Tomato Fruit Photosynthesis Is Seemingly Unimportant in Primary Metabolism and Ripening But Plays a Considerable Role in Seed Development[1][W][OA]

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Fruit of tomato (*Solanum lycopersicum*), like those from many species, have been characterized to undergo a shift from partially photosynthetic to truly heterotrophic metabolism. While there is plentiful evidence for functional photosynthesis in young tomato fruit, the rates of carbon assimilation rarely exceed those of carbon dioxide release, raising the question of its role in this tissue. Here, we describe the generation and characterization of lines exhibiting a fruit-specific reduction in the expression of glutamate 1-semialdehyde aminotransferase (GSA). Despite the fact that these plants contained less GSA protein and lowered chlorophyll levels and photosynthetic activity, they were characterized by few other differences. Indeed, they displayed almost no differences in fruit size, weight, or ripening capacity and furthermore displayed few alterations in other primary or intermediary metabolites. Although GSA antisense lines were characterized by significant alterations in the expression of genes associated with photosynthesis, as well as with cell wall and amino acid metabolism, these changes were not manifested at the phenotypic level. One striking feature of the antisense plants was their seed phenotype: the transformants displayed a reduced seed set and altered morphology and metabolism at early stages of fruit development, although these differences did not affect the final seed number or fecundity. Taken together, these results suggest that fruit photosynthesis is, at least under ambient conditions, not necessary for fruit energy metabolism or development but is essential for properly timed seed development and therefore may confer an advantage under conditions of stress.

Fruit development is a tightly genetically controlled process, unique to flowering plants, which provides both a suitable environment for seed maturation and a mechanism for their dispersal. Given the fundamental nature of both the dietary and biological significance of fruit, the molecular dissection of fruit development has recently received considerable interest (Manning et al., 2006; Vrebalov et al., 2009; Wang et al., 2009). The fruit is the result of the development of the ovary, with fruit organogenesis originating from a flower primordium, with the mature flower either being fertilized (and developing into a fruit) or not (and entering the abscission process; Vivian-Smith and Koltunow, 1999; Wang et al., 2009). Considerable advances have recently been made in understanding key elements of the genetic control of ripening and development (Giovannoni, 2007; Seymour et al., 2008; Matas et al., 2009; Vrebalov et al., 2009; Mathieu-Rivet et al., 2010; Karlova et al., 2011), and the importance of several biochemical pathways, including sugars, organic acids, cell wall, and volatile metabolism, during this process has been demonstrated (Yelle et al., 1991; Rose et al., 2004; Carrari et al., 2006; Klee, 2010). Much of this research has been carried out in tomato (*Solanum lycopersicum*), which is a well-studied model system for fleshy fruit development and is well understood from a hormonal regulatory perspective. That said, despite the fact that the genetic control of pigment metabolism during tomato fruit development is also extremely well studied (Giuliano et al., 1993; Hirschberg, 2001; Fraser and Bramley, 2004), our understanding of the role of fruit photosynthesis

during early stages of organ development is, at best, fragmentary.

Tomato fruit clearly undergo a physiological transition on the differentiation of photosynthetically active chloroplasts into chromoplasts (Büker et al., 1998; Kahlau and Bock, 2008), and this transition would appear to be coupled to a decline in the expression (Piechulla et al., 1987; Wanner and Gruissem, 1991; Alba et al., 2004; Carrari et al., 2006; Kahlau and Bock, 2008) and enzymatic activities (Schaffer and Petreikov, 1997; Steinhauser et al., 2010) associated with carbon assimilation.

Despite the high-level expression of photosynthetic genes, tomato fruit rarely are net assimilators of carbon dioxide (Blanke and Lenz, 1989; Carrara et al., 2001). Moreover, the triose phosphate and Glc phosphate transporters are both active in tomato chloroplasts, indicating that they could, in principle, both import and export phosphoesters. Equally curious are observations of exceedingly high expression of genes associated with photosynthesis in tissues of the fruit, such as the locule (Lemaire-Chamley et al., 2005), which, although capable of photosynthesis (Laval-Martin et al., 1977), are also likely to display higher rates of respiration. Thus, while photosynthesis is occurring in the green fruit, it is clear neither to what extent nor to what avail. Early shading studies analyzing the rate of fruit growth indicated that the fruit contributes by its own fixed carbon between 10% and 15% of the carbon skeletons required (Tanaka et al., 1974). A similar quantitative effect was also more recently observed following the antisense inhibition of the chloroplastic Fru-1,6-bisphosphatase (FBPase; Obiadalla-Ali et al., 2004), while the combined metabolomic and transcriptomic analyses of plants deficient in the expression of the tomato *Aux/IAA* transcription factor *IAA9* were highly suggestive of an important role for photosynthesis in the initiation of fruit development (Wang et al., 2009).

In this study, we generated transgenic tomato plants exhibiting decreased expression of glutamate 1-semialdehyde aminotransferase (GSA), which has previously been documented to contribute to the control of chlorophyll biosynthesis (Höfgen et al., 1994), under the control of the TFM5 promoter, which confers early fruit specificity. GSA catalyzes the transamination reaction to 5-aminolevulinic acid, the first committed metabolite of tetrapyrrole biosynthesis, 5-aminolevulinic acid synthesis being its rate-limiting step. Antisense GSA plants were characterized by a reduced photosynthetic rate, as determined by both gas-exchange measurements and determination of the levels of intermediates of the Calvin-Benson cycle, but few effects on primary or intermediary metabolism and little effect on ripening. By contrast, seed set was dramatically compromised, as was seed morphology and composition during early fruit development. These results are discussed with respect to the proposed roles of photosynthesis during fruit metabolism, ripening, and development, in particular with respect to carbon provision for seed set in tomato.

RESULTS

Generation of Plants Exhibiting a Fruit-Specific Decrease in Chlorophyll Biosynthesis via Expression of an Antisense GSA under the Control of the TFM5 Promoter

Given the major purpose of this work, which was to study the role of fruit photosynthesis and the lack of a suitable enhancer line population analogous to those used in Arabidopsis (*Arabidopsis thaliana*; Janacek et al., 2009), our initial aim was to find a suitable promoter to confer loss of function only in the tissue of choice in tomato. Scanning the literature revealed that the TFM5 promoter isolated by Santino and coworkers (1997) was highly likely to be appropriate for our needs. We confirmed the expression pattern of TFM5 by generating a GUS fusion construct and transforming it into cv MoneyMaker. As can be seen in Figure 1, the promoter is active in all parts of the immature fruit, with intense expression in the inner pericarp and parts of the columella, lower expression in the locular tissues, and almost no expression in the cuticular layer. Importantly, this promoter displays no activity in leaves.

Having established that this promoter was appropriate, we next generated constructs expressing a 1,714-bp fragment of the GSA gene in the antisense orientation. We chose GSA because it has previously been reported to play an essential role in chlorophyll synthesis (Kannangara and Gough, 1978; Höfgen et al., 1994; Ilag et al., 1994; Chen et al., 2003) and leaf chlorophyll content itself has a major impact on the rate of photosynthesis of higher plants (Höfgen et al., 1994; Yaronskaya et al., 2003). Using an established *Agrobacterium tumefaciens*-mediated gene transfer protocol, we were able to generate a total of 22 primary transformants. Growth of these transformants revealed four that displayed a lighter pigmentation of their young fruit, so we amplified these lines and continued to work with them. We attempted many

Figure 1. Fruit-specific GUS expression of the TFM5 promoter. An immature green fruit at 23 DPA is shown on the left.

times to assay the GSA enzyme activity in tomato fruit; however, following multiple attempts, we concluded that this was not currently possible due to the presence of an as yet uncharacterized inhibitor in the tomato extracts. We were able, however, to select two lines that we felt were appropriate for further study on the basis of their dramatically reduced expression levels, their lacking the GSA protein as assessed by western blot (Fig. 2), and importantly, their routinely displaying reduced pigmentation, namely aGSA4 and aGSA8.

Phenotype of Fruit Whose Photosynthesis Is Compromised by Antisense TFM5-Driven Inhibition of GSA

Perhaps unsurprisingly, given previous observations following the inhibition of this enzyme in tobacco (*Nicotiana tabacum*) and *Brassica* species (Höfgen et al., 1994; Hartel et al., 1997; Chen et al., 2003; Tsang et al., 2003), the most obvious phenotype of the transgenic fruit was their very pale color in comparison with the wild-type fruit (Fig. 3); this is true both when looking at the entire fruit and also at the inner tissues of the fruit in cross-section. Given that it has previously been demonstrated that tissues within the fruit display high levels of photosynthetic gene expression (Lemaire-Chamley et al., 2005), this observation was particularly reassuring to us. As a second approach, we confirmed that this lack of pigmentation was due to reduced chlorophyll content by measuring the levels of chlorophylls *a* and *b* and deducing the chlorophyll *a*/*b* and chlorophyll-to-carotenoid ratios of the pericarp of immature green fruit (Fig. 4). We next expanded this analysis to further photosynthetic pigments, revealing dramatic decreases in the levels of neoxanthin, violaxanthin, and lutein in both transgenic lines and of zeaxanthin and antheraxanthin in line aGSA4 (Supplemental Fig. S1). While the magnitude of the changes in chlorophyll content paralleled those of GSA expres-

sion, those of the other pigments did not; however, it is important to note that the differences in expression of the transgenic lines are not greatly different from one another, since the combined changes observed would be anticipated to greatly reduce the photosynthetic capacity of the transgenics. In order to test if that was indeed the case, we next measured two characteristics of photosynthesis. First, we measured the rate of gas exchange using a modified gas chamber cuvette that was adapted in order to allow the gas exchange of an attached fruit to be determined in the absence of interfering signals emanating from the leaves or stem. Using this approach, we were able to obtain accurate measurements of the rate of gas exchange, which is particularly difficult in the fruit, since they additionally display very high rates of respiration (Blanke and Lenz, 1989; Carrara et al., 2001). In our conditions and setup, using fruit approximately 20 DPA, we could not detect net photosynthesis rate in either transgenic or wild-type fruit. Nevertheless, data obtained by this approach revealed a clear increase in the rate of carbon release in both aGSA4 and aGSA8 (Fig. 5A), which in our case was confirmatory of compromised photosynthetic capacity. Second, we assessed chlorophyll fluorescence parameters such as quantum yield and maximum efficiency of PSII, which were also markedly reduced in the transgenic lines (Fig. 5B). This was even more distinctly illustrated by means of the pulse amplitude modulated (PAM) imaging technique, where it was almost impossible to get an image of transgenic fruit (Supplemental Fig. S2). The transgenic lines, however, displayed relatively little difference in these parameters when compared with one another, suggesting that the relationship between GSA activity and photosynthesis may not be strictly linear. We additionally tested the fruit for the ability of the PSII to recover after exposure to high light (over 800 μmol m^{-2} s^{-1}). As expected, antisense fruit were characterized by a reduced recovery rate as

Figure 2. GSA expression. A, Western blot showing the absence of a GSA protein band in antisense fruit (22–27 DPA) in comparison with the wild type (WT; 46.7 kD; arrow). B, GSA transcript expression presented as log$_2$ ratio. Data were taken from microarray analysis.
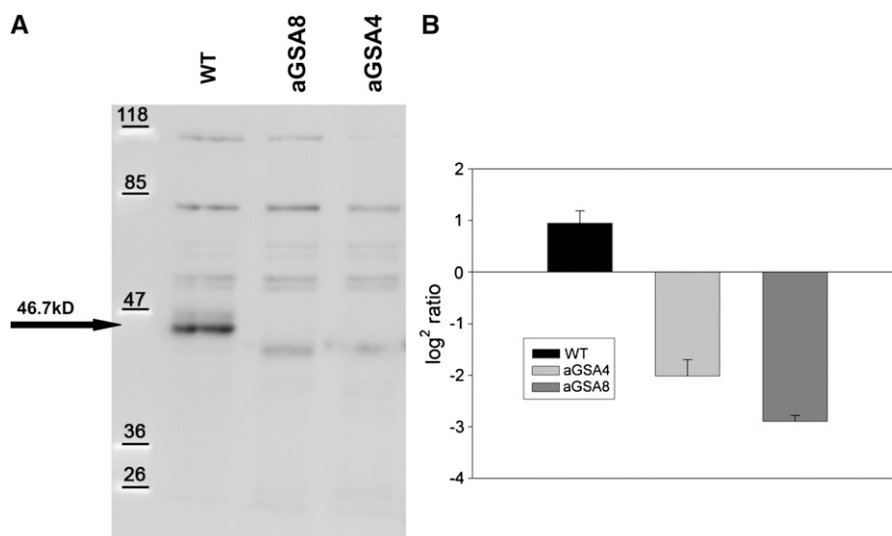
well as much lower electron transfer rate at the higher light irradiance (data not shown). As a further marker of photosynthetic activity, we next used a recently established liquid chromatography-tandem mass spectrometry (MS/MS) method (Arrivault et al., 2009) to quantify several intermediates of the Calvin-Benson cycle. The results presented in Figure 6 revealed a general trend of decreasing levels of the intermediates of the Calvin-Benson cycle, with sedoheptulose 7-phosphate and dihydroacetonephosphate being significantly decreased in both transgenic lines, while Rib 5-phosphate and the combined peak corresponding to xylulose 5-phosphate and ribulose 5-phosphate being decreased only in line aGSA8 and line aGSA4, respectively. However, despite the fact that we repeatedly observed compromised photosynthesis in the transgenic lines, we did not reveal any link (except for slightly delayed ripening) between this perturbation of early fruit photosynthesis and the gross phenotype of the fruit, with total fruit weight at the red ripe stage being invariant between genotypes.

Metabolite Profiling and Microarray Analysis of GSA Antisense Plants

In order to better characterize further the effects of the reduction of chlorophyll content and as a consequence the reduced rates of fruit photosynthesis, we next applied an established gas chromatography (GC)-MS-based metabolite profiling method (Fernie et al., 2004) to pericarp tissue derived from immature green fruit. Perhaps surprisingly, the metabolite profiles of the transgenic lines were remarkably similar to those of the wild type. Indeed, the only exceptions to this statement were the increased levels of Asp displayed by both lines and the increases in malate in line aGSA4 and in inositol in line aGSA8 (Supplemental Table S1). Similarly, application of a recently established liquid

chromatography-ion trap-MS method for the detection of the highly abundant secondary metabolites revealed that these were invariant across the genotypes, as were Suc or starch contents measured spectroscopically (data not shown).

To analyze the genome-wide effects of GSA downregulation in pericarp tissues, next we performed a microarray analysis comparing antisense lines with the wild type using the 12,160-feature TOM2 array representing 11,862 genes from tomato. Using a t test with $P < 0.05$, a total of 138 genes showed differential expression (up- and down-regulated) in both antisense lines by comparing with the wild type, confirming that only relatively minor changes in gene expression were apparent in these lines. Most of these genes were slightly up- or down-regulated with respect to the wild type, and only a few genes showed marked alteration in both lines. The most down-regulated (more
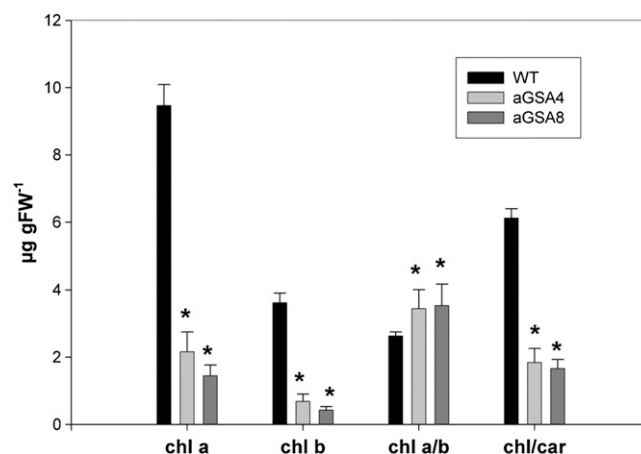

Figure 4. Chlorophyll (chl) contents of the tomato antisense GSA immature green fruit pericarp (22–25 DPA) in comparison with wild-type (WT) fruit. FW, Fresh weight.
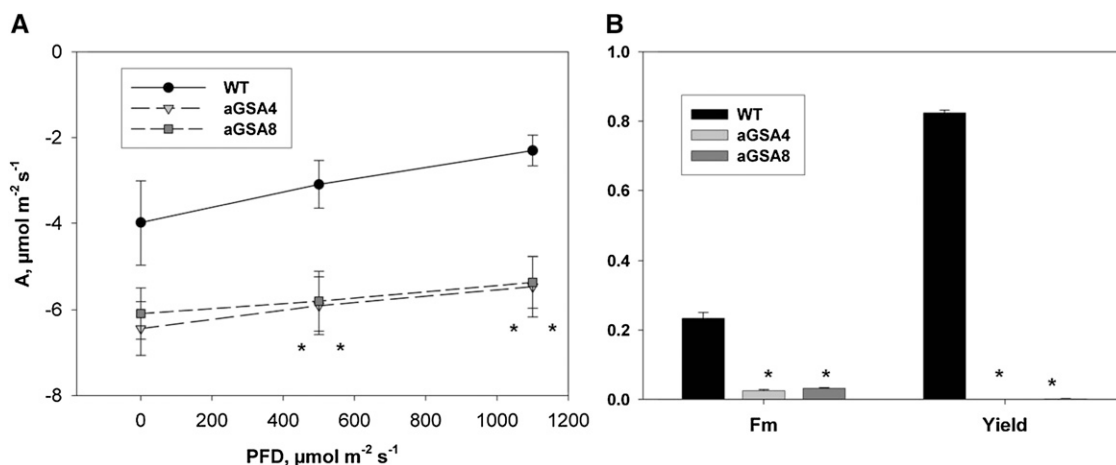
Figure 5. Photosynthetic performance of the immature green fruit (22–25 DPA). A, Assimilation/respiration rates of the fruit attached to the plant. PFD, Photon flux density. B, Chlorophyll fluorescence parameters of the fruit: maximum PSII fluorescence in the dark-adapted state (Fm; left) and quantum yield (right). Note that it was not possible to detect quantum yield in transgenic fruit. WT, Wild type.

than 2-fold reduction) genes included GSA1, urogen III methylase (both involved in tetrapyrrole biosynthesis), a DNA repair protein, a disease-related protein, and two unknown proteins (Supplemental Figs. S2 and S3). The most highly expressed (more than 2-fold up-regulation) genes in both antisense lines included another gene in tetrapyrrole biosynthesis, protochlorophyllide reductase B, ω-6-desaturase, β-ketoacyl-CoA synthase, inositol 1,3,4-trisphosphate 5/6-kinase, an auxin-responsive family protein, SPK1-interacting partner protein 3, and two unknown proteins (Supplemental Figs. S2 and S3). A MapMan overview of the differences in expression between the genotypes of aGSA4 and the wild type is presented in Figure 7A.

The changes in gene expression were relatively mild; however, some of these were unexpected, in particular those of the light reactions or related to PSI, which were surprisingly up-regulated. Closer inspection revealed that the expression of genes encoding the core biochemical reactions of the photorespiratory cycle (Bauwe et al., 2010) were also mostly up-regulated (Fig. 7B). By contrast, many genes related to the Calvin cycle or PSII were down-regulated, as one could expect following compromised photosynthetic performance. A full list of significantly altered transcripts (cutoff of 3-fold changes) is presented in Supplemental Tables S2 and S3. Application of the Wilcoxon rank sum test revealed that changes in the light reaction, cell wall metabolism, amino acid metabolism, and tetrapyrrole biosynthesis were among the most affected in both transgenic lines (Supplemental Tables S4 and S5).

Given that hormones are widely acknowledged to play important roles in normal fruit development and that our previous studies have indicated that the TOM2 array affords broad coverage of genes associ-

ated with biosynthesis and signaling pathways connected to the major phytohormones (Wang et al., 2009), we next evaluated changes in the expression of these genes. Interestingly, genes associated with auxin biosynthesis or function displayed altered expression in the transgenic lines. IAA-amino acid hydrolase 3 (SGN-U322902) was present at 35% and 46% of the levels observed in the wild type for lines aGSA4 and aGSA8, respectively. Similarly, IAA-amino acid hydrolase 1 (SGN-U338277) was present at 91% and 81%, and an IAA-responsive family protein member (SGN-U323951) was present at 40% and 24% of the levels observed in the wild type for lines aGSA4 and aGSA8, respectively. By contrast, another IAA-responsive fam-
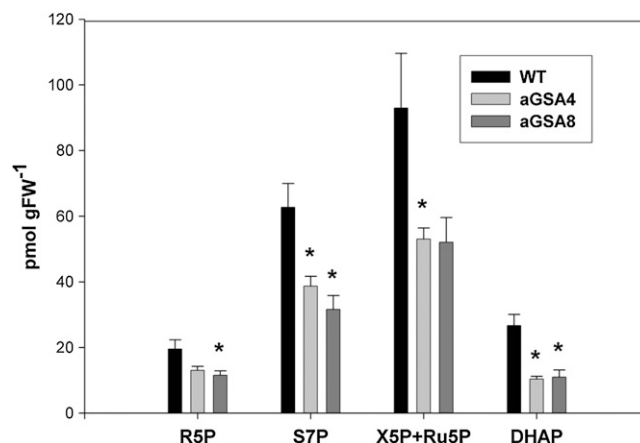
Figure 6. Contents of phosphorylated intermediate compounds of the Calvin cycle in the tomato antisense GSA immature green fruit pericarp (22–25 DPA) in comparison with the wild-type (WT) fruit. DHAP, Dihydroacetonephosphate; FW, fresh weight; R5P, Rib 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, sedoheptulose 7-phosphate.

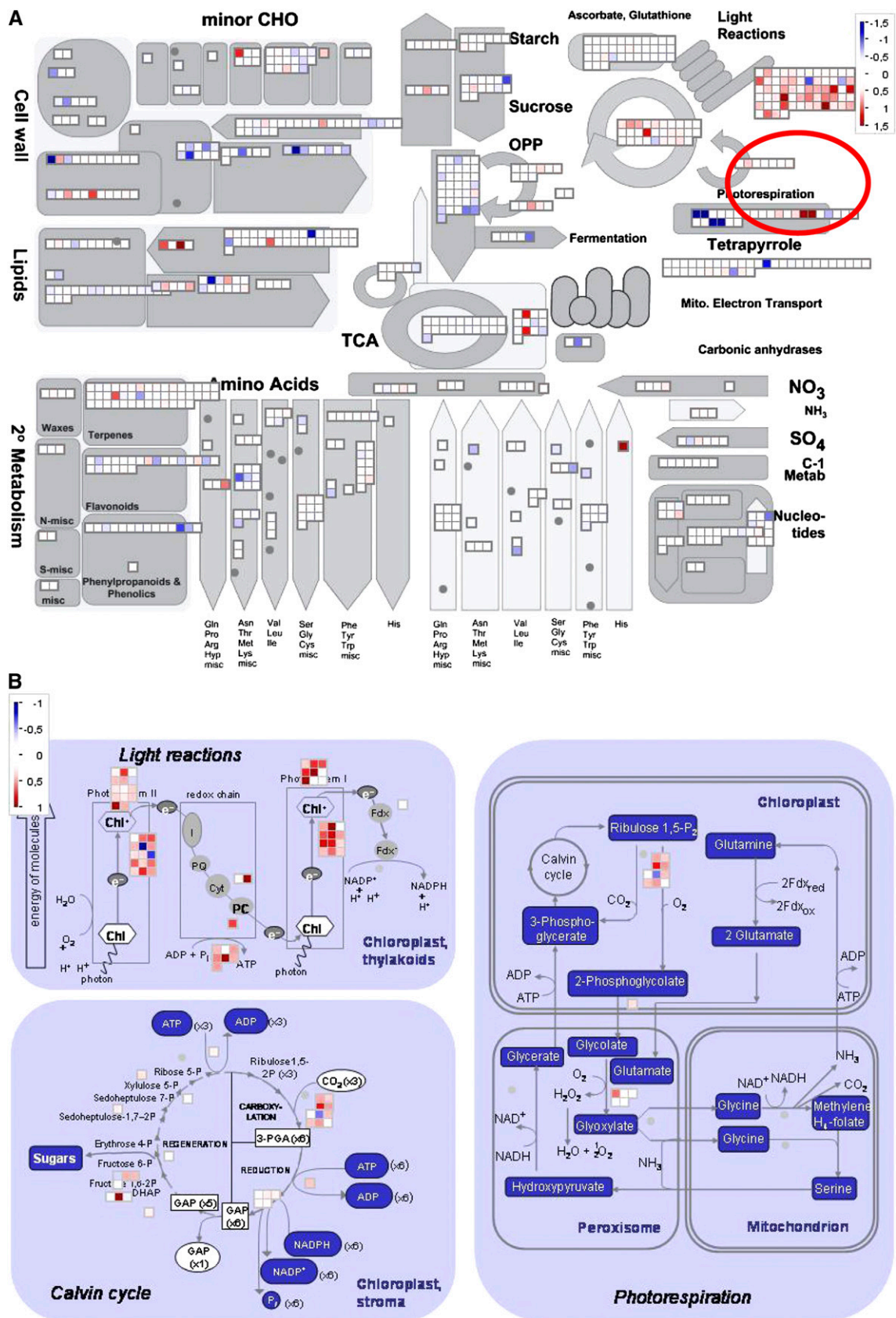**Figure 7.** MapMan representation of transcript changes in the tomato antisense GSA immature green fruit pericarp (22–25 DPA), line aGSA4 in comparison with wild-type fruit. A, General overview. B, Closeup of the most distinct changes related to light reactions and the Calvin cycle (red circle in A). CHO, Carbohydrate; OPP, oxidative pentose phosphate cycle.

ily protein member (SGN-U316711) was 15% and 25% up-regulated for lines aGSA4 and aGSA8, respectively. While these changes were significant in both transgenic lines as assessed by Student's t test using a P value threshold of 0.05, those associated with other phytohormone-associated genes were not. Therefore, we focused direct measurement of the phytohormones on IAA alone, which we found to be present at lower levels in the transgenic lines (1.61 ± 0.55, 1.35 ± 0.44, and 0.88 ± 0.09 ng g^{-1} fresh weight for the wild type, aGSA4, and aGSA8, respectively).

Analysis of Seed Phenotypes of the GSA Lines

Having established that the antisense inhibition of GSA had relatively little impact on the fruit per se yet led to altered IAA levels, we next turned our attention to examining the seed characteristics of these lines. While there were no differences between seed number and subsequent germination rate from ripe fruit of the transgenics in comparison with the wild type (data not shown), the situation during early stages was dramatically different. At this stage, both lines displayed a dramatically reduced rate of seed set and seed-to-embryo ratio (Table I) and, as seen in representative photographs, extreme seed phenotypes (Fig. 8). Such extreme phenotypes accounted for some 20% of all seeds in the transgenic lines, while visibly darker seeds were observed in approximately 30% of seeds of the transgenics. Similar observations were observed in a second independent harvest. Given the perceived importance of tocopherol in seed function (Sattler et al., 2004), we next measured the levels of this vitamin in seeds. The levels of α- and γ-tocopherol were severely compromised in the transformants, particularly the former, which was present at levels between 13% and 15% of those found in the wild type (Table I). We next extended our analysis to analyze a broader range of metabolites in the seed by means of GC-MS (Table II). In contrast to the results obtained from metabolic profiling of the pericarp, we observed considerable changes in metabolism, finding that both antisense lines contained higher levels of the majority of amino acids, threonate, octadecenoic acid, and spermidine but lower levels of pyruvate and raffinose.

These results thus suggest that while fruit photosynthesis has very little impact on fruit development per se, it has a more pronounced effect on seed set, composition, and morphology during early fruit development.

DISCUSSION

While the role of fruit photosynthesis in fruit metabolism and development has been much discussed (Piechulla et al., 1987; Wanner and Gruissem, 1991; Schaffer and Petreikov, 1997; Alba et al., 2004; Carrari et al., 2006; Steinhauser et al., 2010), it has not yet been clearly experimentally defined. Early experiments aimed at addressing this question involved shading individual fruit with aluminum foil (Tanaka et al., 1974). While the reduction in fruit yield was quantitatively similar to that observed following fruit-specific antisense inhibition of the chloroplastic FBPase (Obiadalla-Ali et al., 2004), such experiments probably also have an impact on light receptors, including phytochromes and cryptochomes, which are well documented to have important roles in normal fruit development (Alba et al., 2000; Giliberto et al., 2005; Azari et al., 2010). Moreover, such treatments would also likely elevate the rate of respiration in the fruit, and as such, the results must be interpreted with caution. For this reason, we here chose to assess the impact of modulating photosynthesis by specifically affecting the chlorophyll content of the fruit. Despite the fact that a wide number of fruit mutants have been characterized that contain altered pigmentation (Isaacson et al., 2002; Barry and Giovannoni, 2006; Galpaz et al., 2008; Nashilevitz et al., 2010), we chose to manipulate the chlorophyll content by antisense inhibition of GSA in an attempt to minimize the influence of pleiotropic effects. This same approach has already been successfully applied to a number of species (Kannangara and Gough, 1978; Höfgen et al., 1994; Ilag et al., 1994; Chen et al., 2003), while addressing the functionality of photosynthesis in the cells surrounding the veins of C3 plants was recently achieved using an analogous approach, but different target enzyme, in Arabidopsis (Janacek et al., 2009).

Table I. *Immature green fruit (22–25 DPA) seed characteristics*

Antisense GSA plants were grown in the greenhouse alongside wild-type controls. Values are presented as means $\pm$ SE of determinations from 15 to 20 independent fruit (four in the case of the GC-MS measurements of seed tocopherols; data are normalized to wild-type values). Those determined by the t test ($P < 0.05$) to be significantly different from the wild type are shown in boldface.

Parameter	Wild Type	aGSA4	aGSA8
Seed count per fruit	81.5 ± 5.6	**34.9 ± 4.5**	**50.7 ± 7.9**
Area embryo-seed ratio (%)	63.8 ± 1.5	**39.2 ± 2.9**	**48.1 ± 4.6**
Seed tocopherol contents			
α-Tocopherol	1.00 ± 0.32	**0.15 ± 0.27**	**0.13 ± 0.26**
γ-Tocopherol	1.00 ± 0.18	**0.41 ± 0.29**	**0.38 ± 0.39**
δ-Tocopherol	1.00 ± 0.20	0.55 ± 0.13	0.56 ± 0.33
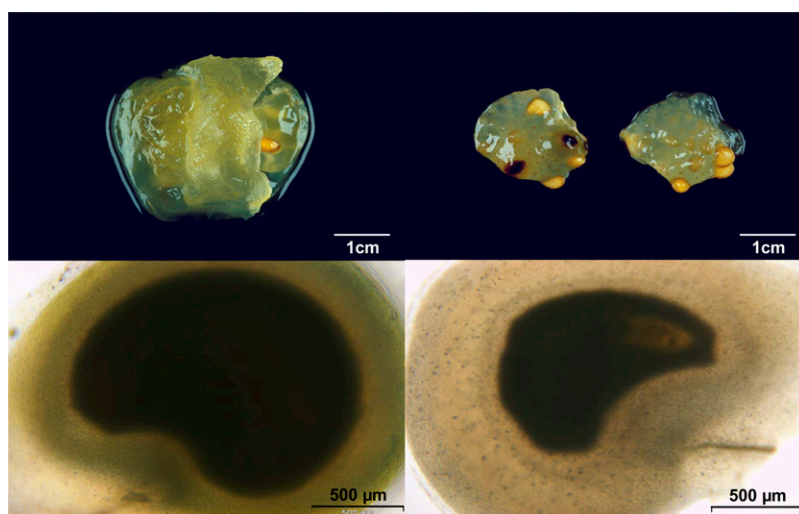
As we anticipated, expressing an antisense GSA construct under the control of the TFM5 promoter to reduce the expression, GSA protein content, and chlorophyll content in an early fruit-specific manner significantly repressed the fruit photosynthetic capacity. However, this had remarkably little effect on fruit morphology or metabolism, with the obvious exception that the fruit of the transgenic lines were distinctly paler than those of the wild type. This is in line with the results of a previous study (Kahlau and Bock, 2008), where it was shown on RNA, translation, and protein accumulation levels that strongly down-regulated expression of all plastid-encoded photosynthesis genes already in the green fruit supports the idea that the contribution of fruit photosynthesis to energy metabolism is a minor one.

The lack of effect on fruit yield, while in contrast to the results reported in the earlier studies mentioned above, is in keeping with results from several other studies in tomato, which imply that the vast majority of photoassimilates are supplied by the leaves rather than produced de novo in the fruit (Hackel et al., 2006; Schauer et al., 2006; Zanor et al., 2009; Do et al., 2010). To summarize these data in brief, it has been demonstrated by a range of studies including both forward and reverse genetics approaches that both composition and yield in fruit are dramatically influenced by the partitioning of assimilates. In light of this vast body of evidence, the lack of effect on fruit yield is perhaps unsurprising, although it remains contradictory to observations made in earlier studies. While the reasons we stated above may explain the discord between the shading results and those obtained here, it is at first sight harder to explain why the deficiency of the chloroplastic FBPase (Obiadalla-Ali et al., 2004) had an effect on final fruit growth and that of GSA did not. It is important to note, however, that the promoters used in both studies have vastly different patterns of expression (in contrast to the GUS expression patterns reported by Frommer et al. [1994] and Santino et al. [1997]) and that the reduction in growth may be a consequence of reducing the activity of the chloroplastic FBPase at a later period of development. Moreover, results of a recent study indicate that disrupting mitochondrial metabolism during early fruit development has a substantial effect on the development of the fruit via an effect on cellular redox balance (Centeno et al., 2011), as does modification of the links of plastid NAD(P)H dehydrogenase complex activity (Nashilevitz et al., 2010), suggesting that the effect of the FBPase inhibition could alternatively be a consequence of altered metabolism rather than a direct effect of altered photosynthesis per se. Given that altering chlorophyll biosynthesis is a more direct way of altering the rate of carbon assimilation in the fruit than the manipulation of the plastidial FBPase, and the fact that the promoter is only active during the period in which the fruit is photosynthetically active, we contend that the strategy taken here is more appropriate to address our aims.

Previous studies have demonstrated that, even within C3 plants, the role of photosynthesis (Janacek et al., 2009) or at least a subset of its reactions (Schwender et al., 2004) varies in a tissue-dependent manner. With restricted photosynthesis in cells surrounding the veins of Arabidopsis, following an analogous approach to that described here, Janacek et al. (2009) suggested a role for photosynthesis in shikimate biosynthesis in this tissue. Similarly, Rubisco was demonstrated to operate, in isolation from the Calvin-Benson cycle enzymes, as part of a more efficient route of fatty acid biosynthesis in developing embryos of *Brassica napus* (Schwender et al., 2004). No such role could be uncovered in the tomato pericarp tissue, which was largely invariant at the metabolite level, with few clear trends in the levels of primary metabolites. Given that antisense inhibition of transketolase resulted in pronounced changes in the levels of phe-

Table II. *Metabolite contents of seed samples taken from immature green fruit (25–30 DPA)*

Normalized to the wild type, fold difference values are presented as means ± sᴇ of determinations from four independent samples. Those determined by *t* test to be significantly different from the wild type are shown in boldface.

Sample	Wild Type	Anti-GSA4	Anti-GSA8
Amino acids			
Ala	1.00 ± 0.11	**1.91 ± 0.09**	**2.00 ± 0.15**
Ala, β-	1.00 ± 0.24	1.72 ± 0.13	2.67 ± 0.16
Arg	1.00 ± 0.37	1.99 ± 0.16	1.65 ± 0.06
Asn	1.00 ± 0.51	**2.85 ± 0.16**	2.50 ± 0.18
Asp	1.00 ± 0.27	**3.04 ± 0.11**	**4.55 ± 0.07**
Butyric acid, 4-amino-	1.00 ± 0.12	**1.49 ± 0.04**	**1.45 ± 0.07**
Glu	1.00 ± 0.18	**2.87 ± 0.08**	**3.56 ± 0.06**
Gln	1.00 ± 0.48	1.71 ± 0.15	1.94 ± 0.24
Gly	1.00 ± 0.23	1.38 ± 0.11	**2.00 ± 0.19**
Ile	1.00 ± 0.30	**2.47 ± 0.19**	**3.84 ± 0.14**
Leu	1.00 ± 0.35	1.73 ± 0.17	**2.64 ± 0.13**
Lys	1.00 ± 0.28	**1.94 ± 0.06**	**1.95 ± 0.06**
Phe	1.00 ± 0.23	2.19 ± 0.24	**2.52 ± 0.09**
Pro	1.00 ± 0.20	1.16 ± 0.15	**1.86 ± 0.16**
Ser	1.00 ± 0.27	1.50 ± 0.14	**1.71 ± 0.07**
Thr	1.00 ± 0.22	1.54 ± 0.11	**2.08 ± 0.09**
Trp	1.00 ± 0.13	**2.61 ± 0.12**	**3.11 ± 0.14**
Tyr	1.00 ± 0.25	**2.23 ± 0.19**	**2.79 ± 0.10**
Val	1.00 ± 0.29	**3.37 ± 0.19**	**4.93 ± 0.14**
Organic acids			
Citrate	1.00 ± 0.31	1.34 ± 0.09	1.46 ± 0.06
Dehydroascorbate	1.00 ± 0.14	**0.52 ± 0.15**	0.68 ± 0.21
Fumarate	1.00 ± 0.13	1.11 ± 0.08	0.90 ± 0.08
Malate	1.00 ± 0.09	0.81 ± 0.04	**0.72 ± 0.06**
Pyruvate	1.00 ± 0.18	**0.54 ± 0.13**	**0.50 ± 0.08**
Saccharate	1.00 ± 0.09	0.82 ± 0.06	0.89 ± 0.10
Succinate	1.00 ± 0.10	**1.48 ± 0.09**	1.20 ± 0.10
Threonate	1.00 ± 0.15	**2.20 ± 0.10**	**3.07 ± 0.08**
Sugars/sugar alcohols			
Fru	1.00 ± 0.12	0.86 ± 0.09	0.87 ± 0.08
Galactinol	1.00 ± 0.31	0.64 ± 0.16	0.83 ± 0.22
Glc	1.00 ± 0.17	0.65 ± 0.18	0.70 ± 0.19
Glycerol	1.00 ± 0.10	0.97 ± 0.14	1.08 ± 0.19
Myoinositol	1.00 ± 0.28	0.82 ± 0.36	1.41 ± 0.29
Maltose	1.00 ± 0.15	0.72 ± 0.17	0.71 ± 0.10
Melezitose	1.00 ± 0.33	0.62 ± 0.31	1.05 ± 0.40
Raffinose	1.00 ± 0.12	**0.60 ± 0.08**	**0.62 ± 0.16**
Rha	1.00 ± 0.10	**1.50 ± 0.04**	1.23 ± 0.10
Suc	1.00 ± 0.08	0.96 ± 0.09	0.74 ± 0.24
Xyl	1.00 ± 0.08	0.91 ± 0.04	0.90 ± 0.09
Others			
Benzoate	1.00 ± 0.13	1.04 ± 0.05	1.13 ± 0.16
Quinate, 3-caffeoyl-, trans-	1.00 ± 0.22	0.76 ± 0.15	0.85 ± 0.21
Phosphoric acid	1.00 ± 0.22	**2.34 ± 0.09**	1.60 ± 0.11
Putrescine	1.00 ± 0.19	0.87 ± 0.13	**0.50 ± 0.16**
Spermidine	1.00 ± 0.19	**1.80 ± 0.07**	**1.74 ± 0.09**
Urea	1.00 ± 0.19	**2.10 ± 0.17**	1.49 ± 0.20
Glc-6-P	1.00 ± 0.19	1.37 ± 0.14	1.13 ± 0.14
Lipophilic compounds			
Hexadecanoic acid 16:0	1.00 ± 0.03	1.07 ± 0.03	1.00 ± 0.07
Hexadecenoic acid 16:1	1.00 ± 0.24	**2.26 ± 0.13**	1.44 ± 0.13
Heptadecanoic acid 17:0	1.00 ± 0.05	1.13 ± 0.03	1.07 ± 0.13
Octadecanoic acid 18:0	1.00 ± 0.02	1.05 ± 0.02	1.03 ± 0.08
Octadecenoic acid 18:1	1.00 ± 0.12	**2.75 ± 0.21**	**2.35 ± 0.21**
Eicosanoic acid 20:0	1.00 ± 0.20	1.24 ± 0.31	1.04 ± 0.14
Docosanoic acid 22:0	1.00 ± 0.24	1.23 ± 0.21	2.21 ± 0.28
Tetracosanoic acid 24:0	1.00 ± 0.29	0.94 ± 0.28	0.50 ± 0.39

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Sample	Wild Type	Anti-GSA4	Anti-GSA8
Octadecanol 1OH-18:0	1.00 ± 0.30	0.92 ± 0.12	0.84 ± 0.23
Eicosanol 1OH-20:0	1.00 ± 0.03	0.98 ± 0.17	0.94 ± 0.16
Hexacosanol 1OH-26:0	1.00 ± 0.20	1.20 ± 0.33	0.96 ± 0.30
Nonacosane C29	1.00 ± 0.17	1.03 ± 0.12	0.89 ± 0.16
β-Sitosterol	1.00 ± 0.19	0.92 ± 0.25	0.79 ± 0.25
Campesterol	1.00 ± 0.24	0.86 ± 0.26	0.65 ± 0.31
Cholesterol	1.00 ± 0.24	0.54 ± 0.15	0.50 ± 0.35
Stigmasterol	1.00 ± 0.17	0.90 ± 0.25	0.90 ± 0.28

nylpropanoids (Henkes et al., 2001), we additionally studied these here. However, with the exception of changes in the levels of the photosynthetic pigments, the only metabolic change shared by both transformants was an increase in Asp. While the transformants displayed a clear reduction in fruit carbon assimilation, they were able to adequately compensate for it. The most likely mechanism by which this can be achieved is the up-regulation of leaf photosynthesis. Our own studies are in keeping with this suggestion, since they have revealed that tomato leaf photosynthesis can be considerably elevated and furthermore that this generally results in a proportional increase in fruit yield (Araújo et al., 2011; Nunes-Nesi et al., 2011). Despite the fact that there was essentially no change in the fruit phenotype, we observed a striking reduction in the rate of seed set as well as an altered seed morphology, which displayed a much reduced embryo-to-seed ratio. This finding suggests that, despite the fact that the plant can compensate for a lack of fruit carbon assimilation at the level of the organ itself, fruit photosynthesis is likely an important source of carbon assimilate for proper seed set and establishment. While there is very weak expression of the promoter used in this study in the vasculature of the stem, petiole, and peduncle, we believe that it is highly unlikely that the seed effects result from altered GSA expression in these tissues; rather, they are likely a result of the restricted fruit chlorophyll biosynthesis. However, the facts that we have previously demonstrated a role for cell wall invertase in this process (Zanor et al., 2009), and that the final seed yield and germination efficiency are the same, suggest that this route is not exclusive and may not be essential. This fact notwithstanding, it is clear that under certain environmental conditions it would be evolutionarily desirable for fruit to exhibit early seed set; therefore, it is highly conceivable that this is one of the functions of fruit photosynthesis. The facts that both the transgenics characterized here and those displaying reduced cell wall invertase expression affect both fruit carbon and energy metabolism, that both result in reduced levels of auxin, and that both exhibit aberrant seed production hint at a mechanistic link between sugar supply and seed set. Indeed, the role of leaf-derived sugar supply has long been studied, with the

miniature mutant of maize (*Zea mays*) being particularly well characterized (Miller and Chourey, 1992; Cheng et al., 1996), and recent studies linked this phenotype to abnormal hormone balance in the seeds (LeClere et al., 2010). Such hormonal changes were also observed for tomato plants in which cell wall invertase was inhibited (Zanor et al., 2009). The results of this study suggest that, while not as vital as the carbon import route is for seed fertility, carbon assimilation by the fruit can also influence early seed set. That said, two further hypotheses for this reduction could be made, both being potentially indirectly linked to the reduced carotenoid content of the transformants. The first of these is that the reduced carotenoid content of the pericarp is responsible for the reduced tocopherol content of the seeds. Since tocopherol has been demonstrated to play an important role in seed development (Sattler et al., 2004), we cannot formally disregard this hypothesis. The second is based merely on the observation that carotenoid cleavage dioxygenase 7 is expressed at very high levels in the fruit, which, given the role of this enzyme in strigolactone formation (Vogel et al., 2010), prompts the question of whether this hormone has an undescribed function in reproductive tissues. It is additionally interesting that despite the apparent competition for common precursors between chlorophyll and tocopherol pathways, the inhibition of biosynthesis of the former does not lead to an accumulation of the latter, suggesting the presence of tight regulatory control at this metabolic juncture.

In summary, while we were able to restrict photosynthetic capacity in a fruit-specific manner, we observed few metabolic or morphological phenotypes beyond the pale coloration of the fruit. That said, a noticeable and dramatic difference was observed in seed set. While this difference was not as dramatic as that observed following the inhibition of import of leaf-derived photoassimilates (Hackel et al., 2006), it did clearly influence the timing of seed set. We conclude that under normal conditions, the repression of fruit photosynthetic capacity could probably be compensated by an enhanced import of photoassimilates from source tissues; nevertheless, fruit photosynthesis is important for the initiation of normal programs of seed formation. It will be interesting in future studies

to evaluate the role of fruit photosynthesis under conditions in which restricted carbon assimilation within the fruit cannot be compensated by an up-regulation of photosynthesis within source leaves as well as to fully understand the roles of sugar- and, indeed, carotenoid-related changes in the hormonal regulation of seed development.

MATERIALS AND METHODS

Materials

Tomato (*Solanum lycopersicum* 'MoneyMaker') seeds were obtained from Meyer Beck. Plants were grown in a growth chamber (250 μmol photons m^{-2} s^{-1}, 22°C) under a 16-h-light/8-h-dark regime before transfer into the greenhouse, where they were grown with a minimum of 250 μmol photons m^{-2} s^{-1} under the same climate conditions. The stage of fruit development was followed by tagging the truss upon appearance of the flower. Pericarp samples were usually harvested from immature green fruit at approximately 25 DPA. Unless stated otherwise, all chemicals and enzymes were purchased either from Sigma-Aldrich Chemical or from Merck KGaG.

Generation of Transgenic Plants

Both constructs described below were independently introduced into plants by an *Agrobacterium tumefaciens*-mediated transformation protocol, and plants were selected and maintained as described before (Tauberger et al., 2000).

TFM5 green fruit-specific promoter (Santino et al., 1997) was provided by Monsanto. The PCR product (1.212 bp) was introduced into pENTR/D/TOPO vector (Invitrogen) and then, using Gateway technology, into binary vector pKGWFS7 (Karimi et al., 2002) to confirm fruit-specific promoter expression using GUS fusion. Thirty-two primary transformant lines were grown, and initial green fruit screening for GUS activity was performed. The three best lines were selected, and complete GUS expression analysis of all plant tissues, especially leaves and various fruit parts at distinct points of fruit development, was performed.

TFM5 promoter was introduced into binary vector pART27 (Gleave, 1992) before the 1,714-bp fragment of tobacco (*Nicotiana tabacum*) GSA in the antisense orientation (Höfgen et al., 1994) and the ocs terminator. Twenty-two primary transformants were selected, and fruit were initially screened with respect to chlorophyll content. Four lines were taken for further analysis, and two of them (aGSA4 and aGSA8) were proven to be stable across generations and used for detailed physiological and biochemical analyses.

Gas-Exchange Analysis

Gas-exchange measurements were performed in a special custom-designed open system (Walz) described by Lytovchenko et al. (2002). The Diagas software package (Walz) was used to calculate the assimilation rates according to Von Caemmerer and Farquhar (1981). Tomato plants were first adapted to gas-exchange phytotron conditions (usually 2 d prior to the measurements), and then intact immature green fruit (approximately 20 DPA) still attached to the plant were put into the cuvette, hermetically sealed around the petiole, and CO_2 assimilation/respiration rates were measured under various light intensities as well as in dark-adapted fruit. After the measurements were completed, fruit was carefully removed and its weight and area were determined. The same series of measurements were repeated for the green parts remaining in the cuvette (calyx and petiole), and afterward, the values for fruit only were estimated by subtracting these nonfruit values from those measured previously for the entire fruit.

Chlorophyll Fluorescence

Chlorophyll fluorescence parameters were measured on immature green fruit (approximately 20 DPA) freshly detached and dark adapted for 30 min, both with a standard PAM chlorophyll fluorometer (Walz), yielding quantitative values, and an imaging PAM (Walz), revealing in vivo images of chlorophyll distribution and functional photosynthetic activity in different parts of the fruit.

At the start of each measurement, fruit was dark adapted for 20 min for the determination of initial (minimum) and maximum PSII fluorescence in the dark-adapted state (Bilger et al., 1995). Then, a series of photon flux densities (4, 10, 50, 100, 225, 365, 585, and 955 μmol m^{-2} s^{-1}) were applied and a set of values were measured.

Protein Expression Analysis Using Western-Blot Hybridization

GSA expression was analyzed in 20 μg of protein from pericarp tissue of immature green fruit (approximately 20 DPA) of antisense transgenic plants (aGSA4 and aGSA8) as well as wild-type fruit. Protein amounts were determined using the Bio-Rad Protein Assay kit. Loading and integrity of proteins were controlled by Coomassie blue staining of SDS-PAGE gels. Transfer and blotting were performed according to the manufacturer's instructions using the Trans-Blot SD semidry blotter (Bio-Rad) on Hybond-C Extra membranes (GE Healthcare). Antisera were diluted 1:1,000 (anti-GSA against *Synechococcus* sp. protein) and 1:10,000 (anti-rabbit; Sigma-Aldrich); signals were detected with a STELLA 3200 CCD camera (Raytest).

Gene Expression Analysis by Microarray Hybridization

RNA was isolated according to Bugos et al. (1995) from tomato fruit pericarp (22–25 DPA) from antisense lines aGSA4 and aGSA8 and wild-type tomato cv MoneyMaker. A pool of equal amounts of RNA from transgenic lines and from wild-type tomato was made and treated as a reference. To obtain differential gene expression values, four biological and technical replicates were hybridized against those of the pool reference. RNA samples for microarray hybridization were amplified using the method of Van Gelder et al. (1990). Briefly, 1 μg of total RNA of each sample and the pool reference was amplified and aminoallyl labeled using the MessageAmp II aRNA kit (Ambion; http://www.ambion.com) and 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (Ambion), following the manufacturer's instructions. Approximately 40 to 70 μg of amplified RNA was obtained. For each sample, 7.5 μg of aminoallyl-labeled amplified RNA was resuspended in 0.1 M Na_2CO_3 (pH 9.0) and labeled with Cy5 Mono NHS Ester (Cy Dye Postlabeling Reactive Dye Pack; Amersham). An equal quantity of RNA from the pool reference was labeled with Cy3. The samples were purified with Mega-clear (Ambion) following the manufacturer's instructions. Incorporation of Cy3 and Cy5 was measured using 1 μL of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies; http://www.nanodrop.com/).

Microarray hybridization of samples and the pool reference to the Tom2 long-mer oligoarray slides (representing 11,862 genes; Instituto de Biología Molecular y Celular de Plantas) was performed manually using Telechem Hybridization Chambers (Corning), following the manufacturer's instructions. Slides were scanned at 532 and 635 nm with a GenePix 4000B scanner (Axon Instruments) at 10-μm resolution, 100% laser power, and different photomultiplicator values to adjust the ratio intensity to 1.0. Microarray images were analyzed using GenePix 4.1 (Axon Instruments) software. Only spots with intensity greater than 2-fold the mean background intensity in at least one channel were selected for analysis. Data files were imported into Acuity 4.0 (Axon Instruments), and background-subtracted intensity was normalized by using the Lowess normalization method within a centered print-pin tip (Yang et al., 2001; Dudoit et al., 2002) using Acuity default values (smoothing filter, 0.4; iterations, 3; δ = 0.01). Finally, only spots with valid values in 80% hybridizations were considered for further analyses. To detect differentially expressed genes, a one-way ANOVA was performed to compare the mean Lowess-normalized values for a gene between experimental groups (antisense and wild type). A P value cutoff of 0.05 was used to flag genes as being differentially expressed. Mean values of differential genes were calculated from each sample as log$_2$ values. A hierarchical cluster (Supplemental Fig. S3) was constructed using the mean of log-normalized expression values for each gene in each line as an input. Pearson correlation centered on 0 was used as a similarity metric. For the visual presentation of the results showing differential expression of the genes between wild-type and antisense GSA lines, as well as for Wilcoxon rank sum test calculation, MapMan software was used (Thimm et al., 2004).

Determination of Metabolite Levels

Fruit pericarp samples were taken at the time points indicated, immediately frozen in liquid nitrogen, and stored at −80°C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. The levels of starch, Suc, and nucleotides were determined exactly as described previously (Fernie et al., 2001). The levels of other polar metabolites in pericarp were quantified by GC-MS exactly following the protocol described by Roessner-Tunali et al. (2003), with the exception that the machine parameters were set as described by Lisec et al. (2006). For the GC-MS analysis, seeds from immature green fruit (approximately 25 DPA) were manually isolated, frozen, and ground in liquid nitrogen, and a 250-mg aliquot was used for extraction. Lipophilic compounds from the same extraction round were determined by GC-MS following the protocol by Lytovchenko et al. (2009). Calvin cycle intermediates were determined as described by Arrivault et al. (2009). Reasonable recovery rates were determined for the Calvin cycle intermediates following the protocol defined by Tohge et al. (2011). Chlorophyll was measured according to Apel and Bock (2009). Other photosynthetic pigments were determined as described by Lohmann et al. (2006). Secondary metabolites were measured according to Tohge and Fernie (2010). IAA was determined exactly as defined by Osorio et al. (2011).

GUS Staining

Tomato tissue fragments were incubated overnight at 37°C in buffer containing 2 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronide cyclohexylamine salt and afterward washed several times in 80% ethanol until full removal of chlorophyll.

Microscopic Evaluation

Seeds from immature green fruit at 25 to 30 DPA were examined using a stereomicroscope (Leica MZ 12,5), and seed area parameters were measured and calculated with the help of corresponding LAS software (Leica).

Supplemental Data

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

Supplemental Figure S1. Pigment contents of the tomato antisense GSA immature green fruit pericarp (22–25 DPA) in comparison with the wild-type fruit.

Supplemental Figure S2. Chlorophyll fluorescence imaging of the antisense GSA immature green fruit of line aGSA4 (right) in comparison with the wild type fruit (left).

Supplemental Figure S3. General hierarchical cluster analysis of transcript changes in the tomato antisense GSA immature green fruit pericarp (22–25 DPA) in comparison with the wild type fruit.

Supplemental Table S1. Full metabolite profiles of the transgenic lines.

Supplemental Table S2. List of significantly changed transcripts in line aGSA4.

Supplemental Table S3. List of significantly decreased transcripts in line aGSA8.

Supplemental Table S4. Wilcoxon rank sum test for transcripts of pericarp samples in line aGSA4.

Supplemental Table S5. Wilcoxon rank sum test for transcripts of pericarp samples in line aGSA8.

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