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Introducing an Antisense Gene for a Cell-Wall-Bound Acid Invertase to Tomato (*Lycopersicon esculentum*) Plants Reduces Carbohydrate Content in Leaves and Fertility

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Abstract

Partial Wiv-1 cDNA for a cell-wall-bound acid invertase isolated from wounded leaves of tomato (*Lycopersicon esculentum*) was introduced into tomato plants in an antisense orientation. The enzyme activity was markedly decreased in wounded leaves of 3 of 9 transformants. The soluble sugars and starch contents in the source leaves of these 3 transformants were reduced compared with control plants. These results suggest that the cell-wall-bound acid invertase encoded by *Wiv-1* regulates the carbohydrate content in source leaves of tomato. Most of the transformants showed low fertility. It is possible that the enzyme encoded by *Wiv-1* participates also in sink metabolism in tomato flowers.

Abbreviations

PCR, polymerase chain reaction.

1. Introduction

Most plants contain multiple isozymes of invertase (EC 3.2.1.26). Cell-wall-bound acid invertases (extracellular or apoplastic) are localized in the apoplast and are ionically bound to cell walls (Hisajima and Arai 1978, Krishnan *et al.*, 1985, Karuppiah *et al.*, 1989). It has been proposed that these enzymes are involved in phloem unloading (Ruan and Patrick 1995, Ho 1996, Brown *et al.*, 1997) and in sink strength (Weber *et al.*, 1995, 1996, Cheng *et al.*, 1996). In tomato plants, 4 distinct genes for the enzymes have been cloned and characterized (Godt and Roitsch 1997). However, the roles of each enzyme in sink and source tissues are still unclear.

We previously cloned Wiv-1 cDNA (Ohyama *et al.*, 1998) from wounded leaves of tomato. Wiv-1 originates from the same gene as *Lin6* cDNA (Godt and Roitsch 1997) and codes for a cell-wall-bound acid invertase. High levels of Wiv-1 (*Lin6*) mRNA are found in young flower buds (Godt and Roitsch 1997), stems, and wounded source leaves (Ohyama *et al.*, 1998). To clarify the roles of the enzyme encoded by Wiv-1 in tomato plants, we introduced an antisense gene to tomato with respect to the heterologous promoter, and analyzed the transgenic tomato plants.

2. Materials and Methods

2.1 Construction of pBWI-2

A partial cDNA, Wiv-2, for a wound-inducible, cell-wall-bound acid invertase in tomato leaves was used for construction of a binary vector. The nucleotide sequence of the Wiv-2 cDNA extends from the 483rd nucleotide of the Wiv-1 (Ohyama *et al.*, 1998) cDNA to 37 bp downstream from the 3′ end of the Wiv-1 cDNA.

The gene for β-glucuronidase on plasmid pBI121 (Clontech Laboratories, Inc., Palo Alto, CA, USA) was excised by digestion with *Sma* I and *Sac* I. A 1.4-kbp Wiv-2 cDNA, excised by *Hinc* II and *Sac* I from recombinant pBluescript KS+ (Stratagene, La Jolla, CA, USA), was ligated to the resultant vector arm in the antisense orientation. The resultant plasmid, pBWI-2 (**Fig. 1**), was used for the transformation.

2.2 Agrobacterium-mediated transformation

Tomato cv. Syugyoku was transformed as described previously (Ohyama *et al.*, 1995) by using *Agrobacterium tumefaciens* LBA4404 harboring pBWI-2. Regenerated plants were grown in a greenhouse and encouraged to self-set fruit by occasional vibration of the inflorescence.

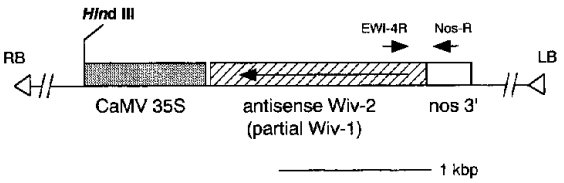

Fig. 1 Partial structure of plasmid pBWI-2. The cauliflower mosaic virus 35S promoter (CaMV 35S) directs expression of an antisense gene (antisense Wiv-2) with the polyadenylation sequences of the gene for nopaline synthase (nos 3′). The other part of the plasmid is identical to the binary vector pBI 121. LB and RB indicate left and right borders of T-DNA respectively. Primers EWI-4R and Nos-R used for detection of the transgene (**Fig. 2**) correspond to the sequences of positions indicated by arrows. The relevant restriction site (*Hind* III) for the Southern blotting analysis (**Fig. 2**) is shown.

2.3 Screening of transformants by PCR

Genomic DNA was isolated from young leaves of regenerated plants by the standard method (Murray and Thompson 1980). The reaction mixture (20μl), which contained *Ex Taq* polymerase (Takara, Kyoto, Japan) and the primers EWI-4R (**Fig. 1**; 5′-AGTATCCATCTCTGCCCATCCAAG-3′) and Nos-R (**Fig. 1**; 5′-ATCATCGCAAGACCGGCAAC-3′), was prepared according to the enzyme manufacturer's protocol. A thermal cycle consisting of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, was repeated 25 times. PCR products were isolated on a 2% agarose gel and stained with ethidium bromide.

2.4 Southern blotting analysis

Genomic DNA was digested with *Hind* III, fractionated by 0.8% agarose gel electrophoresis, and then blotted onto a membrane. The blot was allowed to hybridize with ^{32}P-labeled Wiv-2 cDNA.

2.5 Wounding of leaves and assay of enzymatic activity

Source leaves were wounded and activities of acid invertase in cell-wall-bound fractions were assayed as described previously (Ohyama *et al.*, 1995, 1998).

2.6 RNA blotting analysis

RNA blotting was analyzed as described previously (Ohyama *et al.*, 1998) using ^{32}P-labeled Wiv-2 cDNA as a probe.

2.7 Determination of carbohydrate content

Fully expanded source leaves were sampled at noon between July and September, 1997. Soluble sugars were extracted with 80% ethanol and their concentrations were determined by high-performance liquid chromatography. Insoluble fractions in 80% ethanol were dissolved in a small amount of dimethyl sulfoxide, and then starch contents were determined enzymatically with an F-kit (Boehringer Mannheim, Tokyo, Japan).

Fig. 2 DNA analysis of regenerated plants. PCR was done in a reaction mixture containing primers EWI-4R and Nos-R (**Fig. 1**) and genomic DNA from each regenerated plant as a template. Products of PCR were isolated on a 2% agarose gel and stained with ethidium bromide. The arrow shows a 300 bp fragment amplified from the 3′ part of pBWI-2 (**Fig. 1**). The numbers below show the number of intact copies of the antisense gene estimated by Southern blotting analysis (see **Materials and Methods**).

2.8 Pollen analysis

Pollen grains were stained with acetocarmine. Stained grains were counted under a microscope.

3. Results

3.1 DNA analysis of transformants containing an antisense Wiv-2

Transformants were selected from 13 regenerated plants by PCR (**Fig. 2**). A band of about 300 bp derived from the antisense gene (**Fig. 1**) was detected in 9 plants (**Fig. 2**; T147, T205, T208, T213, T236, T248, T267, T365, T423). These transformants were further analyzed by Southern blotting. All transformants contained at least 1 intact copy of the antisense gene (**Fig. 2**). Four nontransformants (**Fig. 2**; U366, U404, U406, U414) were used as controls.

3.2 Characteristics of the transformants

Growth of all transformants but 1 was normal compared with the control plants (**Fig. 3**); T248 was stunted (data not shown). The percentage of stained pollen grains and the number of fruit were less in most transformants than those in the control plants (**Table 1**).

3.3 Activity of cell-wall-bound acid invertase in wounded leaves of transformants

Although the activity of cell-wall-bound acid inver-

Fig. 3 Growth of tomato plants with an antisense *Wiv-2* gene (pBWI-2). A: nontransformant (U414, control); B: transformant (T236).

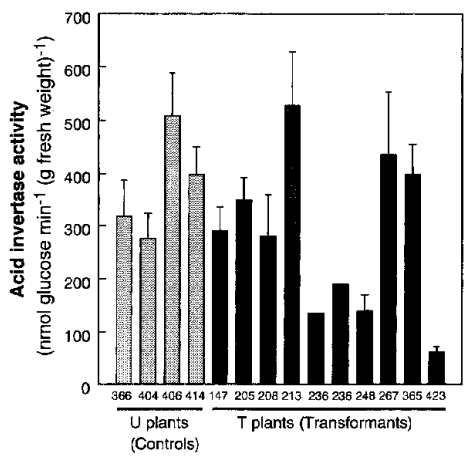

Fig. 4 Activity of cell-wall-bound acid invertase in wounded source leaves of transformants.
Leaf discs cut from nontransformants (U plants) and transformants (T plants) were incubated at 25°C in darkness for 96 h. The activity in extracts of the leaf discs was then measured. Each column represents the mean of results of 3 experiments (2 for T236); vertical bars indicate standard error.

tase in intact leaves of tomato is very low, both the activity and Wiv-1 mRNA levels are raised by wounding (Ohyama *et al.*, 1998). Therefore we compared cell-wall-bound acid invertase activities in wounded leaves of transformants containing the antisense *Wiv -2* (partial Wiv-1) gene. After 96 h, levels of the activity varied (**Fig. 4**). Three transformants (T236, T248, T423) showed substantially less activity than control plants (**Fig. 4**). Activity in T423 was significantly lower ($P<0.05$) than in a control plant, U404, which showed the lowest levels of activity among the controls (**Fig. 4**). These 3 transformants were selected and used for RNA and carbohydrate analyses.

3.4 RNA blotting analysis of leaves of transformants

The levels of Wiv-1 (Ohyama *et al.*, 1998) mRNA

Fig. 5 Levels of Wiv-1 mRNA in leaves of the transformants.
Each lane was loaded with 3μg of total RNA from source leaves. The RNA was fractionated on an agarose gel under denaturing conditions with glyoxal-dimethyl sulfoxide and then blotted onto a membrane. The blot was probed with ^{32}P-labeled Wiv-2 cDNA.

in source leaves of the 3 transformants were less than those in the control plants (**Fig. 5**).

3.5 Carbohydrate content of source leaves

The contents of soluble sugars and starch in leaves of the 3 transformants were lower than in the control plants (**Table 2**). The average contents of the various carbohydrates in the transformants were reduced to about 20% to 60% of those of the control plants (calculated from the data in **Table 2**). The sucrose content of the transformants was markedly decreased (**Table 2**; about 20% of that in the control plants on average).

4. Discussion

4.1 Antisense suppression of cell-wall-bound acid invertase in leaves and analysis of carbohydrates

We introduced an antisense gene for wound-inducible, cell-wall-bound acid invertase (**Fig. 1**; pBWI-2) into cultivated tomato. Enzyme activities in wounded source leaves of transformants T236, T248, and T423 were markedly less than those of the control plants (**Fig. 4**). The level of Wiv-1 mRNA in those transformants was less than in control plants (**Fig. 5**). These results suggest that the antisense modification

Table 1 Fertility of tomato plants transformed with an antisense *Wiv-2* gene (pBWI-2).

Plants	Percentage of stained pollen grains		Relative value of fruit number
	Exp. 1	Exp. 2	
U366* (control)	81.7	84.1	+++
U404* (control)	87.3	82.6	+++
U406* (control)	67.6	69.3	+++
U414* (control)	86.0	N.D.	+++
T147	34.4	6.58	−
T205	27.9	8.47	±
T208	56.5	6.56	−
T213	79.4	83.1	±
T236	69.4	40.5	−
T248**	40.1	N.D.	±
T267	76.8	N.D.	++
T365	20.9	13.5	+
T423	1.10	0.00	−

*nontransformant **stunted N.D.: not determined

reduces the mRNA level and the enzyme activity in leaves of the transformants. It seems unlikely that the expression of soluble acid invertase is also suppressed by the antisense gene (pBWI-2) since the antisense *Aiv-1* gene for soluble acid invertase had no effect on the activity of cell-wall-bound enzyme in tomato leaves (Ohyama *et al.*, 1995). Although 1 transformant, T248, was stunted, growth of the other 8 transformants (including T423, which showed the lowest activity of cell-wall-bound enzyme among the transformants) was normal (**Fig. 3**). We consider that the inhibition of growth observed in T248 could have been caused by somaclonal variation, not by the antisense inhibition of the enzyme synthesis.

Contents of carbohydrates in source leaves of 3 transformants (T236, T248, T423) were less than those in the control plants (**Table 2**). It has been reported that overexpression of yeast invertase in the apoplast leads to an increase in carbohydrates in leaves of tobacco (Sonnewald *et al.*, 1991) and tomato (Dickinson *et al.*, 1991) plants. The increased activity in transformants is thought to degrade sucrose in the apoplast, inhibit phloem unloading, and thus increase sugar levels in the leaves (Dickinson *et al.*, 1991, Sonnewald *et al.*, 1991). The reduced levels of carbohydrates observed in this study (**Table 2**) may support the above hypothesis from the reverse direction.

4.2 Relationships between fertility and cell-wall-bound acid invertase

Most of the transformants containing the antisense gene showed low fertility, whereas the untransformed controls showed normal fertility (**Table 1**). It is possible that the low fertility is due to the effect of the antisense gene, not to somaclonal variation. Cell-wall-bound acid invertase may participate in sink strength (Weber *et al.*, 1995, 1996, Cheng *et al.*, 1996) and phloem unloading (Ruan and Patrick 1995, Ho 1996, Brown *et al.*, 1997). Therefore, the decrease in the number of fruit and stained pollen grains in the transformants (**Table 1**) suggests that the cell-wall-bound invertase encoded by Wiv-1 participates in sink metabolism in flower tissues of tomato. The presence of high levels of *Lin6* (Wiv-1) mRNA in small flower buds of tomato (Godt and Roitsch 1997) may support this hypothesis. Furthermore, the growth of petunia pollen tubes is reported to be accompanied by hexose transport (Ylstra *et al.*, 1998).

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Table 2 Carbohydrate contents in mature leaves of tomato transformed with an antisense *Wiv-2* gene (pBWI-2).

Plants	Carbohydrate content (mg(g fresh weight)$^{-1}$)					
	Sucrose (A)	Glucose (B)	Fructose (C)	Total soluble sugars (A+B+C)	Starch (D)	Total carbohydrate (A+B+C+D)
U366* (control)	1.47±0.13	1.05±0.15	3.70±0.21	6.22±0.42	23.7±4.57	29.9±4.48
U404* (control)	0.45±0.21	0.54±0.17	2.72±0.34	3.70±0.71	7.70±1.68	11.4±1.15
U406* (control)	0.35±0.25	0.53±0.19	2.00±0.40	2.88±0.84	16.6±0.78	19.5±1.04
U414* (control)	1.00±0.19	1.04±0.28	2.88±0.56	4.93±0.99	9.87±1.53	14.8±0.55
T236	0.13±0.06	0.15±0.03	1.40±0.10	1.68±0.17	2.54±0.42	4.22±0.27
T248	0.20±0.08	0.32±0.05	1.70±0.17	2.22±0.30	3.35±0.51	5.57±0.49
T423	0.18±0.13	0.22±0.11	1.90±0.34	2.30±0.56	9.83±3.73	12.1±3.80

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