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Antisense Acid Invertase (*TIV1*) Gene Alters Soluble Sugar Composition and Size in Transgenic Tomato Fruit[1]

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Invertase (β-fructosidase, EC 3.2.1.26) hydrolyzes sucrose to hexose sugars and thus plays a fundamental role in the energy requirements for plant growth and maintenance. Transgenic plants with altered extracellular acid invertase have highly disturbed growth habits. We investigated the role of intracellular soluble acid invertase in plant and fruit development. Transgenic tomato (*Lycopersicon esculentum* Mill.) plants expressing a constitutive antisense invertase transgene grew identically to wild-type plants. Several lines of transgenic fruit expressing a constitutive antisense invertase gene had increased sucrose and decreased hexose sugar concentrations. Each transgenic line with fruit that had increased sucrose concentrations also had greatly reduced levels of acid invertase in ripe fruit. Sucrose-accumulating fruit were approximately 30% smaller than control fruit, and this differential growth correlated with high rates of sugar accumulation during the last stage of development. These data suggest that soluble acid invertase controls sugar composition in tomato fruit and that this change in composition contributes to alterations in fruit size. In addition, sucrose-accumulating fruit have elevated rates of ethylene evolution relative to control fruit, perhaps as a result of the smaller fruit size of the sucrose-accumulating transgenic lines.

Invertases (β-fructosidase, EC 3.2.1.26) are a group of related enzymes that hydrolyze Suc to Glc and Fru. Acid invertase has a pH optimum for activity between 3 and 5, whereas neutral invertase has a pH optimum of about 7. Acid invertases are divided into vacuolar (or soluble) and extracellular (or insoluble) forms, with acid (Unger et al., 1992) and basic (Laurière et al., 1988) pIs, respectively. Neutral (or alkaline) invertase is presumed to be localized in the cytosol (Ricardo, 1974). Plant genes for acid invertase have been cloned from tomato (*Lycopersicon esculentum* Mill.; Klann et al., 1992) and several other plants, all sharing homology to the active site of yeast invertase (Unger et al., 1994). In plants acid invertase is thought to be involved primarily in Suc metabolism for energy in growing tissues (ap Rees, 1974), and increasing extracellular invertase activity results in severely stunted growth and inhibition of photosynthesis (von Schaewen et al., 1990).

In tomato acid invertase activity is present during fruit development, and hexose sugars stored in the vacuole are the primary storage carbohydrate (Manning and Maw, 1975). Acid invertases have been localized both intracellularly and extracellularly in developing tomato fruit (Sato et al., 1993). Suc levels are negatively correlated with the rate of carbon import in tomato fruit. Suc hydrolysis by acid invertase has been proposed to influence the rate and extent of sugar storage in fruit by creating a Suc concentration gradient between the site of phloem unloading and the storage cells (Walker and Thornley, 1977; Walker et al., 1978).

In contrast, a wild tomato relative, *Lycopersicon chmielewskii*, accumulates very high levels of stored sugar in its fruit, primarily as Suc (Yelle et al., 1988). Many plants with high sugar levels accumulate primarily Suc: sugarcane, sugar beet, melon, and carrot. The storage of primarily Suc may favor high soluble sugar levels because it contributes half the osmolarity of the equivalent Glc and Fru (Steingröver, 1983) and may be less metabolically accessible to respiratory loss than hexose sugars (Salerno and Pontis, 1978).

The trait of Suc accumulation (*sucr*) from *L. chmielewskii* is inherited in a monogenic and recessive manner (Yelle et al., 1988; Chetelat et al., 1993) and is tightly linked to the restriction fragment length polymorphism marker TG102 on chromosome 3 of the *Lycopersicon* genome (Chetelat et al., 1993). Suc accumulation is associated with low acid invertase activity and immunologically detectable invertase protein in *L. chmielewskii* and in populations of *L. esculentum* into which the *sucr* allele has been introgressed (Yelle et al., 1988, 1991; Klann et al., 1993). The predominant form of acid invertase in ripening *L. esculentum* fruit is a 52-kD glycoprotein (Yelle et al., 1991), and its corresponding cDNA (*TIV1*) has been cloned (Klann et al., 1992). The *TIV1* gene is tightly linked to the *sucr* locus on chromosome 3 (Chetelat et al., 1993), and its mRNA is abundant in *L. esculentum* fruit but undetectable in *L. chmielewskii* fruit (Klann et al., 1993). These results strongly suggest that acid invertase (*TIV1*) is a component of the *sucr* locus; however, there remains the possibility that the *sucr* locus is comprised of multiple genes, of which *TIV1* is only one. Furthermore, the full role of *TIV1* during *L. esculentum* fruit development is difficult to ascertain in the introgressed populations because of reduced fertility in these lines and because of other unknown contributions from *L. chmielewskii* chromosome fragments.

In this study we investigated the contributions of *TIV1* acid invertase during fruit and plant development by expressing a chimeric antisense *TIV1* gene in transgenic to-

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mato plants. Previous work has established that removing invertase activity from tomato fruit with a chimeric, antisense acid invertase gene results in Suc accumulation in ripe fruit of primary transformants (Ohyama et al., 1995). In the present study we expand on this by analyzing the secondary generation of transformants throughout all stages of fruit development, addressing not only the effects on acid invertase activity and sugar composition but also effects on fruit size and sink capacity. The effect of the chimeric antisense gene on the ripening climacteric of the fruit is also examined.

MATERIALS AND METHODS

Construction of Antisense Transgene

The acid invertase cDNA (*TIV1*) (Klann et al., 1992) was digested internally with *Xho*II and in the polylinker with *Sac*I to remove a 1640-bp fragment of the cDNA that contains the entire 5′ untranslated region and approximately 75% of the coding region. For the *35S*-driven transgene, the GUS-coding sequence was removed from pBI122 (Clontech, Palo Alto, CA) (Bevan, 1984) with *Bam*HI/*Sac*I, and the *TIV1* cDNA fragment was ligated in its place in the antisense orientation. To construct the *E8* transgene, *Eco*RI and *Bam*HI were used to remove approximately 2 kb of the *E8* promoter region (Deikman and Fischer, 1988), including 240 bp of the *E8*-coding sequence. The *nos* terminator region was removed from pBI121 with *Sac*I and *Eco*RI. The *nos* terminator, *E8* promoter, and *TIV1* gene fragment were ligated together simultaneously into *Eco*RI-cut pBluescript (Stratagene). The *Eco*RI cassette was removed from pBluescript and ligated into *Eco*RI-cut pBIN (Clontech). *Agrobacterium tumefaciens* strain LBA4404 was transfected with the *35S* and *E8* chimeric transgenes and with pBIN alone. Cotyledons of tomato (*Lycopersicon esculentum* Mill. var T5 and H100) were transformed as described by McCormick et al. (1986). Plants were grown in the greenhouse.

Genomic DNA Analysis

Genomic DNA was isolated from young leaves (Chetelat and DeVerna, 1991). Genomic Southern blots were prepared according to standard methods (Sambrook et al., 1989). Gels were blotted onto a Hybond-N membrane (Amersham), and the blots were hybridized and probed according to the manufacturer's protocol. ^{32}P-labeled *TIV1* probes were produced by randomly priming (Feinberg and Vogelstein, 1983) part of the fragment of isolated tomato acid invertase cDNA used in transgene construction.

To obtain homozygous plants from the T_1 generation, T_0 plants were self-pollinated, and 20 to 35 of the T_1 progeny were analyzed with genomic Southern blots. A fragment of the TIV1-coding sequence was used for the probe, the sequence of which was also present in the chimeric transgenes. Genonic DNA was prepared for Southern blotting by digesting with *Eco*RI, sites for which are located at each end of the transgene cassettes and not within TIV1. Fragments of the native gene and the chimeric gene obtained were different sizes. Copy number was estimated in the T_0 and T_1 generations by comparing the transgene band with the native gene band using a phosphor imager (Fujix BAS 2000, Fuji, Stamford, CT) and MacBAS software (Fuji). The arrangement of transgene insertions in the host genome was not determined.

Fruit Sampling

During analysis of the T_0 generation, three fruit from one plant were analyzed. Fruit were tagged at the breaker stage, the first sign of color change upon ripening, and harvested 3 d later. The fruit were approximately 7 weeks after anthesis.

During the analysis of the T_1 generation, fruit were harvested from four to five plants of each genotype. Flowers were tagged at anthesis or pollination. Three fruits of the appropriate age were selected at random and analyzed for each data point. The same fruit were analyzed for sugar, size, and enzymatic activity. Sampling continued until the three fruit selected at random were all red (mature). Sampling of the S-T-4 line was continued for an additional 1 week to reach this stage. The azygous line comprised 0 copy segregants from the S-T-4 line.

Statistical analysis was performed with statistical software (CoStat, Cohort Software, Berkeley, CA). Data were analyzed by analysis of variance and the means were compared with the Student-Newman-Keuls procedure. Similar lines were grouped in some cases.

Sugar Measurements

Sugars were extracted from tomato pericarp by boiling tissue in 80% ethanol. They were then analyzed on a carbohydrate column (HPX-87C, Aminex, Hercules, CA) as described by Klann et al. (1993).

Enzyme Analysis

Total protein was extracted from tomato pericarp and desalted as described by Klann et al. (1993) except 0.5 mg mL^{-1} casein was substituted for the 0.5 mg mL^{-1} BSA in the homogenization and desalting buffers. Suc synthase, Suc-P synthase, and acid invertase enzyme activities were analyzed as described by Klann et al. (1993). Neutral invertase reactions and controls contained 50 mM Hepes, pH 7.5, and 120 mM Suc with 20 μL of desalted extract in 100 μL total. Reactions were incubated at 37°C for 30 min and reducing sugars were measured (Klann et al., 1993).

Antibody Preparation

Polyclonal antibodies were raised in rabbits to regions of acid invertase (*TIV1*) expressed as fusion proteins in *Escherichia coli*. The cDNA region corresponding to amino acids 84 to 279 (general I) of the *TIV1* protein was ligated into the appropriate version of the expression vector (pRSET, Invitrogen, San Diego, CA). Proteins were expressed in *E. coli* strain BL21 as recommended by the manufacturer (Invitrogen). Cells were pelleted by centrifugation (10 min at 4,000*g* and 4°C) and resuspended in 50 mM Tris, pH 8, and 5 mM $MgCl_2$. The cells were brought to 200 μg mL^{-1}

lysozyme, incubated for 10 min at room temperature, and sonicated on ice for 4 min. RNase and DNase were added to the cell lysate to 20 μg/mL, and it was then incubated on ice for 45 min. Inclusion bodies were spun out (15 min at 10,000*g*) and the supernatant was removed. The pellet was resuspended in 8 M urea and 50 mM Tris, pH 8, and proteins were separated by SDS-gel electrophoresis (Laemmli, 1970). The fusion protein was electroeluted from the gel and dialyzed overnight against 200 mM sodium bicarbonate and 0.05% SDS. Protein was lyophilized and formulated into two injections of 1 mg each; the first injection was with complete Freund's adjuvant, and the second was at 3 weeks with incomplete Freund's adjuvant. Blood was taken at 5 weeks.

Polyclonal antiserum was also generated to regions of amino acids 279 to 433 (general II) and amino acids 460 to 546 (vacuolar-specific) of the *TIV1* acid invertase protein.

Western Blot Procedure

Total protein was extracted from tomato pericarp and desalted as above (Klann et al., 1993). Protein was precipitated with 1:1 cold acetone and centrifuged (10 min at 16,000*g*). Proteins were separated by SDS-gel electrophoresis and electroblotted to a membrane (Immobilon-P, Millipore) in 10 mM 3-cyclo-lexyl-amino-1-propanesulfonic acid, pH 11.0. Western blot analysis was performed by the Tris-buffered saline-Tween method (Birkett et al., 1985). General I *TIV1* acid invertase polyclonal antiserum at 1:3000 dilution and goat anti-rabbit IgG alkaline phosphatase (Bio-Rad) at 1:3000 were used for all western blots. Blots were developed according to the method of Blake et al. (1984).

Respiration Measurements

Fruits were harvested at the breaker stage when the first sign of ripening appeared, and they were weighed and put into closed 400-mL jars with humidified air flowing at rates adjusted to keep the CO_2 concentration in the jar less than 0.2% (v/v). Temperature was maintained at 25°C. One-milliliter samples of exiting air were taken twice a day and analyzed for CO_2 and ethylene concentrations. Three samples were measured and then averaged at each time point for each gas. CO_2 was measured with an IR absorption detector (PIR-2000, Horiba, Irvine, CA). Ethylene was measured with a gas chromatograph (Carle AGC211, Carle, Anaheim, CA) equipped with a 4', NaCl-modified alumina F1 column, 60/80 mesh.

RESULTS

TIV1 Antisense Transgenic Tomato Plants

Transgenic tomato plants expressing antisense acid invertase (*TIV1*) transgenes were created to assess the function of intracellular soluble acid invertase in tomato fruit and plant development. Chimeric antisense genes were constructed with a 1640-bp fragment of the *TIV1* cDNA (Klann et al., 1992) that contained the entire 5' untranslated region and approximately 75% of the coding region. This *TIV1* fragment was ligated in the antisense orientation to either the constitutive cauliflower mosaic virus 35S promoter (Odell et al., 1985) or the ethylene-inducible *E8* promoter from tomato fruit (Deikman and Fischer, 1988). The *E8* promoter was used to determine the function of acid invertase specifically during fruit ripening and in case the constitutive expression of an antisense *TIV1* gene was lethal. Tomato cv T5 was transformed with chimeric antisense *TIV1* transgenes and an empty vector control using the pBIN *Agrobacterium* vector (Bevan, 1984). Transgenic tomato plants were grown in both the greenhouse and field and were indistinguishable from control plants.

Acid Invertase Repression and Altered Sugar Composition in Primary Transformants

Acid invertase activity, sugar composition, and total soluble sugar levels change throughout tomato fruit development. In wild-type plants acid invertase activity is low throughout most of fruit development, but acid invertase mRNA abundance and enzyme activity increase more than 10-fold during fruit ripening (Klann et al., 1993). Sugar composition and acid invertase protein levels were determined in the pericarp of partially ripe (3 d after the breaker stage) fruit from each T_0 line. This stage provides a good measure of the effectiveness of the antisense gene at repressing *TIV1* gene expression, because at this stage of fruit ripening the gene is normally expressed most abundantly. It should be recognized, however, that acid invertase activity levels at this time do not necessarily reflect levels during the earlier stages of fruit development, when approximately half of sugar accumulation occurs.

Ripening fruit of transgenic tomato lines expressing the *E8/TIV1* antisense gene did not have significantly altered levels of Suc (Fig. 1). It is likely that hexoses accumulate prior to ripening, when the E8 promoter would be inactive. In contrast, ripening fruit of many of the transgenic lines expressing the *35S/TIV1* antisense gene had a significantly higher proportion of Suc (Fig. 1). Several lines exhibited Suc levels approaching 80% of total soluble sugars, which is similar to the composition of the Suc-accumulating wild tomato species *L. chmielewskii* (Yelle et al., 1988; Klann et al., 1993). Several other *35S/TIV1* antisense transgenic lines had levels of Suc similar to control fruit, both untransformed and transformed with the empty pBIN19 vector, in spite of the fact that immunologically detectable invertase was greatly reduced in these lines (data not shown).

Close examination of acid invertase enzyme activity revealed that the transgenic lines with the lowest level of invertase activity in general accumulated the highest percentage of Suc (lines S-T-3 and S-T-15; Fig. 2), but there was not an absolute correlation between acid invertase levels and Suc accumulation. The lack of strict correlation between invertase levels and Suc percentage was also found previously when the acid invertase protein levels were suppressed in tomato by expression of an antisense gene (Ohyama et al., 1995). This lack of correlation is perhaps not surprising since Suc levels are the product of sugar metabolism throughout fruit development, and the acid invertase activity reported here was measured at a single

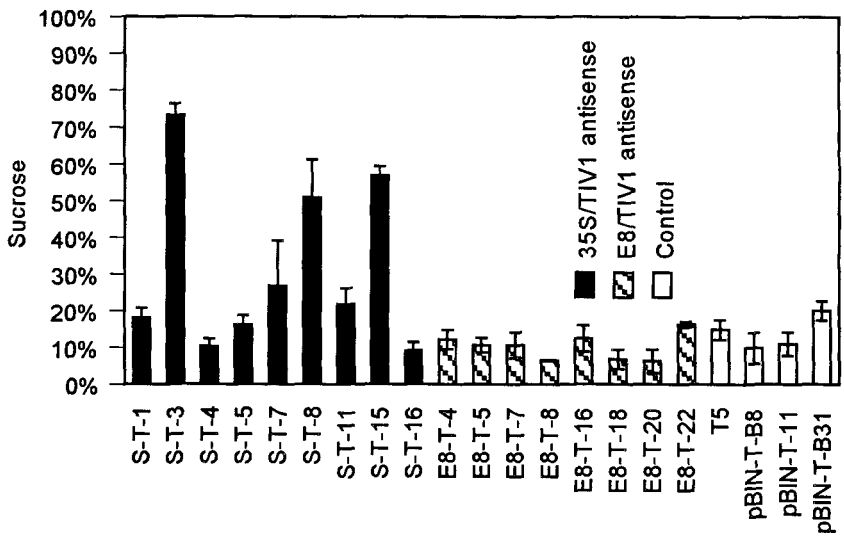

Figure 1. Sugar composition and acid invertase protein in T_0 transformants. Percentage of Suc indicates the proportion of the soluble sugars (Glc, Fru, and Suc) that comprises Suc. S, 35S promoter; E8, E8 promoter; pBIN, empty vector pBIN19; T, *L. esculentum* var T5; the final number indicates the transformant number. Bars indicate SES; $n = 3$. Sugar composition in fruit pericarp was determined 3 d after the breaker stage of T_0 transformants.

time point late in fruit development. It is possible that the capacity of the transgene to suppress endogenous invertase expression may not be uniform throughout development in some transformants. Even very low levels of invertase activity may be sufficient to hydrolyze considerable amounts of stored Suc, and the elevation of Suc probably requires that invertase activity be reduced below some very low critical threshold.

Selected genotypes were self-pollinated and progeny homozygous for the transgene were analyzed in the T_1 generation. Two transgenic lines, S-T-3 and S-T-15, which have the *35S/TIV1* antisense gene and high Suc levels, were selected to investigate the low-acid-invertase phenotype. One transgenic line, E-T-8, containing the *E8/TIV1* antisense gene E-T-8 was analyzed to determine the effect of acid invertase during fruit ripening. Two *35S/TIV1* transgenic lines with low acid invertase protein and low Suc levels, S-T-4 and S-T-11, were included to determine why low acid invertase did not correlate with high Suc levels in fruit from these lines. The copy number of the transgenes in these lines was determined by phosphor image analysis of genomic Southern blots, and the transgene copy number was estimated by reference to the endogenous *TIV1* invertase gene. Estimates of the copy numbers are presented in Table I.

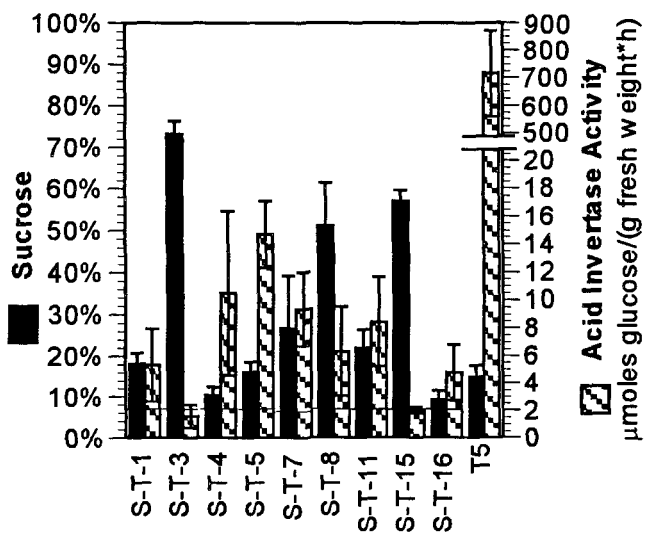

Figure 2. Percentage of Suc and acid invertase activity in fruit from the *35S/TIV1* T_0 transformants and T5. Bars indicate the SES; $n = 3$. Sugar composition and acid invertase activity in fruit pericarp were determined 3 d after the breaker stage.

Sugar Composition in T_1 Fruit

The two *35S/TIV1* genotypes that did not accumulate predominantly Suc in the T_0 generation increased their level of Suc when the transgene copy number was doubled in the T_1 generation (Table I). Fruit from S-T-4 and S-T-11 could therefore be qualified as at least partial Suc accumulators, suggesting that the limited capacity of the antisense genes to repress invertase gene expression was responsible

Table I. *Summary of Suc accumulation in selected transgenic lines*

Copy number of antisense cassette and percentage of Suc accumulation with SE in ripe fruit from T_0 and T_1 generations of selected transgenic genotypes. All fruit were harvested when ripe. Each value is the average of three fruit.

Transgenic Genotype	Copy No.	Suc Composition
		%
S-T-3 T_0	3	73.2 ± 3.1
S-T-3 T_1	6	52.9 ± 3.5
S-T-15 T_0	3	57.1 ± 2.4
S-T-15 T_1	6	41.2 ± 16.4
S-T-4 T_0	2	10.4 ± 2.0
S-T-4 T_1	4	61.2 ± 7.5
S-T-11 T_0	1	22.9 ± 4.4
S-T-11 T_1	2	44.5 ± 18.7
E-T-8 T_0	4	6.5 ± 0.1
E-T-8 T_1	8	6.3 ± 2.2
Azygous T_1	0	2.4 ± 0.2
T5 T_1	0	9.0 ± 3.4

for hexose accumulation in the T_0 generation, which could be partially overcome by doubling the transgene copy number. Surprisingly, doubling the copy number of the antisense transgene in lines S-T-3 and S-T-15 did not further increase the Suc percentage relative to the T_0 lines. It is possible that in these lines, in which Suc accumulation was already high, Suc accumulation was maximal in the T_0 fruit and that variations in stage of fruit development at the time of analysis resulted in an apparent decrease in Suc accumulation.

Sugar composition of the T_1 generation fruit was determined during development for S-T-3 and S-T-4, as well as T5 and azygous segregants of S-T-4. Fruit of the two control genotypes had similar sugar composition throughout development: characteristically low Suc levels and Glc and Fru making up most of the soluble sugar (Fig. 3). Although the sugar concentration when expressed per gram of fresh weight was approximately constant throughout the development of control fruit, it should be pointed out that fruit increased in size approximately 10-fold during this period.

In both transgenic lines expressing the *35S/TIV1* antisense gene, the concentration of Suc in fruit pericarp was elevated 5-fold, and Glc and Fru levels were decreased by approximately 50% during fruit development up to the final week. During these early weeks the transgenic fruit had hexose sugar concentrations of 33 to 50% and Suc concentrations 500 to 1000% of the control fruit. The sugar composition of fruit from the *E8/TIV1* line was similar to the control lines (data not shown). During the last week of development, Suc concentration in S-T-3 fruit doubled, whereas the hexose sugar concentration increased only slightly. In S-T-4 fruit Suc concentration was elevated through the 7th week of green fruit development, but during the final week of development, when the fruit ripened, hexose sugars made up all of the final sugar increase and Suc concentrations declined by 33%. S-T-4 and S-T-3 fruit showed a 100 and 70% increase in sugar accumulation during the last week of development, respectively. The total amount of sugars in transgenic fruits was similar to that in the control fruit in the green stages but exceeded it in the ripening stages by 50% or more.

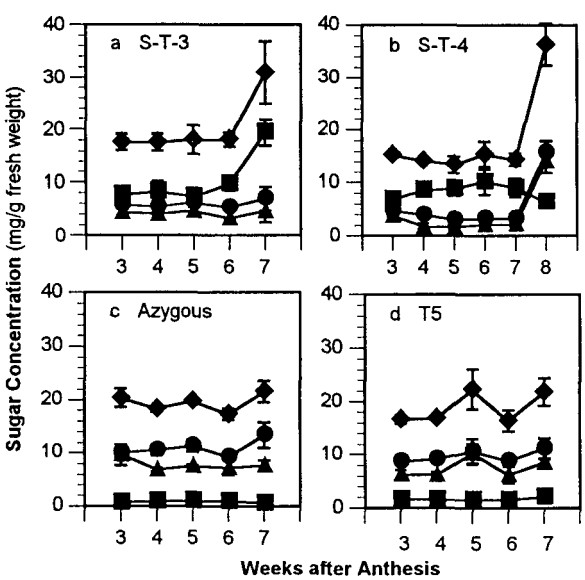

Figure 3. Sugar composition during fruit development. Concentrations of Suc (■), Glc (●), Fru (▲), and total sugar (◆) in *35S/TIV1* transgenic lines S-T-3 (a) and S-T-4 (b), and control lines azygous (c) and *T5* (d) during fruit development. Three fruit were sampled for each time point. Bars indicate SEs.

In the S-T-4 line fruit began to ripen after the 7th week. The reversal of Suc accumulation during this period suggests an increase in acid invertase activity, since expression of the endogenous gene increases dramatically (Klann et al., 1994). It is likely that the effect of the antisense gene began to fail because of high levels of endogenous invertase gene expression during this period.

Fruit Size

The size of the transgenic fruit with elevated Suc levels was significantly reduced ($P = 0.95$, $n = 12$). Control fruit increased in size in two distinct stages: at a slow rate until the final week of development and then at a very rapid rate, nearly doubling their size between weeks 6 and 7 (Fig. 4A). In contrast, the transgenic fruit with elevated Suc (line S-T-3) exhibited only the first, slow-growth stage. The final fruit sizes of all of the transgenic *35S/TIV1* lines in the T_1 generation were reduced by approximately 30% relative to the control fruit (Fig. 4B).

Sink Strength

Sugar accumulation per fruit was measured throughout fruit development. It occurred in two distinct stages in the Suc- and hexose-accumulating fruit in a manner similar to that of *L. esculentum* fruit with the Suc-accumulating trait introgressed from *L. chmielewskii* (Klann et al., 1993): A slow rate of accumulation in the 3rd to 6th week, followed by a rapid accumulation in the final week (Fig. 5a). Despite different fruit sizes, the total sugar content per fruit of S-T-3 and S-T-4 was the same as control fruit, approximately 2.2 g (Fig. 5a). Although previous studies have suggested that the carbohydrate accumulation rate is negatively correlated with Suc concentration in tomato fruit (Walker and Ho, 1977), the similar rates and extent of total soluble carbohydrate accumulation in Suc- and hexose-accumulating fruit does not support this.

The molar concentration of sugars remained approximately constant in the hexose- and Suc-accumulating fruit during most of development, with the Suc-accumulating fruit having a statistically significant lower sugar concentration than the hexose-accumulating fruit ($P = 0.95$, $n = 6$). During the final week of fruit development the concentration of sugars increased by approximately 50% in both Suc- and hexose-accumulating fruit (Fig. 5b), corresponding to the period of rapid fruit expansion. Both the hexose- and Suc-accumulating fruit had similar sugar concentrations at the red-ripe stage (approximately 130 μmol/g fresh weight), indicating a tendency toward a similar osmotic balance in both kinds of fruit.

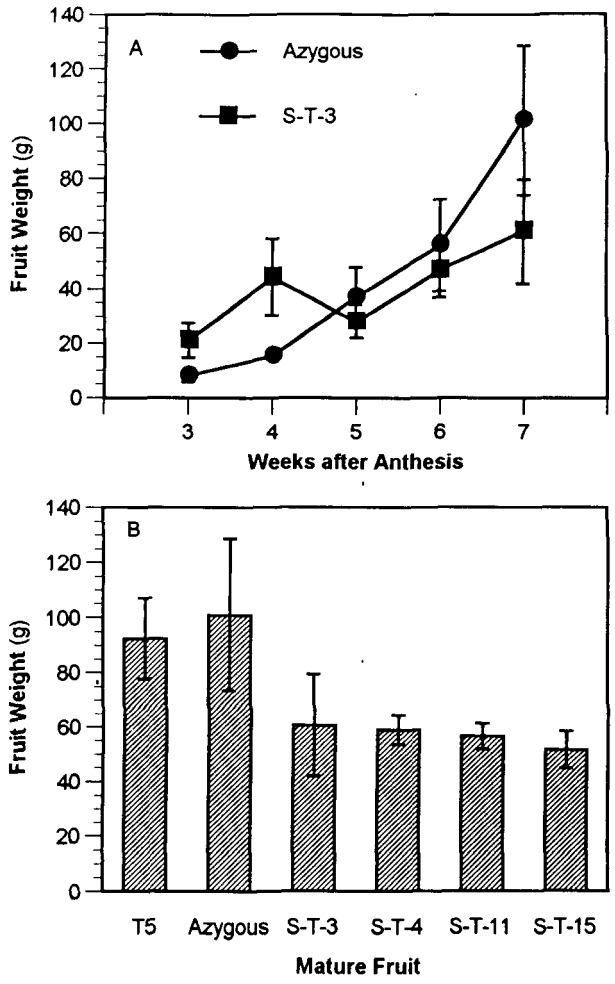

Figure 4. Fruit size. A, Fruit weight during fruit development of *35S/TIV1* antisense lines S-T-3 (■) and azygous control line (●). Three fruit were sampled per data point. Bars indicate SES. B, Weight of mature fruit from *35S/TIV1* antisense lines S-T-3, S-T-4, S-T-11, and S-T-15 and control lines T5 and azygous. Three fruit were sampled per line. Bars indicate SES.

Acid Invertase Activity and Protein

Invertase enzyme activity and immunologically detectable invertase protein were determined in pericarp extracts throughout development (Fig. 6). Acid invertase activity levels throughout most of fruit development until the 6th week after anthesis were not statistically different between transgenic antisense plants (S-T-3 and E-T-8) and controls (azygous segregant and T5). However, in the 7th week after anthesis, acid invertase activity increased in E-T-8 and control fruit to very high levels and decreased in S-T-3 fruit to very low levels (Fig. 6). Acid invertase activity levels at 7 weeks were statistically different between S-T-3 and T5 fruit ($P = 0.95$, $n = 3$). A 52-kD protein was immunologically detected with antibodies raised to a TIV1 fusion protein in all of the genotypes between 3 and 6 weeks after anthesis, in agreement with the similar levels of acid invertase activity found in these genotypes. In the final week of fruit development, large differences in the 52-kD immunologically detected protein and activity were observed. E-T-8 showed a moderate decrease in acid invertase activity and immunologically detectable protein relative to control lines. Invertase activity and immunologically detectable protein fruit were dramatically reduced in the S-T-3 line during the final week of development. The close correlation between acid invertase activity and the immunologically detectable 52-kD protein suggests that this protein is the major form of acid invertase in developing tomato fruit.

These results, demonstrating similar levels of invertase activity and protein in early fruit development, contrast

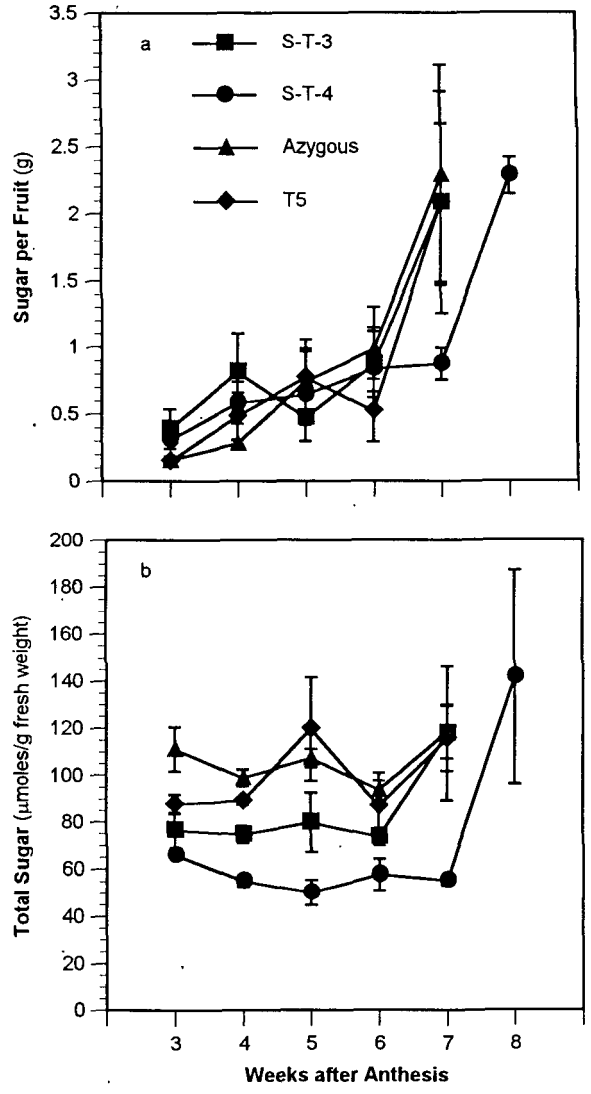

Figure 5. Sugar per fruit and molar sugar concentrations during fruit development. a, Sugar per fruit during fruit development of *35S/TIV1* antisense lines S-T-3 (■) and S-T-4 (●) and control lines azygous (▲) and T5 (♦). Three fruit were sampled per data point. Bars indicate SES. b, Molarity of total sugar in fruit pericarp during fruit development of *35S/TIV1* antisense lines S-T-3 (■) and S-T-4 (●) and control lines azygous (▲) and T5 (♦). Sugar molarity was calculated based on total fruit weight. Three fruit were sampled per data point. Bars indicate SES.

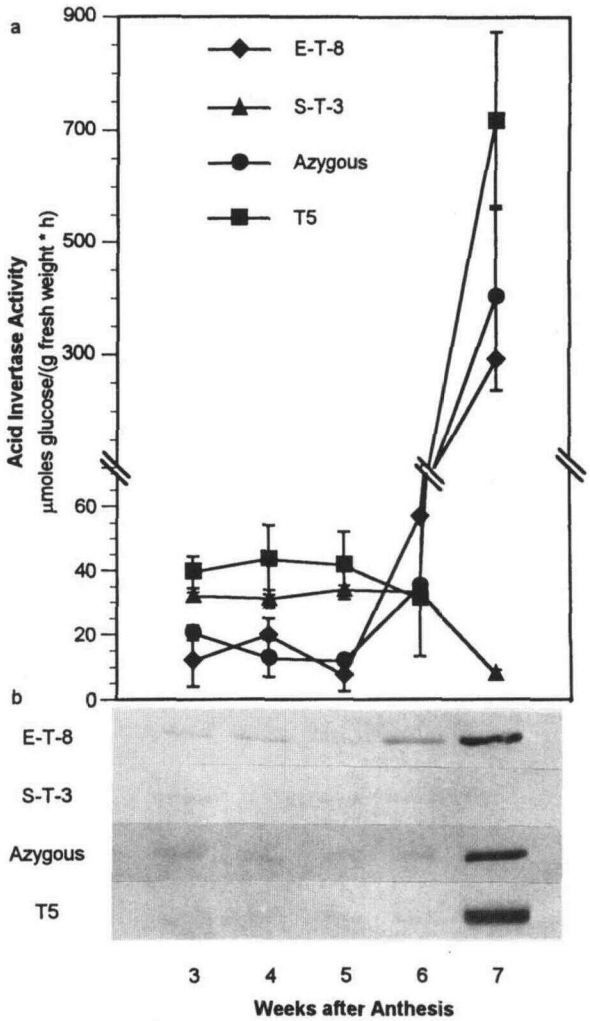

Figure 6. *Acid invertase activity and protein in fruit pericarp during development.* a, Acid invertase enzyme activity assayed in vitro in fruit pericarp during development in the *E8/TIV1* antisense line E-T-8 (♦), *35S/TIV1* antisense line S-T-3 (▲), and control line azygous (●) and T5 (■). Note the split scale on the activity graph. Three fruit were sampled per data point. Bars indicate SEs. b, Acid invertase protein in fruit pericarp during development in *E8/TIV1* antisense line E-T-8 (♦), *35S/TIV1* antisense line S-T-3 (♦), and control lines azygous (●) and T5 (■). Acid invertase protein is indicated as the 52-kD band from a western blot of total protein from 15 mg fresh weight of pericarp pooled from three fruit. Bars indicate SEs.

with analyses of sugar composition, which clearly showed high Suc levels in the S-T-3 line, even in early fruit development. This discrepancy suggests that there are likely to be multiple invertases expressed in early fruit development but that only one, the *TIV1* gene product, controls the Suc/hexose ratio in fruit. We reason that, if the *TIV1* gene product is a relatively minor component of total invertase activity in early fruit development, then assays of total invertase activity during this period apparently do not reveal the relatively small decrease in activity that results from expression of the *35S/TIV1* antisense gene. However, this antisense gene confers a pronounced increase in the Suc/hexose ratio.

Enzymes of Fruit Sugar Metabolism

Changes in Suc and Glc concentrations affect gene activity in potato (Wenzler et al., 1989), soybean (Mason et al., 1992), and maize (Koch et al., 1992). The activities of Suc synthase, Suc-P synthase, and neutral invertase were measured in vitro during fruit development to determine whether gene activities related to Suc metabolism might be affected by altered sugar composition. Suc synthase activity decreased from the highest levels at 3 weeks after anthesis to very low levels at 7 weeks after anthesis (Fig. 7a). The wide variation at 3 weeks was most likely due to

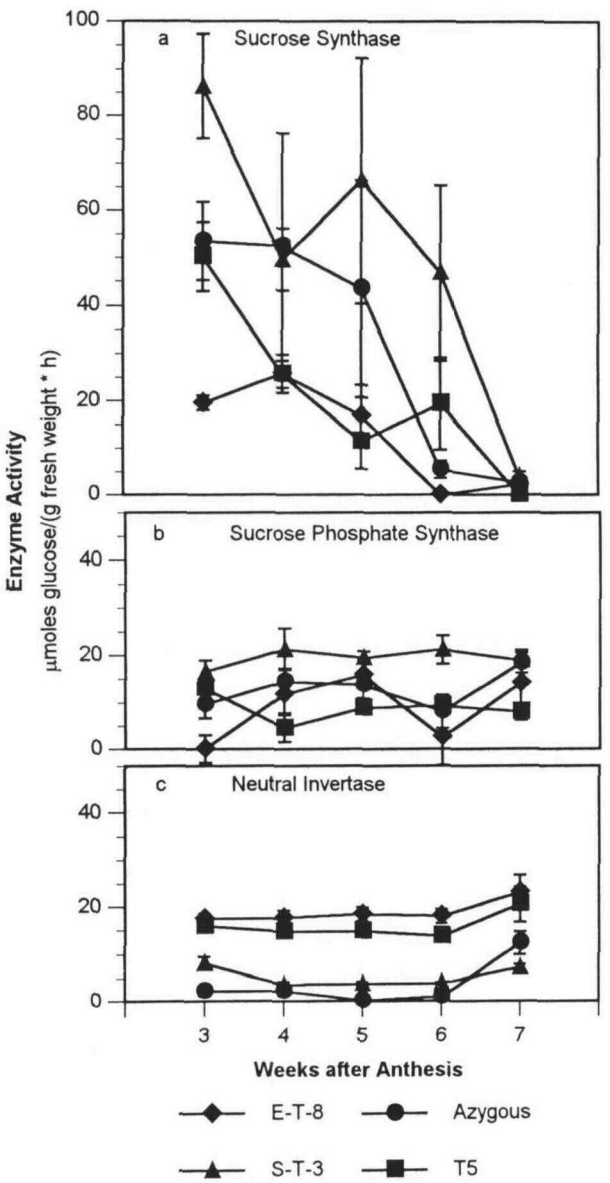

Figure 7. *Activity of enzymes of carbohydrate metabolism in developing fruit.* Activity of enzymes involved in carbohydrate metabolism were assayed in vitro in fruit pericarp during development in *E8/TIV1* antisense line E-T-8 (♦), *35S/TIV1* antisense line S-T-3 (♦), and control lines azygous (●) and T5 (■). a, Suc synthase; b, Suc-P synthase; c, neutral invertase. Three fruit were sampled per data point. Bars indicate SEs.

the lack of complete synchronization of fruit development during this period of rapid change in enzyme activity. Suc-P synthase levels were relatively constant throughout the developmental period assayed (Fig. 7b). The activities of Suc synthase and Suc-P synthase in the S-T-3 antisense were somewhat higher. There were constant levels of invertase activity at pH 7.5 in all of the genotypes (Fig. 7c). It did not appear that Suc percentage had any consistent effect on levels of the enzymes of sugar metabolism that we examined.

Fruit Respiration

One possible advantage of Suc accumulation relative to hexose accumulation is that Suc is theoretically less accessible to metabolic or respiratory losses (Salerno and Pontis, 1978). Tomato fruit undergo a respiratory climacteric during ripening that is triggered by ethylene, and *TIV1* antisense genes altered the respiratory behavior during fruit ripening (Fig. 8). Fruit were picked at the breaker stage, ripened in closed jars with a continuous flow of air, and rates of CO_2 and ethylene production were measured. The Suc-accumulating transgenic fruit differed dramatically from the control fruit during the respiratory climacteric. Respiration was 25 to 100% higher in *35S/TIV1* antisense fruit (Fig. 8a), and ethylene evolution was 100% higher in high Suc *35S/TIV1* antisense and E-T-8 fruit than in control fruit (Fig. 8b). Control fruit carrying the *35S*/antisense transgenes for endoglucanase-1 or endoglucanase-2 did not exhibit any changes in the respiratory climacteric (data not shown), suggesting that transgenes alone do not cause the aberrant physiology.

DISCUSSION

Antisense TIV1 Chimeric Gene Results in Elevated Levels of Suc in Tomato Fruit

Tomato fruit acid invertase levels were previously correlated with Suc accumulation in fruit of the wild tomato relative *L. chmielewskii* (Yelle et al., 1988), as well as *L. esculentum* populations into which the *L. chmielewskii* allele of the *sucr* locus was introgressed (Yelle et al., 1991; Klann et al., 1993). It was also demonstrated that the *TIV1* gene (acid invertase) is tightly linked to the *sucr* locus, leading to the suggestion that the *TIV1* gene controls Suc accumulation in *L. chmielewskii* and its derivatives. In the present study the introduction of a *TIV1* antisense gene in transgenic tomato dramatically altered soluble sugar composition, resulting in an increased Suc concentration. These results agree with those from another recent study in which an antisense acid invertase transgene was used to alter the sugar composition of tomato fruit; however, in that study this resulted in much lower proportions of Suc (Ohyama et al., 1995).

Levels of acid and neutral invertase activity during the early stages of development were not significantly different in T_1 fruit, even in transgenic lines with elevated levels of Suc that approached those of *L. chmielewskii* fruit (Yelle et al., 1988). It is likely that the invertase activity measured in young, developing fruit expressing the *35S/TIV1* antisense transgene may be the product of a second gene that shares physical and immunological similarities with TIV1.

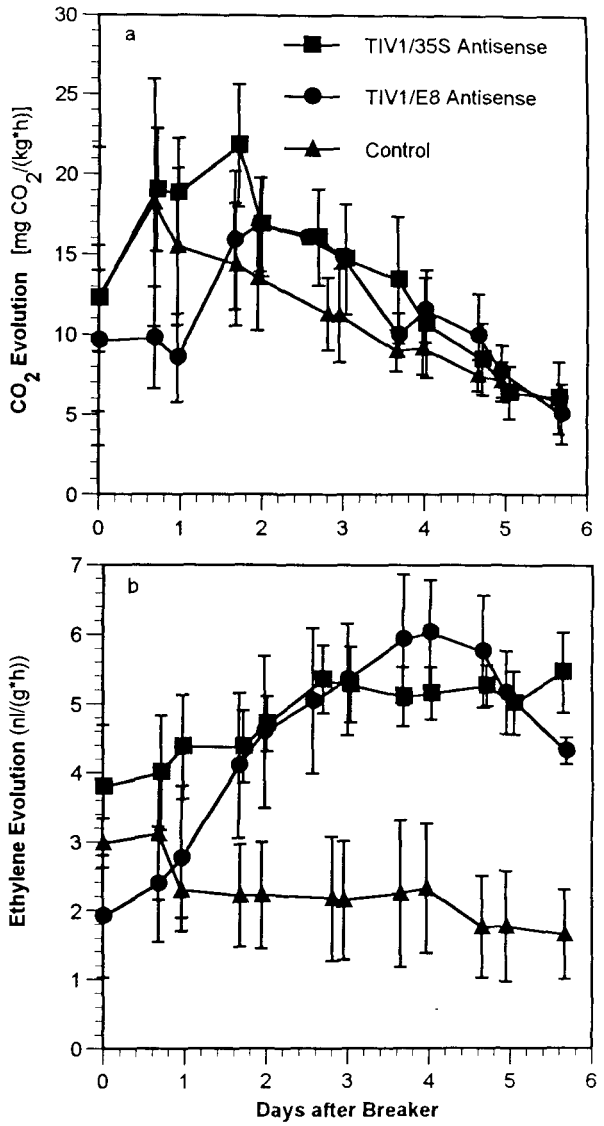

Figure 8. Respiration and ethylene evolution during fruit ripening. a, Respiration during fruit ripening of *35S/TIV1* antisense lines S-T-3 and S-T-15 (■), *E8/TIV1* antisense line E-T-8 (●), and azygous control line (▲) during ripening off the vine. Breaker indicates the 1st d of ripening. Six fruit were sampled for the *35S/TIV1* antisense data point, and three fruit were sampled for the *E8/TIV1* antisense and control data points. Bars indicate SEs. b, Ethylene evolution during fruit ripening of *35S/TIV1* antisense lines S-T-3 and S-T-15 (■), *E8/TIV1* antisense line E-T-8 (●), and azygous control line (▲) during ripening off the vine. Breaker indicates the 1st d of ripening. Six fruit were sampled for the *35S/TIV1* antisense data point, and three fruit were sampled for the *E8/TIV1* antisense and control data points. Bars indicate SEs.

Soluble and insoluble acid invertase activity found in tomato pericarp is thought to represent intracellular and extracellular forms, respectively, of the enzyme. The loss of soluble activity alone correlates with Suc accumulation in *L. hirsutum* and in Suc-accumulating progeny derived from an interspecific cross with *L. esculentum* (Miron and Schaf-

fer, 1991). Antibodies raised to the TIV1 fusion protein described here have been used to localize immunoreactive protein in both the cell wall and vacuole of tomato fruit pericarp (Miron, 1994). Acid invertase activity has been localized in the cell wall in tomato fruit (Sato et al., 1993). These results suggest that further work is needed to elucidate the molecular basis of invertase activity in the early developmental stages of tomato fruit.

Higher Suc Composition Results in Smaller Fruit Size

Expression of the 35S/TIV1 antisense gene and the resulting accumulation of Suc in tomato fruit leads to a 30% reduction in fruit size. Soluble acid invertase activity has been correlated previously with cell expansion in developing leaves. In *Phaseolus vulgaris* the specific activity of soluble acid invertase increases during the early stages of leaf expansion and peaks at the time of most rapid cell expansion (Morris and Arthur, 1984). The rapid phase of lamina expansion is characterized by high concentrations of hexose sugars and low concentrations of Suc. As cell enlargement declines, the concentration of hexose decreases and Suc increases. It is not clear in these studies whether acid invertase promotes cell expansion energetically (Ricardo and ap Rees, 1970) or through increased osmotically active sugars (Howard and Witham, 1983).

During the final week of fruit development, a change in the concentration of osmotically active sugars in hexose- and Suc-accumulating fruit correlates with a change in fruit size. In fully ripe fruit the osmotic concentration of soluble sugars is the same in all genotypes, suggesting that the water influx that drives fruit expansion is closely related to the concentration of osmotically active soluble sugars. Therefore, tomato fruit accumulate water until they reach a similar threshold of soluble sugar concentration or perhaps a similar turgor.

Elevated Ethylene Evolution Rates May Be Due to Smaller Fruit Size

Suc-accumulating tomato fruit had higher rates of ethylene evolution and respiration. Because expression of the antisense *TIV1* gene resulted in reduced acid invertase levels, elevated Suc concentration, and decreased fruit size, it is difficult to ascribe the effects on ethylene evolution and respiration to a single physiological parameter. However, it seems likely that the decrease in fruit size and, by inference, the reduction in cell size could account for these differences. Assuming that the respiratory and ethylene biosynthetic pathways remain constant on a per-cell basis, rates of both ethylene evolution and respiration expressed on a fresh-weight basis would be proportionately higher in the smaller Suc-accumulating fruit.

At odds with this explanation was the *E8/TIV1* antisense-expressing fruit, which also had elevated rates of ethylene production, even though these fruit accumulated hexose and were similar in size to control fruit. Because the native *E8* gene encodes a protein that suppresses ethylene evolution during fruit ripening (Peñarrubia et al., 1992), it is possible that the presence of several copies of the *E8* promoter may have reduced expression of the native *E8* gene through competition for regulatory factors or by co-suppression.

The results presented here demonstrate that the expression of a *TIV1* antisense gene results in higher Suc concentrations and smaller size in tomato fruit. Although the change in sugar composition does not affect the amount of sugar accumulated per fruit, Suc-accumulating fruit appear to acquire less water during development and exhibit a corresponding reduction in expansion. These transgenic lines illustrate the potential of altering soluble sugar composition in fruit tissues through the modification of expression of a single invertase gene.

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