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Research article

Overexpression of a tobacco J-domain protein enhances drought tolerance in transgenic *Arabidopsis*

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ABSTRACT

DnaJ proteins constitute a DnaJ/Hsp40 family and are important regulators involved in diverse cellular functions. To date, the molecular mechanisms of DnaJ proteins involved in response to drought stress in plants are largely unknown. In this study, a putative *DnaJ* ortholog from *Nicotiana tabacum* (*NtDnaJ1*), which encodes a putative type-I J-protein, was isolated. The transcript levels of *NtDnaJ1* were higher in aerial tissues and were markedly up-regulated by drought stress. Over-expression of *NtDnaJ1* in *Arabidopsis* plants enhanced their tolerance to osmotic or drought stress. Quantitative determination of H_2O_2 accumulation has shown that H_2O_2 content increased in wild-type and transgenic seedlings under osmotic stress, but was significantly lower in both transgenic lines compared with the wild-type. Expression analysis of stress-responsive genes in *NtDnaJ1*-transgenic *Arabidopsis* revealed that there was significantly increased expression of genes involved in the ABA-dependent signaling pathway (*AtRD20*, *AtRD22* and *AtAREB2*) and antioxidant genes (*AtSOD1*, *AtSOD2*, and *AtCAT1*). Collectively, these data demonstrate that *NtDnaJ1* could be involved in drought stress response and its over-expression enhances drought tolerance possibly through regulating expression of stress-responsive genes. This study may facilitate our understandings of the biological roles of DnaJ protein-mediated abiotic stress in higher plants and accelerate genetic improvement of crop plants tolerant to environmental stresses.

1. Introduction

J-domain proteins (also called DnaJ proteins) constitute a DnaJ/Hsp40 family and are conserved co-chaperones for HSP70s (Caplan et al., 1993; Silver and Way, 1993; Qiu et al., 2006). DnaJ proteins are involved in a variety of essential cellular processes including protein folding, assembly, translocation, degradation, stabilization and refolding (Wang et al., 2004; Mayer and Bukau, 2005; Craig et al., 2006; Rajan and D'Silva, 2009). Besides their co−chaperone activity, DnaJ proteins function as protein disulfide isomerases to catalyze protein disulfide formation, reduction, and isomerization (de Crouy-Chanel et al., 1995).

DnaJ was originally identified in *Escherichia coli* as a 41 kDa heat shock protein (Goffin and Georgopoulos, 1998). Subsequently, members of the J-protein family were found to function as molecular chaperones by binding Hsp70 to stimulate ATP hydrolysis, and stabilizing the Hsp70 interaction with substrate proteins (Szyperski et al., 1994; Szabo et al., 1996; Cheetham and Caplan, 1998). The J domain is a highly conserved ~70 amino acid α-helical region in DnaJ proteins (Hennessy et al., 2005). Plant J-domain proteins have been classified into four types (I, II, III, and IV) based on the presence of other conserved domains (Rajan and D'Silva, 2009; Miernyk, 2001; Walsh et al., 2004). Traditional type-I J-domain proteins contain four domains including a J domain, a Gly/Phe (G/F) domain, a CXXCXGXG zinc-finger domain and a less conserved C-terminal domain. Type-II J proteins lack the zinc-finger domain, whereas type-III J- proteins contain only the J domain. Type-IV J proteins have been recently described and classified as 'J-like proteins', with significant sequence and structural similarities with the J domain, but they lack the HPD motif (Walsh et al., 2004). The well-characterized *Arabidopsis* genome is known to harbor 120 predicted J-domain proteins (Rajan and D'Silva, 2009).

Abbreviations: DnaJ, J-protein; Hsp40, 40 kDa heat shock protein; HPD motif, Histidine, proline, aspartate motif; MS, Murashige and skoog; qPCR, Quantitative PCR.

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Increasing evidence has shown that J-domain proteins play important roles in growth and development (Kneissl et al., 2009; Yang et al., 2009; Shen et al., 2011; Bekh-Ochir et al., 2013), disease resistance (Bekh-Ochir et al., 2013; Liu and Whitham, 2013; Du et al., 2013), and abiotic stress responses (Yang et al., 2010; Zhou et al., 2012). For example, the *Arabidopsis* J-domain protein OWL1 is involved in the regulation of germination, cotyledon opening, hypocotyl elongation, and perception of very low light influences (Kneissl et al., 2009). TMS1, a DnaJ protein with disulfide isomerase activity, is required for thermotolerance of pollen tubes in *Arabidopsis*, possibly by functioning as a co-molecular chaperone (Yang et al., 2009). The *Arabidopsis* Type-I J-domain protein J3 mediates the integration of flowering signals during the floral transition and regulates the plasma membrane H^+-ATPase through interacting with the PKS5 kinase under high salinity conditions (Yang et al., 2009; Shen et al., 2011). The J-protein AtDjB1 facilitates thermotolerance by protecting cells against heat-induced oxidative damage in *Arabidopsis* (Zhou et al., 2012). Recently, it has been reported that a type-III J domain protein GmHSP40.1 causes HR-like cell death in tobacco (Liu and Whitham, 2013). More interestingly, a tobacco type-I J-domain protein NbMIP1 has been shown to be required for both tobacco mosaic virus infection and plant innate immunity through functioning as co-chaperones (Du et al., 2013).

In spite of progress made in understanding function of DnaJ proteins, few reports have been concerned the role of J-domain proteins in drought stress in plants (Wang et al., 2014). Drought stress often adversely affects plant growth and productivity, thus it is still a serious problem in agriculture worldwide. Tobacco is an important crop as well as a model plant system, and its productivity is vulnerable to drought. To identify genes important in drought stress response in tobacco, we previously identified mRNAs up-regulated by drought stress through microarray analysis (unpublished data). One highly induced mRNA encoding a J-domain protein (*NtDnaJ1*) was characterized in detail. We further characterized the putative *NtDnaJ1* in transgenic *Arabidopsis* to investigate drought tolerance and possible function mechanisms.

2. Materials and methods

2.1. Plant materials and stress treatment

Tobacco (*Nicotiana tabacum* cv. Xanthi) was used throughout this study. *Arabidopsis thaliana* ecotype Col-0 was used for gene transformation. Plants were grown in a growth room as described previously (Xia et al., 2012a). Drought stress in four-week-old plants was realized by replacing the water with 15% PEG 6000 and leaves were sampled at 0, 6, 12, 24, or 48 h for expression analysis as described by us (Xia et al., 2013).

2.2. Cloning of NtDnaJ1 and sequence analysis

The drought-induced EST encoding a putative DnaJ/Hsp40 protein was used to do BLAST (http://www.ncbi.nlm.nih.gov/) and mRNA sequences containing such an EST were downloaded for gene prediction. The gene is highly homologous to DnaJ/Hsp40 family member, and thus is named *NtDnaJ1*. Two primers NtDnaJ1-F and NtDnaJ1-R (Table S1) were designed for amplifying the open reading frame (ORF) of *NtDnaJ1*. The 1257 bp PCR product was verified by sequencing.

The primary structural analysis was performed using InterProScan (http://www.ebi.ac.uk/InterProScan). The alignment of the deduced protein sequences and phylogenetic tree analyses were done by DNASTAR and MEGA 5.1, respectively, using standard parameters (Tamura et al., 2011).

2.3. Real-time PCR analysis

Real-time PCR was used to determine the expression patterns of *NtDnaJ1* in different organs and under drought condition. The qRT-PCR was performed in triplicate with an IQ5 light cycler system (Bio-Rad) using SYBR Premix ExTaq II (Takara, Japan) with gene-specific primers NtDnaJ1-F1 and NtDnaJ1-R1 (Table S1), which produces a 195-bp product. The tobacco *NtActin* transcript was used as an internal control to quantify the relative transcript levels as described by us previously (Xia et al., 2013). The relative level of gene expression was detected using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

To examine the relative expression of *NtDnaJ1* in transgenic *Arabidopsis* plants, the *AtActin2* transcript (gene-specific primers AtActin2-F1 and AtActin2-R1; Table S1) was used as an internal control to quantify the expression levels, and the lowest expression level among transgenic lines was regarded as standard and the relative level of gene expression was computed as described above.

To assay the expression of stress-responsive genes (*AtSOD1, AtSOD2, AtCAT1, AtRD20, AtRD22* and *AtAREB2*) in transgenic *Arabidopsis* plants, qRT-PCR analysis was also performed with the RNA samples isolated from four-week-old transgenic plants harvested in soil under normal conditions. Total RNA isolation and reverse transcription were performed as described above. PCR amplification was performed with gene specific primers (Table S1). Criteria for designing primers were a primer size between 22 and 25, an optimal T_m at 60 °C, and a product size ranging from 150 bp to 250 bp. Amplification of *AtActin2* was used as an internal control, and qRT-PCR experimental procedures were performed as described above. For the entire qRT-PCR assay, three technical replicates were performed for each experiment and the expression of each gene was investigated in three biological replicates.

2.4. Construction of plant expression vectors and development of transgenic Arabidopsis lines

The *NtDnaJ1* coding sequence was amplified and introduced into the pART7 plasmid (Xia et al., 2012b) using primers NtDnaJ1-F2 with *Bam*HI restriction site (underlined) and NtDnaJ1-R2 with *Xba*I restriction site (underlined) (Table S1) and was subsequently inserted downstream of the 35S promoter in the plasmid vector pART7. The resulting expression cassette containing the 35S promoter and *NtDnaJ1* coding sequence was cut and inserted into the binary vector pART27, producing the transformation construct pART27-35S-NtDnaJ1.

The binary construct was introduced into *Agrobacterium tumefaciens* (strain GV3101) and then transformed into *Arabidopsis* (Col-0) via the floral dip method (Clough and Bent, 1998). Transgenic lines were selected by germinating seeds on medium containing Murashige and Skoog (MS) basal salt mixture (Sigma–Aldrich, USA) and 50 mg/L kanamycin. After two weeks on selection medium, green seedlings (T1 plants) were transferred to soil pots and grown to maturity in a growth room. The presence of the transgene in each plant was checked by PCR with genomic DNA from leaves of individual plant using primers 35SP-F and NtDnaJ1-R3 (the forward primer 35SP-F is from CaMV 35S promoter sequence) (Table S1). The PCR-positive plants as transgenes were grown to maturity and seeds were collected (T2 seed). T2 seeds were germinated on kanamycin selection medium again and the one-copy lines were identified by examining the segregation ratio (3:1) of the kanamycin selectable marker. Each one-copy line was maintained growth to set seeds until T_3 generation. Five independent homozygous *NtDnaJ1* transgenic lines (named OE-8, OE-14, OE-18, OE-22 and OE-27) were developed. The expression levels of the transgene