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ORIGINAL ARTICLE

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Fruit-specific V-ATPase suppression in antisense-transgenic tomato reduces fruit growth and seed formation

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Abstract The vacuole is a large, multifunctional organelle related to the processes of cell expansion, solute accumulation, regulation of cytoplasmic ion concentrations, pH homeostasis and osmoregulation, which are directly or indirectly achieved by vacuolar H^+-pumps: vacuolar H^+-ATPase (V-ATPase; EC 3.6.1.3) and vacuolar H^+-pyrophosphatase (V-PPase; EC 3.6.1.1). In this study, we produced antisense-transgenic tomatoes (*Lycopersicon esculentum* L.) of the V-ATPase A subunit, which is under the control of the fruit-specific *2A11* promoter. One β-glucuronidase (GUS)-transgenic line (GUS control) and seven A subunit antisense-transgenic lines were obtained. The amount of V-ATPase A subunit mRNA in fruit decreased in all antisense-transgenic lines, but in leaves showed no difference compared with the GUS control line and the nontransformant, suggesting that suppression of the V-ATPase A subunit by a *2A11* promoter is limited to fruit. The antisense-transgenic plants had smaller fruits compared with the GUS control line and the nontransformant. Surprisingly, fruits from the antisense-transgenic plants, except the fruit that still had relatively high expression of A subunit mRNA, had few seeds. Sucrose concentration in fruits from the antisense-transgenic plants increased, but glucose and fructose concentrations did not change. These results show the importance of V-ATPase, not only in fruit growth, but also in seed formation and in sugar composition of tomato fruit.

Keywords Fruit growth · Gene suppression · Lycopersicon · Seed formation · Tomato · Vacuolar H^+-ATPase

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Abbreviations GUS: β-Glucuronidase · V-ATPase: Vacuolar H^+-ATPase · V-PPase: Vacuolar H^+-pyrophosphatase · WT: Wild type

Introduction

In tomato fruit, cells divide for only 2 weeks after fertilization (Teitel et al. 1985; Bohner and Bangerth 1988) and then the fruit enlarges only by cell expansion. Most of the cell volume of the fruit is occupied by a vacuole, which accumulates many substances related to fruit quality, such as sugar, minerals and organic acids, at high concentrations. This causes high osmotic pressure, and, as a result, water is taken up into the vacuole and the fruit enlarges. To transport substances into the vacuole against their concentration gradient, an electrochemical proton gradient is required; this gradient is generated by two distinct proton pumps in the vacuolar membrane, i.e., vacuolar H^+-ATPase (V-ATPase) and vacuolar H^+-pyrophosphatase (V-PPase).

Vacuolar H^+-pyrophosphatase consists of one polypeptide that is distributed among most land plants, some algae, protozoa, bacteria and archaebacteria (Maeshima 2000). V-ATPase is a universal component of eukaryotic organisms and is a multi-subunit enzyme comprising a membrane sector (V_0) and a cytosolic catalytic sector (V_1) (Ratajczak 2000). Arabidopsis V-ATPase has 13 subunit proteins; a functional unit consists of 21 proteins ($A_3B_3CDEFG_2Hac_5c''de$) (Sze et al. 2002). Eight subunits (A–H) form the V_1 domain, which is responsible for ATP binding and hydrolysis. Five other subunits (a, c, c′, c″ and d) form the V_0 domain, which is responsible for proton transport. The V-ATPase A subunit in the V_1 domain is also called the catalytic subunit and is related to hydrolysis of ATP. Carrot transformed with antisense cDNA of the V-ATPase A subunit has more dissected leaves and shorter taproots, and cell expansion in its root is reduced (Gogarten et al. 1992). In vacuolar membrane vesicles isolated from transformed carrot, 3-

O-methylglucose transport depends on ATP decreases, suggesting that V-ATPase participates in sugar accumulation in sink tissue.

The expression pattern of V-ATPase during fruit growth has been analyzed in tomato (Milner and Smith 1995; Coker et al. 2003). Gene expression and activity of V-ATPase is high in young fruit, decreases in the middle stage of fruit development and then increases again with fruit maturation. Similar tendencies were observed in some fruits, such as pear (Shiratake et al. 1997a), Japanese pear (Suzuki et al. 2000) and grape berry (Terrier et al. 2001). However, the importance of V-ATPase in fruit development has not been shown by using transgenic plants or mutants. Fruit has a large vacuole that accumulates various metabolites at high concentrations; therefore, analysis of the vacuolar function in fruit is interesting, not only physiologically but also horticulturally. In transformed carrot (Gogarten et al. 1992), suppression of V-ATPase appears to affect the whole plant because it uses the 35S promoter and leads to a constant expression. Limiting the effect of a transformed gene on a target tissue or organ is necessary. Therefore, in this study, to find the roles of V-ATPase in fruit, we produced antisense-transgenic tomatoes of the V-ATPase A subunit under the control of a fruit-specific *2A11* promoter (Pear et al. 1989).

Materials and methods

Plant material

Tomato (*Lycopersicon esculentum* L.) varieties "Super First" mRNA were used to isolate the cDNA fragment of the V-ATPase A subunit, "Kantaro Jr." fruits were used for mRNA expression analysis and the miniature-dwarf "Micro-Tom" was used to produce transgenic plants.

Cloning cDNA fragments of V-ATPase A subunit

A single-stranded cDNA was synthesized by using an RNA PCR kit (Takara Shuzo Co., Ltd. Shiga, Japan) with 1 µg of total RNA from tomato fruit and oligo (dT) primer. cDNA fragments of the V-ATPase A subunit were amplified by PCR with two gene-specific primer sets: 1 (forward: VHA-F1, 5′-CCTGTTGCATCAAA(A/G)CTTGC-3′; reverse: VHA-R2, 5′-AGGGTTGCAGATGTTACAGG-3′) and 2 (forward: VHA-F2, 5′-GCAGACAGTGGATATCCTGC-3′; reverse: VHA-R1, 5′-AGAAGTGGATGATGTTGCGC-3′). Amplified DNA fragments (635 bp: *LeVHA-AP1* and 535 bp: *LeVHA-AP2*) were ligated into a pT7Blue plasmid vector (Novagen, Darmstadt, Germany) and were sequenced. *LeVHA-AP1* and *LeVHA-AP2* showed 99% identity with the full-length V-ATPase A subunit cDNA of tomato (accession number: AY177247) and corresponded to the 726–1,360 bp or 1,191–1,725 bp region of the clone, respectively. *LeVHA-AP1* and *LeVHA-AP2* overlapped by 171 bp, and the sequences of this region were identical, suggesting that *LeVHA-AP1* and *LeVHA-AP2* are from the same gene.

Northern blot analysis

Total RNA was extracted by using the phenol sodium dodecyl sulfate (SDS) method (Nakajima et al. 1988) and then by using the cetyltrimethylammonium bromide method (Murray and Thompson 1980). Total RNA (15 µg) was separated on 1% (w/v) agarose gel, denatured with formaldehyde and was blotted onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech Co.). *LeVHA-AP1* was digoxigenin (DIG)-labeled by using a DIG DNA labeling kit (Roche Diagnostics Co., Mannheim, Germany) and was used as a probe. The membranes were incubated with a blocking reagent (Roche) at 68°C for 1 h and were hybridized with the probe at 68°C overnight. Then the membranes were washed twice in 2×SSC containing 0.1% (w/v) SDS at room temperature for 5 min and were washed twice in 0.1×SSC containing 0.1% (w/v) SDS at 68°C for 15 min. The DIG-labeled probes in the membranes were detected by using an anti-DIG antibody (Roche) and the chemiluminescent substrate CDP-Star (Roche).

Plant transformation

The fruit-specific *2A11* promoter was used as described by Chengappa et al. (1999). The 35S promoter in the binary plasmid vector pBI121 (Jefferson et al. 1987) was removed by *Hin*dIII and *Xba*I, and a 4 kb region of the *2A11* promoter was ligated into the vector (p2AGUS). The *β*-glucuronidase (GUS) gene was removed from p2AGUS by using *Xba*I and *Sac*I, and the A subunit cDNA fragment (*LeVHA-AP1*) was ligated into the vector in an antisense orientation (p2AVAA). Binary vectors p2AGUS and p2AVAA were introduced into *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen, Carlsbad, CA, USA).

Plant transformation was done by using a modified method of McCormick et al. (1986). *A. tumefaciens* was cultured in MG/L medium containing 250 mg/l streptomycin, 50 mg/l kanamycin and 50 µM acetosyringone for 48 h. Cotyledons from "Micro-Tom" were co-cultured with *A. tumefaciens* on solid MS medium containing 50 µM acetosyringone for 2 days. Adventitious buds resistant to kanamycin were selected by culturing on MS medium containing 50 mg/l kanamycin, 100 mg/l cefotaxime, 2 mg/l zeatin and 400 mg/l carbenicillin. Regenerated shoots were rooted on MS medium containing 2 mg/l indolebutyric acid and 100 mg/l carbenicillin. Rooted plants were transferred to soil.

Semi-quantitative reverse transcriptase (RT)-PCR Southern blot analysis

Total RNA was extracted from the nontransformant and transformants. A single-stranded cDNA was synthesized by using an RNA PCR kit (Takara) with 1 μg of total RNA and oligo (dT) primer. For Southern blot analysis, the amount of template cDNA and the number of PCR cycles were determined in preliminary experiments to ensure that the amplification was linear. The cDNA (1/20 of the reaction mixture) was amplified semi-quantitatively by using PCR. The primers and optimal cycle numbers for the PCR of the V-ATPase A subunit were forward 5′-TGTTCAGGTCTTCTGGGGTC-3′ and reverse VHA-R1 with 11 cycles for fruit and leaves, and for V-PPase were forward 5′-CATTCCAGAAG ATGATCCCAGAAATCCTGC-3′ and reverse 5′-AG-ATCCAATGGCAAAGCCCTTTCCAATCGC-3′ with 12 cycles for fruit and 16 cycles for leaves. A control gene was 18S rRNA; its primers and optimal cycle numbers for the PCR were forward 18S-F, 5′-TG-TGAAACTGCGAATGGCTCATTAAATCAG-3′ and reverse 18S-R, 5′-TGGTCGGCATCGTTTATGGTT-GAGACTAG-3′ with 8 cycles for fruit and 6 cycles for leaves. The resulting PCR products were segregated on 1% (w/v) agarose gel, were blotted onto nylon membrane (Hybond N+) and were probed with DIG-labeled DNA that was amplified by using the same primer sets as for RT-PCR. Hybridization, membrane washing and detection were done by the same method as for Northern blot analysis.

Measurement of sugar content

Soluble sugar was extracted from fruits at the red stage by grinding in 80% (v/v) ethanol and was heating at 80°C for 30 min. The homogenate was centrifuged at 10,000 *g* for 5 min. The pellet was suspended in 80% (v/v) ethanol and was re-extracted twice more. All supernatants were combined, dried and suspended in water. The suspension was passed through a Sep-Pak cartridge (Waters Co., Milford, MA, USA) and a membrane filter (pore size 0.45 μm, Advantec, Tokyo, Japan). The sample was analyzed by using a high-pressure liquid chromatography system (655A-11LC, Hitachi, Tokyo, Japan) with a Shodex Sugar SP0810 column (Showa Denko Co., Tokyo, Japan) and the sugar content was measured.

Results

Northern blot analysis

The mRNA level of the V-ATPase A subunit during fruit development of tomato was relatively high during the early developmental stage, decreased until the immature green stage and then increased again in the red stage (Fig. 1).

Plant transformation

Four independent plants were regenerated from p2AGUS-transformed cotyledons (GUS control line), and 15 plants were regenerated from p2AVAA-transformed cotyledons (A subunit antisense-transgenic line). Confirmation of the gene introduction by PCR amplification using primers for the NOS terminator region detected a suitable band in one of the four GUS control plants (MP-1) and in 7 of the 15 antisense-transgenic plants (MA-1, MA-2, MA-4, MA-5, MA-8, MA-11 and MA-14) (data not shown). PCR amplification with the primers of the region that included the V-ATPase A subunit sequence and NOS terminator detected a suitable band in the seven antisense-transgenic plants (data not shown). As a result, one GUS control plant and seven antisense-transgenic plants were obtained and were used for further study.

Gene expression of V-ATPase A subunit and V-PPase in transformants

Semi-quantitative RT-PCR Southern blot analysis showed that the amount of V-ATPase A subunit mRNA

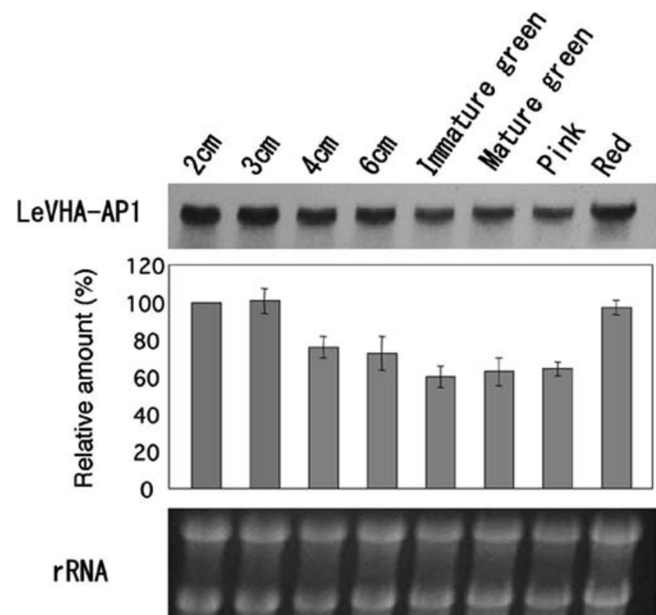

Fig. 1 Changes in amount of V-ATPase A subunit mRNA during fruit development. Total RNA (15 μg) isolated from fruits of 2, 3, 4, 6 cm, immature green, mature green, pink and red stages underwent Northern blot analysis with DIG-labeled *LeVHA-AP* (*top panel*). The strength of the bands in Northern blot analysis was measured by densitometric scan, and the relative amount compared with the 2 cm stage was calculated (*middle panel*). Ethidium bromide-stained RNA was used as a standard control (*bottom panel*). *Vertical bars* are the standard error ($n=3$)

in fruits from the antisense-transgenic plants decreased compared with the GUS control plant and the nontransformant (Fig. 2). The mRNA level of V-PPase hardly changed in most antisense-transgenic plants, but it was lower in MA-4 and MA-5, which also accumulated the lowest A subunit mRNA.

To confirm that the amount of A subunit mRNA decreases only in fruit, mRNA expression of the A subunit and V-PPase in leaves was also investigated. mRNA accumulation of the A subunit and V-PPase differed little between antisense-transgenic plants, GUS control plant and the nontransformant (Fig. 3), indicating that A subunit suppression with the *2A11*-promoter was restricted to fruit.

Morphological characteristics of transformants

Morphological changes due to A subunit suppression were limited to fruit. Fruits from A subunit-suppressed plants were smaller than those of the nontransformant and the GUS control plant. The fruit weight did not differ between the GUS control plant and the nontransformant, but the fruit weight of the A subunit-suppressed plants decreased to 49–79% (Fig. 4a, b). MA-4 and MA-5 lines, which had the lowest A subunit mRNA expression, had the lowest fruit weights. Surprisingly, fruits from the A subunit-suppressed plants had few seeds (Fig. 4a, c), except MA-8 that still had relatively high A subunit mRNA expression (Fig. 2). The decreased fruit weight and seed number seem to correlate with decreasing V-ATPase mRNA level.

Sugar content in transformants

To clarify the importance of V-ATPase in sugar accumulation, the amount of sugar in transgenic tomato fruits was measured (Fig. 5). The total sugar content in each fruit (mg/fruit) of MA11 and MA14 was almost the same as for the nontransformant, but that of other transformants was lower than for the nontransformant (Fig. 5a). The amount of glucose and fructose in each fruit was lower in all transformants compared with the GUS control plant and the nontransformant. However, the amount of sucrose in each fruit from transformants, except MA8, was higher than for the GUS control plant and the nontransformant. Glucose and fructose concentrations showed no marked difference between fruits from the control plants and from the A subunit-suppressed

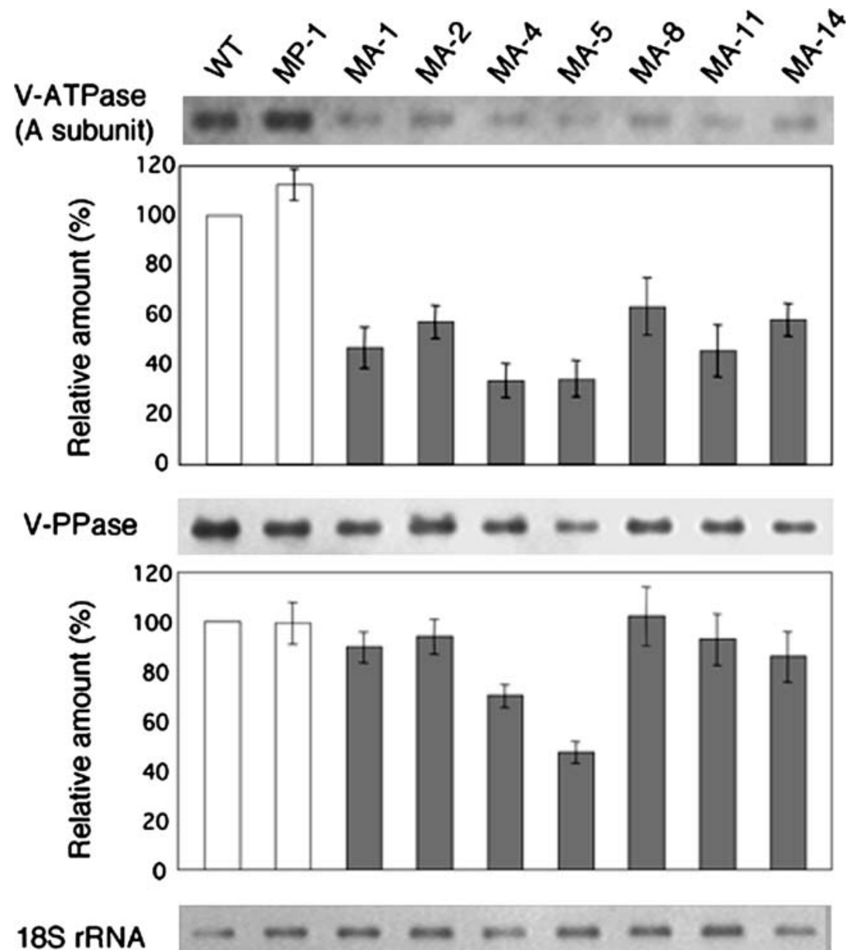

Fig. 2 mRNA amount of V-ATPase A subunit and V-PPase in fruits from transformants. Total RNA isolated from fruits from nontransformant (*WT*) and transformants were used for RT-PCR Southern blot analysis. The strength of the bands in RT-PCR Southern blot analysis was measured by densitometric scan, and the relative amounts compared with WT were calculated. 18S rRNA was used as a control (*bottom panel*). *Vertical bars* are the standard error ($n = 3$)

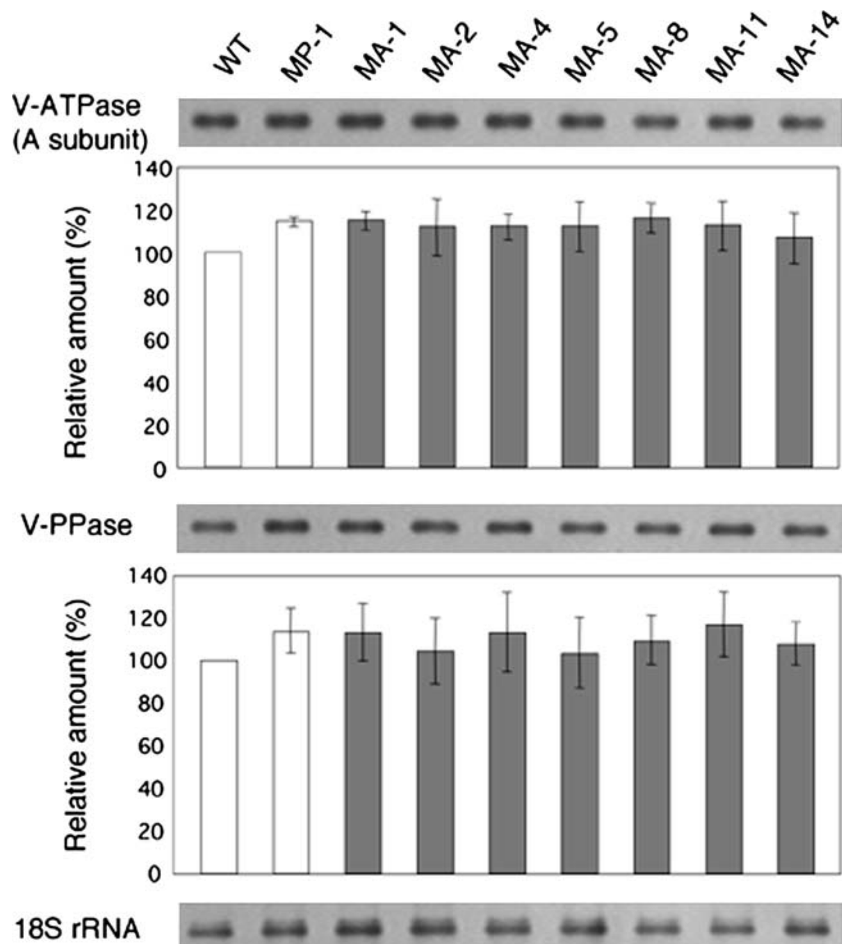

Fig. 3 mRNA amount of V-ATPase A subunit and V-PPase in leaves of transformants. Total RNA isolated from leaves of nontransformant (*WT*) and transformants were used for RT-PCR Southern blot analysis. The strength of the bands in RT-PCR Southern blot analysis was measured by densitometric scan, and the relative amount compared with WT was calculated. 18S rRNA was used as a control (*bottom panel*). *Vertical bars* are the standard error ($n = 3$)

plants (Fig. 5b, c). In contrast, the sucrose concentration of fruits from the A subunit-suppressed plant, except MA-8, was higher than for the GUS control plant and the nontransformant (Fig. 5d). Fruits from MA-4, MA-11 and MA-14 accumulated sucrose 5.6–8.1 times as the sucrose of fruits from the nontransformant. MA-8, which had a relatively high A subunit mRNA expression compared with other transformants, accumulated a similar amount of sucrose as the control lines.

Discussion

Gene transformation is a useful technique to analyze a specific gene function, and many genes have been suppressed or overexpressed in different plant species by various methods. We believe V-ATPase has been used in only one reported experiment (Gogarten et al. 1992) that transformed the antisense gene for the V-ATPase A subunit under the control of the cauliflower mosaic virus 35S promoter to carrot. This transformant had dissected leaves and a shorter taproot. However, the cauliflower mosaic virus 35S promoter promotes ubiquitous gene expression. Therefore, knowing if V-ATPase suppression in the taproot itself or poor growth of the photosynthesis organ causes the shorter taproot is difficult. To avoid this question, the use of a promoter that can specifically control the timing and the tissue of gene expression are needed. Therefore, in this study, we used a promoter region of the fruit-specific gene *2A11* to suppress the V-ATPase A subunit gene.

The *2A11* promoter was isolated from tomato where it is expressed in the fruit, but not in the root, stem and leaf (Pear et al. 1989). Although the function of the 2A11 protein is unidentified, a 4.0 kb 5′ region of this protein promoter confers a high fruit-specific expression (Van Haaren and Houck 1991, 1993). The *2A11* promoter has been used in plant transformation for fruit-specific expression of several genes, such as sucrose synthase, lipoxygenase and isopentenyl transferase (Martineau et al. 1994; Chengappa et al. 1999; Griffiths et al. 1999). In this study, we used about 4 kb of the *2A11* promoter region to express the antisense fragment of the V-ATPase A subunit specifically in fruit.

In this study, we used the miniature-dwarf tomato "Micro-Tom", bred originally for home gardening (Scott and Harbaugh 1989), as plant material for transformation. It can grow at high density (up to 1,357 plants/m^2) and has a short life cycle (70–90 days from sowing to fruit ripening). Gene transformation

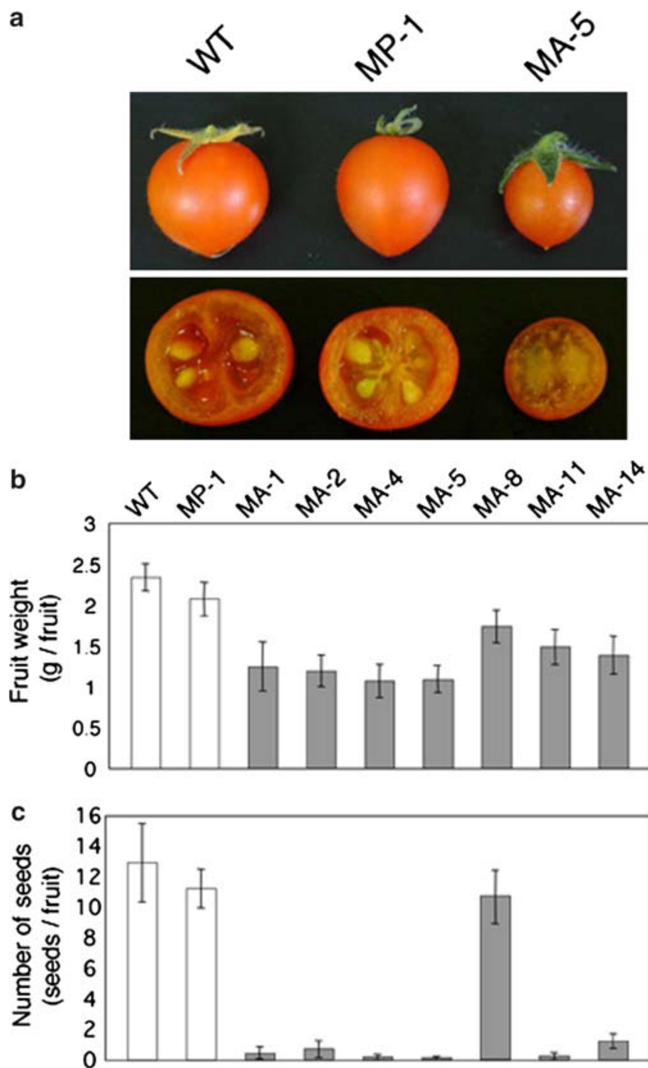

Fig. 4 Fruit morphology in transformants. **a** Fruits from a nontransformant (*WT*), a GUS control plant (*MP-1*) and a A subunit-suppressed plant (*MA-5*). **b** Fruit weight of nontransformant and transformants. **c** Number of seeds in nontransformant and transformants. Average weight of fruit and number of seeds of each fruit of each tomato plant with standard error ($n = 9–12$)

mediated by *A. tumefaciens* is applicable to "Micro-Tom" (Meissner et al. 1997). Therefore, "Micro-Tom" has been recently used as a model plant for research on tomato, solanaceous and fruit-setting plants.

We transformed GUS control vector p2AGUS or a p2AVAA vector, which contains the *2A11* promoter and antisense gene for V-ATPase A subunit mediated by *A. tumefaciens*, in "Micro-Tom", and one GUS control and seven A subunit antisense-transgenic plants were produced. The amount of V-ATPase A subunit mRNA in fruit decreased in all antisense-transgenic plants (Fig. 2), but in leaves it was almost the same as in the GUS control plant and the nontransformant (Fig. 3), suggesting that suppression of the V-ATPase A subunit with the *2A11* promoter is limited in fruit. Therefore, these antisense-transgenic plants are useful to examine the role of V-ATPase in fruit. The amount of mRNA of V-PPase changed little in most antisense-transgenic plants. However, it was much lower in MA-4 and MA-5 that had the lowest A subunit mRNA accumulation (Fig. 2), which might relate to the deterioration of vacuolar development caused by V-ATPase suppression, but the details are unclear.

A subunit-suppressed plants had smaller fruits with few seeds compared with the GUS control plant and the nontransformant (Fig. 4a, b). Fruits from the A subunit-suppressed lines, except MA-8, had few seeds (Fig. 4a, c). MA-4 and MA-5, which showed the lowest expression of A subunit mRNA (Fig. 2), produced the smallest fruit with the lowest number of seeds (Fig. 4). However, MA-8, which had relatively high expression of A subunit mRNA (Fig. 2), showed less reduction in fruit weight and seed number than the other A subunit-suppressed plants (Fig. 4). These results suggested that fruit growth and seed formation correlate with the V-ATPase expression level.

Vacuolar H^+-ATPase expression is relatively high throughout the growth of many fruits, such as *Citrus unshiu* (Takanokura et al. 1998), Japanese pear (Suzuki et al. 2000), peach (Etienne et al. 2002) and tomato (Fig. 1; Milner and Smith 1995; Coker et al. 2003), although it fluctuates during fruit development, suggesting that V-ATPase has important roles throughout fruit development. V-ATPase is localized not only in the vacuolar membrane but also in other endomembranes, such as the endoplasmic reticulum (ER) and golgi membranes (Ratajczak 2000). Because we used a conserved region of the A subunit gene for antisense-transformation, V-ATPase in all organelles might have been suppressed in the antisense-transgenic fruits. In fruit flesh (parenchyma) cells, the most conspicuous organelle is the vacuole that occupies most of the cell volume. Therefore, suppression of the A subunit gene in fruit flesh cells may have affected mostly the vacuolar function that might have led to smaller fruits (Fig. 4a, b). However, seed cells contain a highly developed ER, golgi body and a vacuole, and so deterioration of V-ATPase in these organelles might affect seed development (Fig. 4a, c).

Phytohormones, some of which are synthesized in seeds, are related to growth of tomato fruit (Gillaspy et al. 1993). In this study, smaller fruits of A subunit-suppressed plants were also thought to be due to fewer seeds, that is, a smaller supply of phytohormones from seeds to fruit flesh. Conversely, diminished growth of fruit flesh, including the tissue surrounding seeds, due to A subunit suppression produced fewer seeds, that is, a smaller nutrient supply from fruit flesh to seeds. To understand the relationship between smaller fruit and fewer seeds, we need to determine if V-ATPase suppression with the *2A11* promoter is only in flesh tissue or also in seeds.

Vacuolar H^+-ATPase generates a proton electrochemical gradient across the vacuolar membrane; this gradient acts as a driving force for transport of several

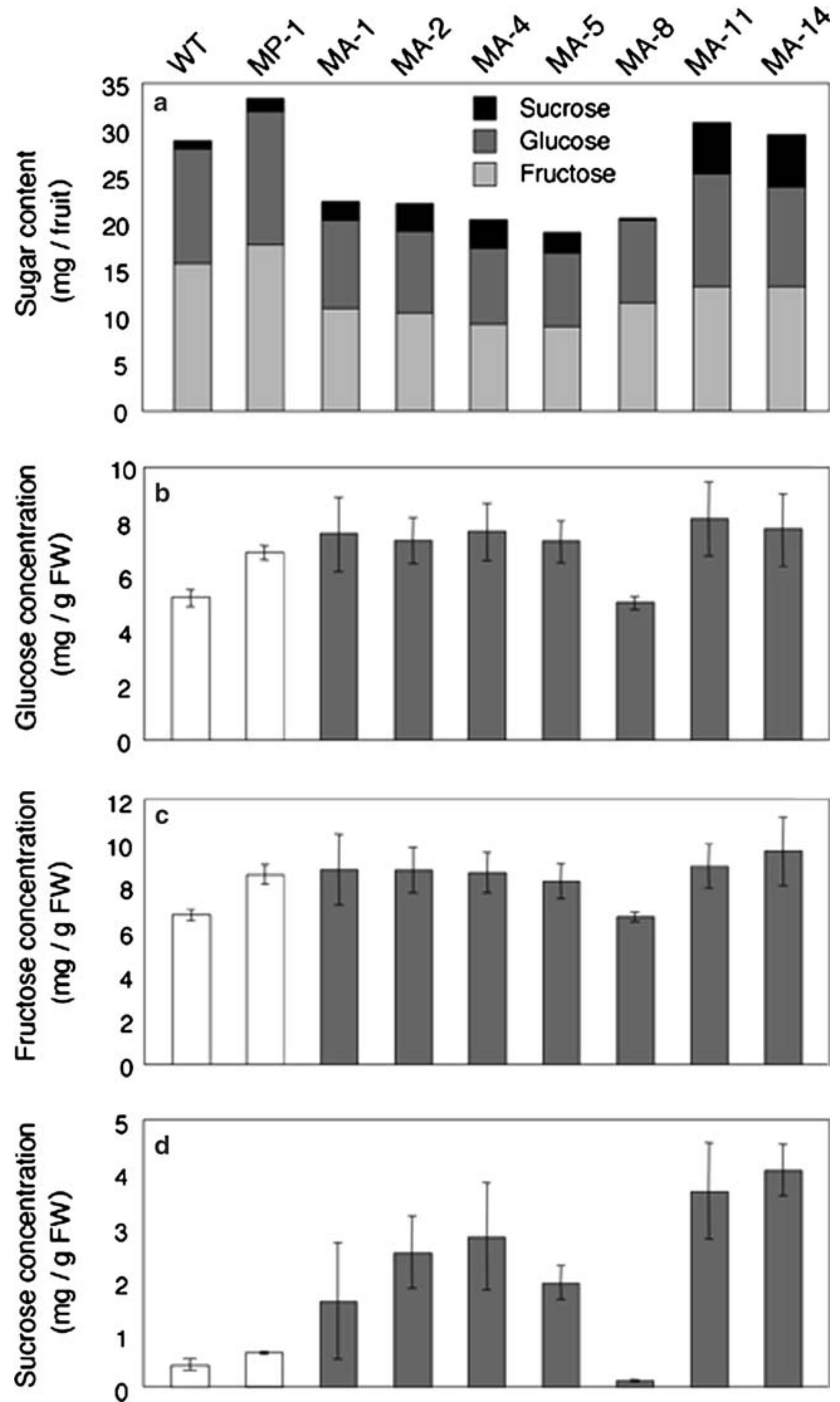

Fig. 5 Sugar content in fruits from transformants. Sugar was extracted from fruits at the red stage and was measured. **a** Sugar content of each fruit (mg/fruit). **b** Glucose, **c** fructose and **d** sucrose concentration in fruits (mg/g FW). *Vertical bars* are the standard error of three replicates based on independent extractions

solutes. H^+/sugar antiport might not participate in sugar uptake into the vacuole of tomato fruit (Milner and Smith 1995). However, whether sugar uptake into and accumulation in fruit vacuoles depends on H^+/sugar antiport or other transport systems, including facilitated transport (Shiratake et al. 1997b) and endocytosis (Etxeberria et al. 2005), is arguable. In this study, although the total amount of sugar in each fruit, in transformants MA11 and MA14, was almost the same as in the nontransformant; the total amount of sugar in the other transformants decreased (Fig. 5a), suggesting that V-ATPase relates to sink strength in tomato fruit. However, the decrease in the total amount of sugar in each fruit mostly depended on decrease in fruit size (Fig. 4b), not on sugar concentration in the fruits. Concentrations of glucose and fructose, the most abundant sugars in tomato fruits, were hardly affected by V-ATPase suppression (Fig. 5b, c). Thus, V-ATPase might not be important for sugar concentration in tomato fruit. However, we could not conclude from our

data that sugar transport into tomato fruit vacuoles is independent of H^+ pumps, because V-PPase still acts like a vacuolar proton pump in A subunit-suppressed fruits.

Although the amount of sucrose is smaller than hexose in the tomato fruits (Fig. 5a), interestingly, sucrose concentration in the A subunit-suppressed fruits was much higher than in fruits from the GUS control plant and the nontransformant (Fig. 5d). Sucrose unloaded in tomato fruit is converted to hexose or its derivatives (Davies and Kempton 1975) and the key enzymes for this reaction are the invertase isoforms. Especially, the activity of vacuolar invertase (soluble acid invertase) is highest in tomato fruit and is correlated to the hexose–sucrose ratio (Nguyen-Quoc and Foyer 2001). V-ATPase is necessary not only for vacuolar acidification, but also for sorting soluble vacuolar proteins (Matsuoka et al. 1997). An acidic condition of the vacuole and the sorting of soluble vacuolar proteins are important for high activity and supply of vacuolar invertase, respectively. V-ATPase suppression might decrease vacuolar invertase activity, which results in an increase in sucrose concentration.

In this study, we showed the importance of V-ATPase in fruit enlargement and in seed formation by fruit-specific suppression of the V-ATPase A subunit in tomato. Many questions exist about seed formation (Gillaspy et al. 1993) and little information exists about V-ATPase roles in seed development, such as the importance of C and E subunits of V-ATPase for embryogenesis (Schumacher et al. 1999; Strompen et al. 2005). This study showed a higher concentration of sucrose in the A subunit-suppressed fruits. Our results suggest unexpected functions of V-ATPase in fruit development and further investigation of V-ATPase-suppressed tomato plants will clarify such V-ATPase functions.

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