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Research Article

Absence of *AVP1* transcripts in wild type watermelon scions grafted onto transgenic bottle gourd rootstocks

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Abstract In this study we confirmed the stable integration of *Arabidopsis AVP1* in the genomes of bottle gourd T_3 homozygous lines and its transcription, and additionally evaluated possibility of translocation of the *AVP1* mRNA from transgenic bottle gourd rootstocks to wild type watermelon scions. Each *AVP1* gene in two bottle gourd T3 lines is abundantly expressed under a field condition. Given the grafting between wild type watermelon scions and AVP1-expressing bottle gourd rootstocks, no translocation of the *AVP1* mRNA was detected in leaves, both sexual flowers, and fruits of the scions.

Keywords Bottle ground, Gene modified, Graft, RT-PCR, Watermelon

Introduction

Grafting is now a popular technique for the cultivation of the horticultural crops including cucurbitaceae fruit vegetables, which has been developed not only to control growth and development of the scion but also to enhance tolerance against soil-borne diseases and/or abiotic stresses, such as salinity, low temperature and drought (Jang et al. 2012; Kubota et al. 2008; Lee 1994). In some special regions, where land utility is extremely limited, the allied crops are repeatedly cultivated all the year round (Kubota et al. 2008; Lee 1994), which increases specific pathogens and salinity of the rhizosphere. To overcome the disadvantages of the intensive cultivation, improvement of rootstocks by using genetic engineering is being attempted as a solution (Han et al. 2009; Smolka et al. 2010; Wang et al. 2012).

Control of abiotic stresses is an important element to increase total yields in modern agriculture. Plants respond to various abiotic stresses by altering their turgor pressure in vacuoles in order to accomplish selective permeation of solutes through proton pumps (Gaxiola et al. 2001; McNeil et al. 1999). A vacuolar H^+-pyrophosphatase encoded by the *AVP1* gene is one of the proton pumps in *Arabidopsis* (Sarafian, et al. 1992) and generates an H^+ electrochemical gradient across the tonoplast (Zhen et al. 1997). Several transgenic plants overexpressing AVP1 have been shown to be more tolerant to salt- and drought-stress than their counterparts (Gaxiola et al. 2001; Jeong et al. 2013; Park et al. 2012; Pasapula et al. 2011). Bottle gourd (*Lagenaria siceraria* Standl.) that gives host-specific resistance against a *Fusarium* spp. is one of the most popular rootstocks for watermelon, although acute disorders such as sudden wilt caused by complicated abiotic stresses appear occasionally under cultivation conditions in year round production systems (Edelstein et al. 1999; Kubota et al. 2008; Lee 1994; Park et al. 2005b). Therefore, transgenic bottle gourd with modified abiotic stress-related traits is being considered as a rootstock of watermelon production (Han et al. 2009). However, the utilization of a transgenic rootstock might bring both sides of the coin owing to feasible long-distance transport of molecules derived from the transgene: to indirectly improve horticultural traits of the scion and to provoke controversy about social acceptance or rejection (Harada 2010; Kim et al. 2001). Here we focused on ascertaining transgene RNA transmission from rootstock to scion, which is one of the most important factors together with protein movement evaluating

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the substantial equivalence (Millstone et al. 1999). Two bottle gourd lines expressing *Arabidopsis* AVP1 were used as rootstocks for watermelon. Molecular characteristics of the *AVP1* and its transcript were analyzed in the two transgenic lines, and the translocation of the transcripts of *AVP1* and *Bar* (a selection marker gene) was tested in each part of watermelon scion.

Materials and Methods

Plant materials and transformation

Bottle gourd (*Lagenaria siceraria* 'G5') transformation was performed by means of the *Agrobacterium*-mediated transformation method using cotyledon explants as described (Han et al. 2004; 2005; 2009). *A. tumefaciens* strain LBA4404, with the pRG521 plasmid that was generated by replacing the selectable marker cassette of pRG395 plasmid (Park et al. 2005a) with the *Nos*-pro/*Bar*/*Nos*-ter of pCB302 plamid (Xiang et al. 1999), was used for this study. Collectively, the T-DNA region of pRG521 plasmid was consisted of *LB*/tandem *35S*-pro/*AVP1*/Poly A/*Nos*-pro/*Bar*/*Nos*-ter/*RB*. The T_3 lines, BGAVP05, was developed through phosphinothricin (Duchefa Biochemie, the Netherlands) at 2 mg/L supplementation for selecting T_0 plants *in vitro*, and herbicide Basta™ (Kyungnoog, Korea) at 0.3%(v/v) treatment and polymerase chain reaction (PCR) analysis for succeeding generations. Final T_3 generation of the BGAVP05 lines and wild type bottle gourd were sown in plastic trays filled with commercial organic soil. After 3 weeks, young plants were transplanted into plastic pots (30×35 cm) and then further grown in a greenhouse at Kyungpook National University located in Daegu, Korea. These plants were then subjected to nucleic acids analyses. Meanwhile, 1 week delayed seedlings of two commercial watermelons (*Citrullus vulgaris* 'prince' and 'speed') were grafted onto the two transgenic and wild type bottle gourd lines. After graft unions were stabilized, grafted plants were also transplanted and grown under the same conditions indicated above for non-grafted bottle gourd lines (Fig. 1).

PCR and Reverse transcription polymerase chain reaction (RT-PCR) analyses

DNAs for PCR analysis and total RNAs for RT-PCR were extracted by using the HiYield™ Genomic DNA Mini Kit (Real Biotech Corporation, Taiwan) and the RNeasy® Plant Mini Kit (Qiagen, Germany), respectively. Up to 0.1 g of young leaves of bottle gourd plants for DNA extraction and up to 0.3 g each of samples from bottle gourds (young leaves and stems, and male and female flowers) and watermelons (young leaves, male and female flowers, and flesh of fruits) for RNA

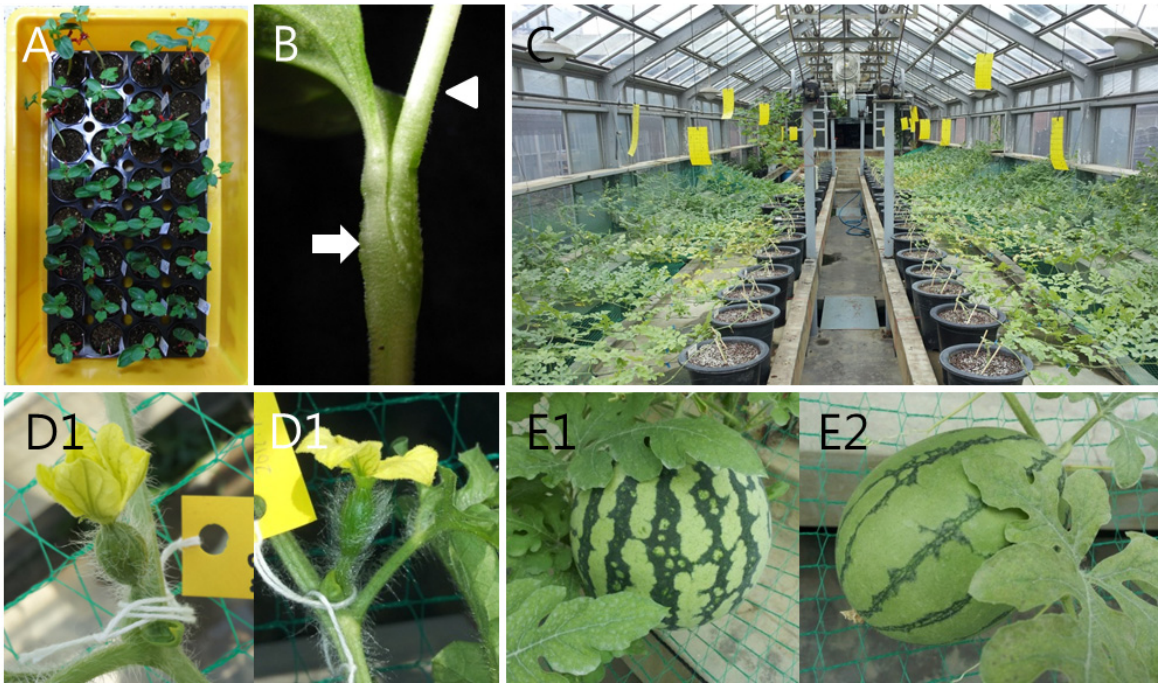

Fig. 1 Growth and development of watermelon plants grafted onto bottle gourd rootstocks expressing AVP1. (A) Acclimatization of grafted watermelon young plants. (B) Formation of graft union between wild type watermelon scion (arrow head) and AVP1-expressing bottle gourd rootstock (arrow). (C) The potted plants of grafted watermelon plants growing in a glasshouse. Female flowers of watermelon, cultivars 'Speed' (D1) and 'Prince' (D2). Fruits of watermelon cultivars 'Speed' (E1) and 'Prince' (E2)

extraction were pulverized after being frozen in liquid nitrogen and then subjected to extraction using the kits according to the manufacturer's instructions. Each DNA (100 ng) was used as a template for PCR amplification. The first strand of cDNA was synthesized from 1 μg of total RNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. Amplification of a 668 bp *AVP1* cDNA fragment and the *Bar* gene were conducted on the Veriti™ Thermal Cycler (Applied Biosystems) using the Solg™ 2× Taq PCR Pre-Mix Kit (SolGent, Korea) with the following primer sets: *AVP1*-PF1 (forward primer: 5`-ATGGTGGCGCCTGCTTTGTT-3`), *AVP1*-PR1 (reverse primer: 5`-TCATCTCCGTAATAGATCTTGAA-3`), *Bar*-PF1 (forward primer: 5`-TCAAATCTCGGTGACGGGCA-3`) and *Bar*-PR2 (reverse primer: 5`-GGTCTGCACCATCGT-CAACC-3`). Amounts of *AVP1* transcripts were normalized in comparison with those of an internal control gene, *Actin*, of which PCR primers, CuAc-PF1 (forward primer: 5`-GTA-TCGTGCTGGATTCTGGT-3`) and CuAc-PR3 (reverse primer: 5`-CCGATGAGAGATGGCTGGAA-3`) were obtained from coding sequences of several *Cucurbitaceae* plants. Expression level was estimated semi-quantitatively by altering the cycles of PCR amplification. PCR was conducted under the following conditions: initial denaturation for 2 min at 95°C, followed by 35 cycles of 95°C for 20 sec, 60°C for 40 sec, and 72°C for 1 min, and then a final extension for 5 min at 72°C. PCR products were separated and visualized on 2% agarose gel.

Southern blot analysis

For Southern blot analysis, preparation of DNAs was performed as described previously (Park et al., 2004). DNAs (10 μg) were digested by the restriction enzymes *Spe* I and *Xba* I (Takara, Japan) according to the manufacturer's procedures. Digested DNAs were separated on 1% agarose gel, and then transferred to a nylon membrane and fixed by using a UV-crosslinker (BLX-313, France). The *AVP1* regions were analyzed by using a probe amplified from the primer set mentioned above. Labeling and detection were conducted by using the DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's procedures.

Results

Nucleic acids analyses of bottle gourd rootstock lines expressing *Arabidopsis AVP1*

We obtained T_0 plants, BGAVP05, from different transformation batch according to the same procedure. To confirm copy numbers of *AVP1* in the T_0 plants, DNA gel blot analysis was conducted. As the recombinant construct for transformation has four multi-cloning sites for total seventeen kinds of restriction enzymes, very limited enzymes were used for the analysis. Among selected four enzymes (*Bam* HI, *Pst* I, *Spe* I, *Xba* I), the T_0 plants showed distinguishable hybridization patterns with single copy each of *AVP1* in only *Xba* I and *Spe* I digestions, respectively: a 3.4 kbp fragment by *Xba* I in

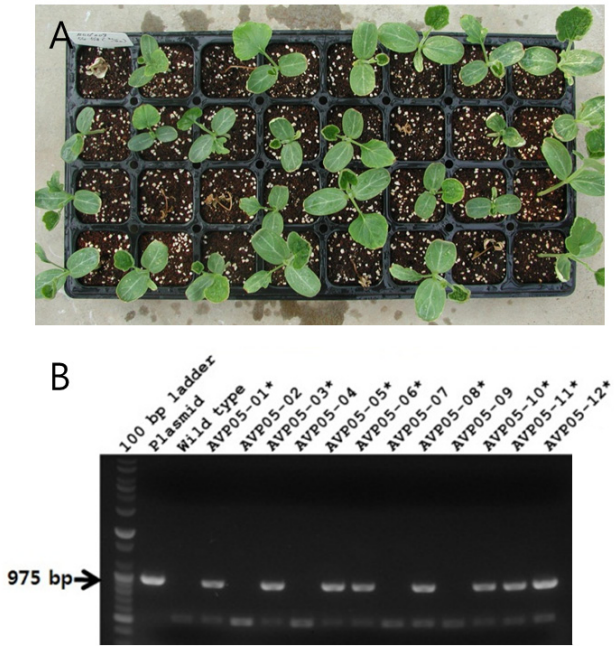

Fig. 2 Herbicide treatment and polymerase chain reaction (PCR) analysis for bottle gourd T_1 plants. (A) Herbicide bioassay for the selectable marker gene *Bar*. Basta™ (Kyungnoog, Korea) was treated at 0.3% (v/v). (B) PCR analysis of the *AVP1*. PCR products were run on 2% agarose gel and stained with ethidium bromide. WT (G5) and P indicate a non-transgenic wild type plant (negative control) and plasmid possessing full-length *AVP1* (positive control), respectively

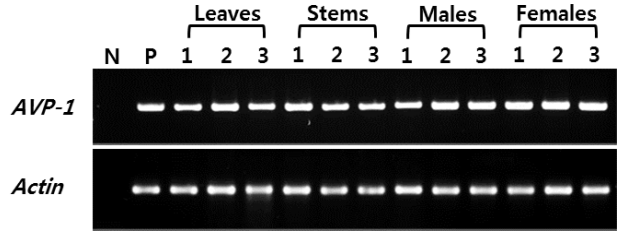

Fig. 3 RT-PCR analysis for bottle gourd T_3 lines, BGAVP05. Genes analyzed are indicated on the left side of the gel photos. The *Actin* (see materials and methods) was used as an internal control for normalizing levels of *AVP1* transcripts. N and P indicate respectively the absence and presence of reverse transcriptase during first strand cDNA synthesis step as using RNAs extracted from leaves of mother T_0 plants. Males and females indicate each unisexual flower

BGAVP18 and a 2.2 kbp fragment by *Spe* I in BGAVP20 were revealed (data not shown). The independent transgenic plants were self-pollinated to obtain each progeny, after which T_1 and T_2 populations were treated with Basta^{TM} and analyzed by PCR to generate non-segregating homozygous lines (Fig. 2).

To confirm transcription of the *AVP1*, semi-quantitative RT-PCR analysis was also conducted in the T_3 progenies. As expected, the target gene was abundantly and uniformly expressed in all tested organs, including leaf, stem, and male and female flowers of both transgenic lines (Fig. 3).

Detection of *AVP1* and *Bar* transcripts in watermelon scions grafted onto transgenic bottle gourd rootstocks

We further performed a grafting experiment to verify whether or not transcripts of the transgenes introduced in bottle gourd rootstocks are translocated to scions. Two kinds of commercial watermelon cultivars, 'Speed' and 'Prince', were grafted on T_3 progenies of transgenic bottle gourd lines (Fig. 1), after which *AVP1* transcripts were detected by RT-PCR in the organs of watermelon scions (Fig. 1, 4). Although the *AVP1* was sufficiently transcribed in the two transgenic rootstock lines (Fig. 3), the transcripts were not detected in the organs of watermelon scions, such as leaves and both flowers (Fig. 4). Moreover, the transcripts of *AVP1* and *Bar* were not detected in the fleshes of watermelon that are edible parts (Fig. 4), which indicating that transcripts of the transgenes introduced in bottle gourd rootstocks were not translocated to the organs of watermelon scion.

Discussion

As rootstocks often help to overcome soil-borne pests and pathogens, many economically important crop species in *Solanaceae* and *Cucurbitaceae* are grafted before being transplanted to open fields or greenhouses in order to promote vigorous growth and enhanced yields of scions (Kubota et al. 2008). Genetic improvement of rootstocks is another important issue in modern crop breeding, as useful horticultural traits are being introduced by transgenic approach (Han et al. 2009; Smolka et al. 2010; Wang et al. 2012). Bottle gourd has long history as a rootstock in watermelon production (Lee 1994). We obtained, in this study, two transgenic bottle gourd lines expressing a *Arabidopsis* vacuolar H^+-pyrophosphatase (AVP1), which had conferred enhanced tolerance to drought- and salt-stress in several crops(Gaxiola et al. 2001; Jeong et al. 2013; Park et al. 2012; Pasapula et al. 2011). The another genetically engineered bottle gourd rootstock that possess *Arabidopsis* Ca^{2+}/H^+ exchanger CAX2B, also allowed more robust growth of their watermelon and melon scions than wild type bottle gourd (Han et al. 2009). These rootstocks with modified tolerance can be potential substitutes for wild type one.

Transgrafting is grafting between genetically engineered rootstocks and non-transgenic scions or vice versa, and it will strengthen the application of grafting by enabling introducing new functional characteristics expressed by the transgene(s). Once a graft union is established, many factors allowing normal plant growth, such as water, hormones, nutrients, biochemicals, RNAs, peptides, and proteins, are capable of long-distance trafficking across the vascular connection between the rootstock and scion (Haroldsen et al. 2012; Koepke and Dhingra 2013). Crosstalks are significantly involved in

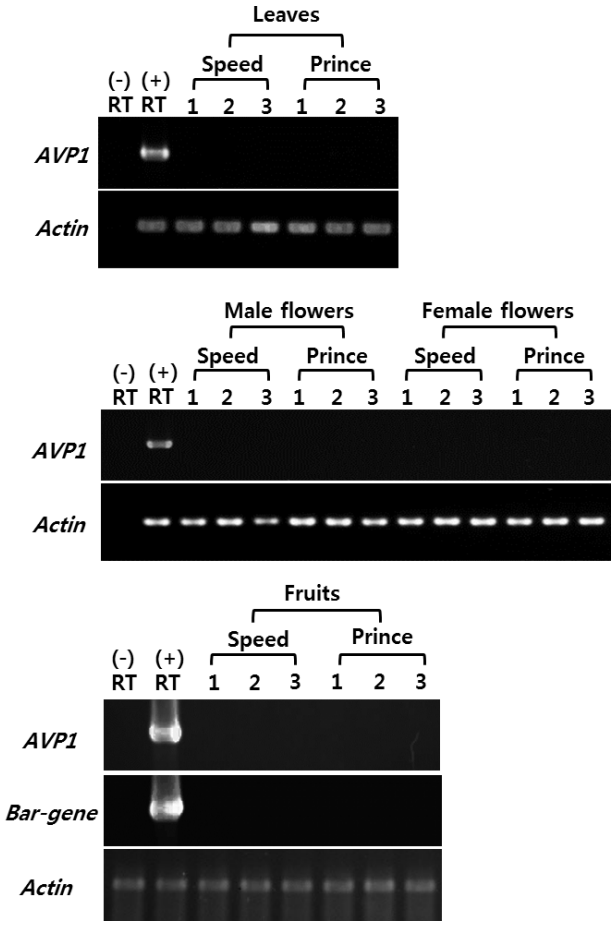

Fig. 4 RT-PCR analyses for transcripts of *AVP1* and *Bar* in wild type watermelon scions grafted onto T_3 bottle gourd rootstocks. Transcripts of the *AVP1* and *Bar* genes were detected in watermelon scions by RT-PCR. *Actin* as an internal control was the same as in Fig. 3. (-) RT and (+) RT indicate respectively the absence and presence of reverse transcriptase during first strand cDNA synthesis step as using RNAs extracted from leaves of T_3 bottle gourd plant

morphological and physiological processes indispensable for plant survival, including flowering, apical meristem growth, senescence, and branching, etc. (Harada 2010; Haroldsen et al. 2012; Koepke and Dhingra 2013). In the case of transgrafted plants, translocation of transgene products can bring about undesirable effects in wild type counterparts, which can limit their application. However, if approved, their use can be economically beneficial in the crop industry (Haroldsen et al. 2012). In this respect, we focused on whether transcripts of transgenes are delivered from genetically engineered rootstocks to wild type scions. Translocation of transgene products from rootstocks to scions has been observed, but the results are still problematic. Several endogenous plant RNAs involved in long distance transport seem to be translocated from transgenic rootstocks to wild type scions, and they are expressed under ectopic conditions as well (Harada 2010; Haroldsen et al. 2012; Kim et al. 2001). Small non-coding RNAs such microRNAs (miRNAs) are also an internal component found in phloem sieve tubes, and they have been shown to mediate systemic RNA silencing through graft unions (Yoo et al. 2004), suggesting that miRNAs play important roles in regulation of gene expression via long distance transport. However, two small RNAs derived from hairpin constructions of genes encoding β-glucuronidase and anthocyanin synthase were shown to not induce systemic silencing in apple transgenic lines grown under greenhouse conditions (Flachowsky et al. 2012). A similar result was also found in a soil bacterium gene, *rooting locus B*, in which mRNA was not detectable in wild type apple scions (Smolka et al. 2010). In this study, the potential translocation of transgene mRNAs was analyzed in scion organs by using RT-PCR. Transcripts of the target gene *AVP1* were not detected in all organs of wild type watermelon scion tested (Fig. 4). Especially, the RT-PCR products were not detected in young reproductive organs and fruits in the harvest, and the product of selectable marker gene *Bar* also was not detected in fruits (Fig. 1, 4). The results suggest that transgene mRNAs in this study might be not transmitted to wild type scion parts.

One possible explanation for this discordance among studies is that long distance transport might be a core system programmed by the plant itself as a survival strategy. As such, it might require some components that form complexes for delivery of target molecules to sink portions, as well as short-term transportation such as cell to cell communication. The system includes genes related to transcriptional regulators, cell fate or cycle controlling elements, hormonal factors, metabolic components, and mRNA-binding proteins (Haroldsen et al. 2012). However, if transgenes are not involved in these systems, then no proteins target or bind to the transgene mRNAs which then could not be translocated to sieve tube cells for systemic transport. This suggests that delivery systems are very limited systems permitted in some special cases, and need proteins related to the movement. Viral infection systems can support this notion, as viruses are not targeted by plant delivery systems. However, once inoculated mechanically, they spread out into all plant parts by virus-encoded nonstructural movement proteins (Citovsky and Zambryski 2000).

Here, we would like to elucidate one of the important factors for the potential use of genetically engineered plants as rootstocks. Our study showed that transgene transcripts are not translocated from rootstocks to scions by using vegetable grafting unions.

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References

Citovsky V, Zambryski P (2000) Systemic transport of RNA in plants. Trends Plant Sci 5:52-54

Edelstein M, Cohen R, Burger Y, Shriber S (1999) Integrated management of sudden wilt in melons, caused by *Monosporascus cannonballus*, using grafting and reduced rates of methyl bromide. Plant Dis 83:1142-1145

Flachowsky H, Tränkner C, Szankowski I, Waidmann S, Hanke M, Treutter D, Fischer TC (2012) RNA-mediated gene silencing signals are not graft transmissible from the rootstock to the scion in greenhouse-grown apple plants *Malus* sp. Int J Mol Sci 13:9992-10009

Gaxiola RA, Li J, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc Natl Acad Sci USA. 98:11444-11449

Han JS, Kim CK, Park SH, Hirschi KD, Mok IG (2005) *Agrobacterium*-mediated transformation of bottle gourd (*Lagenaria siceraria* Standl.). Plant Cell Rep 23:692-698

Han, JS, Oh DG, Mok IG, Park HG, Kim CK (2004) Efficient plant regeneration from cotyledon explants of bottle gourd (*Lagenaria siceraria* Standl.). Plant Cell Rep 23:291-296

Han JS, Park SH, Shigaki T, Hirschi KD, Kim CK (2009) Improved watermelon quality using bottle gourd rootstock expressing a Ca^{2+}/H^+ antiporter. Mol Breed 24:201-211

Harada T (2010) Grafting and RNA transport via phloem tissue in horticultural plants. Sci Hortic 125:545-550

Haroldsen VM, Chi-Ham CL, Bennett AB (2010) Transgene mobilization and regulatory uncertainty for non-GE fruit

products of transgenic rootstocks. J Biotechnol 161:349-353

Haroldsen, V.M., M.W. Szczerba, H. Aktas, J. Lopez-Baltazar, M.J. Odias, C.L. Chi-Ham, J.M. Labavitch, A.B. Bennett, and A.L.T. Powell. 2012. Mobility of transgenic nucleic acids and proteins within grafted rootstocks for agricultural improvement. Front. Plant Sci.3:39.

Jang YA, Park DK, Huh YC, Lee JG, Yeo KH, Lee SG (2012) Incidence of *Monosporascus* wilt and fruit quality of melons influenced by grafting using melon as a rootstock. Kor J Hort Sci Technol. 30 (Suppl. II): 55 (Abstr.)

Jeong M, Kang IK, Kim CK, Choi C, Park KI, Han JS (2013) Physiological responses to drought stress of transgenic Chinese cabbage expressing *Arabidopsis* H^+-pyrophosphatase. J Plant Biotechnol 40:156-162

Kim M, Canio W, Kessler S, Sinha N (2001) Developmental changes due to long-distance movement of a homeobox fusion transcript in tomato. Science 293:287-289

Koepke T, Dhingra A (2013) Rootstock scion somatogenetic interactions in perennial composite plants. Plant Cell Rep 32:1321-1337

Kubota C, McDlure MA, Kokalis-Burelle N, Bausher MG, Rosskopf EN (2008) Vegetable grafting: history, use and current technology status in North America. HortScience 43:1664-1669

Lee JM (1994) Cultivation of grafted vegetables I. current status, grafting methods, and benefits. HortScience 29:235-239

McNeil SD, Nuccio ML, Hanson AD (1999) Betaines and related osmoprotectants. Targets for metabolic engineering of stress resistance. Plant Physiol 120:945-949

Millstone E, Brunner E, Mayer S (1999) Beyond 'substantial equivalence'. Nature 401:525-526

Park KI, Choi JD, Hoshino A, Morita Y, Iida S (2004) An intragenic tandem duplication in a transcriptional regulatory gene for anthocyanin biosynthesis confers pale-colored flowers and seeds with fine spots in *Ipomoea tricolor*. Plant J 38:840-849

Park M, Won HY, Kim CK, Han JS (2012) Increased biomass and enhanced tolerance to salt stress in Chinese cabbage overexpressing *Arabidopsis* H^+-PPase (AVP1). J Plant Biotechnol 39:253-260

Park S, Li J, Pittman JK, Berkowitz GA, Yang H, Undurraga S, Morris J, Hirschi KD, Gaxiola RA (2005a) Up-regulation of a H^+-pyrophosphatase (H^+-PPase) as a strategy to engineer drought-resistant crop plants. Proc Natl Acad Sci USA 102:18830-18835

Park SM, Lee JS, Jegal S, Jeon BY, Jung M, Park YS, Han SL, Shin YS, Her NH, Lee JH, Lee MY, Ryu KH, Yang SG, Harn CH (2005b) Transgenic watermelon rootstock resistant to CGMMV (cucumber green mottle mosaic virus) infection. Plant Cell Rep 24:350-356

Pasapula V, Shen G, Kuppu S, Paez-Valencia J, Mendoza M, Hou P, Chen J, Qiu X, Zhu L, Zhang X, Auld D, Blumwald E, Zhang H, Gaxiola R, Payton P (2011) Expression of an *Arabidopsis* vacuolar H^+-pyrophosphatase gene (AVP1) in cotton improves drought- and salt tolerance and increases fibre yield in the field conditions. Plant Biotechnol J 9:88-99

Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 89:1775−1779

Smolka A, Li XY, Heikelt C, Weiander M, Zhu LH (2010) Effects of transgenic rootstocks on growth and development of non-transgenic scion cultivars in apple. Transgenic Res 19:933-948

Yoo BC, Kragler F, Varkonyi-Gasic E, Haywood V, ArcheEvans S, Lee YM, Lough TJ, Lucas WJ (2004) A systemic small RNA signaling system in plants. Plant Cell 16:1979-2000

Wang L, Yang M, Akinnagbe A, Liang H, Wang J, Ewald D (2012) *Bacillus thuringiensis* protein transfer between rootstock and scion of grafted poplar. Plant Biol 14:745-750

Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol 40:711-717

Zhen RG, Kim EJ, Rea PA (1997) The molecular and biochemical basis of pyrophosphate-energized proton translocation at the vacuolar membrane. Adv Bot Res 25: 297-337