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RESEARCH PAPER

Tomato *SlDREB* gene restricts leaf expansion and internode elongation by downregulating key genes for gibberellin biosynthesis

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Abstract

Plants have evolved and adapted to different environments. Dwarfism is an adaptive trait of plants that helps them avoid high-energy costs under unfavourable conditions. The role of gibberellin (GA) in plant development has been well established. Several plant dehydration-responsive element-binding proteins (DREBs) have been identified and reported to be induced under abiotic and biotic stress conditions. A tomato DREB gene named *SlDREB*, which is a transcription factor and was cloned from cultivated tomato M82, was found to play a negative role in tomato plant architecture and enhances drought tolerance. Tissue expression profiles indicated that *SlDREB* was expressed mainly in the stem and leaf and could be induced by abscisic acid (ABA) but suppressed by GA and ethylene. *SlDREB* altered plant morphology by restricting leaf expansion and internode elongation when overexpressed, and the resulting dwarfism of tomato plants could be recovered by application of exogenous gibberellic acid (GA_3). Transcriptional analysis of transgenic plants revealed that overexpression of *SlDREB* caused the dwarf phenotype by downregulating key genes involved in GA biosynthesis such as *ent*-copalyl diphosphate synthase (*SlCPS*) and GA 20-oxidases (*SlGA20ox1*, *-2*, and *-4*), thereby decreasing endogenous GA levels in transgenic plants. A yeast activity assay demonstrated that SlDREB specifically bound to dehydration-responsive element/C-repeat (DRE/CRT) elements of the *SlCPS* promoter region. Taken together, these data demonstrated that *SlDREB* can downregulate the expression of key genes required for GA biosynthesis and that it acts as a positive regulator in drought stress responses by restricting leaf expansion and internode elongation.

Key words: DREB, dwarfism, *ent*-copalyl diphosphate synthase, gibberellin, gibberellin 20-oxidase, tomato.

Introduction

Drought, high salinity, and low temperature are adverse environmental conditions that affect plant distribution (Yang *et al.*, 2011*c*). When plants encounter adverse ambiance, they exhibit a variety of responses that result in specific changes suited to the particular stress condition encountered. For example, the dwarf phenotype benefits the plant through avoidance of the high-energy cost of producing stress-tolerance proteins (Knight and Knight, 2001).

The endogenous phytohormone gibberellin (GA) is involved in plant growth and development including internode elongation, and is one of the compounds associated with the dwarf phenotype (Hedden and Kamiya, 1997). GA biosynthesis and catabolism

genes in higher plants have been well characterized. Geranyl diphosphate synthase (GPS) has been reported to control the level of bioactive GA by modulating GA precursor biosynthesis in tomato (van Schie *et al.*, 2007). Geranylgeranyl diphosphate (GGDP) is the common precursor for diterpenoids including GAs, and is converted to *ent*-kaurene through successive two cyclization reaction catalyzed by *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS). The *ent*-kaurene is then converted to GA_{12} by the two distinct P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic oxidase (KAO). GA_{12} can be further converted to GA_{53} by 13-hydroxylation. GA_{12} and GA_{53} are converted mainly to various GA intermediates (e.g. GA_9 and GA_{20}) and successively to bioactive GAs (e.g. GA_1 and GA_4) by GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox), respectively. GA3ox catalyses the final step in the synthesis of bioactive GAs. GA 2-oxidase (GA2ox) is the major enzyme that converts active GAs (GA_1 and GA_4) and their precursors (GA_9 and GA_{20}) to inactive forms (Yamaguchi, 2008; Sun, 2011).

Over the past decade, a family of transcription factors known as APETALA2 (AP2)/ethylene-responsive element binding factor (ERF) has been identified in plants. These proteins are involved in a variety of regulatory mechanisms such as fruit ripening (Chung *et al.*, 2010), growth and development (Wilson *et al.*, 1996), and biotic (Thara *et al.*, 1999; Brown *et al.*, 2003) and abiotic stresses (Liu *et al.*, 1998; Sakuma *et al.*, 2002; Fukao and Bailey-Serres, 2008; Li *et al.*, 2011; Wan *et al.*, 2011; Zhang *et al.*, 2011; Shi *et al.*, 2012). The AP2/ERF transcription factors are found only in the plant kingdom and are characterized by the presence of a highly conserved DNA-binding domain (Riechmann and Meyerowitz, 1998; Tournier *et al.*, 2003). ERFs are part of the AP2/ERF superfamily, which also contains AP2 and RAV family genes and is characterized by the presence of the AP2/ERF DNA-binding domain (Sakuma *et al.*, 2002). Based on their ERF domain, the tomato ERFs can be further divided into two subfamilies, ERF and DREB (dehydration-responsive element-binding proteins), and their ERF domain diversity is manifested at amino acid positions 14 and 19 (Sharma *et al.*, 2010). DREBs contain a conserved AP2/ERF domain that binds specifically to the dehydration-responsive element/C-repeat (DRE/CRT) *cis*-acting element (core motif: G/ACCGAC) of the genes that they regulate (Busk *et al.*, 1997; Qin *et al.*, 2004).

DREB-binding factors play crucial roles in the regulation of abiotic and biotic responses in plants. Heterogeneous expression of a DREB gene from *Leymus chinensis* improved drought and salt tolerance of *Arabidopsis thaliana* (Xianjun *et al.*, 2011). Similarly, expression of a DREB gene from *Limonium bicolor* enhanced copper tolerance in transgenic tobacco plants (Ban *et al.*, 2011). A rice DREB gene, *ARAG1*, which is involved in the abscisic acid (ABA) signalling pathway, plays a role in seed germination and drought tolerance (Zhao *et al.*, 2010). Arabidopsis DREBs play a role in the regulation of water homeostasis by regulating multiple aquaporin genes (Rae *et al.*, 2011). The *Arabidopsis* DREB protein DEAR1 has an upstream regulatory role in mediating cross-talk between signalling pathways for biotic and abiotic stress responses (Tsutsui *et al.*, 2009). Moreover, two chrysanthemum DREB genes have been reported to play a regulatory role in abiotic stress responses (Yang *et al.*, 2009), and overexpression of OsDREB1G and OsDREB2B genes in rice significantly improved their tolerance to water deficit stress (Chen *et al.*, 2008).

Here, we demonstrate that *SlDREB* encodes a transcription factor whose expression is regulated by drought, salt, and cold, and by ABA, gibberellic acid (GA_3), and ethylene treatments. Our experimental results revealed that overexpression of *SlDREB* in tomato led to lower endogenous GA levels and consequently a dwarf phenotype consisting of restricted leaf expansion and internode elongation via downregulation of key genes involved in GA biosynthesis. Notably, SlDREB could bind specifically to the DRE/CRT elements of the *SlCPS* promoter region, indicating that *SlDREB* may directly regulate the expression of *SlCPS* in tomato.

Materials and methods

Plant material and plant growth regulator treatment

Tomato (*Solanum lycopersicum*) variety M82 and wild species *Solanum pennellii* (LA0716) were used to compare *SlDREB* nucleotide sequences between wild and cultivated species. *S. lycopersicum* cv. M82 was used to analyse the expression of *SlDREB* in different organs and the response to different plant growth regulators. Tissues from the roots, stems, leaves, flowers, and fruits of M82 plants were collected and immediately frozen in liquid nitrogen and stored at –80 °C until use. For plant growth regulator treatment, four-leaf-stage plants grown in compost plastic trays in a 16 h light/8 h dark regime at 25 °C in a greenhouse were sprayed with 100 μM ABA, 100 μM GA_3, 100 μM ethephon (Eth; an ethylene releaser), or distilled water (control). Three leaves, one from each plant, were collected at the designated times from the different treated and untreated plants and stored as indicated above. Plant morphology was assessed by measuring the internode lengths and number of leaves, and leaf samples were taken 35 d post-inoculation (p.i.). Dwarf phenotypes were rescued by spraying with a 100 μM GA_3 solution containing 0.02% Tween 20 at an interval of 3 d until run-off starting from 30 d p.i.

Isolation of SlDREB*, construction of overexpression vectors, and plant transformation*

In our previous studies on drought stress in tomato introgression lines (ILs), a differential expression profile of the *SlDREB* gene was observed between the drought-tolerant ILs and M82 (Gong *et al.*, 2010). The tomato *SlDREB* gene was PCR amplified from the cDNA of M82 (forward primer: 5'-CCAATTTCCTCTCTCCCAAA-3'; reverse primer: 5'-TGTTCATGAAAATCCAAATGTCT-3') based on the UniGene sequence (SGN-U585938). For transformation, we used the binary plasmid vector pMV2 (Yang *et al.*, 2011*a*), which carries the spectinomycin resistance gene for bacterial selection and the neomycin phosphotransferase II gene for selection of transformed plants. The binary plasmid was constructed by inserting the *SlDREB* cDNA between the *Kpn*I and *Xba*I sites in the sense orientation driven by the cauliflower mosaic virus 35S promoter. The plasmid mediated by *Agrobacterium tumefaciens* strain C58 was transformed in tomato cultivar M82. After screening for regenerated shoots on selection medium containing kanamycin, the transgenic plants were further verified by PCR using genomic DNA as template and 35S forward and gene-specific reverse primers.

RNA isolation and real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, USA). DNase I (Fermentas, USA)-treated RNA was reverse transcribed using a Moloney murine leukaemia virus cDNA reverse transcription enzyme (Invitrogen) and cDNA was used for real-time RT-PCR. Real-time

RT-PCR was performed with a SYBR Premix Ex Taq kit (Takara, Japan) using primers (see Supplementary Table S1 at *JXB* online) specific for *SlDREB*, *SlGPS* (DQ286930), *SlCPS* (AB015675), *SlKS* (AEP82778), *SlKO* (SGN-U583328), *SlKAO* (SGN-U575348), *SlGA20ox1* (AF049898), *SlGA20ox2* (AF049899), *SlGA20ox3* (AF049900), *SlGA20ox4* (EU652334), *SlGA3ox1* (AB010991), *SlGA3ox2* (AB010992), *SlGA2ox1* (EF441351), *SlGA2ox2* (EF441352), *SlGA2ox3* (EF441353), *SlGA2ox4* (EF441354), *SlGA2ox5* (EF441355), *SlGAST1* (X63093), *SlDELLA* (AY269087), and β-actin (SGN-U580609) transcripts as internal controls. The PCR amplification step consisted of an initial incubation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 20 s. Data were collected during the extension step, and melting-curve acquisitions and analyses were also performed on the cycler. PCR products were monitored using a LightCycler 480 (Roche, Switzerland) PCR system.

Subcellular localization assay of SlDREB

The full-length open reading frame (ORF) without the stop codon of *SlDREB* was PCR amplified using primers containing *Kpn*I and *Bam*HI restriction sites (underlined) (forward: 5'-*GGTACC*ATGTCAAAGCGAATAAGAGAGAGTG-3'; reverse: 5'-*GGATCC*TTTCATCATTTCAAAGTTGCTAAG-3'). The PCR product was purified from agarose gel, cloned into a pMD18-T vector (TaKaRa), and sequenced. The plasmid containing the correct sequence of *SlDREB* was digested with *Kpn*I and *Bam*HI and subcloned into a pCAMBIA1391 vector digested using the same restriction enzymes to create a fusion construct (pCAMBIA1391-*SlDREB*-GFP). Both the fusion construct (pCAMBIA1391-*SlDREB*-GFP) and the control vector (pCAMBIA1391-GFP) were bombarded into onion epidermal cells using a Biolistic PDS-1000 (Bio-Rad) system. The onion cells were cultured on MS medium for 24 h in the dark and observed with a Leica TCSST2 confocal laser microscope.

Trans*-activation activity and DRE/CRT binding assay for SlDREB in yeast*

The coding region of *SlDREB* was PCR amplified using specific primers (forward: 5'- *CCCGGG*AATGTCAAAGCGAATAAGAGAGAG-3'; reverse: 5'-*GTCGAC*TTATTTCATCATTTCAAAGTTGC-3') containing *Sma*I/*Sal*I restriction sites (underlined), corresponding to those present in the yeast expression vector pGBKT7 (Clontech, USA), to produce pBD-*SlDREB*. According to the manufacturer's instructions, pBD-*SlDREB*, pGBKT7 (negative control), and pGBKT7-53+pGADT7-RecT (positive control) were transformed separately into the yeast strain AH109. Selection of transformants was done on SD/–Trp or SD/–Ade/–His/–Trp medium, and the *trans*-activation activity of each protein was evaluated according to their status of growth and the activity of X-α-Gal (5-bromo-4-chloro-3-indoxyl-α-D-galactopyranoside).

A yeast activity assay kit (Clontech) was used to investigate whether SlDREB was bound to DRE/CRT of the *SlCPS* promoter region. We isolated the –2500 putative promoter regions of *SlCPS* from the SGN database (http://solgenomics.net/) and confirmed their sequence. The full-length *SlDREB* was fused to the GAL4 activation domain in the vector pGADT7 digested with *Sma*I and *Bam*HI to get pGAD-SlDREB. A 48 bp single-stranded oligonucleotide sequence (5'-*AGCTT*CATGTACCGACTCCCATGTACCGACTCCCATGTACCGACTCC*C*-3') and its reverse complement sequence (5'-*TCGAG*GGAGTCGGTACATGGGAGTCGGTACATGGGAGTCGGTACATG*A*-3'), which contains three tandem repeat copies of 5'-CATG*TACCGAC*TCC-3' (DRE/CRT is underlined) in the *SlCPS* promoter regions and cohesive termini of *Hind*III and *Xho*I was synthesized. The oligonucleotides were annealed as follows: each single-stranded oligonucleotide of 100 μM mixes at a ratio of 1:1, yielding a final concentration of 50 μM each, were heated to 95 °C for 30 s, 72 °C for 2 min, 37 °C for 2 min and 25 °C for 2 min. After annealing, the double-stranded oligonucleotides were cloned into a pAbAi vector linearized using the restriction enzymes *Hin*dIII and *Xho*I, and their sequences were confirmed. The resulting pDRE-AbAi construct and p53-AbAi control vector were digested with *Bst*BI, and the linearized plasmids were transformed into Y1H Gold yeast strain. Selection for transformants was performed on SD/–Ura medium. After PCR confirmation, the minimal inhibitory concentration of aureobasidin A (AbA) for the pDRE-AbAi yeast strain was determined. The p53-AbAi control had a minimal inhibitory concentration of 100 ng ml^{-1} AbA (Clontech). The pGAD-SlDREB and pGADT7 vectors were transformed into the pDRE-AbAi yeast strain, while the pGAD-p53 vector was transformed into p53-AbAi containing Y1H Gold yeast strain as a positive control. A large healthy colony was picked from the yeast strains and suspended in 0.9% NaCl. The optical density at 600 nm was adjusted to 0.002 (for ~2000 cells per 100 μl), and 2 μl of cells was also dotted on the SD/–Ura/–Leu medium with or without AbA to assess DNA–protein interactions. The colonies were then allowed to grow for 2–3 d at 30 °C.

Quantification of endogenous GAs

The leaves of tomato (1 g) were frozen in liquid nitrogen and finely ground follow by extraction with 15 ml of methanol containing 20% water (v/v) at 4 °C for 12 h. Before grinding, the following labelled GAs were added as internal standards: [$^2H_2$]GA_1 (1.00 ng g^{-1}), [$^2H_2$]GA_3 (1.00 ng g^{-1}), [$^2H_2$]GA_4 (2.00 ng g^{-1}), [$^2H_2$]GA_{12} (2.00 ng g^{-1}), [$^2H_2$]GA_{20} (2.00 ng g^{-1}), and [$^2H_2$]GA_{53} (4.00 ng g^{-1}). Further sample was prepared and analysed as described previously (Chen *et al.*, 2011). In brief, the sample was first passed through a C-18 SPE cartridge (12 ml, 1.5 g), and the resulting eluate was evaporated under a nitrogen gas stream and redissolved in 3 ml of water. The solution was acidified with 360 μl of 0.1 M hydrochloric acid and extracted repeatedly with ethyl ether (10 × 0.5 ml). The ether phases were combined, dried under nitrogen gas, and redissolved in 112 μl of acetonitrile. After the addition of 180 μl of Et_3N (20 μmol ml^{-1}) and 108 μl of 3-bromoacetonyltrimethylammonium bromide (20 μmol ml^{-1}), the reaction solution was vortexed for 10 min. The mixture was evaporated under nitrogen gas and the residue was dissolved in 30 μl of water. The prepared samples were injected using 25 kV×1 min and separated by 100 cm amino groups, and coated capillary electrophoresis coupled with electrospray ionization quadrupole-time-of-flight mass spectrometry was performed for analysis.

Scanning electron microscopy for leaf thickness and stomatal observation

For scanning electron microscopy observation, the third leaves from the top were collected from 2-month-old tomato plants overexpressing *SlDREB* and wild-type (WT) plants, all grown in a naturally illuminated glasshouse. The leaves were cut into ~0.1 cm^2 pieces and fixed with 2% glutaraldehyde for 24 h. After washing in 0.1 M cacodylate buffer, the samples were dehydrated in a graded ethanol series, dried in a desiccator (HCP-2; Hitachi), and coated with a film of gold. Observations were carried out on a JSM-6390/LV scanning electron microscope. The stomata were counted in each field of view (400×: ~0.065 mm^2) using an Olympus BHS/BHT System microscope (BH-2).

Results

Tomato SlDREB is a typical transcription factor

The AP2/ERF superfamily is defined by the AP2/ERF domain, which consists of ~60–70 amino acids and is involved in DNA binding. There are 112 AP2/ERFs in tomato, 85 of which are potential ERFs with a single complete AP2/ERF domain. The ERFs have been divided further into two subfamilies, ERF and DREB (Sharma *et al.*, 2010). Based on a phylogenetic analysis based of all tomato ERFs, SlDREB was classified into the DREB subfamily (Fig. 1).

The tomato DREB gene was previously identified as a drought-responsive gene in our microarray experiment (Gong *et al.*, 2010). We confirmed that it is induced by drought, salt, and

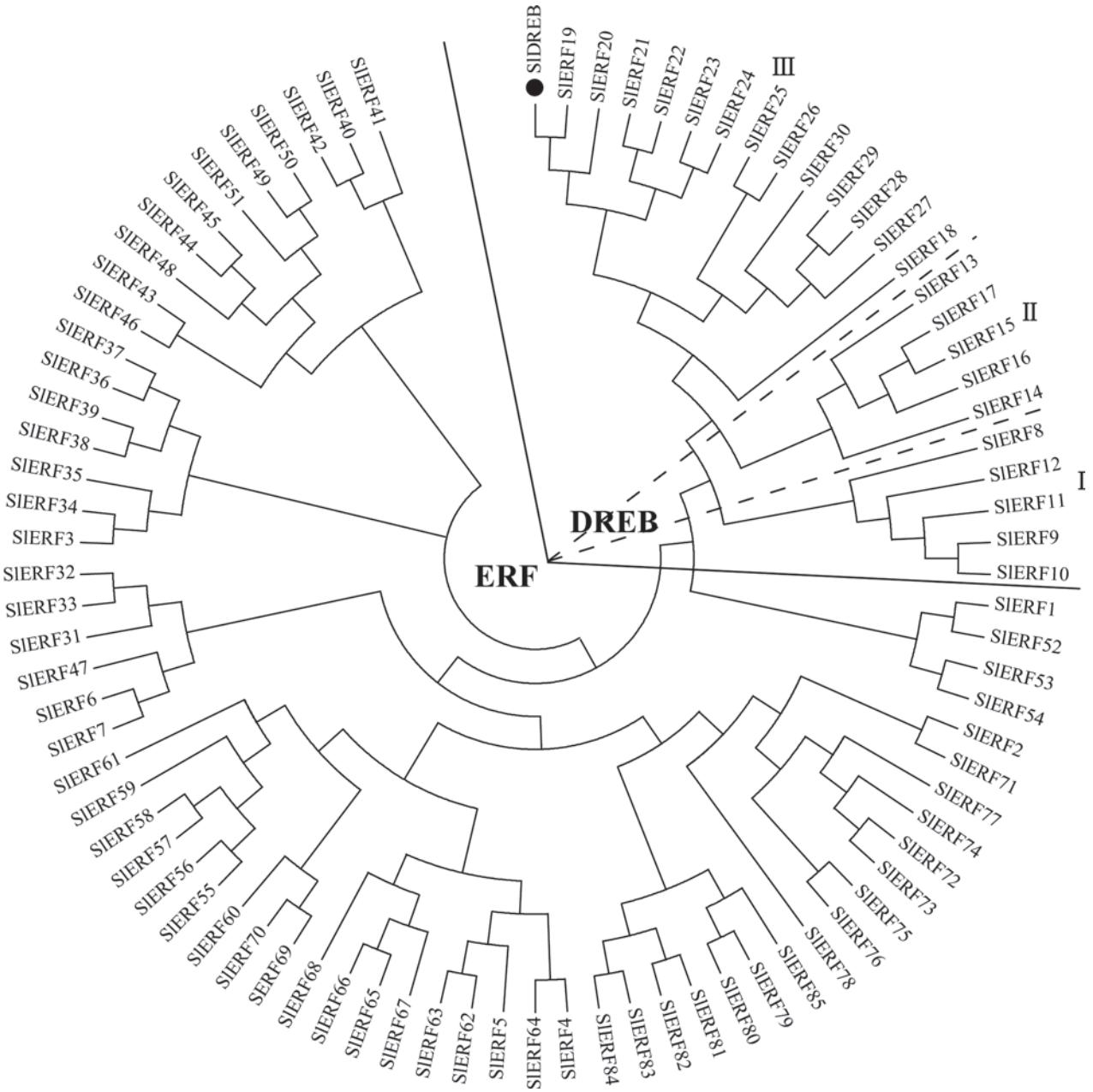

Fig. 1. Phylogenetic analysis of tomato ERFs with SlDREB proteins. Phylogenetic analysis of the ERF domains of 85 unigenes of tomato (Sharma *et al.*, 2010). SlDREB is indicated by a black circle. The phylogenetic tree was generated by ClustalW2 using standard parameters of the neighbour-joining method in MEGA (version 5.05).

cold stresses (Supplementary Fig. S1 at *JXB* online). Using PCR, we isolated and cloned the full-length (579 bp) ORF of *SlDREB* from the cDNA of *S. lycopersicum* cv. M82 and of *SpDREB* from *S. pennellii*. Comparison of their deduced protein sequences revealed that SlDREB shared 97% similarity with SpDREB (from wild tomato) and 60% similarity with StDREB (from *Solanum tuberosum*) but only 53% similarity with AtDREB (from *Arabidopsis*) (Fig. S2A at *JXB* online). A difference of only three amino acids—threonine, serine, and glutamic acid (in M82) in place of serine, arginine, and lysine (in *S. pennellii*) at positions 110, 134, and 144, respectively (Fig. S2B)—was observed between SlDREB and SpDREB.

Bioinformatics analysis revealed a nuclear localization signal (positions 36–42) in the basic region of the AP2/ERF DNA-binding domain, implying that SlDREB may be localized in the nucleus. In order to verify our *in silico* result, subcellular localization of SlDREB was examined by monitoring GFP fluorescence in onion epidermis cells transformed with either a fusion construct (pCAMBIA1391-*SlDREB*-GFP) or a control construct (pCAMBIA1391-GFP). Green fluorescence signals were observed over the entire cell in onion cells transformed with the control construct (Fig. 2A–C). In contrast, fluorescence was exclusively detected in the nuclei of cells transformed with the fusion plasmid (Fig. 2D–H), implying that SlDREB is a nuclear protein.

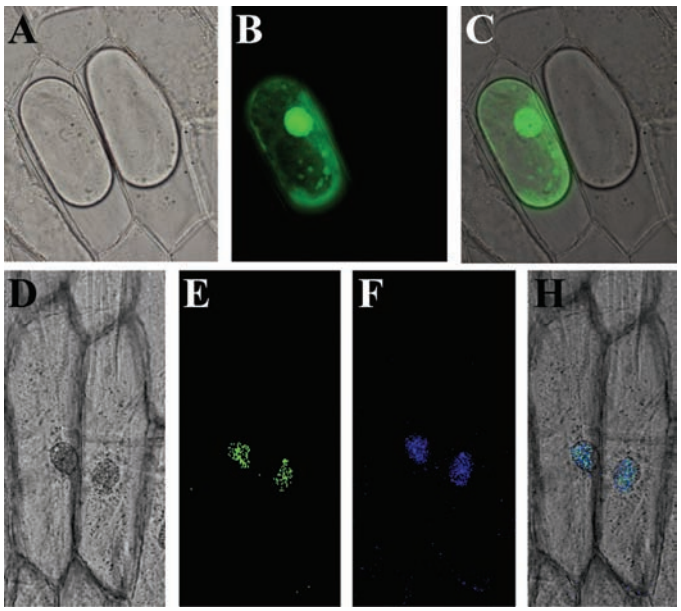

Fig. 2. Subcellular localization of SlDREB in onion epidermal cells. GFP and SlDREB–GFP fused constructs were expressed transiently in onion epidermal cells. Bright-field images (A, D), GFP fluorescent images (B, E), DAPI image (F), and merged images (C, H) of representative cells transformed with GFP (A–C) or the SlDREB–GFP fusion protein (D–G) are shown. (This figure is available in colour at *JXB* online.)

In addition to nuclear localization, transactivation activity is another defining feature of a transcription factor. We used a yeast two-hybrid system to examine the transcriptional activity of SlDREB. A GAL4 DNA-binding domain SlDREB fusion protein was expressed in yeast cells, which were then assayed for their ability to activate transcription from the GAL4 sequence. SlDREB promoted yeast growth in the absence of histidine and adenine, and showed X-α-gal activity, whilst the vector control pGBKT7 did not (Fig. 3). These data confirmed that SlDREB functions as a transcriptional activator in yeast.

SlDREB *is induced by GA_3 and Eth but suppressed by ABA*

Real time-PCR detection results showed that *SlDREB* was highly expressed in tomato stems and leaves (Fig. 4A). By comparing the *SlDREB* expression levels of treated plants relative to those of untreated plants, we found that *SlDREB* expression was significantly suppressed by GA_3 and Eth treatment for up to 6 h after treatment but returned to pre-treatment levels 12 h later. However, under ABA stress, *SlDREB* expression was induced from 1 to 6 h after treatment and returned to pre-treatment levels 12 h later (Fig. 4B, 4C, 4D). These expression patterns indicate that *SlDREB* was downregulated by GA_3 and Eth treatment but upregulated by ABA application.

Transgenic plants overexpressing SlDREB *have shorter internodes and increased drought tolerance*

To investigate the function of *SlDREB*, the plasmid 35S:*SlDREB* was introduced into cultivated tomato M82. Transgenic plants overexpressing (OE) *SlDREB* were obtained after screening for regenerated shoots on selection medium containing kanamycin. The transgenic plants were analysed further by PCR using genomic DNA as template and 35S forward and gene-specific reverse primers. Twenty-three transformants (T_0) regenerated from kanamycin-resistant calli contained *SlDREB*.

The expression level of the *SlDREB* gene in transgenic (T_0, T_1, and T_2) as well as control plants was examined at the five-leaf stage by real-time PCR analyses. Expression of *SlDREB* was 42.84- and 19.33-fold greater in the overexpressing T_2 homozygous line 4 (OE4) and overexpressing line 13 (OE13), respectively, compared with M82 control plants (Fig. 5E).

The *SlDREB* overexpressing transgenic lines showed dwarf phenotypes with obviously shorter internodes. For further experiments, we selected two T_2 homozygous lines: OE4 and OE13 (eight individual plants of each line). The transgenic plants and WT plants were cultivated under the same conditions, and plant height was determined after 2 months. The average plant height of the control, OE4, and OE13 plants was 29.7, 11.3, and

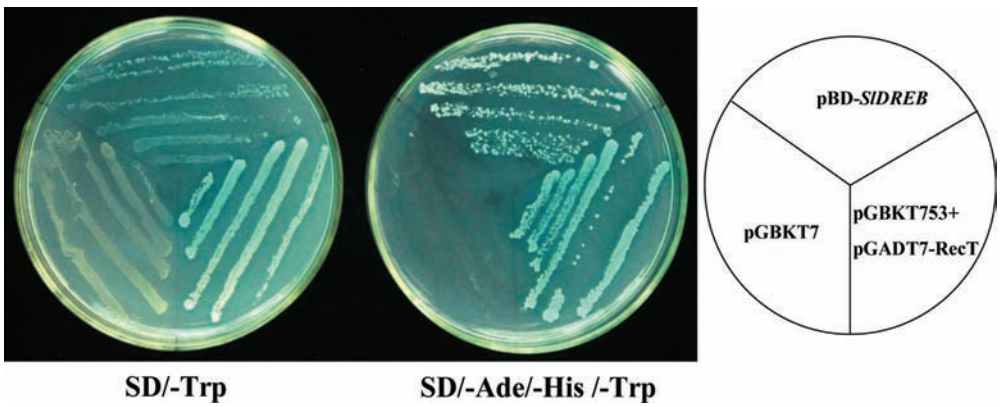

Fig. 3. Analysis of the transactivation activity of SlDREB. SlDREB and GAL4 DNA-binding domain fusion protein were expressed in the yeast strain AH109. Vectors pGBKT7 and pGBKT7-53+pGADT7-RecT were expressed in yeast as a negative and positive control, respectively. The yeast streak was cultured on SD/–Trp and SD/–Ade/–His/–Trp medium; both contained X-α-gal for assaying another yeast reporter (*MEL1*) gene. (This figure is available in colour at *JXB* online.)

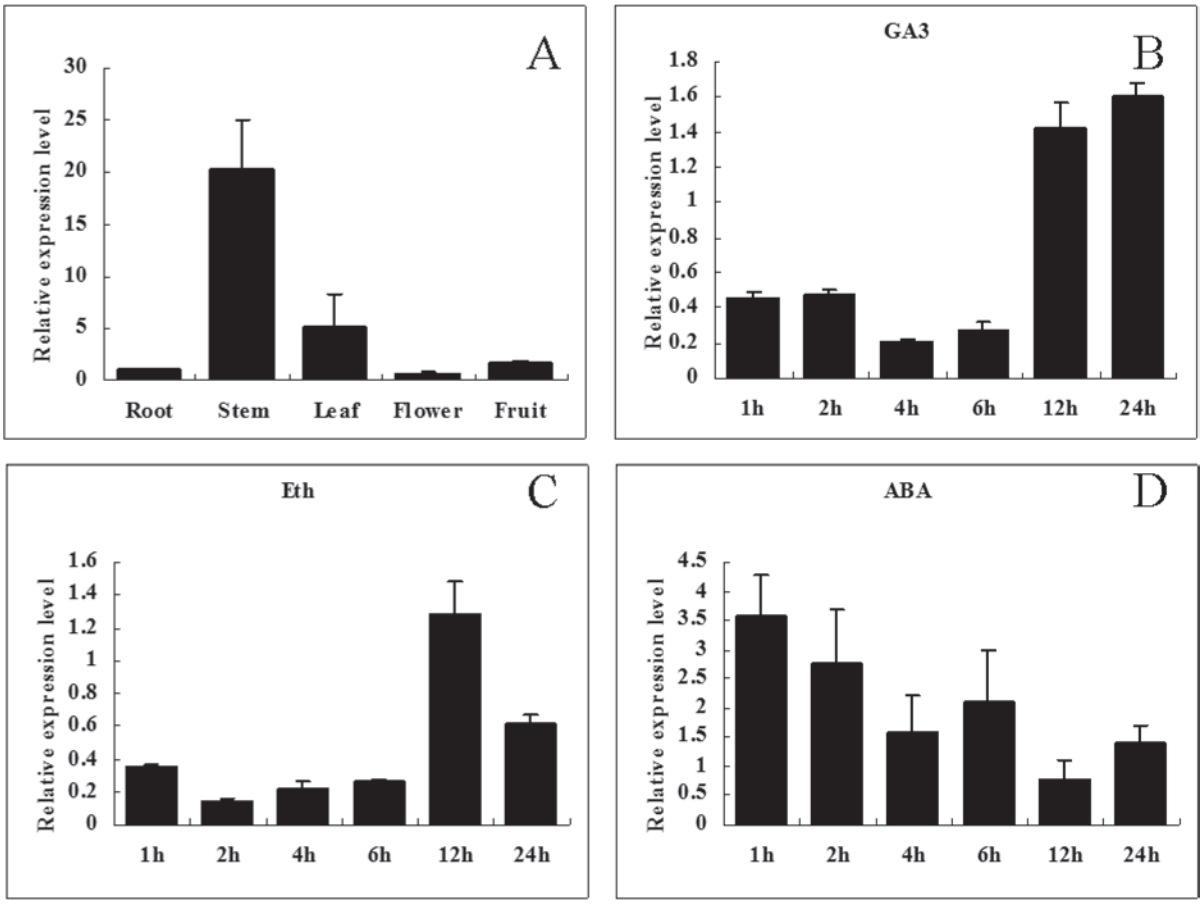

Fig. 4. Real-time RT-PCR analysis of the expression of *SlDREB* in WT tissues (A) and in response to the phytohormones GA_3 (B), ABA (C), and Eth (D). All samples were collected at the indicated time points. Expression of phytohormone-treated WT plants was compared with that in untreated plants after normalization of values with reference to the tomato β-actin gene and is presented as the relative expression level. All samples were collected at the indicated time points from three biological replicates in each treatment group. Error bars indicate the standard error (SE) of three replicates.

10.6 cm, respectively. Thus, OE4 and OE13 plants were 61.95% and 64.30% shorter, respectively, than the control (Fig. 5A). The leaves of the transgenic plants were also smaller: the mean length of the leaflets of the control, OE4, and OE13 lines was 4.55, 2.78 and 2.65 cm, respectively. The leaf width of the transgenic plants was also slightly smaller compared with that of WT plants (Fig. 5B). Furthermore, shortening of the internode was seen in all cases, with the strongest effect on the second internode, which was shortened by a mean of 31.88% (Fig. 5C). On average, an approximate threefold decrease in internode length was observed. Spraying with 100 µM GA_3 on *SlDREB*-overexpressing plants every 3 d rescued shoot elongation back to control levels (Fig. 6A, 6B), confirming that *SlDREB*-overexpressing plants indeed exhibit a classical reduced-GA phenotype (Phinney, 1956).

To investigate the effects of *SlDREB* on leaf structure, the leaves of 2-month-old control and *SlDREB*-overexpressing tomato plants were examined using scanning electron microscopy. The average leaf cross-section was 120.3 µm for control plants, while the thickest cross-section (215.45 µm) was observed in the *SlDREB* OE4 plant, followed by the OE13 plant (201.48 µm) (Fig. 5D, 5F).

The mean number of stomata on functional leaves of *SlDREB*-overexpressing plants was approximately seven compared with nine per unit area (~0.065 mm^2) in WT plants (Fig. 5H). This could reflect specific effects of *SlDREB* on tomato architecture leading to lowered transpiration and energy loss under drought stress. Consistent with this idea, the *SlDREB*-overexpressing plants performed very well, whilst the WT plants showed signs of wilting (Fig. 5G).

Overexpression of SlDREB *downregulates GA biosynthesis genes*

To investigate whether *SlDREB* altered GA metabolism, expression of GA biosynthetic genes was compared between *SlDREB* transgenic and M82 (control) seedlings. Two genes in the GA biosynthetic pathway, *SlCPS* and *SlKS*, showed considerable change in expression in transgenic plants. *SlCPS* was dramatically downregulated in *SlDREB* transgenic seedlings: OE4 exhibited a 3.44-fold decline in *SlCPS* expression compared with M82 (WT) seedlings. In contrast, expression of *SlKS* was 2.18-fold greater in *SlDREB* transgenic seedlings than in M82 WT seedlings (Fig. 7A, 7C). No significant difference in expression

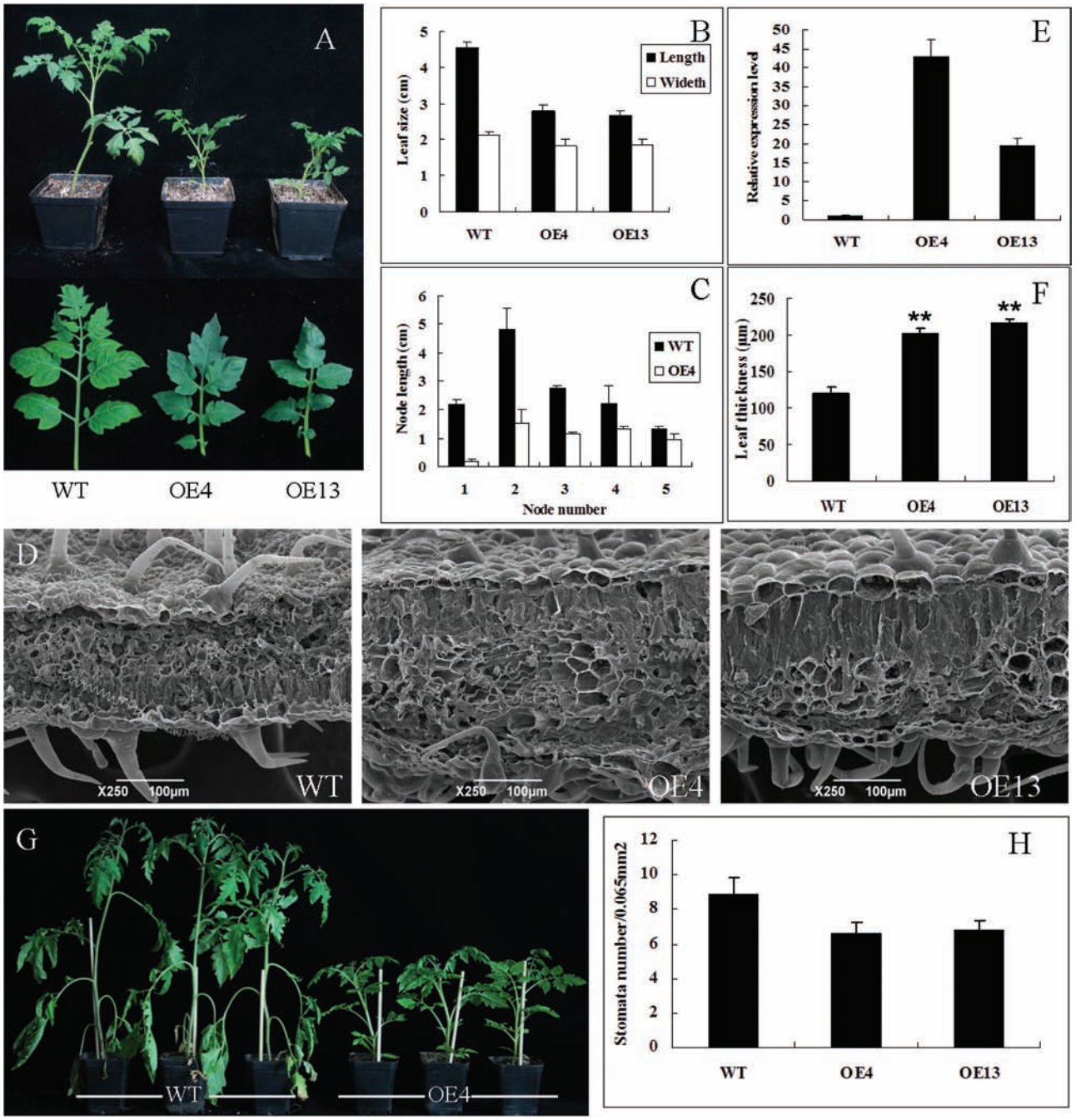

Fig. 5. Overexpression of *SlDREB* restricts tomato leaf expansion and internode elongation. (A) Phenotypic appearance of seedlings (upper) and leaves (lower) of *SlDREB*-overexpressing tomato OE4 and OE13 lines and WT plants. (B) Leaflet size of WT and *SlDREB* lines OE4 and OE13. The third leaf from the top of 2-month-old transgenic and non-transgenic plants was used for leaflet size measurement; the apical leaflet was chosen from the leaf. Error bar indicates SE (n=8). (C) Internode length of 2-month-old WT and *SlDREB* OE4 tomato plants. Error bars indicates SE (n=8). (D) Scanning electron microscopy images showing leaf thickness. Leaves were collected from 2-month-old tomato *SlDREB*-overexpressing and WT plants grown in a naturally illuminated glasshouse. The apical leaflet of the third leaf from the top was collected for analysis. (E) The expression level of *SlDREB* in transgenic and WT control plants was examined at the five-leaf stage by real-time PCR analyses. Error bars indicates SE (n=8). (F) Graphical representation of leaf thickness. Eight individual plants were selected per line and the thickness of every leaf was measured three times. Asterisks indicate significant differences compared with WT (**$P < 0.01$). (G) *SlDREB*-overexpressing plants show drought resistance. At 40 d post-inoculation, watering was stopped for drought treatment. The photographs were taken after 15 d. Treatment was replicated three times under the same conditions ($n > 8$). (H) The mean number of stomata per unit area in *SlDREB* overexpressing and WT leaves. Stomatal number was counted in each field of view (400×, ~0.065 mm^2) on at least 20 microscopes and three plants. The third leaf from the top of 2-month-old transgenic and non-transgenic plants was analysed. (This figure is available in colour at *JXB* online.)

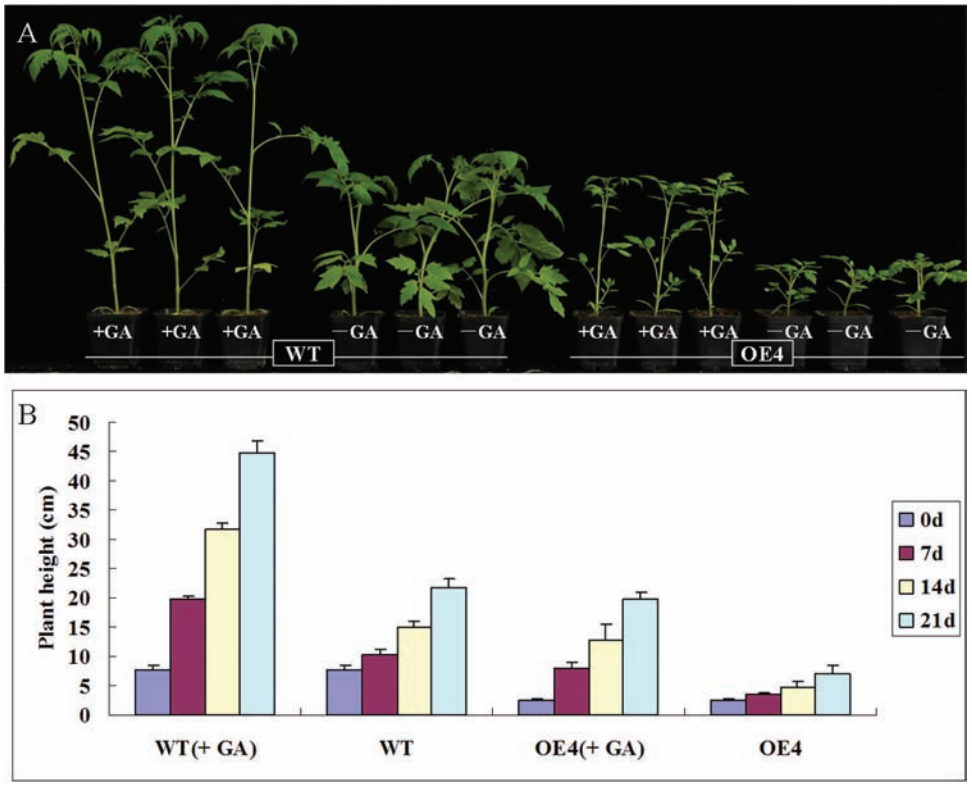

Fig. 6. The dwarf phenotype of *SlDREB*-overexpressing plants was rescued by spraying with GA_3. (A) Pictures were taken 50 d after inoculation. The dwarf phenotype was rescued by spraying GA_3 every 3 d starting from 30 d after inoculation. (B) Plant height is displayed as the mean of eight plants; error bars show SE (n=8). (This figure is available in colour at *JXB* online.)

of other genes responsible for the early steps of GA biosynthesis, such as *SlGPS*, *SlKO*, and *SlKAO*, was seen between *SlDREB* transgenic and WT plants (Fig. 7B).

GA20oxs are also key GA biosynthetic enzymes that determine GA concentration in many plant species, and GA3oxs catalyse the final step to produce bioactive GAs (GA_1, GA_3, and GA_4) (Hedden and Kamiya, 1997; Xiao *et al.*, 2006; Yamaguchi, 2008). We compared transcript levels of GA20oxs and GA3oxs between transgenic plants and WT plants by quantitative RT-PCR. In transgenic plants, *SlGA20ox1*, *SlGA20ox2*, and *SlGA20ox4* were sharply downregulated, but *SlGA3ox1* and *SlGA3ox2* were slightly upregulated when compared with their respective expression in WT plants (Fig. 7A, 7C).

To determine whether *SlDREB* is involved in GA inactivation, transcript levels of genes encoding GA2oxs (the main GA catabolic enzyme) in tomato (i.e. *SlGA2ox1*, *SlGA2ox2*, *SlGA2ox3*, *SlGA2ox4*, and *SlGA2ox5*; Serrani *et al.*, 2008) were quantified by RT-PCR. No significant differences in expression of these genes were seen between transgenic and WT plants (Fig. 7B).

To determine further whether *SlDREB* affects GA signal transduction factors, the expression levels of *SlDELLA*, which has been reported to be a GA repressor in the GA response pathway (Sun and Gubler, 2004), and *SlGAST1*, a GA-responsive gene (Shi *et al.*, 1992), were compared by quantitative RT-PCR. Expression levels of *SlDELLA* and *SlGAST1* were not significantly different between the transgenic and WT plants (Fig. 7B).

Decreased endogenous GA abundance in SlDREB *overexpressing tomato plants*

As our results showed that *SlDREB* repressed the expression of GA biosynthesis genes, we decided to compare endogenous GA levels in *SlDREB* overexpressing transgenic and WT plants. As shown in the schematic representation of GA biosynthesis in Fig. 7C, endogenous levels of several GAs (GA_{53}, GA_{12}, GA_3, GA_{20}, GA_4, and GA_1) in *SlDREB*-overexpressing transgenic plants were significantly lower than in WT plants.

SlDREB specifically binds to the DRE/CRT elements of the SlCPS *promoter region.*

Previous studies have shown that DREBs can bind to the *cis* element of DRE/CRT (Busk *et al.*, 1997; Qin *et al.*, 2004). We found that the promoter region (–1928) of *SlCPS* contains a DRE/CRT element (Fig. S3 at *JXB* online). In order to investigate whether SlDREB binds to the DRE/CRT elements of the promoter region of the *SlCPS* gene, the full-length ORF of *SlDREB* was fused to the GAL4 activation domain of the vector pGADT7. The fused construct thus obtained (pGADT7-*SlDREB*) was co-transformed with the pDRE-AbAi construct containing triple tandem repeats of DRE/CRT from the *SlCPS* promoter region into the Y1H Gold yeast strain (Fig. 8). Although all yeast cells harbouring different constructs could grow on SD/–Ura without AbA, those

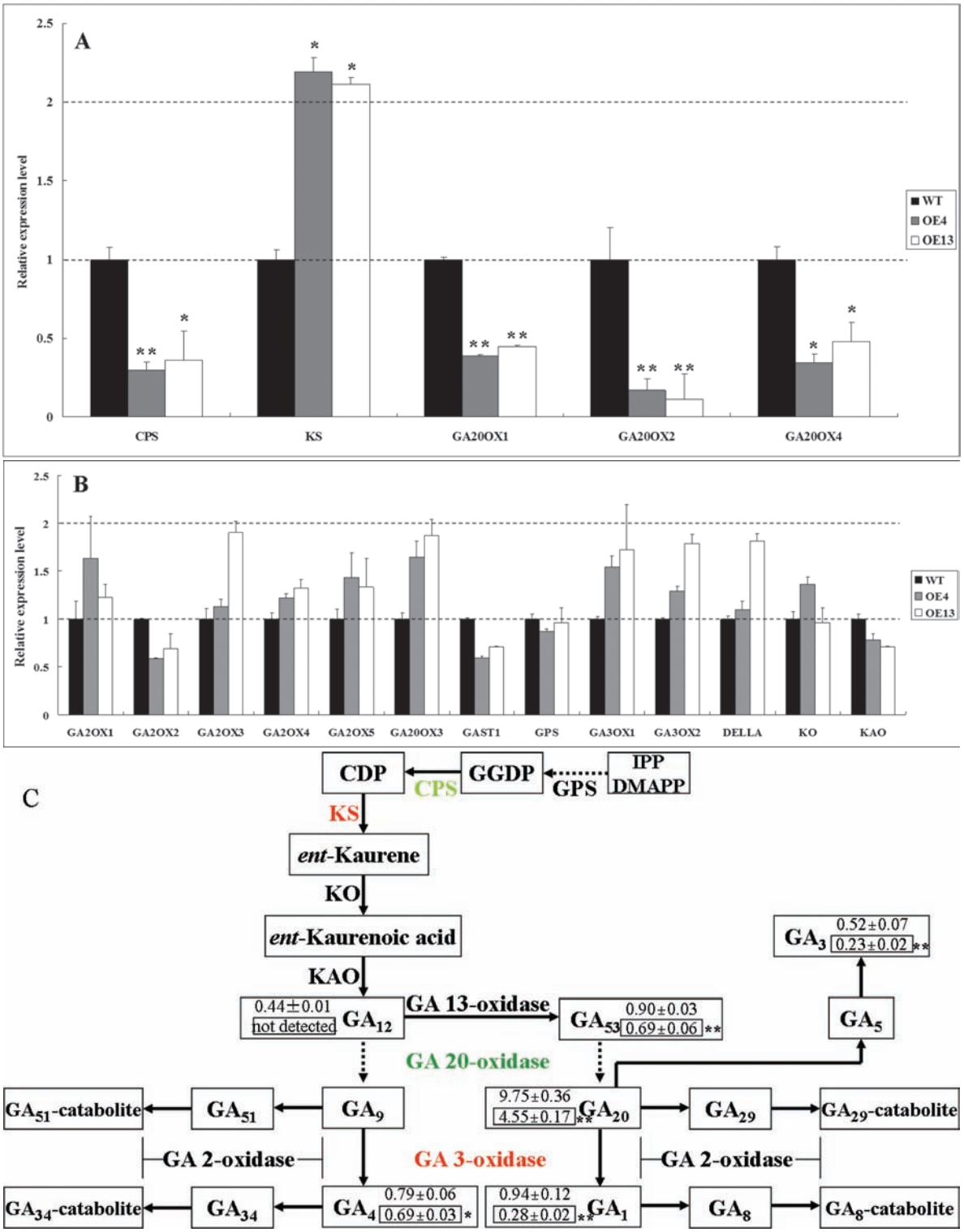

Fig. 7. Decreased GA abundance and GA biosynthesis gene expression in *SlDREB*-overexpressing tomato plants. (A, B) Real-time RT-PCR analysis of the expression of tomato GA biosynthesis genes in OE4, OE13 and WT plants. *$P < 0.05$; **$P < 0.01$. Primer sequences are listed in Supplementary Table S1. All samples were collected at the indicated time points from three biological replicates in each treatment group. Error bar indicates the SE of three replicates. (C) Pathway of GA biosynthesis: green indicates significantly ($P < 0.05$) downregulated expression and red indicates slightly upregulated expression. Numbers indicate endogenous GA levels in the WT (upper) and transgenic plants (lower, boxed). Results that were 'not detected' were due to low abundance; **$P < 0.01$; * $P < 0.05$. Data are presented as means ±standard deviation from three technical replications (ng g^{-1} of fresh weight) using two transgenic lines. IPP, prenyldiphosphates isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPS, geranyl diphosphate synthase; GGDP, geranylgeranyl diphosphate; CPD, copalyldiphosphate; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase, KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase. (This figure is available in colour at *JXB* online.)

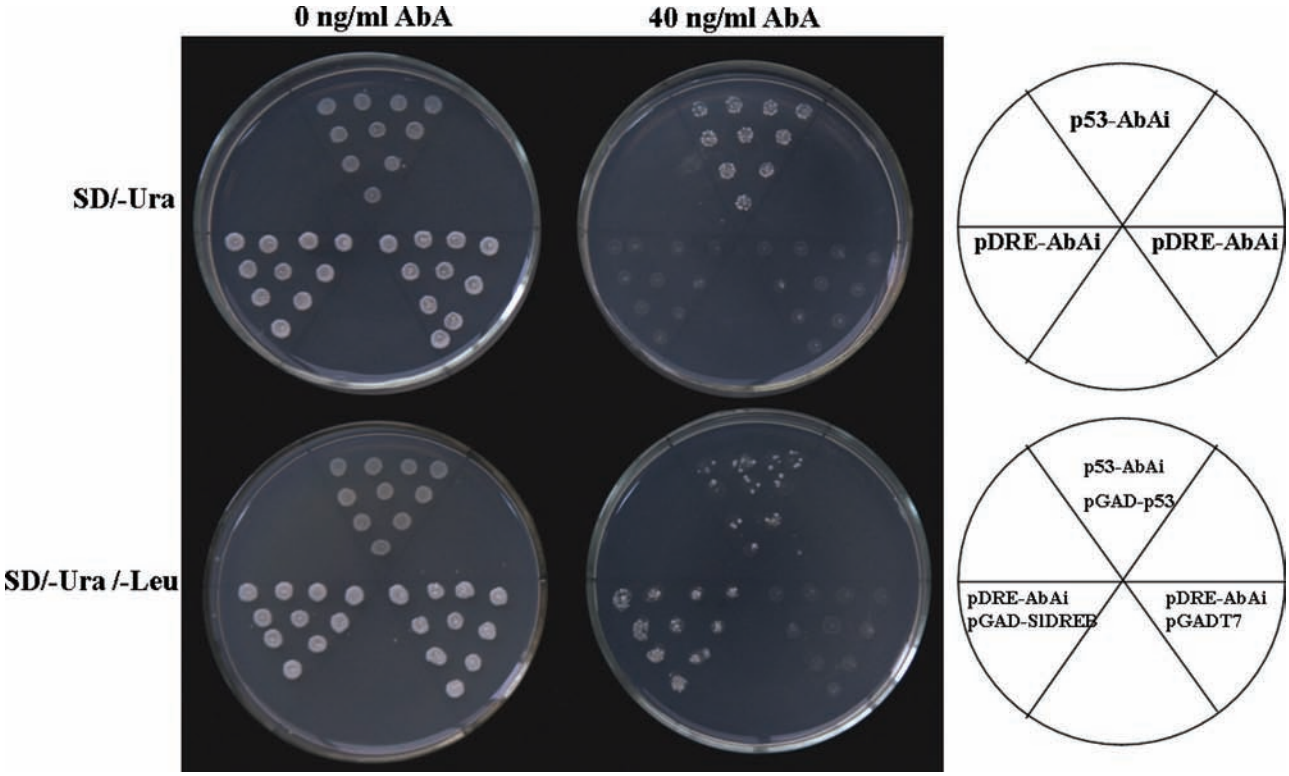

Fig. 8. Yeast one-hybrid assays for binding of SlDREB to the DRE/CRT domains of the *SlCPS* promoter region. Vectors p53-AbAi+pGAD-p53 and pDRE-AbAi+pGADT7 were expressed in yeast as positive and negative controls, respectively. The minimal inhibitory concentration of AbA for the pDRE-AbAi yeast strain is 40 ng ml^{-1}. The photograph shows the growth of yeast cells on SD/–Ura or SD/–Ura /–Leu medium with (40 ng ml^{-1}) or without AbA.

with pDRE-AbAi did not grow in the presence of 40 ng ml^{-1} of AbA (Fig. 8, upper panel). However, cells co-transformed with pGADT7-*SlDREB* and pDRE-AbAi grew normally in the presence of the minimal inhibitory concentration (40 ng ml^{-1}) of AbA. Additionally, the growth of transformants containing constructs lacking SlDREB was completely inhibited (Fig. 8, lower panel), suggesting that SlDREB could bind to the DRE/CRT *cis* element in yeast.

Discussion

SlDREB *is an AP2/ERF-binding factor and is regulated by GA, ABA, and Eth in tomato*

AP2/ERF proteins have been shown to be integrators of biotic and abiotic stress responses through their interaction with *cis*-acting elements, the GCC box, and CRT/DRE (Knight and Knight, 2001; Sakuma *et al.*, 2002; Brown *et al.*, 2003; Li *et al.*, 2011). DREB can be classified into the ERF subgroup of the AP2/ERF superfamily based on its function and group motif (Sharma *et al.*, 2010). DREB transcription factors are involved in abiotic and biotic stress signalling pathways in plants (Agarwal *et al.*, 2006). However, the functions of DREB in tomato remain unclear. *SlDREB* was isolated by screening the results of our previous microarray experiments on drought-tolerant tomato (Gong *et al.*, 2010). *SlDREB* was responsive to drought, salt, and cold stresses and could be classified into subgroup III of the isolated DREBs (Figs 1 and S1). In addition, it was also shown that SlDREB is localized in the nucleus and exhibits transactivation activity in yeast (Figs 2 and 3), suggesting that *SlDREB* encodes a typical transcriptional factor.

It has been reported that AP2/ERF transcription factors can modulate ethylene responses (Chang and Shockey, 1999; Yang *et al.*, 2011*b*). Ethylene has been found to promote GA responsiveness and shoot elongation in submergence-intolerant lines and to confer submergence tolerance in rice (Fukao and Bailey-Serres, 2008). Moreover, internode elongation of rice in deepwater requires the activity of GA and ethylene (Sauter *et al.*, 1995). Under deepwater conditions, ethylene accumulates and triggers remarkable internode elongation via GAs (Hattori *et al.*, 2009). It seems that GA_3 and Eth provide collaboration features in the process of plant internode elongation. This is consistent with our observation that *SlDREB* had the same expression profile under GA_3 and Eth treatment (Fig. 4B, 4C), and it is possible that *SlDREB* can mediate cross-talk between ethylene and GA in plant internode elongation.

GAs regulate various developmental processes including stem elongation, leaf expansion, fruit development, and seed germination (Dill *et al.*, 2004). GA and ABA play antagonistic roles in the regulation of numerous developmental processes: GA is associated with the promotion of germination, growth, and flowering, whilst ABA acts as a competitive inhibitor of GA activity

and inhibits these processes (Razem *et al.*, 2006). This antagonistic relationship was partially confirmed by our observation that expression of *SlDREB* was upregulated by GA application and downregulated by ABA treatment (Fig. 4B, 4D). It appears that *SlDREB* functions as a balance regulator through different networking between GA and ABA signalling pathways.

SlDREB *restricts leaf expansion and internode elongation by negative regulation of GA biosynthesis genes*

Our results demonstrated that the dwarf phenotype of *SlDREB*-overexpressing plants could be attributed to decreased levels of endogenous GAs (Fig. 7C). Accordingly, dwarfism of *SlDREB*-overexpressing plants could be reversed by the application of GA_3 (Fig. 6A, 6B). These results are in agreement with previous studies in GA-deficient mutants of various plants, including *Zea mays* (Fujioka *et al.*, 1988), *Arabidopsis thaliana* (Magome *et al.*, 2004), and *Oryza sativa* (Sakamoto *et al.*, 2004).

To assess further whether the GA-sensitive dwarf phenotype of *SlDREB*-overexpressing plants was due to inhibition of transcription of GA biosynthetic genes, inhibition of GA signal transduction, and/or promotion of expression of GA-inactivating enzyme genes, we analysed the expression level of different genes involved in these pathways. Our results demonstrated that *SlDREB* restricted internode elongation and leaf expansion by downregulating the expression of *SlCPS* and *SlGA20ox1*, *-2*, and *-4* (Fig. 7A), which encode key enzymes in the GA biosynthesis pathway. In fact, a series of examples showed that CPS and GA20ox genes were targeted to lead to alteration of GA level and a dwarf plant. Loss-of-function CPS mutants of several plant species, such as rice (Sakamoto *et al.*, 2004), *Arabidopsis thaliana* (Sun and Kamiya, 1994), and *Pisum sativum* (Ait-Ali *et al.*, 1997), have also been shown to display severe dwarf phenotypes. In contrast, Serrani *et al.* (2008) reported that upregulation of *SlCPS*, *SlGA20oxs*, and *SlGA3ox* by 2, 4-dichlorophenoxyacetic acid treatment led to higher levels of bioactive GA in tomato. GA20ox catalyses the sequential oxidation of C-20 and is responsible for the production of inactive precursors (GA_9, GA_{20}) during bioactive GA synthesis (Yamaguchi, 2008). Overexpression and downregulation of *GA20ox* modified plant height by altering the levels of active GAs (Coles *et al.*, 1999; Carrera *et al.*, 2000; Vidal *et al.*, 2001; Fagoaga *et al.*, 2007). No significant differences were found in the expression of other genes involved in GA biosynthesis (*SlGPS*, *SlKO*, *SlKAO*, *SlGA20ox3*, and *SlGA3ox1* and *-2*), and the GA-inactivating enzyme (*SlGA2ox1–5*) between *SlDREB*-overexpressing lines and WT plants (Fig. 7B). On the other hand, expression of *SlGA20ox3* and *SlKS* was slightly upregulated in transgenic plants, probably for the feedback of GA deficiency (Cowling *et al.*, 1998). Taken together, our results show that *SlDREB* restricts internodal elongation and leaf expansion by downregulating the expression of key GA biosynthesis enzymes. Moreover, the expression of GA response genes (*SlDELLA* and *SlGAST1*) between *SlDERB*-overexpressing and WT plants was not significantly changed. This provides further evidence that *SlDERB* negatively regulates the internode elongation and leaf expansion processes by inhibiting GA biosynthesis and not by signal transduction.

SlDREB may directly regulate expression of SlCPS

Transcription factors bind to either enhancer or promoter regions of DNA to regulate the expression of genes (Latchman, 1997). Most transcription factors act either as activators or repressors (Ikeda *et al.*, 2009); therefore, depending on the transcription factor, the transcription of the adjacent gene is either up- or downregulated (Gill, 2001). Transcriptional regulation is an important mechanism for controlling metabolic pathways. These pathways are generally influenced by environmental stimuli and the plant's developmental programme (Van Moerkercke *et al.*, 2011). As we found that expression of *SlCPS* and *SlGA20ox1*, *-2*, and *-4* was negatively related to *SlDREB* expression in our study (Fig. 7A), we studied the binding of SlDREB with the promoter region of genes it is likely to regulate. Our results showed binding of *SlDREB* to the DRE/CRT domains of the *SlCPS* promoter region (Fig. 8), and thus reveal that SlDREB likely

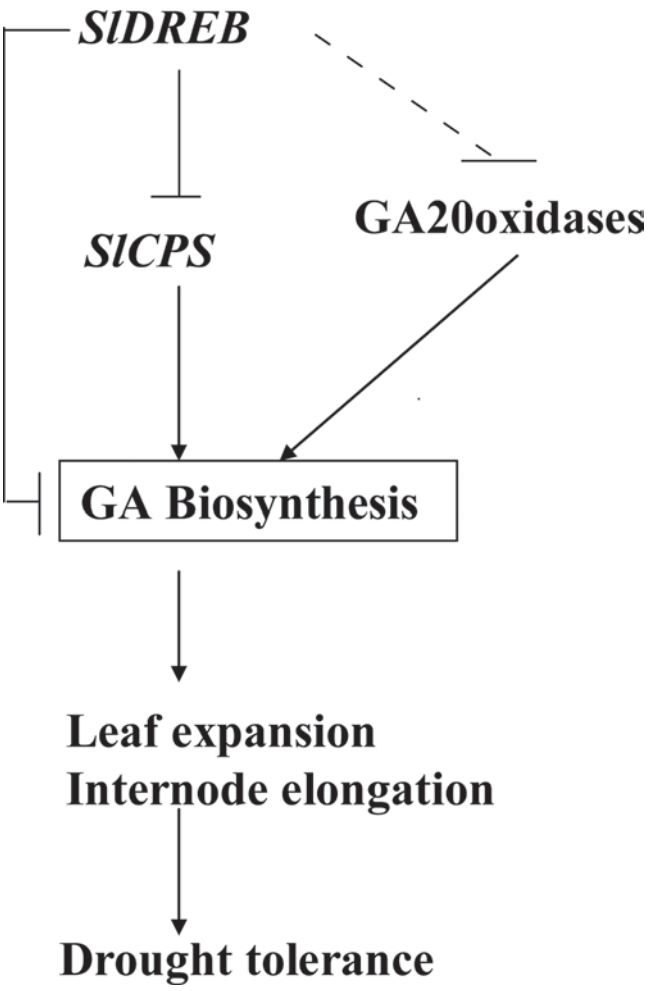

Fig. 9. Model of SlDREB regulation of leaf expansion and internode elongation by downregulating key genes required for gibberellin biosynthesis. *SlDREB* directly or indirectly downregulates the expression of *SlCPS* and *SlGA20oxs*, respectively. Transgenic plants overexpressing *SlDREB* have lower endogenous GA levels, leading to restriction of leaf expansion and internode elongation, which in turn increases the drought tolerance of plants.

acts as a direct repressor of *SlCPS* in plants. We did not find DRE/CRT elements in the putative 2500 bp promoter regions of *SlGA20ox1*, *-2*, and *-4*, which might have been downregulated in the *SlDREB*-overexpressing plants through other mechanisms.

SlDREB *integrates environmental stress signals and reduces endogenous GA levels to avoid abiotic stress*

To sum up, overexpression of *SlDREB* in tomato plants can downregulate the expression of *SlCPS* and *SlGA20oxs*, and lowers endogenous GA levels, leading to restriction in leaf expansion and internode elongation. These effects account for the dwarfism of tomato plants and help in drought tolerance (Fig. 9). It seems that, under stress, decreased GA levels inhibit growth, allowing plants to survive better in unfavourable conditions. This hypothesis was confirmed by our results showing that overexpression of *SlDREB* in tomato leads to lower endogenous GA levels and consequently a dwarf phenotype, which enhances drought tolerance, and it is strengthened by the findings of (Magome *et al.*, 2004), who also observed increased survival of GA-deficient *ga1-3* mutant *Arabidopsis* plants exposed to salt stress and who showed that the mutants exhibited a reduced growth phenotype, which was inhibited by a reduction in GA synthesis. Thus, it can be concluded that plants need adequate GA to promote developmental changes, but limiting GA production is also necessary for them to survive under unfavourable environments. Further studies on *SlDREB* can provide a better understanding of how genes integrate environmental signals to restrict plant internode elongation by balancing ethylene, GA, and ABA signals.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. *SpDREB* expression in response to drought, salt, and cold stress factors.

Supplementary Fig. S2. Multiple alignments of the predicted protein sequence of SlDREB and DREBs from other plants.

Supplementary Fig. S3. Sequence of the promoter region of *SlCPS*.

Supplementary Table S1. Primer sequences used for real-time RT-PCR analysis of *SlDREB* and genes from the GA biosynthesis and GA response pathways in tomato.

Acknowledgements

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