Functional analysis of the GmESR1 gene associated with soybean regeneration

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Abstract

Plant regeneration can occur via in vitro tissue culture through somatic embryogenesis or de novo shoot organogenesis. Transformation of soybean (Glycine max) is difficult, hence optimization of the transformation system for soybean regeneration is required. This study investigated ENHANCER OF SHOOT REGENERATION 1 (GmESR1), a soybean transcription factor that targets regeneration-associated genes. Sequence analysis showed that GmESR1 contained a conserved 57 amino acid APETALA 2 (AP2)/ETHYLENE RESPONSE FACTOR (ERF) DNA-binding domain. The relative expression level of GmESR1 was highest in young embryos, flowers and stems in the soybean cultivar ‘Dongnong 50’. To examine the function of GmESR1, transgenic Arabidopsis (Arabidopsis thaliana) and soybean plants overexpressing GmESR1 were generated. In Arabidopsis, overexpression of GmESR1 resulted in accelerated seed germination, and seedling shoot and root elongation. In soybean overexpression of GmESR1 also led to faster seed germination, and shoot and root elongation. GmESR1 specifically bound to the GCC-box. The results provide a foundation for the establishment of an efficient and stable transformation system for soybean.

Introduction

Plant regeneration is a clonal propagation process in vitro, which may involve a variety of processes, such as exogenous plant hormone signaling response, division of quiescent cells, and formation of a meristem or organ primordia [1]. Overexpression of cyclin-related genes in Arabidopsis thaliana could be an important link between cell proliferation in shoot apical meristems and organogenesis [2]. Shaul et al. (1996) [3] suggested that the expression of cyclin-dependent kinase genes was highly correlated with acquisition of the ability for cell proliferation. In Arabidopsis, the regeneration-associated CLAVATA (CLV) genes may regulate stem cell fate, such as limiting the size of the stem cell population, and the function of CLV3 is dependent on WUSCHEL (WUS) activity in the embryonic shoot meristem [4–6]. A recent
study indicates that changes in molecular patterning scales are associated with organ size in apical stem cell niches in plants [7]. Genes that promote cell proliferation or cell volume increase in soybean have not been studied previously.

Plant transformation is achieved by transferring a DNA fragment to the genome of a plant cell, regenerating a shoot from the transgenic cell, and then generating a root system to produce a genetically modified plant [8, 9]. *Rhizobium radiobacter* (*Agrobacterium tumefaciens*) mediated methods or particle bombardment are the preferred DNA transfer techniques [10]. To achieve high frequencies of plant regeneration, Cheng et al. (1980) [11] used aseptic cotyledon nodes as explants to induce differentiation of multiple shoots clumps. The genetic transformation of soybean (*Glycine max*) has long been of global interest, with successful transformation dependent on the efficient combination of receptor system and transformation method. The poor reproducibility of soybean regeneration represents a bottleneck for transformation of this important crop. Previous reports on soybean regeneration systems focused mainly on aspects such as genetic constitution, hormone types, culture conditions and explant types. Few studies have investigated the molecular basis of regeneration, especially the underlying mechanisms and the genes involved in this process. Detection of the expression of genes associated with soybean regeneration might help to address this problem.

Many *de novo* organogenesis systems are potentially useful as model experimental systems to illustrate the complexity of plant cell differentiation. The molecular mechanisms activated by the auxin to cytokinin ratio, however, are not well characterized. Efficient shoot regeneration involves two consecutive incubation steps: the exophyte is first incubated on an auxin-rich callus induction medium, and then on a cytokinin-rich shoot induction medium. The auxin rich callus induction medium initiates the formation of organogenic callus. More explants could improve the conversion efficiency, thus increasing the rate of emergence. Many regeneration-associated genes are related to the cytokinin signaling pathway, of which *ENHANCER OF SHOOT REGENERATION 1* (*AtESR1*) is one gene. *AtESR1* plays an important role in the regeneration network [12–15].

**APETALA 2/ETHYLENE RESPONSE FACTOR (AP2/ERF)** transcription factors (TFs) are involved in various biological functions, including plant and flower development, fruit and seed maturation, pathogen defense, and responses to damage, high salinity, and drought [16]. The AP2/ERF family belongs to a large group of TFs present in all plant species [17]. In *Arabidopsis*, the AP2/ERF TF family is divided into five subfamilies: AP2, Related to ABI3/VP1 (RAV), Dehydration Responsive Element Binding (DREB), ERF, and others [16]. These TFs are characterized by a conserved DNA-binding domain, the AP2/ERF domain, which was originally identified in *Arabidopsis* and is composed of 57–66 amino acids [18–20]. The ERF subfamily is the largest subgroup in the AP2/ERF family with members containing one or two AP2/ERF domains with specific DNA-binding motifs [21–23]. The three-dimensional structure of the AP2/ERF domain protein showed that the region contains three β fold, which is differences from located in the second β fold in the first 14 (alanine) and 19 (aspartic) amino acid residues, determines the specific binding of such TFs to different *cis*-acting elements [24]. Gutterson et al. (2004) [25] suggested that ten distinct subfamilies accommodate the structural difference in B subgroup. The ERF VIII-b group genes usually function in the early stages of shoot regeneration [22, 26]. The ERF subfamily of TFs participates in biological stress responses through binding to the GCC-box (*AGCCGCC*) [27]. The role of ERF TFs in the regulation of shoot regeneration is complex; both their biological function, and the ERF-mediated signal transduction pathway, are not well characterized.

The *AtESR1* gene (also known as *DORNRÖSCHEN; DRN*) was identified by screening an *Arabidopsis* cDNA library. The ESR1 protein contains a domain that shows sequence homology to the AP2/ERF domain [20, 28, 29]. *ESR1* appears to regulate shoot differentiation, with
overexpression of *ESR1* greatly enhancing the efficiency of shoot regeneration in *Arabidopsis* tissue culture [14]. *ESR1* encodes a TF belonging to the ERF family. The region between the AP2/ERF domain and the ESR motif in *ESR1* is indicated to be essential for enhancement of shoot regeneration [30]. *ESR1* acts as a transcriptional activator [31, 32]. *ESR1* binds to the GCC-box *in vitro* [33]. The GCC-box is an ethylene-responsive element located in the promoter region of many pathogenesis-related genes [34, 35]. Using yeast two-hybrid screening, *ESR1* has been shown to interact with PHAVOLUTA (PHV), while coimmunoprecipitation and bimolecular fluorescence complementation have shown that *ESR1* interacts with BES INTERACTING MYC-LIKE PROTEIN 1 (BIM1), a basic helix-loop-helix (bHLH) protein. BIM1 and PHV also physically interact [36, 37]. In *Arabidopsis*, shoot-related auxin-transport is conducted by *ESR1* and *ESR2*, two partially redundant AP2 TFs that interact during shoot development with PINOID (PID) and PIN-FORMED 1 (PIN1), respectively [38].

An orthologue of *AtESR1* was previously isolated from maize. In the maize shoot apical meristem, similar to *AtESR1*, *ZmESR1* transcriptional activity is associated with the anlage of new lateral organs [39]. In the present study we isolated *GmESR1* (GenBank accession no. JN590243.1, NCBI protein no. AFO52509.2), an AP2/ERF TF containing an ESR motif, from the soybean cultivar 'Dongnong 50'. In this study we examined one of the two gene copies present in the soybean genome. We present expression patterns of the full-length *GmESR1* protein and binding to the GCC-box element and show that the *GmESR1* transcript abundance varies in different organs. *GmESR1* showed organ-specific expression in soybean. Overexpression of *GmESR1* in transgenic soybean and *Arabidopsis* plants was also investigated. Overexpression of *GmESR1* promoted germination and elongation in soybean and *Arabidopsis*.

**Materials and methods**

**Plant materials and cultivation condition**

For plant transformation, seeds of soybean (*Glycine max* (L.) Merr.) cultivar 'Dongnong 50', which shows a high frequency of regeneration, were obtained from the Key Laboratory of Soybean Biology in the Chinese Ministry of Education, Harbin. Seedlings were grown in a growth chamber maintained at 26°C/18°C (day/night) under a 16 h photoperiod and light intensity of 350 μmol m⁻² s⁻¹. Transgenic T1 soybean seeds were sown under the same conditions. Fifteen days after planting, seedlings at the first-node stage (soybean growth phase V1) [40] were used for phenotype analysis and expression analysis using quantitative real-time PCR (qRT-PCR).

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild type (WT). For transgenic *Arabidopsis*, the T3 generation was used. Seeds of the mutant *Arabidopsis atesr1* T-DNA insertion line (Salk_089567) were obtained from The Arabidopsis Information Resource.

**Isolation of GmESR1**

To identify *GmESR1* and homologs in other plant species, the Phytozome 11.0 (https://phytozome.jgi.doe.gov/pz/portal.html) database was searched using the *AtESR1* gene sequence, extracted from the National Center for Biotechnology Information (NCBI) website, as the query sequence. Total RNA was reverse-transcribed into single-stranded cDNA using the ReverTra Ace® qPCR RT Kit (TOYOBO, Japan). Using this cDNA as a template, *GmESR1* gene-specific primers (*GmESR1*-F/R) were used to amplify *GmESR1*. PCR reaction conditions were as follows: 94°C for 4.5 min, then 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min, with final extension at 72°C for 10 min. The PCR products were inserted into the pMDTM19-T vector and ligated together (Takara, Japan). The ligation products were then transformed into *Escherichia coli* DH5α cells (TIANGEN, China) and sequenced (BioMed, China). The sequences were aligned with the *GmESR1* sequence using BLAST.
(http://www.ncbi.nlm.nih.gov/BLAST). The isoelectric point and the molecular weight of the GmESR1 protein were analyzed using the Swiss Institute of Bioinformatics Compute pI/Mw tool (http://web.expasy.org/compute_pi/). Nucleotide and amino acid sequences were compared using the sequence alignment software DNAMAN 6.0 (http://www.lynnnon.com/). To predict the GmESR1 protein structure and discover potential domains, the InterPro online portal was used (https://www.ebi.ac.uk/interpro/). Analysis of homologous protein sequence similarity was performed using the algorithm blastp (protein–protein BLAST) (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis of a multiple sequence alignment of the amino acid sequences of GmESR1 and heterologous AP2/ERF members was performed using MEGA 5.2 software (http://www.megasoftware.net). The three-dimensional structure of GmESR1 was predicted using the Phyre 2 online portal (http://www.sbg.bio.ic.ac.uk/phyre2). The RasMol software 2.7.2.1.1 (http://www.OpenRasMol.org/Copyright.html) was used to generate a graphical representation of the protein structure.

Real-time RT-PCR analysis of GmESR1 expression

The expression of GmESR1 was examined with qRT-PCR using SYBR® Premix Ex Taq™ II Kit (Tli RNaseH Plus, Takara) according to the manufacturers’ instructions (Takara), on an ABI 7500 Real-Time PCR Detection System (ABI, USA). Total RNA was extracted from the pod, root, stem, leaf, flower and immature embryo of soybean ‘Dongnong 50’ plants using TRIzol® Reagent according to the manufacturers’ protocol (Invitrogen, China). Genomic DNA was removed, and reverse transcription carried out, using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). Approximately 1 μg of total RNA was used for each reaction. To remove genomic DNA, samples were incubated at 42˚C for 2 min. For reverse transcription, each reaction used 10 μl of the reaction solution from the first step in a total volume of 20 μl. Samples were incubated at 37˚C for 15 min followed by heat shock at 85˚C for 5 s. A standard two-step PCR amplification protocol of 95˚C for 30 s, followed by 45 cycles at 95˚C for 5 s and 60˚C for 40 s, was used. Gene-specific primers (GmESR1-qF/R) for GmESR1, the soybean internal control gene GmACTIN4 (GenBank accession no. AF049106) and the Arabidopsis internal control gene AtACTIN8 (A. thaliana 18S rRNA gene GenBank accession no. X16077) were used. GmACTIN4 and AtACTIN8 were used as reference genes. The 2−ΔΔCt method was used to determine the relative level of GmESR1 expression in different tissues. Three technical replicates were performed for each real-time RT-PCR experiment.

Expression and purification of recombinant GmESR1 protein

The full-length coding region of GmESR1 was amplified using gene-specific primers (GmESR1-1F/R). The PCR products were digested with BamHI and HindIII and were inserted into the pET-29b vector (EMD Millipore, USA). The recombinant vector pET29b-GmESR1 was transformed into BL21 (DE3) competent E. coli cells, which were then grown in Luria broth (LB) with 50 mg·mL⁻¹ kanamycin at 37˚C to an absorbance of 0.7 at 600 nm. The E. coli liquid medium was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 h induction, the cells were isolated via centrifugation at 5000 × g for 12 min at room temperature. To purify the recombinant protein, bacteria were resuspended in 15 ml of 1× binding buffer and kept on the ice for 25 min. This was followed by cycles of ultrasonification for 20 s and pause for 20 s until the sample was no longer sticky. The sample was then centrifuged at 2000 × g for 8 min at room temperature before being recycled and loaded onto a His-bind Resin column (Novagen, BRD). The pure GmESR1 fusion protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified based on the pET System manufacturer’s protocol (Novagen, BRD).
Electrophoretic mobility shift assay

In soybean and Arabidopsis, members of the ERF family contain a conserved DNA-binding domain (AP2/ERF domain) [41]. A digoxigenin-ddUTP-labeled double-stranded oligonucleotide GCC-box probe has previously been combined with the DNA-binding activity of soybean Ethylene Response Factor 5 (GmERF5) [42]. The sequence of the GCC-box probe and the mutated GCC-box probe are shown in S1 Table. The electrophoretic mobility shift assay (EMSA) was performed as described by Kass et al. (2000) [43].

Identification of transgenic atesr1 plants

Plants homozygous for the T-DNA insert were identified via PCR using a gene-specific primer pair and a T-DNA-specific primer. The left genomic primer (LP), right genomic primer (RP) and the left T-DNA border primer (LB) for atesr1 are shown in S1 Table. After confirmation of the homozygous T-DNA insertion, reduction in relative gene expression level was confirmed using qRT-PCR with a gene-specific primer.

Construction of GmESR1 overexpression vector

To overexpress GmESR1 under the control of the Cauliflower mosaic virus (CaMV) 35S promoter, the pEarleyGate 101 vector, containing the bar gene, was used via the Gateway cloning system. The full-length open reading frame sequence of GmESR1 was used by designing flanking primers for the BP reaction. The reaction mixture was: 1 μl pGWC, 2 μl buffer, 1 μl T4 ligase, 4 μl gene fragment and water to 10 μl, with the ligation carried out at 16°C. Next, the fragment with adapters at each end was cloned into the entry vector pGWC, which contains chloramphenicol resistance. The entry clone pGWC-GmESR1 was used to perform the LR reaction. The extracted pEarleyGate 101 plasmid, 1 μl of each entry clone, 1 μl LR enzyme, and water to make up the volume to 5 μl was incubated at 25°C to facilitate the recombination reaction and clone the desired fragment into the pEarleyGate 101 destination vector. The R. radiobacter strain LBA4404 was transformed with the overexpression vector using the freeze–thaw method as described by Dang et al. (2007) [44].

Arabidopsis transformation and phenotype analysis

Using the method described by Clough et al. (1998) [45], Arabidopsis was transformed with the overexpression vector. Arabidopsis seeds were vernalized in the dark at 4°C. The seeds were sterilized in 10% sodium hypochlorite, vortexed for 10 min and washed six times using distilled sterile water. The seeds were sown on Murashige and Skoog (MS) solid medium and, after 3–4 leaves had developed, the seedlings were transplanted into 1:1 sterilized soil and vermiculite mixture. Transformation via infiltration was carried out during flowering. Rhizobium radiobacter cells from a single colony were suspended in 15 ml Yeast Extract Peptone liquid culture medium containing selection antibiotics and incubated at 28°C, with shaking at 185 rpm, until the absorbance at 600 nm was approximately 0.5. When the absorbance at 600 nm reached 1.6–2.0 the mixture was centrifuged for 15 min at 5000 ×g, and the supernatant was discarded. The flowering Arabidopsis plants were inverted and immersed in the Rhizobium liquid for 30 s. Plants were covered with plastic film and placed in a thermostatic chamber without light for 24 h, then placed upright and left to grow with ambient illumination.

The atesr1 mutant lines, transgenic GmESR1 overexpression lines, and WT Arabidopsis plants were grown in the same growth chamber, maintained at 22°C with a 16 h/8 h (light/dark) cycle and light intensity of 350 μmol·m⁻²·s⁻¹. After 2.5 d, the rates of germination and
elongation in the \textit{GmESR1} overexpression lines, \textit{atesr1} mutant lines, and WT plants were compared and analyzed statistically. After flowering, the height of \textit{Arabidopsis} were compared.

**Soybean transformation and phenotype analysis**

\textit{Rhizobium}-mediated stable soybean transformation was performed using cotyledonary nodes of soybean ‘Dongnong 50’ as explants. Following culturing in the dark, shoot regenerative proliferation, shoot elongation induction, root differentiation multiplication, and plantlet regeneration, the regenerated plants were transferred to pots and grown in the greenhouse [46]. Five T\textsubscript{1} \textit{GmESR1}-overexpressing soybean plants and control lines were grown on soybean seed germination medium. After 5 days, each cotyledon separation and 7–8 wounds were induced in the growing point, to the co-culture medium and dark culture for 3 days, transfer into the bud induction medium; co-culture for 14 d at 25°C under the same conditions. Then the same size buds of \textit{GmESR1}-overexpressing and control group was isolated and used for observation by electron microscopy.

**Results**

**Isolation and molecular characterization of cDNA clone encoding \textit{GmESR1}**

The full-length \textit{GmESR1} cDNA sequence of 1,292 bp, containing an open reading frame of 1,164 bp and encoding a protein of 387 amino acids (GenBank accession no. JN590243.1), was obtained from soybean ‘Dongnong 50’ (Fig 1). The \textit{GmESR1} protein was predicted to have a molecular mass of 42.8324 kDa and an isoelectric point at pH 6.80. The nucleotide sequence showed a 5\textsuperscript{\prime} untranslated region (UTR) of 49 nucleotides and a 3\textsuperscript{\prime} UTR of 79 nucleotides. The NetPhos 2.0 online server (http://www.cbs.dtu.dk/services/NetPhos-2.0/) predicted that \textit{GmESR1} contained 14 serine residues (Ser 15, 30, 72, 116, 159, 190, 205, 213, 232, 257, 260, 261, 263 and 280), five threonine (Thr 83, 120, 148, 304 and 343), and one tyrosine (Tyr 221), which were potential phosphorylation sites (Fig 1).

Analysis of the homolog of \textit{GmESR1} in the soybean genome, based on data obtained from the Phytozome database, indicated that the two genes were clustered in two linkage groups, one each on chromosomes Gm 01 and Gm 02, with one and no introns, respectively. Cladistic analysis of \textit{GmESR1} against other ESR1s, representing a range of species including crops, fruits, and vegetables, grouped \textit{GmESR1} with members of the plant ESR1 family (Fig 2A). The amino acid sequence of \textit{GmESR1} showed 68% and 51% similarity to ESR1 proteins from adzuki bean (\textit{Vigna angularis}; KOM39777) and chickpea (\textit{Cicer arietinum}; XP_004489775), respectively (Fig 2B). The predicted three-dimensional structure of \textit{GmESR1}, based on data from Phyre 2, indicated that the protein contained a long C-terminal \alpha-helix (\alpha) wrapped in a three-stranded anti-parallel \beta-sheet (from \beta\textsubscript{1} to \beta\textsubscript{3}) (Fig 2C) and that the AP2/ERF domain was divided into conserved segments (YRG and RAYD) (Fig 2B) [47]. The predicted structure of \textit{GmESR1} included a conserved region of 57 amino acid residues (residues 51–107) representing the predicted AP2/ERF DNA-binding domain (Fig 2D). Given that residue 14 of the domain was an alanine and residue 19 an aspartic acid, the gene was classified as a member of the ERF subfamily of AP2/ERF TFs. These two amino acids are crucial for specific binding of ERF TFs to the GCC-box in promoter regions and to activate transcription of target genes [16].

**Analysis of \textit{GmESR1} expression in various organs**

To investigate the potential role of \textit{GmESR1}, its expression profiles were analyzed in major organs of the soybean plant using qRT-PCR. \textit{GmESR1} was expressed in all organs analyzed,
with the highest relative expression levels observed in young embryos, flower and stem tissue, and considerably lower relative expression levels observed in the pod, leaf and root (Fig 3). GmESR1 may, therefore, play a role in stem elongation, flower morphogenesis, and embryogenesis.

Purification of the recombinant GmESR1 protein

Expression of the recombinant GmESR1 protein was markedly enhanced after 2–8 h induction with 0.5 mM IPTG at 37˚C, attaining the maximum expression level after 4 h, although the recombinant GmESR1 protein was not detected in the control groups (Fig 4A). The molecular weight of the purified GmESR1 protein was approximately 45 kDa as estimated with SDS-PAGE, consistent with the calculated molecular mass of 42.8324 kDa (Fig 4B).

GmESR1 specifically binds to the GCC-box element in vitro

To confirm binding of GmESR1 to the GCC-box regulatory element in vitro, His-tagged GmESR1 was purified and used in an EMSA alongside a digoxigenin-ddUTP-labeled double-stranded oligonucleotide GCC-box probe. The GCC-box and mGCC-box sequences are

Fig 1. Nucleotide and amino acid sequences of GmESR1. Putative phosphorylation sites are marked in bold italics. The YRG element and RAYD element are highlighted by shading. The α-helix and β-sheets are underlined. Amino acid and base pair numbers are shown on the left.

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shown in Fig 5A. GmESR1 specifically recognized and bound to the GCC-box, but not to the mGCC-box (Fig 5B). When the ratio of unlabeled to labeled GCC probe was 100:1, the labeled probe was not bound, but when 100-fold unlabeled mGCC probe was used as the competitor, the labeled probe was again bound, confirming the specificity of mobility shift (Fig 5B).

Analysis of *Arabidopsis atesr1* mutants and overexpression phenotypes in *Arabidopsis* plants

Using the “double primer” genomic PCR method described by T-DNA Primer Design (http://signal.salk.edu/tdnaprimer2s.2.html), homozygous T-DNA insertion mutants were screened
for the presence of the transgene, resulting in identification of plants homozygous and heterozygous for atesr1 (Fig 6A and 6B). All WT and transgenic plants were grown under the same
conditions. The relative expression level of GmESR1 was examined in WT, mutant atesr1, and three independent GmESR1-ox lines. In the three GmESR1-ox lines, the transcript abundance of GmESR1 was high, whereas no transcripts were detected in the WT or atesr1 Arabidopsis plants (Fig 6C). This finding confirmed that these three lines were overexpression of GmESR1. The germination rates of mutant atesr1 and GmESR1-ox seeds sown on solid medium were compared with those of WT seeds. After 2.5 d, the homozygous mutant atesr1 displayed poor germination. Compared with GmESR1-ox, the germination rates of WT and atesr1 seeds were both reduced, though WT seeds showed slightly better germination rates than atesr1 seeds. All GmESR1-ox seeds successfully germinated (Fig 6D). The germination rates of WT, atesr1 and GmESR1-ox seeds are shown in Fig 6E. The germination rate after 2.5 and 6.5 d was higher in GmESR1-ox seeds compared with WT seeds, and higher in WT seeds compared with atesr1 seeds. This finding indicated that overexpression of GmESR1 promoted and accelerated

Fig 5. Sequence-specific binding of GmESR1 to the GCC-box element. (A) Nucleotide sequences of the GCC-box and mGCC-box probes. (B) Electrophoretic mobility shift assay (EMSA) showed sequence-specific binding to the GCC-box of the recombinant GmESR1 protein. Lane 1, EMSA performed with only the free GCC probes; lane 2, labeled GCC probe and GmESR1 protein; lane 3, titration with a cold GCC sequence as a competitor; lane 4, titration with a cold mGCC-box sequence as a competitor; lane 5, labeled mGCC probe and GmESR1 protein.

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Arabidopsis seed germination, whereas the atesr1 mutation repressed or delayed germination. The elongation rate of GmESR1-ox plants was significantly faster than that of WT plants.

Fig 6. Identification and analysis of mutant atesr1 and GmESR1-ox Arabidopsis plants. (A) Phenotypes of homozygous atesr1 mutant (HM), heterozygous atesr1 mutant (HZ) and wild-type plant (WT). Scale bar = 1.0 cm. (B) PCR results for the genotyping assay to identify atesr1 mutant plants. (C) Identification of transgenic GmESR1-ox Arabidopsis plants using qRT-PCR. Transcript abundances were normalized against the reference gene AtActin8. (D) Germination of mutant atesr1, WT, and GmESR1-ox Arabidopsis seeds. Two independent GmESR1-ox lines are included. (E) Germination of atesr1, WT, and GmESR1-ox seeds after 2.5 and 6.5 d. (F) Comparison of GmESR1-ox, WT, and atesr1 elongation rates on MS medium 2.5 d after planting. (G) Root length in GmESR1-ox, WT, and mutant atesr1 plants 2.5 d after planting. (H) Phenotypes of WT, GmESR1-ox, and atesr1 plants 30 d after transplanting. The experiment was performed on three biological replicates with their respective three technical replicates and statistically analyzed using Student’s t-test (*P<0.05, **P<0.01). Error bars represent the standard error of the mean.

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whereas atesr1 mutants failed to germinate (Fig 6F). After 2.5 d GmESR1-ox seedlings had longer roots than the WT seedlings, and atesr1 seeds had not germinated (Fig 6G). These findings indicated that GmESR1 overexpression promoted germination and root elongation, whereas the atesr1 mutation delayed germination. At 30d after transplanting, it was observed that GmESR1 overexpression in transgenic Arabidopsis plants resulted in dwarfism (Fig 6H).

Identification and analysis of transgenic soybean overexpressing GmESR1

The soybean cotyledons were used for stable transformation. T1 seeds were sown in soil, and transformants were identified at the V1 developmental stage when the first trifoliate leaf appeared. Transgenic soybean plants overexpressing GmESR1 were identified via qRT-PCR as those showing higher relative expression of GmESR1 compared with control soybean plants (Fig 7A). Phenotypic analysis of the transgenic soybean plants showed that GmESR1 overexpression resulted in faster germination and elongation relative to the control soybean plants, indicating that GmESR1 promoted seed germination (Fig 7B). After 15 d, at the seedling stage of development, trifoliate leaves had not yet developed in the control soybean but were fully visible in GmESR1-ox plants (Fig 7C). At the same time point, the root elongation of GmESR1-ox soybean plants was faster and the root length was longer than in control soybean plants (Fig 7D). This finding indicated that GmESR1 promoted shoot and root elongation in soybean. The experimental results showed that GmESR1 overexpression resulted in the increasing number of cells relative to the control soybean clustered bud cells in the same size of the field of vision (Fig 7E). It makes the cell division more exuberant.

Discussion

In this study, we identified GmESR1 in soybean, a novel member of the ERF family of TFs, that promoted soybean germination, and shoot and root elongation when overexpressed. Nakano et al. (2006) [22] investigated phylogenetic relationships, gene structure, and the conserved domain of the ERF gene family in Arabidopsis and rice, but only identified a few members of the ERF family in soybean. At 145 and 420 Mb respectively, Arabidopsis and rice have small genomes compared with the genome of soybean (1115 Mb) [48]. The structure of the AP2/ERF superfamily are relatively similar in all three species [22]. Presently, 98 genes of known function of the ERF family that contain a complete AP2/ERF domain have been identified in the soybean genome [41]. Only six of these genes have been functionally characterized in soybean [42, 47, 49, 50, 51, 52].

GmESR1 is the first soybean ERF family TF expressed in response to germination and shoot and root elongation, although little information on the role of GmESR1 in these processes is available. Although Arabidopsis AtESR1 genes were first identified in 2001 [14], little is known about their protein functions in vivo or in vitro. In the present study, GmESR1 was shown to contain an AP2/ERF domain divided into two conserved segments, the YRG and RAYD elements. The amino acid sequence, protein structure, results of soybean transformation and other biological functions of the GmESR1 protein indicated that GmESR1 belongs to the ERF subfamily of AP2/ERF TFs. The GmESR1 sequence analysis indicated that features such as the molecular mass, predicted eukaryotic protein phosphorylation sites, acidic isoelectric point and lack of introns are conserved. GmESR1 is located on chromosome two and contains no introns. AtESR1 and AtESR2 occur as a duplication on chromosome one, with genetic data indicating that they are highly redundant during embryonic patterning [36, 53]. GmESR1 was analyzed according to publicly available data (http://soybase.org/GlycineBlastPages/) that indicated that 20 genes were clustered into 20 linkage groups.
In embryogenic shoot growth, the cytokinin-induced regenerative genes AtWUSHEL (AtWUS) triggers TOPLESS (TPL) [54], which weakens auxin signaling by interacting with MONOPTEROS/Auxin Response Factor5 (MP/ARF5) and INDOLE-3-ACETIC ACID INDUCIBLE 12/BODENLOS (IAA12/BDL) [55, 56]. Banno et al. (2001) [14] isolated and characterized a novel cDNA of which overexpression promotes ultimate cytokinin-independent shoot regeneration from Arabidopsis explants. Given that the cDNA obtained via screening depended on its overexpression as a substitute of cytokinin essential for shoot regeneration, the cDNA might encode elements involved in cytokinin signaling. Overexpression of AtESR1 in Arabidopsis under the control of the estradiol-inducible XVE system also increases shoot regeneration in the presence of cytokinins [14, 57]. The function of AtESR2 is similar to AtESR1, with plants silenced for AtESR2 displaying weaker regeneration in general, and increased shoot regeneration in the presence of extra cytokinins [58]. The present results indicated that GmESR1 is responsible for regulation of stem elongation and embryogenesis in soybean and Arabidopsis. As plant regeneration is a complex process requiring the interaction of multiple genes, a single gene is insufficient to regulate the entire process and, therefore, additional study of the interactions between GmESR1 and other regeneration-associated genes is required.

Fig 7. Analysis of GmESR1 transgenic soybean plants. (A) Relative expression level of GmESR1 in control group and four independent GmESR1-ox lines. Transcript abundance was normalized against the reference gene GmActin4. (B) Comparison of elongation rate in GmESR1-ox and control soybean plants during germination 5 d after planting. (C) Comparison of shoot elongation rate in GmESR1-ox and control soybean seedlings 15 d after planting. (D) Comparison of root elongation rate in GmESR1-ox and control soybean seedlings 15 d after planting. The experiments were performed on three biological replicates with their respective three technical replicates. Error bars represent the standard error of the mean. Scale bars = 1.0 cm. (E) Comparison of the bud cells in GmESR1-ox and control soybean plants during the bud induction. Scale bars = 5μm. 

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Overexpression of AtESR1 and AtESR2 in Arabidopsis has previously been shown to upregulate CUP-SHAPED COTYLEDON 1 (AtCUC1) expression, with the upregulation of AtCUC1 having a positive effect on shoot regeneration [59]. AtCUC1 overexpression is reported to promote the formation of adventitious buds from callus [60]. AtESR1 is important in the conversion of the young lateral root primordium into a shoot meristem, whereas AtESR2 functions in shoot development [61]. AtCUC1 is involved in the same pathway, and AtESR2 and AtCUC2 are functionally redundant in cotyledon development [58]. A previous study of AtESR1 identified phenotypic changes only during embryonic development, whereas our research analyzed the entire plant growth period. Overexpression of GmESR1 in transgenic soybean promoted germination and elongation, resulting in faster shoot development than in WT soybean plants, suggesting that GmESR1 accelerates soybean development and might be a useful tool for regulation of soybean seedling, stem, and root elongation. Overexpression of GmESR1 in Arabidopsis promoted germination and elongation, resulting in faster shoot development than in WT and mutant atesr1 Arabidopsis plants, suggesting that overexpression of GmESR1 accelerates seedling growth stage in Arabidopsis and might be a useful tool for regulation of germination, seedling development, and promotion of root elongation. Conversely, atesr1 delayed germination and the seedling stage in Arabidopsis. In mature transgenic Arabidopsis plants, overexpression of GmESR1 resulted in a dwarf phenotype, whereas the mutant atesr1 showed reduced stem elongation. We have focused on complementation of the Arabidopsis mutant, the relative experiment is complicated and tedious, and no data have been obtained as yet.

Here we demonstrated that overexpression of GmESR1 in soybean and Arabidopsis plants improved shoot germination and elongation, and that purified GmESR1 protein binds to the GCC-box, which is present in the promoter region of many genes. These observations are further supported by confirmation of the interaction of the AP2/ERF domain of AtESR1 with class III homeodomain-leucine zipper (HD-ZIP) TFs [36]. The binding specificity of the AtESR1 protein can be changed by the interaction of the AP2/ERF domain of AtESR1 and the C-terminal Per/Arnt/Sim (PAS)-like domain of class III HD-ZIP proteins, thus a prolonged sequence containing the GCC-box can be recognized by a combination of ESR1 and class III HD-ZIP TFs [31]. The AP2-type TFs DRN and DRNL interact with the bHLH protein AtBIM1, which supports a role for AtBIM1 in embryonic patterning [37]. The interactions between the soybean homologs of AtBIM1 and AtPID and GmESR1s are currently under investigation. At present, research into GmESR1 is at an early stage and additional investigations are needed to clarify its involvement in regeneration. Furthermore, other reasons should be sought to explain why such a unique proteinic structure is formed. The GmESR1 overexpression resulted in more vacuoles relative to the control soybean clustered bud cells in the same size of the field of vision, so can be divide into multiple cells. The GmESR1 overexpression resulted in larger relative to the control soybean clustered bud cell nucleus. After that, the number of cells increased gradually, which could be showed that soybean plants were growing rapidly at germination and seedling stage. Neighboring cells division increased, and the cells divided repeatedly, and the number of divisions increased linearly [62]. During growth and development, the population of stem cells rapidly proliferates to fill the tissues and organs [63]. The GmESR1 gene is functionally analogous to animal stem cells, and the ability to regenerate can increase the number of cells and increase the volume of cells.

The work reported here may be used to further elucidate the division between the regulation of defense mechanisms and shoot regeneration by the ERF family. In the present study, seed germination, and shoot and root growth of GmESR1-overexpressing transgenic soybean plants were faster than those of non-transgenic soybean plants, suggesting that GmESR1 may be involved in the regulation of seed germination, and shoot and root elongation.
Conclusion
We analyzed the function of the soybean GmESR1 gene. In addition, the relationship between the function of GmESR1 and seed germination, and shoot and root elongation was investigated. In soybean GmESR1 overexpression led to faster seed germination, and shoot and root elongation. And by the observation of cell number under the overexpression of GmESR1, the result support that GmESR1 could promote regeneration. These results indicated that GmESR1 may played an important role in seed germination and elongation of soybean.

Supporting information
S1 Fig. Phylogenetic analysis of GmESR1 and 20 ESR1 proteins from other plant species. (TIF)
S2 Fig. PCR analysis of T1 transgenic soybean plants using bar and GmESR1 gene-specific primers. (TIF)
S3 Fig. Germination of mutant atesr1, wild type, and GmESR1-ox Arabidopsis seeds after 2.5 d. Two independent GmESR1-ox lines are included. (TIF)
S4 Fig. Germination of mutant atesr1, wild type, and GmESR1-ox Arabidopsis seeds after 4.5 d. Two independent GmESR1-ox lines are included. (TIF)
S5 Fig. Germination of mutant atesr1, wild type, and GmESR1-ox Arabidopsis seeds after 6.5 d. Two independent GmESR1-ox lines are included. (TIF)
S1 Table. Oligonucleotide primers used in this study. (DOC)

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References

1. Sugiyama M. Organogenesis in vitro. Curr Opin Plant Biol. 1999; 2(1): 61–64. PMID: 10047565
2. Fletcher JC. Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. BioEssays. 2002; 24(1): 27–37. https://doi.org/10.1002/bies.10020 PMID: 11782948
3. Shaul O, Van Montagu M, Inzé D. Cell cycle control in Arabidopsis. Ann Bot. 1996; 78(3): 283–288.
4. Brand U, Fletcher JC, Hobe M, Meyerowitz EM, Simon R. Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science, 2000; 289(5479): 617–619.
5. Rojo E, Sharma VK, Kovaleva V, Raikhel NV, Fletcher JC. CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. Plant Cell, 2002; 14(5): 969–977. https://doi.org/10.1105/tpc.002196 PMID: 12034890
6. Brand U, Grünewald M, Hobe M, Simon R. Regulation of CLV3 expression by two homeobox genes in Arabidopsis. Plant Physiol, 2002; 129(2): 565–575. https://doi.org/10.1104/pp.001867 PMID: 12068101
7. Gruel J, Landrein B, Tarr P, Schuster C, Refahi Y, Sampathkumar A, et al. An epidermis-driven mechanism positions and scales stem cell niches in plants. Sci Adv, 2016; 2(1): e1500989. https://doi.org/10.1126/sciadv.1500989 PMID: 27152324
8. Rao AQ, Bakhsh A, Kiani S, Shahzad K, Shahid AA, Husnain T, et al. The myth of plant transformation. Biotechnol Adv. 2009; 27(6): 753–763. https://doi.org/10.1016/j.biotechadv.2009.04.028 PMID: 19508888
9. Păcurar DI, Thordal-Christensen H, Păcurar ML, Pamfil D, Botez C, Bellini C. Agrobacterium tumefaciens: From crown gall tumors to genetic transformation. Physiol Mol Plant Pathol. 2011; 76(2): 76–81.
10. Vyacheslavova AO, Berdichevets IN, Tyurin AA, Shakhova ON, Goldenkova-Pavlova IV. Expression of heterologous genes in plant systems: new possibilities. Russ J Genet. 2012; 48(11): 1067–1079.
11. Cheng TY, Saka H, Voqui-Dinh TH. Plant regeneration from soybean cotyledonal node segments in culture. Plant Sci Lett. 1980; 19(2): 91–99.
12. Reinet H, Bajaj YPS, Nitsch C, Clapham DH, Jensen CJ. Springer Berlin Heidelberg, 1977.
13. Motte H, Vereecke D, Geelen D, Werbrouck S. The molecular path to in vitro shoot regeneration. Biotechnol Adv. 2014; 32(1): 107–121. https://doi.org/10.1016/j.biotechadv.2013.12.002 PMID: 24355763
14. Banno H, Ikeda Y, Niu QW, Chua NH. Overexpression of Arabidopsis ESR1 induces initiation of shoot regeneration. Plant Cell. 2001; 13(12): 2809–2818. https://doi.org/10.1105/tpc.010234 PMID: 11732375
15. Valvecens D, Van Montagu M, Van Lijsebettens M. Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana root explants by using kanamycin selection. Proc Natl Acad Sci USA. 1988; 85(15): 5536–5540. PMID: 16593964
16. Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamanouchi-Shinozaki K. DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration-and cold-inducible gene expression. Biochem Biophys Res Commun. 2002; 290(3): 998–1009. https://doi.org/10.1016/S0006-291X(02)00957-1 PMID: 11798174
17. Velasco R, Zharkikh A, Affourtit J, Dingra A, Cestaro A, Kalyanaraman A, et al. The genome of the domesticated apple (Malus [times] domestica Borkh.). Nat Genet. 2010; 42(10): 833–839. https://doi.org/10.1038/ng.654 PMID: 20802477
18. Jofuku KD, Den Boer BG, Van Montagu M, Okamura JK. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell. 1994; 6(9): 1211–1225. PMID: 7919989
19. Weigel D. The APETALA2 domain is related to a novel type of DNA binding domain. Plant Cell. 1995; 7(4): 388. https://doi.org/10.1105/tpc.7.4.388 PMID: 7773013
20. Okamura JK, Caster B, Villarroel R, Van Montagu M, Jofuku KD. The AP2 domain of APETALA2 defines a large new family of DNA binding proteins in Arabidopsis. Proc Natl Acad Sci USA. 1997; 94(13): 7076–7081. PMID: 9192964
21. McGrath KC, Dombrell B, Mannans JM, Schenk PM, Edgar CI, Maclean DJ, et al. 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. 2005; 139(2): 949–959. https://doi.org/10.1104/pp.105.068544 PMID: 16183832

22. Nakano T, Suzuki K, Fujimura T, Shinshi H. Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. 2006; 140(2): 411–432. https://doi.org/10.1104/pp.105.073783 PMID: 16407444

23. Shigyo M, Hasebe M, Ito M. Molecular evolution of the AP2 subfamily. Gene. 2006; 366(2): 256–265. https://doi.org/10.1016/j.gene.2005.08.009 PMID: 16388920

24. Allen MD, Yamasaki K, Ohme-Takagi M, Tateno M, Suzuki M. A novel mode of DNA recognition by a β-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. EMBO J. 1998; 17(18): 5484–5496. https://doi.org/10.1093/emboj/17.18.5484 PMID: 9736626

25. Gutterson N, Reuber TL. Regulation of disease resistance pathways by AP2/ERF transcription factors. Curr Opin Plant Biol. 2004; 7(4): 465–471. https://doi.org/10.1016/j.pbi.2004.04.007 PMID: 15231271

26. Mase H, Hashiba M, Matsuo N, Banno H. 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. 2007; 24(5): 481–486.

27. Hu Y, Zhao L, Chong K, Wang T. Overexpression of OsERF1, a novel rice ERF gene, up-regulates ethylene-responsive genes expression besides affects growth and development in Arabidopsis. J Plant Physiol. 2008; 165(16): 1717–1725. https://doi.org/10.1016/j.jplph.2007.12.006 PMID: 18313797

28. Riechmann JL, Meyerowitz EM. The AP2/EREBP family of plant transcription factors. Biol Chem. 1998; 379: 633–646. PMID: 9687012

29. Chang C, Shockey JA. The ethylene-response pathway: signal perception to gene regulation. Curr Opin Plant Biol. 1999; 2(5): 352–358. PMID: 10508761

30. Nomura Y, Matsuo N, Banno H. A domain containing the ESR motif in ENHANCER OF SHOOT REGENERATION 1 functions as a transactivation domain. Plant Biotechnol. 2009; 26(4): 395–401.

31. Matsuo N, Banno H. The Arabidopsis transcription factor ESR1 induces in vitro shoot regeneration through transcriptional activation. Plant Physiol Biochem. 2008; 46(12): 1045–1050. https://doi.org/10.1016/j.plaphy.2008.07.007 PMID: 18771931

32. Matsuo N, Makino M, Banno H. Arabidopsis ENHANCER OF SHOOT REGENERATION (ESR) 1 and ESR2 regulate in vitro shoot regeneration and their expressions are differentially regulated. Plant Sci. 2011; 181(1): 39–46. https://doi.org/10.1016/j.plantsci.2011.03.007 PMID: 21600396

33. Banno H, Mase H, Maekawa K. Analysis of functional domains and binding sequences of Arabidopsis transcription factor ESR1. Plant Biotechnol. 2006; 23(3): 903–908.

34. Guo H, Ecker JR. The ethylene signaling pathway: new insights. Curr Opin Plant Biol. 2004; 7(1): 40–49. PMID: 14732440

35. Ohme-Takagi M, Shinshi H. Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. Plant Cell. 1995; 7(2): 173–182. https://doi.org/10.1105/tpc.7.2.173 PMID: 7756828

36. Chandler JW, Cole M, Flier A, Grewe B, Werr W. The AP2 transcription factors DORNROŚCHEN and DORNROŚCHEN-LIKE redundantly control Arabidopsis embryo patterning via interaction with PHAVOLUTA. Development. 2007; 134(9): 1653–1662. https://doi.org/10.1242/dev.001016 PMID: 17376899

37. Chandler JW, Cole M, Flier A, Werr W. BIM1, a bHLH protein involved in brassinosteroid signalling, controls Arabidopsis embryonic patterning via interaction with DORNROŚCHEN and DORNROŚCHEN-LIKE. Plant Mol Biol. 2009; 69(1–2): 57–68. https://doi.org/10.1007/s11103-008-9405-6 PMID: 18830673

38. Chandler JW, Cole M, Jacobs B, Comelli P, Werr W. Genetic integration of DORNROŚCHEN and DORNROŚCHEN-LIKE reveals hierarchical interactions in auxin signalling and patterning of the Arabidopsis apical embryo. Plant Mol Biol. 2011; 75(3): 223–236. https://doi.org/10.1007/s11103-010-9721-5 PMID: 21161330

39. Zimmermann R, Werr W. Transcription of the putative maize orthologue of the Arabidopsis DORNROŚCHEN gene marks early asymmetry in the proembryo and during leaf initiation in the shoot apical meristem. Gene Expr Patterns. 2007; 7(1): 158–164.

40. Fehr WR, Caviness CE, Burmood DT, Pennington JS. Stage of development descriptions for soybeans, Glycine max (L.) Merrill. Crop Sci. 1971; 11(6): 929–931.

41. Zhang G, Chen M, Chen X, Xu Z, Guan S, Li LC, et al. Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (Glycine max L.). J Exp Bot. 2008; 59(15): 4095–4107. https://doi.org/10.1093/jxb/ern248 PMID: 18832187
42. Dong L, Cheng Y, Wu J, Cheng Q, Li W, Fan S, et al. Overexpression of GmERF5, a new member of the soybean EAR motif-containing ERF transcription factor, enhances resistance to Phytophthora sojae in soybean. J Exp Bot. 2015; 66(9): 2635–2647. https://doi.org/10.1093/jxb/erv078 PMID: 25779701
43. Kass J, Artero R, Baylies MK. Non-radioactive electrophoretic mobility shift assay using digoxigenin-ddUTP labeled probes. Dros Inf Serv. 2000; 83: 185–188.
44. Dang W, Wei Z. An optimized Agrobacterium-mediated transformation for soybean for expression of binary insect resistance genes. Plant Sci. 2007; 173(4): 381–389.
45. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 1998; 16(6): 735–743. PMID: 10069079
46. Paz MM, ShouHX, Guo ZB, Zhang ZY, Banerjee AK, Wang K. Assessment of conditions affecting Agrobacterium-mediated soybean transformation using the cotyledonary node explant. Euphytica. 2004; 136(2): 167–179.
47. Mazarel M, Puthoff DP, Hart JK, Rodermel SR, Baum TJ. Identification and characterization of a soybean ethylene-responsive element-binding protein gene whose mRNA expression changes during soybean cyst nematode infection. Mol Plant Microbe Interact. 2002; 15(6): 577–586. https://doi.org/10.1094/MPMI.2002.15.6.577 PMID: 12059106
48. Arumuganathan K, Earle ED. Nuclear DNA content of some important plant species. Plant Mol Biol Rep. 1991; 9(3): 208–218.
49. Zhang G, Chen M, Li L, Xu Z, Chen X, Guo J, et al. Overexpression of the soybean GmERF3 gene, an AP2/ERF type transcription factor for increased tolerances to salt, drought, and diseases in transgenic tobacco. J Exp Bot. 2009; 60(13): 3781–3796. https://doi.org/10.1093/jxbерp214 PMID: 19602544
50. Zhang G, Chen M, Chen X, Xu Z, Li L, Guo J, et al. Isolation and characterization of a novel EAR-motif-containing gene GmERF4 from soybean (Glycine max L.). Mol Biol Rep. 2010; 37(2): 809–818. https://doi.org/10.1007/s11033-009-9616-1 PMID: 19597961
51. Zhai Y, Wang Y, Li Y, Lei T, Yan F, Su L, et al. Identification and characterization of GmERF7, a soybean ethylene-response factor that increases salt stress tolerance in soybean. Gene. 2013a; 513(1): 174–183. https://doi.org/10.1016/j.gene.2012.10.018 PMID: 23111158
52. Zhai Y, Li JW, Li XW, Lei TT, Yan F, Zhao Y, et al. Isolation and characterization of a novel transcriptional repressor GmERF6 from soybean. Bioll Plant. 2013b; 57(1): 26–32.
53. Kirch T, Simon R, Grünwald M, Wier W. The DORNROSE/HENochastic SHOOT REGENARIO1 gene of Arabidopsis acts in the control of meristem cell fate and lateral organ development. Plant Cell. 2003; 15(3): 694–705. https://doi.org/10.1105/tpc.009480 PMID: 12615942
54. Kieffer M, Stern Y, Cook H, Cricri E, Maulbetsch C, Davies B. Analysis of the transcription factor WUSCHEL and its functional homologue in Antirrhinum reveals a potential mechanism for their roles in meristem maintenance. Plant Cell. 2006; 18(3): 560–573. https://doi.org/10.1105/tpc.105.039107 PMID: 16461579
55. Long JA, Ohno C, Smith ZR, Meyerowitz EM. TOPLESS regulates apical embryonic fate in Arabidopsis. Science. 2006; 312(5779): 1520–1523. https://doi.org/10.1126/science.1123841 PMID: 16763149
56. Szemenyei H, Hannon M, Long JA. TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis. Science. 2008; 319(5868): 1384–1386. https://doi.org/10.1126/science.1151461 PMID: 18258861
57. Zuo J, Niu QW, Chua NH. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. 2000; 24(2): 265–273. PMID: 11069700
58. Ikeda Y, Banno H, Niu QW, Howell SH, Chua NH. The ENHANCER OF SHOOT REGERNATION2 gene in Arabidopsis regulates CUP-SHAPECDOTYLEDON1 at the transcriptional level and controls cotyledon development. Plant Cell Physiol. 2006; 47(11): 1443–1456. https://doi.org/10.1093/pcri/pcl023 PMID: 17056621
59. Matsuo N, Mase H, Makino M, Takahashi H, Banno H. Identification of ENHANCER OF SHOOT REGENERATION1-upregulated genes during in vitro shoot regeneration. Plant Biotechnol. 2009; 26(4): 385–393.
60. Daimon Y, Takabe K, Tasaka M. The CUP-SHAPECDOTYLEDON genes promote adventitious shoot formation on calli. Plant Cell Physiol. 2003; 44(2): 113–121. PMID: 12610213
61. Matsuo N, Banno H. Arabidopsis ENHANCER OF SHOOT REGERATION2 and PINOID are involved in in vitro shoot regeneration. Plant Biotechnol. 2012; 29(4): 367–372.
62. Burian A, de Reuille PB, Kuhlmeier C. Patterns of Stem Cell Divisions Contribute to Plant Longevity. Curr Biol. 2016; 26(11): 1385–1394. https://doi.org/10.1016/j.cub.2016.03.067 PMID: 27161504
63. Narbonne P, Maddox PS, Labbé JC. DAF-18/PTEN locally antagonizes insulin signalling to couple germline stem cell proliferation to oocyte needs in C. elegans. Development. 2015; 142(24): 4230–4241. https://doi.org/10.1242/dev.130252 PMID: 26552888