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A Multiple Stress-Responsive Gene *ERD15* from *Solanum pennellii* Confers Stress Tolerance in Tobacco

Khurram Ziaf[1,2], Rachid Loukehaich[1], Pengjuan Gong[1], Hui Liu[1], Qinqin Han[1], Taotao Wang[1], Hanxia Li[3] and Zhibiao Ye[1,3,*]

[1]National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, 430070, PR China
[2]Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan
[3]Key Laboratory of Horticultural Plant Biology, Ministry of Education, Huazhong Agricultural University, Wuhan, 430070, PR China
*Corresponding author: E-mail, zbye@mail.hzau.edu.cn; Fax, +86-027-87280016
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Wild species often show more tolerance to environmental stress factors than their cultivated counterparts. An early responsive-to-dehydration gene was cloned from a drought- and salt-tolerant wild tomato *Solanum pennellii* (*SpERD15*). *SpERD15* transcript accumulated differentially in different organs, and was remarkably induced by dehydration, salinity, cold and treatment with plant growth regulators. The protein encoded by *SpERD15* was predominantly localized in the nucleus. Interestingly, we found that the majority of the transgenic tobacco plants were co-suppressed along with the overexpressing line. Overexpressing plants manifested stress tolerance accompanied by the accumulation of more soluble sugars and proline, and limited lipid peroxidation compared with co-suppression lines, which were more sensitive than the wild type. The differential contents of these compatible solutes in different transgenic lines were related to the changes in the expression of the genes involved in the production of some important osmolytes (*P5CS* and *Sucrose synthase*). Reduced lipid peroxidation over a broad range of stress factors was in agreement with increased expression of stress-responsive genes (*ADH* and *GAPDH*). Overexpression of *SpERD15* increased the efficiency of PSII (F_v/F_m) in transgenic tobacco plants by maintaining PSII quinone acceptors in a partially oxidized form. The results show that *SpERD15* augments stress tolerance by enhancing the efficiency of PSII through the protection of cellular membranes, as conferred by the accumulation of compatible solutes and limited lipid peroxidation.

Keywords: Chl fluorescence • Cold • Drought • Osmolytes • Salt • *Solanum pennellii* • Tobacco.

Abbreviations: ADH, alcohol dehydrogenase; CaMV, cauliflower mosaic virus; ERD, Early Responsive to Dehydration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; LAR, leaf area ratio; MDA, malondialdehyde; MS, Murashige and Skoog; NPQ, non-photochemical quenching; ORF, open reading frame; *P5CS*, Δ1-pyrroline-5-carboxylate synthase; RLWC, relative leaf water content; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SLA, specific leaf area; WT, wild type

Introduction

Crop plants are subject to a number of abiotic and biotic stresses due to their sedentary nature. However, these plants have evolved different defense and adaptation measures to cope with such stresses through biochemical and physiological changes, including synthesis and assembly of adaptive molecules into organized structures. All this is achieved through a series of stress-dependent signal transductions involving different types of genes (Chinnusamy et al. 2004). Most abiotic stresses, such as drought, salinity and low temperature, have similar physiological consequences of inducing cellular damage and, therefore, evoke similar signaling pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Osmotic perturbation, the common factor among these abiotic stresses (Munns and Tester 2008), switches on the genes for the synthesis of osmo-protectants, lipid desaturases, mRNA-binding proteins, transcription factors and detoxification proteins (Yamaguchi-Shinozaki and Shinozaki 2005).

Oxidative stress also accompanies most of the abiotic stresses and causes denaturation of structural and functional proteins. Reactive oxygen species (ROS) produced in response to oxidative stress play a central role in signaling networks, starting from stress perception through membrane-localized histidine kinase (Apel and Hirt 2004), leading to the regulation of the antioxidant enzyme system. Response to stress is initiated after its perception by the plasma membranes; the cellular components serve as sensors and are subjected to lipid peroxidation as a result of ROS generation (Gaspar et al. 2002). Tobacco NtC7 (Tamura et al. 2003) and some members of the Arabidopsis histidine kinase family (Tran et al. 2007) have recently been

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reported to be osmosensors. Nevertheless, our knowledge of membrane sensors remains incomplete.

Under stress conditions, the rate of absorption of light energy by Chl exceeds its consumption rate, accelerating the damage to PSII (photoinhibition) due to the generation of ROS. Photoinhibition limits photosynthesis and can be evaluated in terms of Chl fluorescence (Baker 2008). Different strategies to protect the photosynthetic apparatus from this photooxidative damage include photorespiration, cyclic electron transport through PSI or PSII, and the antioxidant system (Asada 2006).

ABA, a sesquiterpenoid, is involved in a number of growth and developmental processes (Xiong et al. 2002). It accumulates in response to water and salt stress, and acts as a signal for acclimation to different stresses (reviewed by Xiong et al. 2002). A number of genes that are positive or negative regulators of ABA have been characterized; some are sensitive to ABA during germination and seedling development (Zhang et al. 2005), and/or are involved in conferring stress tolerance (Xiong et al. 2001). Others are induced in response to more than one phytohormone (Kariola et al. 2006). Early Responsive to Dehydration 15 (*ERD15*) was recently reported to be a negative regulator of ABA; it is induced by ABA, salicylic acid (SA), wounding and pathogenic infection (Kariola et al. 2006). Application of ABA enhances the expression of some members of the same ERD group (ERD10 and 14) (Kiyosue et al. 1994b), while having no effect on others (ERD2, 8 and 16) (Kiyosue et al. 1994a). Some contradictions in the induction, as well as function, of *ERD15*, however, have been reported. Reduced expression in response to wounding was reported by Dunaeva and Adamska (2001), while enhanced transcript levels were observed by Kariola et al. (2006). Moreover, Arabidopsis plants showing increased tolerance to salt stress through overexpression of *AtSAT32* also had higher transcript levels of *ERD15* than control plants (Park et al. 2009). Furthermore, transgenic wheat plants overexpressing *TaDi19A* exhibited increased expression of *ERD15* (Li et al. 2010). In contrast to these findings, Arabidopsis plants overexpressing *ERD15* manifested susceptibility to drought and freezing stress (Kariola et al. 2006).

The diversity in the expression and function of *ERD15*, particularly its feature of conferring tolerance to one genetic background but rendering others sensitive, compelled us to examine the role of *SpERD15* by expressing it in tobacco. Most of the members of the *ERD* group have been characterized for their functional significance, but the physiological functions attributable to *ERD15* have not been fully resolved. In this work, we report on the cloning and functional characterization of *ERD15* from a wild tomato species *Solanum pennellii*. The gene is induced in response to different abiotic stresses and phytohormones. We provide insights into the subcellular localization of *SpERD15*. The response of constitutive expression, leading to both co-suppression and overexpression of *SpERD15* in transgenic tobacco plants, against different abiotic stresses is studied. Overexpression of *SpERD15* improved the stress tolerance of transgenic tobacco plants by regulating the expression of stress-related genes, mainly through osmotic adjustment and protection of PSII, as conferred by the accumulation of compatible solutes and enhanced membrane stability.

Results

Characterization of *SpERD15*

ERD15 has been identified as one of the drought-responsive genes in our microarray experiment (Gong et al. 2010). The full-length open reading frame (ORF) of *ERD15* was amplified from *S. pennellii*. The genomic DNA of *S. pennellii* contains an intron of 105 bp, and its mRNA has an ORF of 486 bp, encoding 162 amino acids. The predicted and actual molecular weight of *SpERD15* is 18.13 kDa (**Supplementary Fig. S1A**), and its isoelectric point (pI) is 4.52. Predicted peptides contain 24 negatively and 14 positively charged residues, totalling 38 charged residues in contrast to 49 in *At*ERD15 (Kiyosue et al. 1994a). *SpERD15* also lacks serine residues, similar to *At*ERD15, and contains a higher proportion of leucine, serine and proline, as well as other amino acids. The multiple alignment of the deduced protein sequences revealed that *SpERD15* shares 98% similarity with *Sl*ERD15 (from cultivated tomato) and 90% similarity with *Nt*ERD15 (from tobacco), but only 38% similarity with *At*ERD15 (from Arabidopsis). Compared with other members of the *ERD* group, *SpERD15* showed closeness to *ERD2*, *ERD4* (membrane protein), *ERD7* (a senescence-related protein involved in the protection of membrane lipids and enzymes), and *ERD8* (heat shock protein hsp8) from Arabidopsis (**Supplementary Fig. S1B**). A difference of only two amino acids, numbered 80 and 135, substituting aspartic acid and leucine (in cultivated tomato) with glycine and proline, respectively (in *S. pennellii*) (**Supplementary Fig. S1C**), was also observed.

Expression analysis of *SpERD15* and subcellular localization

Tissue-specific expression analysis revealed the *SpERD15* mRNA abundances in roots, stems and old leaves of *S. pennellii* (**Fig. 1A**). The transcript regulation of *SpERD15* was studied to dissect its induction in response to various stresses in *S. pennellii*. Real-time reverse transcription–PCR (RT–PCR) analysis showed transcript up-regulation by all the stress treatments. A steady-state increase in *SpERD15* transcription was observed in response to drought (dehydration), salinity, cold, paraquat, ABA, gibberellic acid and ethylene (**Fig. 2**).

To gain insights into its subcellular localization, *SpERD15* ORFs were fused in-frame with *GFP* [cauliflower mosiac virus (CaMV) 35S–*SpERD15*–green fluorescent protein (GFP)] and, together with *GFP* alone (CaMV35S–GFP), was then delivered into tobacco BY-2 cells by particle bombardment. *SpERD15* was localized mainly in the nucleus (**Fig. 3**). Cells transformed with

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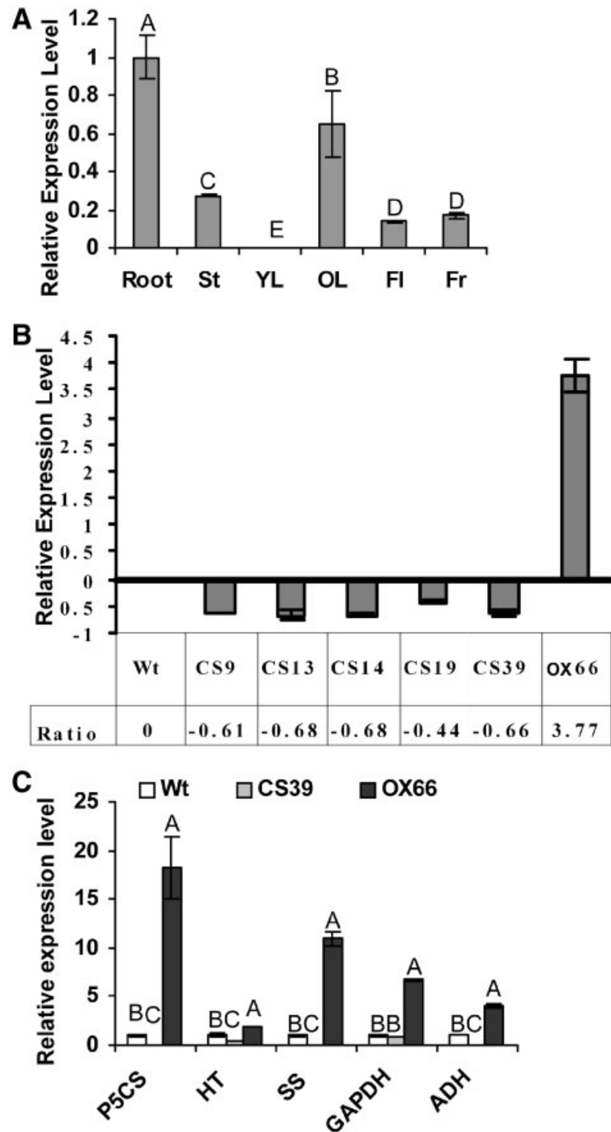

Fig. 1 Expression profile of *SpERD15*. (A) Expression analysis of *SpERD15* mRNA in different organs (root; St, stem; YL, young leaf; OL, old leaf; Fl, flower; Fr, fruit) of *S. pennellii*. (B) Expression of *SpERD15* in different transgenic tobacco lines. Ratio refers to the value of the *SpERD15* transcript in different transgenic lines relative to the control. (C) The transcript levels of Δ1-pyrroline-5-carboxylate synthase (*P5CS*), hexose transporter (*HT*), sucrose synthase (*SS*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and alcohol dehydrogenase (*ADH*) were quantitatively analysed in WT and transgenic tobacco lines. Error bars indicate ± SE of means at $P < 0.05$ ($n = 3$).

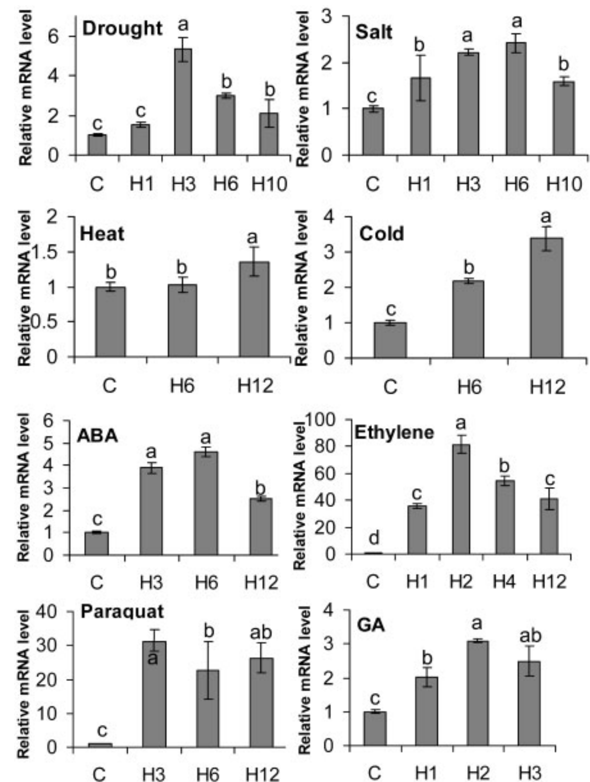

Fig. 2 *SpERD15* expression in response to various stress factors (drought, salt, heat, cold and paraquat) and phytohormones (ABA, ethylene and gibberellic acid). Plants were treated with 100 μM paraquat, ABA and gibberellic acid, and with 1 mM ethephon. All samples were collected at the indicated time points ('C' refers to control and 'H' refers to hours after treatment) from three biological replicates of each treatment. Error bars indicate ± SE of means at $P < 0.05$ ($n = 3$).

the vector containing only *GFP* displayed fluorescence throughout their structures.

Production of transgenic tobacco expressing *SpERD15*

To evaluate the functional significance of *SpERD15* and its effect on the physiology of transgenic plants, transformation was performed in tobacco. A total of 27 kanamycin-resistant T_0 plants were generated and further analyzed through PCR using CaMV35S forward and gene-specific reverse primers. Nine normally growing transgenic lines were analyzed for transcript levels of *SpERD15* through real-time PCR (data not shown). Of these lines, three co-suppression lines (referred to as CS13, CS19 and CS39) and one overexpression line (OX66) were selected for further investigations (Fig. 1B). Wild-type (WT) plants, as well as a vector control (transgenic line with no change in expression level of *SpERD15* compared with WT plants) in some cases, were used as controls wherever mentioned later. Both types of transgenic lines grew normally in T_0 and T_1 generations. Flowering and seed sets were delayed for 2–3 weeks in the transgenic lines compared with the WT plants in T_0.

Drought tolerance of transgenic tobacco lines

We analyzed the drought tolerance of the transgenic lines at different stages of plant growth, i.e. when the seedlings were 4 weeks old, 2 months old, and in the detached leaves from the mature plants prior to flowering. Four-week-old transgenic and

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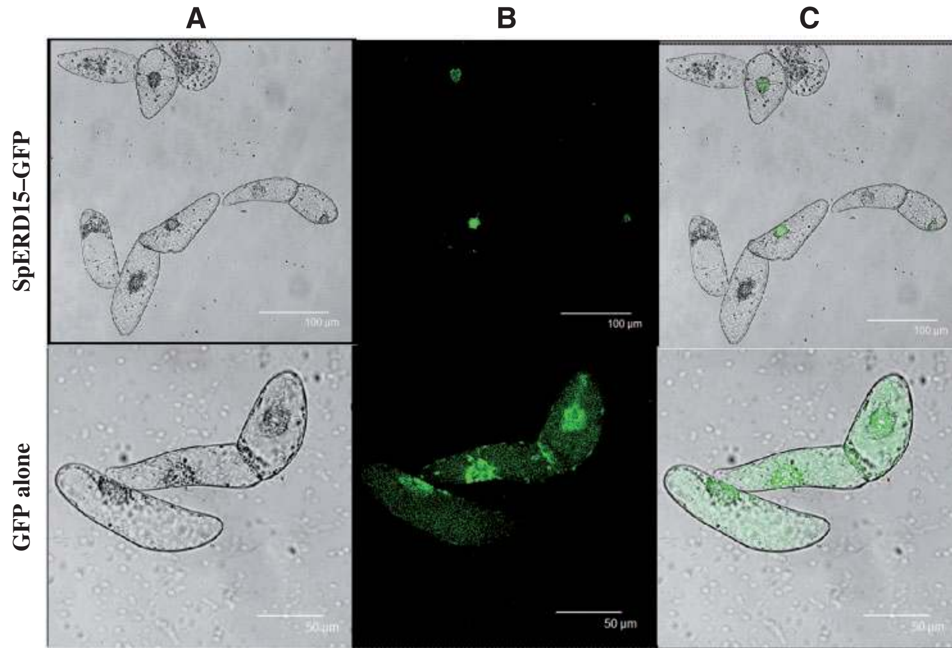

Fig. 3 Subcellular localization of *SpERD15* in tobacco BY-2 cells. The photographs were taken under bright light (A), in the dark field for GFP-derived green fluorescence (B), and merged (C), respectively. Plasmid with GFP alone served as the control.

WT plants were left unwatered for 15 d and observed for phenotypic differences. Co-suppression lines showed more withering compared with WT plants, whereas drought symptoms were less pronounced in OX66 plants (**Fig. 4A**). The relative leaf water content (RLWC) was the same for WT plants and all transgenic lines under control conditions. It was higher in OX66 plants compared with WT plants, but the latter had higher contents than co-suppression lines CS19 and CS39 (**Fig. 4B**) under stress.

Two-month-old plants subjected to drought stress for 21 d also exhibited the same phenotypic differences. The Chl fluorescence of both types of transgenic and WT plants was recorded to reveal changes in the efficiency of PSII. The results indicate that the maximum quantum yield of PSII (F_v/F_m) was not significantly changed under stress until day 12; however, this yield declined in both co-suppression and WT plants. On day 20, OX66 plants retained maximum quantum yields of PSII (F_v/F_m), but a considerable decrease was recorded in the co-suppression lines and WT plants (**Fig. 5A**). We then analyzed other parameters of Chl fluorescence for day 20. The efficiency factor of PSII or photochemical quenching (qP), as well as the PSII operating efficiency (ΦPSII), were significantly higher in OX66 than in WT and co-suppression plants (**Fig. 5B**). Non-photochemical quenching (NPQ) was also high in OX66 compared with WT and co-suppression plants, but the difference was not very significant (**Fig. 5C**). The operating efficiency of open PSII (F'_v/F'_m) was not noticeably affected (**Fig. 5C**).

A water loss assay also indicated more water loss in the co-suppression lines than in the vector control, indicating the decreased tolerance of these transgenic lines to dehydration stress (**Supplementary Fig. S2**). Overall, OX66 plants exhibited more drought tolerance than WT plants, and these WT plants performed better than co-suppressed plants, revealing that the silencing of *SpERD15* renders the plant sensitive to stress, whereas its overexpression confers tolerance.

Salinity and oxidative tolerance of *SpERD15* transgenic plants

The salinity tolerance of transgenic lines was also evaluated at different stages of plant growth and development. The salt tolerance of transgenic lines during seed germination was compared by shifting the germinating seeds (soon after radical emergence but before elongation of the hypocotyl) from selection medium to Murashige and Skoog (MS) medium containing NaCl (300 mM) and then allowing growth. Seeds that completed germination and growth were normal in OX66 seedlings after 5 d of culturing in the medium compared with WT plants, while seedlings of co-suppressed lines showed stunting and yellowing of cotyledons (**Supplementary Fig. S3A**). After 2 weeks, OX66 developed true leaves, as did WT plants. In contrast, most of the co-suppressed lines exhibited stunted growth with no true leaf production. To monitor the effect of salinity on root growth, 2-week-old seedlings were shifted to MS medium with 200 mM NaCl after root excision. After 21 d of culturing, the root growth of both co-suppressed lines, as well as those of the WT seedlings, was suppressed. The OX66 seedlings showed continuous root growth (**Supplementary Fig. S3B, C**). Whole-plant salt tolerance at the young seedling

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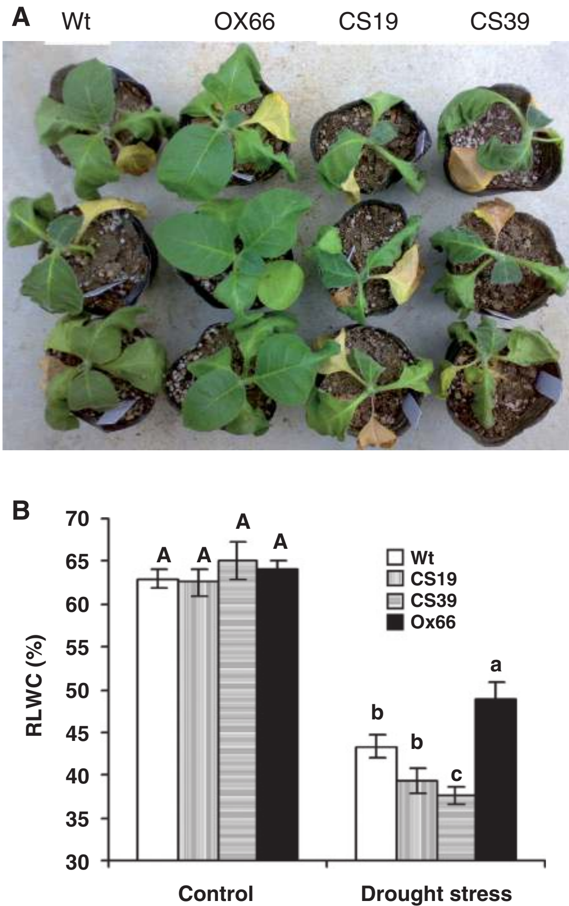

Fig. 4 Evaluation of drought tolerance of different transgenic lines. (A) Phenotypic appearance of drought-stressed transgenic and WT plants. (B) Relative leaf water contents of unstressed and stressed plants.

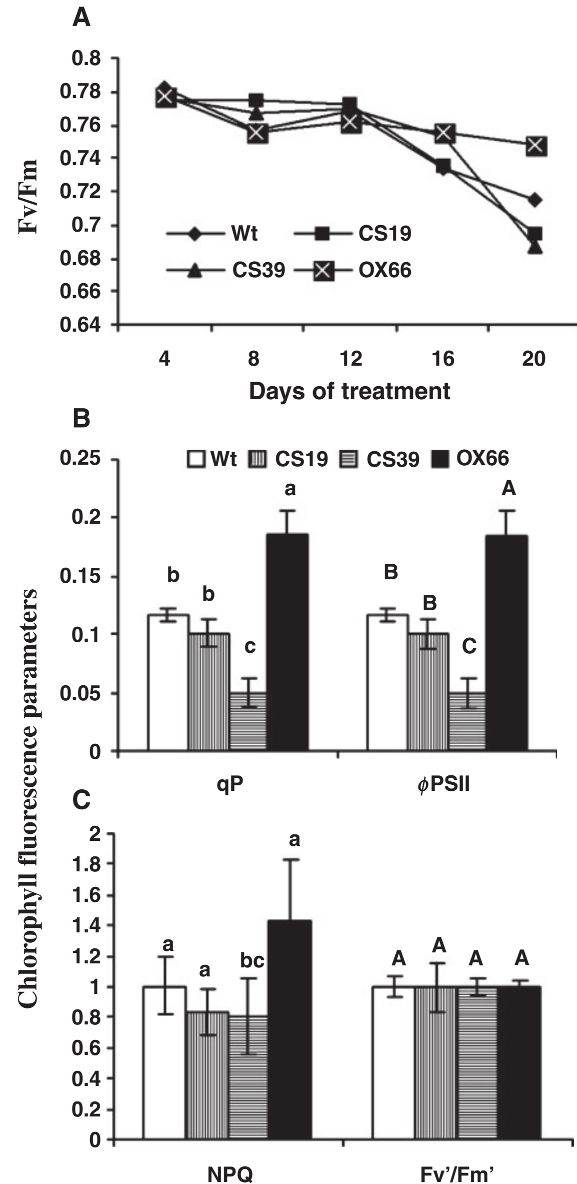

Fig. 5 Changes in the photosynthetic system under drought stress were assessed in terms of (A) maximal quantum yield of PSII (F_v/F_m); (B) photochemical quenching (qP) and the operating efficiency of PSII (ΦPSII); (C) non-photochemical quenching (NPQ) and operating efficiency of open PSII (F'_v/F'_m). Vertical bars bearing different letters for one parameter indicate significant differences at $P < 0.05$ ($n = 4$).

stage was determined by placing the seedlings with intact roots on filter paper wetted with 300 mM NaCl. After 2 weeks, the leaves of all co-suppression lines turned yellow; the degree of yellowing was according to the co-suppression level (**Supplementary Fig. S3D**). OX66 remained green, while WT seedlings showed a slight change in color. SPAD units for Chl content were also high for OX66 and WT seedlings compared with different co-suppression lines (**Supplementary Fig. S3E**). To assess salinity tolerance at the tissue level, leaf disks from transgenic and WT plants were floated in different concentrations of NaCl for 72 h, after which their total Chl content was determined. Differential loss of Chl was observed in different transgenic lines that seemed to be concentration and expression dependent. Loss of Chl was less in OX66 than in WT and co-suppression plants (**Supplementary Fig. S4**).

The effect of salt stress on plant growth was ascertained by irrigating WT and transgenic plants with 300 mM NaCl solution bi-weekly. After 5 weeks, the growth and productivity of co-suppression lines were severely affected by salinity compared with WT and OX66 plants, as evidenced by the biomass production and leaf area per plant (**Table 1**). This increase in leaf area of OX66 plants was not merely due to the increase in the number of leaves. Plant height was considerably higher in the OX66 plants than in both WT and co-suppression lines. Two determinants of plant relative growth rate, specific leaf area (SLA) and leaf area ratio (LAR), were significantly higher in OX66 plants than in WT and the CS19 and CS39 plants.

Both abiotic and biotic stresses caused oxidative bursts due to the production of ROS. Therefore, we evaluated the oxidative stress tolerance of different transgenic lines. Leaf disks from

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transgenic and WT plants were floated in different concentrations of H_2O_2 for 72 h. The Chl content of OX66 was high, whereas bleaching was more conspicuous in the co-suppression lines than in WT tobacco (**Supplementary Fig. S4**). The leaf disks were almost completely bleached in the co-suppression lines, showing a 40–50% reduction in Chl at 2% H_2O_2 concentration. In contrast, the WT and OX66 plants showed 35 and 29% reduction in total Chl contents, respectively, compared with the control (leaf disks kept in de-ionized distilled water) (**Supplementary Fig. S4**).

Cold tolerance of transgenic *SpERD15* plants

The response of *SpERD15* transgenic plants to cold stress was observed in seedlings of different ages. For cold stress in young seedlings, 3-week-old seedlings in MS were exposed to 4°C for 24 h, and then shifted to room temperature. The growth of most of the WT and CS39 seedlings remained stunted after 4 d of recovery, while most of the OX66 seedlings showed restored growth after 24 h of recovery. After 4 d of recovery, 80% of the OX66 seedlings showed healthy growth (**Fig. 6A**).

Table 1 Plant growth, biomass production and leaf area characteristics of WT and transgenic lines after treatment with 300 mM NaCl for 5 weeks

Line	No. of leaves per plant	Plant height (cm)	Biomass (g FW)	Leaf area per plant (cm^2)	SLA ($cm^2\,g^{-1}$)	LAR ($cm^2\,g^{-1}$)
Unstressed						
WT	16.5 ± 0.65 a	52.75 ± 5.72 a	103.45 ± 2.62 a	2507.97 ± 119.69 a	423.48 ± 43.59 a	245.03 ± 22.31 a
CS19	18 ± 1.00 a	58.67 ± 1.85 a	111.32 ± 7.72 a	2707.33 ± 112.40 a	424.13 ± 16.92 a	247.42 ± 13.84 a
CS39	16.67 ± 0.33 a	61.33 ± 1.30 a	116.07 ± 1.58 a	2675.13 ± 21.75 a	411.96 ± 10.79 a	233.88 ± 4.93 a
OX66	18.5 ± 0.5 a	60.25 ± 5.25 a	112.27 ± 6.36 a	2710.2 ± 83.2 a	405.19 ± 26.17 a	233.12 ± 26.20 a
Stressed						
WT	13.25 ± 0.63 ab	24.5 ± 1.66 b	51 ± 2.72 b	1355.63 ± 54.67 b	350.63 ± 8.75 b	235.12 ± 12.24 ab
CS19	12.5 ± 0.29 ab	23.75 ± 0.33 b	44.43 ± 0.19 c	1178.07 ± 13.61 b	326.13 ± 5.60 b	208.80 ± 4.15 b
CS39	11.75 ± 0.48 b	21.82 ± 0.97 b	44.51 ± 1.05 c	1178.5 ± 74.75 b	327.16 ± 5.38 b	215.32 ± 8.62 b
OX66	14.25 ± 0.48 a	34.72 ± 0.91 a	76.74 ± 2.09 a	2058.4 ± 137.98 a	393.59 ± 9.83 a	261.15 ± 12.68 a

Means (±SE) followed by the different letter for each parameter in a column indicate statistical differences at $P < 0.05$ ($n = 4$).

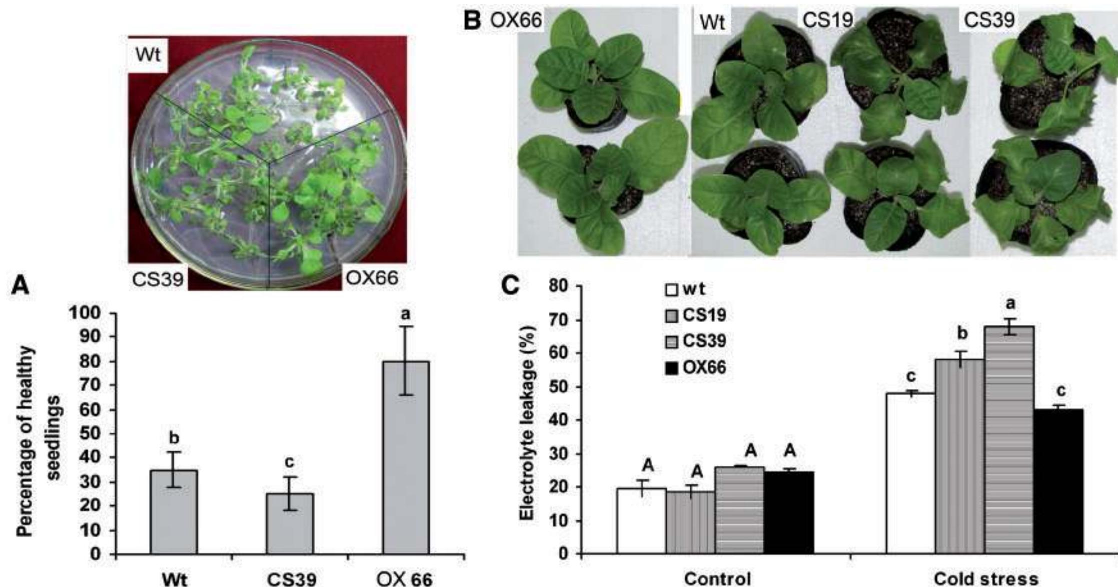

Fig. 6 Response of transgenic lines and WT plants to low temperature (4°C). (A) Seedlings in MS medium were placed in the growth chamber at 4°C for 24 h and then shifted to room temperature. Growth recovery was observed after 4 d. The experiment was repeated twice and, each time, at least eight seedlings in one Petri plate were used. (B) Seedlings grown in pots were exposed to low temperature (4 ± 0.5°C) for 7 d and (C) membrane damage was assessed by electrolyte leakage test. The experiment was repeated twice, with at least eight seedlings each time. Vertical bars bearing different letters in one group of treatment indicate significant differences at $P < 0.05$ ($n = 4$).

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For further analysis of cold tolerance, pot-grown transgenic and WT seedlings were exposed to 4 ± 0.5°C for 7 d. Co-suppression lines showed cold injury symptoms and lost turgidity more rapidly than WT seedlings (**Fig. 6B**). The over-expression plants remained healthy without any symptoms or change in turgidity. Membrane damage in terms of electrolyte leakage is a good indicator of susceptibility and/or tolerance of a genotype to cold stress (Pasquali et al. 2008). Although statistically similar, the membranes of the OX66 lines were comparatively less leaky than those of WT seedlings. The co-suppression plants exhibited more membrane damage than the WT and OX66 plants (**Fig. 6C**).

Differential tolerance of transgenic lines to osmotic stress

The enhanced tolerance of OX66 and conspicuous sensitivity of the co-suppression lines over the WT to drought, salt and cold stress motivated us to perform an osmotic stress assay. Seedlings shifted to MS medium were supplemented with different concentrations of sorbitol after root excision. At 200 mM concentration, no significant difference was observed in the growth of OX66 and WT seedlings; however, growth and leaf color were significantly changed in the co-suppression lines (data not shown). Chlorosis was more prominent in the leaves of co-suppression lines at a sorbitol concentration of 300 mM, whereas OX66 plants displayed less chlorosis than both WT and co-suppression plants (**Fig. 7**). At the same concentration of sorbitol, root growth was inhibited in the co-suppression lines, followed by WT seedlings, compared with the OX66 seedlings. Root length, as well as the number of secondary roots, was higher for OX66 than for WT and co-suppression seedlings (**Fig. 8**).

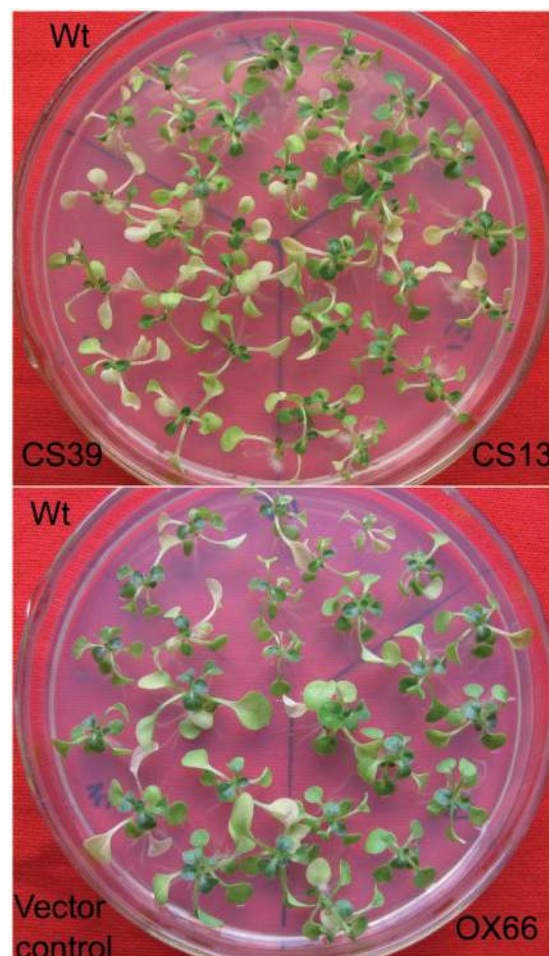

Fig. 7 Effect of osmotic stress on the phenotypic appearance of transgenic lines. Seedlings obtained after root excision were cultured in MS medium supplemented with 300 mM sorbitol. Photographs were taken after 21 d of culture.

Changes in soluble osmolyte content and lipid peroxidation in the transgenic lines

Different chemical compounds, such as proline, soluble sugars, sugar alcohols and quaternary ammonium compounds, are known to accumulate in response to different abiotic stresses (reviewed by Parvanova et al. 2004, Bartels and Sunkar 2005). Therefore, we analyzed the changes in the level of some of these biochemical markers in transgenic and WT plants under stress conditions. The proline content of both WT and transgenic plants was increased in plants subjected to drought and cold stress (**Fig. 9A**). The proline content of OX66 plants was significantly higher than that in WT plants. The proline contents of co-suppression lines CS19 and CS39 were significantly less than in both WT and OX66 plants under drought and cold stress. Soluble sugars were considerably higher in OX66 transgenic plants than in both WT and co-suppression lines under unstressed conditions (**Fig. 9B**). Under stress, soluble sugar contents increased in all transgenic and WT plants; this increase was more observable in OX66 plants, followed by WT plants, under both types of stress. Among the co-suppression lines, the soluble sugar content of CS39 plants under cold stress was the same as that of OX66 under normal growth conditions. The soluble sugar content in both co-suppression lines (CS19 and CS39) under drought stress conditions increased, but it remained considerably lower than in the WT and OX66 plants (**Fig. 9B**).

The malondialdehyde (MDA) content was determined in cold, drought and osmotically stressed WT and transgenic plants as a measure of lipid peroxidation. The MDA content increased in both WT and transgenic seedlings under stress conditions. However, this increase was significantly higher in drought-stressed co-suppression lines than in both WT and *SpERD15*-overexpressing plants (**Fig. 10A**). The transgenic line overexpressing *SpERD15*, when exposed to low temperature (4°C), showed an inhibition of the increase in MDA content, which was maintained at a level similar to that in unstressed plants (**Fig. 10B**). The co-suppression lines had a higher MDA content under cold stress. At a sorbitol concentration of 200 mM, the level of MDA in WT and OX66 plants was significantly less than in the *SpERD15* co-suppression lines. High

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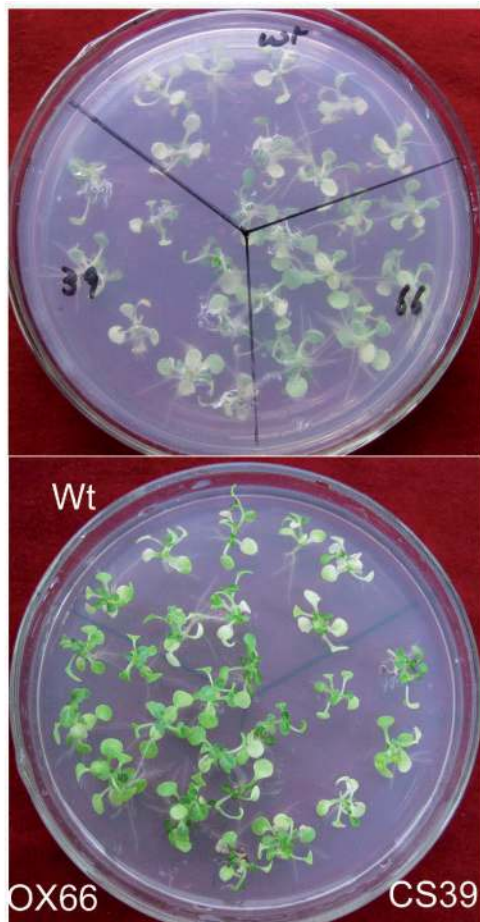

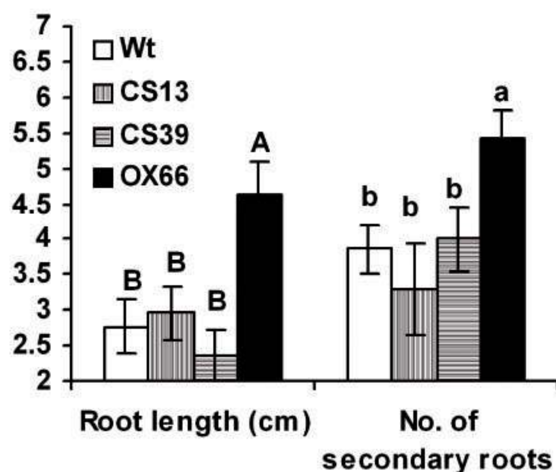

Fig. 8 Root growth in response to osmotic stress (sorbitol 300 mM). Vertical bars bearing different letters for one parameter indicate significant differences at $P < 0.05$ ($n = 5$).

sorbitol concentrations (300 and 400 mM) induced an increase in MDA levels in both WT and co-suppression lines, in contrast to overexpression lines; this increase was more in the co-suppression lines than in the WT plants (Fig. 10C). MDA is a biomarker for oxidative stress, so we sprayed the transgenic and

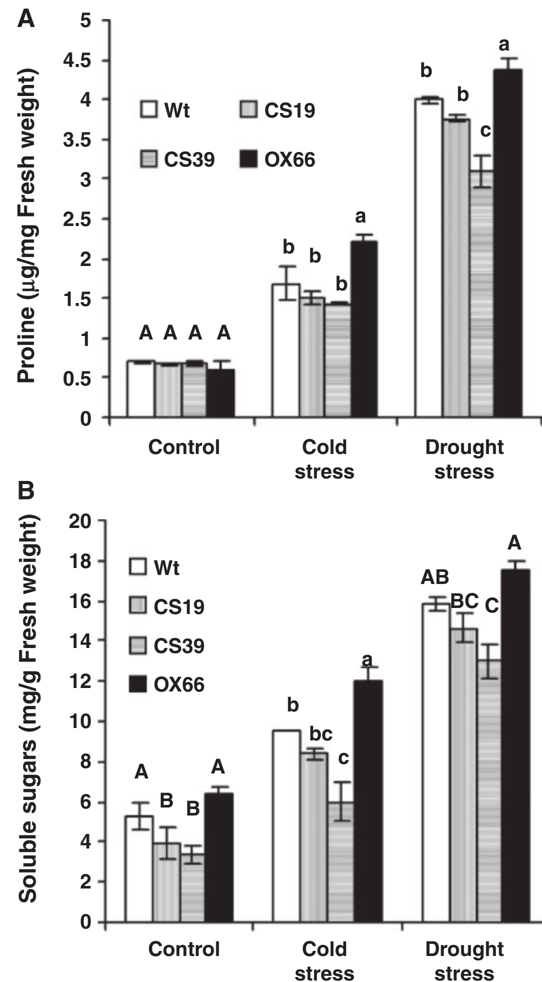

Fig. 9 Changes in the contents of proline (A) and soluble sugars (B) in response to drought and cold stress. Vertical bars bearing different letters in one group of treatment indicate significant differences at $P < 0.05$ ($n = 4$).

WT seedlings with NaCl (200 mM) and ABA (100 µM) to mimic oxidative and drought stress, respectively. The MDA content increased 3-fold in the co-suppression lines in response to salt spray, whereas it increased 2-fold in the WT and OX66 plants (Fig. 10D). Foliar application of ABA (100 µM) induced an almost 4-fold increase in MDA content in CS39, a 3-fold increase in WT plants and only a 2.75-fold increase in OX66 (Fig. 10D). This distinctive change in MDA contents in different transgenic lines indicates that overexpression of *SpERD15* reduces oxidative damage after exposure to stress conditions, in contrast to its co-suppression.

Analysis of gene expression in transgenic plants

To investigate the possible role of *SpERD15* in abiotic stress response, the expression of some stress-related genes [Δ1-pyrroline-5-carboxylate synthase (*P5CS*), *Sucrose synthase*, alcohol dehydrogenase (*ADH*) and glyceraldehyde phosphate dehydrogenase (*GAPDH*)] was analyzed in *SpERD15* transgenic

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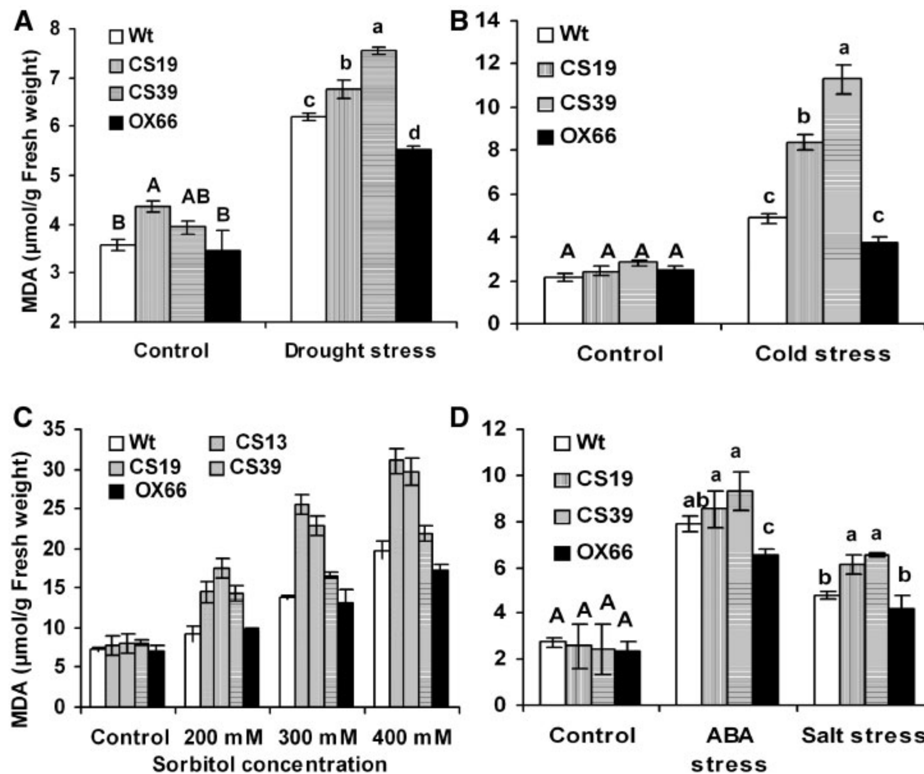

Fig. 10 MDA content in response to drought (A), cold (B), sorbitol stress (C) and foliar application of ABA and salt (200 mM NaCl) (D) in transgenic lines and WT plants under different stress conditions. Vertical bars bearing different letters in one group of treatment indicate significant differences at $P < 0.05$ ($n = 4$).

lines and WT plants growing under normal conditions by real-time RT–PCR analysis (**Fig. 1C**). Overexpression of *SpERD15* induced expression of stress-related genes, particularly those involved in sugar synthesis and transport and those related to lipid peroxidation. In contrast to this observation, expression of these stress-related genes was suppressed in the co-suppression lines. It is possible that *SpERD15* functions in abiotic stress responses by regulating the expression of these genes as a transcriptional regulator or, otherwise, interacting with other genes to alter the expression of transcription factors.

Discussion

In nature, plants are confronted with a number of stresses that induce or suppress the expression of a large number of genes. ABA plays an important role in plant responses against environmental constraints (Yamaguchi-Shinozaki and Shinozaki 2005). Its accumulation starts within 2 h after dehydration (Kiyosue et al. 1994a), and provokes the expression of a large number of genes (Seki et al. 2002). In addition to these genes, some genes are induced prior to the accumulation of ABA; *ERD15* is one of those genes (Kiyosue et al. 1994a). Although *ERD15* has been isolated and characterized in Arabidopsis, its function remains controversial (Kariola et al. 2006, Park et al. 2009, Li et al. 2010). Therefore, we isolated and characterized *ERD15* from *S. pennellii*. Expression profiling revealed differential accumulation of *SpERD15* transcript in plant tissues, which suggests that it plays a role in normal plant growth. In spite of some similarities between *SpERD15* and *AtERD15*, the expression pattern of *SpERD15* is quite different. *SpERD15* mRNA accumulation, in contrast to the rapid (within minutes) and transient increase of *AtERD15* expression (Kariola et al. 2006), is gradually enhanced by drought (dehydration), salinity, cold, ABA, gibberellic acid and ethylene treatments in *S. pennellii*.

To elucidate the physiological role of *SpERD15*, we generated 27 transgenic tobacco lines. However, the expression analysis of the transgenic plants showed down-regulation of the *SpERD15* transcript in most of the transgenic lines, except for one that showed overexpression. Co-suppression is not a rare phenomenon in genetically manipulated plants, overexpressing the genes involved in basic regulatory functions of the cells, to modulate the endogenous expression level of the gene (Jorgensen 1995, Smith 1990). This suppression seems to be due to the highly sensitive nature of the genomes that are able to sense and react to violations of their integrity. The sense co-suppression, found in the present study, could be due to the accumulation of transcripts at high levels, which may be due to single copy insertion (Que 1997), and is common for the genes involved in basal defense systems

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(Ishikawa et al. 2005). The overexpression line possibly avoided co-suppression because of the positional effect of the transgene, which lessens the transcript level of only the transgene without affecting the transcript from the endogenous gene, as reported in a number of studies (Clark 1994, Matzke and Matzke 1998, Pedram 2006).

Functional analysis showed that overexpression plants survived better under drought, salt and cold stress, whereas co-suppression plants were more susceptible to these than WT plants. Physiological analysis revealed high proline and sugar accumulation, particularly the latter, in overexpressing plants. The elevated osmolyte concentration in overexpressing plants, which was higher than in the WT and co-suppression plants, was in line with the expression level of *P5CS* and *sucrose synthase* genes. These solutes work in a variety of ways, such as protection of cellular structures, detoxification of the enzymes and scavenging of ROS alone or in combination with other defense-related enzyme systems (Verma 1999, Xiong et al. 2002). These compounds confer integrity to the membranes (Xiong et al. 2002) and keep the photosynthetic system functioning, as evidenced by the higher F_v/F_m values observed for overexpression plants compared with WT and co-suppression lines. Enhanced photoinhibition in co-suppression lines may be due to lower proline levels because transgenic tobacco producing betaine showed less photoinhibition than WT plants (Holmstrom et al. 2000).

Plasmalemma is known to have a key role in the perception and transmission of signals. Cold stress affects membrane fluidity, while compatible solutes help in protection of membranes (Xiong et al. 2002). Therefore, better performance of overexpression lines under cold stress can be attributed to increased levels of osmolytes, which may have helped in retention of sensing and transference of stress signals from the plasmalemma. Moreover, accumulation of these solutes helped to overcome the osmotic component of the salinity and resulted in the continued growth of *SpERD15* overexpression plants. This additional surface area helped to sequester toxic ions (Bartels and Sunkar 2005), and yielded significantly higher biomass accumulation and leaf area.

Co-suppression plants exhibited more lipid peroxidation, as revealed by the high MDA levels of these plants. *GAPDH* is well known to impart tolerance to oxidative stress (Miyasaka 2000) and, thus, reduces lipid peroxidation. Both *ADH* and *GAPDH*, previously reported to confer drought tolerance in cotton (Zhang et al. 2009), were highly regulated in overexpression plants that had comparatively lower MDA content than in WT and co-suppression plants. The molecular mechanism of the enhanced transcript level of these stress-related genes needs to be unveiled.

Improved tolerance against different stresses by single gene manipulation was reported earlier for genes involved in transcriptional activation and metabolic functions (Hou et al. 2009, Hsieh et al. 2010). Our results demonstrate that increased tolerance of *SpERD15* overexpression plants to different abiotic stresses was possibly due to osmotic adjustment, enhanced cell membrane integrity and detoxification of toxic radicals, as conferred by the compatible solutes accompanied by the increased expression of stress-related genes. The increased expression of stress-related genes may be due to regulation by *SpERD15* overexpression; this aspect requires further experimentation. Further investigations to understand the molecular mechanism of stress tolerance fully in *SpERD15* overexpression plants are in progress.

Materials and Methods

Plant material and stress treatments

Tomato (*Solanum lycopersicum*) variety Ailsa Craig (AC) and one wild species *S. pennellii* (LA0716) were used to compare nucleotide sequences between wild and cultivated species. Expression of *ERD15* was checked in different organs of *S. pennellii* seedlings, as well as in response to different stresses, namely drought (dehydration), salinity (200 mM NaCl), cold (4°C), heat (40°C), paraquat (100 μM), ABA (100 μM), gibberellic acid (100 μM) and ethylene (ethephon 1 mM).

Vector construction and plant transformation

The tomato *ERD15* (referred to *SpERD15*) was amplified from the cDNA and genomic DNA of *S. pennellii* and a cultivated variety, using sequence-specific primers (*SpERD15* Fw and Rv) based on the unigene sequence (SGN-U584748; **Supplementary Table S1**). The amplified PCR product was cloned into the pMD18-T vector, and the correct sequence was identified after sequencing. The pMD18-T vector containing the correct sequence of *SpERD15* cDNA was double digested using *Kpn*I and *Sal*I, and ligated into the plant binary vector pBI121, cleaved with *Kpn*I and *Xho*I. The resulting construct, under the control of a strong constitutive promoter (CaMV35S), was transformed into *Agrobacterium* strain C58 through electroporation and used for transformation in tobacco (*Nicotiana tabacum* L.) using the standard leaf disk protocol (Horsch et al. 1985). The T_0 plants were selfed, and T_1 seeds of the selected transgenic lines were germinated on kanamycin (100 mg l^{-1}) selection medium. For the expression of *SpERD15* proteins in *Escherichia coli*, pMD18-T vector containing the correct sequence of *SpERD15* and pET-28a(+) vector was digested with *Bam*HI and *Sal*I. The *SpERD15* fragment was ligated with digested pET-28a(+) vector and transformed into *E. coli* strain BL21. A 20 μg aliquot of crude protein extracts from isopropyl-β-D-galactopyranoside (IPTG)-induced cultures was separated by 12% SDS–PAGE in triplicate.

Molecular analysis of transgenic plants

Genomic DNA of kanamycin-resistant seedlings was used as a template for further confirmation through PCR using a sense primer for CaMV35S (**Supplementary Table S1**) and an antisense primer specific for *SpERD15*. Total RNA was extracted using TRIzol reagent (Invitrogen) from stressed and

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unstressed plants, treated with DNase I, and then reverse transcribed using MMLV (TOYOBO) reverse transcriptase and oligo(dT). The resulting cDNA was used for the amplification of the target gene (from unstressed plants) and expression analysis (stress treatments) using gene-specific primers for both tobacco and tomato (*QSpERD15*, **Supplementary Table S1**). The β-actin gene was used as a reference gene to optimize semi-quantitative RT–PCR and as internal standard in real-time RT–PCR for tobacco (accession No. X69885; NtActin) and tomato (SGN-U580609, TomActin). Expression of stress-related genes in transgenic tobacco lines was studied using the primer sequences mentioned in **Supplementary Table S1**. Real-time RT–PCR was performed in triplicate in an optical 96-well plate using a LightCycler 480 (Roche) PCR system.

Bioinformatics analysis

Sequencing results of *SpERD15* were used to search for homology, on both a protein and a nucleotide basis, at NCBI (http://www.ncbi.nlm.nih.gov) and the SOL genomics network (http://www.solgenomics.net). The nucleotide and deduced amino acid alignments were computed by the ClustalW program employing standard parameters, and shaded in GENEDOC. The phylogenetic tree was generated in MEGA 4 software, using the Neighbor–Joining method. The theoretical molecular weight, pI and total number of positively and negatively charged residues were predicted using the ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html). Introns in the genomic DNA were computed by the Splign tool at NCBI (http://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi).

GFP fusion construct and transient expression assay

The full-length ORF of *SpERD15* was PCR-amplified using the *SpERD15* overexpression vector as a template. The terminal codon was deleted from the ORF and fused with the GFP sequence. A full-length *SpERD15–GFP* fusion fragment was amplified using a pair of primers (**Supplementary Table S1**) with *Sal*I and *Sac*I restriction sites (underlined) in the forward and reverse primers, respectively. The resultant fusion product was cloned in the modified pBI121 vector, and verified by sequencing and double digestion.

Tobacco BY-2 cells were transiently transformed with a plasmid carrying the CaMV35S–*SpERD15–GFP* fusion construct and CaMV35S–*GFP* as the control, using a Biolistic PDS-1000/He Particle delivery system (Bio-Rad). Gold particles were coated with plasmids and fired into BY-2 tobacco cells, which were cultured on MS medium, under vacuum, using 1,100 p.s.i. rupture disk pressure (Bio-Rad). After incubation for 18 h at 25 ± 2°C, GFP signals were detected using a confocal microscope (Zeiss, LSM510).

Drought tolerance and water loss assay

In all experiments, the T_1 generation seeds of transgenic lines, after surface sterilization and germination on MS medium supplemented with kanamycin (100 mg l^{-1}), were used for assays. Four-week-old plants were watered by placing the pots in a tray containing water before subjecting them to drought stress. Plants were then left unwatered and photographed after 15 d. RLWC, sugar, proline and MDA content of the transgenic and WT seedlings were determined. To monitor the drought tolerance of transgenic lines at the late vegetative growth stage, 2-month-old plants were subjected to drought stress by withholding water for 21 d. The effect of drought stress on the efficiency of the photosynthetic system was analyzed by measuring Chl fluorescence. The relative humidity in the greenhouse varied between 55 and 65%. Each experiment was repeated twice with at least six plants of each of the transgenic lines and the WT. For the water loss assay, young, fully expanded leaves of the WT and transgenic lines were detached and placed on filter paper under white florescent light and weighed periodically at an interval of 30 min up to 6 h. Water loss was expressed as a percentage of the control.

Salt stress tolerance assay

To compare the effect of salt stress on seed germination, germinating seeds (soon after radical emergence but before hypocotyl elongation) were shifted to MS medium supplemented with 300 mM NaCl and allowed to grow for 14 d. The effect of salt stress on root growth was evaluated by shifting 14-day-old seedlings to MS medium with 200 mM NaCl after root excision. Plates were oriented vertically and root length was measured after 3 weeks. To monitor the effect of salt stress on seedlings, positive seedlings selected on kanamycin-containing medium were placed on filter paper wetted with 300 mM NaCl, in sealed Petri plates. After 2 weeks, the Chl content of the third leaf from the top was recorded using a SPAD Chl meter, and the seedlings were photographed. One-month-old transgenic plants were also sprayed with ABA (100 μM) and NaCl (200 mM). Leaf samples were collected after 4 h of treatment and their MDA contents were estimated. In a detached leaf salt stress assay, leaf disks (1 cm diameter) were floated in different concentrations of NaCl (300 and 400 mM) under continuous white light at 25 ± 2°C. Chl content was measured after 72 h. The assay was repeated twice.

To assess the effect of salinity on the growth of transgenic lines, transgenic and WT plants grown in pots were supplied bi-weekly with equal volumes of 300 mM NaCl and water. The control plants were supplied with an equal volume of tap water. After 5 weeks of treatment, plant growth was evaluated in terms of plant height, number of leaves retained, leaf area per plant, SLA and LAR.

Cold stress assay

To evaluate the cold tolerance of different transgenic lines, 3-week-old seedlings in MS medium were placed in a growth chamber at 4°C for 24 h. Petri dishes containing the seedlings were shifted to room temperature (25 ± 2°C) for recovery for 4 d. Seedlings were scored as healthy or stunted on the basis

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of their phenotypic appearance. For further analysis of cold tolerance, WT and transgenic seedlings grown in pots were exposed to low temperature (4 ± 0.5°C). After 7 d of exposure to low temperature, membrane permeability, soluble sugars, proline and the MDA content of both cold-stressed and unstressed seedlings were determined. The experiment was repeated twice, using at least 16 plants of every transgenic line each time.

Osmotic and oxidative stress assay

Fourteen-day-old seedlings were used for the osmotic stress assay. Roots of the seedlings were excised and the shoots were cultured in solid MS medium (containing 0.5% sucrose) supplemented with different concentrations of sorbitol (200, 300 and 400 mM). After 25 d, the root length and the number of secondary roots (for the 300 mM sorbitol concentration) were measured, and the MDA contents of shoots were determined (for all concentrations). The experiment was repeated three times, with two Petri dishes (each containing eight seedlings) as replicates.

To determine osmotic and oxidative stress tolerance in mature plants, leaf disks (1 cm in diameter) from young, fully expanded leaves were suspended in different concentrations of H_2O_2 (1 or 2%) solution and placed under continuous white light at 25 ± 2°C as described previously by Munis et al. (2010). Differences among the transgenic lines and control were observed after 72 h by extracting Chl. The results were expressed as a percentage of the relative reduction in different treatments over the control to clarify the difference among the lines. The experiment was repeated twice.

Determination of MDA, proline and soluble sugar content

MDA was assayed for indirect evaluation of lipid peroxidation using thiobarbituric acid, as described previously by Hodges et al. (1999), and expressed as μmol g^{-1} FW. Proline and soluble sugars were essentially extracted using the procedure described by Li et al. (2004).

Measurement of Chl content and photosynthetic efficiency

Chl content was determined by grinding the samples in 80% acetone. Chl concentrations (μg mg^{-1} FW) were determined using Wellburn's (1994) equation and are presented as the relative change in Chl content over the control (unstressed treatment).

The response of the photosynthetic apparatus to drought stress was analyzed by measuring different fluorescence parameters on the light- and dark-adapted leaves of WT plants and different transgenic lines using a portable Chl fluorescence meter (Handy PEA) as described elsewhere (He et al. 2009).

Supplementary data

Supplementary data are available at PCP online.

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