GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

A *Medicago truncatula* H$^+$-pyrophosphatase gene, *MtVP1*, improves sucrose accumulation and anthocyanin biosynthesis in potato (*Solanum tuberosum* L.)

J.W. Wang[1,3]*, H.Q. Wang[2]*, W.W. Xiang[3] and T.Y. Chai[1]

[1]College of Life Science, University of Chinese Academy of Sciences, Beijing, China
[2]Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, Qinghai, China
[3]College of Life Science, Yulin University, Yulin, Shaanxi, China

*These authors contributed equally to this study.
Corresponding author: T.Y. Chai
E-mail: wangjianwu06@mails.ucas.ac.cn

Genet. Mol. Res. 13 (2): 3615-3626 (2014)
Received March 24, 2013
Accepted September 23, 2013
Published May 9, 2014
DOI http://dx.doi.org/10.4238/2014.May.9.4

ABSTRACT. We recently cloned *MtVP1*, a type I vacuolar-type H$^+$-translocating inorganic pyrophosphatase from *Medicago truncatula*. In the present study, we investigated the cellular location and the function of this H$^+$-PPase in *Arabidopsis* and potato (*Solanum tuberosum* L.). An *MtVP1*::enhanced green fluorescent protein fusion was constructed, which localized to the plasma membrane of onion epidermal cells. Transgenic *Arabidopsis thaliana* overexpressing *MtVP1* had more robust root systems and redder shoots than wild-type (WT) plants under conditions of cold stress. Furthermore, overexpression of *MtVP1* in potato accelerated the formation and growth of vegetative organs. The tuber buds and stem base of transgenic potatoes became redder

than those of WT plants, but flowering was delayed by approximately half a month. Interestingly, anthocyanin biosynthesis was promoted in transgenic *Arabidopsis* seedlings and potato tuber buds. The sucrose concentration of transgenic potato tubers and tuber buds was enhanced compared with that of WT plants. Furthermore, sucrose concentration in tubers was higher than that in tuber buds. Although there was no direct evidence to support Fuglsang's hypothetical model regarding the effects of H^+-PPase on sucrose phloem loading, we speculated that sucrose concentration was increased in tuber buds owing to the increased concentration in tubers. Therefore, overexpressed *MtVP1* enhanced sucrose accumulation of source organs, which might enhance sucrose transport to sink organs, thus affecting anthocyanin biosynthesis.

Key words: H^+-pyrophosphatase; *MtVP1*; Anthocyanin; Sucrose

INTRODUCTION

Vacuolar-type H^+-translocating inorganic pyrophosphatases (V-PPases) are single 75-82-kDa intrinsic membrane protein species that are classified into two distinct types, I and II. Type I V-PPases are primary electrogenic proton pumps that obtain energy from the hydrolysis of inorganic pyrophosphate (PPi). Type I V-PPases, as exemplified by AVP1 from *Arabidopsis,* depend on cytosolic K^+ for their activity, whereas Type II V-PPases, as exemplified by *Arabidopsis* AVP2, share only ~36% sequence identity with Type I proteins and are insensitive to K^+ (Drozdowicz et al., 2000).

Reverse genetics has revealed physiological roles for Type I V-PPases under normal and stress conditions, such as water scarcity, salinity, and nutrient limitations. For example, overexpression of *AVP1* in *Arabidopsis* resulted in plants with enhanced salt tolerance and drought resistance (Gaxiola et al., 2001). In addition, the upregulation of either *Arabidopsis* or *Thellungiella halophila* Type I V-PPases triggered enhanced growth/biomass, photosynthetic capacity, salt tolerance, and drought resistance in a variety of agriculturally important crops grown under drought and salt stress conditions (Park et al., 2005; Yang et al., 2007; Bao et al., 2008; Li et al., 2008; Lv et al., 2008, 2009).

Several studies examining the phloem tissue of *Rhodospirillum rubrum* seedlings indicated that H^+-PPase could be involved in sucrose transport and was localized in close proximity to the plasma membrane of sieve elements in *Ricinus communis* (Long et al., 1995; Robinson et al., 1996; Langhans et al., 2001). These authors suggested that both H^+-pumps were required for sieve element membrane energization to maintain high sucrose, K^+, and amino acid concentrations. Of note, *in vivo* data obtained with the H^+-PPase from *R. rubrum* were consistent with the hypothesis that this enzyme plays two distinct roles depending on its location; it can act as an intracellular proton pump in the acidocalcisomes or as a PPi synthase in the chromatophore membranes during illumination (Davies et al., 1997; Seufferheld et al., 2004). Based on these studies, Fuglsang and colleagues hypothesized that the upregulation of Type I V-PPases enhanced sucrose fluxes from source to sink tissues by improving phloem sucrose-loading capacity (Fuglsang et al., 2011).

The stimulatory effects of sugars on anthocyanin biosynthesis in several plant species have been reported previously. Many anthocyanin biosynthetic genes are induced by sugars (Gollop et al., 2001, 2002; Martin et al., 2002; Hiratsu et al., 2003). Expression of the petunia (*Petunia hybrida*) chalcone synthase (CHS) gene in transgenic *Arabidopsis* leaves is induced by sugars. Interestingly, both the petunia and *Arabidopsis* CHS genes contain sucrose boxes in the 5'-flanking regions of the gene. These sucrose boxes were found in the upstream regions of the sporamin and β-amylase genes, which are induced by sucrose (Tsukaya et al., 1991). Another study demonstrated that sucrose was the most effective inducer of anthocyanin biosynthesis in *Arabidopsis* seedlings (Teng et al., 2005).

We previously reported the cloning of Type I V-PPase genes from *Medicago truncatula* (*MtVP1*) (Wang et al., 2009). In the present study, we investigated the location of this H^+-PPase in cells. Next, *MtVP1* was overexpressed in *Arabidopsis* and in potato to study its function. This is the first study to address the overexpression of H^+-PPase genes in transgenic potatoes. Furthermore, the effects on sucrose accumulation and anthocyanin biosynthesis in transgenic potatoes due to the overexpression of H^+-PPase genes have not been described previously.

MATERIAL AND METHODS

Plant material and growth conditions

The transgenic and wild type (WT) lines used in this study were all in the *Arabidopsis* ecotype Columbia-0 background. For physiological experiments, the seeds of T_3 transgenic and WT *Arabidopsis* were allowed to germinate at 22°C for 7 days and were transferred to agar plates containing half strength Murashig and Skoog's (MS) basal salt mixture (Murashig and Skoog, 1962) medium, where they were cultured for 14 days at 22°C with a 16/8 h light/dark photoperiod.

Potato (*Solanum tuberosum L.* ssp *weishu 4*) seedlings were propagated on culture medium containing MS basal salt mixture medium supplemented with 2% sucrose and solidified with 0.2% Gelrite (Sigma). The cultures were maintained in growth chambers at 22° ± 2°C and a 16/8 h light/dark photoperiod. Shoot tips were excised and subcultured on fresh medium every 2 weeks. The four week-old rooted shoots were planted in pots (10 cm in size) containing compost and humus at a 3:1 ratio and were transferred to a greenhouse for hardening.

Construction of the plasmid pBI121-MtVP1-EGFP and onion epidermal cell transformation

MtVP1 cDNA, containing the complete open reading frame, was obtained as previously described (Wang et al., 2009). The plasmid pBI121-MtVP1-EGFP, with expression driven by the cauliflower mosaic virus (CaMV) 35S promoter, was constructed to investigate the subcellular localization of MtVP1 in onion epidermal cells. The coding region of the *MtVP1* cDNA (stop codon removed) was amplified using a pair of primers (f: 5'-GCGGTACCATGGGAGCAGTAATTCTCCCA-3' and r: 5'-GCGGTACCGATCTTGAAG AGGAGACCAC -3') and inserted into the *Kpn*I site of pBI121-EGFP to be fused in-frame to the N-terminal end of the EGFP coding sequence. Then, pBI121-MtVP1-EGFP was transformed

into the epidermal cells of onion (*Allium cepa*) by *Agrobacterium*-mediated transformation. An *Agrobacterium tumefaciens* strain ($C_{58}C_1$) carrying pBI121-MtVP1-EGFP was cultured from a single colony in YEP medium (5 g/L NaCl, 10 g/L yeast extract, and 10 g/L tryptone, with pH adjusted to 7.0) supplemented with 50 mg/L rifampicin, 50 mg/L kanamycin, and 20 μM acetosyringone overnight at 28°C. Next, *A. tumefaciens* was suspended in YEP medium supplemented with 10 mM $MgCl_2$ and 100 μM acetosyringone. The inner-epidermal cells of onion were incubated at 28°C on solid MS medium for 24 h and inoculated with the *A. tumefaciens* suspension for 30 min. Next, the onion epidermal cells were plated on solid MS medium for 2 days at 25°C with a 16 h photoperiod. The onion epidermal cells were visualized under a fluorescence microscope (Leica DMR).

Construction of plasmid pBI121-MtVP1 and plant transformation

The coding region of the *MtVP1* cDNA was amplified using a pair of primers (f: 5'-GCGGTACCATGGGAGCAGTAATTCTCCCA-3' and r: 5'-GCGAGCTCTTAGATCTTGA AGAGGAGACCAC-3'). Next, the cDNA was subcloned into the plasmid pBI121-GUS by replacing the GUS coding region between the *Kpn*I and *Sac*I sites, resulting in a construct that would overexpress *MtVP1* under the control of the CaMV 35S promoter in plants. The resulting plasmid was transformed into the *A. tumefaciens* strain $C_{58}C_1$ and used for plant transformation.

Adult *Arabidopsis* plants were infected with *A. tumefaciens* by the floral dipping method (Clough and Bent, 1998) and grown in a greenhouse. The seeds collected were screened in MS medium supplemented with 50 mg/L kanamycin.

Potato leaves were transformed following the Banerjee protocol (Banerjee et al., 2006). Transgenic plants harboring 35S::*MtVP1* were screened on MS agar medium containing 50 mg/L kanamycin, and the presence and integrity of the transgenic lines were further confirmed by polymerase chain reaction (PCR) using primers specific to the 35S promoter and *MtVP1* cDNA.

Semi-quantitative reverse transcription (RT)-PCR analysis

Total RNA was isolated from transgenic seedlings using TRIzol Reagent (Invitrogen, USA). For semi-quantitative RT-PCR, the concentration of RNA was accurately quantified by microspectrophotometric measurements and reverse transcription. PCR was performed using *MtVP1* primers (*RTvp1*f: 5'-TGTTGGCAAGGTTGAAAGGAA-3' and *RTvp1*r: 5'-GGCAGAAAACCAGTAAGGGAGC-3') and control *AtActin* primers (*AtActin*f: 5′-CTGG AATGGTGAAGGCTGGTT-3′ and *AtActin*r:5'-CCTGTTAGCTTTAGGGTTAAGAGGTG-3') in *Arabidopsis*. RT-PCR was performed using *MtVP1* primers and control *StActin* primers (*StActin*f: 5'-ACGATTCCGTTGCCCTGAG-3' and *StActin*r: 5'-AAAATAGAACCTCCAAT CCAGACAC-3') in potato. The following cycling parameters were used: 95°C for 3 min followed by 28 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. All RT-PCR analyses were performed in triplicate.

Anthocyanin measurement

The two week-old transgenic *Arabidopsis* seedlings were exposed to cold stress at 4°C for one week. The anthocyanin contents of transgenic *Arabidopsis* seedlings and potato buds

(2-4 cm) were determined following the Mita protocol (Mita et al., 1997). Frozen, homogenized seedlings (20 mg) were extracted for 1 day at 4°C in 1 mL 1% (v/v) hydrochloric acid in methanol. The mixture was centrifuged at 13,000 rpm for 15 min, and the absorbance of the supernatant was measured at 530 nm and 657 nm. Relative anthocyanin concentrations were calculated with the formula: $[A_{530} - (1/4 \times A_{657})]$. The relative anthocyanin amount was defined as the product of the relative anthocyanin concentration and extraction solution volume. One anthocyanin unit equals one absorbance unit $[A_{530} - (1/4 \times A_{657})]$ in 1 mL extraction solution.

Analysis of sucrose concentrations

The tuber buds (2-4 cm) of transgenic potatoes were ground in mortars. The tubers of transgenic potatoes were ground into a pulp using a fruit squeezer. One milliliter juice samples were dissolved in 10 mL 80% ethanol and disrupted by sonication for 30 min; subsequently, the samples were centrifuged at 3000 rpm for 10 min. One milliliter samples were diluted 100-fold with Milli-Q water, passed through a 0.22 μm porosity filter, and were measured for constituent sucrose concentrations using high performance liquid chromatography (HPLC). Ten microliters each sample was injected into a Dionex DX ICS-3000 HPLC system. The HPLC settings were as follows: a CarboPac PA 10 guard (50 x 4 mm), an analytical column (250 x 4 mm), and an ampere electrochemical detector for pulsed amperimetric detection. The column was eluted with Milli-Q water and 250 mM NaOH (88:12, 15 min; 80:20, 2 min; 88:12, 8 min), at a flow rate of 0.80 mL/min, a run time of 25 min, and a column temperature of 30°C. To verify the reproducibility of the HPLC measurements, each sample was measured twice.

Statistical analysis

Statistical analysis was performed using the statistical software package SPSS 19.0 (SPSS Science, Chicago, IL, USA). The two-tailed Student *t*-test was conducted to analyze various indices of the transgenic plants compared to the WT plants at 5 and 1% levels of probability. The experiments were repeated three times. The data are reported as means ± SD.

RESULTS

Subcellular localization of MtVP1

To determine the localization of the MtVP1 protein, the 35S::MtVP1::EGFP DNA construct, encoding an EGFP-tagged MtVP1 protein, was introduced into onion epidermal cells by *Agrobacterium*-mediated transformation. In the control, the EGFP was distributed throughout the cell (Figure 1A, B, and C). The transient transfect assay indicated that the pBI121-MtVP1-EGFP fusion protein was clearly targeted to the plasma membrane (Figure 1D, E and F).

Generation of *MtVP1* transgenic *Arabidopsis* and potato plants

MtVP1 cDNA was inserted into the plant expression vector pBI121 under the control of the CaMV 35S promoter with the plant selective marker gene *Npt*II. The resulting plasmid was named pBI121-MtVP1. This construct was used to transform *Arabidopsis* plants by the

floral dipping method. Fifteen individual kanamycin-resistant plants were obtained. Plants were subsequently selected for three generations to identify transgenic homozygotes.

Potato leaves were transformed with *A. tumefaciens* ($C_{58}C_1$) carrying pBI121-MtVP1. Wounded leaf explants showed initial signs of growth after five days of incubation (Figure 2A). Approximately three shoot buds per explant were produced after 20 days of incubation on shoot induction medium (Figure 2B). Newly formed shoot buds reached a height of 2-3 cm in 1-2 weeks. The buds were later excised and transferred to rooting medium containing 75 mg/L kanamycin (data not shown). Eight individual potato seedlings were produced via *Agrobacterium*-mediated transformation using leaf discs.

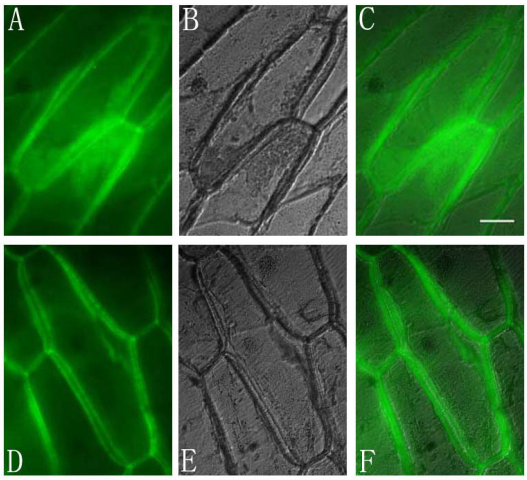

Figure 1. Subcellular localization of MtVP1 protein. A, B and C depict the transient expression of an EGFP fusion protein in onion epidermis. **A.** Fluorescent image; **B.** Bright-field image; **C.** Overlay of images A and B; D, E, and F depict the transient expression of MtVP1-EGFP in onion epidermis. **D.** Fluorescent image; **E.** Bright-field image; **F.** Overlay of D and E images. Bar = 50 μm.

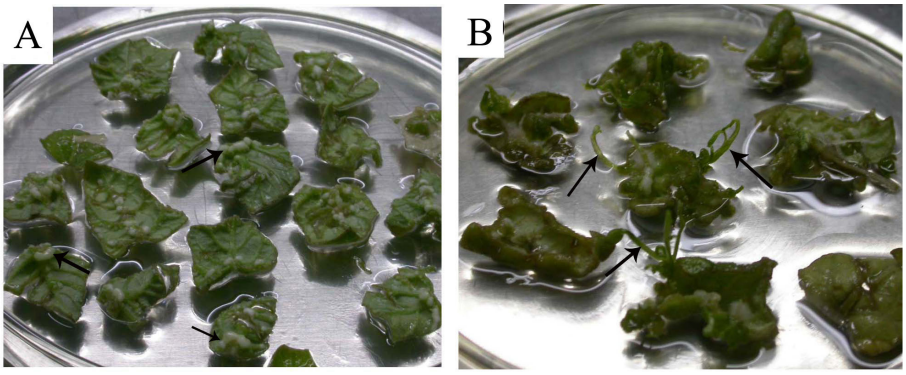

Figure 2. Stages of transformation of potato (*Solanum tuberosum* ssp *weishu 4*). **A.** Callus (arrows) on wounded leaf explants that were cultured on callus induction medium after 5 days of incubation. **B.** Shoot production (arrows) from transformed callus after 20 days on shoot induction medium.

Semi-quantitative RT-PCR analysis

The six transgenic *Arabidopsis* lines were subjected to PCR and semi-quantitative RT-PCR analysis to further test RNA expression. Two transgenic homozygote lines that were tolerant to kanamycin were used for molecular and physiological analyses and subsequent experiments. These lines, named *AtMtVP1* (OE-1) and *AtMtVP1* (OE-2), are described here. Semi-quantitative RT-PCR analysis revealed the presence of *MtVP1* mRNA in these two lines; *AtMtVP1* (OE-1) and *AtMtVP1* (OE-2) are shown in lanes 1 and 2 (Figure 3A).

Five transgenic potato lines that were identified by PCR were subjected to semi-quantitative RT-PCR analysis to further test RNA expression. The lines shown in lanes 1 and 5, which were named *StMtVP1* (OE-1) and *StMtVP1* (OE-2), were used for molecular and physiological analyses and further experiments (Figure 3B).

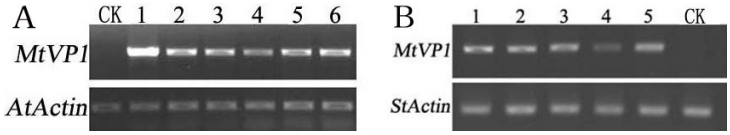

Figure 3. Semi-quantitative RT-PCR analysis of transgenic *Arabidopsis* and potato plants. **A.** *Lane CK* = an untransformed *Arabidopsis* plant; *lanes 1-6* show semi-quantitative RT-PCR analysis of different transgenic *Arabidopsis* plants; **B.** *lanes 1-5* show semi-quantitative RT-PCR analysis of different transgenic potato plants; *Lane CK* = an untransformed potato plant.

Performance of transgenic *Arabidopsis* plants and potato plants

*AtMtVP1*OE plants developed more robust root systems than the WT plants (Figure 4A). The seedlings of the transgenic *Arabidopsis* plant became redder than the WT plants under conditions of cold stress (Figure 4B).

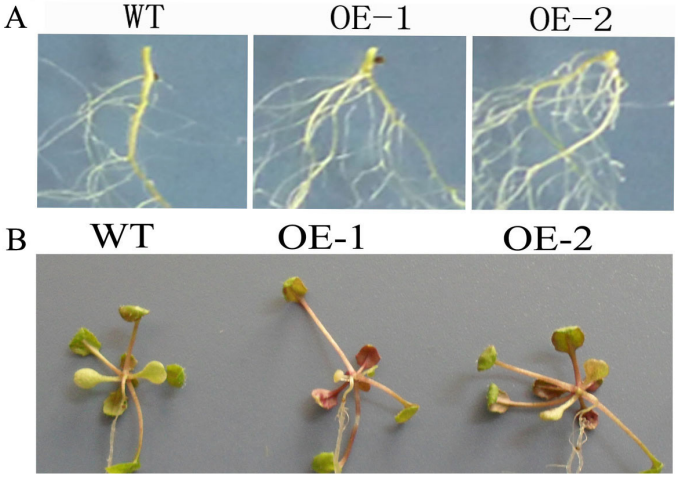

Figure 4. Phenotypic traits of *AtMtVP1*OE plants in root and shoots. **A.** Root system; **B.** 20-day-old seedlings under 4°C stress for a week.

The transgenic potatoes developed more extensive root systems than the WT plants (Figure 5A). The stem base and the tuber buds of the transgenic potatoes became redder than those of the WT plants (Figure 5B and C). The transgenic potatoes exhibited accelerated formation and growth of young leaflets. The six week-old transgenic potato plants generated small compound leaves, whereas there was no obvious change in the WT plants (Figure 5D). Furthermore, the time of flowering was delayed by approximately two weeks (data not shown).

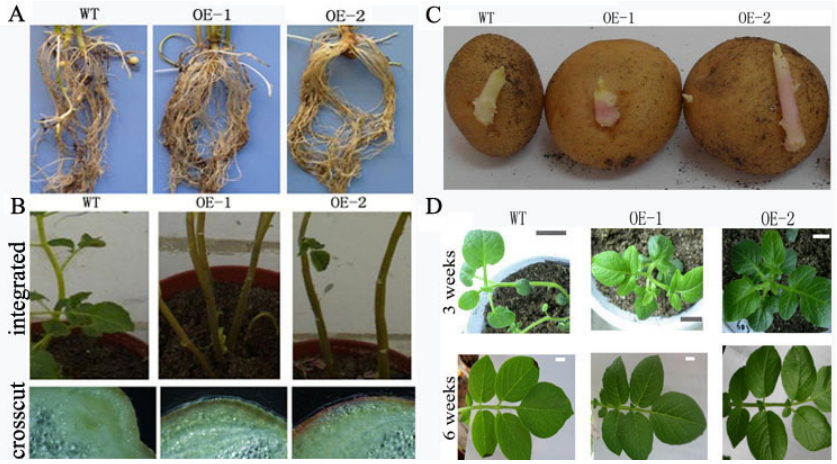

Figure 5. Phenotypic traits of *StMtVP1*OE (OE-1, OE-2) and WT plants in vegetative organs. **A.** Root system; **B.** integrated and crosscut stem; **C.** tuber and the buds; **D.** 3-week-old and 6-week-old leaves.

Analysis of anthocyanin content

The anthocyanin content was enhanced in transgenic *Arabidopsis* shoots and potato tuber buds. The anthocyanin content was 2.95- (OE-1) and 4.57-fold (OE-2) higher in transgenic *Arabidopsis* shoots (Figure 6A) and 1.15- (OE-1) and 1.32-fold (OE-2) higher in transgenic potato tuber buds (Figure 6B) compared to those of the corresponding WT plants. The data represented the mean values of three independent experiments.

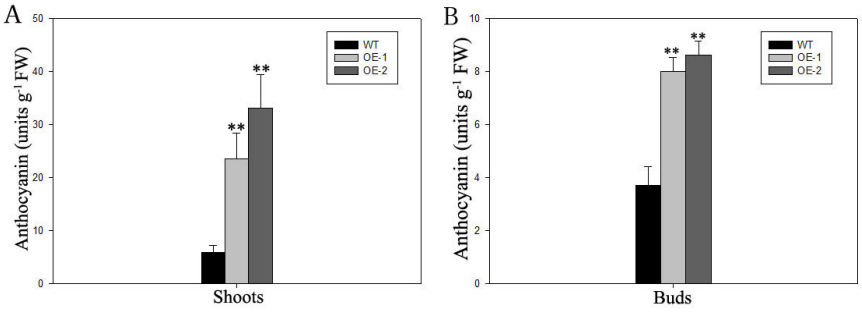

Figure 6. Analysis of the anthocyanin content. **A.** Anthocyanin content of *AtMtVP1*OE (OE-1, OE-2) and WT *Arabidopsis* in shoots; **B.** anthocyanin content of *StMtVP1*OE (OE-1, OE-2) and WT potato in tuber buds. Data are reported as means ± SE of three replicates. **Significant level at 1%.

Analysis of sucrose concentration in buds and tubers

The concentrations of sucrose were 0.36- (OE-1) and 0.53-fold (OE-2) higher in buds and were 17.3- (OE-1) and 13.8-fold (OE-2) higher in tubers of the transgenic lines compared to those of WT potatoes (Figure 7). Thus, the sucrose concentrations in transgenic buds and tubers were higher than those of the WT potatoes.

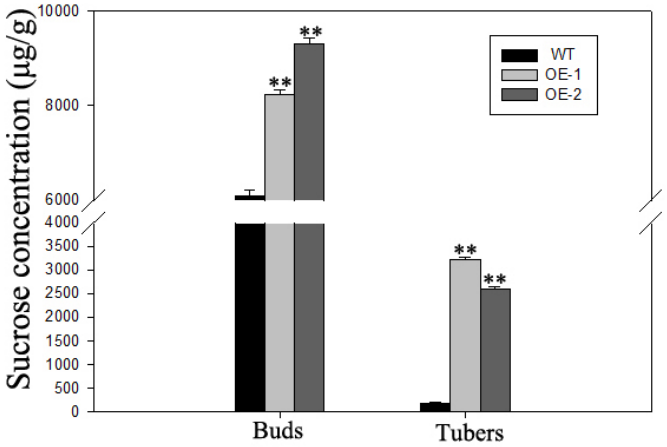

Figure 7. Sucrose concentration of *StMtVP1*OE (OE-1, OE-2) in buds and tubers. Data are reported as means ± SE of three replicates. **Significant level at 1%.

DISCUSSION

Several studies have demonstrated that the subcellular localization of V-PPases is variable in plants. V-PPases can localize to the vacuolar, Golgi, and plasma membranes (Baltscheffsky et al., 1999; Ratajczak et al., 1999; Drozdowicz et al., 2000; Jiang et al., 2001; Mitsuda et al., 2001). Furthermore, H^+-PPases can change their intracellular localization during the invasion of host cells (Drozdowicz et al., 2003). Plant Type I V-PPase was first isolated from vacuoles and was initially considered to be a *bona fide* vacuolar marker (Maeshima, 1991; Rea et al., 1992). However, density gradient centrifugation and phase partitioning of membrane fractions coupled with immunogold electron microscopy showed the presence of H^+-PPases in the plasma membranes of *R. communis* seedlings and cauliflower inflorescences (Ratajczak et al., 1999). Furthermore, proteomic studies confirmed the plasma membrane localization of the *Arabidopsis* H^+-PPase AVP1 (Alexandersson et al., 2004). Our results indicated that the subcellular location of *MtVP*1 was targeted to the plasma membrane in a transient transfection assay.

In our study, the results of cold stress (4°C) showed that the seedlings of transgenic *Arabidopsis* lines overexpressing *MtVP1* became redder than those of WT plants. Previous research demonstrated that low temperatures induced anthocyanin biosynthesis in *Arabidopsis* seedlings (Zhang et al., 2011). Next, we analyzed the anthocyanin content of transgenic *Arabidopsis* shoots, and our results showed that the anthocyanin content was promoted in transgenic *Arabidopsis* shoots.

In the experiment with potatoes, our results showed that the tuber buds and the stem base of potato plants became red in the overexpressed *MtVP1* lines, but that there was no significant change in the WT plants. The anthocyanin content was more than 1-fold higher in the transgenic than in the WT potato tuber buds. This result is consistent with the results from the transgenic *Arabidopsis* lines. The overexpressed *MtVP1* gene may have induced anthocyanin biosynthesis.

It is remarkable that sugar-induced anthocyanin biosynthesis has been observed in many plant species. Sucrose was the most effective inducer of anthocyanin biosynthesis in *Arabidopsis* seedlings (Teng et al., 2005; Solfanelli et al., 2006). In addition, based on a hypothetical model in which the H^+-PPases affected sucrose phloem loading (Fuglsang et al., 2011), we speculated that the overexpression of *MtVP1* increased anthocyanin biosynthesis via sucrose.

To explore the mechanism by which the overexpression of *MtVP1* increased anthocyanin biosynthesis via sucrose, we analyzed the concentration of sucrose in buds and tubers. The results showed that the sucrose concentrations were higher in the buds and tubers of the transgenic potatoes than in those of WT plants. Furthermore, the increased multiples of sucrose in tubers were higher than those in the tuber buds. We speculated that the sucrose concentration was increased in tuber buds owing to the increased sucrose concentration in tubers. Although there is no direct evidence to support Fuglsang's hypothetical model regarding the effects of H^+-PPase on sucrose phloem loading, the overexpressed *MtVP1* enhanced the sucrose accumulation of source organs, which may have then enhanced the sucrose transport to sink organs. Previous studies have demonstrated that sucrose could induce anthocyanin biosynthesis. In the present study, we inferred that the overexpressed *MtVP1* improved sucrose accumulation of source organs, which may enhance the sucrose transport to sink organs, and thus affect anthocyanin biosynthesis.

Sucrose is the main substrate for respiration, biosynthesis, and storage. Thus, an increase in the availability of sucrose for transport could result in both larger and more energized root systems with an enhanced apoplast and rhizosphere acidification capacity. This possibility may explain why our transgenic plants developed more robust root systems, sturdy stems, fat leaves, and hypertrophic tubers.

ACKNOWLEDGMENTS

Research supported by the National Major Special Project on New Varieties Cultivation for Transgenic Organisms (Grant #2009ZX08009-130B) and the National High Technology Planning Program of China (Grant #2006AA10Z407).

REFERENCES

Alexandersson E, Saalbach G, Larsson C and Kjellbom P (2004). *Arabidopsis* plasma membrane proteomics identifies components of transport, signal transduction and membrane trafficking. *Plant Cell Physiol.* 45: 1543-1556.

Baltscheffsky M, Schultz A and Baltscheffsky H (1999). H^+-proton-pumping inorganic pyrophosphatase: a tightly membrane-bound family. *FEBS Lett.* 452: 121-127.

Banerjee AK, Prat S and Hannapel DJ (2006). Efficient production of transgenic potato (*S. tuberosum* L. ssp. *andigena*) plants via *Agrobacterium tumefaciens*-mediated transformation. *Plant Sci.* 170: 732-738.

Bao AK, Wang SM, Wu GQ, Xi JJ, et al. (2008). Overexpression of the *Arabidopsis* H^+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa* L.). *Plant Sci.* 176: 232-240.

Clough SJ and Bent AF (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16: 735-743.

Davies JM, Darley CP and Sanders D (1997). Energetics of the plasma membrane pyrophosphatase. *Trends Plant Sci.* 2: 9-10.

Drozdowicz YM, Kissinger JC and Rea PA (2000). AVP2, a sequence-divergent, K(+)-insensitive H(+)-translocating inorganic pyrophosphatase from *Arabidopsis*. *Plant Physiol.* 123: 353-362.

Drozdowicz YM, Shaw M, Nishi M, Striepen B, et al. (2003). Isolation and characterization of TgVP1, a type I vacuolar H^+-translocating pyrophosphatase from *Toxoplasma gondii*. The dynamics of its subcellular localization and the cellular effects of a diphosphonate inhibitor. *J. Biol. Chem.* 278: 1075-1085.

Fuglsang AT, Paez-Valencia J and Gaxiola RA (2011). Transporters and Pumps in Plant Signaling, Signaling and Communication in Plants. In: Plant Proton Pumps: Regulatory Circuits Involving H^+-ATPase and H^+-PPase (Geisler M and Venema K, eds.). Springer-Verlag Press, Berlin Heidelberg. 7: 39-64.

Gaxiola RA, Li J, Undurraga S, Dang LM, et al. (2001). Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. *Proc. Natl. Acad. Sci. U. S. A.* 98: 11444-11449.

Gollop R, Farhi S and Perl A (2001). Regulation of the leucoanthocyanidin dioxygenase gene expression in *Vitis vinifera*. *Plant Sci.* 161: 579-588.

Gollop R, Even S, Colova-Tsolova V and Perl A (2002). Expression of the grape dihydroflavonol reductase gene and analysis of its promoter region. *J. Exp. Bot.* 53: 1397-1409.

Hiratsu K, Matsui K, Koyama T and Ohme-Takagi M (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J.* 34: 733-739.

Jiang L, Phillips TE, Hamm CA, Drozdowicz YM, et al. (2001). The protein storage vacuole: a unique compound organelle. *J. Cell Biol.* 155: 991-1002.

Langhans M, Ratajczak R, Lutzelschwab M, Michalke W, et al. (2001). Immunolocalization of plasma-membrane H^+-ATPase and tonoplast-type pyrophosphatase in the plasma membrane of the sieve element-companion cell complex in the stem of *Ricinus communis* L. *Planta* 213: 11-19.

Li B, Wei A, Song C, Li N, et al. (2008). Heterologous expression of the TsVP gene improves the drought resistance of maize. *Plant Biotechnol. J.* 6: 146-159.

Long AR, Williams LE, Nelson SJ and Hall JL (1995). Localization of membrane pyrophosphatase activity in *Ricinus communis* seedlings. *J. Plant Physiol.* 146: 629-638.

Lv S, Zhang K, Gao Q, Lian L, et al. (2008). Overexpression of an H^+-PPase gene from *Thellungiella halophila* in cotton enhances salt tolerance and improves growth and photosynthetic performance. *Plant Cell Physiol.* 49: 1150-1164.

Lv SL, Lian LJ, Tao PL, Li ZX, et al. (2009). Overexpression of *Thellungiella halophila* H(+)-PPase (TsVP) in cotton enhances drought stress resistance of plants. *Planta* 229: 899-910.

Maeshima M (1991). H(+)-translocating inorganic pyrophosphatase of plant vacuoles. Inhibition by Ca^{2+}, stabilization by Mg^{2+} and immunological comparison with other inorganic pyrophosphatases. *Eur. J. Biochem.* 196: 11-17.

Martin T, Oswald O and Graham IA (2002). *Arabidopsis* seedling growth, storage lipid mobilization, and photosynthetic gene expression are regulated by carbon:nitrogen availability. *Plant Physiol.* 128: 472-481.

Mita S, Murano N, Akaike M and Nakamura K (1997). Mutants of *Arabidopsis thaliana* with pleiotropic effects on the expression of the gene for beta-amylase and on the accumulation of anthocyanin that are inducible by sugars. *Plant J.* 11: 841-851.

Mitsuda N, Enami K, Nakata M, Takeyasu K, et al. (2001). Novel type *Arabidopsis thaliana* H(+)-PPase is localized to the *Golgi apparatus*. *FEBS Lett.* 488: 29-33.

Murashig T and Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissues. *Plant Physiol.* 15: 473-497.

Park S, Cheng NH, Pittman JK, Yoo KS, et al. (2005). Increased calcium levels and prolonged shelf life in tomatoes expressing *Arabidopsis* H^+/Ca^{2+} transporters. *Plant Physiol.* 139: 1194-1206.

Ratajczak R, Hinz G and Robinson DG (1999). Localization of pyrophosphatase in membranes of cauliflower inflorescence cells. *Planta* 208: 205-211.

Rea PA, Kim Y, Sarafian V, Poole RJ, et al. (1992). Vacuolar H(+)-translocating pyrophosphatases: a new category of ion translocase. *Trends Biochem. Sci.* 17: 348-353.

Robinson DG, Haschke HP, Hinz G, Hoh G, et al. (1996). Immunological detection of tonoplast polypeptide in the plasma membrane of pea cotyledon. *Planta* 198: 95-103.

Seufferheld M, Lea CR, Vieira M, Oldfield E, et al. (2004). The H(+)-pyrophosphatase of *Rhodospirillum rubrum* is predominantly located in polyphosphate-rich acidocalcisomes. *J. Biol. Chem.* 279: 51193-51202.

Solfanelli C, Poggi A, Loreti E, Alpi A, et al. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in *Arabidopsis*. *Plant Physiol.* 140: 637-646.

Teng S, Keurentjes J, Bentsink L, Koornneef M, et al. (2005). Sucrose-specific induction of anthocyanin biosynthesis in *Arabidopsis* requires the MYB75/PAP1 gene. *Plant Physiol.* 139: 1840-1852.

Tsukaya H, Ohshima T, Naito S, Chino M, et al. (1991). Sugar-dependent expression of the CHS-A gene for chalcone synthase from petunia in transgenic *Arabidopsis*. *Plant Physiol.* 97: 1414-1421.

Wang JW, Shen YH, Wang P and Wang HQ (2009). Cloning and characterization of a vacuolar H^+-PPase gene from *Medicago truncatula*. *Acta Bot. Boreal.-Occident. Sin.* 29: 435-442.

Yang H, Knapp J, Koirala P, Rajagopal D, et al. (2007). Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H^+-pyrophosphatase. *Plant Biotechnol. J.* 5: 735-745.

Zhang Y, Zheng S, Liu Z, Wang L, et al. (2011). Both HY5 and HYH are necessary regulators for low temperature-induced anthocyanin accumulation in *Arabidopsis* seedlings. *J. Plant Physiol.* 168: 367-374.

