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Original Paper

Expression patterns of *Arabidopsis* ERF VIII-b subgroup genes during *in vitro* shoot regeneration and effects of their overexpression on shoot regeneration efficiency

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Abstract The *Arabidopsis ESR1*, which encodes a member of the ERF family, is thought to be a key gene for commitment to shoot differentiation in tissue culture. The *Arabidopsis* genome has more than a hundred genes encoding the ERF family and *ESR1* belongs to the ERF VIII-b subgroup. We examined *ESR1* and 5 genes structurally similar to *ESR1* (*ESR2*, *ESL1*, *ESL2*, *ESL3* and *LEP*) on their expression patterns during *in vitro* shoot regeneration. All these genes' transcript levels increased 1–2 days after shoot induction by incubation on shoot-inducing medium and then decayed by day 7. These genes' overexpression demonstrated that *ESR1* and *ESR2* clearly enhanced shoot regeneration when overexpressed, but other genes did not. These results suggest that all 6 examined genes may be involved in early events of shoot regeneration, although only *ESR1* and *ESR2* enhanced shoot regeneration by their overexpression.

Key words: *ESR1*, *ESR2*, ERF, shoot regeneration.

Shoot regeneration is a key step for successful production of transgenic plants in many species. Therefore, it is important for molecular breeding and biotechnology to elucidate the mechanisms regulating shoot differentiation in tissue culture. *Arabidopsis ENHANCER OF SHOOT REGENERATION 1* (*ESR1*) (also known as DORNRÖNSCHEN; *DRN*) is thought to regulate commitment to shoot differentiation, and *ESR1* overexpression greatly enhances 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. The ERF family is the largest branch in the AP2/ERF superfamily transcription factors containing one or two AP2 /ERF domains which are plant-specific DNA-binding motifs (McGrath et al. 2005; Nakano et al. 2006; Shigyo et al. 2006). The *Arabidopsis* genome has more than 100 ERF family genes, which are implicated in many diverse physiological events such as hormone signaling (Ohme-Takagi and Shinshi 1995; van der Fits and Memelink 2000; Guo and Ecker 2004; Rashotte et al. 2006), biotic and abiotic stress tolerance (Gutterson and Reuber 2004; Agarwal et al. 2006), cuticular wax biosynthesis (Aharoni et al. 2004; Broun et al. 2004; Zhang et al. 2005), and development processes (van der Graaff et al. 2000; Banno et al. 2001; Kirch et al. 2003; Ikeda et al. 2006; Chandler et al. 2007). Nakano et al. (2006) analyzed ERF genes using computational methods and classified them into 17 groups based on amino acid sequences in the AP2/ERF domain. *ESR1* is classified in subgroup VIII-b. *ESR2* was identified as the gene encoding a protein most similar to ESR1 in the *Arabidopsis* genome database (Ikeda et al. 2006) and has also been named as *DRNL* (Kirch et al. 2003), *SOB2* (Ward et al. 2006), and *BOLITA* (Marsch-Martinez et al. 2006). Transgenic plants overproducing ESR2 have capacity to develop more shoots than non-transformants in tissue culture. Therefore, *ESR2* appears to have redundant functions to *ESR1* to regulate shoot regeneration. The subgroup VIII-b contains 7 genes and the genes other than *ESR1* and *ESR2* may also possibly be involved in shoot regeneration processes.

In this paper, we report expression patterns of the ERF VIII-b subgroup genes during shoot differentiation processes. Transcript levels of six genes, *ESR1*, *ESR2*, *ESL1*, *ESL2*, *ESL3* and *LEP*, transiently increased 1–2 days after induction of shoot formation, implying that all these genes may be involved in initiation of shoot regeneration. In addition, we investigated effects on efficiency of shoot regeneration when these genes were

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.
This article can be found at http://www.jspcmb.jp/

overexpressed. Whereas *ESR1* or *ESR2* overexpression clearly enhanced shoot regeneration, others did not. These results suggest that among these genes, only *ESR1* and *ESR2* induce initiation of shoot regeneration by their overexpression, although all these genes may be involved in early events of shoot regeneration.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana Wassilewskija (WS) was used for root cultures. Seeds were sterilized and sown on MS medium [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.)] and grown for 2 weeks at 22°C under continuous light. Then roots were cut into approximately 1-cm segments and used for tissue culture. The compositions of the callus-inducing medium (CIM) and the shoot-inducing medium (SIM) were: for CIM, Gamborg's B5 salts, 2% glucose, Gamborg's B5 vitamins, 2 μM 2,4-D, and 0.25 % Gellan gum; for SIM, MS salts, 1% sucrose, Gamborg's B5 vitamins, 12.5 μM 2-ip and 0.25% Gellan gum. C medium is SIM supplemented with 0.4 gl^{-1} of carbenicillin and 50 $mg\,l^{-1}$ of kanamycin.

Construction of plasmids

cDNAs of ERF family genes were amplified by PCR from *A. thaliana* Col-0 genomic DNA, since they do not have any intron, using the following primers: AscI-ESL1F (5′-TAATT ggcgcgccATGTTGAAATCAAGTAACAAGAGAAAAAGCA-3′) and SpeI-ESL1R (5′-TAATTactagtTCACATAAGAAA CTGTGGAGCATCGT-3′) for ESL1, AscI-ESL2F (5′-TAATT ggcgcgccATGGAGTTCAATGGTAATTTGAATGCC-3′) and SpeI-ESL2R (5′-TAATTactagtTTATTGGTAGAAGAATGTG GAGGGTAAAGA-3′) for ESL2, AscI-ESL3F (5′-Aggcgcgcc ATGTCAACCTCCAAAACCCTAGACCA-3′) and SpeI-ESL3R (5′-AactagtCTAAAAGACTGAGTAGAAGCCTGTAG T-3′) for ESL3, AscI-LEP-F (5′-AggcgcgccATGAACA CAACATCATCAAAGAGCA-3′) and SpeI-LEP-R (5′-Tactagt TAGGAGCCAAAGTAGTTGAAACCT-3′) for LEP, AscI-AtERF1F (5′-AggcgcgccATGGATCCATTTTTAATTCAGTCC CCA-3′) and SpeI- AtERF1R (5′-TactagtTCACCAAGTCCCA CTATTTTCAGAAGA-3′) for AtERF1, AscI-AtERF4F (5′A ggcgcgccATGGCCAAGATGGGCTTGAAACCCGA-3′) and SpeI-AtERF4R (5′-TactagtTCAGGCCTGTTCCGATGGAGG A-3′) for AtERF4,AscI-ESR2F (5′-AggcgcgccATGGAAG AAGCAATCATGAGAC-3′) and NotI-ESR2R (5′-Agcggccgc ATTTCTTACAACCCTAAAGAAA-3') for ESR2.

These cDNAs except ESR2 were digested with *Asc*I and *Spe*I and ligated into pER10 between the *AscI* and *Spe*I sites. ESR2 cDNA was digested with AscI after digestion with *Not*I and blunting with T4 DNA polymerase, then inserted into pER10 (Zuo et al. 2000) between the *Asc*I and blunted *Spe*I sites. Construction of pER10-ESR1 was described previously (Banno et al. 2001).

RNA isolation and cDNA synthesis

Total RNAs were prepared from root cultures using RNeasy Plant Mini Kit (Qiagen, Inc.), then digested with DNase using TURBO DNA-free (Ambion). The resulting total RNAs were reverse transcribed using an oligo-dT at 50 μM as a primer with Message Sensor RT Kit (Ambion), according to the manufacture's instructions.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Real-time qRT-PCR reactions were performed in an optical 96-well reaction plate with ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Gene Assays (Applied Biosystems). Assay IDs of TaqMan Genes Assays were as follows: At02284625_s1 for ESR1, At02306690_s1 for ESR2, At02284570_s1 for ESL1, At02308203_s1 for ESL2, At02201815_s1 for ESL3, At02199065_s1 for LEP, At02222707_s1 for AtERF1, At02247829_s1 for AtERF4, At02172492_g1 for WUSCHEL and At02304594_g1 for UBQ5 (At5g25760). Amplification was monitored in real time and results were analyzed with the Relative Quantification method (Applied Biosystems).

Shoot regeneration assay

The procedure for root transformation as described previously (Banno et al. 2001) was modified. Root cultures preincubated on CIM for 2 days were co-cultured with *Agrobacterium tumefaciens* EHA105 carrying an appropriate plasmid for 3 days, washed with sterile water 3 times, then incubated on C medium with or without 5 μM 17β-estradiol. Numbers of shoots generated on the root cultures were counted after 4 weeks.

Results

Sequence similarity in ERF subgroup VIII

The *Arabidopsis* genome has 122 ERF subfamily genes and they were classified into 17 groups based on amino acid sequences in the AP2/ERF domain (Nakano et al. 2006). *ESR1* and *ESR2* are classified into ERF subgroup VIII-b. This subgroup has 7 genes including *ESR1* and *ESR2* and except for *ESR1*, *ESR2*, *LEAFY PETIOLE* (*LEP*) (van der Graaff et al. 2000) they have not yet been investigated. Figure 1A shows the phylogenetic tree of ERF subgroup VIII-b genes. Among these subgroup genes, we chose 6 genes closely branched in the phylogenetic tree and further analyzed them. We named At1g12890, At1g28160 and At5g18560 as *ESL1* (*ESR-like1*), *ESL2* and *ESL3* respectively. Figure 1B shows alignment of amino acid sequences of their AP2/ERF domains. Whereas amino acid sequences in their AP2/ERF domains are very similar (83–93% identical), domains other than AP2/ERF do not display significant structural similarity. However, C-terminal domains of ESR1, ESR2 and ESL3 contain similar short amino acid sequences (Figure1 C) as reported by Nakano et al. (2006).

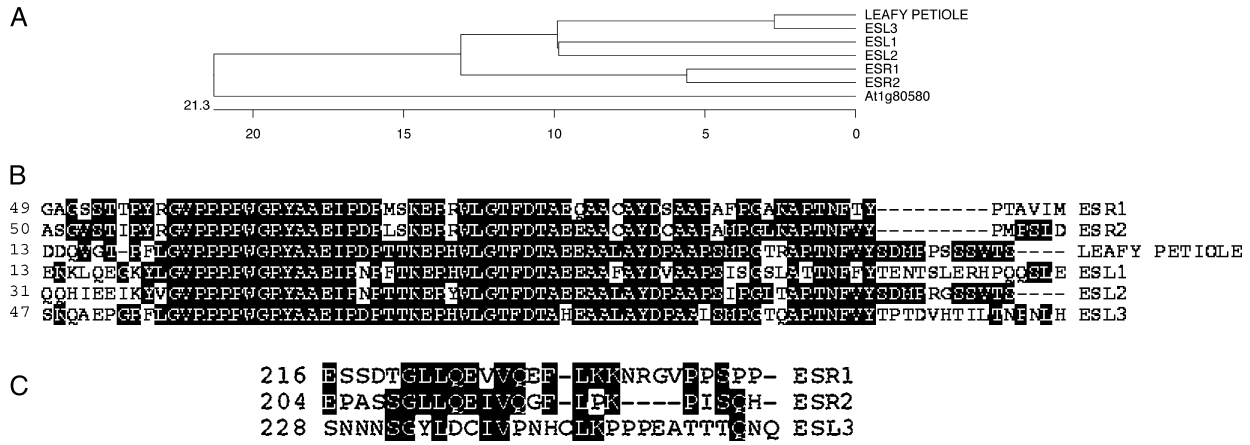

Figure 1. Comparison of amino acid sequences of ERF VIII-b subgroup. (A) The phylogenetic tree was built by using the Clustal method with the MegAlign program ver. 3.1.7 (DNASTAR, Inc.). Sequence distances, which were generated using the PAM250 matrix in MegaAlign program, are indicated by the scale. (B) Alignment of amino acid sequences of the AP2/ERF domains is shown. Black shading indicates identical amino acid residues. Amino acid numbers are indicated on the left. (C) Alignment of similar amino acid sequences in the C-terminal regions of ESR1, ESR2 and ESL3 is shown. Black shading indicates identical amino acid residues. Amino acid numbers are indicated on the left.

Expression profiles of ERF subgroup VIII-b genes during shoot regeneration

In order to investigate whether the *ESL* genes and the *LEP* gene are involved in initiation of shoot regeneration in tissue culture as well as *ESR1* and *ESR2*, we examined expression patterns of the *ESL* and *LEP* genes by quantitative reverse transcription polymerase chain reaction (qRT-PCR) after induction of shoot regeneration by cytokinin. Root segments from wild type *Arabidopsis* were preincubated on CIM for 4 days, then transferred onto SIM. Total RNAs were prepared from the root cultures and subjected to qRT-PCR. Other group genes, *AtERF1* (group IX; B-3) and *AtERF4* (subgroup VIII-a; B-1a), were also examined. The *WUSCHEL* (*WUS*) gene was examined as a marker gene for shoot meristem formation (Mayer et al. 1998). Figure 2 shows the results. *ESR1* transcript level increased transiently 1 day after transfer on SIM as reported previously (Banno et al. 2001). *ESR2* transcripts showed similar profiles to those of *ESR1* transcripts. Not only *ESR1* and *ESR2* transcripts, but also other VIII-b subgroup transcripts examined in this study increased after transfer onto SIM, reached a maximum after 1 or 2 days, and then decayed by day 7, although *ESL1* and *LEP* transcripts levels appear to be higher compared with others. In contrast, *AtERF1* and *AtERF4* transcript levels decreased after transfer onto SIM. Proliferating cells had formed small clusters on day 2, and shoot structures were observed 2 weeks after the transfer onto SIM. *WUS* transcripts were detected at substantial levels after 3 days and increased gradually through shoot regeneration, reflecting increase of *de novo* formation of shoot meristem. These results imply *ESLs* and *LEP* also may function at early stage in shoot regeneration as well as *ESR1* and *ESR2*.

Effects of overexpression of ERF subgroup VIII-b genes on shoot regeneration

Expression patterns of ERF VIII-b subgroup genes in the process of shoot formation led us to ask whether *ESLs* or *LEP* also influence efficiency of shoot regeneration when overexpressed, in addition to *ESR1* and *ESR2*. For this purpose, ESLs and LEP cDNAs were inserted into the estrogen-inducible expression vector pER10. The constructs were transformed into root explants preincubated on CIM for 2 days after cutting from *Arabidopsis* plants. After incubation for 4 weeks on C medium (SIM supplemented with antibiotics) with or without 17 β-estradiol, regenerated shoots were counted (Figure 3B). *AtERF1* and *AtERF4* belonging to another ERF group or subgroup were also examined as controls. Figure 3 shows the results. *ESR1* or *ESR2* clearly enhanced efficiency of shoot regeneration as we had reported (Banno et al. 2001; Ikeda et al. 2006), whereas *ESLs* and *LEP* affected regeneration as little as the empty vector, *AtERF1* or *AtERF4*. These results suggest that *ESLs* and *LEP* do not induce initiation of shoot formation by their overexpression.

Discussion

In this study, we investigated expression patterns of 6 genes, *ESR1*, *ESR2*, *ESL1*, *ESL2*, *ESL3* and *LEP*, belonging to the ERF subgroup VIII-b during *in vitro* shoot regeneration. The results demonstrated that all these genes' transcripts increased transiently at an early stage in the shoot regeneration process (Figure 2). Expression pattern of *ESR2* during shoot regeneration has not yet been examined, although analyses using mutants or overexpression experiments have been carried out (Kirch et al. 2003; Ikeda et al. 2006; Chandler et al. 2007). Transcript profiles of *ESR2* during the shoot

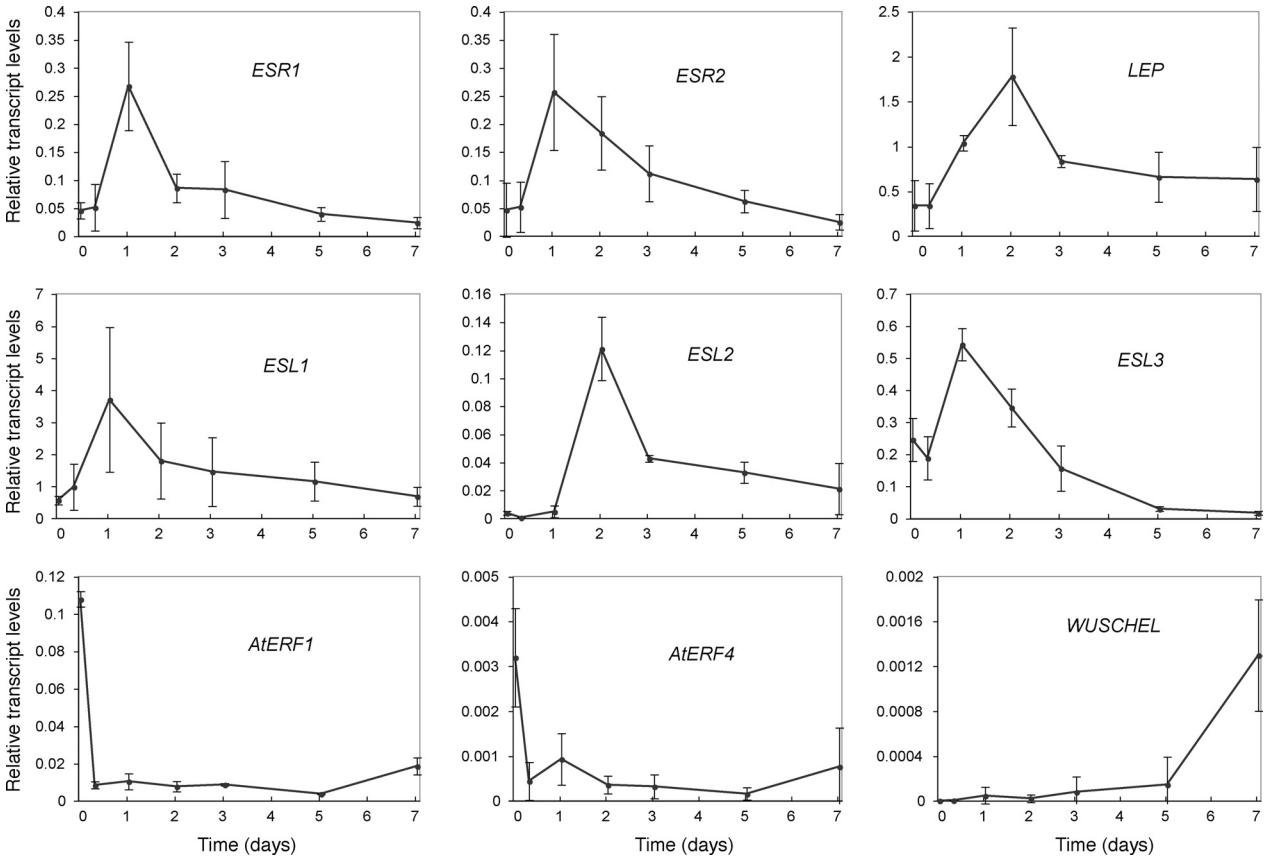

Figure 2. Time course of ERF VIII-b subgroup expressions during shoot regeneration. Root segments were preincubated on CIM for 4 days, then transferred onto SIM. Total RNAs were prepared from root explants at indicated time points after transfer onto SIM. Transcript levels were measured with qRT-PCR. Each value on the vertical axes indicates a relative level calculated by reference to the UBQ5 transcript level. Numbers on the horizontal axes indicate days after transfer onto SIM. Data represent the average of 3 independent experiments and error bars indicate standard deviations.

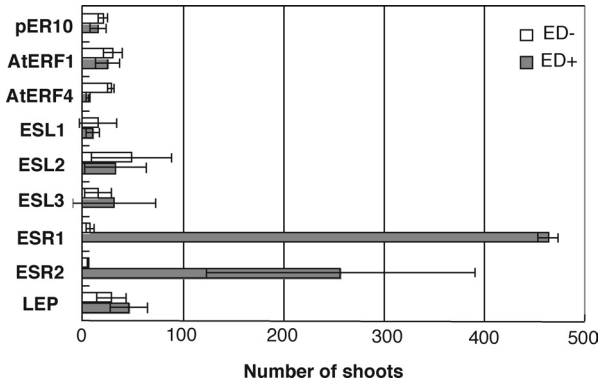

Figure 3. Effects of overexpression of the ERF VIII-b genes on shoot regeneration in root explants. *Arabidopsis* root cultures preincubated on CIM for 2 days were transformed with vector alone (pER10) or the same vectors containing each cDNA, and were then cultured on C medium with (+ED) or without (−ED) a transcription inducer (5 μM 17β-estradiol). After 4 weeks, numbers of transformed shoots per 0.1 g (fresh weight) of root culture were indicated. Data represent the average of 3 independent experiments and error bars indicate standard deviations.

regeneration process were very similar to those of *ESR1*, as demonstrated in this study, suggesting that *ESR1* and *ESR2* have redundant functions in regulation of *in vitro* shoot regeneration. The *WUS* gene plays a central role in shoot meristem organization (Laux et al. 1996; Mayer et al. 1998). *WUS* transcripts started to gradually increase 3 days after transfer onto SIM, following induction of *ESR1* and *ESR2* expression at day 1. The time course supports the idea that the *ESR1* and *ESR2* regulates initiation of shoot regeneration. Additionally, not only *ESR2*, but also *ESL* and *LEP* genes expressed in a similar manner to *ESR1*. It was demonstrated that *ESR1* expression was induced by cytokinin in SIM (Banno et al. 2001). The other VIII-b gene expressions were also likely induced by cytokinin, since our SIM was MS medium supplemented only with 2-ip as the phytohormone. The *LEP* gene was identified in an activation tagging screen, whose overexpression causes ectopic leaf blade formation on the petiole. In wild type *Arabidopsis*, *LEP* expresses in young leaf primodia (van der Graaff et al. 2000). The ERF VIII-b genes may commonly function at an early stage in shoot regeneration, although their roles in regulation of shoot regeneration remain to be elucidated.

Overexpression experiments revealed that only *ESR1*

and *ESR2* enhanced shoot regeneration efficiency among the genes examined in this study (Figure 3). Among these genes only *ESR1* and *ESR2* appear to regulate initiation of shoot regeneration. *ESL3*-overexpression did not affect shoot regeneration, although *ESR1*, *ESR2* and *ESL3* have a similar short amino acid stretch in C-terminal region (Figure 1C). A C-terminal domain including the short stretch is essential for enhancing effects by *ESR1*-overexpression on shoot regeneration, since deletion of the domain abolishes the effects (Banno et al. 2006). The similarity in amino acid sequences of the homologous short stretch in the C-terminal domain between ESL3 and the others is much weaker than that between ESR1 and ESR2 (Figure 1C). ESL3 appears not to have redundant functions with ESR1 and ESR2 in regulation of shoot regeneration. Recently, we found a nuclear localizing sequence of ESR1 located in the AP2/ERF domain (our unpublished results). The C-terminal region of ESR1 appears to have an essential role for its activity other than nuclear localizing or DNA binding, such as interaction with other protein(s). We are attempting to identify ESR1 and ESR2 interacting protein(s). Such study may enable us to elucidate mechanism to regulate shoot regeneration by ESR1.

Note: Recently, At5g18560 (*ESL3*) was identified as *PUCHI* gene whose disruption caused disturbed cell division patterns during lateral root formation (Hirota et al. 2007).

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