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PCR-generated molecular markers for the invertase gene and sucrose accumulation in tomato

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Abstract The green-fruited tomato species, *Lycopersicon hirsutum*, unlike the domesticated red-fruited species, *L. esculentum*, accumulates sucrose during the final stages of fruit development, concomitant with the loss of soluble acid invertase activity. In order to study the genetic linkage of sucrose accumulation to the invertase gene, part of the invertase gene from *L. hirsutum* was cloned, sequenced and the sequence compared with the invertase sequence of the red-fruited *L. esculentum*. Several base changes were found in the coding region of the two invertase genes. Based on these base -pair differences, we developed a species-specific PCR assay capable of determining, in a single PCR reaction, the origin of the invertase gene in segregating seedlings of an interspecific cross. Our results indicate that the invertase gene is genetically linked to sucrose accumulation in the green-fruited *L. hirsutum*.

Key words *Lycopersicon* sp. · Sugar accumulation · β-fructofuranosidase

Introduction

Two subgenera are recognized in the genus *Lycopersicon* (Davies 1966). The *Eulycopersicon* subgenus is red-fruited and predominantly accumulates the hexose sugars fructose and glucose (Davies 1966; Davies and Kempton 1975). This subgenus includes the cultivated *L. esculentum* species, in addition to *L. pimpinellifolium* and *L. cheesmanii*. The *Eriopersicon* subgenus includes the green-fruited species, which predominantly accumulate sucrose (Davies 1966; Yelle et al. 1988; Miron and Schaffer 1991; Yelle et al. 1991; Stommel 1992).

In studies carried out on various sucrose-accumulating tissues it has been shown that such tissues undergo a metabolic transition from their growing stage to their sucrose-storing stage. The growing stage is characterized by high activities of vacuolar acid invertase, a sucrose-hydrolyzing enzyme, while the storage stage is characterized by very low activities of vacuolar acid invertase (Hatch and Glasziou 1963; Kato and Kubota 1978; Giaquinta 1979; Avigad 1982; Schaffer et al. 1987; Hubbard et al. 1989; Schaffer et al. 1989). A low activity of vacuolar acid invertase has also been shown in three green-fruited tomato species – *L. chmielewskii*, *L. hirsutum* and *L. peruvianum* – that accumulate sucrose (Manning and Maw 1975; Yelle et al. 1988; Miron and Schaffer 1991; Stommel 1992). Increased activity of invertase was shown in two red-fruited species, *L. esculentum* and *L. pimpinellifolium*, which have low levels of sucrose (Manning and Maw 1975; Sato et al. 1993). The loss of vacuolar acid invertase activity is presumably a prerequisite for sucrose accumulation, because the enzyme is compartmentalized in the vacuole, as is sucrose (Leigh et al. 1979), and efficiently hydrolyzes imported sucrose to hexoses. Therefore, vacuolar acid invertase plays a key role in sucrose metabolism, either preventing or facilitating its accumulation.

Sucrose accumulation in tomato is a monogenic recessive trait (*sucr*) as was shown in the interspecific crosses of *L. esculentum* (sucrose non-accumulating species) with *L. chmielewskii* and *L. hirsutum* (sucrose-accumulating species) (Yelle et al. 1991; Stommel and Haynes 1993). The invertase gene which appears to be present in one copy per haploid genome in *L. esculentum* (Elliot et al. 1993) and *L. chmielewskii* (Klann et al. 1993) is a natural candidate to control sucrose accumulation. Indeed, a genetic linkage between the invertase gene and sucrose accumulation was demonstrated by a restriction fragment length

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polymorphism (RFLP) analysis in a cross between *L. esculentum* and *L. chmielewskii*, by which *sucr* could be assigned to the right arm of chromosome 3 (Chetelat et al. 1993). The interspecific hybrid between *L. chmielewskii* and *L. hirsutum* accumulates sucrose, suggesting a common genetic basis for sucrose accumulation in both species (Chetelat et al. 1993). Finally, a correlation between increased invertase transcription and low level of sucrose has been shown; invertase transcription increases throughout fruit development in *L. esculentum* and *L. pimpinellifolium* fruits, which do not accumulate sucrose (Elliot et al. 1993; Sato et al. 1993). On the other hand, no recognizable transcription has been observed in *L. chmielewskii* (Klann et al. 1993).

Tomato invertase gene has been cloned from two red-fruited species of tomato, *L. esculentum* (Klann et al. 1992; Elliot et al. 1993; Sato et al. 1993) and *L. pimpinellifolium* (Elliot et al. 1993). The coding sequences of *L. esculentum* and *L. pimpinellifolium* acid invertase are identical (Klann et al. 1992; Elliot et al. 1993), and only minor changes in one intron and in the putative regulatory regions were found between the genomic clones of the two red-fruited species (Elliot et al. 1993). However, a few differences were found in the coding region between two varieties of *L. esculentum*, cv 'Super First' and cv 'UC82B' (Klann et al. 1992; Elliot et al. 1993; Sato et al. 1993), with sequence homology of 98.9%. Cloning of the invertase gene from sucrose-accumulating green-fruited species has not been reported.

In this study we cloned part of the *L. hirsutum* f. *typicum* invertase gene using the polymerase chain reaction (PCR). Based on the sequence differences from the *L. esculentum* invertase gene, we developed a species-specific PCR assay that enables us to determine the origin of the invertase gene in segregating populations from the interspecific cross.

Materials and methods

Plant material

Lycopersicon hirsutum f. *typicum* (LA 1777, kindly supplied by Dr. M. Pilowsky and originally a generous gift to him from Dr. C.M. Rick) and a commercial cultivar and breeding lines of *L. esculentum* Mill were used in this work. Other species used included: *Lycopersicon hirsutum* f. *glabratum* (PI 134418), *L. chilense* (PI 128650), *L. chmielewskii* (PI 379029), *L. peruvianum* (PI 128650), *L. cheesmanii* (PI 365896), and *L. pimpinellifolium* (PI 303662).

All plants were greenhouse-grown according to standard methods. Flowers of *L. hirsutum* f. *typicum* were sib-pollinated and tagged on the day of anthesis; flowers of *L. esculentum* were allowed to self-pollinate and were tagged on the day of anthesis.

Carbohydrate determination and acid invertase assay

Sugar analyses and invertase activity assays were performed as described by Miron and Schaffer, (1991). Fruit portions (about 500 mg fresh weight) were immediately placed in 80% ethanol for sugar determination. An additional sample was frozen at –20°C for the assay of invertase activity. Soluble sugars were extracted 3 times in hot ethanol and separated by HPLC using a Bio-Rad Fast Carbohydrate column with double-distilled H_2O as solvent, according to the manufacturer's directions and refractometric detection. Sucrose, glucose and fructose were identified by their retention times and quantified according to standards.

DNA extraction and Southern analysis

Genomic DNA was isolated from seedling tissue of the various species of tomato. The DNA was prepared by freezing leaves of 2- to 3-week-old seedlings (0.1–1 g) in liquid nitrogen, grinding and thawing in extraction buffer (2% CTAB w/v; 1% PVP 40T; 1.4 *M* NaCl; 100 m*M* TRIS-HCl, pH 8.0; 20 m*M* EDTA pH 4.8; 0.3% β-mercaptoethanol). The slurry was warmed at 60°C for 30 min and extracted with chloroform/isoamylalcohol. Nucleic acids were precipitated with 2/3 volume of cold isopropanol, washed with 76% ethanol and 10 m*M* ammonium acetate for 20 min, precipitated and resuspended in TE.

The nucleic acids were treated with RNase, precipitated again with two volumes of ethanol and resuspended in TE or H_2O according to the procedure of Doyle and Doyle (1990). For Southern blot analysis, 10-μg samples of tomato genomic DNA were digested with various restriction enzymes, electrophorated on a 1% agarose gel and blotted onto nylon membranes (Hybond-N, Amersham).

Hybridization was carried out overnight at 42°C in 25% formamide, 5× SSPE, 6× Denhardts solution and 150 μg/ml denatured herring sperm DNA with a [^{32}P]-labeled probe prepared from the cDNA of tomato invertase obtained from A. B. Bennett (Davis, Calif., USA). The probe was labeled with [^{32}P]-dCTP with a random primer kit according to the manufacturer's instructions. The blot was washed (0.5× SSC, 0.1% SDS in 42°C) and then exposed to Kodak X-ray film at –70°C.

PCR amplification, cloning and sequencing

PCR was carried out in 25 ml of solution containing 50–100 ng of genome DNA; 2 m*M* $MgCl_2$; 10 m*M* β-mercaptoethanol; 16.6 m*M* $(NH_4)_2SO_4$; 0.2 mg/ml BSA; 67 m*M* Tris-HCl, pH 8.4; dNTP (0.3 m*M* each); 3 units of *Taq* DNA polymerase (A.B peptide); 40 mM of each primer, under a drop of mineral oil. Amplification was performed in a DNA Thermal Cycler (Perkin Elmer Cetus) programmed for 1 cycle of 3 min at 94°C, 1.5 min at 60°C and 2 min at 72°C, followed by 35 cycles of 1 min at 94°C and a final incubation of 5 min at 72°C. Aliquots of 10 ml of each PCR reaction was run on a 1% agarose gel and visualized by ethidium bromide staining or blotted and hybridized with [^{32}*P*]-labeled tomato invertase as described before. Lambda DNA digested by *Hin*dIII was used as the molecular weight markers.

Oligonucleotide primers were prepared by a Pharmacia gene assembler and purified with an OPC column. The following primers were used: (1) AGG GAT GGT ACC ACC TTT TT, (2) CGA ATT CCC ATA GTC TAG TCT CAA, (3) GGG TGT CCA TTT GTT CTT TGT, (4) TGT CCA ACC CGT TTG TTT TTT. Primers 1 and 2 were used to clone the *L. hirsutum* invertase gene. Primer 1 includes a native *Kpn*I/Asp718 restriction site while primer 2 includes an *Eco*RI restriction site that was added for cloning purposes. Primer 3 is specific to the *L. hirsutum* invertase gene, and primer 4 is specific to the *L. esculentum* invertase gene.

The PCR product was separated on 1% agarose gel and eluted using the NA45 DEAE membrane (Schleicher and Schull). The fragment was cloned into pBluescript Vector (Stratagene). In one case, the pBluescript was cut with *Eco*RV (blunt end); in a second case, pBluescript was cut with *Eco*RI and Asp718 and ligated to a fragment cut with the same enzymes.

Transformation of *E. coli* JM101 cells and plasmid isolation were done according to standard procedures (Sambrook et al. 1989).

The DNA was sequenced in the laboratory of the DNA Analysis Biological Services at the Weizmann Institute of Science (Rehovot, Israel) using automated DNA sequence analysis with an Applied Biosystems 373 Analyzer.

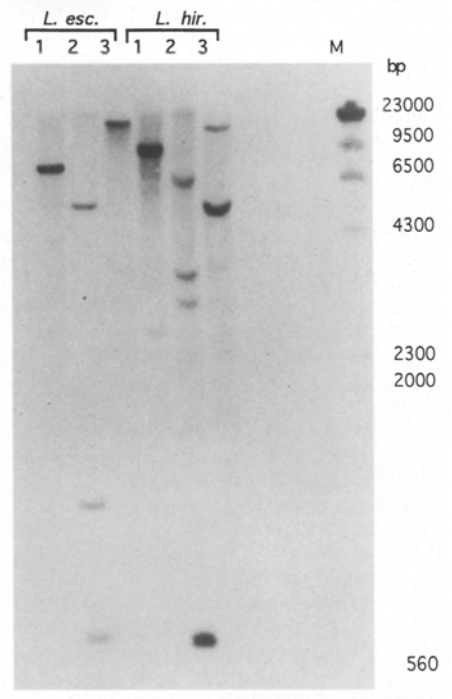

Fig. 1 Southern blot analysis of *L. esculentum* and *L. hirsutum* genomic DNA with *L. esculentum* invertase cDNA as a probe. An autoradiogram is shown following the digestion of tomato genomic DNA with various restriction enzymes: *lane 1 Bam*HI, *lane 2 Hin*dIII, *lane 3 Eco*RI. The DNA was size-fractionated on an agarose gel, blotted and hybridized with [^{32}P]-labeled tomato invertase cDNA. Each lane contains approximately 10 μg of DNA. *M* size marker given in base pairs

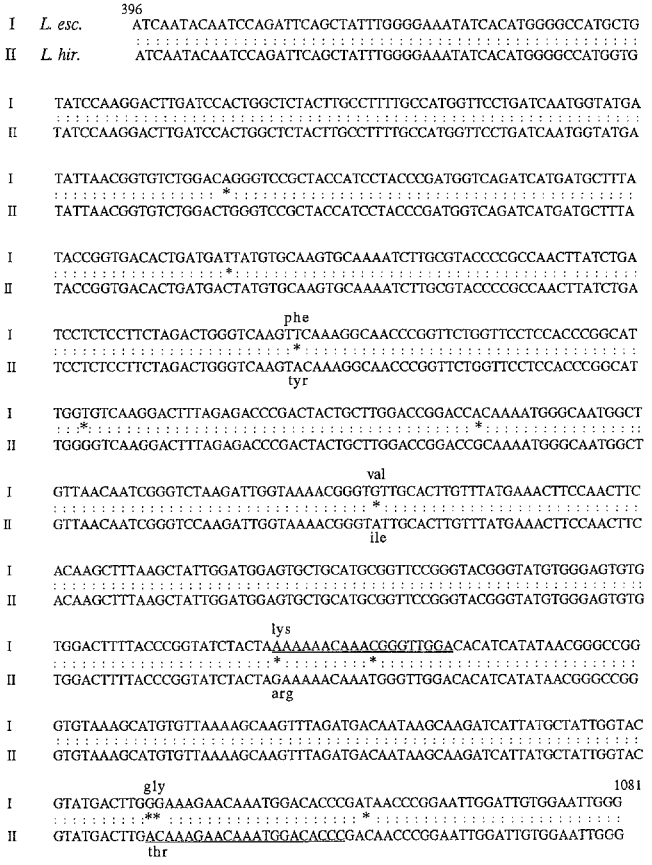

Fig. 2 Sequence comparison of the cloned part of the *L. hirsutum* invertase gene with the *L. esculentum* invertase gene [the *L. esculentum* sequence was taken from Klann et al. (1992)]. The presented sequence of *L. hirsutum* does not include primers 1 and 2, which were used to clone it. The base pair differences are marked with *asterisks* and putative amino acid changes are shown. *L. hirsutum*- and *L. esculentum*-specific primers are *underlined*

Results

Southern blot analysis of *L. esculentum* and *L. hirsutum* invertase genes

In order to estimate the number of invertase genes in *L. hirsutum*, genomic DNA of *L. hirsutum* was cut with three different restriction enzymes and probed with *L. esculentum* invertase cDNA. As shown in Fig. 1, the few strongly hybridizing bands are large enough to accommodate only one or a few copies of the coding sequence, suggesting a single locus for the homologous invertase gene in *L. hirsutum*. A comparison of the restriction patterns of *L. hirsutum* and *L. esculentum* following identical Southern blot analysis (Fig. 1) showed that RFLP was obtained with the two tomato species for each of the enzymes and for almost all of the bands.

Cloning part of *L. hirsutum* invertase gene by PCR

The RFLP obtained with each of the enzymes indicates the existence of several sequence differences within and around invertase gene. In order to develop a PCR assay that would differentiate between the invertase genes of *L. hirsutum* and *L. esculentum*, we cloned part of the *L. hirsutum* invertase gene by PCR. Two synthetic primers homologous to the *L. esculentum* invertase gene were used with total DNA of *L. hirsutum* as a template, as described in the Materials and methods. A fragment of the expected size was obtained, indicating the asence of an intron within this region. The fragment, which cross-hybridized with the *L. esculentum* invertase gene, was cloned from two independent reactions, and the sequence of the two clones was identical. A sequence comparison between the cloned fragment and the *L. esculentum* cv 'UC82B' invertase sequence (Klann et al. 1992) revealed 11 dispersed base changes, none of which overlapped with those found between the two varieties of *L. esculentum*, cv 'Super First' and cv 'UC82B' (Klann et al. 1992; Elliot et al. 1993; Sato et al. 1993). Five of the base-pair changes caused four putative amino acid changes, as shown in Fig. 2.

Species-specific PCR assay for the invertase gene

The sequence differences between the *L. esculentum* and the *L. hirsutum* invertase gene were used to synthesize spe-

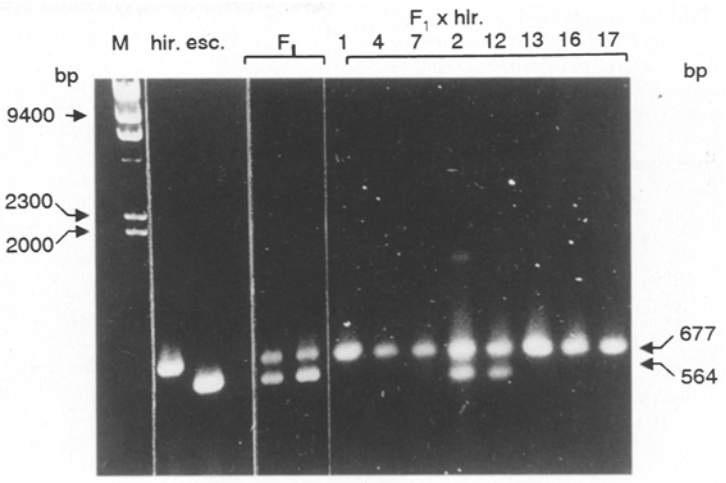

Fig. 3 PCR assay for the invertase gene. *M* size markers in base pairs, *hir. L. hirsutum*, *esc. L. esculentum*, F_1 *L. esculentum* × *L. hirsutum*, F_1 × *hir.* backcross of F_1 to *L. hirsutum* (the *numbers* represent the plant number in the trial)

Table 1 Segregation for invertase gene from progenies of a heterozygous (HE) BC_1F_3 plant

Cross	EE	EH	HH	χ^2
Self of HE	7	16	8	$P>0.99$ (1:2:1)
HE × EE (*L. esc.*)	12	12	0	$P>0.99$ (1:1)

cies-specific PCR primers (Fig. 2). The primers were designed so that when they are used with the upstream primer (primer 1; Materials and methods), different fragment sizes will be obtained with each species. *L. esculentum* invertase gives a 564-bp fragment with the *L. esculentum*-specific primer, while *L. hirsutum* invertase gives a 677-bp fragment with the *L. hirsutum*-specific primer (Fig. 3). The two species-specific primers were used jointly in a single PCR reaction together with the upstream primer (primer 1; Materials and methods) in order to check interspecific F_1 hybrid plants and segregating plants of an F_1 × *L. hirsutum* backcross. As shown in Fig. 3, F_1 hybrids of the interspecific cross between *L. hirsutum* and *L. esculentum* were heterozygous for the invertase gene (marked HE, for Hirsutum/Esculentum) and showed both fragments. Segregating plants of the backcross between the F_1 hybrid and *L. hirsutum* were either heterozygous (HE) and had both fragments, or homozygous (HH) for the *L. hirsutum* invertase gene and had only the *L. hirsutum* invertase fragment. Hence, a single PCR reaction is sufficient to determine the origin of the invertase gene in segregating plants of such an interspecific cross. Although in these early interspecific crosses segregation did not follow a Mendelian ratio for a single gene [consistent with the results of Chetelat et al. (1993)], in more advanced segregating populations Mendelian ratios were observed, as expected (Table 1). Segregation of the invertase gene was consistent with the monogenic 1:1 HE:EE ratio expected for the HE × EE cross and the 1:2:1 ratio for a segregating population from a selfed HE plant.

Genetic linkage of *L. hirsutum* invertase and sucrose accumulation

In order to test the genetic linkage of the *L. hirsutum* invertase gene to sucrose accumulation, sucrose content and invertase activity were measured in fruits of the interspecific F_1 hybrid and in the fruits of segregating plants of the cross F_1 × *L. hirsutum* . The fruits of all of the heterozygous plants had a relatively high level of invertase activity and a low sucrose:hexose ratio compared to the fruits of 2 HH plants (2 and 12) and to those of the *hirsutum* parent (Table 2).

The genetic linkage of the *L. hirsutum* invertase gene to sucrose accumulation was also tested in a more advanced segregating BC_1F_3 population. Here, too, mature fruits of 2 plants (3 and 11) homozygous for the *L. hirsutum* invertase gene had low levels of invertase activity and relatively high sucrose:hexose ratios (Table 2). Fruits of plants which were either heterozygous EH or homozygous EE showed low sucrose:hexose ratios and high invertase activities. Plant 4 appeared to be an exception and showed relatively low invertase activity, similar to that of the HH fruit, although with a low sucrose:hexose ratio. We cannot explain the low enzyme activity in this 1 plant, but it may be unrelated to the invertase gene and may not reflect in vivo invertase activity, as evidenced by the low sucrose:hexose ratio. Nevertheless, the molecular marker was capable of predicting sucrose:hexose ratios.

Comparison of additional *Lycopersicon* species

Additional *Lycopersicon* species were assayed for sugar and invertase activities and analyzed by PCR using the species-specific primers generated from the *L. hirsutum* f. *typicum* sequence. Sibling species *L. hirsutum* f. *glabratum* showed the *L. hirsutum* f. *typicum* allele (Fig. 4). Similarly, the hexose-accumulating red- and orange-fruited *L. pimpinellifolium* and *L. cheesmanii*, respectively, showed the

Table 2 Sugar content and invertase of mature fruit of *L. hirsutum*, *L. esculentum*, the interspecific F_1 and of several plants from the $F_1 \times L.hirsutum$ cross and BC_1F_3. The data are averages of 2–5 fruits per plant

Plant	Genotype[a]	Sucrose	Hexose	Total sugars	Suc/hex	Invertase activity µmol reducing sugar
		[mg (gf.wt)$^{-1}$]		[mg (gf.wt)$^{-1}$]		[mg (gf.wt)$^{-1}$ h^{-1}]
L. esc	EE	0.4	31.3	31.7	0.01	320
L. hir	HH	23.3	11.4	34.7	2.04	1
F_1	HE	1.6	35.7	37.3	0.04	297
$F_1 \times L.$ *hir*						
1	HH	13.2	4.6	17.8	2.80	3
2	HE	2.1	34.6	36.7	0.06	427
4	HH	4.1	5.6	9.7	0.73	5
7	HH	15.2	15.9	31.1	0.95	2
12	HE	1.6	18.6	20.2	0.08	34
13	HH	19.0	6.9	25.9	2.75	1
16	HH	18.5	8.4	26.9	2.20	1
17	HH	20.0	11.0	31.0	1.80	1
BC_1F_3						
1	EE	1.7	26.9	28.6	0.06	360
2	HE	2.2	38.4	40.6	0.05	759
3	HH	8.9	32.6	41.5	0.27	23
4	EE	2.9	36.9	39.8	0.07	24
6	HE	2.8	19.5	22.3	0.14	132
8	HE	0.7	22.7	23.4	0.03	520
9	EE	0.7	23.7	24.4	0.02	472
10	HE	3.3	48.6	51.9	0.06	300
11	HH	8.2	25.0	33.2	0.32	9

[a] *E L. esculentum* invertase allele, *H L. hirsutum* invertase allele

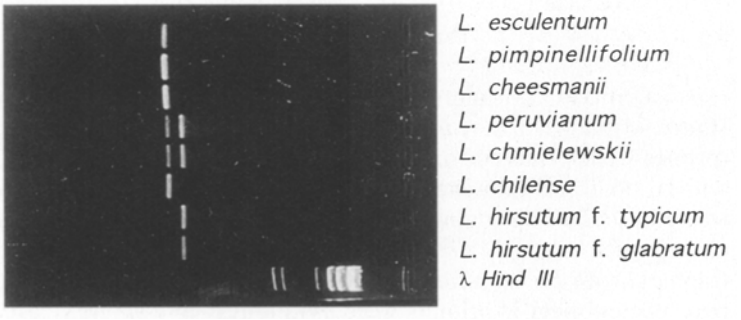

Fig. 4 PCR assay with various *Lycopersicon* species using the species-specific primers

L. esculentum allele. However, two of the green-fruited species, *L. chmielewskii* and *L. peruvianum* reacted with both the *L. esculentum*- and *L. hirsutum*-specific primers, whereas *L. chilense* reacted only with the *L. esculentum* primer.

Discussion

Sucrose accumulation in tomato fruits is a recessive trait that is predominantly dependent upon a low activity of invertase during the last stages of fruit development (Manning and Maw 1975; Yelle et al. 1988; Miron and Schaffer 1991; Stommel 1992; Sato et al. 1993). Low invertase activity has been shown by RFLP to be genetically linked to the single invertase gene of the green-fruited species, *L. chmielewskii* (Chetelat et al. 1993). In this work, instead of RFLP analysis we used a single reaction PCR assay to follow the segregation of the invertase gene within an interspecific cross between *L. hirsutum* and *L. esculentum*. Such a method requires base-pair differences between the two alleles at two distant locations. If only one base-pair difference exists, the analysis has to be done in two separate PCR reactions in order to identify heterozygote plants. The use of a single PCR reaction when possible should be very useful, especially when recessive monogenic traits are to be followed.

The segregation analysis of the invertase gene within the interspecific cross between *L. hirsutum* and *L. esculentum* indicates a genetic linkage of *L. hirsutum* invertase to sucrose accumulation. This extends previous results, based on sugar analyses, obtained with F_1 hybrids of *L. chmielewskii* and *L. hirsutum* (Chetelat et al. 1993). Such hybrids accumulate sucrose, indicating a common genetic basis and hence a monophyletic origin of the trait in these two green-fruited species.

Eleven dispersed base changes were found between *L. esculentum* and *L. hirsutum* within the 686-bp cloned fragment, 5 of which cause four putative amino acid changes. In comparison, the coding sequences of *L. esculentum* and *L. pimpinellifolium* acid invertase are identical (Klann et al. 1992; Elliot et al. 1993). However, 4 changes were found within the same region between two varieties of *L. esculentum*, cv 'Super First' and cv 'UC82B' (Klann et al. 1992; Elliot et al. 1993; Sato et al. 1993), none of which leads to amino acid changes. Nonetheless, it is unlikely that these amino acid changes are responsible for the different invertase activity in the two species, nor do we attribute any physiological significance to the sequence differences upon which the species-specific primers were based. This is further supported by the *L. chilense* PCR pattern which, although reacting specifically with the *L. esculentum*-specific primer, nevertheless has a low invertase activity and a high sucrose:glucose ratio, which is characteristic of the green-fruited species. This emphasizes that the development of species-specific primers for use with species other than *L. hirsutum* will require sequencing of the invertase gene from individual species and, preferably, individual donor parents.

The assumed monophyletic origin of sucrose accumulation in green-fruited tomato species (Chetelat et al. 1993) would suggest a high level of homology between the green-fruited invertase genes. Yet the species comparison with the species-specific primers indicated that, at least for the few base-pair differences utilized, there is no supportive correlation for a common evolutionary origin of sucrose accumulation. Still, a more extensive sequence analysis of the various species may well support a common evolutionary origin. In addition, it is also possible that a monophyletic origin of sucrose accumulation will be revealed within the promoter regions of the various invertase genes and a comparison of these areas may shed light on the molecular control of invertase activity and sucrose accumulation.

Reduced invertase expression and activity is a prerequisite for high fruit sucrose:hexose ratios. Yet the inheritance of the invertase gene is complicated by the wide range of invertase activity observed in populations segregating for the invertase gene. For example, the 2 HE plants analyzed in the $F_1 \times$*L. hirsutum* cross (Table 2) differed greatly in their invertase activity, but the lower extractable activity was sufficient to prevent sucrose accumulation. An even broader range of in vitro activity was observed in the more advanced segregating population, with segregants characterized by extremely high invertase activities, i.e., plant no. 2 of BC_1F_3 (Table 2). There is probably a threshold value of invertase activity above which sucrose does not accumulate; the physiological significance of activity above this value remains to be determined.

The level of fruit sucrose accumulation among the sucrose-accumulating genotypes is highly variable, as pointed out previously by Stommel and Haynes (1993) and Chetelat et al. (1993). In our early generation genetic material ($F_1 \times$*L. hir.* in Table 2), the sucrose:hexose ratio of the HH genotypes is high (from 0.73 to 2.80); however, in the more advanced material (BC_1F_3 in Table 2) HH genotypes has a more modest sucrose accumulation, together with normal hexose levels, leading to sucrose:hexose ratios of 0.27–0.32. This reduction in extent of sucrose accumulation is not necessarily concomitant with the advanced introgression of the *sucr* trait into the *L. esculentum* background and advanced lines can have high sucrose:hexose ratios, similar to that of the wild parent (Klann et al. 1993). Rather, the extent of sucrose accumulation may be related to differences in the developmental pattern of loss of invertase activity. Such variability in sucrose accumulation and hexose levels may be useful and the development of moderate sucrose-accumulating tomatoes with normal hexose levels may serve as an alternative goal to the development of high-sucrose-accumulating, low-hexose tomatoes.

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