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Enhanced tolerance to freezing in tobacco and tomato overexpressing transcription factor *TERF2/LeERF2* is modulated by ethylene biosynthesis

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Abstract Increasing numbers of investigations indicate that ethylene response factor (ERF) proteins play important roles in plant stress responses via interacting with GCC box and/dehydration-responsive element/C-repeat to modulate expression of downstream genes, but the detailed regulatory mechanism is not well elucidated. Revealing the modulation pathway of ERF proteins in response to stresses is vital. Previously, we showed that tomato ERF protein TERF2/LeERF2 is ethylene inducible, and ethylene production is suppressed in antisense *TERF2/LeERF2* tomatoes, suggesting that TERF2/LeERF2 functions as a positive regulator in ethylene biosynthesis. In this paper, we report that regulation of TERF2/LeERF2 in ethylene biosynthesis is associated with enhanced freezing tolerance of tobacco and tomato. Analysis of gene expression showed that cold slowly induces expression of *TERF2/LeERF2* in tomato, implying that TERF2/LeERF2 may be involved in cold response through ethylene modulation. To test the hypothesis, we first observed that overexpressing *TERF2/LeERF2* tobaccos not only enhances freezing tolerance via activating expression of cold-related genes, but also significantly reduces electrolyte leakage. In addition, with treatment of ethylene biosynthesis inhibitor or ethylene receptor antagonist, we then showed that blockage of ethylene biosynthesis or the ethylene signaling pathway decreases freezing tolerance of overexpressing *TERF2/LeERF2* tobaccos. Moreover, the results from tomatoes showed that overexpressing *TERF2/LeERF2* tomatoes enhances while antisense *TERF2/LeERF2* transgenic lines decreases freezing tolerance, and application of ethylene precursor 1-aminocyclopropane-1-carboxylic acid restored freezing tolerance of antisense lines. Therefore our results establish that TERF2/LeERF2 enhances freezing tolerance of plants through ethylene biosynthesis and the ethylene signaling pathway.

Keywords ERF protein TERF2/LeERF2 · Ethylene biosynthesis · Tobacco · Tolerance to freezing · Transcriptional regulation

Introduction

Plants must face with adverse environmental conditions, such as cold, during their life cycle. Cold stress disturbs plant's metabolic reactions and adversely affects their growth and development. To overcome these unfavorable conditions, plants have established mechanism to acclimate to cold by triggering a cascade of events leading to enhance cold tolerance. In this course, transcription factors play a key role (Jaglo-Ottosen et al. 1998; Singh et al. 2002). For example, several types of transcription factor families, including MYB, basic helix-loop-helix (bHLH), basic-leucine zipper (bZIP), ethylene response factor (ERF) and homeodomain transcription factors, are evidenced to be

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involved in regulation of cold response (Shinozaki et al. 2003; Tran et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006; Chinnusamy et al. 2007).

The ERF family that belongs to the AP2/ERF superfamily is a large transcription factor family, which can be divided into two subfamilies: the CBF/DREB and the ERF subfamily (Nakano et al. 2006). CBF/DREB1 subfamily members have been shown to play an important role in cold-stress responses. For example, overexpression of *Arabidopsis CBF1/DREB1B* and *CBF3/DREB1A* both obviously enhance freezing tolerance of transgenic plants (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Gilmour et al. 2000), while the RNA interference and antisense lines of *CBF1/DREB1B* and *CBF3/DREB1A* exhibit reduced induction of downstream cold-responsive genes and impaired freezing tolerance in cold situation (Novillo et al. 2007). GeneChip assay showed that CBF2/DREB1C regulates expression of many cold-induced genes (Vogel et al. 2005). Moreover, *Arabidopsis CBF/DREB1* orthologous genes from other species also confer the regulatory function in cold response (Jaglo et al. 2001). These results indicate that the members of CBF/DREB play an important role in plant cold acclimation.

Earlier studies showed the ERF subfamily members were mainly involved in biotic tresses. For instance, the overexpression of *ERF1* enhances expression of *PDF1.2*, *b-CHI* and *Thi2.1*, resulting in the increased resistance to both *Botrytis cinerea* and *Pseudomonas syringae* tomato DC3000 (Berrocal-Lobo et al. 2002). Recent investigations proved that different members of ERF family may play diverse functions in plant responses to abiotic and biotic stresses (Park et al. 2001; Feng et al. 2005; Nakano et al. 2006). For instance, tobacco Tsi1 enhances tolerance of transgenic tobacco to biotic and abiotic stresses through interacting with both GCC box and DRE/CRT (Park et al. 2001). Overexpressing pepper *CaPF1* in *Arabidopsis* enhances tolerance to pathogens and freezing (Yi et al. 2004). Overexpression of *Medicago truncatula WXP1* increases the drought tolerance of transgenic alfalfa (Zhang et al. 2005a), while expression of tomato *TERF1* enhances the drought and salt tolerance of transgenic tobacco and rice (Zhang et al. 2005b; Gao et al. 2008). However, the regulatory pathway of ERF subfamily in abiotic stress is not well understood.

Ethylene is an important hormone with roles in plant growth, development, and responses to biotic and abiotic stresses (Yu et al. 2001; Kim et al. 2003; Ludwig et al. 2005; Kendrick and Chang 2008). For example, salt and osmotic stresses induce ethylene production (Khan and Huang 1998; Kim et al. 2003), which activates the salt-stress response through the ethylene signaling pathway (Cao et al. 2007). Treatment with ethylene synthesis inhibitor 1-methylcyclopropene decreases cold tolerance of tomato (Zhao et al. 2009), and exogenous ethylene increases while ethylene receptor anatagonist AgNO3 decreased freezing tolerance of winter rye (Yu et al. 2001). In addition, gene profiling studies also showed that ethylene might be involved in cold response (Fowler and Thomashow 2002). Knowledge of the molecular regulatory mechanism of ethylene in cold response is still limited.

Previously, we evidenced that tomato ERF protein TERF2/LeERF2 transcriptionally regulates ethylene biosynthesis in tomato and tobacco (Zhang et al. 2009). In the present paper, we report that overexpressing *TERF2/LeERF2* tobaccos enhances freezing tolerance. In addition, results from the inhibitor treatments further showed that ethylene biosynthesis or the ethylene signaling pathway are associated with freezing tolerance of overexpressing *TERF2/LeERF2* tobacco. Moreover, overexpressing *TERF2/LeERF2* tomatoes enhances while antisense *TERF2/LeERF2* transgenic lines decreases freezing tolerance. And application of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) restored freezing tolerance of antisense lines, establishing that TERF2/LeERF2 enhances freezing tolerance of plants through ethylene biosynthesis and the ethylene signaling pathway.

Materials and Methods

Plant material and growth conditions

Tomato [*Solanum lycopersicum* (*Lycopersicon esculentum*) cv Lichun] and tobacco (*Nicotiana tabacum* cv. NC89) were grown in growth chambers at 25°C with a 16-h light/8-h dark photoperiod. Four-week-old tomato and three-week-old tobacco were used to check gene expression. To analyze expression of *TERF2/LeERF2* in the cold treatment, the tomato plants were transferred into 4°C for different time periods, and the plants were harvested and frozen with liquid nitrogen and stored at −70°C for further isolation of RNA. The total RNA extractions and Northern blots were performed as described previously (Huang et al. 2004; Zhang et al. 2005b).

Generation of transgenic plants

The generation processes of transgenic tobacco and tomato overexpressing *TERF2/LeERF2* and antisense *TERF2/LeERF2* tomato were previously described (Zhang et al. 2009). The wild-type tobacco and overexpressing *TERF2/LeERF2* tobacco were named WT and TERF2-OE, respectively. The wild-type tomato, overexpressing *TERF2/LeERF2* tomato and antisense *TERF2/LeERF2* tomato were named WTM, TERF2-OEm and TERF2-RI, respectively. The number indicates the different transgenic line.

Freezing tolerance assays

For the freezing tolerance assays of tobacco or tomato, the two-week-old tomato or three-week-old tobacco T3 transgenic and wild type plants grown in soil were first kept at 4°C for 2 h, and then transferred to −4°C for tomato or −6°C for tobacco for the indicated time (Gilmour et al. 2000), following a recovery at room temperature. For treatment of ethylene precursor 1-aminocyclopropane-1-carboxylic acid, two-week-old tomatoes grown in soil were sprayed with 50 μM ACC or water (taken as control). After 2 days (for tobacco) or 10 days (for tomato) recovery, the survival seedlings of tobacco or tomato were counted, respectively.

Electrolyte leakage assays

To measure the electrolyte leakage of tobacco, the leaves of three-week-old tobacco grown in soil were placed at 4°C for 2 h and then treated for another 2 h each at −2, −4, −5, −6, −8, −10°C, respectively. For the effects of ACC and inhibitors on electrolyte leakage, tobacco seedlings were first treated for 48 h with 50 μM ethylene precursor ACC, 20 μM ACC synthase inhibitor aminoethoxyvinylglycine (AVG), 200 μM ACC oxidase inhibitor of $CoCl_2$, 50 μM ethylene receptor antagonist $AgNO_3$ or water (as control). Then the seedlings were against freezing treatment at 4°C for 2 h, then −6°C for another 2 h. For the measurement of electrolyte leakage in tomato after ACC treatment, two-week-old tomatoes grown in soil were sprayed with 50 μM ACC or water. After 48 h treatment, the leaves of plants were transferred to 4°C for 2 h, then −4°C for another 2 h.

After the samples were treated as above, electrolyte leakage was measured as described by Gilmour et al. (2000). After the conductivity of samples was measured following freezing treatment, the samples were then autoclaved and the total conductivity of samples was measured again. Percentage of electrolyte leakage was the ratio of conductivity of before autoclaving to that of after autoclaving.

Analyses of gene expression using real time quantitative PCR amplifications

Total RNAs were extracted from three-week-old tobacco using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Real time quantitative polymerase chain reactions (Q-PCR) were performed as (Zhang et al. 2009). All data were normalized to the mRNA level of WT-control as 100, referred to the internal control *actin*. All GenBank accession numbers of genes and primers used in this paper were listed in Supplemental Table 1.

Results

Expression of *TERF2/LeERF2* is cold-inducible in tomato

Increasing numbers of studies have shown that ERF proteins are involved in various signaling pathways such as of ethylene and methyl jasmonic acid (MeJA), and in response to biotic and abiotic stresses (Ohme-Takagi and Shinshi 1995; Kizis et al. 2001; van der Fits and Memelink 2001; Gutterson and Reuber 2004; Karaba et al. 2007). Previously, we showed that the ethylene-inducible ERF gene *TERF2/LeERF2* was a feedback regulator in expression of ethylene synthesis genes and ethylene production (Zhang et al. 2009). To dissect the function of TERF2/LeERF2 in abiotic stresses, we further analyzed the response of *TERF2/LeERF2* to abiotic stress stimuli. Northern blotting showed that expression of *TERF2/LeERF2* can be induced by cold (Fig. 1), but not MeJA and drought (Data not shown). Distinctive to the ethylene treatment that expression of *TERF2/LeERF2* was quickly induced in 30 min and approached the maximum 2 h after ethylene treatment (Zhang et al. 2009); the accumulation of *TERF2/LeERF2* transcripts was detectable within 12 h and remained stable 24 h after cold treatment (Fig. 1). The results suggest that *TERF2/LeERF2* may play a role in cold-stress response. Combined with our previous report (Zhang et al. 2009), it is possible that the expression of *TERF2/LeERF* is involved in cold response through ethylene modulation.

Overexpression of *TERF2/LeERF2* in tobacco enhances tolerance to freezing

Because *TERF2/LeERF2* is cold inducible, we then investigated the function of TERF2/LeERF2 in cold response by overexpressing *TERF2/LeERF2* tobacco (TERF2-OE). The OE tobaccos did not display any difference from wild type tobacco (WT) under normal growth conditions (25°C, 16 h light/8 h dark), however, freezing assays at −6°C showed that >90% of WT seedlings were damaged or dead after 9 h

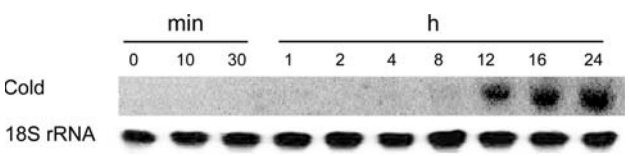

Fig. 1 Cold slowly induces the expression of *TERF2/LeERF2* in tomato. Four-week-old tomato seedlings treated at 4°C for indicated time were used to isolate RNA. Each lane was loaded with 20 μg of total RNA. RNA was analyzed by the gene-specific probe from the 3′ flanking sequences of *TERF2/LeERF2*. 18 s rRNA was used as the loading control. *min* and *h* indicated the treatment minute and hour, respectively

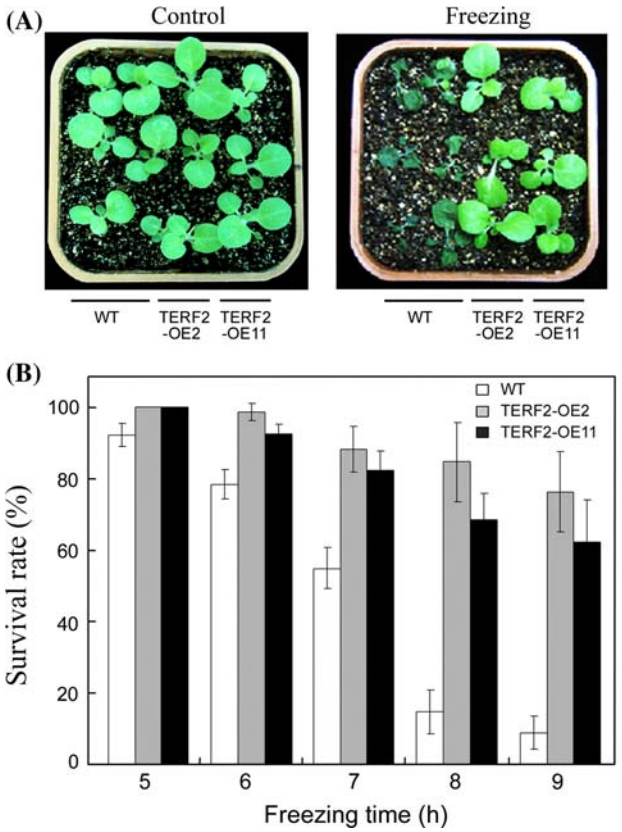

Fig. 2 Overexpression of *TERF2/LeERF2* enhances freezing tolerance in tobacco. **a** Phenotype of transgenic and wild type tobacco under freezing treatment. *Control* Tobacco seedlings were cultured under normal growth conditions; *Freezing* Plants were treated at 4°C for 2 h, then −6°C for 9 h, and recovered for 2 days at 25°C. **b** Survival rate after freezing treatment. Tobacco seedlings were treated at −6°C at indicated time, and survival seedlings were counted after recovery at 25°C for 2 days. WT indicates wild type tobacco; *TERF2-OE* indicates the *TERF2/LeERF2* overexpression transgenic tobacco lines. About 20–30 seedlings of each lines were used in the assay. The assay was repeated three times and the bar represent (±) SE

of freezing, while >65% of TERF2-OE seedlings grew well (Fig. 2a). Observations over time courses of freezing treatment showed that all TERF2-OE plants grew well after 5 h at −6°C, but 10% of WT seedlings died. Almost 50% of WT seedlings were obviously damaged or dead after freezing for 7 h, while >80% of TERF2-OE plants survived. After 8 h of freezing, about 85% of WT seedlings were obviously damaged or dead, while >70% of TERF2-OE plants still grew well (Fig. 2b).

Overexpression of *TERF2/LeERF2* in tobacco reduces ion leakage under freezing stress

During freezing, the plasma membranes of plants are injured and ions leak from the cytoplasm (Gonzalez-Aguilar et al. 2000), suggesting that the ion leakage is an indicator of damage to plasma membranes. To address how

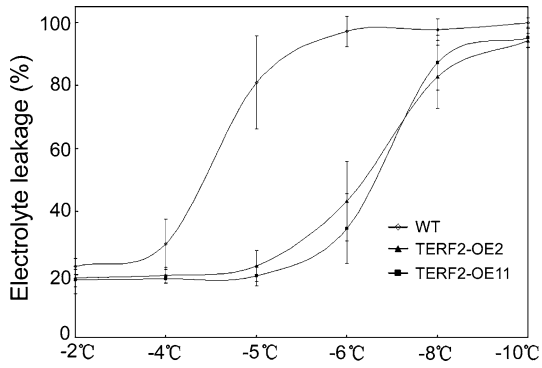

Fig. 3 Overexpression of *TERF2/LeERF2* in tobacco reduces ion leakage under freezing stress. The electrolyte leakage was measured from three-week-old tobacco treated with different freezing temperatures for 2 h. Percentage of electrolyte leakage was the ratio of conductivity of before autoclaving to that of after autoclaving. The assay was repeated three times. *Error bars* indicate (±) SE

TERF2/LeERF2 enhances tolerance to freezing, we first measured changes in electrolyte leakage between TERF2-OE and WT seedlings among temperatures of −2–−10°C. Freezing of −5–−6°C for 2 h gave electrolyte leakages of 80–90% in WT tobacco, but only 20–40% in TERF2-OE plants (Fig. 3), indicating that TERF2-OE may stabilize the plasma membrane to enhance freezing tolerance.

Overexpression of *TERF2/LeERF2* in tobacco activates the expression of stress-responsive genes

It is well known that the responses and acclimation of plants to diverse stresses are closely related to the expressions of specific stress-related genes (Stockinger et al. 1997; Chakravarthy et al. 2003; Aharoni et al. 2004; Sasaki et al. 2007; Andriankaja et al. 2007), via interacting with the GCC box and/or DRE/CRT *cis*-acting elements (Liu et al. 1998; Gutterson and Reuber 2004; Kizis et al. 2001; Yamaguchi-Shinozaki and Shinozaki 2006). Our earlier study reported that TERF2/LeERF2 can identify GCC box and DRE/CRT (Zhang et al. 2009). In order to dissect the modulation of TERF2/LeERF2 in freezing tolerance, we further analyzed whether the enhanced freezing tolerance of TERF2-OE seedlings is attributed to the expression of cold-stress-related genes. Through searching the GCC box- or DRE/CRT-containing stress-related genes in tobacco, we found that *PRB-1b* and *Osmotin* are GCC box containing genes (Sessa et al. 1995; Zhou et al. 1997), while *NtEDR10B*, *NtERD10C* and *NtSPS* are DRE/CRT containing genes (Kasuga et al. 2004; Wu et al. 2008). Analyses with Q-PCR amplifications showed that the expression of these kind genes in TERF2-OE seedlings were increased 3–9-folds (Fig. 4), demonstrating that TERF2/LeERF2 may enhance the freezing tolerance of TERF2-OE tobacco

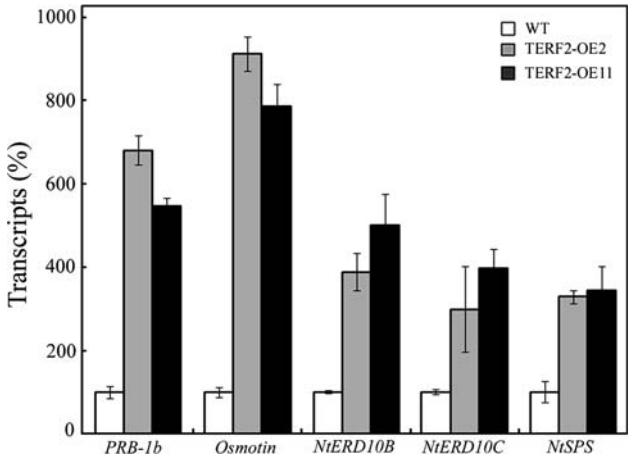

Fig. 4 Overexpression of *TERF2/LeERF2* in tobacco increases expression of downstream stress-related genes. Total RNAs were extracted from three-week-old tobacco grown at 25°C and 16-hr light/8-hr dark. The expression of genes in *WT* and *TERF2-OE* tobaccos was measured by Q-PCR amplifications. The assay was repeated three times and the *bar* represent (±) SE

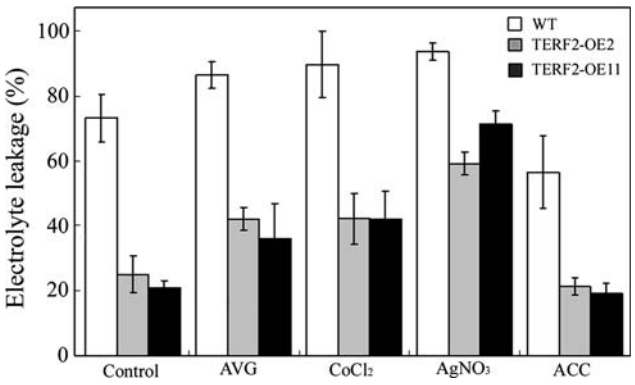

Fig. 5 Overexpression of *TERF2/LeERF2* enhances freezing tolerance of tobacco through the ethylene biosynthesis and signaling pathway. The electrolyte leakage of tobacco was measured after the treatment of the inhibitor of ethylene biosynthesis and ethylene receptor antagonist for 48 h, following freezing treatment described as in "Materials and methods". *Control* Water treatment; *AVG*: 20 μM AVG; *$CoCl_2$* 200 μM $CoCl_2$; *$AgNO_3$* 50 μM $AgNO_3$; *ACC* 50 μM ACC. The assay was repeated three times and the *bar* represent (±) SE

by increasing at least the expression of both GCC box- and DRE/CRT- containing genes.

Ethylene biosynthesis and signaling pathway in tobacco are associated with the TERF2/LeERF2-reduced electrolyte leakage under freezing

It was demonstrated that ethylene plays an important role during plant cold stress response (Ciardi et al. 1997; Yu et al. 2001). Our previous investigations showed that TERF2/LeERF2 can increase ethylene production in tobacco and tomato (Zhang et al. 2009). To address the relationship between overproduction of ethylene and freezing tolerance in tobacco, we used inhibitor of ethylene biosynthesis and ethylene receptor antagonist to treat tobacco seedlings, and monitored changes in electrolyte leakage as an indicator under freezing conditions. After pretreatments of ethylene biosynthesis inhibitors AVG and $CoCl_2$, or ethylene receptor antagonist $AgNO_3$, the WT and TERF2-OE seedlings were used to analyze changes of electrolyte leakage, following 2 h of exposure to −5°C. In such conditions, inhibition of either ethylene biosynthesis or ethylene signaling significantly increased electrolyte leakage from about 20% to 40–65% in TERF2-OE seedlings after freezing exposure. Interestingly, the electrolyte leakage in wild type (∼75%) was much higher than that in TERF2-OE lines (∼20%), while the differences were only about 15% after $AgNO_3$ treatment. And treatment with the ethylene precursor ACC significantly reduced electrolyte leakage in WT (Fig. 5), indicating that TERF2/LeERF2 enhances freezing tolerance through modulating ethylene production and signaling pathway in tobacco.

Overexpressing and antisense *TERF2/LeERF2* tomatoes reversely affect tolerance of tomato to freezing

Our previous investigation showed that overexpressing *TERF2/LeERF2* (TERF2-OEm) lines increases and antisense *TERF2/LeERF2* tomatoes (TERF2-RI) reduces ethylene production (Zhang et al. 2009). Using TERF2-OEm and TERF2-RI tomatoes, we further investigated the regulatory role of TERF2/LeERF2 in freezing tolerance. After freezing treatment at −4°C for 2 h, followed by 10 days recovery at 25°C, about 80% TERF2-RI seedlings, while only about 40% TERF2-OEm plants were died (Fig. 6a). More importantly, with ACC treatment, the survival ratios of both wild type tomato (WTM, about 68%) and TERF2-RI seedlings (about 60%) were obviously increased, close to that of the TERF2-OEm tomatoes (about 72–74%, Fig. 6a). Through monitoring changes of electrolyte leakage, we found that the TERF2-OEm lines displayed a much lower electrolyte leakage, compared to WTM and the TERF2-RI seedlings after freezing treatment. Similar to the seedling survival assay, ACC also reduced electrolyte leakage of the TERF2-RI seedlings under freezing stress, showing the decrease of electrolyte leakage from around 70% to around 35%, very close to that of WTM (Fig. 6b), evidencing that the modulation of transcription factor TERF2/LeERF2 in ethylene production and signaling

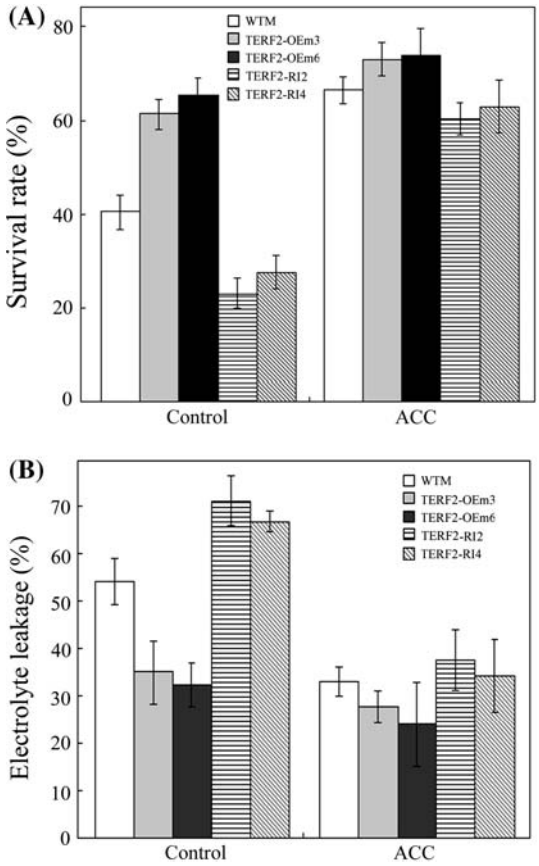

Fig. 6 Overexpressing and antisense TERF2/LeERF2 tomatoes reversely affect freezing tolerance of tomato. **a** The survival rate of tomato seedlings with or without ACC treatment under freezing stress. **b** The electrolyte leakage of tomato seedlings with or without ACC treatment under freezing treatment. Plants were first treated with water or 50 μM ACC, following freezing treatment described as in "Materials and methods". *WTM* indicates wild type tomato; *TERF2-OEm3* and *TERF2-OEm6* indicate different *TERF2/LeERF2* overexpression transgenic tomato lines; *TERF2-RI2* and *TERF2-RI4* indicate different *TERF2/LeERF2* RNA interference transgenic tomato lines. *Control* and *ACC* indicate tomato seedlings were sprayed with water or 50 μM ACC, respectively, for 48 h before freezing treatment. About 20–30 seedlings of each lines were used in the assay. The assay was repeated three times and the *bar* represent (±) SE

pathway plays an important role in freezing tolerance in tomato.

Discussion

Increasing evidence indicates that ERF proteins play vital regulatory roles in response to stress through interacting with *cis*-acting elements, such as GCC box or DRE/CRT, to activate the expression of targeted stress-responsive genes (Kizis et al. 2001; Yamaguchi-Shinozaki and Shinozaki 2006; Wu et al. 2008). Our previous report showed that a tomato ERF protein TERF2/LeERF2 modulates the expression of ethylene biosynthesis genes and ethylene production in tomato and tobacco (Zhang et al. 2009). In the present research, we further reveal that this regulation of TERF2/LeERF2 in ethylene biosynthesis and signaling pathway is closely associated with enhanced freezing tolerance in tobacco and tomato, deepening the understanding of ERF proteins in response to freezing stress.

The ERF proteins play important roles in cold response via regulating the expression of downstream stress-related genes. For example, overexpressing CBF/DREB genes in plants enhance freezing tolerance via regulating the expression of cold-tolerance-related genes (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Gilmour et al. 2000; Kasuga et al. 2004, Ito et al. 2006). Our previous report showed that the TERF2/LeERF2 protein interacts with GCC box and DRE (Zhang et al. 2009), and in the present research, we evidence that the TERF2-OE lines displayed constitutive expression of GCC box- and DRE-containing stress-related genes, indicating that ERF protein TERF2/LeERF2 might regulate the expression of cold-stress responsive genes as transcriptional activator. Thus it is possible that the increased expression of stress responsive genes contributes an important role in the enhanced tolerance of TERF2-OE lines to cold.

It has been demonstrated that the phytohormone abscisic acid (ABA) plays important roles during the abiotic stress responses through regulating the expression of a large number of stress-related transcription factors, such as bZIP, MYB, bHLH and ERF proteins (Abe et al. 1997; Uno et al. 2000; Haake et al. 2002; Yamaguchi-Shinozaki and Shinozaki 2006). For example, ABA can regulate the expression of *Arabidopsis* ERF family genes *CBF4/DREB1*D and *CBF4/DREB1D*, which activate the expression of downstream stress-responsive genes, resulting in the ABA-dependent stress response (Haake et al. 2002). Moreover, some ERF proteins can regulate the stress response via an ABA-independent pathway (Yamaguchi-Shinozaki and Shinozaki 2006). The evidence that the enhanced tolerance to freezing in tobacco and tomato expressing *TERF2/LeERF2* is ethylene-dependent suggests that the ERF proteins have multiple regulation pathways in response to freezing.

The ERF proteins can regulate plant developmental cases and stress responses by binding specific *cis*-element to affect a given metabolism (van der Fits and Memelink 2000; Aharoni et al. 2004; Andriankaja et al. 2007; Sasaki et al. 2007). For example, *Catharanthus roseus* ERF protein ORCA3 can identify the jasmonate- and elicitor-responsive element (JERE) to involve in the plant primary and secondary metabolism regulated by jasmonate (van der Fits and Memelink 2000; 2001). More recent investigations showed that ERF protein can bind to the NF box involved in the nod factor signal transduction in *Medicago truncatula*

(Andriankaja et al. 2007; Middleton et al. 2007). Additionally, overexpression of *Medicago truncatula WXP1* and *Arabidopsis SHINE* increase wax biosynthesis and modify drought tolerance of transgenic alfalfa and *Arabidopsis*, respectively (Aharoni et al. 2004; Zhang et al. 2005a). Most interestingly, hormones play important regulatory roles in stress responses that involve ERF proteins. For example, *Arabidopsis* CBF1/DREB1B not only modulates the expression of cold-responsive genes as discussed above, but also is involved in the regulation of gibberellin (GA) pathway. The constitutive expression of *CBF1/DREB1B* reduces the bioactive GA content through activating the expression of GA-inactivating GA 2-oxidase genes, resulting in the accumulation of DELLA protein RGA and the enhanced cold tolerance (Achard et al. 2008). The rice ERF-like protein Sub1A can also regulate ethylene production and GA-responsiveness to enhance the tolerance to submergence (Xu et al. 2006; Fukao et al. 2006). Consistent with the above researches, tomato ERF protein TERF2/LeERF2 can affect ethylene production (Zhang et al. 2009), and this regulation is closely associated with enhanced freezing tolerance in *TERF2/LeERF2* transgenic tobaccos and tomatoes, demonstrating that TERF2/LeERF2 may have a different regulatory pathway from other reported ERF proteins.

The analyses for the expression of *TERF2/LeERF2* in tomato seedlings is ethylene (Zhang et al. 2009) and cold inducible suggest that the regulation of TERF2/LeERF2 might have a temporal order between ethylene biosynthesis and cold stress response. In addition, ethylene biosynthesis genes *NtACS1/3* and *NtACO1* were induced by cold within 1 h in tobacco seedlings. In accordance with expression of key enzyme genes of ethylene synthesis, the production of ethylene also increased at 6 h induction, then peaked at 12 h induction, then decreased in cold stress (Supplemental Fig. 1). These data indicate that the synthesis of ethylene can be differentially regulated by cold.

Previous reports showed that ethylene-insensitive tomato mutant *Never-ripe* (*Nr*) decreases, while the production of ethylene enhances cold tolerance (Ciardi et al. 1997). Treatment with ethylene synthesis inhibitor 1-methylcyclopropene (1-MCP) decreased cold tolerance of tomato (Zhao et al. 2009), and exogenous ethylene increased while ethylene receptor anatagonist $AgNO_3$ decreased freezing tolerance of winter rye (Yu et al. 2001). In addition, the regulation of CBF/DREB in cold response is possibly related to ethylene (Fowler and Thomashow 2002; Zhao et al. 2009). For example, the ethylene synthesis inhibitor 1-MCP decreased the expression of CBF/DREB gene *LeCBF1*, while endogenous ethylene increased the *LeCBF1* expression level in cold (Zhao et al. 2009), suggesting ethylene is important regulator of cold-stress response. In the present research, we found that the ethylene receptor antagonist $AgNO_3$ obviously increased the electrolyte leakage in TERF2-OE lines, consistent with the above reports that the ethylene signaling is important for the regulation of freezing tolerance. More importantly, the results that the decreased tolerance of TERF2-RI tomatoes to freezing was recovered by ACC application, strongly evidence that the ethylene might play important roles in freezing tolerance.

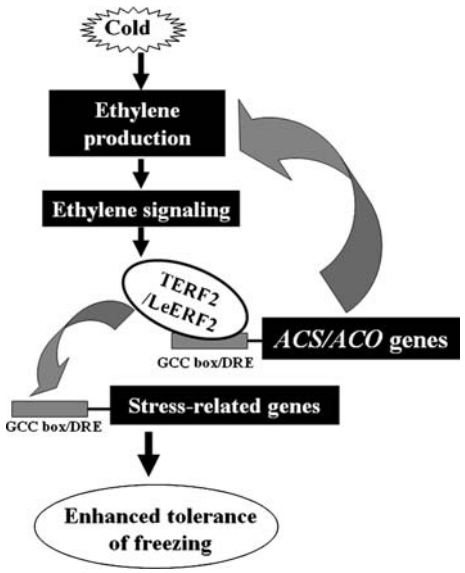

Fig. 7 TERF2/LeERF2 regulates the expression of ethylene biosynthesis genes and the ethylene production in cold response. The model indicates that ERF protein TERF2/LeERF2 as a downstream regulator in ethylene signaling feedback regulates the expression of ethylene biosynthesis genes and maintains higher production of ethylene later in the course of cold response. The increased ethylene activates its signaling pathway, resulting in the transcriptional activation of stress-related genes, and the improved freezing tolerance of plants

Because there is a potential role of ERF proteins in the ethylene signal pathway (Solano et al. 1998), and a different temporal expression of *TERF2/LeERF2* is modulated by ethylene and cold, thus we postulate that ethylene biosynthesis is triggered, through transcript level or posttranscriptional level regulatory, in cold, which further activates the expression of downstream ERF genes, such as *LeCBF1*, *TERF2/LeERF2*. Some ERF genes, such as, *TERF2/LeERF2* playing an important role in the feedback regulation of ethylene synthesis (Zhang et al. 2009), further feedback regulates expression of ethylene biosynthesis genes and maintains higher production of ethylene later in the course of cold response, or indirectly activated the expression of stress-related genes, resulting in the enhanced freezing tolerance of plants (Fig. 7).

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