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MOLECULAR BIOLOGY AND PHYSIOLOGY

Ectopic Expression of *AtNHX1* in Cotton (*Gossypium hirsutum* L.) Increases Proline Content and Enhances Photosynthesis under Salt Stress Conditions

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ABSTRACT

Overexpression of the Arabidopsis gene *AtNHX1* that codes for a vacuolar Na^+/H^+ antiporter in transgenic cotton significantly improved tolerance to 200 mM NaCl as measured by increases in plant height, leaf size, and leaf number. Growth of wild-type plants in 200 mM NaCl had no affect on any photosynthetic parameter measured. Interestingly, most photosynthetic parameters for the *AtNHX1*-expressing plants were enhanced with growth in 200 mM NaCl. Net photosynthesis vs. internal CO_2 concentration curves (*i.e.* A-Ci curves) revealed that transgenic plants exhibited a significant increase in maximum carboxylation, maximum electron transport capacity, and rate of ribulose-1,5-bisphosphate regeneration at high CO_2 levels. Chlorophyll fluorescence analysis during photosynthetic induction revealed that photochemical quenching and electron transport rates for transgenic plants were higher than those for transgenic plants grown with low NaCl (control) or those for wild-type plants grown in 200 mM NaCl; however, non-photochemical fluorescence quenching was not altered. Although proline and soluble sugar contents increased in both genotypes with growth in salt, the transgenic plants accumulated greater amounts of proline. We conclude that photosynthetic factors were not responsible for the negative effect of 200 mM NaCl on wild-type plant growth. Enhancement of photosynthesis in transgenic plants may have contributed to their better growth under salt conditions in addition to their improvement relative to wild-type in mechanisms, such as proline accumulation, to protect cells against NaCl stress.

Salt suppression of growth occurs in all plants, but their tolerance levels and rate of growth reduction at high salt concentrations vary widely among different plant species. Generally, salt stress reduces the water potential in the soil and causes disturbances in ion homeostasis and ion toxicity in plant cells that represses enzymatic activity in critical biochemical reactions (Zhang and Blumwald, 2001; Zhang et al., 2001). Along with these effects, the secondary stresses resulting from high salt stress, such as reactive oxygen species formation, may cause further damage to plant cells (Dat et al., 2000). Since salt stress involves both osmotic stress and ionic toxicity (Hagemann and Erdmann, 1997; Hayashi and Murata, 1998), plant growth suppression is directly related to the total concentration of soluble salts or the osmotic potential of soil water (Flowers et al., 1977; Greenway and Munns, 1980).

Salt damage of leaf tissues is usually the result of Na^+ accumulation in leaf cells that shortens the life span of individual leaves, thus reducing their net photosynthetic productivity and crop yield (Munns, 1993; 2002). Increased NaCl levels result in a significant decrease in root, shoot, and leaf biomass and an increase in the root/shoot ratio in cotton (Meloni et al., 2001; He et al., 2005). Under field conditions, salt stress is a major reason for cotton seed abortion, leading to both yield loss and lower fiber quality (Davidonis et al., 2000).

Although photosynthesis may not always be slowed by salinity and may even be stimulated by low salt concentration in some species (Rajesh et al., 1998; Kurban et al., 1999), there are numerous reports of suppression of photosynthesis under salt stress conditions (Chaudhuri and Choudhuri, 1997; Soussi et al., 1998; Romero-Aranda et al., 2001; Kao et al., 2001). Salinity decreases CO_2 assimilation into carbohydrates through reduction of leaf cell number (Papp et al., 1983;

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Munns et al., 2000), stomatal conductance (Brugnoli and Lauteri, 1991; Parida et al., 2003), mesophyll conductance (Delfine et al., 1998), and the efficiency of photosynthetic enzymes (Seemann and Critchley, 1985; Yeo et al., 1985; Brugnoli and Bjorkman, 1992). Salt stress also inhibits the repair of PSII via suppression of new D1 protein synthesis at the transcriptional and translational levels (Allakhverdiev et al., 2002). Thus, plant growth may be indirectly influenced by the effect of Na^+ on photosynthesis.

Transgenic cotton plants that overexpress the gene *AtNHX1* of Arabidopsis were shown to be more tolerant to salt stress than wild-type cotton plants, likely because of their improved capacity for Na^+/H^+ exchange (Apse et al., 1999; Zhang et al., 2001; Zhang and Blumwald, 2001). In the presence of 200 mM NaCl, the *AtNHX1*-expressing cotton plants produced more root and shoot biomass and greater fiber yield compared with wild-type plants when grown under greenhouse conditions (He et al., 2005). Although the transgenic plants had greater rates of CO_2 assimilation, the biochemical and physiological basis for the observed increase in salt tolerance in these *AtNHX1*-expressing cotton plants is not completely understood. In this study, the photosynthetic parameters associated with gas exchange and electron transport functions were analyzed to investigate the basis for improved CO_2 assimilation exhibited by *AtNHX1*-expressing cotton plants growing in the presence of 200 mM NaCl.

MATERIALS AND METHODS

Plant material and growth conditions. *AtNHX1*-expressing transgenic cotton plants were created by He et al. (2005). Seeds of the transgenic T_1 and its wild-type, Coker 312, were germinated in 50 × 80 cm trays containing Pro-Mix BX peat moss, perlite, and vermiculite medium (Premier Brands; New Rochelle, NY) and grown in a greenhouse at 28 ± 2 °C. The transgenic seedlings resistant to kanamycin were transplanted individually into 11-L pots. Salt stress was applied 2 wk later in an incremental manner, *i.e*. 50mM NaCl for 7 d, then 100 mM for 7 d, 150 mM for 7 d, and finally 200mM for 21 d (He et al., 2005). The experiment was repeated four times. Plant height, the length and width of the first fully-expanded leaf, and leaf number were scored 3 wk after the initiation of the treatment with 200 mM NaCl. The control group was placed in the same growth conditions except with 5 mM NaCl.

Measurement of A-C_i curve and estimation of derived parameters. Leaf net CO_2 assimilation rate (A), stomatal conductance, and transpiration were determined at an ambient CO_2 concentration of 400 μmol mol^{-1}, 60% relative humidity, 25 °C, and a photosynthetic photon flux density (PPFD) of 1500 μmol m^{-2} s^{-1} using a LiCor 6400 photosynthesis system (Li-Cor, Inc.; Lincoln, NE). The response of A to changes in the internal CO_2 concentration (A-C_i curve) was also conducted at 1500 μmol m^{-2} s^{-1}. The reference and sample IRGAs (infra-red gas analyzers) were automatically matched before each measurement. The A-C_i curves were started at 0 Pa CO_2, and the CO_2 was increased step-wise to 200 Pa using 14 different CO_2 values (0, 4, 6, 10, 15, 20, 25, 40, 60, 80, 100, 120, 150, 200 Pa). Parameters derived from the A-C_i curves, such as V_{cmax} (the maximum rate of carboxylation by Rubisco), J_{max} (the light-saturated rate of maximum electron transport), A_{sat} (net photosynthesis at saturating PPFD), and A_{max} (photosynthetic capacity at saturating PPFD and saturating atmospheric CO_2) were calculated using the Photosynthesis Assistant software (ver. 1.1.2; Dundee Scientific; United Kingdom) (Parsons and Ogston, 1999) as described in van Gestel et al. (2005).

Measurement of chlorophyll fluorescence. Chlorophyll fluorescence and gas-exchange parameters were measured for the fully-expanded leaves using the LiCor 6400 portable gas exchange and fluorescence system (Model LI 6400-40 Leaf Chamber Fluorometer; Li-Cor, Inc.; Lincoln, NE) after cotton plants were dark-acclimated for 3 h at dusk. The environmental parameters in the leaf chamber were as follows: (a) the concentration of ambient CO_2 was 400 μmol mol^{-1}; (b) the light source provided a PPFD (red+ blue) of 1500 μmol m^{-2} s^{-1} in which blue light was 10%; (c) the chamber temperature was 25 °C; (d) the air flow rate was 500 μmol s^{-1}. F_0 (minimal fluorescence level when all PSII reaction centers are open) for dark-acclimated leaves was obtained by using modulated light, which was sufficiently low (intensity set at 1 with 0.25 kHz modulation) to prevent photosynthesis. F_m (maximal fluorescence level when all PSII reaction centers are closed) for dark-acclimated leaves was determined by a 0.8 s saturating flash (intensity set at 8 with 20 kHz modulation). The leaves were then continuously illuminated at a PPFD of 1500 μmol m^{-2} s^{-1} of actinic light. F_s (steady-state value of fluorescence) was thereafter recorded and a second saturating flash was imposed to determine F_m' (maximal fluores-

cence level in light-acclimated leaves). The actinic light was turned off and F_0' (minimal fluorescence level in the light-acclimated state) was determined by illuminating the leaf with far-red light for 6 s. Measurements of F_s, F_0', and F_m' were performed at 3-min intervals. By using fluorescence parameters determined in dark-acclimated and light-acclimated leaves, calculations were made of the following parameters: (1) the maximal quantum yield of PSII photochemistry, $(F_m-F_0) / F_m = F_v / F_m$; (2) ΦPSII (actual quantum yield of electron transport in the light-acclimated state) = $(F_m'-F_s) / F_m'$; (3) qP (photochemical quenching coefficient) = $(F_m'-F_s) / (F_m'-F_0')$ and qN (non-photochemical quenching coefficient) = $(F_m-F_m') / (F_m-F_0')$; and (4) ETR (electron transport rate) = $(F_m'-F_s)$ f * I * α_{leaf} / F_m', in which f is the fraction of absorbed quanta that is used by PSII (usually 0.5 for C_3 plants) (Krall and Edwards, 1992), I is the incident photon flux density, and α_{leaf} is an effective leaf absorptance, which was computed based on the fraction of blue light and the user-entered absorptance (0.9 in the blue and 0.84 in the red). Fluorescence nomenclature was previously described by van Kooten and Snel (1990).

Determination of leaf proline and total soluble sugar content. Young, mature cotton leaves were separately prepared for the measurement of free proline content and total soluble sugars. One half gram of frozen plant material was homogenized in 1 mL of sterilized, ion-free water; insoluble material was removed by centrifugation at 3,800 *g*. Proline was measured as described by Bates et al. (1973) with minor modifications. A 100 µL aliquot of the extract was reacted with 1 mL glacial acetic acid and 1 mL acid ninhydrin (1.25 g ninhydrin dissolved in 30 mL glacial acetic acid and 20 mL 6 M phosphoric acid) for 1 h at 100 °C, and the reaction was then terminated in an ice bath. The reaction mixture was mixed with 4 mL toluene. The chromophore-containing toluene was aspirated from the aqueous phase and warmed to room temperature, and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve in the range of 0 to 20 µg/mL of L-proline.

The content of total soluble sugars was determined using the method described by Dubois et al. (1956) with a few modifications. A 25 µL aliquot of the leaf cellular extract was placed into a colorimetetric tube with 50 µL of 80% (v/v) phenol. Five milliliters of concentrated sulfuric acid were then added with rapid mixing. The tubes were allowed to stand for 10 min, and then shaken and placed in a water bath at 28 ºC for 15 min. The absorbance of the yellow-orange solution was measured at 490 nm for total soluble sugars. The amount of sugar was determined by reference to a standard curve obtained using D-glucose.

RESULTS

Under salt stress conditions, cotton plants expressing *AtNHX1* showed higher photosynthetic capacity and carboxylation rate in saturated CO_2 than wild-type plants. The growth of wild-type cotton plants was retarded by the application of 200 mM NaCl compared with the growth of *AtNHX1*-expressing cotton plants (He et al., 2005). Furthermore, most *AtNHX1*-expressing cotton plants displayed higher rates of CO_2 assimilation (A) that were associated with higher stomatal conductance than wild-type plant under salt stress conditions (200 mM NaCl) (He et al., 2005). To investigate further the salinity effects on the photosynthetic process, A-Ci curves for *AtNHX1*-expressing and wild-type cotton plants were obtained at the saturating light intensity of 1500 µmol m^{-2} s^{-1} during 200 mM NaCl treatment. When grown in 5 mM NaCl, there were no substantial differences in the genotypic responses of A to CO_2 (Fig. 1A). When grown with 200 mM NaCl, there was no effect of the salt treatment on the response of A for wild-type plants, but A at Ci > 25 Pa was higher for the *AtNHX1*-expressing cotton plants when grown with 200 mM NaCl (Fig. 1B). These data indicate that the rate of regeneration of the CO_2 acceptor ribulose-1, 5-bisphosphate (RuBP) at high Ci was enhanced for the transgenic plants when grown at 200 mM NaCl; however, at low Ci, there were few differences in A between *AtNHX1*-expressing and wild-type cotton plants (Fig. 1B), indicating that the carboxylation efficiency is not substantially altered by *AtNHX1*-expression when plants were grown in 200 mM NaCl.

The data from the A-Ci curves were applied to the model for photosynthetic response to CO_2 to estimate values of photosynthetic limiting parameters using the Photosynthesis Assistant software (Parsons and Ogston, 1999). Following Farquhar et al. (1980) and van Gestel et al. (2005), the maximum rate of carboxylation by Rubisco (V_{cmax}) at low C_i, the PPFD saturated rate of maximum electron transport (J_{max}), net photosynthesis at saturating irradiance (A_{sat}), and photosynthetic capacity at saturating

light and saturating atmospheric CO_2 (A_{max}) were calculated. Under normal growth conditions, there were no significant differences in A_{sat} (P = 0.61), A_{max} (P = 0.57), V_{cmax} (P = 0.96), and J_{max} (P = 0.55) between wild-type and transgenic lines (Figs. 2A & 2C). When grown with 200 mM NaCl, there was no apparent effect of the salt treatment on the response of A_{sat}, A_{max}, V_{cmax}, and J_{max} for wild-type plants; however, A_{sat}, A_{max}, V_{cmax}, and J_{max} for all transgenic plants were significantly enhanced when grown in 200 mM NaCl (Figs. 2B & 2D).

Under salt conditions, cotton plants expressing *AtNHX1* revealed higher levels of photochemical quenching coefficient and electron transport rate than wild-type plants. The potential maximum quantum yield of PSII photochemistry (F_v/F_m) is the most frequently used parameter to indicate the photoinactivation or damage to the PSII complexes because of stress factors, including salinity (Rees et

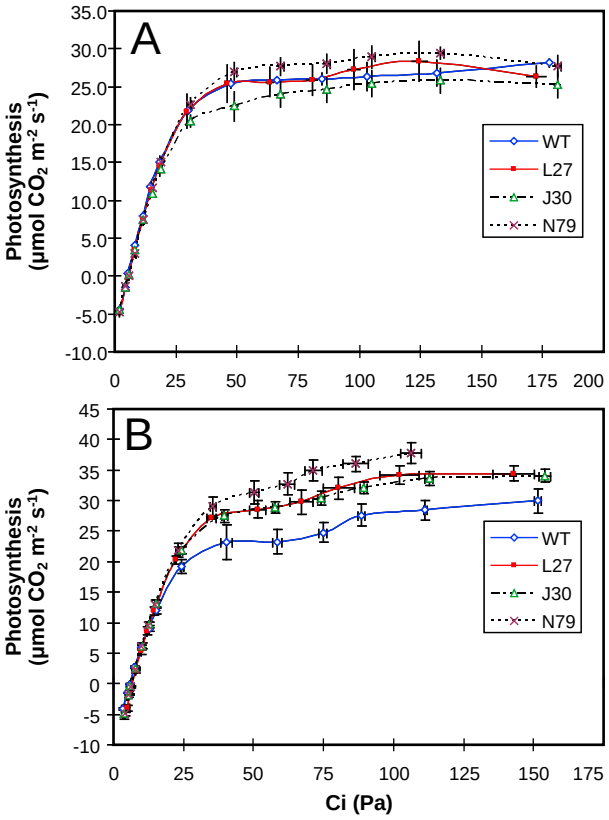

Fig. 1. The A-Ci curves of three independent lines of *AtNHX1*-expressing cotton plants (J30, L27, and N79) and wild-type cotton plants (WT, Coker 312) under 5 mM NaCl (A) and 200 mM NaCl (B) treatment for 4 wk. Values are the mean ± S.D. (n = 3).

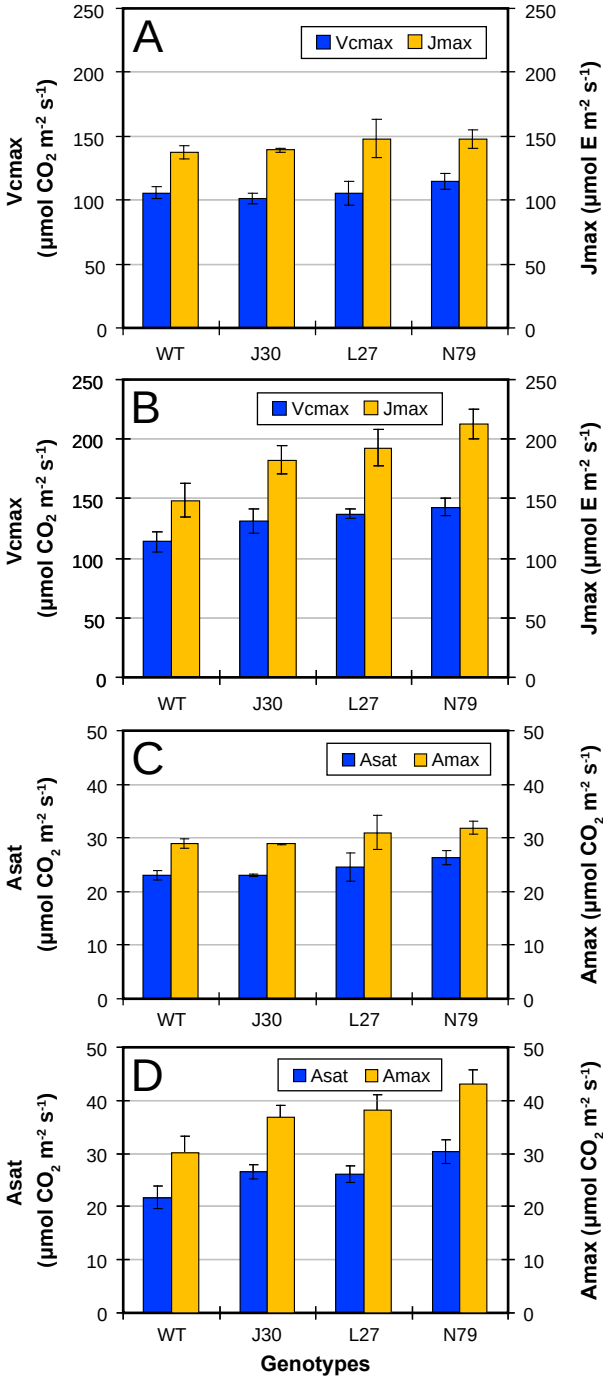

Fig. 2. Estimation of the maximum rate of carboxylation by Rubisco (V_{cmax}), the PAR-saturated rate of electron transport (J_{max}) (A & B), the rate of CO_2 saturated photosynthesis (A_{sat}) and the maximum potential photosynthetic rate (A_{max}) (C & D) for three independent lines of *AtNHX1*-expressing cotton plants (J30, L27, and N79) and wild-type cotton plants (WT, Coker 312) after treatment with 5 mM (A & C) and 200 mM (B & D) NaCl for 4 wk. Values are the mean ± S.D. (n = 3).

al., 1990; Krause and Weis, 1991; Schreiber et al., 1995; Roháček, 2002; Lu et al., 2003). The values of F_v/F_m for dark-acclimated plants of both genotypes grown under control conditions or with 200 mM NaCl for 4 wk and sampled at sunset were all high and not significantly different (data not shown). Thus, no sustained PSII photoinactivation was evident at the end of the day, even for plants grown in a NaCl concentration as high as 200 mM.

To investigate further whether the salinity treatment induced modifications in PSII photochemistry in light-acclimated leaves, chlorophyll fluorescence parameters were measured during a dark-to-light transition. Values for ΦPSII, qP, qN, and ETR were calculated using the values for F_0, F_m, F_0', F_m', and F_s, according to the formulae provided in the LiCor 6400 manual (Li-Cor Biosciences Inc., 2001). When grown in 5 mM NaCl (a normal growth condition), there were no significant differences in ΦPSII, qP, qN, and ETR between wild-type plants and *AtNHX1*-expressing transgenic plants (data not shown). For salt-treated plants during acclimation to light, ΦPSII increased as a result of the initiation of photosynthetic CO_2 assimilation reactions (Fig. 3A). After 6 min of illumination, the proportion of absorbed energy entering photochemistry (ΦPSII) in *AtNHX1*-expressing cotton plants was considerably higher than that in wild-type plants under the 200 mM NaCl treatment (Fig. 3A), a result consistent with the gas-exchange data. Whereas the wild-type plants under this condition did not show a significant depression in ΦPSII compared with growth under normal conditions (i.e. 5 mM NaCl) (data not shown), transgenic lines consistently exhibited 36-39% higher values than values for wild-type plants during photosynthetic induction and at steady state. Accordingly, the fraction of PSII reaction centers that were "open", which was assessed by means of photochemical quenching coefficient (qP), was 32% - 36% higher in *AtNHX1*-expressing transgenic lines than that in wild-type cotton plants (Fig. 3B), whereas thermal dissipation of excitation energy estimated as non-photochemical quenching coefficient (qN) was not significantly different between *AtNHX1*-expressing and wild-type cotton plants (Fig. 3C). The higher portion of "open" PSII reaction centers observed for transgenic vs. wild-type plants implies greater electron flow from PSII to PSI in the leaves of this genotype. Indeed, the electron transport rate, derived from the chlorophyll fluorescence parameters was 35%-40% higher for *AtNHX1*-expressing cotton plants than for wild-type cotton plants (Fig. 3D). These results were consistent with the data from the gas-exchange analyses (He et al., 2005 and Fig. 1).

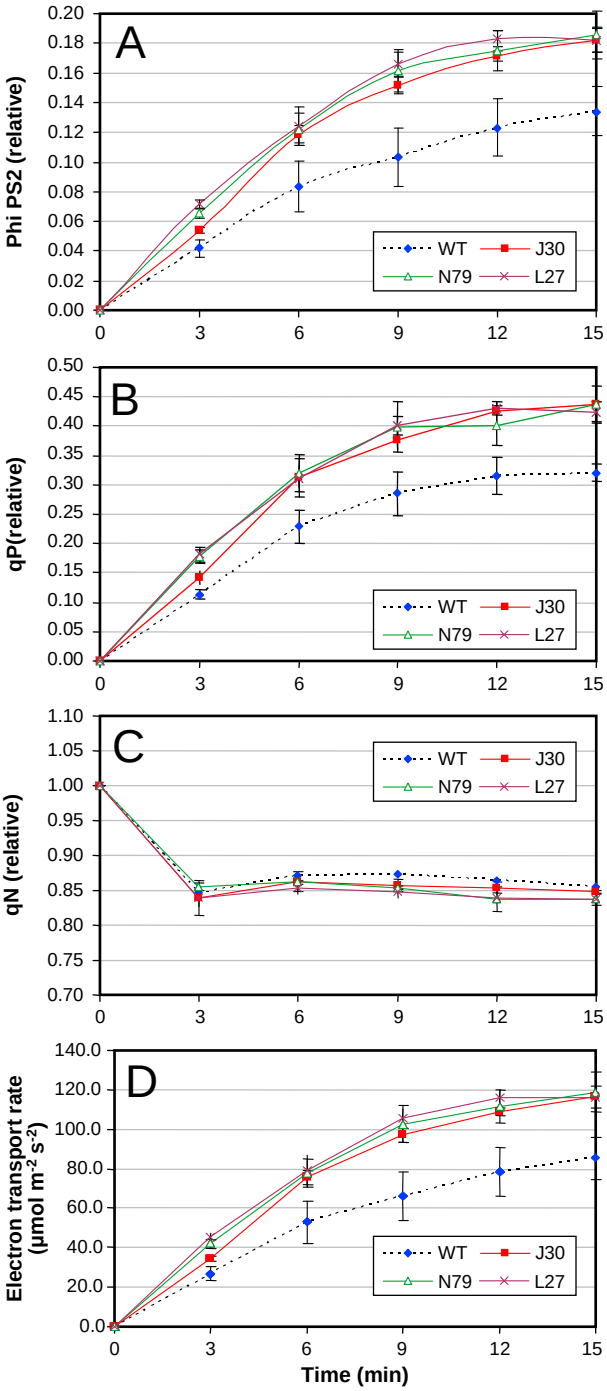

Fig. 3. Photosynthetic parameters for three independent *AtNHX1*-expressing cotton plants (J30, L27, and N79) and wild-type (WT, Coker 312) cotton plants during acclimation to light while being grown in the presence of 200 mM NaCl. A. The efficiency of PSII photochemistry. B. Photochemical quenching coefficient. C. Non-photochemical quenching coefficient. D. Rates of electron transport. Values are the mean ± S.D. (n = 3).

Under salt stress conditions, cotton plants expressing *AtNHX1* contained more proline than wild-type cotton plants. Proline appears to play a vital role in protecting transgenic tomato (Zhang and Blumwald, 2001) and canola plants (Zhang et al., 2001) under salt stress conditions. Soluble sugars, such as glucose, fructose and sucrose, also accumulate under salt stress conditions (Kerepesi and Galiba, 2000; Bohnert and Jensen,1996; Parida et al., 2002) that may contribute to improved ionic balance and osmoprotection (Parida and Das, 2005). Therefore, the content of proline and total soluble sugars of *AtNHX1*-expressing and wild-type cotton plants grown under normal and 200 mM NaCl conditions were determined. Under normal growth conditions, no significant differences in the content of proline and total soluble sugars were observed between *AtNHX1*-expressing and wild-type cotton plants (Figs. 4A & 4B); however, when plants were grown in the presence of 200 mM NaCl, an increase in proline (187%) and soluble sugars (20%) occurred in wild-type plants, and a 251-362% increase in proline content in *AtNHX1*-expressing cotton plants was detected (Fig. 4A), along with an 18-35% increase in soluble sugars (Fig. 4B); however, differences between wild-type and transgenic plants were only substantial for proline content.

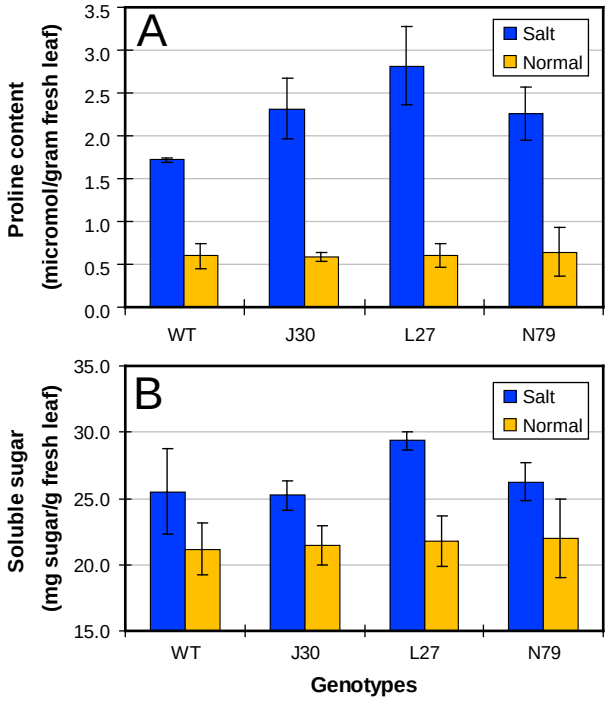

Fig. 4. Proline content (A) and total soluble sugar content (B) of three independent *AtNHX1*-expressing cotton plants (J30, L27, and N79) and wild-type (WT, Coker 312) cotton plants after treatment with 5 mM (Normal) and 200 mM NaCl for 4 wk. Values are the mean ± S.D. (n = 4).

DISCUSSION

It was previously shown that salt-tolerance could be created by boosting vacuolar accumulation of Na^+ through increasing expression of the gene *AtNHX1* that encodes the Na^+/H^+ antiporter (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001). In this research, the growth of wild-type cotton was severely inhibited by the presence of 200 mM NaCl, whereas the growth of *AtNHX1*–expressing cotton plants was significantly less inhibited (He et al., 2005). Furthermore, the *AtNHX1*–expressing cotton plants produced more bolls and fiber (He et al., 2005). Data in this study are consistent with those obtained from other species, such as Arabidopsis (Apse et al., 1999), rapeseed (Zhang et al., 2001), and tomato (Zhang and Blumwald, 2001), which confirms the prediction that expression of *AtNHX1* in cotton could indeed improve cotton's salt tolerance. To explore the biochemical and physiological basis for the observed increase in salt tolerance in the *AtNHX1*–expressing cotton plants, a series of physiological experiments were conducted to analyze the effects of salt on growth and development, as well as photosynthesis of these plants. The data indicate that the shoot growth in *AtNHX1*–expressing cotton plants was much less inhibited than those of wild-type plants in the presence of 200 mM NaCl (He et al., 2005). Furthermore, inhibition of leaf expansion and leaf number by salt was less for *AtNHX1*-expressing plants than for wild-type plants (data not shown).

Growth inhibition of wild-type plants by 200 mM NaCl was not associated with effects of salt on photosynthetic parameters. Photosynthetic parameters measured in this study were not affected by growth in 200 mM NaCl. In a study on barley, salinity induced only small decreases in PSII activity at midday steady-state photosynthesis, indicating that photosynthetic electron transport was little affected by salinity (Belkhodja et al., 1999). Nonetheless, in the case of this cotton experiment, the reduction in leaf length and width (data not shown) resulting from salt stress should have reduced the net carbon gain of the plant. Interestingly, growth in the presence of 200 mM NaCl actually enhanced most photosynthetic parameters for *AtNHX1*-expressing plants. The greatest enhancement appeared to be in the quantum yield of

PSII and overall electron transport; however, some increase over plants grown under control conditions in carboxylation (Fig. 2) and net CO_2 assimilation (A) at ambient CO_2 (He et al. 2005 and Fig. 1) did occur. Thus, the greater values of A and larger leaf size relative to wild-type plants most likely contributed to the greater biomass production when grown in 200 mM NaCl (He et al., 2005). Also, the ability of the transgenic plants to accumulate more proline than wild-type plants during salt stress suggests that the transgenic plants have an enhanced capacity for mechanisms to protect cells and potentially improve growth during salt stress.

The greatest improvement in A for *AtNHX1*-expressing plants grown at 200 mM NaCl was noted at Ci values above those corresponding to ambient external CO_2 concentration (Fig. 1). Below these Ci values, A for all plants was comparable to A under control conditions. Therefore, carboxylation efficiency was not especially enhanced by growth in 200 mM NaCl, but RuBP regeneration at high CO_2 was enhanced for the *AtNHX1*-expressing plants. Improved rates of electron transport and phosphate cycling for ATP synthesis appear to be involved (Figs. 1 and 2).

Because A for wild-type plants was not negatively affected by 200 mM NaCl, no sustained inactivation of PSII (high F_v/F_m) was noted for these plants at the end of the day (data not shown) in accordance with results previously reported in rice and *Suaeda salsa* by Dionisio-Sese and Tobita (2000) and Lu et al. (2003), respectively, suggesting that PSII complexes in cotton are very tolerant of salt stress or they can be effectively repaired during light period. Apparently, the rate of PSII activation/repair along with the rates of electron transport, and the ability to dissipate excess absorbed energy thermally, as reflected in the non-photochemical quenching of chlorophyll fluorescence (qN), were sufficient to maintain population of active PSII reaction centers in wild-type cotton plants. Despite a greater rate of electron transport for the *AtNHX1*-expressing plants, their values of qN were similar to those for wild-type plants (Fig. 3C), suggesting that the same proportion of energy was thermally dissipated in both sets of cotton plants. These results are consistent with results obtained for salt-tolerant rice cultivars (Dionisio-Sese and Tobita, 2000) and *Suaeda salsa* (Lu et al., 2003).

In conclusion, growth at 200 mM NaCl does restrict growth of wild-type cotton stems and leaves; however, this salt treatment does not affect photosynthetic parameters. For the *AtNHX1*-expressing plants, their enhancement of CO_2 assimilation combined with their increased leaf length and width may have contributed to their greater growth rates in 200 mM NaCl than wild-type plants. Also, the transgenic plants may have improved mechanisms of cell protection and growth that would lead to the development of plants larger than wild-type plants whether photosynthesis was enhanced or not.

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