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Differential regulation of tomato ethylene responsive factor *LeERF3b*, a putative repressor, and the activator *Pti4* in ripening mutants and in response to environmental stresses

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Summary

Ethylene responsive transcription factors (ERFs) can be grouped into different classes with either gene activator or repressor activity. We have isolated a tomato ERF cDNA clone (*LeERF3b*) with sequence similarity to class II (repressor class) of the ERF family, which is regulated differently from *Pti4* (a tomato ERF domain-containing gene that activates other genes). *LeERF3b* has similarities to other tomato ERF cDNAs but the DNA or predicted amino acid sequences have significant differences. Northern analysis showed that *Pti4* was highly expressed during fruit ripening, whereas *LeERF3b* accumulated before and declined sharply after the onset of ripening. Furthermore, *Pti4* mRNA was significantly reduced in low-ethylene tomato fruit containing an ACC oxidase sense-suppression transgene and also in the ethylene insensitive mutant never ripe (*Nr*). By contrast, the *LeERF3b* mRNA was markedly increased in those fruits. Environmental stresses including drought, desiccation and low temperature increased significantly the expression level of *LeERF3b*, but markedly reduced the level of *Pti4* mRNA. Conversely, wounding induced the accumulation of *Pti4* mRNA, but had no significant effect on the level of *LeERF3b*. These opposing patterns of regulation of mRNA accumulation are consistent with the activator function of *Pti4* and a repressor function for *LeERF3b* in ethylene responses.

Abbreviations: B, breaker stage of fruit ripening; CaMV, cauliflower mosaic virus; dpa, day post-anthesis; ERF, ethylene-responsive factor; MG, mature green; *Nr*, never ripe

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Introduction

Plants encounter a wide range of abiotic stresses, such as wounding, salinity, UV irradiation, high or low temperature, drought and biotic stresses such as plant pathogen attacks. To adapt to these stresses, plants modulate the expression of specific sets of genes (Ishitani et al., 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Lund et al., 1998) by using diverse and sophisticated signaling strategies. These involve transcription factors that regulate the expression of downstream genes by specifically binding to cis elements or forming transcriptional complexes with other proteins.

Ethylene-responsive factors (ERFs), formerly called ethylene-responsive element binding proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995), are DNA-binding proteins and members of a novel family of transcription factors specific to plants. They contain a highly conserved DNA binding domain (designated as the ERF domain) (Hao et al., 1998) consisting of 58 or 59 amino acids (Ohme-Takagi and Shinshi, 1995) and recognize the GCC box (AGCCGCC; Ohme-Takagi and Shinshi, 1990), which is the unique feature of this protein family. This sequence was determined to be essential for the expression of several pathogenesis related (PR) genes (Sessa et al., 1995; Shinshi et al., 1995; Sato et al., 1996) and to be the core sequence of the ethylene-responsive element (ERE) in tobacco(Ohme-Takagi and Shinshi, 1995). It was suggested that ERFs are factors that respond to extracellular signals to modulate GCC box-mediated gene expression positively or negatively. To date, genes encoding ERF proteins have been found only in higher plants and not in yeast or other fungi.

The ERF proteins were first isolated as GCC box-binding proteins from tobacco (*Nicotiana tabacum*) (Ohme-Takagi and Shinshi, 1995). Based on amino acid sequence identities within the ERF domain, each ERF was categorized into one of three classes (Fujimoto et al., 2000). The class-I ERFs have an acidic domain in the N-terminal region and possess a region rich in basic amino acids (P/L-K-K/R-R-R) that could serve as a putative nuclear localization signal (Raikhel, 1992). Two cysteine residues flanking the ERF domain are also conserved. AtERF1 and AtERF2 from *Arabidopsis* and ERF2 from tobacco (Ohme-Takagi and Shinshi, 1995) belong to this class. The characteristic features of class II ERFs are that the ERF domain is located close to the N-terminus of the protein and that it consists of 58 amino acids, one residue shorter than class-I ERFs. The class-II ERFs possess a C-terminal acidic domain and AtERF3 and AtERF4 from *Arabidopsis* and ERF3 from tobacco belong to this class. The class-III ERF genes have the longest coding region among the ERF proteins isolated thus far, with acidic domains located in both the N- and C-terminal regions flanking the ERF domain. In addition, a putative mitogen-activated protein (MAP) kinase phosphorylation site (PXXSPXSP, in which X represents any amino acid) (Pearson and Kemp, 1991) is conserved in the C-terminal region of class-III ERFs. AtERF5 from Arabidopsis and ERF4 from tobacco belong to this class.

It has been shown that the ability of the class-III proteins to regulate transcription in plant cells is stronger than that of class I (Fujimoto et al., 2000; Ohta et al., 2000). The class II ERF proteins are repressors and downregulate not only the basal transcription level of a reporter gene but also the transactivation activity of other transcription factors (Fujimoto et al., 2000). Furthermore, the class II ERF proteins appear to be more flexible than other ERF proteins with respect to their target sequence preference because they were able to bind to several mutated oligonucleotides to which other ERF proteins did not bind. This binding flexibility implies that class II ERF proteins might interact with genes with which other classes of ERF proteins cannot (Fujimoto et al., 2000).

In *Arabidopsis*, it has been shown that the ethylene signalling component EIN3 binds a primary ERE in the promoter of ERF1. EIN3 expression is both necessary and sufficient for ERF1 transcription and, like EIN3 overexpression in transgenic plants, constitutive expression of ERF1 results in activation of a variety of ethylene response genes and phenotypes (Solano et al., 1998).

A number of tomato genes in the gene bank show homology to the ERF gene family from *Arabidopsis* and from tobacco. Among them, *Pti4*, *Pti5* and *Pti6*, which were identified with the yeast two-hybrid system, physically interact with the Pto kinase, which is involved in the defence system (Zhou et al., 1997). The induction of *Pti4* and *Pti5* in either the compatible or incompatible interactions was originally thought to be independent of salicylic acid, ethylene and jasmonic acid (Thara et al., 1999). Further study confirmed that *Pti4* was induced by ethylene and salicylic acid and was phosphorylated by the Pto kinase (Gu et al., 2000). Overexpression of *Pti4* in *Arabidopsis* induced the expression of GCC box-containing genes (Wu et al., 2002) and conferred enhanced resistance to pathogen attack (Gu et al., 2002). *Pti4* appears to regulate gene expression directly by binding the GCC box and possibly a non-GCC box element and indirectly by either activating the expression of transcription factor genes or interacting physically