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Molecular characterization of two ethylene response factor genes in sweetpotato that respond to stress and activate the expression of defense genes in tobacco leaves

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ABSTRACT

Two ethylene response factor (ERF) genes, *IbERF1* and *IbERF2*, were isolated from a library of expressed sequence tags (EST) prepared from suspension-cultured cells and dehydration-treated fibrous roots of sweetpotato (*Ipomoea batatas*). The deduced IbERFs contained a nuclear localization signal and the AP2/ERF DNA-binding domain. RT-PCR analysis revealed that *IbERF1* was expressed abundantly during the growth of suspension-cultured cells, whereas the expression levels of *IbERF2* transcripts were high in fibrous, thick pigmented roots. Two ERF genes also showed different responses to various types of abiotic stress and pathogen infection. Transient expression of the two ERF genes in tobacco (*Nicotiana tabacum*) leaves resulted in increased transcript levels of the pathogenesis-related 5 (PR5) gene, the early response to dehydration ten gene (ERD10), the CuZn superoxide dismutase gene (CuZnSOD) and the catalase gene (CAT). It is suggested that the two ERF genes play roles in the stress defense-signaling pathway as transcriptional regulators of the PR5, ERD10, CuZnSOD and CAT genes.

Introduction

Abiotic and biotic stresses, such as drought, high salinity and pathogen infection, have adverse effects on plant growth and development. To survive, plants have involved defense mechanisms to perceive signals from their surroundings and to respond to various stresses by modulating the expression of responsive genes (Fujita et al., 2006). Transcription factors function in signal transduction to activate or suppress defense gene expression, as well as in the regulation of interactions between different signaling pathways (Gutterson and Reuber, 2004; Fujita et al., 2006).

In the model plant *Arabidopsis*, more than 1500 genes encoding transcription factors have been reported (Riechmann et al., 2000). Among these, apetala2/ethylene-responsive element-binding protein (AP2/EREBP) transcription factors have important functions in the transcriptional regulation of biological processes related to growth and development, as well as in responses to environmental stimuli (Riechmann and Meyerowitz, 1998; Gutterson and Reuber, 2004). The *Arabidopsis* genome encodes 145 AP2/EREBP proteins. Recently Mizoi et al. (2012) classified these proteins into five families, namely AP2, RAV, DREB (dehydration-responsive element binding protein), ERF (ethylene response factor) and AT4G13040 orthologs, according to their similarities and the number of DNA-binding domains they contain. Plant ERF transcription factors involved in the ethylene signal cascade and the expression of various defense-related genes are regulated directly by their DNA-binding activity to GCC-box (GCCGCC) or the dehydration-responsive element/C-repeat element (DRE/CRT, CCGAC) located in the promoters of various pathogenesis-related (PR) and abiotic stress-responsive genes (Park et al., 2001; Gutterson and Reuber, 2004). For these reasons, it is important to elucidate the mechanisms underlying the transmission of stress signals so that ERF regulation can be manipulated to improve crop stress resistance.

Plant ERF transcription factors are widely involved in abiotic and biotic stress responses. Particularly, many ERF genes are involved in disease resistance responses. Overexpression of some ERF genes, such as *Pti4*, *ERF1*, *OPBP1* and *TSRF1*, confer resistance to fungal and bacterial pathogens in transgenic plants (Berrocal-Lobo et al., 2002; Gu et al., 2002; Onate-Sánchez and Singh, 2002; Guo et al., 2004; Zhang et al., 2004a,b). Interestingly, multiple pathogen resistance

Abbreviations: AP2/EREBP, apetala2/ethylene-responsive element binding protein; CAT, catalase; DREB, dehydration-responsive element binding protein; ERD, early responsive to dehydration; ERF, ethylene response factor; EST, expressed sequence tags; GFP, green fluorescent protein; PR, pathogenesis-related; SOD, superoxide dismutase.

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was also observed following overexpression of tobacco *Tsi1* (Shin et al., 2002), *Arabidopsis TDR1* (Gutterson and Reuber, 2004), and hot pepper *CaPF1* (Yi et al., 2004; Tang et al., 2005). Overexpression of various ERF genes, including *Tsi1* and *OPBP1* of tobacco (Park et al., 2001; Guo et al., 2004), *CaERFLP1* and *CaPF1* of hot pepper (Lee et al., 2004; Yi et al., 2004), and *TERF1* and *JERF1* of tomato (Huang et al., 2004; Zhang et al., 2004a,b) also improved the tolerance of transgenic plants to various abiotic stress, such as high salinity, drought and low temperature conditions. Overexpression of *AtEBP* resulted in resistance to Bax-induced cell death and abiotic stresses such as hydrogen peroxide (H_2O_2) and heat-mediated oxidative stress in tobacco Bright Yellow-2 (BY-2) cultured cells (Ogawa et al., 2005). Therefore, overexpression of plant ERF genes enhanced tolerance to abiotic and biotic stresses, suggesting that they could be used as candidate genes to improve crop resistance.

Sweetpotato (*Ipomoea batatas*) is known to be a relatively drought-resistant crop and it represents one of the most important root crops grown on marginal lands. Although sweetpotato is recognized as a comparatively drought-tolerant plant, the molecular mechanisms underlying drought tolerance are not well defined. Previously, expressed sequence tags (ESTs) from a full-length enriched cDNA library prepared from suspension-cultured cells and dehydration-treated fibrous roots of sweetpotato were isolated and characterized (Kim et al., 2006, 2009a). Expression analysis showed that some sweetpotato genes isolated from the EST library of suspension-cultured cells and dehydration-treated fibrous roots were also induced in response to various environmental stresses. Thus, the investigation of sweetpotato EST pools may yield valuable genetic information about the regulatory networks involved in stress-response processes of sweetpotato.

In this study, we isolated and characterized two *ERF* genes from an EST library of suspension-cultured cells and dehydration-treated fibrous roots of sweetpotato. To better understand the function of these two *ERF* genes, we analyzed their expression under various stress conditions. The results suggest that the two *ERF* genes play a role in responses to abiotic stress and pathogen infection in sweetpotato. Finally, we demonstrate that the two *ERF* genes can act as transcriptional regulators of stress-responsive genes by performing *Agrobacterium*-mediated transient expression of these genes in tobacco leaves.

Materials and methods

Plant materials

Sweetpotato (*Ipomoea batatas* L. *Lam.* cv. White Star) plants were cultivated in a growth chamber in soil at 25 °C under a photocycle of 16 h light/8 h dark for 50 d. For gene expression analysis during plant growth, sweetpotato plants were grown in a greenhouse for 3 months and leaves from shoot apical meristems and fibrous roots were used. Cultured cells (1 g fresh weight), subcultured at 14 d intervals, were inoculated into 50 mL of MS (Murashige and Skoog, 1962) basal medium supplemented with 2,4-dichlorophenoxyacetic acid (1 mg L^{-1}) and sucrose (30 g L^{-1}). The cells were then incubated in darkness at 25 °C (100 rpm).

Analysis of DNA and protein sequences

Sequence identities were determined using the BLAST program available from the NCBI web-server, and multiple sequence alignment was determined using the Clustal X and GeneDoc programs. To predict the isoelectric point (pI), molecular weight and signal peptides of the deduced proteins, the ExPasy (http://www.expasy.org/tools), PSORT (http://psort.ims.u-tokyo.ac.jp) and SoftBerry (http://www.softberry.com) programs were used.

Subcellular localization of ERFs

The coding regions of two ethylene response factor (ERF) genes were amplified by PCR using the following primers with a BamHI restriction site: for *IbERF1*, 5′-CGGATCCATGGCTAGACCTCAGCA-3′ and 5′-GGGATCCATTCTTGAAGAACAG-3′, and for *IbERF2*, 5′-CGGATCCATGTGCGGTGGTGCTAT-3′ and 5′-GGGATCCAGAAGACACCCCCCA-3′. The amplified DNAs were initially cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA, USA). The BamHI fragment was then ligated into the 35S::GFP vector, in which each ERF gene was fused in-frame with GFP at its C-terminus, thereby yielding IbERF1::GFP and IbERF2::GFP. The resultant constructs were then transiently introduced into onion epidermal cells using a biolistic particle delivery system (Bio-Rad, Hercules, CA, USA) described previously by Shieh et al. (1993). After 12–48 h of incubation at 25 °C, the subcellular localization of each ERF::GFP fusion protein was evaluated via fluorescence microscopy (Axioskop, Zeiss, Jena, Germany). The location of a blue precipitate was compared with the location of DAPI-stained nuclei using fluorescence optics.

Stress treatments

Sweetpotato plants grown at 25 °C for 50 d were subjected to stress treatments. For dehydration treatments, sweetpotato leaves and fibrous roots were collected at 0 (untreated control), 1, 2, 4, 8, 16, and 24 h after treatment under light conditions. For treatments with NaCl, the third leaf from the top was detached from each plant, placed into a conical tube containing 30 mL of sterile water (control) or 100 mM NaCl, and then incubated at 25 °C for 24 h under light conditions. For treatments with hydrogen peroxide (H_2O_2, 400 mM), methyl viologen (MV, 0.05 mM), cadmium (Cd, 0.5 mM) and copper (Cu, 0.5 mM), sweetpotato leaves were incubated in conical tubes containing 30 mL of each chemical solution at 25 °C for 24 h under light conditions. Sterile water was used as a control for chemical stress treatments. For bacterial treatment, *Pectobacterium chrysanthemi* (*Erwinia chrysanthemi*, KCTC 2569) was used following the methods of Jang et al. (2004).

Agrobacterium-mediated transient expression

To transiently express each ERF gene in tobacco leaves, a plasmid was constructed for the expression of the two ERF genes by cloning it into the *Bam*HI site of the pCR2.1-TOPO vector (Invitrogen). The coding region of each ERF gene was introduced behind the CaMV 35S promoter in the pCAMBIA2300 plant expression vector. The constructs were verified via sequencing. The CaMV35S::*IbERF1*, CaMV35S::*IbERF2* and control (pCAMBIA2300 vector only) plasmids were introduced into *Agrobacterium tumefaciens* EHA 105, which was transformed into tobacco (*Nicotiana tabacum* cv. Xanthi) leaves via *Agrobacterium*-mediated transient expression. For the *Agrobacterium*-mediated transient expression assay in tobacco leaves, *Agrobacterium* (strain EHA 105) harboring two ERF constructs was grown overnight in Luria Bertani medium containing 25 μg mL^{-1} of rifampicin, 50 μg mL^{-1} of kanamycin and 150 μM acetosyringone. Cells were collected by centrifugation (12,000 rpm), resuspended to an OD_{600} of 1.0 in infiltration medium (10 mM Mes, pH 5.6 plus 10 mM $MgCl_2$) with 150 μM acetosyringone, and infiltrated into the leaves of 4-week-old tobacco plants. Leaf discs were harvested for a 2 d post-inoculation of *Agrobacterium* cells, immediately frozen in liquid nitrogen, and stored at −70 °C until use.