RNA Interference of LIN5 in Tomato Confirms Its Role in Controlling Brix Content, Uncovers the Influence of Sugars on the Levels of Fruit Hormones, and Demonstrates the Importance of Sucrose Cleavage for Normal Fruit Development and Fertility[1][W][OA]

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It has been previously demonstrated, utilizing intraspecific introgression lines, that Lycopersicum Invertase5 (LIN5), which encodes a cell wall invertase, controls total soluble solids content in tomato (*Solanum lycopersicum*). The physiological role of this protein, however, has not yet been directly studied, since evaluation of data obtained from the introgression lines is complicated by the fact that they additionally harbor many other wild species alleles. To allow a more precise comparison, we generated transgenic tomato in which we silenced the expression of LIN5 using the RNA interference approach. The transformants were characterized by an altered flower and fruit morphology, displaying increased numbers of petals and sepals per flower, an increased rate of fruit abortion, and a reduction in fruit size. Evaluation of the mature fruit revealed that the transformants were characterized by a reduction of seed number per plant. Furthermore, detailed physiological analysis revealed that the transformants displayed aberrant pollen morphology and a reduction in the rate of pollen tube elongation. Metabolite profiling of ovaries and green and red fruit revealed that metabolic changes in the transformants were largely confined to sugar metabolism, whereas transcript and hormone profiling revealed broad changes both in the hormones themselves and in transcripts encoding their biosynthetic enzymes and response elements. These results are discussed in the context of current understanding of the role of sugar during the development of tomato fruit, with particular focus given to its impact on hormone levels and organ morphology.

The importance of the supply to, and the subsequent mobilization of Suc in, plant heterotrophic organs has been the subject of intensive research effort over many years (Miller and Chourey, 1992; Zrenner et al., 1996; Wobus and Weber, 1999; Heyer et al., 2004; Roitsch and Gonzalez, 2004; Biemelt and Sonnewald, 2006; Sergeeva et al., 2006; Lytovchenko et al., 2007). While the mechanisms of Suc loading into the phloem have been intensively studied over a similar time period (Riesmeier et al., 1993, 1994; Bürkle et al., 1998; Meyer et al., 2004; Sauer et al., 2004), those by which it is unloaded into the sink organ have only been clarified relatively recently and only for a subset of plants studied (Bret-Harte and Silk, 1994; Viola et al., 2001; Kühn et al., 2003; Carpaneto et al., 2005). In the tomato (*Solanum lycopersicum*) fruit, the path of Suc unloading remains somewhat contentious (Ruan and Patrick, 1995; Hackel et al., 2006). Early studies, utilizing a range of experimental techniques including reverse genetics, suggested that Suc unloading during early stages of fruit development was exclusively symplastic (Ruan and Patrick, 1995; Damon et al., 1998; D'Aoust et al., 1999). However, the results of a range of recent

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studies cast doubt on this claim. A reverse genetics experiment similar to that described by D'Aoust and coworkers (1999) found no evidence in support of their claim for a vital role of the cytosolically localized Suc synthase in young fruit development (Chengappa et al., 1999). Furthermore, evaluation of ripening-induced and ripening-repressed ESTs found in the recently published EST data set are not in keeping with the idea of early fruit exhibiting a symplastic pathway of Suc unloading and older fruit exhibiting an apoplastic pathway (Fei et al., 2004). Finally, evaluation of tomato introgression lines containing an exotic allele of Lycopersicum Invertase5 (LIN5), a cell wall invertase that is exclusively expressed in flower (mainly ovary but also petal and stamen) and in young fruit (Godt and Roitsch, 1997; Fridman and Zamir, 2003), demonstrated that alterations in the efficiency of this enzyme result in significantly increased partitioning of photosynthate to the fruit and hence enhanced agronomic yield (Fridman et al., 2004; Baxter et al., 2005; Schauer et al., 2006). When taken together, these studies provide evidence that the early developmental stages of the tomato fruit are, like those of the potato (*Solanum tuberosum*) tuber (Fernie and Willmitzer, 2001; Kühn et al., 2003; Roessner-Tunali et al., 2003b), characterized by apoplastic unloading of Suc.

In previous studies, the identification and detailed molecular characterization of LIN5 as a quantitative trait locus (QTL) for total soluble solid content in a *Solanum pennellii* introgression line population have been detailed (Fridman et al., 2000, 2002, 2004). These studies revealed that the wild species allele of LIN5 was more efficient than the cultivated allele, due to a single nucleotide substitution that coded for an amino acid residue close to the fructosyl-binding site of the enzyme (Fridman et al., 2004). In this previous study, we were also able to confirm, via site-directed mutagenesis and heterologous expression, that this single nucleotide difference conferred differences in the $K_{m(Suc)}$ of the enzyme. Due to difficulties in the purification of the enzyme, alongside the very narrow time window in which it is the exclusive invertase expressed, we were unable to confirm this finding in planta. That said, we were able to demonstrate that fruit of the introgression line 9-2-5, which harbors, among other genes, the *S. pennellii* allele of LIN5, was capable of achieving a significantly higher rate of sugar uptake than fruit of the cultivated variety (Baxter et al., 2005). However, despite the importance of this confirmation, we cannot exclude that it occurs as the result of allelic variance in one of the many other genes introgressed in the 9-2-5 line. In order to directly study the function of the LIN5 gene, we describe here an analogous approach whereby we reduced the efficiency of the reaction catalyzed by the LIN5 isoform of apoplastic invertase by a specific reduction of the expression of the gene that encodes it. For this purpose, we took an RNA interference (RNAi)-based approach to evaluate the consequences of silencing this enzyme on floral and fruit development as well as performing comprehensive profiling of the transcripts, primary metabolites, and hormones of the fruit. The results obtained are discussed not only in comparison with our previous genetic work but also with respect to other functions of this isoform of invertase within the maintenance of normal fruit metabolism and development.

RESULTS

Generation of Plants Exhibiting Silencing of LIN5

Previous studies have indicated that LIN5 is expressed exclusively at the floral-to-fruit transition, with its transcript detectable in petal, stamen, ovary, and small fruit but not in pollen (Fridman and Zamir, 2003; Fridman et al., 2004), with this expression pattern being unique among its close homologs (LIN6, LIN7, and LIN8; Fridman and Zamir, 2003). A 513-bp fragment of the 3′ end region, chosen to exhibit low homology to the other isoforms of LIN, was cloned, using the RNAi design, into the pART27 vector (Gleave, 1992). We subsequently obtained and transferred 33 transgenic lines obtained by an *Agrobacterium tumefacians*-mediated transformation to the greenhouse. Screening of the lines for reduction in total cell wall invertase activity yielded three lines that exhibited a significant reduction of activity (Fig. 1A). To demonstrate that this reduction was a consequence of a specific reduction of LIN5 expression, we evaluated the level of transcription of this gene by real-time quantitative PCR (qRT-PCR; Fig. 1B) as well as assessing the activity of cell wall invertase in young fruit (Fig. 1D). Importantly, qRT-PCR evaluation of the expression level of LIN7, the closest homolog of LIN5, revealed no changes in the transgenic lines in young fruit (Godt and Roitsch, 1997; Fridman and Zamir, 2003; Fig. 1C). Analysis of the activities of other enzymes of carbohydrate metabolism revealed a decrease in the total activity of Suc synthase (when assayed in the direction of Suc cleavage) in the young fruit of the most strongly silenced lines (Table I). Therefore, we tested the expression levels of the two known isoforms of Suc synthase in tomato, *SuSy2* and *SuSy3*, as well as a highly homologous gene (*SGN-U331270*) in the transformant lines. While line Lin5i-1 displayed somewhat lower expression of *SuSy2* and higher expression of *SuSy3*, the expression level of the genes was essentially unaltered in the transformants (Table II). For this reason, we additionally measured the activity of Suc synthase in the direction of Suc synthesis. The transformants displayed invariant activity from the wild type (data not shown). When taken together, these data suggest that the change in Suc synthase activity appears to be mediated at the posttranslational level. Of the other enzyme activities that we measured, only phosphoglucoisomerase activity was altered, and this change was confined to a single transgenic line (Table I).

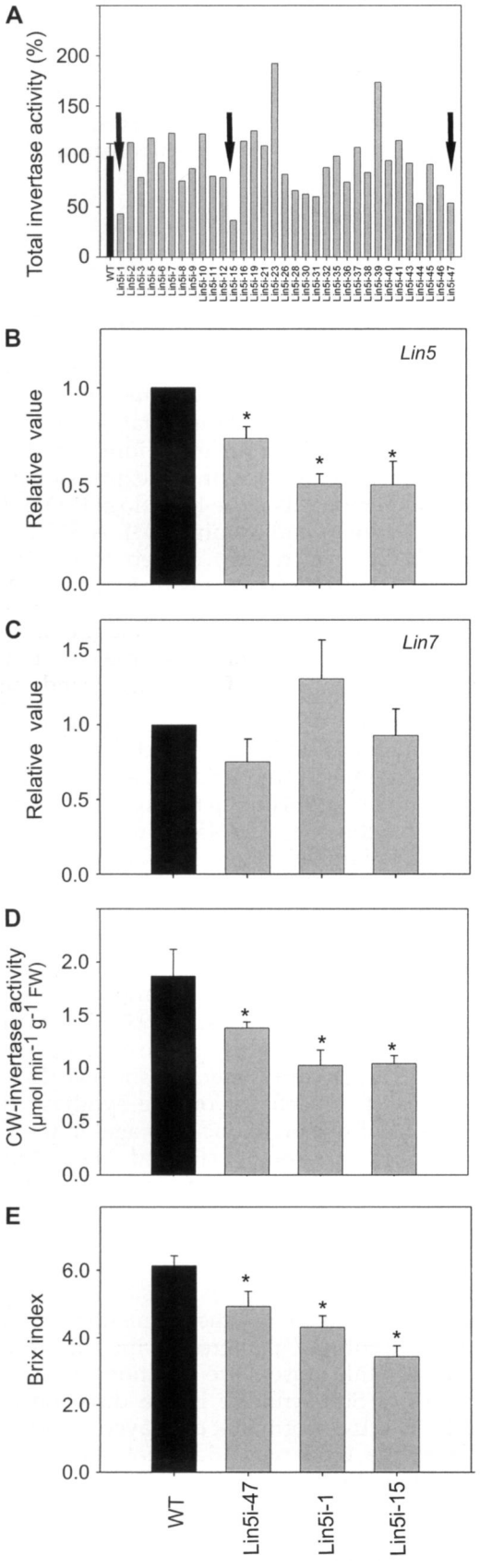

Figure 1. Analysis of tomato RNAi *LIN5*. A, Screening of total invertase activity in primary Lin5i transformants in extracts of tissue sampled from young fruit. B and C, qRT-PCR of *LIN5* and *LIN7*, respectively, in young fruit of wild-type (WT) and Lin5i transgenic lines. The expression of each gene is shown relative to the wild-type value. D, Cell wall activity of extracts from young fruit. FW, Fresh weight. E, Total soluble solids content (Brix index). Asterisks indicate values determined by the *t* test to be significantly different from the wild-type control value ($P < 0.05$).

Transgenic Plants Exhibit Altered Flower and Fruit Morphology

When we grew the transgenic plants in the greenhouse side by side with wild-type controls, clear flower and fruit phenotypes were observed. The transformants exhibited a significantly higher proportion of flowers containing seven or more petals (as opposed to the normal six; Fig. 2A). Close examination of the transformants additionally revealed that although they flowered at a similar time to the wild type, they contained significantly reduced numbers of flowers (59 ± 17, 34 ± 7, 37 ± 11, and 40 ± 10 for the wild type, Lin5i-47, Lin5i-1, and Lin5i-15, respectively; values are means ± SE, $n = 20$). In addition, although occurring at the same time point, fruit abortion was clearly promoted in the transgenics, occurring at more than three times the level seen in the wild type (Fig. 2, B and F), and the fruit were significantly smaller throughout development, reaching approximately 40% of the wild-type size by the red ripe stage (Fig. 2C). Given that such phenotypes can arise due to alterations in ploidy level during transformation and tissue culture processes, we confirmed that all transgenic lines exhibited normal levels (Supplemental Fig. S1). As a combination of these factors, the total fruit yield was dramatically compromised, being approximately 40% of that found in the wild type in all three transgenic lines. To further assess the fruit abortion phenotype, we next evaluated the competence of the transgenics to respond to water stress. We chose to perform this experiment since a number of studies have previously linked changes in invertase expression with abortion in maize (*Zea mays*) under conditions of water stress (Andersen et al., 2002; Boyer and McLaughlin, 2007). As would perhaps be anticipated, the transformants displayed a far greater incidence of additional abortion on the imposition of a 3-d water stress (lines Lin5i-1 and Lin5i-15 showing a far lower proportion of fruit per flower after water stress in comparison with corresponding nonstressed control plants of the same genotype; the wild type showed a ratio of 0.77 ± 0.01, Lin5i-1 of 0.50 ± 0.01, and Lin5i-15 of 0.53 ± 0.01).

We next analyzed the seed yield of nonstressed plants, which revealed a decreased total seed mass per fruit as well as a decreased seed viability (Fig. 3A). In extreme cases, seeds were not developing properly but rather remaining as ovule-like structures (Fig. 3B). Tests of germination efficiency confirmed that the germination rate of the transgenic seeds was reduced from 89% in the wild type to 57%, 41%, and 48% for

Table I. *Enzyme activities in the Lin5i transgenic lines*

Activities were determined in extracts from tissue samples from young fruit. Data are presented as means ± SE. Values shown in boldface were determined to be significantly different from the wild-type value by the *t* test ($P < 0.05$).

Enzyme	Wild Type	Lin5i-47	Lin5i-1	Lin5i-15
		nmol min^{-1} g^{-1} fresh weight		
Phosphoglucoisomerase	24.1 ± 1.4	**43.6 ± 4.2**	31.7 ± 5.2	21.7 ± 4.6
Pyruvate kinase	3.2 ± 0.5	5.9 ± 1.8	5.7 ± 0.4	10.9 ± 4.0
Suc synthase	19.5 ± 3.4	10.8 ± 4.5	**2.1 ± 0.3**	**3.0 ± 0.9**
AGPase	14.3 ± 4.8	9.4 ± 1.0	9.2 ± 0.4	12.2 ± 2.2
Neutral invertase (nmol min^{-1} mg^{-1} protein)	140.5 ± 33.4	190.5 ± 20.8	146.5 ± 42.0	97.4 ± 36.7
Vacuolar invertase (nmol min^{-1} mg^{-1} protein)	121 ± 38.0	140.3 ± 42.7	189.7 ± 64.5	78.7 ± 24.6

lines Lin5i-47, -1, and -15, respectively. Moreover, the seed coat appeared to be affected in the transformants (Fig. 3C). Given that two morphologically distinct seed populations were apparent in the transgenics, we further evaluated whether the rate of germination was different in the different seed types; however, all seeds revealed similarly inhibited rates of germination (data not shown).

Chemical Composition of Fruit of the Transgenics

The analyses of both the *S. pennellii* introgression lines (Fridman et al., 2000; Baxter et al., 2005) and of an allelic series of introgressions of a wide range of tomato species (Fridman et al., 2004) indicate that LIN5 is a highly important determinant of the total soluble solids content (Brix). Therefore, we next evaluated this parameter (which is indicative of agronomical yield in processing cultivars of tomato; Gould, 1992) in the transgenics. In keeping with earlier results, the transformant lines all exhibited a clear reduction in the Brix content of the fruit (Fig. 1E).

In order to gain a deeper comprehension of the chemical basis of the differences in composition indicated by the refractometry measurements, we next determined metabolite levels in the pericarp tissue of the fruit using established enzymatic (Fernie et al., 2001) and gas chromatography-mass spectrometry (GC-MS)-based metabolite profiling (Fernie et al., 2004) methods. The first of these suggested that the levels of carbohydrates in the green fruit (harvested at 35 DPA) were largely unaltered. Indeed, the enzymatic assays revealed that only the levels of Suc were different; however, even this difference, a significant increase, was confined to a single line (Lin5i-1; Table III). When carbohydrate contents were determined in red fruit (harvested at 65 DPA), clearer trends became apparent, with both lines Lin5i-1 and Lin5i-15 displaying reduced levels of Glc and Fru (Table III). GC-MS analyses were carried out on the exact same samples and revealed relatively few changes. The green fruit of the transgenics were characterized by conserved reductions in the levels of Fru and Glc and an increase in the levels of Ara, Xyl, and Ala (Fig. 4B; Supplemental Table S2). A similar pattern of metabolic change was observed following GC-MS evaluation of the pericarp tissue of the red fruit of the transgenics, which still displayed reduced contents of Fru, Glc, and shikimate (Fig. 4C; Supplemental Table S3).

Given that such small changes in metabolite content were observed in later stages of development, we next chose to analyze samples at a very early developmental stage: that of the young fruit. At this time point, the transformants were characterized by reductions in the levels of Fru, Glc, Ara, and shikimate and by an increase in rhamnose (Fig. 4A; Supplemental Table S1).

Table II. *Expression of Suc synthase in the Lin5i transgenic lines*

qRT-PCR results of *SuSy2*, *SuSy3*, and *SGN-U331270* in young fruit are shown. The increase in expression of each gene is shown relative to the wild-type value. Data are presented as means ± SE ($n = 6$). Values shown in boldface were determined to be significantly different from the wild-type value by the *t* test ($P < 0.05$).

Gene	Wild Type	Lin5i-47	Lin5i-1	Lin5i-15
		relative expression		
SuSy2	1.0 ± 0.0	0.9 ± 0.3	**0.4 ± 0.1**	1.1 ± 0.3
SuSy3	1.0 ± 0.0	1.1 ± 0.2	**1.8 ± 0.3**	1.0 ± 0.1
SGN-U331270	1.0 ± 0.0	0.9 ± 0.2	1.2 ± 0.1	0.9 ± 0.3

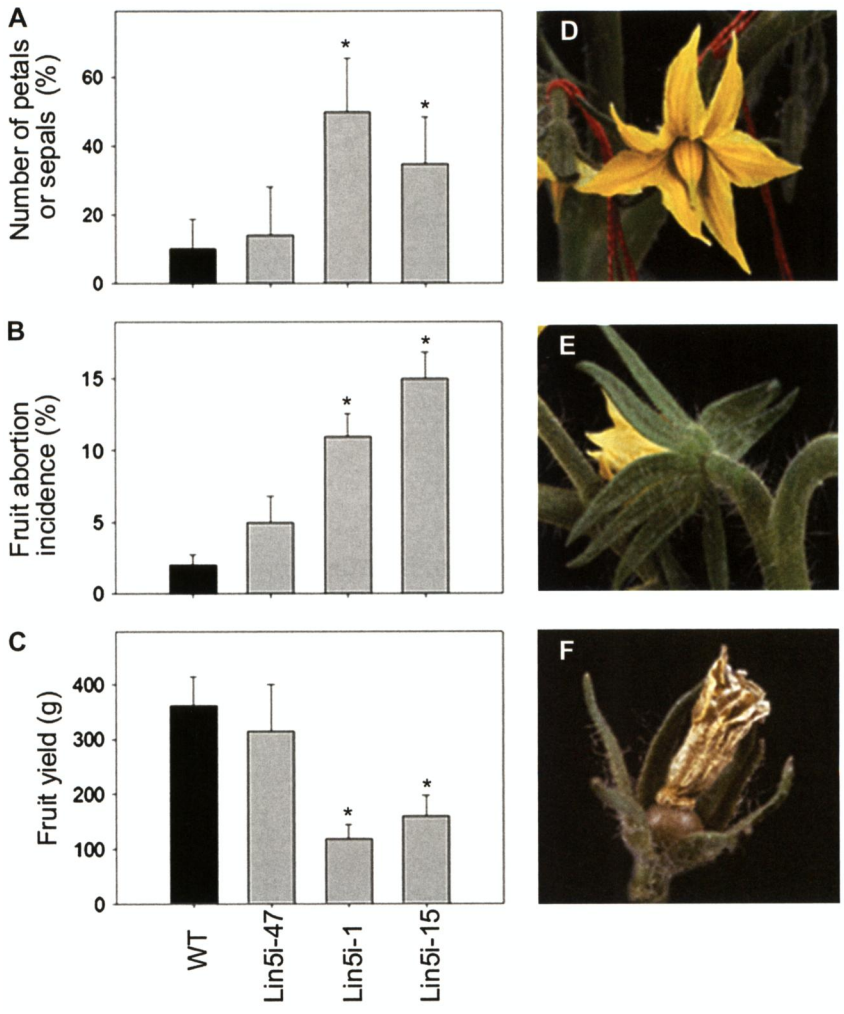

Figure 2. Phenotypic analysis of flowers and fruit of Lin5i RNAi plants. A, Incidence of flowers showing a higher number of petals or sepals in the transgenic lines. B, Fruit abortion incidence. C, Total fruit yield of Lin5i plants in comparison with the wild type (WT) is reduced. D and E, Lin5i flowers showing a higher number of petals and sepals, respectively. F, Aborted Lin5i fruit.

Evaluation of Metabolism in Seeds of the Transgenic Lines

Given the strong seed phenotype observed in the Lin5i transgenic lines and the clear indication of the importance of photoassimilate supply for the development of fertile seeds, we next turned our attention to the metabolite content of seeds of the transformants. Interestingly, the metabolite content of the transgenic seeds was more divergent from that of the wild type than the metabolite content of the fruit (Supplemental Table S4). The most strongly repressed lines (Lin5i-1 and Lin5i-15) both displayed increases in the seed levels of β-Ala, Gly, Ile, Orn/Arg, Tyr, Val, fumarate, succinate, galacturonate, and threonate. All of the transgenic lines, furthermore, displayed elevated seed levels of Fru and Glc (as well as of the minor sugar Gal) and corresponding increases in the levels of Fru-6-P and Glc-6-P. The fact that so many of these metabolites increase in the seeds of the transformants indicates that the changes in seed morphology we described above are unlikely to be due to a modification of seed metabolism per se (which would be anticipated to result in decreased metabolite levels) but rather are the result of the decreased photoassimilate delivery and/or a disruption of normal developmental cues within the transgenic fruit.

LIN5 Inhibition Impairs Pollen (Tube Growth) Development

It is well documented that the number of seeds in tomato fruit, like that in many other species, correlates positively to the final fruit size (Kinet and Peet, 1997; Hackel et al., 2006). It follows, therefore, that the smaller fruit size of the antisense plants might be due to impaired development of fertile seeds. One potential reason for the reduced fertility of the Lin5i lines could be that they exhibit a reduced pollination due to malformation of mature pollen or reduced pollen tube growth. For this reason, we performed evaluation of both the germination rate and viability in the Lin5i lines. The Lin5i lines showed a reduced germination rate (Fig. 5A): whereas the germination rate of wild-type pollen at pH 6 was approximately 35%, the germination rate of pollen in Lin5i-lines was reduced to between 6% and 12%. This result was confirmed by a second, independent study (data not shown). Not only pollen germination but also pollen

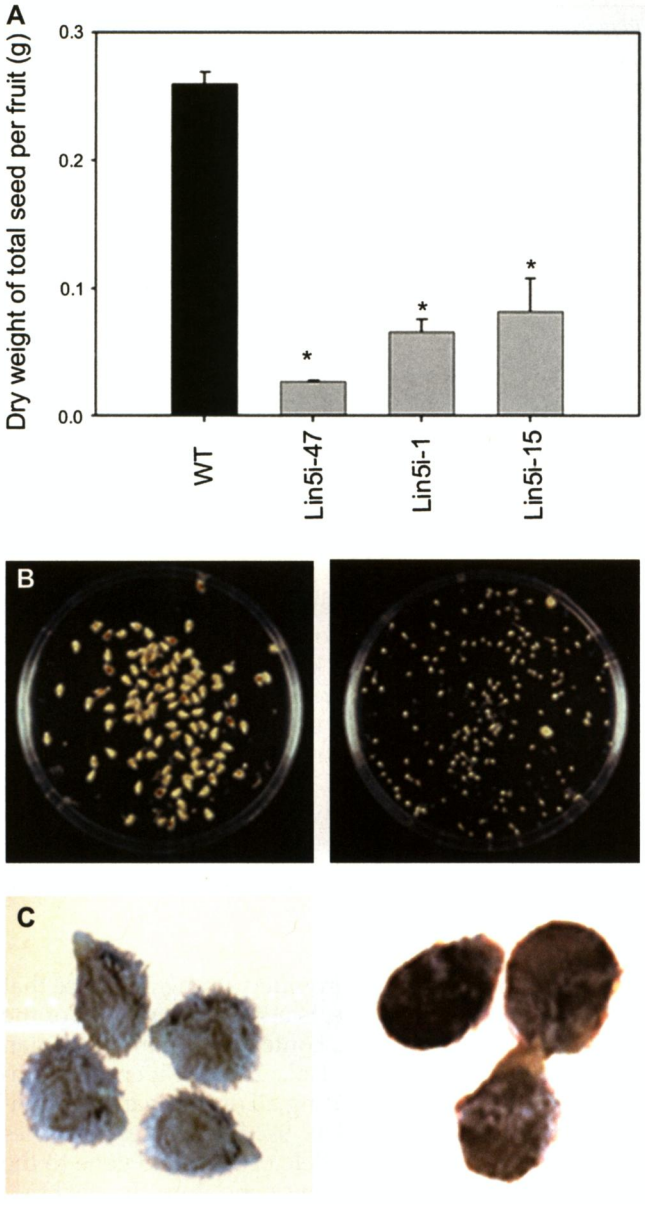

Figure 3. Quantification of seed production per fruit. A, Dry weight of total seeds per fruit of wild-type (WT) and Lin5i transgenic lines. B and C, Phenotypic analysis of wild-type and Lin5i transgenic line seeds. In each case, the wild type is shown on the left side and a representative Lin5i transgenic line is shown on the right side. Ovule-like structures are seen in B, and alterations in the seed coat are observed in C.

viability and pollen morphology were altered in Lin5i plants. Whereas in wild-type plants the pollen viability was around 30%, as shown by fluorescein diacetate staining, the viability of Lin5i lines was reduced to between 7% and 15% (Fig. 5B)

Raster electron microscopic analysis of the transgenic pollen revealed severe morphological distortions (Fig. 5C), indicating that not only pollen tube growth is impaired due to reduced pollen vitality but pollen development is affected at a very early stage of pollen maturation. Obviously, the carbon supply and pollen loading are affected in these transgenic plants at a very early stage of flower development.

Evaluation of Hormone Metabolism

Given that several of the observed phenotypes do not directly correlate with either the change in invertase expression or the changes in sugar content in the transformants, we next decided to evaluate other potential mechanisms that are more directly related to plant morphology. Given the large body of literature suggesting sugar-hormone cross talk (Leon and Sheen, 2003; Moore et al., 2003; Carrari et al., 2004; Loreti et al., 2008) as well as the recent demonstration that the miniature mutant of maize, *mn1*, which harbors a lesion in cell wall invertase, displays altered levels of phytohormones (LeClere et al., 2008), we chose to focus on these.

As a first experiment, we used the TOM1 microarray (Alba et al., 2004) to profile the transcript levels of a wide number of genes in young fruit (3–5 DPA) of the wild type and of line Lin5i-15. From an earlier analysis (van der Merwe et al., 2009), we know that this chip affords relatively broad coverage of genes associated with biosynthesis and/or signaling pathways connected to the major phytohormones. When the genes associated with hormone biosynthesis or function are considered, dramatic changes were seen in indole-3-acetic acid-induced gene, 9-cis-epoxycarotenoid dioxygenase, ethylene-insensitive 3 protein, response regulator 16 (cytokinin, signal transduction), putative lipoxygenase, ethylene-responsive element-binding protein, universal stress protein USP1-like protein (SGN-U315394), and lipoxygenase (SGN-U314559; Fig. 6A). Only minor changes were observed in the GA-associated genes. In order to confirm some of these findings, we chose to perform qRT-PCR analysis across all genotypes for a subset of the genes. For this purpose, we chose indole-3-acetic acid-induced gene (SGN-U319800), 9-cis-epoxycarotenoid dioxygenase (SGN-U316706), ethylene-insensitive 3 protein (SGN-U313902), response regulator 16 (cytokinin, signal transduction; SGN-U320499), putative lipoxygenase (SGN-U315633), GA-regulated family protein (SGN-U317792), and GA metabolism gene (SGN-U315071). All of the qRT-PCR analyses with the exception of that associated with GA metabolism (SGN-U315071) were in close agreement with the microarray data (Fig. 7, A–G). The changes in the levels of those genes associated with GAs and that associated with cytokinin largely paralleled the changes in invertase activity across the genotypes. However, changes in genes associated with auxin, abscisic acid (ABA), ethylene, and jasmonate (JA) were relatively similar in all three transgenic lines.

Having established changes in the levels of transcripts encoding key biosynthetic enzymes or hormone response elements, we next attempted to determine as many of the hormones themselves as possible in plant material harvested in parallel to that used for the qRT-PCR analyses described above. For this purpose, we adopted a recently described protocol to measure

Table III. *Levels of carbohydrates in the pericarp of wild-type and Lin5i transgenic lines*

Values represent means ± SE (n = 6). Values shown in boldface were determined to be significantly different from the wild-type value by the t test ($P < 0.05$).

Metabolite	Wild Type	Lin5i-47	Lin5i-1	Lin5i-15
		μmol g^{-1} fresh weight		
Metabolite content in red fruit				
Suc	17.8 ± 2.4	20.3 ± 2.3	23.7 ± 3.2	19.1 ± 3.7
Glc	291.8 ± 31.8	236.3 ± 43.1	**195.4 ± 32.2**	**205.0 ± 18.5**
Fru	288.5 ± 24.9	251.6 ± 35.2	239.1 ± 13.5	214.7 ± 17.7
Starch (nmol equivalents g^{-1} fresh weight)	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.24 ± 0.08
Metabolite content in green fruit				
Suc	24.0 ± 4.1	24.4 ± 4.8	**34.0 ± 3.3**	28.3 ± 3.9
Glc	158.9 ± 37.8	139.0 ± 12.4	151.9 ± 5.9	151.4 ± 4.7
Fru	150.0 ± 10.6	130.1 ± 8.6	142.8 ± 35.4	151.2 ± 5.6
Starch (nmol equivalents g^{-1} fresh weight)	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.1

phytohormones (Pan et al., 2008) but used ultra performance liquid chromatography Fourier transformion cyclotron resonance mass spectrometry for the determinations. By comparison with spectra obtained from authentic chemical standards, we were able to quantify the relative levels of ABA, JA, and the GAs GA1 and GA3 (Fig. 6B). Wild-type values were similar to those reported previously (O'Donnell et al., 1996; Galpaz et al., 2008; Serrani et al., 2008). The levels of ABA and JA were significantly decreased in all lines, whereas the levels of the GAs closely mirrored the level of inhibition of LIN5, only decreasing in the two most severely inhibited transgenic lines. All observed decreases were dramatic, with ABA being reduced to less than 50% of the wild-type level and JA to as little as 20% of the wild-type level in line Lin5i-47, while GA1 was decreased to as little as 50% and GA3 was only decreased to 90% of the wild-type level in line Lin5i-15.

Given that the transgenic lines exhibited decreased levels of expression of genes associated with hormone biosynthesis as well as altered levels of the phytohormones themselves, we next addressed the question, are these changes in expression sugar mediated? For this purpose, we excised discs from young fruit harvested from wild-type plants in 10 mM MES-KOH buffer (pH 6.5) in the presence or absence of 50 mM Glc, 2-deoxyglucose, Fru, or Suc for a period of 4 h. Following the incubation, discs were rapidly washed and frozen in liquid nitrogen. RNA was subsequently isolated from the discs, and qRT-PCR experiments were carried out using the same primers as described above. Several interesting trends were observable from the study (Fig. 7, H–N). Indole-3-acetic acid-induced gene SGN-U319800 (Fig. 7H) was repressed by Suc but unaffected by the hexoses applied, while the 9-cis-epoxycarotenoid dioxygenase gene SGN-U316706 (Fig. 7I) was repressed by all sugars, including 2-deoxyglucose, which, since 2-deoxyglucose is a nonmetabolizable analog of Glc (Fernie et al., 2002), suggests that this repression is independent of the hexokinase reaction. Expression of the ethylene-insensitive 3 protein (SGN-U313902; Fig. 7J) appears to be repressed by Suc but enhanced by Fru, while both response regulator 16 (cytokinin, signal transduction; SGN-U320499) and putative lipoxygenase (SGN-U315633; Fig. 7, K and L) were repressed by Suc, Glc, and Fru feedings, yet interestingly, not following incubation in 2-deoxyglucose. Finally, the level of only one of the GA-associated genes, the GA-regulated family protein (SGN-U317792; Fig. 7M), was altered following incubation with either Fru or Suc.

DISCUSSION

Previous studies have provided strong evidence that LIN5, a cell wall invertase, is a key determinant of the total soluble solid, or Brix, content in tomato (Fridman et al., 2000, 2004; Baxter et al., 2005). Given the complexities in directly attributing all of the changes previously described in the Brix 9-2-5 introgression lines harboring the *S. pennellii* allele of the LIN5 gene to the allelic variance apparent in this gene alone (Baxter et al., 2005; Schauer et al., 2006), we adopted the RNAi approach here in order to generate transgenic plants that were exclusively altered in the expression of this specific isoform of invertase. This work was carried out with two aims in mind: first, to provide in planta proof of the importance of LIN5 in the control of the total soluble solids content in tomato, and second, to carry out an analysis of other physiological roles of this enzyme in a genetic background that renders such analyses facile.

Confirmation of the Role of LIN5 in the Determination of Brix Content

The use of artificial gene dosage experiments reliant on reverse genetic approaches has been documented previously (Liu et al., 2003) for studies confirming the suggestion from the QTL approach that fw2.2 is an important determinant of tomato fruit size (Frary et al., 2000). However, in this previous example, con-

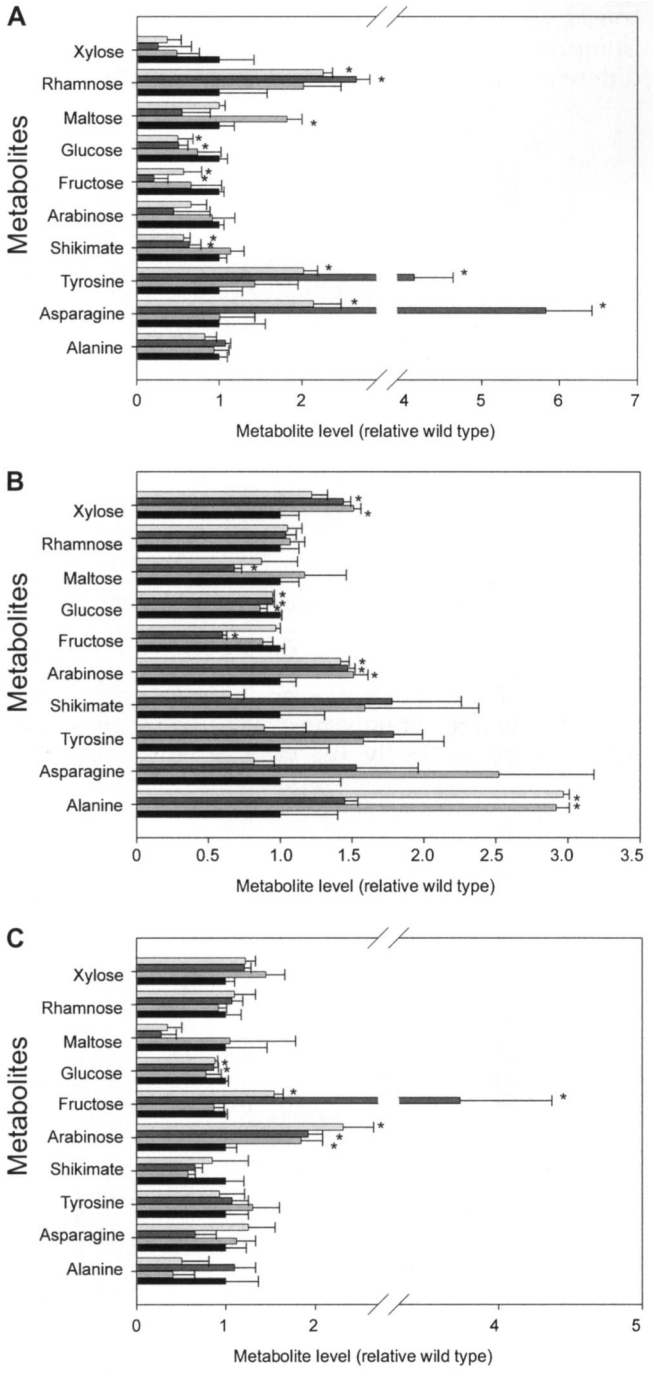

Figure 4. Relative metabolite content in young, green, and red fruit of wild-type and Lin5i transgenic lines. A, Metabolites were determined in pericarp of young fruit. B, Metabolites were determined in pericarp of green fruit. C, Metabolites were determined in pericarp of red fruit. Data are normalized to the mean response calculated for the wild type. The values are representative of the mean ± SE of six individual plants. Asterisks indicate values determined by the *t* test to be significantly different from the wild-type control value ($P < 0.05$). Wild type, Black bars; Lin5i-47, light gray bars; Lin5i-1, dark gray bars; Lin5i-15, white bars.

siderable evidence had accumulated, prior to the generation of the transgenics, that suggested that the fw2.2 QTL exerted its effect at the level of transcription rather than that of the protein function (Cong et al., 2002). This was not the case with the Brix 9-2-5 QTL, since we had previously demonstrated that this was mediated by a conformational change close to the substrate-binding site of the protein, with transcript and protein levels being independent of the allele expressed (Fridman et al., 2004). Despite this fact, we believed that adoption of the RNAi approach would allow us to confirm the results of our previous study. Our reasoning here was that since the heterologous expression of different alleles of LIN5 revealed that they displayed different substrate-binding efficiencies

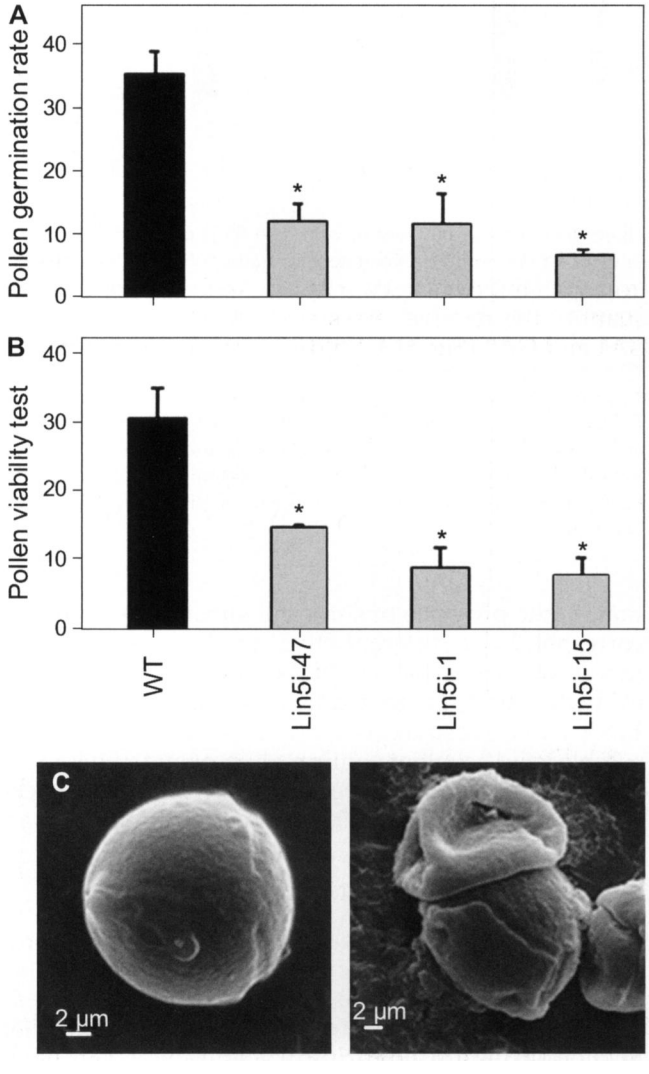

Figure 5. Pollen germination rate, viability, and morphology of wild-type (WT) and Lin5i transgenic lines. A, Pollen germination rate in the transgenic Lin5i lines are decreased. B, Pollen viability on the transgenic lines is also decreased. The values are representative of the mean ± SE of six individual plants. The pollen germination rate was determined from between 50 and 500 pollen grains from each plant. Asterisks indicate values determined by the *t* test to be significantly different from the wild-type control value ($P < 0.05$). C, Raster electron microscopy images of wild-type (left) and Lin5i transgenic plants (right); representative examples were chosen.

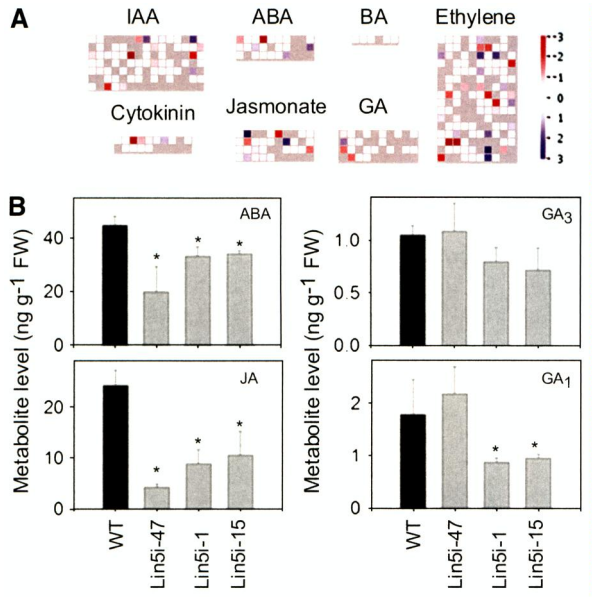

Figure 6. Hormone profiling of wild-type (WT) and Lin5i transgenic lines of young fruit. A, Differences in transcript levels in a Lin5i-1 transgenic line related to the wild type for genes associated with hormone metabolism. Red and blue represent decrease and increase, respectively, of expression with respect to the average of all points. Here, each unigene that has been assigned to a process is represented by a single colored box. BA, Benzyladenine; IAA, indole-3-acetic acid. B, Quantifications include the measurement of ABA, JA, and GAs (GA1 and GA3). The values are representative of the mean ± SE of six individual plants. Asterisks indicate values determined by the *t* test to be significantly different from the wild-type control value ($P < 0.05$). FW, Fresh weight.

that, in the presence of sufficient substrate, effectively correspond to a different efficiency, then reduction of invertase expression could be used as a proxy to evaluate this QTL in planta. That the reduction of LIN5 activity resulted in a linear decrease in the total soluble solids content confirmed, in planta, the importance of this isoform of cell wall invertase in determining agronomic yield in the tomato.

Morphological Effects of the LIN5 Deficiency

In addition to the decrease in soluble solids content, the fruit of the transgenic lines were compromised in size. This observation was also largely to be expected, since several direct genetic studies (Zrenner et al., 1995; Sonnewald et al., 1997; Sturm and Tang, 1999) have implicated Suc mobilization as a key determinant of sink strength in a broad range of species. Within tomato fruit itself, the activities of invertase and Suc synthase have been suggested to play key roles in various aspects of fruit development, including the rate of fruit set and the final fruit size (Sun et al., 1992; Wang et al., 1993; D'Aoust et al., 1999). One surprising consequence of the RNAi inhibition of LIN5 was the dramatic reduction of Suc synthase activity when assayed in the direction of Suc breakdown. Given that we were unable to detect considerable changes in the expression of the known isoforms of Suc synthase in tomato and the fact that this difference was not observable when assayed in the

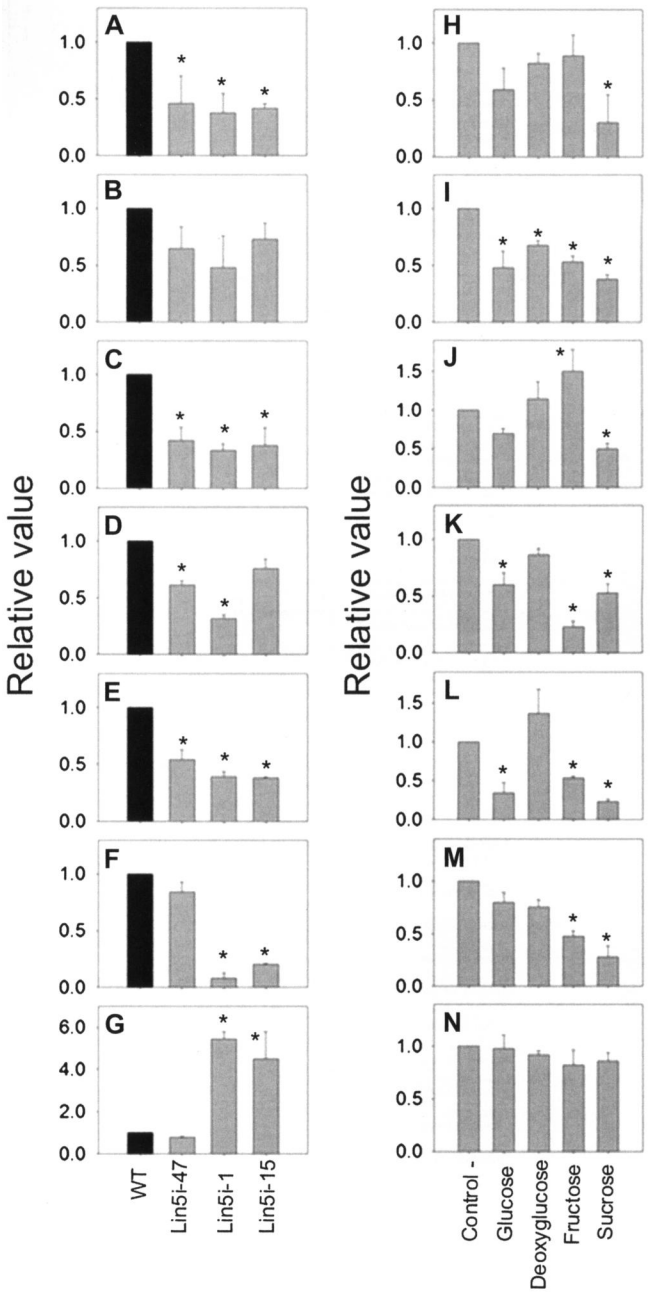

Figure 7. Expression of hormone metabolism genes of wild-type (WT) and Lin5i transgenic lines and in the wild type after feeding with different sugars. Analyses were done in young fruit. A to G, Expression of hormone metabolism genes of wild-type and Lin5i lines in young fruit. H to N, Expression of hormone metabolism genes of the wild type in young fruit incubated for 2 h in 10 mM MES-KOH buffer, pH 6.5, with 50 mM of each sugar. The values are representative of the mean ± SE of six individual plants. Asterisks indicate values determined by the *t* test to be significantly different from the wild-type control value ($P < 0.05$). Genes of hormone metabolism are as follows: A and H, SGN-U319800; B and I, SGN-U316706; C and J, SGN-U313902; D and K, SGN-U320499; E and L, SGN-U315633; F and M, SGN-U317792; G and N, SGN-U315071.

direction of Suc synthesis, we speculate that this is the result of one of the many characterized posttranslational regulations of the enzyme (Huber and Hardin, 2004). Despite the wide changes in morphological parameters, metabolic changes in the transgenic fruit were, by and large, confined to sugar metabolism, with few consistent changes observed in the levels of other metabolic intermediates. While this observation was somewhat surprising at first sight, it was consistent with changes in metabolite profiles for the introgression line harboring (among others) the *S. pennellii* LIN5 allele (Baxter et al., 2005; Schauer et al., 2006). Furthermore, it is in keeping with the prevailing opinion that fruit growth is largely dependent on the import of photoassimilate as opposed to the fruit's own photosynthetic capacity (Tanaka et al., 1974; Obiadalla-Ali et al., 2004).

Deficiency in LIN5 Leads to Clear Alterations in Hormone Metabolism

That the metabolic changes appear to be restricted in breadth is interesting with respect to the morphological parameters that were noted to change in the transgenics. While we currently have no clear explanation for the petal/sepal phenotype, it seems reasonable to suggest that this is the result of an altered hormonal regulation during floral development. Interestingly, a recent report on the *mn1* maize mutant, which is deficient in apoplastic invertase and characterized by miniature seeds, stated that it was characterized by dramatic reductions in auxin and auxin conjugates and alterations in the levels of ABA, JA, and salicylic acid (LeClere et al., 2008). Here, we present multiple lines of evidence that the LIN5 transformants are also affected in hormonal metabolism. First, the expression of genes associated with key biosynthetic enzymes of response factors associated with hormones is markedly reduced in the transgenic lines. Expression of genes associated with indole-3-acetic acid, ABA, ethylene, and JA strongly correlates with the morphological phenotype, while genes associated with cytokinin and GA biosynthesis are in closer accord to the degree of inhibition of invertase expression. Second, the levels of ABA and JA and the levels of the GAs GA1 and GA3 themselves mirror the changes observed at the level of transcription, confirming earlier suggestions that the proteins encoded by these genes play key meditating roles in establishing the observed phenotype. Finally, experiments in which we artificially altered the sugar content of the cell via incubation in Suc or hexoses revealed that the majority of these genes are sugar responsive, hence supplying a clear mechanistic link between the genetic perturbation applied and the observed phenotypes.

Linking Hormonal and Morphological Phenotypes

While it has been commonly noted that tomato fruit size is paralleled by the number of seeds per fruit (Kinet and Peet, 1997), it is accepted that resource allocation between these sink organs is complex, with the majority of studies into parthenocarpy (seedless fruit formation) suggesting a preeminent role for GAs in this process (for review, see Gorguet et al., 2005). That said, evidence resulting from the down-regulation of the *IAA9* gene revealed that auxin is also a major player in the maintenance of normal fertility (Wang et al., 2005, 2009). Given that there seem to be multiple inputs defining this process, as well as the possible impact that seed number itself has on the pericarp hormone levels (Britten, 1950), it is perhaps not surprising that the seed yield does not seem to correlate well to our measurements of changes in GA metabolism. Of particular note is line Lin5i-47, which displays only a relatively minor suppression of invertase expression and unaltered levels of GAs but has a dramatically reduced seed yield. Intriguingly, this seed yield phenotype appears to correlate much more closely to the expression levels of indole-3-acetic acid-induced gene (SGN-U319800), ethylene-insensitive 3 protein (SGN-U313902), response regulator 16 (cytokinin, signal transduction; SGN-U320499), putative lipoxygenase (SGN-U315633), and the levels of ABA and JA. Thus, the biosynthesis of these hormones as well as their associated signal transduction cascades is important in defining this phenotype. By contrast, the total fruit yield correlates much better with the degree of reduction in invertase expression and, therefore, also the levels of GAs and cytokinins. These links are not without precedence, since invertase has long been associated with sink strength (see introduction), while cytokinins have also been clearly demonstrated to be associated with growth and GAs have previously been documented to play a role in growth (Chaudhury et al., 1993; Werner et al., 2003; Biemelt et al. 2004). However, analysis of the pollen vitality and morphology also revealed a defect in pollen growth similar to that previously observed in LeSUT2 antisense tomato plants, which exhibited severely reduced expression of one of the isoforms of the Suc transporter family (Hackel et al., 2006). Although the pollen tubes of the LeSUT2 transformants were also characterized by reduced Suc uptake, the fact that pollen tubes take up both Suc and hexoses (Lemoine et al., 1999) means that an impaired pollen growth does not necessarily implicate a role for LIN5 within the pollen itself. Indeed, the expression pattern of LIN5 (Godt and Roitsch, 1997; Fridman and Zamir, 2003) as well as the metabolic profiles of the larger seeds of the transformants indicate that they exhibit problems in neither uptake nor metabolism of sugars. Our results thus suggest that the LIN5 RNAi lines exhibit aberrant pollen function, which is most likely a consequence of an altered stamen Suc supply. Circumstantial support for this hypothesis is provided by the facts that similar seed phenotypes have been observed in maize (Miller and Chourey, 1992; Roitsch and Gonzalez, 2004) and that an important role for cell wall invertase has been recognized during grain filling in barley (*Hordeum vulgare*) caryopses (Weschke et al., 2003). That said,

given the demonstration of hormonal imbalance in the transformants, we certainly cannot rule out that hormonal factors underlie the phenotype, and further experimentation will be required to clarify the exact mechanism linking these phenotypes.

A Mechanistic Role for LIN5 in Fruit Abortion

A second clear phenotype of the reproductive tissues of the transformants was the enhanced rate of fruit abortion. This is particularly interesting given that invertase has been clearly implicated to be involved in this process on the basis of experiments correlating the level of expression of genes encoding these enzymes with the incidence of abortion following exposure to stress (for review, see Boyer and McLaughlin, 2007). Previous studies have demonstrated that abortion occurs concomitant with low ovary sugar contents in a wide range of species (Dorion et al., 1996; Aloni et al., 1997; Sheveleva et al., 2000; Andersen et al., 2002; McLaughlin and Boyer, 2004). Detailed evaluation of these data sets suggests that low Suc is universally seen prior to abortion but that changes in other sugars appear to be species specific (Aloni et al., 1997; Sheveleva et al., 2000). Several of these studies also demonstrated that the expression of cell wall invertase was reduced in conditions such as drought stress that promote abortion (Andersen et al., 2002; McLaughlin and Boyer, 2004). However, it is important to note that these studies were merely correlative and that changes in hormone levels (Andersen et al., 2002) and in the expression of a diverse range of other genes (Boyer and McLaughlin, 2007) have also been reported to occur coincidently to abortion. The fact that transformants deficient in the expression of LIN5 displayed a higher incidence of abortion provides direct evidence that an inhibition of cell wall invertase is causal to the process of abortion and suggests that it represents an early step in the signal transduction cascade linking perception of stress to the initiation of senescence and membrane degradation events that lead to irreversible abortion. The changes documented here in the expression of genes associated with hormonal synthesis and function, when considered alongside the known functions of phytohormones in fruit set and development of tomato (Wang et al., 2005; Vriezen et al., 2008), provide hints to the nature of this cascade that may ultimately lead to its elucidation.

MATERIALS AND METHODS

Materials

Tomato (*Solanum lycopersicum* 'Moneymaker') was obtained from Meyer Beck. Plants were handled as described in the literature (Carrari et al., 2003; Nunes-Nesi et al., 2005). For the drought stress treatments, well-watered 7-week-old flowering tomato plants were used. The treatments consisted of two different conditions: well-watered plants and plants rewatered after 3 d without water. Before and during the experiment, the new flowers were labeled daily and the number of flowers that developed to fruit (fruit set) was determined after 13 weeks of growth. As a control, the relative water content was determined before rewatering, always after 1 h of illumination. The genotypes used were the wild type, Lin5i-1, and Lin5i-15, with five replicates of each genotype per growth condition. All chemicals and enzymes used in this study were obtained from Roche Diagnostics.

Generation of Transgenic Plants

A 513-bp fragment of SlLIN5 was cloned using the RNAi approach into the vector pART27 (Gleave, 1992) between the cauliflower mosaic virus 35S promoter and the ocs terminator. This construct was introduced into plants by an *Agrobacterium tumefaciens*-mediated transformation protocol, and plants were selected and maintained as described in the literature (Tauberger et al., 2000). Initial screening of 33 lines was carried out on the basis of total acid invertase enzyme activity. This screen allowed the identification of three lines, which were taken to the next generation.

Enzyme Measurement

Enzyme extracts were prepared as described previously (Tauberger et al., 2000). Invertase activities were determined as described by Gibon et al. (2004), and apoplastic invertase activity was measured as described by Baxter et al. (2005). Desalted extracts were used to measure selected enzymes using spectrophotometric assays. Phosphoglucoisomerase and pyruvate kinase were determined as described by Fernie et al. (2001), and Suc synthase (Suc synthesis direction) and AGPase were determined as described by Sweetlove et al. (1996). Suc synthase (hydrolytic direction) was determined as detailed by Zrenner et al. (1995).

RNA Extraction and Quantification and qRT-PCR Analysis

Total RNA was extracted according to Bugos et al. (1995) with minor modifications. Integrity of the extracted RNA was checked by electrophoresis under denaturing conditions after treating the RNA with RNase-free DNaseI (Roche). First-strand cDNA synthesis of 1 μg of RNA in a final volume of 20 μL was performed with Moloney murine leukemia virus reverse transcriptase, Point Mutant RNase H Minus (Promega), according to the supplier's protocol using oligo(dT) T19 primer.

Expression of the *LIN5*, *LIN7*, *SuSy2*, *SuSy3*, *SGN-U331270*, and hormone-related genes was analyzed by real-time qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad; http://www.bio-rad.com/) as described by Schaarschmidt et al. (2006). Relative quantification of the target expression level was performed using the comparative Ct method. The following primers were used: for analysis of *LIN5* transcript levels (GenBank accession no. AJ272304), forward, 5′-TTGGAAGGGATTGAGAATCGA-3′, reverse, 5′-CGGATTCATTTGACCAACCC-3′ (Kortstee et al., 2007); for *LIN7* (GenBank accession no. AF506006), forward, 5′-TTTGGTGCTGGTGGAAAGACA-3′, reverse, 5′-GGCTCCGTTCCGTTGTTAAAC-3′; for *SuSy2* (GenBank accession no. L19762), forward, 5′-TTTGGCTGACTGTTGTCGAAG-3′, reverse, 5′-GTCGAAATGGTTTCCCAATG-3′ (Kortstee et al., 2007); for *SuSy3* (GenBank accession no. AJ011319), forward, 5′-GAACGAAGAACACCTGTGTGT-3′, reverse, 5′-TCTCTGCCTGCTCTTCCAA-3′ (Kortstee et al., 2007); for *SGN-U331270*, forward, 5′-TGGAGGCCGAAATGCTTCT-3′, reverse, 5′-CGTGGTTCCTTTAGCATCAGGT-3′; for *SGN-U319800*, forward, 5′-AGGAGTTTTCTTAGAGGAGCGGAA-3′, reverse, 5′-ACGCGAGTGCTCTTCTTGTAGC-3′; for *SGN-U316706*, forward, 5′-ATTGCATCGTGGCATGTCG-3′, reverse, 5′-ACACCACGTAGCCATCGTCTTC-3′; for *SGN-U313902*, forward, 5′-CACCATGCCTAGTTCTTCAACCTT-3′, reverse, 5′-CTGGTGGATTTTTCATCGCCTA-3′; for *SGN-U320499*, forward, 5′-ACTTCCTCTCCATCGTCCCCTA-3′, reverse, 5′-AAGGCATGGAGGAATCAGTGG-3′; for *SGN-U315633*, forward, 5′-TTGCCTATGGTGCTGAATGGA-3′, reverse, 5′-CAAGCCATGTGGTTCATTTGG-3′; for *SGN-U317792*, forward, 5′-CACATTGCATGCCTCTTCACA-3′, reverse, 5′-GCTTCAATGTCAAGAGCTCAACC-3′; and for *SGN-U315071*, forward, 5′-CTCGAATCCGAAGCCGTTAAA-3′, reverse, 5′-GCCATAACCAAAAGGATCAGCA-3′. To normalize gene expression for differences in the efficiency of cDNA synthesis, transcript levels of the constitutively expressed elongation factor 1α of tomato (GenBank accession no. X14449) were measured using the following primers: forward, 5′-ACCACGAAGCTCTCCAGGAG-3′, reverse, 5′-CATTGAACCCAACATTGTCACC-3′ (Schaarschmidt et al., 2006).

Measurement of Fruit Brix

Ripe fruit tissue was homogenized with a razor blade, and the soluble solids (Brix) content of the resulting juice was measured on a portable refractometer (Digitales Refrktometer DR6000; Krüss Optronic).

Soluble Sugar and Starch Determination

Fruit pericarp samples were harvested as described previously (Carrari et al., 2006; Schauer et al., 2006). In brief, a single fruit was harvested from six independent plants per genotype, and starch and soluble sugars were extracted and determined using established protocols (Fernie et al., 2001).

GC-MS

The relative levels of metabolites were determined on the exact same samples as used for soluble sugar and starch determination using the GC-MS protocol as described by Roessner et al. (2001) with the exceptions that the method was optimized for tomato fruit (Roessner-Tunali et al., 2003a) and the mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka et al., 2005; Schauer et al., 2005). Seed metabolites were extracted following the method of Fait et al. (2006).

In Vitro Pollen Grain Germination

Anthers from tomato plants were collected, opened with a razor blade, placed in Eppendorf tubes, vortexed to remove the pollen, and incubated in the dark at 26°C in 20 mM MES buffer, pH 6.0, containing 15% polyethylene glycol 4000, 2% or 5% Suc, 0.07% $Ca(NO_3)_2 \cdot 4H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% KNO_3, and 0.01% H_3BO_3 according to Jahnen et al. (1989). Photographs were taken with a Zeiss Axiophot microscope, and pollen germination rate was determined with a Fuchs-Rosenthal chamber. Pollen was taken from two to four individual plants for the transformant lines and from six individual plants of the wild type and scored as geminated when the pollen tube was longer than the pollen grain diameter. For each experiment, between 50 and 500 pollen grains were counted. Nuclei were stained with 0.01% 4′,6-diaminophenylindole.

Pollen Viability Assay

Viability assays were performed in medium with fluorescein diacetate (1.75 M Suc, 3.23 mM boric acid, 3.05 mM calcium nitrate, 3.33 mM magnesium sulfate heptahydrate, 1.98 mM potassium nitrate, and 7.21 mM fluorescein diacetate dissolved in acetone; 10 drops per 10 mL of medium) freshly prepared according to Pline et al. (2002). Viability was examined with fluorescence microscopy using a 470-nm excitation filter and a 520-nm emission filter with a Fuchs-Rosenthal chamber. Pollen grains were classified as viable when they showed uniform and bright fluorescence.

Raster Electron Microscopy

Tomato pollen was collected in Eppendorf tubes and attached to double-sided adhesive tape (Plano). Scanning electron microscopy aluminum specimen holders were covered with conducting adhesive tape, and the objects were stuck down onto this. The specimens were coated with 20-nm gold using a SCD 005 sputter coater (BAL-TEC). Scanning electron microscopy was carried out with a LEO 1430 scanning electron microscope (Zeiss). Electron microscopy images were taken from four to 10 pollen grains per line.

Microarray Analysis

Glass slides containing arrayed tomato ESTs were obtained directly from the Center for Gene Expression Profiling at the Boyce Thompson Institute, Cornell University, the Geneva Agricultural Experiment Station, and the U.S. Department of Agriculture Federal Plant and Nutrition Laboratory. The tomato array contains 13,440 spots randomly selected from cDNA libraries isolated from a range of tissues, including leaf, root, fruit, and flowers, and representing a broad range of metabolic and developmental processes. Technical details of the spotting are provided at MIAME (http://www.mpimp-golm.mpg.de/fernie). Fluorescent probe preparation and microarray hybridization and data analysis were exactly as described previously (Urbanczyk-Wochniak et al., 2006). Detailed information is included in MIAME (http://www.mpimpgolm.mpg.de/fernie). The raw expression data were processed using Robin, a graphical application for microarray analysis. Robin facilitates the usage of advanced R/BioConductor packages (i.e. limma and RankProd; Smyth, 2004; Hong et al., 2006) by providing an easy-to-use graphical interface to the underlying R functions. After evaluation of chip quality by visual inspection of background signal intensity, signal intensity distribution per color channel, and red-green signal ratios for each chip, the data were normalized within each array using print-tip group-wise Loess normalization with background subtraction and between the arrays in the experiment by scaling the $\log_2$ ratios to have the same median absolute deviation. The normalized data were statistically analyzed using the linear model-based approach implemented in the limma package using the Benjamini-Hochberg method to correct P values for multiple testing.

Hormone Analysis

Extraction of hormones from young fruit was carried out as described by Pan et al. (2008), with the exception that 10 g of tissue and a lengthened inhibition period were used, as described by van der Merwe et al. (2009). In brief, the liquid nitrogen-frozen pericarp material was ground into a fine powder. Hormones were subsequently extracted by an overnight incubation in 50 mL of 1-propanol:water:concentrated HCl (2:1:0.002, v/v/v) containing 10 to 50 ng of the internal standards ABA, GA (GA1 and GA3), and JA. A total of 50 mL of dichloromethane was then added, and the mixture was incubated for an additional 30 min before two phases were clarified by a 5-min, 13,000-rpm centrifugation. The lower phase, containing the hormones, was collected and concentrated to dryness in a rotary evaporator. The residue was resolubilized in 200 μL of methanol. A total of 2 μL of the methanol extract was separated using an ultra performance liquid chromatography system (Acquity; Waters) connected to a LTQ FT-ICR-Ultra mass spectrometer (Thermo-Fisher). Separation was performed on a HSS T3 C_{18} reverse-phase column (100 × 2.1 mm i.d., 1.8-μm particle size; Waters) at a temperature of 40°C. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate of the mobile phase was 400 μL min^{-1}, and the following gradient profile was applied: 1 min of isocratic run at 75% A, then a linear 6-min gradient was applied to 1% A. This was followed by a 1-min isocratic period at 1% A, before switching back to 75% A. The mass spectra were acquired covering a mass-to-charge ratio from 100 to 400. Resolution was set to 100,000, and the transfer capillary temperature was set to 200°C.

Cytology

The ploidy level of transgenic lines was determined by counting the chromosome number of root tip cells. Small, actively growing root tips from 7-d-old in vitro-grown tomato plantlets were cut and rinsed with tap water to remove residual agar. Root tips were then treated in an aqueous α-monobromonaphthalene-saturated solution for 3 h at room temperature, fixed in a 1:3 mixture of acetic acid:ethanol, and stained in 1% acetoorcein (for 1 d). Root tips were placed on a glass slide and squashed in a drop of lactic acid. Chromosomes in cells undergoing mitosis were counted using an epifluorescence microscope (Olympus AX 70).

Statistical Analysis

The t tests were performed using the algorithm embedded into Microsoft Excel. The term significant is used only in conjunction with the t test result ($P < 0.05$).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ploidy level of the transgenics.

Supplemental Table S1. Metabolite levels in young fruits of wild type and transgenics.

Supplemental Table S2. Metabolite levels in green fruits of wild type and transgenics.

Supplemental Table S3. Metabolite levels in red fruits of wild type and transgenics.

Supplemental Table S4. Metabolite levels in seeds of wild type and transgenics.

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