Transcriptional Modulation of Ethylene Response Factor Protein JERF3 in the Oxidative Stress Response Enhances Tolerance of Tobacco Seedlings to Salt, Drought, and Freezing

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Abiotic stresses such as drought, cold, and salinity affect normal growth and development in plants. The production and accumulation of reactive oxygen species (ROS) cause oxidative stress under these abiotic conditions. Recent research has elucidated the significant role of ethylene response factor (ERF) proteins in plant adaptation to abiotic stresses. Our earlier functional analysis of an ERF protein, JERF3, indicated that JERF3-expressing tobacco (Nicotiana tabacum) adapts better to salinity in vitro. This article extends that study by showing that transcriptional regulation of JERF3 in the oxidative stress response modulates the increased tolerance to abiotic stresses. First, we confirm that JERF3-expressing tobacco enhances adaptation to drought, freezing, and osmotic stress during germination and seedling development. Then we demonstrate that JERF3-expressing tobacco imparts not only higher expression of osmotic stress genes compared to wild-type tobacco, but also the activation of photosynthetic carbon assimilation/metabolism and oxidative genes. More importantly, this regulation of the expression of oxidative genes subsequently enhances the activities of superoxide dismutase but reduces the content of ROS in tobacco under drought, cold, salt, and abscisic acid treatments. This indicates that JERF3 also modulates the abiotic stress response via the regulation of the oxidative stress response. Further assays indicate that JERF3 activates the expression of reporter genes driven by the osmotic-responsive GCC box, DRE, and CE1 and by oxidative-responsive as-1 in transient assays, suggesting the transcriptional activation of JERF3 in the expression of genes involved in response to oxidative and osmotic stress. Our results therefore establish that JERF3 activates the expression of such genes through transcription, resulting in decreased accumulation of ROS and, in turn, enhanced adaptation to drought, freezing, and salt in tobacco.

Regulation of transcription has proved to be a vital aspect of the complex genetic and biochemical networks involved in plant responses to stresses. Understanding the interaction between stress-responsive transcription factors and their corresponding cis-acting elements in promoters of the downstream target genes is a prerequisite to dissecting the transcriptional regulatory network. For instance, investigation of the promoters of COR genes in Arabidopsis (Arabidopsis thaliana) indicated that they are regulated in an abscisic acid (ABA)-dependent or ABA-independent pathway under conditions of cold, dehydration, and high salinity (Liu et al., 1998; Yamaguchi-Shinozaki and Shinozaki, 2006). The subfamily of ethylene response factor (ERF) proteins belongs to the ERF/APETALA2 (AP2) superfamily, which contains one highly conserved AP2 or ERF DNA-binding domain (Kizis et al., 2001; Liu et al., 2006). ERF proteins were first isolated from tobacco (Nicotiana tabacum) as GCC-box binding proteins (Ohme-Takagi and Shinshi, 1995), and consequent investigations indicate that many ERF proteins found in other plants enhance resistance to pathogens by activating the expression of pathogenesis-related genes (Guo et al., 2002; Zhang et al., 2004). Evidence accumulated over the years has proved that ERF proteins bind not only to the GCC box but also to DRE/CRT, CE1, JERE, and CT-rich elements (Liu et al., 1998; Menke et al., 1999; Niu et al., 2002; Xue and Loveridge, 2004; Tang et al., 2005), thereby enhancing plant tolerance to multiple stresses, which suggests...
that ERF proteins are part of the plant’s response to such stresses.

Accumulated evidence indicates that stress-stimulated physiological imbalance increases the level of reactive oxygen species (ROS) in plant cells. The steady-state levels of hydrogen peroxide (H$_2$O$_2$), singlet oxygen, the superoxide anion (O$_2^-$), and the hydroxyl radical depend on the balance between generation and removal, which is facilitated by the ROS-scavenging system of the cell (Bartoli et al., 2004; Gechev et al., 2006). In such a system, H$_2$O$_2$ plays a very important role in mediating signal transduction in response to stress in plant cells. H$_2$O$_2$ diffuses rapidly from its site of synthesis within subcellular microdomains, depending on its concentration, and can transmit intracellular signals to the nucleus by oxidizing various upstream components of the signaling pathway, including transcription factors that ultimately result in changes in gene expression (Mittler, 2002; Gadjev et al., 2006). Further, the expression of the ERF gene CaPF1 in Virginia pine (Pinus virginiana) enhances the level of antioxidant enzyme activities of ascorbate peroxidase, glutathione reductase, and superoxide dismutase (SOD). This results in the improved tolerance to stress (Tang et al., 2005), indicating that ERF proteins might be associated with the regulation of ROS pathway. Although many ERF proteins have been identified in the response of plants to abiotic stress, the details of involvement of an ROS-mediated regulatory module are yet to be established. An ERF protein, JERF3, was isolated from tomato (Solanum lycopersicum) using a yeast one-hybrid screen in our laboratory. It has been shown that JERF3 could be induced by ethylene, jasmonic acid, cold, salinity, and ABA, and that JERF3-expressing tobacco was better adapted to salinity in vitro (Wang et al., 2004); however, the molecular mechanism of that regulatory response remained to be elucidated. In this article, we now report that JERF3 transcriptionally regulates the expression of genes involved in plant responses to osmotic- and oxidative-related stresses; this results in decreased accumulation of ROS, thereby enhancing the adaptation to drought, salt, and freezing in tobacco.

RESULTS

Expression of JERF3 in Tobacco Increases Seed Germination and Root Elongation under Salt and Osmotic Stress, and Tolerance to Drought and Freezing in Seedling Stage

Salinity, drought, and low temperature result in osmotic stress. Our earlier experiments showed that after NaCl treatment, leaf discs from JERF3-over-expressing tobacco (OE) seedlings remained green, whereas those from wild type (wild-type tobacco) were bleached, indicating that JERF3 enhances tolerance to salt in vitro (Wang et al., 2004). This evidence prompted us to test whether JERF3 regulates tolerance to osmotic stress. Previously, we obtained nine independent OE lines, of which four were confirmed to contain one-copy insertions (Wang et al., 2004), and the other five lines two- to three-copy insertions. Three one-copy insertions (T$_3$ generation, OE1, OE3, and OE9) and two two-copy insertions (T$_2$ generation, OE4 and OE24) display distinctive expression of JERF3 (Supplemental Fig. S1A), yet show similar phenotypes during seedling (the controls of Figs. 1 and 2), mature (Supplemental Fig. S1B), and flowering developmental stages (Supplemental Fig. S1C). These were used in the following experiments. To exclude differences in seed germination between the wild-type and OE lines, we first checked the germination in water as control. Our results indicated that wild-type and OE lines showed an equal germination. Then we simulated osmotic stress by exposing wild-type and OE lines to NaCl and mannitol and studied the effects on germination and root elongation. As can be seen in Figure 1A, B, and C, JERF3 overexpression resulted in increased germination and root elongation under salt and osmotic stress, and enhanced tolerance to drought and freezing in seedling stage.
1A, germination in OE lines was clearly higher than that in wild type. After 0.15 M NaCl treatment for 3 d, seed germination in wild type was about 10%, compared to over 40% in OE lines. Similarly, the germination percentage in OE lines was twice that in wild type after 0.2 mM mannitol treatment for 4 d (Fig. 1B). After 0.15 M NaCl treatment for 14 d, the seedling roots in the five independent OE lines were about twice as long than those in wild type (Fig. 1C). Interestingly, when subjected to higher concentrations of 0.2 mM mannitol for the same duration, roots of five independent OE lines were not only about 34% longer than those of wild type but also showed more lateral roots (Fig. 1C). Student's t tests indicate that the above data of seed germination or root elongation are not significantly different among the five independent OE lines at 95% probability, indicating that the distinctive transcript level of JERF3 (Supplemental Fig. S1A) did not correlate with the degree of tolerance in OE lines. However, there are differences (significant at 95% probability) in the seed germination or root elongation between OE lines and wild type. These results indicate that JERF3 increases the adaptation to salt and osmotic stress during seed germination and root elongation in tobacco.

Next we tested the response of wild type and three one-copy insertion OE seedlings to freezing. Initially, we exposed seedlings to −2°C in the growth chamber but found no clear difference between OE lines and wild type. We then set the temperature to −2°C. As can be seen in Figure 2C, wild-type seedlings were injured after a 3-h exposure to −2°C but OE seedlings were not obviously affected. More interestingly, leakage of ions from wild-type seedlings after the treatment was 30% but that from OE seedlings was only 10% (Fig. 2D), which is consistent with the report that plasma membranes of plants are injured because of exposure to cold, resulting in leakage of ions from the cytoplasm (Gonzalez-Aguilar et al., 2000). These results suggest that JERF3 makes tobacco seedlings more tolerant to freezing by increasing the stability of the plasma membrane.

Expression of JERF3 in Tobacco Significantly Activates the Expression of Osmotic- and Oxidative-Related Genes

To investigate how JERF3 modulates plant response to drought, cold, and salt stresses, we compared the expression profiles in two independent OE seedlings with that in wild-type plants using cDNA-amplified fragment length polymorphism (AFLP; Vos et al., 1995). Using 240 primer pairs, we obtained 2,742 fragments, of which 526 were differentially expressed between OE lines and wild type (changes more than
2-fold). Compared to the transcript levels in wild type, there were 277 up-regulated genes and 249 down-regulated genes in OE lines, and approximately 23% of the up-regulated genes are believed to be involved in response to osmotic, photosynthetic carbon assimilation/metabolism, and oxidative stress.

Among the up-regulated genes, we first confirmed the expression of four genes related to osmotic stress in five independent OE lines and wild type using quantitative real-time PCR (qPCR) amplifications, namely NtSAM1, TOBLTP, NtERD10C, and NtSPS. It is known that NtSAM1 encodes S-adenosyl-l-Met synthetase, which was reported to be inducible by ABA, mannitol, and NaCl (Espartero et al., 1994); TOBLTP encodes lipid transfer protein, which is inducible by ABA and drought (Masuta et al., 1992; Torres-Schumann et al., 1992; Trevino and O’Connell, 1998); NtERD10C is induced by cold and drought (Kasuga et al., 2004); and NtSPS encodes Suc-P synthase (Börnke, 2005), which was responsive to cold (Supplemental Fig. S2). The expression of the above four genes in OE seedlings was enhanced 3 to approximately 5 times that in wild type (Fig. 3A), suggesting that JERF3 constitutively activates the expression of genes responding to osmotic stress in tobacco.

Next we analyzed the expression of four genes involved in photosynthetic carbon assimilation/metabolism in OE seedling. It is established that NtRub-SS encodes Rubisco small subunit (Mazur and Chui 1985); ChlGaPA encodes glyceraldehyde-3-P dehydrogenase A-subunit precursor; CyGAP encodes glyceraldehyde-3-P dehydrogenase; and NtRCA342 encodes Rubisco activase precursor (Shih et al., 1986). Our results showed that the expression of these genes except ChlGaPA was increased 3 to approximately 6 times that in wild type (Fig. 3B), indicating that JERF3 confers the expression of genes in the response to photosynthetic carbon assimilation/metabolism in tobacco.

Also we analyzed the expression of seven genes involved in the response to oxidative stress in OE seedling. It has been reported that NtCA encodes carbonic anhydrase, which displays antioxidant activity and functions in the hypersensitive defense response (Slaymaker et al., 2002); NtSOD encodes SOD, which was reported to enhance oxidative stress tolerance in tobacco (Slooten et al., 1995); NtRbohD, a tobacco respiratory burst oxidase homolog, encodes an enzyme similar to the mammalian NADPH oxidase, producing active oxygen species in elicited tobacco cells (Simon-Plas et al., 2002; Morel et al., 2004). And the genes NtCAT1, NtAPX1, NtAPX2, and NtGPX encode catalase, cytosolic ascorbate peroxidase, chloroplastic ascorbate peroxidase, and glutathione peroxidase, respectively, which all are considered to use H₂O₂ as an electron acceptor to catalyze a number of oxidative reactions (Orvar and Ellis, 1995; Pasqualini et al., 2007). Our results confirmed, with the exception of NtCAT1 and NtRbohD, that the expression of these up-regulated genes was increased 3 to approximately 8 times above controls (Fig. 3C). Moreover, the expression of down-regulated genes was consistent with the results of cDNA-AFLP (data not shown). Therefore JERF3 regulates the expression of photosynthetic carbon assimilation/metabolism and oxidative genes as well.

Expression of JERF3 in Tomato Is Responsive to Methyl Viologen But Not H₂O₂ and 3-Aminotriazole

The finding that JERF3 regulates the expression of photosynthetic carbon assimilation/metabolism and
oxidative genes indicates that ROS might be related to the enhanced tolerance to drought, salt, and freezing. The possible involvement of ERF factors in ROS transcriptional regulatory networks that govern plant stress responses has been analyzed in previous studies (Mittler, 2002; Gadjev et al., 2006), although detailed experimental data were not provided. To prove the involvement of ERF gene JERF3 in ROS transcriptional regulation we tested the response of JERF3 in its original source of tomato plants by treatment with 

\[ \text{H}_2\text{O}_2 \] or ROS-generating agents such as methyl viologen (MV) and 3-aminotriazole. qPCR results indicated that MV induced the expression of \( \text{JERF3} \) and 3-aminotriazole. qPCR results indicated that MV induced the expression of \( \text{JERF3} \) in tomato seedlings (Fig. 4), but 3-aminotriazole and \( \text{H}_2\text{O}_2 \) did not (data not shown), indicating that JERF3 might be associated with the ROS response.

**Expression of JERF3 in Tobacco Increases the Activities of SOD But Decreases the Accumulation of ROS**

The data so far indicate that plants accumulate osmotic solutes to regulate osmotic potential under drought, salt, and cold stress (Gilmour et al., 2000). To test whether the expression of JERF3 modulates the accumulation of osmotic solutes under drought, salt, and cold stresses, we checked the Pro content of wild-type and OE seedlings. As anticipated, the contents of osmotic solutes in OE seedlings were approximately 9% higher than those in wild-type seedlings under normal growth conditions, but the differences were not clearly correlated to the stresses of drought, salt, or cold (data not shown). This might suggest that osmotic regulation is not the exclusive regulatory pathway of JERF3 in adaptation to drought, salt, and cold in tobacco. In this case, there must be an alternative regulatory pathway involving JERF3 in the plant’s response to abiotic stress.

The fact that JERF3 increases the expression of genes related to oxidative stress suggests that JERF3 might modulate the levels of ROS. To evaluate the important ROS-scavenging pathways of SOD in cellular compartments, and the possible regulation of individual SOD genes at the transcriptional level, we further measured the activities of SOD in the oxidative response. Our results showed that the SOD activities were 5% to approximately 8% higher in OE seedlings than that in wild type under normal growth conditions, consistent with the significant expression of \( \text{NiSOD} \) in OE seedlings. SOD activities increased 160% to approximately 210% and 7% to approximately 12% in OE and wild-type seedlings, respectively, compared to that in wild type of control, after dehydration, cold, salt, and ABA treatments (Fig. 5A). Student’s \( t \) tests indicate that the significant difference of SOD activities cannot be seen between the two independent OE lines, except between OE lines and wild type at 95% probability, indicating that JERF3 gives rise to the increase of SOD activities in tobacco under abiotic stresses and ABA treatment.

Because \( \text{H}_2\text{O}_2 \), singlet oxygen, \( \text{O}_2 \), and the hydroxyl radical all belong to ROS (Bartoli et al., 2004; Gechev et al., 2006), we then checked the accumulation of \( \text{O}_2 \) in OE and wild-type seedlings under dehydration, cold, salt, and ABA treatments. Under normal growth conditions, \( \text{O}_2 \) accumulated at very low levels and showed no obvious difference between wild-type and OE seedlings. This reduced ROS was not detectable in OE seedlings, while wild type still can be observed along the main vein under normal growth conditions (Fig. 5B). After exposure to dehydration, cold, salt, or ABA treatments, \( \text{O}_2 \) accumulated significantly greater quantities in wild-type plants whereas its levels in OE seedlings were lower (Fig. 5B), suggesting that JERF3 decreases the accumulation of ROS under dehydration, cold, salt, and ABA treatments.

**JERF3 Transcriptionally Regulates Gene Expression through Interacting with Multiple Stress-Responsive cis-Acting Elements**

Our results indicate that JERF3 enhances tolerance to drought, cold, and osmotic stress. Moreover, it has been reported that ERF proteins interact with several elements such as GCC box (Ohme-Takagi and Shinshi, 1995), DRE (Narusaka et al., 2003), and CE1 (Niu et al., 2002) that are involved in plant response to stress. However, no ERF proteins were found to interact with as-1, which is related to oxidative stress and binds to bZIP transcription factors (Despres et al., 2003; Wurmuth et al., 2007). Wang et al. (2004) established that JERF3 could bind to GCC box and DRE in vitro. In this work, GUS reporter vectors driven by GCC, DRE, CE1, or as-1 were constructed (see details in “Materials and Methods”) and *Agrobacterium* harboring these reporter vectors was infiltrated into the leaves of OE lines and wild type. The results showed that GUS activity in OE lines was about 19 to 21, 9 to 15, 6 to 8, and 7 times that in wild type when driven by GCC box, DRE, CE1, or as-1, respectively (Fig. 6). This result demonstrates that

![Figure 4](image-url) Expression of JERF3 in tomato is inducible by MV. The expression of JERF3 in tomato was detected by qPCR. The actin transcripts were used as internal control, and the expression level of JERF3 was standardized as 100 at time 0. The assay was repeated three times. The bars represent ±SE.
JERF3 may not only modulate with cis-acting elements involved in response to osmotic stress, but also with the elements involved in oxidative stress, thereby supporting the possibly transcriptional regulation of JERF3 in plant response to oxidative stress.

To further clarify the direct regulation of JERF3 in the expression of photosynthetic carbon assimilation/metabolism and oxidative genes, we cloned the promoters of Rubisco small subunit gene (the promoter sequence is reported to be 1,041 bp in length; Mazur and Chui 1985), NtCA (1,096 bp), and NtSOD (807 bp), from tobacco genomic DNA (NC89) using direct PCR amplification and thermal asymmetric interlaced (TAIL)-PCR, respectively (Liu et al., 1995). After analyzing the promoters using PLACE software, we found that there is a GCC box (AGCCGCC, 2639 to 2645) in the promoter of NtSOD, but no known cis-element that JERF3 interacts with in the promoters of NtCA and Rubisco small subunit gene. Then the promoters of the Rubisco small subunit gene, NtSOD and NtCA, were inserted into pCAMBIA1381Z upstream of GUS to test their possible interaction with JERF3. Our results indicate that JERF3 does not interact with the promoter of the Rubisco small subunit gene, indicating upstream regulation of JERF3 in the expression of this gene. However, JERF3 modulates the expression of GUS gene driven by the full-length promoter of NtSOD (−1 to −807 bp), but JERF3 does not interact with the promoter of NtSOD in which the GCC-box area (−625 to −658) was deleted (Fig. 7A), indicating that JERF3 activates the expression of reporter genes through interacting with the GCC box of NtSOD promoter. Previously, we proved the physical binding of JERF3 to GCC box (Wang et al., 2004), thus the direct transcriptional activation of JERF3 on the expression of oxidative stress gene NtSOD might contribute to the production of NtSOD activities in tobacco.

Interestingly, JERF3 also interacts with the full-length promoter (−1 to −1,096 bp) of NtCA (NtCA-p1). Concomitant with the deletion of the promoter, GUS activity weakened but not markedly until NtCA-p7 (−1 to −617 bp; Fig. 7B), suggesting that the deleted sequence between NtCA-p6 and NtCA-p7 might interact with JERF3. In fact, removal of the fragment in the promoter of NtCA (NtCA-39 del) significantly reduced the expression of the reporter gene (Fig. 7B). Therefore our results demonstrate that JERF3 might interact with a novel sequence of 39 bp (−617 to −657 bp, ATCTGT-GATCAGCAATAATTGTTGATTTGGAATT) located on the promoter of NtCA to activate gene expression in this manner.

**DISCUSSION**

It has been documented that the stress-induced increase of ROS in plant cells results from an imbalance between generation and removal. The production and accumulation of ROS cause oxidative stress under these abiotic stresses. Our earlier experiments (Wang et al., 2004) and this research with JERF3 have indicated that transcriptional regulation of JERF3 modulates the increased tolerance to drought, salt, and freezing in tobacco during germination and seedling development. Such regulation is not only through
activating the expression of osmotic stress genes, but also through the activation of photosynthetic carbon assimilation/metabolism and oxidative genes. This establishes that the ERF protein JERF3 is involved in a ROS-mediated regulatory module in transcriptional networks that govern plant response to stress.

Chloroplast and mitochondria are the major sites of generation of ROS (Asada, 2006; Wormuth et al., 2007). The generation and removal of ROS is a homeostatic process during plant growth and development under normal conditions. However, this homeostasis is destroyed when plants are exposed to drought, cold, and salinity (Apel and Hirt, 2004; Bartoli et al., 2004). Cellular antioxidants, including enzymatic and non-enzymatic antioxidants, are the key to protecting the cells against damage due to ROS. Among enzymatic antioxidants, SOD plays a pivotal role in clearing away ROS by catalyzing the highly damaging O$_2^-$ into H$_2$O$_2$ (Apel and Hirt, 2004). The results we obtained are consistent with the view that expressing JERF3 in tobacco greatly increases the activities of SOD, resulting in the reduced accumulation of O$_2^-$ under drought, cold, salt, and ABA treatments. To better understand the involvement of JERF3 in ROS transcriptional regulatory network, we found that the expression of JERF3 in its original tomato seedlings was inducible by the ROS-generating agent MV, suggesting that JERF3 might be associated with ROS responses. The enhanced expression of glutathione peroxidase NtGPX and cytosolic and chloroplastic ascorbate peroxidases (NtAPX1 and NtAPX2) in unstressed OE seedlings further supports the idea that JERF3 might transcriptionally regulate the expression of genes related to oxidative reactions. Furthermore, Rubisco, a key enzyme in photosynthesis, catalyzes ribulose-1,5-bisphosphate and assimilates CO$_2$ and also catalyzes the oxygenase reaction of ribulose-1,5-bisphosphate into glycolate, which is metabolized in peroxisomes (Ruuska et al., 2000; Marcus et al., 2005). This process helps scavenge ROS (Apel and Hirt, 2004). Carbonic anhydrase is important to transport because it catalyzes the reversible hydration of CO$_2$ (Smith and Ferry, 2000) and has antioxidant activity (Slaymaker et al., 2002). In our earlier (Wang et al., 2004) and present research, we found that the ERF protein JERF3 significantly enhances tolerance to drought, freezing, and salinity in tobacco. Further research indicated that expression of photosynthetic carbon assimilation/metabolism and oxidative-related genes is up-regulated in JERF3-expressing tobacco, suggesting that JERF3 regulates photosynthesis and eliminates ROS. More importantly, we find that expressing JERF3 in tobacco activates the expression of NtSOD by interacting with the GCC box in the promoter of NtSOD, and NtCa via interaction with a 39-bp novel element, indicating that JERF3 regulates plant response to abiotic stresses mainly by modulating the expression of ROS-related genes.

After being detected by a receptor, ROS continues transferring the signal through two main pathways. In one, a group of transcription factors participating in different cellular pathways is activated by a mitogen-activated protein kinase cascade (Pitzschke and Hirt, 2006). In the other, the level of cellular calcium is changed after a receptor detects ROS and the pathway of calcium/calmodulin is activated (Mittler et al., 2004). For example, ROS may activate the Ca$^{2+}$-permeable channels in plant membranes and induce stomatal closure by increasing the level of cellular calcium (Mori and Schroeder, 2004), which is further modulated by ABA (Mustilli et al., 2002; Apel and Hirt, 2004). The stability of the plasma membrane under cold conditions in JERF3-expressing tobacco indicates that regulation of JERF3 by oxidative stress might occur via a similar ROS-regulatory pathway. The involvement of JERF3 in ROS is also supported by the fact that JERF3 interacts with the ROS-responsive as1 element in a transient assay, consistent with the report that a bZIP-type protein, which interacts with as1/ocs-like elements in the promoters of target genes, might be a component of the ROS-mediated pathway (Cheng et al., 2007). However, the predicted regulatory relationship between ROS-bZIP1 and the as1/ocs element-containing genes awaits further confirmation. Therefore, to our knowledge, it is a novel conclusion that the interaction of JERF3 with the promoters of genes related to oxidative stress regulates the expression of these genes. This results in enhanced SOD activities, consequently decreased accumulation of ROS.

**Figure 6.** Expression of JERF3 in tobacco activates the expression of the GUS gene controlled by GCC box, DRE, CE1, or as-1. The top section displays a schematic diagram of the reporter constructs used in *Agrobacterium*-mediated transient expression assay. Four-times cis-elements (GCC box, DRE, CE1, or as-1) are fused upstream of 35S minimal promoter in pBI121 as reporter. The bottom section shows relative GUS activity in leaves of wild-type and OE seedlings 48 h after *Agrobacterium* infiltration. The data for GUS activity in OE seedling leaves are relative to that in wild type (standardized to 100, inferred to the GUS activity driven by 35S minimal promoter in pBI121). GCC, DRE, CE1, and as-1 indicate that 4× cis-elements of GCC box, DRE, CE1, and as-1 are inserted upstream of the 35S minimal promoter in pBI121 as positive reporters, respectively. Error bars indicate ±SD.
and thereby enhancing tolerance to drought, salt, and freezing.

In Arabidopsis, ethylene receptors including ETR1, ETR2, EIN4, ERS1, and ERS2 (Potuschak et al., 2003) play a negative role in the ethylene pathway (Guo and Ecker, 2004). CTR1, a Ser/Thr kinase downstream of receptors, is also a negative regulator of the pathway (Kieber et al., 1993; Bishopp et al., 2006). Downstream of CTR1 is EIN2, which further regulates EIN3 (Chao et al., 1997; Solano et al., 1998). Although ERF1 is known to be a downstream component of EIN3 in Arabidopsis (Guo and Ecker, 2004) it was unclear whether the ERF protein, JERF3, is modulated by EIN3/EILs. To answer this question, we cloned a 1,595-bp upstream fragment of JERF3 from genomic DNA of tomato (Lichun) using TAIL-PCR. Analysis of the promoter using PLACE software indicates that there is no PERE element that EIN3 interacts with (Guo and Ecker, 2004). However, we observed that tomato LeEIL1, an Arabidopsis EIN3 homolog (Tieman et al., 2001), activates the expression of GUS reporter driven by JERF3 promoter in an Agrobacterium-mediated transient expression assay (Supplemental Fig. S3). The facts that the promoter of JERF3 could be transcriptionally modulated by LeEIL1, that JERF3 is inducible by ethylene, and that JERF3 interacts with GCC box (Wang et al., 2004) suggest that JERF3 may be concerned with ethylene pathway. Furthermore, JERF3 could bind to DRE, CE1, and as-1 or to other elements such as the 39-bp fragment in the promoter of NtCA to directly regulate the expression of oxidative stress genes. Such genes include NtSOD and NtCA, which would decrease the content of ROS. Lastly, JERF3 enhances adaptation to drought, salt, and cold in tobacco not only through binding to the promoters of oxidative stress genes, resulting in the expression of these genes and decreasing the accumulation of ROS, but also through activating the expression of genes responsive to osmotic stress. Therefore, based on our previous report (Wang et al., 2004) and present study, we propose a regulatory model for JERF3 in tobacco abiotic stresses (Fig. 8). In such a model, JERF3, dominantly binding to GCC box, transcriptionally activates the expression of genes related to both osmotic and oxidative stress responses.
oxidative stresses. The expression of these genes subsequently results in the decreased accumulation of ROS, thereby enhancing adaptation to drought, freezing, and salt in tobacco.

MATERIALS AND METHODS

Plant Material and Growth Conditions

JERF3-expressing tobacco (*Nicotiana tabacum; Wang et al., 2004) and wild-type tobacco (cv NC89) were grown in a growth chamber at 25°C under a 16-h photoperiod. T1 (OE4 and OE24 with two-copy insertions) and T2 (OE1, OE5, and OE9 with one-copy insertion) transgenic tobacco plants were used for the experiments. JERF3-expressing tobacco and wild-type tobacco are referred to as OE and wild type, respectively.

Salt, Drought, and Freezing Stress Assays

All seeds in the following assays were surface sterilized and kept at 4°C for 2 d to break dormancy (Mukhopadhyay et al., 2004) before submerging them to any of the experimental treatments. For the germination assay, the tobacco seeds were plated in one-half Murashige and Skoog medium (0.6% agar) plus mannitol or NaCl, and the number of germinated seeds was counted every day. For root elongation assay, the germinated seeds were first placed upright in Murashige and Skoog plates until the roots were about 0.5 cm and the seedlings transferred to the same medium supplemented with mannitol or NaCl and placed upright in the growth chamber. The root lengths were recorded before and after the treatments.

For drought assays, both 3-week-old and 6-week-old OE and wild-type seedlings in pots were adequately watered and then water was withheld for 2 d to break dormancy (Mukhopadhyay et al., 2004) before subjecting them to stress treatments. T2 (OE4 and OE24 with two-copy insertions) and T3 (OE1, OE3, OE5, and OE9 with one-copy insertion) transgenic tobacco plants were used for the experiments. JERF3-expressing tobacco and wild-type tobacco are referred to as OE and wild type, respectively.

Detection of SOD Activities and O$_2^-$ in Tobacco Leaves

The stress treatments were applied as described below. For the drought treatment, OE and wild-type seedlings were removed from Murashige and Skoog medium, washed with water, dried with tissue paper, and then placed on tissue paper for 1 h. For the low-temperature treatment, OE and wild-type seedlings in plates were placed at −2°C in a growth chamber for 2 d. For the ABA and salt treatments, OE and wild-type seedlings in plates were sprayed with 100 μM ABA or 200 mM NaCl and kept under high humidity for 3 h. Activity of SOD was determined using the method of Winterbourn et al. (1975) that is based on the ability of SOD to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxide. In our assays, 0.3 g leaves from 3-week-old tobacco seedlings each sample cultured in Murashige and Skoog medium were used. All of the seedlings were grown in 1.5-l plastic tubes, one in the dark as control, another under light (150 μmol m$^{-2}$ s$^{-1}$). After incubation at 30°C for 20 min, the tubes were placed on ice to stop the reaction. The inhibition of NBT reduction was determined at A560. Leaves of 3-week-old seedlings treated with stresses were also used to determine superoxide using NBT staining as described by Lee et al. (2002).

TAIL-PCR Assay

TAIL-PCR was performed as described by Liu et al. (1995). The genomic DNA of NC89 was used as a template. AP primers were the same as those used by Liu et al. (1995). The sequences of specific primers for NISOD, NICA, and JERF3 were given in Supplemental Table S1.

Modulation of JERF3 in Oxidative Stress Response

Agrobacterium-Mediated GUS Transient Assay

For constructing the reporter vectors, four-times-repeated sequences of cis-acting elements GCC (AGCCGCG), DRE (TACCGCAC), CE1 (TGCCACCAC), or as-1 (TGACG) were inserted upstream of the minimal TATA box (+46 to +10) to replace the cauliflower mosaic virus 35S promoter in pBI212 (CLONTECH). The plasmids were then introduced into the Agrobacterium tumefaciens strain LBA4404. Agrobacterium-mediated transient assay was performed on the leaves of 4-week-old wild-type and OE seedlings as described by Yang et al. (2000). The GUS activity was measured about 48 h later.

To ascertain whether JERF3 interacts with the upstream sequence of the ATG start code of NICA, DNA fragments 1,096 bp (NICA-p1), 870 bp (NICA-p2), 788 bp (NICA-p3), 733 bp (NICA-p4), 692 bp (NICA-p5), 657 bp (NICA-p6), 617 bp (NICA-p7), and 192 bp (NICA-mini as a minimal promoter) upstream of the ATG start code of NICA were inserted separately into pCambia1303 using the enzymes PstI and BglII. Similarly, a DNA fragment 807 bp (NISOD-f), 660 bp (NiSOD-p), 600 bp (NiSOD-mini) upstream of the enzymes PstI and BglII. Sequences of the primers of NICA and NiSOD promoters were listed in Supplemental Table S1. To generate the removal of GCC box or 39-bp fragment from NiSOD-f or NICA-p1, respectively, PCR amplifications were used with special primers listed in Supplemental Table S1 using pCambia1303-NICA-p1 or pCambia1303-NISOD-f-α as a template. Then PCR production was self linked with T4 DNA ligase and was followed by transformation into Escherichia coli. After sequencing confirmation, the above-constructed plasmids were then introduced separately into the A. tumefaciens strain LBA4404. Agrobacterium-mediated transient assay was performed and the GUS activity measured about 48 h later.

The GenBank accession numbers for the sequences used as materials in this article are: AY386360 (JERF3), X66663 (actin), AF127243 (NiSAM), D13952 (TOBLT), A8049357 (NiERD101C), AF194022 (NIP5), X02353 (NIRub-S), M14417 (CthGal4), M14419 (Cgrf), U53111 (NIRuc342), AB003497 (NISOD), AF454759 (NICA), A909006 (NiRuK2D), U93244 (NICT2), A8041518 (NiGPX), AU195933 (NiAPX1), D85912 (NiAPX2), EU432357 (NICA promoter), EU342358 (NISOD promoter), AF328784 (LeEL1), and EU910896 (JERF3 promoter).
Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Expression of JERE3 in tobacco has no effect on the growth at normal growth conditions.

**Supplemental Figure S2.** Expression of NtSPS in response to cold.

**Supplemental Figure S3.** LeELL1 interacts with the promoter of JERE3 in transient expression assay.

**Supplemental Table S1.** The primers used in this article.

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**LITERATURE CITED**


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