Delayed leaf senescence induces extreme drought tolerance in a flowering plant

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Drought, the most prominent threat to agricultural production worldwide, accelerates leaf senescence, leading to a decrease in canopy size, loss in photosynthesis and reduced yields. On the basis of the assumption that senescence is a type of cell death program that could be inappropriately activated during drought, we hypothesized that it may be possible to enhance drought tolerance by delaying drought-induced leaf senescence. We generated transgenic plants expressing an isopentenyltransferase gene driven by a stress- and maturation-induced promoter. Remarkably, the suppression of drought-induced leaf senescence resulted in outstanding drought tolerance as shown by, among other responses, vigorous growth after a long drought period that killed the control plants. The transgenic plants maintained high water contents and retained photosynthetic activity (albeit at a reduced level) during the drought. Moreover, the transgenic plants displayed minimal yield loss when watered with only 30% of the amount of water used under control conditions. The production of drought-tolerant crops able to grow under restricted water regimes without diminution of yield would minimize drought-related losses and ensure food production in water-limited lands.

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grown in the greenhouse under optimal conditions for 40 days. During this period, transgenic plants did not differ in appearance from WT plants (Fig. 1A). After this initial period, plants were subjected to a severe drought treatment that consisted of withholding watering for a period of 15 days. During the drought period, the WT plants wilted and senesced progressively, whereas the transgenic plants partially wilted but did not display drought-induced senescence (Fig. 1A). After the drought treatment, plants were rewatered for 1 week. The WT plants did not recover from the stress and died. However, the transgenic plants recovered, the leaves displayed full turgor, and the plants resumed their growth. The root and shoot biomass of WT plants was severely reduced (65% and 75%, respectively) (Fig. 1B) with respect to transgenic plants after the drought treatment, and transgenic plants displayed a 160% increase in seed yield over that of the WT plants (Fig. 1C). Measurements of soil water potential (Fig. 2A) showed that the soil in both the WT and transgenic plants dried equally, reaching its lowest value (~18 MPa) after 15 days without watering and becoming more positive (~0 MPa) after rewatering. The water content of WT plants was severely reduced during the drought period, reaching a value of ~60% (Fig. 2A). However, the water content of the transgenic plants was merely reduced from 92% to 86% during drought and returned to control levels after rewatering, showing that the transgenic plants were superior to the WT plants in maintaining leaf water potential during drought, even though they partially wilted.

**IPT Expression and Cytokinin and ABA Contents.** To assess the relationship among IPT expression, CK biosynthesis, and drought tolerance, we examined the expression of PSARK::IPT and CK contents in basal, middle, and apical leaves of plants from 15 days after germination until the end of the rewatering treatment (70-day-old plants) (Figs. 2B and 3A–D). When grown under normal conditions, the levels of PSARK::IPT expression were similar in leaves, the expression increasing toward plant maturation (Fig. 2B). In our experiments, PSARK::IPT expression reached maximal levels in all leaves during the drought period, decreasing after 6 and 8 days of growth in apical and basal leaves, respectively, and remaining constant during drought in the middle leaves (Fig. 2B). The expression of PSARK::IPT during drought was not only enhanced in basal leaves but also in the middle and apical leaves, confirming the drought responsiveness of the SARK promoter. After rewatering, PSARK::IPT expression remained high only in the senescent tissue (i.e., basal leaves) and decreased in the actively growing tissue (middle and apical leaves) (Fig. 2B).

During drought, there was a significant increase in transzeatin CK, which is considered to be one of the highly active forms of CK (11), mainly in the older (basal) leaves, its concentrations increasing one order of magnitude with respect to that of WT plants [Fig. 3A and supporting information (SI) Figs. 7–9]. Large increases (up to 100 times) in the concentrations of the N- and O-glycosylated forms,
which are less active or nonactive forms, were seen in the basal and middle leaves during stress, and no clear pattern was seen in the apical leaves (Fig. 3 B–D and SI Fig. 10).

ABA accumulation has been correlated with increased drought tolerance (12). A reciprocal relationship between ABA and CK contents during drought stress has been postulated (13), although data supporting this notion are not conclusive (14). Drought treatments induced an increase in ABA concentrations in both WT and PSARK::IPT transgenic plants, being higher in apical leaves (Fig. 3E). Nevertheless, CK production was not affected by ABA levels, and there were no clear differences between the ABA contents of the WT and PSARK::IPT transgenic plants, indicating that changes in ABA were not associated with the drought tolerant phenotype displayed by our transgenic plants.

Oxidative Metabolism in WT and PSARK::IPT Plants. To gain insight into the mechanism(s) associated with CK-dependent enhanced drought tolerance of the transgenic plants, we performed an array analysis of transcript expression of ~300 stress-related genes (15, 16) in the WT and transgenic plants before, during and after a prolonged drought treatment (Fig. 4 and SI Table 1). There were minimal differences in the expression of different stress-related transcripts between the WT and transgenic plants before drought, in agreement with the lack of phenotypical differences between the WT and transgenic plants grown under the control growth conditions (Fig. 4B). However, a comparison between transcript expression in transgenic plants before and after drought revealed that 66% of the transcripts tested were up-regulated in the transgenic plants, ~20% of these transcripts being related to reactive oxygen species (ROS) metabolism (Fig. 4B and SI Table 1). With the exception of cytosolic superoxide dismutase (cytSOD1) and dehydroascorbate reductase (cytDHAR), all of the transcripts encoding antioxidative enzymes present on the arrays were down-regulated [peroxisomal catalase (CAT); stromal ascorbate peroxidase, and thylakoid-bound APX; cytosolic monodehydroascorbate reductase (MDHAR); glutathione reductase (GR); and glutathione peroxidase] in the WT plants during drought, whereas the expression of all of these transcripts (including cytSOD1 and cytDHAR) was up-regulated in the transgenic plants during drought (Fig. 4D and SI Table 1). Transcript expression correlated well with changes in enzymatic activities for the different ROS-scavenging enzymes (Fig. 4D).

Redox State and H₂O₂ Accumulation During Drought. During stress, disruption of cellular homeostasis is accompanied by the generation of ROS, and the extent of stress-induced damage can be attenuated by the action of the cell’s antioxidant systems, including ascorbate, glutathione, and enzymes capable of scavenging ROS (17, 18). During stress, cellular ascorbate and glutathione pools shift toward their oxidized forms, and the redox state of ascorbate and glutathione is decreased (19, 20). Similar effects on the reducing pools occur during leaf senescence (21). Changes in the ascorbate and glutathione redox state have been shown to affect gene expression, and their involvement in the regulation of metabolic pathways is postulated in refs. 22 and 23. The reduced state of ascorbate and glutathione in the WT plants declined significantly during drought, whereas, in the transgenic plants, it did not change during drought and rewatering (Fig. 5A), indicating that the antioxidative systems were not affected by the severe drought in transgenic plants. To test this conclusion, we measured H₂O₂ concentrations before, during, and after drought (Fig. 5B). Whereas leaves from transgenic plants did not show significant differences in H₂O₂ contents before, during, or after drought, the H₂O₂ contents in leaves from WT plants increased dramatically. These observations were supported by specific staining for H₂O₂ in leaves of WT plants during stress but not in leaves of transgenic plants (Fig. 5C).

Effects of PSARK::IPT on Yields Under Restricted Watering Regimes. Drought-induced decrease in crop productivity is in part due to inhibition of photosynthesis under drought (24) and is brought about by the reduction in both the capacity for ribulose-1,5-bisphosphate regeneration and carboxylation efficiency (25). Whereas after 2–3 days of drought stress photosynthesis was completely inhibited in the WT plants, the transgenic plants expressing PSARK::IPT maintained some photosynthesis during the entire drought treatment and fully recovered their photosynthetic activity after rewatering (SI Fig. 11). Moreover, compared with the WT plants, water use efficiency (WUE) was markedly improved in the transgenic plants, values being two to three times higher than those before drought (SI Fig. 12). The improved water use efficiency shown by the transgenic plants prompted us to test whether these plants could grow and produce significant yields under conditions where their water supply was significantly reduced during growth. Wild-type and the two independent transgenic plants expressing PSARK::IPT were grown under two different
watering regimes: (i) fully watered (1 liter/day) and (ii) a restricted water regime (0.3 liter/day). The experiments started 15 days after germination and continued for 4 months. The biomass and seed yield of the WT plants were severely affected by the restrictive watering treatment; plants showed a reduction of 57% and 60% in biomass and seed yield, respectively (Fig. 6). However, the transgenic plants displayed a minimal reduction, albeit not statistically significant, in biomass and seed yield (8–14%).

Discussion
Most climate-change studies indicate an expansion of arid zones on our planet. This increase in arid land and the world’s growing population will have a direct impact on water resources and water availability. Water scarcity and the concurrent high temperatures create the most significant limitations to crop productivity (26). Our objective is to generate transgenic plants that can survive severe drought episodes and that can grow under restricted water regimes with minimal yield losses. Here, we show that the expression of an IPT gene, driven by a maturation- and stress-induced promoter, induced the synthesis of CKs in the plant and that the production of CKs contributed to the enhanced drought tolerance of the transgenic plants. Steady-state levels of active CK are determined by the rate of release of CK nucleobase from CK-conjugates coupled with CK degradation or conjugation or both (11). The high levels of $P_{SARK}$::IPT expression during stress seen in the younger leaves, together with the lack of accumulation of biologically active forms (IZ, IP, and DZ), would suggest that most of the CKs in these leaves are present in the form of CK-conjugates or were translocated to the bottom leaves. Interestingly, although our results showed a moderate increase in ABA concentrations during drought, there were no clear differences between WT and $P_{SARK}$::IPT transgenic plants.

![Fig. 4](image_url)

**Fig. 4.** Filter arrays hybridization and oxidative metabolism-related genes. (A) Representative filter arrays hybridizations. (B) Relative distribution of changes in transcripts expression in transgenic plants compared with WT. The treatments compared were before drought (B.D.), during drought (D.D.), and after rewatering (A.R.) as indicated in the figures. Yellow, no significant changes; red, up-regulation; blue, down-regulation. (C) Oxidative metabolism transcripts expression and images obtained from stress-related filter arrays hybridization. (D) Activities of the different enzymes participating in oxidative metabolism. Blue and red colors indicate down-regulation and up-regulation of enzymatic activity during drought, respectively (compared with controls). Values are the mean ± SE ($n = 12$).

![Fig. 5](image_url)

**Fig. 5.** Redox state and H$_2$O$_2$ production in WT and $P_{SARK}$::IPT. (A) Redox state of ascorbate and glutathione. Asterisks indicate significant difference ($P < 0.001$) between the transgenic lines and WT. (B) Leaf H$_2$O$_2$ concentrations. Values are the mean ± SE ($n = 12$). (C) H$_2$O$_2$-diaminobenzidine (DAB)-staining in WT and $P_{SARK}$::IPT transgenic plants during drought. Brown spots in the leaves represent H$_2$O$_2$ tissue localization.

![Fig. 6](image_url)

**Fig. 6.** Comparison between WT and transgenic $P_{SARK}$::IPT$_{2,36}$ and $P_{SARK}$::IPT$_{4,24}$ tobacco plants at optimal (1 liter/day) or restricted (0.3 liter/day) watering regimes. (A) Plants after 4 months of treatments. (B) Plant fresh weight at the end of the experiment. (C) Seed fresh weight at the end of the experiment. Asterisks indicate significant differences ($P < 0.001$) between the transgenic lines and WT. Values are the mean ± SE ($n = 24$).
The level of transcripts encoding enzymes associated with the glutathione-ascorbate cycle was greater in plants with elevated CK production (resulting from pSARK::IPT). Pools of reduced ascorbate and glutathione were also greater in these plants, probably accounting for the lower levels of H$_2$O$_2$ during drought. This control of ROS levels in the transgenic plants is likely a contributing factor to survival of the plants during the severe drought period of 15 days and their continued growth after rewatering. Suppression of drought-induced leaf senescence in transgenic plants was also accompanied by enhanced expression of stress-response transcripts, such as dehydrins and heat-shock proteins (SI Table 1), possibly also contributing to the enhanced tolerance and suppressed ROS levels in the transgenic plants during drought.

Studies have shown that the senescence-activated CK production caused the inhibition of chlorophyll breakdown in the basal leaves of tobacco, affecting source/sink relationships and reducing nitrogen mobilization to younger and growing tissues that resulted in an inverted vertical nitrogen profile within the plant canopy (7). In transgenic lettuce, the senescence-activated CK production induced a marked delay in flowering and premature senescence in the apical leaves (8). Our work showed that nitrogen mobilization was not affected in the P$_{SARK}$::IPT plants, because the basal leaves displayed chlorophyll degradation (SI Fig. 13) during the drought episode.

More importantly, no developmental changes were seen in the transgenic plants, and no delay was observed in flowering and seed set, indicating a normal progression of the source/sink relationship in the transgenic plants.

Transgenic plants expressing P$_{SARK}$::IPT maintained relatively high water content and retained photosynthetic activity (albeit at a reduced level). Moreover, the transgenic plants were able to grow under restrictive water supply with a minimal yield penalty. Our data suggest that the efficient scavenging of ROS in the transgenic plants protects the photosynthetic apparatus during drought stress, leading to the improved WUE of the transgenic plants during and after stress. The relationship between CKs and the activation of these mechanisms is not yet identified, and further research is required.

In conclusion, our results show that the suppression of drought-induced leaf senescence resulted in outstanding drought tolerance. The plants had vigorous growth after a long drought period that killed the control plants. These plants had minimal yield loss when watered with only 30% of the amount of water used under control conditions. These results are exciting because they indicate that, in addition to increased drought tolerance, the expression of P$_{SARK}$::IPT in plants, with the concomitant increased WUE, could facilitate the development of transgenic crops able to grow with reduced irrigation without significant yield penalties, contributing to significant savings in irrigation water.

**Methods**

**Plasmid Construct and Plant Transformation.** The 5’ upstream region of the bean SARK gene was isolated by inverse PCR as described by Maniatis et al. (27). Bean (Phaseolus vulgaris) genomic DNA was treated with XbaI, and PCR amplification was carried out by using the following primers: 5’-AGCTTCCCACAAAGACCT-3’ and 5’-TCTGACAGCTAGTGCGATAC-3’.

The PCR was carried out under the following conditions: 30 sec at 94°C, 30 sec at 55°C, 2 min at 72°C for 40 cycles, and then 10 min at 72°C. Nucleotide sequence analysis revealed that it contained 340 bp of the 5’ end of the SARK coding region (28). To obtain a lower DNA fragment containing the SARK gene promoter, a thermal asymmetric-interlaced PCR technique was carried out as described by Liu et al. (29). Three specific PCR primers were used: 5’-TCTGACAGCTAGTGCGATTC-3’, 5’-TTGTCGTCGATGAGAATATGGG-3’, and 5’-TCTACATACGACAAATGGAG-3’.

Three PCRs were performed sequentially to amplify target sequences. The second PCR produced a single product, representing ~820 bases. This fragment was cloned into pUC 57 derivative plasmid (MBI; Fermentas). The Agrobacterium ipt gene was linked to the 840-bp fragment of the SARK promoter and introduced as a HindIII/XbaI fragment into the binary vector pBHi101. Tobacco SR1 transgenic lines containing this construct were treated by Agrobacterium-mediated transformation. Hygromycin-resistant primary transformants were transferred into soil (Metro-Mix 200; Sun Gro) and grown under artificial lighting (50 μmol of photons m$^{-2}$·sec$^{-1}$, 16-h photoperiod, 25°C). Plants were allowed to flower, the flouters were covered to prevent cross-pollination, and T$_1$ seeds were collected. Homozygous lines were selected through two further rounds of selections. Seeds were germinated on plates containing 2.7 g liter$^{-1}$ Phytalg, Murashige, and Skoog medium and 300 mg liter$^{-1}$ hygromycin. T$_1$ seeds giving a 3:1 ratio of survival on hygromycin were selected and grown to maturity, and T$_2$ seeds were collected. One hundred seeds from each line were sown again on soil containing hygromycin, and lines giving 100% survival were deemed homozygous and used for further experiments.

**Plant Growth Conditions.** Seeds of WT (Nicotiana tabacum cv. SR1) and two transgenic lines (P$_{SARK}$::IPT$_{24}$ and P$_{SARK}$::IPT$_{38}$) were sown and grown in soil (Metro-Mix 200, Sun Gro) under growth chamber controlled conditions (500 μmol of photons m$^{-2}$·sec$^{-1}$, 16-h photoperiod, 25°C) during 15 days (appearance of two true leaves). During this time, no differences in germination time and in plant development between WT and both transgenic lines were observed. Forty plants of each transgenic line and WT plants were transferred and transplanted into a greenhouse, using 10-liter pots and grown for 25 days (1,200 μmol of photons m$^{-2}$·sec$^{-1}$, 16-h photoperiod, 28–30/23–25°C day/night). At this point, watering was withheld for 2 weeks, a period after which the WT plants were dead. After 2 weeks of drought, the plants were rewatered during another 2 weeks. At the end of the drought/rewatering experiments (2.5-month-old plants), the plants were harvested. Dry weight of the plants and seeds was obtained after 48 h at 70°C in an air oven.

**Water Potential and Relative Water Content.** For water potential measurements, a Dewpoint PotentialMeter (model WP4-P; Deacon Devices) was used. The relative water content (RWC) was determined as: RWC = (FW - DW)/(Turgid weight - DW). To determine turgid weight, leaves were kept in distilled water in darkness at 4°C to minimize respiration losses until they reached a constant weight (full turgor, typically after 24 h). DW was obtained as described.

**IPT Expression Analysis.** Fresh tissues from basal, middle, and apical leaf leaves were used for total RNA isolation (30), and 1 μg of total RNA was used for first strand cDNA synthesis according to standard methods, using oligo(dT) (31). Internal oligonucleotides, IPT-Forward1 (5’-TTCGACCTTCCTGTTGATGAT-3’) and IPT-Reverse1 (5’-GGGGGAAATTTCTTCTGTTGAT-3’), were designed, and standard RT-PCR was performed. PCRs were analyzed using 1% (wt/vol) Tris/acetate/EDTA/agarose electrophoresis. For the quantification of the relative expression of IPT, ImageQuant software (Molecular Dynamics) was used, and expression levels were normalized against the values obtained for actin, which was used as an internal reference gene. WT RNA was used as a negative control in all of the RT-PCR performed.

**Cytokinins Quantification.** WT and transformant basal, middle, and apical leaf tissues were collected at the times specified and freeze-dried. Extraction and determination of CK contents from ~20 mg of dry tissue were done by using a liquid chromatography–tandem mass chromatography system (model 2695; Quattro Ultima Pt; Waters) as described in ref. 32.

**Glutathione and Ascorbate Determination.** Approximately 0.2 g of leaves per sample was extracted by grinding in liquid N$_2$,
acid-washed sand, and 1.5 ml of 1 M HClO4. After centrifugation (13,000 × g for 10 min at 4°C), supernatants were partitioned into two 400-μl aliquots for ascorbic acid (AsA) and glutathione (GSH) determinations. To these extracts, 200 or 100 μl of 1 M Hepes/KOH buffer (pH 7.0) were added for AsA and GSH determinations, respectively (33). Aliquots of 6 M K2CO3 were incorporated gradually to adjust the pH to 4.0–5.0 for AsA determination or 6.0–7.0 for GSH determination and to precipitate perchorlate. Samples were centrifuged as before, and the pellets were discarded. Reduced AsA, oxidized DHA, and reduced glutathione (GSH) and its oxidized form (GSSG) were determined as described by Griffith (34).

**Antioxidant Enzymes.** Enzymes were extracted by grinding 0.2 g of frozen leaves in liquid N2, acid-washed sand, 50 mM Mes/KOH buffer (pH 7.8), 10% (vol/vol) Triton X-100, 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.5 mM nitroblue tetrazolium, 4 mM xanthine, 50 μl of extract, and 0.04 units xanthine oxidase. After 10 min, absorbance was measured at 500 nm. SOD activity was determined by a standard curve, using horseradish SOD (Sigma–Aldrich). APX activity was assayed by a modified procedure of Nakano and Asada (37) in a reaction mixture of 50 mM KH2PO4 buffer (pH 7.0), 250 μM L-AsA, and 10 μl of extract, with H2O2 added to initiate the reaction. For the preparation of the soluble and membrane fractions, plant tissue powder (50 mg) was homogenized with 500 μl of 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM ascorbate (AsA) and 1 mM EDTA. The homogenate was mixed well and centrifuged at 20,000 × g for 10 min. The supernatant contained the soluble fraction. The pellet was dissolved in 1 ml of 50 mM potassium phosphate buffer (pH 7.8), 10% (vol/vol) Triton X-100, and 1 mM AsA. After centrifugation at 20,000 × g for 10 min, the supernatant containing the solubilized membrane fraction was collected. Change in absorbance was monitored at 290 nm and activity was calculated from the reaction rate, using an extinction coefficient of 2.8 mM⁻¹·cm⁻¹.

MDHAR and DHAR activities were measured as described in ref. 38. GR activity was measured using the nonenzymic NADPH oxidation as described before (39). CAT was measured spectrophotometrically, using the method of Chance and Maehly (40) in a reaction mixture containing 50 mM KH2PO4 buffer (pH 7.0) and 15 mM H2O2, with 100 μl of extract to initiate the reaction. Activity was expressed as the change in absorbance at 240 nm as 50 mM H2O2 was degraded. Catalase activity was calculated by using an extinction coefficient of 39.4 mM⁻¹·cm⁻¹ (41).

**H2O2 Quantification and in Situ Detection.** H2O2 was extracted as described in ref. 42 with modifications (43). The concentration of peroxide in the extracts was determined by comparing the absorbance against a standard curve representing a titanium–H2O2 complex from 0.1 to 1 mM. The hydroperoxides represent the total peroxide. In situ H2O2 production was detected by an endogenous peroxide-dependent staining procedure, using DAB (44).

**Stress-Related Filter Arrays Hybridization.** Clones for the production of filter arrays were ordered from the tomato (Lycopersicon esculentum) expressed sequence tag library at Clemson University (Clemson, SC), or obtained from the laboratories of D. Inze (University of Gent, Gent), B.A. Zilinskas (Rutgers University, Newark, NJ), G. Schuster (Technion, Haifa, Israel). Filter cDNA arrays were prepared from the clones by spotting PCR products in duplicates on Nylon membranes at the Hadassah Medical School DNA Facility of Hebrew University. Filters were hybridized with radiolabeled cDNAs prepared from polya−RNA isolated from the different tissues, using oligo(dT) and SuperScript reverse transcriptase (Life Technologies) as suggested by the manufacturer. Hybridization conditions were as follows: 57°C, 5 × SSC, 5 × Denhart, 0.5% (wt/vol) SDS, and 100 μg ml⁻¹ salmon sperm DNA overnight. Washing conditions were as follows: 57°C, 2 × SSC, and 0.1% (wt/vol) SDS for 20 min, followed by 0.2 × SSC and 0.1% (wt/vol) SDS, 57°C, for 20 min. After hybridization and washes, the signals were assayed with a phosphor imager (Typhoon 8600; Molecular Dynamics) and analyzed with PDQuest software (BioRad). A number of control “housekeeping” genes, animal specific genes (as negative controls), and empty spots (for background) were also spotted on the membrane. These were used to normalize the intensity of signals between the different filters and calculate the changes in gene expression related to oxidative metabolism.

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