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Improvement of Water Stress Tolerance of Tuberous Begonia (*Begonia×tuberhybrida*) by *OsmiR393a* Gene Transformation

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Abstract: The genus *Begonia* is greatly affected by abiotic stresses, which lead to losses in greenhouse production and flower longevity. Obtaining more-tolerant plants is a very important breeding goal in ornamentals. To achieve abiotic tolerant *Begonia×tuberhybrida*, *OsmiR393a*, a miRNA involved in the tolerance of plants to abiotic stresses, was introduced using *Agrobacterium*-mediated transformation. Explants were cultured on modified MS medium supplemented with 1.0 mg/L BA, 0.5 mg/L IAA, and 5 mg/L hygromycin for transgenic selection and regeneration. Stable integration of the transgene was verified in putatively transformed plants by PCR screening. When fresh flower longevity was evaluated by the period from bloom to wilting of 3 petals, flower longevity of transgenic line was 8 d longer than control. Furthermore, 15 days after stress treatment, petals of control plants wilted, whereas transgenic lines remained. This results shows that expressing *OsmiR393a* could extend only flower longevity without affecting flower type under water stress in *Begonia×tuberhybrida.* In addition, the transgenic plants displayed lower transpiration rate, higher proline content and chlorophyll content when subjected to water stress. These results suggest *OsmiR393a* may improve water stress tolerance of *Begonia×tuberhybrida* by regulating different pathways in response to the stress conditions and *miR393a* is conservatized between monocotyledon and dicotyledon.

Keywords: Agrobacterium, *miR393a,* Transformation, Tuberous Begonia, Water Stress Tolerance

1. Introduction

The begonia is one of the most popular ornamental plants in the world and is grown in gardens, pots, hanging baskets, and greenhouses. Over 2000 species are classified into three types such as tuberous, rhizomatous and fibrous rooted [9]. Tuberous begonia (*Begonia×tuberhybrida*) is popular ornamental plant and cultivated widely, of which numerous units are being produced in the world each year. Breeding of begonias has focused on divergence of flower color, stress tolerance and appearance of the shoot and has attempted through the use of interspecific crossing and induced mutations [3]. Stress limits the normal growth and consequently their productivity and quality of flower by preventing the plants from expressing their full genetic potential [26]. An improvement in these characteristics under abiotic stresses is a major challenge for ornamental plant breeders [31]. However, this subject is hard to resolve by conventional breeding approaches because of little documented information about the genomic locus of the traits and the multiple mechanisms of cellular adaptation which lead to abiotic stress tolerance [40]. The achievement of this goal is supported by the genetic engineering. Genetic engineering is providing a valuable means of the expand of the floriculture gene pool to promote the generation of new varieties. Especially, genetic engineering is desirable for the modification of ornamental characteristics such as flower color and shape, floral scent, flower longevity and disease resistance [32, 33]. This would be a powerful tool for producing novel cultivars that cannot be obtained by ordinary

breeding. Several attempts have been made to improve ornamental plants by genetic engineering [25]. For the transformation in begonias, only *Agrobacterium*-mediated transformation has been used successfully up to the present. Successful transformation studies on some begonia species such as *Begonia×tuberhybrida*, *Begonia×hiemalis*, *Begonia rex* and *Begonia×cheimantha* have been reported [20]. Genetic engineering has been applied to transfer different genes associated with abiotic stress tolerance in different plant species including trehalose [23], mannitol [28], galactinol [29], proline [39], glycine-betaine [35], *NAC* [34], *ABF3* [1], *MYB* [7], *DREB* [24] and *API* [30].

MicroRNAs (miRNAs) is non-coding RNAs encoded by *miRNA* genes. MiRNAs are a class of small RNAs that have recently emerged as important regulators of gene expression. In plants, these 21-nucleotide RNAs are processed from stem-loop regions of long primary transcripts by a Dicer-like enzyme and are loaded into silencing complexes. MiRNAs regulate gene expression by targeting mRNAs for degradation or translational repression [4]. MiRNAs play critical roles by regulating numerous biological processes in plants including development, metabolism, signal transduction, protein degradation, pathogen invasion and adaptive responses to environmental stresses, targeting genes such as those encoding transcription factors and F-box proteins [22]. Moreover, many conserved miRNAs were identified and characterized to play regulatory roles [14]. Some of these miRNAs, especially *miR393a* were found to play important roles in abiotic stress tolerance [5]. *MiR393a* is a canonical miRNA conserved across plant species [10]. There is no information regarding the tolerance of *Begonia×tuberhybrida* to abiotic stress associated with overexpressing of *miR393a*. Previously we successfully produced *OsmiR393a* transgenic creeping bentgrass (*Agrostis stolonifera* L.) through *Agrobacterium*-mediated transformation and confirmed the conservation of *miR393a* with monocotyledons [16]. In this study, the tolerance of *OsmiR393a* transgenic *Begonia×tuberhybrida* to abiotic stress was determined. The influences of regeneration rate, fresh flower longevity, the transpiration rate, relative water content (RWC), proline content and chlorophyll content on transgenic line were evaluate, and the conservation of *miR393a* between monocotyledon and dicotyledon was confirmed.

2. Materials and Methods

2.1. Begonia×tuberhybrida Variety and Agrobacterium Strain

Begonia×tuberhybrida '230R', grown in greenhouse was used. They were reproduced predominantly asexually by tissue culture or by tuber. *Agrobacterium tumefaciens* EHA105 (pCAMBIA13011-*OsmiR393a*) maintained in our laboratory were used for transformation. The plasmid pCAMBIA13011 contains the kanamycin resistance gene (*nptII*) under the control of the pnos promoter, the hygromycin resistance gene (*hph*), a gene for GUS activity (*uidA*) and the intron-*uidA*. Both, *hph* and *uidA* genes, were under the control of the 35S promoter of cauliflower mosaic virus.

2.2. Tissue Culture and Transformation via Agrobacterium

Unfolded young leaves were soaked in 70% ethyl alcohol for 15 s, sterilized with 1% sodium hypochlorite for 30 min, and then rinsed twice with sterile distilled water for 10 min each time. Leaves were cut into 5 mm squares and petioles were cut into 8 mm segments for explants, and cultured on solid MS media supplemented with 1.0 mg/L benzylaminopurine (BA), 0.5 mg/L 3-indoleacetic acid (IAA) and 30 g/L sucrose. Cultures were maintained at 2000 Lx for 14 h a day at 25°C. After 4 weeks, vigorous explants were used for transformation experiment. The medium for infection and co-culture used was AAM medium supplemented with 100 mmol/L acetosyringone (AS). For *Agrobacterium* infection, the density of the bacteria was adjusted (A_{600} = 0.05) and the explants were immersed in a *Agrobacterium* suspension for 15 min, then blotted dry on sterilized filter paper. Explants were then placed on another sterilized filter paper on solid medium in the dark at 28°C for 2 days and rinsed with sterilized water.

2.3. Selection and Growing Culture

After co-culture, the explants were placed on MS medium supplemented with 400 mg/L cefotaxim (Cef) and 5 mg/L hygromycin (Hyg). About 2 months after infection, explants that formed shoots were transferred to MS medium (growing medium) containing 0.5 mg/L BA, 0.25 mg/L IAA, 5 mg/L Hyg and 400 mg/L Cef and cultured under a 16 h photoperiod (2000 Lx) at 25°C for regenerated plants to grow. After 3-4 months culture on the growing medium, a single regenerated plantlet was excised from each explant and cultured on growing medium for rooting and further growth (16 h light, 25°C). Plantlets with 30 mm in size were transferred to pots and grown for 4 weeks on acclimatizing chamber with controlled light and humidity.

2.4. Assay for GUS Activity

Histochemical GUS activity was examined by the procedure reported by Jefferson et al. (1987) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as a substrate [18]. Samples, roots about 6 months after infection were incubated with sterile 4 mM X-Gluc solution (Sigma, USA) for overnight at 28°C. Control treatments were samples without *A. tumefaciens* co-culture. GUS activity was estimated from the intensity of the blue staining.

2.5. Genomic DNA Extraction and PCR Analysis

Total DNA was extracted from leaf material according to the method described by Dellaporta el al. (1983) [8]. Polymerase Chain Reaction (PCR) was followed in a C1000 Touch TM thermal cycler (BIORAD) with Taq polymerase (Invitrogen, USA). The following parameters were used: 94°C for 5 min, followed by 30 cycles of 94°C for 30s, 58°C for 30s and 72°C for 1min. Final extension time of 6 minutes at 72°C was used. For identification of *OsmiR393a* transgenic plants, primers

P35S F (5'–ACCACGTCTTCAAAGCAAGTGG–3'), P35S R (5'– CCTCTCCAAATGAAATGAACTTCC–3'), Tnos F (5'–TTGAATCCTGTTGCCGGTCTTG–3'), Tnos R (5'–ACACCGCGCGCGATAATTTATC–3'), *OsmiR393a* F (5'–GGGGTACCTACACAAACCAGGCATCTCCAC–3') and *OsmiR393a* R (5'–CGGGATCCGAGCTTTCTTGCACAACACCTT–3') were used. These primers amplify 123bp fragment of *35S* promotor sequence, 199 bp fragment of *nos* terminator sequence and 599 bp fragment of the *OsmiR393a* gene. PCR products were separated by electrophoresis on a 2% agarose gel.

2.6. RNA Extraction and Quantitative RT–PCR

Small RNA was extracted using an RNAiso kit for small RNA (Takara, China) and the RNA samples were treated with RNAse-free DNAse I (Sangon, Shanghai, China) to remove any trace contamination of genomic DNA. Reverse transcription was performed with a cDNA Synthesis Kit (Promega, China) in combination with a stem-loop RT-PCR technique [6]. The real time PCR were performed according to Pak et al. (2009) [27]. The real-time amplification reactions were performed using the iCycler iQ thermocycler (Bio-Rad, Shanghai, China) and the SYBR Green kit (Takara, Japan) according to the manuals from the manufacturers. Primers used for qRT-PCR are presented in Table 1. Relative RNA levels were calculated and normalized to a reference gene *BurbcL (*Ribulose bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, chloroplast gene). Comparative quantification of transcripts was performed using the $^{\triangle\triangle}$CT method.

Table 1. *Primers used in qRT-PCR analysis.*

Gene name (GenBank ID)	Name of primer	Sequence (5'-3')	Size of PCR product (bp)	Experiment
BurbcL* (U59814.1)	rbcl q-F	CGTTACAAAGGACGATGC	183	RT-qPCR of rbcL (Control)
	rbcl q-R	TCGCAAATCCTCCAGACG		
OsmiR393a (HM139752.1)	*miR393a*-q-F	TGAACCCAACAATGAAGAA	201	RT-qPCR of *OsmiR393a*
	miR393a-q-R	TCCCCAAGACATTGCTGCTAC		

*; Ribulose bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, chloroplast gene encoding chloroplast protein, complete cds in Begonia sp.

2.7. Morphological Observation of Plants

Morphological traits of regenerated plants such as length and thickness of stem, leaf number, leaf area, leaf colour and survival efficiency were examined. Mature plants were evaluated for flower size, flower colour, length and thickness of stem, and leaf number.

2.8. Water-stress Treatment and Evaluation of Transgenic Lines for Water-stress Tolerance

Transgenic plants over-expressing *OsmiR393a* and control plants were maintained in our laboratory. All transgenic plants were clonally propagated with culture. Plants were grown in greenhouse. Plants were watered every day with a nutrition solution. The stress experiment was carried out in pots with plants of similar size. The fresh flower longevity was observed 15 days after the treatments of 55% relative humidity and 16°C day temperature.

2.9. Measurement of RWC, Transpiration Rate, Free Proline and Chlorophyll

Controlled plants were watered every day and stressed plants were not watered for 6d. Each treatment was replicated three times with three pots per line for a replicate. Leaves and petals were collected at the 6d after water withholding and RWC was measured as described by Gonzalez et al. (2001) [13]. The transpiration rate of the leaves and petals was measured during 10-11 a.m. at the end of the water-stress treatment (6 days). 200 mg of the leaves and petals were subjected to dry at 100°C for 24 h and then free proline content was determined using ninhydrin reaction [12].

Chlorophyll was extracted from a fresh leaves using 80% acetone in the dark. Absorbance was measured at 646, 663 and 710 nm, and the total chlorophyll concentration (μg/mL) of the samples was determined according to Lichtenthaler et al. (1985) [21].

2.10. Statistical Analysis

Each treatment replicated three times. Treatment means and the standard error of the means were calculated using SAS (Statistical Analysis System, Cary, NC) software. Statistical analysis was performed with the Student's *t* test.

3. Results

3.1. Agrobacterium-mediated Transformation and Assay of Transformed Plants

We performed the experiment for determination of Hyg selection concentration to select transgenic tissues. Explants were transferred to selection medium supplemented with different Hyg concentrations and the growth of explants was investigated (Table 2).

As shown in Table 2, no significant change was observed in growth of explants upto 3 μg/mL Hyg, but explants cannot survive and becomes brown in above 5 μg/mL concentration. This shows that Hyg resistant explants (Hygr explants) can be selected in this Hyg concentration. After Agrobacterium infection, explants undergo a selection process in above condition. Most explants gradually turned brown and died about 2 months after infection, except for putative transformants. Green adventitious buds of putative transformants appeared at the cut surfaces of explants about 2 months after *Agrobacterium* infection. Six months after infection, 59 putatively transformed explants showed Hyg-resistant phenotype were obtained from 297 explants (Table 3).

Table 2. *The growth of explants according to the Hyg concentration.*

Hyg (μg/mL)	No of total	Color	Growth	No of Hyg^r explants	Rate of Hyg^r (%)
0	60	Green	+	60	100
1	75	Green	+	75	100
3	90	Green	+	83	92
5	80	Brown	-	0	0

+: The growth of explants is very good, -: The growth of explants is not very well.

Table 3. *The efficiency of Hygr explants Culture period-30d.*

	No of total	No of Hyg^r explants	Efficiency of Hyg^r explants (%)
Control	85	0	0.0
Transgenic	297	59	19.8

Culture period 30d.

On the contrary, Hyg-sensitive explants showed a white color and characteristic brown spots that indicate necrotic tissue as a consequence of selection pressure. Green adventitious buds were sub-cultured growing medium and regeneration medium, and then obtained the regenerated plants (Figures 1b, 1c, 1d) from the Hyg resistant explants (Figure 1a).

Figure 1. *Plant transformation and essay of transgenic plants.*

(a) Proliferation of explants in 30 d culture, *(b)* Regenerated plants (Right is controls and left is transgenics.), *(c, d)* Pot plants from Hygr explants, *(e)* Histochemical GUS assay in root of transgenic plant (Left is control and right is transgenic.).

We assayed expression of the β-glucuronidase (GUS) gene to indicate gene transfer in root tissue. The GUS expression in roots of regenerated plants was a result of transgene expression in the plant cells. There was no detectable GUS activity in the control tissues (Figure 1e). This result showed that the GUS gene was transferred to the begonia cell and was successfully expressed. Fifteen putative transgenic plants derived from Hygr explants were tested by PCR to have a first confirmation of integration of the *35S* promoter and *nos* terminator sequence (Figure 2a). No band was found in control plant (Figure 2a, lane 1). The expected fragments of 123 bp and 199 bp were identified in selected plant of the putative transgenic plant tested (Figure 2a lane 2). Moreover, stable integration of 599bp fragment of the transgene was confirmed using PCR analysis (Figure 2b), suggesting integration of the *OsmiR393a* gene.

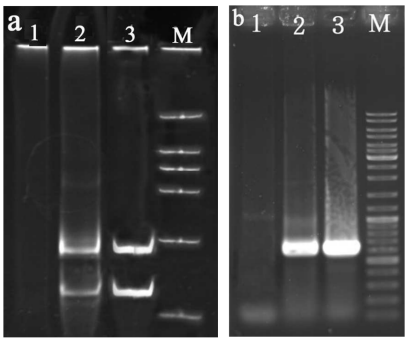

Figure 2. *PCR analysis of putative transgenic plant.*

(a) PCR analysis of transgenic plant using *35S* promoter and *nos* terminator primers. Lane 1 is nontransgenic negative control, lane 2 is transgenic line, lane 3-positive control and M is molecular weight DNA Ladder 2kb. 123bp and 199bp amplification bands are visible for transgenic line in lane 2, *(b)* PCR analysis of transgenic plant using *OsmiR393a* primers. M is molecular weight DNA Ladder 100bp, lane 1 is non-transgenic negative control, lane 2 is transgenic line and lane 3 is positive control. A 599bp amplification band is visible for transgenic line in lane 2.

3.2. Characteristics of Transformed Plants

Characteristics of young regenerated plants were investigated (Table 4, Figure 3).

Table 4. *Characteristics of young regenerated plants.*

	No of leaves	Stem Length (cm)	Stem Thickness (cm)	Ca. of Leaf (cm^2)	Survival rate (%)	Leaf color
Control	2.8±0.2	1.2±0.1	1.6±0.2	2.3±0.3	67	Green
Trans genic	3.4±0.2	1.6±0.2	2.1±0.3	3.7±0.3*	94*	Dark green

*: $P<0.05$, Regeneration period-30d.

The growth of transgenic plants was significantly better than WT control in regeneration stage. The leaf area and survival rate of transgenic plants were respectively 1.6 and 1.4 times higher compared to control (Figure 3b). *OsmiR393a* transcript accumulation of leaves of regenerated plant increased about twofold compared to the control (Figure 3a). Furthermore, some morphological characteristics of early mature transgenic plants were observed (Table 5).

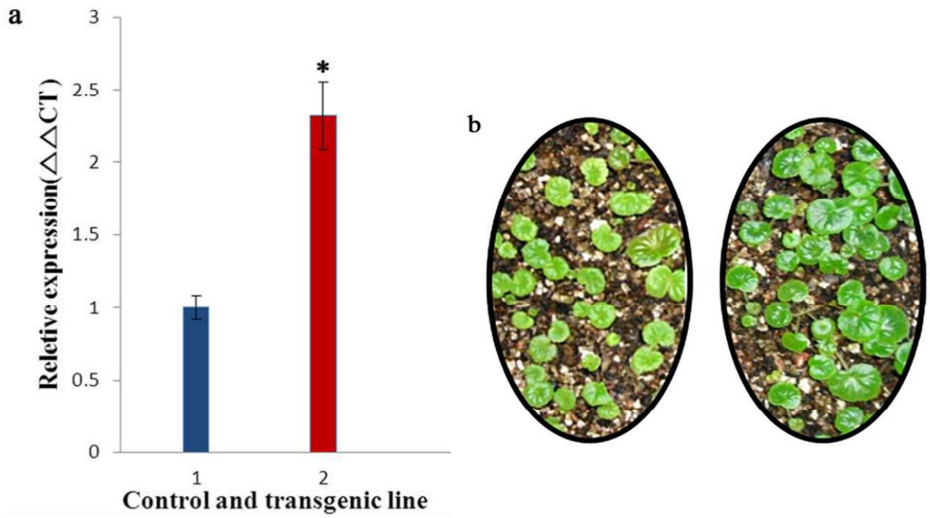

Figure 3. *Characteristics of regenerated plants.*

(a) Quantitative RT-PCR analysis of *OsmiR393a* transcript accumulatio n in control and transgenic line. The y axis shows the fold increase in *OsmiR393a* expression compared to control plants. Three replicates were performed from each sample. Asterisks values significantly different from the WT ($P < 0.05$), (b) Left is controls and right is transgenic plants.

Table 5. *Morphological characteristics of transgenic plants.*

	Flower diameter (cm)	Flower deep (cm)	Flower color	Stem length (cm)	Stem thickness (cm)	No of leaves
control	13.1±2.1	6.3±1.6	Bright red	18.5±2.1	1.9±0.1	9±1
1	12.5	6.0	Bright red	19.5	2.0	10
2	13.0	8.1	Bright red	20.0	1.9	10
3	13.3	8.2	Bright red	18.7	2.1	8
4	14.0	7.0	Bright red	20.5	2.0	9
5	13.2	7.4	Bright red	20.5	2.1	9

Day temperature: 23±2°C. Night temperature: 18±2°C. Relative humidity: 70± 5%.

As shown in Table 5, morphological characteristics of transgenic plants didn`t reveal differences compared with control. Transgenic plants and untransformed controls had similar morphology and flowering patterns.

3.3. Evaluation of Transgenic Lines for Stress Tolerance

Stress experiment was carried out in a pot environment with transgenic lines and the control plants. Flower longevity of transgenic lines was investigated in stress condition of low humidity and lower temperature of day and night (Table 6, Figure 4).

Fresh flower longevity was evaluated by the period from bloom to wilting of 3 petals. Flower longevity of transgenic line was 8 d longer than control, showing that expressing *OsmiR393a* could extend only flower longevity without affecting flower type. Flower and petal of the WT control and transgenic lines 15d after the treatment were shown in Figure 4. As shown in Figure 4b and Figure 4c, 15 days after stress treatment, petals of control plants wilted, whereas transgenic lines remained turgid, healthy and red without obvious wilting. Gene expression data were also collected for *OsmiR393a* genes under water stress. In the petal of control and transgenic line, a fourfold increase in *OsmiR393a* transcript accumulation was observed when plants experienced water stress (Figure 4a).

Table 6. *Flower longevity of transgenic line of stress treatment.*

	Flower diameter (cm)		Flower deep (cm)		Flower longevity (d)
	1	2	1	2	
Control	16.5±0.3	19.3±0.1	8.3±0.1	9.4±0.2	15±2
Transgenic	16.9±0.3	21.5±0.1	8.5±0.1	10.3±0.2	23±2*

1: Before stress treatment. 2: After stress treatment. Day temperature: 16±2°C. Night temperature: 12±2°C. Relative humidity: 55±5%. n=20. $P<0.05$.

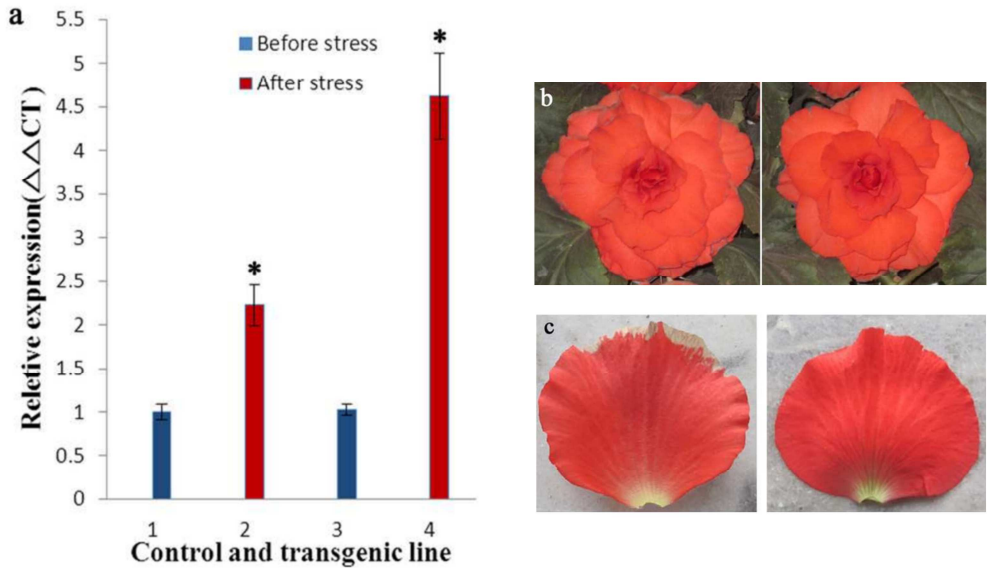

Figure 4. *Results of stress experiment.*

(a) Quantitative RT-PCR analysis of *OsmiR393a* transcript accumulation in petal of control (1 and 3) and transgenic line (2 and 4) before and after 15 d stress treatment. The y axis shows the fold increase in *OsmiR393a* expression compared to control plants before and after stress treatment. Three replicates were performed from each sample. Asterisks values significantly different from the WT ($P < 0.05$), *(b, c)* Flower and petal of Transgenic and control plant 15 d after stress treatment. Left is control and right is transgenic.

3.4. Effects on the Relative Water Content (RWC), Transpiration Rate, Proline Content and Chlorophyll Content of Stress Treatment

We first evaluated relative water content (RWC) and transpiration rate to gain more insight into plant responses to water stress. Under control condition, there was no difference between RWC and transpiration rate of control and transgenic line. However, transgenic line showed significant difference in relative water content (RWC) and transpiration rate than the control after 7 days of water stress. The leaves and petals of transgenic line showed higher RWC than the controls under complete water deprivation (0% water) (Figure 5a). The water stress resulted in a decrease of the transpiration rate of both control and transgenic plants. There was no difference between transpiration rate in the leaves and petals of control and transgenic line under control condition, versus transgenic line maintained significantly lower transpiration rate than the controls at the end of treatment (Figure 5b). Next, leaves and petals of transgenic plants accumulated more free-proline than control plants under stress treatment (Figure 6a). After 100% water treatment, free proline contents in the leaves of the transgenic line and control plants were respectively 93.7 μg/g and 69.1 μg/g, increased 36% in the leaves of the transgenic line compared with control plants ($P<0.05$), versus in the petals were respectively 226.7 μg/g and 165.7 μg/g, increased 37% ($P<0.05$). After 7 days of drought treatment, proline contents in the leaves of the transgenic line and control plants were respectively 133.4 μg/g and 97.7 μg/g, increased 33% in the leaves of the transgenic line compared with control plants ($P<0.05$), versus in the petals were respectively 333.7 μg/g and 238.6 μg/g, increased 40% ($P<0.05$).

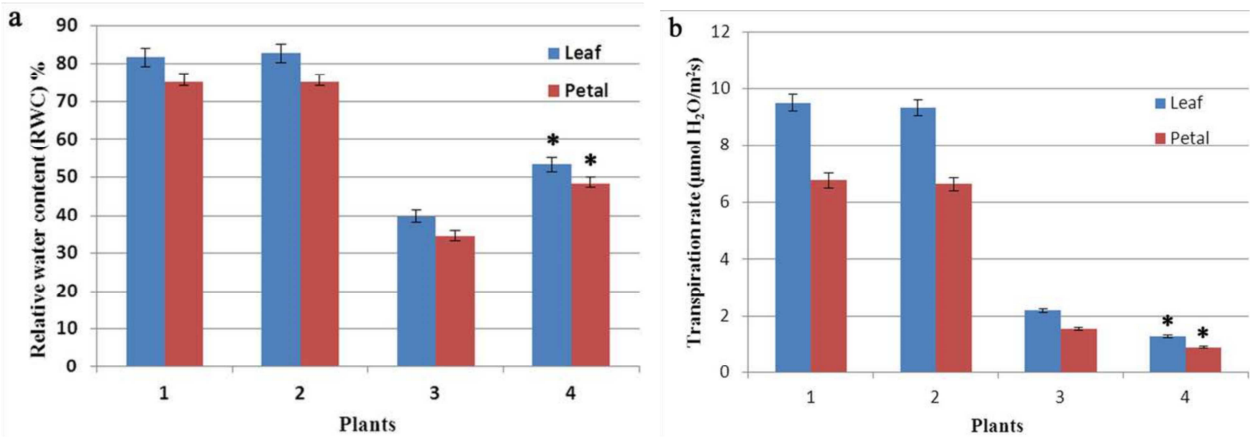

Figure 5. *Response of transgenic and control plants to water stress.*

Transgenic and control plants were subjected to water stress by withholding water. One set of plants was well watered (100% watering). After 7 days of water-stress treatment, relative water content. *(a)* and transpiration rate, *(b)* were measured. Relative water content and transpiration rate from control (1) and transgenic line (2) 7 days after the control treatment. Relative water content and transpiration rate from control (3) and transgenic line (4) 7 days after water withholding. Transgenic line showed significantly less water loss and transpiration rate than the control ($P<0.05$).

After 7 days under drought, a increase of about 1.4 times in chlorophyll content of the transgenic line compared to the control lines was noted (Figure 6b). In contrast, significant difference in Chl content was not observed between transgenic line and control line under well-watered conditions. There was a clear superiority of transgenic line in decreasing the transpiration rate, increasing the free proline contents and in maintaining relative water content and the chlorophyll content compared to control under water stress. These results showed that decreased transpiration rate, increased relative water content (RWC), proline content and chlorophyll content in transgenic lines over-expressing *OsmiR393a* may be responsible for the improved tolerance observed in this line under conditions of water stress.

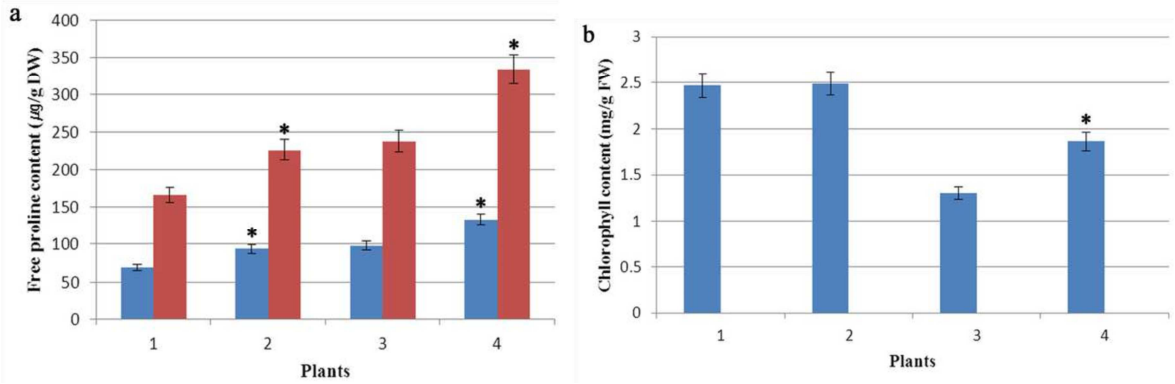

Figure 6. *Free proline contents and chlorophyll contents of transgenic and control plants.*

Free proline contents (a) and chlorophyll contents (b) from control (1) and transgenic line (2) 7 days after the control treatment. Free proline contents (a) and chlorophyll contents (b) from control (3) and transgenic line (4) 7 days after water withholding. Transgenic line showed significantly high free proline content and chlorophyll contents than the control ($P<0.05$).

4. Discussion

Begonia has the advantages of growing well in glasshouse culture, being genetically tractable and transformable. Plant resistant to abiotic stress are of interest to floricultural crop scientists and have been characterized using various approaches, including transformation. Application of the technology to crops has used the many useful genes shown to function in protecting cells from abiotic stresses [17]. The number of successful transformations is increasing every year in floricultural crop according as the development of Agrobacterium-mediated techniques and appropriate tissue culture techniques [15].

In this study, we used *OsmiR393a* gene that may play a role in abiotic stress response in plant. Our results indicated that the transgene is stably integrated, and accumulation of the *OsmiR393a* transcript increases in leaves and petals when *Begonia×tuberhybrida* plants experienced water stress, suggesting that miR393a itself and the regulation pathway of miR393a might be conserved among the rice and *Begonia×tuberhybrida* (Figure 4a). Also, we found that stress tolerance of transgenic *Begonia×tuberhybrida* increases when plants experienced water stress. This indicated that *OsmiR393a* may have a physiological role in *Begonia×tuberhybrida* water stress responses. In the regeneration stage, transgenic plants exhibited a better growth (Figure 3). No obvious change in flower shape was observed in any of the transgenic plants, but shift in flower longevity was showed when compared to the wild type under water stress (Figure 4b, Figure 4c). Measurement of RWC also showed enhancement in water retention in *OsmiR393a* transgenic *Begonia×tuberhybrida* compared with control plants (Figure 5). Transgenic plants also exhibited increased proline and chlorophyll content compared with the control (Figure 6), suggesting that the improvement of water stress tolerance mediated by expression of *OsmiR393a* in *Begonia×tuberhybrida* may be partially attributable to increased proline content and chlorophyll content in these plants under conditions of water stress. An understanding of gene function is an essential first step in engineering novel traits. To our knowledge, *OsmiR393a* gene has not been studied in *Begonia×tuberhybrida*. Although our results demonstrated that overexpressing *OsmiR393a* can enhance the ability of *Begonia×tuberhybrida* to tolerate water stress, many questions remain with respect to the target genes. The identification of target genes of *miR393a* is very important to understanding *miR393a* function in water stress responses. Over the years, target genes of microRNAs involved in the regulatory roles have been identified [19]. The action of some of these microRNAs responsive to stress involves auxin

[11, 36]. The *miR393*-dependent degradation of *TIR1* and *AFB2* transcripts contributes to osmotic stress-mediated inhibition of lateral root growth by regulating auxin signaling [37, 38]. However, the *miR393* was predicted to target genes which encode a transport inhibitor like protein, TFs, DNA-binding proteins, and a GRR1-like protein. These target genes play an important role in various biological processes like responses to cold, salt stress and water deprivation [2]. Although the targets of plant *miR393a* are not known fully, studies on *miR393a* in other systems may provide some guidance on the potential targets of this gene in plants.

5. Conclusion

In summary, this study demonstrated for the first time that through a transgenic approach, manipulation of *OsmiR393a* could lead to improved water stress tolerance in *Begonia×tuberhybrida* plants. Our results provide a means of targeting fresh flower longevity in *Begonia×tuberhybrida* and also can contribute to an understanding of a multiple mechanism of abiotic stress tolerance, and the evolution and the conservation of *miR393a* between monocotyledon and dicotyledon.

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