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Research article

Combination of the endogenous promoter-intron significantly improves salt and drought tolerance conferred by TdSHN1 transcription factor in transgenic tobacco

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ABSTRACT

Recent years have witnessed a renewed interest in introns as a tool to increase gene expression. We previously isolated *TdSHN1* gene encoding a transcription factor in durum wheat. Here we show that *TdSHN1* intron contains many CT-stretches and the motif CGATT known to be important for IME. When subjected to bioinformatics analysis using IMEter software, *TdSHN1* intron obtained a score of 17.04 which indicates that it can moderately enhance gene expression. *TdSHN1* gene including its intron was placed under the control of *TdSHN1* endogenous salt and drought-inducible promoter or the constitutive 35S promoter and transferred into tobacco. Transgenic lines were obtained and designated gD (with 35S promoter) and PI (with native promoter). A third construct was also used in which intron-less cDNA was driven by the 35S promoter (cD lines). Results showed that, gD lines exhibited lower stomatal density than cD lines. When subjected to drought and salt stresses, gD lines outperformed intron-less cD lines and WT. Indeed, gD lines exhibited longer roots, higher biomass production, retained more chlorophyll, produced less ROS and MDA and had higher antioxidant activity. qRT-PCR analysis revealed that gD lines had higher *TdSHN1* expression levels than cD lines. In addition, expression of ROS-scavengering, stress-related and wax biosynthesis tobacco genes was higher in gD lines compared to cD lines and WT. Interestingly, under stress conditions, PI transgenic lines showed higher *TdSHN1* expression levels and outperformed gD lines. These results suggest that *TdSHN1* intron enhances gene expression when used alone or in combination with *TdSHN1* endogenous promoter.

1. Introduction

Abiotic stresses, particularly drought and salinity, represent serious problems to agriculture and threaten food security. Their negative impact on crops productivity is expected to be intensified by the global climate change with increased temperature and significant alteration in rainfall pattern (Trenberth, 2011). In developing countries, water scarcity aggravated by increased salinity resulted in limited crop performance and insufficient food supply (Loescher et al., 2011). Thus, drought and salt tolerant crops are urgently needed to meet increasing food demand (United Nations, 2015). Genetic engineering of plants holds great potential to help address the constraints of abiotic stress and global climate change very efficiently (Kissoudis et al., 2016). The identification and characterization of genes related to plant stress responses is an important step towards engineering plants endowed with stress tolerance. Success has been achieved following the overexpression of single stress responsive genes however, stress resilience remains below the level required for practical agriculture production. Several excellent publications reported the achievements of genetic engineering approach in the creation of transgenic plants capable of withstanding abiotic stress more efficiently than their wild-type counterparts (Jewell et al., 2010). These plants were produced by overexpression of genes known to be involved in abiotic stress tolerance. These genes can be grouped into two main categories: functional and regulatory genes. The latter, comprises transcription factors (TFs) (Djemal and Khoudi, 2016) which play key roles in abiotic stress tolerance. The use of TFs in genetic engineering of plants to increase their tolerance to abiotic stress represents an elegant and powerful strategy for crops improvement. In fact, TFs govern the expression of various stress-inducible genes in a cooperative or separate manner. Thus, TFs

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Abbreviations: DREB, DRE-binding protein; DRE, dehydration-responsive element; ERF, ethylene-responsive factor; TF, transcription factor; IME, intron-mediated enhancement; SD, stomatal density

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are ideal candidates for enhancing drought and salt tolerance known to be very complex and multi-genic in nature (Agarwal and Jha, 2010; Tran et al., 2010; Wang et al., 2016).

The Ethylen-responsive transcription factor (ERF) family is one of the largest families of transcription factors in plants (Mizoi et al., 2012). It is involved not only in developmental processes and signal transduction, but also in plant response to abiotic stress (Feng et al., 2005; Sharoni et al., 2011). Members of this family bear the AP2/ERF domain of about 60–70 amino acid residues (Riechmann and Meyerowitz, 1998; Sakuma et al., 2002). This domain has a dual capacity to bind to the GCC box (AGCCGCC) and the dehydration-responsive element (DRE) (Ohme-Takagi and Shinshi, 1995). The AP2/ERF family comprises the AP2, DREB, ERF and RAV subfamilies (Sharoni et al., 2011; Sakuma et al., 2002). However, DREB and ERF members have been the subject of intensive research due to their implication in both biotic and abiotic stresses. Interestingly, members of the ERF subfamily, isolated from *Arabidopsis* and known as the WAX INDUCER1/SHINE, are involved in the accumulation of cuticlar wax (Aharoni et al., 2004; Broun et al., 2004). *Arabidopsis thaliana* contains there SHINE genes: *AtSHN1*, *AtSHN2* and *AtSHN3* (Dietz et al., 2010). In plants, cuticle plays an important protection role against both biotic and abiotic stresses (Jenks and Ashworth, 1999). The overexpression of SHINE-type transcription factor genes was found to increase both drought and salt tolerance in transgenic plants (Djemal and Khoudi, 2016; Aharoni et al., 2004).

Almost all previous reports about the overexpression of transcription factors in plants were performed using cDNA sequences placed under the control of the 35S promoter, but there are no reports on the characterization of full-length gene with regards to drought and salinity stresses. In the past years, the choice of promoter elements received much attention, but little was paid to the choice of other elements such as introns. The latter, are characteristics of eukaryotic gene structures. In plants, introns are ubiquitous elements found in 80% of the genes (The *Arabidopsis* Genome Initiative, 2000). When they were discovered in 1977, introns were considered as junk DNA (Sambrook, 1977). It later turned out, that they are actively involved in different aspects of gene regulation. The pre-mRNA splicing, which consists of the excision of introns and the ligation of exons, represents a key mechanism of eukaryotic gene expression (Johnson and Vilardell, 2012). The process is under the control of multiple cis-acting elements and is performed by a ribonucleoprotein complex known as splicesome (Wahl et al., 2009). The presence of introns and their processing by the spliceosome determines the pattern of gene expression through transcription, polyadenylation, mRNA stability, mRNA localization and translation efficiency (Le Hir and Moore, 2003).

Among the most important roles attributed to introns is their enhancement of gene expression. This phenomenon is known as intron-mediated enhancement (IME) and was reported in several studies dealing with gene expression in various plant species such as *Arabidopsis*, rice and maize (Clancy and Hannah, 2002; Jeon et al., 2000; Jeong et al., 2006; Rose and Beliakoff, 2000; Rose et al., 2008; Gallegos and Rose, 2015). From a biotechnological point of view, introns are very interesting elements and are included into gene expression cassettes to enhance the expression of foreign genes in plants (Emami et al., 2013). One of the major problems for the creation of transgenic plants is silencing of introduced genes. Interestingly, it was shown that the use of introns prevents transgene silencing (Christie et al., 2011).

On the other hand, the use of the constitutive 35S promoter to drive the expression of TFs such as DREBs resulted into severe growth retardation (Kasuga et al., 1999). To circumvent this problem, the *RD29A* promoter, which is stress-inducible promoter, was used as replacement of the 35S promoter and normal phenotype was restored in many plants such as cotton, *Arabidopsis*, tobacco, wheat and barley (Kasuga et al., 2004; Morran et al., 2011).

In this study, we transformed tobacco with the full-length *TdSHN1* gene which encodes a WIN1/SHN1-type transcription factor in durum wheat and contains a promoter region, one intron, and two exons (PI lines). Transgenic plants (PI lines) were subjected to abiotic stress and their tolerance was compared to that of WT, transgenic plants harbouring the intron-less cDNA (cD lines), and transgenic plants containing the *TdSHN1* intron and two exons placed under the control of the duplicated 35S promoter (gD lines).

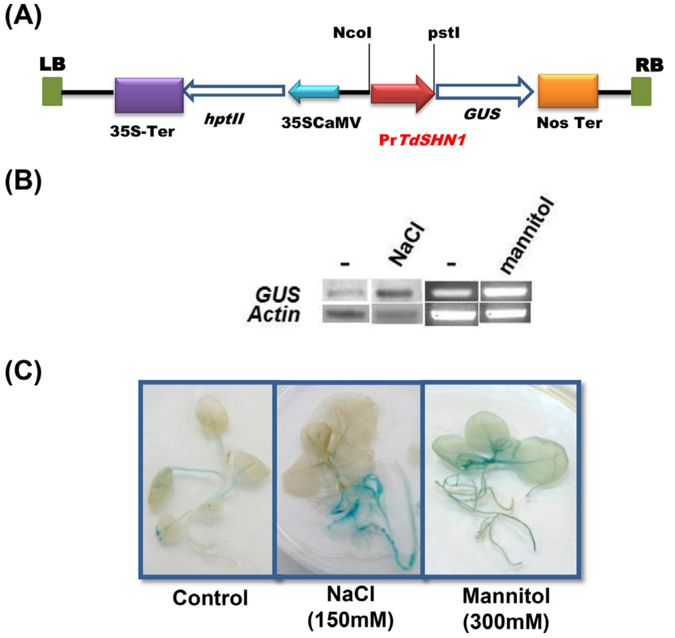

Fig. 1. RT-PCR and histochemical analysis of *GUS* reporter gene driven by Pr*TdSHN1* promoter in transgenic tobacco plants. (A) Linear map of T-DNA region of genetic construct used to generate transgenic tobacco Pr*TdSHN1*:GUS. (B) RT-PCR analysis of Pr*TdSHN1*:GUS transgenic seedlings grown under control condition (−) or NaCl (150 mM) or mannitol (300 mM) for 24 h. (C) Histochemical staining of Pr*TdSHN1*:GUS Pr*TdSHN1*:GUS transgenic seedlings grown under control condition or NaCl (150 mM) or mannitol (300 mM) for 24 h.

2. Materials and methods

2.1. Construction of TdSHN1 promoter::GUS and assessment of its activity and induction by salt and mannitol in stable transgenic tobacco

To test the ability of *TdSHN1* gene promoter, designated here as Pr*TdSHN1*, to drive gene expression and its induction by salt and drought stresses, promoter fragment was liberated from full length *TdSHN1* gene (Djemal and Khoudi, 2015) using *NcoI* and *PstI* restriction enzymes and cloned in pCAMBIA1391Z vector upstream of *GUS* gene (Fig. 1A). Transgenic tobacco lines were obtained as described below and verified by PCR analysis using *hptII* and *GUS* gene specific primers (See Supplementary Table 1). To test the activity of Pr*TdSHN1* under normal and stress conditions, seeds of transgenic lines were surface-sterilised and germinated on MS solid medium (Murashige and Skoog, 1962) supplemented with hygromycin (15 mg/L) and kept for 25 days in a growth chamber at 22 °C under 16/8 light/dark photoperiod. For stress treatments, seedlings were transferred to liquid MS medium supplemented with 150 mM NaCl or 300 mM mannitol for 24 h. After treatments, seedlings were used to extract RNA using Trizol method and RNA was used for RT-PCR to determine *GUS* expression levels using *GUS* gene specific primers. For GUS histochemical staining, seedlings were incubated O/N in GUS staining buffer (50 mM $NaPO_4$ (pH 7), 10 mM EDTA (pH 8), 2 mM K_4FeCN, 2 mM K_3FeCN, Triton X-100, X-Gluc 0.5 mg/ml**)** and chlorophyll was removed in 70% ethanol following (Jefferson et al., 1987).

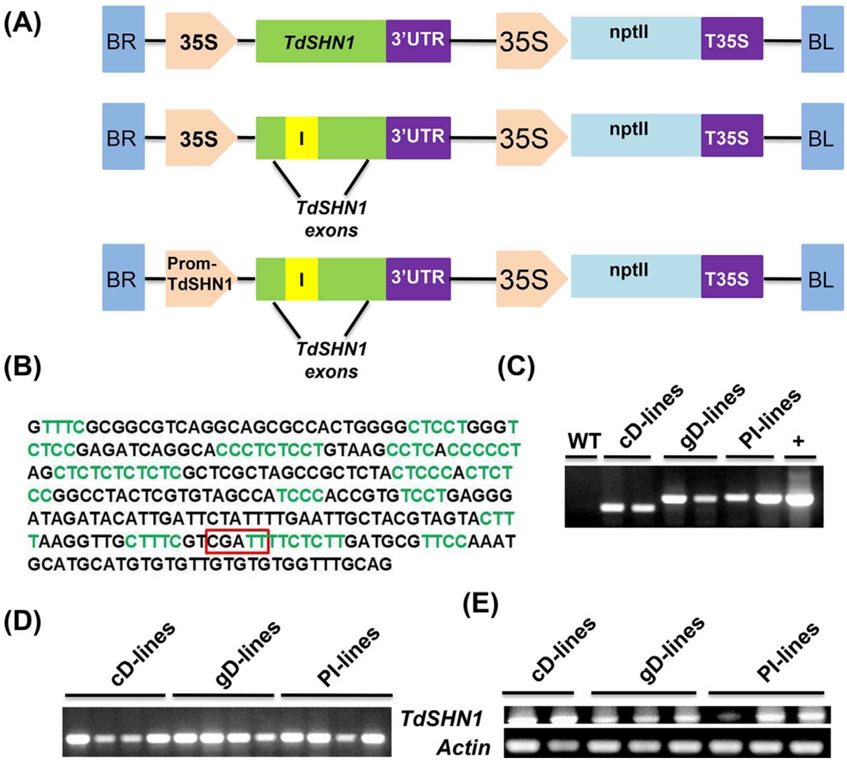

Fig. 2. Molecular characterization of transgenic lines. (A) Linear map of T-DNA regions of different genetic constructs used in this study in plasmid pCAMBIA2300. I, *TdSHN1* intron; *Prom-TdSHN1*, Pr*TdSHN1* native promoter. (B) IME related motif in *TdSHN1* intron. Green letters, C/T-streches; red box CGATT motif (Parra et al., 2011). (C) PCR products obtained following amplification of genomic DNA derived from various transgenic tobacco lines using *TdSHN1* gene-specific primers flanking the intron. (D) PCR products obtained following amplification of genomic DNA with *nptII* specific primers. WT, amplification from WT plant; +, positive control: amplification from plasmid DNA harboring the *TdSHN1* gene. (E) RT-PCR analysis of various transgenic lines using *TdSHN1* gene-specific primers flanking the intron. The tobacco actin gene was used in RT-PCR as internal control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.2. Genetic constructs with TdSHN1 gene and generation of transgenic tobacco plants

Three genetic constructs in pCambia 2300-35S vector (Gouiaa et al., 2012) were prepared for the present study. The first harbors *TdSHN1* intron-less cDNA sequence which was put under the control of the duplicated 35S promoter and the 3′UTR of the *TdSHN1* genomic sequence (Djemal and Khoudi, 2016). The second harbors the *TdSHN1* genomic sequence starting from the ATG initiation codon and includes the unique intron, the two exons as well as the 3′UTR of the *TdSHN1* ligated as a single fragment in the correct orientation downstream the 35S promoter using the *EcoRI* restriction site present in pCambia2300-35S vector (Gouiaa et al., 2012). The third construct is identical to the second construct except that it contains *TdSHN1* native promoter, Pr*TdSHN1* instead of the duplicated 35S promoter. All these constructs are illustrated in Fig. 2A. The expression vectors were mobilized into *Agrobacterium tumefaciens* strain GV3101 and their stability was checked prior to tobacco (*Nicotiana tabacum* var Xanthi) transformation using the leaf disc method (Horsch et al., 1985). Transgenic lines were produced for different constructs. For clarity purposes, we have designated cD, gD and PI lines the lines obtained following transformation of tobacco with first, second and third construct, respectively.

2.3. Molecular analysis of transgenic tobacco plants

PCR and RT-PCR were used for molecular analysis of transgenic plants. Total tobacco genomic DNA was isolated from various transgenic tobacco lines and WT according to Dellaporta et al. (1983). To verify the presence of the corresponding transgenes, PCR amplifications were carried out using specific primers for *npt*II and *TdSHN1* genes (See Supplementary Table 1). PCR amplifications were performed in 25 µl total volume containing 0.15 mM of each dNTP, 2.5 µl of 10 x *Taq* DNA polymerase buffer, 2.5 mM $MgCl_2$, one unit of *Taq* DNA polymerase, 2.5 µM gene specific primers. The PCR steps were as follows: 94 °C, 5 min for initial denaturation, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 1 min 30 s at 72 °C. A 10 min extension period at 72 °C was added at the end of the 35 cycles. The PCR products were run on 1% agarose gels and photographed.

For determination of expression level of *TdSHN1* and stress-related genes in various transgenic lines using qRT-PCR, seeds of various transgenic lines were surface-sterilised and germinated on MS solid medium supplemented with kanamycin (250 mg/L) for transgenic lines, or MS devoid of antibiotic for WT seeds and kept for 25 days in a growth chamber at 22 °C under 16/8 light/dark photoperiod. For stress treatments, seedlings were transferred to liquid MS medium supplemented with 200 mM NaCl or 300 mM mannitol for 24 h. Thereafter, Trizol method was used to extract total RNA. qRT-PCR was used to examine the level of expression of *TdSHN1* gene in transgenic lines derived from various constructs. In addition, the expression level of a total of 11 stress-related genes known to harbor GCC box or DRE elements, the binding sites of TdSHN1 transcription factor, in their promoter regions was also determined. RNA samples derived from various transgenic lines and WT were subjected to amplifications using SYBR Green fluorescent dye according to manufacturer's recommendations and flurometric thermal cycler (DNA Engine Opticon 2; MJ Research, Walthan, MA, USA). The SDS2.2 software integrated in Applied Biosystem 7900 HT Fast Real-Time PCR system was used to display the results. The C_T value among replications was used for the comparison of repeated samples. The tobacco *actin* gene was also amplified and its C_T value was used for normalization of linear data. The relative expression was determined using formula $2^{-\Delta\Delta CT}$. Primers used in this experiment are listed in Supplementary Table 1.

2.4. Verification of presence of alternative splicing events in genomic sequences of TdSHN1

To check for the presence of alternative splicing events, total RNA was isolated from gD and PI transgenic lines as described above. cDNA was produced using MML-Reverse transcriptase (InVitrogen) and oligo-dT and used for RT-PCR. RT-PCR amplification products of gD and PI transgenic lines were purified from agarose gel, cloned in pGEM-Teasy vector and sequenced.

2.5. Inheritance of nptII gene in transgenic tobacco lines

To study the segregation of the *npt*II gene, which is closely linked to *TdSHN1* sequences, T_1 seeds from various transgenic lines (cD, gD and PI lines) were surface sterilised and cultured on MS medium (Murashige and Skoog, 1962) supplemented with 250 mg/L kanamycin in petri dishes. For each transgenic line, three petri dishes containing 50–60 seeds each were prepared. After incubation for 2 weeks in growth chamber at 23 °C and 16 h light/8 h dark, the numbers of kanamycin-resistant seedlings were counted. The segregation ratio was tested using the X^2 test and the number of integrated copies of the transgene was estimated.

2.6. Toluidine blue (TB) staining, chlorophyll leaching and stomatal density (SD) determination

Stomatal density was determined as described elsewhere (Djemal and Khoudi, 2016). TB staining was performed as follows: leaves were collected from 2-month-old in *vitro*-grown tobacco seedlings and incubated, with gentle agitation, O/N in 0.05% TB solution (Sigma) prepared in water. Thereafter, leaves were washed thoroughly in water to get rid of excessive dye and photos were taken. For chlorophyll leaching determination, leaf discs of 1 cm (5 discs) were cut from various transgenic lines and WT, immersed in ethanol 80% and chlorophyll content was determined at 60 and 90 min.

2.7. Evaluation of salt and drought tolerance of transgenic plants

To evaluate the tolerance of transgenic plants to salt and mannitol stress, T_1 seeds from WT and various transgenic lines were surface sterilised as described (Djemal and Khoudi, 2016) and germinated on MS medium for WT seeds or MS medium supplemented with 250 mg/L kanamycin for transgenic tobacco seeds. Fifteen days later, WT and the surviving transgenic seedlings on kanamycin were transferred to MS medium supplemented with 200 mM NaCl or 300 mM mannitol. Plates were held vertically and maintained in growth chamber with controlled environment (T: 22 °C and 16/8 light/dark cycles) for 15 days. To test abiotic stress tolerance of transgenic lines at adult stage, one-month-old tobacco plants were produced as described above and transferred to pots filled with garden soil substrate and kept in phytotron chamber under controlled conditions (T: 22 °C and 16/8 light/dark cycles) for 15 days before the onset of salt treatment. Salt stress was imposed gradually by irrigation of plants every day with 50 mM increments of NaCl until achieving 200 mM NaCl concentration. Thereafter, plants were irrigated twice a week using 200 mM NaCl for 15 days. Drought stress was imposed by withholding water for 15 days. At the end of these treatments adult plants were used for biochemical analysis (CAT and SOD activities, MDA, and H_2O_2 contents).

2.8. Chlorophyll content determination

Leaf discs of 0.5 cm were cut from WT and transgenic tobacco plants of the same age. They were floated (at least five discs per line) on 10 ml of stressful agents (400 mM NaCl or 400 mM mannitol) or sterile water for 5 days. At the end of the incubation period, they were transferred individually to 1.5 ml Eppendorf tubes filled with 80% acetone solution to extract total chlorophyll (Arnon, 1949) and content was calculated using formulas established by McKinney (1941).

2.9. Malondialdehyde (MDA) and H_2O_2 content estimations and detection of O_2^- and H_2O_2

Approximately 200 mg of fresh leaf material was homogenized in the presence of TCA (1%) and the mixture was centrifuged (20 000 g, 20 min) at 4 °C. H_2O_2 contents were determined according to Velikova et al. (2000). MDA content was estimated according to Draper and Hadley (1990). For visual detection of H_2O_2 and O_2^- produced following drought and salinity stresses, leaves of adult plants were incubated overnight into solutions of 3,3-diaminobenzidine (DAB) (1 mg/ml DAB, 10 mM Na_2HPO_4, pH 3.8) or nitroblue tetrazolium (NBT) (1 mg/ml NBT, 25 mM HEPES, pH 7.6). Thereafter, chlorophyll was removed using absolute ethanol and leaves were photographed.

2.10. Catalase (CAT) and superoxide dismutase (SOD) activities determination

Fresh leaf tissue was collected from plants and homogenized in mortar on ice using (50 mM phosphate (pH 7.0), 2% PVP and 1 mM PMSF). The homogenate was centrifuged at 10 000 g for 20 min at 4 °C. Total soluble proteins were determined using the Bradford method (Bradford, 1996) and BSA was used for standard curve. CAT activity was calculated according to (Lück, 1965) and expressed as units per mg protein per min. The total SOD activity was determined according to Scebba et al. (1999) and expressed as units per mg protein.

2.11. IMEter score calculation

To evaluate the potential of *TdSHN1* intron to stimulate gene expression, IMEter score was calculated using IMEter algorithm available at http://korflab.ucdavis.edu/cgi-bin/web-imeter2.pl.

2.12. Statistical analysis

The difference of means among WT and different transgenic lines was analysed using one-way analysis of variance (ANOVA). 5% level of significance was used for hypothesis testing. More than 4 plants for each transgenic line and WT were used for calculations. For q-RT-PCR, 5 seedlings were pooled and used as one biological replicate. Three biological replicates were used for each line and data were analysed using Student's *t*-test.

3. Results

3.1. PrTdSHN1 promoter is active in transgenic tobacco and is stress-inducible

To test the ability of Pr*TdSHN1* promoter to direct gene expression, it was cloned upstream *GUS* reporter gene (Fig. 1A) and introduced into tobacco. Stable transgenic lines were obtained. Seedlings were subjected to NaCl and mannitol treatments and analysed by RT-PCR and GUS histochemical staining. As illustrated in Fig. 1B, Pr*TdSHN1* was able to drive the transcription of *GUS* gene both under control and stress conditions. Interestingly, Pr*TdSHN1* was strongly induced by salt and mannitol in the 24 h treatments as shown by the enhancement of *GUS* transcript levels (Fig. 1B). In addition, histochemical analysis of transgenic seedlings showed that transgenic seedlings had stronger staining after treatments particularly in their roots following salt stress treatment. These results indicate that Pr*TdSHN1* is active in transgenic tobacco plants and is stress-inducible.

3.2. Analysis of TdSHN1 intron sequence reveals potential enhancing effect

The *TdSHN1* intron is 207 bp long (Djemal and Khoudi, 2015) and contains characteristic features found in other plant introns such as the sequence GTAAG at the 5’ splicing site and the consensus sequence TGCAAG. It has a % GC of 48.7% and % T of 33.8% and contains many CT-stretches (Fig. 2B). Importantly, it contains the motif (CGATT) (Fig. 2B) known to be important for IME (Parra et al., 2011) which prompted us to subject *TdSHN1* sequence to bioinformatics analysis using IMEter software. *TdSHN1* intron obtained a score of 17.04 using IMEter version 2 trained on *Arabidopsis* (Table 1). According to this IMeter version 2, introns that moderately enhance expression tend to

Table 1
IMEeter scores and ability to increase expression of *TdSHN1* intron and other plant introns that were determined experimentally (Schwab et al., 2013).

Intron name	IMEter score V2	Fold increase
i*TdSHN1*	17.04	3.7
i*UBQ*	53.95	14.9
i*TRP1*	15.73	4.5
i*TCH 3*	4.96	1.4

have IMEter v2.0 scores above 10 and introns that strongly enhance expression tend to have scores above 20. Thus, the positive score (17.04) obtained for *TdSHN1* intron indicates that it has the potential to moderately enhance gene expression when used in a genetic construct.

3.3. Production of transgenic tobacco lines harbouring different TdSHN1 genetic constructs

Several transgenic tobacco lines were generated using *Agrobacterium*-mediated transformation harbouring one of the genetic constructs illustrated in Fig. 2A. As shown in Fig. 2C, PCR products of the expected size were obtained from genomic DNA amplifications of various transgenic lines. Indeed, using PCR specific primers flanking *TdSHN1* intron, a difference in size of 207 bp, corresponding to the size of the intron, was observed between PCR products of gD-lines and PI-lines, on the one hand and the intron-less cD-line, on the other hand (Fig. 2C). For the *npt*II gene, the unique expected PCR product was obtained for all transgenic lines (Fig. 2D). These results confirm the correct integration of the transgenes into the genomic DNA of transgenic tobacco. In addition, RT-PCR analysis using total RNA isolated from various transgenic lines revealed that all constructs produced a single band of the same size (Fig. 2E). PCR products obtained from gD and PI lines were cloned and sequenced. Sequence analysis of cloned RT-PCR products confirmed that no alternative splicing events occurred in genomic sequences of *TdSHN1* when expressed in gD and PI transgenic tobacco lines.

To estimate the copy number of transgenes in various transgenic lines, we studied the segregation of the *npt*II gene which is linked to *TdSHN1* (Fig. 2A). Results revealed that kanamycin resistance followed a Mendelian phenotype segregation of 3:1 which is typical of single copy gene integration (Supplementary Table S2).

3.4. Cuticle permeability and stomatal density assessment

The effect of overexpression of *TdSHN1* in different transgenic lines on cuticle permeability was evaluated using TB staining of leaves derived from different transgenic lines and WT grown under non stress conditions. TB is a hydrophilic dye used in general for histological staining. In plants, TB is used to reveal cuticlar defects. Plants with normal cuticle repel TB whereas plants with deficient cuticle allow TB to permeate the epidermal surface. As illustrated in Fig. 3A, leaves derived from transgenic line gDL-4 exhibited the strongest staining compared to those derived from intron-less line cDL-1 and PI-L11 transgenic line. By contrast, leaves derived from WT plants had not been dyed in 0.05% TB which indicates an easier penetration of TB molecules through gD and PI transgenic line cuticles (Fig. 3A). Chlorophyll leaching assay was also used to confirm the higher permeable cuticle exhibited by *TdSHN1* transgenic lines. Results showed that at 60 and 90 min, transgenic lines harboring *TdSHN1* gene with its intron (lines gDL-4, gDL-7, PI-L1 and PI-L13) showed significantly higher chlorophyll leaching in 80% ethanol than lines harboring intron-less cDNA (cDL-1 and cD-L7) and WT (Fig. 3B). This difference suggests that expression of full-length *TdSHN1* gene is capable of altering the cuticle permeability. In addition, microscopic examination of the leaf epidermal peels of abaxial side of the leaves derived from various transgenic lines grown under control conditions revealed that, though a significant reduction of stomatal densities was observed in transgenic lines compared to WT, the transgenic line gDL-4 exhibited the lowest stomatal density (Fig. 3C).

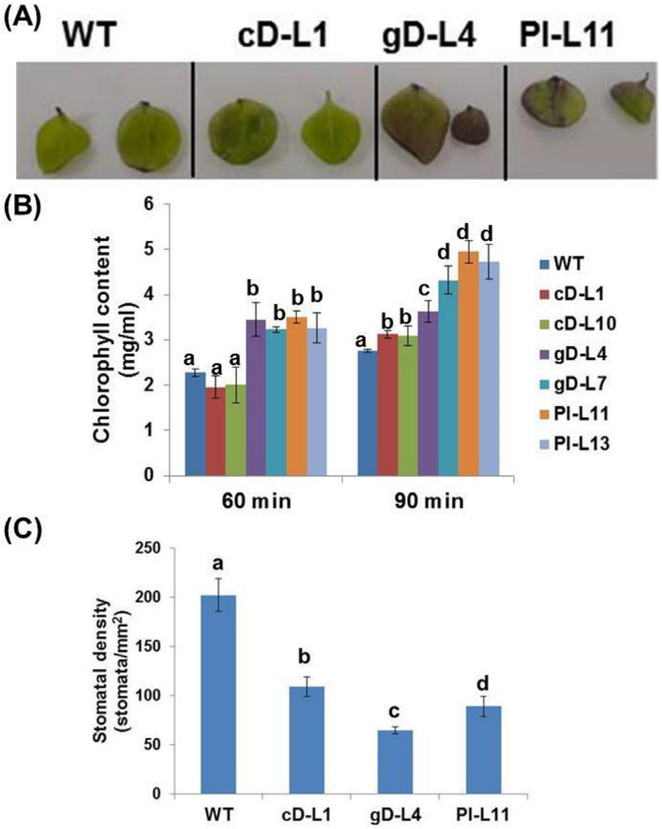

Fig. 3. Effect of TdSHN1 expression by various constructs on cuticle permeability and stomatal density. (A) Photos of abaxial side of leaves derived from two month-old plants of *in vitro*-grown WT and *TdSHN1*-overexpressing lines stained with Toluidine blue. (B) Chlorophyll leaching assays using leaf discs derived from mature leaves (of same age) of various transgenic lines and WT, immersed in 80% ethanol for different time intervals. Three plants for each transgenic line were used. Data are mean ± SD. (C) Stomatal density measurements using leaf epidermal peels at three different microscopic fields under light microscope. Plants used for stomatal densities measurements were maintained in controlled conditions. Column with different letters indicate significant differences ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.5. Induced TdSHN1 expression stimulates growth and enhances salt and mannitol tolerances

The overexpression in tobacco of *TdSHN1* either as a cDNA or full-length gene seems to stimulate the plant growth. In fact, as illustrated in Fig. 4A and B, and Supplementary Fig. 1A, plants of all transgenic lines exhibited significantly longer roots than WT plants when grown on MS medium. Compared to a root length of 3.3 cm ± 0.2 in WT plants, *TdSHN1* transgenic lines showed a root lengths of 4.4 ± 0.1 (line cD-L1), 5.06 ± 0.13 (line gD-L4) and 6.34 ± 0.13 (line PI-L15). This increase under control conditions in root lengths varies between 35 and 50% over WT plants. Notably, all transgenic lines harboring *TdSHN1* under control of Pr*TdSHN1* promoter had significantly longer roots than other transgenic lines and WT. In addition to the positive effect of the overexpression of *TdSHN1* on root growth, all *TdSHN1* transgenic lines exhibited better biomass production than WT plants under control conditions (Fig. 4C). The trend was maintained also under salt and mannitol stresses where all transgenic lines produced significantly more

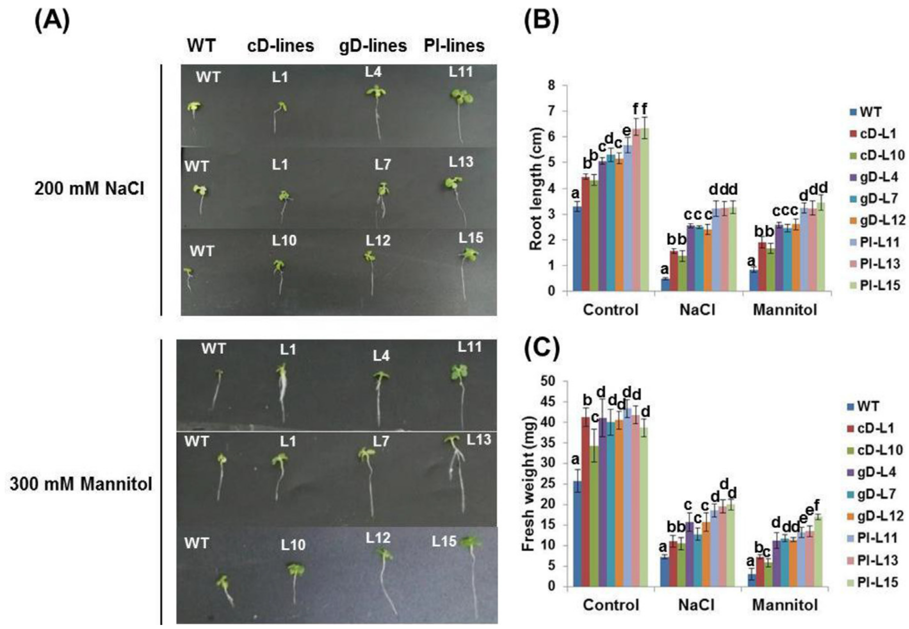

Fig. 4. Phenotype of transgenic tobacco seedlings grown in the presence of NaCl and mannitol. Shown are WT and various transgenic lines that were grown on MS medium containing indicated concentrations of mannitol and NaCl (A) for 15 days in plates maintained vertically. (B) Primary root lengths and (C) aerial fresh weights of the seedlings grown in the presence of NaCl and mannitol. The data are the means (n = 5) ± SD. Columns with different letters indicate significant differences ($P < 0.05$).

biomass than their WT counterparts Fig. 4. Noteworthy, transgenic lines harboring the *TdSHN1* gene including intron (lines gDL-4, gD-L7, gD-L12, PI-L11, PI-L13 and PI-L15) exhibited better biomass production than intron-less counterparts (cD-L1 and cD-L10). Interestingly, transgenic PI lines harboring *TdSHN1* gene including its native promoter Pr*TdSHN1* and intron (lines PIL-11, PIL-13 and PIL-15), showed the longest root and the highest biomass production across the various treatments used in the present study (Fig. 4). In fact, PI transgenic lines showed 7 and 4 times longer roots than WT under salt and mannitol stress, respectively. However, cD lines showed 3 times longer roots than WT under salt treatment and 2.5 times under mannitol treatment. Finally, gD lines showed 5 times longer roots than WT under salt treatment and 3 times longer roots than WT under mannitol treatment (Fig. 4B).

3.6. Chlorophyll, H_2O_2 and MDA contents of various transgenic lines subjected to abiotic stresses

For further assessment of abiotic stress tolerance imparted by various genetic constructs, leaf disc senescence assay was performed. For this, leaf discs of same size were cut from healthy leaves derived from various transgenic lines and WT and floated on NaCl (400 mM) and mannitol (400 mM) solutions. Total chlorophyll was extracted from stressed discs and quantified. At 400 mM NaCl, leaf discs derived from WT lost 54% of their total chlorophyll compared to their corresponding content in water, while leaf discs from cD lines (cD-L1, cD-L10), gD lines (gD-L-4, gD-L7) and PI lines (PI-L11, PI-L13) lost 30–38%, 26–27%, 19–21% of their control content, respectively (Fig. 5A). A similar trend was also observed in the case of mannitol (400 mM) treatment. Indeed, WT discs lost 60% of their control chlorophyll content while cD-lines, gD-lines, PI-lines, lost only 34–36%, 27–32% and 19–24%, respectively of their control chlorophyll contents (Fig. 5A).

To estimate ROS production and membrane damage following abiotic stress exposure, H_2O_2 and MDA contents were determined in adult plants subjected to salt and drought stresses (Supplementary Fig. 1). Results revealed that, even under control conditions, transgenic lines accumulated significantly less H_2O_2 and MDA than their WT counterparts (Fig. 5B and C). Under salt stress, for example, transgenic cD-lines, gD-lines and PI-lines showed 1.3–1.6-fold, 1.7–2.2 fold and 1.9–2.2 fold less H_2O_2 respectively, than WT. Under drought stress, cD-lines, gD-lines and PI-lines showed 1.23–1.37 fold, 1.82–1.83 fold and 1.83 fold less H_2O_2, respectively than WT (Fig. 5B). Furthermore, intense NBT and DAB staining in leaves of transgenic cD and gD lines compared to transgenic PI-lines was observed (Supplementary Fig. 3) which indicates higher levels of free radicals and H_2O_2 accumulation in transgenic cD and gD lines than in transgenic PI lines when adult plants were subjected to salt and drought stresses.

3.7. Evaluation of antioxidant enzyme activities in different transgenic lines under stress

Due to their importance in abiotic stress tolerance, SOD and CAT activities were monitored in this study in adult plants (Supplementary Fig. 1). Results showed that under all tested conditions, the activities of both enzymes in various transgenic lines were significantly higher than WT (Fig. 6). Under salt and drought stresses, the activity of SOD enzyme in transgenic lines harbouring *TdSHN1* intron, gD (lines L4 and L7) and PI (lines L11 and L13), was significantly higher than cD lines (Fig. 6A). Although SOD activity decreased under salt and drought stress, cD, gD and PI lines retained 60%, 72–80% and 80%–96%, respectively of their control SOD activity, under salt stress. Similar trend was also observed under drought stress (Fig. 6A). In contrast, CAT activity increased following salt and drought stress. However, this increase was more important in gD and PI lines, under drought stress, compared to WT and cD lines (Fig. 6B). Overall, these results demonstrate that gD and PI transgenic lines showed the best enzymatic antioxidant capacity compared to cD lines and WT which might help to lower ROS levels under stress conditions (Fig. 5).

3.8. Combination of native promoter and intron significantly increased expression level of TdSHN1 as well as stress-related genes in transgenic tobacco lines

To investigate the effect of various *TdSHN1* constructs at molecular level, we performed qRT-PCR analysis to estimate the transcription levels of *TdSHN1* in five independent transgenic tobacco lines from each construct (15 independent transgenic lines in total). These lines were subjected to salt and mannitol stress treatments. Results showed that the presence of *TdSHN1* intron in the genetic constructs significantly increased the level of expression of *TdSHN1* in gD and PI transgenic lines compared to intron-less cD lines under salt and mannitol treatments (Fig. 7 and Supplementary Fig. 2). Notably, the highest expression level of *TdSHN1* was found in PI transgenic lines which

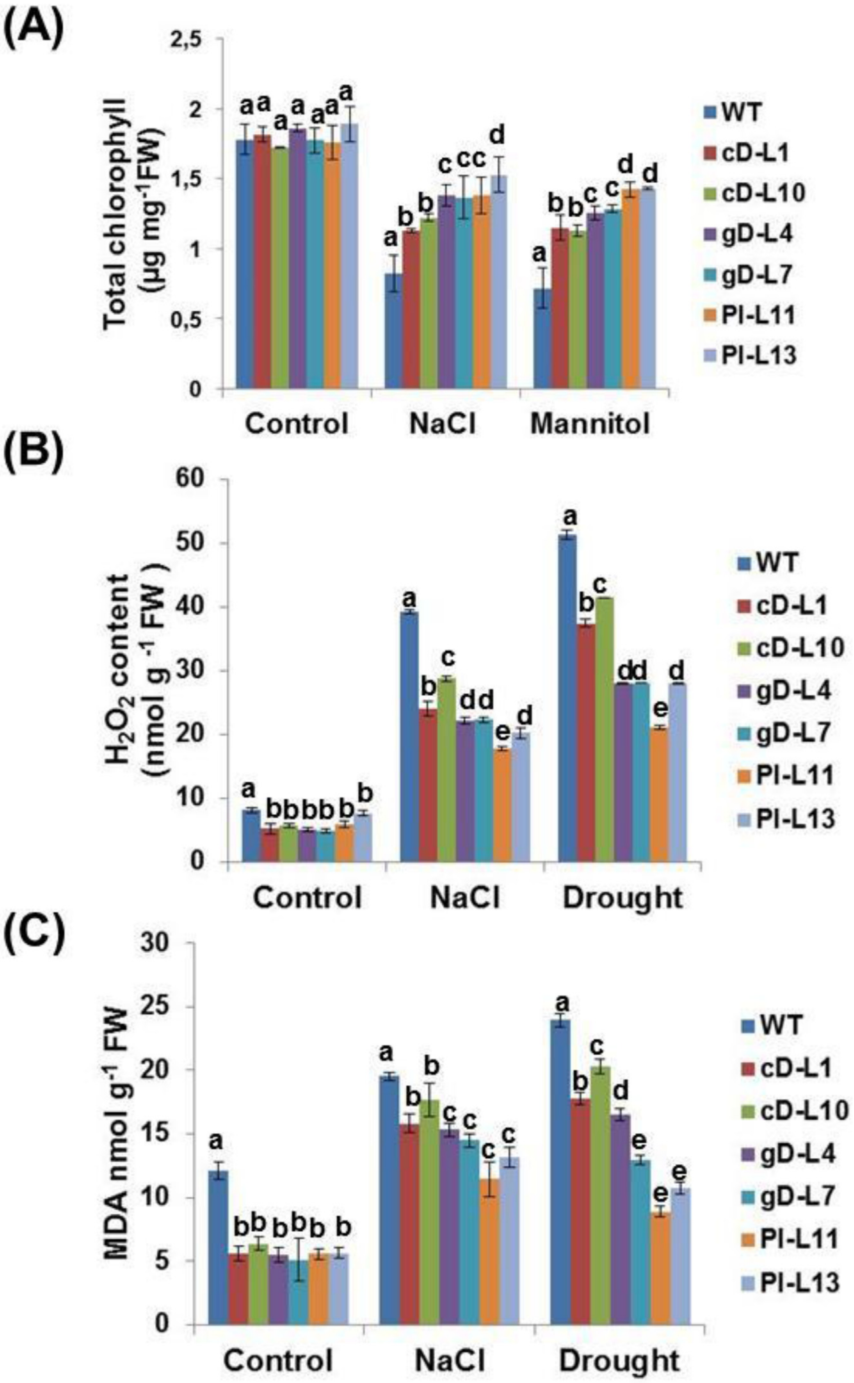

Fig. 5. Biochemical analysis of transgenic plants. (A) Total chlorophyll contents in the leaf discs derived from two independent lines from each construct and subjected to senescence assay under salt and mannitol treatments for 5 days. (B) H_2O_2 and MDA (C) contents in adult plants derived from various transgenic lines and subjected to salt (continuous irrigation with 200 mM NaCl) or drought by withholding water for 15 days. Data are means (n = 5) ± SD. Columns with different letters indicate significant differences ($P < 0.05$).

harbor the combination of Pr*TdSHN1* and intron. Two transgenic lines having the highest expression level of *TdSHN1* in each construct were chosen to perform a quantitative RT-PCR analysis on a set of 11 genes known to harbor GCC box or DRE elements in their promoter regions. These genes include ROS detoxification genes (*NtSOD*, *NtCAT1* and *NtGPX*) genes related to osmotic stress (*NtSAM* and *NtERD10C*), a lipid-transfer protein (*TOBLTP)*, wax biosynthesis gene (*NtCER1)*, sucrose biosynthesis gene (*NtSPS*), raffinose biosynthesis gene (*NtGol*) and pathogenesis-related gene (*PR5)*. Results showed that, compared to WT, the expression level of most of genes was significantly increased in all transgenic lines following either salt or mannitol treatments (Fig. 7, Supplementary Fig. 2). Interestingly, fold of gene up-regulation was significantly higher in transgenic lines harboring the *TdSHN1* intron (gD-L4 and PI-L11 lines) than in the intron-less line (cD-L1) (Fig. 7). More importantly, the highest level of induction, both under salt and mannitol treatments, was found in PI transgenic lines.

4. Discussion

In our previous report (Djemal and Khoudi, 2015), we described the

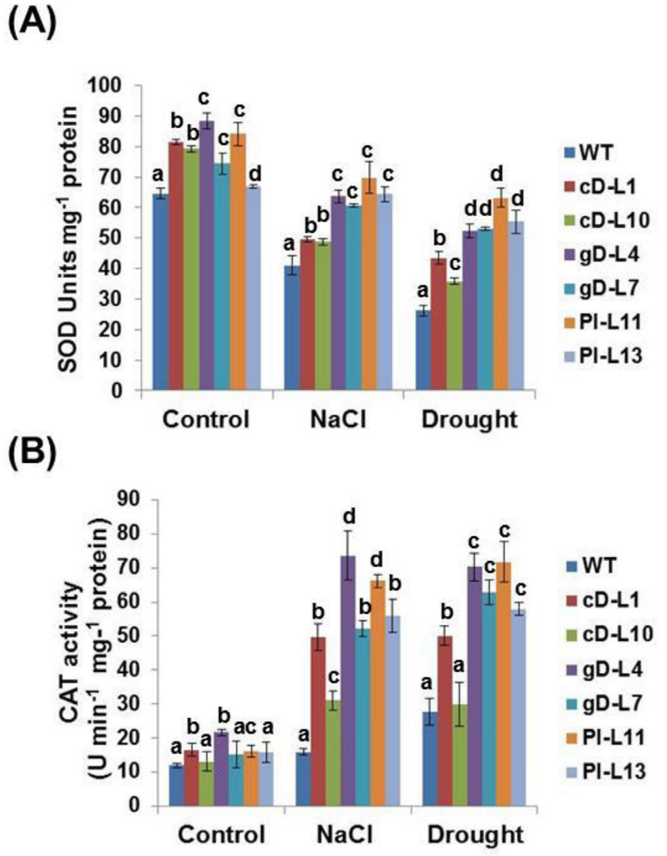

Fig. 6. Enzymatic antioxidant activity of adult transgenic and WT tobacco plants subjected to drought and salt stress. (A) CAT activity and (B) SOD activity in various transgenic tobacco lines. Data are means (n = 4) ± SD. Columns with different letters are significantly different at $P < 0.05$.

isolation of genomic clone and the cDNA of *TdSHN1* gene using chromosome walking method and RT-PCR, respectively. The genomic clone consisted of a single fragment of 1.82 kb composed of 0.5 kb promoter region, the two *TdSHN1* exons and one intron. The 0.5 promoter region sequence, designated here as Pr*TdSHN1*, was subjected to bioinformatics analysis using PLACE and was found to contain several abiotic stress boxes (cis-elements) such as abscisic acid responsive element ABRE involved in salt stress response which suggest that it can drive stress-induced gene expression. Here, we first assessed the ability of Pr*TdSHN1* promoter to drive *GUS* reporter gene in stably transformed tobacco plants. Results showed that Pr*TdSHN1* promoter is sufficient to drive *GUS* expression. In addition, Pr*TdSHN1* is strongly induced by salt and mannitol treatments (Fig. 1B and C). In addition, we confirmed experimentally the IME effect of *TdSHN1* intron predicted by IMEter software. In fact, under constitutive expression, *TdSHN1* intron was able to increase gene expression. Moreover, we showed that the induced overexpression of *TdSHN1* using a combination of endogenous Pr*TdSHN1* promoter and *TdSHN1* intron imparts significantly better drought and salinity tolerances than the constitutive overexpression of *TdSHN1* gene using 35S promoter in combination with *TdSHN1* intron.

The cuticle, coating the outer surfaces of land plants, plays an important role in limiting water loss and is involved in both biotic and abiotic stress tolerances as well as plant development and growth (Yeats and Rose, 2013). The involvement SHINE-type transcription factor in cuticle formation is well established (Djemal and Khoudi, 2016; Aharoni et al., 2004; Broun et al., 2004). In the present work, the expression of *TdSHN1* using genomic sequences altered the cuticle permeability. In fact, as illustrated in Fig. 3A, leaves derived from gD (line L1) and PI (line L11) lines were stained more intensely compared to those from intron-less cD-L1 line and WT tobacco plants. In addition, chlorophyll leaching assay showed higher chlorophyll leaching in gD and PI transgenic lines than WT and cD lines (Fig. 3B). However, our results showed that SD was significantly decreased in transgenic line gD-L4 compared to transgenic line cD-L1 and PI-L11 lines (Fig. 3C). The results obtained for chlorophyll leaching are contradictory to what is

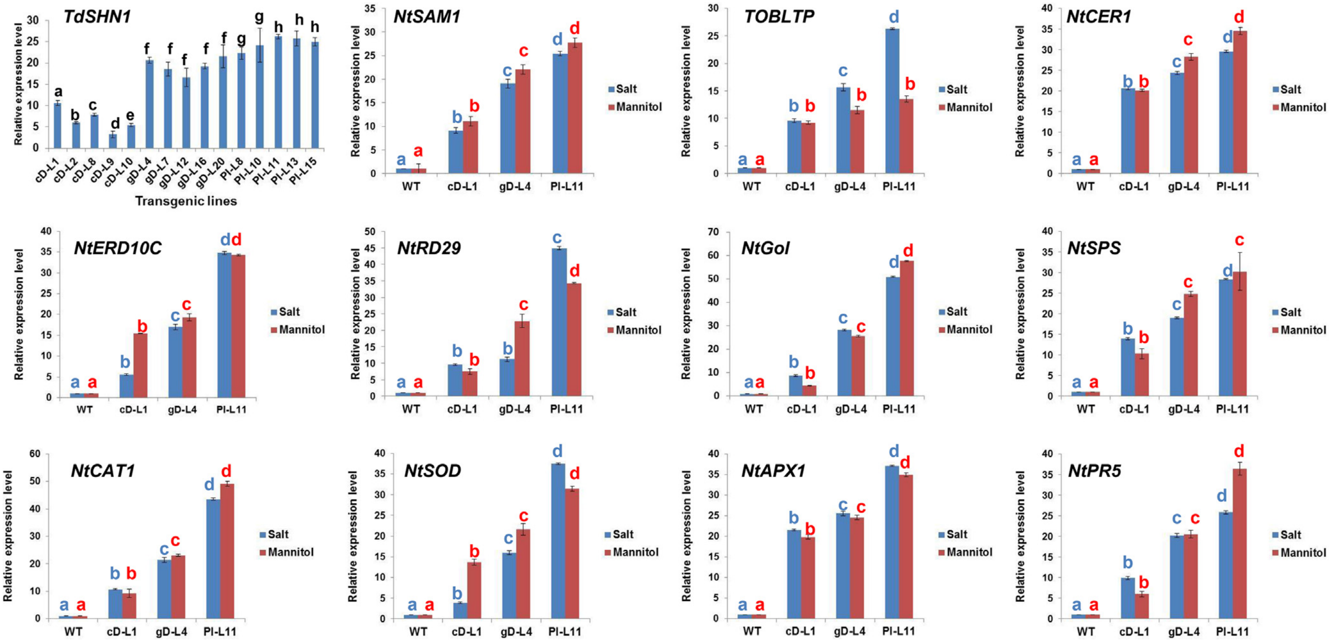

Fig. 7. qRT-PCR analysis of *TdSHN1* and stress-related genes in transgenic tobacco lines harboring various *TdSHN1* genetic constructs. Total RNA was extracted from three week-old WT and transgenic tobacco plants grown *in vitro* and subjected for 24 h to NaCl (200 mM) and mannitol (300 mM) treatments. *TdSHN1* expression level, shown here, is determined in tobacco seedlings subjected to 24 h salt stress and the actin gene was used as reference. For stress-related genes, the relative expression levels in transgenic lines are indicated relative to that of WT set to one, using the transcript of actin in the same sample. Results are from three biological replicates each involved five plants from each transgenic line and WT. Columns of the same treatment but with different letters are significantly different at $P < 0.05$ using Student's *t*-test.

expected. In fact, ethanol is used in chlorophyll leaching assay since it has the ability to dissolve cuticular waxes and hence facilitates chlorophyll leaching through the epidermis (Aharoni et al., 2004). Moreover, ethanol can diffuse into mesophyll tissue following stomatal route and elutes chlorophyll. Thus, chlorophyll leaching is expected to be more important in transgenic plants with higher stomatal densities and less wax biosynthesis. However, in this study as well as many reports (Aharoni et al., 2004), results were contrary to this expectation. This type of contradiction is common following the expression of transcription factors such as TdSHN1. In fact, the overexpression of *medicago truncatula* L. *WXP1* gene encoding an AP2/ERF transcription factor led to 37% increase in leaf wax biosynthesis and to reduction of chlorophyll leaching (Yeats and Rose, 2013). In contrast, overexpression of *WXP1 Arabidopsis* homolog (WIN1/SHN1) in *Arabidopsis* led to similar increase in wax biosynthesis but increased rate of chlorophyll leaching although stomatal density was decreased similar to our study (Aharoni et al., 2004). It is possible that the increased wax biosynthesis caused by the overexpression of SHN1-type transcription factors displaces normal wax packing in the cutin framework causing an increase in the number and size of diffusion pathways. Although many studies described the effect of SHN1-type transcription factors on chlorophyll leaching, the degree to which the chlorophyll assay measures chlorophyll leaching through stomata pores has not been determined.

Given the fact that plants used for SD determination were grown under non-stress conditions, the differences between gD-L4 and PI-L11 in cuticle permeability and stomatal density could be related to the difference in the promoter used to drive *TdSHN1* expression; the constitutive 35S promoter in the case of cD-L1 and gD-L4 lines and Pr*TdSHN1* stress-inducible promoter in the case of PI-L11 line (Fig. 2A). In fact, the 35S promoter is a constitutive promoter and confers high levels of gene expression. Thus when used to drive the expression of an SHN1-type transcription factor such as TdSHN1 it will reduce stomatal density at all stages of the plant growth and development in the absence or presence of stress. In contrast, stress-inducible promoter such as *PrTdSHN1* seems to reduce stomatal density to a lesser extent than that caused by the 35S promoter, under control conditions, due to probably weak background activity of *PrTdSHN1*. After the onset of stress conditions, important reduction of stomatal density is expected to take place in young/newly-formed leaves of transgenic plants harboring the stress-inducible *PrTdSHN1* promoter.

Interestingly, when subjected to drought stress, gD-L4 and PI lines exhibited less wilting phenotype than WT and cD-L1 line (data not shown). This could be attributed to the fact that cD-L1 line has the highest SD value compared to gD-L4 and PI lines (Fig. 3). In plants, stomata are the main source of water loss despite the fact that they represent only 1–2% of the total leaf area (Backley, 2005). Thus, the manipulation of SD has the potential to become an interesting tool for increasing drought tolerance. Indeed, recent studies reported that transgenic *Arabidopsis* plants engineered to have reduced SD were drought tolerant (Hepworth et al., 2015).

One important finding in the present study is the enhanced root development and biomass production following the stress-induced expression of *TdSHN1*. In fact, under control conditions, root length of seedlings of PI transgenic lines was twice as long as WT roots. Interestingly, PI transgenic lines showed 7 and 4 times higher root length than WT under salt and mannitol stress, respectively, whereas gD-lines showed 5 and 3 times higher root length than WT under salt and mannitol stress, respectively. TdSHN1 belongs to AP2/ERF family of transcription factors. Several studies demonstrated that this family is involved not only in abiotic stress tolerance but also in plant development. Recently, Nie et al. (2018) found that CmERF053, an ERF transcription factor of chrysanthemum, stimulates shoot branching and lateral root development. Lee et al. (2015) reported that *Arabidopsis* AtERF71/HRE2 transcription factor is involved in root development. AtERF71/HRE2 was found to bind both GCC and DRE elements which is similar to TdSHN1 (Djemal and Khoudi, 2015). Here, a strong GUS staining was observed in roots of transgenic tobacco line harboring Pr*TdSHN1-GUS* construct particularly following salt treatment (Fig. 1C). Thus, the possibility that *TdSHN1* overexpression stimulates root growth besides its role in abiotic stress tolerance cannot be excluded.

Abiotic stresses inflict significant damage to the most sensitive processes such as redox homeostasis, photosynthesis and carbon metabolism (Wang et al., 2015). Thus, the contribution of the expression of various constructs in salt and drought tolerance was investigated further using biochemical and physiological analyses of adult transgenic plants. Salt and drought stresses are known to decrease chlorophyll content (Jagtap et al., 1998). Interestingly, when leaf discs derived from various transgenic lines and the WT were subjected to NaCl or mannitol, leaf discs of transgenic lines gD and PI lines retained significantly more chlorophyll than transgenic cD lines (Fig. 5). The highest chlorophyll content was registered in transgenic line PI-L13. Chlorophyll content is commonly used as a marker of photosynthetic capacity which has an important role in plant growth and development (Nageswara et al., 2001).

Abiotic stresses such as salinity and drought lead to overproduction of ROS whose primary targets are membrane lipids. Particularly, H_2O_2 is considered as the main element causing membrane lipid peroxidation. In this study, we measured H_2O_2 and MDA levels in various transgenic lines subjected to salt and drought stresses. Interestingly, transgenic gD and PI lines showed significantly lower H_2O_2 and MDA contents than cD lines and WT (Fig. 5B and C). In addition, abiotic stress causes redox imbalance, therefore plants activate enzymatic antioxidant defense. The latter, comprises many enzymes among which we evaluated CAT and SOD activities. Results showed that activity of both enzymes was significantly higher in gD and PI lines compared to cD lines and WT (Fig. 6). In this study, gD and PI lines exhibited the highest levels of SOD and CAT activities, when grown under salt and drought stress conditions (Fig. 6). Several studies reported that the overexpression of genes encoding for ROS detoxification enzymes leads to decrease of cellular damage; better photosynthetic capacity and an overall better shoot and root growth under stress conditions (Konstantinos et al., 2010; Ray et al., 2012).

Despite tremendous work, the mechanism of IME in plants is still unclear. Careful examination of *TdSHN1* intron sequence revealed some important features which suggest that it might be involved in enhancing gene expression through IME mechanisms (Parra et al., 2011; Callis et al., 1987; Rose, 2004). In fact, besides the presence of C/T stretches, *TdSHN1* intron contains the CGATT motif shown to be linked to IME (Parra et al., 2011). Previous studies reported the presence of a pentamer of CGATT motif in the *Arabidopsis UBQ10* intron which were found to play an important regulatory role in IME (Rose et al., 2008; Parra et al., 2011). In addition, *TdSHN1* intron harbors T-rich regions that were shown, in the case of maize *SH1* first intron, to be required for maximum enhancement effect (Clancy and Hannah, 2002). In this study, we used IMEter algorithm to estimate the potential of *TdSHN1* intron to enhance gene expression trough IME. The IMeter score of *TdSHN1* intron is 17.04 (Table 1), which is similar to the score of the intron iTRP1. Interestingly, introns reported in Table 1 have been shown, experimentally, to strongly (iUBQ), moderately (iTRP1) or weakly (iTHC3) enhancing PAT1:GUS mRNA accumulation through IME (Schwab et al., 2013). Based on these reports, *TdSHN1* intron has the potential to moderately boost the *TdSHN1* mRNA accumulation through IME effect. Indeed, qRT-PCR results showed that though *TdSHN1* intron has IMeter score of 17.04, its inclusion in genetic constructs significantly increased *TdSHN1* expression level in gD and PI transgenic lines compared to intron-less cD lines (Fig. 7). In addition, the combination of TdSHN1 promoter and intron led to the highest level of TdSHN1 mRNA accumulation in PI transgenic lines. Our results are in line with Schwab et al. (2013) who demonstrated that *MIR163* and *MIR172a* introns contributed to a very significant increase of miRNA accumulation although they produced very low scores in IMEter test.

Accumulated evidence shows that IME is strictly related to splicing (Agrawal and Ansari, 2016). In fact, several studies showed that mutations at the intron splice sites abolish intron enhancement effect at protein or mRNA levels (Schwab et al., 2013). Large deletion or point mutations at 5′and 3′ splice site, which are involved in splicing, of maize *Adh 1* and *Hsp82* gene introns, abolished splicing and simultaneously decreased the IME effects of these introns (Sinibaldi et al., 1992; Luehrsen and Walbot, 1991). Similar results were also reported for the intron of *ATMHX* gene of *Arabidopsis* (Akua et al., 2010). *TdSHN1* intron was efficiently and correctly spliced by tobacco cells (Fig. 2E). In addition, sequences of cloned cDNA amplification products derived from gD and PI lines confirmed the absence of alternative splicing in genomic sequence of *TdSHN1* in tobacco. These results indicate that *TdSHN1* intron may have exerted an IME effect through, at least, increasing expression at RNA level (Laxa, 2017). The use of intron sequences in genetic construct to improve an agronomic trait is rare. For instance, only few reports in literature described this approach. Interestingly, a significant improvement was achieved over the use of intron-less constructs. In fact, Lang et al. (2004) demonstrated that expression of *SBgLR* genomic sequence in transgenic maize allowed a higher lysine and protein content than the expression of the corresponding intron-less cDNA. Cabello et al. (2007) demonstrated that transgenic plants bearing chimerical construct between plant transcription factor Hahb-4, Hahb-4 promoter and the leader intron of *Arabidopsis Cox5c* gene enhanced the drought tolerance imparted by Hahb-4 transcription factor. Recently, Sato et al. (2014) have shown that resistance to cucumber mosaic virus is dependent on the expression of RCY1 protein and achieved higher resistance, of transgenic plants, by using the genomic clone including its native intron for *Arabidopisis* and tobacco transformation. Our study demonstrates further that the expression of genomic sequences in transgenic plants is an effective strategy for enhancing gene expression and thereby significantly impacting a desirable agronomic trait.

In the present study, the IME effect applies only to the expression with the constitutive promoter 35S (cD and gD lines). A comparison between PI transgenic lines and transgenic tobacco lines harboring a construct in which intron-less cDNA is driven by the endogenous promoter will be useful to further confirm the IME effect of *TdSHN1* intron. In fact, Emami et al. (2013) demonstrated that IME positive effect of *UBQ10* gene intron was preserved when used in combination with either constitutive, tissue specific or light regulated promoters although magnitude of the change and the level of expression varied.

TdSHN1 TF is known to bind GCC box and DRE elements present in the promoter regions of many stress-related genes and to up-regulate their expression (Djemal and Khoudi, 2015, 2016). In this study, the expression level of a set of 11 genes known to harbor one of these cis-elements in their promoter regions was estimated in various transgenic lines and compared to that of WT plants, subjected to salt and drought stresses. Indeed, ROS-scavengering, stress-related and wax biosynthesis tobacco genes (*NtSOD*, *NtCAT1, NtGPX, NtSAM, NtERD10C, NtRD29, NtGol, TOBLTP, PR5* and *NtCER1*) were up-regulated by overexpression of either genomic or cDNA versions of *TdSHN1*. Interestingly, for most of the tested genes, this up-regulation was highest in PI transgenic lines compared to gD and the intron-less cD lines (Fig. 7). The up-regulated genes selected in the present study were shown to play a significant role in salt and drought tolerances in plants. This explains the best resilience showed by PI lines compared to gD, cD-transgenic lines and WT.

The choice of promoter to drive the expression of transgene, based on its qualitative and quantitative proprieties, is particularly critical in the case of transcription factors. The results obtained in the present work clearly show that use of a combination of stress-inducible promoter Pr*TdSHN1* and intron to drive expression of TdSHN1 transcription factor imparted the better salt and drought tolerances compared to a combination of the 35S promoter and the native intron. Many researchers found that the use of the 35S promoter to drive the expression of plant transcription factors in various plant species resulted in phenotypic variations compared to WT. The problem was solved when stress-inducible promoters such as *RD29* promoter were used to replace the constitutive 35S promoter. Besides these effects, constitutive expression may require high input of energy and nutrients. Furthermore, the 35S promoter affects not only its associated transgene but also other genes located thousands of base pairs away. Pr*TdSHN1* promoter used in the present work contained many cis-elements involved in abiotic stress responses (Djemal and Khoudi, 2015) and is stress-inducible. These characteristics clearly contributed to the better resilience of the PI transgenic lines to salt and drought stresses.

5. Conclusion

In conclusion, we clearly showed here that the use of *TdSHN1* intron significantly improved abiotic stress tolerance over the intron-less cDNA. This improvement is brought about by the IME effect of *TdSHN1* intron which led to higher induction of the expression of many stress-related genes as well as genes involved in SD formation. In addition, we showed that use of a combination of stress-inducible promoter Pr*TdSHN1* and intron to drive expression of TdSHN1 transcription factor imparted better salt and drought tolerances compared to a combination of the 35S promoter and the native intron. Therefore, endogenous Pr*TdSHN1* promoter and *TdSHN1* gene intron are promising tools for improving important traits in plants especially productivity and abiotic stress tolerance.

Author contributions

H.Khoudi conceived the project and designed the experiments. R. Djemal designed and performed the experiments. H. Khoudi and R. Djemal analysed the data. H. Khoudi and R. Djemal wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.009.

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