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AlSRG1, a novel gene encoding an RRM-type RNA-binding protein (RBP) from *Aeluropus littoralis*, confers salt and drought tolerance in transgenic tobacco

Rania Ben Saad[a,*], Nihed Ben Halima[a], Mouna Ghorbel[a], Nabil Zouari[a], Walid Ben Romdhane[a,c], Emmanuel Guiderdoni[b], Abdullah Al-Doss[c], Afif Hassairi[a,c]

[a] Biotechnology and Plant Improvement Laboratory, Centre of Biotechnology of Sfax, University of Sfax, B.P "1177" 3018, Sfax, Tunisia
[b] CIRAD, UMR AGAP, 34398 Montpellier Cedex 5, France
[c] College of Food and Agriculture Sciences, King Saud University, PO Box 2460, Riyadh 11451, Saudi Arabia

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ABSTRACT

We characterized a novel stress tolerance gene from *Aeluropus littoralis* encoding a protein designed as AlSRG1 (***A****. littoralis* **S**tress-**R**elated **G**ene 1). Phylogenetic and conserved domain prediction identified AlSRG1 as an uncharacterized protein of unknown function. The conserved domain database revealed that AlSRG1 contains an RNA-recognition motif (RRM). This domain was highlighted in AlSRG1 primary, secondary and tertiary structures with the two conserved motifs RNP1 and RNP2 being part of it. These two conserved motifs contain the active site for RNA-binding and a 3D-model of conserved domain (AlSRG1p) was built from known X-ray structures and sequence alignments. The analysis with RT-qPCR revealed higher *AlSRG1* transcript accumulation under salt and osmotic stresses. Overexpression of *AlSRG1* in transgenic tobacco plants improved both salt and osmotic tolerance and was accompanied by higher seedling survival rates, better growth parameters and ROS accumulation than non-transgenic plants (NT). Furthermore, the transgenic tobacco plants were able to complete their life cycle and to produce viable seeds while the NT plants died at the vegetative stage. The *AlSRG1* transgenic lines compared to NT tobacco, showed a significantly lower water loss rate in senescent-basal leaves. *AlSRG1* overexpression also enhanced the transcript levels of ROS-scavenging genes and some stress-related transcription factors under salt and osmotic stresses. Taken together, these results show that *AlSRG1* may play an important positive modulation role in abiotic stress tolerance.

1. Introduction

Abiotic stresses are the primary causes of crop loss worldwide, causing poor productivity or plant death in extreme conditions (Vinocur and Altman, 2005). The negative impacts of various environmental stresses are expected to be aggravated due to the forecasted climate instability. Therefore, there is an urgent need to improve crops' yield by developing stress tolerant varieties to cope with the upcoming problem of food security (Mahajan et al., 2008). Plants can be divided into salt sensitive "glycophytes" and salt tolerant "halophytes". Halophytes plants that are adapted to grow on saline environments represent a very important genetic resource for isolating novel genes and promoters underlying salt stress adaptation (Gong et al., 2005). Genetic engineering of abiotic stress responsive genes from halophytes represents a very promising approach for improving salt and drought tolerant in glycophyte crops (Rajalakshmi and Parida, 2012; Himabindu et al., 2016). Experimental studies in our laboratory have focused on the halophyte C4 grass *Aeluropus littoralis* with a small haploid genome of 349 Mb (2n = 2x = 20), in an effort to identify and characterize novel/unknown genes that enable salt tolerance (Zouari et al., 2007; Ben Saad et al., 2010). In addition to salt tolerance, *A. littoralis* is also capable to live in extreme drought and heat conditions without showing any detrimental symptoms. This plant proved to be a precious genetic resource to isolate and characterize salt-stress-regulated genes such as *AlSAP* and its promoter (Ben Saad et al., 2011). Despite the availability of many stress inducible genes that are known to induce salinity and drought tolerance upon introduction into susceptible plants, research is still ongoing to discover novel genes from any genetic sources. In addition to known functional genes, unknown and hypothetical genes provide a good candidate pool to find novel stress related genes.

Post-transcriptional processes are crucial to not only plant growth

* Corresponding author.
E-mail address: raniabensaad@gmail.com (R. Ben Saad).

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and development regulation but also to the stress responses (Ambrosone et al., 2012; Jung et al., 2013). RNA-binding proteins (RBPs) have important roles in post-transcriptional gene regulation by directly binding to single/double strand RNA (ssRNA/dsRNA) molecules (Ambrosone et al., 2012). They are defined as proteins that directly interact with RNA molecules via protein domains called RNA binding domains (Lukong et al., 2008). Obviously, RBPs have key roles as regulatory proteins in plant stress response and adaptation. They could be involved in the post-transcriptional processes of gene expression in plants under various stress conditions (Mittal et al., 2011; Yeap et al., 2012; Meyer et al., 2015; Lee and Kang, 2016). RBPs are widely distributed among plant species (Peal et al., 2011; Lewinski et al., 2016). Approximately 250 putative RBP genes have been identified in *Oryza sativa* and more than 200 in *Arabidopsis thaliana* (Lorkovic, 2009; Cook et al., 2011; Ambrosone et al., 2012).

Yeap et al., 2012 reported the characterization of *EgRBP42* gene from oil palm (*Elaeis guineensis* Jacq.) encoding a RBP which responds to various abiotic stresses such as salinity and drought. Recently, Xu et al. (2017) have reported a novel stress associated RNA-binding protein 1 (SRP1) repressed by ABA, salt and cold and encodes a C2C2-type zinc finger protein in *Arabidopsis*. SRP1 is involved in the ABA signaling by post-transcriptionally repressing *ABI2* expression in *Arabidopsis* (Xu et al., 2017). RBPs contain RNA-binding domains (RBDs) and several common motifs such as RNA recognition motifs (RRMs), K homology (KH) domain (Lorkovic and Barta, 2002), zinc finger (ZnF) domains (mainly CCCH type) (Kim et al., 2007; Qu et al., 2014), double-stranded RNA-binding domain (DS-RBD) (Tam et al., 2010), RNA helicase DEAD/DEAH box (Owttrim, 2006), Pumilio/FBF (PUF) domain, Piwi/Argonaute/Zwiklle (PAZ) domain (Song et al., 2003), and the auxiliary domains (glycine-rich, arginine-rich, arginine-glycine, or serine-arginine repeats) (Alba and Pagès, 1998; Ambrosone et al., 2012). The most widespread RBDs in plants are RRMs and KH (Chen and Varani, 2005; Lorkovic, 2009). The RBP repertoire of *A. thaliana* was studied recently by Marondedze et al. (2016). A survey of its genome revealed 196 RRM and 26 KH containing RBPs (Lorkovic and Barta, 2002). RRMs are found in a wide range of RBPs including small nuclear ribonucleoprotein (snRNP)-associated proteins, chloroplast RNA-binding proteins, poly(A)-binding protein, heterogeneous ribonucleoproteins, and SR proteins (splice factors with serine-arginine rich domains) (Lunde et al., 2007). Generally, RRM contains two short consensus sequences highly conserved motifs named RNP-1 and RNP-2 (Maris et al., 2005; Dominguez and Allain, 2006; Cléry et al., 2008).

To further understand the genetic basis of salt and drought tolerance mechanisms in *A. littoralis* at the molecular level, we have isolated, sequenced and annotated 492 salt stress regulated transcripts with a size ranging from ESTs to full length cDNAs using SSH technology (Suppression Subtractive Hybridization) (Zouari et al., 2007). In the present study, we reported the isolation and characterization of a novel gene, *AlSRG1*, encoding a stress related protein from *A. littoralis*. *AlSRG1* transcript accumulation was monitored under different salt and drought stress conditions and its overexpression in tobacco enhanced stress tolerance.

2. Materials and methods

2.1. Plant growth and stress treatments

A. littoralis seeds were collected from salt marshes near "Sfax", a coastal town in the South-East of Tunisia. Seeds of *A. littoralis* were surface sterilized, germinated on half strength MS (Murashige and Skoog, 1962) solid medium and grown in nutrient solution as described by Zouari et al. (2007). Later, the seedlings were grown for two months before treating them with different stress factors: high salinity (350 mM NaCl) and osmotic pressure (10% PEG 8000). Plants of *A. littoralis* were sampled at 0, 6, 24, 48 and 72 h after each treatment, frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Leaves of *Nicotiana tabacum* var. Xanthi were used to produce transgenic plants over-expressing *AlSRG1* gene.

2.2. AlSRG1 gene isolation from A. littoralis

We have previously isolated, sequenced and annotated 492 salt stress regulated transcripts with a size ranging from ESTs to full length cDNAs using SSH technology (Suppression Subtractive Hybridization) (Zouari et al., 2007). Basing on the hybridization profile using the unsubtracted and subtracted probes we have identified some ESTs which are the most abundant differentially expressed genes in roots and leaves (Zouari et al., 2007). The functional analysis of their full-length genes in tobacco can allow the identification of the key candidate genes to be used for crop stress tolerance improvement. As a demonstration of this strategy, we previously reported the isolation of the *AlSAP* gene and its promoter (Ben Saad et al., 2010, 2011). The constitutive expression of *AlSAP* in tobacco, wheat, and rice has resulted in enhanced tolerance to drought, salinity, cold, heat, and oxidative stresses (Ben Saad et al., 2010, 2012a,b; Ghneim-Herrera et al., 2017). Recently we studied the function of another gene *AlTMP1* encoding the plasma membrane protein 1 in transgenic tobacco (Ben Romdhane et al., 2017). In this work another EST "*AlSRG1*" (cloned in pDNR-LIB vector, CLONTECH) from this group was isolated from the *A. littoralis* cDNA library prepared from RNA extracted from roots stressed during 15 days with 300 mM NaCl (Zouari et al., 2007). The full-length cDNA clone was sequenced by using an ABI 3100 automatic DNA sequencer (Applied Biosystems) with M13F and M13R primers (Table S1). The sequence has been deposited in GenBank (KY522980).

2.3. In silico analysis

The *AlSRG1* sequence was compared with the reference sequences from NCBI (National Center for Biotechnology Information, USA) databases using BLAST (Altschul et al., 2005) and the multiple sequence alignment was performed using ClustalW algorithm (Thompson et al., 1994). Primary structure analysis using predicted aa sequences was performed with the ExPASy Proteomics tools. The ProtParam tool was used to calculate the theoretical parameters of the protein (Bairoch et al., 2005). Molecular Evolutionary Genetics Analysis (MEGA) package version 7 (Kumar et al., 2016) was used to generate the phylogenetic tree. A dendrogram was constructed using 20 sequences by the Neighbor-Joining method (Saitou and Nei, 1987). Then the evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992). Finally, the robustness of the inferred tree was evaluated by bootstrap (1000 replications). The prediction of the protein secondary structure was performed using The PSIPRED Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/). A three dimensional structure of AlSRG1 conserved domain (named AlSRG1p) was modeled using the PHYRE PROTEIN FOLD RECOGNITION Server (http://www.sbg.bio.ic.ac.uk/phyre2/) (Kelley et al., 2015). Various others automated protein structure homology-modeling servers such as Geno3D server (Combet et al., 2002) and MUSTER (Wu and Zhang, 2008), were also used to verify the reliability of AlSRG1 3-D models.

2.4. RT-qPCR

Total RNA was extracted from tissues of *A. littoralis* and of tobacco transgenic lines using the RNAeasy Plant mini kit (Qiagen) according to the manufacturer's protocol. Synthesis of first-strand cDNA was performed using 5 µg treated total RNA, SuperScript™ III reverse transcriptase (Invitrogen), oligo-(dT_{18}), and random hexamer primers according to manufacturer's instructions. Total cDNA from *A. littoralis* or from transgenic tobacco lines prepared as described above were used in RT-qPCR reactions. Transcript accumulation of *AlSRG1* was monitored in six independent transgenic lines by RT-qPCR using qAlSIP-F and