Journal of Plant Physiology **161** (2004) 1177–1184

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JOURNAL OF PLANT PHYSIOLOGY

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Engineered sorbitol accumulation induces dwarfism in Japanese persimmon

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Received 18 February 2004; accepted 27 April 2004

KEYWORDS
Dwarfism;
Japanese persimmon;
Sorbitol;
Sorbitol-6-phosphate dehydrogenase

Summary

A cDNA encoding sorbitol-6-phosphate dehydrogenase (S6PDH), which is a key enzyme in sorbitol biosynthesis in Rosaceae, was introduced into the Japanese persimmon (*Diospyros kaki*) to increase the environmental stress tolerance. Resultant transformants exhibited salt-tolerance with dwarfing phenotypes. Therefore, we studied two transgenic lines to understand the physiological mechanism of this dwarfism: lines PS1 and PS6 accumulated high and moderate levels of sorbitol, respectively. The average length of shoots was significantly shorter as compared with the wild-type in line PS1, while no such decrease was observed in line PS6. The *myo*-inositol and glucose 6-phosphate (G6P) contents were measured in the transgenic lines because previous work with tobacco transformed with *S6PDH* had suggested that growth inhibition was due to depletion of these metabolites. Although the *myo*-inositol content was decreased in PS1 plants, the decrease was much smaller than that observed in transgenic tobacco that accumulates sorbitol. The G6P contents were the same in PS1 plants and phenotypically normal PS6 plants. The level of indole-3-acetic acid (IAA), which affects stem elongation, in line PS1 was similar to the levels in the other lines. A decrease in gibberellin (GA) content generally induces dwarfism in plants. However, GA was not decreased in PS1 plants compared with wild-type or control plants. Therefore, we focused on sorbitol accumulation as the most remarkable feature of

Abbreviations: ABA, abscisic acid; F6P, fructose 6-phosphate; GA, gibberellin; G6P, Glucose 6-phosphate; IAA, indole-3-acetic acid; S6PDH, sorbitol-6-phosphate dehydrogenase

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0176-1617/$ - see front matter
doi:10.1016/j.jplph.2004.04.009

PS1 plants. As one possibility, the observed growth inhibition was likely caused by an osmotic imbalance between the cytosol and vacuole.

Introduction

Sorbitol plays a key role in the translocation of carbohydrate in some important fruit trees in the Rosaceae family. Sorbitol-6-phosphate dehydrogenase (S6PDH), a key enzyme in the sorbitol biosynthesis pathway, was purified and its cDNA isolated (Kanayama et al., 1992; Kanayama & Yamaki, 1993; Sakanishi et al., 1998). As sorbitol can function as a compatible solute, *S6PDH* cDNA has been introduced into plants to increase their tolerance to environmental stress. In tobacco transformed with *S6PDH*, a low sorbitol concentration increases tolerance to boron deficiency by facilitating boron mobility (Brown et al., 1999). In contrast, a high sorbitol concentration induces stunted growth and necrosis in transgenic tobacco (Sheveleva et al., 1998).

Gao et al. (2001) introduced *S6PDH* cDNA into Japanese persimmon. The transgenic Japanese persimmon producing high levels of sorbitol did not develop necrotic lesions, but showed increased salt tolerance. Interestingly, they also reported that engineered sorbitol biosynthesis induced dwarfism, which is an important phenotype for fruit production. As it is very difficult to produce transgenic fruit trees, the production of a stress-tolerant, dwarf Japanese persimmon is a rare successful case of a transgenic fruit tree.

Engineered sorbitol biosynthesis induced dwarfism, stunted growth, and necrosis, as described above. Growth inhibition was also observed in tobacco with genetic-transformation induced enhanced mannitol-biosynthesis (Karakas et al., 1997). Therefore, it is necessary to study the effect of polyol accumulation on the growth of transgenic plants when genetic engineering of polyol production is used to increase stress tolerance. We studied two transgenic lines of Japanese persimmon to understand the physiological mechanism of the dwarfism: one line accumulated high levels of sorbitol, while the other showed accumulation of moderate amounts.

Materials and methods

Plant materials

Japanese persimmon were transformed with apple *S6PDH* cDNA under control of the cauliflower mosaic virus *35S* promoter and several shoots each of individual lines were transplanted to soil after rooting, as described previously (Gao et al., 2001). Shoots and roots were harvested from three plants from each line in mid-June and late-August four years after transplanting and stored at $-80^{\circ}C$ until analysis. Lines PS1 and PS6 accumulated high and moderate amounts of sorbitol, respectively. Control plants were obtained from a transgenic line that lacked S6PDH activity. This control line is previously described as a line PS7 (Gao et al., 2001). In addition, non-transformed wild-type plants (cv. Jiro) were also used for analysis four years after transplanting from in vitro culture, as with the transgenic lines. These lines and wild-type plants were produced and described previously (Gao et al., 2001).

Extraction and analysis of soluble carbohydrate and hexose phosphate

Soluble carbohydrate was extracted and analyzed as described previously (Gao et al., 2001). Glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P) were extracted from frozen tissues according to the method of Tobias et al. (1992). Pigments were removed using Sep-Pak C18 Cartridges (Waters) or activated carbon. The G6P and F6P contents were determined according to the method of Tobias et al. (1992), except the reaction buffer was the same as that used to measure fructokinase and hexokinase activity described by Martinez-Barajas and Randall (1996).

Plant hormone analyses

Endogenous GA, IAA, and abscisic acid (ABA) in leaves was extracted and quantified according to the method of Koshita et al. (1999). Briefly, samples of 10 g of leaves were homogenized, extracted in 80% (v/v) acetone containing 100 mg L^{-1} butylhydroxytoluene (BHT), and concentrated to give aqueous solutions, which were subjected to solvent partitioning to obtain fractions soluble in acidic ethyl acetate. The fractions were further purified by HPLC. The HPLC column was a PEGASIL ODS (6 mm i.d. $\times$ 150 mm; Senshu Scientific Co. Ltd.). [$^{13}C_6$]IAA and d6ABA were used as internal standards for the IAA and ABA analyses, respectively.

IAA and ABA were quantified by gas chromatography–mass spectrometry (QP5000; Shimadzu Co., Ltd.). The $GA_{1/3}$ and $GA_{4/7}$ contents were quantified using an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies against GA_4 prepared by Nakajima et al. (1991). $GA_{1/3}$ and $GA_{4/7}$ were identified tentatively based on the HPLC retention time.

The cytokinins, trans-zeatin (Z), trans-zeatinriboside ([9R]Z), isopentenyladenine (iP), and isopentenyladenosine ([9R]iP), were extracted and quantified by ELISA according to the method of Ito et al. (1999). Briefly, samples of 10 g of leaves were homogenized, extracted in 80% acetone containing 100 mg L^{-1} BHT, and concentrated to give aqueous solutions. Then, they were subjected to solvent partitioning to obtain fractions soluble in *n*-butyl alcohol. The fractions were dried in vacuo and purified by HPLC, as described above. Z and [9R]Z were analyzed using a monoclonal antibody against [9R]Z, and iP and [9R]iP were analyzed using a monoclonal antibody against [9R]iP.

The antibodies were purchased from Ide Tech and the tracers were synthesized according to the method described by Eberle et al. (1986).

Results

Plant growth

The numbers of shoots in line PS1, which accumulated large amounts of sorbitol (Table 1), were approximately twice those in the control and wild-type plants (Fig. 1). The length of the shoots in line PS1 was less than half of those in the control and wild-type plants. In contrast, the number and length of shoots in line PS6, which accumulated moderate amounts of sorbitol, were similar to those in the control and wild-type plants.

Soluble carbohydrate content

Sorbitol was detected in lines PS1 and PS6 in June and August (Tables 1, 2). The sorbitol concentration in line PS1 was higher than that in PS6. Especially in June, when shoots grew better, the stems and leaves of PS1 plants showed accumulation of more than 40 μmol g^{-1} FW of sorbitol. The sorbitol content in the stems reached 56% of the total soluble carbohydrate. The sorbitol contents were very low in the roots of PS1 and PS6 plants.

The fructose contents in the leaves of PS1 and PS 6 plants were lower than those in wild-type and control plants in June. However, decreases in lines PS1 and PS6 were not observed in August. Although the fructose and glucose contents in the stems and roots of PS1 plants were lower than those in other lines in August, this was not the case in June.

The *myo*-inositol contents in the leaves and stems of PS1 and PS6 plants were lower than those in wild-type and control plants in June. In August, the *myo*-inositol content was lower only in the stems of PS1 plants.

The sucrose content in the leaves of PS1 plants was higher than those in wild-type and control plants in June, while in August PS1 plants showed levels similar to those of wild-type and PS6 plants.

The total soluble carbohydrate contents in the leaves and stems of PS1 plants were higher than

Table 1. Soluble carbohydrate content in the leaves and stems of current shoots and roots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon in June

		Soluble carbohydrate content (μmol g^{-1}FW)						
Line	Organ	Fructose	Glucose	*myo*-Inositol	Mannitol	Sorbitol	Sucrose	Total
WT	Leaf	12.0±1.2	9.0±0.9	16.3±2.5	—	—	27.2±3.4	64.5±4.5
PS1	Leaf	4.2±0.7	3.6±0.4	13.2±1.8	2.6±0.2	40.5±4.8	43.3±5.5	107.4±5.4
PS6	Leaf	2.9±0.3	3.2±0.3	11.4±0.8	1.8±0.1	27.5±2.7	29.0±3.2	75.8±3.0
Control	Leaf	8.3±2.4	4.8±1.0	19.1±0.8	—	—	28.9±0.6	61.1±3.2
WT	Stem	10.8±3.1	9.8±2.5	4.2±0.8	—	—	7.8±1.6	32.7±6.8
PS1	Stem	14.8±1.7	11.9±2.4	1.8±0.3	—	49.3±2.3	10.9±3.4	88.7±8.7
PS6	Stem	19.8±4.7	12.4±3.2	2.6±0.3	0.4±0.2	21.9±0.9	10.7±3.4	67.9±7.7
Control	Stem	12.4±2.2	10.4±1.2	6.4±0.8	—	—	14.9±0.6	44.1±3.5
WT	Root	4.7±1.0	5.7±1.7	0.9±0.3	—	—	13.4±4.0	24.7±4.8
PS1	Root	5.3±2.3	3.5±0.8	1.2±0.4	—	0.8±0.1	11.9±3.8	22.7±4.4
PS6	Root	3.7±0.7	4.6±0.5	1.0±0.3	—	0.4±0.1	7.9±1.9	17.7±2.4
Control	Root	4.6±0.4	3.0±0.1	0.9±0.3	—	—	13.2±3.4	21.7±4.1

Means±SE values were calculated from three plants in each line.—, not detected.

those in the other lines in June, when the shoots grew better. The total soluble carbohydrate contents in the roots were similar in all lines in June, although the contents in the roots of PS1 and PS6 plants were lower than those in wild-type and control plants in August.

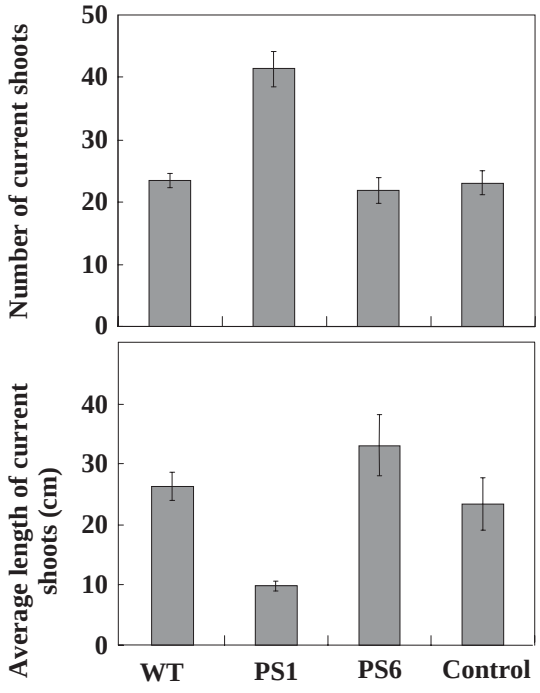

Figure 1. The number and average length of shoots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon. The values were measured after the end of the shoot growth. Bars represent±SE calculated from three plants in each line.

Mannitol was detected in the leaves of PS1 and PS6 plants. Mannitol can be synthesized in the transgenic lines because S6PDH has weak mannose-6-phosphate reductase activity (Negm and Loescher, 1981).

G6P and F6P content

The G6P contents in the leaves were similar in lines PS1 and PS6 (Fig. 2). The G6P content was decreased in the stems of PS1 and PS6 plants in June, although the G6P content in line PS1 was increased to the levels of wild-type and control plants in August (Fig. 3). The G6P contents in the roots were similar in lines PS1 and PS6 (Fig. 4).

The F6P content was not decreased in any of the organs of PS1 plants, as compared with PS6 plants.

Plant hormone contents

The levels of IAA and ABA in line PS1 were similar to those in the other lines (Figs. 5A and B). It was thought that the GA content might be decreased in line PS1 because a decrease in GA induces dwarfism. However, GA was not decreased in line PS1 as compared with the wild-type and control lines (Figs. 5C and D). Z was the major cytokinin in wild-type plants (Fig. 5E). The Z content was lowest in line PS1, although it was also decreased in PS6 plants as compared with wild-type and control plants.

Table 2. Soluble carbohydrate content in the leaves and stems of current shoots and roots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon in August

		Soluble carbohydrate content (μmol g^{-1}FW)						
Line	Organ	Fructose	Glucose	*myo*-Inositol	Mannitol	Sorbitol	Sucrose	Total
WT	Leaf	4.1±1.1	3.2±1.2	10.9±2.2	—	—	32.6±8.5	50.7±12.5
PS1	Leaf	7.9±2.7	3.6±0.8	15.5±4.5	2.9±0.2	17.3±2.5	28.3±0.8	75.6±8.4
PS6	Leaf	5.4±1.7	4.9±1.1	14.4±1.8	1.8±0.9	5.1±2.3	29.3±2.6	60.8±7.3
Control	Leaf	7.7±0.7	5.8±0.7	14.3±0.5	—		41.8±0.8	69.6±1.4
WT	Stem	15.0±2.6	17.1±3.2	7.1±0.6	—	—	20.1±0.7	59.3±6.3
PS1	Stem	8.3±0.9	10.4±0.9	2.1±0.2	—	9.3±3.4	28.0±2.6	58.1±7.0
PS6	Stem	21.1±5.9	20.7±4.1	9.0±1.4	—	1.7±0.6	32.8±9.3	85.3±11.5
Control	Stem	15.4±3.9	18.5±3.5	8.8±0.8	—	—	30.5±2.0	73.3±8.3
WT	Root	5.4±1.1	4.5±0.8	3.6±0.2	—	—	63.9±9.6	77.3±11.1
PS1	Root	2.0±0.3	2.5±0.5	1.5±0.3	—	0.3±0.0	27.2±2.1	33.6±1.3
PS6	Root	4.0±1.1	3.7±0.7	1.9±0.2	—	0.1±0.0	31.0±1.3	40.1±3.1
Control	Root	4.1±0.9	3.5±0.4	1.5±0.1	—	—	60.2±8.5	69.3±8.6

Means±SE values were calculated from three plants in each line. —, not detected.

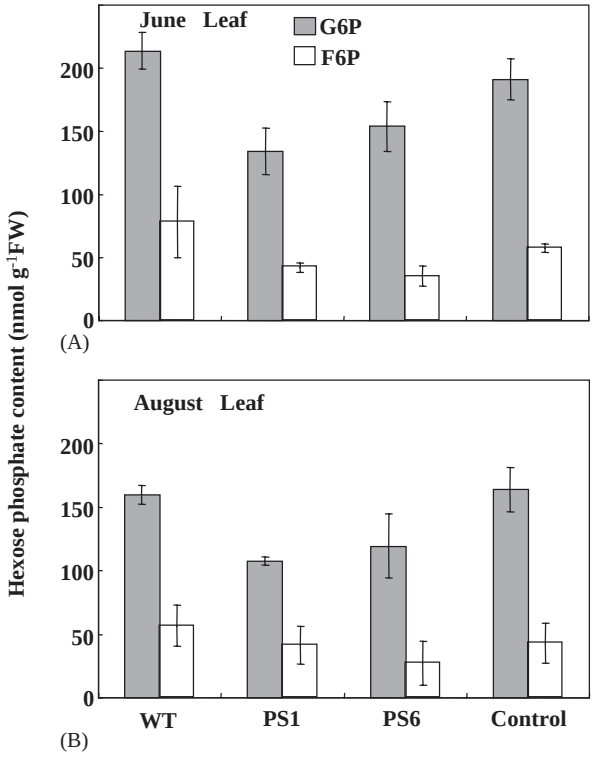

Figure 2. Hexose phosphate contents in the leaves of shoots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon. Hexose phosphate content was determined in June (A) and August (B). Gray and white columns indicate G6P and F6P contents, respectively. Bars represent±SE calculated from three plants in each line.

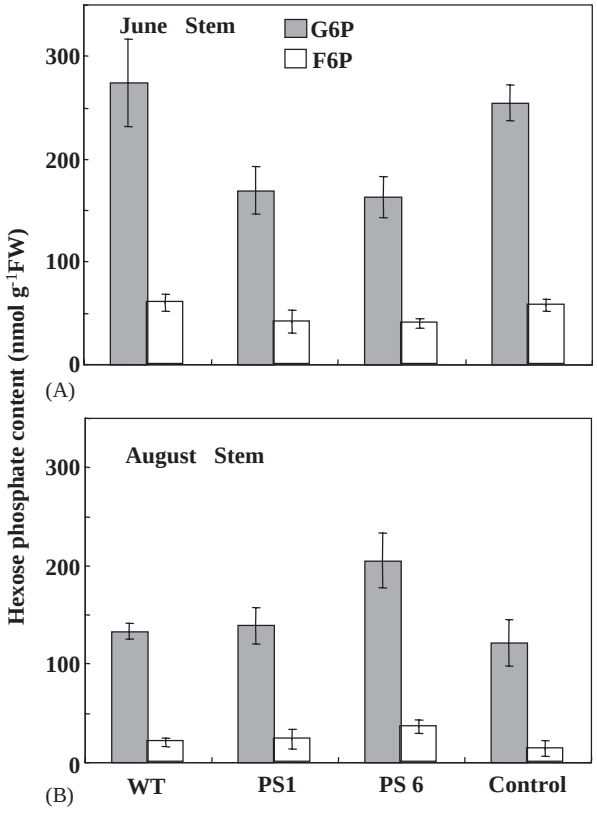

Figure 3. Hexose phosphate contents in the stems of shoots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon. Hexose phosphate content was determined in June (A) and August (B). Gray and white columns indicate G6P and F6P contents, respectively. Bars represent±SE calculated from three plants in each line.

Discussion

PS1 plants showed dwarfism, as described previously (Gao et al., 2001). Some other transgenic lines show similar dwarfism. In this study, the average length of shoots was significantly reduced in line PS1, as shown in Fig. 1, confirming the dwarf phenotype of PS1 plants. As the average length of shoots was not decreased in PS6 plants, which accumulate moderate amounts of sorbitol, we focused on PS1-specific physiological parameters to understand the mechanism of dwarfism caused by introduction of the *S6PDH* gene.

Interference with *myo*-inositol biosynthesis might lead to growth inhibition in *S6PDH* transformants (Sheveleva et al., 1998). *myo*-Inositol levels in the stems were decreased in line PS1 as compared with wild-type and control lines in both June and August. The *myo*-inositol contents in the stems were also lower in line PS6 in June, when shoots grew better. However, PS6 plants did not show dwarfism. The *myo*-inositol content in tobacco plants accumulating high amounts of sorbitol is a hundredfold lower than that in control plants (Sheveleva et al., 1998). The decrease in *myo*-inositol in transgenic tobacco is marked compared with that in transgenic Japanese persimmon. Therefore, it is unlikely that the small decrease in *myo*-inositol content in PS1 plants induced dwarfism.

Sheveleva et al. (1998) assumed that the increased hexose in transgenic tobacco accumulating high levels of sorbitol led to altered sugar sensing. This altered sugar sensing might inhibit photosynthesis and cause lesion formation by inducing PR protein. In this study, however, the hexose content was not increased in PS1 plants. This suggests that the phenotype of line PS1 is not related to alterations in sugar sensing.

The G6P content was determined in the transgenic Japanese persimmon because G6P is the substrate of S6PDH. In fact, transgenic alteration of hexose kinase activity, which supplies hexose phosphate, affects vegetative growth in tomato (Dai et al., 1999; Odanaka et al., 2002). Although

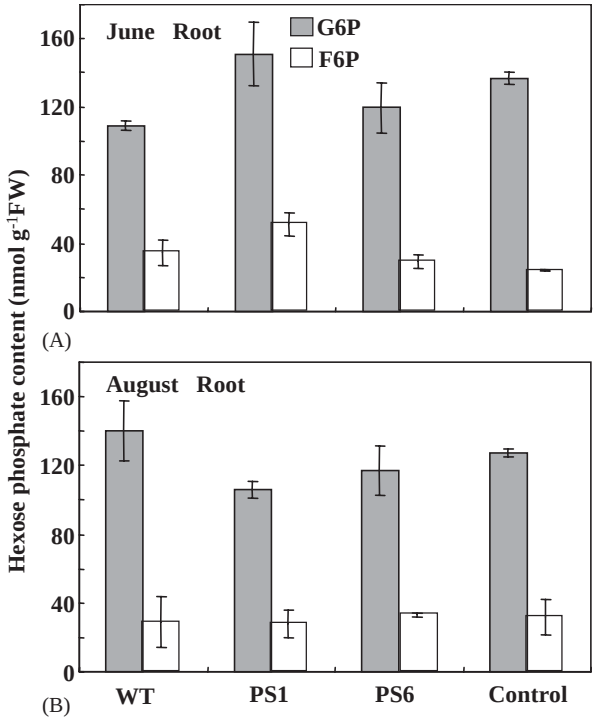

Figure 4. Hexose phosphate contents in roots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon. Hexose phosphate content was determined in June (A) and August (B). Gray and white columns indicate G6P and F6P contents, respectively. Bars represent±SE calculated from three plants in each line.

the G6P contents in the stems and leaves of PS1 and PS6 plants were lower than those in wild-type and control plants in June, the contents in PS1 were similar to those in PS6, which was phenotypically normal. G6P can be supplied from F6P via the excess activity of phosphoglucose isomerase after phosphorylation of fructose by fructokinase (Kanayama et al., 1998; Suzuki et al., 2001). The F6P contents in PS1 plants were similar to those in PS6 plants. Therefore, it is unlikely that G6P depletion was the cause of dwarfism in the transgenic Japanese persimmon.

In this study, plant hormone content was determined in leaves. The stem hormone content should also be measured to understand the mechanism of dwarfism. However, it was difficult to collect sufficient stem tissue to analyze the plant hormone contents because the growth and propagation of fruit trees is very slow. Therefore, leaves were used for the analyses. In fact, the leaves in line PS1 were clearly smaller than those in the other lines, suggesting that the accumulation of high levels of sorbitol affected leaf growth as well as stem elongation. Moreover, the plant hormone levels in leaves were shown to be altered in dwarf transgenic plants in previous studies (Nilsson et al., 1993; Sano et al., 1994; Kusaba et al., 1998).

The level of IAA, which is related to stem elongation, in line PS1 was similar to the levels in the other lines. A decrease in GA content generally induces dwarfism in plants. However, GA was not decreased in PS1 plants. These results suggest that sorbitol accumulation does not affect the levels of GA or IAA, which play key roles in shoot growth.

There have been a few reports on growth inhibition due to ABA (Zeevaart and Creelman 1988), which inhibits coleoptile elongation and leaf expansion. In this study, the ABA content was not changed in line PS1.

Transgenically enhanced cytokinin synthesis can induce abnormal phenotypes (Klee and Estelle, 1991). Transgenic tobacco plants with the *rgp1* gene show increased tillering and dwarfism (Kamada et al., 1992). The phenotype is associated with elevated levels of Z and [9R]Z in leaves (Sano et al., 1994). As PS1 plants were morphologically similar to the transgenic tobacco plants, endogenous cytokinin was measured in this study. The level of Z, the major cytokinin in persimmons, was decreased in PS1 plants, and no increases were observed in other cytokinin levels in PS1 plants. Although it is interesting that the level of Z was correlated with the sorbitol content in the four lines, this observation was opposite to the expected result.

A large amount of sorbitol accumulated in PS1 plants, especially in June when shoot growth was comparatively active. As Japanese persimmon does not synthesize sorbitol, it probably lacks a system to transport sorbitol into vacuoles. Therefore, sorbitol likely accumulates in the cytosol, leading to a high osmotic pressure in the cytosol. Heineke et al. (1992) suggested that increased osmotic pressure in the cytosol caused lesion formation via apoplastic expression of yeast-derived invertase in potato plants. The expression of the vacuolar water channel is inhibited in *Arabidopsis acl* mutants, which have internodes with reduced lengths, suggesting a relationship between the elongation growth of organs and the transport of water into vacuoles (Hanzawa et al., 1997). In our study, it is possible that the sorbitol concentration reached several hundred mM in the cytosol of stem and leaf tissues in line PS1. As one possibility, the osmotic imbalance between the cytosol and vacuoles in PS1 plants was likely above the threshold for dwarfism.

The growth of PS6 plants, which accumulated approximately 20 μmol g^{-1} FW sorbitol in the stems, was phenotypically normal. No necrotic

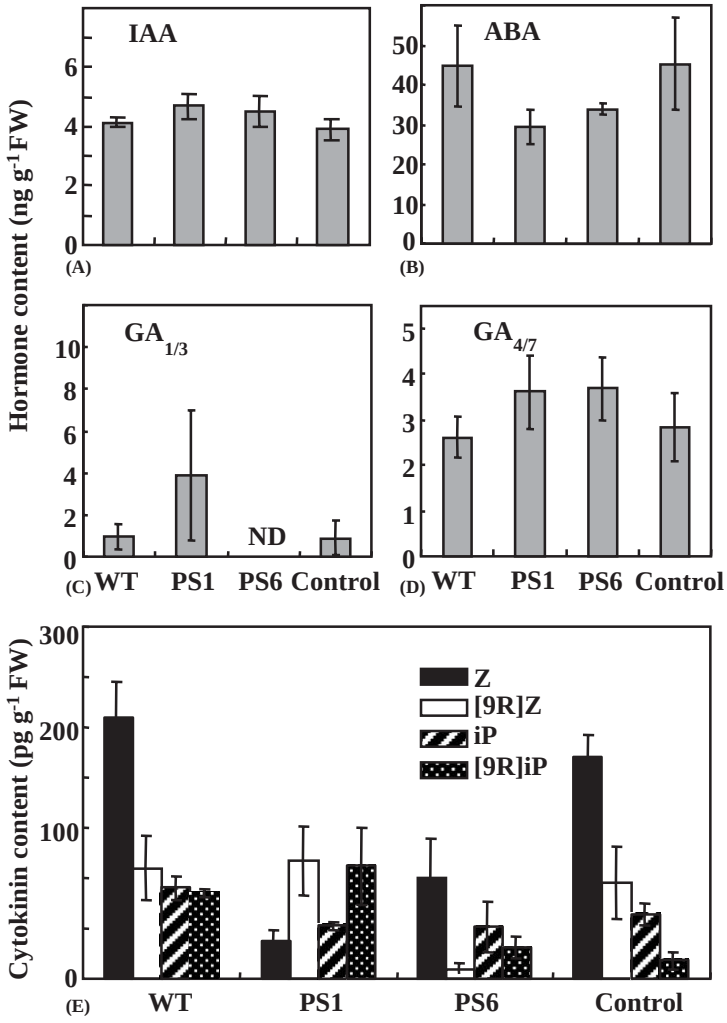

Figure 5. IAA, ABA, GA, and cytokinin contents in the leaves of shoots in sorbitol-accumulating lines (PS1, PS6), wild-type (WT), and control line of Japanese persimmon. IAA (A), ABA (B), $GA_{1/3}$ (C), $GA_{4/7}$ (D), and cytokinin (E) contents were determined in June. Bars represent±SE calculated from three plants in each line. ND, not determined.

lesions were observed, even in PS1 plants in which the sorbitol content was almost 50 μmol g^{-1} FW in the stems. In contrast, tobacco plants expressing *S6PDH* accumulating 10 μmol g^{-1} FW sorbitol show necrosis and growth inhibition, and those accumulating 50 μmol g^{-1} FW die (Sheveleva et al., 1998). These observations indicate that Japanese persimmon is a comparatively sorbitol-tolerant plant. *myo*-Inositol level decreases to one hundredth when *S6PDH*-expressing tobacco accumulates approximately 30 μmol g^{-1} FW of sorbitol (Sheveleva et al., 1998). However, the *myo*-inositol content in line PS1 was the same order of magnitude as those in the other lines. This also suggests that Japanese persimmon is a sorbitol-tolerant plant. Therefore, the enhanced stress-tolerance caused by introducing *S6PDH* appears to be a useful technique that depends on the species.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research (no. 12556005) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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