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Research article

CsWRKY46, a WRKY transcription factor from cucumber, confers cold resistance in transgenic-plant by regulating a set of cold-stress responsive genes in an ABA-dependent manner

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ABSTRACT

Plant WRKY transcription factors are trans-regulatory proteins that are involved in plant immune responses, development and senescence; however, their roles in abiotic stress are still not well understood, especially in the horticultural crop cucumber. In this study, a novel cucumber WRKY gene, *CsWRKY46* was cloned and identified, which was up-regulated in response to cold stress and exogenous abscisic acid (ABA) treatment. CsWRKY46 is belonging to group *II* of the WRKY family, CsWRKY46 was found exclusively in the nucleus, as indicated by a transient expression assay. Yeast one-hybrid assay shown that CsWRKY46 interact with the W-box in the promoter of *ABI5*. Transgenic *Arabidopsis* lines over-expressing *CsWRKY46*, WRK46-OE1 and WRK46-OE5 had higher seedling survival rates upon freezing treatment compared with that of the wild-type. The above over-expression lines also showed much a higher proline accumulation, less electrolyte leakage and lower malondialdehyde (MDA) levels. Furthermore, the *CsWRKY46* overexpression lines were hypersensitive to ABA during seed germination, but the seedlings were not. Quantitative RT-PCR analyses revealed that the expression levels of the ABA-responsive transcription factor *ABI5* were higher in the WRKY46-OE lines than in wild-type and that the overexpression of *CsWRKY46* increased the expression of stress-inducible genes, including *RD29A* and *COR47*. Taken together, our results demonstrated that CsWRKY46 from cucumber conferred cold tolerance to transgenic plants and positively regulated the cold signaling pathway in an ABA-dependent manner.

1. Introduction

It is common for plants to encounter different types of environmental stress during their lifetime, and low temperature is a major abiotic stress that has a substantial impact on the growth and development of plants (Chinnusamy et al., 2003). Plants adapt to chilling stress by a variety of complex mechanisms that rapidly the sense and transduce the stress signals and initiate a chilling tolerance response to protect the plants from cold stress (Beck et al., 2007). A number of genes, such as transcription factors, that respond to various environmental stimuli have been characterized using biochemical, molecular and genetic tools (Kong et al., 2011; Sobkowiak et al., 2014; Wang et al., 2014).

WRKY transcription factors in plants have been studied extensively. The first WRKY transcription factor was found in sweet potato under chilling stress (Ishiguro and Nakamura, 1994); thereafter, a great number of WRKY genes were cloned from various plant species, forming a large gene family. For instance, there are more than 70 WRKYs in Arabidopsis, and more than 100 WRKYs

Abbreviations: ABA, abscisic acid; CsWRKY46, *Cucumis sativus* group *II* WRKY transcription factor; MDA, malondialdehyde; WRKY46-OE, CsWRKY46 over-expression transgenic line; WT, wild type.

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have been identified in rice to date (Wu et al., 2005). A similar repertoire of genes was observed in poplar and cucumber (Dharmawardhana et al., 2010; Ling et al., 2011). The WRKY transcription factors contain a 60 amino acid WRKY domain and a zinc finger motif. The conservation of the WRKY domain is mirrored by a remarkable conservation of its cognate binding site, the W box (TTGACC/T) (Eulgem et al., 2000). Previous studies showed that WRKY transcription factors play vital roles in various biotic stress responses and during plant development (Dong et al., 2003; Ryu et al., 2006; Marchive et al., 2007; Zou et al., 2010; Meng et al., 2016), however, their roles in certain other processes, especially in response to abiotic stresses, remain unclear (Rushton et al., 2010). Recently, WRKYs have been shown to have important functions in abiotic stress tolerance and ABA response (Zhou et al., 2015; Li et al., 2015). Ren et al. (2010) demonstrated that ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis, and AtWRKY40, AtWRKY18 and AtWRKY60 were found to interact with the magnesium-protoporphyrin IX chelatase H subunit (CHLH/ABAR) to relieve ABA-responsive genes of inhibition (Shang et al., 2010). Downstream signaling of the core pathway involves regulation of ABA-responsive element binding factors (ABFs/AREBs) through phosphorylation, ubiquitination, and sumoylation in the case of ABI5 (abscisic acid-insensitive 5). The *Arabidopsis* abscisic acid (ABA)-insensitive abi5 mutants have pleiotropic defects in ABA response, including decreased sensitivity to ABA inhibition of germination and altered expression of some ABA-regulated genes. ABFs/AREBs are themselves subjected to transcriptional regulation, and some transcription factors harboring the WRKY domain appear to regulate their expression through W-box sequences present in the promoters of ABFs/AREBs (Antoni R, 2011).

Cucumber is one of the most popular greenhouse-cultivated crops and is generally a thermophilic species; therefore, low temperature is the limiting factor for the yield and quality of greenhouse-grown cucumber in winter. The completion of the draft genome sequence of *Cucumis sativus var. sativus* L. provides a good opportunity to characterize the cucumber WRKY genes in response to various environmental stresses. Our previous study indicated that there were 57 WRKY genes in the cucumber genome (Ling et al., 2011). To elucidate the roles of cucumber WRKY transcription factors under cold stress further, we found the expression of *CsWRKY46* was induced by both chilling stress and ABA treatment, so we isolated and characterized *CsWRKY46* in the present study. The over-expression of *CsWRKY46* in *Arabidopsis* conferred tolerance to cold stress and hypersensitivity to ABA during seed germination.

2. Materials and methods

2.1. Plant materials, growth conditions and treatments

Line 9930, a typical cucumber variety of Northern China, was used for the study. The seeds were germinated in pots containing a mixture of vermiculite and peat, and grown in illumination incubator for 3 weeks, the temperature is 28 °C/18 °C (day/night), the light is 14 h/10 h (day/night), the light intensity is 300 μmol $m^{-2} \cdot s^{-1}$.3-week old seedlings were used in the following treatments. For the cold treatment, the seedlings were incubated at 4 °C in an illuminated incubator for 6 h; for the ABA treatments, the seedlings were sprayed with water or 100 μmol L^{-1} ABA, and samples for RNA extraction were collected after 12 h. The roots, stems, cotyledon, leaves, female flowers, male flowers, tendrils, ovary 2 day fruit and 10 day fruit of mature plants were collected separately for RNA isolation, which was used for the tissue-specific expression analysis. And the primers used are specific to the spliced mRNA.

2.2. Identification of CsWRKY46 gene

Total RNA were extracted according to the instructions of TRIzol reagent (Invitrogen) from leave of cucumber seedlings. The first-strand cDNA were synthesized using a 5 μg of total RNA and a Fermentas Revert Aid™ First Strand cDNA Synthesis Kit in reaction volume of 20 μL. The primers were designed according to the cucumber *CsWRKY46* sequence (Ling et al., 2011) to amplify the full-length *CsWRKY46* by RT-PCR. The expected PCR fragment was cloned into the *p*EASY-T1 vector and sequenced.

2.3. Transient expression in Arabidopsis protoplasts for assaying subcellular localization CsWRKY46 protein

Transient expression in *Arabidopsis* protoplasts was essentially performed according to the procedures described by Walter et al. (2004). The full-length *CsWRKY46* coding region was amplified with primers CW46-s-H-X-F (5′CCAAGCTTATGTCGGATGAAATG, *Hind*III site underlined) and CW46-s-H-X-R (5′GCTCTAGACGGCTGTCGGTTG, *Xba*I site underlined). The coding sequence of the gene was inserted into the 16318-hGFP vector (donated by Dr. Xianguo Cheng); the expression of the cloned gene was driven by the CaMV35S promoter and C-terminally fused to GFP. The recombinant plasmid CsWRKY46-GFP and vector (GFP alone) were introduced into *Arabidopsis* protoplasts, which were isolated from the leaves of 3- to 4-week-old plants (ecotype Col) and transiently transformed using polyethylene glycol, essentially according to Sheen's protocol (http://genetics.mgh.harvard.edu/sheenweb/). After transformation, the protoplasts were incubated under dark conditions at 23 °C for 16 h. The transformed protoplasts were observed using a confocal microscope (Olympus, Tokyo, Japan).

2.4. Yeast one-hybrid assay

Yeast one-hybrid assay was performed essentially as Shang et al. (2010) described with the kit provided by Clontech (Matchmaker™ One-Hybrid Library Construction & Screening Kit catalogue no. 630304) using the Y187 yeast strain according to the manufacturer's instructions. The related cDNAs or promoter DNAs were amplified by PCR using the following primer pairs: forward primer 5′- AAAGTAACTAAAGAGGCCAATATGAT-3′ and reverse primer 5′-CTAAGAAGAGAGGCGTGAAGGTCA-3′ for the *ABI5* promoter; forward primer5′-CATATGTCGGATGAAATGTTTAAAG-3′ and reverse primer5′-CTCGAGTCACGGCTGTCGGTT-3′ for *CsWRKY46* cDNA; The promoter fragments of *ABI5* were subcloned into the *Eco*RI/*Mlu*I sites of the *p*HIS2 vector. The one-hybrid assays were performed using the Y187 yeast strain according to the manufacturer's instructions. Yeast cells were co-transformed with *p*HIS2 bait vector that harboured the promoter of target genes and *p*GADT7 prey vector that carried the ORF of *CsWRKY46*. As negative controls, the yeast cells were transformed with the empty *p*GADT7 vector with *p*HIS2 harbouring the corresponding promoter. Transformed yeast cells were first grown in tryptophan (Trp) and leucine (Leu)-deficient SD medium (SD−Trp−Leu) to ensure that the yeast cells were successfully co-transformed, and then the yeast cells were grown on plates of the Trp, Leu, and histidine (His)-deficient SD medium (SD−Trp−Leu−His) supplemented with 3-aminotriazole (3-AT; Sigma) at 25 mM (for the *p*His2 vector with the *ABI5* promoter). The plates were then incubated at 30 °C for 3 day prior to investigations.