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Original Paper

Identification of *ENHANCER OF SHOOT REGENERATION 1*-upregulated genes during *in vitro* shoot regeneration

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Abstract The *Arabidopsis ENHANCER OF SHOOT REGENERATION 1* (*ESR1*) is thought to be a key gene for commitment to *in vitro* shoot regeneration in tissue culture. *ESR1* encodes a member of the ETHYLENE RESPONSIVE FACTOR (ERF) family of transcription factors. Here, we report identification of genes downstream of *ESR1* during *in vitro* shoot regeneration. We previously demonstrated that the ESR1 protein functions as a transcriptional activator; in the present study, genes upregulated after induction of *ESR1* overexpression were screened by microarray experiments. Seven genes, including *CUP-SHAPED COTYLEDON 1* (*CUC1*), *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED PEPTIDE 2* (*CLE2*), and *GCN5-related N-acetyltransferase 1* (*GNAT1*) were identified as *ESR1*-upregulated genes by screening. *CUC1*, *CLE2*, and *GNAT1* were also upregulated by translocation of ESR1-ER (estrogen receptor) fusions to the nucleus in the presence of cycloheximide, suggesting that these genes are possibly the direct target of the ESR1 protein. Transcript levels of *CUC1*, *CLE2*, and *GNAT1* as well as *ESR1* increased during the early *in vitro* shoot regeneration process, although their time courses were not necessarily similar. Thus, these genes may function downstream of *ESR1* and may be involved in the shoot differentiation process.

Key words: *CLE2*, *CUC1*, *ESR1*, *ESR2*, GNAT protein, shoot regeneration.

In many species, organogenesis or somatic embryogenesis *in vitro* is the first step in the production of transgenic plants from single-transformed cells. Although organogenesis in tissue culture is directed by the appropriate plant hormones, i.e., auxins, and cytokinins, optimal conditions, including hormone and nutrient concentrations, vary widely among plant species and even within varieties or cultivars of the same species. Despite recent advances in the understanding of the molecular mechanisms of auxin and cytokinin actions, less is known about the developmental events downstream. Therefore, for molecular breeding and biotechnology, it is important to elucidate mechanisms downstream of hormones that regulate shoot differentiation in tissue culture.

ENHANCER OF SHOOT REGENERATION 1 (*ESR1*) (also known as *DORNRÖSCHEN; DRN*) is thought to regulate commitment of *Arabidopsis* root cells to shoot differentiation. *ESR1* overexpression greatly enhances the efficiency of shoot regeneration in *Arabidopsis* tissue culture (Banno et al. 2001; Kirch et al. 2003). *ESR1* encodes a transcription factor belonging to the ethylene responsive factor (ERF) family (McGrath et al. 2005; Nakano et al. 2006). The *Arabidopsis* genome has another gene, *ESR2* (Ikeda et al. 2006) [also named *DRNL* (Kirch et al. 2003), *SOB2* (Ward et al. 2006), and *BOLITA* (Marsch-Martinez et al. 2006)]; this gene has redundant functions with *ESR1* during *in vitro* shoot regeneration (Mase et al. 2007). Chandler et al. (2007) reported that the double mutants *drn* (*esr1*)/*drnl* (*esr2*) showed embryo cell patterning defects, implicating *DRN* (*ESR1*) and *DRNL* (*ESR2*) in embryonic development.

The ESR1 protein functions as a transcriptional activator; this transactivation is essential as an enhancer of *in vitro* shoot regeneration by ESR1 (Matsuo and Banno 2008), suggesting that sequential expression of the downstream genes is required for commitment to shoot differentiation. In the present study, we identified genes that were upregulated by ESR1 overexpression during *in vitro* shoot regeneration.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) plants were used for root culture. The Murashige-Skoog (MS) medium contained

Abbreviations: CIM, callus-inducing medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2-ip, N^6-Δ^2-isopentenyladenine; MS salts, Murashige and Skoog salts; SIM, shoot-inducing medium; PCR, polymerase chain reaction.
This article can be found at http://www.jspcmb.jp/

MS salts (Wako Pure Chemical Industries Ltd.), Gamborg's B5 vitamins (Sigma-Aldrich), 1% sucrose, and 0.25% gellan gum (Wako Pure Chemical Industries Ltd.). CIM contained Gamborg's B5 salts (Sigma-Aldrich), 2% glucose, Gamborg's B5 vitamins, 2 μM 2,4-D, and 0.25% gellan gum. SIM contained MS salts, Gamborg's B5 vitamins, 1% sucrose, 12.5 μM 2-ip, and 0.25% gellan gum. Plants were grown at 22°C under continuous light.

Construction of plasmids and transformation

For pERH::ESR1 construction, ESR1 cDNA was amplified from pSK-ESR1 (Banno et al. 2001) using the following primers: AscI-ESR1N (5′-GGCGCGCCATGGAAAAAGCC-TTGAGAAACTTCACCG-3′) and vector primer 1 (5′-AACGATCGGGGAAATTCGAGCTGCGG-3′). The amplified fragments were digested with *Asc*I and ligated into pER8 (Zuo et al. 2000) between the *Asc*I and blunted *Spe*I sites.

To construct pSK::ESR1-ER, fragments encoding *ESR1* were amplified from pSK-ESR1 by polymerase chain reaction (PCR) using the primers vector primer 2 (5′-CTCTATA-TAAGGAAGTTCATTTCATTTGG-3′) and ESR1-C-BamHI (5′-GAGAGAGAGGATCCTCCCCACGATCTTCGGCAAG-3′). The fragments were then digested with *Asc*I and *Bam*HI. The estrogen receptor region was amplified by PCR from pER8 using primers Est-F (5′-AGGATCCCCGTCTGCTGGAGA-CATGAG-3′) and Est-R (5′-TGCGGCCGCACTAGTTAG-ACTGTGGCAGGGAAACCCT-3′) and then digested with *Bam*HI and *Not*I. These fragments were co-inserted into pSK34 between the *Asc*I and *Not*I sites to produce pSK::ESR1-ER.

To generate pESR1::GFP, a 3.7-kb region of *ESR1* promoter and a 0.8-kb *ESR1* terminator regions were amplified by PCR from Col-0 genomic DNA. The primers for the *ESR1* promoters were ESR1P-F (5′-AACGGTACCAGAAGC-TTACCAGGTACCAAATCCATATCCATCCATGTTGTT-3′) and ESR1P-R (5′-AAGAACCGTGTCGACTTCCACCTG-CAGGCCATTTTTGGTTTCTAGGGTTTTGGTTTGA-3′). The primers for the *ESR1* terminator were ESR1T-F (5′-TGGAAGTCGACACGGTTCTTGGCGCGCCGAAGATCGT GGGGATAGCTAGAT-3′) and ESR1T-R (5′-ACGAAGAG-CTCGAATTCTGGATCCGACGGATATACATGTTACATCAA CGCA-3′). These fragments were fused with green fluorescent protein (GFP) (Sheen et al. 1995) and then inserted into modified pPZP200 (Hajdukiewicz et al. 1994). These constructs were then transformed to *A. thaliana* (Col-0) mediated by *Agrobacterium tumefaciens* EHA105.

RNA isolation and cDNA synthesis

Total RNA isolation and cDNA synthesis were performed as described previously (Mase et al. 2007).

Quantitative reverse transcription-PCR (qRT-PCR) analysis

Real-time qRT-PCR reactions were performed in a Fast 96-well Reaction Plate (0.1 ml) using the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems), TaqMan Universal PCR Master Mix (Applied Biosystems), and TaqMan Gene Expression Assays (Applied Biosystems) or *Power* SYBR Green PCR Master Mix (Applied Biosystems). The assay IDs of the TaqMan Gene Expression Assays were as follows: At02284625_s1 for *ESR1*, At02306690_gl for *ESR2*, At02247793_g1 for *CUC1*, and At02304595_g1 for *UBQ5*. The primers used were as follows: *CLE2* (CLE2-F 5′-CGAGTCCGACTGTAGAGGATGA-3′ and CLE2-R 5′-CGG-GTCCTCCTGGGCTTA-3′) and *GNAT1* (GNAT1-F 5′-CTGT-GAGCCGCTCAGAGTTG-3′ and GNAT1-R 5′-AGTCTA-CGCCGTCATCGTCAT-3′). Amplification was monitored in real time, and the results were analyzed according to the standard curve method (Applied Biosystems).

Microarray experiment

A GeneChip Arabidopsis ATH1 Genome Array (Affymetrix) was used for the microarray experiments. Double-stranded cDNAs were synthesized from total RNAs using the GeneChip expression 3′-Amplification Reagents One-Cycle cDNA Synthesis Kit (Affymetrix) according to the manufacturer's instructions and purified using the Affymetrix GeneChip sample cleanup module. Biotin-labeled cRNAs were synthesized using the Affymetrix GeneChip IVT labeling system. The biotin-labeled cRNAs were fragmented and hybridized to the array according to the manufacturer's instructions. Each array was scanned with a GeneChip Scanner (Affymetrix).

Microscopy

GFP expressions were observed under a fluorescence stereoscopic microscope (model SZX7, Olympus Ltd.). Images were captured using a Canon EOS 40D digital camera (Canon Ltd.).

Results

Screening of genes induced by ESR1 overexpression

Ikeda et al. (2006) reported identification of genes that were upregulated by *ESR2* overexpression without *de novo* protein synthesis by using microarray assays. Although many genes were listed as potential direct target genes of the ESR2 protein, their functions as ESR2 recipients were unclear, except for *CUP-SHAPED COTYLEDON 1* (*CUC1*). We attempted to identify *in vitro* shoot regeneration enhancer genes that were downstream of the ESR1 protein but not necessarily direct targets. For this purpose, we produced a transgenic *Arabidopsis* plants carrying an estrogen-inducible *ESR1* construct (XVE-ESR1) (Figure 1A). To confirm the functionality of this construct, shoot regeneration efficiencies from root cultures of the transgenic lines were examined. We previously showed that *ESR1* overexpression enhanced shoot regeneration. Root segments from a transgenic line preincubated on CIM for 4 days were transferred onto SIM with or without an estrogen (17β-estradiol) as a transcription inducer. We previously demonstrated that *ESR1* expression was induced by cytokinin on SIM and that the induction required preincubation on CIM with 2,4-D [coincident with the fact that preincubation on CIM is required for efficient shoot regeneration (Banno et al. 2001)]. Figure

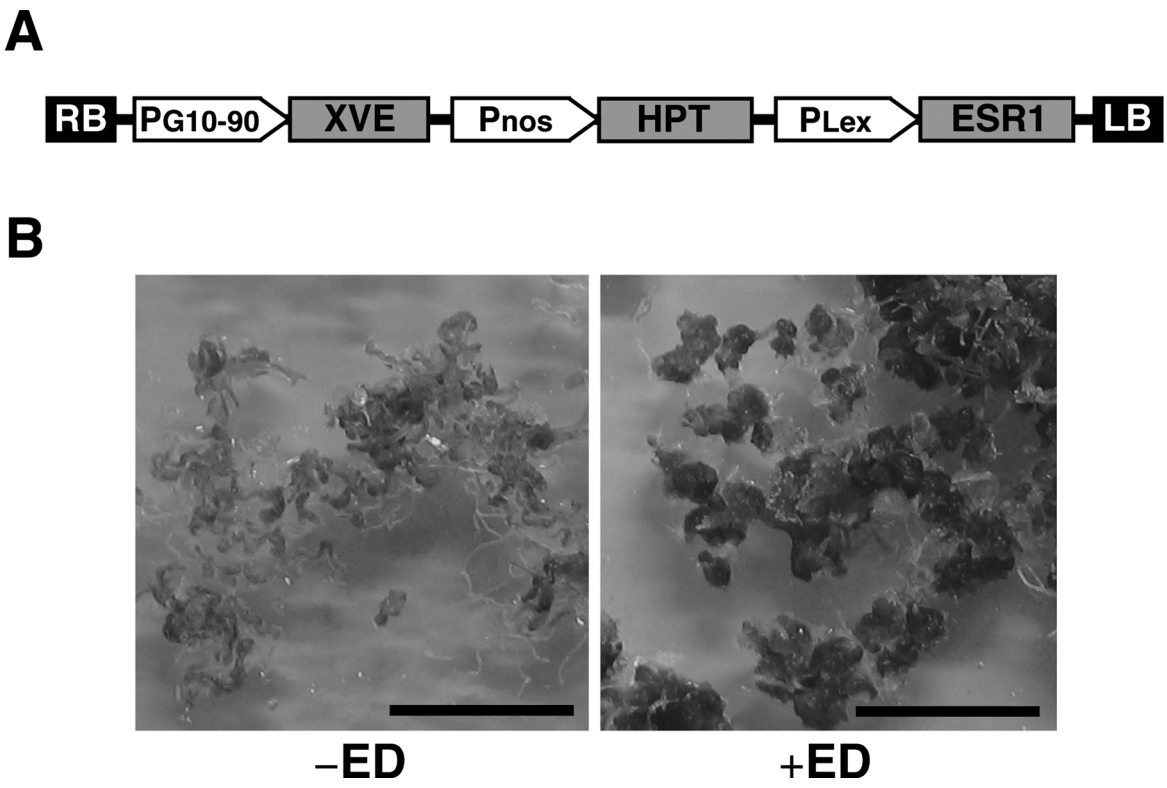

Figure 1. Enhancement of shoot regeneration by induced *ESR1* expression. (A) Schematic representation of T-DNA region in pERH::ESR1 from the left border (LB) to the right border (RB). Expression of an artificial transcription factor, XVE, is under the control of a constitutive synthetic promoter, G10-90 ($P_{G10\text{-}90}$). HPT (HYGROMYCIN PHOSPHOTRANSFERASE II) was expressed under the control of nopaline synthase promoter (P_{nos}). Lex promoter (P_{Lex}) is a fusion promoter of eight copies of the LexA operator sequence and 35S minimal promoter. (B) Shoot regeneration from transgenic plants harboring pERH::ESR1. After root segments were incubated on callus-inducing medium (CIM) for 4 days, they were incubated on shoot-inducing medium (SIM) with (+ED) or without (−ED) 10 μM 17β-estradiol for 3 weeks. Bars=1 cm.

1B shows shoot regeneration from the transgenic root segments after incubation on SIM for 3 weeks. Application of estrogen onto the medium clearly enhanced shoot regeneration efficiency.

For microarray experiments, root segments from transgenic plants carrying XVE-ESR1 preincubated on CIM for 4 days were transferred onto SIM with or without estrogen and incubated for 24 h. Total RNAs were prepared from these root cultures and subjected to analyses using the Affymetrix ATH1 GeneChip. The scanned array data were processed by Affymetrix GeneChip Operating Software (GCOS), which scaled the average intensity of all the genes on each array to a target signal of 200. Each array comprised 22,746 plant genes (probe sets). We calculated the expression signal for the two sample sets and the log 2 ratio for the +ED (with an estrogen) sample against the −ED (without an estrogen) sample by using GCOS software. In this experiment, we selected those genes detected as present (P) by GCOS in the +ED sample. We excluded those genes for which GCOS found no difference in expression signal in the +ED sample compared with the −ED sample. In the +ED sample, the probes that expressed at a signal intensity of less than 200 were excluded. It is empirically difficult to reproduce expression by RT-PCR for genes that have a signal intensity of less than 200 after scaling to 200. In the present analysis, the probes that expressed at a log 2 ratio of less than two were also excluded. Seven genes were identified as upregulated by *ESR1* overexpression (Table 1). We focused on two genes, namely, At4g18510 and At5g67430, with the highest induction levels in the presence of estrogen (as indicated by their log 2 ratio), along with *CUC1*, which was identified by Ikeda et al. (2006) as a direct target of ESR2. At4g18510 and At5g67430 encode CLAVATA3/EMBRYO SURROUNDING REGION-RELATED PEPTIDE 2 (CLE2) (Sharma et al. 2003; Strabala et al. 2006) and the GCN5-related *N*-acetyltransferase (GNAT) family protein (Dyda et al. 2000; Vetting et al. 2005), respectively. We designated At5g67430 as *GNAT1*. To confirm induction of these genes by *ESR1* overexpression, qRT-PCR was performed. Root explants for the transgenic line used for microarray analyses were preincubated on CIM for 4 days and then transferred onto SIM. Total RNAs were prepared at the indicated times and subjected to qRT-PCR (Figure 2). *ESR1*, *CUC1*, *CLE2*, and *GNAT1* expression was induced on SIM without estrogen. *CUC1*,

Table 1. Genes upregulated by induction of ESR1 overexpression.

Probe ID	−ED		+ED		Signal log 2 ratio	Change P-value	Transcript ID	Description
	Signal	Signal call	Signal	Signal call				
254644_at	48.8	P	681.6	P	3.7	2.00E-05	At4g18510	CLAVATA3/ESR-RELATED PEPTIDE 2 (CLE2)
246992_at	30.9	A	386.8	P	3.6	2.00E-05	At5g67430	GCN5-RELATED N-ACETYLTRANSFERASE 1 (GNAT1)
260335_at	77.4	P	448.1	P	2.5	2.00E-05	At1g74000	STRICTOSIDINE SYNTHASE 3
260406_at	41.3	P	211.8	P	2.4	3.07E-04	At1g69920	GLUTATHIONE S-TRANSFERASE TAU12 (ATGSTU12)
256857_at	70	P	290.9	P	2.4	2.70E-05	At3g15170	CUP-SHAPED COTYLEDON 1 (CUC1)
253880_at	96	P	392	P	2	2.00E-05	At4g27590	Copper-binding protein-related, similar to metal ion binding
253493_at	179.6	P	832.4	P	2	2.00E-05	At4g31820	ENHANCER OF PINOID/MACCHI-BOU4/NAKED PINS IN YUC MUTANTS 1

Signal values, signal calls, signal log 2 ratios, and change *P*-values were determined by GCOS software. Signal values and signal log 2 ratios were calculated by One-Step Tukey's Biweight Estimation (Hoaglin et al. 1983). Signal calls were categorized on the basis of the default GCOS setting for signal *P* values (P: *P* values <0.04, M: 0.04≤*P* values <0.06, A: *P* values≥0.06). Signal *P* values were calculated by Wilcoxon signed-rank test (Wilcoxon 1945) on the basis of the differences between perfect match probes and mismatch probes. Change *P*-values for signal log 2 ratios were calculated by Wilcoxon signed-rank test on the basis of the differences between the target array and the control array.

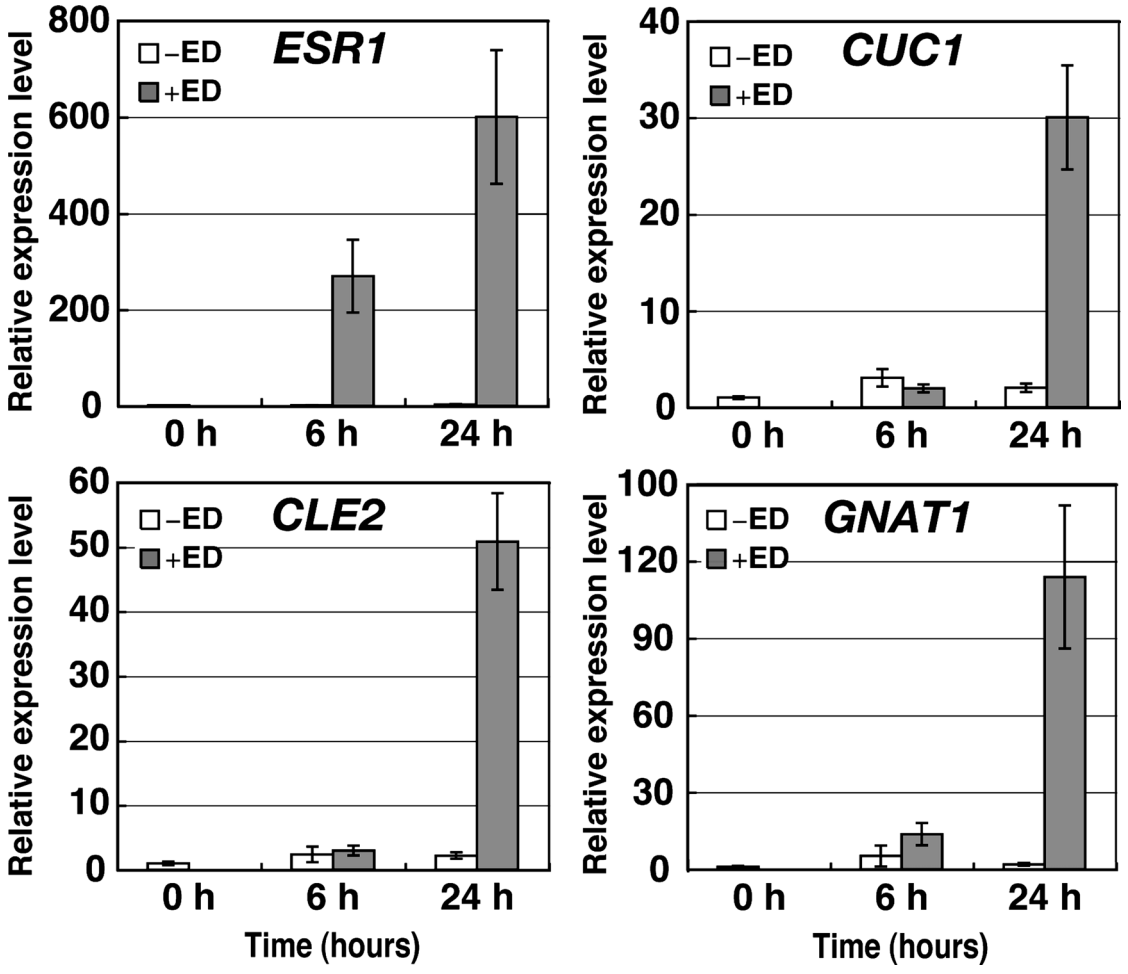

Figure 2. Upregulation of *ESR1*, *CUC1*, *CLE2*, and *GNAT1* expression by ESR1 overexpression during *in vitro* shoot regeneration. Root segments were incubated on callus-inducing medium (CIM) for 4 days and then transferred onto shoot-inducing medium (SIM) with (+ED) or without (−ED) 10 μM 17β-estradiol. Total RNAs were extracted at 0 h, 6 h, and 24 h after transfer onto SIM. Each value on the vertical axes indicates a relative level calculated by reference to the *UBQ5* transcript levels. Data represent the average of three independent polymerase chain reaction runs, and error bars indicate standard deviations.

CLE2, and *GNAT1* also appeared to be upregulated by incubation on SIM. *ESR1* transcript level increased 167-fold after 6-h incubation with the estrogen and 171-fold after 24-h incubation, compared with *ESR1* transcript levels after incubation without estrogen. *CUC1* and *CLE2* mRNA levels clearly increased after 24 h on SIM with estrogen compared with mRNA levels measured after incubation on SIM without estrogen. However, *CUC1* and *CLE2* mRNA did not increase significantly after only 6 hours' incubation on SIM with estrogen compared with their mRNA levels after incubation on SIM without estrogen. *GNAT1* mRNA increased 2.6-fold after 6 hours' incubation and by 58-fold after 24 hours' incubation on SIM with estrogen. These results suggest that *CUC1*, *CLE2*, and *GNAT1* expression was upregulated in an *ESR1* overexpression-dependent manner. Furthermore, *GNAT1* expression appeared to be induced by *ESR1* earlier than *CLE2* or *CUC1*.

Direct induction of CUC1, CLE2, and GNAT1 by ESR1 overexpression

GNAT1, *CLE2*, and *CUC1* were upregulated by ESR1 overexpression. To examine whether induction of these genes by *ESR1* required *de novo* protein synthesis, we used transgenic plants expressing *ESR1* with an estrogen receptor-mediated nuclear translocation system. We produced transgenic *Arabidopsis* plants expressing ESR1-ER (ESR1 fused to the hormone-binding domain of the estrogen receptor), which facilitated import of a fusion protein into the nucleus in response to estrogen. Root explants from the transgenic lines were preincubated on CIM and then transferred onto SIM with or without estrogen to promote nuclear translocation of the ESR1-ER. Treatment with estrogen clearly enhanced shoot regeneration (Figure 3A). Cycloheximide was applied to SIM to inhibit *de novo* protein synthesis in order to examine direct induction by ESR1 protein. The transgenic roots preincubated on CIM for 4 days were incubated on SIM with cyclohexamide with or without estrogen. After 2 h, total RNAs were prepared and qRT-PCR was carried out (Figure 3B). *CLE2* transcript level elevated 5.2-fold by adding estrogen onto SIM, although induction levels of *CUC1* (3.1-fold) and *GNAT1* (2.6-fold) by the estrogen were not very high. These results suggested that these genes were directly upregulated by *ESR1*.

Expression patterns of ESR1, ESR2, CUC1, CLE2, and GNAT1 during in vitro shoot regeneration

We also investigated expression patterns of candidates for *ESR1*-downsteam genes during shoot regeneration. Root segments from wild-type *Arabidopsis* were preincubated on CIM for 4 days and then transferred onto SIM. Total RNAs were prepared after incubation on SIM. Transcript levels of *ESR1*, *ESR2*, *CUC1*, *CLE2*, and *GNAT1* were determined by qRT-PCR (Figure 4). *ESR1* transcript levels gradually increased until day 5 and then decreased slightly, while *ESR2* transcript levels increased later than those of *ESR1*. *CUC1* transcript levels increased rapidly after transfer of the explants onto SIM, and high transcript levels were maintained through day 7. *CLE2* and *GNAT1* transcript levels increased until day 2 or day 3 and then gradually decreased. These results suggested that all the genes examined in these experiments may function in early shoot regeneration events, although their expression patterns were not necessarily similar or parallel.

Although we had demonstrated using RNA blot

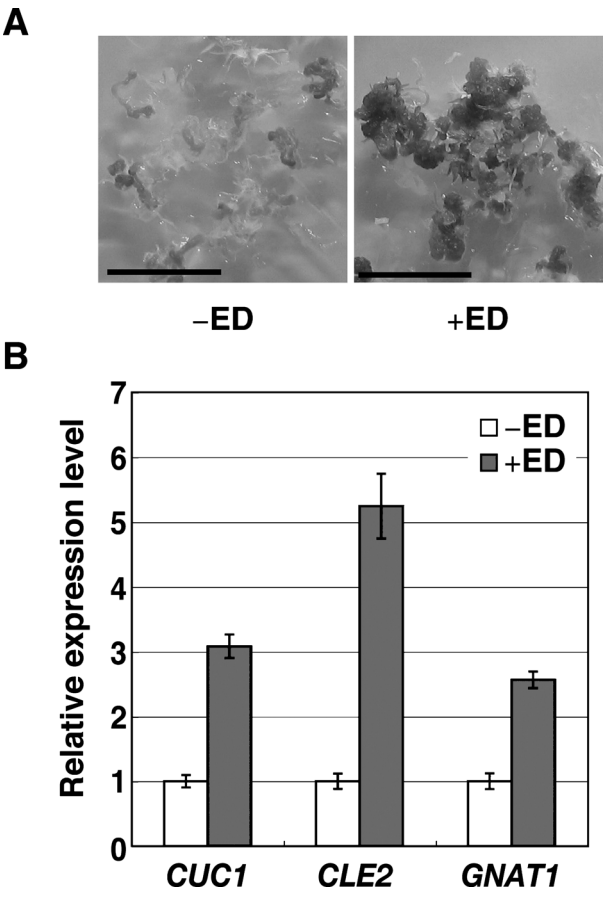

Figure 3. Direct upregulation of *CUC1*, *CLE2*, and *GNAT1* expression by ESR1-ER during *in vitro* shoot regeneration. (A) Shoot regeneration from transgenic plants expressing ESR1-ER. Root segments preincubated on CIM for 4 days were transferred onto SIM with (+ED) or without (−ED) 10 μM 17β-estradiol and incubated for 2 weeks. Bars = 1 cm. (B) Root segments preincubated on callus-inducing medium (CIM) for 4 days were transferred onto CIM with 10 μM cycloheximide and incubated for 1 h. They were subsequently incubated on shoot-inducing medium (SIM) with 10 μM cycloheximide with (+ED) or without (−ED) 10 μM 17β-estradiol for 2 h. Transcript levels were determined by quantitative reverse transcription-polymerase chain reaction (qTR-PCR). Each value on the vertical axes indicates relative level of +ED sample to −ED sample. Data represent the average of three independent PCR reactions, and error bars indicate standard deviations. Three biological replicates were carried out and showed similar results (data not shown).

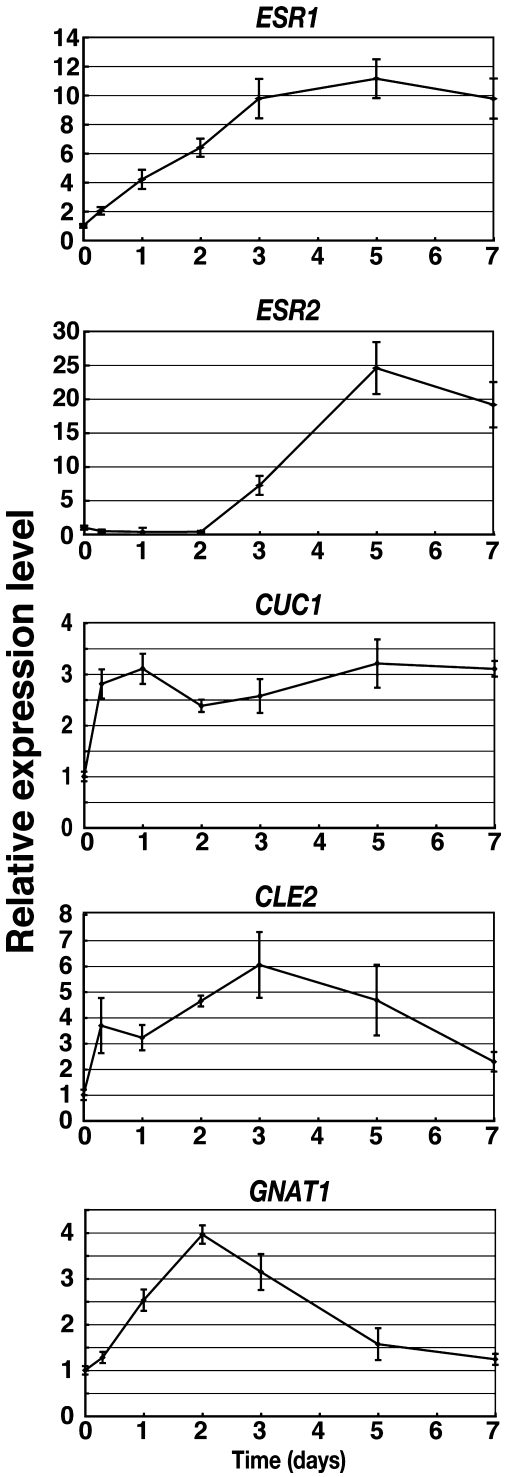

Figure 4. Time courses of *ESR1*, *ESR2*, *CUC1*, *CLE2*, and *GNAT1* expressions during shoot regeneration. Root segments were incubated on callus-inducing medium (CIM) for 4 days and then transferred onto shoot-inducing medium (SIM). Total RNAs were prepared from root explants at indicated time points after transfer onto SIM. Transcript levels were measured with quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Each value on the vertical axes indicates a relative level calculated by reference to the *UBQ5* transcript levels. Numbers on the horizontal axes indicates days after transfer onto SIM. Data represent the average of three independent PCR reactions, and error bars indicate standard deviations. Three biological replicates were carried out and showed similar results (data not shown).

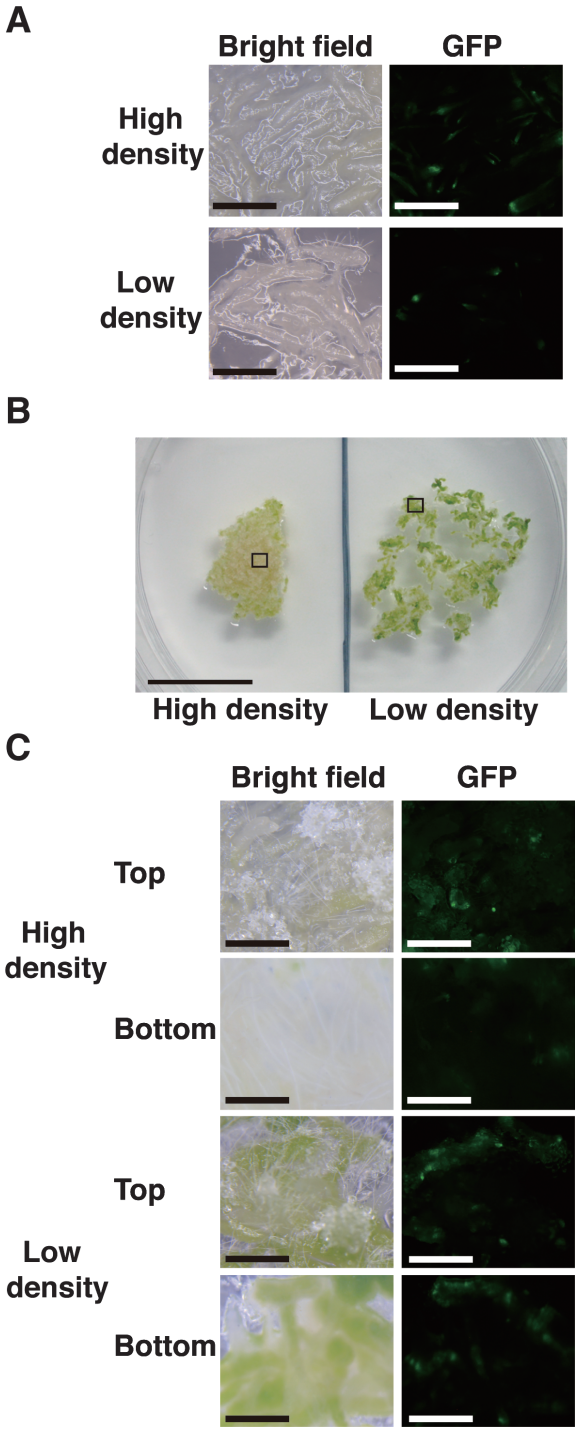

Figure 5. *ESR1*::GFP expression during shoot regeneration. (A) *ESR1*::GFP expression after 2 days on shoot-inducing medium (SIM). Root segments from transgenic plants harboring *ESR1*::GFP were preincubated on callus-inducing medium (CIM) for 4 days and then transferred onto SIM. Root cultures were incubated on SIM in high density (400 mg root explants/4 cm^2) or in low density (80 mg root segments/10 cm^2). Left panels are bright-field images and right panels are green fluorescent protein (GFP) fluorescence images. Each picture was taken from the table side. Bar = 1 mm. (B) Shoot regeneration after 7 days on SIM. Areas in the black frames represent the localized regions shown in (C). Bar = 2 cm. (C) *ESR1*::GFP expression after 7 days on SIM. Left panels are bright-field images and right panels are GFP fluorescence images. Each picture was taken from the table side (Top) or the other side (Bottom). Bars = 1 mm.

analysis that *ESR1* transcript levels increased until day 2 and then declined after transfer onto SIM (Banno et al. 2001), different results were obtained by incubation of explants at a lower density (Figure 5). In the previous experiments, root segments were incubated in cultures with a density of approximately 100 mg/cm^2 to obtain sufficient RNAs for RNA blot analyses. However, it is possible to obtain sufficient RNAs for qRT-PCRs from root segment cultures incubated at a lower density, a process that confers more efficient shoot regeneration. In the present study, we examined RT-PCR expression patterns in this low-density culture (Figure 4). To confirm that a different density of tissue culture produced a different expression pattern of *ESR1*, we used a GFP reporter system. To accomplish this, the *ESR1* promoter-GFP fusion (*ESR1*::GFP) construct was transformed to *Arabidopsis*. Root segments from the transgenic plants were incubated on CIM for 4 days, followed by transfer onto SIM at two different culture densities. Cells grew and filled spaces between root segments in the dense culture by day 7 (Figure 5B). While most of the green calli and shoot formation was observed in the peripheral region of the dense culture, green calli and shoots emerged throughout the low-density culture. Similar patterns of GFP expression were observed on day 2 in both culture densities (Figure 5A). Although cell densities are very different, GFP expression on day 7 in the high-density culture was not very different from that in the low-density culture when observed from the top side and even declined in the high-density culture when observed from the bottom side. In contrast, GFP expression in the low-density culture extended throughout the tissues, especially in the developing shoots (Figure 5C) as was evident by the appearance of many green calli and shoots. These results indicate that GFP expression decreased when cultured in high cell density. These GFP expression patterns coincided with *ESR1* transcript levels as determined by qRT-PCR. Thus, we concluded that different tissue culture densities produced different expression patterns of *ESR1*.

Discussion

ESR1-upregulated genes were screened to identify candidate genes functioning downstream of ESR1; 3 candidate genes were identified: *CUC1*, *CLE2*, and *GNAT1*. *CUC1* expression was found to be upregulated by *ESR2* overexpression in the presence of cycloheximide, and the phenotypes of *ESR2* overexpression in a *cuc1-1* mutant background were suppressed. On the basis of these results, *CUC1* is thought to be a direct target of the ESR2 protein. Moreover, *ESR1* overexpression was found to upregulate *CUC1* expression in the presence of cycloheximide (Figure 3B). *ESR1* and *ESR2* upregulated *CUC1* expression in a redundant manner; we believe that this upregulation of *CUC1* expression may induce shoot regeneration. Indeed, it has been reported that *CUC1* overexpression promotes the formation of adventitious shoots on calli (Daimon et al. 2003).

CLE2 and *GNAT1* expressions were also upregulated by *ESR1* overexpression (Figure 2). Direct upregulation of *CUC1* (3.1-fold) and *GNAT1* (2.6-fold) expressions by *ESR1* were less clear compared with that of *CLE2* expression (5.2-fold). It is difficult to determine whether leaky translation of other ESR1-upregulated genes in the presence of cycloheximide resulted in the expression of *CLE2* and *GNAT1* at low levels or whether *ESR1* induced the expression of *CLE2* and *GNAT1* at low levels.

The optimal binding sequence of the ESR1 protein is GCCGCC (GCC box), which is known as the ethylene-responsive element (Banno et al. 2006). *CUC1* and *CLE2* genes contain a few GCCGCC-related sequences within 2 kbp upstream of their initiation codons; however, no GCCGCC sequences are found in their upstream sequences, and a GCCGCC exists in each coding region. A GCCGCC sequence exists at −808 bp upstream from the initiation codon of *GNAT1*. Although we conducted chromatin immunoprecipitation assays to confirm that ESR1 was bound directly to the promoters of these genes, we were unable to recover the ESR1 protein by using an antibody against c-myc from the tissue culture of *Arabidopsis* expressing the ESR1-myc fusion protein. Therefore, we were unable to prove that these genes are direct targets of ESR1.

It is remarkable that *GNAT1* was also listed as an *ESR2*-upregulated gene (Ikeda et al. 2006); however, this has not been further investigated. We believe that *GNAT1* should be further investigated as a candidate gene present downstream of *ESR1* and *ESR2* that induces shoot differentiation; however, it is unclear whether *GNAT1* is a direct target of ESR1 or ESR2. *CLE2* encodes a CLV3-like small peptide (Sawa et al. 2006; Strabala et al. 2006). The *Arabidopsis* genome has 31 CLE genes, and it has been suggested that some of them are involved in various developmental events (Fiers et al. 2007; Mitchum et al. 2008). Among the proteins that are closely related to the *HOOKLESS1* (*HLS1*) product, the GNAT1 protein is most closely related to it. *HLS1* is required for apical hook formation and encodes a putative *N*-acetyltransferase (Lehman et al. 1996), but the biochemical function of HLS1 is unknown. Recently, Chatfield and Raizada (2008) reported that *hls1-1* is capable of enhancing shoot regeneration. If the HLS1 and GNAT1 proteins share substrates for acetylation, a negative feedback loop may regulate the shoot regeneration process. We have found that overexpression of *CLE2* or *GNAT1* does not affect the efficiency of shoot regeneration (our unpublished results).

Downstream of ESR1, shoot regeneration appeared not to be a single pathway. Currently, we are examining shoot regeneration in mutants with T-DNA in the *CLE2* or *GNAT1* gene. We think that the relationship between *ESR1* and these genes can be elucidated with the help of such experiments.

The time courses of *ESR1*, *ESR2*, *CUC1*, *CLE2*, and *GNAT1* expression were not necessarily similar, although all these genes were upregulated after transfer onto SIM (Figure 4). The levels of *CUC1* transcripts increased more rapidly than those of *ESR1* or *ESR2*. It has been reported that *CUC1* expression increased in calli incubated on CIM, and that its expression increased further on incubating the calli on SIM (Cary et al. 2002; Daimon et al. 2003). The expression of *CUC1* appears to be differentially regulated by multiple transcription factors according to the stage of development. ESR1 may be one of the transcription factors that cooperatively regulate *CUC1* expression during shoot regeneration. Although the *CLE2* transcript was detected by RT-PCR analysis of seedlings, roots, and reproductive shoot apices (Sharma et al. 2003), its detailed expression pattern during development or tissue culture have not been investigated. The GNAT1 sequence and its expression patterns in various organs are available in databases; however, it has not been further investigated. The *CLE2* and *GNAT1* transcript levels decreased after day 3, although the *ESR1* and *ESR2* transcripts were maintained at high levels through day 7. Therefore, we think that negative feedback regulations may modulate the expression of these genes. Recently, we produced an *esr1*/*esr2* double-mutant line. However, we have not yet examined the expression patterns of ESR1-downstream candidates during *in vitro* shoot regeneration because of the lethality of this mutant.

In this study, we demonstrated that *ESR1* expression increased in the early stages of shoot formation in low-density cultures. We have reported that the *ESR1* transcript levels increased transiently after the culture was transferred onto SIM (Banno et al. 2001). However, experiments using transgenic *Arabidopsis* harboring the *ESR1*::GFP fusion gene revealed that *ESR1* promoter activity was inhibited by the overgrowth of calli, and that this inhibition coincided with decreased shoot formation in dense cultures (Figure 5). Therefore, we concluded that transient expression was observed previously because the cell cultures were overly dense. In the future, we will investigate the relationship between the location of shoot formation and the location of *ESR1* and *ESR2* expressions by using the *ESR1* promoter-GFP fusion gene and the *ESR2* promoter-CFP fusion gene.

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References

Banno H, Ikeda Y, Niu QW, Chua NH (2001) Overexpression of *Arabidopsis ESR1* induces initiation of shoot regeneration. *Plant Cell* 13: 2609–2618

Banno H, Mase H, Maekawa K (2006) Analysis of functional domains and binding sequences of *Arabidopsis* transcription factor ESR1. *Plant Biotechnol* 23: 303–308

Cary AJ, Che P, Howell SH (2002) Developmental events and shoot apical meristem gene expression patterns during shoot development in *Arabidopsis thaliana*. *Plant J* 32: 867–877

Chandler JW, Cole M, Flier A, Grewe B, Werr W (2007) The AP2 transcription factors DORNROSCHEN and DORNROSCHEN-LIKE redundantly control *Arabidopsis* embryo patterning via interaction with PHAVOLUTA. *Development* 134: 1653–1662

Chatfield SP, Raizada MN (2008) Ethylene and shoot regeneration: *hookless1* modulates *de novo* shoot organogenesis in *Arabidopsis thaliana*. *Plant Cell Rep* 27: 655–666

Daimon Y, Takabe K, Tasaka M (2003) The *CUP-SHAPED COTYLEDON* genes promote adventitious shoot formation on calli. *Plant Cell Physiol* 44: 113–121

Dyda F, Klein DC, Hickman AB (2000) GCN5-related N-acetyltransferases: a structural overview. *Annu Rev Biophys Biomol Struct* 29: 81–103

Fiers M, Ku KL, Liu CM (2007) CLE peptide ligands and their roles in establishing meristems. *Curr Opin Plant Biol* 10: 39–43

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol Biol* 25: 989–994

Hoaglin DC, Mosteller F, Tukey JW (1983) *Understanding Robust and Exploratory Data Analysis.* John Wiley & Sons Inc, New York

Ikeda Y, Banno H, Niu QW, Howell SH, Chua NH (2006) The *ENHANCER OF SHOOT REGENERATION 2* gene in *Arabidopsis regulates CUP-SHAPED COTYLEDON 1* at the transcriptional level and controls cotyledon development. *Plant Cell Physiol* 47: 1443–1456

Kirch T, Simon R, Grunewald M, Werr W (2003) The *DORNROSCHEN/ENHANCER OF SHOOT REGENERATION 1* gene of Arabidopsis acts in the control of meristem ccll fate and lateral organ development. *Plant Cell* 15: 694–705

Lehman A, Black R, Ecker JR (1996) *HOOKLESS1*, an ethylene response gene, is required for differential cell elongation in the *Arabidopsis* hypocotyl. *Cell* 85: 183–194

Marsch-Martinez N, Greco R, Becker JD, Dixit S, Bergervoet JH et al. (2006) BOLITA, an *Arabidopsis* AP2/ERF-like transcription factor that affects cell expansion and proliferation/differentiation pathways. *Plant Mol Biol* 62: 825–843

Mase H, Hashiba M, Matsuo N, Banno H (2007) Expression patterns of *Arabidopsis* ERF VIII-b subgroup genes during *in vitro* shoot regeneration and effects of their overexpression on shoot regeneration efficiency. *Plant Biotechnol* 24: 481–486

Matsuo N, Banno H (2008) The *Arabidopsis* transcription factor ESR1 induces *in vitro* shoot regeneration through transcriptional activation. *Plant Physiol Biochem* 46: 1045–1050

McGrath KC, Dombrecht B, Manners JM, Schenk PM, Edgar CI et

al. (2005) Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of *Arabidopsis* transcription factor gene expression. *Plant Physiol* 139: 949–959

Mitchum MG, Wang X, Davis EL (2008) Diverse and conserved roles of CLE peptides. *Curr Opin Plant Biol* 11: 75–81

Nakano T, Suzuki K, Fujimura T, Shinshi H (2006) Genome-wide analysis of the ERF gene family in Arabidopsis and rice. *Plant Physiol* 140: 411–432

Sawa S, Kinoshita A, Nakanomyo I, Fukuda H (2006) CLV3/ESR-related (CLE) peptides as intercellular signaling molecules in plants. *Chem Rec* 6: 303–310

Sharma VK, Ramirez J, Fletcher JC (2003) The *Arabidopsis CLV3-like* (*CLE*) genes are expressed in diverse tissues and encode secreted proteins. *Plant Mol Biol* 51: 415–425

Strabala TJ, O'Donnell PJ, Smit AM, Ampomah-Dwamena C, Martin EJ et al. (2006) Gain-of-function phenotypes of many *CLAVATA3*/*ESR* genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. *Plant Physiol* 140: 1331–1344

Vetting MW, LP SdC, Yu M, Hegde SS, Magnet S et al. (2005) Structure and functions of the GNAT superfamily of acetyltransferases. *Arch Biochem Biophys* 433: 212–226

Ward JM, Smith AM, Shah PK, Galanti SE, Yi H et al. (2006) A new role for the *Arabidopsis* AP2 transcription factor, LEAFY PETIOLE, in gibberellin-induced germination is revealed by the misexpression of a homologous gene, *SOB2*/*DRN-LIKE*. *Plant Cell* 18: 29–39

Wilcoxon F (1945) Individual Comparisons by Ranking Methods. *Biometrics Bull* 1: 80–83

Zuo J, Niu QW, Chua NH (2000) An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant J* 24: 265–273