Several geranylgeranyl diphosphate synthase isoforms supply metabolic substrates for carotenoid biosynthesis in tomato

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Short title: The tomato GGPPS family

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1 **ABSTRACT**

2 Geranylgeranyl diphosphate (GGPP) produced by GGPP synthase (GGPPS)
3 serves as a precursor for many plastidial isoprenoids, including carotenoids. Here
4 we show that five different GGPPS isoforms exist in tomato (*Solanum*
5 *lycopersicum*). From these, SIGGGPPS1, 2 and 3 (or SIG1-3 in short) produce
6 GGPP in plastids and exhibit similar kinetic parameters. Phytoene synthase
7 (PSY) converts GGPP into phytoene, the first committed intermediate of the
8 carotenoid pathway. Gene expression and co-expression network analyses
9 showed a preferential association of individual GGPPS and PSY isoforms in
10 processes linked to carotenoid biosynthesis such as root mycorrhization,
11 seedling deetiolation and fruit ripening. Co-immunoprecipitation experiments
12 showed that SIG2, but not SIG3, physically interacts with PSY proteins. By
13 contrast, CRISPR-Cas9 mutants defective in SIG3 showed a stronger impact on
14 carotenoid levels and derived metabolic, physiological and developmental
15 phenotypes than those impaired in SIG2. Double mutants with a simultaneous
16 knockout of both genes could not be found. Our work demonstrates that the bulk
17 of GGPP production in tomato chloroplasts and chromoplasts relies on two
18 cooperating GGPPS paralogs, unlike other plant species such as *Arabidopsis*
19 *thaliana*, rice or pepper, which produce their essential plastidial isoprenoids using
20 a single GGPPS isoform.

21 INTRODUCTION

22 Isoprenoids are essential biological molecules in all living organisms. In
 23 particular, plants are the main source of the enormous structural and functional
 24 variety that characterizes this family of compounds (Pulido et al., 2012; Tholl,
 25 2015). The building blocks for the biosynthesis of all isoprenoids are isopentenyl
 26 diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate
 27 (DMAPP). These five-carbon (C5) universal isoprenoid units are produced in
 28 plants by the mevalonic acid (MVA) pathway in the cytosol and the
 29 methylerythritol 4-phosphate (MEP) pathway in plastids (Vranová et al., 2013;
 30 Rodríguez-Concepción and Boronat, 2015). Short-chain prenyltransferases (SC-
 31 PTs) subsequently condense one or more molecules of IPP to one molecule of
 32 DMAPP giving rise to C10, C15, C20 and C25 prenyl diphosphates known as
 33 geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl
 34 diphosphate (GGPP), and geranylfarnesyl diphosphate (GFPP), respectively.
 35 These molecules are the immediate precursors for downstream pathways leading
 36 to the production of the main groups of isoprenoids. GPP is produced in plastids
 37 as the precursor of C10 monoterpenes. FPP is mainly produced in the cytosol
 38 and used to synthesize C15 sesquiterpenes and C30 triterpenes (including
 39 phytosterols). GGPP is mostly produced in plastids, serving as the precursor of
 40 gibberellins and photosynthesis-related isoprenoids such as chlorophylls,
 41 carotenoids, tocopherols, plastoquinone and phyloquinones. However, GGPP is
 42 also used for the production of C20 diterpenes in the cytosol, and both FPP and
 43 GGPP are produced in mitochondria for ubiquinone and diterpenoid biosynthesis.
 44 GFPP is used to produce C25 sesterterpenes in different cell compartments. SC-
 45 PTs are encoded by gene families in most plants and they are typically found in
 46 different cell compartments, consistent with the requirement of their specific
 47 prenyl diphosphate products in different subcellular locations. Prediction of
 48 specific products and cell targeting based solely on their protein sequences is still
 49 a challenge, making experimental evidence necessary to ascertain their
 50 biological role (Cunillera et al., 1996, 1997; Gaffe et al., 2000; Beck et al., 2013;
 51 Jones et al., 2013; Nagel et al., 2015; Wang et al., 2016a; Zhou et al., 2017;
 52 Zhou and Pichersky, 2020).

53 Carotenoids are one of the most studied groups of plant isoprenoids. These C40
54 tetraterpenes are greatly demanded by cosmetic and agro-food industries as
55 natural red to yellow pigments and provide benefits for human health, e.g. as
56 precursors of vitamin A and other biologically active molecules (Sandmann,
57 2015; Rodriguez-Concepcion et al., 2018). In plants, carotenoids have different
58 functions. In photosynthetic tissues, they are required for the assembly of the
59 photosynthetic apparatus, contribute to light harvesting and are essential for
60 photoprotection by dissipating excess light energy as heat and by scavenging
61 reactive oxygen species. They are also fundamental in growth regulation, since
62 they are the precursors of retrograde signals and phytohormones such as
63 abscisic acid (ABA) and strigolactones. As a secondary role, carotenoids provide
64 distinctive colors to flowers and fruits to attract pollinators and seed dispersal
65 animals (Nisar et al., 2015; Yuan et al., 2015). In plants, carotenoids are
66 produced and stored in plastids, including chloroplasts and chromoplasts (Ruiz-
67 Sola and Rodríguez-Concepción, 2012; Sun et al., 2018). MEP-derived IPP and
68 DMAPP are converted into GGPP by plastidial GGPP synthase (GGPPS)
69 isoforms and then GGPP is transformed into phytoene by phytoene synthase
70 (PSY) enzymes. The production of phytoene, the first committed intermediate of
71 the carotenoid pathway, is considered to be a major rate-determining step
72 regulating the metabolic flux through this pathway (Fraser et al., 2002). In tomato
73 (*Solanum lycopersicum*), three PSY-encoding genes control carotenoid
74 biosynthesis in different tissues. *PSY1* expression is boosted during ripening to
75 produce carotenoids involved in the pigmentation of the fruit (Bartley et al., 1992;
76 Fray and Grierson, 1993; Giorio et al., 2008; Kachanovsky et al., 2012). *PSY2* is
77 expressed in all tissues, including fruits, but transcript levels are much higher
78 than those of *PSY1* in photosynthetic tissues, where carotenoids are required for
79 photosynthesis and photoprotection (Bartley and Scolnik, 1993; Giorio et al.,
80 2008). Lastly, *PSY3* is mainly expressed in roots and it is induced during
81 mycorrhization (Walter et al., 2015; Stauder et al., 2018), when carotenoid
82 biosynthesis is up-regulated to produce strigolactones and apocarotenoid
83 molecules essential for the establishment of the symbiosis (Fester et al., 2002,
84 2005; Baslam et al., 2013; Ruiz-Lozano et al., 2016; Stauder et al., 2018).
85 Whether the corresponding PSY isoforms use GGPP supplied by different
86 GGPPS isoforms remains unknown.

87 Several GGPP synthase (GGPPS) paralogs have been retained in plants during
 88 evolution (Beck et al., 2013; Zhang et al., 2015; Ruiz-Sola et al., 2016a, 2016b;
 89 Zhou et al., 2017; Wang et al., 2018). However, a single GGPPS isoform appears
 90 to produce the GGPP substrate needed for the production of carotenoids and
 91 other plastidial isoprenoids in the three plant species whose GGPPS families
 92 have been best characterized to date: *Arabidopsis thaliana*, rice (*Oryza sativa*)
 93 and pepper (*Capsicum annuum*) (Ruiz-Sola et al., 2016a, 2016b; Zhou et al.,
 94 2017; Wang et al., 2018). While tomato has become one of the best plant
 95 systems to study the biosynthesis of carotenoids and its regulation, we still have
 96 a very incomplete picture of the GGPPS family in this plant. In particular, the
 97 GGPPS isoform(s) required for the production of carotenoids in photosynthetic
 98 tissues (e.g. for photoprotection), fruits (e.g. for pigmentation) or roots (e.g. for
 99 mycorrhization) remain unknown. Here we identify the plastidial GGPPS set in
 100 tomato and provide clues to understand how the supply of plastidial GGPP for
 101 the synthesis of carotenoids with different biological functions in particular tissues
 102 is regulated in this important crop plant.

103

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105

106 RESULTS

107 The tomato GGPPS protein family includes three plastidial isoforms

108 Several genes encoding proteins with homology to GGPPS enzymes can be
 109 found in the tomato genome (Table S1). Two of them were previously reported
 110 and named *GGPPS1* (*Solyc11g011240*) and *GGPPS2* (*Solyc04g079960*) (Ament
 111 et al., 2006; Fraser et al., 2007; Stauder et al., 2018; Zhou and Pichersky, 2020).
 112 Besides them, herein referred to as *SIG1* and *SIG2*, three more putative GGPPS
 113 paralogs were found using as a query the *Arabidopsis thaliana* *GGPPS11*
 114 (*AtG11*) gene (*At4g36810*). We named them *SIG3* (*Solyc02g085700*), *SIG4*
 115 (*Solyc02g085710*), and *SIG5* (*Solyc02g085720*). In our phylogenetic analyses
 116 (Figure 1), *SIG1* was found to cluster with *Arabidopsis* *GGPPS1* (*AtG1*), a
 117 mitochondrial isoform found to produce both GGPP and GFPP, whereas *SIG2*
 118 and *SIG3* were more closely related to *GGPPS2* (*AtG2*) and *GGPPS11* (*AtG11*),
 119 the two plastid-targeted *Arabidopsis* isoforms that mainly produce GGPP (Nagel
 120 et al., 2015; Wang et al., 2016a). *SIG4* and *SIG5* grouped in a separated cluster
 121 (Figure 1). All five tomato GGPPS candidates harbored the two essential catalytic
 122 motifs required for prenyltransferase activity (FARM and SARM) and a
 123 methionine (M) residue in the fifth position upstream to the FARM shown to be
 124 essential for GGPP production (Figure 1) (Figure S1) (Nagel et al., 2015; Wang
 125 et al., 2016a).

126 Next, we carried out experiments to ascertain the subcellular localization and
 127 enzymatic parameters of the identified GGPPS candidates, *SIG1-5* (Figure 2).
 128 First, we fused their complete sequences to a C-terminal green fluorescent
 129 protein (GFP) reporter and cloned the resulting constructs under the control of
 130 the 35S promoter. Following agroinfiltration of tobacco (*Nicotiana benthamiana*)
 131 leaves, the green fluorescence emitted by each chimeric protein was analysed by
 132 confocal microscopy at 3 dpi (days post-infiltration) (Figure 2A). The GFP fusions
 133 of *SIG1-3* showed a clear chloroplast localization as their GFP fluorescence
 134 overlapped with chlorophyll autofluorescence. By contrast, the signal
 135 corresponding to the *SIG5*-GFP fusion protein was detected as a punctate
 136 pattern typical of mitochondrial proteins (Figure 2A). This subcellular
 137 compartmentalization is in agreement with *in silico* predictions by *TargetP* and
 138 *ChloroP* algorithms (Emanuelsson et al., 2007). Fusions of the N-terminal 120 aa

Figure 1. Phylogenetic tree of GGPP and GFPP enzymes. Arabidopsis and tomato protein sequences lacking their predicted transit peptides were used to generate an unrooted Neighbor-Joining tree. The percentage of trees in which the associated sequences clustered together is indicated in each branch. The scale bar represents the mean number of substitutions per site. The chain-length determination (CLD) region (*i.e.* the FARM and the previous five residues) is indicated for each enzyme lineage. Residues determining the product length are shown in blue and aspartate (D) residues are shown in black; 'x' represents any amino acid. See Table S1 for accessions.

sequence of the tomato GGPPS candidates to GFP were also reported to localize in chloroplasts (SIG1/GGPPS1, SIG2/GGPPS2 and SIG3/GGPPS3) or mitochondria (SIG5/TPT2) when expressed in Arabidopsis protoplasts (Zhou and Pichersky, 2020). In the case of SIG4, the indicated algorithms predicted a plastidial targeting but we observed a predominantly cytosolic localization (Figure 2A) with only some cells occasionally exhibiting a plastidial localization of the SIG4-GFP reporter (Figure S2). Strikingly, a mitochondrial pattern was observed in Arabidopsis protoplasts using an N-terminal fragment of the SIG4/TPT1 protein fused to GFP (Zhou and Pichersky, 2020). These data, together with the observation that only SIG1, SIG2 and SIG3 produce GGPP *in vitro* (Zhou and Pichersky, 2020), led us to discard SIG4 and SIG5 and focus on the three tomato GGPPS enzymes with a clear plastidial location for further experiments.

151

SIG1, SIG2 and SIG3 are GGPP-producing enzymes with similar kinetic properties.

153

Figure 2. Subcellular localization and activity of tomato GGPPS candidates. (A) Representative confocal microscopy images of *N. benthamiana* leaf cells transiently expressing the indicated GFP fusion proteins. For each construct, GFP fluorescence (green, first column), chlorophyll autofluorescence (red, second column) and merged images of the same field (green and red, third column) are shown. White arrowheads mark stromules. Bars, 10 μ m. **(B)** LC-MS chromatograms of reaction products. Extracts of *E. coli* cells overproducing the indicated recombinant proteins (with an N-terminal 6x-His tag instead of their predicted plastid-targeting peptide) were incubated with IPP and DMAPP and the products were analyzed by LC-MS. Prenyl diphosphates were detected using mass-to-charge (m/z) ratios: 244.998 (IPP), 313.061 (GPP), 381.123 (FPP), 449.186 (GGPP) and 518.254 (GFPP). Retention times of available standards is also shown.

154 To investigate whether SIG1-3 proteins produce GGPP with similar efficiency, we
 155 set up *in vitro* activity assays followed by the analysis of the reaction products by
 156 LC-MS (Figure 2B). For expression in *Escherichia coli* cells, the three tomato
 157 isoforms were separately cloned without their predicted plastid-targeting
 158 sequences to aid solubility and fused to a N-terminal 6x-histidine tag to facilitate
 159 purification (Figure S3). As positive and negative controls, we used the
 160 Arabidopsis AtG11 (active) and AtG11s (inactive) proteins (Ruiz-Sola et al.,
 161 2016b). *E. coli* transformants were induced to produce the corresponding
 162 recombinant proteins (Figure S3) and whole-cell protein extracts were directly
 163 used for activity assays in the presence of IPP and DMAPP. LC-MS analysis of

164 the reaction products showed that SIG1, SIG2, SIG3 and AtG11 (but no AtG11s)
165 produced only GGPP (Figure 2B). Other prenyl diphosphate such as GPP, FPP
166 or GFPP were not detected in the assays. These results confirmed that these
167 three tomato proteins are true GGPPS enzymes, in agreement with recently
168 reported data (Zhou and Pichersky, 2020).

169 In order to analyze the kinetic properties of the identified tomato GGPPS
170 isoforms, we then purified the recombinant proteins taking advantage of their 6x-
171 histidine tag, using again AtG11 as a positive control (Figure S3). Enzymatic
172 assays with the purified enzymes (Barja and Rodríguez-Concepción, 2020)
173 showed that all tested GGPPS proteins exhibited a similar optimal pH around 7.5
174 (Figure S3), as expected for stromal enzymes (Höhner et al., 2016). The kinetic
175 parameters K_m (an estimator of the apparent affinity for the IPP and DMAPP
176 substrates) and V_{max} exhibited very similar values among the three tomato
177 enzymes (Table 1). They are also similar to those obtained for AtG11 here and
178 elsewhere (Wang and Dixon, 2009; Camagna et al., 2019). We can therefore
179 conclude that tomato SIG1, SIG2 and SIG3 and Arabidopsis AtG11 are true
180 plastidial GGPPS enzymes with very similar kinetic properties.

181

182 **Gene expression profiles suggest a major role of SIG2 and SIG3 in** 183 **chloroplasts and chromoplasts**

184 Analysis of public gene expression databases showed that the genes encoding
185 SIG1-3 enzymes were expressed in roots, leaves and flowers (Figure S4). Of
186 these, the most highly expressed gene is *SIG3* followed by *SIG2*, while *SIG1*
187 transcripts are present at very low levels. *SIG2* and *SIG3*, but not *SIG1*, are also
188 expressed at high levels in fruit pericarp and seed tissues (Figure S4). As an
189 initial approach to get an insight on the possible functions of these individual
190 isoforms, we performed a gene co-expression network (GCN) analysis. This is a
191 powerful tool to infer biological functions by applying the simple concept of “guilt-
192 by-association”, which means that a gene significantly associated with a
193 molecular pathway at transcriptional level may share similar functions (Oliver,
194 2000). Because we were interested in understanding the contribution of SIG1,
195 SIG2 and SIG3 to the production of carotenoids and related isoprenoids in
196 plastids, we performed targeted GCN analyses with GGPP-related plastidial

Figure 3. Gene co-expression analysis of tomato genes encoding plastidial GGPPS isoforms. Positive co-expression relationships ($p \geq 0.55$) are depicted in tissue-specific networks as edges. *SIG1*, *SIG2* and *SIG3* are depicted as central green nodes. Surrounding smaller nodes represent genes from the indicated isoprenoid pathways. Red, green and black edges indicate positive co-expression with *SIG1*, *SIG2* and *SIG3* genes, respectively. See Table S2 for gene accessions and Table S3 for p values.

197 isoprenoid biosynthetic pathways in different tomato tissues (Figure 3) (Figure

198 S5). By using publicly available databases for plant comparative genomics
 199 (*PLAZA 4.0*, *Phytozome*), we searched for tomato homologs of the pathways that
 200 supply GGPPS substrates (MEP pathway) and consume GGPP to produce
 201 carotenoids, chlorophylls, tocopherols, phylloquinone, plastoquinone,
 202 gibberellins, strigolactones and ABA (Table S2). Then, we retrieved their
 203 expression data from *TomExpress* (Zouine et al., 2017) in vegetative (i.e.
 204 photosynthetic) and fruit tissues and calculated their correlation with those of
 205 *SIG1*, *SIG2* and *SIG3* using pairwise Pearson correlations. It was not possible to
 206 obtain correlation data for tomato roots since only two expression values are
 207 available for each gene in the *TomExpress* database. GCN analyses are shown
 208 in Figure 3 and Figure S5, and positive significant correlations ($p \geq 0.55$) are listed
 209 in Table S3. The GCN analysis showed that *SIG1* is poorly co-expressed with the
 210 query genes (Figure 3). By contrast, *SIG2* and, to a lower extent, *SIG3* are highly
 211 connected to plastidial isoprenoid biosynthetic genes in green tissues.
 212 Connectivity was lower in fruit, and in this case it was a bit higher for *SIG3*
 213 (Figure 3). Remarkably, the majority of significant correlations occurs with genes
 214 of the carotenoid biosynthesis pathway in both vegetative and fruit tissues. These
 215 co-expression analyses confirm the importance of *SIG2* and *SIG3* in the tomato
 216 isoprenoid network and suggest that both isoforms are important to meet the
 217 demand of GGPP in chloroplasts and chromoplasts.

218 In tomato, carotenoids contribute to mycorrhizal associations, photoprotection,
 219 and fruit pigmentation and, hence, the levels of these GGPP-derived metabolites
 220 increase during root mycorrhization, seedling de-etiolation, and fruit ripening. In
 221 agreement with the rate-determining role of *PSY* for carotenoid synthesis (Fraser
 222 et al., 2002), the expression levels of *PSY*-encoding genes also increase during
 223 such carotenoid-demanding developmental processes. By using real-time
 224 quantitative PCR (qPCR) analysis, we experimentally confirmed the up-regulation
 225 of *PSY1* during fruit ripening and *PSY3* in mycorrhized roots (Figure 4).
 226 Furthermore, we found that the *PSY2* gene was more strongly upregulated than
 227 *PSY1* during tomato seedling de-etiolation (Figure 4). Using the same samples,
 228 we observed that only *SIG1* was upregulated during root mycorrhization, similarly
 229 to the expression pattern observed for *PSY3* (Figure 4). During fruit ripening,
 230 *SIG2* and, to a lower extent, *SIG3* were up-regulated, but not as much as *PSY1*

Figure 4. Expression profiles of genes encoding tomato PSY and GGPPS paralogs during processes involving increased carotenoid production. First column corresponds to non-mycorrhized (-) and mycorrhized roots (+) at 6 weeks post-inoculation. Transcript levels were normalized using the tomato *EXP* gene and are shown relative to untreated root samples. Central column samples correspond to 7-day-old dark-grown seedlings at 0, 6 and 24 h after exposure to light and to seedlings continuously grown in the light (L). Transcript levels were normalized to the *EXP* gene and are represented relative to etiolated (0 h) samples. Third column depicts different fruit ripening stages: MG, mature green; B, breaker; O, orange; and R, red ripe. Levels were normalized using *ACT4* and are shown relative to MG samples. Expression values represent the mean±SD of three independent biological replicates (n=3). Asterisks indicate statistically significant differences relative to untreated (-), etiolated (0 h) or MG samples (t-test or one-way ANOVA with Dunnett's multiple comparisons test, *p<0.05, **p<0.01).

(Figure 5). *SIG2* was also the most strongly upregulated GGPPS-encoding gene during seedling de-etiolation, paralleling *PSY2* induction. Interestingly, *SIG3* and *PSY1* were also induced with a similar profile during this process, even though induction levels were much lower than those observed for *SIG2* and *PSY2* (Figure 4). Together, these data suggest that *SIG1* might provide GGPP for *PSY3* to produce carotenoids in roots, particularly when needed during mycorrhization, whereas both *SIG2* and *SIG3* would be required in leaves and fruits to support carotenoid production for photosynthesis (mostly via *PSY2*) and fruit pigmentation (via *PSY1*).

SIG2, but not SIG3, can interact with PSY1 and PSY2

A coordinated role for *SIG1* and *PSY3* in mycorrhization has been already proposed (Stauder et al., 2018) but the possible connection between the other

Figure 5. Co-immunoprecipitation analyses. *N. benthamiana* leaves were co-agroinfiltrated with the indicated proteins tagged with C-terminal Myc (in blue) or HA (in red) epitopes. Controls agroinfiltrated only with the HA-tagged protein as indicated as (-). A fraction of the protein extracts (INPUT) was used to test protein production by immunoblot analyses using antibodies against Myc (αMyc) and HA (αHA). After immunoprecipitation (IP) of the remaining protein extracts using αMyc, samples were used for immunoblot analyses with αMyc (to confirm successful IP) and αHA (to detect the presence of co-immunoprecipitated HA-tagged proteins).

244 plastidial GGPPS and PSY isoforms remains unclear. GGPPS proteins can
 245 physically interact with PSY and other enzymes catalyzing both upstream and
 246 downstream biosynthetic steps in the plastids of different plant species (Dogbo
 247 and Camara, 1987; Camara, 1993; Maudinas et al., 1977a; Fraser et al., 2000;
 248 Ruiz-Sola et al., 2016a; Zhou et al., 2017; Camagna et al., 2019; Wang et al.,
 249 2018). This mechanism may facilitate channeling of precursors towards specific
 250 groups of plastidial isoprenoids. In particular, protein complexes containing both
 251 GGPPS and PSY enzymes were isolated from tomato chloroplasts and fruit
 252 chromoplasts (Maudinas et al., 1977a; Fraser et al., 2000). Nevertheless, the
 253 specific isoforms forming these protein complexes were never identified. Given
 254 the co-regulation of *SIG2* and *SIG3* with *PSY1* and *PSY2* genes in chloroplasts
 255 (i.e. photosynthetic tissues) and chromoplasts (i.e. fruits), we decided to test
 256 possible interactions of these isoforms (Figure 5). Constructs harbouring C-
 257 terminal Myc-tagged GGPPS and HA-tagged PSY sequences were combined
 258 and transiently co-expressed in *N. benthamiana* leaves. After 3 dpi, the
 259 recombinant *SIG2*-Myc and *SIG3*-Myc proteins were immunoprecipitated with
 260 αMyc antibody and the presence of *PSY1*-HA or *PSY2*-HA proteins in the

261 samples was detected by immunoblot analyses using α HA antibody. As negative
262 control, we used a Myc-tagged version of Arabidopsis phosphoribulokinase
263 (PRK-Myc), a stromal enzyme of the Calvin cycle. Using this approach, we found
264 that both PSY1-HA and PSY2-HA could be co-immunoprecipitated with SIG2-
265 Myc, suggesting that they are present in the same complexes *in vivo* (Figure 5).
266 By contrast, none of these PSY isoforms could be detected in the samples co-
267 immunoprecipitated with either SIG3-Myc or PRK-Myc. The same Myc-tagged
268 SIG2 and SIG3 proteins used in these experiments were able to co-
269 immunoprecipitate their HA-tagged counterparts (Figure 5). This result,
270 consistent with the ability of GGPPS proteins to form homodimers and also
271 heterodimers, confirms that the observed lack of interaction of SIG3 with PSY
272 enzymes was not due to SIG3-Myc having lost its capacity to interact with other
273 proteins.

274

275 **Loss of function mutants defective in SIG3, but not those impaired in SIG2,** 276 **show lower photosynthetic activity**

277 To further understand the biological roles of SIG2 and SIG3, we generated
278 CRISPR-Cas9 mutants defective in these enzymes (Figure 6). We designed two
279 single guide RNAs (sgRNA) for each gene with the aim of creating deletions
280 encompassing unique restriction sites for rapid screening (Figure 6A). Tomato
281 plants of the variety MicroTom (MT) were transformed via *A. tumefaciens*, and
282 two independent deletion alleles that created premature translation stop codons
283 were selected for each gene and named *slg2-1*, *slg2-2*, *slg3-1* and *slg3-2* (Figure
284 6A) (Figures S6-S8). To confirm that the truncated proteins lacked GGPPS
285 activity, we tested them in *E. coli* strains that synthesize the red carotenoid
286 lycopene only when a source of GGPP is supplied (Figure 6B). While colonies
287 harboring complete SIG2 or SIG3 enzymes produced lycopene, those
288 transformed with the truncated versions of the tomato proteins were unable to
289 synthesize GGPP for carotenoid biosynthesis (Figure 6B). Once confirmed that
290 the selected mutant alleles produced non-functional proteins, homozygous lines
291 without Cas9 were obtained and used for further experiments.

Figure 6. CRISPR-Cas9 mutagenesis of *SIG2* and *SIG3* genes. (A) Scheme representing the designed strategy to generate deletions on *SIG2* and *SIG3* genes and resulting proteins in selected mutant alleles (See Figures S6-S8 for further details). Green, pink and black boxes represent transit peptides, protein-protein interaction motifs, and catalytic domains (FARM and SARM), respectively. Blue arrowheads indicate the position of the designed sgRNAs encompassing specific restriction sites, and black arrows represent primer pairs used for genotyping. (B) Activity assays of WT and mutant GGPPS enzymes in *E. coli* strains expressing bacterial genes for lycopene biosynthesis (*crtB* and *crtI*) but lacking GGPPS activity. Lycopene production after transformation with constructs harboring the indicated sequences or empty plasmid controls (C) is shown relative to the levels obtained with the bona-fide GGPPS enzyme AtG11. Values represent the mean±SD of at least three independent transformants (n=3).

292 The most obvious phenotype among the selected lines was the pale color of *sg3*
 293 mutants compared to *sg2* alleles or azygous (WT) plants (Figure 7). This
 294 phenotype was clear in emerging and young leaves but it weakened as leaves
 295 grew and became mature (Figure 7A). The pale color correlated with significantly
 296 reduced levels of carotenoids and chlorophylls in young leaves of *sg3-1* and
 297 *sg3-2* lines compared to those of *sg2* mutant alleles, which exhibited WT levels
 298 of photosynthetic pigments (Figure 7B) (Table S4). Tocopherols, other GGPP-
 299 derived plastidial isoprenoids, were also reduced in young leaves from SIG3-
 300 defective plants compared to WT or *sg2* samples (Table S4). This change was
 301 statistically significant in the *sg3-2* allele (Figure 7B) but also when samples from
 302 both *sg3-1* and *sg3-2* alleles were considered as a whole (Figure 8). By
 303 contrast, similar levels of carotenoids, chlorophylls and tocopherols were
 304 detected in mature leaves of WT, *sg2* and *sg3* plants (Figure 7B) (Table S4).

305 To test whether the reduced accumulation of carotenoids and other
306 photosynthesis-related isoprenoids in *sig3* lines had an impact on
307 photosynthesis, we quantified effective quantum yield of photosystem II (Φ PSII) in
308 both young and mature leaves from WT and mutant plants (Figure 7C). A 30%
309 reduction in Φ PSII was observed in young leaves from *sig3* plants compared to
310 those of WT or *sig2* lines, consistent with the *sig3*-specific reduction of GGPP-

Figure 8. Metabolic changes in *slg2* and *slg3* mutants. Colors represent statistically significant fold-change (FC) values (t-test, $p < 0.05$) of metabolite levels in young leaves or ripe fruit (B+10) from mutant plants relative to those in WT controls. See Table S5 (leaves) and S7 (fruit) for quantitative data.

311 derived metabolites. Despite similar levels of photosynthetic pigments
 312 accumulated in the mature leaves of all genotypes tested, Φ PSII was slightly
 313 reduced in those from the *slg3* mutants relative to WT and *slg2* lines (Figure 7C).
 314 This result suggests that impaired production of photosynthesis-related
 315 isoprenoids in SIG3-defective young leaves might have an impact on

316 photosynthesis that lasts even after recovering normal pigment levels in mature
317 leaves.

318 We further explored possible effects that the loss of SIG2 or SIG3 function might
319 have on other metabolic pathways using the same samples of young leaves used
320 for isoprenoid and ϕ PSII determination (Figure 8) (Tables S5 and S6). GC-MS
321 metabolite profiling showed strongly decreased levels of sucrose, glucose and
322 fructose in SIG3-defective leaves, likely due to photosynthetic impairment. Mutant
323 *slg3* leaves also displayed increased levels of amino acids derived from glycerate
324 (Ser and Gly), shikimate (Phe, Trp and Tyr), pyruvate (Val, Ile and Ala), 2-
325 oxoglutarate (Glu, Orn, His and GABA) and malate (Asp, Asn, Lys, Thr, Met,
326 homoserine and beta-alanine). In line with some of these amino acid changes,
327 SIG3-defective leaves displayed altered accumulation of tricarboxylic acid cycle-
328 related intermediates (citrate and 2-oxoglutarate). Only a few common changes
329 were detected in both *slg2* and *slg3* leaves. They included a decrease in
330 putrescine and ascorbate levels (more pronounced in *slg3* leaves), as well as an
331 altered accumulation of metabolites produced by the plastidial shikimate
332 pathway, including the above mentioned aromatic amino acids and
333 phenylpropanoid derivatives such as caffeate and 3-caffeoyl-quinic acid (Figure 8)
334 (Tables S5 and S6). The levels of the carotenoid-derived hormone ABA were
335 similar in WT and mutant samples (Figure 8 and Table 2).

336

337 **Ripening-associated fruit pigmentation is altered in *slg2* and *slg3* mutants** 338 **in correlation with their carotenoid profile**

339 Lines with reduced levels of plastidial GGPPS activity also showed alterations in
340 reproductive development (Figure 9). Flowering time was similar in WT, *slg2* and
341 *slg3* plants (Figure 9A). However, pigmentation changes associated to fruit
342 ripening were visually delayed in mutant fruits (Figure 9B). Tomato fruits reach
343 their final size at the mature green (MG) stage and then start to ripe. The first
344 visual symptoms of ripening define the breaker (B) stage, when chlorophyll
345 degradation and carotenoid biosynthesis change the fruit color from green to
346 yellow (Figure 9C). As ripening advances, accumulation of orange and red
347 carotenoids (β -carotene and lycopene, respectively) progressively change the
348 fruit color and define the orange (O) and eventually red (R) stages (Figure 9C).

Figure 9. Flowering and fruit phenotypes of mutant lines defective in SIG2 or SIG3. (A) Flowering time measured as days after germination (left) or number of leaves (right). Values correspond to the mean $\pm$ SD of at least n=4 independent biological replicates. **(B)** Number of days to reach the indicated ripening stages represented as days post-anthesis on-vine (DPA, left) and days post-breaker off-vine (DPB, right). **(C)** Representative images of fruit from WT and mutant lines harvested at the breaker stage. **(D)** RT-qPCR analysis of *E8* and *ACS2* transcript levels in B+10 fruits of the indicated lines. Expression values were normalized using *ACT4* and represent the mean $\pm$ SD of n=3 independent biological replicates. **(E)** Relative levels of individual carotenoids (phytoene, lycopene and β -carotene) and total tocopherols in B+10 fruits of WT and mutant lines. Values are represented relative to those in WT samples and correspond to the mean $\pm$ SD of n=3 independent biological replicates. In all plots, asterisks indicate statistically significant differences among means relative to WT samples (one-way ANOVA with Dunnett's multiple comparisons test, *p<0.05, **p<0.01).

349 The time from anthesis to B was similar in WT and SIG2-defective fruits but it
 350 was longer in the *slg3-1* and *slg3-2* mutants (Figure 9B) (Figure S9). Fruits from
 351 lines defective in SIG3, but also those defective in SIG2, showed a pigmentation

352 delay in the transition from B to O. The delay was observed both on-vine (i.e. in
353 fruits attached to the plant) and off-vine (i.e. in fruits detached from the plant at
354 the B stage) (Figure 9B) (Figure S9). Both on-vine and off-vine measurements
355 revealed that *slg2* mutants also took longer to reach the R stage compared to WT
356 fruits (Figure S9), whereas *slg3* mutants did not reach a proper R stage as they
357 developed a dark-orange color when ripe and never turned fully red (Figure 9C).

358 We next analyzed the levels of individual carotenoids and other GGPP-derived
359 plastidial isoprenoids in fruits at MG (Figure S10) and B+10, i.e. 10 days after B
360 (Figures 8 and 9E) (Table S4). WT and mutant fruits showed similar levels of
361 carotenoids, chlorophylls and tocopherols at the MG stage, but clear differences
362 were detected in ripe (B+10) fruits. Phytoene and lycopene were significantly
363 decreased in all mutants, although the impact was higher in the case of *slg3*
364 fruits. No significant differences were found for β -carotene, although the levels of
365 this orange carotenoid tended to be higher in *slg3* mutants. This, together with
366 the lower levels of the red carotenoid lycopene may explain the dark orange color
367 of B+10 *slg3* fruits (Figure 9C). Tocopherols also showed a trend towards higher
368 abundance in *SIG3*-deficient fruits, a change that was statistically significant in
369 the *slg3-1* allele (Figure 9E) or when *slg3-1* and *slg3-2* samples were considered
370 together (Figure 8).

371 Unlike that observed in young leaves, ABA levels were reduced in B+10 fruits of
372 *slg2* and, most strongly, *slg3* mutants compared to WT controls (Figure 8 and
373 Table 2). A role for ABA in promoting tomato fruit ripening has been proposed
374 based on the analysis of mutants or external application of hormones and
375 inhibitors (Seymour et al., 2013; Quinet et al., 2019). While decreased ABA levels
376 in *slg2* and *slg3* fruits might be expected to result in slower ripening, analysis of
377 ripening marker genes such as *E8* and *ACS2* (Figure S4) (Estornell et al., 2009;
378 Llorente et al., 2016; D'Andrea et al., 2018) did not show a substantial ripening
379 delay in mutant fruits (Figure 9D). At the level of primary metabolites, B+10 fruits
380 from both *slg2* and *slg3* mutants exhibited increased levels of raffinose,
381 galacturonate, pyruvate and Asp, and lower levels of Ser, Gly, Tyr, Val, Ala, Glu
382 and GABA than WT controls (Figure 8) (Tables S7 and S8). The observation that
383 metabolite changes are very similar in both *slg2* and *slg3* fruits suggests that
384 *SIG2* and *SIG3* have redundant roles during fruit ripening. The strongest

385 decrease in carotenoids and derived ABA detected in the *slg3* mutant further
386 indicates that SIG3 likely has a more prominent role for the production of
387 ripening-related carotenoids.

388

389 **Double mutants defective in both SIG2 and SIG3 are not viable**

390 To assess the impact of simultaneous disruption of both *SIG2* and *SIG3* genes,
391 alleles *slg2-2* and *slg3-1* were crossed using the former as female parent and the
392 latter as male parent or vice versa. Double heterozygous F1 plants from each
393 cross were allowed to self-pollinate and the resulting seeds were used to screen
394 the F2 population for double homozygous plants, which were expected to occur
395 at a frequency of 6.25% (1 in 16). PCR genotyping of 200 F2 seedlings (100 from
396 each cross), however, found no double homozygous mutants (Table 3).
397 Interestingly, single azygous plants (i.e. *SIG2 slg2 slg3 slg3* and *slg2 slg2 SIG3*
398 *slg3*) were found at lower frequencies than expected (Table 3), suggesting a
399 gene dosage effect on the production of essential GGPP-derived isoprenoids.
400 Hence, the absence of both *SIG2* and *SIG3* appears to result in a lethal
401 phenotype that is partially rescued by incorporating one copy of any of these two
402 genes (as in single azygous plants) and fully rescued when two copies are
403 present in the genome (as in double heterozygous or single homozygous
404 mutants). These results, together with the similar expression levels of both genes
405 in developing tomato seeds (Figure S4), suggest that SIG2 and SIG3 contribute
406 similarly and additively to embryo or/and seed development.

407

408

409

410 **DISCUSSION**

411 GGPP synthesis and allocation to different downstream pathways in plants is
 412 controlled by GGPPS enzyme families, whose members are regulated at
 413 transcriptional and post-transcriptional level (Beck et al., 2013; Ruiz-Sola et al.,
 414 2016a, 2016b; Zhou et al., 2017; Stauder et al., 2018; Wang et al., 2018).
 415 Several efforts have been done since the late 90's to characterize the GGPPS
 416 protein family in Arabidopsis, establishing the fundamental basis for our
 417 knowledge of the regulation of GGPP biosynthesis in plants (Zhu et al., 1997a,
 418 1997b; Okada et al., 2000; Beck et al., 2013; Nagel et al., 2015; Ruiz-Sola et al.,
 419 2016a, 2016b; Wang et al., 2016b). In this model plant, two GGPPS paralogs
 420 (AtG2 and AtG11) have been shown to produce GGPP and to be targeted to
 421 plastids. However, only AtG11 appears to be required for the production of
 422 plastidial isoprenoids (Beck et al., 2013; Nagel et al., 2015; Ruiz-Sola et al.,
 423 2016a, 2016b). The gene encoding AtG11 is ubiquitously expressed at high
 424 levels and can generate long transcripts encoding the plastid-targeted isoform
 425 but also short transcripts encoding a cytosolic enzyme that retains enzymatic
 426 activity and is essential for embryo development (Ruiz-Sola et al., 2016b). The
 427 production of GGPP has also been studied in a few crop plants (Wang and
 428 Dixon, 2009; Zhang et al., 2015; Zhou et al., 2017; Wang et al., 2018, 2019).
 429 Similar to Arabidopsis, rice and pepper contain only one enzymatically active
 430 GGPPS isoform localized in plastids, named OsGGPPS1 (OsG1 in short) and
 431 CaGGPPS1 (CaG1), respectively (Zhou et al., 2017; Wang et al., 2018).
 432 Strikingly, little information is available on the tomato GGPPS family despite this
 433 species being a well-established model plant that accumulates high amounts of
 434 GGPP-derived metabolites of human interest such as carotenoids in fruits. Here
 435 we have gathered information on the tomato GGPPS family, providing evidence
 436 of a coordinated role of two plastidial isoforms (SIG2 and SIG3) on supplying
 437 GGPP to produce carotenoids and other isoprenoids essential for
 438 photosynthesis, fruit pigmentation, and seed viability.

439 All the GGPPS paralogs identified here (SIG1-5) harbored the seven domains
 440 known to be highly conserved in SC-PTs (Koike-Takeshita et al., 1995) and all
 441 other features known to be required for GGPPS activity (Figure S1). Moreover,
 442 they were differentially located in plastids, cytosol and mitochondria (Figure 2A),

consistent with the need of GGPPS isozymes in these cell compartments to feed the different organelle-specific isoprenoid pathways (Bick and Lange, 2003; Beck et al., 2013; Ruiz-Sola et al., 2016a, 2016b; Zhou et al., 2017; Wang et al., 2018). We confirmed that the three plastid-targeted GGPPS homologs (SIG1-3) produce GGPP with similar kinetic parameters (Figure 2B and Table 1) and an optimal pH around 7.5 (Figure S3), similar to the stromal pH (Höhner et al., 2016). SIG5-GFP was targeted to mitochondria, whereas the GFP-fused SIG4 protein exhibited a major preference for cytosolic location but it was also observed in the chloroplasts of some *N. benthamiana* cells (Figure 2A) (Figure S2). Further illustrating a complex localization pattern for the SIG4 protein, fusion of its N-terminal sequence to GFP generated a mitochondrial fluorescence pattern when expressed in Arabidopsis protoplasts (Zhou and Pichersky, 2020). Multiple subcellular localization of many isoprenoid biosynthetic enzymes has been widely described (Cunillera et al., 1997; Phillips et al., 2008; Sapir-Mir et al., 2008; Ruiz-Sola et al., 2016b). In many cases, including Arabidopsis *AtG11* (Ruiz-Sola et al., 2016b), the production of two differentially-targeted proteins from the same gene is due to a weak transcription initiation site on the corresponding gene, giving rise to some transcripts that lack the first ATG codon of the coding sequence. Thus, translation starts from a second in-frame ATG codon producing a shorter protein lacking the N-terminal region (which usually corresponds to the organelle-specific transit peptide). A similar transcriptional regulation could generate long versions of the SIG4 protein that would be targeted to chloroplasts or mitochondria, as well as short versions that would be retained in the cytosol. Despite the clear plastidial localization observed here (Figure 2A) and elsewhere (Zhou and Pichersky, 2020) for GFP fusions of the SIG1-3 isoforms, we cannot exclude that shorter versions of these proteins could also be produced *in vivo*, paralleling that observed in the case of *AtG11* (Ruiz-Sola et al., 2016b). This possibility is strongly supported by the observation that no double mutants defective in both SIG2 and SIG3 could be identified (Table 3), suggesting that the loss of these two GGPPS isoforms might cause an embryo-lethal phenotype similar to that reported in Arabidopsis mutants lacking the short (i.e. cytosolic) version of *AtG11* (Ruiz-Sola et al., 2016b). Indeed, several M residues can be found in the N-terminal region of both SIG2 and SIG3 enzymes (Figure S8); they could be used

476 as alternative translation start sites to produce catalytically active GGPPS
477 enzymes with an absent or shorter (i.e. dysfunctional) plastid-targeting domain.

478 SIG1-3 are the only GGPPS-related sequences in tomato shown to actually
479 produce GGPP as their main product (Figure 2B) (Zhou and Pichersky, 2020).

480 The presence of these three GGPPS isoforms in tomato plastids may be caused
481 by the acquisition of specific roles during evolution (subfunctionalization).

482 Besides localization in distinct subcellular compartments, new functions can be
483 acquired either through a differential spatio-temporal gene expression, triggered
484 by developmental and environmental signals, or through specific interactions with
485 other proteins. Mining of public tomato gene expression databases, GCN
486 analyses and qPCR assays led us to conclude that SIG1 is likely contributing to
487 carotenoid biosynthesis in roots together with PSY3. This conclusion is supported
488 by a recent study showing that the expression of *PSY3* and *SIG1* coordinately
489 responds to tomato root mycorrhization and phosphate starvation (Stauder et al.,
490 2018). The SIG1-PSY3 tandem might be channeling the flux of MEP-derived
491 precursors towards the synthesis of carotenoid-derived molecules that are crucial
492 for the establishment of symbiosis, such as strigolactones and apocarotenoids,
493 including C13 α -ionol and C14 mycorradicin (Stauder et al., 2018). The
494 observation that *SIG1* was significantly co-expressed with genes from the ABA
495 synthesis pathway in vegetative and fruit tissues (Figure 3) is consistent with a
496 possible involvement of this GGPPS isoform in the production of ABA under
497 certain environmental conditions. *SIG1* expression was also found to be induced
498 in tomato leaves after herbivore-feeding or treatment with jasmonates, correlating
499 with an increase in the emissions of GGPP-derived volatile isoprenoids (Ament et
500 al., 2006). It is therefore possible that this isoform specializes in the formation of
501 GGPP in response to biotic interactions not only in roots, but also in other plant
502 tissues such as leaves.

503 Unlike *SIG1*, *SIG2* and *SIG3* are constitutively expressed, with *SIG3* being the
504 paralog with the highest expression level in all plant tissues (Figure S4). In green
505 tissues, *SIG2* is more strongly co-expressed than *SIG3* with genes from
506 photosynthesis-related isoprenoid pathways, exhibiting higher correlations than
507 with genes involved in carotenoid biosynthesis (Figure 3). Additionally, *SIG2* was
508 much more upregulated than *SIG3* during seedling de-etiolation (Figure 4) and

509 leaf development (Figure S4), when an enhanced production of carotenoids and
 510 other photosynthesis-related isoprenoids contributes to assemble a functional
 511 photosynthetic machinery. *S/G2* was also much more induced than *S/G3* during
 512 fruit ripening, when carotenoid biosynthesis is boosted. The production of
 513 carotenoids during ripening relies on the *PSY1* isoform, whose strong induction
 514 during fruit ripening has been recently explained as a probable consequence of
 515 its poor enzyme activity (Cao et al., 2019). While we did not find a significant
 516 correlation of *S/G2* or *S/G3* with *PSY1* expression in fruits, *PSY1* and *S/G2*, but
 517 not *S/G3*, are coordinately regulated by FUL and RIN transcription factors that
 518 control the expression of ripening-related genes, including many of the MEP and
 519 carotenoid pathways (Fujisawa et al., 2013, 2014). All these expression data
 520 show that *S/G2* expression is more responsive to increased demands of
 521 precursors to support carotenoid biosynthesis. In contrast, *S/G3* expression is
 522 higher and does not change so much, suggesting a house-keeping role to
 523 maintain a continuous supply of GGPP in plastids for carotenoids and other
 524 isoprenoids. According to this model, *SIG1* and *SIG2* would help *SIG3* to supply
 525 GGPP when needed. The very low and restricted expression level of *SIG1*,
 526 however, strongly suggests that *SIG2* is the main helper isoform for *SIG3* in
 527 chloroplasts and chromoplasts.

528 Besides diverging gene expression profiles, subfunctionalization of GGPPS
 529 paralogs might also involve isoform-specific interactions with other proteins. The
 530 interaction of different plant GGPPS enzymes with other proteins involved in
 531 isoprenoid biosynthesis has been demonstrated through different protein-protein
 532 interaction approaches (Dogbo and Camara, 1987; Camara, 1993; Maudinas et
 533 al., 1977b; Fraser et al., 2000; Ruiz-Sola et al., 2016a; Zhou et al., 2017;
 534 Camagna et al., 2019; Wang et al., 2018). The enzymatic properties of GGPPS
 535 proteins change to produce GPP upon heterodimerization with members of the
 536 GPP synthase small subunit type I (SSU-I) subfamily (Orlova et al., 2009; Wang
 537 and Dixon, 2009). Thus, the tomato SSU-I protein (Soly07g064660) interacts
 538 with *SIG1-3* enzymes in plastids to change their product specificity from GGPP to
 539 predominantly GPP (Zhou and Pichersky, 2020). Other protein-protein
 540 interactions forming multienzymatic complexes appear to be particularly
 541 important for metabolic channeling of GGPP into the different pathways that

consume it, as its high amphipathicity makes it unlikely that GGPP could diffuse in monodisperse form (Camagna et al., 2019). In particular, spatial proximity of GGPPS and PSY enzymes appears to be a precondition for efficient phytoene synthesis, because PSY cannot access freely diffusible GGPP or time-displaced GGPP supply by GGPPS (Camagna et al., 2019). Arabidopsis AtG11 and pepper CaG1 can directly interact with PSY proteins (Ruiz-Sola et al., 2016a; Camagna et al., 2019; Wang et al., 2018). We found that tomato SIG2, but not SIG3, is able to interact with PSY1 and PSY2 *in planta* (Figure 5). However, tomato SIG3 might deliver GGPP to PSY enzymes via heterodimerization with PSY-interacting SIG2 (Figure 5). An alternative possibility involves interaction with members of another catalytically-inactive SSU subfamily named type II (SSU-II). Similar to AtG11 and CaG1, OsG1 is the only GGPPS enzyme producing GGPP for carotenoid biosynthesis in rice. Strikingly, OsG1 does not interact with PSY but heterodimerizes with a SSU-II homolog named GGPPS Recruiting Protein, OsGRP (Zhou et al., 2017). While binding of SSU-I proteins changes the product specificity of GGPPS enzymes from GGPP to GPP (Orlova et al., 2009; Wang and Dixon, 2009; Zhou and Pichersky, 2020), interaction with the SSU-II protein OsGRP enhanced OsG1 catalytic efficiency to produce GGPP. Additionally, OsGRP binding delivered the OsG1 protein to a large protein complex in thylakoid membranes with enzymes involved in chlorophyll biosynthesis (Zhou et al., 2017). A plastid-targeted and catalytically inactive SSU-II protein was also shown to interact with pepper CaG1 and enhance its GGPP-producing activity (Wang et al., 2018). Interestingly, the pepper SSU-II protein also interacts with PSY, suggesting that it might stimulate both GGPPS activity and interaction with PSY (Wang et al., 2018). The tomato SSU-II homolog (Soly09g008920) is an ubiquitously expressed and catalytically inactive protein targeted to plastids, as its rice and pepper counterparts (Zhou and Pichersky, 2020). In agreement with the conclusion that tomato, pepper and rice SSU-II homologs share similar functions, heterodimers formed between tomato SSU II and SIG1-3 catalyzed the formation of GGPP with improved efficiency (Zhou and Pichersky, 2020). It is possible that SIG3 might be delivered to PSY-containing protein complexes through interaction with tomato SSU-II. This protein might also enhance interaction of SIG2 with PSY isoforms, similar to that proposed for PSY-interacting CaG1 (Wang et al., 2018). A role for SSU-II in promoting interaction

576 between GGPPS and PSY enzymes might be key to enhance carotenoid
577 biosynthesis in plants.

578 The biochemical characterization of purified SIG2 and SIG3 enzymes together
579 with the phenotypes of *slg2* and *slg3* mutants supported the conclusion that
580 these are very similar enzymes with functionally interchangeable functions whose
581 differential role as helper or house-keeping is basically determined by their
582 expression pattern. Analysis of single tomato mutants defective in either SIG2 or
583 SIG3 suggested that absence of any of the two individual enzymes decreases
584 GGPP production and hence delays the production of photosynthetic isoprenoids
585 in leaves (Figure 7) and carotenoids in fruits (Figure 9). Consistent with the
586 higher expression levels, these phenotypes were stronger in *slg3* alleles.
587 However, the effects of reduced isoprenoid synthesis could also be directly or
588 indirectly detected in *slg2* plants. Thus, a carotenoid-associated pigmentation
589 delay was more subtle but still detected in ripening fruit from *slg2* alleles (Figure
590 9). Our HPLC analysis could not detect reduced levels of carotenoids or any
591 other GGPP-derived isoprenoid in SIG2-defective leaves but the GC-MS analysis
592 showed higher levels of all aromatic amino acids derived from the shikimate
593 pathway (Trp, Tyr and Phe) as well as Phe-derived phenylpropanoids caffeate
594 (caffeic acid) and 3-caffeoyl-quinic acid (chlorogenic acid) in both *slg2* and *slg3*
595 mutant lines (Figure 8). This might be a physiological response to cope with
596 photooxidative stress caused by lower levels of carotenoids in the mutants, as
597 phenylpropanoids (including Phe-derived flavonoids and anthocyanins) can also
598 function as photoprotective metabolites (Muñoz and Munné-Bosch, 2018).
599 Reduced levels of well-known metabolites associated with oxidative stress such
600 as ascorbate and putrescine in leaves from both mutant lines would also support
601 this view. When levels of GGPP-derived photoprotective isoprenoids such as
602 carotenoids and tocopherols fell below a certain threshold (and hence the
603 decrease became detectable, as in young leaves of the SIG3-defective alleles),
604 photosynthesis was eventually impacted (Figure 7C), causing sugar starvation
605 and the subsequent metabolic changes observed only in the *slg3* mutant (Figure
606 8) (Tables S5 and S6). In agreement, the increased accumulation of most amino
607 acids in *slg3* leaves suggested a high proteolytic activity to generate an
608 alternative respiratory source, a likely response to sugar starvation derived from

609 reduced photosynthesis and/or photooxidative stress (Araújo et al., 2011; Obata
610 and Fernie, 2012; Galili et al., 2016). In ripe (i.e. non-photosynthetic) fruits,
611 increased levels of 3-caffeoyl-quinic acid, citrate and tocopherols were detected only
612 in *slg3* samples. The rest of metabolic changes detected in fruits were also
613 similar in *slg2* and *slg3* lines (Figure 8) (Tables S7 and S8), again supporting the
614 conclusion that these enzymes are redundant and interchangeable.

615 Because ABA is synthesized from carotenoids, its reduced levels in GGPPS-
616 defective ripe fruits but not in leaves (Table 2) may be the result of a more
617 substantial reduction in carotenoid contents in mutant fruit (Figure 9) compared to
618 leaves (Figure 7) (Table S4). When the metabolic profile of ABA-defective B+10
619 fruit from *slg2* and *slg3* lines was compared with that of ripe fruit from other
620 tomato lines with altered ABA levels, no correlation was found. For example, Glu
621 and GABA were downregulated in our samples (Figure 8) (Tables S7 and S8)
622 and in transgenic lines in which a constitutively silenced β -carotene desaturase
623 (ZDS) prevents transformation of phytoene into lycopene and hence results in
624 reduced ABA levels (McQuinn et al., 2020), but also in ABA-overaccumulating
625 transgenic fruits with a constitutively expressed lycopene β -cyclase (LCYB) that
626 converts lycopene into β -carotene (Diretto et al., 2020). Asp increased in ripe
627 fruits with reduced ABA levels either by decreasing plastidial GGPPS (Figure 8)
628 or ZDS activity (McQuinn et al., 2020) while it decreased in those with higher
629 ABA levels (Diretto et al., 2020). By contrast, levels of Ser, Gly, Tyr, Val and Ala
630 were decreased in ABA-defective *slg2* and *slg3* mutants (Figure 8) and ABA-
631 accumulating LCYB overexpressors (Diretto et al., 2020) but increased in ZDS-
632 silenced lines with lower ABA levels (McQuinn et al., 2020). Together, the results
633 indicate that most of the observed amino acid changes in these mutant and
634 transgenic fruits cannot be explained by altered ABA levels. Although reduced
635 ABA might delay ripening (Seymour et al., 2013; Quinet et al., 2019), a slower
636 production of carotenoids in GGPP-depleted mutant fruit could explain by itself
637 the longer time required for these fruits to advance in color-based ripening
638 phases (Figure 9B). Additionally, metabolic roles of SIG2 and SIG3 besides their
639 GGPPS activity in plastids might play a role in fruits but also in developing seeds,
640 hence explaining why we could not isolate a double *slg2 slg3* mutant (Table 3). If
641 SIG2 and SIG3 play the same role in tomato that AtG11 plays in Arabidopsis, as

642 it appears to be the case based on the data discussed above, it would be
643 expected that the tomato double mutant was not rescued because an embryo-
644 lethal phenotype results from an impaired activity of these enzymes in the cytosol
645 (Ruiz-Sola et al., 2016b). Further experimental evidence would be required to
646 confirm whether the use of alternative ATG codons to produce shorter (i.e.
647 cytosolic) versions of otherwise plastidial GGPPS enzymes is a general
648 mechanism in plants, and also to identify what is the product essential for embryo
649 development supplied by the cytosolic enzymes (either alone or in combination
650 with interacting proteins). In any case, the observation that the lethal phenotype
651 is dose-dependent in an isoform-independent fashion (i.e. can be rescued by a
652 single genomic copy of either *SIG2* or *SIG3*) reinforces our conclusion that *SIG2*
653 and *SIG3* are enzymes with functionally interchangeable roles.

654 In summary, this work demonstrates that the bulk of GGPP production in tomato
655 leaf chloroplasts and fruit chromoplasts relies on two redundant but cooperating
656 GGPPS paralogs, *SIG2* and *SIG3*. Additionally, the *SIG1* isoform might contribute
657 to GGPP synthesis in root plastids. This scenario contrasts with that described to
658 date in other plant species such as *Arabidopsis thaliana*, rice or pepper, which
659 produce their essential plastidial isoprenoids using a single GGPPS isoform.
660 Deciphering how different plants regulate plastidial GGPP production and
661 channeling will be useful for future metabolic engineering approaches targeted to
662 manipulate the accumulation of specific groups of GGPP-derived isoprenoids
663 without negatively impacting the levels of others. In the case of tomato
664 carotenoids, understanding how GGPPS and PSY isoform pairs interact should
665 allow to specifically improve the nutritional quality of fruits without interfering with
666 other vital processes such as photoprotection, mycorrhization, or stress
667 tolerance.

668

669

670 **MATERIALS AND METHODS**

671 **Plant material and growth conditions**

672 Tomato (*Solanum lycopersicum* var. MicroTom) plants were used for most
673 experiments. Seeds were surface-sterilized by a 15 min incubation in 25 mL of

674 40% bleach containing a drop of Tween-20 followed by 3 consecutive 10 min
675 washes with sterilized milli-Q water. Sterile seeds were germinated on plates with
676 solid 0.5x Murashige and Skoog medium without vitamins or sucrose. The
677 medium was supplemented with kanamycin (100 µg/mL) when required to select
678 transgenic plants. After stratification at 4 °C in the dark for at least 3 days, plates
679 were incubated in a climate-controlled growth chamber at 24 °C with a
680 photoperiod of 10 h of darkness for 14 h of fluorescent white light at a
681 photosynthetic photon flux density of 140 µmol m⁻² s⁻¹. After 1 to 2 weeks, old
682 seedlings were then transferred to soil and grown under standard greenhouse
683 conditions (14 h light at 27 ± 1 °C and 10 h dark at 22 ± 1 °C). For the analysis of
684 flowering time, at least five independent plants of each genotype were used.
685 Flowering time was assessed by counting the number of days from germination
686 until the first flower was fully opened (anthesis) or the number of leaves in the
687 plant at this first anthesis day.

688 For deetiolation experiments, seeds were sown on sterile water-soaked cotton in
689 plastic containers. After stratification, seeds were exposed to fluorescent white
690 light for 2-4 hours at 22°C to induce germination. The containers were then
691 covered with a double layer of aluminum foil and kept in darkness at 22 °C. After
692 one week, seedlings were exposed to light and samples were harvested after 0,
693 6 and 24 h. Control samples were germinated and grown under continuous light
694 and collected at the 0 h time point. Leaf samples were collected from four-week-
695 old plants. Young leaf samples correspond to growing leaflets from the fifth and
696 sixth true leaves, and mature leaf samples correspond to fully expanded leaflets
697 from the third or fourth leaf. Tomato fruit pericarp samples were collected at four
698 ripening stages based on days post-anthesis (DPA) or days post-breaker (DPB):
699 mature green (~30 DPA), breaker (~35 DPA), orange (~38-40 DPA) and red
700 (~45-50 DPA or 10 DPB). Full seedlings, leaflets, and pericarp samples were
701 frozen in liquid nitrogen immediately after collection, freeze-dried and stored at -
702 80 °C. *Nicotiana benthamiana* plants were grown and used for transient
703 expression assays (agroinfiltration) as previously described (Llorente et al.,
704 2019).

705

706 **Constructs**

707 Full-length cDNAs encoding SIG1-5 and PSY1-2 proteins without their stop
708 codons were amplified by PCR and cloned via BP clonase into pDONR207 entry
709 plasmid using Gateway (GW) technology (Invitrogen). Full-length sequences
710 were then subcloned through an LR reaction into pGWB405 plasmid for
711 subcellular localization assays, or into pGWB414 and pGWB420 plasmids for co-
712 immunoprecipitation experiments. Constructs in pGWB405, pGWB414 and
713 pGWB420 vectors harbor GFP, 3x-HA and 10x-Myc tags, respectively. These tag
714 sequences are fused to the C-terminus of each cloned element and the
715 expression module is controlled by the CaMV 35S promoter. For recombinant
716 protein production in *E. coli*, SIG1-3 versions lacking the predicted transit peptide
717 for plastid import were amplified from pGWB405 constructs, cloned into
718 pDONR207 plasmid, and then subcloned into pET32-GW plasmid (fusing a 6x-
719 His tag at the N-terminal end of the cloned fragments) under the control of the T7
720 promoter. pET32-GW constructs containing Arabidopsis AtG11 versions are
721 described in (Ruiz-Sola et al., 2016b).

722 For CRISPR-Cas9-mediated disruption of *SIG2* and *SIG3*, two single guide RNAs
723 (sgRNA) were designed for each gene to create short deletions using the
724 CRISPR P 2.0 online tool (<http://crispr.hzau.edu.cn/CRISPR2/>) (Liu et al., 2017).
725 The selected sgRNA sequences encompass an *EcoRI* and a *PstI* restriction site
726 for *SIG2* and *SIG3* genes, respectively (Figures S6 and S7). The cloning of the
727 sgRNA sequences was performed as described (Schiml et al., 2016) but using a
728 pDE-Cas9 plasmid providing kanamycin resistance. A pair of primers for each
729 guide was designed, denaturalized and assembled into pENC1.1 (pENTRY)
730 vector previously digested with *BbsI*. The entry vectors contained the
731 corresponding sgRNA expression cassette flanked by *Bsu36I* and *MluI* restriction
732 sites, and by GW recombinant sites to allow both types of interchange with the
733 pDE-Cas9 vector (pDESTINY). The final binary vectors were generated in a two-
734 step cloning process that involved *Bsu36I* and *MluI* digestion-ligation of the first
735 sgRNA into the pDE-Cas9 vector followed by an LR reaction to subclone the
736 second sgRNA of each gene into the pDE-Cas9 vector already containing the
737 first sgRNA.

738 For activity assays in *E. coli*, full-length *SIG2*, *SIG3*, *slg2-1*, *slg2-2*, *slg3-1* and
739 *slg3-2* sequences were amplified from genomic DNA of the corresponding lines

740 and cloned into the *Sma*I site of the pBluescript SK+ plasmid. All constructs were
741 confirmed by restriction mapping and DNA sequencing. Information about
742 primers used and cloning details are described in Tables S9 and S10,
743 respectively.

744

745 **Phylogenetic analyses**

746 Arabidopsis GGPPS and GFPP protein sequences were retrieved from *The*
747 *Arabidopsis Information Resource* (<https://www.arabidopsis.org/>) and used as
748 queries to search for tomato homologs on the *Solanaceae Genomics Network*
749 (<https://solgenomics.net/>) and the *National Center for Biotechnology Information*
750 websites (<https://www.ncbi.nlm.nih.gov/>) using BLAST. The accession numbers
751 of the identified homologs are listed in Table S1. The presence of transit peptides
752 was predicted using ChloroP and TargetP algorithms (Emanuelsson et al., 2007).
753 Protein and DNA alignments of Arabidopsis and tomato sequences were
754 performed using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) with
755 default settings (Sievers et al., 2011; Sievers and Higgins, 2014). An unrooted
756 phylogenetic tree of Arabidopsis and tomato GGPPS and GFPPS protein
757 sequences was constructed using MEGA6 (Hall, 2013; Tamura et al., 2013).
758 Evolutionary connections among proteins were predicted by the Neighbor-Joining
759 method based on the Poisson model, where pairwise deletion was selected for
760 gap deletions. Bootstrapping was performed on 2,000 pseudoreplicates.

761

762 **Subcellular localization assays**

763 Subcellular localization assays were performed by *Agrobacterium tumefaciens*-
764 mediated transient expression in *N. benthamiana* leaves (Sparkes et al., 2006).
765 *A. tumefaciens* GV3101 strains were transformed with pGWB405-based
766 constructs (Table S10) and grown on LB plates at 28 °C for 3 days. A single
767 PCR-confirmed colony per construct was grown overnight at 28 °C in 5 mL
768 antibiotic-supplemented LB media and 500 µL of the grown culture were then
769 inoculated in 20 mL of fresh medium. After another overnight incubation, bacterial
770 cells were pelleted and resuspended in infiltration buffer (10 mM MES pH5.5-6,
771 10 mM MgSO₄, 150 µM acetosyringone) to a final OD₆₀₀ of 0.5. To prevent

772 silencing, leaves were co-infiltrated with an *Agrobacterium* strain harboring a HC-
773 Pro silencing suppressor (Goytia et al., 2006). A 1:1 mixture of the two cultures
774 was infiltrated with a syringe in the abaxial part of leaves from 4 to 6-week old *N.*
775 *benthamiana* plants. Subcellular localization of GFP fusion proteins was
776 determined three days post-infiltration with an Olympus FV 1000 confocal laser-
777 scanning microscope. GFP signal and chlorophyll autofluorescence were
778 detected using an argon laser for excitation (at 488 nm) and a 500–510 nm filter
779 (for GFP) or a 610–700 nm filter (for chlorophyll). All images were acquired using
780 the same confocal parameters.

781

782 **GGPPS activity determination**

783 Constructs to produce different truncated GGPPS protein versions were
784 generated in the pET32-GW vector (Table S10). Competent *E. coli Rosetta 2*
785 (DE3) cells (Novagen) were separately transformed with each construct and
786 single transformants were grown overnight at 37 °C in 5 mL of LB medium
787 supplemented with appropriate antibiotics. Then, 250 µL of each overnight
788 culture were diluted in 25 mL 2xYT medium with the required antibiotics and
789 incubated at 37 °C and 250 rpm until reaching an OD600 between 0.5 and 0.8.
790 After inducing the production of the recombinant proteins with 1 mM IPTG, the
791 cultures were grown overnight at 18 °C and 250 rpm. Bacterial cells were
792 harvested by centrifugation at 3,400 rpm for 15 min and pellets were
793 resuspended in 1 mL Assay buffer (15 mM MOPSO, 12.5% v/v glycerol, 1 mM
794 ascorbic acid, pH 7.0, 1 mM MgCl₂, 2 mM DTT). About 0.2 g of zirconium/silica
795 beads 0.1 mm (BioSpec Products) were added and bacterial lysis was carried out
796 in two rounds of shaking for 10 s at a speed of 6.5 in a FastPrep machine (FP120
797 Bio101 Savant). Cell lysates were subsequently centrifuged for 10 min at 13,000
798 g and 4 °C, and supernatants were collected for SDS-PAGE and GGPP activity
799 assays. Enzymatic assays were performed in Eppendorf tubes in a final volume
800 of 200 µL containing 25 µL of cell extract, 150 µM IPP and 50 µM DMAPP in
801 Assay buffer supplemented with 5 mM Na₃O₄V. The reaction mix was incubated
802 for 2 h at 30 °C in mild agitation and stopped by adding 800 µL of 100%
803 methanol / 0.5% formic acid. After vortexing, samples were sonicated for 15 min
804 and centrifuged at maximum speed for 10 min. Supernatants were then

805 evaporated in a SpeedVac concentrator and 80 μ L of 100% methanol / 0.65%
806 formic acid were added to the remnant sample. After centrifugation at maximum
807 speed for 15 min, the supernatants were transferred to glass vials. The detection
808 of prenyl diphosphate products by Liquid Chromatography-Mass Spectrometry
809 (LC-MS) was carried out as described (Ruiz-Sola et al., 2016b). Data acquisition
810 and visualization was performed using XcaliburTM software (ThermoFischer
811 Scientific). Kinetic parameters were calculated as detailed (Barja and Rodríguez-
812 Concepción, 2020) using 3 μ g of purified SIG1, SIG2, SIG3 and AtG11 enzymes.
813 pET32 constructs were used to produce 6xHis-tagged recombinant enzymes
814 (Table S10) and protein purification from *E. coli* Rosetta cells was carried out
815 using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) as described in Barja
816 and Rodríguez-Concepción (2020). Protein concentration was determined
817 according to Bradford method (Bradford, 1976). IPP and DMAPP substrates and
818 FPP and GGPP standards were obtained from Echelon Biosciences. Activity
819 assays in *E. coli* were carried out as described (Beck et al., 2013).

820

821 **Gene Co-expression Network (GCN) analyses**

822 GCN analyses were performed as previously described (Ahrazem et al., 2018).
823 Briefly, an ad hoc list of tomato GGPPS and plastidial isoprenoid biosynthetic
824 genes was compiled (Table S2), and used to retrieve all the expression data
825 available for different tomato cultivars/tissues/treatments in the TomExpress
826 database (Maza et al., 2013). Subsequently, pairwise Pearson correlations
827 between each GGPPS gene and each selected isoprenoid biosynthetic input
828 gene was computed for vegetative and fruit tissues and Fisher's Z-transformation
829 was used to test the statistical significance of pairwise correlations. Finally,
830 pairwise correlations were used to build a heatmap and positive significant
831 correlations ($\rho \geq 0.55$) were specifically used to draw gene co-expression networks
832 (Table S3).

833

834 **RT-qPCR analyses**

835 Total RNA was isolated from tomato freeze-dried tissue (seedlings, leaves or fruit
836 pericarp) using the Maxwell[®] RSC Plant RNA Kit with the Maxwell[®] RSC

Instruments (Promega) following the manufacturer's instructions. RNA was quantified using a NanoDropTM 8000 spectrophotometer (ThermoFischer Scientific) and checked for integrity by agarose gel electrophoresis. The Transcriptor First Strand cDNA Synthesis Kit (Roche) was used to reverse transcribe 0.5 µg of extracted RNA into 20 µL of cDNA, which was subsequently diluted ten-fold and stored at -20 °C for further analysis. Relative mRNA abundance was evaluated via Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) in a reaction volume of 20 µL containing 10 µL of the LightCycler 480 SYBR Green I Master Mix (Roche), 0.3 µM of each specific forward and reverse primer (Table S9) and 5 µL of cDNA. The RT-qPCR was carried out on a LightCycler 480 Real-Time PCR System (Roche). Three independent biological replicates of each condition and at least two technical replicates of each biological replicate were performed. Normalized transcript abundances were calculated as described previously (Simon, 2003) using tomato *ACT4* (*Solyc04g011500*) or *EXP* (*Solyc07g025390*) as endogenous reference genes. Primer efficiencies were calculated using serial dilutions of genomic or plasmidic DNA. Three biological replicates of cDNA samples from roots of non-mycorrhized and mycorrhized tomato plants (Ruiz-Lozano et al., 2016) were kindly provided by Dr. Juan Antonio López-Ráez.

856

857 **Co-immunoprecipitation and immunoblot analyses**

Co-immunoprecipitation (Co-IP) assays were performed by transient expression assays in *N. benthamiana* leaves via *A. tumefaciens* as described (Muñoz and Castellano, 2018). Constructs encoding Myc- and HA-tagged tomato GGPPS and PSY proteins (Table S10) were transformed into *A. tumefaciens* GV3101 strains as detailed above. A plasmid containing the Arabidopsis phosphoribulokinase protein with a Myc tag (pGWB417_AtPRK-Myc) was kindly provided by Dr. Ernesto Llamas and used as a negative control. Different *Agrobacterium* infiltration mixtures were prepared and infiltrated into *N. benthamiana* leaves as described (Muñoz and Castellano, 2018). After 3 days, 1.2 g of leaf tissue infiltrated with the same *Agrobacterium* mixtures were frozen in liquid nitrogen and directly stored at -80 °C until use. For crude extracts preparation, frozen leaf samples were ground in liquid nitrogen and incubated in

870 4 ml lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 5% glycerol, 0.05% NP-
871 40, 1 mM MgCl₂, 0.5 mM PMSF, 1X Sigma protease inhibitor, 10 mM DTT, 2%
872 PVPP) at 4 °C for 15 min using a rotator to form a homogeneous suspension,
873 that was then pre-clarified at 3,000 g for 15 min. Supernatants were cleaned by
874 centrifugation at 16,000 g for 30 min and used for protein quantification
875 (Bradford, 1976). Crude extracts were then adjusted to the same volume and
876 protein concentration with lysis buffer lacking PVPP. An aliquot of each adjusted
877 crude extract was boiled for 10 min in SDS-loading buffer and stored at -20 °C as
878 input sample. 500 µL of each crude extract were incubated overnight with 1 µL of
879 monoclonal αMyc antibody (Sigma) in a rotator at 4 °C. Immunoprecipitation of
880 αMyc interacting protein/complexes was carried out using Pierce Protein A/G
881 Magnetic Beads (ThermoFischer Scientific). After pre-washing the magnetic
882 beads (50 mM Tris-HCl pH7.5, 500 mM NaCl, 5% glycerol, 0.05% NP-40, 1 mM
883 MgCl₂, 0.5 mM PMSF, 1X Sigma protease inhibitor, 10 mM DTT), the Co-IP
884 sample (crude extract + αMyc antibody) was added and incubated with the beads
885 at room temperature for 1 h with shaking. The beads were then collected with a
886 magnetic stand and repeatedly washed with washing buffer and water. After
887 removing the water from the last washing step, 100 µL of SDS-PAGE loading
888 buffer were added to the beads and boiled for 10 min. Afterwards, the beads
889 were magnetically removed from the supernatants containing the
890 immunoprecipitated complexes and stored at -20 °C. The presence of Myc- and
891 HA-tagged proteins in input and Co-IP samples were detected by immunoblot
892 analyses as described (Pulido et al., 2013) using 1:5000-diluted αMyc (Sigma)
893 and 1:1000-diluted αHA (Roche) as primary antibodies. Horseradish peroxidase
894 (HRP)-conjugated secondary antibodies against mouse and rat IgGs were used
895 in a 1:10000 dilution. WesternBright ECL Western blotting detection kit
896 (Advansta) and Amersham ECL Prime Western Blotting Detection Kit (GE
897 Healthcare) were used for detection and the signal was visualized using the
898 ChemiDoc Touch Imaging System (Bio-Rad).

899

900 **Generation of tomato CRISPR-Cas9 mutant plants**

901 *A. tumefaciens* GV3101 strain was used to stably transform tomato MicroTom
902 cotyledons with pDE-Cas9 plasmids harboring two sgRNAs to disrupt *SlG2* and
903 *SlG3* genomic sequences as described (Fernandez et al., 2009). *In vitro*
904 regenerated T1 tomato mutant lines were identified based on kanamycin
905 resistance (100 µg/ml), genotyping PCR and restriction analyses of the amplified
906 regions (Figures S6 and S7). Four T1 lines of each transformation showed
907 biallelic deletions in the designed region. Homozygous T2 lines lacking Cas9
908 were obtained after segregation. Stable T3 offspring was used for further
909 experiments. For the isolation of double mutants, *slg2-2* and *slg3-1* homozygous
910 plants were manually crossed. Double heterozygous F1 plants were confirmed by
911 PCR genotyping and restriction analyses, and allowed to self-pollinate. The
912 resulting seeds were used to screen the F2 population for double homozygous
913 plants by PCR genotyping and restriction analyses.

914

915 **Photosynthetic measurements**

916 Photosynthetic activity was assessed by measuring chlorophyll *a* fluorescence
917 with a MAXI-PAM fluorometer (Walz). The effective quantum yield ϕ_{PSII} ($\Delta F/F_m'$)
918 of young and mature tomato leaves was measured as $(F_m' - F_s)/F_m'$, where F_m'
919 and F_s are the maximum and the minimum fluorescence of light exposed plants,
920 respectively. The light intensity chosen was 21 PAR (actinic light, AL=2) as
921 previously reported (Llorente et al., 2019). The results are presented as the
922 average of three biological replicates and four different leaf areas for each
923 replicate.

924

925 **Metabolite analysis**

926 Carotenoids, chlorophylls and tocopherols were extracted as follows. A mix was
927 prepared in 2 mL Eppendorf tubes with ca. 4 mg of freeze-dried leaf tissue, 375
928 µL of methanol as extraction solvent and 25 µL of a 10 % (w/v) solution of
929 canthaxanthin (Sigma) in chloroform as internal control. After vortexing the
930 samples for 10 s and lysing the tissue with 4 mm glass beads for 1 min at 30 Hz
931 in the TissueLyser II (Qiagen), 400 µL of Tris-HCl pH:7.5 were added and the
932 samples were again mixed for 1 min in the TissueLyser. Next, 800 µL of

933 chloroform were added and the mixture was again shaken for 1 min in the
934 TissueLyser. Samples were then centrifuged for 5 min at maximum speed at 4
935 °C. The lower organic phase was placed in a new 1.5 mL tube and evaporated
936 using a SpeedVac. Fruit isoprenoids were extracted using 15 mg of freeze-dried
937 tissue and 1 ml of hexane/acetone/methanol 2:1:1 as extraction solvent. After
938 vortexing and lysing the tissue with the TissueLyser as described for leaves, 100
939 µL of milli-Q water were added. Then, 1 min of TissueLyser was carried out again
940 and samples were centrifuged for 3 min at 3,000 rpm and 4 °C. The organic
941 phase was transferred to a 1.5 mL tube and the rest was re-extracted by adding
942 1 mL of hexane/acetone/methanol 2:1:1 solvent, TissueLyser-mixing for 1 min
943 and centrifuging for 5 min at maximum speed and 4 °C. The new organic phase
944 was mixed with that previously extracted and evaporated using the SpeedVac
945 system. Extracted metabolites from leaf and fruit pericarp samples were
946 resuspended in 200 µL of acetone by using an ultrasound bath (Labolan) and
947 filtered with 0.2 µm filters into amber-colored 2 mL glass vials. Separation and
948 detection was next performed using an Agilent 1200 series HPLC system (Agilent
949 Technologies) as previously reported (Fraser et al., 2000). Eluting chlorophylls
950 and carotenoids were monitored using a photodiode array detector whereas
951 tocopherols were identified using a fluorescence detector. Peak areas of
952 chlorophylls (650 nm), carotenoids (470 nm for lycopene, lutein, β-carotene,
953 violaxanthin, neoxanthin and canthaxanthin or 280 nm for phytoene), and
954 tocopherols (330 nm) were determined using the Agilent ChemStation software.
955 Quantification was performed by comparison with commercial standards (Sigma).
956 ABA levels were determined as described (Diretto et al., 2020). Primary
957 metabolites were extracted, annotated and quantified as described previously
958 (Llorente et al., 2019) using approximately 10 mg of lyophilized tissue.
959 Parameters used for peak annotation are provided in Table S6 for leaves and
960 Table S8 for fruits.

961

962

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982

983

984 **AUTHOR CONTRIBUTIONS**

985 MVB, ME and MRC designed the research; MVB, ME, GD, IFS, EF, and AF
 986 performed research; RK, ARF and JB contributed analytic tools; MVB, ME, GD,
 987 IFS, EF, AF, RK, ARF, JB and MRC analyzed data; MVB and MRC wrote the
 988 paper.

TABLES

Table 1. Kinetic parameters of plastidial GGPPS enzymes. Values correspond to the mean $\pm$ SD of three independent experimental replicates (n=3).

	DMAPP (+100 μ M IPP)		IPP (+100 μ M DMAPP)	
	Km (μ M)	Vmax (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)	Km (μ M)	Vmax (nmol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$)
SIG1	31.82 $\pm$ 2.92	47.47 $\pm$ 1.40	74.18 $\pm$ 7.55	59.87 $\pm$ 2.73
SIG2	49.55 $\pm$ 5.31	38.87 $\pm$ 1.53	79.75 $\pm$ 8.33	36.73 $\pm$ 1.73
SIG3	45.75 $\pm$ 6.81	26.13 $\pm$ 1.40	45.92 $\pm$ 4.86	29.13 $\pm$ 1.13
AtG11	32.86 $\pm$ 4.86	21.53 $\pm$ 1.07	38.49 $\pm$ 4.94	24.13 $\pm$ 1.07

Table 2. ABA levels in GGPPS-defective leaves and fruit. Values (μ g/g dry weight) correspond to the mean $\pm$ SD of four independent samples (n=4). Statistically significant changes in mutants compared to WT samples (t-test, p<0.01) are indicated in bold.

	Young leaves	B+10 fruit
WT	1.67 $\pm$ 0.19	0.63 $\pm$ 0.13
<i>slg2-1</i>	1.69 $\pm$ 0.10	0.55 $\pm$ 0.12
<i>slg2-2</i>	1.98 $\pm$ 0.39	0.30 $\pm$ 0.08
<i>slg3-1</i>	1.96 $\pm$ 0.09	0.16 $\pm$ 0.04
<i>slg3-2</i>	1.61 $\pm$ 0.29	0.08 $\pm$ 0.01

Table 3. Expected and observed frequencies of the F2 population from the crosses of *slg2-2* and *slg3-1*. Mutant alleles are marked in red. Percentages in bold indicate observed frequency lower than expected.

Genotypes	Expected	Observed	Plants
G2g2 G3g3	25%	26%	52
G2g2 G3G3	12.5%	13%	26
G2G2 G3g3	12.5%	17.5%	35
g2g2 G3g3	12.5%	9%	18
G2g2 g3g3	12.5%	8%	16
g2g2 g3g3	6.25%	0%	0
g2g2 G3G3	6.25%	8.5%	17
G2G2 g3g3	6.25%	7%	14
G2G2 G3G3	6.25%	11%	22

Parsed Citations

Ahrazem, O., Argandoña, J., Fiore, A., Aguado, C., Luján, R., Rubio-Moraga, Á., Marro, M., Araujo-Andrade, C., Loza-Alvarez, P., Diretto, G., and Gómez-Gómez, L. (2018). Transcriptome analysis in tissue sectors with contrasting crocins accumulation provides novel insights into apocarotenoid biosynthesis and regulation during chromoplast biogenesis. *Sci. Rep.* 8: 1–17.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ament, K., Van Schie, C.C., Bouwmeester, H.J., Haring, M.A., and Schuurink, R.C. (2006). Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways. *Planta* 224: 1197–1208.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Araújo, W.L., Tohge, T., Ishizaki, K., Leaver, C.J., and Fernie, A.R. (2011). Protein degradation - an alternative respiratory substrate for stressed plants. *Trends Plant Sci.* 16: 489–498.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Barja, M.V. and Rodríguez-Concepción, M. (2020). A Simple In Vitro Assay to Measure the Activity of Geranylgeranyl Diphosphate Synthase and Other Short-Chain Prenyltransferases. In *Methods in Molecular Biology*, M. Rodríguez-Concepción and R. Welsch, eds, pp. 27–38.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Bartley, G. and Scolnik, P. (1993). cDNA Cloning, Expression during Development, and Genome Mapping of a second phytoene synthase. *Biochemistry* 268: 25718–25721.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Bartley, G.E., Viitanen, P. V, Bacot, K.O., and Scolnik, P.A (1992). A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway. *J. Biol. Chem.* 267: 5036–9.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Baslam, M., Esteban, R., García-Plazaola, J.I., and Goicoechea, N. (2013). Effectiveness of arbuscular mycorrhizal fungi (AMF) for inducing the accumulation of major carotenoids, chlorophylls and tocopherol in green and red leaf lettuces. *Appl. Microbiol. Biotechnol.* 97: 3119–3128.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Beck, G., Coman, D., Herren, E., Ruiz-Sola, M.A., Rodríguez-Concepción, M., Gruissem, W., and Vranová, E. (2013). Characterization of the GGPP synthase gene family in *Arabidopsis thaliana*. *Plant Mol. Biol.* 82: 393–416.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Bick, J.A and Lange, B.M. (2003). Metabolic cross talk between cytosolic and plastidial pathways of isoprenoid biosynthesis: Unidirectional transport of intermediates across the chloroplast envelope membrane. *Arch. Biochem. Biophys.* 415: 146–154.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–54.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Camagna, M., Grundmann, A., Bär, C., Koschmieder, J., Beyer, P., and Welsch, R. (2019). Enzyme Fusion Removes Competition for Geranylgeranyl Diphosphate in Carotenogenesis. *Plant Physiol.* 179: 1013–1027.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Camara, B. (1993). Plant phytoene synthase complex: Component enzymes, Immunology and Biogenesis. *Methods Enzymol.* 214: 352–365.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Cao, H., Luo, H., Yuan, H., Eissa, M., Thannhauser, T., Welsch, R., Hao, Y.-J., Cheng, L., and Li, L. (2019). A neighboring aromatic-aromatic amino acid combination governs activity divergence between tomato phytoene synthases. *Plant Physiol.* 180: pp.00384.2019.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Cunillera, N., Arró, M., Delourme, D., Karst, F., Boronat, A., and Ferrer, A (1996). *Arabidopsis thaliana* Contains two differentially expressed farnesyl-diphosphate synthase genes. *J. Biol. Chem.* 271: 7774–7780.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Cunillera, N., Boronat, A., and Ferrer, A. (1997). The Arabidopsis thaliana FPS1 gene generates a novel mRNA that encodes a mitochondrial farnesyl-diphosphate synthase isoform. J. Biol. Chem. 272: 15381–15388.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

D'Andrea, L., Simon-Moya, M., Llorente, B., Llamas, E., Marro, M., Loza-Alvarez, P., Li, L., and Rodriguez-Concepcion, M. (2018). Interference with Clp protease impairs carotenoid accumulation during tomato fruit ripening. J. Exp. Bot. 69: 1557–1568.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Diretto, G. et al. (2020). Manipulation of β -carotene levels in tomato fruits results in increased ABA content and extended shelf life. Plant Biotechnol. J. 18: 1185–1199.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Dogbo, O. and Camara, B. (1987). Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from Capsicum chromoplasts by affinity chromatography. Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab. 920: 140–148.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Emanuelsson, O., Brunak, S., von Heijne, G., and Nielsen, H. (2007). Locating proteins in the cell using TargetP, SignalP and related tools. Nat. Protoc. 2: 953–971.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Estornell, L.H., Orzáez, D., López-Peña, L., Pineda, B., Antón, M.T., Moreno, V., and Granell, A. (2009). A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits. Plant Biotechnol. J. 7: 298–309.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fernandez, A.I. et al. (2009). Flexible Tools for Gene Expression and Silencing in Tomato. Plant Physiol. 151: 1729–1740.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fester, T., Schmidt, D., Lohse, S., Walter, M.H., Giuliano, G., Bramley, P.M., Fraser, P.D., Hause, B., and Strack, D. (2002). Stimulation of carotenoid metabolism in arbuscular mycorrhizal roots. Planta 216: 148–154.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fester, T., Wray, V., Nimtz, M., and Strack, D. (2005). Is stimulation of carotenoid biosynthesis in arbuscular mycorrhizal roots a general phenomenon? Phytochemistry 66: 1781–1786.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fraser, P.D., Enfissi, E.M.A., Halket, J.M., Truesdale, M.R., Yu, D., Gerrish, C., and Bramley, P.M. (2007). Manipulation of Phytoene Levels in Tomato Fruit: Effects on Isoprenoids, Plastids, and Intermediary Metabolism. Plant Cell 19: 3194–3211.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fraser, P.D., Romer, S., Shipton, C.A., Mills, P.B., Kiano, J.W., Misawa, N., Drake, R.G., Schuch, W., and Bramley, P.M. (2002). Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner. Proc. Natl. Acad. Sci. U. S. A. 99: 1092–7.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fraser, P.D., Schuch, W., and Bramley, P.M. (2000). Phytoene synthase from tomato (Lycopersicon esculentum) chloroplasts--partial purification and biochemical properties. Planta 211: 361–369.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fray, R.G. and Grierson, D. (1993). Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. Plant Mol. Biol. 22: 589–602.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fujisawa, M., Nakano, T., Shima, Y., and Ito, Y. (2013). A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. Plant Cell 25: 371–386.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Fujisawa, M., Shima, Y., Nakagawa, H., Kitagawa, M., Kimbara, J., Nakano, T., Kasumi, T., and Ito, Y. (2014). Transcriptional regulation of fruit ripening by tomato FRUITFULL homologs and associated MADS box proteins. Plant Cell 26: 89–101.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Gaffe, J., Bru, J.-P., Causse, M., Vidal, A., Stanitti-Bert, L., Carde, J.-P., and Gallusci, P. (2000). LEFPS1, a Tomato Farnesyl Pyrophosphate Gene Highly Expressed during Early Fruit Development. *Plant Physiol.* 123: 1351–1362.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Galili, G., Amir, R., and Fernie, A.R. (2016). The Regulation of Essential Amino Acid Synthesis and Accumulation in Plants. *Annu. Rev. Plant Biol.* 67: 153–178.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Giorio, G., Stigliani, A.L., and D'Ambrosio, C. (2008). Phytoene synthase genes in tomato (*Solanum lycopersicum* L.) - New data on the structures, the deduced amino acid sequences and the expression patterns. *FEBS J.* 275: 527–535.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Goytia, E., Fernández-Calvino, L., Martínez-García, B., López-Abella, D., and López-Moya, J.J. (2006). Production of plum pox virus HC-Pro functionally active for aphid transmission in a transient-expression system. *J. Gen. Virol.* 87: 3413–23.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Hall, B.G. (2013). Building phylogenetic trees from molecular data with MEGA *Mol. Biol. Evol.* 30: 1229–1235.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Höhner, R., Aboukila, A., Kunz, H.-H., and Venema, K. (2016). Proton Gradients and Proton-Dependent Transport Processes in the Chloroplast. *Front. Plant Sci.* 7: 1–7.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Jones, M.O., Perez-Fons, L., Robertson, F.P., Bramley, P.M., and Fraser, P.D. (2013). Functional characterization of long-chain prenyl diphosphate synthases from tomato. *Biochem. J.* 449: 729–740.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Kachanovsky, D.E., Filler, S., Isaacson, T., and Hirschberg, J. (2012). Epistasis in tomato color mutations involves regulation of phytoene synthase 1 expression by cis-carotenoids. *Proc. Natl. Acad. Sci. U. S. A.* 109: 19021–6.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995). Molecular cloning and nucleotide sequences of the genes for two essential proteins constituting a novel enzyme system for heptaprenyl diphosphate synthesis. *J. Biol. Chem.* 270: 18396–18400.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K., and Chen, L.L. (2017). CRISPR-P 2.0: An Improved CRISPR-Cas9 Tool for Genome Editing in Plants. *Mol. Plant* 10: 530–532.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Llorente, B., D'Andrea, L., Ruiz-Sola, M.A., Botterweg, E., Pulido, P., Andilla, J., Loza-Alvarez, P., and Rodriguez-Concepcion, M. (2016). Tomato fruit carotenoid biosynthesis is adjusted to actual ripening progression by a light-dependent mechanism. *Plant J.* 85: 107–19.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Llorente, B., Torres-Montilla, S., Morelli, L., Florez-Sarasa, I., Ezquerro, M., D'andrea, L., Majer, E., Troncoso, A., Fernie, A.R., Daròs, J.A., and Rodriguez-Concepcion, M. (2019). Synthetic biogenesis of chromoplasts from leaf chloroplasts. *bioRxiv*: 819177.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Maudinas, B., Bucholtz, M.L., Papastefanou, C., Katiyar, S.S., Briedis, A V., and Porter, J.W. (1977a). The partial purification and properties of a phytoene synthesizing enzyme system. *Arch. Biochem. Biophys.* 180: 354–362.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Maudinas, B., Bucholtz, M.L., Papastefanou, C., Katiyar, S.S., Briedis, A V., and Porter, J.W. (1977b). The partial purification and properties of a phytoene synthesizing enzyme system. *Arch. Biochem. Biophys.* 180: 354–62.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Maza, E., Frasse, P., Senin, P., Bouzayen, M., and Zouine, M. (2013). Comparison of normalization methods for differential gene expression analysis in RNA-Seq experiments. *Commun. Integr. Biol.* 6: e25849.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

McQuinn, R.P., Gapper, N.E., Gray, A.G., Zhong, S., Tohge, T., Fei, Z., Fernie, A.R., and Giovannoni, J.J. (2020). Manipulation of ZDS in tomato exposes carotenoid- and ABA-specific effects on fruit development and ripening. Plant Biotechnol. J.: 1–15.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Muñoz, A. and Castellano, M.M. (2018). Coimmunoprecipitation of Interacting Proteins in Plants. In Two-hybrid systems: Methods and Protocols, Methods in Molecular Biology, L. Oñate-Sánchez, ed, pp. 279–287.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Muñoz, P. and Munné-Bosch, S. (2018). Photo-oxidative stress during leaf, flower and fruit development. Plant Physiol. 176: 1004–1014.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nagel, R., Bernholz, C., Vranová, E., Košuth, J., Bergau, N., Ludwig, S., Wessjohann, L., Gershenzon, J., Tissier, A., and Schmidt, A. (2015). Arabidopsis thaliana isoprenyl diphosphate synthases produce the C 25 intermediate, geranylgeranyl diphosphate. Plant J. 84: 847–859.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Nisar, N., Li, L., Lu, S., Khin, N.C., and Pogson, B.J. (2015). Carotenoid metabolism in plants. Mol. Plant 8: 68–82.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Obata, T. and Fernie, A.R. (2012). The use of metabolomics to dissect plant responses to abiotic stresses. Cell. Mol. Life Sci. 69: 3225–3243.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., and Kamiya, Y. (2000). Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in Arabidopsis. Plant Physiol. 122: 1045–1056.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Oliver, S. (2000). Guilt-by-association goes global. Nature 403: 601–3.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Orlova, I. et al. (2009). The small subunit of snapdragon geranyl diphosphate synthase modifies the chain length specificity of tobacco geranylgeranyl diphosphate synthase in planta. Plant Cell 21: 4002–4017.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Phillips, M. a, D'Auria, J.C., Gershenzon, J., and Pichersky, E. (2008). The Arabidopsis thaliana type I Isopentenyl Diphosphate Isomerases are targeted to multiple subcellular compartments and have overlapping functions in isoprenoid biosynthesis. Plant Cell 20: 677–96.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Pulido, P., Perello, C., and Rodriguez-Concepcion, M. (2012). New Insights into Plant Isoprenoid Metabolism. Mol. Plant 5: 964–967.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Pulido, P., Toledo-Ortiz, G., Phillips, M. a, Wright, L.P., and Rodríguez-Concepción, M. (2013). Arabidopsis J-protein J20 delivers the first enzyme of the plastidial isoprenoid pathway to protein quality control. Plant Cell 25: 4183–4194.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Quinet, M., Angosto, T., Yuste-Lisbona, F.J., Blanchard-Gros, R., Bigot, S., Martinez, J.P., and Lutts, S. (2019). Tomato Fruit Development and Metabolism. Front. Plant Sci. 10: 1–23.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rodríguez-Concepcion, M. et al. (2018). A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. Prog. Lipid Res. 70: 62–93.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Rodríguez-Concepción, M. and Boronat, A. (2015). Breaking new ground in the regulation of the early steps of plant isoprenoid biosynthesis. Curr. Opin. Plant Biol. 25: 17–22.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only](#) [Title Only](#) [Author and Title](#)

Ruiz-Lozano, J.M., Aroca, R., Zamarreño, A.M., Molina, S., Andreo-Jiménez, B., Porcel, R., García-Mina, J.M., Ruyter-Spira, C., and López-Ráez, J.A. (2016). Arbuscular mycorrhizal symbiosis induces strigolactone biosynthesis under drought and improves drought tolerance in lettuce and tomato. *Plant, Cell Environ.* 39: 441–452.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ruiz-Sola, M.Á. et al. (2016a). Arabidopsis GERANYLGERANYL DIPHOSPHATE SYNTHASE 11 is a hub isozyme required for the production of most photosynthesis-related isoprenoids. *New Phytol.* 209: 252–264.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ruiz-Sola, M.Á., Barja, M.V., Manzano, D., Llorente, B., Schipper, B., Beekwilder, J., and Rodriguez-Concepcion, M. (2016b). A Single Arabidopsis Gene Encodes Two Differentially Targeted Geranylgeranyl Diphosphate Synthase Isoforms. *Plant Physiol.* 172: 1393–1402.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Ruiz-Sola, M.Á. and Rodríguez-Concepción, M. (2012). Carotenoid Biosynthesis in Arabidopsis: A Colorful Pathway. *Arab. B.* 10: e0158.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sandmann, G. (2015). Carotenoids of Biotechnological Importance. *Adv. Biochem. Eng. / Biotechnol.* 148: 449–467.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sapir-Mir, M., Mett, A., Belausov, E., Tal-Meshulam, S., Frydman, A., Gidoni, D., and Eyal, Y. (2008). Peroxisomal Localization of Arabidopsis Isopentenyl Diphosphate Isomerases Suggests That Part of the Plant Isoprenoid Mevalonic Acid Pathway Is Compartmentalized to Peroxisomes. *Plant Physiol.* 148: 1219–1228.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Schirml, S., Fauser, F., and Puchta, H. (2016). CRISPR/Cas-Mediated Site-Specific Mutagenesis in Arabidopsis thaliana Using Cas9 Nucleases and Paired Nickases. *Methods Mol. Biol.* 1469: 111–22.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Seymour, G.B., Chapman, N.H., Chew, B.L., and Rose, J.K.C. (2013). Regulation of ripening and opportunities for control in tomato and other fruits. *Plant Biotechnol. J.* 11: 269–278.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sievers, F. and Higgins, D.G. (2014). Clustal omega. *Curr. Protoc. Bioinforma.* 48: 3.13.1-16.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., and Higgins, D.G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7: 1–6.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Simon, P. (2003). Q-Gene: Processing quantitative real-time RT-PCR data. *Bioinformatics* 19: 1439–1440.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sparkes, I.A., Runions, J., Kearns, A., and Hawes, C. (2006). Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nat. Protoc.* 1: 2019–25.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Stauder, R., Welsch, R., Camagna, M., Kohlen, W., Balcke, G.U., Tissier, A., and Walter, M.H. (2018). Strigolactone Levels in Dicot Roots Are Determined by an Ancestral Symbiosis-Regulated Clade of the PHYTOENE SYNTHASE Gene Family. *Front. Plant Sci.* 9: 1–18.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Sun, T., Yuan, H., Cao, H., Yazdani, M., Tadmor, Y., and Li, L. (2018). Carotenoid Metabolism in Plants: The Role of Plastids. *Mol. Plant* 11: 58–74.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30: 2725–2729.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Tholl, D. (2015). Biosynthesis and Biological Functions of Terpenoids in Plants. In *Advances in biochemical engineering/biotechnology*,

pp. 63–106.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Vranová, E., Coman, D., and Grussem, W. (2013). Network analysis of the MVA and MEP pathways for isoprenoid synthesis. *Annu. Rev. Plant Biol.* 64: 665–700.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Walter, M.H., Stauder, R., and Tissier, A. (2015). Evolution of root-specific carotenoid precursor pathways for apocarotenoid signal biogenesis. *Plant Sci.* 233: 1–10.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, C., Chen, Q., Fan, D., Li, J., Wang, G., and Zhang, P. (2016a). Structural Analyses of Short-Chain Prenyltransferases Identify an Evolutionarily Conserved GFPPS Clade in Brassicaceae Plants. *Mol. Plant* 9: 195–204.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, C., Chen, Q., Fan, D., Li, J., Wang, G., and Zhang, P. (2016b). Structural Analyses of Short-Chain Prenyltransferases Identify an Evolutionarily Conserved GFPPS Clade in Brassicaceae Plants. *Mol. Plant* 9: 195–204.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, G. and Dixon, R. a (2009). Heterodimeric geranyl(geranyl)diphosphate synthase from hop (*Humulus lupulus*) and the evolution of monoterpene biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* 106: 9914–9919.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, J., Lin, H.-X., Su, P., Chen, T., Guo, J., Gao, W., and Huang, L.-Q. (2019). Molecular cloning and functional characterization of multiple geranylgeranyl pyrophosphate synthases (ApGGPPS) from *Andrographis paniculata*. *Plant Cell Rep.* 38: 117–128.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Wang, Q., Huang, X.-Q., Cao, T.-J., Zhuang, Z., Wang, R., and Lu, S. (2018). Heteromeric Geranylgeranyl Diphosphate Synthase Contributes to Carotenoid Biosynthesis in Ripening Fruits of Red Pepper (*Capsicum annuum* var. *conoides*). *J. Agric. Food Chem.* 66: 11691–11700.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Yuan, H., Zhang, J., Nageswaran, D., and Li, L. (2015). Carotenoid metabolism and regulation in horticultural crops. *Hortic. Res.* 2.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zhang, M., Su, P., Zhou, Y.-J., Wang, X.-J., Zhao, Y.-J., Liu, Y.-J., Tong, Y.-R., Hu, T.-Y., Huang, L.-Q., and Gao, W. (2015). Identification of geranylgeranyl diphosphate synthase genes from *Tripterygium wilfordii*. *Plant Cell Rep.* 34: 2179–88.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zhou, F. and Pichersky, E. (2020). The complete functional characterisation of the terpene synthase family in tomato. *New Phytol.*

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zhou, F., Wang, C.-Y., Gutensohn, M., Jiang, L., Zhang, P., Zhang, D., Dudareva, N., and Lu, S. (2017). A recruiting protein of geranylgeranyl diphosphate synthase controls metabolic flux toward chlorophyll biosynthesis in rice. *Proc. Natl. Acad. Sci. U. S. A.* 114: 6866–6871.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zhu, X.F., Suzuki, K., Okada, K., Tanaka, K., Nakagawa, T., Kawamukai, M., and Matsuda, K. (1997a). Cloning and functional expression of a novel geranylgeranyl pyrophosphate synthase gene from *Arabidopsis thaliana* in *Escherichia coli*. *Plant Cell Physiol.* 38: 357–61.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zhu, X.F., Suzuki, K., Saito, T., Okada, K., Tanaka, K., Nakagawa, T., Matsuda, H., and Kawamukai, M. (1997b). Geranylgeranyl pyrophosphate synthase encoded by the newly isolated gene GGPS6 from *Arabidopsis thaliana* is localized in mitochondria. *Plant Mol. Biol.* 35: 331–41.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)

Zouine, M., Maza, E., Djari, A., Lauvernier, M., Frasse, P., Smouni, A., Pirrello, J., and Bouzayen, M. (2017). TomExpress, a unified tomato RNA-Seq platform for visualization of expression data, clustering and correlation networks. *Plant J.* 92: 727–735.

Pubmed: [Author and Title](#)

Google Scholar: [Author Only Title Only Author and Title](#)