Genetic Analysis of the Trait of Sucrose Accumulation in Tomato Fruit Using Molecular Marker

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Summary

The trait of sucrose accumulation in some wild species of tomato such as *L. chmielewskii*, *L. hirsutum* and *L. peruvianum* is thought to be monogenic and recessive. We hypothesized that this trait may be linked to acid invertase activity, and examined the relationship between sucrose accumulation and the acid invertase gene. We previously cloned a cell wall-bound form of acid invertase cDNA in tomato (*L. esculentum*) fruit. As a result of PCR analysis of the acid invertase gene in *L. esculentum* and *L. chmielewskii*, a difference in amplified fragment length was detected using one pair of primers. Each fragment was cloned and sequenced, identifying a difference in the sequence of the sixth (last) intron of the invertase gene. A total deletion of 11 bp was found in *L. chmielewskii* including the continuous sequence of the 10bp long “AAAAAGTTT” sequence. PCR analysis enabled us to characterize and distinguish the invertase allele between these species.

In the F2 population, all the sucrose accumulating plants were homozygous for the *L. chmielewskii* allele. This strongly suggested that the trait of sucrose accumulation was determined by the acid invertase gene at the molecular level. Another possible enzyme linked to the trait of sucrose accumulation, sucrose synthase, was examined, but no relationship was detected.

Key Words: tomato, sucrose, invertase, sucrose synthase, PCR, molecular marker.

Introduction

Sugars and acids as major components of soluble solids are important determinants of tomato fruit quality. Sugars especially affect tomato processing quality and flavor in fresh tomato. Melon and banana and so on accumulate large amounts of sugars including sucrose, while tomato (*L. esculentum*) accumulate lower amounts of sugars not including much sucrose. On the other hand, wild species of green-fruit tomatoes belonging to the subgenus *Eriopersicon* such as *L. chmielewskii*, *L. hirsutum* and *L. peruvianum* accumulates large amounts of sugars including sucrose. By introducing these traits of wild species to cultivated species (*L. esculentum*), more valuable tomatoes could be bred. It is thought that the trait of high sugar content may be associated with many genes, however the trait of sucrose accumulation may be monogenic and recessive (Yelle et al. 1991, Chetelat et al. 1993a, Chetelat et al. 1993b, Stommel 1993, Stommel and Haynes 1993). In tomato, carbon is translocated from leaves to fruits in the form of sucrose, and sucrose is hydrolyzed to glucose and fructose in fruits (Walker and Ho 1977, Damon et al. 1993). Sucrose in fruits is thought to be hydrolyzed by acid invertase, and low levels of acid invertase activity are thought to be associated with high levels of sucrose accumulation in *L. chmielewskii* (Yelle et al. 1988), *L. hirsutum* (Miron and Schaffer 1991) and *L. peruvianum* (Stommel 1992).

The level of acid invertase activity is controlled by the extent of acid invertase protein (Yelle et al. 1991, Endo et al. 1990), therefore it is thought that the trait of sucrose accumulation may be determined by the acid invertase gene of *L. chmielewskii*. Introducing the trait of sucrose accumulation into cultivated tomatoes can make it possible not only to improve the sweetness quality, but also to increase the total sugar content (Yelle et al. 1991). However, because the trait of sucrose accumulation is recessive and wild species also possess many undesirable traits, the breeding of sucrose accumulating tomatoes with desirable qualities in other traits may be difficult.

We have found that the level of acid invertase activity is controlled by the extent of acid invertase protein (Endo et al. 1990). We also previously determined that most of the acid invertase exist in the intercellular, cell wall-bound fraction in fruits of *L. esculentum*. Moreover the nucleotide sequence of this acid invertase cDNA has already been determined (Sato et al. 1993).

In this study we proved at the molecular level that the trait of sucrose accumulation is determined by the acid invertase gene, and acquired the useful molecular marker for the trait of sucrose accumulation.

Materials and Methods

**Plant material**

F2, BC1F2, BC2F2 and BC3F2 population were used in this study. F2 progeny were derived from a cross between *Lycopersicon esculentum* (cv PK 169) and *L. chmielewskii* (LA 1028) and backcrossed with *L. esculentum* (cv PK 169) several times, selfed to produce the BC3F2 generation.

For PCR analysis, six *L. esculentum* varieties, that is,
Kagome 70, Kagome 77, Kandenbanzo, Morioka No. 20 and two small fruited varieties (PK 165, PK 169) were used. For other wild species, L. pimpinellifolium (PI 344102), L. hirsutum var. glabratum (WIR 924), L. peruvianum (LA 2153), L. chilense (PI 128650, PI 128652) were analyzed.

The plants were grown in a green house and the fruit formation was induced by spraying 10 ppm GA3 and 150 fold dilution of TOMATO TONE® which includes 0.15 % β-Chlorophenoxyacetic acid.

Sugar concentration measurements
Whole tomato fruits were washed by water, squeezed, and then the aqueous fraction was filtered through a 0.45 μm nylon filter before HPLC analysis. 75 % acetonitrile solution was used as eluent. Sugars were identified and quantified by chromatography on an ASAIH-PAK NH2P-50 column (4.6 φ-250 L) and detected with a reflective index detector (Shimadzu RID-6 A). Concentrations were calculated from peak areas using glucose, fructose and sucrose standards, and shown by percentage gram per 100 ml of squeezed tomato juice.

Acid invertase gene analysis
From the cloned acid invertase cDNA sequence (Sato et al. 1993), PCR primers for amplifying the invertase gene were synthesized using a DNA Synthesizer Cyclone Plus (Milligen). Plant DNA was prepared by a modified procedure of Klimyk et al. (1993). Genomic DNA of L. esculentum, L. chmielewskii, F1, F2, BC1F2, BC2F2, BC3F2 plants were used as templates. Amplification was carried out in 25 μl volumes containing 10 mM Tris-HCl (pH 8.9), 1.5 mM MgCl2, 80 mM KCl, 500 μg/ml BSA, 0.1 % Sodium Cholate, 0.1 % Triton X-100, 240 μM dNTP (dATP, dCTP, dGTP, dTTP), 0.2 μM primer, approximately 200 ng of genomic DNA, and 1.0 unit of Tth DNA Polymerase (TOYOBO). Amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed for an initial cycle of five minutes at 95 °C for template denaturation and five minutes at 55 °C for primer annealing. This was followed by 40 cycles of three minutes at 72 °C for primer extension, one minute at 95 °C, and two minutes at 55 °C, with a final ten minutes incubation at 72 °C. Fragments generated by amplification were separated according to size on 3 % NuSieve 3 : 1 agarose (FMC BioProducts) gels, stained with ethidium bromide and visualized by illumination with ultraviolet light (254 nm). Some amplified fragments were cloned into pUC 118 and the sequence were determined by a A. L. F. DNA sequencer (Pharmacia).

Sucrose synthase gene analysis
A homology comparison of the sucrose synthase cDNA sequence of potato (Salanoubat and Belliard 1987) and corn (Werr et al. 1985) was performed to identify two pairs of primers for PCR amplification which were synthesized using a DNA Synthesizer Cyclone Plus (Milligen). Plant DNA was prepared by a modified procedure of Klimyk et al. (1993). Genomic DNA of L. esculentum, L. chmielewskii and F2 polulations were used as templates. Amplification was carried out as described above.

Fragments generated by amplification were digested with MboI, AflII, HapII, and separated according to size on 3 % NuSieve 3 : 1 agarose (FMC BioProducts) gels, stained with ethidium bromide and visualized by illumination with ultraviolet light (254 nm).

Results and Discussion
Sugar accumulation
F2 plants produced by crossing L. esculentum and L. chmielewskii didn’t accumulate much sucrose (less than 0.5 %) as in L. esculentum. Of the 95 plants analyzed in the F2 generation, 23 plants accumulated sucrose, and 72 plants didn’t accumulate much sucrose. Thus, the ratio of sucrose to hexose accumulating plants was approximately 1 : 3. As expected from other reports, the trait of sucrose accumulation is controlled by a single recessive gene. According to the report of Yelle et al. (1991), F2 segregating ratio was distorted to 1 : 14 by close linkage between the sucrose accumulating locus and a locus conferring sterility in crosses between L. esculentum and L. chmielewskii. In our study, all the plants were fruited by parthenogenesis treating with plant hormones, so F2 plants were segregated 1 : 3 as expected. Comparing the total sugar content between sucrose accumulator and hexose accumulator, sucrose accumulator accumulated about 14 % higher sugar than hexose accumulator (Table 1). This tendency was also found in the BC1F2 generation, and support the report of Yelle et al. (1991). That is, sucrose-accumulating fruit can accumulate more soluble carbohydrate than

<table>
<thead>
<tr>
<th>Population</th>
<th>Number</th>
<th>Fructose (%)</th>
<th>Glucose (%)</th>
<th>Sucrose (%)</th>
<th>Total sugar (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexose accumulator</td>
<td>36</td>
<td>4.55 ± 1.11</td>
<td>2.82 ± 0.91</td>
<td>0.12 ± 0.16</td>
<td>7.52 ± 1.00</td>
</tr>
<tr>
<td>Sucrose accumulator</td>
<td>15</td>
<td>1.25 ± 0.53</td>
<td>0.52 ± 0.29</td>
<td>6.80 ± 2.26</td>
<td>8.57 ± 2.53</td>
</tr>
</tbody>
</table>

1) Each point represents the mean ± SE of different plants of the F2 population. Values are shown by the percentage (g/100ml of squeezed tomato juice × 100).
2) Significant at the 10% level between values.
hexose-accumulating fruit while maintaining an equivalent osmotic potential, and also sucrose is metabolically less active than hexose and may be less accessible for loss through respiration (Pontis 1978). We also expected that L. esculentum could accumulate more sugar by accumulating sucrose.

**Acid invertase gene analysis**

The trait of sucrose accumulation in *L. chmielewskii* is controlled by a single recessive gene, and it is located on chromosome 3 linked to the RFLP marker TG 102 (Chetelat et al. 1993, Yelle et al. 1991). We hypothesized that this recessive gene is the acid invertase gene.

We have already cloned a cell wall-bound form of acid invertase cDNA in *L. esculentum*, and its sequence was almost identical to the vacuolar acid invertase cDNA (Elliott et al. 1993), and other acid invertase gene sequences previously reported (Klann et al. 1992, Ohyama et al. 1992). Using one pair of primers (Fig. 1 5'-1, 3'-1) specific for nucleotide sequence within the cloned acid invertase cDNA sequence, a difference in amplified fragment length by PCR using genomic DNA of *L. esculentum* and *L. chmielewskii* as a template was detected. Each fragment was cloned into pUC 118, and the sequence was determined. As a result, a difference in the sequence of the sixth (last) intron of the invertase gene was detected (Fig. 1). A total deletion of 11 bp was found, although other sequences were almost the same. Within the 11 bp deletion the continuous sequence of a 10 bp long fragment was of interest, because of its palindromic sequence "AAAGGTTTTTT". This sequence in *L. esculentum* could be deleted easier than other sequences (Kurosawa and Tonegawa 1982), so it may have been deleted in *L. chmielewskii* over the course of evolution. It is not clear whether this sequence plays any specific roles, and it is difficult to speculate that this difference influences the expression of the invertase gene. However, invertase allele of *L. esculentum* and *L. chmielewskii* can be distinguished by this PCR analysis. Using the genomic DNAs of F2 population as templates and 5'-2, 3'-2 primers (Fig. 1) for PCR, the fragment generated in 23 sucrose accumulating plants was the same size as that of *L. chmielewskii*. And in 72 hexose accumulating tomatoes, the generated fragment was the same size as that of *L. esculentum* (28 plants) or that of both species (heterozygous, 44 plants) (Fig. 2). In heterozygous plants, an additional third fragment was identified which migrated more slowly on gel electrophoresis. This fragment may have been produced by annealing of the *L. esculentum* allele with *L. chmielewskii* allele. The palindromic sequence forming a stem-loop structure may have caused altered mobility.

Acid invertase gene analysis in F2, BC1F2, BC2F2 and BC3F2 showed the segregating ratio almost followed mendelian fashion (Table 2). All the plants homogysous for the *L. chmielewskii* allele of the acid invertase gene accumulated sucrose, and any other plants didn't accumulate much sucrose. Also, all the progeny of sucrose accumulating plants accumulate sucrose without exception.

The results mentioned above proved at the molecular level that the trait of sucrose accumulation is determined by a single recessive acid invertase gene of *L. chmielewskii*.

Using the genomic DNAs of several *L. esculentum*
varieties including small fruited varieties and *L. pimpinellifolium, L. hirsutum, L. peruviana*, and *L. chilense* as templates, DNA fragments of all *L. esculentum* varieties and *L. pimpinellifolium* were generated and found to be the same size. The *L. hirsutum* amplified DNA fragment, the *L. peruviana* DNA fragment was a little longer than the *L. esculentum* DNA fragment, and two fragments were found in *L. chilense* (Fig. 3). Moreover, different fragments size was found in different lines of *L. chilense*. So sequence of this DNA region is very variable in tomato species and it is expected that sucrose accumulating and non-accumulating tomatoes can be also distinguished in these species.

As described above, we confirmed that the trait of sucrose accumulation is determined by the acid invertase gene. Recently, Klann *et al.* (1993) reported about RFLP analysis of the trait of sucrose accumulation using invertase DNA probes. The results of this study are supported by their genomic DNA analysis results. Also, Ohyama *et al.* (1995) reported that the suppression of invertase activity by the antisense gene caused the increase of sucrose content in fruits.

**Another possible enzyme**

It can be assumed that the trait of sucrose accumulation is mostly determined by the acid invertase gene. However, because the ratio of sucrose content to total sugar content in BC$_1$F$_2$ generation was distributed from 44% to 92%, other sucrose hydrolyzing enzymes or synthesizing enzymes may affect the trait of sucrose accumulation. To gain further insight into this possibility the relationship between sucrose accumulation and the sucrose synthase gene, which is thought to act as a sucrose hydrolyzing enzymes, (Yelle *et al.* 1988, Stommel 1992) was examined.

Since the nucleotide sequence of sucrose synthase gene in tomato was not determined yet, a homology comparison of the sucrose synthase gene in potato (*Dicondylae*) and corn (*Monocotyledoneae*) was performed. About 67% homology in nucleotide sequence was found between them. Perfectly matched sequences were selected for the synthesis of a pair of primers for PCR DNA amplification (Fig. 4). Using genomic DNAs as a template, a single fragment was detected in *L. esculentum* and *L. chilenses*. A 17 bp long oligonucleotide specific for DNA sequence between the two primers was synthesized and used as a probe for southern hybridization (Fig. 4). Southern probe hybridization strongly suggested that the amplified fragment was the sucrose synthase gene fragment. Recently, Wang *et al.* (1993) reported about the cloning of sucrose synthase cDNA in tomato. Our primers and probe used in this study perfectly matched their sequence data, suggesting each amplified fragment in *L. esculentum* and *L. chilenses* must be derived from the sucrose synthase gene.

Since the clear difference of amplified fragments length between *L. esculentum* and *L. chilenses* wasn’t detected, fragments were digested with some restriction enzymes. Restriction analysis with *Afa I, Mbo I* detected clear polymorphisms (PCR-RFLP). Next the sucrose synthase gene of sucrose accumulating 19 F$_2$ plants were analyzed. There were three types of plants, (1)3 plants homozygous for the *L. chilenses* allele of

**Table 2. Segregation for the invertase allele in F$_2$, BC$_1$F$_2$, BC$_2$F$_2$**, BC$_3$F$_2$ generation**

<table>
<thead>
<tr>
<th>Generation</th>
<th>invertase genotype</th>
<th>observed ratio</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c/c</td>
<td>c/e</td>
<td>e/e</td>
</tr>
<tr>
<td>F$_2$</td>
<td>23</td>
<td>44</td>
<td>28</td>
</tr>
<tr>
<td>BC$_1$F$_2$</td>
<td>60</td>
<td>115</td>
<td>65</td>
</tr>
<tr>
<td>BC$_2$F$_2$</td>
<td>51</td>
<td>146</td>
<td>63</td>
</tr>
<tr>
<td>BC$_3$F$_2$</td>
<td>48</td>
<td>107</td>
<td>61</td>
</tr>
</tbody>
</table>

Values represent the number of plants in each category. $^{ab}$ represents the allele of *L. chilenses*, e represents the allele of *L. esculentum*.

$^b$$^c$not significant

All the plants showing c/c invertase genotype mainly accumulated sucrose, plants showing c/e or e/e invertase genotype didn't accumulate much sucrose.

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**Fig. 3. PCR analysis of invertase gene in several varieties of L. esculentum, L. pimpinellifolium, L. hirsutum and L. peruviana.** 5’-2 and 3’-2 primers in Fig. 1 were used for PCR.

**Fig. 4. Comparison of the sucrose synthase gene in corn and potato.** Only the sequence around the primer and probe used in this study are shown; they show the same base as in corn.
the sucrose synthase gene, (2)6 plants homoygous for the L. esculentum allele and (3)10 plants heterozygous for both species alleles (Fig. 5). Therefore it was found that the sucrose synthase gene itself is not associated with the sucrose accumulating trait. This results support the reports of Yelle et al. (1991) and Dali and Yelle (1992) about the relationship between the level of sucrose synthase activity and sucrose accumulation. It was also found that the sucrose synthase gene is not associated with the ratio of sucrose content to total sugar content (data not shown). We are presently sequencing the amplified fragment to identify the sucrose synthase gene.

Application of molecular analysis to tomato breeding

By distinguishing the invertase allele between L. esculentum and L. chmielewskii using PCR analysis, sucrose accumulating tomato plants can be selected. Compared with the previously reported RFLP method, the method in this study is easier, more efficient, quicker and enables us to select more samples, from a smaller sample quantity at an earlier time. The main purpose of studying molecular markers is to apply them to breeding. A molecular marker using the invertase gene in this study was very effective in breeding sucrose accumulating tomatoes. Especially in this case, because the invertase gene in L. chmielewskii is recessive, we can shorten the time of backcrossing by using this molecular marker.

Literature Cited


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