Original Paper

Analysis of functional domains and binding sequences of *Arabidopsis* transcription factor ESR1

Hiroharu Banno*, Hiromi Mase, Koji Maekawa

Department of Environmental Biology, College of Bioscience and Biotechnology, Chubu University, 1200 Matsumoto-cho, Kasugai, Aichi 487-8501, Japan

* E-mail: bannoh@isc.chubu.ac.jp Tel: +81-568-51-6242 Fax: +81-568-52-6594

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Abstract The *Arabidopsis ESR1* is thought to be a key gene for commitment to *in vitro* shoot regeneration in tissue culture. The *ESR1* gene encodes a putative transcription factor. We examined the DNA-binding potential for specific sequences. Recombinant ESR1 protein specifically bound to the GCC box, known as the ethylene-responsive element. In addition, ESR1 was localized to the nucleus when expressed in onion epidermal cells. Deletion experiments revealed that the DNA-binding domain or the C-terminal region of ESR1 is essential for ESR1 overexpression to enhance shoot regeneration. The C-terminal region may be involved in transcriptional regulation by ESR1. These results suggest that ESR1 functions as a transcription factor and that its transcriptional regulation is important for the initiation of shoot regeneration.

Key words: AP2/ERF protein; ESR1; shoot regeneration; transcription factor.

In many species, organogenesis or somatic embryogenesis *in vitro* is the first step in the production of transgenic plants from single transformed cells. Therefore, the efficiency of organogenesis or somatic embryogenesis is often critical for the successful production of transgenic plants.

Overexpression of arabidopsis ESR1 cDNA enhances the efficiency of shoot regeneration in tissue culture (Banno et al. 2001). The expression pattern of the *ESR1* gene during *in vitro* shoot regeneration suggests that *ESR1* plays a key role in its initiation. *ESR1* encodes a putative transcription factor belonging to the AP2/ERF family (Weigel 1995; Okamuro et al. 1997; Riechmann and Meyerowitz 1998). The AP2/ERF domain of ESR1 is most similar to those of AtERFs that are thought to be involved in ethylene signalling (Ohme-Takagi et al. 2000). AtERFs bind to the GCC box known as the ethylene-responsive element. Arabidopsis has several genes encoding AtERFs and some of these act as transcriptional activators, while some act as repressors (Riechmann and Meyerowitz 1998; Fujimoto et al. 2000). In contrast to the similarity of the AP2/ERF domain, ESR1 did not display sequence homology to any known protein outside of the AP2/ERF domain. Therefore, based on structural features alone, it is uncertain whether ESR1 is a transcriptional activator or a repressor. Although several reports have suggested that ethylene negatively affects *in vitro* regeneration in various plants (Lakshmanan et al. 1997; Amor et al. 1998; Naik and Chand 2003), the mechanism by which ethylene affects shoot regeneration in tissue culture is uncertain.

In this study, we analyzed the functional domains of ESR1 using cDNA mutants. The AP2/ERF domain and the C-terminal region of ESR1 are indispensable for its enhancing effects on shoot regeneration when overexpressed in tissue cultures. The N-terminal region of ESR1, including the AP2/ERF domain, specifically bound to the GCCGCC sequence *in vitro*. In addition, an ESR1-GFP fusion protein was localized exclusively in the nuclei of onion epidermal cells. These results suggest that ESR1 functions as a transcription factor and the transcriptional regulation by ESR1 controls the initiation of shoot regeneration.

Materials and methods

Construction of plasmids

The N-terminal region of ESR1 was amplified by PCR using pSK-ESR1 (Banno et al. 2001) as a template with the following primers; 5′-GAGGATCCATGGAAAAAGCCTTGAGAAACTTCAC-3′ and 5′-GCAAGACCGGCAACAGGATTC-3′. The amplified fragment was blunted with T4 DNA polymerase after digestion with

Abbreviations: ESR1, Enhancer of Shoot Regeneration 1; FITC, Fluorescein isothiocyanate; GFP, green fluorescent protein; MS salts, Murashige and Skoog salts.

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SphI, and was then digested with *Bam*HI. pGEX-ESR1N and pMAL-ESR1 were constructed by inserting the resulting fragment into pGEX4T-2 between the *Bam*HI and blunted *Not*I sites, or into pMAL-C2 between the *Bam*HI and blunted *Hind*III sites.

Expression vector pTH9 for a GFP fusion protein was constructed by replacing 35S-GUS in pBI221 (Clonthech, USA) with 35S-GFP, which was excised from pTH-2 with *Bam*HI and *Xba*I (Sheen et al. 1995). ESR1 cDNA was amplified from pSK-ESR1 as a template using following primers; 5′-CTCTATATAAGG-AAGTTCATTTCATTTGG-3′ and 5′-GAGAGAGAGG-ATCCTCCCCACGATCTTCGGCAAG-3′. The amplified fragments were digested with *Xba*I and *Bam*HI and the resulting fragments were inserted between the *Xba*I and *Bam*HI sites in pTH9 to give pTH-ESR1.

ESR1ΔN cDNA was produced by PCR using pSK-ESR1 with following primers; 5′-ATGGCGCGCCAT GGGCAGCAGCACGACGAGGTAC-3′ and 5′-GCA AGACCGGCAACAGGATTC-3′. In order to construct ESR1ΔAP2 cDNA, ESR1 cDNA was excised from pSK-ESR1 by digestion with *Xba*I and *Not*I, and was then reinserted into a modified version of pBluescript II SK^+ (Stratagene, La Jolla , CA, USA), in which the *Kpn*I site was deleted, resulting in pBS-ESR1. pBS-ESR1 was blunted with mung bean nuclease after digestion with *Kpn*I and *Sty*I, and was then self-ligated, resulting in ESR1ΔAP2 cDNA. To give ESR1ΔC cDNA, pBS-ESR1 was blunted with T4 DNA polymerase after digestion with *Sph*I and *Not*I, and was then self-ligated. These cDNAs were inserted into pER10 (Zuo et al. 2000) between the *Asc*I and blunted *Spe*I sites.

Purification of recombinant proteins

pGEX-ESR1N, pMAL-C2 and pMAL-ESR1N were transformed into an *Escherichia coli* strain, BL21-CodonPlus (DE3)-RIL (Stratagene, La Jolla, CA, USA). Lysate from *E. coli* carrying pGEX-ESR1N was cleared by centrifugation, was mixed with Glutathione-Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) and was washed with TBS [25 mM Tris-HCl (pH 7.4), 250 mM NaCl]. GST-ESR1N-bound Glutathione-Sepharose beads were subjected to binding sequence selection assay. Maltose-binding protein (MBP) and MBP-ESR1N were purified from *E. coli* harboring pMAL-C2 and pMAL-ESR1N, respectively using Amylose resin (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions.

Binding Sequence Selection Assay (BSSA)

Approximately 100 ng of GST-ESR1N bound to Glutathione-Sepharose was mixed with 1 μg of double-stranded oligonucleotides (5′-AACGGTACCAGAAGC-TTACCNNNNNNNNNNNNNNNNNNNNCCAGA-ATTCGAGCTCTTCGT-3′) and 10 μg of poly[dI-dC] (Sigma-Aldrich, St. Louis, MO, USA) in 100 μl of Binding Buffer [10 mM HEPES-KOH (pH 7.6), 50 mM KCl, 0.1% Triton-X100], and was incubated for 15 min at room temperature. After the sepharose beads were washed with Binding Buffer three times, double-stranded oligonucleotides were eluted with Elution Buffer [10 mM HEPES (pH 7.6), 1 M NaCl, 1 mM EDTA]. The eluted double-stranded oligonucleotides were recovered by ethanol precipitation, and were then amplified by PCR using following primers; 5′-AACGGTACCAGAAGCT-TACC-3′ and 5′-ACGAAGAGCTCGAATTCTGG-3′. Next, 100 ng of amplified DNA was mixed with approximately 100 ng of GST-ESR1N bound to Glutathione-Sepharose, and the above procedures were repeated. After 5 repetitions, the final amplified DNA was cloned into a pT7-Blue TA vector (Novagen, San Diego, CA, USA) and was subjected to sequencing analyses.

Gel mobility-shift assay

Fluorescent probes were produced by annealing following the oligonucleotides: for wt-GCC, 5′-FITC-TAAGAGCCGCCAC-3′ and 5′-GTGGCGGCTCTTA-3′; or for m-GCC, 5′-FITC-TAAGATCCTCCAC-3′ and 5′-GTGGAGGATCTTA-3′. Each probe was incubated with MBP or MBP-ESR1N in Binding Buffer containing 50 μM of poly[dA-dT] (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 26°C. The reaction mixture was then subjected to 8% PAGE in 0.5×TBE, and was analyzed using a Typhoon 9200 (Amersham Biosciences, Piscataway, NJ, USA).

Transient transformation with GFP fusion constructs

Biolistic transformation of onion epidermis was performed according to previously published procedures (Scott et al. 1999). Cells in the epidermal layer of onion bulbs were transformed with pTH9 or pTH-ESR1 by bombardment using a particle bombardment gene delivery system (IDERA GIE-III; Tanaka Co. Ltd., Hokkaido, Japan). After bombardment, the onion pieces were incubated on MS plates for 24 h at 22°C, and fluorescence signals were then observed under a fluorescence microscope (Axioplan2; Carl Zeiss, Oberkochen, Germany).

Shoot regeneration assay

Arabidopsis thaliana Wassilewskija (WS) was used for root transformation. The procedure for root transformation was as described previously (Banno et al. 2001). 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-dichlorophenoxyacetic acid (2,4-D), 0.25 μM kinetin and 0.25% Phytagel (Sigma-Aldrich, St. Louis, MO, USA); and for SIM, MS salts, 1% sucrose,

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Gamborg's B5 vitamins, 0.8 μM indole-3-acetic acid (IAA), 12.5 μM N^6-Δ^2-isopentenyladenine (2-ip) and 0.25% Phytagel. C medium is SIM supplemented with 0.4 g l^{-1} of carbenicillin and 50 mg l^{-1} of kanamycin.

Results

Identification of optimal binding sequences of recombinant ERS1 protein

The predicted amino acid sequence of ESR1 strongly suggests that it is an AP2/ERF class transcription factor. In order to identify the optimal binding sequences of ESR1 protein, Binding Sequence Selection Assay (BSSA) was employed. The N-terminal region (ESR1N; amino acid residues 1–200) containing an AP2/ERF domain of ESR1 was fused to the C-terminus of glutathione-S-transferase (GST) and the fusion protein (GST-ESR1N) was used for BSSA, as full-length of ESR1 protein was insoluble when expressed in *E. coli*. GST-ESR1N protein was mixed with random sequence probes, which consisted of 20 random-nucleotide sequences and primer sequences for amplification by PCR at both ends, and then bound sequences were amplified by PCR after unbound sequences were washed away. After these procedures were repeated five times, the final amplified sequences were cloned into a cloning vector and their sequences were analyzed. Among the 30 sequences analyzed, 10 contained GCCGCC and 14 sequences contained GCCGCC-related sequences (Figure 1). The consensus sequence was the GCC box, A/TGCCGCC, which is almost identical to the ethylene-responsive element, AGCCGCC (Ohme-Takagi and Shinshi 1995; Shinshi et al. 1995). These results were expected as the amino acid sequence of theAP2/ERF domain in ESR1 is most similar (66–82% identical) to those of arabidopsis ethylene-responsive element binding factors, AtERFs, and tobacco ethylene-responsive element binding proteins, EREBPs (Banno et al. 2001), and all amino acid residues within the AP2/ERF domain that interact directly with the GCC box (Allen et al. 1998; Fujimoto et al. 2000) are conserved in ESR1.

Binding specificity of ESR1 to the GCCGCC sequence

In order to confirm the binding specificity of ESR1 to the GCCGCC sequence, gel mobility-shift assay was carried out (Figure 2). The N-terminal region (amino acid residues 1–200) of ESR1 was fused with maltose-binding protein (MBP) at its C-terminus and was expressed in *E. coli* cells, as MBP-ESR1N has better stability than GST-ESR1N after purification. MBP-ESR1N protein was purified and mixed with wild-type probe (GCCGCC) or mutant probe (TCCTCC) (Figure 2A). Whereas MBP-ESR1N bound to the GCCGCC sequence, it did not bind to the TCCTCC sequence (Figure 2B). Furthermore, the intensity of the bands increased depending on the amount of MBP-ESR1N added, while a retarded band was not observed, even when MBP alone was added at higher concentrations (Figure 2C). These results demonstrate that the N-terminal region containing the AP2/ERF domain of ESR1 specifically binds to the GCCGCC sequence.

GCCGCC

CC**AGCCGCC**TT
AC**TGCCGCC**AC
TT**TGCCGCC**AG
CG**TGCCGCC**TC
TA**AGCCGCC**AG
AC**TGCCGCC**AC
CG**AGCCGCC**TT
AA**TGCCGCC**GT
TGG**GCCGCC**CC
AGC**GCCGCC**AA

GCCGCC-related

CT**T**A**CCGCC**TG
CG**A**A**CCGCC**TT
TT**A**T**CCGCC**AG
CG**A**T**CCGCC**GG
CT**TG**T**CGCC**TG
GA**TGC**A**GCC**GA
TA**AGCC**A**CC**CA
TA**TGCCG**A**C**GT
AG**TGCCG**T**C**CG
GC**AGCCGC**ACT
GCGA**CCGCC**GA
ACC**GCC**A**CC**AC
TTG**GCCG**G**C**AG
GTC**GCCG**A**C**TT

Consensus

a/t G C C G C C
19/20/24/24/24/21/24

Figure 1. Alignment of ESR1-binding sequences determined by Binding Sequence Selection Assay. Final amplified PCR fragments were cloned into a cloning vector and sequences of the 30 inserts were analyzed. 'GCCGCC' indicates the sequences containing the GCCGCC sequence and 'GCCGCC-related' indicates sequences similar to the GCCGCC sequence. Consensus sequence is labeled with the numbers of matching bases.

ESR1-GFP fusion protein targets the nucleus

Because ESR1 protein specifically binds to the GCC box, ESR1 probably functions as a transcription factor. To verify this, we investigated the subcellular localization of ESR1. For this purpose, 35S::GFP (pTH-2) or 35S::ESR1-GFP (pTH-ESR1) was transiently expressed in onion epidermal cells after introducing the constructs by a particle bombardment. Onion cells were observed under a fluorescence microscope 24 h after bombardment. Figure 3 shows the results. Whereas GFP

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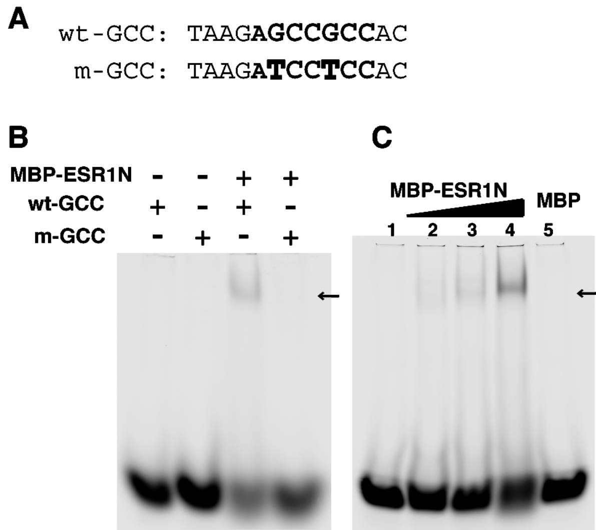

Figure 2. Binding affinity of MBP-ESR1N to GCC box by Gel mobility-shift assay. (A) Sequences of wild-type GCCGCC probe (wt-GCC) and mutant GCCGCC probe (m-GCC). Each probe is labeled with FITC at the 5′-end. Nucleotides corresponding to the GCC box are written in bold and mutated nucleotides are boxed. (B) Binding Specificity of MBP-ESR1N. Double-stranded oligo-DNA probes (20 pmol) were mixed with 70 pmol of MBP-ESR1N in a 10-μl volume. After 30 min of incubation, reaction mixtures were analyzed by native PAGE. The absence or presence of MBP-ESR1N protein and probes is indicated by − or +, respectively. (C) Dose dependency of intensity of retarded bands on amount of MBP-ESR1N. Twenty picomoles of wt-GCC was mixed with various amounts of MBP-ESR1N: lane 1; 0 pmol, lane 2; 3 pmol, lane 3; 10 pmol, and lane 4; 50 pmol. Seventy picomoles of MBP was mixed with 20 pmol of wt-GCC in lane 5. All combinations were carried out in 10-μl volumes. After 30 min of incubation, reaction mixtures were analyzed by native PAGE. Retarded bands are indicated by arrows.

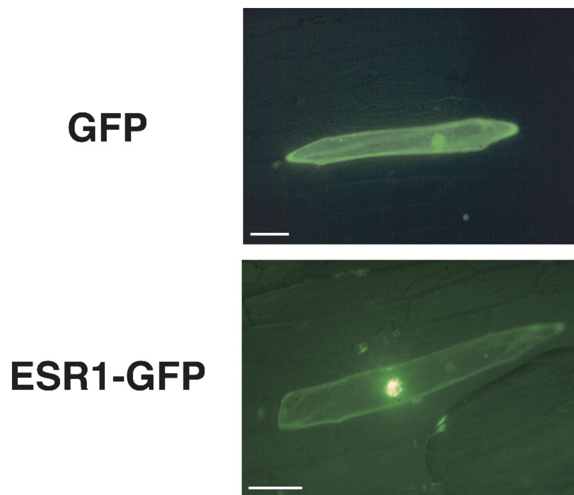

Figure 3. Subcellular localization of ESR1-GFP fusion protein in onion cells. 35S::GFP or 35S::ESR1-GFP constructs were transiently introduced into onion epidermal cells. Fluorescence was observed under a fluorescence microscope at 24 h after bombardment. Upper panel (GFP) indicates fluorescence for bombardment with the 35S::GFP construct and the lower panel indicates fluorescence for bombardment with the 35S::ESR1-GFP construct. Bars=50 μm.

alone was distributed throughout the cells, ESR1-GFP specifically targeted the nuclei. These results indicate that ESR1 protein is localized in the nuclei, although ESR1 has no typical nuclear localization signal that could be detected by searching a database of nuclear localization signals, NLS db (Nair et al. 2003). Taken together with the ability of ESR1 to bind specific sequences, these results strongly suggest that ESR1 functions as a transcription factor.

Shoot regeneration induced by overexpression of wild-type and mutant ESR1 cDNAs

In order to identify the functional domains of ESR1, we constructed mutant ESR1 cDNAs. Figure 4A

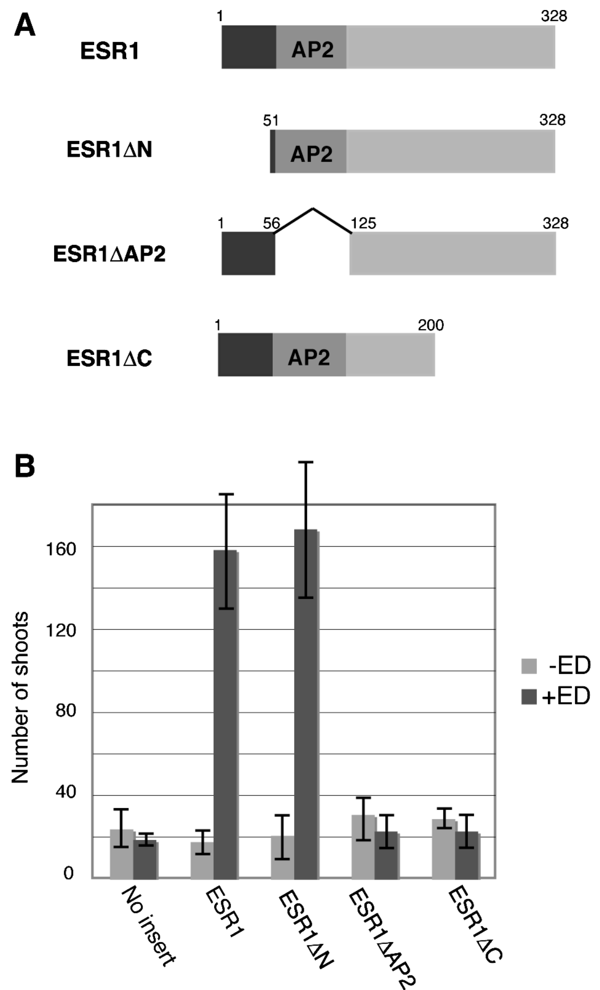

Figure 4. Effects of overexpression of mutant cDNAs on shoot regeneration in root explants. (A) Schematic representations of the wild-type and mutant versions of ESR1 protein are shown. “AP2” indicates the AP2/ERF domain. Amino acid numbers corresponding to wild-type ESR1 protein are indicated. (B) Numbers of transformed shoots from 0.1 g (fresh weight) of root culture. Arabidopsis root cultures preincubated on CIM for 3 days were transformed with vector alone (pER10) or the same vectors containing wild-type or mutant cDNAs, and were then cultured on C medium with (+ED) or without (−ED) a transcription inducer (5 μM 17β-estradiol) for 4 weeks. Data represent the average of 3 independent experiments and error bars indicate standard deviations.

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illustrates the mutant cDNAs used in these experiments. These cDNAs were inserted into the inducible binary vector pER10 (Zuo et al. 2000), and expression of cloned inserts was induced by adding 17β-estradiol (ED) to media. The constructs were transformed into arabidopsis 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), regenerated shoots were counted (Figure 4B). Overexpression of full-length ESR1 cDNA strongly enhanced efficiency (6.7-fold) of shoot regeneration in an ED-dependent manner, as reported previously (Banno et al. 2001). Deletion of the AP2/ERF domain or the C-terminal region abolished the enhancing effects on shoot regeneration, but deletion of the N-terminal region had no effect on that. These results indicate that the AP2/ERF domain and the C-terminal region are essential to the shoot regeneration enhancing effects of ESR1.

Discussion

ESR1 cDNA encodes a putative transcription factor (Banno et al. 2001). To examine whether the ESR1 product has any features common to transcription factors, we examined whether ESR1 is able to bind to specific DNA sequences. ESR1 was found to bind specifically to the GCC box (Figure 1, 2). In addition, ESR1-GFP targeted the nucleus when expressed in onion epidermal cells. These results support the notion that ESR1 functions as a transcription factor. Overexpression of ESR1 cDNA effectively enhances shoot regeneration, as shown previously. Deletion experiments indicated that the AP2/ERF domain of ESR1 is indispensable for the shoot regeneration enhancing effects (Figure 4), which suggests that ESR1 enhances shoot regeneration through transcriptional regulation of downstream genes involved in shoot formation.

The optimal binding sequences of ESR1 were almost identical to the GCC box. The GCC box is thought to be a regulatory element for ethylene signaling (Shinshi et al. 1995; Ohme-Takagi et al. 2000). One simple interpretation of the shoot regeneration enhancing effects by ESR1 overexpression is that the effects are caused by repression of ethylene signaling through ESR1 binding to the GCC box in the promoters of ethylene-regulated genes, as it is known that expression of an antisense ACC oxidase gene stimulates shoot regeneration in *Cucumis melo* (Amor et al. 1998) and overexpression of AtEBP, an ethylene-induced member of the AP2/ERF family that is thought to be a transcriptional activator (Ogawa et al. 2005), strongly inhibits shoot regeneration in arabidopsis (Banno 2004). The C-terminal region of ESR1 is also essential for the shoot regeneration enhancing effects. The region is likely to function in transcriptional regulation of downstream genes. We examined whether ESR1 activates or represses expression of a reporter gene under control of 4 repeats of the GCCGCC enhancer sequence using a transient expression system involving bombardment of arabidopsis leaves. ESR1 did not activate reporter expression or repress basal expression of the reporter (unpublished results). Transcriptional regulation by ESR1 may require some co-factors that are not active in leaves. Further research is necessary to confirm whether ESR1 is a transcriptional activator or a repressor.

We are continuing to search for target genes of transcriptional regulation by ESR1 using DNA microarrays probed with labeled mRNAs from transformed plants expressing inducible nuclear-targeting ESR1 proteins under induced or non-induced conditions. Such experiments may assist in the investigation of ESR1-mediated signaling pathways.

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