# Transcription Factor FUS3 Counteracts ETR1 Overexpression-induced Salt Tolerance in Plant Cells

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**Abstract:** The ethylene receptor 1 (ETR1) of Arabidopsis (*Arabidopsis thaliana* L.) plays critical roles in modulating expression of defense response genes during the developmental processes of plants. To examine the function of the *ETR1* gene in NaCl stress tolerance, cell lines of *A. thaliana*, white pine (*Pinus strobes* L.), and rice (*Oryza sativa* L.) overexpressing *ETR1* were generated using *Agrobacterium*-mediated genetic transformation. Physiological analysis of transgenic cell lines showed that overexpression of *ETR1* increased cell viability and growth rate and decreased the level of thiobarbituric acid reactive substance (TBARS). Biochemical analysis of transgenic cell lines demonstrated that overexpression of *ETR1* enhanced tolerance to NaCl stress by regulating expression of a set of defense response genes including of *CTR1, EIN2, MPK11, EIN3, ERF1, BREB2A, NAC6, PDF1.2, WRKY13, bZIP23, ABI5*, and *LEA3*. In rice cells, overexpression of *FUS3* counteracts *ETR1* enhanced expression of defense response genes under NaCl stress, and overexpression of $SCF^{TIR1}$ reduces *ETR1* enhanced expression of defense response genes under NaCl stress. Altogether, our results suggest that overexpression of *ETR1* enhanced NaCl stress tolerance of transgenic plant cells by decreasing lipid peroxidation and by regulating expression of defense response genes.

**Keywords:** *Agrobacterium*-mediated genetic transformation, Defense response genes, The ethylene receptor 1, Lipid peroxidation, NaCl stress, *Pinus.*

## INTRODUCTION

The ethylene receptors are important for plant growth and plays important roles in different developmental processes of plants [1, 2]. Receptors localized on the plasma membrane are essential for cells to regulate gene expression through hormone signaling transduction pathways [2, 3]. Among different hormones, ethylene has been reported to regulate growth, ripening, senescence, abscission, and wound responses [4, 5]. In *A. thaliana*, there are five members of ethylene receptor family including ETR1, ERS1, ETR2, ERS2, and EIN4 [4, 6]. The ethylene receptor ETR1 of *A. thaliana* was the first member of the receptor family identified [7-9]. ETR1 contains transmembrane domains responsible for ethylene binding and membrane localization. Biochemical analysis by aqueous two-phase partitioning, sucrose density-gradient centrifugation, and immunoelectron microscopy indicates that ETR1 is predominantly localized to the endoplasmic reticulum [1, 10-12]. The ethylene precursor 1-aminocyclopropane- 1-carboxylic acid has been used to analyze the function and location of the ETR1 receptor and the results demonstrated that the amino-terminal of ETR1 is essential to target the endoplasmic reticulum, indicating the involvement of the endoplasmic reticulum in the ETR1 receptor related signal transduction [5, 13-16].

ETR1 has three predicted transmembrane domains including a GAF domain [15, 17], a histidine kinase domain [17, 18], and a receiver domain [5, 13-16]. GAF domains are involved in cGMP binding [15, 19-21]. Histidine kinase and receiver domains are signaling components that have also identified in bacteria, fungi, protists, and plants [15, 19-21]. Histidine kinase activity has been demonstrated for ETR1 [19, 20]. The ETR1 transmembrane domains have two functions that are involved in various developmental processes. One function is to determine membrane localization of the receptor; another function is to bind the signal molecules ethylene [15, 19, 22]. ETR1 is a disulfide-linked homodimer with each dimer containing a single ethylene-binding site [15, 19, 22]. The amino acids Cys-65 and His-69 that located in the second transmembrane domain coordinated with a copper co-factor to confer the high affinity binding of ethylene [15, 19, 22].

The transmembrane domains of the ETR1 receptor are important in signal transduction and regulation of plant development and response to abiotic stress. The features of amino acid conservation in the transmembrane domains of the ETR1 receptor are similar with other members of the ethylene receptor

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E-ISSN: 2311-858X/18 

family and are essential in ethylene binding [23-25]. The receptor of ETR1 regulates different aspects of plant growth, development, and responses to NaCl, drought, and cold stresses using a membrane-bound receptor for signal transduction [17, 18, 26]. Although some members of the ethylene receptor family have specific signal sequences for ethylene binding and signal transduction, no signal sequences that are associated with subcellular localization have been defined in ETR1 [17, 27, 28].

The receptors of ethylene affect many aspects of abiotic stress responses through responding to endogenous signals and environmental factors [24]. Expression of the ethylene receptors may be regulated by abiotic factors. For example, exposure of *A. thaliana* plants to NaCl reduced expression of the ethylene receptor ETR1, suggesting that plant responses to abiotic stress are modulated by changes in the expression level of ethylene receptors [29-37]. Results from a cross talk between the abscisic acid and ethylene signal transduction pathways in *A. thaliana* wild-type plants, mutant (*eto1-1*), and mutants (*etr1-1* and *ein3-1*) demonstrated that ethylene delays stomatal closure by inhibiting the ABA signaling pathway in drought condition [38-41]. The receptor of ethylene negatively regulates plant responses to cold stress in plants. Genetic and biochemical analyses demonstrated that EIN3, CBFs, ARR5, ARR7, and ARR15 are important factors that are associated with the ETR1-related freezing tolerance of plants. The receptor of ETR1 negatively regulates cold signaling through the direct transcriptional control of cold-regulated CBFs and type-A ARR genes by EIN3 [32, 39, 42-46].

The FUS3 transcription factor plays important role in plant growth, development, and stress responses [48-54]. FUS3 controls lateral organ development and phase transitions, activates triacylglycerol accumulation, and regulates embryogenesis through interaction with the epidermal regulator TTG1 in *A. thaliana* [53-60]. In this paper, we first demonstrate that overexpression of *ETR1* enhanced NaCl stress tolerance of transgenic plant cells by decreasing lipid peroxidation and regulating expression of defense response genes in *A. thaliana*, *P. strobes*, and *O. sativa*. We demonstrated that overexpression of *FUS3* counteracts *ETR1* enhanced expression of defense response genes under NaCl stress, and overexpression of $SCF^{TIR1}$ reduces *ETR1* enhanced expression of defense response genes under NaCl stress, indicating diverse interactions between ETR1 and defense response genes. These results have implications for our understanding of the receptor ETR1 that has a function in NaCl stress tolerance.

## MATERIALS AND METHODS

### Expression Vector

To construct the expression vectors for overexpression of *ETR1*, *FUS3*, and $SCF^{TIR1}$, the cDNAs of *ETR1*, *FUS3*, and $SCF^{TIR1}$ and the pBI121 binary vector were digested by *Kpn* I and *Xba* I (Promega, Madison, WI, USA) at 37°C. The digested and purified pBI121 was ligated with the fragments of the *ETR1*, *FUS3*, and $SCF^{TIR1}$ genes to produce the expression vectors pBI-*ETR1*, pBI-*FUS3*, and pBI-$SCF^{TIR1}$. Vectors pBI-*ETR1*, pBI-*FUS3*, and pBI-$SCF^{TIR1}$ were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *Agrobacterium tumefaciens* strains GV3101 carrying pBI-*ETR1*, pBI-*FUS3*, or pBI-$SCF^{TIR1}$ were used for transformation [47].

### *Agrobacterium*-Mediated Transformation

The *ETR1* transgenic cell lines of Arabidopsis (*Arabidopsis thaliana* L.), white pine (*Pinus strobes* L.), and rice (*Oryza sativa* L.) were generated as described before [48, 49], using *Agrobacterium tumefaciens* strain GV3101 carrying the expression vector pBI-*ETR1*. The *ETR1* and *FUS3* double transgenic cell lines of rice were generated by transforming the *ETR1* transgenic cell lines using *Agrobacterium tumefaciens* strain GV3101 carrying the expression vector pBI-*FUS3*. The *ETR1* and $SCF^{TIR1}$ double transgenic cell lines of rice were generated by transforming the *ETR1* transgenic cell lines using *Agrobacterium tumefaciens* strain GV3101 carrying the expression vector pBI-$SCF^{TIR1}$. Six weeks after *Agrobacterium tumefaciens* strain GV3101-mediated transformation, the selected cell lines were used for PCR, Southern blotting, and northern blotting analysis.

### Polymerase Chain Reaction Analyses of Transgenic Cells

Polymerase chain reaction (PCR) analysis of transgenic cells was conducted as previously described [48]. One gram of control cells and transgenic cells were used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). The primers used to amplify the *NPTII* gene are forward primer 5'-GTCGACATGGCGGAGGAATTTGGAAGCATAG-3' and the reverse primer 5'-CCATGGTAGACTCC-

TGCTTCGACATCATGG-3'. The primers used to amplify the *ETR1* gene are forward primer (zfp) 5'-GTTAAACCCA ACCAATTTTGACTTGA-3' and the reverse primer 5'- TGTTGTTGTTCTAC TAAGCGG-CGCAAAG -3'. The primers used to amplify the *FUS3* gene are forward primer 5'- TGTTGTTGTTCTACTAA-GCGGCGCAAAG-3' and the reverse primer 5'-AACACTAAATAACATTTATTCATCAAATAC -3'. The primers used to amplify the $SCF^{TIR1}$ gene are forward primer 5'- TTTGAAACTAGATTATCTCCGTTAATGG-CGCC -3' and the reverse primer 5'- CATTGTCTTTT-AAAAAAATCTCTTATCCTC-3'. The PCR mixture, the PCR conditions, and gel electrophoresis were carried out as described previously [48, 50].

**Southern Blot Analysis of Transgenic Cells**

Southern blotting analysis of transgenic cells of *A. thaliana*, *P. strobes*, and *O. sativa* was conducted as previously described [48, 50]. Five grams of control cells and transgenic cells were used to isolate genomic DNA, using a Genomic DNA Isolation Kit (Sigma). Twenty-five micrograms of DNA were digested 16 hours with the enzymes *Xba* I (Boehringer Mannheim) at 37 $^{o}$C. The molecular probes (2217 pb fragment of *ETR1*) were labeled by Digoxigenin (DIG) (Roche Diagnostics, Indianapolis, IN, USA).

**NaCl Treatment of Transgenic Cell Lines**

NaCl treatment was applied by transferring transgenic and control cells to culture media supplemented with 200 mM NaCl for different time (0d, 1d, 2d, and 4d), as previously described [49, 50]. The cell viability, growth rate, and changes of thiobarbituric acid reactive substance (TBARS) of transgenic and control cells were determined 3 days after 200 mM NaCl treatment.

**Measurement of Cell Viability**

The staining chemical 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide (MTT) was used to cell viability. Cell suspension samples (1 ml) were wash aseptically with 50 mm phosphate buffer, pH 7.5, for three times. Cells were re-suspended in 1 ml of the same buffer. MTT was added to samples at a final concentration of 1.25, then incubate samples for 8 h in the dark at 25°c and solubilize formazan salts with 1.5 ml 50% methanol, containing 1% SDS, at 60°c for a period of 30 min. Samples were centrifuged at 1875**G** for 5 min and recover the supernatant for three times. Pool the supernatants and quantify absorbance at 570 nm. Fluorescein will accumulate in cells, which possess intact membranes, so the green fluorescence can be used as a marker of cell viability. The cell viability was expressed as percentage of live cells in total cells assayed [49, 50].

**Measurement of Cell Growth Rate**

Samples of 10-ml transgenic and control cell cultures were withdrawn from the culture flasks using sterile de-tipped pipettes. For determination of fresh cell weight, the centrifuged (at 1000 **G** for 5 min) cells were washed once with 10 ml of distilled water, filtered on a pre-washed and pre-weighed 0.45-μm Millipore filter (Millipore, Bedford, MA**)** and the weight was determined. The average growth was expressed as mg/g FW/day [49, 50].

**Measurement of Thiobarbituric Acid Reactive Substances (TBARS)**

Lipid peroxidation was determined as the amount of thiobarbituric acid reactive substances (TBARS) measured by the thiobarbituric acid (TBA) reaction as described previously (Tang and Page 2013). Transgenic and control cells (1 g) were homogenized in 3 ml of 20 % (w/v) trichloroacetic acid (TCA), then centrifuged at 5,000 rpm for 20 min, following by mixing with 20 % TCA containing 0.5 % (w/v) TBA and100 μl 4 % BHT in ethanol at 1:1. The absorbance of the extracts of different cell lines was measured at 532 nm. The value of TBARS was calculated using the method described previously [47].

**Determination of Defense Response Gene Expression**

Expression of Expression levels of *CTR1, EIN2, MPK11, EIN3, ERF1, BREB2A, NAC6, PDF1.2, WRKY13, bZIP23, ABI5*, and *LEA3* in transgenic cells was examined using qPCR as previously described [51]. Total RNA was extracted from frozen sample cells using TRIzol reagent by following the description in the manufacturer's protocol (Invitrogen). Synthesis of cDNA of samples were prepared using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems). Samples were analyzed in triplicate on the Applied Biosystems 7900HT System by following the description in the manufacturer's manual. The primers used for qPCR are listed in Table **1**.

**Statistical Analyses**

Statistical analysis was performed using the General Linear Model procedure of SAS (Cary, NC,

**Table 1: Primer Sets used for qRT-PCR**

| Primers | Sequences |
|---|---|
| *OsCTR1*-Forward | 5′-GCCTTACGTTACATGTCAGCAAGAGTCG-3′ |
| *OsCTR1*-Reverse | 5′-TTCAGATATGGATAACTCTCACCGGG-3′ |
| *OsEIN2*-Forward | 5’-CGGATAGGTACTATGATGGC-3’ |
| *OsEIN2*-Reverse | 5’-GCACTCGACACACCAAACAG-3 |
| *OsMPK11*-Forward | 5′-CTCCTGCTTCCGAAATCCTCAACT-3′ |
| *OsMPK11*-Reverse | 5′-TATTGCAACCTTTTCTCCAGTA-3′ |
| *OsEIN3*-Forward | 5′-ATCTTCCCGGCAACCTACAA-3′ |
| *OsEIN3*-Reverse | 5′-CATGATCGTGGCATTGTCGT-3′ |
| OsERF1-Forward | 5′-GGTGCAGGCATGGTACCCC-3′ |
| OsERF1-Reverse | 5’-CCCTCACAAACTCACTCGG-3′ |
| *OsDREB2A* -Forward | 5′-AGGGCAATGTATGGTCCCACAG-3′ |
| *OsDREB2A* -Reverse | 5′-TGCTGATGTGCAGCCAGAGTTG-3′ |
| *OsNAC6*-Forward | 5′-AGAAGAACAGCCTCAGGTTGGATG-3′ |
| *OsNAC6*-Reverse | 5′-AGCCCGCCCTTCTTGTTGTAAATC-3′ |
| *OsPDF1.2*-Forward | 5′-TCAGGCAAGCTCTCCTCTTC-3′ |
| *OsPDF1.2*-Reverse | 5′-ATGCAGCGTCGAGTCAAGTA-3′ |
| *OsWRKY13*-Forward | 5′-GTGATGGCGGCAGGAGAG-3' |
| *OsWRKY13*-Reverse | 5′-TGAACACGACGGCGCACTC-3' |
| *OsbZIP23*-Forward | 5′-GGAGCTGAACGATGAACTCCAG-3′ |
| *OsbZIP23*-Reverse | 5′-TCGGCTCATTCTCTCTAGAACCTC-3′ |
| OsABI5-Forward | 5′-ATGGCATCGGAGATGAGCAAGAAC-3′ |
| OsABI5-Reverse | 5’-GCTTCTTTGTCAGTAGAACCGTCTTC-3′ |
| *OsLEA3*-Forward | 5’-TTTCTGACGGGTGTGGGTGATG-3’ |
| *OsLEA3*-Reverse | 5’-AACACAGACGAGAAACTCTGACG-3’ |

USA), employing ANOVA models. Each value of different cell lines was presented as the standard deviations of the mean.

## RESULTS

### Production and Confirmation of Transgenic Cells

To examine the function of the *ETR1* gene in NaCl stress tolerance, cell lines of *A. thaliana*, *P. strobes*, and *O. sativa* overexpressing *ETR1* were generated using *Agrobacterium tumefaciens* strain GV3101 carrying the expression vector pBI-*ETR1* (Figure **1a**). Three transgenic cell lines from each of *A. thaliana* (At1, At2, and At3), *P. strobes* (Ps1, Ps2, and Ps3), and *O. sativa* (Os1, Os2, and Os3) overexpressing *ETR1* were selected and confirmed by PCR analysis of the *NPT II* gene (Figure **1b**) and the *ETR1* gene (Figure **1c**), as well as Southern blot analysis (Figure **1d**) and northern blot analysis (Figure **1e**). Transgenic cell lines of At1, At2, At3, Ps1, Ps2, Ps3, Os1, Os2, and Os3 were sub-cultured on growth medium for 5 weeks to get large number of cells before using for experiment of NaCl treatment.

### Cell Viability, Growth Rate, and Changes of Thiobarbituric Acid Reactive Substance (TBARS) of Transgenic Cell Lines

Transgenic cell lines of At1, At2, At3, Ps1, Ps2, Ps3, Os1, Os2, and Os3 were treated with 200mM NaCl [49, 50] for different time points (0 d, 1 d, 2 d, and 4 d). Three days after treatment of 200 mM NaCl, cell viability of transgenic *A. thaliana* cell lines At1, At2, and At3 (Figure **2a**), *P. strobes* cell lines Ps1, Ps2, and Ps3 (Figure **2b**), and *O. sativa* cell lines Os1, Os2, and Os3 (Figure **2c**) were examined. Transgenic cells overexpressing *ETR1* increased cell viability 116% (*O. sativa*), 228% (*P. strobes*), and 236% (*A. thaliana*) at the time point 4 d of 200 mM NaCl treatment. Growth rate of transgenic *A. thaliana* cell lines At1, At2, and

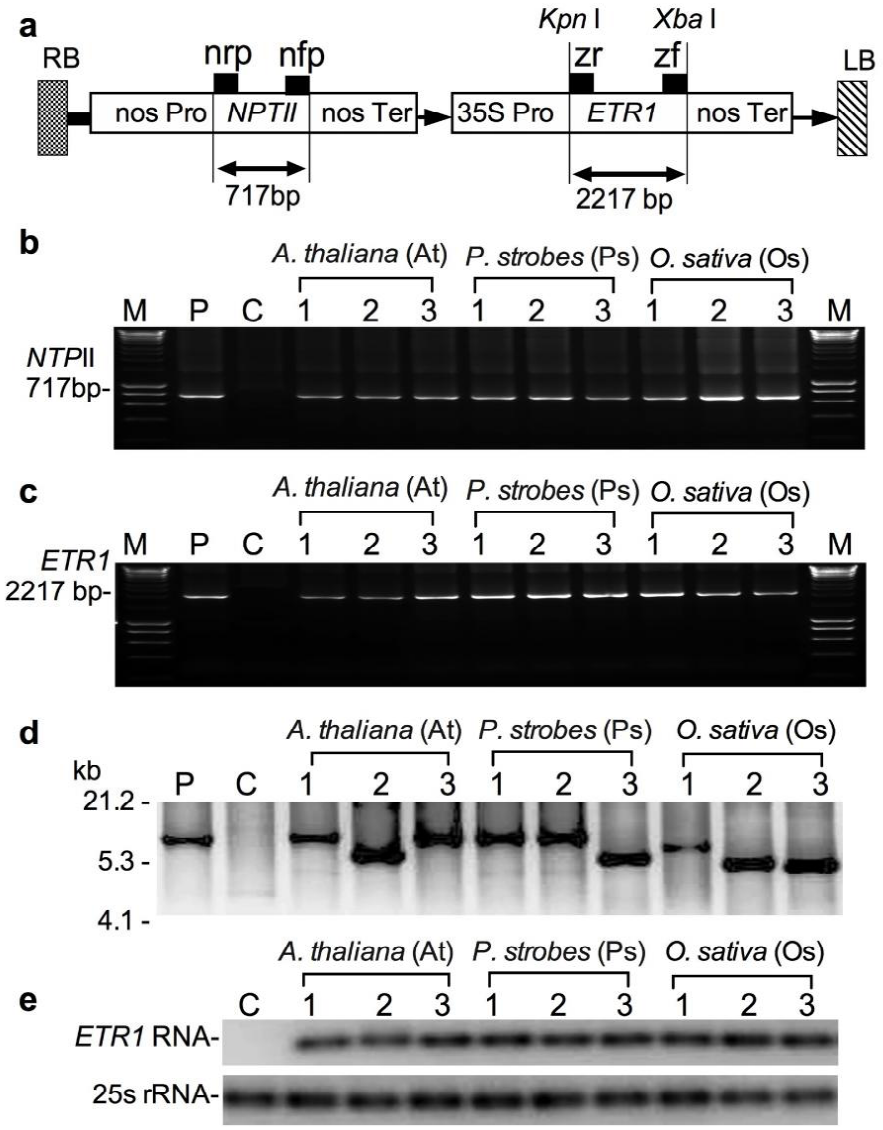


**Figure 1:** A linear map of the expression vector and confirmation of transgenic cells.

(**a**) A linear plasmid map including the neomycin phosphotransferase II gene (*NPTII*), the ethylene receptor ETR1 of *Arabidopsis* (*ETR1)*, the promoter of nopaline synthase gene (*nosPro*), the Cauliflower Mosaic Virus 35S promoter (*35S Pro*), the terminator from nopaline synthase gene (*nos Ter*), T-DNA right border (RB), and T-DNA left border (LB). (**b**) PCR analysis of the *NPT II* gene in transgenic cell lines of Arabidopsis (left lines 1, 2, and 3), pine (middle lines 1, 2, and 3), and rice (right lines 1, 2, and 3). Lanes: M HyperLadder I DNA molecular markers (Bioline), P plasmid control, C non-transgenic control. (**c**) PCR analysis of the *TRE1* gene in transgenic cell lines of Arabidopsis (left lines 1, 2, and 3), pine (middle lines 1, 2, and 3), and rice (right lines 1, 2, and 3). Lanes: M HyperLadder I DNA molecular markers (Bioline), P plasmid control, C non-transgenic control. (**d**) Southern blot analysis of transgenic cell lines of Arabidopsis (left lines 1, 2, and 3), pine (middle lines 1, 2, and 3), and rice (right lines 1, 2, and 3). Lanes: P plasmid control, C non-transgenic control. (**e**) Northern blot analysis of transgenic cell lines of Arabidopsis (left lines 1, 2, and 3), pine (middle lines 1, 2, and 3), and rice (right lines 1, 2, and 3). Lane: C non-transgenic control. The tobacco 25S rRNA was used as a loading control.

At3 (Figure **2d**), *P. strobes* cell lines Ps1, Ps2, and Ps3 (Figure **2e**), and *O. sativa* cell lines Os1, Os2, and Os3 (Figure **2f**) increased 118%-249% under 200 mM NaCl treatment for 4 d. TBARS levels of transgenic *A. thaliana* cell lines At1, At2, and At3 (Figure **2g**), *P. strobes* cell lines Ps1, Ps2, and Ps3 (Figure **2h**), and *O. sativa* cell lines Os1, Os2, and Os3 (Figure **2i**) decreased by 108% (*P. strobes*), 116% (*A. thaliana*), and 128% (*O. sativa*) under 200 mM NaCl treatment at time point 4 d.

### Gene Expression in ETR1 Transgenic Rice Cell Lines

To examine the effect of *ETR1* overexpression on the expression levels of defense response genes,

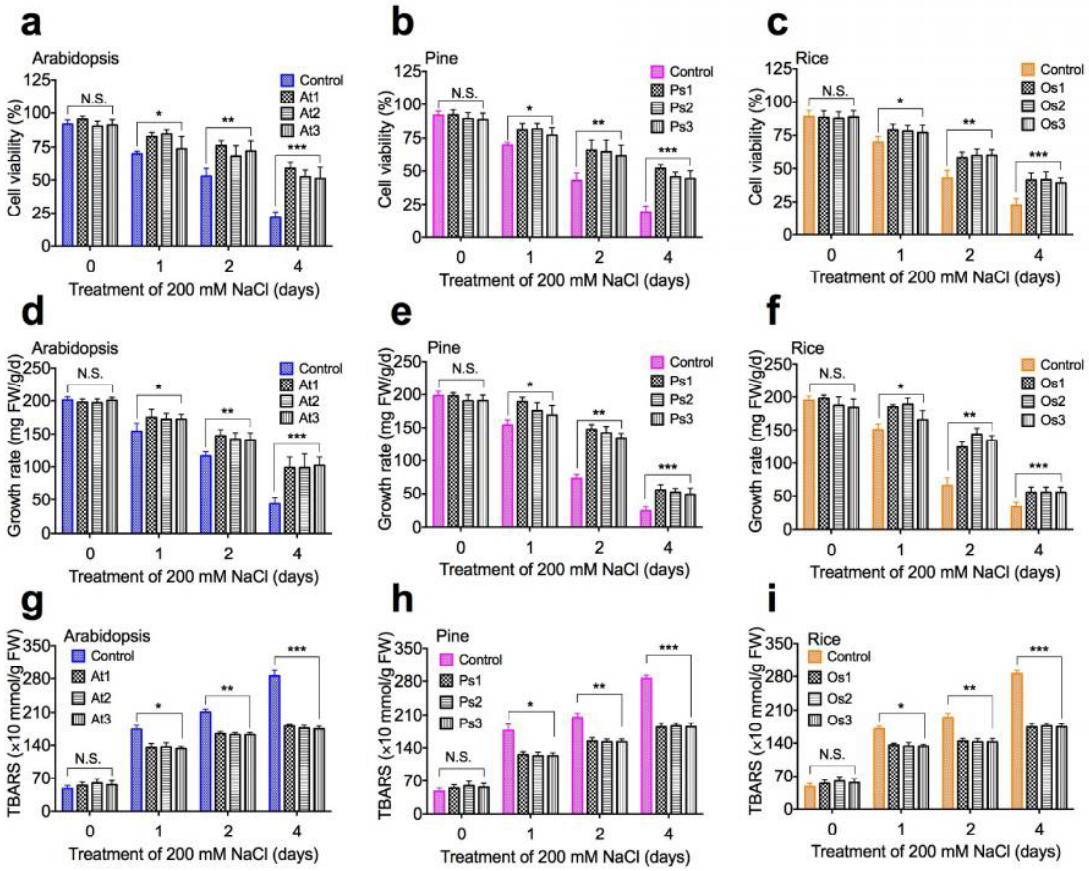


**Figure 2:** Cell viability, growth rate, and changes of thiobarbituric acid reactive substance (TBARS) of transgenic cell lines.

(**a-c**) Cell viability of transgenic Arabidopsis cell lines At1, At2, and At3 (**a**), pine cell lines Ps1, Ps2, and Ps3 (b), and rice cell lines Os1, Os2, and Os3 (**c**) under 200 mM NaCl treatment at different time (0 d, 1 d, 2 d, and 4 d). (**d-f**) Growth rate of transgenic Arabidopsis cell lines At1, At2, and At3 (d), pine cell lines Ps1, Ps2, and Ps3 (**e**), and rice cell lines Os1, Os2, and Os3 (**f**) under 200 mM NaCl treatment at different time (0 d, 1 d, 2 d, and 4 d). (**g-i**) TBARS levels of transgenic Arabidopsis cell lines At1, At2, and At3 (**g**), pine cell lines Ps1, Ps2, and Ps3 (**h**), and rice cell lines Os1, Os2, and Os3 (**i**) under 200 mM NaCl treatment at different time (0 d, 1 d, 2 d, and 4 d). The cell viability, growth rates, and TBARS were measured 3 days after treatment of 200 mM NaCl. Each experiment was replicated three times, and each replicate consisted of five to ten 250-ml flasks of transgenic cell cultures. Values represent the means ± S.D.

qPCR was used to examine the expression of *CTR1* (Figure **3a**), *EIN2* (Figure **3b**), *MPK11* (Figure **3c**), *EIN3* (Figure **3d**), *ERF1* (Figure **3e**), *BREB2A* (Figure **3f**), *NAC6* (Figure **3g**), *PDF1.2* (Figure **3h**), *WRKY13* (Figure **3i**), *bZIP23* (Figure **3j**), *ABI5* (Figure **3k**), and *LEA3* (Figure **3l**) in *ETR1* transgenic rice cell lines Os1, Os2, and Os3 and rice control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The levels of gene expression were measured 3 days after treatment of 200 mM NaCl. *ETR1* overexpression increased expression of *EIN2* (Figure **3b**) and *MPK11* (Figure **3c**) by 86-112% under 200 mM NaCl treatment at time point 4 d. *ETR1* overexpression increased expression of *CTR1* (Figure **3a**), *EIN3* (Figure **3d**), *ERF1* (Figure **3e**), *BREB2A* (Figure **3f**), *NAC61* (Figure **3g**), *PDF1.2* (Figure **3h**), *WRKY13* (Figure **3i**), *bZIP23* (Figure **3j**), *ABI5* (Figure **3k**), and *LEA3* (Figure **3l**) by 288-332% under 200 mM NaCl treatment at time point 4 d.

### Gene Expression in *ETR1* and *FUS3* Double Transgenic Rice Cell Lines

To investigate the effect of *FUS3* on *ETR1* gene-induced expression of defense response genes, *ETR1* and *FUS3* double transgenic *O. sativa* cell lines were produced. qPCR was used to examine the expression levels of *CTR1* (Figure **4a**), *EIN2* (Figure **4b**), *MPK11* (Figure **4c**), *EIN3* (Figure **4d**), *ERF1* (Figure **4e**), *BREB2A* (Figure **4f**), *NAC6* (Figure **4g**), *PDF1.2* (Figure **4h**), *WRKY13* (Figure **4i**), *bZIP23* (Figure **4j**), *ABI5* (Figure **4k**), and *LEA3* (Figure **4l**) in *ETR1* and *FUS3* double transgenic *O. sativa* cell lines Os1, Os2, and Os3 and *O. sativa* control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The

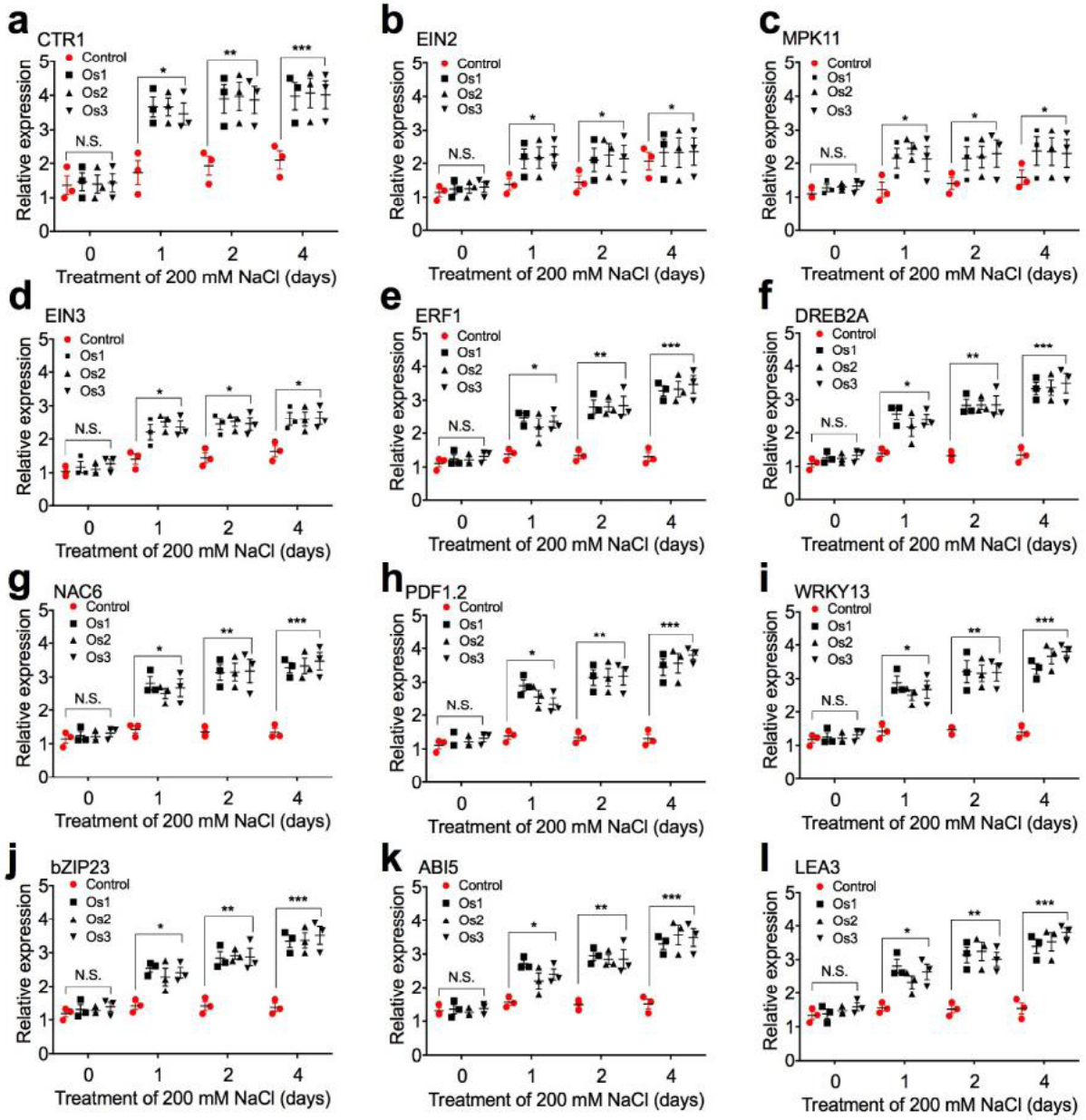


**Figure 3:** Gene expression in *ETR1* transgenic rice cell lines.

Expression levels of *CTR1* (**a**), *EIN2* (**b**), *MPK11* (**c**), *EIN3* (**d**), *ERF1* (**e**), *BREB2A* (**f**), *NAC6* (**g**), *PDF1.2* (**h**), *WRKY13* (**i**), *bZIP23* (**j**), *ABI5* (**k**), and *LEA3* (**l**) in ETR1 transgenic rice cell lines Os1, Os2, and Os3 and rice control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The levels of gene expression were measured 3 days after treatment of 200 mM NaCl. Each experiment was replicated three times, and each replicate consisted of three 250-ml flasks of transgenic cell cultures. Values represent the means ± S.D.

levels of gene expression were measured 3 days after treatment of 200 mM NaCl. The results showed that *FUS3* counteracts *ETR1* gene-induced expression of defense response genes *CTR1* (Figure **4a**), *EIN2* (Figure **4b**), *MPK* 11(Figure **4c**), *EIN3* (Figure **4d**), *ERF1* (Figure **4e**), *BREB2A* (Figure **4f**), *NAC6* (Figure **4g**), *PDF1.2* (Figure **4h**), *WRKY13* (Figure **4i**), *bZIP23* (Figure **4j**), *ABI5* (Figure **4k**), and *LEA3* (Figure **4l**).

### Gene Expression in *ETR1* and *SCF$^{TIR1}$* Double Transgenic Rice Cell Lines

To investigate the effect of *SCF$^{TIR1}$* on *ETR1* gene-induced expression of defense response genes, *ETR1* and *SCF$^{TIR1}$* double transgenic *O. sativa* cell lines were produced. qPCR was used to examine the expression levels of *CTR1* (Figure **5a**), *EIN2* (Figure **5b**), *MPK11* (Figure **5c**), *EIN3* (Figure **5d**), *ERF1* (Figure **5e**),

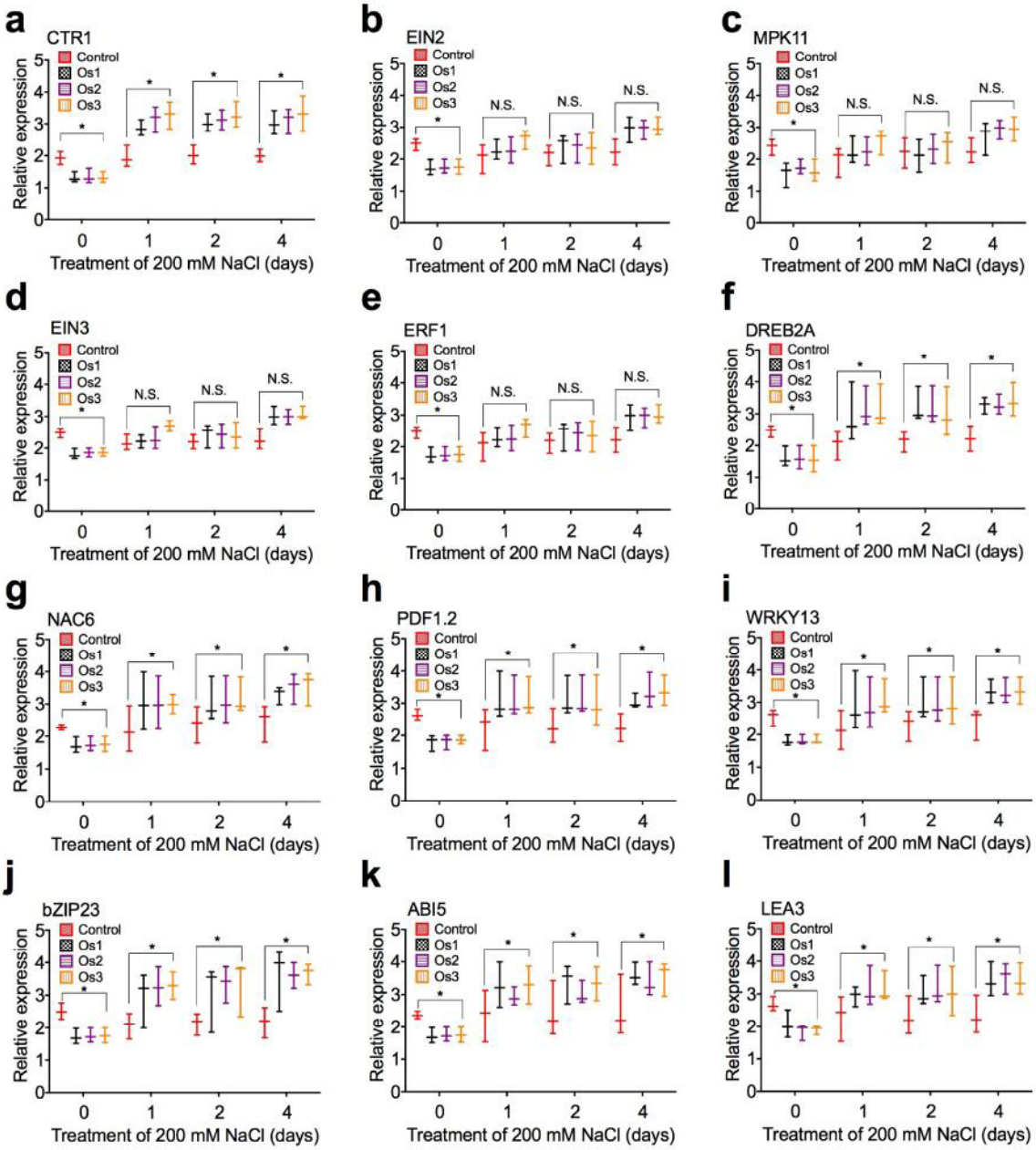


**Figure 4:** Gene expression in *ETR1* and *FUS3* double transgenic rice cell lines.

Expression levels of *CTR1* (**a**), *EIN2* (**b**), *MPK11* (**c**), *EIN3* (**d**), *ERF1* (**e**), *BREB2A* (**f**), *NAC6* (**g**), *PDF1.2* (**h**), *WRKY13* (**i**), *bZIP23* (**j**), *ABI5* (**k**), and *LEA3* (**l**) in ETR1 and FUS3 double transgenic rice cell lines Os1, Os2, and Os3 and rice control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The levels of gene expression were measured 3 days after treatment of 200 mM NaCl. Each experiment was replicated three times, and each replicate consisted of three 250-ml flasks of transgenic cell cultures. Values represent the means ± S.D.

*BREB2A* (Figure **5f**), *NAC6* (Figure **5g**), *PDF1.2* (Figure **5h**), *WRKY13* (Figure **5i**), *bZIP23* (Figure **5j**), *ABI5* (Figure **5k**), and *LEA3* (Figure **5l**) in *ETR1* and $SCF^{TIR1}$ double transgenic *O. sativa* cell lines Os1, Os2, and Os3 and *O. sativa* control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The levels of gene expression were measured 3 days after treatment of 200 mM NaCl. The results showed that $SCF^{TIR1}$ counteracts *ETR1* gene-induced expression of defense response genes *CTR1* (Figure **5a**), *EIN2* (Figure **5b**), *MPK11* (Figure **5c**), *EIN3* (Figure **5d**), *ERF1* (Figure **5e**), *BREB2A* (Figure **5f**), *NAC6* (Figure **5g**), *PDF1.2* (Figure **5h**), *WRKY13* (Figure **5i**), *bZIP23* (Figure **5j**), *ABI5* (Figure **5k**), and *LEA3* (Figure **5l**).

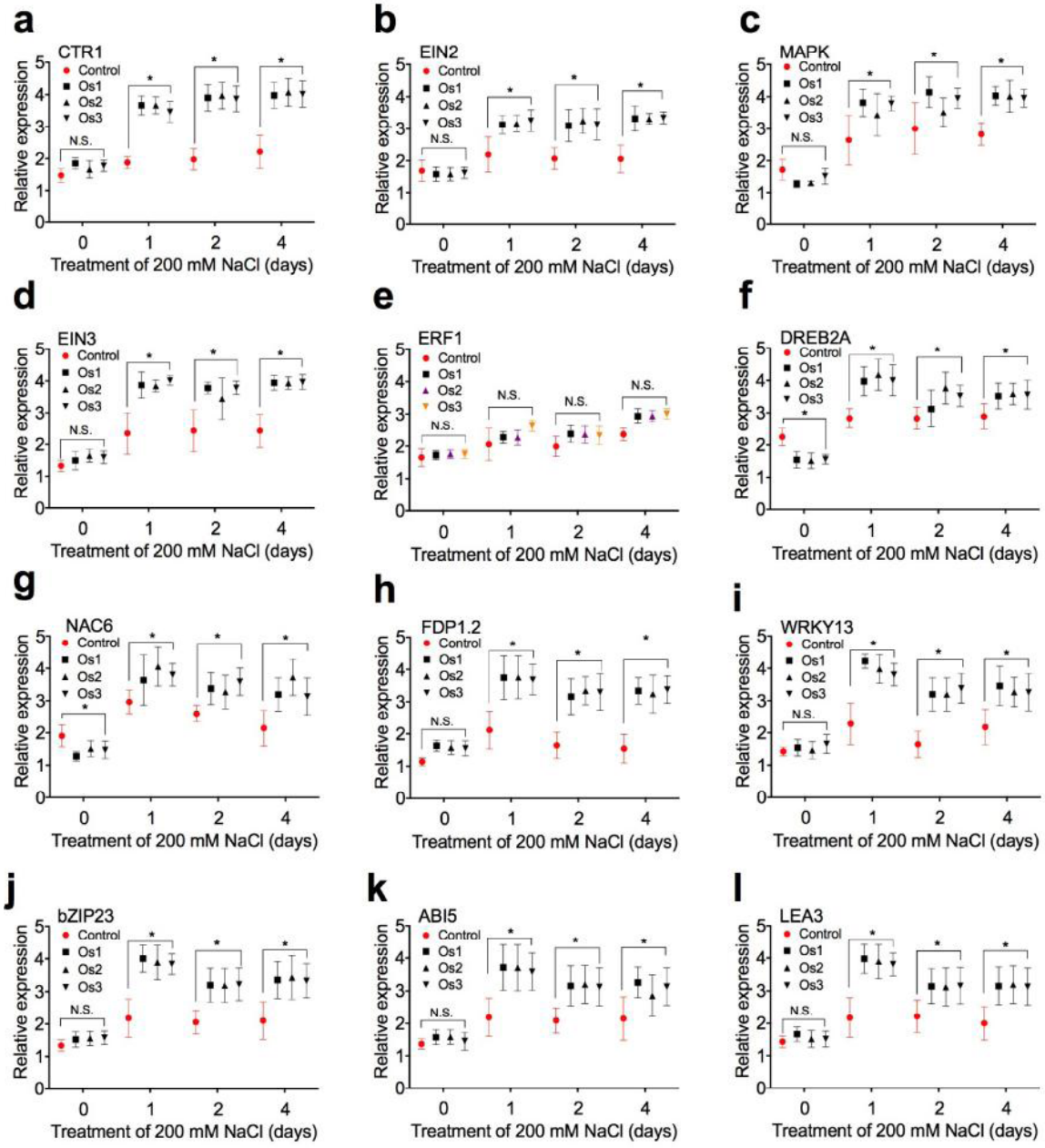


**Figure 5:** Gene expression in *ETR1* and *SCF*$^{TIR1}$ double transgenic rice cell lines.

Expression levels of *CTR1* (**a**), *EIN2* (**b**), *MPK11* (**c**), *EIN3* (**d**), *ERF1* (**e**), *BREB2A* (**f**), *NAC6* (**g**), *PDF1.2* (**h**), *WRKY13* (**i**), *bZIP23* (**j**), *ABI5* (**k**), and *LEA3* (**l**) in ETR1 and SCF$^{TIR1}$ double transgenic rice cell lines Os1, Os2, and Os3 and rice control cells under different time (0 d, 1 d, 2 d, and 4 d) of 200 mM NaCl treatment. The levels of gene expression were measured 3 days after treatment of 200 mM NaCl. Each experiment was replicated three times, and each replicate consisted of three 250-ml flasks of transgenic cell cultures. Values represent the means ± S.D.

### Morphological Changes of Transgenic Cell Lines

To examine the effect of *ETR1* overexpression on morphological changes of transgenic cell lines, cells images were taken for cell morphology of transgenic *A. thaliana* cell line At1 under treatment of 200 mM NaCl for 0 d (Figure **6a**), 1 d (Figure **6b**), 2 d (Figure **6c**), and 4 d (Figure **6d**). Cell morphology of transgenic *P. strobes* cell line Ps1 under treatment of 200 mM NaCl for 0 d (Figure **6e**), 1 d (Figure **6f**), 2 d (Figure **6g**), and 4 d (Figure **6h**) and cell morphology of transgenic *O. sativa* cell line Os1 under treatment of 200 mM NaCl for 0 d (Figure **6i**), 1 d (Figure **6j**), 2 d (Figure **6k**), and 4 d (Figure **6l**) were similar as cell morphology of transgenic *A. thaliana* cell line At1. Cell images were taken 3 days after treatment of 200 mM NaCl.

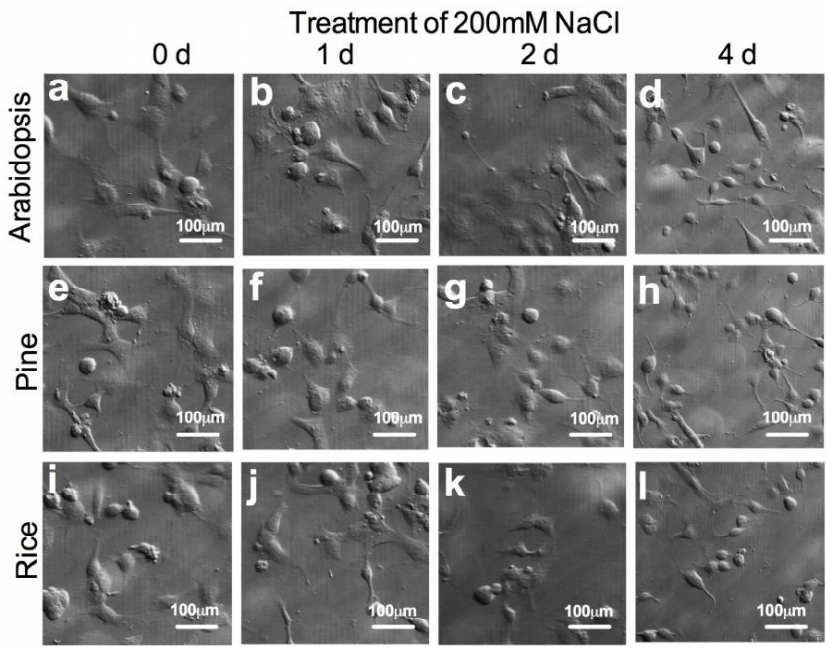


**Figure 6:** Morphological changes of transgenic cell lines.

(**a-d**) Cell morphology of transgenic Arabidopsis cell line At1 under treatment of 200 mM NaCl for 0 d (**a**), 1 d (**b**), 2 d (**c**), and 4 (**d**). (**e-h**) Cell morphology of transgenic pine cell line Ps1 under treatment of 200 mM NaCl for 0 d (**e**), 1 d (**f**), 2 d (**g**), and 4 d (**h**). (**i-l**) Cell morphology of transgenic rice cell line Os1 under treatment of 200 mM NaCl for 0 d (**i**), 1 d (**j**), 2 d (**k**), and 4 d (**l**). Cell images were taken 3 days after treatment of 200 mM NaCl.

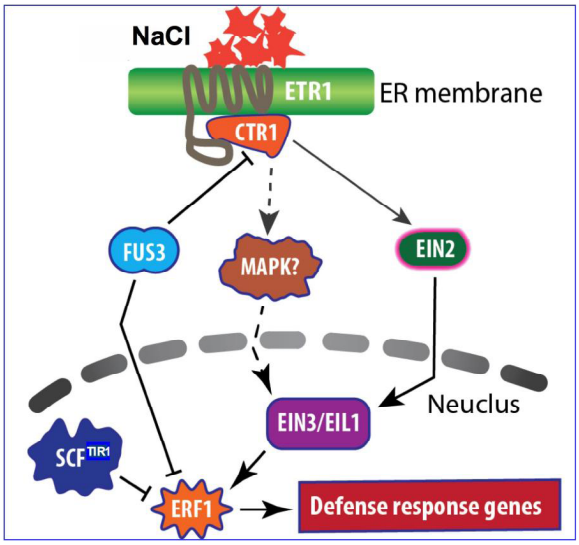


**Figure 7:** A mechanistic map of function of ETR1 over-expression in transgenic Arabidopsis, pine, and rice cell lines under NaCl treatment.

Overexpression of *ETR1* enhances NaCl stress tolerance through increasing expression of deference response genes. *FUS2* and $SCF^{TIR1}$ over-expression counteracts *ETR1* over expression-enhanced NaCl stress tolerance through inhibiting expression of deference response genes.

## Function of *ETR1* Over-expression in Transgenic Arabidopsis, Pine, and Rice Cell lines under NaCl Treatment

Overexpression of *ETR1* enhances NaCl stress tolerance through increasing expression of deference response genes including *BREB2A*, *NAC6*, *PDF1.2*, *WRKY13*, *bZIP23*, *ABI5*, and *LEa3* (Figures **4** and **5**). *FUS2* and $SCF^{TIR1}$ over-expression counteracts ETR1 overexpression-enhanced NaCl stress tolerance Through inhibiting expression of deference response genes.

## DISCUSSION

The receptors of the simple gas ethylene modulate some of the physiological processes in the growth, development, and stress response of higher plants. How plants perceive ethylene and how this signal is transduced are not fully understood. The ETR1 gene encodes a homolog of two-component regulators that are known almost exclusively in prokaryotes. The discovery of *A. thaliana* genes encoding proteins related to ETR1 suggests that the failure to recover recessive etr1 mutant alleles may be due to the presence of redundant genes [3, 7, 12, 14, 52-56]. In this investigation, the effect of the *ETR1* gene on NaCl stress tolerance, three-plant species *A. thaliana*, *P. strobes*, and *O. sativa* were used to generate *ETR1* transgenic cell lines using *Agrobacterium tumefaciens* strain GV3101-mediated genetic transformation (Figure **1a**). After confirmation of transgenic cells by PCR, Southern blotting, and northern blotting analysis, three transgenic cell lines from each of *A. thaliana* (At1, At2, and At3), *P. strobes* (Ps1, Ps2, and Ps3), and *O. sativa* (Os1, Os2, and Os3) overexpressing *ETR1* were selected to examine the cell viability, growth rate, and changes of thiobarbituric acid reactive substance (TBARS). Nine transgenic cell lines At1, At2, At3, Ps1, Ps2, Ps3, Os1, Os2, and Os3 were treated with 200 mM NaCl for different time points (0 d, 1 d, 2 d, and 4 d). Compared to the controls, cell viability of transgenic *A. thaliana*, *P. strobes*, and *O. sativa* (Figure **2a**-**c**) increased during the time course of 200 mM NaCl treatment. Transgenic cells overexpressing *ETR1* increased cell viability 116% (*O. sativa*), 228% (*P. strobes*), and 236% (*A. thaliana*) at the time point 4 d of 200 mM NaCl treatment. Growth rate of transgenic *A. thaliana*, *P. strobes*, and *O. sativa* (Figure **2d**-**f**) increased 118%-249% under 200 mM NaCl treatment for 4 d. These results demonstrated that overexpression of the *ETR1* gene enhanced NaCl stress tolerance in *A. thaliana*, *P. strobes*, and *O. sativa*.

Expression of the ethylene receptors in plants could be regulated by abiotic factors. Exposure of *A. thaliana* plants to NaCl reduced expression of the ethylene receptor ETR1. Immunoblot analysis indicated a general effect of osmotic stress upon the expression level of ETR1 and suggested that plant responses to abiotic stress are modulated by changes in the expression level of ethylene receptors [29-37]. Expression of the ethylene receptors in plant cells is also related to drought stress tolerance. In *A. thaliana*, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) delays stomatal closure by inhibiting the ABA signaling pathway [38-41]. In this study, we identified that TBARS levels of transgenic *A. thaliana* cell lines At1, At2, and At3 (Figure **2g**), *P. strobes* cell lines Ps1, Ps2, and Ps3 (Figure **2h**), and *O. sativa* cell lines Os1, Os2, and Os3 (Figure **2i**) decreased by 108% (*P. strobes*), 116% (*A. thaliana*), and 128% (*O. sativa*) under 200 mM NaCl treatment at time point 4 d. TBARS levels decreased by overexpressing *ETR1* may enhance NaCl stress tolerance in *A. thaliana*, *P. strobes*, and *O. sativa*.

It has been reported that the receptors of ethylene negatively regulate plant responses to environmental stress in *A. thaliana* [32, 46]. Freezing tolerance was decreased in ethylene overproducer1 and by the application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid but increased by the addition of the ethylene biosynthesis inhibitor aminoethoxyvinyl glycine [32, 46]. In addition, ethylene-insensitive mutants, including etr1-1, ein4-1, ein2-5, ein3-1, and ein3 eil1, displayed enhanced freezing tolerance. Genetic and biochemical analyses demonstrated that the type-A *A. thaliana* response regulators (ARRs) function as key nodes to integrate ethylene and cytokinin signaling in regulation of plant responses to environmental stress [32, 39, 42-49]. It has been reported that the *A. thaliana* FUSCA3 (FUS3) gene results in various abnormalities during the process of embryogenesis by controlling accumulation of chlorophyll and the sensitivity to abscisic acid [57]. The function of FUS3 was normally activated during late embryo development. Results from differential display indicate that FUS3 negatively regulate a particular set of genes during late embryo development [58, 66]. We examined the effect of FUS3 on ETR1-related NaCl stress tolerance in this study. Overexpression of *FUS3* counteracts *ETR1* enhanced expression of defense response genes under NaCl stress.

**T**he E3 ubiquitin-ligase Skip-Cullin-F-box complex (SCF) plays important role in ETR1 signaling transduction. **T**he function of the SCF complex is related to hormones. Salicylic acid and jasmonic acid are the defense hormones that play an essential role in the regulation of the plant immune signaling network. Suppression of the jasmonic acid pathway by salicylic acid functions downstream of the E3 ubiquitin-ligase Skip-Cullin-F-box complex. Functional study revealed that the 1-kb promoter regions of jasmonic acid-responsive genes are suppressed by salicylic acid. Salicylic acid is enriched in the jasmonic acid-responsive GCC-box motifs, indicating that the salicylic acid pathway inhibits jasmonic acid signaling

downstream of the E3 ubiquitin-ligase Skip-Cullin-F-box complex by targeting GCC-box motifs in jasmonic acid-responsive promoters [67]. In this investigation, we showed that overexpression of $SCF^{TIR1}$ reduces *ETR1* enhanced expression of defense response genes under NaCl stress.

## CONCLUSION

Overexpressing *ETR1* enhances NaCl stress tolerance by increasing cell viability and growth rate and decreasing the level of thiobarbituric acid reactive substance. Transgenic cell lines overexpression of *ETR1* enhance tolerance to NaCl stress by regulating expression of a set of defense response genes including of *CTR1, EIN2, MPK11, EIN3, ERF1, BREB2A, NAC6, PDF1.2, WRKY13, bZIP23, ABI5*, and *LEA3*. In rice cells, overexpression of *FUS3* counteracts *ETR1* enhanced expression of defense response genes under NaCl stress, and overexpression of $SCF^{TIR1}$ reduces *ETR1* enhanced expression of defense response genes under NaCl stress. These results suggest that overexpression of *ETR1* enhanced NaCl stress tolerance of transgenic plant cells by regulating expression of defense response genes.

## ACKNOWLEDGMENTS

The author appreciates Nicole Moskvin, Kuribayashi Bloom, and Ambrosia Nishimura for their help in maintaining transgenic cell cultures. The authors are grateful to Dr. Thompson for their critical reading of the manuscript. This work was supported by a grant from the Education Committee of Hubei Providence of China (Grant No. D20101306).

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Received on 09-09-2018 Accepted on 16-10-2018 Published on 31-12-2018

DOI: https://doi.org/10.12974/2311-858X.2018.06.01.6