Manipulation of ZDS in tomato exposes carotenoid- and ABA-specific effects on fruit development and ripening

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Summary

Spontaneous mutations in fruit-specific carotenoid biosynthetic genes of tomato (Solanum lycopersicum) have led to improved understanding of ripening-associated carotenogenesis. Here, we confirm that ZDS is encoded by a single gene in tomato transcriptionally regulated by ripening transcription factors RIN, NOR and ethylene. Manipulation of ZDS was achieved through transgenic repression and heterologous over-expression in tomato. CaMV 35S-driven RNAi repression inhibited carotenoid biosynthesis in all aerial tissues examined resulting in elevated levels of \( \zeta \)-carotene isomers and upstream carotenoids, while downstream all-trans-lycopene and subsequent photoprotective carotenones and xanthophylls were diminished. Consequently, immature fruit displayed photo-bleaching consistent with reduced levels of the photoprotective carotenones and developmental phenotypes related to a reduction in the carotenoid-derived phytohormone abscisic acid (ABA). ZDS-repressed ripe fruit was devoid of the characteristic red carotenoid, all-trans-lycopene and displayed brilliant yellow pigmentation due to elevated 9,9'-di-cis-\( \zeta \)-carotene. Over-expression of the Arabidopsis thaliana ZDS (AtZDS) gene bypassed endogenous co-suppression and revealed ZDS as an additional bottleneck in ripening-associated carotenogenesis of tomato. Quantitation of carotenones in addition to multiple ripening parameters in ZDS-altered lines and ABA-deficient fruit-specific carotenoid mutants was used to separate phenotypic consequences of ABA from other effects of ZDS manipulation and reveal a unique and dynamic \( \zeta \)-carotene isomer profile in ripe fruit.

Introduction

Carotenoids play pivotal roles throughout plant development, among the most observable being coloration of ripe fruit and flowers for attraction of seed dispersing frugivores and pollinators. Carotenoids are additionally requisite to maintain proper function of the photosynthetic apparatus. Photoprotective carotenones (i.e. \( \beta \)-carotene) and xanthophylls (i.e. lutein, zeaxanthin, violaxanthin and neoxanthin) are localized in photosystems I and II where they absorb excess light energy and limit damage from adjacent excited chlorophylls through quenching of reactive singlet oxygen (Amunts et al., 2007; Jahns and Holzwarth, 2012). Numerous aspects of plant development, dormancy and stress responses are dependent on the synthesis of the carotenone-derived phytohormone abscisic acid (ABA) (Cutler et al., 2010; Hauser et al., 2011; Mauch-Mani and Mauch, 2005; Vishwakarma et al., 2017; Zhang et al., 2006). Recent evidence indicates additional roles of carotenones as precursors for important regulatory functions via strigolactones (reviewed in Waters et al., 2017) in addition to signalling functions with other organisms through volatile carotenoid metabolites (reviewed in Hou et al., 2016; McQuinn et al., 2015).

Utilization of available natural tomato (Solanum lycopersicum), carotenoid biosynthetic mutants in combination with studies in other model species (e.g. Arabidopsis thaliana and Zea mays) elucidated a nearly comprehensive carotenoid biosynthetic pathway (Figure S1). Moreover, duplication of genes in a majority of biosynthetic steps in tomato illuminated chloroplast- and chloromplast-specific pathways clarifying ripening-associated carotenogenesis (Galpaz et al., 2006; Sato et al., 2012; Figure S1 and Table S1). Natural mutations in genes involved in chromoplast-specific carotenogenesis (e.g. \( r \), PSY1; tangerine, CRTISO; Beta, CYC-B/CLR-B2) have allowed researchers to functionally define many steps of the pathway relevant to fruit ripening (Fray and Grierson, 1993; Isaacson et al., 2002; Ronen et al., 2000) and led to some of the colour variation in tomato (yellow and orange fruit varieties) appreciated by consumers. However, the lack of gene duplication within the poly-cis-transformation of 15-cis-phytoene to all-trans-lycopene limits the assessment of ripening roles for these genes.

In the poly-cis-transformation of 15-cis-phytoene to all-trans-lycopene, 15-cis-phytoene undergoes four desaturation steps carried out by two desaturases (phytoene desaturase/PDS and zeta-carotene desaturase/ZDS) in conjunction with two intermediary isomeric conversions carried out by two isomerases (zeta-carotene isomerase/ZISO and carotenoid isomerase/CRTISO; Figure S1; Beltrán et al., 2015; Brausmann et al., 2017; Chen et al., 2010b; Dong et al., 2007; Isaacson et al., 2002; Qin et al., 2007). Unlike ZISO and CRTISO which carry out reactions that can
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alternatively be catalysed by light in photosynthetic tissues (Isaacson et al., 2002; Li et al., 2007, respectively), mutations in the single copy genes PDS and ZDS manifest in dramatically reduced plant fitness reflecting highly unstable photosynthetic tissues (Dong et al., 2007; Qin et al., 2007). This is likely the reason that, until recently, PDS and ZDS remained uncharacterized in terms of tomato fruit ripening (McQuinn et al., 2017).

Initial characterization of PDS and ZDS was limited to their transient repression via virus-induced gene silencing (VIGS) due to their indispensable nature in photosynthetic tissues (Fantini et al., 2013). More recently, we further characterized PDS via heterologous over-expression of the Arabidopsis PDS in tomato and investigated its impact on the carotenoid accumulation and gene expression throughout the aerial tissues of the tomato plant (McQuinn et al., 2017).

Genes involved in synthesis of 15 cis-phytoene and its poly-cis-transformation to all trans-lycopene are induced transcriptionally at the onset of fruit ripening giving the tomato its characteristic red colour. It is well established that the initiation of physiological changes associated with tomato ripening, including carotenoid accumulation, is induced by a climacteric burst of ethylene, and is dependent on an elaborate combination of epigenetic and transcriptional dynamics influenced to varying degrees by ripening-specific transcriptional regulators RIN (a MADS-box transcription factor), NAC-NOR (a NAC domain transcription factor) and CNR (a Squamosa promoter binding protein; Eriksson et al., 2004; Gallusci et al., 2016; Giovannoni et al., 2017; Giovannoni et al., 2004; Ito et al., 2017; Lü et al., 2018; Manning et al., 2006; Vrebalov et al., 2002).

As the main rate-limiting step of carotenogenesis, phytoene synthase (PSY) has garnered much attention regarding its transcriptional and post-transcriptional regulation across various plant species (i.e. Arabidopsis, Zhou et al., 2015; Melon, Chayut et al., 2017). This holds true in tomato, where PSY1 has been well studied and shown to be transcriptionally regulated by ethylene perception and signalling (Gapper et al., 2013), and a direct target of RIN-mediated transcriptional regulation (Martel et al., 2011), which remains dependent on demethylation of its promoter prior to ripening (Zhong et al., 2013). Further, numerous strategies targeting PSY1 have been deployed to enhance carotenoid accumulation in tomato (reviewed in Fraser et al., 2009). Recently, researchers enhance carotenoid accumulation in tomato upon ectopic expression of the Arabidopsis ORANGE protein, which has dual roles in post-transcriptionally regulating PSY as well as inducing chloroplast biogenesis (Yazdani et al., 2019). While such strategies have proven successful, new bottlenecks emerge in subsequent desaturase steps carried out by PDS and ZDS, thereby limiting their effectiveness. These secondary bottlenecks present additional targets for manipulation to enhance carotenoid content, one of which, PDS, has been successfully exploited to increase downstream health-promoting carotenoids demonstrating its potential in carotenoid biotechnology strategies (McQuinn et al., 2017).

Regarding the enzymes carrying out the poly-cis-transformation of 15-cis-phytoene to all trans-lycopene, very little is known regarding their regulation, other than their induction by ethylene perception and signalling upon initiation of tomato fruit ripening (Alba et al., 2005). Further, while ZISO and CRTISO have been characterized via natural mutations (Gonda et al., 2019; Isaacson et al., 2002, respectively) and now PDS through transgenic approaches (Fantini et al., 2013; McQuinn et al., 2017), ZDS remains the least characterized step in tomato fruit ripening-associated carotenogenesis. Therefore, transgenic manipulation of ZDS and exploration into its regulation is essential and may aid future strategies aimed at enhancing carotenoid content beyond levels achieved via PSY exploitation.

Given the limited knowledge of ZDS in terms of its regulation and phenotypic consequences on fruit development and ripening, we explored the consequences of transgenic manipulation of ZDS throughout tomato fruit development, investigating its function via RNAi-guided repression and over-expression using a heterologous gene from Arabidopsis (AtZDS). The heterologous over-expression of AtZDS was implemented to reduce potential co-suppression of the endogenous tomato ZDS gene, a strategy that has proven useful for the manipulation of PDS in tomato (McQuinn et al., 2017). Herein, we identify ZDS as a heavily regulated gene in carotenogenesis by multiple ripening regulatory factors. Additionally, we highlight the consequences of ZDS manipulation on fruit ripening carotenogenesis and its potential for the enhancement of carotenoid content in tomato fruit. Lastly, we confirm diminished ABA and carotenoid accumulation throughout tomato fruit development reduces fruit growth and expansion, delays the initiation of fruit ripening and alters fruit quality (i.e. primary metabolites and brix<sup>®</sup> content).

**Results**

ZDS is a single copy gene in tomato strongly regulated by ripening transcription factors and ethylene

In tomato, multiple steps of carotenoid biosynthesis are represented by multigene families (e.g. Solanum lycopersicum PSY1, SIPSY2, SIPSY3, and SILCY-BI CRTL-B1; SIBCYC1 CRTL-B2; Table S1), the origins of which can be traced in some cases to a whole-genome triplication event Tomato Genomics Consortium (TGC, 2012). These genes have been further categorized according to chloroplast and chromoplast specificity (Table S1; Galpaz et al., 2006). Chromoplast- or fruit ripening-specific genes have been functionally characterized in tomato through the study of natural mutations that have minor effects, if any, on non-fruit photosynthetic tissues. Given that a tomato ZDS mutant has not yet been reported, and consistent with the many deleterious phenotypes of Arabidopsis and Zea mays ZDS mutants, it is likely that ZDS is represented by a single copy gene in tomato and many other widely studied plant species. Queries of available protein sequences using tomato and Arabidopsis ZDS (SIZDS and AtZDS, respectively) as the query in multiple sequence/genome databases (NCBI, http://www.ncbi.nlm.nih.gov; TAIR, www.arabidopsis.org; Solgenomics, Solgenomics.net) indicates this is the case for tomato and many other plant genomes. It is noteworthy that genome duplication in apple and orange may have led to an increase from one to two or more paralogous copies of ZDS, respectively, in these lineages (Chen et al., 2010a; Velasco et al., 2010).

Analysis of ZDS transcript accumulation in above ground tissues of tomato via qRT-PCR demonstrates ZDS is expressed in all tissues analysed, consistent with available microarray and RNA-seq data (TGC, 2012; Fernandez-Pozo et al., 2017; Shinozaki et al., 2018; Zouine et al., 2017; ted.bti.cornell.edu). ZDS was most highly expressed in the leaves, ripening fruit and flowers, in increasing order (Figure 1a). 9,9’-di-cis-ß-carotene is converted to all trans-lycopene by ZDS in conjunction with carotenoid isomerase (CRTISO), whose expression pattern for the most part paralleled ZDS (Figure 1a). ZDS expression analysis through a comprehensive fruit developmental time course reveals ZDS
transcripts accumulate during early stages of fruit development, after which ZDS transcript levels decreased until the onset of ripening at which point expression increases substantially through early ripening (Figure 1b).

Because ZDS expression is induced upon ripening initiation, ZDS transcript levels were quantified in tomato ripening transcription factor (ripening inhibitor, rin, and non-ripening, nor) and ethylene insensitive (Green ripe, Gr, and Never ripe, Nr) mutants at the 7 days post-breaker (7DPB) stage (Barry et al., 2005; Giovannoni et al., 2004; Hackett et al., 2000; Vrebac et al., 2002). For accurate fruit staging, wild-type and mutant flowers were all tagged at anthesis and ripening mutant fruits were collected at the same number of days after pollination as that of 7DPB wild-type fruit where ‘breaker’ was determined by the first sign of colour change. ZDS transcripts are reduced in all four mutants with the greatest reduction observed in nor fruit. These results highlight NOR, a NAC domain transcription factor, as influential in the regulation of ZDS, and remain consistent with the Rin transcription factor and ethylene all as ripening-related ZDS regulatory components (Figure 1c).

**ZDS-RNAi lines demonstrate inhibited carotenogenesis in flowers and fruits**

ZDS expression was suppressed via RNA interference driven by the constitutive CaMV 35S promoter in the pHELLSGATE2 vector containing cDNA targeting the gene-specific 3’UTR of ZDS. *Agrobacterium tumefaciens*-mediated transformation of wild-type tomato (*S. lycopersicum* cv Ailsa Craig) yielded eight independent transgenic ZDS-RNAi lines, three of which (ZDS.2, ZDS.5 and ZDS.7) were selected for further characterization based on insert number, and limited deleterious effects on photosynthetic tissues ensuring flower and fruit set (Figure S2). A number of chlorotic RNAi lines were removed from the analysis due to their inability to propagate. Quantitative PCR confirmed lines ZDS.5 and ZDS.7 as single insertion lines, whereas ZDS.2 contains two transgene insertions. Plants heterozygous for both inserts (in the case of ZDS.2) and for the single insert in lines ZDS.5 and ZDS.7 were used for subsequent characterization as homozygous plants were severely dwarfed and incapable of proliferation and propagation (Figure S3). RNAi resulted in significant repression of ZDS in young leaves, anthesis flowers and ripe fruits of the three independent RNAi lines as compared to wild-type and all three lines displayed phenotypes consistent with altered carotenogenesis (Figure 2). The strongest repression of ZDS was observed in anthesis flowers (~8-fold decrease) and in ripe fruit (~16-fold decrease), while expression was repressed approximately twofold in leaves of repression lines (Figure 2a).

Leaves of two-month-old plants began to photobleach when removed from low light conditions and placed in natural light (Figure S2). Interestingly, this susceptibility to photobleaching was temporary, as newly formed leaves became visually indistinguishable when compared to wild-type throughout the remainder of the growing cycle. Similar results were reported by Dong et al. (2007) for the weaker Arabidopsis ZDS-mutant allele of spontaneous cell death 1 (spc1-1) when grown in short-day conditions. These observations are consistent with photoprotective carotenoids and xanthophylls accumulating to wild-type levels in the leaves of the ZDS-RNAi lines (Table 1). Further, given the accumulation of β-carotene and xanthophylls remains unchanged in leaves, it is likely that carotenoid-derived hormones, strigolactone and ABA are not substantially impacted in those tissues as well.

In contrast, photobleaching is prevalent throughout fruit development of ZDS-RNAi lines, with the double-insert ZDS.2 line being the most severe (Figure 2b). Accumulation of all trans-lycopene, the characteristic red pigment of ripe tomato fruit, is inhibited throughout ripening of ZDS-RNAi tomato fruit as observed in fruit at the full-ripe stage (i.e. 7DPB; Figure 2b). Reduced pigmentation was also observed in ZDS-RNAi anthesis flowers compared with wild-type (Table 1 and Figure S2). Additional phenotypes were observed consistent with reduced synthesis of the carotenoid-derived hormone ABA in seeds (Figure S4).

Carotenoid profiles in ZDS-RNAi and wild-type control tissues were assessed using high-performance liquid chromatography (HPLC). Wild-type leaves, flowers and ripe fruit accumulated undetectable or low levels of linear carotenoids upstream of ZDS,
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Arabidopsis thaliana ZDS (AtZDS) in tomato driven by the CaMV 35S promoter was employed using the pART27 vector system (Gleave, 1992). As noted above, we deployed heterologous over-expression rather than use of the tomato ZDS gene to reduce potential for co-suppression of the endogenous gene, a strategy proven useful for manipulation of tomato PDS (McQuinn et al., 2017). Nine independent transgenic lines were identified and one, AtZDS.OE.8, demonstrated phenotypes similar to ZDS-RNAi lines, suggesting ZDS co-suppression (Figure S5). Of the remaining eight lines, four (AtZDS.OE.1A, AtZDS.OE.4, AtZDS.OE.7, AtZDS.OE.9), each containing a single insert and presenting consistent phenotypic variation, were carried to the T2 generation.

The effectiveness of AtZDS.OE was determined via qRT-PCR of the AtZDS transgene in young leaves, anthers, flowers and 7DPB fruit from each independent transgenic line (Figure 3). CaMV 35S-driven expression of AtZDS was successful with all tissues analysed displaying substantial transgene mRNA accumulation. The native Solanum lycopersicum ZDS, SlZDS, is slightly reduced in some of the tissues analysed in the AtZDS.OE lines, possibly reflecting a response to the transgene (Figure 3a). Nevertheless, total ZDS mRNA was greatly increased in all tested tissues and lines. Young leaves showed the lowest relative increase in AtZDS transcript levels ranging from approximately 0.5-fold to 4-fold higher when compared to wild-type (Figure 3a). In chromoplast rich tissues (mature petals and ripe fruits), AtZDS transcript levels were elevated to greater than 10 on a log 2 scale when compared to wild-type (Figure 3a). Despite successful over-expression in multiple tissues, visual phenotypes were limited to the ripe fruit at 7DPB (Figure 3b).

Carotenoid profiles of young leaves, flowers and ripe fruits from AtZDS.OE lines were investigated via HPLC analysis and compared to carotenoid content in wild-type control plants. Carotenoid levels remained unchanged in young leaves and flowers in the AtZDS.OE lines in agreement with the lack of visual differences (Table 2), suggesting ZDS activity is not limiting. A modest increase in total lycopene content was observed in the 7DPB fruit of the AtZDS.OE lines with no significant change in total cis-carotene amount (Table 2).

Manipulation of ZDS exposes a dynamic ζ-carotene isomer profile in ripe tomato fruit

While total ζ-carotene remained unchanged in the AtZDS.OE lines, the ζ-carotene isomer profile was altered greatly in fruit either repressing the endogenous SlZDS or over-expressing AtZDS (Figure 4). In ZDS-RNAi fruit, ζ-carotene isomers 9,15,9′-trans-ζ-carotene, 9,9′-cis-cis-ζ-carotene, 9 cis-ζ-carotene, and all trans-ζ-carotene were elevated in a manner consistent with that observed in fruit with ZDS repressed by VIGS (Fantini et al., 2013; Figure 4a). In AtZDS.OE fruit, the ZDS substrates, 9,9′ di-cis-ζ-carotene and 9 cis-ζ-carotene were depleted and undetectable while all trans-ζ-carotene was significantly increased (Figure 4b). This change in the ζ-carotene isomer profile is consistent with the lack of reduction in the total ζ-carotene content of AtZDS.OE lines (Table 2).

Reduced carotenoid content and ABA in ZDS-repressed fruit impacts tomato development

In contrast to vegetative tissues of ZDS-RNAi plants, developing fruit displayed clear phenotypes evident of ZDS repression and the resulting inhibition of carotenogenesis (Figures 2b and 5). The

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**Figure 2** Phenotypic characterization of tomato fruit from ZDS-RNAi lines. (a) Tissue-specific repression of ZDS mRNA levels in ZDS-RNAi lines relative to wild-type (AC++) (n = 3 and performed in triplicate). (b) Visually apparent alteration of chlorophyll and carotenoid content during fruit development in ZDS-RNAi lines compared to wild-type (AC++). MG, mature green; BR, breaker; RR, red ripe. Photographs are not to scale and are relevant only to fruit pigmentation.
carotenoid contents are presented as μg/g FW (±SEM). Values represent a mean of a minimum of 3 biological replicates. “n.d.” denotes “not detected”. Bold numbers – indicates significance (P < 0.05).

Table 1 Carotenoid content in young leaves, anthesis flowers, and ripe fruits of ZDS-RNAi lines compared to wildtype (Ailsa Craig) (n≥3)

| Genotype | Total Phytoene | Total Phytofluene | Total β-carotene | Total Lycopene | Total ζ-carotene | Lutein | Other Xanthophylls | Total Carotenoids |
|----------|----------------|-------------------|-----------------|----------------|-----------------|-------|------------------|-----------------|
| Leaf     |                |                   |                 |                |                 |       |                  |                 |
| AC++     | 0.1 (0.01)     | n.d.              | 102.8 (2.26)    | 168.2 (6.27)   | 79.8 (5.23)     | 357.9 (13.15) |                   |                 |
| ZDS.2    | 1.0 (0.29)     | 0.3 (0.08)        | 110.0 (4.49)    | 165.7 (7.89)   | 77.2 (6.23)     | 360.7 (18.88) |                   |                 |
| ZDS.5    | 4.0 (0.46)     | 1.4 (0.18)        | 89.3 (4.81)     | 145.1 (8.88)   | 70.6 (3.40)     | 317.3 (16.91) |                   |                 |
| ZDS.7    | 2.4 (0.40)     | 0.7 (0.14)        | 99.5 (3.02)     | 150.9 (4.68)   | 70.4 (2.17)     | 329.6 (9.38)  |                   |                 |
| Anth. Flowers |        |                   |                 |                |                 |       |                  |                 |
| AC++     | 0.1 (0.02)     | 0.1 (0.01)        | 4.6 (0.37)      | 8.4 (0.71)     | 82.6 (7.88)     | 96.3 (8.87)  |                   |                 |
| ZDS.2    | 17.8 (2.07)    | 5.9 (0.82)        | 11.7 (1.20)     | 1.7 (0.17)     | 6.0 (0.69)      | 34.2 (3.53)  | 84.8 (9.11)      |                 |
| ZDS.5    | 5.9 (0.77)     | 2.4 (0.27)        | 7.08 (0.69)     | 2.3 (0.18)     | 7.5 (0.63)      | 45.6 (4.62)  | 75.9 (6.79)      |                 |
| ZDS.7    | 14.2 (1.12)    | 5.5 (0.45)        | 10.5 (0.79)     | 2.4 (0.20)     | 7.1 (0.57)      | 49.1 (3.88)  | 94.6 (7.34)      |                 |
| Ripe Fruit |           |                   |                 |                |                 |       |                  |                 |
| AC++     | 2.2 (0.21)     | 1.3 (0.11)        | 0.3 (0.06)      | 105.7 (4.15)   | 7.0 (6.2)       | 1.7 (0.16)   | 120.4 (3.89)     |                 |
| ZDS.2    | 7.6 (1.47)     | 3.4 (0.64)        | 21.7 (4.26)     | 1.1 (0.23)     | 2.9 (0.34)      | 0.5 (0.07)   | 39.6 (6.56)      |                 |
| ZDS.5    | 21.6 (2.73)    | 11.4 (1.47)       | 78.9 (12.01)    | 2.0 (0.83)     | 8.0 (0.87)      | 1.2 (0.23)   | 130.5 (15.37)    |                 |
| ZDS.7    | 18.1 (2.04)    | 9.3 (1.00)        | 71.5 (5.47)     | 0.3 (0.20)     | 4.0 (0.21)      | 0.7 (0.10)   | 107.0 (8.95)     |                 |

Figure 3 Effectiveness of the AtZDS over-expression transgene in tomato. (a) Quantitative RT-PCR comparing tomato and Arabidopsis ζ-carotene desaturase (SIZDS and AtZDS, respectively) transcript levels in AtZDS over-expression lines relative to the wild-type (AC++). Ripe fruits are 7 days post-breaker (7DPB) (n = 3 and performed in triplicate). (b) Visual phenotypes of chromoplast rich ripe fruit (7DPB) from AtZDS over-expression lines compared to wild-type. Chlorophyll a oxygenase (CAO) expression was elevated significantly in not and ZDS-RNAi lines relative to wild-type (Figure S7a). Expression of
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Table 2 Carotenoid content in young leaves, anthesis flowers, and ripe fruits of AtZDS.OE lines compared to wildtype (Ailsa Craig) (n=3)

| Genotype      | Total Phytene | Total Phytolene | Total β-carotene | Total Lycopene | Total Χ-carotene | Lutein | Other Xanthophylls | Total Carotenoids |
|---------------|---------------|-----------------|------------------|----------------|-----------------|--------|-------------------|------------------|
| Leaf          |               |                 |                  |                |                 |        |                   |                  |
| AC++          | 1.0 (0.06)    | 0.23 (0.03)     | 68.4 (2.65)      | 5.4 (0.14)     | 1.1 (0.08)      |        |                   | 77.9 (2.84)       |
| AtZDS.OE.1A   | 1.9 (0.18)    | 0.24 (0.02)     | 86.2 (4.02)      | 5.9 (0.40)     | 1.3 (0.06)      |        |                   | 96.5 (4.19)       |
| AtZDS.OE.4    | 2.1 (0.07)    | 0.24 (0.01)     | 81.9 (3.59)      | 6.4 (0.14)     | 1.0 (0.05)      |        |                   | 92.8 (3.74)       |
| AtZDS.OE.7    | 2.0 (0.13)    | 0.26 (0.01)     | 81.7 (1.50)      | 5.1 (0.18)     | 1.2 (0.06)      |        |                   | 91.2 (1.47)       |
| AtZDS.OE.9    | 1.7 (0.13)    | 0.21 (0.01)     | 81.3 (3.65)      | 4.9 (0.15)     | 1.2 (0.07)      |        |                   | 90.2 (3.84)       |

Carotenoid contents are presented as μg/g FW (±SEM). Values represent a mean of a minimum of 3 biological replicates. “n.d.” denotes “not detected”. Bold numbers – indicates significance (P < 0.05).

protochlorophyllide oxidoreductase (POR-B1 and POR-B2), which catalyses the reduction of protochlorophyllide to chlorophyll, showed a >2-fold increase in expression in all ABA-deficient lines with the largest and most prolonged in the ZDS-RNAi lines (Figure 7b). Despite the elevation of chlorophyll biosynthetic gene expression in the ZDS-RNAi lines, ZDS.2 and ZDS.7 fruit did not accumulate chlorophyll at levels comparable to wild-type during development, consistent with reduced photoprotective carotenoids and xanthophylls (Figure 2c and 5d) and similar to the effect of reduced ZDS on leaves early in development (Figure 52).

We further explored the effects of reduced photosynthetic pigments on attributes of fruit quality (i.e. primary metabolism and brix²). A comprehensive assessment of primary metabolism in mature green (MG) and ripe (7DPB) fruit of ZDS-RNAi lines was performed via GC-MS (Figure 6a). Total soluble solids are negatively influenced in the MG fruit with strong decreases in major brix² components (e.g. fructose, glucose, citric acid; Figure 6a). However, as the fruit ripens the brix² components approach wild-type levels, though remain lower (Figure 6a). In addition, both stages showed elevated amino acid content consistent with abiotic stress (i.e. photooxidative stress; Obata and Fernie, 2012; Figure 6a). Interestingly, when comparing total brix² levels of 7DPB fruit of ZDS-RNAi lines with same stage fruit of fruit-specific carotenoid mutants (i.e. r and tangerine), it is apparent that carotenoids are linked to fruit soluble sugar content during ripening (Figure 6b). It was observed that ripe fruit of r and tangerine, which lack any visible defect in photoprotection during early fruit development, displays a similar and significant reduction in total brix² levels (Figure 6b).

In addition to influencing chloroplasts and chlorophyll levels during fruit development, reduced ABA levels appear to also impact fruit size and the number of days between pollination and the initiation of ripening (Figure 6c-f). Post-breaker fruit were weighed and ZDS-RNAi fruits showed a significant reduction in mass as did the ABA-deficient not mutant (Figure 6c). In AtZDS.OE fruit, a significant increase in fruit size was observed in only one of the three lines compared to wild-type (Figure 6d and Table S2). Ripening initiation was delayed in ZDS-RNAi lines, ZDS.2 and ZDS.7, and the ABA-deficient mutant, not, by approximately 6 days (Figure 6e), consistent with prior observations that exogenous ABA can promote ripening (Zhang et al., 2006). It is noteworthy that this significant ripening impairment has not previously been attributed to tomato ABA-deficient mutants, presumably because they eventually ripen and the phenotype has not been carefully monitored. Consistently, significant reduction of two days in time to ripening initiation was observed in two of three AtZDS.OE lines analysed (Figure 6f and Table S2). Minimal changes in other fruit developmental phenotypes observed in AtZDS.OE lines may be indicative of ZDS not being rate-limiting in photosynthetic tissues, consistent with no detectable levels of ZDS substrates recorded in wild-type leaves and developing fruit.

ABA and ethylene have been demonstrated to have an antagonistic relationship during plant development including leaf epinasty and adventitious root growth as observed in the ABA-deficient tomato not mutation, and during fruit ripening in the ABA-deficient tomato hp3 mutant (Galpaz et al., 2008; Thompson et al., 2004). During fruit ripening of ZDS-RNAi lines, ripening fruit displayed elevated or prolonged ethylene production (Figure 7a). However, no complementary effect was observed in AtZDS.OE ripening fruit where ethylene levels remain comparable to wild-type (Figure 7b).
Discussion

Current understanding of ripening-associated carotenoid biosynthesis has been largely established through the cloning and functional characterization of genes underlying natural fruit-specific carotenoid mutants in tomato (Fray and Grierson, 1993; Galpaz et al., 2006; Isaacson et al., 2002; Ronen et al., 2000). Both desaturase genes (PDS and ZDS) remain undefined by tomato mutations, likely due to their single copy gene status and anticipated deleterious effects on photosynthesis impeding survival and consistent with Arabidopsis and Zea mays ZDS mutants (Dong et al., 2007; Matthews et al., 2003). We implemented transgenic repression of tomato ZDS and heterologous overexpression of AtZDS to provide insight into how the manipulation of ZDS and carotenogenesis may alter tomato fruit development and quality.

The single copy ZDS is up-regulated at ripening initiation by NAC-NOR, yet represents a second bottleneck in ripening-associated carotenogenesis

While most steps of the carotenoid biosynthetic pathway are associated with small gene families including members expressed specifically in chloroplast- or chromoplast-rich tissues, we confirm that ZDS is a single copy gene in tomato crucial for carotenoid biosynthesis throughout plant development. Ripe fruit accumulate elevated levels of carotenoids as attractants for seed dispersing frugivores and are largely dependent upon transcriptional induction of ZDS (Figure 1a). ZDS transcript levels are dynamic and tightly regulated in developing and maturing fruit. Prior work on tomato ripening mutants (i.e. rin, nor, Nr, Gr) has demonstrated that key steps in the carotenoid pathway are targets of enhanced transcription as fruits ripen to increase flux.

Figure 4 Differential accumulation of \(\zeta\)-carotene isomers in ZDS-RNAi and AtZDS.OE ripe fruit (7DPB). (a) Carotenoid content (µg/g FW) of 7 \(\zeta\)-carotene isomers found in ZDS-RNAi ripe fruit compared to Ailsa Craig wild-type (AC++) (n ≥ 3). (b) Carotenoid content (µg/g FW) of 4 \(\zeta\)-carotene isomers most commonly found in wild-type (AC++) ripe fruit (7DPB) compared to AtZDS.OE 7DPB fruit (n ≥ 5). The legend in A is the same for B. *, \(P<0.05\); **, \(P<0.01\); ***, \(P<0.001\).
towards lycopene (Alba et al., 2005; Barry et al., 2005; Vrebalov et al., 2009; Vrebalov et al., 2002). PSY1 is well described as the rate-limiting step in fruit carotenogenesis and positively up-regulated by ethylene perception and signalling and the RIN transcription factor (reviewed in Gapper et al., 2013). We demonstrate that ZDS transcription is controlled via a complex array of ripening regulatory components in a manner similar to PSY1, including ethylene signal transmission and two ripening transcription factors, the MADS-box transcription factor RIN and NAC domain transcription factor NAC-NOR (Gapper et al., 2013; Giovannoni et al., 2004; Wang et al., 2019; Figure 1c).

ZDS transcripts were significantly reduced in ripe fruit of Nr and Gr, both dominant mutations in an ethylene receptor and a receptor-associated protein, respectively, conferring ethylene insensitivity suggesting ethylene signalling is critical for ZDS transcriptional regulation via a potential interaction with ethylene response transcription factors or ERF’s (Adams-Phillips, Barry & Giovannoni, 2004 and Gapper et al., 2013). Similar and stronger repression of ZDS was observed in 7DPB fruit of rin and nor, respectively (Figure 1c). At first glance, this repression of ZDS may reflect an indirect regulation through RIN’s and NAC-NOR’s influence on ethylene (Adams-Phillips et al. 2004; Ito et al., 2017; Wang et al., 2019). On the contrary, upon mining available databases of RIN and NAC-NOR ChIPSeq data (ted.bti.cornell.edu and www.epigenome.cuhk.edu.hk/encode.html), both transcription factors bind to the promoter region immediately upstream of the transcriptional start site, suggesting a role in the direct regulation of ZDS (Vrebalov et al., 2002, Wang et al., 2019 and reviewed in Giovannoni et al., 2017). Herein, we demonstrate that ZDS represents an additional carotenoid biosynthetic gene heavily regulated at the onset of and throughout tomato fruit ripening by ripening transcription factors and ethylene signalling either separately or in combination.

Manipulation of ZDS in tomato

Figure 5 Decreased synthesis of photoprotective carotenoids and ABA in ZDS-repressed tomato fruit. (a) Changes in accumulation of photoprotective carotenoids, lutein; b-carotene; neoxanthin; and violaxanthin, in ZDS-RNAi lines and not compared to wild-type (AC++) controls through early fruit development. 1 cm, 7–10 days post-anthesis (DPA); 15 DPA; and 25 DPA (n ≥ 3). (b) Decreased ABA synthesis in ZDS-RNAi lines and not compared to wild-type (AC++) through early stages of fruit development (n ≥ 3). (c) Elevated FtsZ mRNA levels in ZDS-RNAi lines, ZDS.2 and ZDS.7, and not relative to wild-type (AC++) in 3 early stages of fruit development (n = 3 and performed in triplicate). (d) Altered chlorophyll a and chlorophyll b content of ZDS-RNAi lines and not compared to wild-type (AC++) during early stages of fruit development (n ≥ 3). Inset picture of photobleached ZDS-RNAi 25 DPA fruit compared to not and AC++ 25 DPA fruit. Scale bar = 1 cm. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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Figure 6 Manipulation of tomato ZDS alters fruit quality and development. (a) Primary metabolite profiling of AC++ control and ZDS-RNAi fruit. Intensity of fold change to wild-type (AC++) is visualized by indicating colour (log2FC > 2.0 shows red, log2FC < –2.0 shows blue). Fruit stages analysed were mature green (MG) and red ripe (RR) (n = 6). For metabolite guidelines check list and overview of metabolite list see Table S3 and S4, respectively. (b) Brix^o content in 7 DPF fruit pericarp and locule of ZDS-RNAi lines, ZDS.2 and ZDS.7, compared to other carotenoid mutants, r/r and tangerine (t/t), and wild-type (AC++) (n ≥ 5; *, P < 0.05). (c) Decrease in fruit weight of ZDS-RNAi lines, ZDS.2, ZDS.5 and ZDS.7, compared to wild-type (AC++) (n ≥ 3; *, P < 0.05; **, P < 0.01). (d) Fruit weight of AtZDS.OE lines, AtZDS.OE.1A, 7 and 9 compared to wild-type (AC++) (n ≥ 3; *, P < 0.05; **, P < 0.01). (e) Number of days to reach the initiation of ripening in ABA-deficient ZDS-RNAi lines, ZDS.2 and ZDS.7, and not compared to wild-type (AC++) (DPA, days post-anthesis). (f) Number of days post-anthesis (DPA) to reach the initiation of ripening (breaker) in AtZDS.OE lines compared to wild-type (AC++) (n ≥ 3; *, P < 0.05; **, P < 0.01).
Manipulation of ZDS in tomato

Table 1, similar to the effects of the weak ZDS breaker, 3DPB and 7DPB fruit of a consequence of ZDS manipulation. (a) Ethylene content (ng/g/hr) in ®

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observed mature fruit weight was approximately 75% of control fruit for all three mutants. 30%–40% reductions in fruit weight were also observed for transgenic tomatoes with repressed NCED1 expression (Sun et al., 2012). The 30%–50% reduction in fruit weight of the ZDS-RNAi lines is consistent with these observations and their resulting decrease inABA(Figure 6c).

Taken together, these results demonstrate thatABAplays an important role in fruit size determination and levels of the hormone are influenced by changes in upstream carotenoid synthesis enzymes including ZDS.

Climacteric fruit such as tomato produce a characteristic ethylene burst at the onset of ripening, which is necessary for maturation to proceed (Gapper et al., 2013; Giovannoni et al., 2017). The synthesis and perception ofABAduring fruit development has been proposed as an early ripening regulator upstream of ethylene (Zhang et al., 2009). Inhibition of ABAbiosynthesis in developing tomato fruit via a carotenoid synthesis inhibitor (NNGDA) delayed initiation of ripening for 3 days (Zhang et al., 2009). The ABA-deficient pinalate mutant inCitrus sinensisdisplays a similar effect in that de-greening is prolonged (Rodrigo et al., 2003). The 7-day delay of ripening initiation in ZDS-RNAitomato fruit further supports a strong relationship betweenABAand fruit ripening (Figure 6e). While the ABA-deficient mutantnotabilisis previously shown to inhibit fruit expansion, we show here that it is also ripening delayed (Figure 6e), while fruit from two out of the three AtZDS over-expression lines tested ripened on average 2 days earlier than wild-type fruit (Figure 6f and Table S2). Further, altered plastid levels are known to associate with fruit sugar/TSS content (Nguyen et al., 2014) but the effect of altered carotenoid flux on ABA and downstream signalling molecules may indicate a more direct role of carotenoid metabolism on ripening control. Indeed, other carotenoid impaired mutants such aslutescent2(I2)that fail to accumulate carotenoids at the onset of ripening due to impaired plastid accumulation are significantly ripening delayed (Barry et al., 2012) further supporting a role of carotenoids in coordination of ripening process and beyond their effects on ripe fruit pigmentation and associated appearance and nutrient quality.

In short, we confirm thatABAinfluences fruit development and ripening at many junctures. Initially as a mediator of fleshy fruit expansion and later as a component of the complex set of genetic and hormonal interactions that contribute to ripening induction. As the fruit matures, ABA influences ethylene synthesis via its well-documented antagonistic relationship (Cheng et al., 2009). In ZDS-RNAirepression lines, ethylene synthesis is maintained at a higher level for a longer period consistent with observations from the ABA-deficient tomatohp3mutant (Galpaz et al., 2008; Figure 7a). Together, these results further suggest that in contrast to observations in leaves ofArabidopsisZDS mutants (chloroplast biogenesis 5(clb5) andspc1), the overaccumulation of upstream carotenoids (i.e. phytofluene andζ-carotene) does not produce any identifiable alterations in tomato fruit development that differ from the expected phenotypes attributed to ABA and carotenoid deficiency.

Experimental procedures

Plant materials and growth conditions

Wild-type (Solanum lycopersicum cv Ailsa Craig, LA2838A) and homozygous notabilis (LA3614); Never ripe (Barry et al., 2005); Green ripe (LA2435); ripening-inhibitor (LA3754); and non-ripening (LA3770) mutant seed were obtained from the Tomato Genetics Resource Center, UC Davis (http://tgrc.ucdavis.edu). Plants were grown in greenhouses at the Guterman Bioclimate Laboratory and Greenhouse Complex, Cornell University, Ithaca, NY. All plants were grown under low light conditions consisting of 12-h daytime and under a mesh shade cloth for the first month after which plants were transplanted, removed from shade and grown under long day conditions (16-hr days and 8-hights). ZDS-RNAi transgenic lines were carried on to the T1 generation where heterozygotes were selected for further analysis. Analysis of the AtZDS over-expression lines was carried out to the T2 generation.

Young leaf tissue was collected off the 4th, 5th and 6th leaves from the meristem of two-month-old plants. Whole petals and anthers were harvested from flowers at anthesis/pollination (the day a flower fully opened). Developing fruit was staged relative to pollination by tagging flowers at anthesis and collecting fruit at specific days post-anthesis (i.e. 10, 15, 25 and 30DPA/MG). Ripening stages were determined as days post-breaker where breaker is the initiation of overt ripening – initial colour change at the blossom end of the fruit. Zygosity and copy number of transgenic lines were determined in the T1 generation and confirmed in the T2 generation.

RNAi and over-expression constructs for plant transformation

The ZDS-RNAi construct was made in the pHellsgate 2 vector (provided by Peter Waterhouse, University of Sydney, Australia). The ZDS cDNA sequence used in construct development spans 285bp of the 3′UTR starting from the most 5′bp of the stop codon. PCR amplification of the RNAi fragments was accomplished using the FastStart High-fidelity PCR system (Cat. No. 04-738-292-001, Roche Applied Sciences, IN) using the EST clone 738-292-001, Roche Applied Sciences, IN) using the EST clone TUS-69-K8 with primers ZDSRNAi-for and ZDSRNAi-rev containing recombination sequence specified in the Gateway BP Clonase kit (Cat No. 11789-020, Invitrogen, CA; Table S3). The ZDS-RNAi fragment was Gel purified (Cat No. 28706, Qiagen, MD) and cloned into pHellsgate 2 according to BP Clonase kit instructions. The resulting construct was sequence verified and transformed into S. lycopersicum cv Ailsa Craig by Agrobacterium tumafaciens (strain LBA-4404) as described in Van Eck et al. (2006). The AtZDS over-expression (AtZDS. OE) construct was generated as described previously (Gleave, 1992; McQuinn et al., 2017). RNA from Arabidopsis thaliana (accession Columbia-0) leaf tissue was converted to cDNA via iScript™cDNA synthesis kit (Cat. No. 170-8891, Bio-Rad, CA). Resulting cDNA was used to amplify the full length AtZDS ORF via FastStart High-fidelity PCR system (Cat. No. 04-738-292-001, Roche Applied Sciences, IN) with AtZDS-OE-KpnI.for and AtZDS-OE-XbaI.rev primers (Table S3). The resulting AtZDS.OE construct was sequence verified and transformed into S. lycopersicum cv. Ailsa Craig by Agrobacterium tumafaciens (strain LBA-4404) as described in Van Eck et al. (2006).

DNA isolation and zygosity/copy number analysis

Genomic DNA was isolated from fresh meristematic leaf tissue as previously described Barry et al. (2005). Verification of insertion events in the ZDS-RNAI T0 plants was carried out via PCR using primers specific for the 3SS promoter in the pHellowgate 2 vector (Table S3). Insertion of AtZDS was validated using internal primers within the 3SS promoter and the AtZDS ORF (Table S3). Zygosity and copy number was determined in the T1 generation via
quantitative PCR relative to the single copy polygalacturonase 2a gene (PG2a, GenBank accession No. X004583) as previously described (McQuinn et al., 2017).

RNA isolation and quantitative RT-PCR analysis

Total RNA was isolated using a modified protocol from the RNeasy Minikit (Cat. No. 74106, Qiagen Sciences, MD) as described in McQuinn et al. (2017). Quantitative real-time PCR was performed using the Power SYBR® Green RNA-to-Ct™ 1-Step Kit (Cat No. 4309169, Applied Biosystems, NJ) in a 5 µL reaction volume (2.5 µL 2X Master Mix; 1 µM forward and reverse primers; 1 µL of total RNA; 0.46 µL DEPC-treated water; 0.04 µL RT enzyme mix). All tissue samples were represented by a minimum of 3 biological replicates, each with triplicate technical replicates. Gene-specific primers were checked for efficiency using wild-type or reference RNA (for primer sequence, see Table S3).

To be able to apply the standard curve method described in User Bulletin #2 (Applied Biosystems, 1997), a standard curve was included on each plate for the specific gene being analysed using wild-type or reference RNA (serial dilutions: 50 ng; 5 ng; 0.5 ng; 0.05 ng; 0.005 ng) in triplicate. For each gene analysis, template-free and negative-RT controls were included. Real-time PCRs were carried out using an ABI PRISM™ 7900HT Sequence Detection System (Applied Biosystems, CA) under the following reaction conditions: reverse transcription at 48°C for 30 min; enzyme activation at 95°C for 10 min; followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation curve was added at the end of the run for verification of primer specificity.

ABI PRISM™ SDS version 2.3 software (Applied Biosystems, California) was used to determine gene-specific threshold cycles (Ct) using the endogenous reference (18S rRNA) for every sample. Cts were extracted, and the standard curve method (Applied Biosystems, 1997) was applied to calculate relative mRNA levels in comparison to the wild-type control or the reference sample. Gene-specific primers were checked for efficiency using wild-type or reference RNA (for primer sequence, see Table S3).

Carotenoid and chlorophyll extraction and analysis

Carotenoids and chlorophylls were extracted from 200 mg of leaf tissue and frozen tomato pericarp, while carotenoid and xanthophyll contents of anthesis flowers were extracted from replicates of petals and anthers of a single flower. Tissue prep, pigment extraction and analysis were carried out using a modified protocol from Alba et al. (2005) as described previously (McQuinn et al., 2017). All solvents used were HPLC grade.

Brix® (TSS) content quantification

Brix® content was measured from pericarp juice and pulp separately in freshly harvested ripe fruit with a digital refractometer (Artisan™ HR200 Digital Refractometer; APT Instruments; IL).

Ethylene measurements

Ethylene was measured from fruits by sealing the fruit in airtight jars for 3 h at 22°C, after which a 1ml aliquot of headspace gas for each fruit was injected on to an Agilent 6850 Network GC System equipped with a flame ionizing detector (Agilent technologies; CA). Samples were compared to standards of known concentration and normalized for fruit mass.

ABA extraction and quantification

Abscisic acid (ABA) levels were extracted from fruit via a modified extraction method described in Thaler et al. (2010) designed for the extraction and quantification of ABA and 3 additional plant hormones (i.e. salicylic acid (SA), jasmonic acid (JA) and auxin/indole-3-acetic acid (IAA)). Modification was addition of 100 µL of internal standard containing 0.8 ng/mL each of D5-ABA, D5-SA, D5-JA and D5-IAA to each sample prior to homogenization. ABA levels were quantified upon application of a 10ml aliquot of each sample on a triple-quadrupole LC-MS/MS (Thermo Scientific®, Waltham, MA, USA) equipped with a C18 reverse-phase HPLC column (Gemini-NX, 3 mm, 150 x 2.00 mm, Phenomenex, Torrance, CA) using the method described in Thaler et al. (2010). ABA was analysed by negative electrospray ionization (spray voltage: 3.5 kV; sheath gas: 15; auxiliary gas: 15; capillary temperature: 350°C) with a collision-induced dissociation (CID) energy of 13.0 eV (argon CID gas pressure 1.3 mTorr [1.3 micron Hg]) and selected reaction monitoring (SRM) of compound-specific [parent → product ion] transitions: ABA[263 m/z → 153 m/z] D5-ABA as described in Rasmann et al. (2012).

Primary Metabolite analysis using GC-TOF-MS

Metabolite analysis of primary metabolites by GC-MS was performed as described by Lise et al. (2006) and optimized for tomato fruit pericarp as described in Rohrmann et al. (2011). Chromatograms and mass spectra were evaluated with Chroma TOF 1.0 (Leco) and TagFinder 4.0 software (Luedemann et al., 2008). Further supporting information regarding the metabolite analysis is available in Tables S4 and S5.

Statistical analysis

Statistical analysis and comparison of mean values (i.e. carotenoid, xanthophyll and chlorophyll amounts, ABA, brix®, fruit size, days to ripening and ethylene) from each mutant genotype to the control genotype were performed using the Student’s t-test.

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Conflicts of Interest

All authors declare there is no conflict of interest.

Authors Contributions

RPM and JIG conceived the research project and designed the experiments. RPM, NEG, ASG, SZ and TT carried out the experiments. ZF, ARF and JIG supervised the experiments. RPM wrote the article, under the supervision and complementary writing of JIG. JIG agrees to serve as the author responsible for contact and ensures communication.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1.** The carotenoid biosynthetic pathway noting S. *lycopersicum* mutations.

**Figure S2.** Additional phenotypes in ZDS-RNAi lines.

**Figure S3.** ZDS repression construct homozygosity results in severe deleterious phenotypes.

**Figure S4.** Evidence of reduced ABA accumulation in ZDS-RNAi seeds.

**Figure S5.** To generation ripe fruit images reveals co-suppression in line A2ZDS.Œ.8
Figure S6. Transgene efficiency in ZDS-RNAi developing fruit.
Figure S7. Low ABA induced chlorophyll biosynthesis during early stages of fruit development in ZDS-RNAi lines.
Table S1. Carotenoid gene families with chloroplast and chromoplast specificity.
Table S2. Fruit weight (g) and days to breaker of AtZDS.OE lines compared to AC++ (±SE).
Table S3. Primer list.
Table S4. Metabolite Reporting Guidelines (Checklist table).
Table S5. Overview of the metabolite reporting list.