Plant Cell Physiol. 46(3): 425–437 (2005)
doi:10.1093/pcp/pci040, available online at www.pcp.oupjournals.org

Fruit Carbohydrate Metabolism in an Introgression Line of Tomato with Increased Fruit Soluble Solids

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A tomato line (IL9-2-5) of the cultivated species, *Lycopersicon esculentum*, carrying a 9 cM introgression from the wild species, *Lycopersicon pennelli*, produces fruit with high soluble solids content (Brix), an important determinant of fruit quality for processing. Two quantitative trait loci (QTLs) relating to fruit soluble solids content have been identified within the introgressed segment. One of these QTLs (*PW-9-2-5*) is silent under the growth conditions used in this study, while a second (*Brix-9-2-5*) has been shown to encode a fruit apoplastic invertase (Lin5) with altered kinetic properties. In this study, we have undertaken a detailed biochemical analysis of the introgression line to attempt to gain an understanding of the metabolic changes associated with increased fruit soluble solids. Increased Brix in ripe fruit was shown to be the result of increased sucrose and glucose, with a more minor contribution from aspartate and alanine. The introgression leads to a pronounced increase in apoplastic invertase activity in the columella tissue that extends throughout fruit development. Furthermore, columella tissue from IL9-2-5 fruit has a greater capacity to take up exogenously supplied sucrose, an observation that is consistent with the kinetic properties of the introgressed *Lin5* allele. Apart from the increase in mature fruit sugar and increases in some amino acids, metabolite profiling revealed few other metabolic perturbations in fruit from IL9-2-5. The only other major change was a dramatic increase in starch accumulation at earlier stages of fruit metabolism. This occurred without any increase in the activity of the enzymes of sucrose metabolism or starch synthesis and may therefore be driven by increased availability of sucrose. We conclude that the major factor that leads to increased fruit sugar in IL9-2-5 is an increase in the capacity to take up sucrose unloaded from the phloem.

Keywords: Apoplastic invertase — Brix — Introgression — Tomato.

Abbreviations: DAA, days after anthesis; IL, introgression line; PCR, polymerase chain reaction; QTL, quantitative trait locus.

Introduction

There are two main markets for tomato as a food crop—as a fresh fruit product and as a processed foodstuff (primarily tomato pastes and sauces) (Gould 1992). Economic success in the latter market is dictated in part by a combination of total fruit yield and fruit soluble solids content. Fruit with high soluble solids contain less water and therefore require less processing to generate pastes of the appropriate consistency for consumer tastes. In addition, since sugar is a major constituent of total soluble solids, such fruit are also likely to be sweeter and therefore require the addition of less sugar during processing. These processing savings can have a significant bearing on the profitability of processed tomato products and, thus, from a commercial standpoint, there is considerable interest in manipulating the soluble solids content of tomato varieties.

The cultivated variety of tomato, *Lycopersicon esculentum*, has large, red fruit that predominantly accumulate hexose sugars. In contrast, the wild relative, *Lycopersicon pennellii*, has small, green fruit that predominantly accumulate sucrose and have higher soluble solids content (Yelle et al. 1988). In an attempt to gain an insight into the underlying genetic factors that govern these differences between the cultivated and wild varieties, Zamir and colleagues generated a series of introgression lines in which defined genomic segments of the *L. pennellii* genome replaced homologous regions in an *L. esculentum* background (Eshed and Zamir 1995). A total of 76 lines were produced that collectively contained introgressions that covered the entire tomato genome. In a series of field studies, a number of phenotypic traits in these lines were quantified and a series of quantitative trait loci (QTLs) identified (Eshed and Zamir 1995, Gur et al. 2004). Recently, these lines have been used to map candidate genes involved in fruit size and composition (Causse et al. 2004). Of particular interest was introgression line 9-2-5 (IL9-2-5) that produced ripe fruit with increased Brix (a refractive index measure that is commonly used as an indicator of total soluble solids) (Eshed and Zamir 1995). A subsequent mapping study delimited this QTL to a region of *Lin5* (Fridman et al. 2000), a gene encoding an apoplastic

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invertase expressed exclusively in fruits and flowers (Godt and Roitsch 1997, Fridman and Zamir 2003). More recently, the QTL has been delimited to a single nucleotide that leads to a single amino acid change in the invertase protein resulting in an enzyme with more efficient kinetics (Fridman et al. 2004).

Lin5 is one of a family of genes that encode invertases—enzymes that cleave sucrose to form glucose and fructose. The enzymes are classified according to their subcellular localization; invertases are present in the vacuole, cytoplasm and in the apoplast (often referred to as extracellular invertase) (Sturm and Tang 1999). Those enzymes present in the vacuole and cytosol control the sucrose : hexose ratio in the cell (Zrenner et al. 1996, Sturm 1999). The invertase in the apoplast is thought to play two main roles. First, by cleaving sucrose in the apoplast it helps to maintain a favourable sucrose gradient for unloading of sucrose from the phloem (Sturm and Tang 1999). In doing so, it increases the total capacity for uptake of sugar into the sink organ (due to the presence of both sucrose and monosaccharide transporters) (Bush 1993). Secondly, the action of apoplastic invertase is thought to be a mechanism by which sugar signals are used to integrate source and sink metabolism (Roitsch 1999). In this regard, invertase may also act to amplify sugar signals since its expression is induced by glucose, the product of its activity (Roitsch et al. 1995). The importance of apoplastic invertase in sink organ development is demonstrated by mutant and transgenic plants deficient in this enzyme that have retarded or aborted sink organ formation (Miller and Chourey 1992, Tang et al. 1999). Transgenic manipulations of invertase expression also highlight the signalling role of apoplastic invertase; expression of a heterologous invertase in potato tuber apoplast in a temporally inappropriate manner has a profound impact upon tuber morphology (Sonnewald et al. 1997).

The 9-2-5 introgression line provides a useful tool to investigate the mechansims by which genetic changes impact on fruit metabolism in such a way as to result in increased fruit soluble solids. The 9-2-5 line contains a 9 cM introgression of *L. pennellii* DNA which contains two linked QTLs that contribute to the increased fruit soluble solids trait: the fruit-specific *Brix9-2-5* (which maps to the *Lin5* gene) and *PW9-2-5* which causes semi-determinate plant growth (Fridman et al. 2002). It is argued that both QTLs contribute to increased fruit Brix; the semi-determinate growth habit linked to *PW9-2-5* increases shoot mass such that more photoassimilate is generated, while the *Lin5* allele facilitates uptake of this photoassimilate into the fruit (Fridman et al. 2002). Although a 9 cM region of DNA contains many hundreds of genes, *Brix9-2-5* and *PW9-2-5* are the only loci that could be quantitatively linked to the high fruit soluble solids trait (Eshed and Zamir 1995, Fridman et al. 2002). Other genes in the introgressed segment therefore do not contribute directly to the trait. Moreover, an extensive array of genes involved in carbohydrate metabolism [including those involved in sugar transport and breakdown, starch metabolism, glycolysis and the tricarboxylic acid (TCA) cycle] have been mapped and none of these genes falls in the 9-2-5 mapping region (Causse et al. 2004). Thus, it is very likely that changes in carbohydrate metabolism in IL9-2-5 are the result of the *Brix9-2-5* and *PW9-2-5* QTLs. An investigation of these changes will thus provide insight into the regulation of fruit carbohydrate metabolism and the mechanisms by which genetic changes can modulate sugar metabolism. Therefore, here we present a detailed biochemical analysis of the 9-2-5 introgression line.

Results

Growth and fruit-Brix of IL9-2-5

When grown in the field, IL9-2-5 has increased fruit Brix, no yield penalty and increased total plant weight (Eshed and Zamir 1995). The Brix QTL was restricted to a 9 cM subintrogression (IL9-2-5) which contains two linked QTLs that contribute to the trait: the fruit-specific *Brix9-2-5* (which maps to the *Lin5* gene) and *PW9-2-5* which causes semi-determinate plant growth (Fridman et al. 2002). It is argued that both QTLs contribute to increased fruit Brix; the semi-determinate growth habit linked to *PW9-2-5* increases shoot mass such that more photoassimilate is generated, while the *Lin5* allele facilitates uptake of this photoassimilate into the fruit (Fridman et al. 2002).

We wished to exploit this genetic resource to investigate the biochemical factors that underpin the accumulation of fruit soluble solids. To prevent excessive variability in measured parameters due to environmental fluctuation, the parent line, M82 *L. esculentum*, and IL9-2-5 were grown in a controlled-environment glasshouse. Under these conditions, we observed a significant increase in fruit Brix in IL9-2-5 (Fig. 1a), demonstrating that the Brix trait is still apparent under glasshouse conditions. Total sugar content (sucrose + glucose + fructose) was also increased (*L. esculentum*, 136 ± 8 μmol hexose g FW^{-1}; IL9-2-5, 166 ± 13 μmol hexose g FW^{-1}). However, under glasshouse conditions, we could find no evidence for a difference in the growth habit of this line compared with *L. esculentum*, with both genotypes producing the same number of inflorescences per plant and with total leaf area remaining unchanged (Fig. 1b, c). In addition, when IL9-2 (which contains a larger introgressed segment, of which IL9-2-5 is a subintrogression) was grown under the same glasshouse conditions, plant weight was not significantly different from that of the *L. esculentum* parent (data not shown).

Increased fruit Brix is not driven by increased photosynthetic capacity

The *Lin5* allele is fruit specific in its expression and is therefore likely to mainly affect fruit metabolism. However, it is possible that secondary effects (perhaps as a consequence of altered sugar signalling) or other alleles in the introgressed segment could influence photosynthetic metabolism in the source tissues. To assess whether there are changes in photosynthesis in IL9-2-5 plants that could contribute to changes in fruit sugar

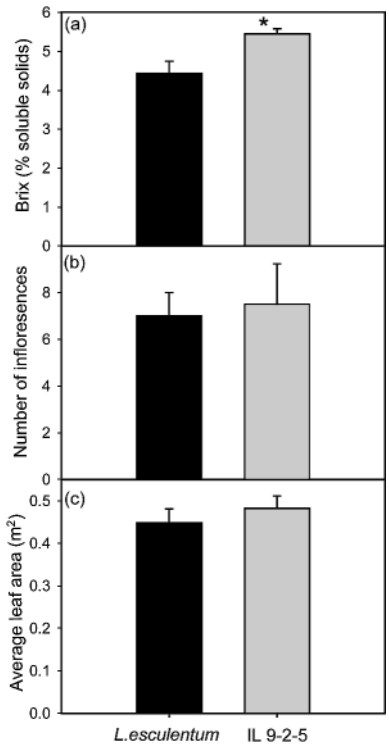

Fig. 1 Growth and fruit Brix content of *L. esculentum* plants and plants from introgression line 9–2-5. *L. esculentum* and IL9-2-5 plants were grown as described in Materials and Methods. (a) Brix value of ripe fruit. (b) Flowering (number of inflorescences) was assessed after 6 weeks growth and (c) average leaf area in plants just prior to flowering. Results presented are the mean of measurements from a minimum of five plants per line from two harvests. * indicates values in IL9-2-5 that are significantly different from the *L.esculentum* parent ($P < 0.05$).

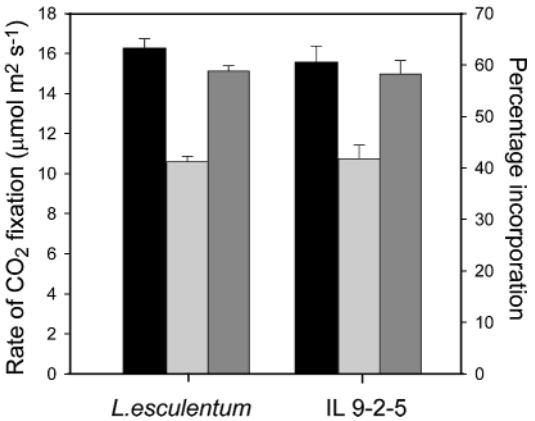

Fig. 2 The rate of $^{14}CO_2$ fixation (black bars) and the proportion of ^{14}C recovered in starch (insoluble fraction, light gray bars) and sucrose (soluble fraction, dark grey bars). Measurements of CO_2 fixation (under saturating light intensity) and carbon partitioning were calculated from $^{14}CO_2$ feeding of leaf disc harvested from plants just prior to flowering. Leaves were sampled during the first 4 h of the photoperiod. Values are the mean ± SE; $n = 5$ plants per group

content, we measured the rate of photosynthetic CO_2 fixation and the partitioning of fixed photoassimilate (Fig. 2). Leaf discs were supplied with $^{14}CO_2$ at saturating light intensity and, as such, the rate of CO_2 fixation calculated from incorporated ^{14}C is a reflection of maximum photosynthetic capacity. There were no significant changes in the rate of CO_2 fixation per unit leaf area between the parent and IL9-2-5 leaves, nor was the partitioning of fixed carbon into soluble or insoluble components altered (Fig. 2). This suggests that neither the photosynthetic capacity nor the partitioning of photoassimilate within leaves is altered in IL9-2-5.

Detailed analysis of metabolic changes in fruit from introgression line 9-2-5

Brix is a refractive index measure that reflects the total soluble solids of the fruit. In tomato fruit, the dominant soluble metabolites are sugars and organic acids (Grierson and Kader 1986, Roessner-Tunali et al. 2003), but other metabolites may contribute. In order to gain a more detailed understanding of the metabolic changes that lead to altered soluble solids in ripe fruit, we measured the amounts of a broad range of metabolites in *L. esculentum* and IL9-2-5 fruit (Fig. 3–5). We also measured metabolite contents at four earlier stages of development [10, 15 and 20 d after anthesis (DAA) and Breaker (the point at which fruit undergo the green–red colour transition] to investigate changes in metabolism that underpin the metabolic state of the ripe fruit.

Of the total soluble metabolites measured, sugars account for 33%, organic acids for 13% and amino acids for 15%. The data for the main soluble carbohydrates (sucrose, glucose and fructose) as well as starch, the main insoluble carbohydrate, are presented in Fig. 3. For these measurements, fruit were divided into the two main tissue types—pericarp and columella. In ripe fruit, there were significant increases in both sucrose and glucose in pericarp but not in columella tissue. There were no significant differences in ripe fruit fructose content in either tissue. The increased pericarp sucrose and glucose contents of ripe IL9-2-5 fruit were not preceded by any change in these sugars at earlier stages in development. However, there were significant increases in sucrose content of columella tissue at several earlier points of development (10 and 20 DAA and Breaker stage). There were also significant increases in starch content in IL9-2-5 fruit, with these changes being most pronounced in columella tissues. In tomato fruit, starch is a transitory carbohydrate reserve, accumulating during early fruit development and then being turned over such that it is undetectable in ripe fruit (Schaffer and Petreikov 1997). In pericarp tissue, starch content declined steadily from 7 DAA but had accumulated to a significantly higher level in IL9-2-5 fruit at the 7 DAA point. The pattern of starch accumulation in columella tissue was quite different, with a peak much later in development at 20 DAA. The amount of starch in IL9-2-5 columella tissue at this developmental time point was dramatically

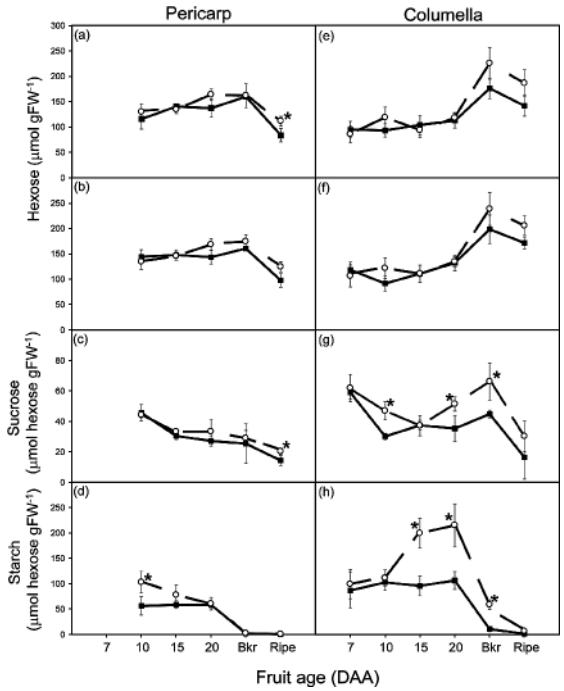

Fig. 3 Changes in the abundance of carbohydrates in fruit from *L. esculentum* and IL9-2-5 tomato plants. The abundance of glucose (a and e), fructose (b and f), sucrose (c and g) and starch (d and h) was measured in the pericarp (a–d) and columella (e–h) of tomato fruit from *L.esculentum* (filled squares) and IL9-2-5 (open circles) plants. Values represent the mean ± SE; $n = 3$–5 fruit per time point, harvested from different plants. * indicates values in IL9-2-5 that are significantly different from the *L. esculentum* parent ($P < 0.05$).

higher, with approximately twice as much starch as the *L. esculentum* parent per g FW of tissue.

A number of minor sugars were also measured in fruit pericarp (Fig. 4). In general, there were few significant differences between *L. esculentum* and IL9-2-5 fruit, but a significant decrease in maltose content in IL9-2-5 fruit at 15 and 20 DAA stands out. In addition, there was a significant increase in arabinose at 20 DAA. Since arabinose is a component of the cell wall, we prepared cell wall material and determined its monosaccharide composition (Fig. 5). There was a small but significant increase in cell wall arabinose, but this was out of phase with the soluble arabinose pool, the former increase occurring only at the ripe stage. There were no significant changes in other cell wall-derived monosaccharides.

Organic acids are also considered to be a significant contributer to Brix. There was a significant increase in citrate in IL9-2-5 fruit at 10 and 20 DAA (Fig. 4). However, none of the other organic acids measured (aconitate, isocitrate, α-ketoglutarate, succinate, fumarate and malate) showed changes that were consistent throughout development, although both aconitate and malate were significantly increased in IL9-2-5 fruit at 20 DAA (Fig. 4). In general, it can be concluded that organic acids do not contribute to increased Brix of ripe IL9-2-5 fruit since there were no significant increases in any of the organic acids at this developmental stage. Organic acids are metabolically linked to amino acids, so we also measured the content of all 20 protein amino acids, a selection of which is shown in Fig. 4. An increase in citrate may feed carbon skeletons into the glutamate branch of amino acid metabolism. The abundance of glutamate itself was not significantly altered. However, other related amino acids were significantly increased, particularly in ripe fruit. Of note, are increases in alanine ($P < 0.1$) and aspartate. These two amino acids may therefore contribute to increased Brix, particularly when one considers that they are two of the most abundant amino acids in tomato fruit, with absolute values in ripe IL9-2-5 fruit of 11.0 ± 5.0 and 29.0 ± 6.7 μmol g FW^{-1} for alanine and aspartate, respectively. We also observed a dramatic increase in proline in ripe IL9-2-5 fruit. This amino acid generally accumulates in response to water stress (Hare et al. 1999). The full metabolite data set can be viewed in Supplementary Table 1.

Increased sucrose and starch accumulation is not caused by changes in the maximum catalytic activities of key enzymes of sucrose and starch metabolism

The observed increases in accumulation of sucrose, glucose and starch at different stages of development of IL9-2-5 fruit suggest that either the rate of synthesis of these compounds has increased, or the rate of turnover has decreased (or a combination of both). Since the patterns of metabolite accumulation during organ development are largely determined by altered amounts of key enzymes (Ruuska et al. 2002), a relevant question is whether the pattern of expression and activity of enzymes of carbohydrate metabolism during development differs between wild-type and IL9-2-5 fruit. Given that apoplastic invertases are thought to be involved in the interpretation of source sugar signals (Roitsch 1999) and many of the genes encoding enzymes of starch metabolism are regulated by sugar (Jang and Sheen 1994, Koch 1996), this is a distinct possibility. We therefore decided to measure the maximum catalytic activity of a range of enzymes involved in carbohydrate synthesis and degradation. We measured activity rather than expression (transcript abundance) for two reasons. First, transcript abundance does not always correlate with protein amount and enzyme activity, and only a change in activity can account for altered patterns of carbohydrate accumulation. Secondly, some sugar-regulated changes are effected through post-translational changes in enzyme activity (Hendriks et al. 2003) and thus would not be apparent at the transcript level.

The activities of enzymes of sucrose synthesis and degradation, hexose metabolism and starch synthesis were determined throughout fruit development in both pericarp and columella tissue (Fig. 6). In general, there was good agreement between the activity profiles of these enzymes during development presented here and those reported previously (Robinson et al. 1988, Miron and Schaffer 1991, Schaffer and Petreikov 1997, Husain et al. 2003). As one might expect, given that the

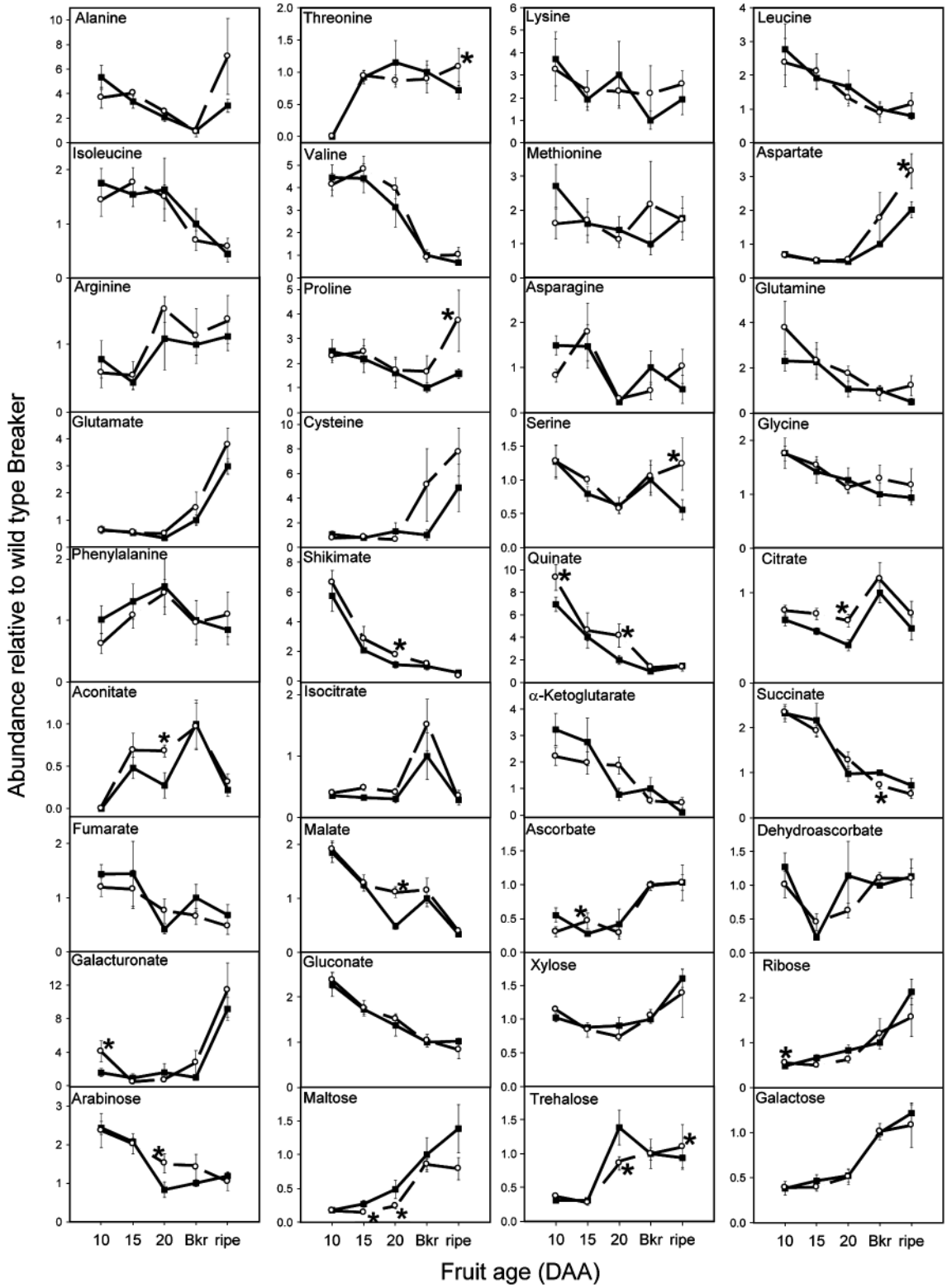

Fig. 4 The relative abundance of metabolites in fruit tissue from *L. esculentum* and IL9-2-5. Fruit tissue was extracted in methanol/chloroform and metabolite contents determined by GC–MS. Data are the mean relative abundance of each metabolite in *L. esculentum* (filled squares) and IL9-2-5 (open circles) compared with wild-type Breaker fruit (±SE). Measurements were made on fruit from each developmental stage harvested from six plants. * indicates values in IL9-2-5 that are significantly different from the *L. esculentum* parent ($P < 0.05$)

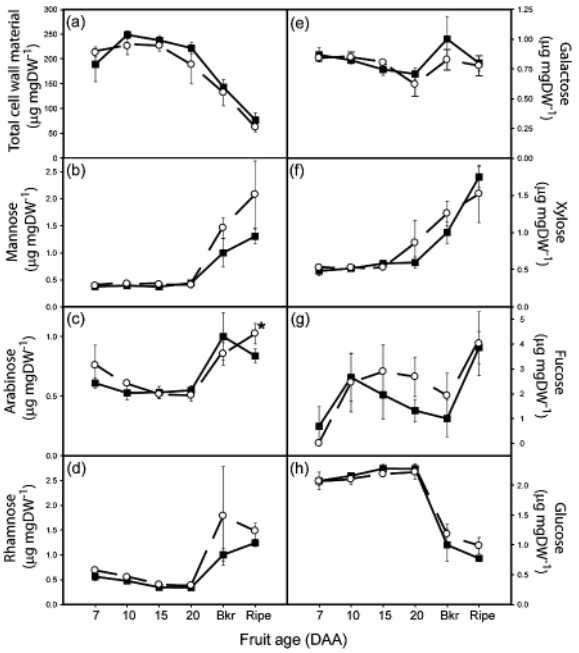

Fig. 5 Changes in the abundance and monosaccharide content of cell wall material during development in fruit from *L. esculentum* and IL9-2-5 tomato plants. The abundance of (a) total cell wall material, (b) mannose, (c) arabinose, (d) rhamnose, (e) galactose, (f) xylose, (g) fucose and (h) glucose was measured in fruit from *L.esculentum* (filled squares) and IL9-2-5 (open cirlces) plants. Values represent the mean ± SE; $n = 6$ fruit per time point, harvested from different plants. * indicates values in IL9-2-5 that are significantly different from the *L. esculentum* parent ($P < 0.05$)

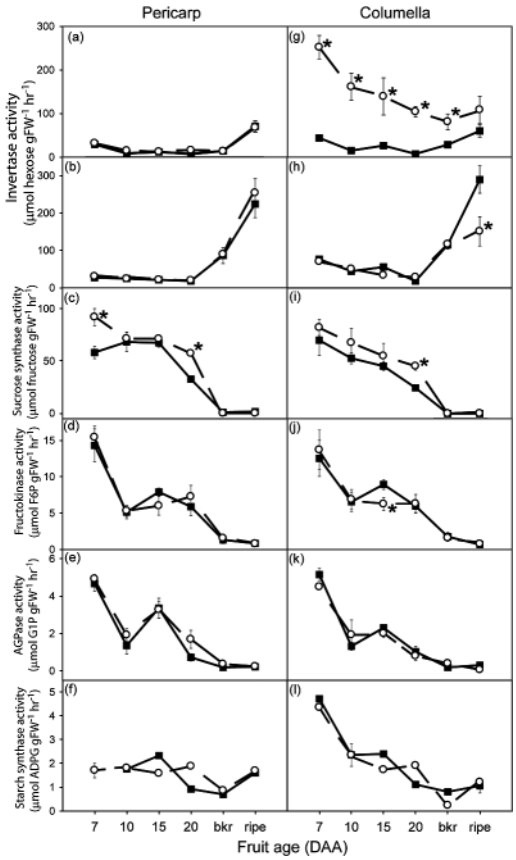

Fig. 6 Maximum catalytic activities of enzymes of sucrose and starch metabolism during development in fruit from *L. esculentum* and IL9-2-5 tomato plants. The maximum catalytic activity of (a and g) apoplastic invertase, (b and h) soluble acid invertase, (c and i) sucrose synthase, (d and j) fructokinase, (e and k) ADP glucose pyrophosphorylase and (f and l) starch synthase was measured in the pericarp (a–f) and columella (g–l) of tomato fruit from *L. esculentum* parent (filled squares) and IL9-2-5 (open circles) plants. Values represent the mean ± SE; $n = 3$–5 fruit per time point, harvested from different plants. * indicates values in IL9-2-5 that are significantly different from the *L. esculentum* parent ($P < 0.05$).

Brix QTL in IL9-2-5 maps to an apoplastic invertase (*Lin5*), we found a dramatic increase in the maximum catalytic activity of apoplastic invertase in the introgression line in comparison with the *L. esculentum* parent (Fig. 6). This change was apparent throughout fruit development and was confined to columella tissue (there was no significant difference in apoplastic invertase activity in pericarp of IL9-2-5 fruit at any stage of development). In contrast, however, there were no major differences in the maximum catalytic activities of other enzymes of carbohydrate metabolism between wild type and IL9-2-5 (Fig. 6). Vacuolar invertase (soluble, acid invertase) activity was not significantly different between *L. esculentum* and IL9-2-5 at any stage of development in either pericarp or columella tissue, except for a decrease in activity in IL9-2-5 ripe fruit columella. Sucrose synthase activity showed some small increases in IL9-2-5, but these were not apparent in a consistent pattern during development; there were significant increases in sucrose synthase in IL9-2-5 pericarp tissue at 7 and 20 DAA but not at intervening developmental stages. Similarly, there was a small but significant increase in activity in IL9-2-5 columella tissue at 20 DAA but not at other stages of development. Fructokinase showed no significant changes apart from a small significant decrease in activity in IL9-2-5 columella tissue at 15 DAA. There were no significant differences in ADP glucose pyrophosphorylase or soluble starch synthase activity between wild-type and IL9-2-5 fruit.

IL9-2-5 fruit have an increased capacity for sucrose assimilation

In tomato fruit, sucrose is unloaded from the phloem into either the symplasm or apoplasm of sink organs. In the pericarp tissues, unloading is symplastic until 14 DAA and apoplastic at later stages of development (Ruan and Patrick 1995). The pattern of phloem unloading in columella tissue, which contains the bulk of the vasculature, is unknown. When sucrose is unloaded into the apoplasm, active uptake across the plasma membrane of sink cells occurs. This can either be by direct uptake of sucrose via a sucrose/proton symporter or by apoplastic invertase-catalysed conversion to hexose and uptake of

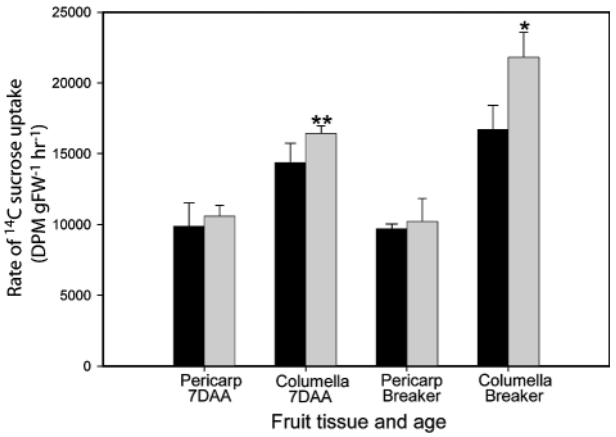

Fig. 7 The rate of uptake of ^{14}C sucrose by pericarp and columella fruit tissue from *L. esculentum* and IL 9-2-5 tomato plants. The apoplasm of tissue discs from pericarp and columella was flooded with a buffered solution containing ^{14}C sucrose and the rate of uptake of ^{14}C sucrose was measured. Fruit tissue was harvested at 7 DAA and Breaker stage from *L. esculentum* parent (black bars) and IL9-2-5 (gray bars) plants. The values represent the mean rates of uptake ± SE; $n = 3$ plants. * indicates values in IL9-2-5 that are significantly different ($P < 0.05$) from the *L. esculentum* parent. ** indicates values that are significantly different at $P < 0.1$.

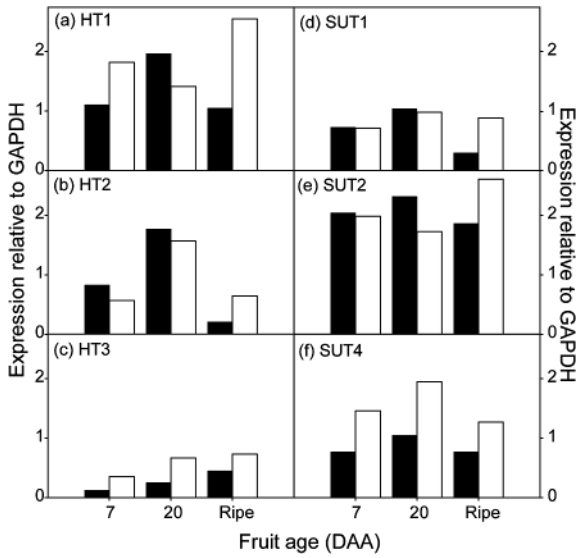

Fig. 8 Transcript abundance of hexose (a, b and c) and sucrose (d, e and f) transporters in developing fruit tissue from *L. esculentum* (black bars) and IL9-2-5 (white bars) tomato plants. The abundance of hexose and sucrose transporter mRNA was measured using semi-quantitative RT–PCR with glyceraldehyde dehydrogenase as an internal standard. The relative expression levels of *Le*HT1 (a), *Le*HT2 (b), *Le*HT3 (c), *Le*SUT1 (d), *Le*SUT2 (e) and *Le*SUT4 (f) were determined for fruit columella tissue from *L.esculentum* and IL9-2-5 tomato plants. Values represent the mean quantification of PCR band intensity from experiments carried out on pooled RNA ($n = 3$).

hexose via monosaccharide transporters (Bush 1993, Ruan and Patrick 1995, Kuhn et al. 2003). Thus, the activity of apoplastic invertase may be a key component in the transfer of sucrose from source to sink tissues. Fruit from IL9-2-5 show a large increase in the activity of apoplastic invertase which may result in an increased capacity for sucrose uptake into fruit. To test this, we measured the rate of uptake of [^{14}C]sucrose into discs of pericarp and columella tissue from *L. esculentum* and IL9-2-5 fruit (Fig. 7). Fruit were sampled at either 7 DAA or at Breaker stage. Fruit discs were washed thoroughly to remove intracellular invertase that may have leaked from the cut surfaces and were incubated for a time course with 200 mM sucrose containing tracer [^{14}C]sucrose. The concentration of sucrose supplied was higher than found in fruit apoplasm (Ruan et al. 1996) to ensure that sucrose supply is not limiting, and therefore [^{14}C]sucrose recovered in the tissue is a reflection of the maximal capacity for uptake. To ensure that the movement of [^{14}C]sucrose from the incubating solution into the apoplast did not limit the rate of uptake into the tissue, the incubating solution was infiltrated into the fruit discs under vacuum, such that the apoplast was flooded with [^{14}C]sucrose. There were no differences in apoplast volumes between *L. esculentum* and IL9-2-5 fruit (data not shown) so the total amount of [^{14}C]sucrose available to each genotype was the same. After an initial lag of 30 min, the rate of incorporation of ^{14}C into the tissue was linear for at least 3 h (data not shown). The data in Fig. 7 show the rate of uptake of [^{14}C]sucrose into tissue calculated from a 2 h incubation. There was a significant increase ($P < 0.05$) in the rate of uptake of sucrose into columella tissue of IL9-2-5 Breaker-stage fruit in comparison with *L. esculentum*. There was a smaller increase in the rate of sucrose uptake in fruit at 7 DAA ($P < 0.1$). In contrast to the changes in columella tissue, there were no significant differences in the rate of uptake of [^{14}C]sucrose into pericarp tissue of the two lines.

Hexose sugars produced in the apoplast by invertase activity are taken up into the fruit cells by monosaccharide transporters, and it has been observed previously that the expression of apoplastic invertase and monosaccharide transporters in sink tissues is co-regulated (Fotopoulos et al. 2003, Weschke et al. 2003). The presence of the introgressed *Lin5* allele might therefore lead to an increase in the expression of monosaccharide transporters. To test this, we used semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) to assess the expression patterns of known genes that encode both monosaccharide and sucrose transporters in tomato. Primers used to amplify the monosaccharide transporter genes, *LeHT1*, *LeHT2* and *LeHT3*, were as detailed in Gear et al. (2000). Primers were also designed to amplify specifically three tomato sucrose transporter genes; *LeSUT1*, *LeSUT2* and *LeSUT4* (see Materials and Methods for details). The relative transcript abundance of hexose and sucrose transporters in columella tissue from wild type and IL9-2-5 is shown in Fig. 8. In general, the overall pattern of expression of these transporters is similar in the two

lines. However, there are some alterations in expression of both hexose and sucrose transporters. In particular, there is a consistent increase in expression of the hexose transporter, *Le*HT3, and the sucrose transporter, *Le*SUT4, throughout development in IL9-2-5 fruit.

Discussion

Under glasshouse conditions, it is unlikely that PW9-2-5 makes a major contribution to increased fruit Brix

IL9-2-5 harbours a 9 cM homologous segment of the *L. pennellii* genome that contains two QTLs, one of which has been mapped to *Lin5*, an apoplastic invertase, and the other is associated with an altered growth habit (Fridman et al. 2000, Fridman et al. 2002). The *L. esculentum* parent is determinate in growth, with the shoot terminating after the production of five inflorescences. The presence of the *PW9-2-5* introgression leads to plants with semi-determinate growth—the shoot terminating after the production of eight inflorescences (Fridman et al. 2002). This results in increased plant weight and an associated increase in total leaf area that may contribute to increased fruit Brix by increasing the total photoassimilate that is partitioned to the fruit. However, under glasshouse growth conditions, neither the wild type nor IL9-2-5 demonstrated a determinate growth pattern, with both lines going on to produce an average of seven inflorescences after 80 d of growth (Fig. 1). It is not clear why this is the case, but it may be related to altered light quality in the greenhouse in comparison with the field. Nevertheless, since the *PW9-2-5* introgression only has an effect in a determinate background (Fridman et al. 2002), it is unlikely that this allele makes a major contribution to the metabolic changes we observe. Further evidence in support of this assertion was the fact that total leaf area was not increased in IL9-2-5 (Fig. 1), nor was plant weight increased in IL9-2.

Increased fruit Brix is not driven by increased photosynthetic capacity

Because the *Lin5* allele is expressed only in non-photosynthetic tissues (Godt and Roitsch 1997, Fridman and Zamir 2003), it is most likely that the introgressed *Lin5* allele affects fruit sugar principally through a direct influence of fruit metabolism. However, apoplastic invertases operate at a point of communication between source and sink tissues, and it is conceivable that the introgressed *Lin5* allele may alter sugar signalling in such a way as to alter source metabolism. In particular, an increase in photosynthetic capacity could result in increased carbohydrate being partitioned to sink tissues, and this could contribute to increased fruit sugar content. Other alleles present in the introgressed segment could also have an effect on photosynthesis. However, we could find no evidence that photosynthetic capacity per unit leaf area (under saturating light and CO_2) was altered in IL9-2-5 leaves (Fig. 2). Moreover, since total leaf area per plant was also unchanged in IL9-2-5 (Fig. 1), it follows that total photosynthetic capacity per plant was similar between IL9-2-5 and the *L. esculentum* parent. The fact that partitioning of photoassimilate between soluble and insoluble products (mainly sucrose and starch, respectively) was unchanged also suggests that it is unlikely that the rate of export of photoassimilate to the sink organs is increased (although we made no direct measures of export rates). Although maximal photosynthetic capacity was unchanged in IL9-2-5, it is possible that photosynthetic rates could be higher at subsaturating light intensities. However, measurements of ambient CO_2 assimilation rates on intact plants suggest this is not the case (data not shown). Collectively, these data suggest that changes in photosynthesis are unlikely to make a major contribution to altered fruit sugars in the fruit of IL9-2-5.

Increased fruit Brix is underpinned by increased sugars and some amino acids

Metabolite profiling of *L. esculentum* and IL9-2-5 fruit was carried out to identify the component metabolites that are responsible for the increased Brix value of IL9-2-5 fruit. Metabolites analysed included a range of sugars, organic acids, amino acids and secondary metabolites, as well as insoluble polysaccharides (starch and cell wall polysaccharides). The complete data set can be viewed in Supplementary Table 1. In general, the metabolite profiles of *L. esculentum* and IL9-2-5 fruit throughout development were remarkably similar, with most metabolites following the same pattern in the two genotypes. The notable exceptions to this were sucrose and glucose which were significantly increased in ripe fruit pericarp and at earlier time points in columella tissue (Fig. 3) and appear to be the main contributor to increased Brix. In relating these changes to the apoplastic invertase activity of IL9-2-5 fruit (Fig. 6), it is apparent that the largest changes in carbohydrate occur in the columella tissue which is where the increased invertase activity is apparent. However, there is also a small but significant increase in both sucrose and glucose in the pericarp of ripe fruit and yet the extractable invertase activity is unchanged in this tissue. There are two possible explanations for this apparent discrepancy. First, the extractable apoplastic invertase activity does not accurately reflect in vivo activity, possibly due to the presence of the proteinaceous invertase inhibitor (Bracho and Whitaker 1990). However, since we made no effort to inactivate the inhibitor protein, we consider this unlikely. Alternatively, it is possible that symplastic transport of carbon from columella tissue to pericarp occurs during fruit maturation. Indeed, the increases in sucrose and starch apparent in columella tissue at earlier stages in development have disappeared in ripe fruit.

The other main storage carbohydrate, starch, does not contribute directly to Brix since it is insoluble. However, significant quantities of starch accumulate in both pericarp and columella. The starch accumulation peaks early in development (at 10 DAA in pericarp and 20 DAA in columella) and declines rapidly thereafter to undetectable levels in ripe fruit (Fig. 3).

The degradation of starch will contribute to the pools of glucose and sucrose in ripe fruit. Fruit of IL9-2-5 accumulate significantly more starch in both pericarp and columella tissues (at the peak of starch accumulation in the latter tissue there is more than twice as much starch in IL9-2-5 than in the *L. esculentum* parent). The extent of active starch accumulation has been argued to be an important contributory factor in determining the soluble solids content of mature fruit (Dinar and Stevens 1981, Schaffer and Petreikov 1997). Therefore, an increased accumulation of starch may be a crucial factor that underpins the subsequent increased sugar content of IL9-2-5 fruit at maturity. Presumably, conversion of imported sugar into starch is important in maintaining a favourable sugar concentration gradient during symplastic unloading which occurs at early stages of tomato fruit development (Ruan and Patrick 1995). At first inspection, it appears that the quantitative increase in starch in the columella tissue is much greater than the subsequent increases in pericarp sugar content. However, the data are presented per g FW and the pericarp tissue constitutes a greater proportion of the mass of the total fruit than the columella. Moreover, as well as being converted to sugar, degraded starch can have other fates, for example as a respiratory substrate to support the climacteric ripening of the fruit (Adams-Phillips et al. 2004).

Other potential contributers to Brix such as organic acids do not appear to be responsible for increased Brix in ripe IL9-2-5 fruit as there were no significant increases in any of the measured organic acids at the ripe stage of development (Fig. 4). However, an increase in the abundance of the two major protein amino acids, aspartate and alanine, may be significant in terms of the Brix increase (Fig. 4).

Mechanism of altered carbohydrate accumulation in IL9-2-5

In an extensive and comprehensive analysis of metabolic changes in fruit of the 9-2-5 introgression line throughout development, we were able to observe two major changes in comparison with the *L. esculentum* parent. First, an increase in sugar content in ripe fruit which is responsible for the high soluble solids trait that has been observed previously in this line. Secondly, a dramatic increase in starch accumulation earlier in fruit development. The soluble solids trait is linked to two QTLs, one of which (*PW9-2-5*) we have argued is silent under our growth conditions. Within 9–2-5, the high soluble solids trait has been delimited further to a single nucleotide polymorphism within the coding region of the *Lin5* gene (Fridman et al. 2000). Furthermore, this polymorphism has been demonstrated to result in an invertase with altered kinetics—the *L. pennellii* allele encoding an enzyme with a greater affinity for its substrate (Fridman et al. 2004). This provides an attractive mechanism by which the presence of the *L. pennellii Lin5* allele could lead to increased fruit soluble solids: by improving the efficiency of sucrose cleavage in the apoplast, more sugar could be taken up into the fruit. However, to date, the effect of the *L. pennellii Lin5* allele has not been demonstrated in planta. An increased insoluble invertase activity has been reported in ovaries of IL9-2-5 (Fridman et al. 2004). We have confirmed that IL9-2-5 fruit have a dramatically increased insoluble invertase activity and showed that this increase is confined to columella tissue and persists well beyond anthesis (Fig. 6). To address the issue of the effect of this increased invertase activity, we measured the capacity of IL9-2-5 fruit slices to take up exogenously supplied ^{14}C-labelled sucrose. A significant increase in the rate of uptake of sugar was observed into columella but not pericarp fruit tissue (Fig. 7), suggesting that the presence of a more efficient apoplastic invertase does indeed enhance the capacity of tomato fruit to take up sugar. The fact that the increased capacity for sucrose uptake is only observed in columella tissue is entirely consistent with the observation that the increase in apoplastic invertase activity in IL9-2-5 is confined to the columella tissue (Fig. 6). The contribution of apoplastic invertase activity to uptake of sugar into the sink is probably to make available a second route of sugar transport into fruit cells (i.e. carbon can be imported via both sucrose and monosaccharide transporters). We observed some increased expression of both monosaccharide and sucrose transporter genes (Fig. 8) that may contribute to the increased capacity for sugar uptake in IL9-2-5 fruit. However, it is worth emphasizing that the genes encoding these transporters are not present in the 9-2-5 region (Causse et al. 2004) and this must therefore be viewed as a secondary effect, possibly as a result of altered sugar signalling.

One aspect of our data set that is not entirely consistent with the argument that invertase activity is responsible for sucrose uptake capacity is that the increase in apoplastic invertase activity in IL9-2-5 fruit is at its greatest early in development whereas the rate of uptake of sugar into fruit tissue is greater later on in development (Fig. 6, 7). However, this is not as surprising as it first seems if one considers that invertase activity is not the only factor that contributes to sucrose uptake. In particular, it is highly likely that sugar uptake is also co-limited by sucrose and hexose transporter activity. Thus, at early stages in development, even though the activity of apoplastic invertase is several fold higher in IL9-2-5 fruit, there is insufficient capacity for transport of the hexose produced into the fruit cells. Consistent with this suggestion is the observation that the expression of the monosaccharide transporters is generally low at early stages in development and rises at later developmental points (Fig. 8). Alternatively, the lesser effect of apoplastic invertase activity on sucrose uptake in young tissue may be related to the fact that symplastic unloading of the phloem is thought to predominate in young fruit (Ruan and Patrick 1995). Since this pathway does not involve apoplastic invertase, the more active enzyme encoded by the *Lin5* allele would have little effect until apoplastic unloading begins at later stages in development. However, it should be borne in mind that the pathway of unloading in the columella (where the increase in sugar uptake is observed) is not known.

The second major change that we observed was a dramatic accumulation of starch early in development (Fig. 3).

There are two explanations for this. First, starch accumulation could be effected by other genes in the introgressed segment and therefore cannot be directly linked to the high soluble solids QTL. Alternatively, the increase in starch could be seen as a consequence of increased sugar uptake as a result of altered invertase activity and can therefore be viewed as part of the mechanism of the invertase QTL. Without genetic experimentation, it is not possible to discriminate conclusively between these two possibilities. However, we provide the following evidence in support of the latter possibility. First, the extent of active starch accumulation has been argued to be an important contributory factor in determining the soluble solids content of mature fruit (Dinar and Stevens 1981, Schaffer and Petreikov 1997) and it is driven primarily by sugar supply (N'tchobo et al. 1999). Secondly, of an extensive range of genes encoding enzymes of primary carbohydrate metabolism, including those of sugar and starch metabolism, none maps within the 9-2-5 region (Schaffer et al. 2000, Causse et al. 2004). Thirdly, the change in starch accumulation occurred without any change in the maximum catalytic activities of the enzymes of sucrose metabolism and starch synthesis (Fig. 6), arguing against the presence of *L. pennelli* alleles of these enzymes in IL9-2-5. Whatever the exact relationship between this increase in starch accumulation and the final fruit soluble solids content, it does provide a new insight into the regulation of starch accumulation in tomato fruit. The pertinent facts are that the increased starch content occurred without any changes in the activities of the enzymes involved in its synthesis and in a genetic background with an increased capacity for sucrose uptake into the fruit. This suggests that the capacity for starch synthesis is in excess of that which actually occurs and that the rate of starch synthesis is controlled by sucrose availability. Interestingly, the sucrose transporter, SUT1, lies in a QTL for tuber starch content in potato, suggesting that starch accumulation in potato tubers is also strongly influenced by sugar supply (Chen et al. 2001).

Conclusion

We have undertaken a detailed biochemical characterization of a line (IL9-2-5) of the cultivated tomato variety, *L. esculentum*, containing an introgressed segment of *L. pennellii* DNA that harbours two QTLs relating to fruit soluble solids content. One of these QTLs (*PW9-2-5*) is silent under the growth conditions used in this study, while a second (*Brix9-2-5*) has been shown to encode a fruit apoplastic invertase (Lin5) with altered kinetic properties. Here we provide evidence that the 9-2-5 introgression leads to a pronounced increase in apoplastic invertase activity in the columella tissue that extends throughout fruit development. Furthermore, columella tissue from IL9-2-5 fruit has a greater capacity to take up exogenously supplied sucrose, an observation that is consistent with the kinetic properties of the introgressed allele of *Lin5* (Fridman et al. 2004). Apart from the increases in mature fruit sugar and amino acid content that relate to the trait of high fruit soluble solids previously observed in this line, we could find few other metabolic perturbations in fruit from IL9-2-5. The only other major change was a dramatic increase in starch accumulation at earlier stages of fruit metabolism. This occurred without any increase in the activity of the enzymes of sucrose metabolism or starch synthesis and may therefore be driven by increased availability of sucrose. We conclude that the major factor that leads to increased fruit sugar in IL9-2-5 is an increase in the capacity to take up sucrose unloaded from the phloem.

Materials and Methods

Plant growth conditions and fruit sampling

The *L. esculentum* parent (cv. M82 accession LA3475) tomato plants and plants from an introgression line containing a defined portion of the *L. pennellii* genome on chromosome 9 (IL9-2-5) were grown in a greenhouse, with supplementary lighting providing an irradiance of 250–400 μmol m^{-2} s^{-1}. Individual tomato plants were grown in pots (20 cm diameter) containing compost (Levingtons M3; 6 kg per pot) supplemented with Osmocote slow release fertilizer (30 g per pot). Plants were watered daily and prior to flowering given liquid fertilizer (Phostrogen plant food) on a weekly basis. Fruit were tagged at anthesis and harvested when they had reached the appropriate developmental stage. Individual fruit were removed from the plant, divided into tissue sections (pericarp or columella), snap-frozen in liquid nitrogen and stored at –80°C until required.

CO_2 fixation and carbon partitioning

Leaf discs (1.1 cm^2) were cut from each of the first three fully expanded source leaves and placed on wire gauze (six discs per chamber) in an oxygen electrode leaf chamber (25 ml volume, maintained at 25°C via a temperature-controlled water bath). Leaf discs were placed above moistened matting containing 250 μl of a labelled solution of $^{14}CO_2$ (2 M $KHCO_3$, 2 M K_2CO_3 containing 1.25 MBq $^{14}CO_2$ ml^{-1}) providing saturating CO_2 and illuminated for 20 min at an irradiance of 500 μmol m^{-2} s^{-1} at 25°C. Following incubation, leaf discs were snap-frozen in liquid nitrogen and extracted in trichloroacetic acid (Sweetlove et al. 1996b). Total incorporation of radioactivity in the soluble and insoluble fractions was determined by liquid scintillation counting.

Uptake of ^{14}C sucrose by tomato fruit tissue

The capacity for sucrose uptake by isolated sections of pericarp and columella tissue was assessed by incubation in a solution of radioactively labelled sucrose. Discs of pericarp or columella tissue were cut from the fruit using a cork borer (1.1 cm^2) and sectioned longitudinally to a uniform thickness. Discs were washed three times in buffer A (25 mM MES pH 6.5, 300 mM mannitol), blotted dry and transferred to 250 ml conical flasks containing 60 ml of buffer B (25 mM MES pH 6.5, 100 mM mannitol, 200 mM sucrose, 5 μCi of [U-^{14}C]sucrose). The apoplasm of the tomato fruit tissue was flooded with incubation buffer by vacuum infiltration of the discs for 2×1 min. Following vacuum infiltration, tissue was incubated at 25°C with shaking (100 rpm) for 2 h. Samples of tissue were removed at 0, 30, 60 and 120 min after infiltration, washed in buffer A, blotted dry and snap-frozen. Frozen tissue was extracted in trichloroacetic acid and the incorporation of radioactivity was determined by counting appropriate aliquots on a liquid scintillation counter. The incorporation of radioactivity during the experiment was expressed by subtracting the initial radioactivity associated with the apoplasm (initial samples) from the

label present over time. In control experiments, the volume of the apoplasm in the tomato fruit studied was measured (Damon et al. 1988, Sweetlove et al. 1998).

RNA extraction

Total RNA was isolated from homogenized, powdered tomato fruit tissue using a CTAB (hexadecyltrimethylammonium bromide) method (Chang et al. 1993).

cDNA synthesis

A 10 μg aliquot of total RNA was DNase treated using an Ambion DNA-free kit (Ambion Ltd, Huntingdon, U.K.). RNA was recovered according to the manufacturer's protocol and used for cDNA synthesis. A 1 μg aliquot of RNA was denatured at 65°C for 5 min in a reaction containing 0.5 μg of oligo(dT)12–18 primer (Invitrogen, Paisley, U.K.) in a final volume of 20 μl. The reaction was cooled on ice and cDNA synthesized after the addition of 4 μl of 5× first strand buffer (Roche, Lewes, U.K.), 10 mM dithiothreitol (DTT; final concentration), 1 mM of each dNTP (final concentration) and 50 U of Expand Reverse Transcriptase (Roche, Lewes, U.K.) at 43°C for 60 min. The reaction was heat inactivated at 95°C for 2 min and samples diluted 1 : 10 in H_2O prior to use.

RT–PCR

To determine the expression patterns of tomato hexose transporter genes, PCR primers and reaction conditions were as described in Gear et al. (2000). For the amplification of sucrose transporter transcripts, a pair of gene-specific primers was designed from the following sequences: *LeSUT1*-x82275, *LeSUT2*-AF166498 and *LeSUT4*-AF176950, resulting in the following forward and reverse primers: for *LeSUT1*, 5′-ACCCAAAATTAAAACAGAGC-3′and 5′-ATCGTACAGTTTCGCATCAC-3′; for *LeSUT2*, 5′-GAGGAATCCGACGCCGTTTC-3′ and 5′-GCGTCAGCTGTCAACTCTGC-3′; and for *LeSUT4*, 5′-ACCACCTCGCCACCTTCTTC-3′ and 5′-GACAAGCCTTGTCCAAGCCC-3′. To allow the semi-quantitative analysis of gene expression, transcript abundance was determined relative to the expression of the tomato glyceraldehyde 3-phosphate dehydrogenase gene (AB110609) using the following primers: 5′-TGGAATCAGGAACCCTGAAG-3′ and 5′-GATCGACAACGGAGACATCA-3′. PCR conditions were 30 s at 94°C followed by 35 cycles of 20 s at 94°C, 20 s at 55°C (*LeSUT1* and *GAPDH*) or 20 s at 60°C (*LeSUT2* and *LeSUT4*) or 40 s at 68°C, and an additional 2 min at 68°C. A 10 μl aliquot of the PCR was separated on a 1% (w/v) agarose gel, stained with ethidium bromide and visualized and captured using a Bio-Rad Molecular image FX scanner and Quantity One, 1D analysis software (Bio-Rad laboratories Inc., CA, U.S.A.).

Measurement of fruit Brix

Ripe fruit tissue was homogenized with a razor blade, and the soluble solids (Brix) content of the resulting juice measured on a portable refractometer (Bellingham and Stanley Ltd, Kent, U.K.)

Carbohydrate assays

Frozen fruit powder was extracted with trichloroacetic acid (Sweetlove et al. 1996b). Carbohydrates were assayed spectrophotometrically using the methods described in Baxter et al. (2003).

Protein extraction and enzyme assays

Frozen fruit powder was extracted with 4 vols (w/v) of extraction buffer [50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 5 mM ε-amino-*n*-caproic acid, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 1% (w/v) PVPP]. Samples were allowed to thaw, transferred to chilled Eppendorf tubes and centrifuged at 4°C, 12,000×*g* for 2 min. An aliquot of the resulting supernatant was then desalted on a NAP 5 Sephadex G25 column (Amersham Pharmacia Biotech, Bucks, U.K.), pre-equilibrated with ice-cold extraction buffer minus Triton X-100 and PVPP. The pellet from the initial extraction was washed in extraction buffer, resuspended in 50 mM HEPES-NaOH (pH 7.5), 5 mM $MgCl_2$, 1 mM Na EDTA and the homogenate used to assay apoplastic invertase activity (Weschke et al. 2003). The desalted supernatant was used to measure the activity of selected enzymes in spectrophotometric assays according to the following reaction mixtures and accompanying references. Acid invertase (EC 3.2.1.26): 50 mM $C_6H_5O_7Na_3$, 100 mM Na_2HPO_4 pH 5.0, 240 mM sucrose in 300 μl at 37°C (Husain et al. 2003). ADPglucose pyrophosphorylase (EC 2.7.7.27): 40 mM HEPES pH 8.0, 5 mM $MgCl_2$, 2.5 mM ADPglucose, 1 mM 3-PGA, 1.5 mM NaH_2PO_4 in 200 μl at 25°C (Sweetlove et al. 1996a). Sucrose synthase (EC 2.4.1.13): 80 mM MES pH 6.0, 300 mM sucrose, 10 mM UDP in 200 μl at 25°C (Nguyen-Quoc et al. 1999). Fructokinase (EC 2.7.1.4): 100 mM HEPES pH 8.0, 5 mM $MgCl_2$, 200 mM fructose, 2.5 mM ATP in 300 μl at 25°C (Sweetlove et al. 1996a). Soluble starch synthase (EC 2.4.1.21): 150 mM bicine, pH 8.4, 400 mM sodium citrate, 0.1 mg of potato amylopectin and 1.4 mM ADP[U-^{14}C]glucose (5.3 kBq μmol^{-1}) in 200 μl at 25°C (Sweetlove et al. 1996a). Phosphoglucomutase (EC 5.4.2.2): 50 mM HEPES, pH 7.6, 1 mM $MgCl_2$, 0.25 mM glucose-1-phosphate, 0.024 mM glucose 1,6-bisphosphate, 0.4 mM NAD^+ and 1.5 U of glucose-6-phosphate dehydrogenase (NAD^+ specific) (Sweetlove et al. 1996a).

Cell wall analysis

The insoluble residue after metabolite extraction was washed with ice-cold 70% (v/v) ethanol followed by a washing step with a 1 : 1 (v/v) mixture of methanol/chloroform. The dried residue was then subjected to 2 M trifluoroacetic acid hydrolysis for 1 h at 121°C to release the monosaccharides of the wall matrix polysaccharides. The released monosaccharides were quantified as their alditol acetate derivatives by gas chromatography–mass spectroscopy (GC–MS) (Albersheim et al. 1967).

GC–MS

The relative levels of metabolites were determined using the GC–MS protocol exactly as described in Roessner et al. (2001). Data are presented normalized to wild-type Breaker fruit as detailed by Roessner-Tunali et al. (2003).

Statistical analysis

Data were analysed by *t*-test using the algorithm contained with Microsoft Excel software. Unless otherwise stated, all instances of the word 'significant' in the text denote a statistical significance of $P < 0.05$ as determined by the *t*-test.

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

Acknowledgments

The authors would also to acknowledge Markus Pauly (Max Planck Institute for Molecular Plant Physiology, Golm, Germany) for providing access to facilities for measurement of cell wall components, and Ewa Urbanczyk-Wochniak (Max Planck Institute for Molecular Plant Physiology, Golm, Germany) for help with metabolite measurements. Financial support to L.J.S., S.A.H. and W.P.Q. by the BBSRC and to A.R.F. by BMBF in the frame of a DIP grant is acknowledged.

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(Received August 13, 2004; accepted 18 December, 2004)