Running title: A role for fruit photosynthesis?

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Tomato fruit photosynthesis is seemingly unimportant in primary metabolism and ripening but plays a considerable role in seed development

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ABSTRACT

Fruits of tomato, like those from many species, have been characterized to undergo a shift between partially photosynthetic to truly heterotrophic metabolism. Whilst 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 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. When 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 may therefore confer an advantage under conditions of stress.
INTRODUCTION

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; Wang et al., 2009; Vrebalov et al., 2009). The fruit is the result of the development of the ovary with fruit organogenesis originating from a flower primordial, with the mature flower either being fertilized (and developing into a fruit) or not (and entering the abscission process; Wang et al., 2009; Vivian-Smith and Koltunow, 1999). Considerable advances have recently been made in understanding key elements of the genetic control or ripening and development (see Karlova et al., 2011; Mathieu-Rivet et al., 2010; Seymour et al., 2008; Vrebalov et al., 2009; Mattas et al., 2009; Giovannoni, 2007) and the importance of several biochemical pathways including sugars, organic acids, cell wall and volatile metabolism during this process has been demonstrated (Klee, 2010; Carrari et al., 2006; Rose et al., 2004; Yelle et al., 1991). Much of this research has been carried out in tomato, 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 (Fraser and Bramley, 2004; Hirschberg et al., 2001; Guiliano et al., 1993), our understanding of the role of fruit photosynthesis during early stages of organ development is, at best, fragmentary.

Tomato fruits clearly undergo a physiological transition on the differentiation of photosynthetically active chloroplasts into chromoplasts (Kahlau and Bock, 2008; Büker et al., 2004) and this transition would appear to be coupled to decline in the expression (Kahlau and Bock, 2008; Carrari et al., 2006; Alba et al., 2004; Wanner and Gruissem, 1991; Piechulla et al., 1987) and enzymatic activities (Steinhauser et al., 2010; Schaffer and Petreikov, 1997) associated with carbon assimilation. Despite the high level expression of photosynthetic genes, tomato fruits rarely are net assimilators of carbon dioxide (see for example, Carrara et al., 2001; Blanke and Lenz, 1989). Moreover, the triose phosphate and glucose 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, whilst photosynthesis is occurring in the green fruit, it is neither clear to what extent nor to what avail. Early shading studies analysing the rate of fruit growth indicated that the fruit contributes by it’s 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 FBPase (Obiadalla-Ali et al., 2004), whilst 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 the current 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 catalyses the transamination reaction to 5-aminolevulinic acid (ALA), the first committed metabolite of tetrapyrrole biosynthesis; ALA synthesis being its rate limiting step. Antisense GSA plants were characterized by a reduced photosynthetic rate as determined by both gas exchange measurements and by 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 proposed roles of photosynthesis during fruit metabolism, ripening and development, and 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 glutamate-1-semialdehyde aminotransferase 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 (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 co-workers (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 *S. lycopersicum* 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 collumella, but 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 1714bp fragment of the glutamate-1-semialdehyde aminotransferase (GSA) gene in the antisense orientation. We chose GSA since it has previously been reported to play an essential role in chlorophyll synthesis (Chen et al., 2003; Höfgen et al., 1994; Ilag et al., 1994; Kannangara and Gough, 1978), and leaf chlorophyll content itself has a major impact on the rate of photosynthesis of higher plants (Yaronskaya et al., 2003; Höfgen et al., 1994). Using an established Agrobacterium-mediated gene transfer protocol we were able to generate a total of 22 primary transformants. Growth of these transformants revealed four which displayed a lighter pigmentation of their young fruits so we amplified these lines and continued to work with them. We attempted many times to assay the GSA enzyme activity in tomato fruits, however, following multiple attempts we concluded that this was not currently possible due to the presence of an, as yet uncharacterised inhibitor in the tomato extracts. We were, however, able to select two lines, on the basis of their dramatically reduced expression levels, and lacking the GSA protein as assessed by Western blot (Figure 2), and importantly routinely displaying reduced pigmentation, which we felt were appropriate for further study, namely aGSA4 and aGSA8.
Phenotype of fruits whose photosynthesis is compromised by antisense TFM5 driven inhibition of GSA

Perhaps unsurprisingly, given previous observations following the inhibition of this enzyme in tobacco and brassica (Tsang et al., 2003; Chen et al., 2003; Hartel et al., 1997; Höfgen et al., 1994), the most obvious phenotype of the transgenic fruit was their very pale colour in comparison to the wild type fruit (Figure 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 (Figure 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 zeaxanthin and antheraxanthin in line aGSA4 (Supplementary Figure S1). Whilst the magnitude of the changes in chlorophyll content paralleled those of GSA expression 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 (Carrara et al., 2001; Blanke and Lenz, 1989). In our conditions and set up, using fruits approximately 20 DPA, we could not detect net photosynthesis rate in either transgenic or wild type fruits. Nevertheless, data obtained by this approach revealed a clear increase in the rate of carbon release in both aGSA4 and aGSA8 (Figure 5a), which in our case was confirmatory of compromised photosynthetic capacity. Secondly, we assessed chlorophyll
fluorescence parameters such as quantum yield and maximum efficiency of photosystem II, which were also markedly reduced in the transgenic lines (Figure 5b). This was even more distinctly illustrated by means of the PAM imaging technique, where it was almost impossible to get an image of transgenic fruit (Supplementary Figure 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 fruits for the ability of the photosystem II to recover after exposure to the high light (over 800 µmol m\(^{-2}\) s\(^{-1}\)). As expected, antisense fruits were characterised by a reduced recovery rate, as well as much lower ETR at the higher light irradiance (data not shown). As a further marker of photosynthetic activity we next used a recently established LC-MS/MS method (Arrivault et al., 2008) to quantify several intermediates of the Calvin-Benson cycle. The results presented in Figure 6 revealed a general trend to decreasing levels of the intermediates of the Calvin-Benson cycle with sedoheptulose 7-phosphate (S7P) and dihydroacetonephosphate (DHAP) being significantly decreased in both transgenic lines, whilst ribose 5-phosphate (R5P) 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 characterise further effects of the reduction of chlorophyll content and as a consequence the reduced rates of fruit photosynthesis we next applied an established 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 being the increased levels of aspartate displayed by both lines and the increase in malate in line aGSA4 and inositol in line aGSA8 (Supplementary Table SI). Similarly, application of a recently established LC-ion trap MS method for the detection of the majorly abundant secondary metabolites revealed that these were invariant across the genotypes, as
well as were sucrose or starch contents measured spectroscopically (data not shown).

To analyze the genome wide effects of GSA down regulation in pericarp tissues, next we performed a microarray analysis comparing antisense lines to the wild type using the 12,160 feature TOM2 array representing 11,862 genes from tomato. Using a t-test with a p-value<0.05, a total of 138 genes showed differential expression (up and down regulated) in both antisense lines by comparing to WT, 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 than two-fold reduction) genes included GSA1, urogen III methylase (both involved in tetrapyrrole bionsynthesis), a DNA repair protein, a disease-related protein and two unknown proteins (Supplementary Figures SII and SIII). The highest expressed (more than two-fold up-regulation) genes in both antisense lines included another gene in tetrapyrrole biosynthesis protoclorophyllide reductase B, omega-6-desaturase, beta-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 (Supplementary Figures SII and SIII). A Mapman overview of the differences in expression between the genotypes aGSA4 and 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 photosystem I, which were surprisingly upregulated. Closer inspection revealed that expression of genes encoding the core biochemical reactions of the photorespiratory cycle (Bauwe et al., 2010) were also mostly upregulated (Figure 7b). By contrast, many genes related to Calvin cycle or photosystem II were down regulated, as one could expect following compromised photosynthetic performance. A full list of significantly altered transcripts (cut-off three-fold changes) is presented in Supplementary Tables SII and SIII. Application of the Wilcoxon rank sum test revealed that changes in the light reaction, cell wall metabolism, amino acid metabolism and tetrapyrrole biosynthesis were amongst the most affected in both transgenic lines.

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 associated with biosynthesis and signalling 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 level observed in wild type for lines aGSA4 and aGSA8, respectively. Similarly, IAA amino 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 wild type for lines aGSA4 and aGSA8, respectively. By contrast, another IAA-responsive family protein member (SGN-U316711) was 15 and 25% upregulated in for lines aGSA4 and aGSA8, respectively. Whilst these changes were significant in both transgenic lines as assessed by the Students t-test using a P value threshold of 0.05 those associated with other phytohormone associated genes were not. We therefore focussed 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 gFW⁻¹ for 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 lead to altered IAA levels, we next turned our attention to examining the seed characteristics of these lines. Whilst there were no differences between seed number and subsequent germination rate from ripe fruit of the transgenics in comparison to 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 (see Table I) and representative photographs of extreme seed phenotypes (Figure 8). Such extreme phenotypes accounted for some 20% of all seeds in the transgenics lines whilst 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 (Salter et al., 2004), we next measured the levels of this vitamin in seeds. The levels of alpha- and gamma-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).
next extended our analysis to analyse a broader range of metabolites in the seed by means of GC-MS (Table II). By 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 whilst 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

Whilst the role of fruit photosynthesis in fruit metabolism and development has been much discussed (Carrari et al., 2006; Alba et al., 2004; Wanner and Gruissem, 1991; Piechulla et al., 1987; Steinhauser et al., 2010; Schaffer and Petreikov, 1997), it has as yet not been clearly experimentally defined. Early experiments aimed at addressing this question involved shading individual fruit with aluminium foil (Tanaka et al., 1974). Whilst the reduction in fruit yield was quantitatively similar to that observed following fruit-specific antisense of the chloroplastic fructose 1,6-bisphosphatase (Obiadalla-Ali et al., 2004), such experiments will probably also have an impact on light receptors including phytochromes and cryptochromes, which are well documented to have important roles in normal fruit development (Azari et al., 2010; Gilberto et al., 2005; Alba et al., 2000). 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 characterised which contain altered pigmentation (Nashilevitz et al., 2010; Galpaz et al., 2008; Barry and Giovannoni, 2006; Isaacson et al., 2002), we chose to manipulate the chlorophyll content by antisensing GSA in an attempt to minimize the influence of pleiotropic effects. This self-same approach has already been successfully applied to a number of species (Chen et al., 2003; Höfgen et al., 1994; Ilag et al., 1994; Kannangara and Gough, 1978), whilst 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).

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 of the fruits of the transgenic lines were distinctly paler than those of the wild type. This is in line with the results of previous study (Kahlau and Bock, 2008), where it was shown on RNA, translation and protein accumulation levels that strongly downregulated 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, whilst 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 is supplied by the leaves rather than produced _de novo_ in the fruit (Hackel et al., 2008; 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, however, it remains contradictory to observations made in earlier studies. Whilst the reasons we stated above may explain the reason for the discord between the shading results and those obtained here, it is at first sight harder to explain why 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 (contrast the GUS expression patterns in Frommer et al., 1994 and Santori et al., 2005) 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 at which the fruit is photosynthetically active, we contest 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., 2010) 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. (2010) suggested a role for photosynthesis in shikimate biosynthesis in this tissue. Similarly, Rubisco was demonstrated to operate, in isolation to 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 phenylpropanoids (Henkes et al., 2003), 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 aspartate. Whilst 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 upregulation 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 (Nunes-Nesi et al., 2011; Araujo 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. Whilst 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 but rather that it is likely a result of the restricted fruit chlorophyll biosynthesis. However the fact 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 is the same, suggests 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 fruits to exhibit early seed set, and it is therefore highly conceivable that this is one of the functions of fruit photosynthesis. The fact that both the transgenics characterized here and those displayed reduced cell wall invertase...
expression both affect fruit carbon and energy metabolism, both result in reduced levels of auxin and both exhibit aberrant seed production hints to a mechanistic link between sugar supply and seed set. Indeed the role of leaf derived sugar supply has long been studied with the miniature (min) mutant of maize being particularly well characterized (Cheng et al., 1996; Millar and Chourey, 1992), 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., 2010). The results of the current study suggest, whilst 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 (Salter et al., 2010) we cannot formally disregard this hypothesis. The second is based merely on the observation that carotenoid cleavage dioxygenase 7 (ccd7) is very highly 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 as to whether this hormone has as yet undescribed function in reproductive tissues. It is additionally interesting to note 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 later suggesting the presence of tight regulatory control at this metabolic juncture.

In summary, whilst 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 fruits. That said, a noticeable and dramatic difference was observed in seed set. Whilst this difference was not as dramatic as that observed following an inhibition of import of leaf-derived photoassimilates (Hackel et al., 2008), it did clearly influence the timing of seed set. We conclude that under normal conditions repression of fruit photosynthetic capacity could probably be compensated by enhanced import of photoassimilates from source tissues, but 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 upregulation of photosynthesis within source leaves, as well as
to fully understand the roles of sugar-, and indeed carotenoid-related changes in
hormonal regulation of seed development.
MATERIALS AND METHODS

Materials
Tomato (Solanum lycopersicum) cv 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 16h light/8h 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 fruits approximately 25DPA. Unless stated otherwise, all chemicals and enzymes were purchased either from Sigma-Aldrich Chemical Company 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 company. PCR product (1212 bp) was introduced into pENTR/D/TOPO vector (Invitrogen) and then using Gateway® technology into binary vector pKGWFS7 (Karimi et al., 2002) for confirming fruit-specific promoter expression using GUS-fusion. 32 primary transformant lines were grown, and initial green fruit screening for GUS-activity was performed. 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 1714bp fragment of tobacco GSA in antisense orientation (Höfgen et al., 1994) and ocs terminator. 22 primary transformants were selected, and fruits 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 were used for detailed physiological and biochemical analyses.

Gas-exchange analysis
Gas-exchange measurements were performed in a special custom-designed open system (Walz, Effeltrich, Germany; described in 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 days prior to the measurements), and then intact immature green fruit (approximately 20DPA) still attached to the plant were put into the cuvette, hermetically sealed around petiole, and CO₂ assimilation/respiration rate was measured under various light intensities, as well as in dark-adapted fruits. After the measurements were completed, fruit was carefully removed, and its weight and area were determined. The same series of measurements was repeated for the remaining in the cuvette green parts (calyx and petiole), and afterwards the values for fruit only were estimated through subtracting these non-fruit values from those measured previously for the entire fruit.

**Chlorophyll fluorescence**

Chlorophyll fluorescence parameters were measured on freshly detached and dark-adapted for 30min immature green fruits (approximately 20DPA), both with standard PAM chlorophyll fluorometer (Walz, Effeltrich, Germany) yielding quantitative values, and imaging PAM Walz, Effeltrich, Germany) revealing in vivo picture 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 determination of F₀ and Fm (Bilger et al. 1995). Then series of PFD (4, 10, 50, 100, 225, 365, 585 and 955 µmol m⁻² s⁻¹) was applied and a set of values was measured.

**Protein expression analysis using Western-blot hybridisation**

GSA expression was analysed in 20µg of protein from pericarp tissue of immature green fruits (approximately 20DPA) of antisense transgenic plants (aGSA 4 and aGSA8) as well as wild type fruits. Protein amounts were determined using the Bio-Rad Protein Assay (Bio-Rad). Loading and integrity of proteins were controlled by Coomassie staining of SDS PAGE gels. Transfer and blotting were performed according to manufacturer’s instructions using Trans-Blot SD semi-dry blotter (Bio-Rad) on Hybond-C Extra membranes (GE Healthcare). Antisera were diluted 1:1000 (anti-GSA against Synechococcus sp. protein) and 1:10000 (anti-Rabbit, Sigma-
Aldrich), respectively; signals were detected with a STELLA 3200 CCD camera (Raytest).

Gene expression analysis by microarray hybridisation

RNA was isolated according to Bugos et al. (1990) modified, from tomato fruit pericarp (22-25DPA) from antisense lines aGSA4 and aGSA8 and wild type tomato cv. Money Maker. A pool of equal amounts of RNA from transgenic lines and from wild type tomato was made and treated as a pool reference. To obtain differential gene expression values, 4 biological and technical replicates were hybridized against those of the pool reference. RNA samples for microarray hybridisation were amplified using the method of Van Gelder et al. (1990). Briefly, 1 µg of total RNA of each sample and pool reference was amplified and aminoallyl-labelled using MessageAmp® II aRNA kit (Ambion, http://www.ambion.com) and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (aa-dUTP, Ambion), following manufacturer’s instructions. Approximately 40–70 µg of amplified RNA (aRNA) was obtained. For each sample, 7.5 µg of aminoallyl-labelled aRNA was re-suspended in 0.1 M Na2CO3 (pH 9.0) and labelled with Cy5 Mono NHS Ester (CyTM Dye Postlabelling Reactive Dye Pack, Amersham). Equal quantity of RNA from Pool reference was labeled with Cy3. The samples were purified with MegaclearTM (Ambion) following manufacturer instructions. Incorporation of Cy3 and Cy5 was measured using 1 µl of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.; http://www.nanodrop.com/).

Microarray hybridisation of samples and pool reference to the Tom2 long-mer oligo array slides (representing 11,862 genes, Instituto de Biología Molecular y Celular de Plantas, Valencia, Spain) was performed manually using Telechem Hybridisation Chambers (Corning), following manufacturer 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 PMT values to adjust the ratio intensity to 1.0. Microarray images were analysed using GenePix 4.1 (Axon Instruments) software. Only spots with intensity greater than two-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 normalised by using the Lowess normalisation method within centred print-pin tip (Yang et al 2001, Dudoit et al 2002) using Acuity default values.
(smoothing filter: 0.4, iteration:3 and δ= 0.01). Finally, only spots with valid values in 80% hybridisations were considered for further analyses. To detect differentially expressed genes, a one-way ANOVA was performed to compare the mean lowess normalised values for a gene between experimental groups (antisense and wild type). A p-value cut-off of 0.05 was used to flag genes as being differentially expressed. Mean values of differential genes were calculated from each sample as log2 values. A hierarchical cluster (Supplementary Figure 3) was constructed using the mean of log-normalised 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 point 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 and sucrose 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 in Lisec et al. (2006). For the GC-MS analysis seeds from immature green fruits (approximately 25DPA) were manually isolated, freezed and grinded in liquid nitrogen, and aliquote of 250mg 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 in 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 in Osorio et al. (2011).

**GUS-staining**
Tomato tissue fragments were incubated overnight at 37°C in buffer containing 2mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronide cyclohexylamine salt and afterwards washed several times in 80% ethanol until full removal of chlorophyll.

**Microscopic evaluation**

Seeds from immature green fruit 25-30DPA were examined using stereomicroscope Leica MZ 12.5, and seed area parameters were measured and calculated with the help of corresponding software LAS (Leica, Wetzlar, Germany).

**Acknowledgments**

We are grateful to Dr. Eugenia Maximova for help in organisation of microscopic measurements and discussion of the results. We thank Anna Zbierzak for initial measurements of pigment contents. We are additionally indebted to Helga Kulka (all – MPI-MP) for excellent care of the plants.
LITERATURE CITED


SICCDD7 controls strigolactone biosynthesis, shoot branching and mycorrhiza-induced apocarotenoid formation in tomato. Plant J 61: 300-311.


Figure legends

Figure 1. Fruit-specific GUS-expression of TFM5 promoter. Immature green fruit 23DPA is shown on the left.
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Figure 7. MapMan representation of transcript changes in the tomato antisense GSA immature green fruits pericarp (22-25DPA), line aGSA4 in comparison to the wild type fruits: a - general overview; b – close up of the most distinct changes related to light reactions and Calvin cycle (labelled with red circle at Figure 7a).

Figure 8. Seed phenotype of immature green fruits (25-30DPA). Upper panel: abnormalities in seed development in the fruits of antisense line aGSA4; lower panel: wild type (left) and antisense line aGSA4 (right) individual seed under the light microscope, bar 500µm.

Supplementary Figure legends

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

Supplementary Figure S2. Chlorophyll fluorescence imaging of the antisense GSA immature green fruit of the line aGSA4 (right) in comparison to the wild type fruit (left). Quantum yield following a saturating pulse light of darkened fruit is represented.

Supplementary Figure S3. General HCA of transcript changes in the tomato antisense GSA immature green fruits (22-25DPA) pericarp in comparison to the wild type fruits
Table I. Immature green fruit (22-25DPA) seed characteristics. Antisense GSA plants were grown in the greenhouse alongside wild type controls. Values are presented as mean ± SE of determinations from 15-20 independent fruits (four in the case of the GC-MS measurements of seed tocopherols, data are normalised to the wild type values). Those determined by the t-test (P<0.05) to be significantly different from wild type are set in bold type.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>aGSA4</th>
<th>aGSA8</th>
</tr>
</thead>
<tbody>
<tr>
<td>seed count per fruit</td>
<td>81.5 ± 5.6</td>
<td><strong>34.9 ± 4.5</strong></td>
<td><strong>50.7 ± 7.9</strong></td>
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<tr>
<td>area ratio embryo/seed, %</td>
<td>63.8 ± 1.5</td>
<td><strong>39.2 ± 2.9</strong></td>
<td><strong>48.1 ± 4.6</strong></td>
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<td><strong>Seed tocopherol contents</strong></td>
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<td>alpha-tocopherol</td>
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<td><strong>0.15 ± 0.27</strong></td>
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<td>gamma-tocopherol</td>
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<td><strong>0.41 ± 0.29</strong></td>
<td><strong>0.38 ± 0.39</strong></td>
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<td>delta-tocopherol</td>
<td>1.00 ± 0.20</td>
<td>0.55 ± 0.13</td>
<td>0.56 ± 0.33</td>
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Table II. Metabolite contents of seed samples taken from immature green fruits (25-30DPA). Normalised to the wild type fold difference values are presented as mean ± SE of determinations from four independent samples, those determined by the t-test to be significantly different from wild type are set in bold type

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<tr>
<th></th>
<th>WT</th>
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<th>antiGSA8</th>
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<td>Alanine</td>
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<td>1.91 ± 0.09</td>
<td>2.00 ± 0.15</td>
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<td>Arginine</td>
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<td>Asparagine</td>
<td>1.00 ± 0.51</td>
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<td>2.50 ± 0.18</td>
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<td>Aspartate</td>
<td>1.00 ± 0.27</td>
<td>3.04 ± 0.11</td>
<td>4.85 ± 0.07</td>
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<td>Butyric acid, 4-amino-</td>
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<td>1.45 ± 0.07</td>
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<td>Glutamate</td>
<td>1.00 ± 0.18</td>
<td>2.87 ± 0.08</td>
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<td>1.71 ± 0.15</td>
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<td>1.95 ± 0.06</td>
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<td>Succinate</td>
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<td>3.07 ± 0.08</td>
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<td>0.79 ± 0.25</td>
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<td>Stigmasterol</td>
<td>1.00 ± 0.17</td>
<td>0.90 ± 0.25</td>
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</tr>
</tbody>
</table>
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Figure 7 continued
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