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Expression of apoplastically secreted tobacco osmotin in cotton confers drought tolerance

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Abstract Osmotin or osmotin-like proteins have been shown to be induced in several plant species in response to various types of biotic and abiotic challenges. The protein is generally believed to be involved in protecting the plant against these stresses. Although some understanding of the possible mechanism underlying the defense function of osmotin against biotic stresses is beginning to emerge, its role in abiotic stress response is far from clear. We have transformed cotton plants with a tobacco-osmotin gene, lacking the sequence encoding its 20 amino acid-long, C-terminal vacuolar-sorting motif, under the control of CaMV 35S promoter. Apoplastic secretion of the recombinant protein was confirmed and the plants were evaluated for their ability to tolerate drought conditions. Under polyethylene glycol-mediated water stress, the osmotin-expressing seedlings showed better growth performance. The transformants showed a slower rate of wilting during drought and faster recovery following the termination of dry conditions in a greenhouse setting. During drought, the leaves from transgenic plants had higher relative water content and proline levels, while showing reduced H_2O_2 levels, lipid peroxidation and electrolyte leakage. Importantly, following a series of dry periods, the osmotin transformants performed better in terms of most growth and developmental parameters tested. Most relevant, the fiber yield of transgenic plants did not suffer as much as that of their non-transgenic counterparts under drought conditions. The results provide direct support for a protective role of osmotin in cotton plants experiencing water stress and suggest a possible way to achieve tolerance to drought conditions by means of genetic engineering.

Keywords Abiotic stress · Cotton · Drought · Osmotin · Transgenic

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Introduction

Osmotin is one of the unique proteins that are induced in response to both abiotic and biotic stresses in plants. Singh et al. (1985) showed that a cationic 26 kDa protein was induced in cultured tobacco cells adapted to grow under salt stress or polyethylene

glycol (PEG)-mediated water stress. This protein was also induced in cultured tobacco cells and in roots in response to treatment with abscisic acid (Singh et al. 1987). Osmotin exhibits considerable similarity in its size and amino acid sequence to the thaumatin protein from katemfe (*Thaumatococcus daniellii* Benth.) plant, but it does not have the sweet taste of thaumatin (Singh et al. 1987). The tobacco protein was named Osmotin due to its induction by low water potential of the growth medium and a correspondence between the level of osmotin protein produced and the degree of osmotic stress (Singh et al. 1985, 1987). Transcription of osmotin was induced by ABA but low water potential was required for the accumulation of the protein, and the level of osmotin transcript increased with increasing endogenous ABA content (Singh et al. 1987, 1989). Besides its induction by ABA, osmotin expression is controlled by a host of complex developmental-, tissue specific- and various hormonal signals indicating substantial transcriptional and post-transcriptional regulation of osmotin mRNA (Kononowicz et al. 1992; LaRosa et al. 1992; Raghothama et al. 1993). Grillo et al. (1995) showed that in tomato, both salt- and water stress caused increase in the levels of osmotin transcripts and protein. The enhanced transcript levels were accompanied by an increase in endogenous ABA. Interestingly, osmotin transcripts were not induced by salinity or osmotic stress in an ABA deficient mutant of tomato suggesting involvement of ABA in osmotin mRNA accumulation (Grillo et al. 1995). Two cotton osmotin genes have been also cloned by probing the genomic DNA library of *Gossypium hirsutum* with osmotin gene from tobacco (Wilkinson et al. 2005). Based on the putative N-terminal 24 amino acid-long signal sequence of the preprotein, the mature forms of the proteins are believed to be targeted for extracellular secretion (Wilkinson et al. 2005). Many of the above mentioned studies established a correlation between abiotic stress and the induction/accumulation of osmotin and suggest that this protein may play a role in protecting the plant against stresses.

Confirmation of the protective abilities of osmotin against abiotic stresses comes from some overexpression studies published relatively recently. Constitutive expression of an osmotin gene in transgenic tobacco improved their tolerance to salinity and drought stress (Barthakur et al. 2001). Transgenic strawberry expressing the tobacco-osmotin showed improved germination of seeds and higher growth rates of plants under high salinity (Husaini and Abdin 2008). Osmotin has been also proposed to play a role in cold acclimation (Angeli and Altamura 2007). In this study, overexpression of a tobacco osmotin gene in olive tree demonstrated its involvement in cold acclimation-related programmed cell death, blocking cold-induced calcium signaling, and cold-induced cytoskeleton alterations. However, the underlying mechanism of the protection provided by osmotin to tolerate various abiotic stresses is still unclear.

Besides osmotic stress, osmotin is induced in response to viral and fungal pathogen infections in tobacco and tomato (Stintzi et al. 1991; Woloshuk et al. 1991). Osmotin proteins from both species were found to cause lysis of sporangia and growth inhibition of *Phytophthora infestans* (Woloshuk et al. 1991). Tobacco-osmotin was also shown to inhibit the growth of *Candida albicans*, *Neurospora crassa*, and *Trichoderma reesei* (Vigers et al. 1992). In a large-scale, in vitro study, Abad et al. (1996) demonstrated antifungal properties of osmotin against a wide range of fungal pathogens. Osmotin has been classified as a plant defense related protein under the PR5 protein family. Overexpression of tobacco osmotin delayed the development of late blight disease in potato (Liu et al. 1994). In a subsequent study, this group showed that when a C-terminal truncated tobacco osmotin was expressed in potato, it was secreted to the extracellular matrix, the protein remained functional, and the transgenic potato plant showed resistance to *P. infestans* (Liu et al. 1996).

Based on various reports demonstrating antifungal properties of tobacco osmotin, our original intent was to test whether expression of the truncated osmotin (Liu et al. 1996) in cotton would confer resistance to fungal pathogens. Our study with osmotin transformants did not show a significant improvement in disease tolerance when challenged with *F. oxysporum*, *Verticillium dahliae*, and *Alternaria alternata*. A moderate degree of resistance was observed against *Rhizoctonia solani*. While studying disease resistance in the transgenic plants, we observed that the transformants were able to tolerate dry soil better than their wild-type counterparts. This observation was all the more surprising because the C-terminal-truncated, recombinant osmotin was expected to be

secreted into the extracellular space and not in the vacuoles, where its accumulation is believed to confer protection to water- and salinity stresses. This unexpected observation led us to conduct an in-depth investigation on transgenic cotton lines, constitutively expressing the tobacco osmotin, to evaluate their ability to withstand salt- and drought stress. A number of physiological, biochemical, and developmental parameters were examined and the results from these studies are presented in this report.

Materials and methods

Vector construction and cotton transformation

A truncated tobacco osmotin gene lacking the sequence encoding the C-terminal 20 amino acids (Singh et al. 1989; Liu et al. 1996) was amplified using PFU turbo® DNA polymerase (Stratagene, Cat. #600250-51) using the primer pair: Osm1-F: 5′-GCCG ccatggGCAACTTGAGATCTTCT-3′; and Osm1-R: 5′-ACCcccgggCTAAGGACAAAAGATAACCCT-3′ with *Nco*I and *Sma*I restriction sites (indicated in lower case) incorporated into the forward and reverse primers, respectively. The PCR product was digested with *Nco*I and *Sma*I and ligated into the binary vector pRTL2 (Restrepo et al. 1990). The expression cassette from the resulting construct contained the CaMV 35S promoter with a double enhancer, a translational enhancer from tobacco etch virus (TEV), the osmotin gene and the CaMV 35S terminator. This cassette was isolated and ligated into pCAMBIA2300 to create the plant transformation vector pCam-RTL-Osm. Transformation of cotton (*G. hirsutum* L.), cv. Coker 312, was performed following the protocol established by Sunilkumar and Rathore (2001) and Rathore et al. (2006). Transgenic plants were grown in a greenhouse. The presence of transgene in the transformants was confirmed by PCR and Southern blot analyses.

Extraction of total, intracellular, and apoplastic proteins and Western blotting

Methods described by Liu et al. (1996) and Nishizawa et al. (1999) were used to extract apoplastic and intracellular proteins from the leaves of plants grown in a greenhouse. The first true leaf from a 14-day-old cotton plant was used for apoplastic and intracellular protein extraction. To obtain the apoplastic protein fraction, 2 g leaves from ten plants were cut into 1-cm strips, infiltrated under vacuum with 0.1 M sodium borate buffer (pH 7.6) containing 10 mM 2-mercaptoethanol and 0.6 mM phenylmethylsulfonyl fluoride (PMSF) for 20 min, blotted dry with filter paper and placed in an empty syringe (10 ml size). The syringe was placed in 50 ml centrifuge tube and centrifuged at 4,000×*g* for 10 min. The apoplastic protein fraction collected at the bottom of the centrifuge tube was lyophilized and redissolved in borate buffer before use for Western analysis. The leaf segments from which the apoplastic fluid had been removed were used to extract the intracellular protein fraction in borate buffer.

Transgenic plants were screened by Western blot analysis for the expression of tobacco osmotin gene. Forty micrograms of total soluble proteins (TSP) from cotton leaf tissues were separated on 12.5% polyacrylamide SDS gel and Western blotting was performed as described by Sunilkumar et al. (2009). Antibody raised against tobacco osmotin protein in chicken was used as the primary antibody (1:500). Anti-osmotin antibodies were purified from the chicken eggs using Eggcellent™ chicken IgY purification kit (Pierce, Cat. #44918). Goat anti-chicken IgY HRP (Santa Cruz Biotechnology, Cat. #SC-2428) was used as the secondary antibody (1:5000). Antibody binding was detected by ECS chemiluminescence method as described by Sunilkumar et al. (2009). Osmotin levels were quantified by comparing the band intensity on a Western blot with that of a known quantity of osmotin using the AlphaEase v5.5 software (Alpha Innotech, USA).

PEG-mediated water stress

Cottonseeds, soaked overnight, were germinated vertically within a rolled-up wet filter paper for 2 days at 28°C. The seedling was placed on a filter paper wick in a test tube (17 × 100 mm, VWR International, LLC, Cat. #60818-667) containing 12 ml of 1/2 strength MS medium with or without polyethylene glycol (PEG 6000) in such a manner that the radicle remained submerged in the medium as the seedling grew. Ten such tube assemblies were kept in a 2 l glass beaker covered with a loose-fitting plastic Petri dish (150 × 15 mm), sealed with parafilm, and placed under light at 25°C in an incubator.

After 3 days, cotton seedlings were gently pulled out from the tubes and total fresh weight of the seedling, fresh weight of the cotyledons, and lengths of hypocotyl and root were recorded.

Short-term drought treatment

For short-term drought exposure, ten untransformed, wild-type plants and ten progeny plants each from two transgenic lines (#61-53 and #61-63) were grown under optimal conditions in a greenhouse for two and a half weeks in small plastic pots containing 500 ml of commercial soil mixture (Metromix® 700 series, SUN GRO Horticulture Ltd., Bellevue, WA, USA). Care was taken to ensure that all the pots contained equal amount of soil. For one set of plants, watering was withheld for 8 days to create drought conditions, while another set of plants were watered regularly. The plants were monitored daily for wilting. Following the drought treatment, plants were re-watered and observed for recovery.

Determination of relative water content

Eighteen-day-old plants grown in a greenhouse in soil (Metromix® 700) were subjected to water stress by withholding watering for 8 days. The first fully expanded leaves from well-watered as well as water-stressed plants were collected and their fresh weights (FW) were recorded. Then, each leaf was soaked in water in a Petri dish, sealed with parafilm and incubated for 24 h at room temperature. After this incubation, leaves were blotted on filter paper to remove excess water and weighed to obtain turgid weights (TW). Dry weights (DW) were determined after drying the leaves in an oven for 72 h at 70°C. Relative water content (RWC) was calculated according to the following formula as described by Schonfeld et al. (1988):

$$\mathrm{RWC}(\%) = (\mathrm{FW} - \mathrm{DW})/(\mathrm{TW} - \mathrm{DW}) \times 100.$$

Estimation of leaf H_2O_2 content

Eighteen-day-old plants grown in soil (Metromix® 700) in a greenhouse were subjected to water stress by withholding watering for 8 days. A protocol described by Zhou et al. (2006) was used to estimate H_2O_2 level in the leaf tissue. A leaf sample (excluding the midrib) weighing 300 mg from water-stressed or unstressed cotton plant was collected in a 2 ml microfuge tube containing 1.5 ml 5% trichloro acetic acid (TCA) and 0.15 g activated charcoal. Samples were homogenized with the aid of 10 mm stainless steel beads using a '2000 Geno/Grinder® Mill' (SPEX CertiPrep, Metuchen, NJ). The mixture was centrifuged at 10,000 rpm for 10 min. The supernatant from each sample was aliquoted (400 µl) to two microfuge tubes and the pH was adjusted to 8.4 by adding ammonium hydroxide. One of these tubes was used as a blank after adding 3.6 µg catalase to remove all H_2O_2. Both the tubes (with and without catalase) were incubated at room temperature for 10 min. After adding 400 µl color reagent (10 mg 4-aminoantipyrine; 10 mg phenol, 700 U of peroxidase, dissolved in 50 ml of 0.1 M acetic acid buffer, pH 5.6) to each aliquot, the reaction mixture was incubated at 30°C for 30 min and the absorbance was measured spectrophotometrically at 505 nm. A standard curve for H_2O_2 was used to determine endogenous concentration in the leaf tissue.

Estimation of malondialdehyde content in leaf tissue

Eighteen-day-old plants grown in a greenhouse in soil (Metromix® 700) were subjected to water stress by withholding watering for 8 days. A 150 mg piece obtained from the first, fully expanded leaf (excluding the midrib) was homogenized in 5 ml of 10% TCA. The extract was centrifuged at 10,000 rpm for 10 min. The reaction was set up by adding 2 ml of supernatant to 3 ml of 0.6% thiobarbaturic acid dissolved in 10% TCA and the mixture was then incubated at room temperature for 2 h. The reaction mixture was boiled at 100°C for 1 h. After cooling to room temperature, the OD was measured at 450 nm, 532 nm and 600 nm (Quan et al. 2004). The malondialdehyde (MDA) content was estimated by the following formula:

$$\mathrm{C}(\mu\mathrm{mol/l}) = [6.45(\mathrm{OD}_{532} - \mathrm{OD}_{600})] - 0.56\,\mathrm{OD}_{450}.$$

Measurement of ion leakage in cotton leaf tissue

Eighteen-day-old plants grown in a greenhouse in soil (Metromix® 700) were subjected to water stress by

withholding irrigation for 8 days. A modified method of Fan et al. (1997) was followed to measure the conductivity of the incubating solution bathing an individual leaf sample. A leaf disk (14 mm in diameter) from the first fully expanded leaf of a well-watered or a drought-stressed plant was collected in a 50 ml centrifuge tube containing 5 ml of 0.4 M mannitol. Tubes containing the leaf samples were incubated (with gentle shaking) for 3 h at room temperature and the conductivity of the bathing solution was measured with a conductivity meter (Ultrameter™ 6P, Myron L. Co., USA). Following this reading, total conductivity of the bathing solution was determined after boiling the sample for 10 min. The conductivity due to membrane ion leakage was expressed in terms of initial conductivity of the bathing solution as a percentage of the total conductivity.

Estimation of proline content of leaf tissue

Eighteen-day-old plants grown in a greenhouse in soil (Metromix® 700) were subjected to water stress by withholding watering for 8 days. Proline levels in tissues were determined according to Bates (1973) and Ringel et al. (2003). A sample weighing approximately 100 mg obtained from the first fully expanded leaf of a drought-stressed or unstressed control plant was collected in a 2 ml microfuge tube containing 1.7 ml of 3% sulfosalicylic acid. Samples were homogenized with the aid of 10 mm stainless steel beads using a '2000 Geno/Grinder® Mill'. Ground samples were centrifuged and 1 ml of the supernatant was mixed with 1 ml of acid ninhydrin (1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid) and 1 ml of glacial acetic acid in a fresh glass tube (20 ml size). Tubes were capped and incubated at 100°C in a water bath for 1 h. The reaction was terminated by chilling the tubes on ice. Two milliliter of toluene was added to each tube and the mixture was vortexed for 10 s. One milliliter of the upper, toluene phase containing the chromophore was aspirated and read at 520 nm in a quartz cuvette. Tissue proline concentrations were estimated based on a standard curve (0–100 μg/ml) for proline and are presented as μg proline/g FW.

Multiple drought treatments

In a long-term experiment, effects of three cycles of drought, imposed at different stages of plant development, were determined on various parameters related to the yield. Ten untransformed, wild-type cotton plants and ten progeny plants from the transgenic line #61-63 were grown in 3-gallon pots containing commercial soil mixture (Metromix® 700) in a greenhouse. Three drought cycles at various stages of plant growth and development were applied. The timings were based on the criteria for the rate of water use in relation to the developmental stage of cotton plant (McWilliams 2003; Basal and Unay 2006). The first drought cycle lasting 10 days was imposed at the onset of the squaring stage, the second one lasting 6 days after the opening of first bloom and the third one for 3 days after opening of the first boll. Drought stress was applied by withholding watering until severe wilting of the leaves in non-transformants was observed. Normal watering was resumed after each drought cycle. Another set of plants (10 wild-type and 10 transgenic progeny from #61-63) were well-watered throughout their growth and development. Plants were fertilized once a week. Data on boll number, yield (seed and lint), plant height, and root biomass were recorded following the harvest, approximately 5 months after the seeds were first planted (seed planting date: October 8, 2007; experiment termination date: March 20, 2008).

Results

Generation of cotton transformants, osmotin-expression analysis and screening for stress tolerance

We produced 174 transgenic plants from 114 independent transgenic lines using the *Agrobacterium*-mediated cotton transformation method. Expression of osmotin was analyzed by Western blotting in the leaf tissue from T_0 transgenic cotton plants. Osmotin expression was observed in 87 T_0 transgenic plants derived from 64 independent transgenic lines at levels ranging from 0.073 to 2.743 μg/mg TSP. Proteins from non-transgenic control plants did not react with the anti-osmotin antibody. Expression of

osmotin was followed in seven independent transgenic lines over three generations and two (#61-53 and #61-63) were selected for disease/drought tolerance studies as each of these continued to maintain high osmotin levels up to the T_3 generation. Although each of these lines had two copies of the transgene as determined by Southern hybridization analysis (data not shown), segregation analysis revealed that these copies were linked (data not shown). T_3 progeny from line #61-53 had 3.1 μg/mg TSP, while #61-63 had 4.1 μg/mg TSP. Presence of recombinant osmotin in the apoplastic space was confirmed by analyzing the extracellular fluid and intracellular contents of leaves from transgenic lines by Western blot analysis (Fig. 1a, b).

While establishing conditions for infection assays with *R. solani*, we observed that the transgenic lines fared better than non-transgenic controls in one of the experiments in which soil had become overly dry. This observation and the fact that osmotin accumulates in tobacco cells under high salinity and water stress conditions (Singh et al. 1987; LaRosa et al. 1989) prompted us to examine the ability of osmotin-expressing transgenic cotton to tolerate various abiotic stresses. The transgenic lines did not show any tolerance to salinity either in terms of germination or their ability to grow on a medium containing 200 mM NaCl (data not shown). We further examined the ability of the transformants to tolerate water stress applied in various ways and the results from these experiments are described below.

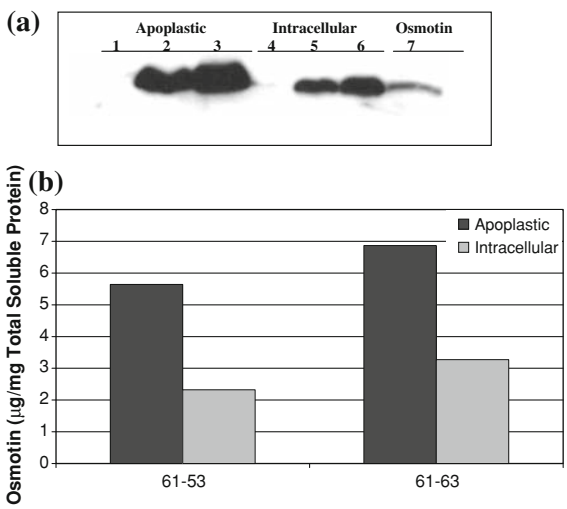

Fig. 1 **a** Immunodetection of recombinant tobacco osmotin in the apoplastic fluid and intracellular fraction of transgenic cotton lines, #61-53 and #61-63. *Lanes 1 and 4* correspond to untransformed wild-type plants. *Lanes 2 and 5* correspond to #61-53 and *lanes 3 and 6* correspond to #61-63. Twenty microgram of total protein was loaded in each lane. *Lane 7* contains positive control (50 ng purified tobacco osmotin protein). **b** Quantitative estimation of tobacco osmotin, based on the band intensity on the Western blot, in the apoplastic fluid and intracellular fraction of transgenic lines #61-53 and #61-63

Plant growth under PEG-mediated water stress

Water stress imposed by PEG is a convenient and widely used method for a preliminary evaluation of the drought-tolerant nature of the plants (Verslues et al. 1998, 2006). We examined the ability of osmotin-expressing cotton plants to tolerate water stress by growing T_3 progeny from the two selected transgenic lines in 1/2 strength MS medium supplemented with 15% PEG 6000 and comparing their growth with wild-type plants. After 3 days of growth, with or without PEG-mediated stress, various developmental parameters were evaluated. The results from this analysis are presented in Table 1. It is noteworthy that, in the absence of stress, the seedlings of both the transgenic lines were superior in terms of growth parameters tested compared to the wild-type. As expected, PEG did have an adverse effect on all of the growth parameters tested in the non-transgenic plants. Similarly, the transgenic seedlings were also affected negatively in their growth; however, the reductions in many of the growth parameters were less severe compared to those observed in the untransformed controls. Reduction in the root length caused by PEG was higher in the case of line #61-53 compared to wild-type and the second transgenic line. However, this line was superior to the wild-type plants in terms of the remaining growth parameters.

Wilting during drought stress

The two transgenic lines and wild-type control plants grown in small pots were subjected to drought stress in a greenhouse by withholding watering for 8 days. Wild-type plants began to show wilting by the 6th day of the drought treatment, while the transgenic lines were unaffected at this stage (Fig. 2a). On the 7th day, one out of ten plants from the transgenic line #61-53 showed wilting while the other transgenic

Table 1 Growth performance of cotton seedlings in 1/2 strength MS medium with or without 15% polyethylene glycol (Mol. Wt 6,000)

Parameters	Wild-type			61-53			61-63		
	−PEG	+PEG	% Reduction	−PEG	+PEG	% Reduction	−PEG	+PEG	% Reduction
Hypocotyl length (mm)	58.3 ± 1.37	40.7 ± 1.38	30.18	58.8 ± 1.87	48.7 ± 1.01**	17.17	62.4 ± 1.59	48.9 ± 0.90**	21.63
Root length (mm)	81.7 ± 1.70	72.2 ± 0.66	11.62	104.7 ± 1.05	83.8 ± 0.58**	19.96	95.1 ± 1.53	88.6 ± 1.58**	6.83
Seedlings fresh weight (mg)	844.1 ± 37.01	589.1 ± 20.41	30.20	929.5 ± 29.00	689.2 ± 24.44*	25.85	898.1 ± 40.80	665.7 ± 19.67*	25.86
Cotyledons fresh weight (mg)	458.8 ± 22.02	321.5 ± 13.42	29.92	501.6 ± 13.08	362.5 ± 14.77	27.73	498.2 ± 18.02	359.9 ± 10.84*	27.75

The data represent mean ± SE ($n = 10$); the mean values for transgenic lines are significantly different from wild-type values at * $P < 0.05$ and ** $P < 0.005$

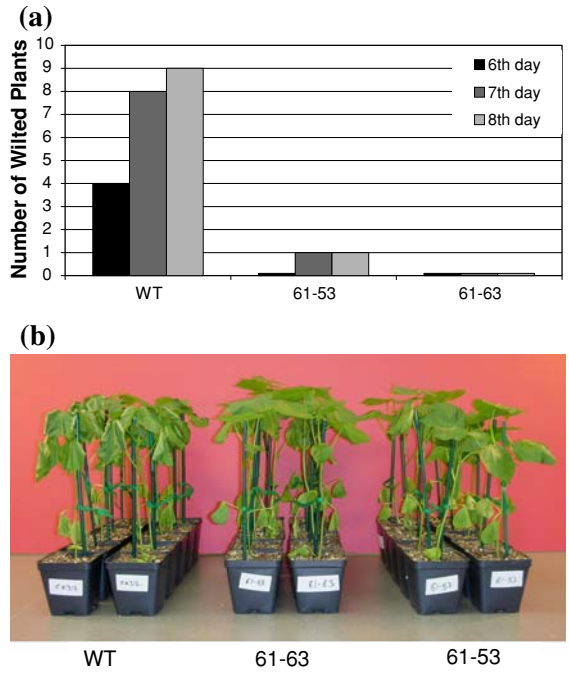

Fig. 2 **a** Observations on leaf wilting in wild-type plants and transgenic cotton lines (#61-53 and #61-63) subjected to water stress for 8 days. A total of ten plants were used in each category. **b** Wilting status of wild-type and two transgenic lines subjected to water stress by withholding watering for 8 days

line, #61-63, did not exhibit any visible symptoms of stress. On 8th day following the withholding of watering, leaves of nine out of ten wild-type plants were wilted, while no leaf wilting was observed in transgenic line #61-63 (Fig. 2a, b). On the 9th day of the drought, even the transgenic plants began to show wilting, however, these recovered more rapidly following re-watering.

Relative water content of leaves under water stress

After 8 days of withholding of watering, RWC of the transgenic cotton lines #61-53 and #61-63 were significantly higher ($P < 0.05$ and $P < 0.001$, respectively) than that of the wild-type plants. Transgenic lines #61-53 and #61-63 had 35 and 39.8% higher RWC values, respectively, when compared to the wild-type control plants (Fig. 3a). No significant difference was observed between the RWC values of unstressed transgenic plants and the wild-type plants.

H_2O_2 levels in the leaves of plants under water stress

H_2O_2 levels were estimated in fully expanded leaves from 18-day-old cotton plants after subjecting them to drought stress by withholding watering for 8 days. Under these conditions, the leaves from untransformed control plants had significantly higher levels ($P < 0.05$) of H_2O_2 compared to either of the transgenic lines (Fig. 3b). Compared to optimal watering conditions, drought caused about threefold rise in the H_2O_2 level in control plants, while only a twofold increase was observed in the transgenic lines.

Lipid peroxidation (MDA level) estimation in the leaves of plants under water stress

MDA is the product of lipid peroxidation caused by reactive oxygen species (ROS) generated during oxidative stress (Davey et al. 2005; Zlatev et al. 2006; Shulaev and Oliver 2006). MDA is considered a marker of oxidative lipid injury and its levels are widely used as a measure of damage due to various biotic and abiotic stresses (Pastori and Trippi 1992; Sairam et al. 1998; Kenton et al. 1999; Scarpari et al. 2005; Zlatev et al. 2006). Results presented in Fig. 3c show that MDA levels of the two transgenic cotton lines and wild-type controls were comparable when the plants were watered regularly. On the other hand, MDA level in the leaves of control plants under water stress was substantially higher (Fig. 3c). The MDA values in the transgenic lines #61-53 and #61-63 were 18.9 and 18.6 nmol/g FW, respectively, and were significantly lower ($P < 0.05$) compared to the wild-type level of 24.5 nmol/g FW.

Electrolyte leakage in the leaves of plants under water stress

Various stresses including drought cause generation of ROS that result in lipid peroxidation, ultimately leading to membrane damage (Chowdhury and

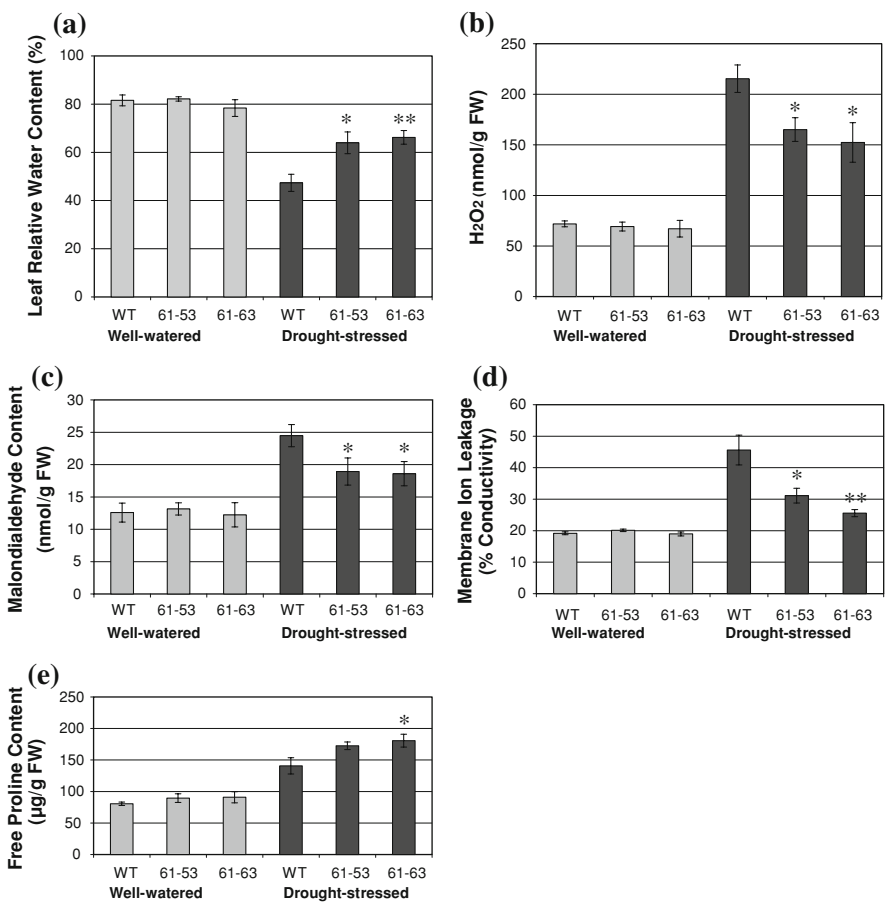

Fig. 3 Measurement of various physiological parameters in the leaves of wild-type plants and two transgenic lines (#61-53 and #61-63), with or without the imposition of water stress for 8 days. **a** Relative water content (%) ($n = 10$); **b** H_2O_2 levels ($n = 5$); **c** degree of membrane lipid peroxidation (malondialdehyde content) ($n = 10$); **d** degree of membrane ion leakage (measured as % conductivity of the bathing medium) ($n = 10$); **e** free proline content ($n = 6$). The data represent mean ± SE. Results are significantly different from wild-type control under the same treatment conditions (*$P < 0.05$, **$P < 0.001$)

Choudhuri 1985; Apel and Hirt 2004; Luna et al. 2005; Khanna-Chopra and Selote 2007). Such membrane damage can be assessed by quantifying ion leakage from plant tissues. This leakage was determined by measuring the conductivity of the medium, bathing the leaf tissue, with an ion conductivity meter. The results show that unstressed transgenic plants were not significantly different from their wild-type counterparts in terms of ion leakage under optimal growth conditions. However, wild-type plants subjected to water stress showed a substantial increase in ion leakage (Fig. 3d). The conductivity values obtained for transgenic lines, #61-53 and #61-63 were 31.7% ($P < 0.05$) and 43.9% ($P < 0.001$) lower compared to wild-type controls suggesting lesser damage to membrane systems in the transgenic plants under drought conditions.

Free proline content in the leaves of plants under water stress

Accumulation of proline is believed to be an indicator of drought tolerance in plants. Higher proline accumulation negatively correlates with susceptibility to drought (Dib et al. 1994; Ramanjulu and Sudhakar 2000; Kavi Kishor et al. 1995; Yamada et al. 2005). Interestingly, Barthakur et al. (2001) observed higher proline levels in osmotin-expressing tobacco plants under both unstressed and water-stress conditions. This prompted us to examine the proline levels in our transformants. Proline content of a fully expanded leaf was determined and the results are shown in Fig. 3e. No significant increase in proline content was observed in osmotin transformants under the optimal watering regime. However, water stress caused an increase in proline levels in all the plants, with the osmotin transformants showing higher levels of proline. Compared to the wild-type control plants, the transgenic lines #61-53 and #61-63 had 22.6 and 28.3% higher proline content, respectively.

Yield performance of transgenic plants subjected to multiple drought cycles in a greenhouse

Based on the positive results from the short-term water-stress experiments on young plants from the transgenic lines, we conducted a long-term study by subjecting the cotton plants to three cycles of drought stress during various stages of plant development. Since line #61-63 performed slightly better than line #61-53 with respect to many of the parameters evaluated, it was selected for this particular experiment. After the first cycle of water stress at the squaring stage, transgenic plants showed less severe wilting compared to the controls (Supplementary Fig. 1). In fact, two of the ten control plants that were subjected to water stress showed extreme wilting and death of most of their leaves including the terminal bud. Upon rewatering, majority of the control plants showed slower recovery. The two severely wilted control plants lost their apical dominance and grew axillary branches near the base of the plant and eventually did recover and produced bolls. In contrast, the osmotin transformants exhibited slower and lesser wilting compared to the wild-type plants during the drought and showed rapid and full recovery upon rewatering. Plants were subjected to two more rounds of water stress and allowed to grow to maturity. Various growth, development and yield parameters were measured at the end of the experiment. Number of bolls retained at maturity is an important economic parameter in cotton. Results presented in Fig. 4a show that although water stress reduced the number of bolls in both types of plants, the transgenic plants retained 57.6% more bolls compared to the control plants. We observed an interesting and unexpected change in the relative seed to lint ratio resulting from the expression of tobacco osmotin gene in cotton. The seed:lint ratio for Coker 312 is $\sim$1.54 under optimal watering conditions in our greenhouse. While the amount of fiber produced by transgenic plants was similar to the control plants under optimal watering conditions, the amount of seed produced was reduced, resulting in a seed:lint ratio of 1.11. Under drought conditions, there were substantial reductions in the yield of these two products in the wild-type cotton plants while still maintaining the usual seed to lint ratio. Compared to these control plants, the transgenic plants produced about the same amount of seeds, but significantly higher yield ($P < 0.001$) of lint under drought conditions (Fig. 4b). In order to examine whether osmotin expression affected seed development, 100-seed weight was determined for plants in each category. No significant differences in 100-seed weight were observed amongst plants regardless of their transgenic nature and whether or not stress was imposed (data not shown). This result suggests that

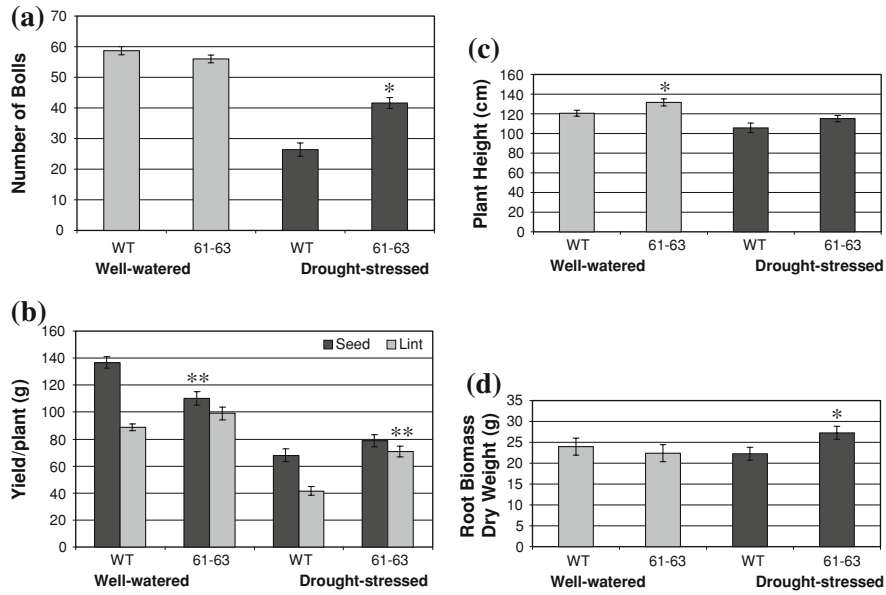

Fig. 4 Growth and yield data for wild-type and transgenic line #61-63 that were either unstressed or subjected to three cycles of drought stress. **a** number of bolls; **b** seed and lint weights per plant; **c** plant height; and **d** root biomass. The data represent mean ± SE ($n = 10$). Results are significantly different from wild-type control under the same treatment conditions ($*P < 0.05$, $**P < 0.001$)

the slight reduction in the yield of seeds is probably due to lower fertility or seed set in the transgenic plants. After harvesting cotton from plants in this experiment, plant height and dry weight of root biomass was determined for plants from all categories. The height of transgenic plants under well-watered conditions was slightly greater than the wild-type plants. Since two of the non-transgenic controls had lost apical dominance and grew axillary branches, the final height measurements in these plants were not considered meaningful and were not taken into account. No significant differences in height were observed between transgenic plants and the eight non-severely injured control plants that were subjected to drought conditions (Fig. 4c). Surprisingly, the root biomass of transgenic plants was significantly higher ($P < 0.05$) compared to that of the non-transgenic plants under water stress conditions (Fig. 4d).

Discussion

Results obtained in this study demonstrate clearly that overexpression of apoplast-targeted tobacco osmotin gene in cotton confers tolerance to water stress. Various physiological and biochemical parameters including seedling growth (on PEG-supplemented medium), leaf RWC, leaf-wilting, leaf H_2O_2 level, leaf MDA content, membrane ion leakage in leaf tissue and leaf free-proline content were examined in plants subjected to water stress. The results demonstrate that overexpression of apoplast-targeted tobacco osmotin in transgenic cotton conferred tolerance to water stress. A final confirmation of the drought tolerance properties of the transformants was obtained by subjecting the plants to three cycles of water stress in a greenhouse during three critical stages of development. Under the drought stress, the transgenic cotton performed better in terms of number of bolls set, lint yield, plant height, and root biomass.

We utilized some well-established assays to evaluate the drought tolerance characteristics of osmotin-expressing transgenic lines. PEG treatment is routinely used to measure drought tolerance in plants. Solutions containing PEG of molecular weight 6,000 or higher can create a cytorrhytic low water potential environment around the roots and are therefore used to simulate dry soil conditions for examining drought tolerance in plants (Verslues et al. 2006). Our observations showing better growth performance of the transgenic seedlings on medium supplemented with 15% PEG 6000 indicate that osmotin-transformants can better withstand water stress (Table 1). A similar observation was made when thaumatin, which shares biochemical properties with osmotin, was overexpressed in tobacco with respect to seed germination and survival rates in transgenic tobacco exposed to water stress (Rajam et al. 2007). Our findings with the PEG treatment were confirmed by

results from an experiment that involved induction of drought stress in plants growing in soil under greenhouse conditions (Fig. 2a, b). Transgenic plants exhibited less wilting in these experiments. Slower and lower rates of leaf wilting have been shown to correlate with the ability of plants to tolerate drought stress (Abraham et al. 2004; Ouvrard et al. 1996; Ober et al. 2005). Quisenberry et al. (1985) had demonstrated higher seed cotton yield in drought tolerant cultivars, which showed lower or no leaf wilting during drought stress. RWC is an important and a major determinant of metabolic activity and leaf survival. It was proposed to be a better indicator of the health status of plant cell than water potential (Sinclair and Ludlow 1985). Schonfeld et al. (1988) demonstrated that RWC could be used as an indicator of drought resistance by demonstrating a positive relationship between RWC and yield under drought conditions. In the present study, transgenic cotton lines #61-53 and #61-63 showed 35 and 39.8% higher RWC compared to the untransformed controls under drought conditions (Fig. 3a). It has been hypothesized that osmotin may be involved in osmotic adjustment of cells either by facilitating accumulation or compartmentation of solutes or by regulating metabolic or structural alterations during osmotic adjustment (Singh et al. 1987).

Generation of ROS during various oxidative stresses is a well-established fact. H_2O_2 is one of the important ROS, accumulation of which leads to oxidative damage in the plant cell and also plays a role in ROS mediated cell signaling (Mittler 2002; Neill et al. 2002; Mittler et al. 2004; Miller et al. 2008). Lower H_2O_2 accumulation has been shown to correlate with drought tolerance in a study conducted on three bean cultivars (Zlatev et al. 2006). In an investigation on field-grown sage, Munne-Bosch et al. (2001) showed an increase in H_2O_2, other ROS, and MDA levels in drought-stressed senescing leaves, thus suggesting a relationship between drought and oxidative stress (Munne-Bosch et al. 2001). Lower lipid peroxidation and higher membrane stability (lower electrolyte leakage) has been reported in drought-tolerant cultivars of maize (Pastori and Trippi 1992), wheat (Sairam et al. 1998) and bean (Zlatev et al. 2006). Our results (Fig. 3b, c, and d) show that osmotin transformants have lower levels of H_2O_2 accumulation, MDA content, and membrane ion leakage under drought stress conditions, suggesting better membrane stability in these transgenic plants.

Several studies have demonstrated a correlation between proline accumulation and drought tolerance in plants (Dib et al. 1994; Kavi Kishor et al. 1995; Ramanjulu and Sudhakar 2000; Yamada et al. 2005). It has been shown that proline acts as a potent ROS scavenger during oxidative stress (Chen and Dickman 2005; Kaul et al. 2008). Higher accumulation of free proline during drought is one of the characteristic features of drought tolerant cultivars of wheat (Dib et al. 1994). Petunia plants transformed with Arabidopsis and rice Δ1-pyrroline-5-carboxylate-synthetase genes, which accumulated free proline, survived drought stress for 14 days (Yamada et al. 2005). Free proline content of osmotin-overexpressing tobacco and strawberry plants was found to be higher than their untransformed counterparts and its accumulation was greater after drought or salinity stress (Barthakur et al. 2001; Husaini and Abdin 2008). The turnover of osmotin protein was hypothesized as the basis for higher free proline content in transgenic tobacco plant (Barthakur et al. 2001). In contrast to these two reports, we did not observe a significant difference between the free proline content of the transgenic lines and untransformed control plants under optimal growth conditions (Fig. 3e). However, in the present study, free proline content of the leaves of drought-stressed transgenic lines was higher compared to their untransformed counterparts. Although the basis for higher proline levels in the stressed transformants is not known at this stage, this may well be one of the factors contributing to the drought tolerance of the transformants.

A long-term drought experiment under greenhouse conditions was undertaken to determine whether the better performance of transgenic lines in terms of various stress-related parameters would translate into better plant growth and higher yield. This experiment involved three drought cycles at various stages of plant growth, including square stage, first bloom opening, and first boll opening. Restricting the water supply to cotton plants at these three stages is believed to result in yield loss and affect the fiber quality (Basal and Unay 2006). We obtained 36.5% higher yield (combined weight of seed and lint) in the transgenic line compared to the untransformed controls under drought conditions as indicated by the results presented in Fig. 4b. However, even under

optimal growth conditions, the seed:lint ratio was reduced to 1.11 in the transgenic line when compared to the usual ratio of 1.54 for the untransformed control plants (Fig. 4b). Under drought conditions, a reduction in plant height was observed in both transgenic as well as control plants (Fig. 4c). In a manner similar to many other plant species, cotton plants growing under field conditions fulfill their water requirement by extending root length during water stress (Basal and Unay 2006). Based on the information available on osmotin and osmotin-like proteins from various species, we did not anticipate osmotin expression to affect the root growth. In addition, because the plants were growing in pots and not in the field, there was no expectation for an increase in the root tissue due to water stress. However, we did observe significantly higher (22.5%) root biomass in transgenic plants compared to the untransformed plants under drought-stress conditions (Fig. 4d). These results are very encouraging and suggest that osmotin transformants may be better able to tolerate drought under field conditions and suffer lesser penalty in terms of fiber yield.

Previous reports suggested that full-length tobacco osmotin could confer tolerance to NaCl in transgenic tobacco (Barthakur et al. 2001); strawberry (Husaini and Abdin 2008); and wheat (Noori and Sokhansanj 2008). However, our experiments did not show any improvements in the ability of transgenic lines to tolerate salt stress. It is possible that intracellular accumulation of osmotin is required to confer such a tolerance.

This is the first report on conferral of drought tolerance in plants by the expression of tobacco osmotin gene that lacks the sequence encoding 20 amino acids at the C-terminal end. This C-terminal sequence functions as a vacuolar-sorting motif and it is believed that the protein lacking it will be secreted extracellularly (Liu et al. 1996). Our results from Western blot analysis showing a higher level of osmotin in the apoplastic fraction confirmed that the recombinant protein is secreted extracelluarly in the transgenic cotton plants. Immunocytochemical detection can be used to more accurately pinpoint the localization of a given protein within the cell wall (Santen et al. 2005; Sujkowska et al. 2007). Thus, using the immuno-electron microscopy, Garcia-Casado et al. (2000) showed that a basic isoform of PR5 protein, CsTL1 (without C-terminal polypeptide), was secreted to the apoplast in chestnut seeds. It is interesting that an acidic isoform of PR5 protein from soybean (*Gm*OLPa), that lacks the C-terminal polypeptide, is secreted into extracellular space, and is induced by salt stress as well as dehydration (Onishi et al. 2006). It was hypothesized that *Gm*OLPa localized in the extracellular space protects soybean roots from salinity and dehydration stress. Taken together, these reports and our results suggest that apoplastically localized osmotin or osmotin-like proteins also play a role in protecting the plant from dehydration stress.

In an investigation involving osmotin-promoter characterization, Kononowicz et al. (1992) found high-level promoter activity in the mature pollen grains and in the pericarp tissue at the desiccating stage of fruit development. The authors speculated that osmotin may be involved in the protection of membranes during dehydration. Earlier reports on osmotin from this group had implied that it is the intracellular accumulation of this protein by cells under salt- or drought stress conditions that somehow enhances their ability to withstand these abiotic stresses (Singh et al. 1987; LaRosa et al. 1989). Thus, our demonstration of drought tolerance in transgenic cotton as a result of the expression of apoplast-secreted osmotin was somewhat unexpected. As described earlier, support for the involvement of apoplastically secreted osmotin that is induced in response to abiotic stresses comes from results on soybean *Gm*OLPa (Onishi et al. 2006). Thus, it is possible that both intracellular and extracellular osmotin may be involved in plant response to abiotic stresses.

Extensive research has been conducted over the past two decades to understand the basis for antifungal properties of osmotin (Abad et al. 1996; Yun et al. 1997, 1998; Narasimhan et al. 2001, 2005) and some understanding of osmotin's role as an antifungal agent is beginning to emerge. However, the biochemical/physiological mechanisms underlying its role in the protection against abiotic stress remain unexplored. The results from our study and a few other reports (Barthakur et al. 2001; Husaini and Abdin 2008; Noori and Sokhansanj 2008) showing the beneficial effects of osmotin overexpression in abiotic stress tolerance may prompt future explorations on whether and how the induction of osmotin by water- and salt stress provides protection to the

plants. Whatever the mechanism, the results presented in this report demonstrate clearly that the expression of apoplast-targeted tobacco-osmotin in cotton confers a significant degree of tolerance to water stress as indicated by better growth performance of the transformants as well as their fiber yield. With the exception of a small reduction in the yield of seeds, osmotin-expression did not result in any other penalty, either in terms of growth and development of the plant or the yield of fiber when the plants were grown under optimal greenhouse conditions. The use of a constitutive promoter may be responsible for this slightly lower yield of seeds in the transgenic plants. With the increasing choice of tissue-specific and stress-induced promoters, it may even be possible to fine-tune osmotin expression to obtain the desired drought tolerance while avoiding any undesirable consequences of constitutive expression. In addition, further studies under the rigors of field conditions will be required to ascertain the feasibility of incorporating this transgene into cotton for commercial exploitation.

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