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Molecular cloning and characterization of novel WIN1/SHN1 ethylene responsive transcription factor *HvSHN1* in barley (*Hordeum vulgare* L.)

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ABSTRACT

Barley (*Hordeum vulgare* L.) is the fourth major cereal crop and shows high adaptive capabilities to diverse environments. Thus, it might represent a potential reservoir of novel genes to improve abiotic stress tolerance. In this study, a novel AP2/ERF transcription factor gene designated as *HvSHN1* was isolated from barley. Protein sequence analysis showed that the HvSHN1 protein contained a nuclear localization signal and the conserved AP2/ERF domain. Phylogenetic analysis showed that *HvSHN1* belongs to the group Va protein in the ERF subfamily which contains the *Arabidopsis* genes (*SHN1, 2* and *3*) and the wheat gene *TdSHN1* with which it has 94.7% protein sequence identity. Expression profile analysis revealed that *HvSHN1* is strongly induced by heat, cold, salt and drought. Transient expression using tobacco BY-2 protoplast coupled to confocal microscopy analysis revealed that HvSHN1 is exclusively targeted to the nucleus. Interestingly, when constitutively expressed in transgenic tobacco, *HvSHN1* up-regulated stress responsive genes known to harbor GCC or DRE motif in their promoter regions. Therefore, *HvSHN1* might represent a potential candidate for improvement of abiotic stress tolerance in economically important crops.

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth major cereal crop in the world after rice, maize and wheat and has a significant economic value. It was domesticated from a wild ancestor (*H. vulgare* spp. *Spontaneum*) and cultivated barley was spread from the Near Eastern region and become acclimated to various ecogeographic conditions (Pankin and von Korff, 2017). It is widely used for animal feeding, human food and in malting as well as in other industrial purposes. In the United States and Canada, for example, 50% and 80%, respectively of the barley production is used to feed livestock (Blake et al., 2011; Albertabarley, 2017). Barley is an important source of human nutrients such as proteins, B vitamins, niacin, minerals and fibers. It is also known for its health benefits. In fact, barley consumption participates in the reduction of cholesterol levels and in the maintenance of healthy blood sugars (Lupton et al., 1994; Hallfrisch et al., 2003a,b).

Compared to other cereals, barley is considered as salt, drought and heat tolerant and shows high adaptive capabilities in addition to its natural resistance to fungal diseases (Munns et al., 2006). Therefore, it could represent an important staple food in regions most vulnerable to climate change. Due to these characteristics and to the fact that it is considered as a model crop, barley has become the focus of intensive research around the globe aiming at identifying and characterizing major stress responsive genes using modern tools such as omics and genomics as well as genetic transformation. Genetics (QTLs) and transcriptomic approaches were applied in barley and allowed the generation of wealth of data very useful for the identification of major pathways and protein components involved in stress tolerance (Li et al., 2013; Bedada et al., 2014). The characterization and use of barley genes governing these interesting traits such as adaptability to diverse environments, high yield and nutritive value can significantly contribute to yield increase, and ultimately, food security threatened by climate change, fast growth of world population, decrease in arable land and water scarcity.

Due to their sessile nature, plants have evolved very complex mechanisms to withstand abiotic stress. These mechanisms are based on signal transduction pathways which relate stress perception to plant molecular responses consisting of the expression of several classes of stress responsive genes (Hirayama and Shinozaki, 2010). These genes encode two main categories of proteins: the first category is directly involved in plant protection while the second is implicated in the control of expression of downstream genes. The latter, comprises

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transcription factors (TFs) which have the capacity to recognize and bind specifically to *cis*-elements in the promoter regions of plethora of stress responsive genes. In addition to binding domains, TFs harbor domains allowing them to up or down-regulate the expression of many downstream genes and hence impart stress tolerance in plants (Agarwal and Jha, 2010; Ahmad and Prasad, 2012).

APETALA2/ethylene response factor (AP2/ERF), which contains from 119 to 220 members, is considered as one of the most important superfamilies of TFs in the plant kingdom. This family is involved in the regulation of many aspects, such as growth, development, fruit ripening, and defense responses (Pirrello et al., 2006, 2012; Du et al., 2013; Rao et al., 2015). In addition, AP2/ERF TFs play a key role in connecting the multiple phytohormonal signals (Zhang et al., 2008; Li et al., 2011; Qi et al., 2011). This family is characterized by the presence of a common domain of about 60–70 amino acids residues known as AP2 domain. A simple classification based on the copy number of AP2 yielded four families: AP2, ERF, RAV and soloist (Nakano et al., 2006; Licausi et al., 2010). Recently, ERF members that harbor a single AP2 domain were split into DREB (Drought Response Element Binding proteins) and ERF based on the ability of the AP2 domain to bind one of the two characteristic binding elements of ERFs (Sakuma et al., 2002). In another classification, ERF and DREB were split into ten groups known as group I to X or group A to *J* (Nakano et al., 2006; Pirrello et al., 2012). AP2/ERFs have strong capacity to bind to cis-acting elements namely the GCC (AGCCGCC) and DRE/CRT (RCCGCC) boxes present in the promoter region of their target genes (Ohme-Takagi and Shinshi, 1995; Shinozaki and Yamaguchi-Shinozaki 2000; Ohta et al., 2000; Sasaki et al., 2007; Djemal and Khoudi, 2015). However, the degree of activation of downstream genes is dependent on the transcription factor and the flanking region of the GCC box (Pirrello et al., 2012).

The involvement of AP2/ERF in mediating various stress tolerance responses has been demonstrated in several studies (Park et al., 2011; Licausi et al., 2013; Klay et al., 2014). The subfamily ERF contains WAX INDUCER1/ SHINE1 (WIN1)/SHN1-type of TFs which is an interesting positive regulator of resistance to drought (Aharoni et al., 2004). In fact, these TFs increase the accumulation of cuticular wax, which has been associated with drought tolerance. Cuticular wax accumulation resulted from the up-regulation, by SHINE1-type transcription factors, of many genes involved in wax biosynthesis pathways (Kannangara et al., 2007; Shi et al., 2011). Recently, we have demonstrated that overexpression of *TdSHN1* in transgenic tobacco improved salt and drought tolerance (Djemal and Khoudi, 2016). An in-depth analysis of transgenic tobacco plants revealed that this tolerance resulted not only from alteration of cuticular development, but also from significant reduction of stomatal density and the activation of several genes involved in wax biosynthesis as well as in abiotic stress tolerance. SHINE genes were first reported in *Arabidopsis*. This species comprises three well-studied SHINE genes, namely *AtSHN1*, *AtSHN2* and *AtSHN3*, which belong to the ERF-B6 (Ethylene Responsive Factor B6) clade, a subgroup from the AP2/ERF TFs family (Dietz et al., 2010). In recent years, several SHINE genes were isolated from various plant species such as wheat (Djemal and Khoudi, 2015), Sorghum (Bao et al., 2017) and tomato (Shi et al., 2013; Al-Abdallat et al., 2014). However, no SHINE transcription factor involved in abiotic stress tolerance in barley has been reported so far.

In this study, we isolated a cDNA sequence, orthologous to durum wheat gene *TdSHN1*, from a barley genotype. In addition, we studied its expression profile under abiotic stress, subcellular localization and transactivation activity *in vivo*.

2. Materials and methods

2.1. Plant material and stress treatments

The Tunisian cultivar of barley (Rihane), used in this study, was provided by the Tunisian Agronomic Research Institute. To isolate the cDNA sequence of *HvSHN1* from barley, seeds were sterilized in commercial bleach for 15 min, thoroughly washed with sterile water and germinated on sheet of Whatman filter imbibed with MS medium (Murashige and Skoog, 1962) and placed in Magenta boxes. Germination was performed in a growth chamber at 25 °C and 14/10 light/dark cycles for 10 days. To determine the expression profile of *HvSHN1* under salt stress, the same steps were undertaken, except that after germination, seedlings were transferred to MS solution supplemented with 400 mM NaCl and kept for 6 h, 24 h and 48 h. For drought stress treatment, seedlings were transferred to dry sheet of Whatman filter and kept at room temperature for 6 h, 24 h and 48 h. For heat and cold treatments, seedlings were transferred to 37 °C and 4 °C growth chamber, respectively, for 6 h, 24 h and 48 h. Plant tissues were harvested, immersed in liquid nitrogen and stored at −70 °C for RNA isolation and expression analysis.

2.2. Cloning of HvSHN1 from barley and expression analysis with quantitative RT-PCR

Two hundred milligrams of leaf tissue derived from barley seedlings were used to isolate total RNA. The commercial Trizol method (Invitrogen) was used according to manufacturer's instructions. RNA was treated with RNase-free DNase to get rid of DNA contamination. After quantification using nanodrop, reverse transcription reactions were performed for 1 h at 37 °C using MML-Reverse transcriptase (Invitrogen) and oligo-dT. To isolate putative *TdSHN1* orthologous in barley, designated here *HvSHN1*, the first strand cDNA was used as template for PCR amplifications using a couple of specific primers derived from *TdSHN1* sequence (Djemal and Khoudi, 2015). PCR amplifications were performed in 25 µl total volume containing 0.15 mM of each dNTP, 2.5 µl of 10x *Taq* DNA polymerase buffer, 2.5 mM $MgCl_2$, one unit of *Taq* DNA polymerase, 2.5 µM gene specific primers. The PCR steps were as follows: 94 °C, 5 min for initial denaturation followed by 35 cycles of 30 s at 94 °C, 1 min.s at 55 °C and 1 min.30 s at 72 °C A 10 min extension period at 72 °C was added at the end of the 35 cycles. The PCR products were run on 1% agarose gels and the unique amplified band was cut, purified from agarose gel, cloned in pGEM-T easy vector and sequenced using ABI PRISM automated sequencer.

The expression profile of *HvSHN1* in barley was determined using quantitative expression analysis and specific primers (HvSHN1FW 5′-tccaaccaaaagggaaggct-3′, HvSHN1REV 5′-gcttccatttttacatcagcactca-3′). For this, RNA samples derived from various treatments were subjected to amplifications using SYBR Green fluorescent dye according to manufacturer's recommendations and flurometric thermal cycler (DNA Engine Opticon 2; MJ Research, Walthan, MA, USA). The SDS2.2 software integrated in Applied Biosystem 7900 H T Fast Real-Time PCR system was used to display the results. The C_T value among replications was used for comparison of repeated samples. The barley ACT2 gene (accession number MLOC_7453.1) was amplified using primer set (ACTFW 5′-ttcccaggtatcgctgaccg-3′, ACTRW 5′-cactgtacttcctctcaggtg-gagc-3′) and its C_T value was used for normalization of linear data. The relative expression was determined using the formula $2^{-\Delta\Delta CT}$.

To test the capacity of HvSHN1 to up-regulate stress responsive genes in transgenic tobacco, we selected four tobacco genes which are known to harbor GCC box (*NtCER1* and *PR5*) or DRE elements (*NtSAM* and *NtERD10*) in their promoter regions. Total RNA was isolated from leaves of stably-transformed tobacco plants expressing the *HvSHN1* transcription factor. Trizol method was used on 200 mg of fresh leaves derived from 40-days old transgenic tobacco plants grown *in vitro* in Magenta vessels at 22 °C under 16/8 light/dark photoperiod. qRT-PCR was performed as described above using gene-specific primers as follows: The *NtCER1* primer set (5′-gcaactaacttggtcctgtc-3′, 5′-gttacca-cactcgtgctc-3′), the *PR5* (5′-atgagaaagacccacgtc-3′, 5′-atgccttctttg-cagcag-3′) the *NtSAM* (5′-cagaccaataaacaagcttca-3′, 5′-cctgaaggactctttca-3′) and the *NtERD10* (5′-ggaagaagagaaggcgggtga-3′,

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