Ectopic expression of *GmNHX3* and *GmNHX1*, encoding two *Glycine max* Na⁺/H⁺ vacuolar antiporters, improves water deficit tolerance in *Arabidopsis thaliana*

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Abstract

The importance of Na⁺/H⁺ antiporters in salt tolerance in plants has been demonstrated in many studies, but much less is known about their protective role during drought stress. To study their possible contribution to water deficit tolerance, two closely related soybean Na⁺/H⁺ antiporters belonging to the intracellular NHX exchanger protein family, GmNHX3 and GmNHX1, were evaluated in transgenic *Arabidopsis thaliana*. *A. thaliana* plants ectopically expressing *GmNHX3* or *GmNHX1* displayed a more drought-tolerant phenotype compared to wild-type plants, which was accompanied by an increase in relative water content and chlorophyll content during stress conditions. Both *GmHNX1* and *GmHNX3* transgenic lines accumulated higher amounts of Na⁺ and K⁺ cations, showed increased antioxidant enzyme activities and less membrane damage due to lipid peroxidation under water deficit, as compared to non-transformed plants. Furthermore, plants expressing *GmNHX3* showed an increased sensitivity to abscisic acid as deduced from stomatal closure and seed germination inhibition studies. Finally, a significant up-regulation of abiotic stress-related genes was observed in both transgenic lines compared to wild-type plants in response to abscisic acid and mannitol treatments. These results demonstrate that GmNHX3 and GmNHX1 antiporters confer protection during drought stress in *A. thaliana* and hence are potential genetic targets to improve drought tolerance in soybean and other crops.

Keywords: abscisic acid, antioxidant enzymes, chlorophyll, drought tolerance, lipid peroxidation, mannitol, soybean, stomata.

Introduction

Water deficiency is probably the most important environmental factor limiting plant growth and reducing crop yields worldwide, a problem that is thought to be exacerbated by the effects of climate change (Fuganti-Pagliarini *et al.* 2017). As a consequence, there is an urgent need to develop more drought-tolerant crop cultivars to counteract the negative impact of less water availability in most crop production areas. But, to achieve this, a better understanding of the underlying molecular mechanisms of plant responses to water stress is needed.

Soybean (*Glycine max* L. Merr) is a major staple food and the most important legume crop. Almost all soybean production is rain-fed and low water availability is responsible for an average yield loss of 13 %, with much

higher losses in years with prolonged periods with little or no precipitation (Zipper *et al.* 2016).

Plant adaptations to water scarcity include avoiding cellular dehydration by different physiological responses such as synthesis of abscisic acid (ABA), stomatal closure, and accumulation of osmolytes (proline and soluble sugars) as well as by increasing drought tolerance through the induction of protective mechanisms against cell damage, such as the production of antioxidants and activation of redox-regulating enzymes together with synthesis of dehydrins and late-embryogenesis abundant (LEA) proteins (Fang and Xiong 2015). ABA is also known to be involved in shoot growth inhibition while maintaining or stimulating root growth, to explore access to water under low water availability (Sharp *et al.* 2004), and to control seed germination and dormancy (Garciarrubio *et al.* 1997), which are inhibited under adverse environmental conditions such as cold and drought (Rodriguez-Gacio *et al.* 2009).

The cation/proton (monovalent cation/H^+) exchangers (NHX) constitute an important family of proteins in plant abiotic stress responses, which have been shown to play an important protective role under salinity (Li *et al.* 2017, Ma *et al.* 2017, Wang *et al.* 2018, Wu *et al.* 2018) and to a lesser extent under drought (Brini *et al.* 2007, Li *et al.* 2011, Bao *et al.* 2016). In plants, NHX antiporters are ubiquitous and catalyze the electroneutral exchange of H^+ for Na^+ or K^+ (Bassil *et al.* 2019). These antiporters are generally found in the cellular plasma membrane, tonoplast, or endosomal compartments and they play a central role in diverse cellular processes, including pH homeostasis, Na^+ and K^+/H^+ movement, cell elongation, vesicle trafficking and fusion, and to generate an electrochemical gradient of H^+ across the tonoplast and plasma membrane to transport Na^+ or K^+ into the vacuole or Na^+ outside the cell (Adabnejad *et al.* 2015, Dong *et al.* 2019). A recent report revealed specific contributions to both vacuolar pH and K^+ and Na^+ uptake/selectivity of different tonoplast NHX isoforms in *A .thaliana*, shading light to specific roles of NHXs in ion homeostasis (Bassil *et al.* 2019).

Numerous studies describe transgenic plants expressing *NHX* genes of different origin (Brini *et al.* 2007, Li *et al.* 2010, Li *et al.* 2011, Bao *et al.* 2016, Dong *et al.* 2019, Guo *et al.* 2020), but there are only a few reports describing the effects of ectopic expression of *NHX* genes from soybean. In the first study, the soybean antiporter GmNHX1, coded by glyma.20g229900 gene, was shown to enhance salt tolerance in transgenic *Lotus corniculatus* plants. GmNHX1 showed high similarity to other plant vacuolar NHXs such as AtNHX1 (75.8 %) in *A. thaliana*, OsNHX1 (75.3 %) in *Oryza sativa* and AgNHX1 (78 %) from the halophyte *Atriplex gmelini* (Sun *et al.* 2006). In accordance with the result in *L. corniculatus*, transgenic bright yellow (BY)-2 cells expressing a putative chloride channel gene (*GmCLC1*) together with *GmNHX1*, displayed increased NaCl tolerance (Li *et al.* 2006). Further evidence for the role of soybean NHX proteins in salinity tolerance comes from a study of a second soybean antiporter, GmNHX2, coded by glyma.15g124100 gene, which improved salt stress tolerance when expressed in *A. thaliana* plants

(Zhou *et al.* 2009). Recently, it was reported that *GmNHX1* expression increased under salt stress in salt-susceptible and tolerant soybean cultivars, but that expression was more pronounced in salt-tolerant genotypes (Ning *et al.* 2018). However, no additional abiotic stress tolerance studies, including drought, have been conducted with soybean GmNHX antiporters.

The aim of this study was to evaluate the possible protective role during water scarcity of two soybean antiporters, the aforementioned GmNHX1 and the not previously studied homolog, GmNHX3, encoded by gene glyma.10g158700. For this purpose, physiological, biochemical and genetic responses were studied in *A. thaliana* homozygous transgenic plants expressing either *GmNHX1* or *GmNHX3* exposed to water stress conditions.

Materials and methods

***GmNHX1* and *GmNHX3* cDNA cloning:** Cloning of *GmNHX1* and *GmNHX3* cDNAs from soybean (*Glycine max* L. Merr) cv. Munasqa was performed using the *Gateway* cloning system (*Life Technologies*, USA). Coding sequences (CDS) for both genes were amplified by PCR (DNA polymerase *Kapa HiFi*, *Kapa Biosystems*, USA) using primers listed in Table 1 Suppl. Amplified PCR products were sequenced (*Macrogen*, Seoul, Korea) and cloned in the pDONOR entry vector (*Invitrogen*, USA) by a recombination reaction using the BP clone II enzyme (*Invitrogen*), according to the manufacturer´s instructions. Donor vector was transformed and amplified in competent *Escherichia coli* Top10 strain and the presence of cloned genes was verified by restriction of DNA with enzymes *Xho*I/*Hind*III and further sequencing. Sub-cloning, behind the constitutive ubiquitin-10 (UBI 10) promotor from *Arabidopsis thaliana*, in the target vector pUB-DEST binary vector (Grefen *et al.* 2010) (Fig. 1 Suppl.) was carried out by recombination using the LR clone enzyme (*Invitrogen*). *E. coli* Top10 competent cells were transformed and the insertion of correct genes was confirmed by *Xho*I/*Hind*III restriction analysis and by DNA sequencing. Clones with complete soybean cDNAs were used to transform *Agrobacterium tumefaciens* strain C58C1 and used for genetic transformation of *A. thaliana* (Col-0) plants.

***Arabidopsis thaliana* transformation and molecular characterization of transgenic lines:** The *Agrobacterium*-mediated floral dip transformation method was used to produce transgenic *A. thaliana* plant (Clough and Bent 1998). Four independent lines (T2 generation) were obtained for each antiporter cDNA sequence. Transgenic NHX plants were identified by PCR with primers listed in Table 1 Suppl. Homozygous transgenic T3 plants were selected by 3:1 segregation of *BASTA* resistance (glufosinate ammonium, *Sigma Aldrich*, St. Louis, USA). Expression of transgenes was tested in 2 independent T3 lines from all selected antiporter-transformed lines by reverse transcription (RT) semi-quantitative (sq)PCR

(*GmNHX1* L1 and L2, *GmNHX3* L1 and L2) and RT-quantitative (q)PCR (Fig. 2 Suppl.) using specific primers for *GmNHX3* and *GmNHX1* (Table 1 Suppl). The PCR was performed using an annealing temperature of 57 °C for 30 s and 30 cycles, with an extension time of 40 s at 72 °C. *A. thaliana* elongation factor 1 (*EF1c*) gene (At1g18070) was employed as an internal control for constitutive expression. *BLAST*, *TMpred*, and *ClustalW* (*EMBL-EBI*) online software were used for sequence analysis.

Growth conditions and treatments: *A. thaliana* (Col-0) wild type (WT) and transgenic plants were grown in Petri dishes containing Murashige and Skoog (MS) medium supplemented with 1 % sucrose in a growth cabinet with a 12-h photoperiod, the photon flux density of 90 μmol m^{-2} s^{-1}, and a temperature of 22 $\pm$ 1 °C. After one week, seedlings were transferred to plastic pots containing a mixture of commercial peat (*Grow mix Multipro*, *Terrafertil*, Argentina) and *Perlite* (5:1). For RT-qPCR gene expression assays, 5-d-old seedlings grown on MS agar were transferred to liquid MS medium for two days before mannitol- or ABA-treatments were applied.

For drought treatment, 4-week-old WT and transgenic plants were treated by withholding water for 12 d where after watering was resumed. Photographs were taken before the drought, after drought treatment for 12 d and 2 d after rewatering, respectively. The outlined experiment was performed four times with similar results.

Relative water content (RWC) and chlorophyll measurements: Leaves from plants subjected to 12 d of water stress or well-watered plants were weighed (FM) and thereafter placed in Petri dishes containing distilled water, and incubated at room temperature for 12 h where after leaves were reweighed to obtain a water-saturated mass (WSM). For dry mass (DM) determination, each sample was dried at 80 °C until a constant mass was obtained. RWC was calculated according to Antolín *et al.* (1995). Total chlorophyll content in leaves for all treatments was measured as previously described Ni *et al.* (2009). The experiments were repeated four times using two biological replicates per treatment and experiment ($n = 8$).

Na^+ and K^+ content measurements: Four-week-old soil-grown plants were subjected to either water stress or well-watered conditions for 12 d. Leaves from WT and transgenic lines were harvested, dried at 80 °C, and then incinerated. The corresponding ashes were dissolved in 0.1 M HCl, and the Na^+ and K^+ content was determined by atomic absorption spectrophotometry (*Hitachi*, University of Buenos Aires, Argentina). Two independent experiments were performed using two biological replicates ($n = 4$).

Stomatal aperture assays were performed as previously described (Gudesblat *et al.* 2009). Epidermal peels from WT and transgenic 4-week-old plants were floated in 10:10 buffer (10 mM KCl and 10 mM MES-KOH, pH 6.15) under irradiance of 90 μmol m^{-2} s^{-1} for 2.5 h, after which ABA (0.1, 1, and 20 μM, *Sigma*) was added to the medium and peels were incubated under irradiance for a further 1.5 h.

Forty apertures were measured for each treatment. Data are presented as the average from 120 aperture measurements, collected from three independent experiments.

Seed germination assay: After surface sterilization, WT and transgenic seeds were sown on MS plates supplemented or not with ABA (0.3 μM). Sown plates were placed in the dark at 4 °C for 2 d to break seed dormancy and then transferred to a plant growth chamber with a 12-h photoperiod, a photon flux density of 90 μmol m^{-2} s^{-1}, and at 22 $\pm$ 1 °C, to let seeds germinate. Seed germination rate was determined every 24 h and a seed was considered as germinated when its radicle had penetrated the seed coat.

RT-qPCR gene expression assays: Gene expression assays were performed on five 7-d-old seedlings grown in a liquid medium (four biological replicates per treatment). Seedlings were then placed on different medium with H_2O (control), ABA (50 μM), or mannitol (300 mM, *Biopack*, Buenos Aires, Argentina) for 3 h. Plant material at indicated time points was frozen in N_2. Total RNA was extracted using *Trizol* reagent (*Invitrogen*) according to the manufacturer's instructions. RNA samples were treated with *RQ-1* DNA-free DNase (*Promega*, USA) and quantified with a *Nanodrop* spectrophotometer (*Biophotometer Plus*, *Eppendorf*, Germany). The cDNA synthesis was performed using the M-MLV reverse transcriptase enzyme (*Promega*) according to the manufacturer's instructions. All RT-qPCR experiments were carried out in a *StepOne Plus* real-time PCR system (*Applied Biosystems*). Elongation factor 1 gene (*EF*1c) was used as an internal constitutive reference gene. Primers used for RT-qPCR are listed in Table 1 Suppl. The RT-qPCR data analysis and primer efficiencies were obtained using *LinReg PCR* software (Ramakers *et al.* 2003). *EF*1c gene was used to standardize the expression of a given target gene; then a ratio between treatments was calculated using the algorithm previously developed (Pfaffl 2001). Relative expression ratios and statistical analysis were performed using *fgStatistics* software (Di Rienzo *et al.* 2009). The cut-off for statistically significant differences was set as a *P* value < 0.05.

Biochemical analyses: Plant leaves from 4-week-old plants were frozen in liquid N_2, weighed and stored in 2-cm^3 Eppendorf tubes in aliquots of 70 mg of tissue at -70 °C. A method of uniform extraction (Singh *et al.* 2015) and protein content quantification (Bradford 1976) was carried out. The determination of the activity of superoxide dismutase (SOD, EC 1.15.1.1) was performed by the pyrogallol self-oxidation method (Li *et al.* 2012), meanwhile, ascorbate peroxidase (APX, EC 1.11.1.11), phenol peroxidase (POX, EC 1.11.1.7), and catalase activities (CAT, EC 1.11.1.6) were determined according to previously published protocols (Chance and Maehly 1955, Kar and Mishra 1976, Nakano and Asada 1987). Malondialdehyde content (MDA) quantification was performed according to the method of Hodges *et al.* (1999) and free proline was determined according to Bates *et al.* (1973). All spectrophotometric readings were performed in

triplicate in a UV-VIS Model U-1800 Spectrophotometer (Hitachi).

Statistical analysis: Statistically significant differences were determined based on *ANOVA* and Student´s tests (for RWC, chlorophyll content, Na$^+$ and K$^+$ determinations, and seed germination assay) performed with *Infostat* software (Di Rienzo *et al.* 2018).

Results

To study the possible role played by soybean NHX proteins in drought tolerance, gene *GmHNX1*, and its closely related homolog *GmNHX3* were both cloned and transformed into *A. thaliana* plants. Two T3 homozygous transgenic lines for each antiporter, *GmNHX1* L1 and L2 and *GmNHX3* L1 and L2, were selected and molecular analyses performed. The presence of the two antiporter genes in the *A. thaliana* genome was confirmed by PCR analysis (Fig. 1*A*) and constitutive gene expression of the two transgenes was studied by semi-quantitative RT-PCR (Fig. 1*B*) and RT-qPCR (Fig. 2 Suppl.). A difference in transgene expressions between *GmNHX3* and *GmNHX1* lines was observed, where lines transformed with *GmNHX3* showed higher expressions of the transgene compared to *GmNHX1* lines.

The cDNA sequences (Fig. 3*A* Suppl.) of *GmNHX3* and *GmNHX1* isolated from soybean cv. Munasqa showed a 93 % of similarity between them (Fig. 3*B* Suppl.). Comparison with the CDS sequences of the cv. Williams 82 soybean reference genome showed a high similarity of *GmNHX3* with Glyma.10g158700 (97 %) and *GmNHX1* with Glyma.20g229900 (96 %) (*Phytozome v12.1*). Sequence analysis of *GmNHX3* and *GmNHX1* isolated from Munasqa revealed putative ORFs of 1 101 and 951 bp, encoding for proteins of 367 and 317 amino acids, respectively (Fig. 3*C* Suppl.). Protein alignments showed that these NHXs presented high similarity to orthologues from other legumes such as *Vigna unguiculata* (94 %), *Medicago sativa* (92 %), and *Lupinus angustifolius* (89 %) (Fig. 4*A* Suppl.). A phylogenetic tree analysis demonstrated a higher similarity between the two NHX3

antiporters from cultivars Munasqa and Williams 82 than between the two NHX1 proteins (Fig. 4*B* Suppl.).

When analyzing the corresponding coding regions using the *TMpred* prediction program, 9 putative transmembrane segments were found for GmNHX3 and 8 for GmNHX1 (Fig. 4*C* Suppl.). The closely related *GmNHX1* cDNA product isolated from soybean cv. Kefeng 34 showed 12 transmembrane segments and exhibited high similarity to vacuolar antiporters AtNHX1, OsNHX1, and AgNHX1 (Sun *et al.* 2006). The high homology found between the three soybean NHXs and known plant vacuolar antiporter proteins supports that Munasqa GmNHX1 and GmNHX3 belong to the gene family of vacuolar NHXs.

To evaluate plant growth and survival under water deficit, 4-week-old soil-grown transgenic and WT plants were withheld of water for 12 d. This treatment caused severe tissue damage to WT plants while transgenic *GmNHX* lines showed less tissue damage and dehydration symptoms such as bleaching and withering (Fig. 2*A* and *B*). When watering was resumed for 2 d after drought treatment, 25 % of WT plants were able to recover from the stress treatment, while 83.3 % of *GmNHX3* L1 and 75 % of *GmNHX1* L1 plants resumed growth. Lines *GmNHX3* L2 and *GmNHX1* L2 displayed an intermediate response with 50 and 41.6 % of plants resuming growth, respectively (Fig. 2*C*, Table 2 Suppl.).

RWC and chlorophyll content showed no significant differences between WT and transgenic lines when plants were grown under well-watered conditions, but in plants exposed to drought stress a significantly higher reduction in RWC (~50 %) was observed in WT plants as compared to *GmNHX* expressing lines (~30 %) (Fig. 2*D*).

Chlorophyll content decreased more than 50 % in drought-stressed WT plants and a similar reduction was observed in transgenic lines *GmNHX3* L2 and *GmNHX1* L2 (Fig. 2*E*). In contrast, a very low reduction in total chlorophyll content was observed for transgenic lines, *GmNHX3* L1 and *GmNHX1* L1, when exposed to water stress (Fig. 2*E*). This is in agreement with the dehydration tolerant studies where these two lines demonstrated the highest survival rates.
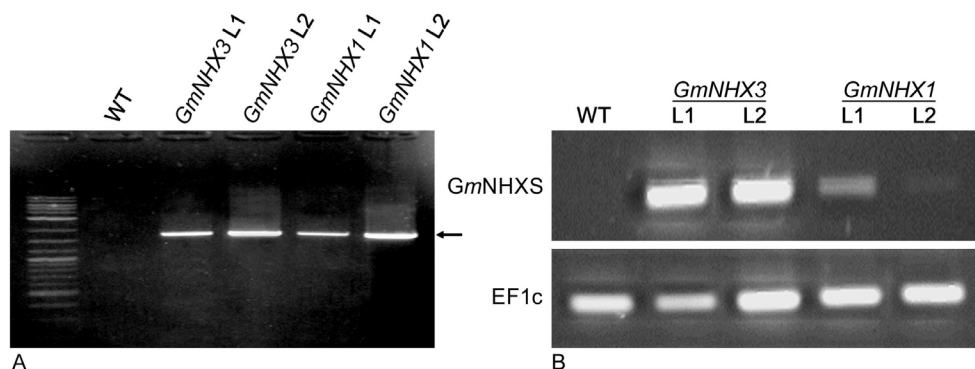
To investigate the capacity to accumulate cations in

Fig. 1. *A* - PCR analysis of *A. thaliana* genomic DNA confirms the presence of *GmNHX1* (L1 and L2, 1661 bp) and *GmNHX3* (L1 and L2, 1657 bp) in transgenic lines. DNA size marker (NEB, UK) is indicated on the left. *B* - Semi-quantitative RT-PCR analysis of *GmNHX1* and *GmNHX3* expression in transgenic *A. thaliana* lines: *EF*1c constitutive expressions (151 bp; *below panel*), *GmNHX1* (151 bp; *upper panel*) and *GmNHX3* (150 bp; *upper panel*) are indicated for 4 transgenic lines and WT as a control.
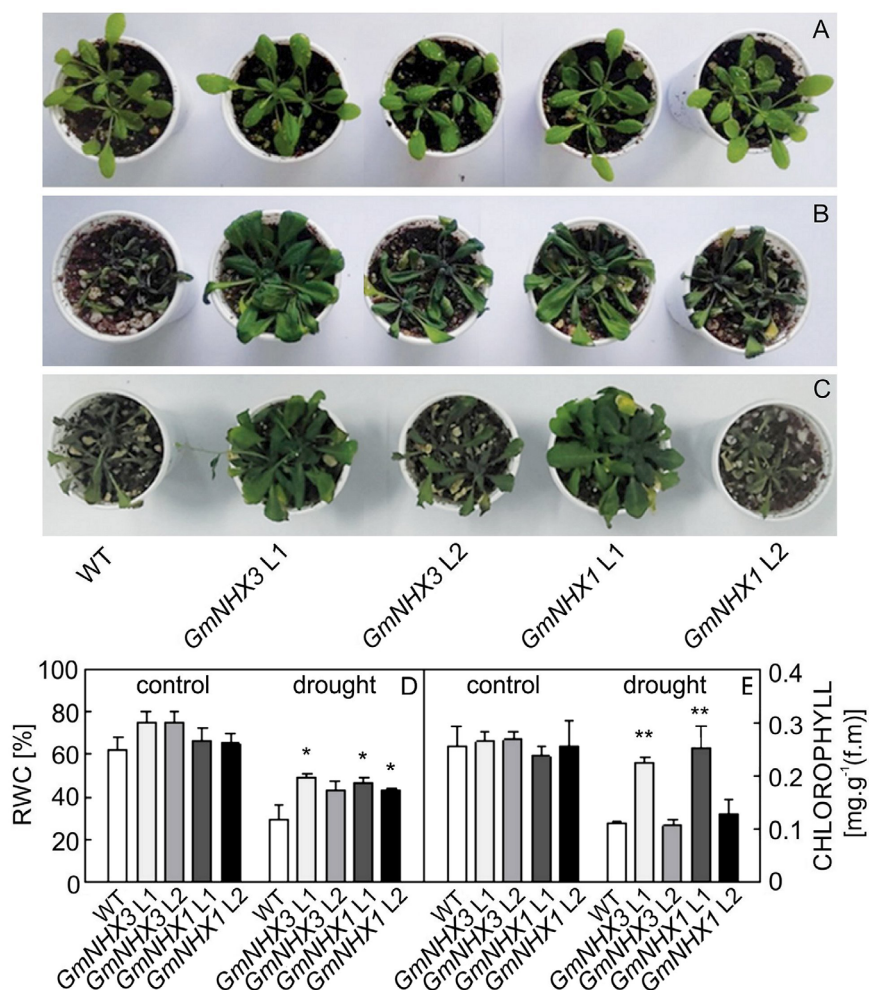
Fig. 2. *A* - Wild type (WT) and transgenic 4-week-old plants grown under well-watered conditions prior to drought treatment; *B* - after 12 d of drought treatment; *C* - recovering plants after being rewatered for 2 d; *D* - relative water content, and *E* - chlorophyll content measurements in plants under normal conditions and after 12 d of drought stress. *A*, *B*, *C* were performed four times with similar results (four independent experiments with three biological replicates each, *n* = 12). *D* and *E* are presented as the average from 8 measurements per WT and transgenic lines, collected from four independent experiments with two biological replicates each, *n* = 8). Means ± SEs. Significant differences between transgenic and WT samples are indicated with *asterisks* (* - $P < 0.05$, ** $P < 0.01$; Student's *t*-test).

Fig. 3. Na$^+$ (*A*) and K$^+$ (*B*) content measured in *A. thaliana* leaves from plants treated by withholding water for 12 d or grown under well-watered conditions. Means ± SEs from 4 individual plants for each treatment (*n* = 4). Significant differences between transgenic and WT samples are indicated with *asterisks* (* - $P < 0.05$, ** $P < 0.01$; Student's *t*-test).

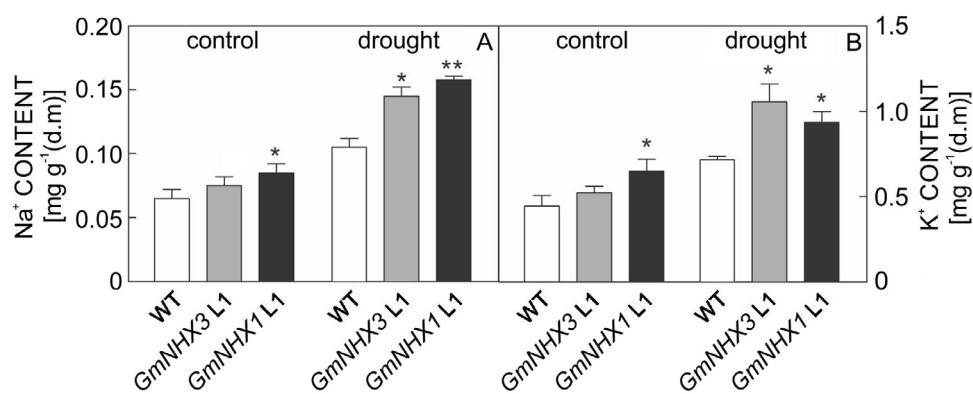*GmNHXs* transgenic plants exposed to drought, Na$^+$ and K$^+$ content was determined in *A. thaliana* leaves after withholding water for 12 d. Results showed that *GmNHX3* L1 and *GmNHX1* L1 plants exposed to drought stress, accumulated significantly more Na$^+$ and K$^+$ than WT plants (38 and 50 % for Na$^+$ and 47 and 30 % for K$^+$, respectively)
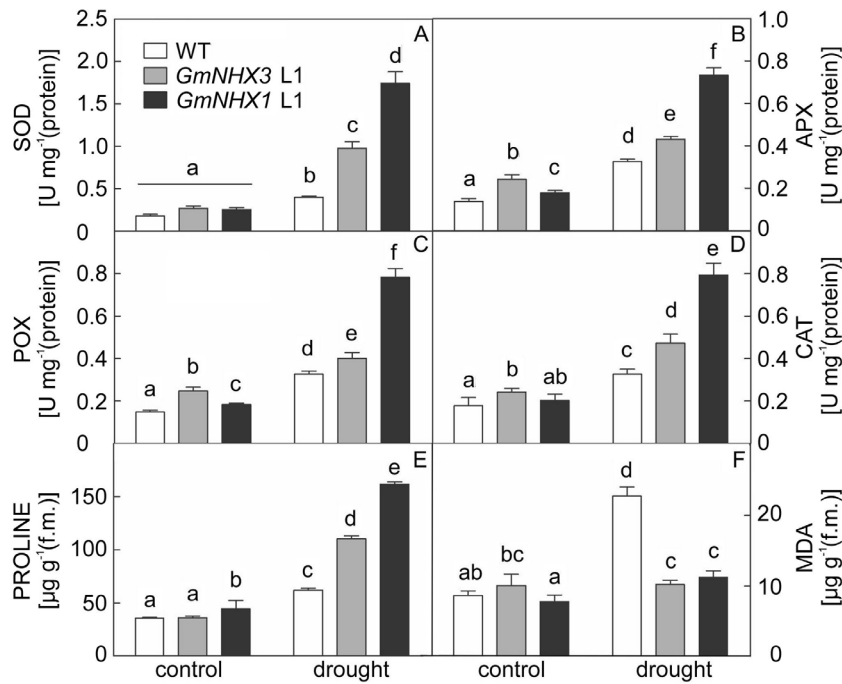
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Fig. 4. Specific activity of SOD (*A*), APX (*B*), POX (*C*), and CAT (*D*) as well as free proline (*E*) and MDA (*F*) content in WT and transgenic plants grown under well-watered conditions and drought (12 d treated with withholding water). The enzyme specific activities are represented as units of enzyme per mg of protein. Means from 5 measurements per WT and transgenic lines, collected from two independent experiments (*n* = 2 per trial). Means ± SDs. Different letters indicate significant differences at *P* < 0.05 in all experiments (two-way *ANOVA*, Bonferroni´s test).

(Fig. 3*A* and *B*).

Improved oxidative stress protection in transgenic lines exposed to water stress was evident by increased enzymatic activities of SOD, APX, POX, and CAT as compared to WT plants (Fig. 4*A-D*). Similarly, a more pronounced proline accumulation was observed in both *GmNHX* lines under drought although especially notable in the *GmNHX1* L1 line (Fig. 4*E*). When exposed to drought treatment, transgenic lines maintained similar MDA values as seen in non-stressed plants, which was in sharp contrast to WT plants where a significant increase of MDA was observed under drought conditions (Fig. 4*F*).

To determine the effects of constitutive expression of *GmNHX*s on stomatal regulation, plants were treated with different ABA concentrations and stomatal aperture determined. No difference in stomatal aperture was observed between WT and *GmNHX*s without ABA treatment, indicating that there is no stomata phenotype, *a priori*, in transgenic lines (Fig. 5*A* Suppl). However, at 0.1 μM ABA, *GmNHX3* L1 exhibited a significant increase in stomatal closure (~42 %) compared to WT stomata (30 %). At 1 μM ABA, *GmNHX3* L1 still exhibited a more ABA-sensitive phenotype (70 % of stomata closure) compared to 42 % for WT and *GmNHX1* L1 (Fig. 5*A* Suppl). At 20 μM ABA, no difference was observed in stomatal closure between WT and *GmNHX*s, indicating that, only at lower ABA concentrations, *GmNHX3* L1 plants are more sensitive to ABA-induced stomatal closure.

To evaluate if other known ABA-responses in plants were affected, a seed germination inhibition assay was performed. Again, no significant difference was observed

between WT and transgenic lines (Fig. 5*B* Suppl.) without hormone treatment. However, ABA-treated seeds showed significantly delayed germination rates for both transgenic lines *GmNHX3* L1 and *GmNHX1* L1 as compared to WT. The effect was most noticeable after 3 d of treatment, where 80 - 90% of WT seeds germinated as compared to only 60% of seeds from line *GmNHX3* L1 and 60 - 70% of seeds from *GmNHX1* L1. However, after 4 d of treatment all seeds from WT and transgenic lines germinated (Fig. 5*B* Suppl.).

To determine whether there were any differences in abiotic stress-responsive gene regulation between transgenic *GmNHX* lines and WT, we analyzed expressions of five known *A. thaliana* abiotic stress-inducible genes (*RD29A*, *RAB18*, *P5CS*, *COR15A,* and *RD22*), in plants treated with ABA or exposed to osmotic stress (mannitol) (Fig. 5). Under non-stressful conditions, none of the analyzed genes was markedly expressed in any of the plants tested. However, under ABA- and mannitol-treatments all genes were clearly up-regulated in both WT and transgenic plants. Interestingly, gene expression induced by ABA or mannitol was observed to be several-fold increased in both *GmNHX*s expressing lines ranging from 1.5 to a 5-fold induction as compared to WT. In general, expressions were found to be higher in *GmNHX1* L1 than in *GmNHX3* L1.

Discussion

Several studies have described the role of *NHX*s genes from soybean in plants/cells exposed to salt or osmotic
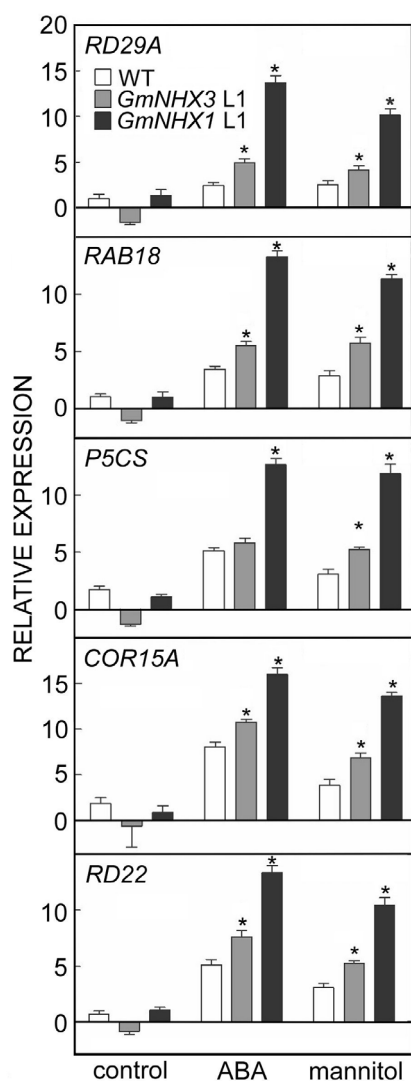
Fig. 5. Relative expressions of *RD29A*, *RAB18*, *P5CS*, *COR15A*, and *RD22* genes determined by RT-qPCR. Seven-day-old seedlings were treated with ABA (50 µM and mannitol (300 mM) for 3 h. The relative expressions for all 5 genes after ABA or mannitol treatment are shown. Gene expression values are relative to the *EF*1c housekeeping gene and are normalized to untreated WT plants. Means ± SDs from 4 samples (biological replicates) per treatment. The experiment was conducted twice with very similar results. *Asterisks* indicate statistically significant differences (* - $P < 0.05$).)

stress (Sun *et al.* 2006, Zhou *et al.* 2009, Li *et al.* 2017, Ning *et al.* 2018, Wang *et al.* 2018), but no detailed study reported their role in drought stress.

In a previous study, we identified soybean genotypes with contrasting drought tolerance both under controlled growth and field conditions (Pardo *et al.* 2015). Later, in a transcriptomic assay (Pardo 2015) gene expression profiles were compared under control and mild drought stress conditions in a tolerant (Munasqa) and a susceptible (TJ2049) genotype. Interestingly, both antiporter genes, *GmNHX3* and *GmNHX1,* showed a markedly induced expression pattern during stress in Munasqa (data not

shown). Due to their significant stress-inducible expression pattern and previous information regarding their roles in abiotic stress tolerance, such as salinity and osmotic stress, we hypothesized that these genes could play a relevant role during drought protection in soybean and decided to study the effect of *GmNHX1* and its homolog *GmNHX3* in plant drought tolerance by overexpressing these genes individually in *A. thaliana* plants.

Expression of either *GmNHX1* or *GmNHX3* increased tolerance to water deficit in *A. thaliana,* where especially the two lines *GmNHX3* L1 and *GmNHX1* L1 displayed significantly improved tolerance to low water supply (Fig. 2) although a noticeable stress tolerance also was observed for lines *GmNHX3* L2 and *GmNHX1* L2. Differences in stress tolerance among transgenic lines were probably due to the *GmNHX1* expressions, as line *GmNHX1* L2, which exhibited the lowest drought tolerance phenotype, also demonstrated a much lower *GmNHX1* expression compared to L1 and both *GmNHX3* lines. However, the difference in drought tolerance between the two lines expressing *GmNHX3* was probably due to other reasons, as no major variation in transgene expression was observed. Possible explanations for this could be attributed to differences of active protein amount either by total protein accumulation or due to post-translational effects or a secondary effect due to the insertion site of the transgene (Bassil *et al.* 2012). Further studies would have to be conducted to define the exact reason for this variance.

The constitutive expression of *GmNHX*s genes in *A. thaliana* helped to maintain RWC and chlorophyll content in water-stressed plants, corroborating the visual drought-tolerant phenotypes for lines *GmNHX3* L1 and *GmNHX1* L1. These results could be associated with increased ROS regulation and subsequent membrane protection, evidenced by results obtained for enzymatic ROS control and MDA accumulation, which would help to maintain cell water content and pressure potential and so create a non-hostile environment for a wide range of biological processes in the cell.

High Na^+ content in the cytosol is deleterious to cellular functions and Na^+-toxicity can be alleviated by Na^+ compartmentation into vacuole mainly due to tonoplast NHX proteins, which contribute to both ion homeostasis maintenance and osmoregulatory capacity of cells under saline conditions (Maathuis *et al.* 2014, Flowers *et al.* 2015). Several transgenic plants expressing tonoplast NHX have demonstrated improved salt tolerance, which has been correlated with an increased vacuolar Na^+ accumulation capacity (Apse *et al.* 1999, Galvez *et al.* 2012, Joshi *et al.* 2013, Bassil and Blumwald 2014, Guo *et al.* 2020). Consistently, *GmNHX3* L1 and *GmNHX1* L1 plants accumulated more Na^+ compared to WT plants after water stress treatment. Further support of a correlation between Na^+ accumulation and plant drought tolerance comes from a study where a positive correlation between up-regulation of *Zygophyllum xanthoxylum ZxNHX* antiporter expression and tolerance to drought, is related to higher Na^+ accumulation in stressed plants (Wu *et al.* 2011). Finally, a study in alfalfa where co-expression of the tonoplast *ZxNHX* and a *ZxH⁺-PPase* genes result in

plants with higher Na$^+$ and K$^+$ accumulation and with improved tolerance to both salt and drought stress (Bao *et al.* 2016).

It has been demonstrated that tonoplast NHXs facilitate K$^+$ transport into the vacuole regulating intracellular K$^+$ homoeostasis (Bassil and Blumwald 2014, Reguera *et al.* 2014, Guo *et al.* 2020). Coincidentally, overexpression of tonoplast *NHX*s generate vacuolar K$^+$ accumulation and increased K$^+$ uptake in transgenic plants (Rodriguez-Rosales *et al.* 2008, Peleg and Blumwald 2011, Gouiaa *et al.* 2012, Huertas *et al.* 2013, Guo *et al.* 2020). In this work, both *GmNHX3* L1 and *GmNHX1* L1 accumulated a higher amount of K$^+$ than WT plants when exposed to drought, indicating enhanced vacuolar compartmentation of K$^+$ and increased uptake leading to improved regulation of intracellular K$^+$ homeostasis during stress. Furthermore, we observed higher K$^+$ content in *GmNHX3* overexpressing plants whereas *GmNHX1* plants seemed to exhibit more affinity for Na$^+$, which could imply different biochemical functions of the two proteins as has recently been shown for NHX in *A. thaliana* (Bassil *et al.* 2019). Interestingly, a recent study of *Iris lactea* NHX in transgenic tobacco demonstrated markedly higher vacuolar H$^+$-ATPase (V-ATPase) activity compared to WT when subjected to salinity, which suggests that IlNHX plants could compartmentalize more Na$^+$ into vacuoles *via* enhanced V-ATPase activity, which further contributes to maintaining K$^+$ and Na$^+$ homeostasis (Guo *et al.* 2020).

A well-functioning antioxidant activity is crucial for withstanding low cellular water content and its importance in plant drought tolerance has been extensively reported (Rahdari and Hoseini 2012). Both *GmNHX1* L1 and *GmNHX3* L1 lines demonstrated a significant increase in SOD and APX activity. SOD is the enzyme with the highest catalytic activity in the antioxidant system and APX is the first in the glutathione-ascorbate cycle that detoxifies H$_2$O$_2$ and both enzymes have been shown to play important roles for water stress tolerance (Marok *et al.* 2013, Padmavathi and Rao 2013, Pyngrope *et al.* 2013, Sekmen *et al.* 2014). Additionally, a significant increase in POX and CAT activity was also found, which suggest that GmNHXs partly influence stress-response processes like biosynthesis of phenolic compound and photorespiration rate.

Proline is an important osmoprotectant in higher plants that accumulates in response to cellular water loss (Garde-Cerdán *et al.* 2014, Jday *et al.* 2016) and is involved in maintaining subcellular structures and ROS-scavenging (Mafakheri *et al.* 2010). Our study showed a significant increase in proline content in *GmNHX1* L1 and *GmNHX3* L1, which in addition displayed lower lipid peroxidation than in WT plants as revealed through MDA measurements. These findings suggest that cellular oxidation by ROS is alleviated, at least partially, by the enzymatic antioxidant machinery in transgenic lines expressing *GmNHXs*.

The plant hormone ABA regulates many physiological processes in response to drought, including stomatal closure (Beguerisse-Diaz *et al.* 2012). Surprisingly, stomatal closure in *GmNHX3* L1 was found to be more sensitive to ABA as compared to WT and *GmNHX1* LI, indicating that *GmNHX3* expression could confer an increased ABA-sensitivity. Additional support was seen in seed germination inhibition assays where seeds from *GmNHX3* L1 and *GmNHX1* L1 showed significantly less germination percentage in the presence of ABA as compared to WT during the first 3 d of treatment. Interestingly, an increased ABA-sensitivity in stomatal regulation was only observed in line *GmNHX3* L1 while germination inhibition assays revealed higher ABA-sensitivity for both *GmNHX*s lines. Further studies are needed to elucidate the exact reason behind this difference which could include differences in Ca^{2+} content, an effect observed in transgenic alfalfa plants overexpressing a *ZxNHX,* which exhibited higher Ca^{2+} content correlated with increased water stress tolerance (Bao *et al.* 2016).

Finally, we monitored gene expression of the *RD22*, *RD29A*, *COR15A*, *RAB18*, and *P5CS* genes, which are known to be induced by drought, salinity, low temperatures, ABA, and osmotic stress in *A. thaliana* (Yamaguchi-Shinozaki and Shinozaki 1993a,b, Baker *et al.* 1994, Mantyla *et al.* 1995, Strizhov *et al.* 1997, Xiong *et al.* 1999, Msanne *et al.* 2011). Interestingly, expressions of all 5 genes were significantly higher in both *GmNHX3* L1 and *GmNHX1* L1 compared to WT in plants exposed to high mannitol concentrations. A very similar result in transgenic sweet potato demonstrated that overexpression of a tonoplast Na$^+$/H$^+$ antiporter gene from *Ipomoea batatas,* promoted both drought and salt tolerance and that stress-related genes such as *P5CS* and *LEA* were significantly up-regulated in these transgenic plants as compared to WT plants (Guo *et al.* 2020).

In summary, our results demonstrate that constitutive expression of the two soybean cation/H$^+$ antiporters *GmNHX1* and *GmNHX3* in *A. thaliana* improved drought tolerance in soil-grown plants, which was supported by significant differences in several important cellular and biochemical stress-responsive markers, when compared to WT plants, strengthening the idea of NHX antiporter as promising candidates for improving abiotic stress tolerance in various crops including soybean.

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