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Mini Review

Regulation of Ethylene-Induced Transcription of Defense Genes

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Ethylene-induced gene expression has been studied in systems in which the biosynthesis of ethylene is stimulated during developmental process such as ripening of fruit, senescence of flower petals, or during pathogen infection. Functional analysis of the promoters of these genes revealed that the ethylene-responsive *cis*-elements of fruit ripening genes and senescence genes differed from that of defense genes whose expression is induced by ethylene in response to pathogen infection. The ethylene-responsive element identified as the GCC box (AGCCGCC) is commonly found in the promoter region of the ethylene-inducible defense genes. The ethylene responsive element binding factors that interact with the GCC box were demonstrated to be the transcription factors, which respond to extracellular signals to modulate GCC box-mediated gene expression positively or negatively.

Key words: Ethylene — *Cis*-element — DNA-binding domain — Transcription factors — Defense response.

Abbreviations: ERE, ethylene-responsive element; ERF, ethylene-responsive transcription factor; PR, pathogenesis-related; TvX, xylanase from *Trichoderma viride*.

Introduction

Ethylene is an endogenous plant hormone that influences many aspects of plant growth and development, such as germination, senescence, epinasty, abscission, and fruit ripening and also participates in a variety of biotic and abiotic stresses (Abeles et al. 1992). Control of these processes by ethylene involves regulation of ethylene biosynthesis and perception followed by signaling to transcription factors that regulate ethylene-responsive genes. Molecular genetic studies using *Arabidopsis* have revealed a number of mutations that affects responses to ethylene throughout the life cycle and defined components of a common signal transduction pathway for ethylene-induced responses in plants (Ecker 1995, Bleecker 1999, Chang and Shockey 1999). An alternative approach is to use ethylene-inducible genes to define the regulatory DNA elements that mediate transcriptional activation and subsequently to identify the transcription factors that bind to the critical elements. Ethylene-responsive *cis*-regions and proteins that interact with these *cis*-regulatory regions have been identified in the tomato fruit *E4* gene (Montgomery et al. 1993, Coupe and Deikman 1997), the carnation senescence-related glutathione-*S*-transferase (*GST1*) gene (Itzhaki et al. 1994) and the tobacco defense genes (Meller et al. 1993, Ohme-Takagi and Shinshi 1995). These provide tools to link ethylene signal transduction pathway to gene transcription.

Ethylene-responsive cis-regulatory element

The biosynthesis of ethylene is stimulated prior to several developmentally programmed events and in response to environmental stresses, such as infection by pathogen. Ethylene-induced gene expression has been investigated in systems in which a rise of ethylene occurs during natural processes such as ripening of fruits, senescence of flower petals, or during pathogen infection (Deikman 1997). Among the different classes of ethylene-responsive genes, the most studied are defense genes whose expression is activated by ethylene in response to pathogen infection. Exogenous application of ethylene induces transcription of genes encoding class I basic chitinases, class I β-1,3-glucanase and other basic-type pathogenesis-related (PR) proteins. An analysis of the promoters of the defense genes revealed the presence of a conserved sequence referred to as a GCC box in their respective promoter regions (Ohme-Takagi and Shinshi 1990, Eyal et al. 1993, Hart et al. 1993). The GCC box functions as the ethylene-responsive element (ERE) that is necessary and sufficient for transcriptional regulation by ethylene in tobacco (Ohme-Takagi and Shinshi 1995, Shinshi et al. 1995).

The GCC box motif is not found in the regulatory regions of fruit ripening genes and flower petal senescence genes. Therefore, distinct *cis*-elements are likely to be involved in ethylene-regulated transcription during ripening and senescence. An 126 bp sequence within the promoter region of carnation *GST1* gene was shown to be necessary and sufficient for ethylene regulation during senescence of flower petals (Itzhaki et al. 1994). The ethylene-responsive region of fruit ripening gene was identified in tomato *E4* gene, and E4/E8BP-1 that interacts with the sequence was isolated (Montgomery et al. 1993, Coupe and Deikman 1997). However, the E4/E8BP binding site alone was not sufficient to confer the ethylene responsiveness, and it was concluded that at least two *cis*-element are

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required for ethylene-responsive transcription of the *E4* gene (Xu et al. 1996).

Transcription factors involved in ethylene-induced transcription

The GCC box (AGCCGCC) sequence that is commonly found in the 5′ upstream regions of ethylene-inducible defense genes was identified to be the core sequence for ethylene responsive transcription of the genes (Ohme-Takagi and Shinshi 1990, Ohme-Takagi and Shinshi 1995, Shinshi et al. 1995). Four different cDNAs encoding the ethylene-responsive transcription factors (ERF1 to ERF4, previously known as EREBP1 to EREBP4) that specifically interact with GCC box were isolated in tobacco (Ohme-Takagi and Shinshi 1995). ERFs contain a highly conserved DNA binding domain consisting of 58 or 59 amino acid residues that designated as ERF domain (Ohme-Takagi and Shinshi 1995, Hao et al. 1998). The solution structure of the ERF domain revealed that it is novel both in structure and in its mode of DNA recognition (Allen et al. 1998). Sequences made available through the Arabidopsis genome project indicates that numerous plant genes encode proteins that possess ERF domain (ERF protein), representing a large multigene family with many members in both dicots and monocots. Genes encoding ERF proteins have been found only in higher plants and not in yeast or other fungi.

It has been argued that the ERF domain is closely related to the AP2 domain (Weigel 1995, Okamuro et al. 1997). However, knowledge accumulated thus far has indicated that the AP2 and ERF domains belong to distinct families. Sequence alignment of extended family members between these two domains show <30% amino acid identity, whereas members of each family exhibit >60% sequence identity (Büttner and Singh 1997, Hao et al. 1998, Riechmann and Meyerowitz 1998). The recent report of the solution structure of the ERF domain has contributed direct evidence that amino acid residues within the ERF domain that interact directly with the GCC box are not present in the AP2 domain. Although detailed studies regarding this aspect have not been reported for the AP2 domain, the AP2 domain is likely to possess a mode of DNA recognition distinct from that of the ERF domain as well as a different DNA target sequence (Fig. 1). ERF proteins shown to bind to GCC box include ERFs of tobacco, Pti4/5/6 of tomato, AtERFs, AtEBP and ERF1 of *Arabidopsis* (Zhou et al. 1997, Büttner and Singh 1997, Solano et al. 1998, Fujimoto et al. 2000). The phylogenetic tree based on the ERF domain shows that these proteins can be divided into four subfamilies (Fig. 2).

Arabidopsis cDNAs encoding five different ERF proteins (AtERF1 to AtERF5) were isolated and their structure, DNA binding preference, transactivation ability and mRNA expression were analyzed (Fujimoto et al. 2000). Each AtERF was categorized into one of three classes based on the amino acid sequence identities within the ERF domain and in the region outside the ERF domain. AtERF1 and AtERF2 have a high degree of amino acid identity to ERF2 from tobacco and to Pti4

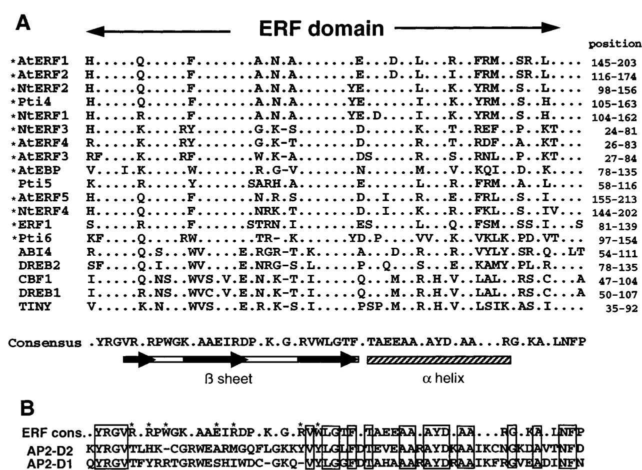

Fig. 1 ERF domain from various ERF proteins. (A) Alignement of the amino acid sequence of ERF domain from various ERF proteins. The asterisks represent those proteins that were shown to bind to the GCC box. Dots indicate amino acid identities; dashes indicate gaps introduced to maximize alignment. Numbers at right indicate the amino acid position of the ERF domain in each protein. The bar and black arrows indicate the β sheet and the β strands. The cross-hatched box indicates the α helix (Allen et al. 1998). DREB1, DREB2, CBF1, TINY, ABI4, AtEBP, ERF1, AtERF1, AtERF2, AtERF3, AtERF4, and AtERF5 are from *Arabidopsis*. Pti4, Pti5 and Pti6 are from tomato. NtERF1, NtERF2, NtERF3 and NtERF4 are from tobacco. (B) Comparison of the ERF domain consensus sequence with the AP2 domain. The consensus amino acid sequence of the ERF domain (ERF cons.) is compared with the D2 (AP2-D2) and D1 (AP2-D1) domains of APETALA2. The amino acids with asterisks in the ERF consensus indicate residues that interact with nucleotides within the GCC box. Amino acids identical to the ERF consensus are boxed.

from tomato (Ohme-Takagi and Shinshi 1995, Zhou et al. 1997, Fujimoto et al. 2000). Pti5 of tomato and *Arabidopsis* ERF1 contain similar sequence in the amino-terminal region that are conserved among this class of ERFs. AtERF3 and AtERF4 possess substantial amino acid identity within ERF domain to tobacco ERF3 and share a conserved sequence in the carboxy-terminal region. AtERF5 possesses sequence similarity to tobacco ERF4. All five AtERFs displayed GCC box-specific binding activity, but detailed binding experiments revealed that AtERF1, AtERF2 and AtERF5 are more sensitive to change in the GCC box sequence whereas AtERF3 and AtERF4 appear to be more flexible in target sequence recognition than other AtERFs (Fujimoto et al. 2000). Functional analyses revealed that AtERF1, AtERF2 and AtERF5 function as activators of GCC box-dependent transcription in plant cells. By contrast, AtERF3 and AtERF4 act as active repressors that can downregulate the transactivation activities of other transcription factors (Fujimoto et al. 2000). These indicate that a dynamic system utilizing antagonistic mechanisms for controlling GCC box-dependant transcription operate in plants.

The ETHYLEN-INSENSITIVE3 (EIN3) is an ethylene

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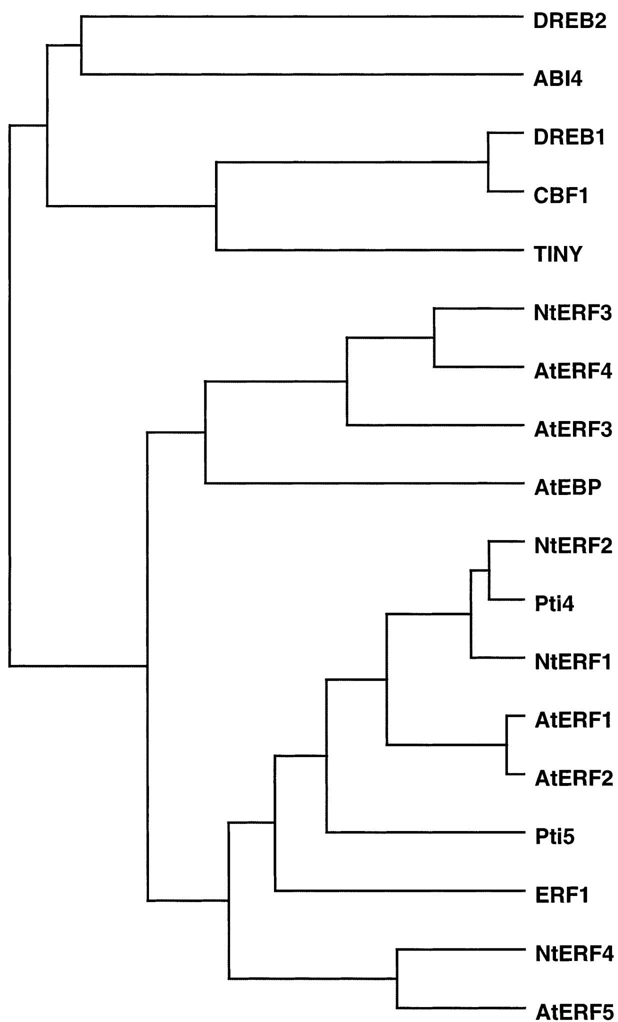

Fig. 2 Phylogenetic tree of ERF-domain proteins. A phylogenetic tree of the ERF proteins based on their ERF domains was generated by GENEWORKS software (IntelliGenetics). DREB1, DREB2, CBF1, TINY, ABI4, AtEBP, ERF1, AtERF1, AtERF2, AtERF3, AtERF4, and AtERF5 are from *Arabidopsis*. Pti4, Pti5 and Pti6 are from tomato. NtERF1, NtERF2, NtERF3 and NtERF4 are from tobacco.

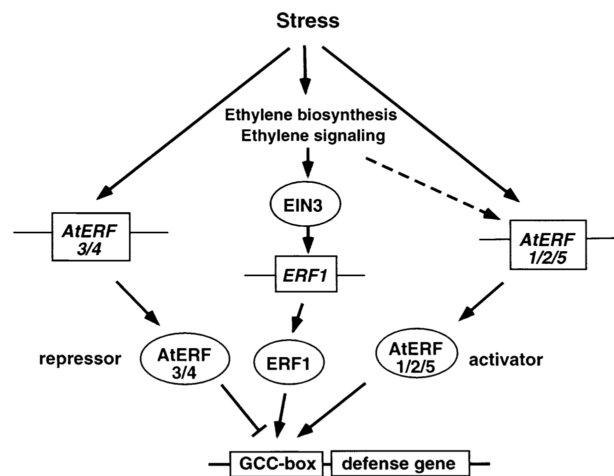

Fig. 3 GCC box-dependent ethylene inducible gene expressions mediated by ERF transcription factors in *Arabidopsis*. ERFs are ethylene-responsive element (GCC box) binding transcription factors. ERFs genes are upregulated via an ethylene-dependent pathway or an ethylene-independent pathway. Expression of ERF1 is directly regulated under ethylene signal mediator EIN3, while expressions of other AtERFs are partially regulated by ethylene signaling. ERF1, AtERF1, AtERF2, and AtERF5 activate a subset of GCC box-containing genes, whereas AtERF3 and AtERF4 repress the expression of these genes.

response regulator that controls ethylene-dependent transcription. In *ein3* mutant plants ethylene-inducible genes are not induced by exogenous ethylene, while overexpression of *EIN3* in transgenic *Arabidopsis* plants results in phenotype similar to that of the constitutive triple response1 (*ctr1*) mutant, indicating that EIN3 is an essential downstream component of ethylene signaling pathway (Chao et al. 1997). EIN3 and tobacco EIN3 homologue (TEIL) were identified to be sequence-specific DNA-binding protein (Solano et al. 1998, Kosugi and Ohashi 2000). EIN3 binds to the 5′ upstream region of *Arabidopsis ERF1* gene that is so called primary ethylene response element and the *ERF1* is considered as an immediate target of *EIN3* (Solano et al. 1998). Ectopic expression of the *ERF1* in transgenic *Arabidopsis* plants results in a constitutive activation of several ethylene-inducible genes that contains the GCC box in the promoter and in phenotype similar to that of the ctr1 mutant (Solano et al. 1998), indicating that the *Arabidopsis* ERF1 can activates the ethylene-inducible genes in vivo.

The *AtERF* genes respond differently not only to ethylene but also to various forms of abiotic stress, such as wounding, cold, high salinity, or drought (Fujimoto et al. 2000). *AtERF1*, *AtERF2* and *AtERF5* were induced by ethylene 12 h after treatment, whereas the ethylene response of *Arabidopsis ERF1* is observed within 2 h (Solano et al. 1998). Thus, the AtERFs may function later in the induction of ethylene-inducible genes. In contrast, responses of *AtERF* genes to abiotic stress occur much more quickly than those to ethylene. Induction of the *AtERFs* by wounding, cold, and drought appears to be independent from the ethylene signaling pathway because responses to these abiotic stresses were observed in the ethylene-insensitive2 (*ein2*) mutant plants. By contrast, induction of the genes encoding AtERF3 and AtERF4 by high salinity stress seems to be regulated by EIN2 (Fujimoto et al. 2000). Thus, *AtERF* expression appears to be controlled by a complex pathway that is independent from or dependent on ethylene signal transduction. AtERF proteins may function as transcription factors by activating or repressing the expression of GCC box containing genes dependent on or independent of the ethylene signal. Not all the ethylene-inducible GCC box containing

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genes are activated in the *Arabidopsis ERF1*-overexpression plants. *HOOKLESS1*, which contains a GCC box in its promoter is not induced in the *ERF1*-transgenic plants, suggesting that ERFs activate only a subset of GCC box containing genes (Solano et al. 1998). These suggest that each of ethylene-inducible gene may be regulated differentially and that multiple transcription factors are involved in GCC box-mediated transcription (Fig. 3).

Regulation of transactivation activities of ERF transcription factors

Multiple classes of ERFs with virtually identical DNA-binding properties are encoded in the plant genome. The sequence similarity among members of each class is restricted to the DNA-binding domain, and the limited similarities outside this domain suggest the possibility that each ERF exert its transactivation function differently. The transactivation functions of tobacco ERFs were examined by transient expression assays using tobacco protoplast and a heterologous system in yeast (Ohta et al. 2000). The tobacco ERF2 and ERF4 enhanced the GCC box-mediated transcription of a reporter gene in tobacco protoplasts. When fused to the DNA-binding domain of yeast GAL4, both amino- and carboxy-terminal regions of ERF4 functioned as transactivation domains, but only the carboxy-terminal region of ERF2 functioned as a transactivation domain in tobacco protoplasts. These results suggest that the mechanism of transactivation by ERF2 must be different from that by ERF4. The tobacco ERF2 and ERF4 functioned as transcriptional activators in yeast, and the amino terminal-regions of ERF2 and ERF4 functioned as transactivation domains in yeast, suggesting that ERF2 and ERF4 have basal transactivation activity which do not require ethylene-dependent modifications or plant-sepecific factors. In contrast to ERF2 and ERF4, ERF3 reduced the transcription of the reporter gene in tobaccco protoplasts and did not function as a transciptional activator in yeast. The ERFs were shown to be localized to nucleus in transient transfection experiments (Ohta et al. 2000). Thus, it appears that the tobacco ERFs exert their functions in different ways, with ERF2 and ERF4 being activators and ERF3 being a repressor of transcription. As the carboxy-terminal regions of ERF2 and ERF4 had transactivation activity in tobacco protoplasts while not in yeast cells, some cofactors specific to plant cells are likely to be required for the function of the carboxy-terminal regions. Alternatively, the activities of the carboxy-terminal regions might be regulated in signal-dependent manner.

The GCC box-mediated transcription of a reporter gene in transgenic tobacco plants is regulated by ethylene signal (Ohme-Takagi and Shinshi 1995, Suzuki et al. 1998). Molecular genetic studies of the ethylene signaling pathway in *Arabidopsis* have suggested that the plants sense ethylene signal through a protein kinase cascade (Ecker 1995). This suggestion is consistent with the results that an inhibitor of protein kinases prevented the ethylene induced activation of GCC box-mediated transcription of a reporter gene in tobacco plants (Suzuki et al. 1998). Thus, ERF proteins are also candidate targets in the ethylene signal-transduction pathway. Accumulation of mRNA for ERFs is induced by ethylene and wounding, and the genes for individual ERFs exhibit different patterns of expression. Therefore, increased rates of transcription of gene for ERFs might be involved in the ethylene-responsive regulation of GCC box-mediated transcription. The ethylene-responsive activation of the transcription of the GCC box reporter gene in transgenic tobacco plants was blocked by cycloheximide, while the wound induced accumulation of mRNA for the ERFs was not. However, transcription of the GCC box reporter gene was not activated by wounding, even though transcription of genes for the tobacco ERFs were activated (Suzuki et al. 1998). These results suggest that the expression of the ERFs might be insufficient for activation of the GCC box-mediated transcription of genes and, moreover, that the synthesis of protein factors possibly including ERFs might be required for the GCC box-mediated transcription of genes that is dependent on ethylene. Ethylene might act by converting ERFs to active forms that are capable of activating transcription when associated with the GCC box. This activation could involve changes in the extent of phophorylation and/or interaction of the ERFs with coactivators. A putative site for phosphorylation by proline-directed protein kinases such as mitogen-activated protein kinase (MAPK) can be found in the carboxy-terminal regions of tobacco ERF4 and AtERF5 (Fujimoto et al. 2000, Ohta et al. 2000).

By contrast, the constitutive expression of *Arabidopsis ERF1* in transgenic plants is shown to be sufficient to activate transcription of a subset of the ethylene-responsive GCC box containing target genes (Solano et al. 1998), which suggest that ethylene signal might be not required for the modification of transactivation activity of the ERF1 to activate transcription of a subset of endogenous genes. Transient transactivation assays showed that AtERF1, AtERF2 and AtERF5 activate the GCC box-mediated transcription of a reporter gene in the leaves of the ethylene-insensitive2 (*ein2*) mutant (Fujimoto et al. 2000). The results suggest that ethylene signal is not required for the AtERFs to activate transcription from a transiently transfected reporter gene template, however, it is necessary to study whether the modification of transactivation function is not required to activate transcription from a stably integrated reporter gene in transgenic plants.

Although the regulatory phosphorylation of ERFs by the components of the ethylene signal-transduction pathway has not been demonstrated, several types of protein-protein interaction involving members of the ERF family of proteins have been demonstrated in physical interactions and yeast two-hybrid systems (Büttner and Singh 1997, Zhou et al. 1997, Xu et al. 1998). ERF proteins of Pti4, Pti5 and Pti6 interact with Pto kinase encoded by disease resistance gene, suggesting that the Pto kinase may regulate the function of ERF proteins (Zhou et al. 1997). *Arabidopsis* ethylene-responsive element binding

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protein (AtEBP) interacts with an octopine synthetase element binding factor (OBF4; Büttner and Singh 1997), suggesting that defense gene expression is regulated via interaction between ERF protein and the basic-region leucine zipper protein. It is likely that multiple signal-transduction pathways converge on ERFs through a variety of protein-protein interactions in which different types of protein regulate different members of the ERF family.

Involvement of GCC box and ERFs in elicitor-responsive expression of defense genes

The GCC box has been found in the promoters of a number of defense genes, which are responsive to pathogen infection and elicitor treatment (Eyal et al. 1993, Hart et al. 1993, Ohme-Takagi and Shinshi 1990, Sessa et al. 1995, Zhou et al. 1997, Manners et al. 1998). Interestingly, however, the GCC box has yet to be found in the promoters of ethylene-regulated genes involved in some of the other ethylene responses, such as fruit ripening (Deikman 1997). These suggest the involvement of GCC box in transcriptional activation of defense genes during plant-pathogen interaction. The hypothesis was supported by the observation that the binding activity to DNA sequence of GCC box motif in a promoter region of gene for a class I β-1,3-glucanase was more abundant in nuclear extracts from plants showing the bacterial pathogen-induced hypersensitive reaction compared to uninfected controls (Alonso et al. 1995).

To date, it is clear that the GCC box has a key role as *cis*-acting element in ethylene-responsive transcription of genes (Shinshi et al. 1995, Ohme-Takagi and Shinshi 1995, Suzuki et al. 1998). Pathogen infection is known to induce the biosynthesis of ethylene (Abeles et al. 1992) and expression of the defense genes (Kombrink and Somssich 1995). Exogenous ethylene also induces the expression of such genes (Broglie et al. 1989, Felix and Meins 1987, Eyal et al. 1993). Therefore, it has been often proposed that ethylene, which is synthesized de novo during plant-pathogen interaction, acts through the GCC box to induce the transcription of a subset of defense genes (Kitajima and Sato 1999, Rushton and Somssich 1998, Deikman 1997). However, this has been hardly demonstrated.

Several recent reports showed that the GCC box-mediated transcription of genes does not always requires ethylene. A fungal elicitor, xylanase derived from *Trichoderma viride* (TvX), activates the GCC box-mediated transcription in tobacco cells, whereas the activation is shown to be independent of the ethylene biosynthesis even though the elicitor induced ethylene biosynthesis (Yamamoto et al. 1999). More recent report demonstrated that ERF homologs of *Arabidopsis* transactivated the GCC box-mediated transcription of reporter gene in leaves of an ethylene-insensitive mutant, *ein2* (Fujimoto et al. 2000). Yamamoto et al. (1999) showed that TvX also induced the expression of genes for ERFs in cultured tobacco cells. Interestingly, not only activation of GCC box-mediated transcription but also expression of *ERF2* gene induced by TvX required both protein phosphorylation and dephosphorylation (Yamamoto et al. 1999). It was demonstrated that Pti4 and Pti5, tomato homologues of the tobacco ERF2, interact directly with a protein kinase encoded by the *Pto* gene, which confers resistance to the bacterial pathogen *Pseudomonas syringae* pv. tomato (Zhou et al. 1997). In addition, it was shown that the Pti4 protein is phosphorylated by the Pto kinase and that this phosphorylation enhances binding of Pti4 to the GCC box (Gu et al. 2000). Infection with TMV induced expression of gene for ERF1 in tobacco (Horvath et al. 1998), and the expression of *Pti4* and *Pti5* in tomato was induced by infection with *Pseudomonas* (Thara et al. 1999). It was also shown that salicylic acid induced the expression of *ERF1* gene in tobacco cultured cells (Horvath et al. 1998) and of *Pti4* and *Pti5* genes in tomato leaves (Thara et al. 1999). However, induction of expression of genes for Pti4 and Pti5 by *Pseudomonas* infection was independent of ethylene and salicylic acid (Thara et al. 1999). Therefore, it is very likely that the GCC box, ERF1/ERF2 and their orthologs play a pivotal role in the elicitor-responsive activation of transcription of defense genes mediated by protein kinase cascade independently of ethylene and salicylic acid during early defense response of plants to pathogen infection.

Responses of ERF genes to various extracellular stimuli

After the first finding by Ohme-Takagi and Shinshi (1995), several reports also showed ethylene-inducible expression of genes for ERF proteins, including AtEBP, *Arabidopsis* ERF1, and AtERF1, 2, 4, and 5 in *Arabidopsis* (Büttner and Singh 1997, Solano et al. 1998, Fujimoto et al. 2000), and Pti4 in tomato (Thara et al. 1999). By contrast, it was shown that immediate early induction of *ERF* genes was triggered by cutting and cycloheximide independently of ethylene in tobacco leaf strips (Suzuki et al. 1998). Wound-responsive expression of *ERF*-related genes was also shown in *Arabidopsis* (Fujimoto et al. 2000) and tomato (Thara et al. 1999). By using an ethylene-insensitive mutant, *ein2*, of *Arabidopsis*, it was also demonstrated wound-inducible and ethylene-independent expression of genes for AtERFs (Fujimoto et al. 2000). We have also found that wounding rapidly activates local and systemic expression of genes for ERF2, ERF3, and ERF4 in tobacco plant, and that the wound- and cycloheximide-responsive expression of gene for ERF3 is regulated by rapid activation of transcription in a tobacco plant (Nishiuchi, Suzuki, Kitajima, Sato and Shinshi, submitted). The results also indicate that these ERF genes are responsive to touch, hyperosmotic condition, salinity stress and treatment with cycloheximide. Interestingly, wound-responsive expression of ERF3 and ERF4 seems not to require biosynthesis of jasmonic acid, while jasmonic acid and methyl jasmonic acid induce expression of these ERF genes (Nishiuchi, Suzuki, Kitajima, Sato and Shinshi, submitted, Horvath et al. 1998). Similarly, differential induction of expression of AtERFs in response to several external stimuli, such as cold, drought, cycloheximide, and NaCl, was demonstrated (Fujimoto et al. 2000). The expression of *ERF* genes is also developmentally regulated. During seed germination of tobacco,

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expression of genes for ERF3 and ERF4 but not ERF1 was induced and such expression of *ERF* genes seemed to require gibberellin (Leubner-Metzger et al. 1998).

The expression of genes for ERFs are regulated by a divers array of extracellular stimuli, including biotic and abiotic stress and possibly developmental signals as well as ethylene. Multiple signal-transduction pathways seem to converge on ERFs and GCC box element through a variety of protein–protein and protein–DNA interactions. These suggest important roles of ERFs in control of transcription of genes via target *cis*-element for acclimation to environmental changes.

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