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Research article

Overexpression of wheat *NF-YA10* gene regulates the salinity stress response in *Arabidopsis thaliana*

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ABSTRACT

The nuclear factor Y (NF-Y) transcription factor is formed by the interaction of three distinct subunits (NF-YA, -YB and -YC). It targets the CCAAT box, a common *cis*-element in eukaryotic promoters. Here, the bread wheat gene *TaNF-YA10-1* has been isolated from the salinity tolerant cultivar SR3. Recombinant TaNF-YA10-1 was heterologously produced in *Escherichia coli*, and the purified protein successfully bound to the CCAAT motif *in vitro*. *TaNF-YA10-1* was down-regulated by the imposition of salinity and abscisic acid (ABA). The constitutive expression of *TaNF-YA10-1* in *Arabidopsis thaliana* significantly increased the plant's sensitivity to salinity and repressed its sensitivity to ABA as judged from the seed germination, cotyledon greening and the relative root growth. The transcription of stress-related genes *AtRAB18*, *AtRD29B*, *AtABI5*, *AtCBF1* and *AtCBF3* was downregulated in *TaNF-YA10-1* overexpression transgenic plants. The data provide supportive evidence that *TaNFYA10-1* is involved in the regulation of growth under salinity stress conditions.

1. Introduction

Soil salinity is among the most severe environmental constraints on plant growth and crop production (Munns and Tester, 2008). It affects plant function as a result of both the toxicity of excessive sodium (and other) ions, and the osmotic stress it imposes on the root. The *Arabidopsis thaliana* response to salinity stress has been intensively studied, leading to the identification of a number of key pathways; some, but not all, of these are regulated by the phytohormone abscisic acid (ABA) (Zhu, 2001). Activation of the Salt Overly Sensitive (SOS) pathway helps to maintain ionic homeostasis, while both ABA-independent and -dependent signaling pathways regulate an array of genes for response to osmotic stress caused by high salt or other stresses (Munns and Tester, 2008; Wang et al., 2003; Zhu, 2002). Many of the pathways are dependent on the activation of a transcription factor, such as a CBF/DREB, a NAC or a RING-H2 zinc finger protein (Hu et al., 2006; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Ko et al., 2006).

NF-Y transcription factors, also called CCAAT-binding factor or "heme-activated protein", are ubiquitous in eukaryotic genomes. It is composed of three subunits, namely NF-YA (=HAP2), -YB (=HAP3/CBF-A) and -YC (=HAP5/CBF-C) (Frontini et al., 2004; Kahle et al., 2005). The NF-YB-YC heterodimer is suggested to be assembled in the cytoplasm, which subsequently translocates into the nucleus and interacts with NF-YA to form an active heterotrimer (Frontini et al., 2004; Steidl et al., 2004). This complex has high affinity and sequence specificity for the CCAAT box, which is a cis-element that exists in approximately 25% of eukaryotic gene promoters (Cai et al., 2007; Ceribelli et al., 2008; Maity and de Crombrugghe, 1998). The NF-YA subunit confers the sequence specificity to the complex via an unknown mechanism (Dolfini et al., 2012; Mantovani et al., 1994).

In contrast to animal and yeast *NF-Y* genes, which are single copy, plant homologs are typically present in the form of multigene families (Edwards et al., 1998). In *A. thaliana*, for example, there are, respectively 10, 13 and 13 copies of *NF-YA*, *NF-YB* and *NF-YC* (Siefers et al., 2009), while in the model grass species *Brachypodium distachyon* and in bread wheat (*Triticum aestivum*), the number of *NF-Y* genes represented is, respectively, 36 and 35 (Cao et al., 2011; Stephenson et al., 2007). Due to the potential combinatorial diversity among numerous NF-Y factors, as well as the high affinity

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and sequence specificity for the extensive CCAAT box in the eukaryotic genomes, these members are involved in the regulation of various developmental processes and stress responses. It has been demonstrated that AtNF-YB9 (LEC1) plays a pivotal role in embryo development (Lee et al., 2003a). Altered expression of *AtNF-YB1* and *AtNF-YB2* could greatly affect the flowering time (Cai et al., 2007; Chen et al., 2007; Wenkel et al., 2006). Some NF-YA subunits such as AtNF-YA2, AtNF-YA3 and AtNF-YA5 were reported to participate in nitrogen nutrition (Laloum et al., 2013). Moreover, NF-Ys have been identified as regulators of drought tolerance in different plant species. The over-expression of *AtNF-YA5* and *AtNF-YB1* in *A. thaliana*, and of *ZmNF-YB2* in maize, improves plant performance and survival under drought conditions (Li et al., 2008; Nelson et al., 2007). Broader functions of NF-Y factors like regulation of light signaling, ER stress, chloroplast biogenesis etc were also revealed (Laloum et al., 2013; Petroni et al., 2012). As yet, the biological roles of most of the NF-Y family members in the salinity stress response are poorly understood.

The salinity tolerant bread wheat cultivar Shanrong No. 3 (SR3) is a derivative of an asymmetric somatic hybrid between the bread wheat cultivar Jinan 177 (JN177) and tall wheatgrass (*Thinopyrum ponticum*) (Wang et al., 2003). It out-yields JN177 in salinity-affected soil, and it has been planted commercially in China (http://www.seedsd.com/news/news_view.asp?id=511) (Xia, 2009). Here, it is shown that in the SR3 plant, *TaNF-YA10-1* was down-regulated by a number of abiotic stress factors, including salinity. The constitutive expression of *TaNF-YA10-1* in *A. thaliana* resulted in a marked increase in sensitivity to salinity stress and a decrease in sensitivity to ABA, as well as an altered transcription profile with respect to a number of known stress response genes. The data contribute to the elucidation of the role of NF-YA subunits in the salinity stress response.

2. Materials and methods

2.1. Plant materials and treatments

Grain of SR3 and JN177 were germinated on moist filter paper at 20 °C for two days. Uniform seedlings were raised in half-strength Hoagland's liquid medium under a 16 h photoperiod at 22 °C. Plants were subjected to abiotic stress by adding one of 18% (w/v) PEG6000, 200 mM NaCl, 100 mM H_2O_2 or 100 μM ABA to the culture solution after two weeks, and seedlings were harvested after exposure to the stress agent for either 1, 3, 12 or 24 h. *A. thaliana* ecotype Col-0 was used as the transformation target for either *pSTART::TaNF-YA10-1* or an empty *pSTART* vector, using the floral dip method (Clough and Bent, 1998). Progeny of the primary transgenics were germinated in the presence of kanamycin and the inheritance of the transgene in surviving seedlings was confirmed using both genomic PCR and RT-PCR. Two homozygous T_4 selections were retained (OE 3–6 and 5–7) for functional analysis. *A. thaliana* plants were raised under a 14 h photoperiod, a light intensity of 120 μmol m^{-2} s^{-1}, a day/night temperature of 22/18 °C and a relative humidity of 70%.

2.2. Gene structure and phylogeny

The fragment of the NF-YA homolog identified via microarray analysis (Liu et al. 2012) was used as a BLASTN query against the wheat (*T. aestivum*) EST database held at the National Center for Biotechnology Information. All matching ESTs were assembled using CAP3 software, and a pair of gene-specific primers designed from this assembly (Supplementary Table 1) was used to amplify a full-length cDNA from a cv SR3 cDNA library. Gene structure was predicted using SMART software (Schultz et al., 1998), and peptide sequences were aligned using ClustalW as implemented by Molecular Evolutionary Genetics Analysis (MEGA) software, version 5.0 (Tamura et al., 2011). A phylogenetic analysis was conducted using the neighbor-joining method, applying the following parameters: bootstrap replicates: 1000, Poisson mode and pairwise deletion.

2.3. Reverse transcription (RT)-PCR and quantitative (Q)-PCR analysis

Total RNA was isolated from both *A. thaliana* and wheat seedlings using the TRIzol reagent (Invitrogen), then treated with RNase-free DNase I (Promega). RT-PCR was conducted following the supplier's protocol (Takara). Each qPCR was repeated for three biological replicates, and the reactions were based on FastStart Universal SYBR Green Master (Roche). The reference sequences comprised segments of the wheat *actin* (AB181991) or the *A. thaliana actin* (GI:145338402) genes. Primer sequences for RT-PCR and qPCR were designed using PRIMER PREMIER 5.0, and are listed in Supplementary Table 1.

2.4. Subcellular localization of TaNF-YA10-1

To generate the p*35S::TaNF-YA10-1-GFP* plasmid, the *TaNF-YA10-1* open reading frame was amplified by PCR using the primer pair NF-YA10-1GF/R (Supplementary Table 1). The amplicon was inserted into the *Bam*HI/*Sal*I cloning site of a modified CaMV35S-GFP vector (Liu et al., 2014). The plasmids CaMV35S-GFP and CaMV35S:TaNF-YA10-1-GFP were introduced separately into both onion epidermal cells (Lee et al., 2003b) and wheat cv. Yangmai11 protoplasts of suspension cell lines from embryogenic calli (Sheen, 2001). After a 16 h incubation at 23 °C in the dark, the GFP signal and chlorophyll autofluorescence were detected by confocal laser-scanning microscopy (LSM700; Carl Zeiss), using excitation wavelengths of 488 nm and 647 nm, respectively.

2.5. Electrophoretic mobility shift assay

To express and purify TaNF-YA10-1 for electrophoretic mobility shift assay, the full-length coding regions of *TaNF-YA10-1* was PCR amplified with the primer pairs *TaNF-YA10-1*-GF and *TaNF-YA10-1*-GR (Supplementary Table 1) and cloned into pET-30a vector. The recombinant plasmids were transformed into *Escherichia coli* strain BL21 (DE3), and the subsequently expressed fusion protein was purified from homogenised bacterial cells using a His-tagged protein purification kit (R&D Systems) according to the manufacturer's protocol. A DIG Gel Shift kit (Roche) was used to detect DNA-protein interaction. Digoxigenin (DIG) was labeled at the 3′ end of the double-stranded oligonucleotides. DIG labeled (0.4 ng/μl), unlabeled (0.1 μg/μl) or mutated CCAAT oligonucleotides (0.1 μg/μl) was mixed with TaNF-YA10-1-His protein or a crude protein extracts of wheat in binding buffer and incubated at room temperature for 15 min. The reaction products were fractionated through a 7.5% non-denaturing polyacrylamide gel, then electrophoretically transferred to a positively charged nylon membrane (Roche) by applying a constant current of 50 mA for 4 h. The DNA was cross-linked to the membrane using a UV stratalinker (Stratagene). The membrane was blocked for 1 h at room temperature in 1× blocking reagent, then challenged with a 1:20,000 dilution of anti-DIG antibody coupled to alkaline phosphatase for 30 min. The membrane was rinsed twice in 0.1 M maleic acid (pH 7.5), 0.15 M NaCl, 0.3% (v/v) Tween 20 for 20 min, then after a 5 min equilibration in 100 mM Tris–HCl (pH 9.5), 100 mM NaCl, a 1:100 dilution of the substrate CSPD in the same buffer was added. After a 10 min incubation at 37 °C, the membrane was exposed to an X-ray film for 20 min to capture the chemiluminescent signal.