namely HECT, APC, RING/U-box and SCF (a complex of Skp1/Cullin or CDC53/F-box protein, Deshaies *et al*., 1999) have been identified in plants (reviewed by Smalle and Vierstra, 2004). SCF^{TIR1} complex is the first E3 isolated in the lateral root development of *Arabidopsis* (Gray *et al*., 1999). It composes of Cullin (AtCUL1), the RING-H2 protein RBX1, an SKP1-like protein (ASK1 or ASK2) and the F-box protein TIR1 (Gray *et al*., 1999). TIR1 is specific for the substrate recognition. Mutation in *TIR1* gene led to the declination in the lateral root formation and resistance to exogenous auxin in *Arabidopsis* (Ruegger *et al*., 1998). TIR1 has been identified to be the auxin receptor (Kepinski and Leyser, 2005; Dharmasiri *et al*., 2005). With the presence of auxin, TIR1 interacts with the AUX/IAA (AUXIN-RESPONSIVE PROTEIN/INDOLEACETIC ACID-INDUCED PROTEIN) proteins and directs their degradation (Gray *et al*., 2001), thus promotes the auxin signaling. The *Arabidopsis* SINAT5 (SEVEN IN ABSENTIA of *Arabidopsis thaliana* 5) is a RING finger-containing E3 (Xie *et al*., 2002). Transgenic plants overexpressing SINAT5 showed a reduction in the lateral roots, whereas the plants overexpressing a dominant-negative allele of *SINAT5* produced more lateral roots. SINAT5 interacts with a transcription activator NAC1 in the yeast two-hybrid assay. Overexpression of *NAC1* resulted in more lateral roots. In contrast, reduction of *NAC1* by antisense led to a decrease in the emergence of lateral roots. SINAT5 was thought to attenuate the auxin signal by targeting NAC1 for degradation. (Xie *et al*., 2002). XBAT32 (XB3 ortholog 2 in *Arabidopsis thaliana*) is also a RING finger-containing E3. Mutation in *XBAT32* resulted in a decreased lateral root production. As a positive regulator, it may be involved in the lateral root development by interfering with the auxin transport (Nodzon *et al*., 2004).

Additionally, Class III HD-Zip gene family members (*PHABULOSA* [*PHB*], *PHAVOLUTA* [*PHV*], *REVOLUTA* [*REV*], *ATHB8*, and *ATHB15*) and *KANADI* genes encode two classes of transcription factors. Phenotypic and expression analysis of members of the two gene

families in the root revealed that they antagonistically function in the lateral root ontogeny. Class III HD-Zip proteins act to promote the meristematic activity in the pericycle, possibly in response to auxin maxima. Upon initiation, *KANADI* genes function to attenuate the activity of *Class III HD-Zip*s through inhibiting their transcription (Hawker and Bowman, 2004). *AtMDR1* and *AtPGP1*, the two closely related ABC (ATP-binding cassette) transporter genes, are also required for the auxin-mediated lateral root formation. Both *atmdr1–100* and *atpgp1–100* mutants, possessed fewer lateral roots than did wild-type, and showed resistance to auxin and auxin transport inhibitor NPA, suggesting that AtMDR1 and AtPGP1 are required for auxin transport necessary for normal lateral root formation (Lin and Wang, 2005).

Because most of lateral root mutants can hardly block the lateral root formation completely, it is anticipated that additional factors are involved in this process in *Arabidopsis*. Recently, we characterized an *Arabidopsis* F-box gene family *AtSFL* (*Arabidopsis thaliana S*-locus F-box-Like) related to *AhSLF-S*$_2$ (Wang *et al*., 2004), which controls the pollen function of *S*-RNase-based self-incompatibility (Lai *et al*., 2002; Qiao *et al*., 2004a, b). Several AtSFL proteins were shown to be capable of directly interacting with one or several ASK (*Arabidopsis* Skp1) proteins, suggesting that they could be part of SCF complexes. Except *SON1* (*Suppressor Of Nim-1*) implicated in defense response (Kim and Delaney, 2002), no specific biological function is known for members of this gene family. In this report, we provide evidence to show that CEGEDUO (CEG, AtSFL61) is a new negative regulator involved in the auxin-mediated lateral root production in *Arabidopsis*.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used. The *ceg* mutant was obtained from SALK T-DNA insertion collection (http://signal.salk.edu). The original line is Salk-019864. The *tir1-1* mutant seeds were a gift from Mark Estelle. *Arabidopsis* plants were grown on MS medium containing 2% (W/V) sucrose and 0.8% (W/V) agar or grown in soil at 22 °C with a 16/8 h light/dark cycle.

RT-PCR (reverse transcription polymerase chain reaction) analysis

Total RNA was prepared as previously described (Lai *et al*., 2002) and was digested with DNase I (TaKaRa, Dalian, China). Reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used to synthesize the first strand cDNA. After 1/10 dilution, 1 μl of the synthesized cDNA was used for RT-PCR. PCR conditions were 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 5 min. Specific primers of *CEG* were BX325 (5′-ATGGCTTCATGTGAA CGTTCGTTG-3′) and BX326 (5′-TTATTCTG GAACT GGAACCAGAC-3′). Specific primers of *Tubulin* were BX163 (5′-TTTGGAGCCTGGGAC TATGGAT-3′) and BX164 (5′-ACGGGGGAAT GGGATGAGAT-3′).

RT-qPCR (quantitative real-time reverse transcription-polymerase chain reaction) analysis

Reverse transcription was performed using TaqMan Reverse Transcription Regents Kit (Applied Biosystems, CA, USA). The cDNA samples were diluted to 10, 5, and 1 ng/μl. Triplicate quantitative assays were performed on 1 μl of each cDNA dilution using the SYBR Green Master mix with an ABI 7900 sequence detection system according to the manufacture's protocol (Applied Biosystems). Gene-specific primers BX351 (5′-GAACG ATAA TATCATGGTATGGTGTGA-3′) and BX352 (5′-TTTACTAATCATGCAAACGCTA ACG-3′) were designed by using PRIMEREXPRESS 1.0 software (Applied Biosystems, CA, USA). The relative quantization method ($\Delta\Delta$CT) was used to evaluate quantitative variation between the replicates examined. The amplification of 18S rRNA was used as an internal control to normalize all data.

Plasmid construction and Arabidopsis *transformation*

CEG::GUS (CEGENDUO:: β-glucuronidase) construct

Genomic DNA sequences corresponding to 906 and 2050 bp upstream the predicted ATG codon

of *CEG* ORF were cloned into pBI121.2 binary vector between *Sal*I and *Bam*HI sites (Clontech, CA, USA). Junction sequences of the resulting construct *CEG::GUS* were sequenced.

CEG overexpression (CEG-OX) construct
The ORF of *CEG* gene was introduced into the *Kpn*I and *Sal*I sites of the pCAMBIA1301 vector, droved by the 35S promoter.

CEG RNA interference (CEG-Ri) construct
The partial sequence of *CEG*, corresponding to the 775–1152 nucleotides, was amplified with the primer BX335 (5′-CAACTCGAGGATCCGA GAAACAATGAAGATCGAAG-3′) and BX336 (5′-CAAGTCGACTGCAGTTATTCTGGAACT GGAACCAGAC-3′). Two versions of the fragment were cloned into the estrogen-inducible vector pER8 (Zuo *et al.*, 2001) in the opposite directions. A tubulin intron of 408 bp was recombined between them. These constructs were introduced into *Agrobacterium* strain GV3101 and transformed into wild type *Arabidopsis* (Col-0) plants, respectively, according to Bechtold and Pelletier (1998).

Promoter analysis

Kanamycin-resistant T_1 plants of *CEG::GUS* were used for GUS activity detection as described by Weigel and Galzebrook (2002) for whole mount observations. For the transverse observation, the roots were embedded in the Historesin (Leica Microsystems Nüssloch GmbH, Heidelberg, Germany) and sectioned at 6 μm.

DNA gel blotting analyses

Genomic DNA was isolated according to Liu *et al.* (1995). DNA (1 μg) was digested, separated on 0.8% agarose gel, and transferred onto Hybond Nt (Amersham, Buckinghamshire, UK) membrane. Prehybridization, hybridization, and washing of the blot were performed as recommended by the manufacturers. Probes were labeled with ^{32}P by random primers using the Prime-a-Gene labeling system (Promega, Madison, WI).

Auxin, CHX and TIBA experiments

For NAA induction of *CEG::GUS*, 14-day-old seedlings were transferred to MS media supplemented with 2 μM NAA for 1–24 h, respectively, then assayed for GUS staining or RT-qPCR. For inhibition of protein synthesis coupled with NAA induction, CHX (Cycloheximide) (Sigma-Aldrich, St. Louis, USA) of 100 μM was added to media simultaneously. For induction of lateral roots, 7-day-old seedlings were transferred to MS media supplemented with NAA, IAA or 2,4-D of various concentration (10^{-7}–10^{-4}M). Lateral root production was scored 5 days later with a microscope. For TIBA treatment, seeds were germinated on MS medium supplemented with 5 μM TIBA (Sigma-Aldrich, St. Louis, USA), seedlings of 9 day after germination were collected for observation.

Quantification of GUS activity by fluorescent MUG assay

Fluorometric assay of GUS activity was performed essentially as described by Gallagher (1992). Total protein of roots was extracted using 300 μl MUG (4-methyl umbelliferone glucuronide. Sigma-Aldrich, St. Louis, USA) assay buffer. After centrifugation, the supernatant was assayed for GUS activity and for protein quantification. Samples were assayed using 1 mM MUG at 37 °C, and the reaction stopped with 0.2 M Na_2CO_3 after 30 and 60 min, respectively. Fluorescence was measured on FLUOstar OPTIMA (BMG LABTECH, Offenburg, Germany). Activity was presented as nmol 4-MU per min and μg protein (nM MU min^{-1} μg^{-1}). Total protein content of samples was measured using Bradford reagent (BioRad, Hercules, CA).

Decapitation of Arabidopsis *seedlings*

Arabidopsis seedlings were decapitated by removal of the shoot apex at 4 and 7 day-old, respectively. Another 5 days later, the root development was observed.

Results

The CEG is expressed specifically in the root vascular system

AtSFL61 (*At3g22650*) was identified as a member of the Class A of AtSFLs (Wang *et al.*, 2004) and

encoded an uninterrupted ORF of 383 amino acids. Initial phenotypic observation revealed that mutation in *AtSFL61* resulted in an increased lateral root production, so that the gene was renamed as *CEG* (CEG*ENDUO*, meaning "more lateral roots" in Chinese).

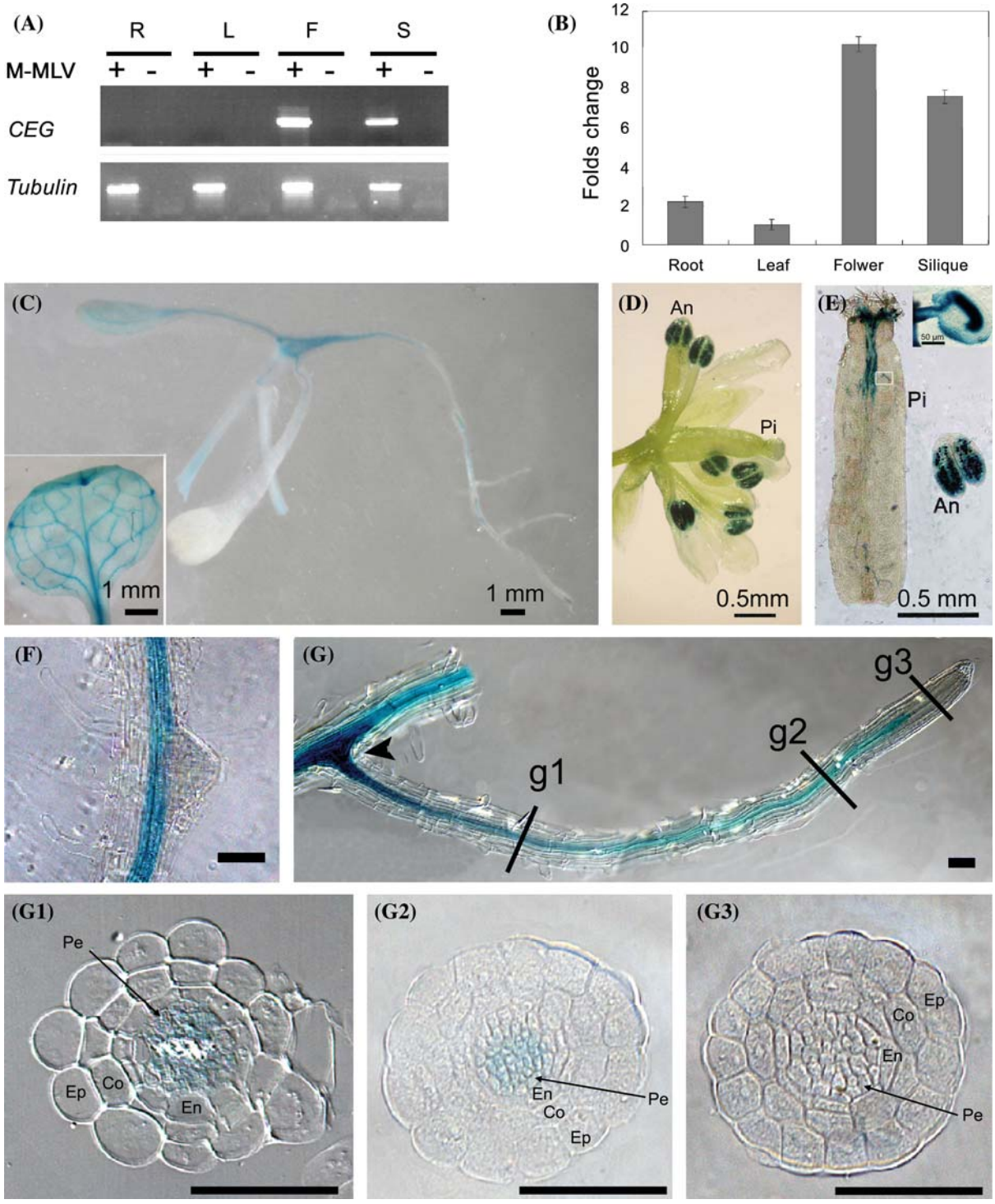
To examine the *CEG* function, its expression pattern was analyzed in detail. RT-PCR was performed in four major organs including roots, leaves, flower buds and siliques. As shown in Figure 1A, the *CEG* was transcribed specifically in flower buds and siliques. Using RT-qPCR (quantitative real-time reverse transcription-polymerase chain reaction), weak signals could be detected in leaves and roots in addition to the flower buds and siliques (Figure 1B). To further examine the *CEG* expression, the *CEG* promoter was fused to the β-*Glucuronidase* (GUS) reporter gene. Two versions (906 or 2050 base pairs) sequences upstream of start codon were adopted. The two constructs were transformed into wild-type *Arabidopsis* plants, respectively, and both gave similar results (Figure 1C–G). In leaves, GUS staining was detected weakly in the vascular tissue through the leaf blade (Figure 1C). In flowers, the *CEG::GUS* expression was found in pollen (Figure 1D). After pollination and fertilization, the GUS activity was detected in the pollen tubes and embryo sacs (Figure 1E), respectively. In the roots, the *CEG::GUS* activity was observed in the stele of roots (Figure 1A, F), particularly in the zone where lateral roots had grow out (Figure 1G). In contrast, no GUS signal was detected in the newly formed lateral root primordia (Figure 1F) and the root apical meristem (Figure 1G). The transverse sections showed that the GUS activity only appeared in the pericycle and cells within them in the mature roots. None of the endomermis, cortex and epidermis layers exhibited the positive signal (see Figure 1 g1–3). We speculated that the results from the *CEG::GUS* transgenic plants might represent the *CEG* transcription *in vivo*. Taken together, the *CEG* gene is mainly expressed in the stele of the roots as well as in pollen.

More lateral roots are produced in the ceg *mutant*

Wang *et al*. (2004) have identified a mutant line (Salk_019864) from the Salk insertion mutant database (http://signal.salk.edu), carrying a T-DNA insertion in the *CEG* gene in the Col-0 background. The insertion was located between nucleotide positions 163 and 164 downstream of the start codon (Figure 2A) and further confirmed by sequencing the T-DNA border flanking regions and DNA gel blot analysis (Figure 2B). Using the resistant marker gene *NPT II* as a probe, two hybridizing bands of 7.7 kb *Bgl*II and 3.6 kb *Hin*dIII fragments were detected in the mutant plants (Wang *et al*., 2004). When using the full-length *CEG* cDNA as a probe, 7.7 kb *Bgl*II and 3.6 kb *Hin*dIII fragments were detected again in the mutant line, whereas, in the wild-type line, 3.5 kb *Bgl*II and 5.4 kb *Hin*dIII fragments were detected. The lengths of all the hybridizing bands were consistent with the prediction from a single T-DNA insertion in the mutant line (see Figure 2A). These results revealed that the T-DNA was indeed inserted into the cording region of the C*EG* gene in the mutant lines, and both lines were homozygous and referred to as *ceg*. RT-PCR analysis further showed that no *CEG* transcripts were detectable in the mutant line (Figure 2C). Therefore, the *ceg* is a null allele and was subsequently used for detailed phenotypic analyses.

During most of the developmental stages, there was no obvious difference found between the wild-type Col-0 and the *ceg* mutant, except the lateral root production in *ceg* mutant was substantially increased. As shown in the Table 1, the length of the primary roots, the production of adventitious roots (nAR) and the lateral roots (nLR), as well as the primordia (nP) were counted for the 14-day-old seedlings. The nLR/cm (number of lateral roots per

Figure 1. Tissue-specific expression of the *CEG* gene. (A) RT-PCR analysis of the expression of *CEG* in different organs, and *Tubulin* was used as the control. R, roots; L, leaves; F, flowers; S, siliques. "+" and "−" indicate that M-MLV reverse transcriptase was present or absent in the process of reverse transcription reaction. (B) RT-qPCR detection of the *CEG* transcripts in the same organs as (A). (C–G) Detection of the *CEG::GUS* expression in transgenic *Arabidopsis*. The GUS activity was traced in the whole seedling(C), leaf (Inset in the (C)) flower (D), silique (E) and root (F and G). Inset in (E) is a magnified image of the ovule framed in the rectangular. g1–g3 represent transverse sections corresponding to the position g1, g2 and g3 in (G). The arrowhead in (G) indicates the position that the lateral root had grown out, where the activity of *CEG::GUS* showed the highest level. An, anther; Pi, pistil; Ep, epidermis; Co, cotex; En, endodermis; Pe, pericycle. The scale bars in (C–E) are shown. Scale bars in F, G and g1–g3 are 50 μm.



cm), the nP/cm (number of root primordia per cm) and the (nLR + nP)/cm (number of lateral roots plus the premordia per cm) were calculated subsequently. The primary root of mutant plant (31.5 ± 5.8 mm) was shorter than that of the wild-type plant (43.1 ± 3.9 mm). Whereas, the lateral

Table 1. Lateral root development in the different mutant lines related to the *CEG*.

	WT	*ceg*	*35S::CEG/ceg*	*CEG RNAi*–estradiol	*CEG RNAi*+estradiol	*35S::CEG*
Primary root Length (mm)	43.1 ± 3.9	31.5 ± 5.8*	52.1 ± 4.1*	42.5 ± 3.3	29.3 ± 3.6*	51.3 ± 4.0*
No. adventitious root (nAR)	1.9 ± 0.4	2.0 ± 0.9	1.6 ± 1.0	1.9 ± 0.9	2.1 ± 1.0	1.8 ± 0.8
No. lateral root (nLR)	4.1 ± 0.9	8.3 ± 1.9*	1.9 ± 0.7*	4.0 ± 0.9	8.8 ± 1.3*	2.0 ± 0.7*
No. primodium (nP)	2.7 ± 0.7	3.0 ± 1.0	1.6 ± 0.7*	2.7 ± 0.9	3.1 ± 1.0	1.4 ± 0.9*
nLR + nP	6.8 ± 1.2	10.3 ± 2.0*	3.5 ± 1.1*	6.4 ± 1.3	11.9 ± 1.7*	3.4 ± 1.1*
nLR/cm	1.0 ± 0.3	2.7 ± 0.8*	0.4 ± 0.1*	1.0 ± 0.2	3.1 ± 0.7*	0.4 ± 0.1*
nP/cm	0.6 ± 0.1	0.9 ± 0.1*	0.3 ± 0.2*	0.6 ± 0.1	1.2 ± 0.2*	0.3 ± 0.1*
nLR + nP/cm	1.6 ± 0.4	3.7 ± 1.0*	0.7 ± 0.2*	1.6 ± 0.4	4.1 ± 0.9*	0.7 ± 0.3*

The primary root length, the number of adventitious root (nAR) and lateral root (nLR) as well as premodium (nP) were counted. nLR plus nP (nLR + nP), nLR per centimeter (nLR/cm), nP per centimeter (nP/cm) and nLR plus nP per centimeter [(nLR + nP)/cm] were calculated. Thirty seedlings of each sample were used for the statistics analyses. The significance of the differences of the lateral production between the wild-type and the mutant or the treated samples was analyzed by *t*-test. * indicates the significant difference ($P < 0.05$).

root production was increased (see Figure 2D), which could be deduced from the result of nLR/cm (Table 1), in other words, the root spacing. The nLR/cm in the mutant was 2.7 ± 0.8, significantly more than that in wild-type (1.0 ± 0.2). The production of adventitious root (nAR) and premordia (nP) showed no significant differences between the mutant and wild-type plants. Therefore, the (nLR + nP)/cm exhibited the similar changes as the nLR/cm. Taken together, the phenotype of the *ceg* mutant indicated that *CEG* play a role in the lateral root formation, especially in the process of its outgrowth.

The wild-type CEG gene can restore normal lateral root formation in the ceg *plants*

To confirm that the increase of the lateral root number was indeed due to the mutation in *CEG*, we constructed a *CEG* overexpression vector (*CEG-OX*) driven by the 35S promoter (Figure 3A) and transformed it into the *ceg* mutant plants. Three independent transgenic lines were obtained, namely *CP-1*, *CP-2* and *CP-3*. DNA gel blot was performed to examine the genotype of the transformed lines. In addition to the 7.7 kb *Bgl*II and the 3.6 kb *Hin*dIII bands representing the mutant allele, we detected 1–5 bands in *Bgl*II and *Hin*dIII lanes of the three different lines. The bands detected in the *Bgl*II lanes was less than that in *Hin*dIII, which may be due to that some *Bgl*II restriction fragments were similar to each other in length, so it was impossible to be clearly separated by the agarose gel electrophoresis. The result revealed that there were at least 4–5 T-DNA copies inserted into the genome of the individual lines. *CP-1* and *CP-2* had four and *CP-3* five (Figure 3B). RT-PCR was performed to detect the *CEG* expression. As shown in Figure 3C, the *CEG* were transcribed highly in all of the three transformed lines, in contrast to the wild-type control. The T_2 generation of these lines was grown on MS media and the lateral roots production was determined. In the 14-day-old seedlings of the *ceg* plants, there were 2.7 ± 0.8 lateral roots per cm primary roots, whereas there were only 0.4 ± 0.1 in the *CEG-CP1* plants (namely (*35S::CEG/ceg*) in Table 1), less than that of the *ceg* mutant and the wild-type (1.0 ± 0.2). *CEG-CP2* gave a similar result (Figure 3D and Table 1). These results showed that the *ceg* mutant phenotype in these two transgenic lines was fully complemented for the lateral root abnormality, indicating that the *CEG* gene acts as a negative regulator of the lateral root formation.

Overexpression and RNA interference of CEG *in transgenic lines confirm the negative regulation of* CEG *in the lateral roots formation*

The phenotype of the *ceg* mutant and the complemented plants revealed that the *CEG* likely functions as a negative factor for the lateral root formation. However, additional evidence is required to confirm this possibility. Therefore, we transformed the *CEG* overexpression vector (see Figure 3A) and an RNA interference vector driven by an estrogen-inducible promoter (Figure 4A) into the wild-type plants, respectively.

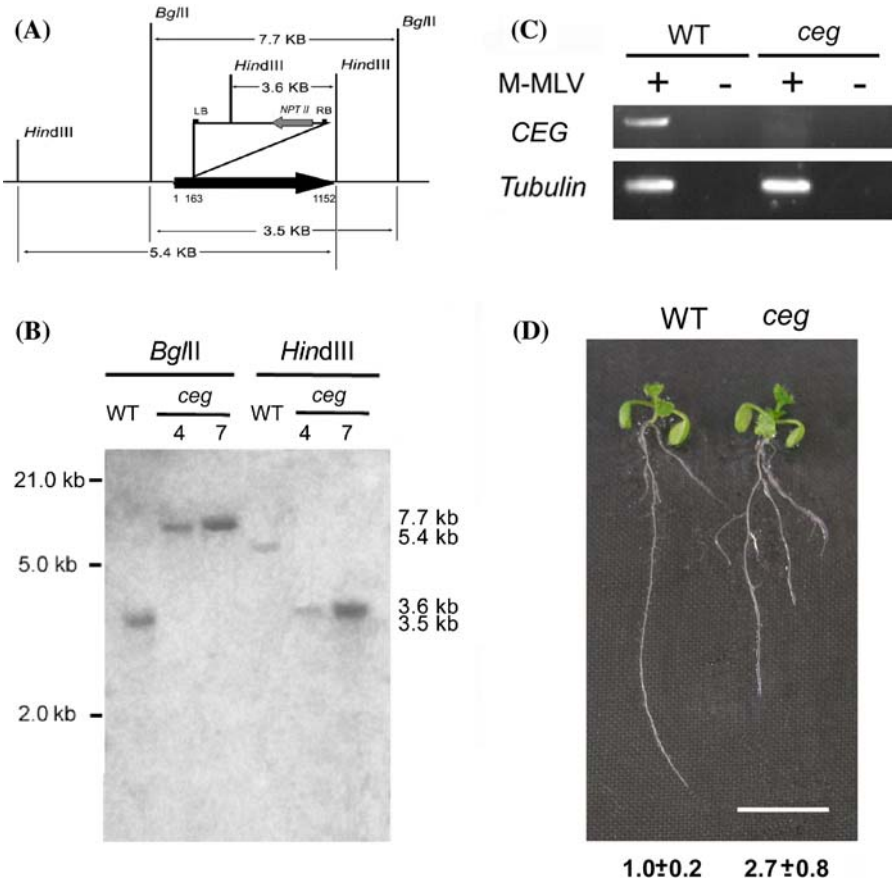


Figure 2. Increased lateral root production in the *ceg* mutant. (A) A schematic representation of T-DNA insertion of the *ceg* mutant allele. Closed arrow represents the coding region; upstream and downstream regions are shown as thick lines; the inverted triangle above the coding region denotes the structure of T-DNA and the insertion site is marked. Restriction sites and the length between them are shown. *NPT II* is the T-DNA-specific marker. (B) Genotype identification of the *ceg* mutant line by DNA gel blot analysis. Genomic DNA from wild-type and two T-DNA insertion-containing plants were hybridized with the full length *CEG* coding region. The enzymes used, as well as the length of DNA molecular markers and the hybridized bands are shown. (C) RT-PCR analysis of the *CEG* expression in the *ceg* mutant line. No detectable *CEG* transcripts were found in the mutant. Expression of *Tubulin* is used as the control. "+" and "−" indicate whether M-MLV reverse transcriptase was present or absent in the process of reverse transcription reaction. (D) Phenotypes of wild-type and the *ceg* plants. The plants were grown on MS media. Photographs were taken from 14-day-old plants. Numbers of the lateral roots per centimeter of primary root of each seedling (average ± SE) for wild-type and *ceg* lines are shown at bottom ($n = 30$). Scale bars, 1 cm.

We obtained three independent lines transformed with the *CEG* overexpression vector (*CEG-OX*), namely *OX-1*, *OX-2* and *OX-3*. DNA gel blot analysis was carried out to determine the copy number of the transgene using the *CEG* cDNA as a probe (Figure 4B). In addition to 21 kb *Eco*RI and 5.4 kb *Hin*dIII bands also detected in wild-type, we detected 1–3 bands in *Eco*RI and *Hin*dIII-digested genomic DNA of these lines. The bands detected in the *Eco*RI digested DNA were less than that in *Hin*dIII digested DNA, which may be due to that the sizes of some *Eco*RI restricted fragments were similar to each other in length. Therefore, all the lines had 2–3 *CEG* copies that were inserted into the genome. *OX-1* had two copies, *OX-2* three, and *OX-3* two. With the same method, we examined the copy number of the introduced transgene in the four lines transformed with RNA interference vector (*CEG-Ri*, namely *Ri-1*, *Ri-3*, *Ri-8* and *Ri-9* in abbreviation). Besides 2.5 *Bgl*II and 5.2 *Spe*I bands also present in wild-type, there were 1 or 3 copies of the *CEG-Ri* in these lines. *Ri-1* had three *CEG Ri* insertions, and all of the other lines had only one (Figure 4C).

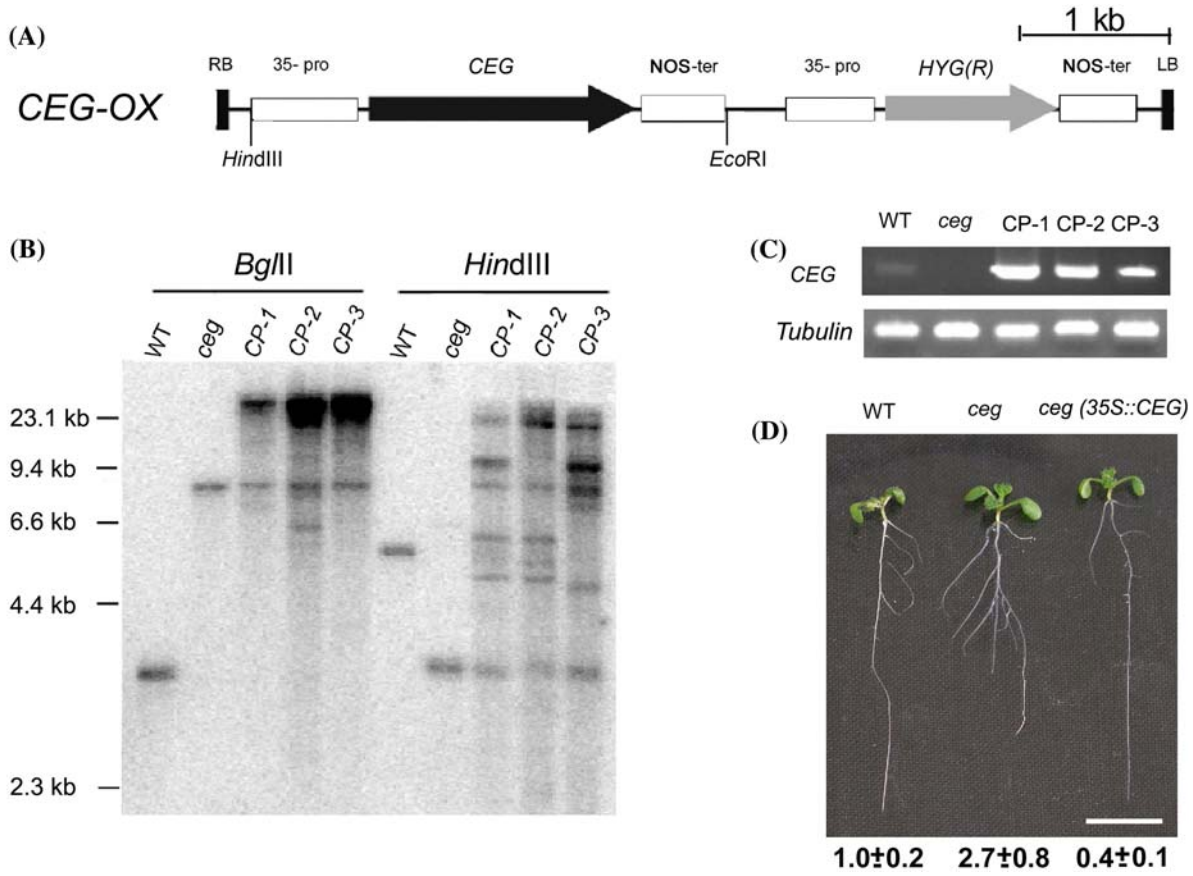


Figure 3. Complementation of *ceg* in lateral root production by the wild-type *CEG* gene. (A) Schematic diagram of the *CEG* overexpression (*CEG-OX*) construct. The closed black arrow represents the full length *CEG*. RB and LB, right and left borders of the T-DNA; *HYG(R)*, hygromycin resistance gene. 35S-pro, 35S promoter; NOS-ter, NOS terminators. Restriction sites used are shown. (B) DNA gel blot analysis of the *CEG* complemented lines. DNA of wild-type, *ceg* and three transformed lines (*CP-1*, *CP-2* and *CP-3*) were hybrid with the full length *CEG*. The DNA molecular marker and restriction enzymes used are shown. (C) RT-PCR analysis of the *CEG* expression in the transformed lines. Expression of *Tubulin* is used as the control. (D) The lateral root production in the complemented plant [*ceg* (*35S::CEG*)]. Numbers of lateral roots per centimeter of primary root (average ± SE) were determined as described for Figure 2D. Scale bars, 1 cm.

To examine the expression of the transgene in the transgenic lines, RT-PCR analysis was performed. As shown in Figure 4D, three *CEG-OX* lines expressed the *CEG* in great abundance, whereas the transcripts were hardly detected in the *CEG-Ri* lines. We further determined the transcription level using the quantitative real-time PCR. Consistent with the RT-PCR result, the *CEG* expression was greatly increased in all of the *CEG* overexpression lines. In contrast, little *CEG* transcripts were detected in the *CEG-Ri* lines, significantly lower than that in wild-type (Figure 4E).

We further examined the phenotypes of the transformed plants. All of the *CEG-OX* lines showed a decrease in the lateral root production, in contrast to the wild-type, whereas the lateral roots were increased in the *CEG-Ri* plants after the treatment of 10 μM estradiol (Figure 5A), especially the *Ri-8* and *Ri-9* lines, mirroring the *ceg* mutant plants.

To investigate the impact of the different mutant alleles on the root development, the lateral root production of 14-day-old seedlings were examined in the wild-type and various *CEG* mutant lines (Figure 5B and Table 1). There were only 0.4 ± 0.1 lateral roots per cm primary root evenly in the *CEG-OX* plants (*35S::CEG*), less than that in the wild-type plants (1.0 ± 0.2). With the presence of 10 μM β-estradiol, the *CEG-Ri* plants showed a

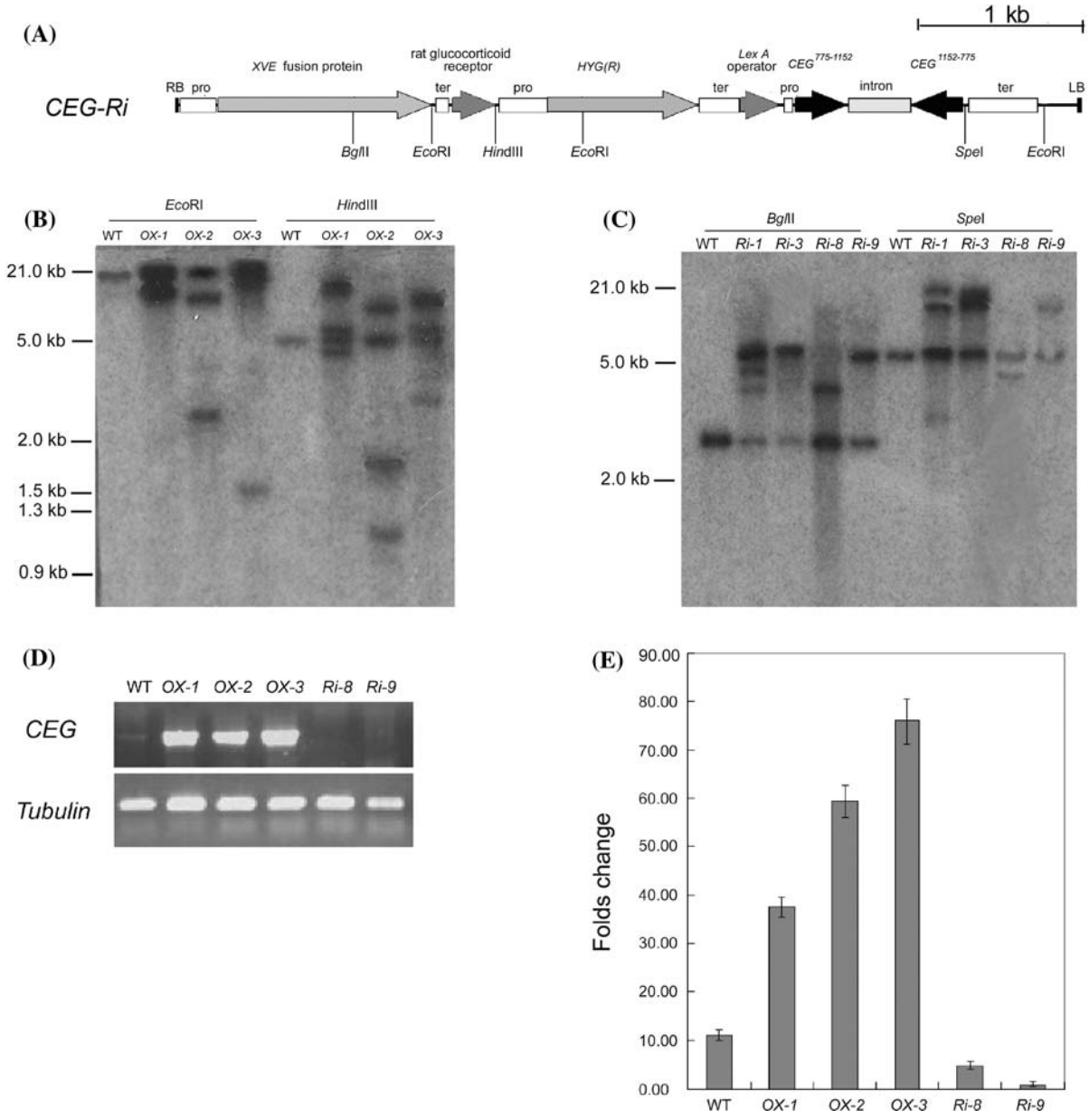


Figure 4. Molecular analyses of the *CEG* overexpression (*CEG-OX*) and RNA interference (*CEG-Ri*) transgenic plants. (A) Schematic diagram of the *CEG-Ri* construct. The closed black arrows represent the partial sequence of *CEG*. For the detail of the abbreviations, see Figure 3A. For the information of the *CEG-Ri* backbone, refer to Zuo *et al*. (2001). (B) DNA gel blot analysis of the *CEG-OX* transgenic lines. (C) DNA gel blot analysis of the *CEG-Ri* transgenic lines. WT, the wild-type control. Both blots were probed with the full length *CEG* cDNA. (D) RT-PCR analysis of RNA isolated from roots of the three of *CEG-OX* (*OX-1, OX-2* and *OX-3*) and two of the *CEG-Ri* (*Ri-8* and *Ri-9*) transgenic lines, as well as wild-type (WT) plants, using specific primers of *CEG* with (+) or without (−) reverse transcriptase in the synthesis of cDNA. RT-PCR of *Tubulin* is used as the control (bottom panel). (E) RT-qPCR analysis the *CEG* expression in roots of the *CEG-OX*, *CEG-Ri* transgenic lines and wild-type plants. The relative abundance of each sample is shown.

significant increase (3.1 ± 0.7), nearly two folds of lateral roots per cm primary root more than the untreated control (1.0 ± 0.2). Taken together, the results from the transgenic plants indicated that the *CEG* indeed plays a negative role in regulating the lateral root formation.

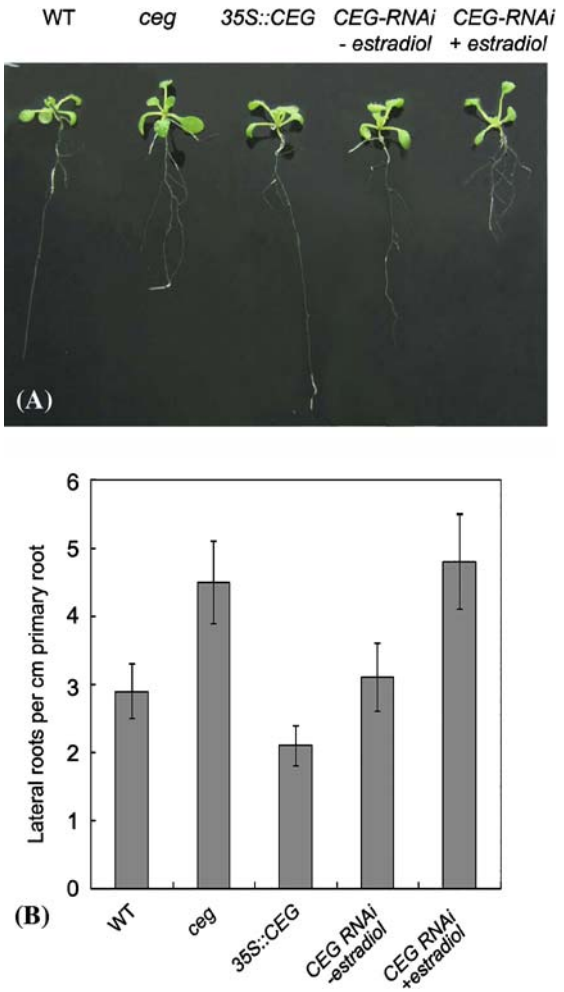


Figure 5. Phenotypes of the *35S::CEG* (*CEG-OX*) and *CEG-RNAi* transgenic plants. (A) Seedlings of the wild-type (WT), *ceg*, *35S::CEG* and *CEG-RNAi* transgenic plants were grown on the MS medium and photographed 14 days later. The medium for the *CEG-Ri* plants were supplemented with or without 10 μM β-estradiol. (B) The lateral root production in the different mutant lines of the *CEG* gene. Numbers of lateral roots per cm of primary root were determined as described for Figure 2D.

The ceg *mutant exhibits hyposensitivity to auxin*

In wild-type *Arabidopsis*, the lateral root production was increased by exogenous NAA or IAA treatment (Laskowski *et al*., 1995). Therefore, we examined the response of the *ceg* plants to auxin. We first grew the *ceg* seeds on MS media. In the seventh day after the germination, the seedlings were transferred to the same media supplemented with NAA, IAA or 2,4-D of different concentrations (10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M). Five days later, the primary root length and the production of lateral roots were examined (Table S1). To investigate the impact of the auxin treatments, the ratio to the untreated controls of the root length and the lateral root production (nLR/cm) were calculated (Figure 6). The growth of primary root was inhibited in most cases (Figure 6A, D, G). However, the ratio of the root length to the untreated control in the *ceg* mutant was obviously higher than that in wild-type, except the samples that were supplied with 10^{-7} M or 10^{-6} M NAA, which showed similar variation curve (Figure 6A). It indicated that the *ceg* plants were less sensitive to these auxin analogs. On the other hand, the lateral root production (presented by indicators of nLR and nLR/cm. Both of them exhibited the similar variation curves) was generally increased. Whereas, it was found that the treatments showed differential responses to different auxin analogs. As for the NAA treatment, 10^{-7}, 10^{-6} and 10^{-5} M concentrations, especially 10^{-5} M, promoted the lateral root formation, and the 10^{-4} M acted to inhibit (Figure 6B, C). In the IAA assay, all of the concentrations that were applied appeared to promote the lateral root formation, and the effect of 10^{-4} M was the most remarkable (Figure 6E, F). In contrast, 10^{-7} and 10^{-6} M 2,4-D played a promotive role and 10^{-5} and 10^{-4} M a inhibitive role in the lateral root production (Figure 6H, I). In most of the auxin-induction experiments, the ratio of the nLR and nLR/cm increased in the *ceg* plants were much lower than that in the wild-type. These results indicated that the *ceg* plant was hyposensitive to auxin.

The CEG *expression can be induced by auxin*

It is well known that the transcription of several genes that are associated with root development can be induced by auxin (Reed, 2001; Xie *et al*., 2000, 2002). To test whether auxin regulates the *CEG* expression, we transferred 14-day-old *CEG::GUS* seedlings, grown on MS media, to the same media supplemented with the auxin 2 μm NAA. Twenty-four hours later, the seedlings were subjected to the GUS staining. As shown in Figure 7A, the expression of the *CEG::GUS* was increased in the vascular tissues of the root in the presence of NAA. In addition, the GUS signal was expanded to the root tips over several stages of the lateral root

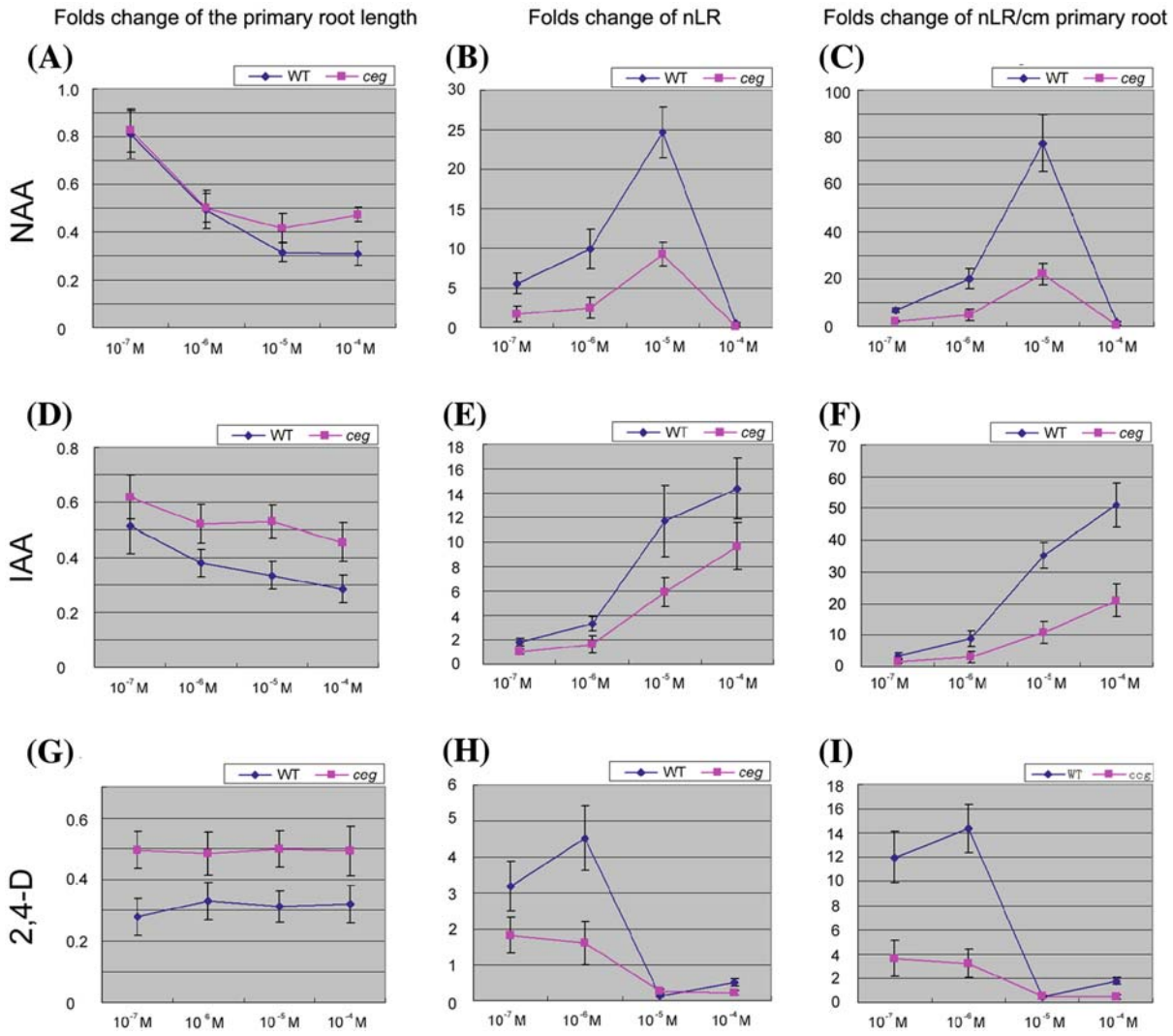


Figure 6. The lateral root development of the wild-type and *ceg* plants in response to three auxin analogs. A serial concentration of NAA (A, B and C), IAA (D, E and F) and 2,4-D (G, H and I) were applied to the 7-day-old seedlings. The ratios of root length (A, D and G), the number of lateral root (B, F and H) and the lateral root per cm (C, F and I) were calculated after another 5-day-cultivation. nLR, number or lateral root; nLR/cm, the number of lateral root per cm.

development, compared to the untreated controls. The quantification of GUS activity was performed further to confirm the effect of NAA induction to the *CEG* promoter. The 14-day-old seedlings were treated with 2 μM NAA for 1, 3, 6, 12, and 24 hours (h), respectively. The total proteins of roots were extracted and subjected to GUS activity quantification. Obviously, the GUS activity began to increase after the NAA induction for 3 h with nearly two-fold increase and maintained the similar level until 24 h later (Figure 7B). Therefore, the *CEG* transcription could be induced in the root vascular tissues, mimicking the *CEG::GUS* plants after the NAA treatment.

To confirm the induction of the *CEG* transcripts by NAA, we tested its expression in response to auxin treatment *in vivo*. The wild-type plants were treated with auxin at different time points and concentrations, and the samples were analyzed by RT-qPCR (Figure 7C, D). An increase of the *CEG* transcription was detected after 1 h of the auxin exposure and reached a maximum level after 3 h (Figure 7C), similar to that obtained with the GUS reporter gene. Different concentrations of NAA treatments revealed that the *CEG* expression was induced substantially after the treatment of 0.5 μM NAA and remained

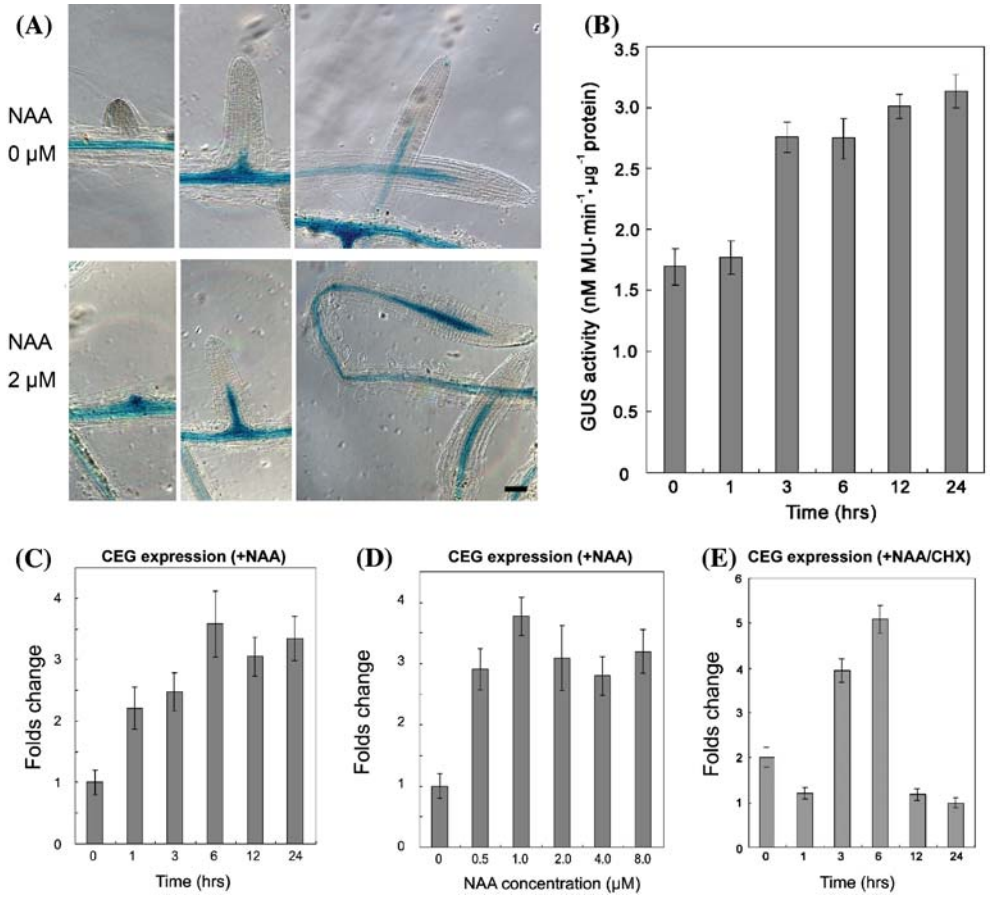


Figure 7. The *CEG* gene is associated with the auxin response pathway. (A) The *CEG::GUS* expression in the roots of the transgenic seedlings with (upper panel) or without (lower panel) treatment of 2 μM NAA for 24 h. (B) Quantification of the GUS activity in the *CEG::GUS* transformed plants. The total protein from roots with 2 μM NAA treatment of different time periods was extracted. The nM MU min^{-1} μg^{-1} total protein were measured. (C) qRT-PCR analysis of the *CEG* expression with the treatment of NAA at different time points. (D) qRT-PCR analysis of the *CEG* expression with the treatment of NAA at different concentrations. (E) qRT-PCR analysis of the *CEG* expression with the treatment of NAA and CHX (cycloheximide) at different time points.

at a similar level when the concentration was increased up to 8 μM (Figure 7D). The expression kinetics indicates that the *CEG* is an auxin-responsive gene. Consistent with these results, we found one TGTCTC element at position −437 to −442 in the *CEG* upstream region, which had been identified as an early auxin response element and the target for the ARFs binding to mediate the transcriptional responses to auxin. (Ulmasov *et al*., 1997a).

To examine if *CEG* is a primary auxin-response gene, the protein synthesis inhibitor cycloheximide was introduced to the induction system to see if the increase in mRNA abundance was affected. As shown in Figure 7E, the induction of *CEG* expression was inhibited transitionally from the beginning of the treatment. During the period of 3–6 h, the induction effect of NAA was restored. However, after the 6-h incubation, the transcription of *CEG* was declined to its original level. These results suggested that the *CEG* may not be a primary auxin-responsive gene and additional factors, such as ARFs, likely to mediate the auxin response of the *CEG* expression.

The ceg *mutant is hyposensitive to the auxin transport inhibition*

The wild-type Col-0 seedlings grown on the media containing auxin transport inhibitors, such

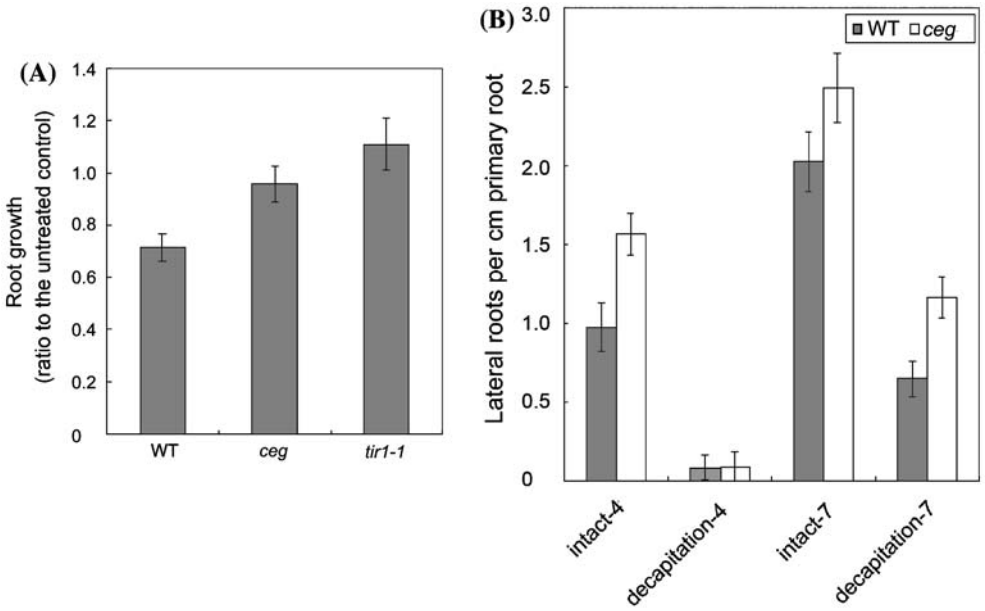


Figure 8. Responses of the *ceg* mutant plants to TIBA and decapitation during the lateral root development. (A) Effects of the auxin-transport inhibitor TIBA(5 μM) on wild-type, *ceg* and *tir1-1* seedlings. The primary root growth ratios of the TIBA treated plants to the untreated controls were calculated. (B) The impact of the decapitation on the 4 DAG (day after the germination) and 7 DAG seedlings of wild-type and the *ceg* mutant. Numbers of lateral roots per centimeter of primary root were determined as described for Figure 2D.

as NPA (N-1-naphthylphthalamic acid), CPD (2-carboxyphenyl-3-phenylpropane-1,2-dione) and TIBA (3,3,5-triiodobenzoic acid), exhibit an inhibition of the lateral root formation and primary root elongation (Ruegger *et al*., 1997; Geldner *et al*., 2001). In fact, *tir1* was initially identified as a mutant resistant to the auxin transport inhibitor (Ruegger *et al*., 1998). To examine the response of the *ceg* to the auxin transport inhibitor, we grew the plants in the presence of 5 μM TIBA. Similar to the Col-0, the lateral root production in the *ceg* mutant seedlings were blocked, whereas, the primary root elongation of the *ceg* mutant showed a partially resistance to the TIBA treatment (Figure 8A). As a control, the *tir1-1* mutant displayed an expected TIBA-resistance growth under the same condition. It is possible the *ceg* partially affected the inhibition of the primary root elongation mediated by the auxin-transport inhibitor TIBA in the root development, especially in the *Arabidopsis* seedlings older than 7 DAG. Alternatively, the resistance to TIBA may be the result of the altered sensitivity to the auxin accumulated by TIBA.

To further examine the relationship between *CEG* and the auxin transport, the source of IAA from the shoot apex was removed by decapitation. As the IAA pulse always reaches the root between 5 and 7 DAG (Bhalerao *et al*., 2002), we removed the apical tissues of 4- and 7-day-old seedlings, respectively, and observed the root development in 5 days later (Figure 8B). The lateral roots per cm (nLR/cm) was calculated. In the *ceg* seedlings decapitated at 4 DAG, the lateral roots were barely formed, similar to the wild-type. However, in the *ceg* seedlings decapitated at 7 DAG, the ratio of the lateral root decreased was different from that in wild-type plants. The nLR/cm primary root was 2.49 ± 0.22 and 2.02 ± 0.19 in the intact *ceg* and the wild-type seedlings, respectively. In contrast, they were 1.16 ± 0.15 and 0.65 ± 0.11 in the decapitated plants (Figure 8B). Therefore, the ratio of the change was 46.6% in the *ceg* seedlings and 32.2% in wild-type. It was suggestive that the *ceg* mutation may be involved in auxin transport. However, this phenotype also could be due to the altered auxin sensitivity in the *ceg* roots rather than the altered auxin transport.

Discussion

CEG *acts as a negative regulator of the lateral root formation in* Arabidopsis

Molecular studies have revealed that there is a complex molecular regulation network controlling the lateral root development in *Arabidopsis*. Components of the network function to either promote or attenuate the lateral root formation. TIR1, as the auxin receptor, may be the most important member that is involved in the auxin response process promoting the lateral root initiation. It acts by mediating the degradation of AUX/IAAs (Gray *et al*., 2001). Most recently, microRNAs also have been implicated in the regulation of the lateral root development. *NAC1* mRNA is a target of microRNA164 (MiR164) *in vivo* to down-regulate auxin signals for the lateral root development (Rhoades *et al*., 2002; Mallory *et al*., 2004; Guo *et al*., 2005).

Although many factors have been identified to mediate the lateral root development, most of them act as positive regulators, such as TIR1, NAC1 and Class III HD-ZIPs. Only few negative factors, such as several Aux/IAA proteins, HY5 and SINAT5, have been found. The gain-of-function *axr5*/*iaa1, shy2*/*iaa3, slr*/*iaa14, msg2*/*iaa19* and *iaa28* mutations block lateral root formation, indicating that these IAA proteins negatively regulate lateral root formation (Tian and Reed, 1999; Rogg *et al*., 2001; Fukaki *et al*., 2002; Tatematsu *et al*., 2004; Yang *et al*., 2004). HY5, a transcriptional factor, is an important negative regulator for lateral root formation (Oyama *et al*., 1997). CEG, as a subunit of SCF E3 ligase, plays a negative role in the auxin-mediated lateral root formation through the proteolysis pathway similar to SINAT5. *CEG* is mainly expressed in the vascular system of roots with strong accumulation in the areas where the lateral roots have grown out, but there is no observable expression in the newly formed lateral roots or apical meristem of the primary root (Figure 1F, G). In contrast, high levels of *SINAT5* expression are observed in the primary root apical meristem and in developing lateral root primordia (Xie *et al*., 2000). Secondly, SINAT5 is involved in attenuating the auxin response downstream of TIR1 (Xie *et al*., 2000), whereas CEG may function to influence the auxin transport in the root (see below). Thus, CEG represents a new negative regulator implicated in the lateral root formation. But, the loss of *CEG* function had a modest effect on the lateral root formation, supporting the notion that a complicated regulatory pathway is involved. Furthermore, *CEG* may also play a role in regulating the pollen development and/or pollination and leaf development since the *CEG* transcripts were accumulated highly in pollen and vascular tissues of leaves (Figure 1C, D).

CEG *is involved in the auxin-mediated lateral root formation*

Most of the genes involved in the lateral root development, such as *TIR1, AUX*/*IAA* members and *NAC1*, are identified to function in the auxin signal-transduction pathway. The transcription level of these genes were increased with the treatment of auxin, regardless of positive or negative regulators (Xie *et al*., 2000, 2002; Kepinski and Leyser, 2004). Mutation in several genes of them led to the reduced sensitivity to the auxin (Rugger *et al*., 1998; Yang *et al*., 2004).

Nevertheless, it is still unclear how auxin controls the lateral root formation. Himanen *et al*. (2002) reported that the auxin mediates the cell cycle activation during early lateral root initiation, probably via the transcriptional regulation of *KRP2* (Kip-Related Protein 2). KRP2 protein is one of the CDK inhibitors and its transcripts accumulated in pericycle cells not implicated in lateral root initiation. Overexpression of *KRP2* reduced the number of lateral roots by more than 60%. Its expression is induced in roots treated with NPA and dramatically declined upon auxin treatment. KRP2 plays a role in controlling the branching system of root through inhibiting permanently present CDK complexes and thus the division activity of the meristematically competent pericycle (Stals *et al*., 2001).

The *CEG* expression was induced by exogenous auxin NAA, and mutation in *CEG* resulted in hyposensitivity to three auxin analogs (NAA, IAA and 2,4-D), indicating that CEG is involved in the auxin-mediated pathway for the lateral root formation. *CEG* is highly expressed in the position where the root branching has taken place, implying that it might act during both the initiation and outgrowth process of the lateral root. We propose that CEG may play a role in inhibiting the activity of pericycle cells, thus confining the lateral root formation. Because

CEG is induced by auxin, a feedback regulation loop likely occurs between them. Identification of possible target(s) for CEG will help to reveal the molecular mechanism for such a regulation.

We have shown that the expression of *CEG* is induced by auxin NAA, and the *ceg* mutant was hyposensitive to three auxin analogs: NAA, IAA and 2,4-D. These results indicate that the *CEG* plays a role in the auxin response. On the other hand, the *ceg* mutant exhibited a hyposensitivity to auxin transport inhibition, such as TIBA treatment and decapitation experiment, suggesting that the *CEG* gene is likely involved in the auxin transport. However, further direct evidence is required to support this notion.

So far, three ubiquitin-mediated proteolytic pathways have been implicated in the auxin-dependent lateral root formation, represented by TIR1, SINAT5 and XBAT32, respectively. Both TIR1 and SINAT5 are involved in the auxin response. XBAT32 is proposed to be associated with the auxin transport (Nodzon *et al*., 2004). CEG was initially identified as one of AtSFL (*Arabidopsis* thaliana *S*-locus F-box-Like) proteins capable of physically interacting with ASK1, 2, 9, 11, 14, 16, 18 and 19 based on yeast two-hybrid assay, indicating that it may function as a subunit of SCF E3 ligase in the ubiauitin/26S-mediated proteolysis pathway (Wang *et al*., 2004).

Our results strongly suggest that CEG may function in the auxin response during the lateral root development, representing a novel negative regulator in the auxin-mediated lateral root formation in *Arabidopsis* and revealing an extra possible protein-degradation pathway mediating the lateral root formation. It is worthy to investigate whether these pathways interconnect and the role of CEG in the auxin response, if any, in the auxin transport in *Arabidopsis*.

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