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SodERF3, a Novel Sugarcane Ethylene Responsive Factor (ERF), Enhances Salt and Drought Tolerance when Overexpressed in Tobacco Plants

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The molecular signals and pathways that govern biotic and abiotic stress responses in sugarcane are poorly understood. Here we describe *SodERF3*, a sugarcane (*Saccharum officinarum* L. cv Ja60-5) cDNA that encodes a 201-amino acid DNA-binding protein that acts as a transcriptional regulator of the ethylene responsive factor (ERF) superfamily. Like other ERF transcription factors, the SodERF3 protein binds to the GCC box, and its deduced amino acid sequence contains an N-terminal putative nuclear localization signal (NLS). In addition, a C-terminal short hydrophobic region that is highly homologous to an ERF-associated amphiphilic repression-like motif, typical for class II ERFs, was found. Northern and Western blot analysis showed that *SodERF3* is induced by ethylene. In addition, *SodERF3* is induced by ABA, salt stress and wounding. Greenhouse-grown transgenic tobacco plants (*Nicotiana tabacum* L. cv. SR1) expressing *SodERF3* were found to display increased tolerance to drought and osmotic stress.

Keywords: Abiotic stress — ABA — Ethylene responsive factors — EAR motif — Salt and drought tolerance — Sugarcane.

Abbreviations: AP2, APETALA 2; CaMV, cauliflower mosaic virus; CRT, C-repeat; DRE, dehydration-responsive element; EAR, ERF-associated amphiphilic repression; EMSA, electrophoretic mobility shift assay; ERF, ethylene responsive factor; EST, expressed sequence tag; NLS, nuclear localization signal; SA, salicylic acid; TF, transcription factor.

The *SodERF3* nucleotide sequence reported in this paper has been submitted to the EMBL database with the accession number AM493723.

Introduction

Plants devote a large portion of their genome to genes that are involved in transcription, as it can be illustrated by the *Arabidopsis thaliana* genome that encodes about 1,500 transcription factors (TFs) (Ratcliffe and Riechmann 2002). Most of these TFs are grouped in large families, some of which are unique to plants (Riechmann et al. 2000). One group of plant-specific transcription factors encompasses the so-called ethylene-responsive factors (ERFs) that act at the last step of ethylene signaling pathways, the first member of which was identified in tobacco (Ohme-Takagi and Shinshi 1995). To date, in different plant species ERFs have been found to be involved in growth, development and regulation of metabolism (van der Fits and Memelink 2000, van der Graaff et al. 2000, Banno et al. 2001), but also in the response to biotic and abiotic stress (Stockinger et al. 1997, Liu et al. 1998, Yamamoto et al. 1999, Fujimoto et al. 2000, Gu et al. 2000, Berrocal-Lobo et al. 2002, Gu et al. 2002, Dubouzet et al. 2003, Aharoni et al. 2004, Broun et al. 2004, Zhang et al. 2005, Xu et al. 2007).

ERF proteins contain a very characteristic and highly conserved plant-specific DNA-binding domain that consists mainly of 58–59 amino acids structured as a three-stranded antiparallel β-sheet and an α-helix in parallel to the β-sheet (Allen et al. 1998). Two similar *cis*-elements have been identified as binding sites in the promoters of ERF-controlled genes: the GCC box that is typically identified in pathogenesis-related (*PR*) genes, and the C-repeat (CRT)/dehydration-responsive element (DRE) motif in dehydration- and low-temperature-responsive genes (Singh et al. 2002).

ERFs belong to the large APETALA2 (AP2)/ERF TF superfamily that is unique to plants. The AP2 domain group comprises >150 ERF genes classified into two subgroups A and B, each of which is further divided into six clusters based on sequence conservation (Sakuma et al. 2002). Preliminary analysis of the A and B subgroups based on data from overexpression experiments and transcriptional activation suggests that TFs belonging to subgroup A are involved in abiotic stress responses, while those ones involved in disease resistance responses are found in the B subgroup (Sakuma et al. 2002, Gutterson and Reuber 2004). Recently, the 122 ERF genes identified in Arabidopsis were reclassified into 12 groups (Nakano et al. 2006). Several of these groups were further divided into

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subgroups and a comparative analysis between the phylogenetic relationships among the genes within these different groups was performed. This classification largely agrees with earlier reports on ERF phylogeny (Riechmann and Meyerowitz 1998, Sakuma et al. 2002).

Several well-studied ERFs, such as tobacco *ERF2* and *ERF4*, Arabidopsis *ERF1*, *ERF5*, *CBF1*, *DREB1* and *DREB2*, periwinkle *ORCA2* and *ORCA3*, and tomato *Pti4*, *Pti5*, *Tsi1* and *JERF3*, for example, function as transcriptional activators that, when overexpressed, lead to the activation of downstream genes (Stockinger et al. 1997, Zhou et al. 1997, Liu et al. 1998, Solano et al. 1998, Menke et al. 1999, Fujimoto et al. 2000, Ohta et al. 2000, van der Fits and Memelink 2000, He et al. 2001, Park et al. 2001, Wang et al. 2004). These transcriptional activators fit well within subgroup IX (Nakano et al. 2006) with their Arabidopsis homologs. In contrast, tobacco *ERF3*, and Arabidopsis *ERF3*, *ERF4*, for example, firstly categorized as class II ERFs (Ohta et al. 2000), act as transcriptional repressors. These repressors share a conserved ERF-associated amphiphilic repression-like motif (L/F)DLN (L/F)xP (Fujimoto et al. 2000, Ohta et al. 2000, McGrath et al. 2005) and could be gathered in subgroup B cluster VIII according to Nakano et al. (2006). Although repressor-like TFs, other than ERFs, have been identified in plants, yeast, mammals and *Drosophila* (Miller et al. 1985, Hanna-Rose and Hansen 1996, Yin et al. 2005), only a few ERF TFs able to bind the GCC box have been reported to be repressors in plants (Singh et al. 2002).

Sugarcane (*Saccharum officinarum* L.) is a tropical grass that has been cultivated for >4,000 years. In recent years, sugarcane cultivation occurs worldwide in tropical and subtropical regions and contributes >60% of the world sugar production. Importantly, sugarcane biomass is also increasingly used for the production of bioethanol as an alternative to petroleum-derived fuels. Despite its economic importance, sugarcane genetics is still in its infancy, which is largely due to the poor availability of genetics tools for *Saccharum* spp. (Grivet and Arruda 2001). This may be explained by the complexity of the sugarcane genome, which exceeds that of any other crop plant (D'Hont and Glaszmann 2001). Although a large amount of DNA sequence information for sugarcane was released into the public domain as expressed sequence tags (ESTs) derived from cDNA libraries (Casu et al. 2004), few genes that govern biotic or abiotic stress responses have been molecularly characterized from this crop to date.

It is well known that phytohormones mediate development and stress response by modulating the expression of specific subsets of hormone-responsive genes (Klee 2004). Ethylene, for example, affects the expression of a group of genes related to pathogen attack, wounding, abnormal temperatures and drought stress

Our search for sugarcane genes involved in ethylene responses led to the identification of a new sugarcane member of the ERF transcription factor family, named SodERF3, with the predicted characteristic DNA-binding domain, a nuclear localization signal (NLS) and a C-terminal ERF-associated amphiphilic repression (EAR)-like motif. A detailed phylogenetic analysis with other members of the AP2/ERF TFs indicates that SodERF3 belongs to subgroup VIII together with class II ERFs containing an EAR motif. Constitutive expression of the *SodERF3* gene in tobacco did not lead to phenotypical changes in plant growth and development, but enhanced drought and salt tolerance, a desired trait for crop engineering.

Results

SodERF3 encodes a novel ERF protein from sugarcane

By single-pass sequencing of randomly selected clones from a λ ZAP-cDNA library generated from ethephon-treated young sugarcane leaves, we identified an EST encoding a putative protein with a DNA-binding domain that is typically found in EREBP/AP2-type transcription factors. This cDNA fragment was subsequently used as a probe to isolate the corresponding full-length cDNA clone from the mass-excised library. The full-length cDNA clone, named *SodERF3* (EMBL accession No. AM493723), contains an open reading frame encoding a polypeptide of 201 residues with a predicted molecular mass of 20.7 kDa (Fig. 1). The N-terminal region of the predicted protein contains a short stretch of basic amino acids in its N-terminus that might function as an NLS, and a putative DNA-binding domain (Fig. 1). These elements are highly conserved in members of the ERF family of TFs. In addition, a short C-terminal hydrophobic region appears highly similar to an EAR motif (L/F)DLN(L/F)xP typical of class II ERFs (Fujimoto et al. 2000, Ohta et al. 2000). However, as shown in Fig. 2A the seventh amino acid in the EAR motif of SodERF3 corresponds to a leucine (L) instead of the strictly conserved proline (P) described previously by Ohta and co-workers (2000) in class II ERF repressors and zinc finger proteins.

A phylogenetic analysis based on the structural organization of members of the subgroup B of the single AP2 domain is shown in Fig. 2B. The overall distribution of the sequences corresponding to 161 ERF DNA-binding domains (see Materials and Methods), gathered in clusters B1–B6 according Sakuma et al. (2002) or subgroups V–X according to a recent reclassification by Nakano et al. (2006), agrees with that reported earlier by Gutterson and Reuber (2004). However, in our phylogenetic tree, some sequences are assigned to different clusters and their classification varies, in relation to other previous phylogenetic trees (Sakuma et al. 2002, Gutterson and Reuber 2004)

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GAA TTC GGC ACG AGC GGA GCT CCA CGG CGG CAA ACA AGC AGG AGA AGA CAA GGC     54
AAG GCA AGA AGC CTG CGA GCG GCG ACT TCG TGC GCG ACA CCC CGG ATT ACT GGT    108

          M   A   P   K   R   S   T   S   P   T   G   S   S   S   G   G     54
CCA TCC ATG GCG CCC AAG AGA TCG ACG TCG CCG ACC GGG AGC AGC AGC GGC GGC    162

 V   R   A   A   A   T   E   Q   P  R  L  R  G  V  R  K  R  P             72
GTC CGC GCG GCG GCG ACG GAG CAG CCG AGG CTC CGC GGC GTG CGG AAG CGG CCG    216

 W   G   R   Y   A   A   E   I   R   D   P   V   L   K   A   R   V   W     90
TGG GGC CGG TAC GCG GCG GAG ATC CGG GAC CCG GTG CTG AAG GCG CGC GTG TGG    270

 L   G   T   F   D   T   P   E   Q   A   A   R   A   Y   D   A   A   A    108
CTG GGC ACC TTC GAC ACG CCC GAG CAG GCG GCG CGG GCG TAC GAC GCC GCC GCG    324

 R   R   L   R   G   P   G   A   T   T   N   Y   P   A   A   S   E   P    126
CGC AGG CTC CGC GGG CCC GGC GCC ACC ACC AAC TAC CCC GCC GCC TCG GAG CCG    378

 M   V   P   A   V   S   G   S   A   V   L   S   E   S   S   S   S   S    144
ATG GTT CCA GCG GTG AGC GGC AGC GCC GTG CTG TCT GAG TCG TCG TCG TCG TCC    432

 S   W   S   L   L   P   E   S   V   T   A   A   V   A   A   P   P   P    162
TCG TGG TCC CTG CTG CCG GAG TCG GTG ACG GCG GCG GTC GCG GCG CCG CCC CCG    486

 S   L   D   L   S   L   A   L   P   A   S   A   A   A   A   N   T   Y    180
TCG CTG GAC CTC AGC CTG GCG CTG CCG GCT TCT GCG GCG GCC GCA AAC ACG TAC    540

 Q   V   F   M   D   P   T   P   A   L   L   Q   F   L   P   P   K   S    198
CAG GTG TTC ATG GAC CCG ACG CCG GCG CTG CTG CAG TTC CTG CCG CCG AAG AGC    594

 E   E   E   Q   S   C   S   G   S   L   S   S   S   S   G   V   F   D    216
GAG GAG GAG CAG AGC TGC TCT GGG TCG CTG TCG TCA TCG TCT GGG GTG TTC GAC    648

 A   A   P   P   V   G   L   G  L  D  L  N  L  A  L   L   P   P          234
GCG GCG CCG CCC GTG GGC CTG GGG CTG GAC CTC AAC CTG GCG CTG CTG CCG CCG    702

 A   E   M   V   M   *                                                   252
GCC GAG ATG GTC ATG TGA TTG CCG CCC GCG TTC CAG GAG ACT AAT CGA TTG CTT    756


AAT TAA TTA ATT AGT CAT TAT TAG TAG TAG CTA ATC GAT GGA GGC AGT CGT TCA    810
GGT GTG TGT AAA TAG GAT GCA GAT TTG TGT GTG TGC AGT GCA GGC GCG TAG CCC    864
CCT CTT TTC TTT GTT TGT ACT GCC AGA TTT GCG CCT TTG GTT GGT GCA AGT GAT    918
GCA ACA ATG AAA AAA AAA AAA CCG AGG ATA ATT ATG CCC CCA GCG GTT CTC ATT    972
TTA AAA AAA AAA AAA AAA AAA CTC GAG
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Fig. 1 Nucleotide and predicted amino acid sequence of SodERF3, EMBL accession No. AM493723. The ERF or DNA-binding domain is shown in bold and underlined. Amino acid residues comprising a putative nuclear localization signal are indicated with asterisks, and the C-terminal ERF-associated amphiphilic repression (EAR) motif (L/F)DLN(L/F)xP is indicated in bold and italics.

constructed with other methods or distance models. Our analysis confirmed that SodERF3 clustered within subgroup VIII (Nakano et al. 2006), in a novel subcluster together with the sugarcane EST CA164762 of unknown function. The subgroup IX (Nakano et al., 2006) includes several ERFs that activate downstream genes when they are overexpressed in transgenic plants, while subgroup VIII/B1 comprised TFs of class II ERFs containing conserved EAR repression-like motifs. A few of them, labeled with a star in Fig. 2B, have been tested *in vitro* and found to act as transcriptional repressors (Ohta et al., 2001).

Sequences CA130476 and CA101530 (Cavalcante et al. 2007) and five sugarcane EST sequences that encode putative ethylene-binding domains (CA239762, CA149330, CA282648, CA212910 and CA106080) available in public databases all belong to different clusters others than VIII (Fig. 2B). Sequence comparison analysis at the amino acid level showed that the SodERF3 DNA-binding domain is highly homologous with both dicotylenedous and monocotylenedous subgroup VIII members of the AP2/ERFs TFs (Fig. 3). The six amino acids (R, W, E, R, R and W) that have previously been identified to interact with the DNA bases (Allen et al. 1995) are well conserved in the VIII cluster members, including SodERF3 (Fig. 3). The few differences between SodERF3 amino acids and the reported consensus binding sequence are not expected to account for differences in binding activity since these regions are not reported to be directly involved in the interaction with DNA.

Interestingly, a protein–protein BLAST search of SodERF3 without the ERF domain showed in general a very low similarity score (<9%) of the flanking sequences with other members of the ERF family; including, besides sugarcane genes CA130476 and CA101530 reported by Cavalcante et al. (2007), EST sequences of unknown function available in public databases (see Supplementary Material 1). Only the sugarcane CA164762 EST is very close to SodERF3.

A **Class II ERFs**

	EAR motif
SodERF3*	APPVGLG LDLN LAL LPPAEMVM
CA164762*	VEAPPPT LDLN LAL PAF
NtERF3	AKNNGRG LDLN EP PPQEMA
OsERF3	NKAAAFD LDLN RP PPVEN
LeERF3	STTAGLN LDLN FP PENM
AtERF7	RRKTPFQ FDLN FP PLDGV
AtERF8	KISPPLD LDLN LA PPAE
AtERF9	EPRRELN LDLN LA PPVVDV
AtERF10	KLRMEPD LDLN AS P
AtERF11	GRRVVLD LDLN FP PPPEN
AtERF12	NFPSPLS LDLN HL PSAPSAAT

B

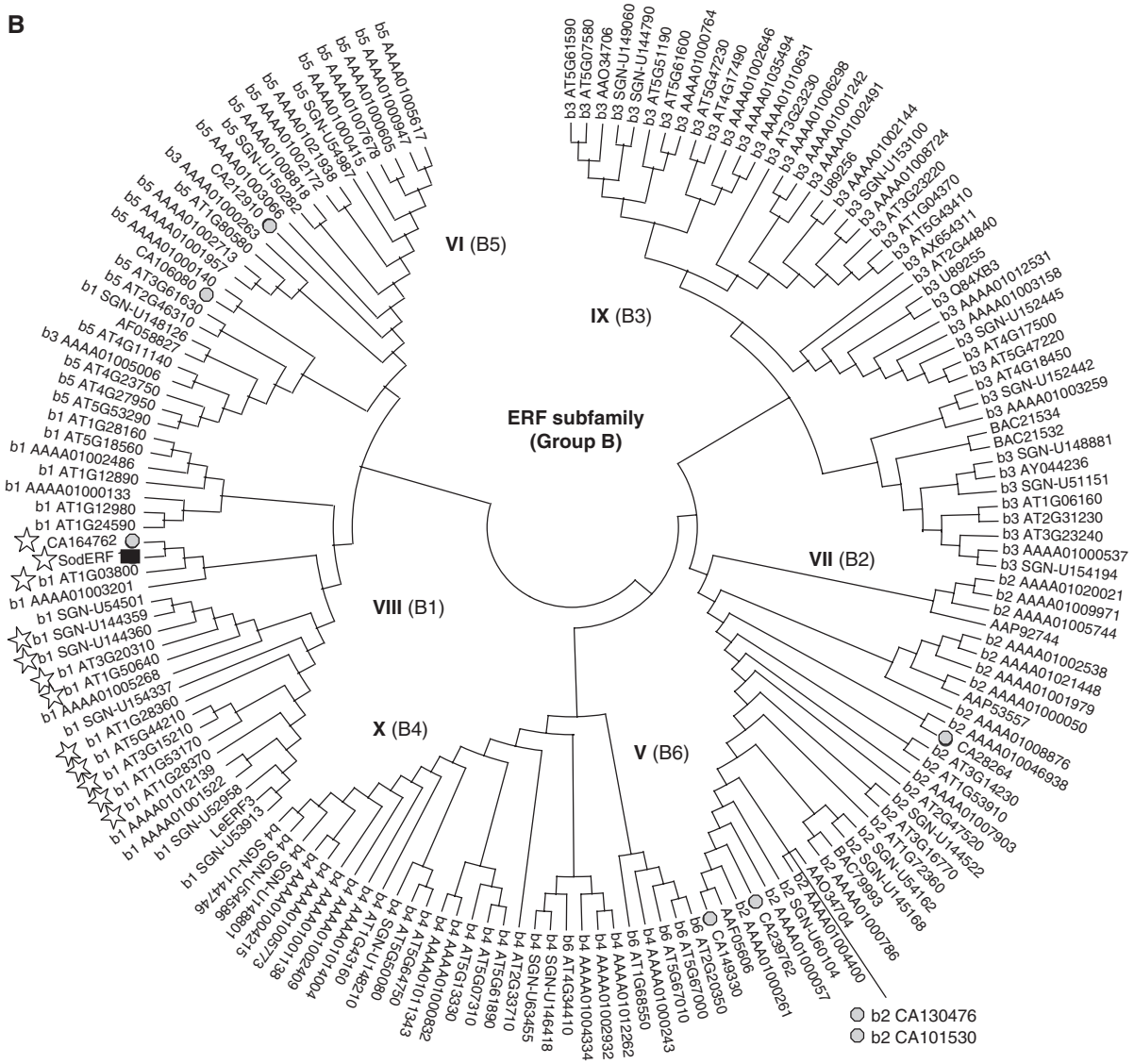

Fig. 2 (A) Comparison of the amino acid sequences of the C-terminal regions of class II ERF proteins from various plants. Asterisks indicate SodERF3 and sugarcane EST CA164762. (B) Phylogenetic tree of the AP2 B-subgroup of ERF TFs. AT, SGN, AAAA and CA correspond to identifiers for Arabidopsis, tomato or tobacco homologous, rice and sugarcane EST genes, respectively. The ERF classification according to Sakuma et al. (2002) is indicated at the beginning of each classifier. The classification according to Nakano et al. (2006) is indicated outside the parenthesis. *SodERF3* is indicated with a black square. Other known sugarcane genes and ESTs are indicated in gray circles. Indicated with a star are some members of the VIII/B1 cluster containing a defined EAR-like motif: SodERF3, CA164762, At1g28370 (AtERF11), At1g53170 (AtERF8), At3g15210 (AtERF4), At1g03800 (AtERF10), At1g50640 (AtERF3), At3g20310 (AtERF7), At1g28360 (AtERF12), At5g44210 (AtERF9), SGN-U144360 (ERF3), SGN-U144359 (ERF4).

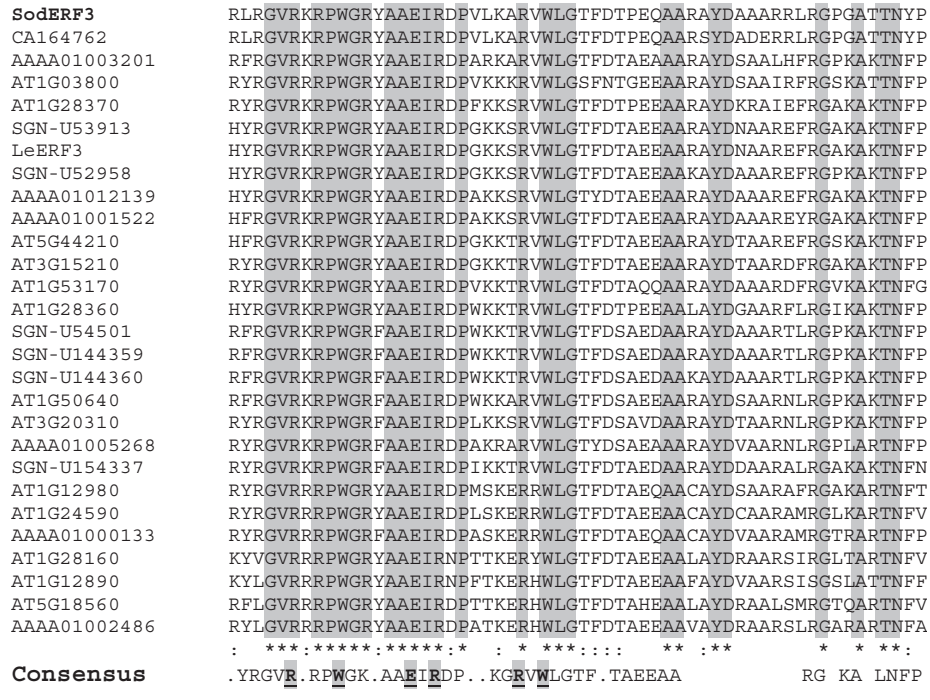

Fig. 3 Multiple alignments of the ERF DNA-binding domain of SodERF3 and other members of the VIII cluster. AT, SGN, AAAA and CA correspond to identifiers for *Arabidopsis*, tomato, rice and sugarcane genes, respectively. Boxes in gray and with asterisks represent 100% identity with respect to the consensus. Double dots indicate partial lack of homology. The six amino acids previously identified to interact with promoter elements are in bold and underlined.

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

SodERF3 expression in unchallenged sugarcane plantlets under normal physiological growth conditions was almost undetectable by Northern blot analysis (Fig. 4A). Contrasting with the high level of *SodERF3* transcripts easily detected in plantlets treated with ethephon, ABA, NaCl or wounding, a weak induction of *SodERF3* was observed upon treatment with salicylic acid. Time-course experiments showed that *SodERF3* expression can be observed as rapidly as 1 h after treatment with ethephon, ABA and NaCl (Fig. 4B). The transcript abundance gradually increased over 48 h of the experiment. The protein synthesis and accumulation also occurred gradually after ethephon treatment of sugarcane leaf discs as monitored by Western blot analysis using antibodies raised against recombinant SodERF3 (Fig. 4C).

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 (Ohme-Takagi and Shinshi 1995). To analyze whether SodERF3 has GCC box-binding activity *in vitro*, recombinant SodERF3 was used in an electrophoretic mobility shift assay (EMSA). To this end, the *SodERF3* coding region was overexpressed 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 (CATAAGAGCCGCCACT), but not with a labeled probe carrying a mutated GCC box (CATAAGAtCCtCCACT) (Fig. 5). The SodERF3 binding capacity was severely reduced in a competition assay with excess unlabeled probe (Fig. 5). According to this experiment, SodERF3 displays specific GCC box-binding activity *in vitro*.

Ectopic expression of SodERF3 in tobacco enhances salt and drought tolerance

The presence of a functional DNA-binding domain and an uncommon EAR consensus sequence indicates that SodERF3 might modulate the transcriptional activation/repression of a group of genes. To evaluate the role of *SodERF3* in transcriptional responses, gene functional analysis in sugarcane would be obviously highly desirable; however, genetic tools in this crop are limited because of the generally polyploid genomes for most varieties, and chromosomal mosaic and gene functional redundancy. Therefore, we generated transgenic tobacco lines expressing *SodERF3* under the transcriptional control of the

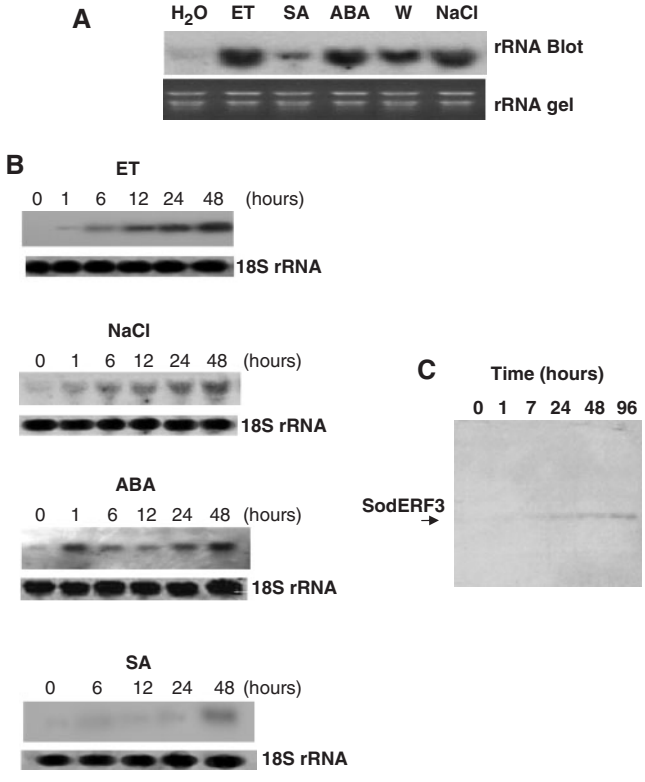

Fig. 4 Expression of the *SodERF3* gene in response to hormones and abiotic stress. (A) Northern blots showing the induction of the *SodERF3* gene upon wounding (W), or after 48 h treatment with ethephon (ET), salicylic acid (SA), abscisic acid (ABA), or NaCl. (B) Time course of the accumulation of *SodERF3* transcripts upon treatment with ethephon (ET), NaCl, ABA and salicylic acid (SA). Equal RNA loading was verified by ethidium bromide staining of the agarose gel or by using 18S rRNA as probe. (C) Immunodetection of SodERF3 by Western blotting. Total proteins isolated from a time series of ethephon-treated sugarcane leaf discs were separated on a 12.5% SDS–polyacrylamide gel, transferred to a nitrocellulose membrane and probed with polyclonal antibodies raised against *E. coli*-produced recombinant SodERF3.

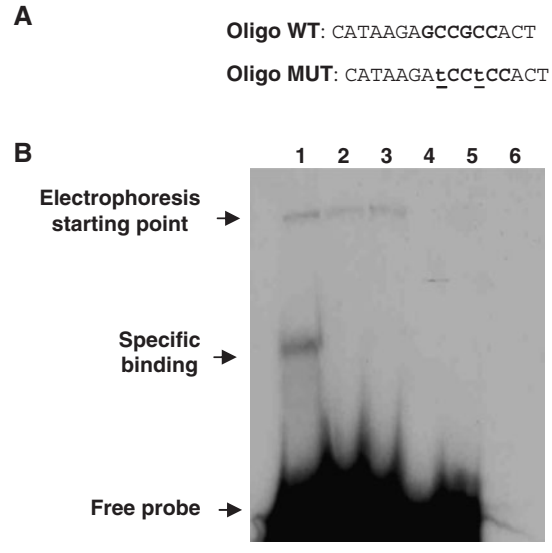

Fig. 5 SodERF3 specifically binds the regulatory GCC *cis*-element. (A) Oligonucleotides containing wild-type (WT) and mutated (MUT) GCC box sequences that were used in the electrophoretic mobility shift assays (EMSAs). Mutated positions are in lower case and underlined. (B) EMSAs showing sequence-specific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

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 (results not shown). Since *SodERF3* expression in sugarcane leaves was induced upon NaCl treatment (Fig. 4A), we performed a germination assay using 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 (results not shown). However, we focused our attention on lines 16, 28 and 34 because their germination rate in 350 mM NaCl was quite near to that displayed by wild-type seeds on MS medium without NaCl (Fig. 6A). When these *SodERF3*-transformed seedlings were subsequently grown on MS medium supplemented with 350 mM NaCl, a significant root elongation and vegetative development initiated ($P < 0.001$; χ^2 test), contrasting with the severely retarded growth of the non-transformed seedlings (Fig. 6B).

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 d, the percentage of plants reaching the adult stage and flowering was significantly higher ($P < 0.001$; χ^2 test) for the transgenic lines than for the wild type (Fig. 7A, D).

Furthermore, when a 500 mM NaCl solution was injected in the leaves of 5-week-old soil-grown plants ($n = 15$), leaves of the control line drastically changed their phenotype while *SodERF3* transgenic leaves kept their normal shape significantly ($P < 0.001$; χ^2 test), although occasionally chlorosis appeared (Fig. 7B).

Drought tolerance was also tested and we found that after 30 d without watering, all the plants ($n = 15$) from clones 16, 28 and 34 were significantly taller ($P < 0.001$) than wild-type control plants (Fig. 7C, D). In contrast to the wild type, these drought-tolerant plants were also able to flower when growth was extended to 60 d (Fig. 7D).

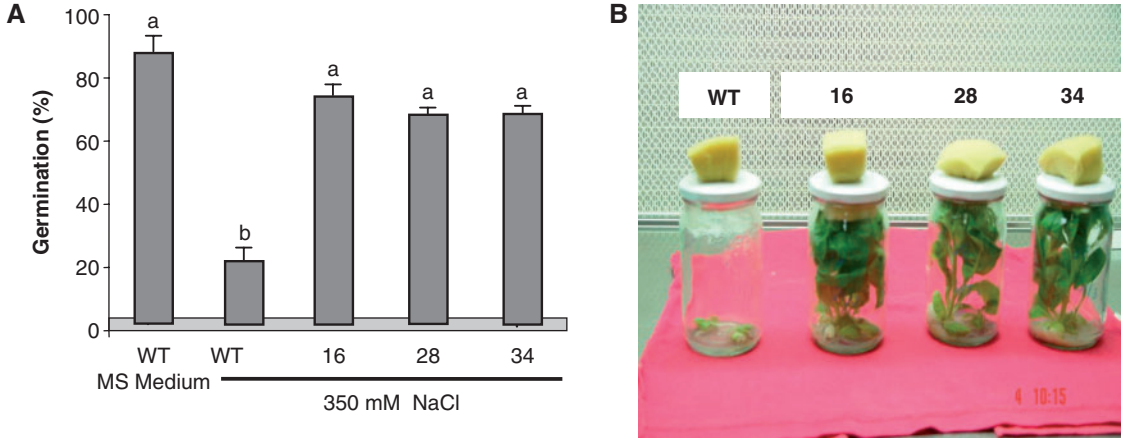

Fig. 6 Expression of *SodERF3* in tobacco (lines 34, 16 and 28) enhances salt tolerance in: (A) seed germination assay. A total of 50 seeds (T_2 generation) from the wild type (WT) or each of the transgenic tobacco lines 34, 16 and 28 were placed on plates containing MS medium (Murashige and Skoog 1962) supplemented with 350 mM NaCl. Wild-type seeds were also germinated in MS medium without NaCl. Germinated seeds were evaluated until 15 d after sowing. (B) Vegetative stages: root elongation and vegetative development of transgenic lines and wild type (WT) seedlings were evaluated in MS medium supplemented with 350 mM NaCl. The Microsoft Excel statistical ANOVA package was used for data statistical analysis. Statistical significance compared against the value of the control plants was determined by Bartlett's χ^2 test. Different letters over the bars indicate significative differences.

Since it has been reported that overexpression of heterologous ERFs in tobacco may cause deleterious effects and phenotypic changes (Kasuga et al. 2004), we evaluated phenotypic parameters in the transgenic lines compared with 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 *SodERF3* transformants and control plants (Fig. 8A).

A Northern blot analysis showed different accumulation levels of *SodERF*3 transcripts among unstressed lines 16, 28 and 34 compared with other recombinant lines (Fig. 8B). According to this result, it is clear that the levels of *SodERF*3 transcripts correlate with the increased salt/drought tolerance observed in these clones. Despite the fact that a probe matching with the 3′-untranslated region (UTR) region of *SodERF3* was used, still a non-specific endogenous background that is not salt/drought inducible was detected in the wild type.

Ectopic expression of SodERF3 in tobacco does not enhance resistance against Phytophthora parasitica

To evaluate whether the *SodERF3* gene is involved in antifungal resistance, greenhouse-grown wild-type and transgenic tobacco plantlets were inoculated with the oomycete pathogen *Phytophthora parasitica* var. *nicotianae*, the causal agent of tobacco black shank disease. Since the development of disease symptoms occurred similarly in inoculated transgenic tobacco plants and the wild type, we concluded that *SodERF3* expression is not involved in resistance, at least, against this pathogen.

Many pathogenesis-related (PR) genes, such as *PR-2*, *PR-3* and *PR-5* are induced upon abiotic and biotic stress stimuli, and have been found to carry a GCC box sequence at their promoter region. Since SodERF3 specifically binds the GCC box (Fig. 5), we assayed whether SodERF3 is able to induce the expression of well characterized tobacco PR genes, such as β-1,3-glucanase (*PR-2*), osmotin (*PR-5*) and *rd29A* in the leaves of greenhouse-grown plants. Northern analysis showed that *SodERF3* does not activate the expression of these genes.

Discussion

In this paper we describe the isolation and characterization of SodERF3, a novel sugarcane ERF-like protein. Phylogenetic analysis of the predicted SodERF3 sequence locates this protein in the AP2 family, B-subgroup, cluster VIII, according to the classification of Nakano et al. (2006). This cluster comprises mainly transcriptional repressors containing the consensus EAR sequence (L/F)DLN(L/F)xP at the C-terminal region. The EAR motif has been shown to function as a conserved repression domain in repressor-type ERF proteins (Fujimoto et al. 2000, Ohta et al. 2000, Ohta et al. 2001, McGrath et al. 2005) and also in TFIIIA-type C2H2 zinc-finger proteins (Ohta et al. 2001, Hiratsu et al. 2004). Interestingly, the seventh amino acid in the EAR-like motif of SodERF3 and the sugarcane EST CA164762 that formed a well-supported branch (bootstrap value of 98%)

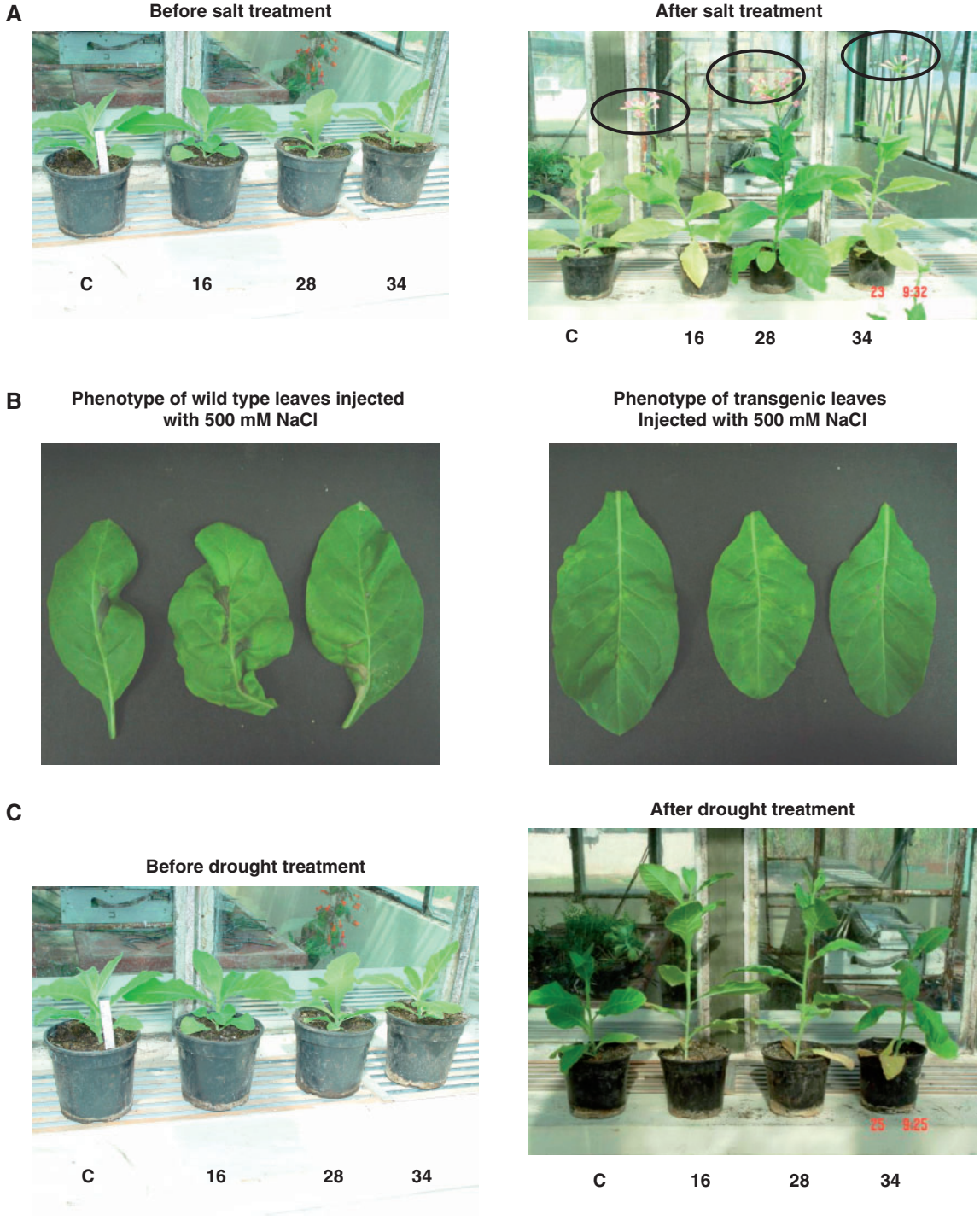

Fig. 7 (A) Evaluation of salt tolerance of greenhouse-grown transgenic plants. Five-week-old soil-grown 35:*SodERF3* lines were watered with a 350 mM NaCl solution for 30 d. (A and B) correspond to transgenic lines 16, 28, 34 and (C) control wild-type (WT) plants before and after salt treatment. (B) Phenotype of WT and transgenic leaves after 500 mM NaCl injection in the middle vein with a 30-gauge needle. (C) Evaluation of drought tolerance of greenhouse-grown transgenic tobacco lines. Five-week-old soil-grown 35:*SodERF3* lines 16, 28 and 34, or the WT (C) as control ($n = 15$), were left for 20 d without watering. (D) Phenotypical changes after NaCl or drought treatments in transgenic or WT plants. Data gathered from these experiments were statistically processed using the Microsoft Excel statistical ANOVA analysis package. Statistical significance compared with the value of the control plants was determined by Bartlett's χ^2 test. Values in the graphics correspond to the means ± SD of 15 independent samples. Different letters over the bars indicate significant differences.

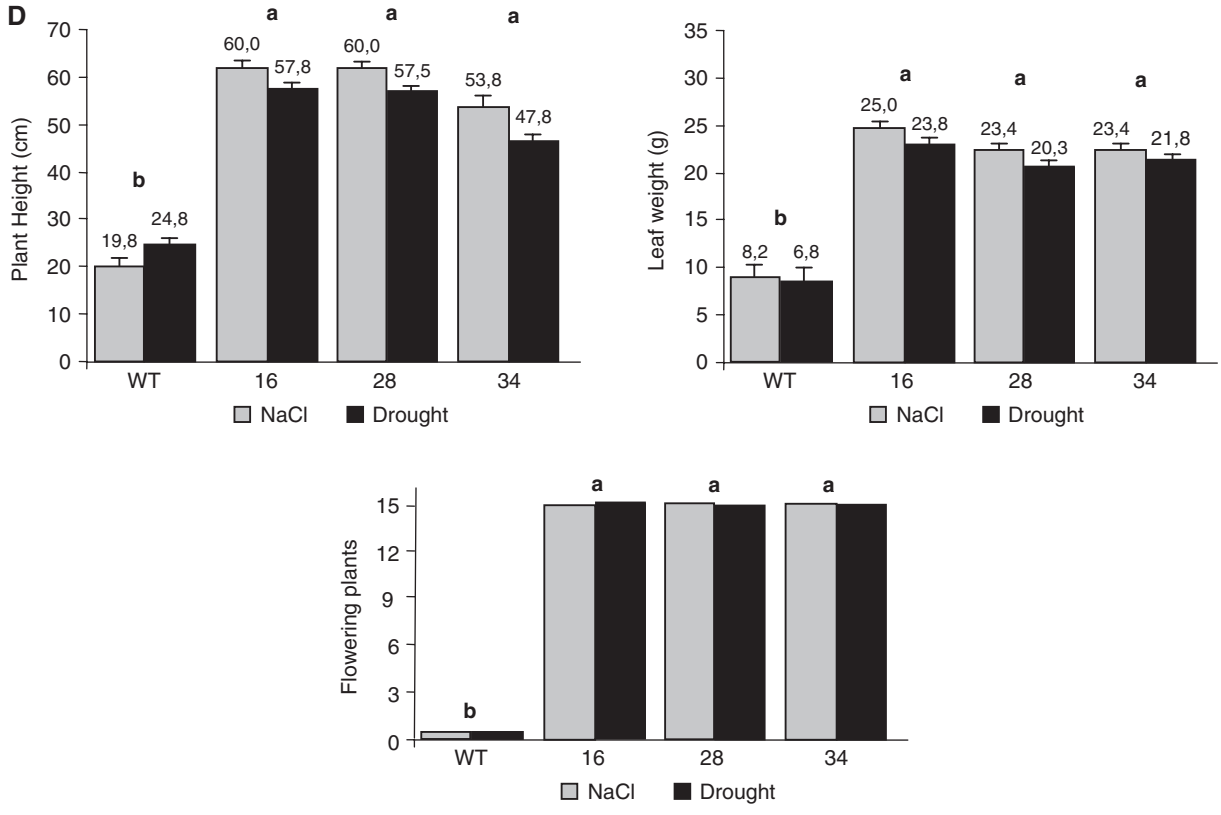

Fig. 7 Continued.

corresponds to leucine (L) instead of the proline (P) found strictly conserved in other repressor ERF proteins gathered in cluster VIII (Fujimoto et al. 2000, Ohta et al. 2001) (Fig. 2A, B). Ohta and co-workers (2001) and Tsukagoshi et al. (2005) convincingly demonstrated that a single amino acid change mutation within the conserved bases of the EAR motif is enough to abolish completely the repression capacity of these proteins.

ERFs have been found to modulate activation or repression of GCC box-mediated gene expression in tobacco and Arabidopsis, indicating that they respond to extracellular signals in distinct ways (Fujimoto et al. 2000, Ohta et al. 2000, Ohta et al. 2001). *SodERF3* is up-regulated in sugarcane by salt stress, wounding and different hormones. The fact that *SodERF3* is induced by ABA and wounding could indicate the possibility that this ERF plays an integral role in both biotic and abiotic signaling pathways and may be responsible for regulating the possible antagonisms between them (Anderson et al. 2004). In contrast, other known sugarcane ERFs (*ScERF1* and *ScERF2*) are not induced by wounding (Cavalcante et al. 2007). ABA is the major plant hormone related to water stress signaling and regulates plant water balance and osmotic stress tolerance (Denekamp and Smeekens 2003). Wounding is another stress factor often closely related to osmotic stress. Tissue damage is usually associated with decompartmentalization, release of cellular contents and a loss of water. Such damage induces local osmotic stress responses similar to those occurring in water-stressed intact plants (Reymond et al. 2000). On the other hand, *SodERF3* was activated slowly under salicylic acid (SA) treatment. This result is in sharp contrast to the expression pattern of other ERF genes such as *Tsi1* (Park et al. 2001), whose induction by SA was achieved in <1 h exposure.

In vitro experiments confirmed that SodERF3 interacts with the GCC box as it has been previously reported for other ERFs (for a review, see Oñate-Sanchez and Singh 2002). In addition, other ERFs that displayed improved tolerance to salt stress as well as to pathogen infection in transgenic tobacco plants, such as *Tsi1* (Park et al. 2001) and *CaERFLP1* (Lee et al. 2004), also bound specifically to the DRE/CRT *cis*-element, albeit with significantly lower affinity. Despite differences in binding affinity for different *cis*-elements *in vitro*, Lee and co-workers (2004) found that genes containing GCC or DRE/CRT *cis*-elements in their promoters were overexpressed in the analyzed transgenic plants.

Genetic tools in sugarcane are limited because of the generally polyploid genomes for most varieties, and

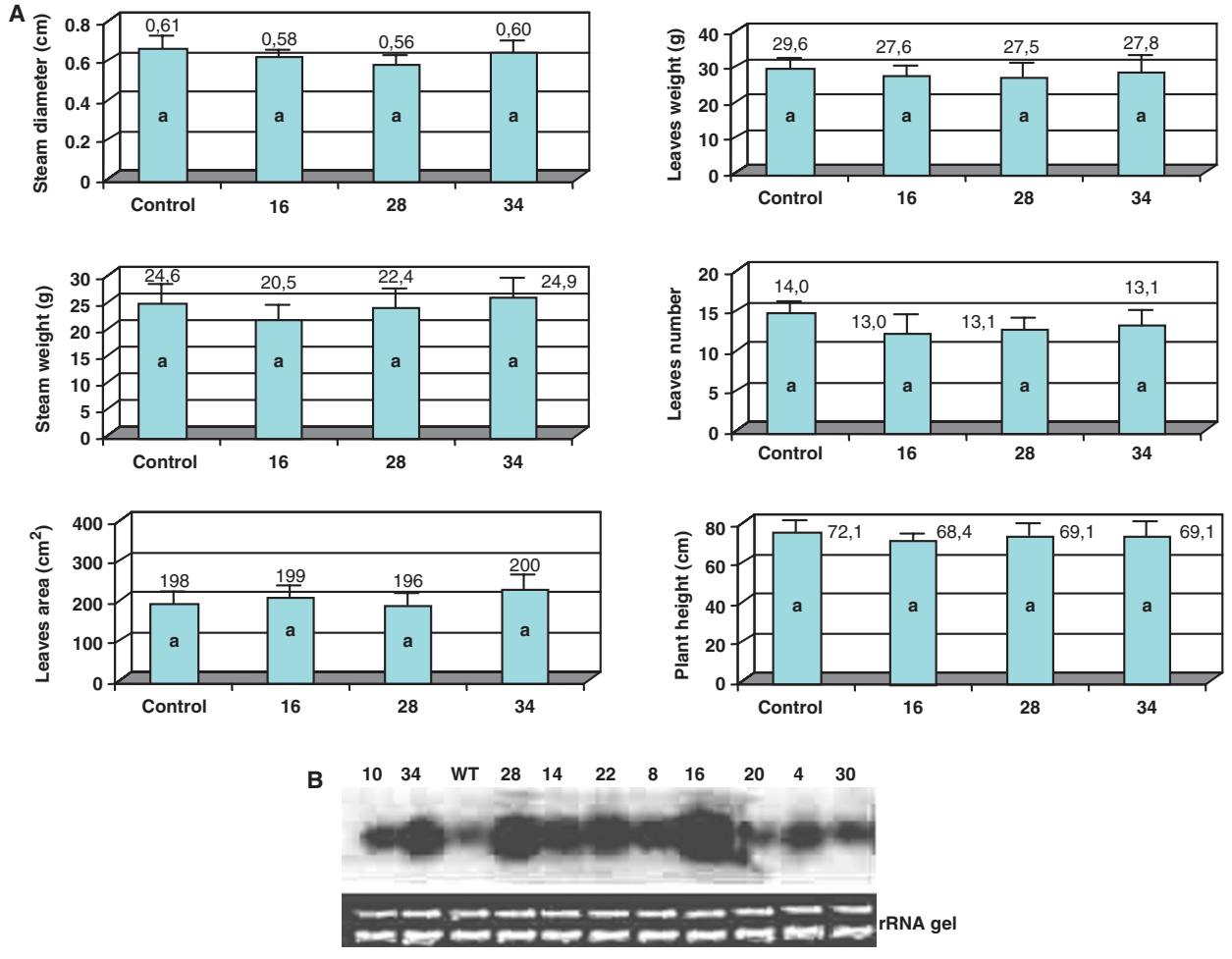

Fig. 8 (A) Phenotypic evaluation of wild-type (WT) and transgenic plants grown in a greenhouse without any stress. Evaluated parameters were: plant height, stem diameter, leaf weight, stem weight, leaf number and leaf area. Data gathered from these experiments were statistically processed using the Microsoft Excel statistical ANOVA analysis package, and the significance of sifferences between the means was determined by using the χ^2 test. Values in the graphics correspond to the means ± SD of 15 independents samples. Similar letters over the bars indicate no significant differences. (B) RNA blots for constitutive *SodERF3* detection in unstressed WT and different transgenic lines. A 20 µg aliquot of RNA isolated from leaves of 1-month-old WT tobacco plants and different recombinant clones including 16, 28 and 34 were blotted and probed with 3′-terminal labeling of *SodERF3* cDNA.

chromosomal mosaic and gene functional redundancy. Furthermore, genetic transformation of native sugarcane varieties is not straightforward. Therefore, to investigate the biological function of *SodERF3* we used tobacco plants as an experimental model. *In vitro* and *in vivo* experiments conducted with three 35S:*SodERF3* independent tobacco lines revealed their tolerance to salt and drought treatments. This result suggests that SodERF3 is functionally relevant in tobacco cells and interacts with tobacco genes or proteins thought to activate a subset of osmotic stress response mediators. However, the expression level of the PR genes β-1,3-glucanase (*PR-2*) and osmotin (*PR-5*) remained the same in the wild type as in SodERF3-transgenic plants. Two explanations for this observation are possible. First, the assayed PR genes controlled or not by SodERF3 might not be directly involved in salt/drought tolerance. Secondly, some modifications including protein–protein interactions, e.g with other TFs, could be required during or after transcription to induce the expression of these downstream target genes in transgenic tobacco. Overexpression of DREB2A, an ERF member of the EREBP/AP2 family, did not result in significant induction of downstream genes in Arabidopsis (Umezawa et al. 2006).

Reports dealing with the characterization of ERF members of subgroup VII or IX containing acidic activation domains in the C-terminus have been widely published (Ohta et al. 2000, van der Fits and Memelink 2000, Park et al. 2001, Wang et al. 2004). However, 35S:*SodERF3* phenotypes *in vivo* have not yet been reported for any VIII

ERF member. It is a fact, as explained above, that we were not able to detect clearly overexpression of PR genes in transgenic SodERF3 tobacco plants when compared with the wild type. However, three lines of strong evidences point to SodERF3 as a transcription activator. One of them has been previously mentioned above regarding the fact that SodERF3 has an important amino acid change (L instead of P) within the conserved sequence of the EAR motif. This change, according to previous reported research, is enough to abolish completely the repression capacity of these proteins (Ohta et al. 2000, Ohta et al. 2001, McGrath et al. 2005, Tsukagoshi et al. 2005). The other two pieces of evidence also strongly reinforce the activator role of SodERF3. First, tobacco plants expressing *SodERF3* were phenotypically similar to those reported in previous studies overexpressing ERF transcriptional activators that conferred salt/pathogen tolerance. Secondly, overexpression of the transcriptional repressors AtERF7 or AtERF4, clustered very close to SodERF3 (Fig. 2B), induced hypersensitivity to salt in Arabidopsis (Song et al. 2005, Yang et al. 2005).

Transgenic 35S:*SodERF*3 lines displayed a desirable phenotype indicating, according to the discussion above, that SodERF3 could confer drought or salt tolerance to the transgenic plants through a cross-talk with an additional pathway other than ethylene or ABA, probably interacting with other proteins responsible, for example, for increasing the plant Na^+ efflux and thereby reducing the Na^+ accumulation in the cells.

Extremely interesting from the agronomical point of view is the fact that transgenic tobacco plants grown under unstressed greenhouse conditions were phenotypically similar to wild-type tobacco plants despite the fact that the level of transcripts of the constitutively expressed *SodERF3* was higher in lines 16, 28 and 34 than in the other seven analyzed recombinant lines as judged by Northern blot assays. In contrast to our results, transgenic Arabidopsis plants expressing the AP2/ERF *ZmDREB1A*, a TF that confers tolerance to abiotic stress, displayed a dwarf and growth retardation phenotype, being more severe with the higher expression of this gene (Qin et al. 2004). The use of stress-inducible promoters minimizes the negative effects of DREB/CBF overexpression on plant growth (Kasuga et al. 1999, Kasuga et al. 2004).

For a better understanding of the role of SodERF3 in transcription, new lines of research must be addressed to generate transgenic sugarcane overexpressing *SodERF3* and then 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; therefore, in the near feature, these studies should assist the manipulation of plants in order to improve their stress tolerance.

Materials and Methods

Mass excision of a sugarcane cDNA library

A sugarcane library was generated from ethephon-treated young sugarcane leaves, using the Lambda ZAP-cDNA synthesis kit (Stratagene, La Joya, CA, USA). After mass excision of the cDNA library following the manufacturer's instructions, a single-pass sequencing of randomly selected clones was carried out using the dideoxynucleotide chain termination method (^{T7}Sequencing Kit, Pharmacia, Uppsala, Sweden). One of the sequenced cDNA fragments (EST) encoding a putative protein with a DNA-binding domain typically found in EREBP/AP2-type TFs was used as probe to isolate the corresponding full-length cDNA clone from the mass-excised library. The full-length cDNA was named *SodERF3* (EMBL accession No. AM493723).

Plant materials and treatments

All plants were grown under controlled greenhouse conditions (28°C, 70% relative humidity, approximately 2,000 Lux with a 12 h natural light/12 h darkness photoperiod).

Six-week-old sugarcane (*S. officinarum* L. cv. Ja60-5) and tobacco (*N. tabacum* L. cv. SR1) plants were used for Northern analyses. To determine the expression pattern of *SodERF3* under different stress stimuli, plantlets uprooted from the soil were rinsed with water and placed in a beaker containing 40 ml of water as the control treatment, or 0.1 mM ABA, 200 mM NaCl, 2 mM SA or 1 mM ethephon for 48 h. Subsequently, the plants were flash-frozen in liquid nitrogen and leaf tissues were harvested. To evaluate the effect of wounding on *SodERF3* expression, sugarcane plantlets were rinsed with water, manually macerated, and flash-frozen after 8 h. For the time-course of *SodERF3* expression, plantlets were treated as described and harvested at the indicated time points.

Sequence analysis of ERF DNA-binding domains

The sugarcane SodERF3 protein was compared with 161 ERFs from Arabidopsis, tomato, sugarcane and rice, described previously and classified within the AP2 superfamily subgroup B (Sakuma et al. 2002, Gutterson and Reuber 2004, Nakano et al. 2006). Arabidopsis, tomato and rice sequences were obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org), The Solanaceae Genomics Network (http://www.sgn.cornell.edu/index.html) and GenBank (Benson et al. 2004), respectively. In addition, a tBLASTn search was performed using the *S. officinarum* L. databank (SOFUNIGENE) available at the INFOBIOGEN Sequence Retrieval System (SRS) server (http://www.infobiogen.fr), revealing six entries that display significant homology to *SodERF3* (CA164762, CA239762, CA149330, CA282648, CA212910 and CA106080). The multiple protein sequence alignment of ERF DNA-binding domains was performed using ClustalW (Thompson et al. 1994).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al. 2001). A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei 1987), in which gap positions were excluded. Pairwise distances were calculated according to the p-distance model that analyzes the proportion (p) of different amino acids per site.

RNA gel blot analyses

Total RNA was isolated as described previously (Borrás-Hidalgo et al. 2005). A 20 μg aliquot of total RNA was separated

on a 1.5% formaldehyde–agarose gel, and transferred onto nylon membranes (Amersham Pharmacia-Biotech, Uppsala, Sweden). For RNA dot blots, the same amount of RNA was vacuum-blotted using a Biorad Dot Blot device (Bio-Rad, Richmond, CA, USA).

The 3′-terminal region of the *SodERF3* cDNA labeled with [^{32}P]dCTP was used as probe, and the membrane was hybridized as described previously (Borrás-Hidalgo et al. 2005).

Western blot analysis

After SDS–PAGE on a 12.5% gel, total sugarcane leaf protein from plants treated with ethephon as described above and harvested at the indicated time points (0,1, 7, 24, 48 and 96 h) was electro-transferred onto nitrocellulose membranes (Amersham Pharmacia-Biotech) using a Mini Trans-Blot Electrophoresis Transfer (Bio-Rad) at a constant current of 350 mA for 1.5 h. The immunological detection of SodERF3 was achieved using polyclonal rabbit antibodies generated with *E. coli*-expressed SodERF3, and the ProtoBlot AP system (Promega, Madison, WI, USA).

In vitro binding assay

The *SodERF3* coding sequence was fused to a C-terminal hexa-histidine tag and expressed in *Escherichia coli*. The recombinant protein was purified with nickel affinity chromatography (His-tag Purification and Detection Kit 1, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The oligonucleotides containing the wild-type (CATAAGAGCCGCCACT) and mutated (CATAAGAtCCtCCACT) GCC box sequences were end-labeled with [^{32}P]dATP and used as probes. The assay mixtures contained 0.1 μg of recombinant SodERF3, 2 ng of binding probe (8×10^4 c.p.m.), 2 μg of salmon DNA, 20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 10% glycerol and 0.5 mM dithiothreitol (DTT) in a 10 μl reaction volume. The mixtures were incubated at room temperature for 15 min and separated on a 5% polyacrylamide gel in 0.5× TBE buffer. For competition assays, 0.1 μg of recombinant SodERF3 was incubated for 10 min with the unlabeled oligonucleotide before addition of the labeled oligonucleotide probe.

Generation of tobacco plants expressing SodERF3

SodERF3 cDNA was placed after the tobacco mosaic virus (TMV) translational enhancer Ω under the control of the CaMV 35S promoter, resulting in the vector pBinΩ-*SodERF3*. This binary plant expression vector was introduced into tobacco (*N. tabacum* L. cv. SR1) via *Agrobacterium tumefaciens*-mediated transformation following the procedure of Horsch et al. (1985) with minor modifications. After selection on MS medium (Murashige and Skoog 1962) amended with 40 μg ml^{-1} kanamycin, 36 independent recombinant lines were obtained. The presence of the transgene was verified by Northern blot in 30 of the 36 tested recombinant lines.

T_1 seeds were collected and sown on medium supplemented with 40 μg ml^{-1} kanamycin. Surviving T_1 plantlets were transferred to soil to obtain T_2 seeds that were used for further analysis.

Seed germination assays

To evaluate the germination rate of the 30 Northern blot-positive *SodERF3*:35S lines in NaCl, 50 seeds (T_2) from each recombinant and wild type line were placed on MS medium supplemented with 150, 250 and 350 mM NaCl, respectively, and incubated at 23°C, 2,000 Lux, with a 16 h photoperiod in an incubation chamber. Germinated seeds in 350 mM NaCl from clones 16, 28 and 34 were scored till 15 d after sowing. Data were quantified and statistically analyzed using the Microsoft Excel statistical simple ANOVA analysis package. Values are represented in the graphics as percentage relative to control ± SD. Root elongation and vegetative development of 15 germinated plantlets corresponding to the wild type and the three recombinant clones, respectively, were also evaluated after growing under salt conditions. Wild-type and recombinant plants able or not able to develop well under those conditions were scored and statistically analyzed as described above.

Salt and drought-stress tolerance of transgenic lines

Seeds ot the wild type and recombinant lines 16, 28 and 34 were germinated in soil. After 2 weeks, 15 individual plants corresponding to the wild type or each of the three clones mentioned above were transferred to 20 cm pots filled with soil.

Plants were grown under greenhouse conditions as described previously. Five-week-old soil-grown transgenic and wild-type lines were watered with a 350 mM NaCl solution for 30 d.

In another experiment, drought stress was imposed by withholding water for 30 d. Phenotypic parameters were then evaluated and compared against the wild-type non-transgenic line. Data gathered from both experiments were statistically processed using the Microsoft Excel statistical ANOVA analysis package. Statistical significance compared with the value of the control plants was determined by the χ^2 test. Similar letter indicates no significant differences.

Phenotypic analyses of transgenic lines without stress

Wild-type and transgenic plants were germinated and grown under greenhouse conditions as described above, but without any stress. The parameters evaluated after 50 d were: plant height, stem diameter, leaf weight (FW), stem weight (FW), leaf number and leaf area. Statistical analysis was conducted as described above.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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