REPORT

Engineering drought and salt tolerance in plants using SodERF3, a novel sugarcane ethylene responsive factor

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ABSTRACT

The ability of plants to tolerate salt and drought conditions is crucial for agricultural production worldwide. The increased understanding of the regulatory networks controlling drought stress response has led to practical approaches for engineering salt and drought tolerance in plants. By a single-pass sequencing of randomly selected clones from a ë ZAP-cDNA library generated from ethephon-treated young sugarcane leaves, we identified an expressed sequence tag encoding a putative protein with a DNA-binding domain that is typically found in EREBP/AP2-type transcription factors. The full-length cDNA clone, named SodERF3 (EMBL accession number AM493723) was further isolated from the excised library. SodERF3 encodes a 240 amino acid DNA-binding protein that acts as a transcriptional regulator of the Ethylene Responsive Factor (ERF) superfamily, but also contains a C-terminal short hydrophobic region resembling an ERF-associated amphiphilic repression-like motif, typical for class II ERFs. This protein binds to the GGC box, and its deduced amino acid sequence contains an N-terminal putative nuclear localization signal. SodERF3 is induced in sugar cane leaves by ethylene, abscisic acid, salt stress and wounding as judged by Northern and Western blots assays. Greenhouse grown transgenic tobacco plants (Nicotiana tabacum L. cv. SR1) expressing SodERF3 were found to display increased tolerance to drought and osmotic stress without any visible phenotypic change in growth and development. According to our results SodERF3 will be a valuable tool to assist the manipulation of plants to improve their stress tolerance

*I*ntroduction

Growing in their natural environment, plants often encounter unfavorable environmental conditions that interrupt their normal growth and productivity. Among such environmental stresses drought and salinity are among the most important environmental constraints for agriculture in the world [1].

About 20% of the arable land in Cuba is affected by drought and salinity [2]; however, to date the strategies are inadequate to increase the stability of crop production under these conditions [3]. Therefore, engineering drought and salt tolerance in plants is of enormous economic importance.

Recent progress has been made in our understanding of gene expression, transcriptional regulation and signal transduction in plant responses to salinity and drought. On the other hand, molecular and genomic analyses have facilitated gene discovery and enabled genetic engineering using several functional or regulatory genes to activate or repress specific or broad pathways related to salinity and drought tolerance in plants. Also, the understanding of regulatory networks controlling drought/salinity stress response has led to practical approaches for engineering drought/salinity tolerance in plants.

Transcription factors (TF) involved in the response to ethylene, the so-called ethylene responsive factors (ERF-TF), confer tolerance to different biotic and abiotic stresses [4, 5]. These transcriptional regulators once activated by the ethylene biosynthesis pathway, increase or repress the activation of genes related to stress and vegetative development.

The main objectives of this study were: 1) The identification, isolation, cloning and characterization of a new sugarcane gene encoding a transcription factor linked to the response to ethephon (ethylene analogue) treatment called *SodERF*3; 2) To determine how the *SodERF*3 gene is regulated in sugarcane in the response to different hormones involved in biotic and abiotic stress, and 3) To assess tolerance to salinity and drought in transgenic 35S: *SodERF*3 tobacco lines used as an experimental model.

The working strategy included: 1) The identification and isolation of the gene encoding for an ERF-TF from a λ ZAP-cDNA library generated from ethephon-treated young sugarcane leaves; 2) The cloning and analysis of the S*odERF*3 DNA sequence; 3) The location of *SodERF3* phylogeny and the introduction of the new DNA sequence in the database EMBL-Gene Bank; 4) Showing the binding capacity of this protein to DNA *in vitro*; 5) Describing the regulation of this gene in sugarcane plants subjected to the treatment of different hormones related to abiotic factors; 6) Assessing the biological function of *SodERF*3 in transgenic model plants (*Nicotiana tabacum* L. cv. SR1) without and under salt stress and drought; and 7) A detailed phenotypic study of transgenic plants overexpressing *SodERF*3.

*R*esults and discussion

SodERF3 encodes a novel ERF-TF protein from sugarcane

Using a λ ZAP-cDNA library generated from ethephon-treated young sugarcane (*Saccharum officinarum* L. cv Ja60-5) leaves we isolated a cDNA encoding *SodERF3* (GeneBank No. AM493723), a 201 resi-

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dues polypeptide with a predicted molecular mass of 20,7 kDa. The N-terminal region of the predicted protein contains a short stretch of basic amino acids in its N-terminal that may function as a nuclear localization signal (NLS) and a putative DNA-binding domain that is highly conserved in all members of the APETALA 2 (AP2) ERF TF superfamily. Sequence analysis also revealed that this protein contains a short C-terminal hydrophobic region similar to an ERF-associated amphiphilic repression (EAR) motif (L/F)DLN(L/F)xP typical of Class II ERFs [5, 6]. However, in the case of *SodERF3* and other sugarcane EST as shown in red in figure 1, the seventh amino acid in the motif corresponds to a leucine (L) instead of the strictly conserved proline (P) described previously by Ohta *et* al. [6] in other ERFs proteins (Figure 1). This new finding may have an evolutionary, structural and physiological meaning. Sequence comparison analysis at the amino acid level showed that the *SodERF3* binding domain is highly homologous with both dicotylenedous and monocotylenedous members of the AP2/ERFs TFs superfamily so, according to the phylogeny, *SodERF3* is gathered in the B-subgroup; cluster VIII of the AP2 super family according to the classification of Nakano *et al.*[7].

Interestingly, the protein-protein BLAST search of *SodERF3* without the ERF domain showed in general very low similarity scores (less than 9%) of the flanking sequences with other members of the ERF family.

According to these findings *SodERF*3 encodes for a new sugarcane protein that belongs to the B-subgroup; cluster VIII of the AP2 super family and therefore, it is a new member of this family.

SodERF3 expression in sugarcane is induced by hormones involved in biotic and abiotic stress

Contrasting with the high presence of *SodERF3* transcripts easily detected in sugarcane leaves treated 48 hs with ethephon (ET), ABA, NaCl or wounding (W), a weak induction of *SodERF3* was observed upon the treatment with salicylic acid (SA) as shown in figure 2A. Time-course experiments showed that *SodERF3* expression can be observed as early as 1 h after the treatment with ethephon, ABA and NaCl (Figure 2B). The transcript amount gradually increased over 48 hs in the experiment. The protein synthesis and accumulation also occurred gradually after the ethephon treatment of sugarcane leaf discs as monitored by western blot analysis using antibodies against recombinant *SodERF3* (Figure 2C). The fact that *SodERF3* is induced by ABA and wounding could indicate the possibility that this ERF plays an integral role in both the biotic and abiotic signaling pathways.

		EAR Motive	
*SodERF3**	APPVGLG	LDLN LA L	LPPAEMVM
Ca164762*	VEAPPPT	LDLN LA L	PAF
NtERF3	AKNNGRG	LDLN EP P	PQEMA
OsERF3	AKNNGRG	LDLN RP P	PVEN
LeERF3	STTAGLN	LDLN FP P	PENM
AtERF7	RRKTPPQ	FDLN FP P	LDGV
AtERF8	KISPPLD	LDLN LA P	PAE
AtERF9	EPRRELN	LDLN LA P	PVVDV
AtERF10	KLRMEPD	LDLN AS P	
AtERF11	GRRVVLD	LDLN FP P	PPEN
AtERF12	NFPSPLS	LDLN HL P	SAPSAAT

Figure 1. Comparison of the amino acid sequences of the C-terminal EAR motives (marked in gray) of different class II ERF proteins from various plants. Asterisks indicate *SodERF3* and sugarcane EST CA164762. CA, Nt, Os, Le and At are referred to sugarcane, tobacco, rice, tomato and arabidopsis respectively.

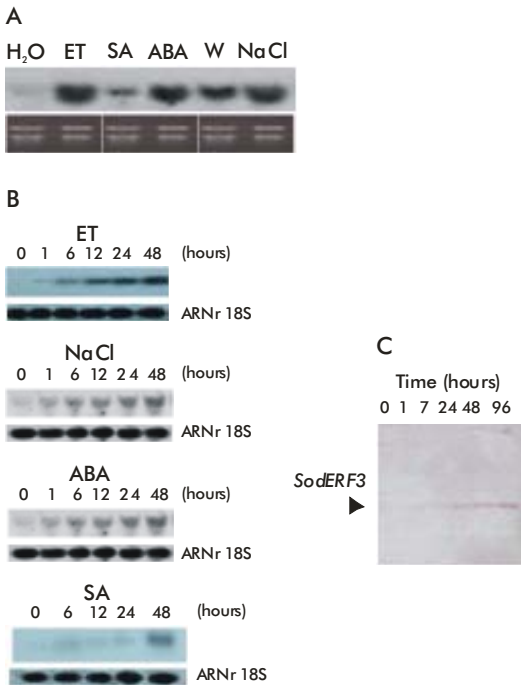

Figure 2. Expression of the *SodERF3* gene in response to hormones and abiotic stress. A) Northern blots showing the induction of the *SodERF3* gene upon wounding (W), or after a 48 hour treatment with ethephon (ET), salicylic acid (SA), abscisic acid (ABA), or NaCl. B) Time course of the accumulation of *SodERF3* transcripts after the treatment with ethephon (ET), NaCl, salicylic acid (SA) and abscisic acid (ABA). Equal RNA loading was verified by ethidium the bromide staining of the agarose gel or by using 18S rRNA as the probe respectively. C) Immunodetection of *SodERF3* by Western Blot. Total proteins isolated from a time series of ethephon-treated sugarcane leaf discs were separated on 12.5% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with polyclonal antibodies raised against *E. coli*-produced recombinant *SodERF3*.

ABA is the major plant hormone related to water stress signaling and regulates plant water balance and osmotic stress tolerance [8].

SodERF3 is a GCC box-binding protein

It has been demonstrated that ERF proteins interact through the EREBP/AP2 binding domain with the GCC box *cis* element present in the promoter region of the genes whose expression they control [9]. To analyze if *SodERF3* has GCC box-binding activity *in vitro*, recombinant *SodERF3* was used in an electrophoretic mobility shift assay. To this end, the *SodERF3* coding region was over-expressed as a fusion protein with a hexa-histidine tag in *Escherichia coli*.

The recombinant *SodERF3* produced a gel shift when the labeled probe with a wild-type GCC box was used (CATAAGA**GCCGCC**ACT), but not with a labeled probe carrying a mutated GCC box (CATA AGA**T**CC**T**CCACT) (Figure 3A). The *SodERF3* binding capacity was severely reduced in a competition assay with an excess of the unlabelled probe (Figure 3B). According to this experiment, *SodERF3* displays specific GCC box-binding activity *in vitro*.

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Ectopic expression of *SodERF3* enhances salt and drought tolerance in model tobacco plants

To evaluate the role of *SodERF3* in transcriptional responses, gene functional analysis in sugarcane would obviously be highly desirable; however, genetic tools in this crop are limited because of the generally polyploid genomes for most varieties, chromosomal mosaic and gene functional redundancy. Therefore, we generated transgenic tobacco lines expressing *SodERF3* under the transcriptional control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter, to investigate its biological function *in vivo*.

After a Northern Blot analysis we found that 30 independent transgenic tobacco lines showed different levels of *SodERF*3 transcripts (data not shown). Since *SodERF3* expression in sugarcane leaves was induced with the NaCl treatment (Figure 2A and B), we performed a germination assay in seeds collected from the 30 northern blot-positive transgenic tobacco lines. Twenty two lines were found to be tolerant to NaCl concentrations of 150 and 250 mM indicating that *SodERF3* expression enhances salt tolerance during seed germination (data not shown). However, we focused our attention on lines 16, 28 and 34 because their germination rate in 350 mM NaCl was quite close to that displayed by WT seeds grown in the MS medium without NaCl (Figure 4A). When these *SodERF*3-transformed seedlings were subsequently grown on the MS medium supplemented with 350 mM NaCl, they started a significant root elongation and vegetative development ($p < 0.001$; X^2 test), contrasting with the severely retarded growth of the WT non-transformed seedlings.

When 5-week-old soil-grown *SodERF* transgenic lines and control plants (n = 15 for each case) were watered with a 350 mM NaCl solution for 30 days, the percentage of plants reaching the adult stage and flowering was significantly higher ($p < 0.001$; X^2 test) for the transgenic lines than for WT (Figure 4B, 5A).

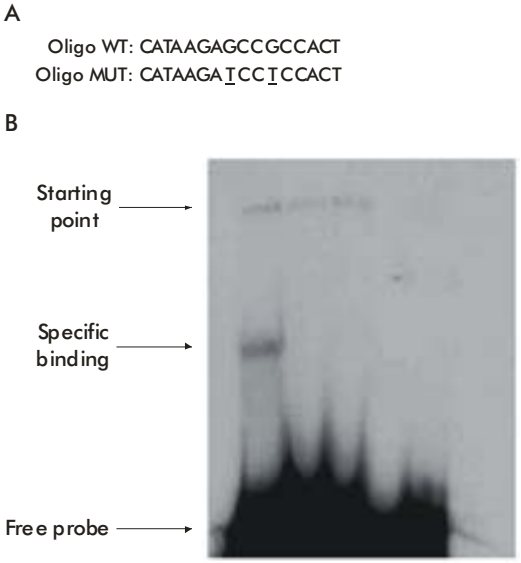

Figura 3. *SodERF3* specifically binds the regulatory GCC cis element. A) Oligonucleotides containing wild-type (WT) and mutated (MUT) GCC-box sequences that were used in the electrophoretic mobility shift assays. Mutated positions were low case and underlined. B) Electrophoretic mobility shift assays showing sequence-specific binding of the *SodERF3* fusion protein to the GCC box. Numbers indicate: 1- *SodERF3* incubated with the labelled wild-type oligonucleotide, 2- *SodERF3* incubated with the labelled wild-type oligonucleotide in the presence of excess unlabeled wild-type oligonucleotide, 3- *SodERF3* incubated with the labelled mutated oligonucleotide, 4- mutated oligonucleotide only, 5- wild-type oligonucleotide only, 6- *SodERF3* only.

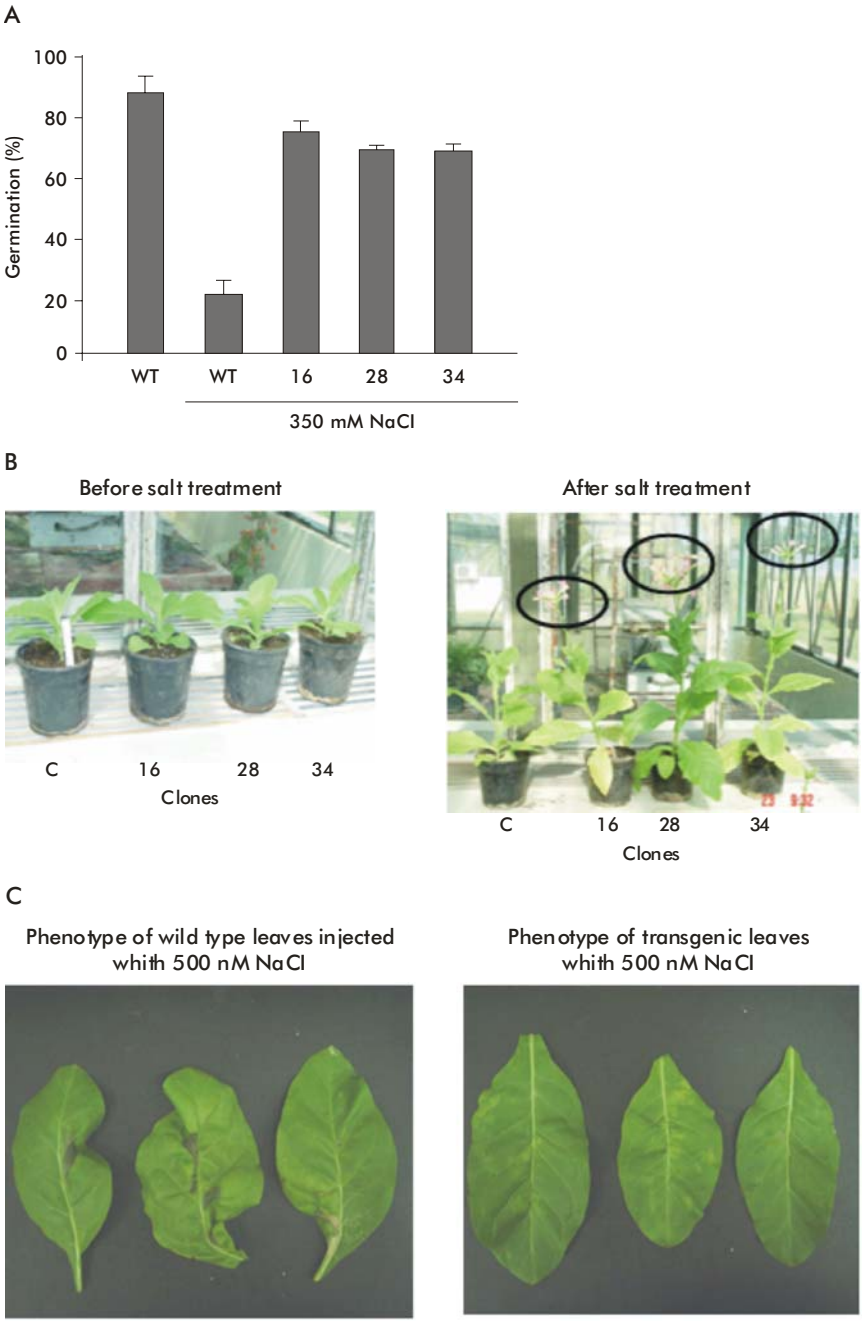

Figure 4. Expression of SodERF3 in tobacco (lines 34, 16, 28) enhances salt tolerance in: (A) Seed germination assay. 50 seeds (T2 generation) from wild type (WT) or each one of the transgenic tobacco lines 34, 16 and 28 were placed on plates containing MS medium (Murashige and Skoog, 1962) supplemented with 40 μg/mL kanamycin and 350 mM NaCl. Wild type seeds were also germinated in the MS medium without NaCl. Germinated Vegetative seeds were evaluated up to 15 days after sowing. (B) Evaluation of salt tolerance of greenhouse grown transgenic plants. 5-week-old soil-grown 35:SodERF3 lines were watered with a 350 mM NaCl solution for 30 days. A) and B) panels correspond to transgenic lines 16, 28, 34 and (C) control WT plants before and after salt treatment. C) Phenotype of wild type (WT) and transgenic leaves after 500 mM NaCl injection in the middle vein with a 30-gauge needle. Microsoft Excel ANOVA package was used for statistical analysis. Statistical significance compared against the value of the control plants was determined by Bartlett´s x^2 test.

Furthermore, leaves from the control line drastically changed their phenotype while *SodERF3* transgenic leaves significantly maintained their normal shape ($p < 0.001$; X^2 test), although occasionally chlorosis appeared, when a 500 mM NaCl solution was injected in the leaves of 5-week-old soil-grown plants (n = 15) (Figure 4C).

Drought tolerance was also tested and we found that after 30 days without watering, all the plants (n = 15) from clones 16, 28 and 34 were significantly taller ($p < 0.001$) than the WT control plants (Figure 5A, B). In contrast to WT, these drought tolerant plants were also able to flower when growth was extended to 60 days (Figure 5A).

Since it has been reported that the over-expression of heterologous ERFs in tobacco may cause deleterious effects and phenotypic changes [9,10], we evaluated phenotypic parameters in the transgenic lines compared to empty-vector transformants grown under greenhouse conditions without stress. These studies included plant height, number of leaves, leaf area, leaf weight and stalk weight. No significant phenotypic differences ($p < 0.001$) were found between *SodERF*3 transformants and control plants. Also, Northern blot analysis showed different accumulation levels of *SodERF*3 transcripts among unstressed lines 16, 28, and 34 compared to other recombinant lines (results not shown). According to these results, it is clear that the levels of *SodERF*3 transcripts correlate with the increased salt/drought tolerance observed in these clones.

For a better understanding of the *SodERF3* role in transcription, new lines of research must be addressed to generate transgenic sugarcane over-expressing *SodERF*3 and then, to determine other possible interactions and the battery of target genes related to this protein *in vivo*.

Soil salinity is a major factor reducing plant growth and productivity worldwide so, in the near future, these studies should assist in the manipulation of plants for improving stress tolerance.

Relevance of this research

SodERF3 is a new member of the FT-ERF family in sugar cane, closely related to salinity and drought tolerance. The C-terminal repression (EAR) motive in *SodERF3* is different from that described to date for other plants transcription factors. Proline (P), the seventh amino acid strictly conserved in the motive sequence (H / F) DLN (L / F) x P in other plant transcription factors, is a leucine (L) in *SodERF3*. This new FT-ERF is regulated in sugarcane plants by various hormones and abiotic factors. *SodERF3* is functional in tobacco plants used as the experimental model, and unstressed tobacco plants expressing the *SodERF*3 gene grown under greenhouse conditions have a normal phenotype. This fact makes *SodERF3* a potential candidate for the improvement of varieties of economic importance.

Acknowledgements

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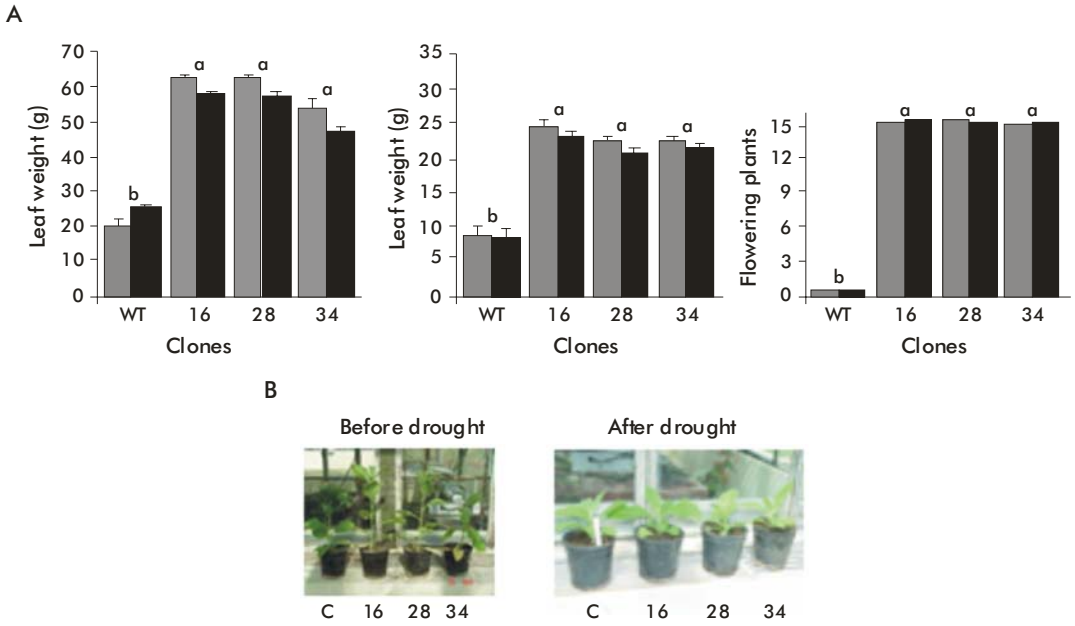

Figura 5. A) Phenotypic changes after NaCl (■) or drought (■) treatments in transgenic or WT plants. B) Plants under drought treatment for 30 days. Data gathered from these experiments were statistically processed using the Microsoft Excel ANOVA analysis package and the significance between means was determined by the x2 test. Values in the graphs correspond to the means +/- SD of 15 independents samples. Similar letters over the bars indicate no significant differences.