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Research article

Overexpressing *IbCBF3* increases low temperature and drought stress tolerance in transgenic sweetpotato

Rong Jin [a, b, c], Beg Hab Kim [a], Chang Yoon Ji [a, b], Ho Soo Kim [a], Hong Min Li [c], Dai Fu Ma [c], Sang-Soo Kwak [a, b, *]

[a] Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Daejeon 34141, South Korea
[b] Department of Environmental Biotechnology, KRIBB School of Biotechnology, Korea University of Science and Technology (UST), 217 Gajeong-ro, Daejeon 34113, South Korea
[c] Sweetpotato Research Institute, Chinese Academy of Agricultural Sciences (CAAS), Xuzhou 221121, Jiangsu, China

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ABSTRACT

Dehydration-responsive element-binding/C-repeat-binding factor (DREB/CBF) proteins regulate the transcription of genes involved in cold acclimation in several species. However, little is known about the physiological functions of CBF proteins in the low temperature-sensitive crop sweetpotato. We previously reported that the *DREB1/CBF-like* sweetpotato gene *SwDREB1/IbCBF3* is involved in responses to diverse abiotic stresses. In this study, we confirmed that IbCBF3 is localized to the nucleus and binds to the C-repeat/dehydration-responsive elements (CRT/DRE) in the promoters of cold-regulated (*COR*) genes. We generated transgenic sweetpotato plants overexpressing *IbCBF3* under the control of the CaMV 35S promoter (referred to as SC plants) and evaluated their responses to various abiotic stresses. *IbCBF3* expression was dramatically induced by cold and drought but much less strongly induced by high salinity and ABA. We further characterized two SC lines (SC3 and SC6) with high levels of *IbCBF3* transcript. The SC plants displayed enhanced tolerance to cold, drought, and oxidative stress on the whole-plant level. Under cold stress treatment (4 °C for 48 h), severe wilting and chilling injury were observed in the leaves of wild-type (WT) plants, whereas SC plants were not affected by cold stress. In addition, the *COR* genes were significantly upregulated in SC plants compared with the WT. The SC plants also showed significantly higher tolerance to drought stress than the WT, which was associated with higher photosynthesis efficiency and lower hydrogen peroxide levels. These results indicate that IbCBF3 is a functional transcription factor involved in the responses to various abiotic stresses in sweetpotato.

1. Introduction

Sweetpotato (*Ipomoea batatas* [L.] Lam), an important food crop that is also used to produce animal feed and biofuels, is widely cultivated in Asia and Africa. This crop can grow on marginal lands due to its relatively high tolerance to abiotic stress, and has high nutritional value (Bovell-Benjamin, 2007). Therefore, sweetpotato plays a critical role in both food security and bio-industrial development (Mukhopadhyay et al., 2011; Mohanraj and Sivasankar, 2014). Nevertheless, severe environment stresses such as cold and drought seriously reduce crop productivity and affect the expansion of sweetpotato cultivation (Fan et al., 2012, 2015). In particular, sweetpotato is highly sensitive to low temperatures due to its tropical and subtropical origins.

Abbreviations: ABA, abscisic acid; AP2/ERE, APETALA2/ETHYLENE-responsive factor; COR15A, cold-regulated 15A; CRT/DRE, C-repeat/dehydration-responsive element; COR, cold-responsive; DAB, 3,3'-diaminobenzidine; DREB1/CBF, dehydration-responsive element-binding/C-repeat-binding factor; GFP, green fluorescence protein; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; KIN2, COLD-RESPONSIVE 6.6; MDA, malondialdehyde; MV, methyl viologen; NBT, nitroblue tetrazolium; O^2, superoxide; PSII, photosystem II; qRT-PCR, quantitative real-time polymerase chain reaction; Rd29A, responsive to dehydration 29A; ROS, reactive oxygen species; TBA, thiobarbituric acid.

* Corresponding author. Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Daejeon 34141, South Korea. Tel.: 042-062-3551/010-8156-3551.
E-mail address: sskwak@kribb.re.kr (S.-S. Kwak).

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Over the past 20 years, genetic engineering has been used to improve abiotic and biotic stress resistance in sweetpotato using transgenic technology. Plants overexpressing many genes from sweetpotato, such as *IbLEA14*, *IbTPS*, *IbNAC1*, *IbMIPS1*, and *IbOR*, display enhanced tolerance to various abiotic stresses (Kim et al., 2013; Jiang et al., 2014; Chen et al., 2016; Park et al., 2016; Zhai et al., 2016). In addition, transgenic sweetpotato plants with improved resistance to abiotic and biotic stress have been produced via the overexpression of foreign genes. For instance, transgenic sweetpotato plants with increased tolerance to stem nematode stress due to overexpression of rice *OCI* have been developed, and sweetpotato transformants expressing *Arabidopsis thaliana NHX1* or spinach *BADH* are highly resistant to salt and cold stress conditions (Gao et al., 2011; Fan et al., 2012, 2015). In addition, overexpression of the *Arabidopsis HDG11* gene improved drought tolerance in sweetpotato (Ruan et al., 2012).

Dehydration-responsive element-binding/C-repeat-binding factor (DREB1/CBF) proteins, which belong to the APETALA2/ETHYLENE-responsive factor (AP2/ERE) transcription factor family, play important roles in cold stress tolerance in *Arabidopsis* (Chinnusamy et al., 2007). These transcription factors activate the expression of their target genes by binding to cis-acting C-repeat/dehydration-responsive element (CRT/DRE, core sequence A/GCCGAC) motifs in their promoters, including many cold-regulated genes (COR genes) (Zhao et al., 2015, 2016). Overexpression of *DREB1/CBF* genes increases tolerance to drought, heat, high salinity, and freezing stress in *Arabidopsis* and other plants (Oraby and Ahmad, 2012; Dou et al., 2015; Sarkar et al., 2016; Wei et al., 2016), which indicates the importance of *DREB1/CBF* genes and CRT/DRE elements in stress responses (Mizoi et al., 2012). Moreover, homologs of *DREB1/CBF* genes play essential roles in a variety of other plants, such as rice, soybean, and wheat (Choi et al., 2002; Chen et al., 2009; Dou et al., 2016). Transgenic plants overexpressing these genes exhibit enhanced tolerance to multiple abiotic stresses, implying that *DREB1/CBF* genes help plants adapt to harsh environments.

We previously identified a DREB1/CBF-like gene from sweetpotato (*SwDREB1/IbCBF3*) encoding a deduced protein with a conserved AP2/EREBP domain (Kim et al., 2008). In this study, with the aim of improving the adaptability of sweetpotato plants to abiotic stresses, especially cold and drought stress, we generated and evaluated transgenic sweetpotato plants overexpressing *IbCBF3* under the control of the CaMV 35S promoter (referred to as SC plants). We used the Xushu18 cultivar, the most widely planted sweetpotato cultivar in China, for the transformation to increase its tolerance to various abiotic stresses (Yang et al., 2011a). The results indicate that transgenic sweetpotato plants overexpressing *IbCBF3* had significantly improved tolerance to both cold and drought stress compared with the wild type (WT).

2. Materials and methods

2.1. Plants materials and growth conditions

Sweetpotato (cv. Xushu18) plants were grown on soil at 25 °C under a 16 h light/8 h dark photoperiod. Three-week-old sweetpotato plants were subjected to cold (4 °C), drought (25% PEG), salinity (350 mM NaCl), or ABA (10 μM) stress. The third leaves from the tops of plants harvested at 0, 3, 6, 12, 24, and 48 h after treatment were used for the analysis of *IbCBF3* expression. For tissue-specific expression analysis, the fourth leaves from the tops of plants, as well as petioles, stems, fibrous roots, pencil roots, and storage roots, were prepared from 10-week-old sweetpotato plants. *Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in soil at 22 °C under a 16 h light/8 h dark photoperiod after vernalization for 48 h. The *cbf3* homozygous mutant was kindly provided by Dr. Dae-Jin Yun (Konkuk University, Korea).

2.2. Gene isolation and plasmid construction

The full-length cDNA of *IbCBF3* was obtained via PCR from total RNA isolated from sweetpotato (cv. Xushu18) using a specific *IbCBF3* forward and reverse primer pair. The PCR products were purified and initially cloned into the T-blunt vector (BioFACT, Daejeon, Korea) and sequenced. To generate the pBD-IbCBF3, GST-IbCBF3, and 35S::IbCBF3-GFP plasmids, the full-length cDNA of *IbCBF3* was fused with the yeast GAL4 DNA-binding domain in the pDEST32 vector, the glutathione s-transferase (GST) gene in the pDEST15 vector, and the green fluorescence protein (GFP) gene under the control of the 35S promoter in the pGWB5 vector, respectively, using Gateway cloning technology (Curtis and Grossniklaus, 2003). The specific primers are listed in Supplemental Table S1.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the indicated plant samples with TRIzol reagent (Invitrogen, MA, USA), and cDNA synthesis was performed using TopScript™ RT DryMIX (dT18) (Enzynomic, Daejeon, Korea) according to the manufacturer's instruction. The qRT-PCR analysis was performed with a Bio-Rad CFX96 thermal cycler (BioFACT, Daejeon, Korea) and CFX system software (Bio-Rad, CA). The *UBQ5* and *Ibtubulin* genes were used for the *Arabidopsis* and sweetpotato internal controls, respectively. The specific primers for qRT-PCR are listed in Supplemental Table S1.

2.4. Subcellular localization analysis

Subcellular localization analysis of 3-week-old tobacco (*N. benthamiana*) leaves was conducted as described previously (Kim et al., 2016). The plasmid 35S::IbCBF3-GFP was transformed into *Agrobacterium tumefaciens* strain GV3101 and used for *Agrobacterium*-mediated transient expression analysis. After 3 d, the infiltrated parts of tobacco leaves were sampled and subjected to GFP analysis under a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg, Germany).

2.5. Transcriptional activation assay in yeast

For the transcriptional activation assay, *IbCBF3* was fused in-frame to the GAL4 DNA-binding domain in the pDEST32 vector to generate the fusion construct pBD-IbCBF3. The construct was transformed into yeast strain PJ69-4A, and the transformants were examined for their ability to activate transcription from the GAL4 upstream activation sequence and to promote yeast growth in SD medium lacking leucine and histidine (SD/-Leu/-His). Transformants harboring pBD-AtCBF3 and pDEST32 were used as positive and negative controls, respectively. A colony-lift filter assay using SD/-Leu plates supplied with X-gal (SD/-Leu/X-gal) was used to analyze β-galactosidase activity. The procedure was performed according to the Yeast Protocols Handbook (Clontech, USA).

2.6. Electrophoretic mobility shift assay (EMSA)

For the electrophoretic mobility shift assay (EMSA) assay, GST-IbCBF3 was expressed in *E. coli* strain BL21 (DE3) and the recombinant protein was purified by affinity chromatography using a Glutathione Sepharose 4B column (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocols. Oligonucleotide