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Overexpression of *StDREB1* Transcription Factor Increases Tolerance to Salt in Transgenic Potato Plants

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Abstract It has been established that drought-responsive element binding (DREB) proteins correspond to transcription factors which play important regulatory roles in plant response to abiotic and biotic stresses. In this study, a novel cDNA encoding DREB transcription factor, designated *StDREB1*, was isolated from potato (*Solanum tuberosum* L.). This protein was classified in the A-4 group of DREB subfamily based on multiple sequence alignments and phylogenetic characterization. Semi-quantitative RT-PCR showed that *StDREB1* is expressed in leaves, stems, and roots under stress conditions and it is greatly induced by NaCl, drought, low temperature, and abscisic acid (ABA) treatments. Overexpression of *StDREB1* cDNA in transgenic potato plants exhibited an improved salt and drought stress tolerance in comparison to the non-transformed controls. The enhanced stress tolerance may be associated with the increase in *P5CS*-RNA expression (δ^1-pyrroline-5-carboxylate synthetase) and the subsequent accumulation of proline osmoprotectant in addition to a better control of water loss. Overexpression of *StDREB1* also activated stress-responsive genes, such as those encoding calcium-dependent protein kinases (*CDPK*s), in transgenic potatoes under standard and high salt conditions. These data suggest that the StDREB1 transcription factor is involved in the regulation of salt stress tolerance in potato by the activation of different downstream gene expression.

Keywords DREB · Potato · Salt stress · Stress-responsive genes · Transcription factor

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Introduction

Environmental stresses have several adverse effects on plant growth. Therefore, plants have developed defense mechanisms to perceive signals from their surroundings and appropriately respond to different stresses by modulating the expression of response genes [1, 2]. Transcription factors regulate such gene expression via binding to specific elements (*cis*-elements) of target genes, and consequently enhance stress tolerance in plants [3, 4]. Drought-responsive element binding factors (DREBs)/C-repeat binding factors (CBF) and ethylene response factors (ERFs) are important factors that regulate differential gene expressions in the diverse signaling pathways due to their different DNA-binding specificity [5–7]. All these transcription factors harbor a DNA-binding domain, the so-called AP2/ERF domain, of approximately 60 amino acids [8]. The highly conserved 14th and 19th amino acids differ between DREB and ERF and have proved to be essential for specific binding to DRE [8]. DREBs are characterized by valine residue at position 14 and glutamic acid at position 19. However, ERFs display an alanine and

aspartic acid residue at position 14 and 19, respectively. In addition to these two residues, a conserved alanine at position 37 in the AP2/ERF domain has also been shown to be essential in binding to DRE elements [9]. Moreover, the DNA-binding domain of DREB factors play central roles in consensus recognition and binding to the DRE *cis*-element [6, 8].

It was reported that some DREB factors such as *AtDREB2A* [8], *TINY2* [10], *Tsi1* [11], and BnDREBIII [9] can bind to both GCC and DRE boxes.

DREB genes form a large multigene family that were classified into six subgroups (A-1 to A-6) by Sakuma et al. [8].

Subgroups A-1 and A-2, harboring the *DREB1*-type and *DREB2*-type genes, respectively, are the largest ones and are involved in abscisic acid (ABA)-independent pathways [6, 12]. The *DREB1*-type genes (*AtDREB1A*, *AtDREB1B*, and *AtDREB1C*) are thought to be involved in cold-responsive pathway, whereas *DREB2*-type genes (*AtDREB2A* and *AtDREB2B*) are mainly involved in osmotic-responsive pathways [13].

Up to date, most reports about DREB/CBFs have focused mainly on DREBA1 and DREBA2, while investigation of other groups is very limited. *TINY2* (A-4), *PpDBP1* (A-5), *GhDBP1* (A-5), and *ZmDBP1* (A-6) were identified as stress-response regulation genes [10, 14–16].

Arabidopsis TINY factor that is involved in both abiotic- and biotic-stress signaling pathways belongs to the A-4 subgroup of the DREB subfamily. It is characterized by a serine residue at position 15 in the AP2/ERF domain that is essential for specific binding of TINY to ethylene responsive element (ERE, AGCCGCC) [17]. Another *TINY* gene (*GmTINY*) isolated from Soybean, seems to play some roles in plant reproductive organs and roots, but not in leaves [18].

Dubouzet et al. [19] isolated five rice *DREB* homologs: *OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D*, and *OsDREB2A*. The expression of *OsDREB1A* and *OsDREB1B* was induced by cold, whereas OsDREB2A was induced by drought and salt stress. The *OsDREB1C* showed constitutive expression, while no expression of *OsDREB1D* was detected in any case [20]. Recently, three other *DREB* homologs namely *OsDREB1E*, *OsDREB1G*, and *OsDREB2B* were isolated. The overexpression of *OsDREB1G*/*OsDREB2B* significantly enhanced drought tolerance, while overexpression of *OsDREB1E* in rice led to improved tolerance to a moderate water deficit stress [21]. Moreover, Wang et al. [22] reported that rice OsDREB1F factor was induced by salt, drought, cold stress, and ABA treatment. However, pathogen, wound, and H_2O_2 treatment did not have any effect on this DREB factor expression. Overexpression of this gene in both *Arabidopsis* and rice transgenic plants increased salt, drought, and low temperature tolerance.

Interestingly, expression of rice and maize DREB in *Arabidopsis thaliana* improved tolerance to abiotic stress and induced stress marker genes such as *RD17* and *RD29* (responsive to dehydration) and *COR15A* (cold responsive) [19, 23]. Moreover, *DREB1A* isolated from rice and *Arabidopsis* and *DREB1B* isolated from rice were overexpressed in rice and enhanced tolerance to dehydration. Rice plants overexpressing *DREB1A* isolated from *Arabidopsis* proved to be more effective for salt tolerance [24].

In Wheat (*Triticum aestivum*), 117 putative AP2/ERF genes were identified via *in silico* analysis based on the presence of the conserved AP2/ERF domain. One hundred and four genes were predicted to encode proteins containing a single AP2-domain and were assigned to the *DREB* and *ERF* family. Fifty-seven genes were identified as possibly encoding members of the DREB factors, and 47 genes were predicted to encode ERF proteins. The putative AP2/ERF family genes from *T. aestivum* were detected in nine tissue types and most of them showed some tissue specificity [25].

Transgenic wheat and barley plants, overexpressing the TaDREB2 and TaDREB3 transcription factors isolated from wheat grain, exhibited a significant increase in drought and frost tolerance [26].

In the durum wheat (*Triticum durum*), novel alleles in *DREB* gene family (*DREB1–DREB5*) were identified in drought-tolerant and susceptible lines by a new method called high-resolution melting (HRM) [27]. The identification of single-nucleotide polymorphisms (SNPs) mutation in tolerant wheat genotypes was reported. This technique measures temperature-induced strand separation, and is therefore able to detect variations as small as one base difference among samples [28].

A detailed study of *GmDREB2* and *GmDREB3* isolated from soybean [29, 30] showed that they bind to the DRE/CRT element. *GmDREB2* gene was induced by cold, drought, salt, and ABA treatments, whereas *GmDREB3* was induced only by cold.

Overexpression of these genes in transgenic tobacco has resulted in higher proline levels under drought stress, while an improved tolerance to both salt and drought stresses was observed in transgenic *A. thaliana* [29, 30].

Potato which world production reached 324 million tons in 2010 [31] is the world's fourth-largest food crop, following rice, wheat, and maize [32]. It is not only an important food source but also a vital raw material in the starch-processing industry, a source of animal feed that makes use of potato vines and a potentially important resource in medicine owing to the compounds found in its seeds [33]. As potato is relatively vulnerable to abiotic stresses such as drought and high salinity, it is classified as an environmental sensitive crop [34]. Potato tuber initiation, bulking, and growth are sensitive to drought stress [35].

Transcriptomic and targeted metabolite approaches allowed the identification of drought-responsive compounds in potato [34, 35].

Salinity stress generally causes osmotic stress, water loss, and ionic imbalance [36, 37]. Salt and drought-responsive mechanisms involve several physiologic and biochemical changes due to the activation of different signal transduction pathways. These signaling mechanisms involve gene regulation by several factors, particularly abscisic acid (ABA) [38] and calcium [39]. ABA modulates the expression of stress-related gene, its accumulation in guard cells triggers an increase in cytosolic Ca^{2+} leading to the activation of calcium-dependent protein Kinases (CDPKs, [40]).

Cytosolic calcium regulated oxidative burst via CDPKs and induced many intracellular signaling pathways [41, 42].

A number of studies in potato were made to overcome crop yield loss under environmental stress conditions through different molecular biologic approaches [35, 43, 44]. However, little is known about stress-tolerance mechanisms in potato plants. This species is highly stress sensitive and its stress-tolerance signal-regulating mechanisms are poorly understood. Nevertheless, potato cDNA microarray allowed identification of genes encoding signal transduction factors, heat-shock proteins, and transcription factors [45].

Potato transcription factors such as WRKY [46] and MYB [47] were identified. However, no DREB homologs have as yet been identified in this plant species, while increased salt tolerance was observed in transgenic potato lines harboring *Arabidopsis rd29A::DREB1A* [48, 49]. Moreover, *Arabidopsis rd29A::DREB1A* seems to improve the response of transgenic potato plants to freezing [50], suggesting that the control of tolerance to abiotic stress, in potato, is mediated by DREB factors.

We have isolated four *StDREB* cDNA from *Solanum tuberosum* cv. Nicola (data not shown). Two of these *StDREB* factors showed a significant increase in their expression by salt and drought stresses. The *StDREB2* gene was overexpressed in potato cv. Spunta and this ectopic expression conferred enhanced tolerance to salt stress in transgenic potato plants [51].

In this paper, we report the cloning and characterization of the *StDREB1* cDNA. Its expression pattern was investigated in response to exogenous ABA, salt, cold, and drought stress treatments. Overexpression of this cDNA in transgenic potato plants cv. *Belle* de *Fontany* led to enhanced tolerance to salinity and dehydration stresses.

Materials and Methods

Plant Materials and Stress Treatments

Two potato plants (*S. tuberosum* L.) cultivars, i.e., Nicola and Belle de Fontenay (BF15) were used in this study. Potato plants were cultivated in vitro and propagated in solid MS basal medium [52] in a growth chamber at 21 °C for 16/8 h photoperiod, under 250 $\mu E/m^2/s$ light intensity.

Stress treatments were performed on 3 weeks old in vitro cultivated plants as follows: for salt stress and ABA treatments, plants were transferred in aqueous MS medium supplemented with 100 or 200 mM NaCl or 50 μM ABA. For dehydration stress, plants were transferred in MS liquid medium containing 10 % (w/v) polyethylene glycol with an average molecular weight of 8,000 (PEG 8000). Low temperature treatments were performed by transferring plants to a growth chamber set to 4 °C under the light and the photoperiodic conditions described above. ABA, PEG, and cold treatments were applied to Nicola cv. Salt stress was applied to Nicola and BF15 commercial cultivars as well as to five independents transgenic lines overexpressing the *StDREB1* cDNA. For each plant line, three individuals were used for each treatment that was replicated twice. All stress treatments were performed for 24 h since a previous study revealed that RNA expression of *StDREB1* in potato leaves reached its maximum upon 24 h and decreased after 72 h of salt treatment (data not shown).

Isolation and Cloning of *StDREB1* cDNA

The full coding DREB sequence (CDS) was found in the SGN (http://www.sgn.cornell.edu) website. Specific primers designed from potato unigen (accession no. SGNU281030) were chosen to amplify the full-length coding sequence from the ATG to the STOP codon. The corresponding full-length cDNA was amplified from a mixture of cDNAs from different tissues (stem, roots, and leaves). The cloning of the *StDREB* cDNA was achieved by the Gateway cloning technology (Invitrogen). To generate overexpressing constructs, gene-specific primers were designed spanning the open reading frame (ORF) with adapters at the 5′ and 3′ ends (Table 1) and the primary amplicon was PCR amplified using the ISIS *Taq* DNA polymerase (Q-Biogene). For the addition of the attB sites, primary amplicons were used as templates and were amplified using attB primers (Table 1). The PCR product was inserted in pDONR207 entry vector (Invitrogen) by BP reaction. Cloning of the amplicon (Gateway BP reaction) was carried out by adding 150 ng of pDONR207 vector, 2 μL of BP clonase II mix (Invitrogen) to 1 μL of amplicon in 10 μL of reaction mixture adjusted to the desired volume with Tris–EDTA buffer. The reaction was left overnight at 25 °C and then stopped by the addition of 1 μL of proteinase K and incubated for 10 min at 37 °C. One to 10 μL of the reaction mix were used to transform 50 μL of competent DH-5α cells. A PCR was carried out with vector-specific primers to check the presence of the insert.

The *DREB* cDNA was transferred from the pDONR207 into the binary vector pMDC32 (Gateway LR reaction). This reaction was carried out by adding 4 μL of a mix containing

Table 1 Sequences of primers used for *StDREB1* cDNA cloning and PCR analyses

Names	Forward primers 5′–3′	Revers primers 5′–3′	T_m °C	No of cycles
StDREB1	*AAAAAGCAGGCTTC***ATGGCGGAACTTGTACAATC**	*CAAGAAAGCTGGGTC***TCAAGCCCATTTCATCGTCTCG**	60	30
attB	ACAAGTTTGTACAAAAAAGCAGGCT	ACCACTTTGTACAAGAAAGCTGGGT	65	35
pDONR207	TCGCGTTAACGCTAGGGATCTC	GTAACATCAGACATTTTGAGACAG	50	35
pMDC32	TGTTTGAACGATCGGGGAAATTCGAGCTCC	GGATCCCCGGGTACCGGGCC	55	35

In the *StDREB1* primers, italics indicated adapters and the bold indicated specific genes primers

150 ng of pMDC32 [53], 1 μL of left border (LR) clonase mix II (Invitrogen) to 1 μL (150 ng) of the attL1–*DREB*–attL2 cassette DNA. The LR reaction was followed by the transformation and screening of *Escherichia coli DH5α* strain.

Database searches were performed using the National Center for Biotechnology Information (NCBI) BLAST search program. Alignment of the potato DREB protein with other structurally related AP2/ERF proteins was performed by means of the COBALT multiple alignment Tool from NCBI (www.ncbi.nlm.nih.gov).

Phylogenetic analysis was performed by the UPGMA method [54] with the aid of MEGA5.0 Version software.

Generation of Transgenic *StDREB1* Potato Plants

Solanum tuberosum L. cv. *Belle* de *Fontany* (BF) cultivar was used to produce transgenic plants. The recombinant binary vector (*pMDC32::StDREB1*) was transferred in *Agrobacterium tumefaciens* LBA4404 that was subsequently used for leaf transformation. Potato transformation was performed as previously described [55]. These putative transformants were multiplied by node culture in MS basal medium [52] containing 2 mg/L hygromycin at 25 °C.

Identification of Transgenic Potato Lines

Genomic DNA was extracted from potato leaves as described by Dellaporta et al. [56] and was used in PCR-based identification of transgenic plants. To detect positive lines, a couple of primers from pMDC32 binary vector flanking the transgene was used (Table 1).

The PCR was performed in a final volume of 25 μL containing 50 ng of genomic DNA, 100 ng of each primer, and 1 U of Taq DNA polymerase (Promega). The amplified product of 850 bp specific to the pMDC32-*StDREB1* gene was resolved on a 1 % agarose gel and visualized by ethidium bromide staining.

Five positive transgenic lines were randomly selected to analyze the expression of the *StDREB1* gene. Total RNA was isolated using the EZ-10 Spin column "Total RNA Mini preps Super Kit" (Bio Basic Inc), and cDNA was synthesized by means of the reverse transcriptase M-MuLV RT (Bio Basic Inc). The *StDREB1* expression gene was analyzed by RT-PCR using the *StDREB1* specific primers (Table 1). The elongation factor 1α (*ef1α*) gene (GenBank accession no AB061263) was used as constitutive gene marker and the sequences of its specific primers are presented in Table 2.

Semi-Quantitative RT-PCR Analyses

Total RNA was isolated, as described above, from in vitro cultivated potato plants for the analysis of *StDREB1* expression. RNA concentration and quality were based on

Table 2 Sequences of primers used for semi-quantitative RT-PCR analyses of *StDREB1* putative target genes

Genes	Forward primers 5′–3′	Reverse primers 5′–3′	T_m °C	No of cycles
ef1α	ATTGGAAACGGATATGCTCCA	TCCTTACCTGAACGCCTGTCA	60	25
StCDPK4	TGGGCAACACATGCCGTGGAT	TGACACGCCTCCACAACCCC	59	30
StCDPK5	GGCTCTGCGGGTGATTGCTGA	CCGGGCGCATCTCTCATGCT	59	30
P5CS	CCCACAGCAGCACAA	TTCGCAAGGGTATGAAG	60	30

absorbance measurement at 260 and 280 nm. Semi-quantitative RT-PCR analysis was carried out on DNaseI-treated RNA (2 µg) using 200 U of M-MuLV RT. The expression pattern of *StDREB1* putative target genes, including *StCDPK4* (AB279737), *StCDPK5* (AB279738), and *P5CS* (SGN-U271255; PGSC0003DMG402026767 code in the genome of potato) was performed. Their expression was investigated in transgenic and wild-type (WT) potato plants by RT-PCR using specific primers (Table 2). Each cDNA sample was diluted to 1:10 and 1 µL of the diluted cDNA was used for PCR. RT-PCR of the *ef1α* constitutive gene was performed under the same conditions to normalize the amount of template added. PCR-amplified products were visualized on ethidium bromide-stained 1.5 % agarose gels and quantified using the Gel DocXR Gel Documentation System (Bio-Rad). This software was used to calculate average band density which was recorded and used in graphic analyses. Band density was determined by this software and was given in arbitrary units and graphed using Microsoft Excel. The error bar was determined from three separate biologic replicates.

Analysis of Salt Tolerance of Transgenic Plants and Determination of Chlorophyll Content

WT Belle de Fontany (BF) and *StDREB1* transgenic in vitro cultivated potato plants were transferred in MS medium containing 200 mM NaCl for salt tolerance assays, and were incubated for 1 month, in this salty medium. Changes in leaf morphology and the degree of root elongation were measured. The chlorophyll content determination was performed on healthy and fully expanded leaves from WT and transgenic plants (five plants from each line were used). These leaves were cut and incubated for 5 days into half-strength MS medium containing 400 mM NaCl. Leaves from the same plants were incubated in the MS/2 medium without any NaCl supplementation and were used as control. The treatment was performed under continuous light at 25 °C and was replicated twice. The chlorophyll content was then measured as described by Arnon [57].

Greenhouse Planting

Three- to 4 week-old in vitro cultivated plants (12 individual plants from each line) from five transgenic lines (BF1-5) and the WT control that had developed roots in MS medium were transferred to a greenhouse under temperature conditions varying between 20 and 30 °C and in the presence of daylight. They were sprinkled with tap water daily during the first week and then every 3 days. Three weeks later, the plants were sprinkled with tap water supplemented with 100 mM NaCl. Dehydration stress was applied by withholding water for 25 days.

Measurement of Relative Water Content

The relative water content (RWC) of the WT and *StDREB1* transgenic potato plants was determined as described by Yamasaki and Dillenburg [58]. Leaves were cut from in vitro cultivated potato plants and their fresh weight (FW) was measured. To determine turgid weight (TW), the leaves were floated in deionized water for 7 h. Finally, the leaf samples were dried in an oven at 80 °C for 48 h to obtain the dry weight (DW). The RWC was then calculated by the following formula:

$$\text{RWC}\ (\%) = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100.$$

Determination of Proline Content

After being subjected to salt stress, leaves from transgenic and control plants were collected every 5 days to measure free proline as described by Bates et al. [59].

Results

Isolation and Phylogenetic Analysis of the *StDREB1* cDNA

A full-length cDNA sequence, designated as *StDREB1* (GenBank accession no. JN125862) was isolated from potato (Nicola cultivar). This cultivar was chosen since it exhibited a better salt tolerance than the other ones from our collection [60].

This *StDREB1* cDNA is 657-bp long corresponding to a protein of 218 amino acids. The sequence alignment of the AP2/ERF domain of StDREB1 and those of known DREB factors, such as TINY, TINY2, and ZmDBF2, which bind both DRE and ERE elements, was performed. A comparison with the CBF2/DREB1C and CBF3/DREB1A domains that only bind to DRE element was also investigated. As shown in Fig. 1a, the AP2/ERF domains of these proteins,

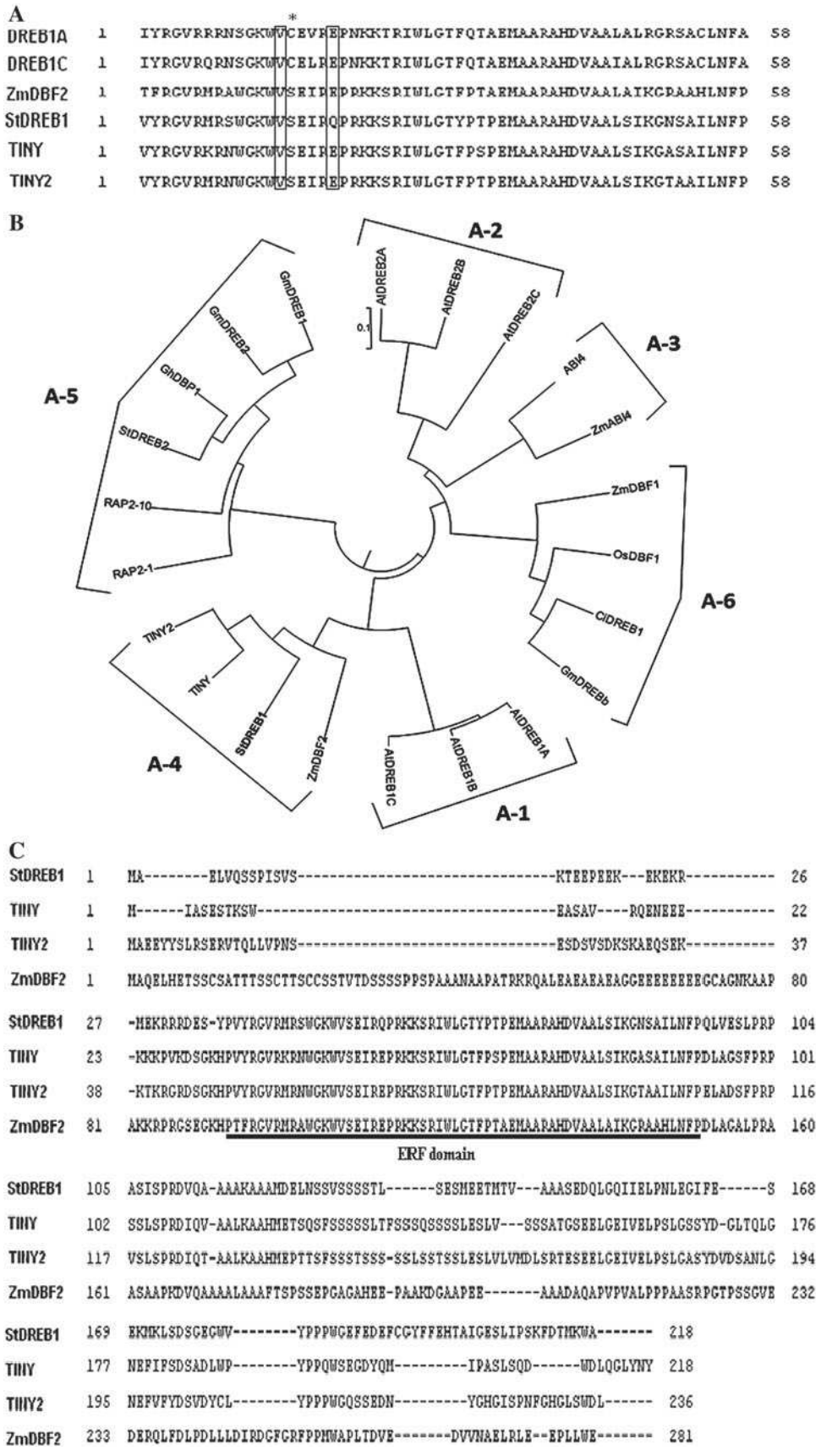

Fig. 1 Sequence comparison and phylogenetic relationship of potato StDREB1 with other plant DREBs. **a** Alignment of the amino acid sequences of the AP2/ERF domains of StDREB1, TINY (Q39127), TINY2 (NM_121197), ZmDBF2 (AF493799), DREB1C/CBF2(AB013817), and DREB1A/CBF3 (AB007787), the *star* (*) corresponds to the 15th amino acid. The 14th valine and the 19th glutamic acid inside the AP2/ERF domain are presented in *boxes*, **b** phylogenetic tree of StDREB1 full-length protein with DREBs from other plants. The phylogenetic tree was constructed by the MEGA program. The accession number of each appended protein is as follows: StDREB2: JN125858, OsDBF1: BAD37688, CiDREB1: ABR53728, ZmDBF1: AAM80486, ZmDBF2: AF493799, ABI4:AF085279, ZmABI4: AY125490, GhDBP1: AY174160, AtDREB1A: AB007787, AtDREB1B: AB007788, AtDREB1C: AB007789, AtDREB2A: AB007790, AtDREB2B: AB007791, AtDREB2C: NM_129594,GmDREBb: AAQ57226, GmDREB2: ABB36645, GmDREB1: AF514908, RAP2.10: Q9SW63, RAP2.1: Q8LC30, TINY: Q39127, TINY2 (NM_121197). StDREB1 is indicated in *bold*. **c** Amino acid alignment of full-length DREB proteins. StDREB1, TINY (Q39127), TINY2 (NM_121197) and ZmDBF2 (AF493799), *black bar* indicate the AP2/ERF domain

made up of 58 amino acid residues in StDREB1, are homologous with 62.1 % identity. The highest identity of StDREB1 was observed with TINY2 (91 %) from *A. thaliana*. The most noticeable difference between these sequences was observed for the 15th amino acid which is Ser in StDREB1, TINY, TINY2, and ZmDBF2, while it is a Cys in CBF2/DREB1C and CBF3/DREB1A. These results suggest that StDREB1 is able to bind both DRE and ERE *cis*-elements and it can be then classified in the A-4 group of the DREB subfamily because of its homology to the *Arabidopsis* TINY [17], TINY2 [10] and the *Zea mays* ZmDBF2 [14] genes (Fig. 1b).

The sequence comparison of StDREB1 with the other known A-4 group members of the DREB subfamily (Fig. 1c) showed that the AP2/ERF domains of StDREB1 share 89.7, 91.4, and 82.8 % amino acid identity with the *Arabidopsis* TINY and TINY2 and Zea mays ZmDBF2 domains, respectively. The overall amino acid sequence identity between StDREB1 and these proteins is 45, 42.4, and 30.2 %, respectively.

Abiotic Stresses and the Exogenous ABA Phytohormone Upregulate the Expression of *StDREB1*

In plants, *DREB*s play a direct regulatory role in response to multiple signal stimulations. In order to determine the putative function of *StDREB1* in response to environmental stresses, transcript accumulation was examined under different stress conditions: two salt stress conditions (100 and 200 mM NaCl), 10 % PEG, 4 °C, and 50 µM ABA treatments. Expression levels of *StDREB1* were measured in three plant organs (leaves, stems, and roots) after 24 h of treatment. Such stress duration was chosen since a preliminary study revealed that *StDREB1* transcripts amount increased progressively from 3-h salt treatment to reach a maximum level upon 24 h (data not shown).

The *StDREB1* mRNA levels measured under control conditions were very low in leaves, stems, and roots (Fig. 2a) while they increased in plants submitted to salt stress in all tested organs. The induction rate differed with the NaCl concentration applied on plants. Indeed, a higher induction of *StDREB1* was observed in leaves of plants submitted to 200 mM NaCl compared to 100 mM NaCl. However, both NaCl treatments led to a high accumulation in stem and roots.

Since ABA plays an important role in the regulation of gene expression in response to various abiotic stresses [61], transcription of the *StDREB1* gene was assessed after exogenous application of 50 µM ABA (Fig. 2b). Under these conditions, *StDREB1* transcript accumulation was significantly induced, indicating that *StDREB1* may function in an ABA-dependent signaling pathway. Moreover, cold treatment at 4 °C induced *StDREB1* expression in leaves and stems while in roots a slight induction was observed (Fig. 2b).

PEG treatment suggests that dehydration induced transcription of *StDREB1* to much higher levels in all tissues compared to the other abiotic constraints tested (Fig. 2b).

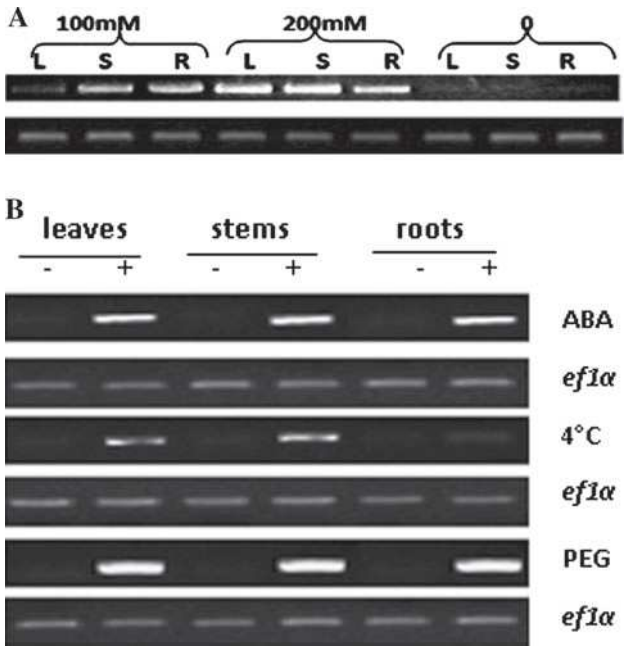

Fig. 2 **a** StDREB1 gene transcription is induced by salt stress in an organ-specific way. Total RNA was isolated from leaves (*L*), stems (*S*), and roots (*R*) after 24 h of NaCl treatment at two concentrations 100 and 200 mM, **b** organ-specific expression of the *StDREB1* gene in potato after 24 h exposure to treatment with ABA, cold, and drought (PEG). *ef1α* constitutive gene expression was used as control to normalize the amount of templates in PCR samples

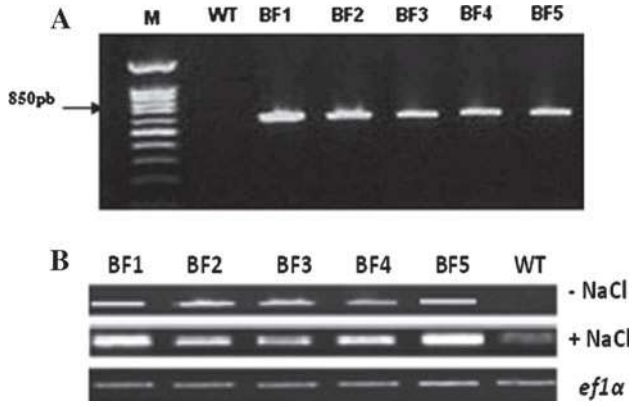

Fig. 3 **a** PCR amplification of the specific *StDREB1* gene from genomic DNA of the different hygromycin resistant plants (BF1; BF2; BF3; BF4; and BF5). *M* 100 bp DNA Ladder, **b** RT-PCR analysis of *StDREB1* expression in transgenic lines (BF1; BF2; BF3; BF4; and BF5) and the WT, in plants cultivated under standard (−NaCl) or salt stress conditions (+NaCl). *ef1α* a constitutive gene was used as internal marker

Analysis of Transgenic Potato Plants Expressing *StDREB1*

In order to analyze the biologic function of StDREB1 as transcription factor, it was overexpressed in transgenic potato plants (cv BF15) generated by transforming potato leaf disks with the recombinant *pMDC32-StDREB1* binary vector. The *StDREB1* cDNA was inserted upstream of the CaMV35S promoter.

Potato is a clonal crop multiplied by tuber seeds and because of its tetraploid status and the lack of plant flowering under greenhouse conditions, transgenic plants from To generation were used in this study [62].

The presence of the *StDREB1* transgene in the genome of putative transformed plants was confirmed by PCR analysis using primers the pMDC32 binary vector corresponding to sequences flanking the *StDREB1* cDNA. As expected, a PCR product of 850 bp was obtained (Fig. 3a).

Semi quantitative RT-PCR analyses (Fig. 3b) showed that the *StDREB1* transcript was overexpressed in the five positive transgenic lines tested, i.e., BF1, BF2, BF3, BF4, and BF5.

No phenotypic modification such as dwarfism was noticed in these *StDREB1* transgenic plant lines.

StDREB1 Expression Enhances In Vitro Tolerance to Salt in Transgenic Potato Plants

In order to test the role of *StDREB1* in salt stress tolerance, leaves from in vitro cultivated transgenic and WT potato plants were cut and incubated for 5 days into half-strength MS medium supplemented with 400 mM NaCl and salt tolerance was estimated by measuring chlorophyll contents [57]. After 5 days of treatment, the WT leaves appeared bleached, whereas those from *StDREB1* transgenic plants remained green (Fig. 4a). Measurements of the chlorophyll content in these leaves confirmed the phenotypic

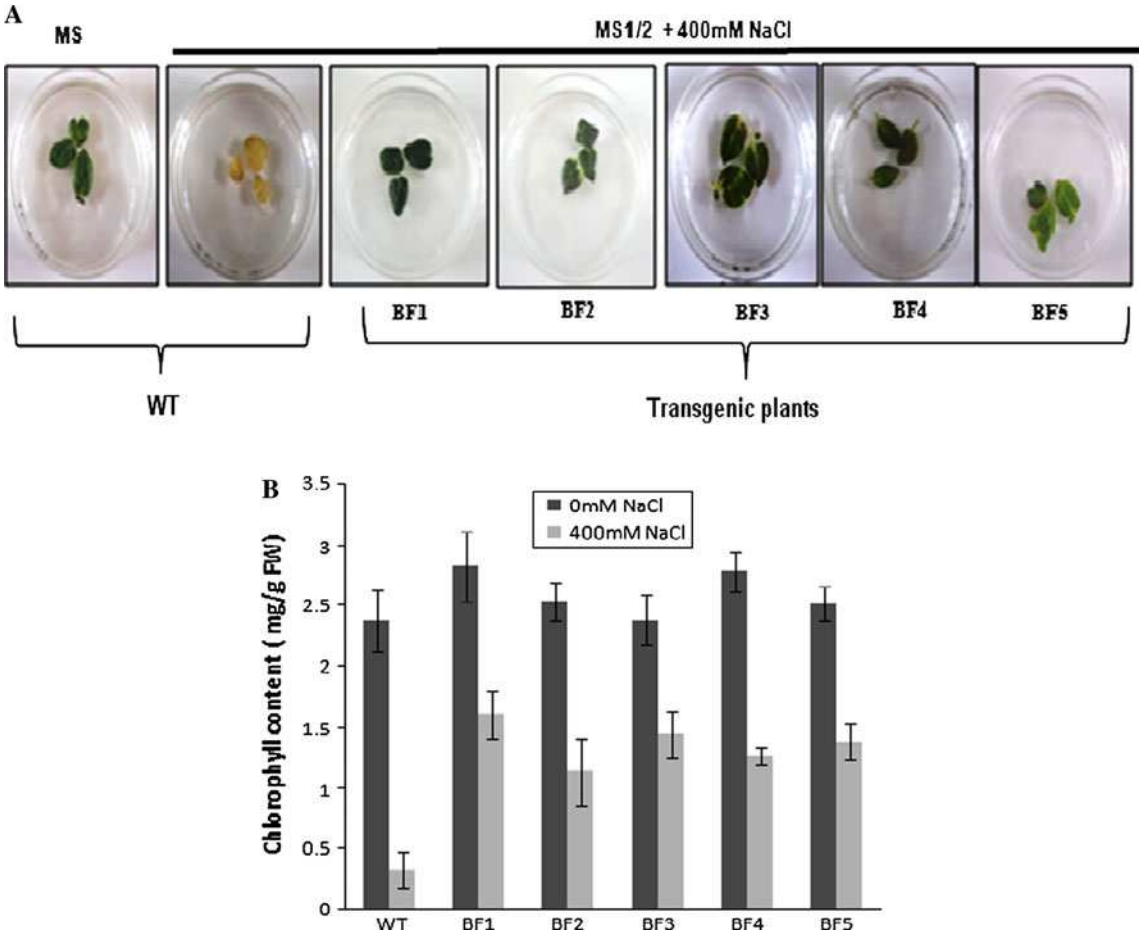

Fig. 4 Overexpression of *StDREB1* enhances tolerance to salt in potato. **a** Phenotypic comparison between 35S:: *StDREB1* transgenic and WT potato plants. Leaf disks from transgenic plants (BF1; BF2; BF3; BF4; and BF5) carrying the *StDREB1* gene and WT plants were floated on half-strength MS liquid medium containing 400 mM NaCl for 5 days. As a control, WT leaf disks were floated on half-strength MS liquid medium, **b** chlorophyll contents of transgenic potato leaf tissues incubated under standard and salt stress conditions. Chlorophyll contents (mg/g fresh weight) were measured from NaCl-treated leaf disks. Values represent mean from chlorophyll content of each leaf with standard deviation

differences (Fig. 4b). Although there were differences between them, all transgenic lines showed higher chlorophyll content than that of WT plants suggesting a better salt tolerance. In contrast, no significant difference in chlorophyll content was observed in leaves from transgenic and WT plants incubated in salt free MS/2 medium.

In a second experiment, shoot tips excised from aseptic seedlings of both WT and transgenic *StDREB1* potato plants were transferred to MS medium containing 200 mM NaCl and plant culture was pursued for 30 days. Significant phenotypic differences between WT and transgenic lines were observed after 30 days of salt treatment (Fig. 5a). During this period, leaves of WT gradually lost their green color, and root elongation was severely delayed, whereas leaves of the transgenic plants, overexpressing *StDREB1*, remained green and root development was observed (Fig. 5b).

Overexpression *of StDREB1* Enhances Tolerance to Salt and Drought Stresses

Investigation of plant behavior under greenhouse culture conditions was performed to further evaluate their robustness and their degree of tolerance to salinity and drought. Therefore, 3–4 weeks old in vitro cultivated transgenic and non transgenic plants were transferred to the greenhouse, and treated with tap water supplemented with 100 mM NaCl. After two to three weeks of treatment, transgenic plants showed a different phenotype compared to the controls. Indeed, their leaves increased in size, while the margins of leaflets in WT plants became necrotic after 5 days of salt treatment. Later on, the WT plants displayed visible necrotic lesions in leaves and then in the entire plant that finally died after 20 days of salt treatment. In contrast, leaves of all transgenic plants remained green (Fig. 6a). Stem length of transgenic plants showed an increase especially in BF1, BF2, BF4, and BF5 lines when evaluated after 5, 10, and 20 days of salt treatment (Fig. 6b). However, in BF3 transgenic line and the WT one (Fig. 6b), the plant growth stopped completely after 5 days of salt treatment.

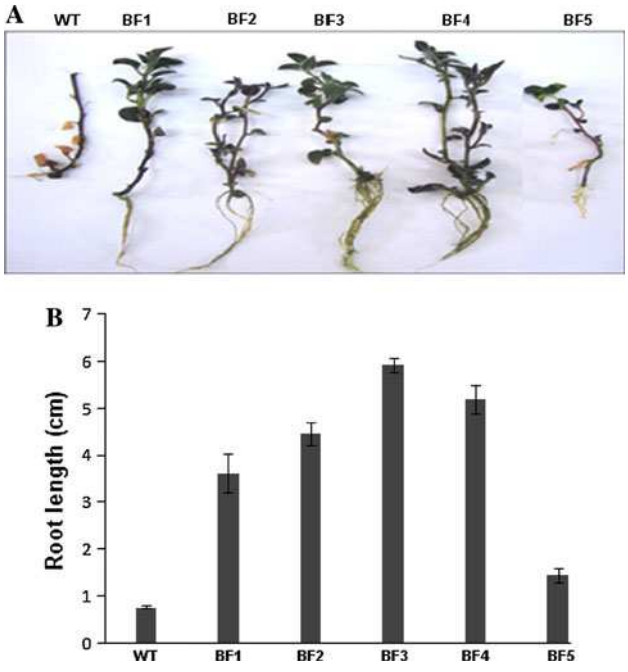

Fig. 5 **a** Morphological aspect of transgenic plants (BF1; BF2; BF3; BF4; and BF5) and the WT ones after 30 days of culture in the presence of 200 mM NaCl, **b** root lengths of seedlings grown for 30 days on MS medium containing 200 mM NaCl. Values represent means of root length from the different plants + standard deviation

Dehydration treatment was applied for two transgenic lines (BF1 and BF2) and the WT plant that was used as control (Fig. 6c). Five plants from each line were used. After withholding water for 25 days, a clear difference between transgenic and WT plants was observed. Indeed, the control plants withered and their growth stopped, while the transgenic lines grew well and their leaves remained green.

In order to better evaluate the response of these plants to drought stress, the growth of BF1 and BF2 transgenic lines and the WT ones was tested by measuring the aboveground length after 7, 15, and 25 days of drought stress (Fig. 6d). A retardation in growth was observed for WT plants, while for BF1 and BF2 transgenic plants overexpressing *StDREB1* gene a significant increase of stem length was noticed especially after 15 days and 25 of drought stress. These results suggest that overexpression of *StDREB1* in potato can greatly enhance plant tolerance to salt and drought stresses. They corroborate those described above about the effect of dehydration on *StDREB1* mRNA expression.

Chlorophyll contents of the WT and transgenic potato lines were measured after 20 days of salt treatment. As shown in Fig. 7, a significant decrease of chlorophyll content was observed in WT plants submitted to salt stress, while in transgenic plants this decrease was less important, but still statistical significantly different. Indeed, the total chlorophyll content loss in WT plants was about 62 %, whereas in transgenic lines overexpressing *StDREB1*, the chlorophyll loss ranged between 16 % in BF1 to 53 % in BF2. The BF4 line displayed a very interesting phenotype since it showed an increase in leaf chlorophyll content in the presence of salt. Although the behavior of the BF3 transgenic line resembled that of WT plants since it stopped growing in the presence of salt, it displayed a chlorophyll loss of 43 % under salt stress conditions.

To further examine the osmotic adjustment capacity of transgenic potato plants, the relative water content (RWC) was measured after 20 days of salt treatment (Fig. 8).

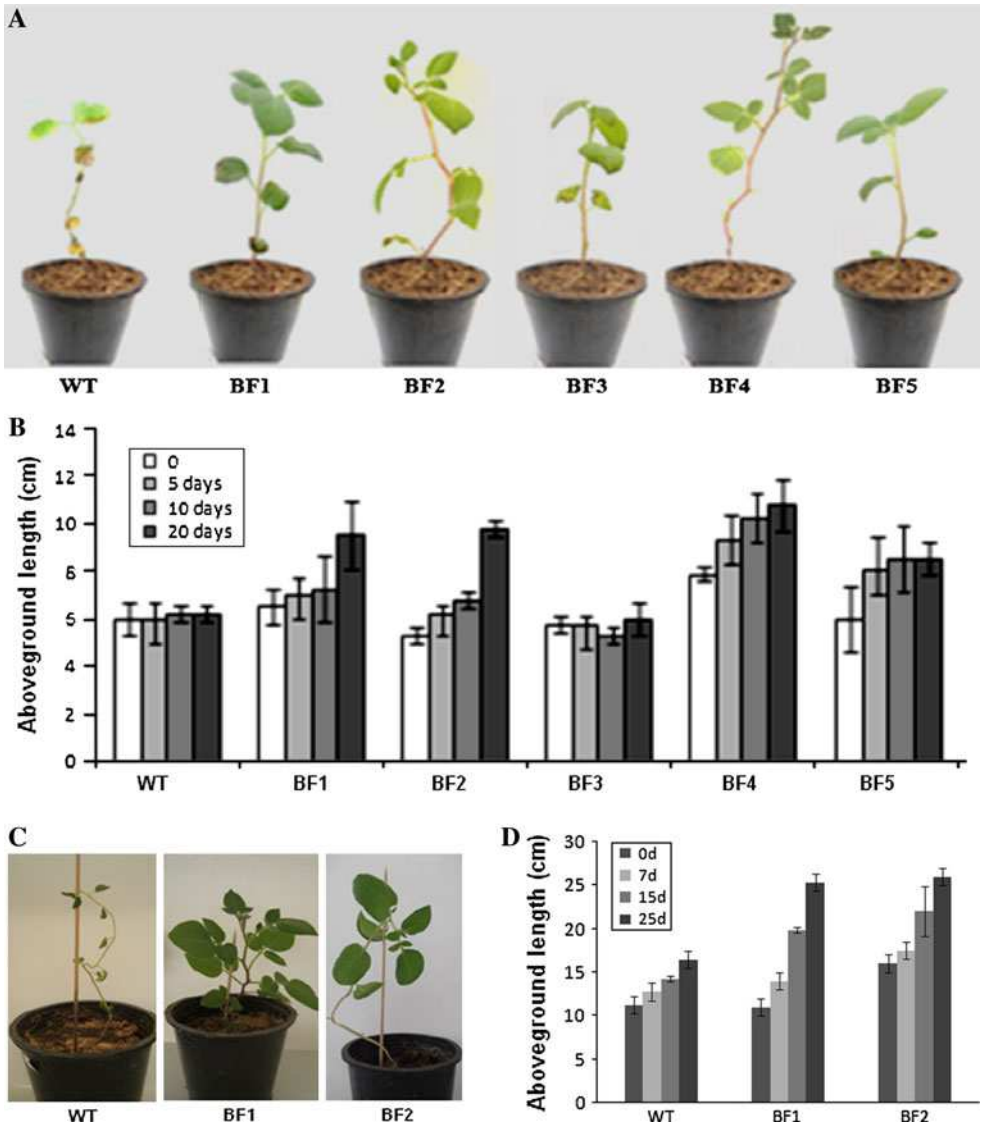

Fig. 6 Effect of salt and drought treatment on plant growth in the greenhouse. **a** Plant morphology under stress salt treatment, **b** length above ground of transgenic lines (BF1; BF2; BF3; BF4; and BF5) and the WT taken at different time of culture under salt treatment, **c** plant morphology under drought treatment of two transgenic lines (BF1and BF2) and the WT, **d** length above ground of transgenic lines (BF1 and BF2) and WT under drought treatment

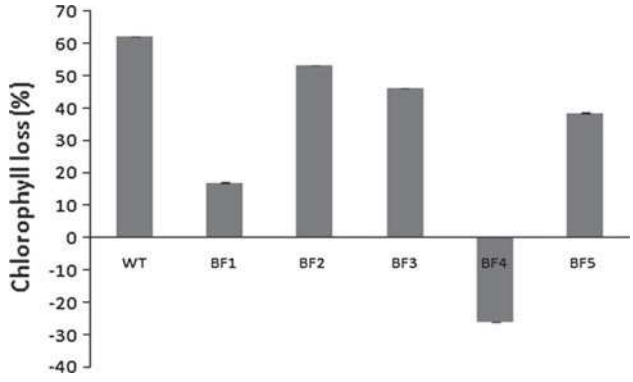

Fig. 7 The percentage of Chlorophyll contents loss in transgenic potato plants (BF1; BF2; BF3; BF4; and BF5) and the WT plants submitted to salt treatment for 20 days

A decrease in the RWC of 41 % was observed in WT plants under salt stress, while this decrease of RWC did not exceed 25 % in BF1 transgenic line. Interestingly, all the other transgenic lines overexpressing the *StDREB1* gene exhibited a very low decrease of the RWC suggesting that these plants are able to control their RWC under salt stress conditions. All these data confirm that overexpression of *StDREB1* gene in transgenic potato plants can confer a significant tolerance to salinity compared to WT plants.

Overexpression of StDREB1 Activates the Expression of Stress-Responsive Genes

Since the DREB family members play central roles in the regulation of stress-response gene expression, the overexpression of *StDREB1* gene in transgenic plants may regulate the expression of downstream stress-induced genes containing the CRT/DRE in their promoter sequence as reported for other DREB genes [63–65]. Thereby, three putative target genes were chosen here. They encode the δ^1-pyrroline-5 carboxylate synthase (P5CS) and two

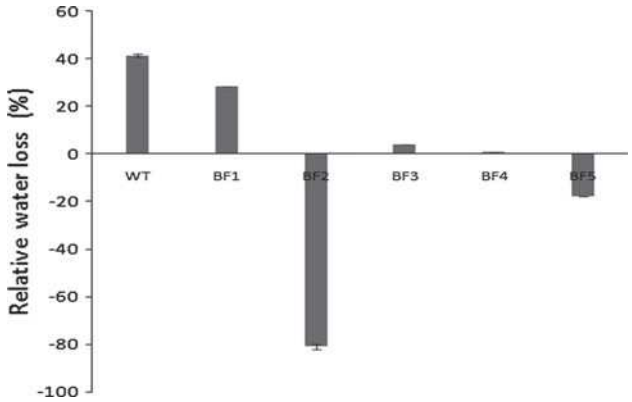

Fig. 8 The percentage of relative water contents loss of transgenic potato (BF1; BF2; BF3; BF4; and BF5) and the WT plants submitted to salt treatment for 20 days

calcium-dependent protein kinases (StCDPKs; StCDPK4; and StCDPK5). *In silico* analysis of their promoter sequences revealed that P5CS and StCDPK4 genes harbor the DRE (ACCGAGA) motif. However, StCDPK5 harbors the GCC box (GCCGCC) target sequence. Such genes have been identified as *DREB* downstream regulated genes in tomato (*LeP5CS*) [66] and rice (*OsCDPK7* and *OsCDPK13*) [67]. Their ectopic expression in transgenic plants enhanced tolerance to drought and high salinity.

Semi-quantitative RT-PCR analyses of these target genes were performed for the WT BF15 and Nicola cultivar and for the *StDREB1* transgenic potato plants. An increase in transcription level of these genes was noticed in almost all transgenic plants cultivated under standard growth conditions in comparison to those in WT ones. This increase was higher under salt stress conditions (Fig. 9; supplementary materials). BF1 and BF5 lines exhibited the highest levels of *StCDPK4* and *StCDPK5* transcripts in plants cultivated in standard conditions. This expression increased to a much higher level in plants submitted to salt stress (Fig. 9a, b). An increase in *P5CS* gene transcription was also observed in these transgenic *35S:StDREB1* lines cultivated under stress conditions (Fig. 9c). A high increase of P5CS transcripts level was observed in BF2 and BF5 lines.

All these findings strongly suggest that StDREB1 may upregulate the expression of stress-related functional genes such as those involved in the calcium-dependent pathway via the activation of calcium-dependent protein Kinase such as CDPK4 and CDPK5. Similarly, the overexpression of *GmDREB1* in alfalfa [68] and *GmDREB2* in tobacco [25] have led to an increase of free proline contents in transgenic plants in comparison to WT plants submitted to salt stress.

Since the P5CS gene is involved in proline osmoprotectant synthesis pathway, the proline was measured in *StDREB1* transgenic plants. Indeed, proline is involved in

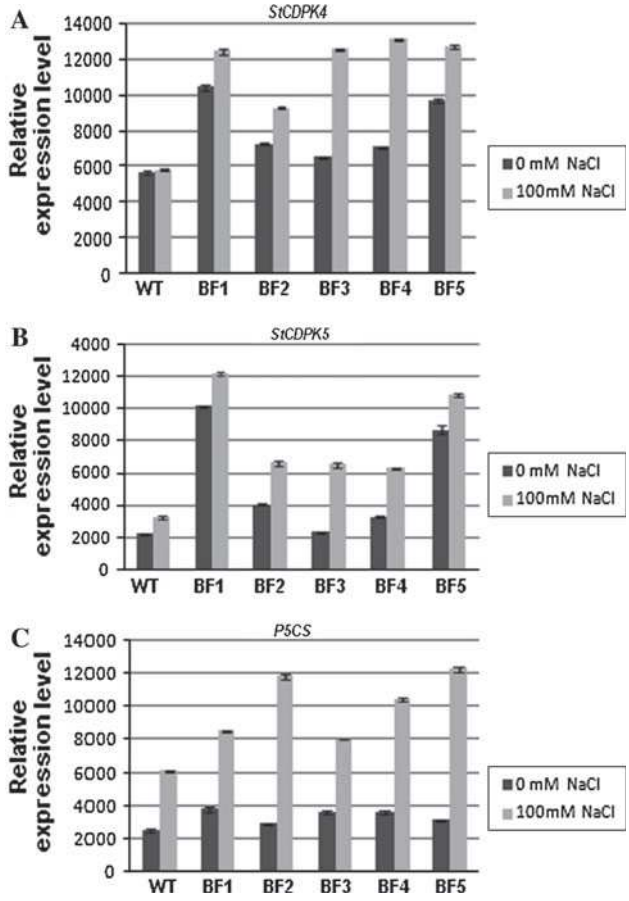

Fig. 9 RT-PCR analysis of the expression of the stress-induced genes. **a** StCDPK4, **b** StCDPK5, **c** P5CS in transgenic lines (BF1; BF2; BF3; BF4; and BF5) and the WT under standard and salt stress conditions. The standard error was determined from three independent biologic replicates; band densities in the gels are expressed in arbitrary units calculated by the analysis Gel DocXR software

osmotic adjustment in plants and plays an adaptive role in enhancing plant tolerance to a wide range of environmental stresses [69]. To investigate the possible involvement of proline osmolyte accumulation in the salt stress tolerance observed in transgenic plants, proline contents were measured in the WT and *35S::StDREB1* transgenic potato lines (Fig. 10). No significant difference in the proline content was detected in WT and *StDREB1* transgenic lines under standard conditions. However, after 1 day of salt treatment, the proline level in transgenic lines increased, while it remained unchanged in the WT (Fig. 10).

During salt treatment, the proline contents in both *StDREB1* transgenic plants and the control ones increased progressively, but this increase was much higher in transgenic plants especially after 20 days of treatment. Indeed, the levels of proline in transgenic lines were approximately 45.7–66.8 times higher than those measured under standard conditions, whereas under stress conditions the WT proline content increased only 17.5-fold compared to standard

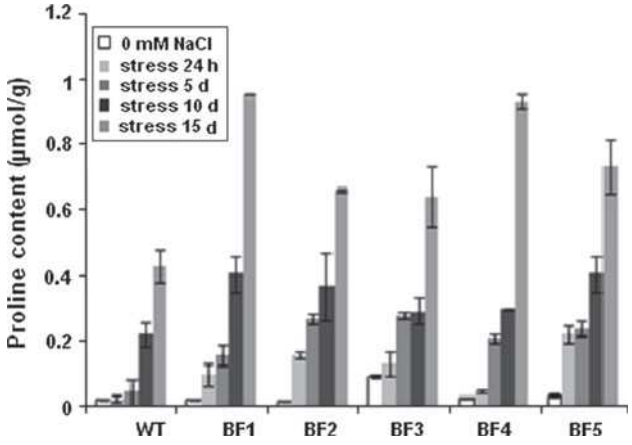

Fig. 10 Effects of salt stress on free proline contents of (BF1; BF2; BF3; BF4; and BF5) transgenic plants and the WT plant grown under salt stress during 20 days

conditions (Fig. 10). These results imply that StDREB1 may activate the proline level increase in transgenic potato submitted to salt conditions by the activation of *P5CS* gene.

Discussion

This study describes the isolation and characterization of a DREB factor from potato, termed StDREB1. The sequence analysis identified an AP2/ERF domain of 58 amino acids that is predicted to fold into a structure containing three anti-parallel β-sheets and one α-helix. This structure is thought to play a key role in recognizing and binding to specific *cis*-elements [8]. The second β-sheet and the α-helix contained conserved amino acid residues corresponding to valine at the 14th position and alanine at the 37th position characterizing DREB factors. Alignment analysis and domain comparison suggest that StDREB1 belongs to DREB A-4 protein family.

A number of reports have suggested that Val14 and Glu19 in the AP2/ERF domain are essential for specific binding to DRE [6, 8], while, in StDREB1, Glu (E) 19 is replaced by Gln (Q). Similar amino acid changes have also been observed in other plant species including soybean, rice, and maize. Furthermore, in rice, wheat, and barley, the DREB1-type factors harbor a valine residue at position 19 in the AP2/ERF domain [19, 70]. These observations suggest that the function of the 14th amino acid is probably more important than that of the 19th amino acid for a specific DNA-binding activity. Moreover, and for the first time in potato, we showed here that StDREB1 contains in the AP2/ERF domain a Ser-15 which was reported as a crucial amino acid for specific binding to ERE in *Arabidopsis* TINY factor [17].

Various abiotic stresses, specifically high salinity, dehydration, and low temperature, upregulated the expression of *StDREB1*. The ABA phytohormone which is involved in several physiologic processes and in the adaptation of plants to different environmental stresses seemed also to upregulate the expression of *StDREB1* gene. Similar data were reported by other studies [65, 71], in which exogenous ABA induced the expression of *DREB* genes. This suggests that *StDREB1* is involved in abiotic stress response through an ABA-dependent signaling pathway. These data are in agreement with previous reports describing the role of DREB factors in plant response to abiotic stress [20, 27]. The overexpression of StDREB1 factor in potato plants also revealed a significant improvement in salt and drought tolerance of these plants.

Similar results were described for *Arabidopsis* plants overexpressing the *AtDREB1A* and *OsDREB1A* that exhibited an enhanced tolerance to freezing and dehydration [6, 19, 72]. However, these plants showed growth retardation when compared to WT ones.

In potato, the overexpression of *Arabidopsis rd29A::DREB1A* enhanced tolerance to salt [48, 49] and freezing [50]. These data correlate our result that showed that transgenic potato lines harboring the *StDREB1* transgene exhibited also an enhanced tolerance to salt and drought stress. The data also suggest that the enhanced tolerance of *35S::StDREB1* transgenic plants may result from *StDREB1* mediated regulation of expression of downstream target genes involved in response to abiotic stresses. These target genes may be ABA and/or calcium dependent.

Despite the case of a constitutive 35S promoter for the expression of *StDREB1* transgene, the downstream genes showed an increased expression under stress condition and proline accumulation was significantly higher in *StDREB1* transgenic plants cultivated under stress conditions. Similar data were observed by Zhang et al. [73] who reported that in transgenic rice overexpressing the tomato ERF3 gene under control of 35S promoter, the expression of downstream gene increased significantly under drought stress condition and not in normal growth. These authors suggested that the ERF factor may interact with other protein factor upon stress that triggers the observed increase in downstream gene expression. This hypothesis would be also effective in our case. Moreover, it is well known that a proline catabolism mechanism is active in plants cultivated under normal condition which is controlled by mitochondrial proline dehydrogenase enzyme [74].

These data are similar to those described by Celebi-Toprak et al. [48] who showed that overexpression of *Arabidopsis rd29:: DREB1A* in a polyploid crop like potato can lead to stress tolerance through relevant expression of the gene. These transgenic plants showed increased tolerance to elevated levels of salinity. Thus, our results and

those described by Celebi-Toprak et al. [48] are convergent and confirm that the effectiveness of overexpression of DREB factor in transgenic potato plant can improve abiotic stress tolerance.

In contrast to the data reported by Yamaguchi-Shinozaki and Shinozaki [75], the overexpression of *St DREB1* gene in transgenic plants did not show any phenotypic changes such as dwarfism.

Moreover, these data are also in agreement with other reports that revealed that ERF family members can regulate the expression of numerous stress-related functional genes [20]. Similarly, this report clearly showed that StDREB1 can activate the expression of stress-inducible genes (*StCDPK4*, *StCDPK5*, and *StP5CS*) in transgenic potato plants which are homologous to *LeP5CS*, *OsCDPK7*, and *OsCDPK13*. The expression of these latter genes is known to be induced by external stimuli and they play important roles in plant response to abiotic stresses [41, 42, 76]. It was suggested that *StCDPK4* and *StCDPK5* expression may contribute to the oxidative burst and activate plasma membrane Ca^{2+} channels. Consequently, calcium may function not only as an inducer of the oxidative burst but also as a signaling molecule downstream of the oxidative burst leading to the release of the generated ROS into the apoplast [77].

In this context, StDREB1 may provide protection against damaging attack of ROS under salinity or drought stress via the expression *StCDPK4* and *StCDPK5* putative genes. They seemed also capable of activating proline osmoprotectant accumulation by the activation of P5CS gene.

All these data suggest that *StDREB1* may be involved in the regulation of multiple stress-responsive signaling transduction pathways.

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