Transcriptional Regulation of the Ethylene Response Factor LeERF2 in the Expression of Ethylene Biosynthesis Genes Controls Ethylene Production in Tomato and Tobacco\(^1\)\[^{[W]}\]\[^{[OA]}\]

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Fine-tuning of ethylene production plays an important role in developmental processes and in plant responses to stress, but very little is known about the regulation of ethylene response factor (ERF) proteins in ethylene biosynthesis genes and ethylene production. Identifying cis-acting elements and transcription factors that play a role in this process, therefore, is important. Previously, a tomato (\(Solanum lycopersicum\) [s. sp. \(Lycopersicon esculentum\)]) ERF protein, LeERF2, an allele of TERF2, was reported to confer ethylene triple response on plants. This paper reports the transcriptional modulation of LeERF2/TERF2 in ethylene biosynthesis in tomato and tobacco (\(Nicotiana tabacum\)). Using overexpressing and antisense LeERF2/TERF2 transgenic tomato, we found that LeERF2/TERF2 is an important regulator in the expression of ethylene biosynthesis genes and the production of ethylene. Expression analysis revealed that LeERF2/TERF2 is ethylene inducible, and ethylene production stimulated by ethylene was suppressed in antisense LeERF2/TERF2 transgenic tomato, indicating LeERF2/TERF2 to be a positive regulator in the feedback loop of ethylene induction. Further research showed that LeERF2/TERF2 conservatively modulates ethylene biosynthesis in tobacco and that such regulation in tobacco is associated with the elongation of the hypocotyl and insensitivity to abscisic acid and glucose during germination and seedling development. The effects on ethylene synthesis were similar to those of another ERF protein, TERF1, because TERF1 and LeERF2/TERF2 have overlapping roles in the transcriptional regulation of ethylene biosynthesis in tobacco. Biochemical analysis showed that LeERF2/TERF2 interacted with GCC box in the promoter of \(NtACS3\) and with dehydration-responsive element in the promoter of \(LeACO3\), resulting in transcriptional activation of the genes for ethylene biosynthesis in tomato and tobacco, which is a novel regulatory function of ERF proteins in plant ethylene biosynthesis.

Ethylene is the simplest but a very important phytohormone, which participates in major developmental processes, including seed germination, cell elongation, flowering, fruit ripening, organ senescence, abscission, and responses to stress (Johnson and Ecker, 1998). Increasing evidence suggests that controlling the biosynthesis of ethylene and regulating the activation of the ethylene pathway are important in mediating plant developmental processes and stress responses (Yu et al., 2001; Zhao and Schaller, 2004; Achard et al., 2006; Cao et al., 2006). The key components of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid synthesis (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO), play the important roles of catalyzing the conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC) and of ACC to ethylene, respectively (Wang et al., 2002). A number of studies have demonstrated that plants can regulate the activity of ACS at both transcriptional and posttranscriptional levels (Chae et al., 2003; Kim et al., 2003; Wang et al., 2004b; Joo et al., 2008). For example, Arabidopsis (\(Arabidopsis thaliana\)) ethylene overproduction mutants \(eto1\) and \(eto2\) are associated with the 26S proteasome pathway that determines the activity of ACS5 (Chae et al., 2003; Wang et al., 2004b; Joo et al., 2008; Christians et al., 2009). Additionally, mitogen-activated protein kinase proteins, cytokinin, and brassinosteroid can enhance ethylene production through their effects on the activity or stability of ACS proteins (Liu and Zhang, 2004; Hahn and Harter, 2009; Joo et al., 2008; Xu et al., 2008; Hansen et al., 2009). On the
other hand, the expression of ACS and ACO is related to ethylene production in most cases. For instance, during fruit ripening in tomato (Solanum lycopersicum [f. sp. Lycopersicon esculentum]), accumulation of ACS and ACO mRNA in the fruit accelerates the production of ethylene (Maunders et al., 1987; Oeller et al., 1991; Picton et al., 1993; Barry et al., 1996; Blume and Grierson, 1997; Liu et al., 1998a; Giovannoni, 2001; Alexander and Grierson, 2002). Stresses such as cold, drought, and salt also induce high levels of ACS transcripts, the level of which is associated with the amount of ethylene production (Wang et al., 2002; Achard et al., 2006). Matarasso et al. (2005) demonstrated that tomato Cys protease, LeCP, when pathogenically induced, regulates the expression of LeACS2 by directly binding to the LeACS2 promoter. Recently, Lin et al. (2008) found that an HD-Zip homeobox protein, LeHB-1, can bind to the homeobox cis-elements of the tomato LeACO1 promoter and regulate the gene’s expression in developing fruits (Lin et al., 2008). And the transcription factor RIN modulates the expression of LeACS2 by binding to the CarG motif in fruit ripening (Ito et al., 2008), indicating that transcriptional regulation plays a key role in ethylene biosynthesis.

Ethylene can be captured by its receptors in Arabidopsis (Hua and Meyerowitz, 1998), which remove the block of CTR1 on EIN2 (Solano et al., 1998; Huang et al., 2003). The release of EIN2 further activates the ethylene-signaling primary transcription factor EIN3/EIL1 (Chao et al., 1997), resulting in the expression of secondary transcription factors such as Arabidopsis ERF1. These secondary factors regulate the expression of downstream stress-responsive genes, consequently enhancing the plant’s tolerance to stress (Solano et al., 1998; Berrocal-Lobo et al., 2003). ERF1 belongs to the ethylene response factor (ERF) family, members of which contain an ERF DNA-binding domain (Nakano et al., 2006). A growing number of investigations suggest that ERF proteins are important in plant responses to stress and prevent developmental changes by interacting with multiple cis-acting elements, including GCC box and dehydration-responsive element/C-repeat (DRE/CRT; Ohme-Takagi and Shinshi, 1995; Liu et al., 1998b; Guttenson and Reuber, 2004; Oñate-Sánchez et al., 2007). For instance, Pti4 and ERF1 can bind to GCC box and affect the response to biotic stress (Berrocal-Lobo et al., 2003; Chakravarthy et al., 2003). Additionally, CBF1/DREB2A and DREB1A can bind to DRE/CRT and affect plant responses to abiotic stress (Liu et al., 1998b), whereas tobacco (Nicotiana tabacum) Tsi1 can bind to both GCC box and DRE/CRT (Park et al., 2001). Moreover, ERF proteins, Pti4 and TERF1, have been found in plants that exhibit the ethylene triple response (Wu et al., 2002; Huang et al., 2004), strongly suggesting that ERF proteins are involved in ethylene signaling or biosynthesis. However, very little is known about the regulation of ERF proteins in ethylene biosynthesis at the transcriptional level, which makes it vitally important to identify elements and transcription factors involved in ethylene biosynthesis.

In tomato, more ERF proteins have been isolated by several research groups (Zhou et al., 1997; Tournier et al., 2003; Chen et al., 2008). For instance, ERF proteins Pti4, Pti5, and Pti6 were isolated using a yeast two-hybrid system; they can interact with Pto kinase and are involved in plant response to pathogens (Zhou et al., 1997). LeERF1 to LeERF4 were isolated using degenerate primers that target the highly conserved ERF domain (Tournier et al., 2003). We earlier isolated 14 positive clones from the tomato cDNA library using the yeast one-hybrid system (Wang et al., 2004a). One of these clones with full-length cDNA encodes a putative protein, namely the tomato ethylene-responsive factor2 (TERF2), which contains 260 amino acids and has a predicted molecular mass of 29.6 kD. National Center for Biotechnology Information BLAST and data analyses showed the putative protein to be identical to LeERF2 (Tournier et al., 2003), except for the substitution of Val-253 with Leu-253. This difference in a single amino acid is caused by the exchange of guanine (G) in LeERF2 to cytosine (C) in TERF2 in the cDNA sequence. In order to analyze the gene structure, PCR amplifications based on the known cDNA sequence using genomic DNA of the tomato as a template were performed, and the results indicated that there is an intron of 74 bp in the gene, which is consistent with the report of Pirrello et al. (2006). Based on the similarities of TERF2 to LeERF2, it should be possible that TERF2 is an allele of LeERF2. Functional investigations showed that overexpressing LeERF2 in tomato elicited the typical triple response, with exaggerated apical hook formation in the absence of exogenous ethylene treatment, whereas treatment with 1-methylcyclopene, a potent inhibitor of ethylene perception, prevented the formation of a complete hook (Pirrello et al., 2006), indicating that LeERF2 may have a role in ethylene biosynthesis. In this study, we found that TERF2, the allele of LeERF2, interacted with GCC box and DRE/ CRT and affected ethylene levels through transcriptional activation of expression of the genes for ethylene biosynthesis in tomato and tobacco. The fact that another ERF protein, TERF1, also regulates ethylene biosynthesis through a similar molecular mechanism shows a novel regulation of ERF proteins in plant ethylene biosynthesis.

RESULTS

The ERF Protein LeERF2/TERF2 Is Possibly a Regulator Associated with Ethylene Responses

In order to distinguish the regulatory functions of LeERF2/TERF2, we first analyzed the response to ethylene. Expression of LeERF2/TERF2 was induced within 0.5 h of treating with ethylene, and the transcripts peaked after 2 h (Fig. 1), which is different from the report that LeERF2 transcripts were unaffected by

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ethylenic treatment (Tournier et al., 2003), suggesting that LeERF2/TERF2 may participate in the ethylene response.

**LeERF2/TERF2 Has a Regulatory Role in Activating the Expression of Biosynthesis Genes and Ethylene Production in Tomato**

Overexpressing LeERF2 in tomato is known to elicit the typical triple response in the form of exaggerated apical hook formation in the absence of exogenous ethylene treatment, whereas treatment with a potent inhibitor of ethylene perception, 1-methylcyclopropene, prevented the formation of a complete hook (Pirrello et al., 2006), suggesting a role for LeERF2 in ethylene biosynthesis. To test this assumption, we generated overexpressing (TERF2-OEm) and antisense (TERF2-RI) LeERF2/TERF2 in tomato (cv Lichun). The expression of LeERF2/TERF2 in transgenic tomato was confirmed by reverse transcription (RT)-PCR amplification, showing enhanced and decreased expression of LeERF2/TERF2 in TERF2-OEm and TERF2-RI, respectively (Fig. 2A). The production of ethylene by TERF2-OEm as measured by a gas chromatogram was nearly doubled, whereas that by TERF2-RI was halved compared with that in plants that had not been exposed to exogenous ethylene (Fig. 2B), demonstrating that reduced expression of LeERF2/TERF2 in TERF2-RI tomatoes obviously affects the production of ethylene. This result points to LeERF2/TERF2 as a potential feedback regulator of ethylene biosynthesis.

**Tobacco Phenotypes Expressing LeERF2/TERF2 Display Ethylene-Associated Responses**

To evaluate the effects of LeERF2/TERF2-modulated ethylene production, we planned to conduct physiological experiments on transgenic tomato plants. It proved very difficult to raise tomato seedlings on Murashige and Skoog (MS) plates for monitoring hypocotyl elongation and seedling development. Germination assays, however, showed that sensitivity to abscisic acid (ABA) and Glc during tomato seed germination was enhanced by overexpressing LeERF2/TERF2 and lowered by silencing LeERF2/TERF2 (data not shown). Therefore, we produced tobacco lines overexpressing LeERF2/TERF2 (TERF2-OE) to investigate the regulation of LeERF2/TERF2-modulated ethylene biosynthesis in plant development. In the following assays, two to three independent transgenic lines each with one copy insertion were used.

Glc is known to inhibit the ethylene signal pathway by destroying the stability of a protein, namely EIN3, and ethylene is known to be capable of countering the effect of Glc and of making the protein stable again (Guo and Ecker, 2003; Potuschak et al., 2003; Yanagisawa et al., 2003). In addition, the ethylene overproduction mutant eto2 and the constitutive activation mutant ctr1 display resistance to Glc (Zhou et al., 1998), indicating that components of the ethylene and Glc pathways share an antagonistic relationship. To further confirm whether LeERF2/TERF2 regulates ethylene response by modifying its biosynthesis, we examined the response of TERF2-OE to Glc during germination and...
growth. Our results show that when seeds were germinated in half-strength MS medium containing 7% Glc, germination was about 50% in TERF2-OE but only 7% in the wild type (Fig. 3A). In addition, the effect of ethylene during germination is known to be opposite that of ABA (Ghassemian et al., 2000). In our investigation, TERF2-OE also decreased the sensitivity to ABA during germination: germination in TERF2-OE seeds was twice as high as that in wild-type seeds on half-strength MS medium containing 0.5 to 1 mM ABA (Fig. 3B).

Our results also showed that, under light, hypocotyls of TERF2-OE lines were nearly twice as long as those in the wild type (Supplemental Fig. S2A). To analyze whether TERF2-OE brings about hypocotyl elongation by up-regulating the production of ethylene, we analyzed the effects of inhibitors of ethylene biosynthesis and activation on hypocotyls in TERF2-OE seedlings. We found that the ethylene biosynthesis inhibitors aminoethoxyvinylglycine and CoCl₂ and the ethylene activation inhibitor AgNO₃ significantly decreased hypocotyl elongation in transgenic tobacco when grown under light (Supplemental Fig. S2A), indicating that LeERF2/TERF2 might affect the response of ethylene by regulating its biosynthesis. This observation supports the reports that ethylene can promote elongation of hypocotyls by activating the ethylene-signaling pathway (Smalle et al., 1997; Alonso et al., 1999). In addition, we found that TERF2-OE seedlings showed greater resistance to Glc and ABA.

**Figure 2.** TERF2-OEm increases while TERF2-RI decreases the production of ethylene by regulating the expression of ethylene biosynthesis genes. A, Expression of LeERF2/TERF2 in transgenic tomato. After reverse transcription, the synthesized cDNA was used as a template for RT-PCR amplifications. The mRNA levels were detected with ethidium bromide staining. B, Production of ethylene in wild-type (WTM), TERF2-OEm, and TERF2-RI plants was measured in three independent assays with three plant samples each. The data show ethylene production by wild-type, TERF2-OEm, and TERF2-RI plants, expressed in terms of the increase over the control (sealed vials without any plant). FW, Fresh weight. C, Expression of LeACS and LeACO in the wild type, TERF2-OEm, and TERF2-RI by Q-PCR. The expression level of each gene in the wild type was standardized to 100, referring to the internal control of Actin. The assay was repeated three times. Error bars represent s.
than wild-type seedlings did (Supplemental Fig. S2B). Moreover, the ethylene synthesis inhibitor CoCl$_2$ restored the sensitivity to Glc and ABA (Supplemental Fig. S2B). These results strongly imply that LeERF2/TERF2 is involved in regulating ethylene biosynthesis.

**TERF1 and LeERF2/TERF2 Have Overlapping Roles in Activating the Expression of Biosynthesis Genes and Ethylene Production**

To confirm whether the responses associated with ethylene described above can be attributed to ethylene biosynthesis, production of ethylene by TERF2-OE was further analyzed using a gas chromatogram. As Figure 4A shows, the production of ethylene in TERF2-OE seedlings was 50% more than that in wild-type seedlings. Student’s $t$ tests indicated that the production of ethylene between wild-type and TERF2-OE seedlings was significantly different at 90% probability, indicating that TERF2-OE lines overproduce ethylene.

To find out how LeERF2/TERF2 regulates the production of ethylene, the expression of ACS and ACO in TERF2-OE and wild-type plants was analyzed by Q-PCR. This approach showed that TERF2-OE increased the expression of $NtACS1$ and $NtACS3$ by 3.5-fold and of $NtACO1$ by nearly 2-fold (Fig. 4B), suggesting that LeERF2/TERF2 ectopically regulates the expression of specific ACS genes in tobacco.

We reported earlier that tobacco overexpressing *TERF1* (TERF1-OE) displays the phenotype of an ethylene-associated response, such as the triple response (Huang et al., 2004), implying that TERF1 may also affect ethylene production. In fact, TERF1-OE plants also produced about 3 to 4 times as much ethylene as wild-type plants did (Fig. 4A). TERF1-OE also increased the expression of $NtACS1$ and $NtACS3$ by 4-fold to 6-fold (Fig. 4B). Thus, our results indicate that TERF1 and LeERF2/TERF2 have overlapping roles in ethylene production and that both act by regulating the expression of genes responsible for ethylene biosynthesis.

**GCC Box of the ACS3 Promoter Is Essential for Transcriptional Interaction of TERF1 or LeERF2/TERF2**

To further elucidate the regulatory mechanism of the expression of $NtACS3$, we cloned the upstream sequences of $NtACS3$ with a 1,239-bp length from the translation start code ATG by inverse PCR amplification. By analyzing the promoter sequence using PLACE, we found that the $NtACS3$ promoter contains a GCC box (AGCCGCC) between –276 and –272 bp and a DRE/CRT-like sequence (ACCGCA) between –1,097 and –1,092 bp. Based on these characteristics of the sequence, we found that the interaction of TERF1 or LeERF2/TERF2 with the $NtACS3$ promoter enhanced the activity of GUS by 35-fold or 20-fold, respectively, compared with that in the control (Fig. 5A), indicating that TERF1 and LeERF2/TERF2 activate the expression of $NtACS3$ in tobacco, which is also consistent with the observation that TERF1-OE produced more ethylene in tobacco (Fig. 4A). We then generated promoters of different lengths by deleting GCC box and the DRE/CRT-like sequence and transforming the resulting promoter:GUS plasmids into *Agrobacterium tumefaciens*. TERF1 or LeERF2/TERF2 binding to the promoter of $NtACS3$ was characterized by transient expression assays in tobacco leaves. We found that GUS activity was highest in the full-length promoter and lowest in the promoter in which GCC box sequence had been deleted, whereas deletion of the DRE/CRT-like sequence of the $NtACS3$ promoter had little effect on the expression of the GUS reporter after the interaction of TERF1 or LeERF2/TERF2 with the promoter of $NtACS3$ (Fig. 5A). These results suggest that GCC box is the most important for the activation of TERF1 and LeERF2/TERF2 in the expression of $NtACS3$. To examine the intensity of the interaction of TERF1 or LeERF2/TERF2 with GCC box, we cloned the $NtACS3$ promoter with the GCC box of the ACS3 Promoter Is Essential for Transcriptional Interaction of TERF1 or LeERF2/TERF2
deletion of the 56-bp fragment containing GCC box into pCAMBIA1303. The transient expression assay showed that the deletion of GCC box prevented TERF1 and LeERF2/TERF2 from activating the expression of the GUS reporter gene (Fig. 5A). Our earlier study had shown that TERF1 binds physically to GCC box in vitro to activate the expression of such genes in tobacco (Huang et al., 2004). Accordingly, we focused our research on the interaction of LeERF2/TERF2 with GCC box. To confirm these findings, a synthetic and a mutated GCC box of the \textit{NtACS3} promoter, each replicated four times, were inserted upstream of the minimal promoter of 35S in pBI121. LeERF2/TERF2 binding to GCC box was then characterized by transient expression assays. Our results showed that LeERF2/TERF2 can activate the expression of GCC box but not of that driven by the mutant GCC box (Fig. 5B), indicating that LeERF2/TERF2 can directly identify GCC box of the \textit{NtACS3} promoter and regulate the expression of \textit{NtACS3} at the transcriptional level.

\textbf{LeERF2/TERF2 Specifically Interacts with GCC Box of the ACS3 Promoter in Vitro and in Vivo}

To identify the physical interaction of LeERF2/TERF2 with GCC box or DRE, we first produced a recombinant His-TERF2 fusion protein containing a six-His tag in \textit{Escherichia coli}. Through electrophoretic mobility shift assays, we established that LeERF2/TERF2 binds physically to GCC box in vitro to activate the expression of such genes in tobacco (Fig. 6A). To clarify the interaction of LeERF2/TERF2 with GCC box in tobacco further, a chromatin immunoprecipitation assay was performed to support our conclusion. First, we transiently expressed the TERF2-His fusion protein in tobacco in which the GFP-His fusion protein was used as the negative control. Specific primers were designed for the \textit{NtACS3} promoter from –508 to –223 bp containing the GCC box area, whereas primers from tobacco \textit{NtActin} were used as the negative control. Our results showed that fragments of the \textit{NtACS3} promoter and \textit{NtActin} were present in the input samples of TERF2-His and GFP-His. After affinity selection, only the \textit{NtACS3} promoter was detectable in the eluate of TERF2-His but no amplification occurred in the extract of GFP-His (Fig. 6B), demonstrating that, in tobacco, LeERF2/TERF2 specifically binds to GCC box of the \textit{NtACS3} promoter in vivo.

An earlier study had reported that amino acids of the ERF domain play a key role in DNA-binding activity (Allen et al., 1998). To further establish the specific binding of LeERF2/TERF2 to GCC box of the \textit{NtACS3} promoter, we fused the deleted ERF domain and full-length LeERF2/TERF2 to pB42AD (Clontech). GCC box of \textit{NtACS3} was cloned upstream of the reporter vector \textit{pLacZ}. In the yeast one-hybrid system, full-length LeERF2/TERF2 strongly activated the expression of the \textit{LacZ} reporter, but LeERF2/TERF2 in which the ERF domain had been deleted did not (Fig. 7A). Following the analysis of the interaction of the ERF DNA domain with GCC box reported by Allen et al. (1998), we further analyzed the characteristics of the binding of LeERF2/TERF2 to GCC box of the \textit{NtACS3} promoter by amino acid mutation of the LeERF2/TERF2 ERF domain. In total, 13 amino acids of the LeERF2/TERF2 ERF domain were mutated to Ala. The results demonstrated that R84A and W86A evidently resulted in the loss of binding activity of LeERF2/TERF2 to GCC box of the \textit{NtACS3} promoter.
in yeast, although this ability was retained in other mutations, including V85A (Fig. 7A).

To confirm this result, we examined the effect of the LeERF2/TERF2 ERF mutation on binding to GCC box of the NtACS3 promoter in tobacco using transient expression analysis. Consistent with the results from the yeast system, except R84A, which retained a weak signal in the assays (Fig. 7B), we found that Trp-86 is the essential amino acid involved in the interaction with GCC box of the NtACS3 promoter.

**DISCUSSION**

Regulation of ethylene production plays an important role in plant development and in plant responses to stress, but very little is known about how the regulation of ethylene biosynthesis and expression of ACS and ACO genes are modulated at the transcriptional level. Therefore, it is important to identify the elements and transcription factors involved in this process. Our finding that the LeERF2/TERF2 transcription factor regulates the expression of ACS genes in tobacco and of both ACS and ACO genes in tomato offers new evidence of the regulatory functions of ERF proteins in ethylene biosynthesis.

Ethylene gas is difficult to preserve in plants; therefore, control of ethylene biosynthesis plays a key role in its physiological function. Accumulated evidence shows that the expression of ACS and ACO is induced by various factors, including indole-3-acetic acid, physical injury, cytokinins, ABA, brassinosteroid, ethylene, and other forms of stress (Tsuchisaka and Theologis, 2004; Wang et al., 2005; Hansen et al., 2009). A growing number of studies have reported that multiple regulations are involved in the expression of ACS and ACO (Matarasso et al., 2005; Ito et al., 2008; Lin et al., 2008). For example, tomato LeCp is a dual-function protein that acts as a Cys protease in the cytoplasm and can be SUMO modified to enter the nucleus to directly activate the expression of tomato LeACS2 following attack by a pathogen (Matarasso et al., 2005). The MADS box transcription factor RIN plays an important role in regulating the expression of

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**Figure 5.** LeERF2/TERF2 identifies GCC box of the NtACS3 promoter in in vivo transient expression assays. A, Interaction of LeERF2/TERF2 or TERF1 with the promoter of NtACS3 in vivo. P1, P2, P3, and dGCC indicate that the GUS reporter gene was driven by 1,239-, 508-, and 248-bp sequences and the removal of GCC box of the NtACS3 promoter, respectively. B, GCC box of the NtACS3 promoter is essential for interaction with LeERF2/TERF2. 4GCC box and 4mGCC box indicate that four repeated GCC box or mutated GCC box sequences of the NtACS3 promoter, respectively, were inserted upstream of the minimal promoter of 35S in pBI121. Mini is the reporter containing the basic promoter TATA box upstream of the GUS reporter gene. TERF2 indicates the constitutive expression of full-length LeERF2/TERF2. None, as the negative control of the effector, indicates the empty vector plasmid. *Agrobacterium* containing the reporter vector and effector vector was simultaneously infiltrated into tobacco leaves. Relative GUS activity is the ratio of GUS activity in 35S:TERF2 to that in the empty vector. The assay was repeated three times. Error bars indicate s.e.
LeACS2 through both indirect and direct regulation during fruit ripening (Ito et al., 2008). Moreover, our research here shows that ERF proteins may also play an important role in regulating the expression of ACS and ACO.

Increasing studies have shown that ERF proteins play many roles in plant responses to biotic and abiotic stresses and in development and metabolism (Chakravarthy et al., 2003; Gutterson and Reuber, 2004; Huang et al., 2004; Wang et al., 2004; Zhang et al., 2005; Wu et al., 2008). For example, the ERF proteins SHINE and WAX1 are involved in the synthesis of wax in Arabidopsis and alfalfa (Medicago sativa) and enhance tolerance to salt and drought (Aharoni et al., 2004), demonstrating that, in addition to affecting the expression of genes involved in plant responses to stress, the proteins are also involved in the regulation of plant metabolism. Arabidopsis AtERF14 not only enhances resistance to Fusarium oxysporum but also affects flowering and seed development (Oñate-Sánchez et al., 2007). Moreover, the barley (Hordeum vulgare) ERF protein NUD can regulate lipid biosynthesis and thus affect the form of the hull (Taketa et al., 2008).

More detailed studies have shown that a wide range of functions of ERF proteins are based on their ability to bind to different DNAs that regulate the expression of different downstream genes (Ohme-Takagi and Shinshi, 1995; Park et al., 2001; Niu et al., 2002; Chakravarthy et al., 2003; Acevedo-Hernandez et al., 2005; Sasaki et al., 2007). For example, tobacco Tsi1 identifies GCC box and DRE/CRT in responding to biotic and abiotic stresses (Park et al., 2001), whereas ABI4 binds to GCC box, CE1, and S box in ABA, sugar, and light signal pathways (Niu et al., 2002; Acevedo-Hernandez et al., 2005). Our earlier studies have shown that TERF1 can identify GCC box and DRE/CRT (Huang et al., 2004). This study supplies additional evidence that LeERF2/TERF2 binds to GCC box of the NtACS3 promoter in vitro, an observation consistent with the report of Tournier et al. (2003). We also show that LeERF2/TERF2 activates the expression of NtACS3 by interacting with GCC box in vitro and in vivo. Furthermore, through biochemical and molecular analyses, we observed that the ERF domain of LeERF2/TERF2 is essential to the interaction with GCC box that activates the expression of NtACS3, which consequently leads to ethylene production in tobacco. Most importantly, LeERF2/TERF2 transcriptionally activates the expression of tomato ethylene biosynthesis genes, possibly through interacting with GCC box or DRE/CRT. More interestingly, some studies have analyzed the structure and regulatory elements of ACS and ACO genes (Lincoln et al., 1993; Blume et al., 1997; Blume and Grierson, 1997; Ito et al., 2008; Lin et al., 2008), showing that both a 10-bp sequence [AATA(AA)TATT] and a 9-bp sequence [AATA(A)TATT] in homeobox cis-elements play important roles in regulating the expression of LeACS2 (Ito et al., 2008).

Our research here shows that GCC box and DRE/CRT cis-elements are also involved in regulating the expression of ACS and ACO genes. Although the effect of LeERF2/TERF2 on GCC box and DRE/CRT is common in proteins of this kind, the involvement of ERF proteins in ethylene biosynthesis is a novel finding.

The observations that TERF1 and LeERF2 confer the triple response (Huang et al., 2004; Pirrello et al., 2006) and that TERF2-OE promotes hypocotyl elongation under light are similar to those made with the Arabi-
The expression of the lacZ reporter gene driven by four repetitions of GCC box was used to confirm the interaction of essential amino acids of LeERF2/TERF2 with the promoter of NtACS3. The reporter was the pCAMBIA 1303 vector, in which the full-length promoter of NtACS3 substituted for the 35S to drive the expression of the GUS reporter gene. Effectors TERF2, dERF, W84A, V85A, and W86A indicate the native and LeERF2/TERF2 ERF mutants in which the ERF domain had been deleted and mutations of W84A, V85A, and W86A, respectively. CaMV, *Cauliflower mosaic virus*; NOS, nopaline synthase.

Further research points to feedback regulation of ethylene signaling that results from direct up-regulation of EBF2 expression by EIN3 in Arabidopsis (Konishi and Yanagisawa, 2008). Moreover, accumulated evidence suggests that ethylene can activate or limit its own biosynthesis through positive or negative regulation of ACS gene expression and activity as well (McMurchie et al., 1972; Liu et al., 1985; Nakatsu et al., 1997, 1998; Barry et al., 2000; Alexander and Grierson, 2002). For example, ethylene treatment reduced the expression of LeACS1 and LeACS6 in seedlings and mature green fruit in tomato, indicating that high ethylene levels can down-regulate the expression of ethylene biosynthesis genes and ethylene production as well (Barry et al., 2000). Meanwhile, during

dopsis ethylene constitutive response mutant *ctr1* (Zhou et al., 1998; Alonso et al., 1999), indicating that TERF1 and LeERF2/TERF2 are both associated with the ethylene pathway. As discussed in the introduction, ERF proteins might be a downstream element in the ethylene pathway (Solano et al., 1998). Considering the novel regulation of ERF proteins in ethylene biosynthesis, it is possible that ERF proteins are involved in feedback regulation of ethylene production. Feedback regulation plays a very important role in phytohormone pathways. It has been proved that proteolysis of EIN3 is mediated by the E3 ubiquitin ligase SCF that contains either of the two closely related F-box proteins, EBF1 or EBF2 (Guo and Ecker, 2003; Potuschk et al., 2003; Yanagisawa et al., 2003).
biosynthesis of ethylene compared with wild-type
the regulation of ERF proteins in ethylene pathways
several genes that might be involved in ethylene
responses in ERF gene mutants, we have identified
Therefore, the observations that ethylene can induce
expressed antisense
ethylene. In our research here, we found that tomato
proteins may be involved in the feedback regulation
of ethylene. In ERF pathway (Solano et al., 1998); therefore, ERF
genes (Liu et al., 1985; Maunders et al., 1987; Barry et al., 1996;
Blume and Grierson, 1997; Nakatsuka et al., 1998; Alexander and Grierson, 2002). Of ERF proteins, ERF1
has proved to be a downstream element in the ethylene
pathway (Solano et al., 1998); therefore, ERF
proteins may be involved in the feedback regulation of ethylene. In our research here, we found that tomato expressing antisense LeERF2/TERF2 significantly decreased the expression of LeACS and LeACO and the biosynthesis of ethylene compared with wild-type tomato and tomato overexpressing LeERF2/TERF2. Therefore, the observations that ethylene can induce the expression of LeERF2/TERF2 and that LeERF2 activates the biosynthesis of ethylene through the expression of ACS and ACO genes in tomato support the contention that LeERF2/TERF2 is a positive regulator in the feedback loop of ethylene production.
Although both TERF1 and TERF2 have similar effects on the expression of genes involved in ethylene synthesis in tobacco, several differences between them may affect ethylene production. One difference is that the expression of promoter:GUS driven by TERF1 in tobacco is much higher than that driven by LeERF2/ TERF2, supporting the observation that TERF1-OE produced more ethylene in tobacco. Additionally, some transcription factors can form homodimers or heterodimers to regulate the expression of tagged genes. For example, the ethylene-responsive transcription factor Tspl1 can interact with the DnaJ-type zinc finger protein Tsip1 to increase Tspl1-mediated transcription, resulting in greater resistance to pathogens and greater tolerance to salt (Ham et al., 2006), while the HD-Zip protein LeHB-1 can regulate the expression of downstream genes in homodimers (Lin et al., 2008). Similarly, we found that TERF1 and LeERF2/TERF2 have different cofactors, because, in vitro, LeERF2/TERF2 interacts with the tobacco bZIP transcription factor BZI-3/TBZF, whereas TERF1 does not (data not shown), suggesting that LeERF2/TERF2 may need cofactors to regulate the expression of ACS genes.
For the limitation of available mutants that show ethylene overproduction or deficit in tobacco and tomato, we searched the Arabidopsis database but did not find any homolog genes of TERF1 and LeERF2/ TERF2. However, by screening ethylene-related responses in ERF gene mutants, we have identified several genes that might be involved in ethylene biosynthesis and will continue our investigation of the regulation of ERF proteins in ethylene pathways using genetic and biochemical approaches.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All plants were grown in growth chambers maintained at 25°C with 16 h of light per day from cool-white fluorescent lights of about 150 μmol m–2s–1. For ethylene treatment, 4-week-old wild-type tomato plants (Solanum lycopersicum [f. sp. Lycopersicon esaculentum] ‘Lichun’) were sealed in an incubator containing 100 μL L–1 ethylene (Tanaka et al., 2005). For the analyses of ethylene biosynthesis genes, 6-week-old tobacco plants (Nicotiana tabacum ‘NC89’) were used. Total RNA from the above material was extracted following the Trizol protocol (Invitrogen), and northern blotting was performed as described earlier (Huang et al., 2004; Zhang et al., 2005).

Quantification of Ethylene Biosynthesis

Ethylene production by tobacco and tomato was measured with a gas chromatograph (Hitachi) as described by Kim et al. (2003). Two 4-week-old tomato seedlings or 2-week-old tomato seedlings were used for determination of ethylene. For inducing ethylene, tomato seedlings were first sealed in 20-mL vials containing 5 mL of MS medium. Vials with the medium but without the seedlings served as controls. All of the capped vials were injected with 100 μL L–1 ethylene. After fumigation for 24 h, 0.5-mL samples of the air inside the vials were used for determination of ethylene content with a gas chromatogram. Levels of ethylene in the vials that contained wild-type, TERF2-OEm, and TERF2-RI plants were compared with those in the control vials.

Screening with Yeast One-Hybrid Analysis

The construction of reporter plasmids and the tomato cDNA library, and the screening procedure with yeast one-hybrid analysis, followed the protocol described by Wang et al. (2004a).

Generation of Transgenic Tobacco and Tomato

The generation of tobacco overexpressing TERF1 was described by Huang et al. (2004). Similarly, full-length sense and antisense LeERF2/TERF2 cDNA was separately cloned into pROK2 by Xhol and KpnI under control of the cauliflower mosaic virus 35S promoter. The resulting pROK-TERF2 was separately introduced into tobacco (cv NC89) and tomato (cv Lichun), while pROK-TERF2-antisense was introduced into tomato (cv Lichun), through Agrobacterium tumefaciens-mediated transformation. The transformed plants were selected on the basis of their resistance to kanamycin. For tobacco transformation, seven lines overexpressing LeERF2/TERF2 were confirmed by RT-PCR from 12 independent lines, and five of these showed a consistent phenotype of ethylene. For tomato transformation, five lines overexpressing LeERF2/TERF2 were confirmed by RT-PCR from eight independent lines, and three of these showed consistent ethylene production; six lines expressing antisense LeERF2/TERF2 were confirmed by RT-PCR from nine independent lines, and two of these showed consistent ethylene production.

In order to exclude the interference of antisense LeERF2/TERF2 on other ERF genes, we analyzed the expression of LeERF1, LeERF3, LeERF4, and TERF1 in different TERF2-RI lines. Our results indicate that their expression was not obviously different compared with those in wild-type tomato. Thus, we assume that the antisense expression of LeERF2/TERF2 may specifically affect its self expression. In this study, TERF2-OE and TERF2-RI were used to designate tobacco overexpressing TERF1 and LeERF2/TERF2, respectively. Similarly, TERF2-OEm and TERF2-RI were used to designate tomato overexpressing LeERF2/TERF2 or expressing antisense LeERF2/TERF2, respectively. The numbers indicate the different transgenic lines.

Germination and Seedling Growth Assays

All seeds in the following assays were first surface sterilized and kept at 4°C for 3 d to break their dormancy. For the germination assay, seeds were sown on plates containing half-strength MS medium and different concentrations of ABA and Glc. Germinated seeds were counted every day by observing the emerging root. For the seedling growth assay, germinated seeds were placed on plates of half-strength MS medium with or without 5 μM ACC, 3 μM aminooxyacetic acid, 50 μM CoCl2, or 10 μM AgNO3, and incubated for 10 d.

RT-PCR and Q-PCR Assay

Total RNA was extracted from 6-week-old tobacco or 4-week-old tomato plants using Trizol (Invitrogen) according to the manufacturer’s recommen-
Thermal Asymmetric Interlaced PCR Assay

Thermal asymmetric interlaced PCR was performed using the same arbitrary degenerate primers as described by Liu et al. (1995). The genomic DNA of NC89 was used as a template. The specific primers for NtACS3 are listed in Supplemental Table S1.

Vector Construction and Generation of Mutations

To construct the reporter vector driven by the NtACS3 promoter, fragments of different lengths were produced by PCR amplification. The primers used for generating NtACS3 promoters of different lengths are listed in Supplemental Table S1. The resulting fragments of the promoter were cloned into pCAMBIA1303. The effector vector of LeERF2/TERF2 is described above, and the empty vector pROK2 was used as the negative effector vector control.

To produce the 4-fold native GCC box reporter, the synthetic 4-fold native GCC box (`5'-ACATAAGAAGCCGCCGACCCCTGCAT-3') and mutant GCC box (`5'-ACATAAGAAGCCGCCGACCCCTGCAT-3') with 5'-end restriction site EcoRI and 3'-end restriction site XhoI were first annealed and purified and then subcloned into the reporter vector pLaZi containing the minimal promoter of the Cyclin gene. Subsequently, DNA fragments containing 4×GCC box and the minimal Cyclin promoter were ligated into pBlB121 between the HindIII and BamH1 enzyme sites. To construct the vector for the TERF2-His fusion protein, the overexpressing vector of TERF2-His was constructed with PCR amplification using the plasmid pB42AD-TERF2 as a template. Full-length TERF2-His was then cloned into pCAMBIA 1303 by BgIII and HindIII under control of the 35S promoter. The GFP-His fusion protein in pCAMBIA 1303 was used as the negative control.

Mutations of the LeERF2/TERF2 ERF domain were produced by PCR with special primers listed in Supplemental Table S1 using pB42AD-TERF2 as a template. PCR production was self-linked with T4 DNA ligase and was followed by transformation into Escherichia coli. The resulting mutations of the LeERF2/TERF2 ERF domain were separately constructed into pROK2.

Transient Expression Assays, Fluorimetric Measurement, and Histochemical GUS Staining

Tobacco leaves were first infiltrated simultaneously with Agrobacterium containing the reporter vector and the effector vector. After growing for 36 h in the dark, the infected leaves were used for analyzing the activity of GUS as containing the reporter vector and the effector vector. After growing for 36 h in the dark, the infected leaves were used for analyzing the activity of GUS as containing the reporter vector and the effector vector. After growing for 36 h, the infected leaves were used for analyzing the activity of GUS as described by Yang et al. (2000). The mixtures containing 12 μg of recombinant LeERF2/TERF2, 2 ng of binding probe (8 × 10^4 cpm), 2 μg of salmon DNA, 20 μg HEPEs-KOH (pH 7.5), 50 μM KCl, 0.1 mM EDTA, 10% glycerol, and 0.5 μM dithiothreitol in a 10-μL reaction volume were incubated at room temperature for 15 min and then separated on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA buffer. Subsequently, the gel was exposed to Imaging Screening-K and examined with Molecular Imager FX (Bio-Rad).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation was conducted as described by Bowler et al. (2004). Briefly, tobacco leaves (2 g) transiently infiltrated for 36 h with Agrobacterium containing pCAMBIA1303-GFP-His or pCAMBIA1303-TERF2-His were fixed with 1% formaldehyde in phosphate-buffered saline at room temperature for 10 min with gentle agitation. After two washes with 40 mL of ice-cold phosphate-buffered saline, the samples were grown to extract the proteins and DNA. The chromatin solution was then sonicated to shear the DNA into fragments. After centrifuging, the chromatin pellet was resuspended in 300 μL of buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. The above solution was divided into three portions, and 900 μL of buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl [pH 8.0], and 167 mM NaCl) was added for every 100 μL of solution. After adding 40 μL of salmon sperm-sheared DNA to each chromatin sample with gentle agitation and overnight incubation at 4°C, the pellet was rinsed three times and resuspended in a buffer containing 50 mM NaPO4 (pH 8.0), 167 mM NaCl, 10 mM imidazole, and protease inhibitors. The input solution was then used for performing immunoprecipitation using anti-His antibodies in a process known as technical blunting of His-Select Nickel affinity gel (Sigma P6611). After washing first with a high-salt buffer (500 mM NaCl, 0.2% SDS, 0.5% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl [pH 8.0]) and then with a LiCl buffer (0.25 mM LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), the column was eluted twice with TE buffer (10 mM Tris-HCl [pH 8.0]), and the eluates were concentrated and incubated at 65°C for 15 min with gentle agitation. After spinning, the supernatant was carefully transferred to a fresh tube. The precipitated DNA fragments were then extracted with phenol and precipitated with ethanol. PCR analysis was performed using equal amounts of DNA from the inputs and eluates using 5'-TCAATGCCCCTATTCCACTA-3' and 5'-CGTAAAGGATTTCCCATGAA-3' as primers for the NtACS3 promoter and the Actin gene in order to normalize sample loading and as the negative control. The primers of the Actin gene are given in Supplemental Table S1.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers Actin (X63606), NtACS3 (X69692), NtACS2 (AJ055002), NtACS3 (X98492), NtACO1 (Z55929), NtACO2 (X83229), NtACO3 (Z46349), LeActin (U60480), LeACO1 (U18057), LeAC5 (X95139), LeAC5 (U18055), LeAC5 (M88447), LeACO6 (AF167428), LeACO1 (X58273), LeACO2 (Y00478), LeACO3 (Z54199), and LeAC04 (A80131).

Supplemental Data

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

Supplemental Figure S1. Comparison of cis-acting elements in LeERF2/TERF2-targeted ethylene biosynthesis genes in tomato.

Supplemental Figure S2. Effects of overexpressing TERF2 on the elongation of hypocotyls and the sensitivity of ABA and Glc in tobacco seedlings.

Supplemental Table S1. The primers used in this article.

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