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RESEARCH ARTICLE

Overexpression of *miR408* influences the cotton response to boron toxicity

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ABSTRACT

Boron toxicity is a severe abiotic stress affecting plant growth and productivity in semi-arid regions of the world including Anatolian plateau. In this study, we investigated antioxidative responses of cotton (*Gossypium hirsutum* L.) as well as four different B transporter and aquaporin gene activities, which are early B stress responders, by using *miR408* overexpression strategy. *miR408* is a conserved microRNA in plant kingdom involved in almost all physiological responses of the plants as well as responses under abiotic and biotic stresses. Our results showed that, *miR408* overexpression improved overall antioxidative status of cotton plants under B stress, in terms of reduced ion leakage and malondialdehyde levels and increased proline, chlorophyll and relative water contents. We also detected strong B transporter *BOR1*, *BOR2* and water channel *PIP1;1* and *PIP2;1* gene expression in cotton leaf tissues under almost all treatments. *miR408* overexpressing plants exhibited differential gene expression patterns when compared to control plants, always increasing with stress duration, revealing direct or indirect regulation of the genes by *miR408* under B toxicity. Our results showed that, *miR408* overexpression can be used as a strategy to improve B toxicity tolerance of cotton plants to increase agricultural production or for phytoremediation purposes on B rich soils.

Key words: Boron toxicity, *Gossypium hirsutum*, *miR408*, overexpression.

INTRODUCTION

Cotton (*Gossypium* spp.) is the most important naturally produced textile fiber crop worldwide. Its byproducts are also used in various industries and as animal feed. Cotton is also the second major oil seed crop after soybeans (Shang et al., 2017). The most widely cultivated cotton is *G. hirsutum* L., which is a tetraploid species originated from two close relatives, *G. arboreum* L. and *G. raimondii* Ulbr. Cotton is grown in arid and semi-arid regions of the world and frequently encounters unfavorable growth conditions. As a glycophyte, it shows higher tolerance to abiotic stresses than other major crops (Ullah et al., 2017). It is moderately tolerant to drought and salt stress and also presents good tolerance. However, severe abiotic stress restricts cotton growth and development by reducing plant height, fresh and dry weights of shoots and roots, leaf area index, node number, photosynthesis and transpiration rate, stomatal conductance, yield, fiber quality, canopy and root development (Sun et al., 2021). Due to its relative stress tolerance, large biomass and being a non-edible culture plant, cotton is a potential candidate for the phytoremediation of heavy metal contaminated soils. “Heavy metals” is a general collective term that applies to the group of metals and metalloids with atomic density greater than 4 g cm^{-3} (Edelstein and Ben-Hur, 2018). Boron is a metalloid and an essential plant micronutrient with the narrowest optimal range in the soil of any micronutrient. Boron is needed by all plants as a component of cell walls where it serves to cross-link pectic polysaccharides by bonding to apiose residues in the rhamnogalacturonan II complex (Onuh and Miwa, 2021). However, B is a potent toxin for the plants in high concentrations. Controlling B transport is in the best interest of

developing plants since its toxicity affects various metabolic and physiological processes beyond cell wall reinforcement.

Boron toxicity occurs in dry environments, especially in Southern Australia and in parts of the Middle East, often where plants are grown on alkaline soils of marine or volcanic origin, but sometimes also as a consequence of irrigation, especially with treated wastewater (Edelstein and Ben-Hur, 2018). Translocation of B from soil to plant roots was believed to take place via passive diffusion until the identification of two B transporters at early 2000's. One of the most evident roles of *BOR1* type transporters is to efflux borate toward the xylem and the contribution to the translocation of B from roots to shoots. This type of transporters, that exports borate out of cells, is expressed in various cell types including epidermis and endodermis (Brdar-Jokanović, 2020). On the other hand, *BOR2*, which is another efflux type borate transporter, is usually expressed in the epidermal cells and plays a critical role in pectin cross-linking under low B conditions (Onuh and Miwa, 2021). Another potential B transport mechanism includes aquaporin channels.

A majority of abiotic stresses, including salt, drought and heavy metal, disturb plant-water homeostasis which is attributed to non-coordinated regulation between expression and activity of different aquaporin isoforms. Although PIP, TIP, NIP and SIP aquaporin subfamilies are conserved in plants, homology comparisons demonstrate that plant aquaporins have divergent sequence and function. Research which are mostly based on functional genomic approaches identified various substrates other than water, transferred by different aquaporin isoforms including H_2O_2, urea, ammonia, glycerol, B, silica, arsenic and gases including CO_2 (Maurel et al., 2015). Among the MIP subfamilies, members of the PIP subfamily are the most studied. The PIP subfamily is further divided into two groups: PIP1s and PIP2s. The PIP1 isoforms have very low water channel activity, whereas, PIP2s isoforms have been shown to possess high water channel activity (Kapilan et al., 2018). The interaction between PIP1 and PIP2 isoforms to form heterotetramers also alters the aquaporin affinity and efficiency.

The miRNAs are a class of single-stranded RNA molecules of about 21-24 nucleotides at length that are produced by Dicer enzymes acting on precursors with intramolecular stem-loop structures and they act as negative regulators of gene expression in eukaryotes (Sun et al., 2019). Currently, miRBase database (Release v22) (University of Manchester, Manchester, UK; https://mirbase.org/) holds the record of 10 120 mature miRNA sequences belonging to 81 different plant species. The *miR408*, identified in more than 30 plant species, is one of the most common miRNA species appearing in almost all plant transcriptome studies. It is conserved between monocots and dicots and appears to take significant roles in almost all physiological plant responses (Song et al., 2018). Its regulatory roles were identified in apple rootstocks during rooting (Li et al., 2019), leaf growth in maize (Aydinoglu and Lucas, 2019), in vitro embryo development and regeneration, pollen tube germination and growth in rice (Zhang et al., 2018), heading in wheat (Zhao et al., 2016) and cotton fiber development (Ayubov et al., 2019). The *miR408* is also one of the most responsive microRNAs in abiotic stress and takes important roles in oxidative stress in various plant species. The *miR408* was reported to respond to H_2O_2 and also known to be down regulated upon drought stress in peach and under drought and salt stress in rice and cotton (Xie et al., 2015; Gao et al., 2022). However, its expression was strongly up regulated under drought stress in *Medicago truncatula* and barley (Hajyzadeh et al., 2015). The *miR408* expression was also found to be responsive to γ-aminobutyric acid (GABA) and heat stress in creeping bent grass. In *Arabidopsis* and cold resistant *Populus simonii* × *P. nigra* hybrid, its expression strongly up regulated under cold stress (Zhou et al., 2019). The *miR408* expression also responded to nutrient stress in *Arabidopsis* (Gao et al., 2022). Copper binding proteins plastocyanin and laccases, as well as a proteolytic enzyme DegP9 and *DEAD-box helicase* genes are among the reported targets of *miR408* (Li et al., 2019; Gao et al., 2022). The *miR408* overexpression studies revealed the improvement of overall plant photosynthetic efficiency, growth, biomass, fertility and seed yield as well as plant stress tolerance (Hajyzadeh et al., 2015; Ma et al., 2015; Guo et al., 2018; Song et al., 2018; Sun et al., 2018; Gao et al., 2022).

Recent studies revealed that *miR408* was among the few microRNAs which are very responsive to cadmium stress in *Brassica* and also Cr and As stress in rice (Sharma et al., 2015; Fu et al., 2019; Dubey et al., 2020). Significant upregulation was observed under Cd stressed *Brassica* tissues and As stressed rice

tissues, while significant downregulation was noted under Cr stress in rice. Overexpression of *miR408* in *Arabidopsis* leaded to improved tolerance to salinity and cold stress, while enhanced sensitivity to drought stress (Ma et al., 2015). Very similar results were also obtained for rice, which exhibited enhanced cold tolerance and decreased drought tolerance with *miR408* overexpression, while improved drought tolerance was also reported in chickpea (Hajyzadeh et al., 2015; Sun et al., 2018). Overexpression of *miR408* also enhanced salt stress tolerance in *Nicotiana benthamina* and wheat, while it also increased cupric stress and stripe rust tolerance in wheat (Guo et al., 2018). Altered *miR408* expression also influenced *Arabidopsis* response to Fe deficiency (Carrió-Seguí et al., 2019).

Boron toxicity is a common abiotic stress in Anatolian plateau. Heavy metal levels in the environment are also continuously increasing with anthropogenic activities, parent-material weathering and precipitation of sea spray in coastal areas including Mediterranean cost (Edelstein and Ben-Hur, 2018). Heavy metal contaminated soils pose an increasing problem to human and animal health, since heavy metals accumulate in the biomass of agriculturally important plants (Yaashikaa et al., 2022). Heavy metals also decrease the growth and yields of many industrial crops by way of inducing severe oxidative damage. Metal accumulating plants with increased phytoremediation capacity is important both for cleaning up of contaminated agricultural soils and also indirectly for the improvement of local economies. The *miR408* is an important abiotic stress regulating plant miRNA and were reported to take ameliorative roles in various abiotic stresses, including heavy metals in previous studies. In this study, *miR408* overexpressing cotton lines were produced and tested for their physiological responses and also for their molecular B transport machinery under severe B toxicity.

MATERIALS AND METHODS

Seeds of cotton (*Gossypium hirsutum* L.) 'GSN-12' were kindly provided by Cotton Research Institute, Aydın, Turkey. EHA105::*pEarlyGate103* carrying *pre-miR408* in T-DNA region was used as bacterial strain and the binary vector. *pre-miR408* fragment was amplified from genomic DNA of *Arabidopsis thaliana* Col-5. The vector was prepared by Hajyzadeh et al. (2015) using Gateway cloning system with the insertion of *pre-miR408* fragment under the control of *CaMV35S* promoter. The vector contained *nptII* and *bar* genes for bacterial and plant selection, respectively.

Transformation, selection and regeneration of cotton plants

The cotton seeds were surface sterilized with 70% ethanol instantly followed by three times rinsing with 10% sodium hypochlorite solution for 7 min and finally rinsed with autoclaved distilled water. The seeds were grown on half strength MS/MS medium for 5 d. The shoot apices of germinating embryos were used for *Agrobacterium* mediated transformation. *Agrobacterium tumefaciens* cells were prepared for transformation on bacterial inoculum density increased in the ratio of 1/3, to 0.8 at OD_{600}. Bacterial suspension was used for transformation of the explants under the vacuum pressure of 200 mm Hg for 20 min.

The inoculated explants were transferred to MS/MS medium including 0.1 mg L^{-1} kinetin (KIN), 100 µM acetosyringone and 10 µM 2-(*N*-morpholino)ethanesulfonic acid (MES) at 23 ± 1 °C in dark for 3 d. After co-cultivation, inoculated explants were taken to MS/MS selection medium which contains 0.1 kinetin, 100 mg L^{-1} cefotaxime, 3 mg L^{-1} ammonium glufosinate, 10 mg L^{-1} $AgNO_3$ and 1.5 g L^{-1} active charcoal.

Analysis of putative transgenic plants

Total DNA isolation was performed by use of Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Limburg, Holland) according to the manufacturer's instructions. Genomic DNAs were digested with SacI restriction enzyme (Thermo Fisher Scientific, Waltham, Massachusetts USA) which was selected from pEarlyGate103 vector multiple cloning site (MCS). Southern blot analysis was performed meticulously. Genomic DNA fragments were separated by 1% agarose gel electrophoresis. Blotting was performed by using 10x SSC solution and DNA samples in the gel were transferred to nylon membrane (Hybond, Amersham,

Buckinghamshire, England). The DNA fragments that were transferred to the nylon membrane were fixed for 2 h at 80 °C. Hybridization was performed for 16 h at 42 °C using digoxigenin (DIG) labeled CaMV35S-specific DNA probe. After hybridization, the membrane was washed and the signal on the membrane was prepared for visualization using Anti-DIG alkaline phosphatase (Roche, Mannheim, Germany) and CDP-Star (Biolabs, Ipswich, Massachusetts, USA). The imaging was performed in the darkroom using standard X-ray film development and fixer solutions.

Growth of T_1 generation transgenic cotton plants and application of B stress

The T_1 generation transgenic cotton seeds were germinated on plastic pots containing perlite and ½ strength Hoagland's solution, in a growth chamber at 28 ± 1 °C with 8 h dark and 16 h light (400 μmol m^{-2} s^{-1}) photo-cycle. Germinated plants were first exposed to 3 mg L^{-1} ammonium glufosinate for 15 d. Twenty-four putative transgenic plants that survived under bar selection were used for DNA isolation and subsequently PCR amplification for CaMV35S promoter region by use of forward (5'-GCCATCATTGCGATAAAAGG-3') and reverse primers (5'-GGTCTTGCGAAGGATAGTG-3') (data not shown). Glufosinate resistant and PCR positive plants were subsequently transferred to new pots and after 25 d of recovery period, boric acid treatments (½ strength Hoagland's solution containing 7.5 mM H_3BO_3) were started for two different durations, namely 1 and 5 d. Concentration of boric acid, which imposes severe stress, was determined by preliminary experiments in which the responses of the wild type cotton plants were tested under different boric acid concentrations. Stress treatments were performed on 21 40 d old T1 generation transgenic cotton plants as well as 21 control plants (wild type cotton plants) in the growth chamber. Control sets were also prepared with non-transgenic seedlings that were irrigated with ½ strength Hoagland's solution with and without the addition of 7.5 mM H_3BO_3. The same pot transfer steps were also performed for the control plants at exactly the same way and durations. Samples were collected at the same time of the day to prevent potential daily fluctuations in particular gene expressions at the sample collection days.

Semi quantitative RT-PCR analyses

Fresh leaf tissues of control, B treated control and B treated transgenic cotton plants were used for total RNA isolation. The isolation was performed by using the GF-1 Total RNA Extraction Kit (Vivantis Tech, Malaysia). Three biological replicates from each treatment, namely three different non-transgenic controls, three different B treated non-transgenic controls and three different T1 progenies of transgenic T0 line 5 were prepared during RNA isolation. The quality of RNA was evaluated by 1% agarose gel electrophoresis and quantity of the RNA samples was determined by Nanodrop 2000 (Thermo Scientific). The RNAs were reverse transcribed using oligo (dT) primer according to the instructions of Thermo RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The expression level of *PIP1;1* (EF079900), *PIP2;1* (EF079901), *Boron transporter 1* (XM_016886698), *Boron transporter 2* (XM_016863428) and *Translation elongation factor 1A-8* (DQ174257) genes in control, B treated control and B treated transgenic cotton leaves were carried out by semi quantitative RT-PCR using Biorad thermal cycler (Biorad, Hercules, California, USA). *Translation elongation factor 1A-8* (*EF1A8*) was used as internal control of gene expression and the expression patterns are presented at Supp. Data 3. PCR reactions, performed for the same biological replicates of RNA isolation, were initiated with an initial denaturation at 95 °C for 5 min followed by 28 cycles denaturation at 95 °C for 1 min, annealing at specific annealing temperature for the particular primer for 45 s, and elongation at 72 °C for 45 s followed by final elongation at 72 °C for 10 min.

Biochemical analysis of B tolerance

Leaf tissues were used to calculate the relative water content (RWC) values. Wet weights were recorded before the incubation in distilled water for 24 h at 23 °C. After turgid mass determination, leaf tissues were dried at 60 °C for 48 h and weighed to assess the dry masses. Relative water contents were determined according to the formula RWC (%) = (WM-DM)/(TM-DM)×100 (Akcay and Erkan, 2016).

Membrane damage was evaluated by quantification of the electrolytes leaked from leaf tissues. Thermo Scientific Orion 013016MD conductivity meter was used for the measurements of electrical conductance of tissues incubated in 0.4 M mannitol for 3 h (Akcay and Erkan, 2016).

Lipid peroxidation was evaluated by measurement of the malondialdehyde (MDA) content. Leaf tissues were homogenized in 5% trichloroacetic acid (TCA) solution. The homogenates were centrifuged at 12 000 rpm for 15 min. The pellets were suspended with thiobarbituric acid added in trichloroacetic acid (1/4 w/w) and incubated for 25 min at 96 °C. After centrifugation at 10 000 rpm for 5 min, absorbances of the supernatants were recorded at 532 nm optical density (Akcay and Erkan, 2016).

Proline contents were determined. Initially 0.2 g sample was homogenized in liquid nitrogen and mixed with 1 mL 3% sulfosalicylic acid. 0.1 mL supernatant was then mixed with 0.2 mL acid ninhydrin, 0.2 mL 96% acetic acid and 0.1 mL 3% sulfosalicylic acid. The mixtures were incubated at 96 °C for 1 h, followed by centrifugation and addition of 1 mL toluene to the supernatant. The absorbance values were recorded at 520 nm optical density.

Total chlorophyll contents were determined by using methanol. Leaf tissues were homogenized in liquid nitrogen and centrifuged at 4000 rpm for 5 min at 23 °C. Supernatants were mixed with methanol and absorbances were read at 653 and 666 nm. After calculation of chlorophyll *a* and chlorophyll *b*, the total chlorophyll contents were also determined.

Data analysis

Experiments were performed with three to five replicates per analysis. The significance of the treatments was determined at 5% probability level by using Tukey test of one-way ANOVA by the help of SPSS 15.0 (IBM, Armonk, New York, USA).

RESULTS AND DISCUSSION

Agrobacterium transformation and regeneration studies were performed on 1160 cotton explants with a stable transformation efficiency of 0.69%. To increase regeneration efficiency of putative transformants, activated charcoal and silver nitrate were always included on the selection media. A non-transformed control cotton plant and eight putative transgenic lines from T_0 generation were used for the molecular confirmation of transgenic status by Southern blot analysis (Figure 1). While the non-transformed control plant did not exhibit any hybridization signal, a single signal was obtained for the lines 2, 3, 4, 5 and 8. The analysis also confirmed that three different lines 1, 6 and 7 carried two inserts of T-DNA.

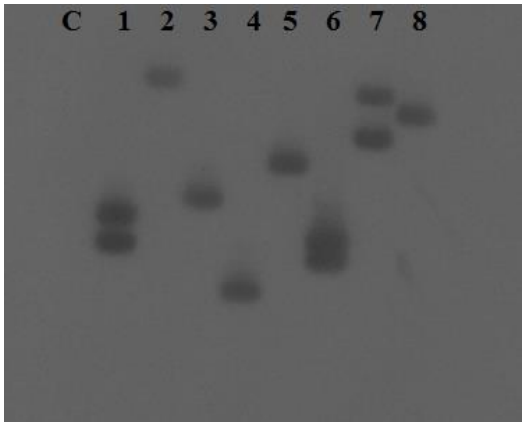

Figure 1. Southern blot analysis of non-transformed control cotton plant (C) and eight transgenic lines (1-8). CaMV35S-specific DNA probe revealed the presence of one or two copies of T-DNA in transgenic plants.

All transgenic lines showed normal phenotype and produced seeds (data not shown). Randomly selected *miR408* overexpression Line 5 was used for the analysis of B tolerance. The T_1 generation putative transgenics were germinated under 3 mg L^{-1} ammonium glufosinate selection and successful seedlings were tested for the presence of *bar* gene and *CaMV35S* promoter, followed by their analysis for B tolerance. Boron tolerance was tested by the investigation of membrane integrity parameters; ion leakage and MDA, relative water and total chlorophyll content of the tissues in addition to proline levels (Table 1).

Table 1. Levels of various physiological stress indicators in control and transgenic cotton leaf tissues, measured after 1 d and 5 d long B treatments. RWC: Relative water content; MDA: malondialdehyde; C: non-transgenic, wild type cotton plant; T: transgenic cotton plant; +B: 7.5 mM B application. Values designated by different letters on the same column are significantly different on 5% significance level.

Treatment	Total chlorophyll content	RWC	Ion leakage	MDA	Proline
	mg mL^{-1}	%		nmol g^{-1}	µmol g^{-1}
1 day B treatment					
C	12.08 ± 0.60^{a}	90.16 ± 2.57^{a}	23.27 ± 0.97^{b}	20.47 ± 4.19^{b}	34.19 ± 0.78^{b}
C+B	11.67 ± 0.63^{a}	77.60 ± 0.84^{c}	29.01 ± 0.48^{a}	34.12 ± 2.95^{a}	44.84 ± 2.86^{a}
T+B	12.88 ± 0.27^{a}	82.57 ± 1.09^{b}	24.17 ± 0.25^{ab}	25.51 ± 2.99^{b}	38.83 ± 2.59^{b}
5 days B treatment					
C	12.33 ± 0.97^{a}	87.55 ± 1.95^{a}	24.33 ± 1.14^{b}	22.32 ± 3.12^{b}	32.30 ± 6.86^{b}
C+B	10.45 ± 0.48^{b}	71.45 ± 3.22^{b}	29.05 ± 2.24^{a}	36.39 ± 1.16^{a}	46.04 ± 2.32^{a}
T+B	11.43 ± 0.25^{ab}	77.02 ± 3.12^{b}	27.55 ± 1.11^{ab}	26.13 ± 1.79^{b}	39.17 ± 3.21^{ab}

There are limited numbers of studies investigating cotton response to B toxicity and they are generally focused on major growth and yield parameters, as well as elemental concentrations in cotton tissues (de Souza Junior et al., 2019). In this study, we determined that relative water contents of the B treated cotton leaves decreased significantly as early as 1 d long treatment. The *miR408* overexpression lines maintained water contents close to untreated control plant levels, although significant reductions were also observed in transgenic tissues. Leaf total chlorophyll contents of B treated control plants decreased significantly under 5 d long B stress, while the decrease was insignificant under 1 d B treatment. The *miR408* overexpressing transgenic plants exhibited higher chlorophyll contents compared to control plants under B toxicity. Ion leakage and MDA levels, which are indicators of cell membrane integrity, showed similar patterns with significant increases upon B treatment and levels in *miR408* overexpressing plants remained at the same range with untreated control plants. Therefore, all stress indicator physiological parameters revealed superior self-protection status of *miR408* overexpressing cotton leaf tissues under two durations of B stress. Levels of proline increased significantly in tissues under B stress for both durations, whereas transgenic plants showed insignificant increases compared to non-treated control plants. The results showed that proline has little effect on the physiological superiority of the transgenic plants under B stress, indicating the presence of alternative osmoprotection mechanisms for B tolerance of *miR408* overexpression lines.

Boron transporter gene expressions should be among the first responses under B toxicity similar to the aquaporin gene expressions which take immediate roles in plant water homeostasis during abiotic stress. Their investigation under *miR408* overexpression lines has a potential to help understanding of transporter proteins' functions and potential microRNA control imposed upon them. Blast of the cotton genes investigated in this study to the well characterized transporters from *Arabidopsis*, *Triticum* and *Hordeum* taxid showed 45% to 92% query coverage and 65.25% to 78.16% identity to particular genes. Both cotton B transporter genes showed homologies to *BOR1* and *BOR4* transporters of *Arabidopsis* and *BOR1* transporter of *T. aestivum* and *H. vulgare*. Studies on various plant species suggested the potential roles of the B transporter proteins under B toxicity conditions as the removal of excess B out of cells into apoplast,

redistribution of excess B in tissues for establishing homeostasis, and the disposal of B from leaves via hydathode guttation (Akcay and Erkan, 2016). We detected strong *BOR1* and *BOR2* expression in cotton shoot tissues under all treatments (Figure 2). During early stress, non-transgenic plants exhibited stronger expression compared to control cotton plants grown under normal conditions; however, transgenic shoots exhibited reduced expression compared to both controls. This pattern might be resulted from overall oxidative stress status of the transgenic plants, which showed milder stress symptoms under both durations revealed by the physiological stress indicators (Table 1). That is, lesser B accumulation or detoxification of B in *miR408* overexpressing transgenic plants possibly made early exclusion or redistribution of B by *BOR1* and *BOR2* unnecessary. In transgenic plants, comparison of 1 d and 5 d long B treatment also showed that expression of both genes increased under prolonged stress, which might be attributed to the protective roles of B transporters including exclusion or homeostasis facilitated by *miR408* overexpression.

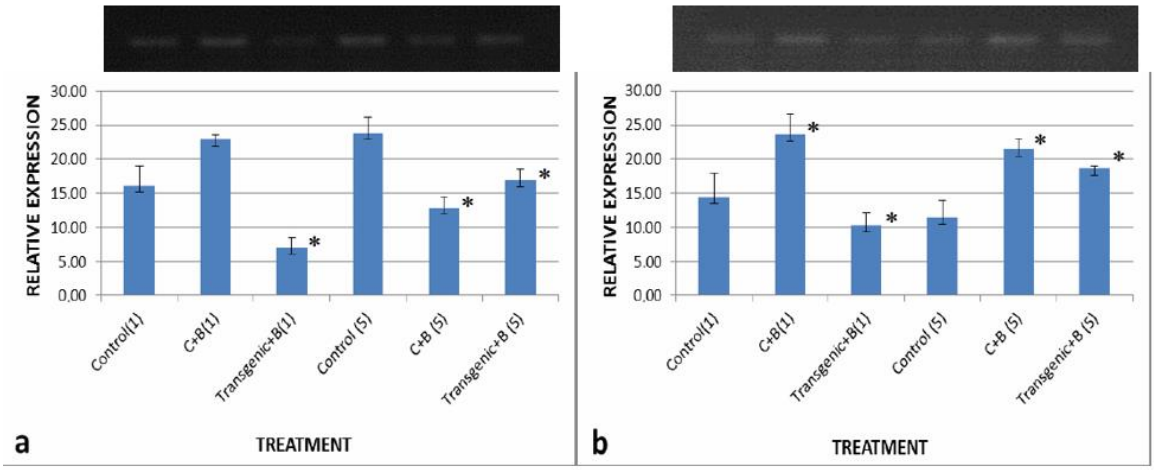

Figure 2. *Boron transporter 1* (a) and *Boron transporter 2* gene expressions (b). Control is wild type cotton. C+B stands for B treated control plants. Transgenic+B stands for B treated transgenic plants. Numbers in brackets indicates duration of the stress, which are 1 and 5 d. Lanes in agarose gel images given at the top of the figures are in the same order of treatments in the bar graphs. *Significant differences compared to control plants.

Plant aquaporins are intrinsic membrane proteins that have been shown to regulate mainly water homeostasis, however different isoforms are also known to take roles in transferring various substrates including B and other metalloids. Among the MIP subfamily aquaporins, PIP subfamily has been demonstrated to take role in B transport. The expression of maize Zm-PIP1 in *Xenopus laevis* oocytes resulted in increased B permeability (Mosa et al., 2016). Yeast complementation assays have confirmed that *HvPIP1;3* and *HvPIP1;4* can facilitate B transport across the membranes into yeast cells (Jia et al., 2021). Rice PIP proteins, *OsPIP1;3*, *OsPIP2;4*, *OsPIP2;6* and *OsPIP2;7* have also been shown to be involved in mediating B permeability and provided tolerance in overexpressed *Arabidopsis* plants most likely by internal redistribution of toxic B (Kumar et al., 2014; Mosa et al., 2016). We observed reduced *PIP1;1* gene expression in non-transgenic cotton plants under short-term B stress and total lack of expression in transgenic plants, which are in consistency with previous reports (Figure 3). Since PIP1 class aquaporins are known to possess very low water channel activity when compared to PIP2 class aquaporins, shut down of *PIP1;1* but not *PIP2;1* during early stress exposure seems to be a response against accumulation of B into toxic concentrations. This response, which is intense in *miR408* overexpression line, possibly both to conserve water and prevent access B accumulation appeared to give a survival advantage to the transgenic plants, revealed by the investigated stress indicators. The response did not last long since PIP transporters

are known to mobilize various vital molecules other than B. During long term stress, B treatment increased *PIP1;1* expression 4-fold in non-transgenic leaves and 6-fold in transgenic cotton leaves. The *PIP2;1* gene expression did not change significantly under short term B stress, however expressions significantly increased under long term stress in a similar pattern to *PIP1;1* gene expression, which should be related to the regulation of water and B distribution in leaf cells. In general, during early abiotic stresses, plants reduce the expression and activity of aquaporins to conserve their water contents. This is very important for the efficient induction of various defense responses. Contrary to this, long-term stress exposure leads to higher expression of aquaporins to meet daily water requirement of plant (Chaumont and Tyerman, 2014). Macho-Rivero et al. (2018) also observed a decrease in the transcript levels of shoot and root *PIP* aquaporins in *Arabidopsis* after 24 and 48 h of B toxicity, which are in consistency with our results, especially when considering *miR408* overexpressing lines that showed a distinct pattern of decrease in expression after 1-d long stress treatment and increase in expression at the 5th day of B stress.

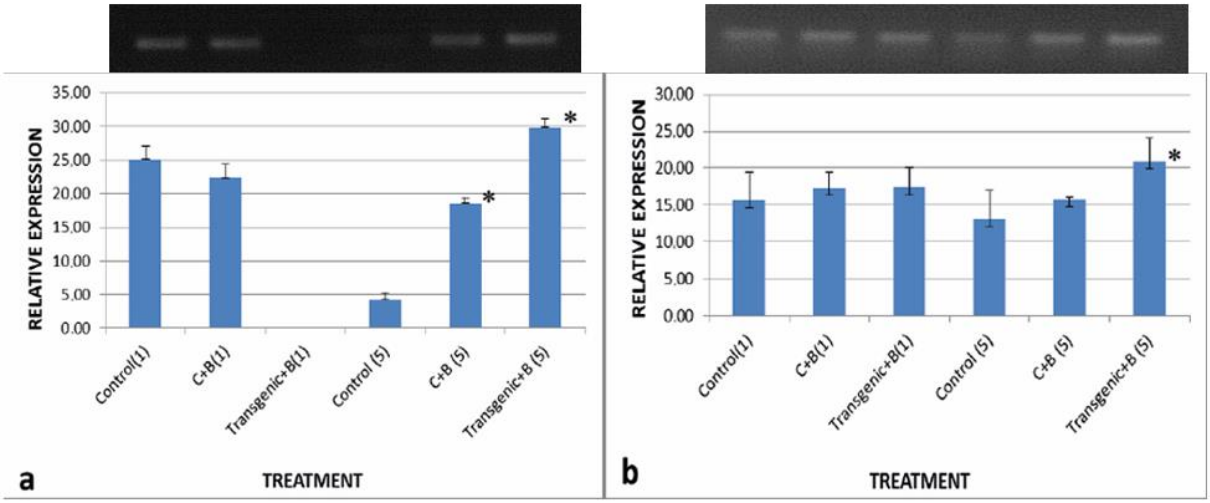

Figure 3. *PIP1;1* (a) and *PIP2;1* gene expressions (b), agarose gel images are given at the top of figure with same order of treatment. Control is wild type cotton. C+B stands for B treated control plants. Transgenic+B stands for B treated transgenic plants. Numbers in brackets indicates duration of the stress, which are 1 and 5 d. Lanes in agarose gel images given at the top of the figures are in the same order of treatments in the bar graphs. *Significant differences compared to control plants.

Although its biological functions have many unknowns, *miR408* is known to regulate genes encoding copper binding proteins that are members of phytocyanin family, which mainly take role in photosynthesis. It was also proposed that *miR408* has control on the gene encoding laccase, which take role in lignification. Both processes are fundamental for plant survival and should be sustained effectively in altered environmental conditions, which might explain the situation of *miR408* being the most conserved microRNA identified in more than 30 plant species and detected in almost all transcriptome studies on plants. Our results also demonstrated involvement of *miR408* in B toxicity stress by way of altered cellular responses and improved overall antioxidant capacity of cotton plants.

CONCLUSIONS

Although the presence of immense effort to reveal the function and define the vast range of substrates of plant aquaporins, and many studies to describe the individual functions of B transporters since their discovery, both proteins are found to be multifunctional with wide variety of isoforms, which make their expression patterns complicated. It is also possible that the functions of different isoforms change with changing tissue type, developmental stage of the tissue and different types and magnitudes of external stimuli. Our results showed that *miR408* overexpression improved overall tolerance of cotton plants under B stress in terms of reduced ion leakage and malondialdehyde levels and increased proline, chlorophyll and relative water contents. The *miR408* overexpression also significantly affected the expression levels of *boron transporter 1* and *2*, as well as *PIP1;1* and *PIP2;1* aquaporins, which exhibited different expression patterns under B toxicity compared to stressed and non-stressed control plants. Although further studies are required to elucidate exact functions, our results helped to obtain a better insight into the biological roles of particular cotton transporter genes as well as *miR408* under B toxicity stress. Our results also present evidence that *miR408* overexpression can be an effective strategy for the improvement of cotton tolerance under B stress, which also make transgenic plants potential candidates for B phytoremediation.

Author contribution

Ibrahim Ertan Erkan and Ufuk Celikkol Akcay contribute equally to this work. Authors read and approved the manuscript.

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