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ORIGINAL ARTICLE

A novel *Dreb2*-type gene from *Carica papaya* confers tolerance under abiotic stress

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Abstract The ethylene-responsive element-binding factors AP2/ERF compose one of the largest families of transcription factors in plants. *Dreb2*-type gene from *Carica papaya* L. cv. Maradol was found to be a member of the AP2/ERF family and contains a conserved APETALA 2 (AP2) domain located within the group IV of the AP2/ERF superfamily. *CpDreb2*-type gene is differentially expressed under stress by extreme temperatures. Moreover, genetic transformation of tobacco plants that overexpress the *CpDreb2*-type gene showed an increase amount of proline and a greater tolerance level to low and high temperature as well as drought experiments. CpDREB2-type protein::GFP is localized mainly in the nuclei of cells from specific organs such as roots and leaves in tobacco seedlings. Our results indicate that *CpDreb2*-type gene can be used to gain tolerance to extreme conditions of temperature and drought in other plants.

Keywords Abiotic stress tolerance · *Carica papaya* · *DREB2-type gene* · Transcription factor

Ana Arroyo-Herrera and Luis Figueroa-Yáñez authors contributed equally to this work.

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Introduction

Transcription factors (TFs) are proteins that modulate transcriptional activity of the RNA polymerases to change the level of transcripts being generated (Udvardi et al. 2007). Stress-related TFs up-regulate the expression of many downstream genes that provide abiotic stress tolerance, like extreme temperatures, drought and high salinity (Agarwal and Jha 2010; Lata and Prasad 2011; Tang et al. 2014). Some TFs involved in abiotic stress tolerance belong to the superfamily APETALA2 (AP2)/ethylene-responsive element-binding (ERF). The AP2/ERF TFs bind to cis-DRE/CTR (A/GCCGAC) sites located in specific promoter regions that regulate transcriptional expression of stress responsive genes (Bouaziz et al. 2015). The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of about 60–70 amino acids involved in DNA binding. This superfamily is composed of three families: The AP2 family proteins that contain two AP2/

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ERF domain, the ERF family proteins containing a single AP2/ERF domain, and the RAV family proteins with a B3 domain (DNA-binding domain conserved in other plant-specific transcription factors, including VP1/ABI3) in addition to a single AP2/ERF domain (Riechmann et al. 2000; Sakuma et al. 2002; Nakano et al. 2006).

The ERF family is sometimes further divided into two major subfamilies, the ERF subfamily and the CBF/DREB subfamily (Sakuma et al. 2002). Many DREB subfamily transcription factors have been isolated from various plants and their involvement in stress tolerance has been proposed (Mizoi et al. 2012). Overexpression of DREB homologous genes have been isolated from various plants such as Arabidopsis, rice (*Oryza sativa* L.), and maize (*Zea mays* L.), these genes could show an enhanced tolerance to abiotic stress in transgenic plants (Liu et al. 1998; Dubouzet et al. 2003; Qin et al. 2007). DREB2A is induced by drought or heat shock as well as a combination of drought and heat shock (Rizhsky et al. 2004), suggesting that DREB2 genes play a role in regulating gene expression under various combinations of drought and heat stresses. Over-expression of wheat TaDREB2 and maize ZmDREB2A in Arabidopsis and tobacco resulted in a particular phenotype unlike overexpression of rice OsDREB2A which yields no particular phenotypic response in Arabidopsis (Dubouzet et al. 2003; Kobayashi et al. 2007; Qin et al. 2007). Transgenic Arabidopsis plants over-expressing maize ZmDREB2A were dwarf, but exhibited improved drought and heat stress tolerance (Qin et al. 2007). Transgenic tobacco plants over-expressing PgDREB2A exhibited enhanced tolerance to both hyperionic and hyperosmotic stresses. The transgenic plants also showed higher expression of downstream genes NtERD10B, HSP70-3, Hsp18p, PLC3, AP2 domain TF, THT1, LTP1, NtHSF2, and pathogen-regulated (NtERF5) factors with different stress treatments (Agarwal et al. 2010). Sakuma et al. (2006a) found that removal of a negative regulatory domain (NRD) downstream of the DNA-binding domain converts this protein into a constitutively active form (DREB2A CA). The overexpression of DREB2A CA in transgenic Arabidopsis resulted in the induction of genes during dehydration or heat shock and resulted in an enhanced tolerance to both drought and heat shock (Sakuma et al. 2006a, b).

Papaya tree is a fruit crop known for its nutritional benefits and medical applications. However there are no reports on the AP2/ERF transcription factor gene in *Carica papaya*. Here we report a novel DREB transcription factor from *C. papaya* cv. Maradol named *CpDreb2*-type gene that is expressed under stress conditions in *C. papaya* and we tested the phenotype of tobacco plants that overexpress this gene under 3 different abiotic stress conditions.

Materials and methods

Retrieval of DREB family sequences

The complete proteomes of *Arabidopsis thaliana* (TAIR10), *C. papaya* (ASGPBv0.4), *Physcomitrella patens* (v3.0), and *O. sativa* var. japonica (RAP-DB), were used to retrieve the DREB's proteins by means of a Hidden Markov model (HMM, Hidden Markov Model). The HMM model was constructed by the HMMER software package v3.1 (Eddy 2011) from an alignment of 19 DREB sequences reported by Nakano et al. (2006) and several functional DREB2 genes reported by Lata and Prasad (2011). The alignment was performed by Clustal Omega (Sievers et al. 2011), and then manually corrected. The alignment was tested with the statistical package ProtTest 2.4 (Abascal et al. 2005) to find the best evolutionary model. The resulting profile of 81 amino acids (AP2/ERF domain and CMIV-1 motif) was calibrated default by HMMER v3.1, and was fixed with cut-off values. The annotations of the *A. thaliana* genome were downloaded from TAIR (The Arabidopsis Information Resource, http://www.arabidopsis.org/). The gene files of *C. papaya*, and *P. patens* were recovered from Phytozome V.9.0 (Goodstein et al. 2012). *Oryza sativa* var. japonica was recovered from EnsemblPlants (http://plants.ensembl.org/index.html, EMBL-EBI). The resulted HMM profile was used to detect DREB proteins into the selected plant species. A phylogenetic reconstruction was performed by RAxML version 7.0.4 (Stamatakis 2006). The tree was estimated by Maximum Likelihood (ML) by the substitution model JTT + I + Γ, with 1000 replicates re-sampling and 12,345 random seeds. The topology of the trees was visualized by Figtree v1.4 (http://www.molecularevolution.org). Sequence alignments were performed using ClustalW2 (http://www.ebi.ac.uk/) and similar amino acids were identified by BOXSHADE v3.31C (http://boxshade.sourceforge.net/). Statistically significant conserved motifs and the AP/ERF domain of *CpDreb2*-type gene were used to create sequence logos by WebLogo (http://weblogo.berkeley.edu/logo.cgi).

Plant growth conditions and stress treatment

Seeds from papaya (*C. papaya* cv. Maradol) were obtained from "Semillas del Caribe" (http://www.semillasdelcaribe.com.mx/) and were surface-sterilized using 1.05 % sodium hypochlorite and 200 µl of Tween-20 for 1 h at 27 °C. Subsequently, the papaya's seeds were shaken in 50 ml of 1.0-M KNO_3 for 24 h at 27 °C. Floating seeds were discarded, and submerged seeds were shaken in 100 ml of sterile water at 32 °C for 3 days or until the testae cracked.

Once the seeds germinated, seedlings were planted in a potting mixture of Horti Pearl, Sunshine, Vermiculite, and Peat moss (2:2:2:1, w/w/w/w). Plants were grown under a photoperiod of 16 h light/8 h dark at 25 °C for 6 weeks. For the temperature stress treatments, papaya's plants were incubated at 4 and 40 °C for different exposure times for each specific extreme temperature stress.

Wild type and transgenic tobacco plants (*Nicotiana tabacum* L. cv. SRI*)* showed an identical phenotype and flowered after 10 weeks of transfer in a potting mixture of Horti Pearl-Sunshine-Vermiculite-Peat moss (2:2:2:1, w/w/w/w). The flowers were covered with a mesh bag to prevent cross pollination, after 4 weeks the seed pods were collected dried at 37 °C and the seeds were removed for storage and further processing. The seeds were surface-sterilized using 1.05 % sodium hypochlorite and 200 µl of Tween-20 for 1 h at 27 °C. Remaining seeds were shaken in 50 ml of 1.0 M KNO_3 for 24 h at 27 °C (Nagao and Furutani 1987). Floating seeds were discarded, and submerged seeds were shaken in 100 mL of sterile water at 32 °C for 3 days or until the testae cracked (Fitch 1993). Once the seeds germinated, seedlings were planted in a potting mixture of Horti Pearl-Sunshine-Vermiculite-Peat moss (2:2:2:1, w/w/w/w). Plants were grown under photoperiod of 16 h light/8 h dark (25 °C) with a photon flux density 180 µmol m^{-2} s^{-1} for 8 weeks.

For high temperature stress treatment, plants of tobacco were transferred to 40 °C and maintained at this temperature according to the indicated times (Matsukura et al. 2010), low temperature stress treatment plants were transferred to 4 °C for 24 h and for drought treatment the plants were kept to 25 °C without irrigation for 24 h.

Nucleic acid extraction

Total RNA was isolated using TriZol Reagent (Invitrogen, Ca) according to the manufactureŕs protocol instructions. In the case of total DNA extraction, 5 g of leaf tissue were ground and mixed with 15 mL of extraction buffer [0.1 M Tris–HCl (pH 8.0); 1.0 M NaCl; 0.02 M EDTA (pH 8.0); 2 % (w/v) CTAB; 2 % (w/v) polyvinlypyrrolidone-40; 0.2 % (v/v) 2-mercaptoethanol], and incubated for 1 h at 70 °C. The solution was twice extracted with equal volumes of chloroform:isoamyl alcohol (CIA, 24:1, v/v) and centrifuged at 15,000×*g*, 4 °C for 10 min. The aqueous phase was transferred to a fresh tube and DNA was precipitated with an equal volume of isopropanol and kept at −80 °C for 1 h. DNA was then spooled out and washed with 80 % ethanol, 15 mM ammonium acetate, and 100 % ethanol sequentially, each for 20 min with gentle shaking. The DNA pellet was air dried and dissolved in 100 µl Tris–EDTA buffer (10 mM Tris (pH 8.0); 1 mM EDTA (pH 8.0); 1 M NaCl) incubating at 65 °C for 1 h.

cDNA synthesis and semiquantitative RT-PCR

For semi-quantitative RT-PCR, 1 µg of total RNA was used for cDNA synthesis. Reverse transcription was performed using SMARTer^{TM} PCR cDNA Synthesis Kit (Clontech), according to the manufacturer's instructions. Each cDNA was diluted 1/5 (v/v) and 2 µL of diluted sample was used in 35 cycle PCR amplification using specific CpDREB2 gene primers (forward: 5′-ATG GGT ACT TTT GAT CAA G-3′, reverse: 5′-TCA AAA GTC TAA ATC TTC CAA-3′). The housekeeping 18S gene was amplified from each sample to normalize the level of each test gene using 18S universal primers (forward: 5′-CGG CTA CCA CAT CCA AGG AA-3, reverse: 5′-GCT GGA ATT ACC GCG GCT-3′PCR products were run on a 2 % agarose gel.

Construction of binary vector for transformation

PCR product of *CpDreb2*-type gene was cloned into pGEM-T Easy vector (Promega). Subcloning of *CpDreb2* was performed using the following primers: attB-*CpDreb2* forward (5′-AAA AAG CAG GCT TCA CCA TGG GTA CT-3′) and attB-*CpDreb2* reverse (5′-AGA AAG CTG GGT GAA AGT CTA AAT CT-3′) were used to recombine the *CpDreb2* gen with the flanking *att*B sites into the *att*P pDONR^{TM}221 site (Invitrogen^{TM}) by the Gateway® BP Clonase® (Invitrogen^{TM}) for the generation of supported sites. Subsequently, the *CpDreb2* fragment in the entry clone was transferred to the binary vector pK7FWG2.0 (Karimi et al. 2002) as described in http://www.uoguelph.ca/~jcolasan/pdfs/gateway_protocols_and_plasmids.pdf.

Nicotiana tabacum transgenic plants overexpressing the *CpDreb2*-type gene and stress tolerance tests

The constructed binary vector 35S:*CpDreb2*::*GFP* was introduced into *Agrobacterium tumefaciens* EHA 105 cells. *N. tabacum* L. cv. SR1 plants were transformed according to the method described by Clemente (2006). Leaf disks of *N. tabacum* were prepared using a sterile cork border (8 mm) and placed onto the preculture medium (MS salts, 3 % sucrose, 10 ml l^{-1} Vitamins B_5, 1 mg l^{-1} Benzilaminopurine (BAP) and 3 g l^{-1} Gelzan. The explants were incubated at 25 °C under photoperiod of 16 h light/8 h dark. The explants were transformed by vacuum infiltration method and cocultived at 25 °C in darkness for 3d and then were transferred to a selection medium (MS salts, 3 % sucrose, 10 ml l^{-1} Vitamins B_5, 1 mg l^{-1} Benzilaminopurine (BAP), 0.1 mg l^{-1} NAA, 3 g l^{-1} Gelzan, 200 mg l^{-1} Timetin and 150 mg l^{-1} kanamycin). *N. tabacum* transgenic seeds were grown onto a selection medium (MS salts, 3 % sucrose, 10 ml l^{-1} Vitamins B5, 200 mg l^{-1} thimentin, 150 mg l^{-1} kanamycin and 3 g l^{-1} Gelzan) according to the

protocol described by Clemente (2006). Transgenic and non-transformed seedlings were transferred to a potting mixture of Peat moss, Sunshine, Vermiculite, and Soil (2:2:2:1, w/w/w/w), and then grown under photoperiod (16 h light/8 h dark) with a photon flux density 180 μmol m^{-2} s^{-1}at 25 °C for 6 weeks. For low temperature treatment, plants of 42 days old were transferred to the same potting mixture and grown for additional 35 days under temperature of 4 °C; plants were irrigated every 2 days. In the case of high temperature treatment, plants were subjected to 40 °C for 6 days, where plants were irrigated every 2 days. For drought treatment, control plants were maintained well-watered by daily irrigation, and the treatment group was subjected to drought stress by withholding irrigation for 8 days at room temperature (25 °C).

PCR and Southern blot analysis of putative transgenic plants

Approximately 200 ng of genomic DNA template was used for a 50 μl PCR reaction. Putatively transformed *N. tabacum* F1 plants were analyzed for *nptII* transgene presence using forward primer (5′-ATG ATT GAA CAA GAT GGA TTG C-3′) and the reverse primer (5′-TCA GAA GAA CTC GTC AAG AAG G-3). Specific *CpDreb2* gene primers (forward: 5′-ATG GGT ACT TTT GAT CAA G-3′reverse: 5′-TCA AAA GTC TAA ATC TTC CAA-3′were used for PCR amplification. For Southern blotting assays, 20 μg of plant genomic DNA was digested with restriction enzyme *HindIII*, and *CpDreb2* PCR product was used as a hybridization probe. Probe labeling and hybridization was carried out following the instructions of Amersham AlkPhos Direct Labelling and Detection System (GE Healthcare Life Sciences). The hybridized membranes were exposed to Kodak X-OMAT BT X-ray film for autoradiography.

Cellular localization of the green fluorescent signals in transgenic plants

GFP fluorescence (excitation filter 488 nm, emission filter band pass of 505-530 nm) was analyzed by a confocal laser-scanning microscope FV100 Olympus. DAPI staining of *N. tabacum* transgenic plants was made in order to determine the location of the nuclei in cells. Untransformed plants were used as control reference.

Photosynthesis measurements in transgenic plants

The photosynthetic net rate (*P*n) and stomatal conductance (*G*s) were measured using a portable infrared gas analyzer (Li-6400XT, Licor, Lincoln, NE, USA) at 0, 2, 4, 6, 15, 20, 25, and 30 days after exposure of cold treatments (4 °C). High temperature (40 °C) was measured at 0, 2, 4, and 6 days. The analyzer was equipped with a clamp-on leaf cuvette that exposed 6 cm^2 of leaf area. Light, temperature, and humidity were 180 μmol m^{-2} s^{-1} (Li-6400-02B LED), 25 ± 1 °C and 70 %, respectively and a CO_2 concentration of 400 μmol mol^{-1}. The readings were determined within 60 s of having locked up the leaf chamber. Measurements were made on four randomly selected plants of each treatment.

Proline quantification in plants

Proline quantification was performed as described by Schweet (1953). 0.25 g of *N. tabacum* leaves were macerated and added to 5 ml of 3 % (w/v) sulfosalicylic acid. The mash was filtered through Whatman paper. Minutes before testing a mixture of ninhydrin were made (20 ml of phosphoric acid 6 M, 30 ml of glacial acetic acid and 1.25 g ninhydrin). 1 ml of the filtrate was reacted with 1 ml of the ninhydrin mixture. Then 1 ml of glacial acetic acid was added, stirred vigorously and then covered with foil tubes. The mixture was incubated at 94 °C for 45 min and then cooled in cold water for 5 min. Then 2 ml of toluene was added with vigorous vortex stirring until they acquired a milky appearance. The upper (organic) phase was extracted with a Pasteur pipette and then absorbance at 520 nm was determined.

CpDREB2-type protein recombinant purification

Full length CpDreb2-type gene was cloned into the pDEST17 Gateway vector (Invitrogen) and expressed in *Escherichia coli* BL21 as described (Guo and Ecker 2003). His-tagged recombinant CpDREB2 protein was purified using nickel-nitrilotriacetic acid agarose columns (Qiagen) according to the manufacturer's instructions.

DNA probe and DNA binding Assays (DBA)

DBA was carried out as previously published with minor modifications (Castaño et al. 1997). We tested three DRE core motif as probes for DBA, containing different bases next to the core sequence CCGAC: (Forward DRE1 core primer: 5′-TAT TTA TTT A**A**C CGA C**TA** AAA AAA TA-3′ and Reverse DRE1 core primer: 5′-TAT TTT TTT AGT CGG TTA AAT AAA TA-3′; Forward DRE2 core primer 5′-CAA AAT AGT T**A**C CGA C**AT** AAT TTT AA-3′ and Reverse DRE2 core primer: 5′-TTA AAA TTA TGT CGG TAA CTA TTT TG-3′; Forward DRE3 core primer: 5′-TTT CTA CTC G**G**C CGA C**AT** GCG GAA TT-3′ and Reverse DRE3 core primer: 5′-AAT TCC GCA TGT CGG CCG AGT AGA AA-3′). We used NAC TF core motif as probe control for DBA (CACG) (Forward

NAC core primer: 5′-TAC AAA CGT GTT CAC GAA TCG TAA TA-3′ and Reverse NAC core primer: 5′-ATG TTT GCA CAA GTG CTT AGC ATT AT-3′). End-labeled DNA probe was incubated for 30 min with 10 ng of purified CpDREB2 protein and loaded into a 6 % native PAGE. Autoradiography was carried out after electrophoresis.

Statistical analyses

For each point, independent measurements of three samples from *CpDreb2*-type transgenic gene and wild type tobacco plants were taken and indicated by different letters depending on their differences. The data was analyzed by one-way analysis of variance (ANOVA) followed by the comparison of mean values using the Tukey's test at $P < 0.01$ in Statgraphics Centurion (StatPoint Technologies, Inc.,Warrenton, VA). Data were presented as mean ± SD error of three determinations per sample. Figures were created using Sigma Plot 10.0 (Systat Software, Inc. Germany).

Results

In silico characterization of Dreb2-type gene in papaya tree

DREB2 protein sequences from four plants were obtained by means of a HMM: ten sequences from *A. thaliana*, six sequences from *C. papaya*, five sequences from *P. patens*, and five sequences from *O. sativa*. All sequences were clustered by CD-HIT (Huang et al. 2010), with a cut-off value of 0.9 of identity, and only one sequence of *O. sativa* was identical (100 %); the final number of sequences in the study was reduced to four sequences for this species. Phylogenetic analysis was performed using DREB2 protein sequences from *O. sativa* (monocot), *A. thaliana* (dicot), *C. papaya* (dicot), and *P. patens* (Bryophyta) to define their evolutionary relatedness and divergence for this AP2/ERF subgroup.

Six Putative *DREB2* genes from *C. papaya* were identified into their protein annotation file (Phytozome database), and called as CpDREB_IV proteins, according to the classification of Nakano et al. (2006). CpDREB_IV1 protein from *C. papaya* was named as CpDREB2-type protein in this study (Fig. 1; S1 Table).

The *CpDreb2*-type gene encoded a complete protein containing the conserved AP2/ERF domain (S1 Fig. and S2 Fig.); the sequence was submitted to GenBank under the accession number KJ432868.

The 24 four proteins of the subgroup IV belong to the DREB2 subfamily, where TFs were divided into three clades by the phylogenetic tree (Fig. 1). This agrees with the previous assumption that DREB2 genes are ubiquitous in all flowering plants (Mizoi et al. 2012). We found a 57 % of identity between CpDREB_IV1 and OsDREB2A proteins, 61 % between CpDREB_IV1 and AtDREB2E, 62 % between CpDREB_IV1 and CpDREB_IV6, 40 % between CpDREB_IV1 and AtDREB2A, and 47 % between CpDREB_IV1 and AtDREB2B. The CpDREB_IV1 sequence is more related to the OsDREB2A sequence from *O. sativa* than PpDREB_IV4 from *P. patens* (84 % identity). All the sequences presented in this study show low query against CpDREB_IV1 protein, one possibility is that *CpDREB2*-type gene may have acquired unique characteristics (S2 Table). Furthermore, CpDREB2 protein structure contains an AP/ERF domain and therefore belongs to the A-2 group previously reported by Sakuma et al. (2002). It also has the motifs CMIV-1, CMIV-2, and CMIV-3 reported by Nakano et al. (2006; S1 Fig. and S2 Fig.). A multiple sequence alignment showed that CpDREB2 protein does not share a high level of identity in the region between amino acids 136–165 with any other DREB2-related protein from different plants (Sakuma et al. 2006a, b), besides that CMIV-1 motif, and AP2/ERF domain showed several conserved amino acids at a specific position. These results suggest a novel DREB2-type transcript factor from *C. papaya*.

Expression analysis of CpDreb2 gene under abiotic stress conditions

Expression profile of a gene under a particular condition provides evidence for its further functional characterization. Therefore, an expression profile was generated for the *CpDreb2*-type gene under three abiotic stress conditions (drought, low- and high-temperatures). Untreated papaya seedlings were used as control (Fig. 2). Total RNA was extracted from *C. papaya* leaves at different time intervals after drought, high- (40 °C), and low temperature (4 °C) treatment. Semi quantitative RT-PCR was performed to evaluate the expression of the *CpDreb2*-type gene. Results showed that *CpDreb2* expression was induced by high-, low temperature and drought treatment and its expression increased after 24 h exposure to these stresses (Fig. 2). These findings suggest a possible signaling role of *CpDreb2* triggered by abiotic stress conditions.

Generation of *Nicotiana tabacum* plants overexpressing CpDreb2 gene

The system overexpressing *CpDreb2* in *N. tabacum* was selected to show the functionality of this protein in plants growing under stress conditions. Transformation of *CpDreb2*-type gene::GFP was carried out using *A.*

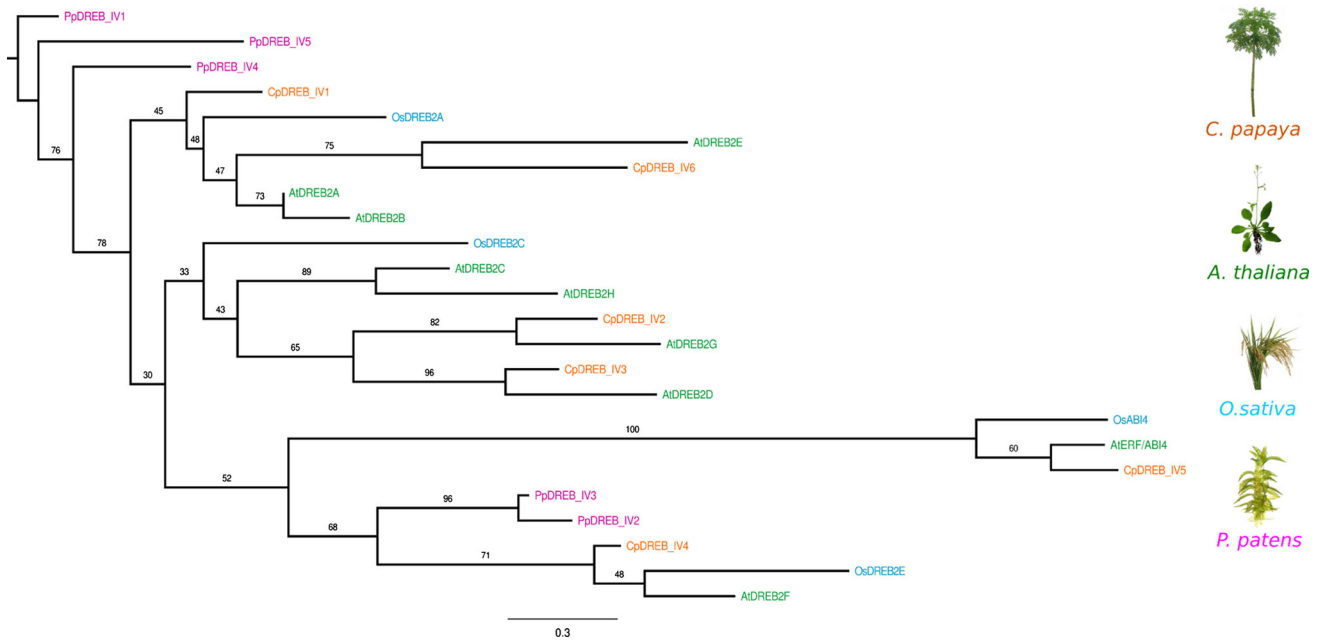

Fig. 1 Phylogenetic tree of DREB2-related proteins in four different plants. The tree was estimated by Maximum Likelihood (ML) analysis, using the JTT + I + Γ substitution model, with 1000 replicates re-sampling. The topology of the tree was visualized by Figtree v1.4 (http://www.molecularevolution.org)

tumefaciens strain EHA 105. The putative F1 transgenic lines were transferred into soil and their leaves were used for genotyping by PCR to check transformed tissue, using the forward and reverse primer of *CpDreb2* and also primers against the *nptII* gene (S3 Fig.). Southern blot analyses were performed to determine the presence and copy number of *CpDreb2*-type gene in F1 *N. tabacum* transgenic plants (Fig. 3a). The results of Southern blot analysis showed that the copy numbers of the transgenic plants varied from 1 to 4 (Fig. 3a; line 3, 4, and 5). One independent transgenic plant with single-copy insertion was selected (Fig. 3a; line 5). Tobacco non transgenic plants were used as control and signal was detected in the blot analysis (Fig. 3a; line 2). We conclude from this experiment that the probe was specific for the *CpDreb2* gene and did not cross-react with *Dreb2* type genes from *N. tabacum* (Fig. 3a, line 2). Southern blot from *C. papaya* showed several bands indicating that other members of the *Dreb2*-type genes from papaya subfamily are very similar in their nucleotide sequence (Fig. 3a, line 6). Transformed plants of *N. tabacum* of 7 weeks old were used to test the ability to acquire tolerance to abiotic stress including: low- temperature, high-temperature stress, and drought stress treatment. Overexpression of *CpDreb2*-type gene in *N. tabacum* showed a specific tolerance to low temperature stress (Fig. 3b). Wild type tobacco plants were also compared to overexpressing *CpDreb2* under high-temperature stress. Control plants showed a stress phenotype after 5 days of the treatment. This included developing a large number of yellowish-green leaves with reduced stems and finally abscessing from the plant. In comparison, transgenic plants survived the treatment and maintained a vigorous stem green color and their flaccid leaves readily recovered upon addition of water at room temperature, this recovery was not observed in wild type plants (Fig. 3b). Similar results were obtained when wild type plants were treated for drought stress, control plants died after the fifth day, while the transgenic plants containing *CpDreb2*-type gene survived the drought stress treatment until the eighth day. A tolerant phenotype was observed in tobacco transgenic plants with a significant reduction in stem size, internodes and peduncle (Fig. 3b). Moreover, the most dramatic difference was under cold temperature stress in which transgenic plants continue to show their normal appearance (Fig. 3b).

Cellular localization of green fluorescent signals in transgenic plants

CpDREB2-type protein::GFP was analyzed for its subcellular location. The GFP fluorescence was observed on a confocal laser microscope. DAPI staining was used to localize the nuclei in cells of monitored plants. All the CpDREB2-type protein::GFP have the same cellular co-localization pattern with DAPI staining in leave and roots of *N. tabacum* seedlings (Fig. 4a, b). The signal majority of CpDREB2-type protein::GFP in the nucleus corresponds with its role as a transcription factor.

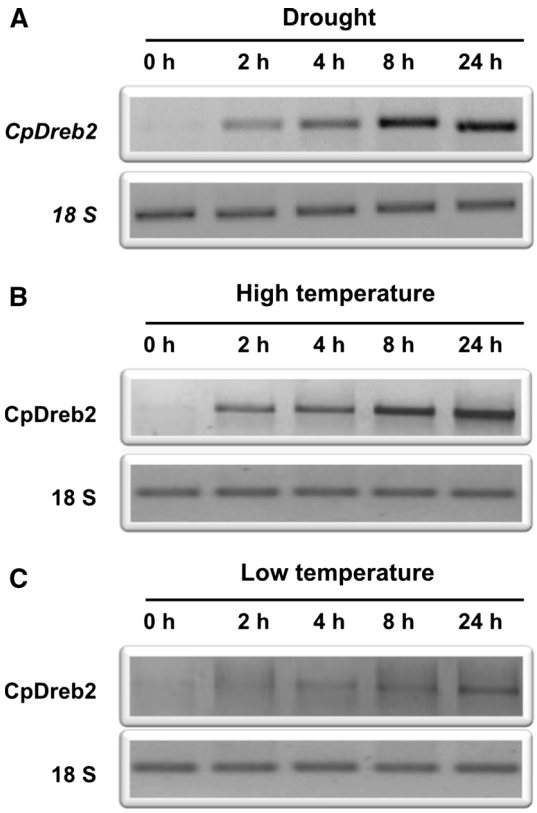

Fig. 2 Semi-quantitative reverse transcription PCR (RT-PCR) analysis of *CpDreb2*-type gene under different stress conditions from *C. papaya* seedling during 24 h. **a** RT-PCR analysis of *CpDreb2*-type gene under treatment of drought stress (air dried). **b** High temperature stress. **c** Low temperature stress. The quantification values of *CpDreb2*-type genes were normalized with 18S gene. All analyses were performed with three biological replicates

Photosynthesis measurements, stomatal conductance and proline content in plants

Compared with the wild type controls at 25 °C (Ct25WS) the net rate of CO_2 assimilation decreased gradually in transformed (Tg4) and non-transformed tobacco plants (Ct4) at 4 °C after 30 days exposure (Fig. 5a). Within 30 days of exposure the photosynthetic net (*P*n) of controls (Ct25WS) kept a linear condition, while *P*n was lowered in the control (Ct4) by 0.18 µmol CO_2 m^{-2} s^{-1} and 2.3 µmol CO_2 m^{-2} s^{-1} compared to transgenic plants (Tg4) that had been treated at 4 °C for 30 days (Fig. 5a). This indicates that photosynthetic capacity of transgenic plants is maintained during low temperature stress. High temperature treatment of 40 °C decreased as the maximum observed values of photosynthetic rate in transformed plants (Tg40) by 2 µmol CO_2 m^{-2} s^{-1} compared to control plants (Ct40) (Fig. 5c). To determine the effect of drought on photosynthetic activity the rates of photosynthesis were determined at 0, 3, 5, and 8 days in drought stressed plants. The photosynthetic efficiency was measured during water stress treatment both in control (Ct25D), and transgenic plants (Tg25D). Leaf photosynthetic traits did not change appreciably in well-watered control plants (Ct25WS) during the experimental period. Compared to non-stressed plants, the net rate of CO_2 assimilation decreased gradually in all plants after 8 days of exposure under water stress. After 5 days of drought exposure, control plants (Ct25D) *P*n was 0.1 µmol CO_2 m^{-2} s^{-1} lower and transgenic plants (Tg25D) showed 1.3 µmol CO_2 m^{-2} s^{-1} as compared with well-watered control plants (Ct25WS) (Fig. 5e).

Stomatal conductance (*G*s) was measured in leaves from transgenic plants at low- (Fig. 5b), high- temperature (Fig. 5d) or drought conditions (Fig. 5f). Measurements from transgenic tobacco plants remained below controls, even under CO_2 concentration of 400 µmol mol^{-1} inside a chamber. Stomatal conductance (gs) in leaves of transgenic tobacco plants treated at low temperature (Tg4 plants) (Fig. 5b) or high temperature (Tg40 plants) (Fig. 5d) or a water deficit (Tg25D plants) (Fig. 5f), show that stomatal conductance remained close in wild type plant (Ct25WS) (almost around 83.3 % for low-temperature, 81.6 % for high-temperature and 76.32 % for drought, respectively) of the numeric value of *G*s (400 µmol mol^{-1}).

The content of free proline as osmolyte was used as a physiological related parameter for this type of abiotic stresses. Proline from wild type and CpDREB2 overexpressing transgenic tobacco plants were measured following high-temperature (HT), Low-temperature (LT), and drought (D) treatment (Fig. 6). The proline content compared under high-temperature treatment (40 °C) showed that wild type tobacco plants had a 5.3-fold less amount of free proline as compared with transformed plants. Low-temperature stress treatment (4 °C) also showed a two fold increase in proline concentration from the transformed plants over wild type plants. More significantly was the difference by 4.1 fold increase in transgenic plants over wild type plants under drought stress.

CpDREB2-type protein specifically binds to core DRE motif sequence

DRE sequence binding ability was tested in vitro with purified recombinant CpDREB2-type protein and DNA fragments containing the ACCGACTA (DRE1), ACCGA CAT (DRE2) and GCCGACAT (DRE3) sequences. These DRE sequences were reported by Sakuma et al. (2006a). The sequence (A/G)CCGACNT was found in the promoter regions of 75 % of the DREB1A-upregulated genes. However, only 36 % of DRE sequences were in the promoter regions of the DREB2A-upregulated genes. Although the last T of the sequence (A/G)CCGACNT is critical for DREB1A binding, DREB2A does not require T

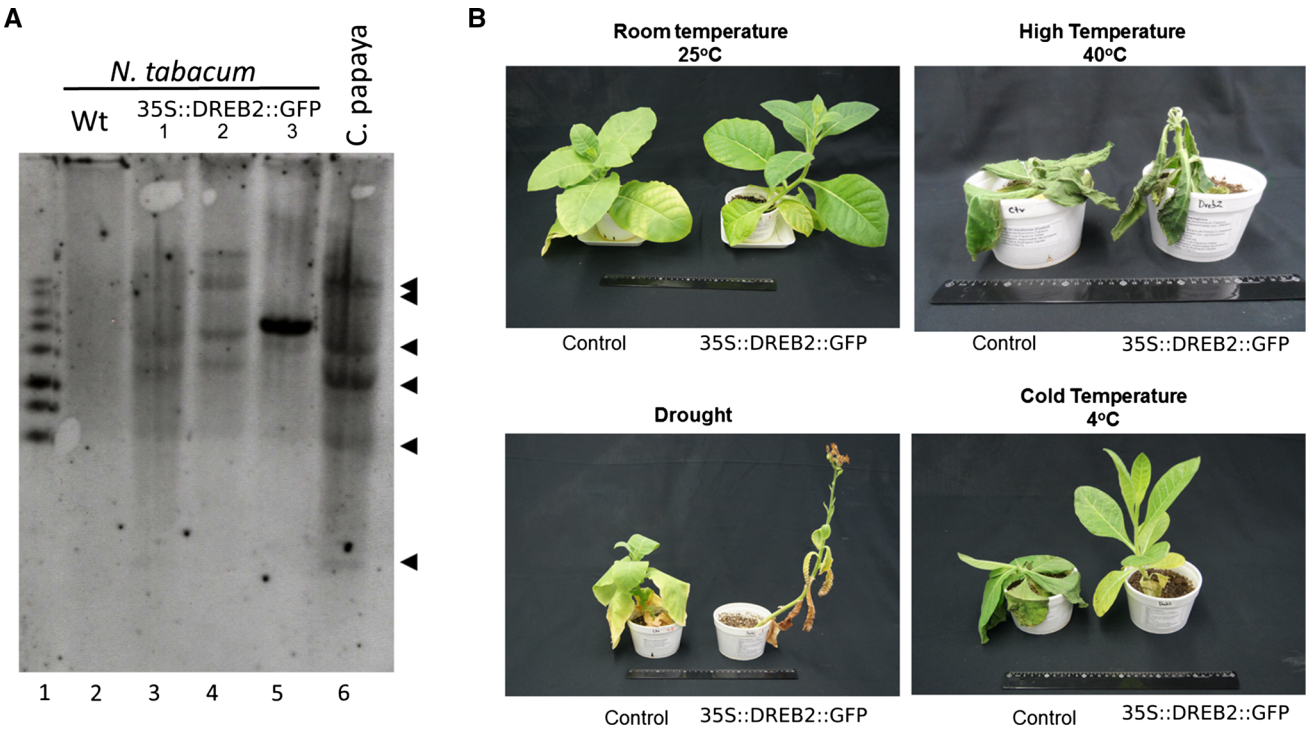

Fig. 3 Abiotic stress tolerance of 35S::CpDreb2-type gene transgenic tobacco plants. **a** Southern blot analysis of CpDreb2-type gene transgenic tobacco plants. *Lane 1* 1-Kb DNA Ladder (Promega), *2* non-transformed tobacco plant, *3–5* different transgenic tobacco plats of CpDreb2-type gene, *6* non-transformed *C. papaya* plant. The *arrows* show the specific binding of the DREB2 probe to *C. papaya*. Transgenic plant from *lane 5* was used for all further experiments. **b** Phenotypes of transgenic tobacco plants with *CpDreb2*-type gene under different temperature treatments and drought stress

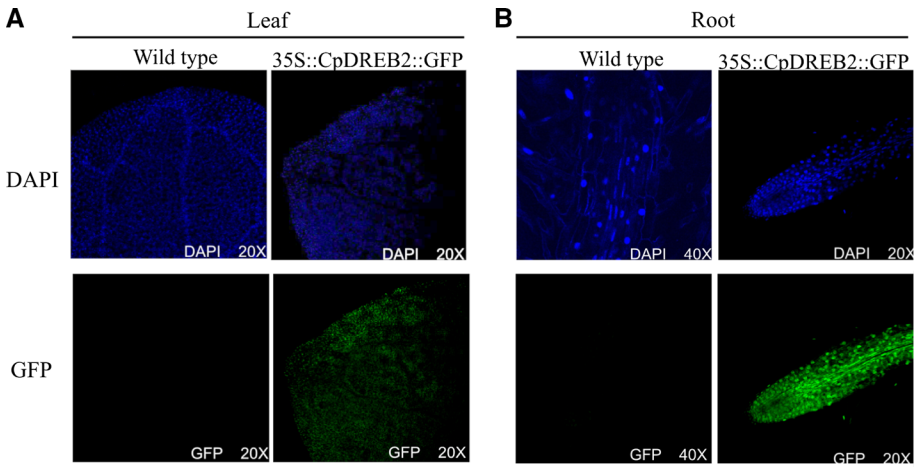

Fig. 4 Subcellular accumulation patterns of CpDREB2-type protein fused to GFP in *N. tabacum* transgenic plants. **a** DAPI staining from transformed (35S::*CpDreb2*::GFP) or not transformed leaf of tobacco plants. **b** DAPI staining from transformed (35S::*CpDreb2*::GFP) or not transformed root of tobacco plants

for binding (Maruyama et al. 2004). We found that CpDREB2-type protein binds primarily to DRE3 core sequence instead of DRE1 or DRE2 motifs (Fig. 7). The recombinant CpDREB2-type protein was used as control, incubated in presence of NAC Transcription Factor core motif as probe; we observed that the recombinant CpDREB2-type protein did not bind with the NAC Transcription Factor core motif.

Discussion

DREB2 subfamily is known to be induced by drought and high-salinity stress and as such it represents an important family of genes to study stress-responsive gene expression. DREB2A and DREB2B were first isolated as DRE/CRT-binding proteins in Arabidopsis (Liu et al. 1998). DREB2 homologous genes have been isolated from economically

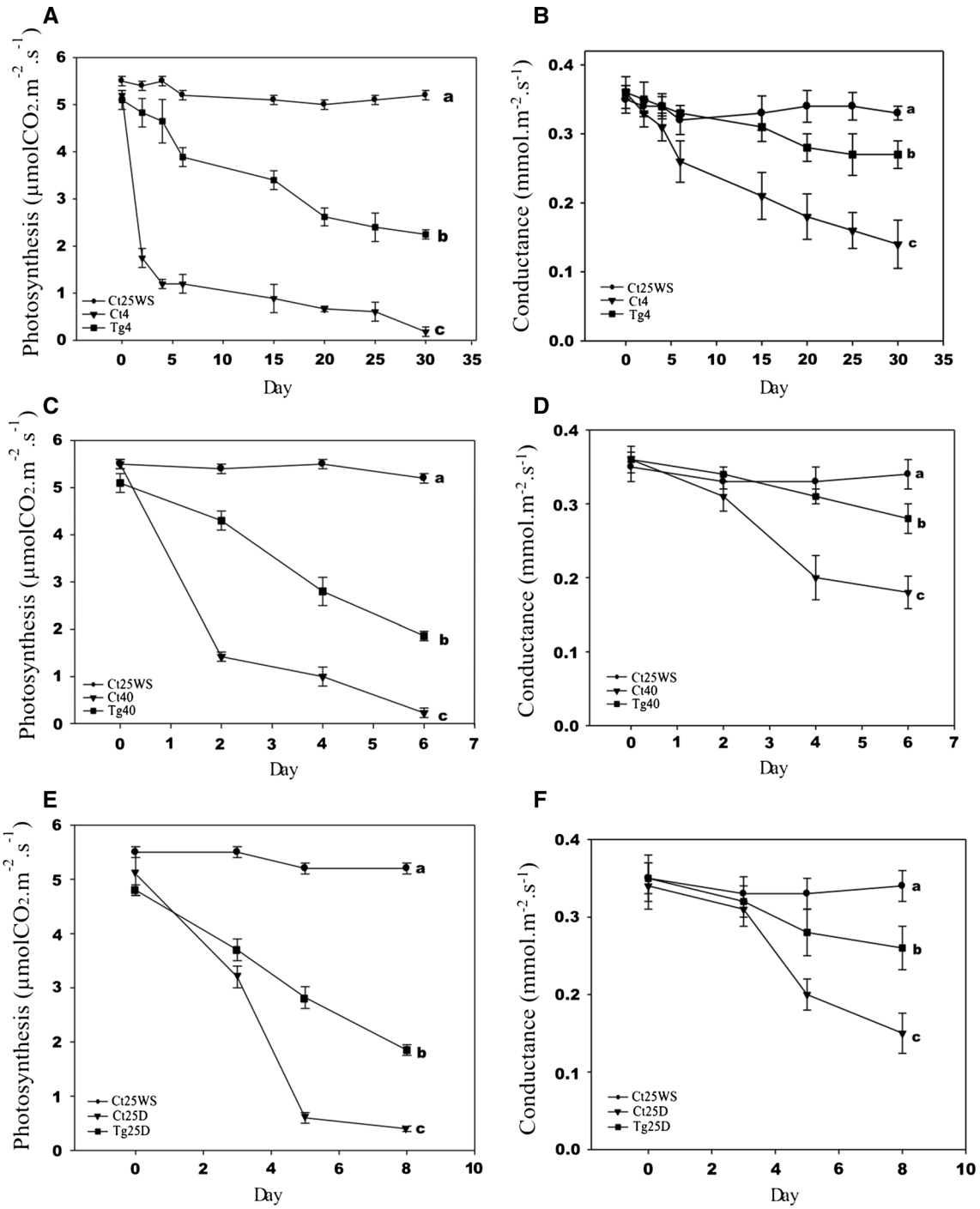

Fig. 5 Physiological effects of CpDreb2-type overexpressing tobacco plants under different abiotic stresses. Effects of low-, high temperature treatments, and drought stress in the photosynthetic activity (**a**, **c** and **e**, respectively), and stomatal conductance (**b**, **d** and **f**, respectively) from leaves of the wild type, and *CpDreb2*-type gene transgenic tobacco plants. *N. tabacum* (wild type) plants (Ct25WS) were grown continuously at 25 °C and used as control. *N. tabacum* transgenic (Tg4) and wild type (Ct4) plants were treated at 40 °C during different times. *N. tabacum* transgenic (Tg40) and wild type (Ct40) plants were treated at 4 °C during different times. *N. tabacum* transgenic (Tg25D) and wild type (Ct25D) plants were treated at drought stress during different times. Data was analyzed by one-way analysis of variance (ANOVA) followed by the comparison of mean values using the Tukey's test at $P < 0.01$. Data were presented as mean ± SD error of at least three determinations for sample. *Different letters* indicate significant differences. Units: [photosynthesis: μmol CO_2 m^{-2} s^{-1}]; [Stomatal conductance: mmol m^{-2} s^{-1}]

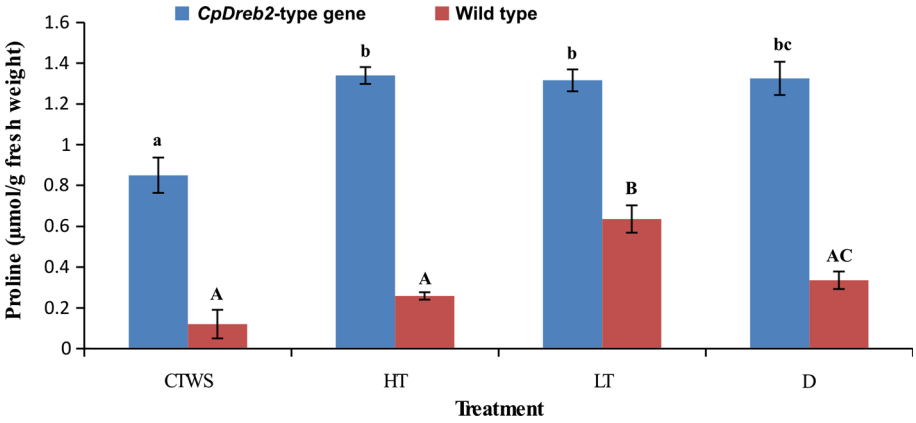

Fig. 6 Effects of low-, high-temperature and drought stress on free proline content from leaves of the wild type and CpDREB2 overexpressing tobacco plants. (CTWS) *N. tabacum* wild type and CpDreb2-type plants were grown continuously at 25 °C and they were irrigated every 2 days. These plants were used as control. (HT) Tobacco transgenic and wild type plants were treated at 40 °C and free proline contents were measured on the last day of treatment after 5 days of high-temperature stress. (LT) Tobacco transgenic and wild type plants were treated at 4 °C and free proline contents was measured on the last day of treatment after 35 days of low-temperature stress. (D) Tobacco transgenic and wild type plants were treated at drought stress and free proline contents was measured on the last day of treatment after 8 days of drought stress. Data was analyzed by one-way analysis of variance (ANOVA) followed by the comparison of mean values using the Tukey's test at $P < 0.01$. Data was presented as mean ± SD error of at least three determinations for sample. *Different letters* indicate significant differences. Units: [proline: µmol of proline per gram of fresh weight]

important cereal crops such as rice, wheat, barley, maize, pearl millet, and foxtail millet (Dubouzet et al. 2003; Xue and Loveridge, 2004; Egawa et al. 2006; Agarwal et al. 2007; Qin et al. 2007).

Multiple alignment analyses were performed to define the phylogenetic relationships between *CpDreb2*-type gene and Dreb2 genes found in different plants species. We found 24 genes from other plant species that where phylogenetically related proteins to *CpDreb2*-type gene. CpDREB2-type protein sequence (CpDREB_IV1) shows homology with AtDREB2A protein sequence (query cover of 99 % with an identity of 40 %). AtDREB2A and AtDREB2B have been identified as transcription factors involved in DRE-mediated transcription (Liu et al. 1998). DREB2-related proteins and ABI4 belong to the Group IV which is divided into two subgroups (IVa and IVb) according to Nakano et al. (2006). The N-terminal region outside the AP2/ERF domain is divided into two blocks, referred to as motifs CMIV-1 and CMIV-2. The CMIV-2 motif contains a putative nuclear localization signal (Liu et al. 1998). ORCA1 (Menke et al. 1999) and OsDREB2A (Dubouzet et al. 2003) belong to subgroup IVa in *Catharanthus roseus* and rice, respectively. The CMIV-1 motif is completely conserved in the proteins of group IV. ABI4 has shown to be involved in germination-related ABA signaling (Finkelstein et al. 1998) and sugar response (Arenas-Huertero et al. 2000; Huijser et al. 2000). The phylogenic tree shows that the CpDREB2-type protein is found in the same clade as DREB2A protein from Arabidopsis and rice.

CpDreb2-type gene respective transcript amount changed significantly over a temporal course in response to stress caused by drought or high-temperature, and slightly by low-temperature stress. These results are similar to *DREB2A* from *A. thaliana* where they showed a similar pattern of accumulation following dehydration, or exposure to high-temperature. Sakuma et al. (2006b) performed quantitative RT-PCR to confirm the expression of *DREB2A* from *A. thaliana* during high temperature. They found the expression of *DREB2A* was rapidly induced by high temperature treatment followed by a rapid decreased to basal level that was maintained 10 h after treatment. In contrast, *DREB2A* was gradually induced by drought and salt stress during 10 h. Our transgenic tobacco plants overexpressing *CpDreb2* gene exhibited enhanced tolerance when the plants where treated with low temperatures and drought stress. Different results were obtained when DREB2A related proteins were overexpressed in Arabidopsis plants. In particular *OsDreb2A* gene from rice, *TaDreb2* gene from wheat and *ZmDreb2A* gene from *Z. mays* exhibit enhanced tolerance to drought, heat stress, and cold stress (Dubouzet et al. 2003; Kobayashi et al. 2007; Qin et al. 2007).

The localization of CpDREB2 in transgenic tobacco plants to ensure its normal localization as transcription factors which are typically localized in cell nucleus. Previous studies have shown that overexpressed transcription factors have a similar localization as the native protein (Joshi et al. 2014; Shi et al. 2015). We observed a co-localization of the DAPI stain signal with the well-

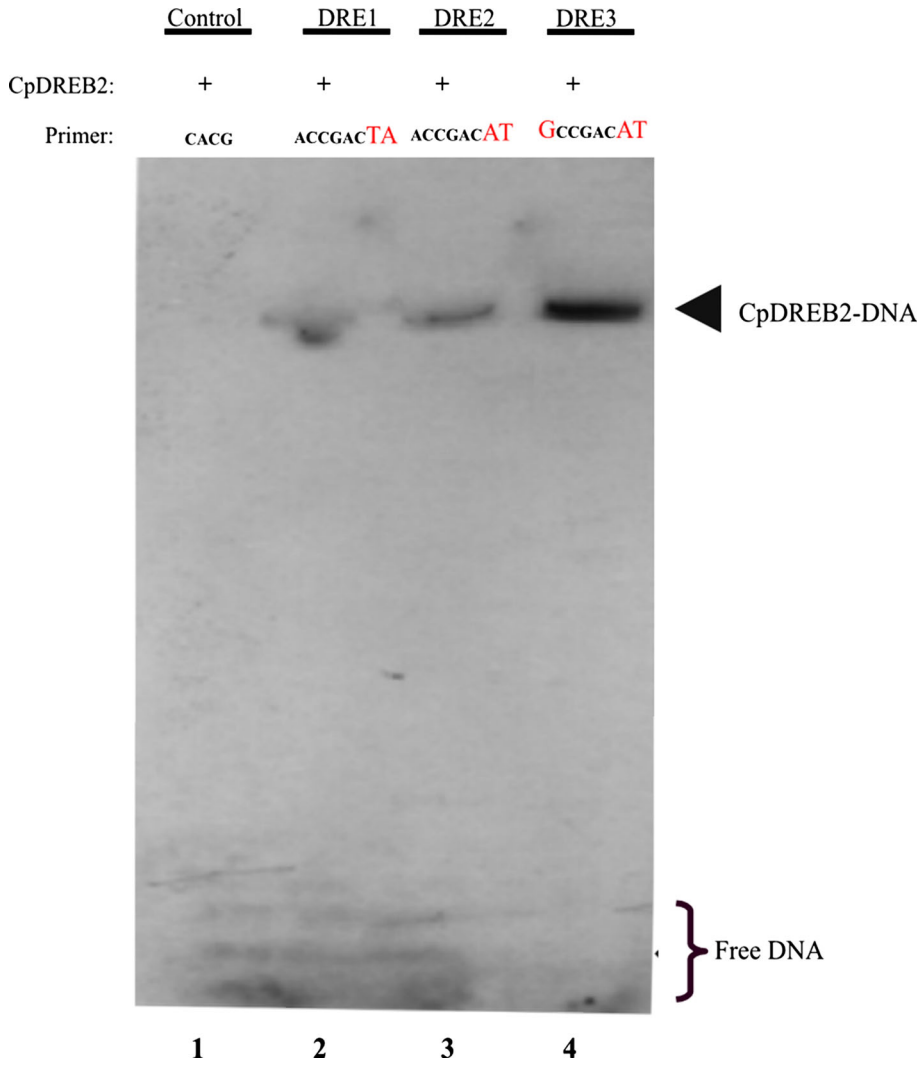

Fig. 7 DNA binding assay of the CpDREB2-type protein using three different DRE motifs. Synthetic sequence of DRE1, DRE2 and DRE3 was Radiolabeled with 33P and used as probe. Core sequence of DRE motifs are shown under the identifier DRE names. The recombinant CpDREB2-type protein was used as control, incubated in presence of NAC core motif as probe. The *retarded bands* are indicated by *arrowheads*

organized distribution pattern of the green fluorescence of the CpDREB2::GFP protein in the nucleus. The signal of CpDREB2::GFP protein was higher in leaf and root of transgenic tobacco plants, especially in root tips and cotyledons. Similar results were obtained by Sakuma et al. (2006a, b) where they found that DREB2A proteins are accumulated in the nucleus. The protein accumulation and localization patterns that were identified in leaves were not observed in guard cells.

Here we characterized the response of transgenic tobacco plants to different specific abiotic stresses since drought stress and heat shock may affect plant metabolism in different manner. The very high photosynthetic rates exhibited by the well-watered plants can be explained by their high based-area concentration of soluble proteins, chlorophylls and Rubisco. However, under water stress conditions, tobacco plants showed a strong stomatal regulation of gas exchange, associated with reductions in CO_2 assimilation. Decreased photosynthetic rate is the result of stomatal and non-stomatal (biochemical) limitations (Wise et al. 1992; Yordanov et al. 2003). The plant reacts to water deficit with a rapid closure of stomata to avoid further loss of water through transpiration (Flexas et al. 2006). As a consequence of this closure, the diffusion of CO_2 into the leaf is restricted (Chaves 1991; Flexas et al. 2006). Stomatal closure is frequently pointed out as one of the first limitations for photosynthesis under drought stress, as well as for low or high temperatures. Thus, low *P*n values during exposure to low temperatures were not caused by CO_2 restriction at the carboxylation sites due to stomatal closure. Instead, they would be related to metabolic limitations caused by low temperature or heat shock. The alterations on mesophyll capacity depend on the activity of Rubisco and capacity of photosynthetic electron transport to regenerate Rubisco (Crafts-Brandner et al. 1997; Feller et al. 1998; Daymi et al. 2005). Heterologous expression of SlERF36 under the CaMV35S promoter in tobacco leads to a 25–35 % reduction in stomatal density but without any

effect on stomatal size or sensitivity. Reduction in stomatal density leads to a marked reduction in stomatal conductance as well as transpiration and is associated with reduced CO_2 assimilation rates, reduction in growth, early flowering, and senescence (Upadhyay et al. 2014).

Many other studies have shown that decreased photosynthesis under drought stress can be attributed to perturbations of biochemical processes (Lauer and Boyer 1992). There are several reports that underline the stomatal limitation of photosynthesis as a primary event, which is then followed by the adequate changes of photosynthetic reactions (Chaves 1991; Zlatev and Yordanov 2004). The reduction in *P*n due to high temperature could be attributed to both stomatal and non-stomatal limitations. Stomatal closure usually occurs before inhibition of photosynthesis and restricts CO_2 availability at the assimilation sites in chloroplast. The decrease in photosynthetic rate under stressful conditions (drought, low- and high-temperature) is normally attributed to suppression in the mesophyll conductance and the stomata closure at severe stress (Chaves et al. 2009; Flexas et al. 2004). The effects of high temperature and drought on photosynthesis are attributed directly to the stomatal limitations for gases diffusion. These stresses apparently reduce photosynthesis by a similar mechanism. In fact, it is known that photosynthesis is often limited by low CO_2 diffusion conductance under drought stress conditions (Cornic et al. 1992; Kaiser 1987) Low CO_2 diffusion under high temperature or drought conditions is generally attributed to reduced stomatal conductance (Cornic and Fresneau 2002; Lauteri et al. 1997). The adverse effects of high temperature with severe drought are mainly attributed to non-stomatal limitation such as weakened PSII function and low Rubisco activity. Thus, the decrease in photosynthesis net at high temperatures is partially caused by a faster increase of respiration in relation to photosynthesis. In addition, Rubisco affinity for O_2 increases at high temperature when compared to the affinity for CO_2, thereby causing a reduction in photosynthesis through higher rates of RuBP oxygenation (Bernacchi et al. 2001).

The observed decreases of the *P*n under high temperature stress, indicated a reduction in CO_2 assimilation, can be partly attributed to stomatal conductance in tobacco plants. It is known that the limitation to CO_2 assimilation imposed by stomatal closure promotes an imbalance between photochemical activities at photosystem II (PSII). It is well documented that upon abiotic stress, most plants respond rapidly by stomatal closure to avoid excessive water loss by establishing physiological and molecular responses to prevent irreversible damage to the photosynthetic machinery (Chaves et al. 2003). These two processes are closely linked since stomatal closure results in a decline in the rate of photosynthesis (Pelleschi et al. 1997).

The electron requirement for photosynthesis leads to an over-excitation and subsequent photo-inhibitory damage of the PSII reaction centers (Souza et al. 2004). Here the plants that were under stress showed a decrease in photosynthetic capacity under abiotic stress. These decreases may be due to an increase in Rubisco hydrolysis and/or a decline in its catalytic ability.

Furthermore, this photosynthesis limitation also results in a reduced carboxylation efficiency (Wise et al. 1992), reduced ribulose-1,5-bisphosphate (RuBP) regeneration (Tezara and Lawlor 1995), or to inhibition in chloroplast activity (Shangguan et al. 1999). When Yordanov et al. (1997) exposed maize plants to temperatures above 40 °C there was less damage in water-stressed plants, indicating that drought stress counteracts the negative effects of high temperature.

The well-known transcription factors involved in stress response like DREB1A, DREB2A, and CBF1 proteins specifically bind to the DRE/C repeat sequence containing the core sequence (A/G)CCGACNT, however it has been found that some DREB2A downstream target genes were different from those of DREB1A (Liu et al. 1998). The reason for this can be due to DNA-binding specificity of DREB2A differing from DREB1A. Specifically, DREB1A has a high affinity to (A/G)CCGACNT sequences where the last T is crucial for the binding of DREB1A protein to DRE while the binding of DREB2A protein is not affected by a base substitution at the T position of DRE (Maruyama et al. 2004). DREB2A protein could recognize not only (A/G)CCGACNT but also (A/G)CCGACN(A/G/C), and prefers ACCGAC motifs (Sakuma et al. 2006a). On the other hand, Sakuma et al. (2006a) performed a gel mobility shift assay using 75-bp DNA fragments containing (A/C/G/T)CCGAC. The addition of unlabeled ACCGACAT or GCCGACAT competitors inhibited the binding of the DREB1A protein equivalently. By contrast, the addition of an unlabeled ACCGACAT fragment was more effective at binding the DREB2A protein than the addition of an unlabeled DNA fragment containing (C/G/T)CCGACAT. We found that CpDREB2-type protein from *C. papaya* bind to DRE3 core sequence (GCCGACAT) with greater efficiency than DRE1 and DRE2 motifs. This result suggests the CpDREB2-type protein is a transcription factor capable to bind the promoters containing the GCCGACAT core sequence (S5 Fig). These different binding abilities between CpDREB2-type protein from *C. papaya* and DREB2A from *A. thaliana* may explain why these proteins control different downstream genes.

In general, these observations coincide with previous reports that DREB proteins are important TFs in regulating abiotic stress-related genes and play a crucial role in imparting stress tolerance to plants. Our results clearly show that CpDREB2 type protein can be used to improve the tolerance to stress in other plants, which may be useful

to various kinds of agriculturally important crop plants that are affected by drought, high-temperatures, and freezing stresses.

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Author contributions Conceived and designed the experiments: AA, LF, EC, APS, JEA, FE, LDA, RL, LR. Performed the experiments: AA, LF, EC, APS, JEA, FE. Analyzed the data: AA, LF, EC, APS, JE, FE, RL, LR. Contributed reagents/materials/analysis tools: AA, LF, EC, JS, FE, LDA, FS, LS, LR. Wrote the paper: AA, LF, EC, APS, JEA, LR.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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