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Specific sets of geranylgeranyl diphosphate synthases and phytoene synthases control the production of carotenoids and ABA in different tomato tissues

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Abstract

Plant carotenoids are plastid-synthesized isoprenoids with roles as photoprotectants, pigments, and precursors of bioactive molecules such as the hormone abscisic acid (ABA). The first step of the carotenoid biosynthesis pathway is the production of phytoene from geranylgeranyl diphosphate (GGPP), catalyzed by phytoene synthase (PSY). GGPP produced by plastidial GGPP synthases (GGPPS) is channeled to the carotenoid pathway by direct interaction of GGPPS and PSY enzymes. Three plastid-localized GGPPS isoforms (referred to as SlG1-3) and three PSY enzymes (PSY1-3) are present in tomato (*Solanum lycopersicum*). Our previous work showed that SlG1 and PSY3 function together in the roots, whereas the rest of the isoforms are required in aerial tissues. Here we generated and analyzed combinations of double mutants lacking PSY1 or PSY2 and SlG2 or SlG3 to investigate the contribution of specific GGPPS and PSY pairs to the production of carotenoids and ABA in different tissues of the tomato plant. Despite that the loss of individual enzymes was found to trigger compensatory mechanisms that complicate interpretation of the results, the results confirm a major role for SlG3 in providing GGPP to PSY2 for housekeeping carotenoid biosynthesis in leaves, whereas SlG2 and PSY1 become most relevant when a more active production is required in flowers and breaker fruits, i.e., at the onset of ripening. We could also confirm that ABA production in the fruit pericarp is more dependent on PSY1 activity than on total carotenoid levels and that fruit size correlates with ABA levels accumulated in ripe rather than breaker fruits.

1 | INTRODUCTION

Carotenoids are lipophilic pigments synthesized by all photosynthetic organisms and some non-photosynthetic bacteria and fungi (Rodriguez-Concepcion et al., 2018). With only a few exceptions, animals are unable to produce carotenoids but need to take them in from their diet as an essential source of retinoids, including vitamin A. In plants, carotenoids play roles as photoprotectants, pigments, and precursors of bioactive molecules such as the hormones abscisic acid (ABA) and strigolactones (Rodriguez-Concepcion et al., 2018). Plant carotenoids are synthesized in plastids from precursors supplied by the methylerythritol 4-phosphate (MEP) pathway. The five-carbon

(C5) products of the MEP pathway, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), are the universal building blocks for all isoprenoids (Rodríguez-Concepción and Boronat 2015). Condensation of three IPP molecules and one DMAPP produces C20 geranylgeranyl diphosphate (GGPP), a common precursor for carotenoids and other plastidial isoprenoids such as chlorophylls, tocopherols, plastoquinone, phylloquinone, diterpenes and gibberellins. In most plants, GGPP is produced by a family of GGPP synthase (GGPPS) enzymes with different expression patterns, kinetic properties, interacting partners, and subcellular locations (Barja and Rodriguez-Concepcion 2021; Song et al., 2023). The production of C40 phytoene from two GGPP molecules catalyzed by the enzyme phytoene synthase (PSY) is the first committed and main rate-determining step of the carotenoid biosynthesis pathway. PSY is also typically encoded by small gene families in plants (Zhou et al., 2022). Desaturation and isomerization reactions transform the colorless phytoene into red lycopene. Cyclization of the two ends of the linear lycopene molecule then produces either β-carotene, when two β-rings are formed, or α-carotene, with one β-ring and one ε-ring. Oxidation of the rings leads to the formation of xanthophylls such as zeaxanthin, violaxanthin and neoxanthin from β-carotene (referred to as β,β-xanthophylls) or lutein from α-carotene (Rodriguez-Concepcion et al., 2018). The carotenoid content of chloroplasts is quite similar in most plant species, with lutein, β-carotene and β,β-xanthophylls being the most prominent in order of abundance (Rodriguez-Concepcion et al., 2018). In non-photosynthetic tissues such as flower petals and ripe fruits, carotenoid pigments can also be produced and accumulated at very high levels in specialized plastids called chromoplasts (Sun et al., 2018; Sadali et al. 2019). During tomato fruit (*Solanum lycopersicum*) ripening, the carotenoid profile changes as the chloroplasts present at the mature green (MG) stage differentiate into chromoplasts. At the breaker (B) stage, chlorophylls start to degrade, and lycopene starts to accumulate. Later stages of ripening result in the progressive removal of xanthophylls and the overaccumulation of lycopene in chromoplasts, which gives the characteristic red color to ripe (R) fruits. By contrast, petal chromoplasts mainly accumulate β,β-xanthophylls that provide a yellow color to tomato flowers (Ezquerro et al., 2023a).

Direct interaction of GGPPS and PSY enzymes has been proposed to facilitate channeling of MEP-derived GGPP into the carotenoid pathway (Ruiz-Sola et al., 2016; Camagna et al., 2019; Barja and Rodriguez-Concepcion 2021). In *Arabidopsis thaliana*, the single PSY present interacts with plastid-localized GGPPS11, the only GGPPS isoform involved in carotenoid biosynthesis (Ruiz-Sola et al., 2016). By contrast, the genome of most crops typically harbors multiple copies of differentially expressed genes encoding GGPPS and PSY paralogs. While several GGPPS-encoding genes have been found in tobacco (*Nicotiana tabacum*), pepper (*Capsicum annuum*), and rice (*Oryza sativa*), only one paralog appears to be responsible for the production of GGPP for carotenoid biosynthesis in different tissues of these plants (Zhou et al., 2017; Wang et al., 2018a; Dong et al., 2022). The tobacco and pepper enzymes have been found to interact with specific isoforms of PSY present in these crops (Zhou et al., 2017; Wang et al., 2018a; Dong et al., 2023). Unlike the described examples, several GGPPS paralogs contribute to carotenoid biosynthesis in tomato. The tomato genome contains three plastid-localized GGPPS isoforms, referred to as SlG1-3, and three PSY enzymes, named PSY1-3 (Giorio et al., 2008; Stauder et al., 2018; Zhou and Pichersky 2020; Barja et al., 2021; Ezquerro et al., 2023a; Ezquerro et al., 2023b). Our previous results with CRISPR mutants defective in individual genes suggested a housekeeping role for SlG3 and a helper role for SlG2 in the production of carotenoids for photoprotection in leaves and for pigmentation and ABA synthesis in the pericarp of ripening fruits (Barja et al., 2021). Loss of SlG1 function had no impact in carotenoid or ABA levels but led to a reduction in the production of strigolactones in roots (Ezquerro et al., 2023b). Single CRISPR mutants defective in either PSY1 or PSY2 confirmed that PSY1 is the main isoform for carotenoid and ABA biosynthesis in the fruit pericarp with a minor contribution of PSY2; by contrast, PSY1 only appears to contribute to, the production of leaf carotenoids when an extra supply is required, making PSY2 the main PSY isoform providing carotenoids for photosynthesis and photoprotection (Ezquerro et al., 2023a). PSY3 appears to only function in the roots, specifically interacting with SlG1 to produce strigolactones (Stauder et al., 2018; Ezquerro et al., 2023b). On the other hand, PSY1 and PSY2 interact with SlG2 but not with SlG1 or SlG3 (Barja et al., 2021; Ezquerro et al., 2023b). However, it remains unknown, whether PSY1 and PSY2 use GGPP supplied by SlG3, SlG2, or both, in a tissue-specific manner.

Here we generated and analyzed double mutants defective in pairs of individual GGPPS and PSY isoforms to confirm the contribution of specific GGPPS and PSY pairs to the production of carotenoids and ABA in different tissues of tomato plants. It was expected that removing the activity of the particular GGPPS isoform supplying GGPP to a particular PSY for carotenoid biosynthesis in a specific tissue would result in exacerbated carotenoid-dependent phenotypes of the PSY-defective mutant. However, the data obtained by our analyses were often puzzling and difficult to interpret, illustrating the complex interactions taking place among these enzymes.

2 | MATERIALS AND METHODS

2.1 | Plant material

All the experiments were carried out using tomato plants (*Solanum lycopersicum*) of the MicroTom background. Single mutant alleles defective in SlG2 (*slg2-1*), SlG3 (*slg3-1*), PSY1 (*psy1-2*) or PSY2 (*psy2-1*) were previously available and used for the generation of double mutants (Barja et al., 2021, Ezquerro et al., 2023a). Double mutants were identified by PCR amplification of target sequences using gene-specific primers followed by sequencing (Barja et al., 2021; Ezquerro et al., 2023a). Seed sterilization and plating in solid 0.5x MS medium containing 1% (w/v) agar without vitamins or sucrose was done as described in Barja et al., (2021). Five days after germination (DAG), seedlings that had germinated at the same time were transferred to soil and grown in a climate-controlled walk-in Fitotron growth chamber (8 h of darkness at 22 ± 1°C and 16 h of fluorescent

white light at a photosynthetic photon flux density of 140 µmol m^{-2} s^{-1} at 25 ± 1°C). Young leaflets were collected from 15 DAG plants, and petals were collected from fully opened flowers at anthesis. Fruits at the breaker stage were used to collect pericarp tissues. Immediately after collection, tissue samples were snap-frozen in liquid nitrogen and stored to −80°C. Fruits from plants grown for six months in the chamber were used to calculate ripe fruit volume and weight (Ezquerro et al., 2023a).

2.2 | Quantification of metabolite levels and photosynthetic parameters

For ABA extraction and quantification, 10 mg of freeze-dried pericarp tissue was used as described in Sánchez-Bel et al., (2018). ABA-d6 was used as internal standard in a final concentration of 5 ppb. External calibration curves of pure standards were prepared for precise quantification using an Acquity ultra performance liquid chromatography system (Waters) coupled to a Xevo TQS triple quadrupole mass spectrometer (Waters). A UPLC Kinetex 2.6 µm EVO C18 100 Å, 2.1 × 50 mm (Phenomenex) column was used for the chromatographic separation. Conditions and solvent gradients used in this chromatographic analysis were the same as described in Sánchez-Bel et al., (2018). Chlorophylls, carotenoids and tocopherols were measured by HPLC-DAD (Barja et al., 2021). Chlorophyll levels were also estimated in the youngest fully expanded leaflets from 75 DAG plants using a SPAD 502 Plus device (Konica-Minolta). The same leaves were used to quantify chlorophyll fluorescence-related parameters using the LI-600 (LI-COR) porometer / fluorometer. All non-destructive measurements were

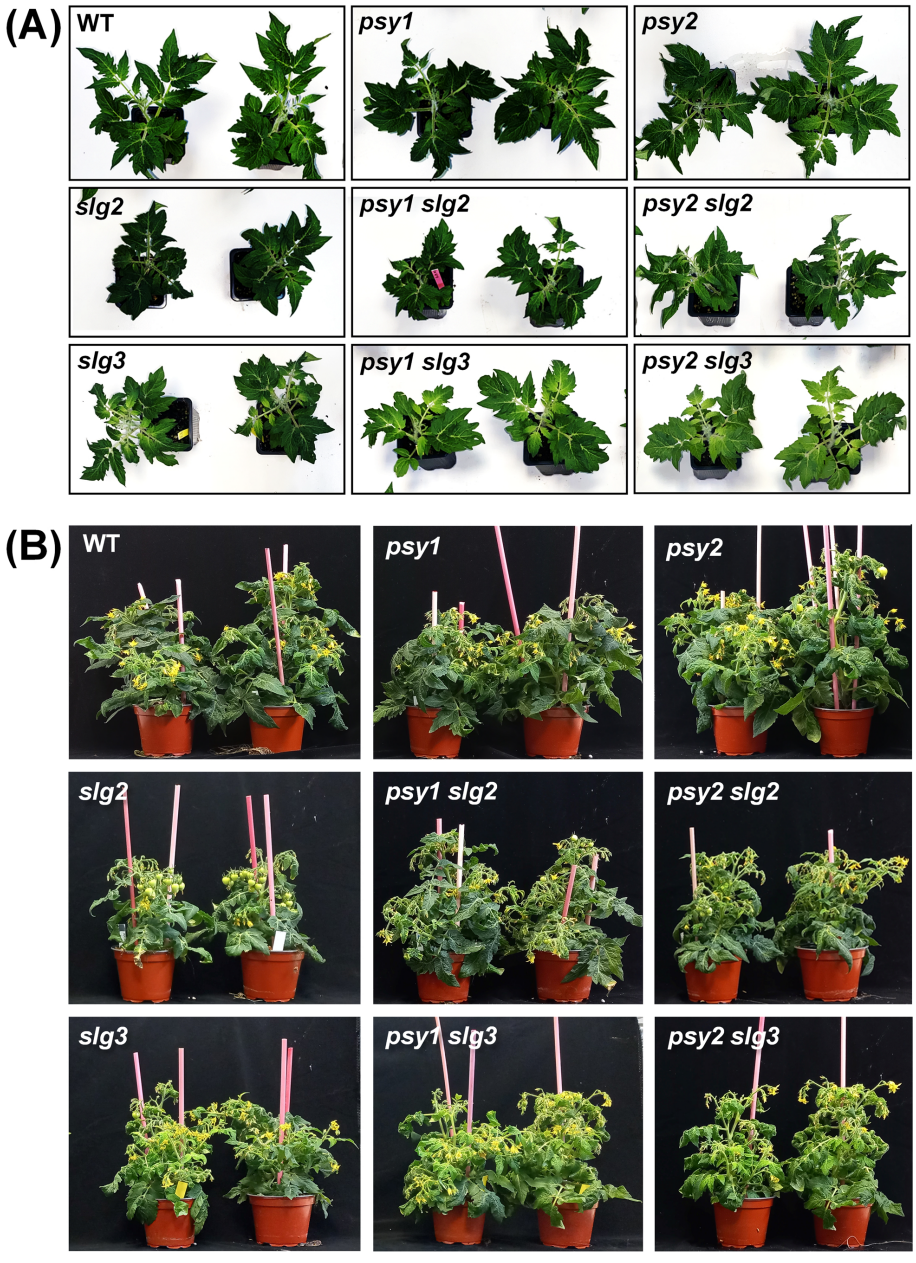

FIGURE 1 Representative tomato plants of the indicated genotypes. (A) Plants grown for 25 days after germination (DAG). All panels are to the same scale. (B) Plants at 75 DAG. All panels are to the same scale.

done between 10 and 12 am, i.e., between 8 h and 10 h after lights were switched on.

2.3 | RNA extraction and RT-qPCR analyses

The Purelink RNA mini kit (Thermo Fisher Scientific) was used for isolation of total RNA from frozen tissue samples. Determination of RNA integrity and quantity as well as cDNA synthesis were carried out as described (Ezquerro et al., 2023a). *SlG2* and *SlG3* transcript abundance was assessed using gene-specific primers (Barja et al., 2021). The tomato *Solyc07g025390* gene was used as endogenous reference gene for normalization (Gonzalez-Aguilera et al., 2016). The RT-qPCR was carried out on a QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific) using three technical replicates of each sample.

2.4 | Statistical analyses

One way ANOVA followed by Duncan multiple range tests were carried out using the statistical software R v4.1.0 (https://www.R-project.org/).

3 | RESULTS

3.1 | Generation of double mutants

For the generation of double mutants, we crossed homozygous plants harboring the following knock-out alleles: *psy1-2*, *psy2-1*, *slg2-1* or *slg3-1*, all of them in the MicroTom background (Barja et al., 2021; Ezquerro et al. 2023a). Plants of the F1 generation were allowed to self-pollinate and double mutants (*psy1 slg2*, *psy1 slg3*, *psy2 slg2* and *psy2 slg3*) were identified by PCR-based genotyping of individuals from the segregating F2 population. Seeds of azygous plants (considered as the wild-type, WT) together with mutant parental lines (*psy1*, *psy2*, *slg2* and *slg3*) and double mutants were germinated on plates, and five days later the corresponding seedlings were moved to soil. Plants were then grown in pots under long-day conditions for experiments. The phenotype of different lines 25 and 75 days after germination (DAG) is shown in Figure 1.

Single *psy1* and *psy2* mutants were similar to the WT but *slg2* and *slg3* lines grew more slowly (Figure 1). Double mutants were also smaller than the WT and PSY-defective parentals and similar to their respective GGPPS-defective parental (Figure 1). The most striking phenotype was the paler color of young leaves from single and double mutants defective in SlG3 (Figure 1). Consistently, estimation of chlorophyll levels (SPAD units) and photosynthetic performance (effective quantum yield of photosystem II, φPSII) in young leaves of 75 DAG plants showed lower values in all SlG3-defective lines, i.e., *slg3*, *psy1 slg3*, and *psy2 slg3* (Figure 2).

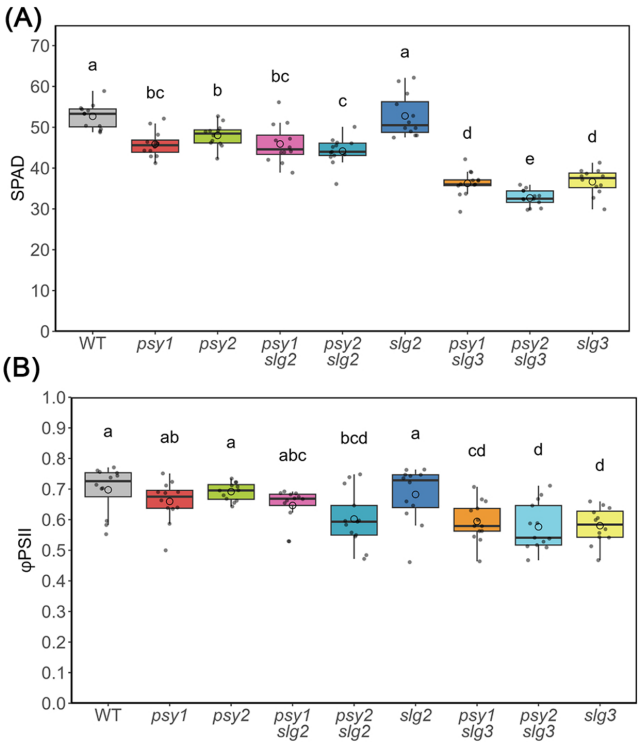

FIGURE 2 Chlorophyll levels (SPAD units) and effective quantum yield of photosystem II (φPSII) in young leaves of 75 DAG plants of the indicated genotypes. The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the hollow circle in the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Dots mark data values and whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (one-way ANOVA followed by Duncan's multiple range test, $p < 0.05$).

3.2 | Leaf isoprenoid phenotype of double mutants

To analyze the impact of decreased GGPPS and PSY activity in carotenoid production, we next investigated the carotenoid profile of different tissues of tomato WT and single and double mutant lines (Figure 3). First, we quantified the levels of different carotenoids (Figure 3A) in leaves of 15 DAG seedlings by HPLC (Figure 3B). Young leaves of single *psy1* and *psy2* mutants were previously reported to contain slightly decreased levels of carotenoid pigments compared to WT controls (Ezquerro et al. 2023a). Leaves of *psy1* and *psy2* seedlings also showed a trend towards lower carotenoid levels compared to WT controls (Figure 3B). Also as described in Barja et al. (2021), the pale color of young *slg3* leaves (Figure 1) correlated with strongly decreased levels of carotenoids in the mutant whereas WT levels of these pigments were found in the *slg2* mutant (Figure 3B). Consistent with the predominant role of SlG3 in the supply of GGPP for carotenoid biosynthesis in leaves, carotenoid levels tended to be lower in *psy1 slg3* and *psy2 slg3* leaves compared to single PSY-defective mutants (Figure 3B). However, leaf carotenoid levels in double mutant seedlings tended to be higher than those in the single *slg3*

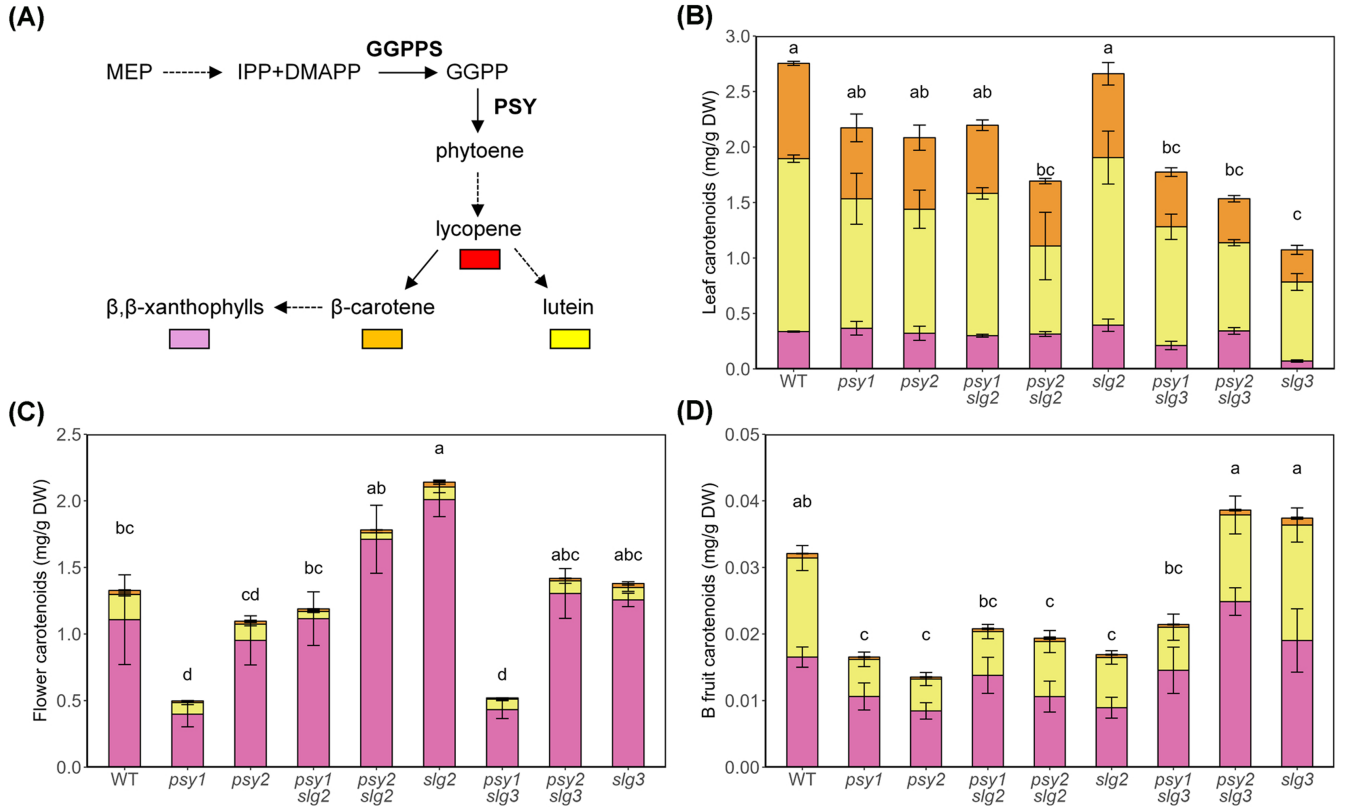

FIGURE 3 Carotenoid levels in different tissues of the indicated tomato genotypes. (A) Simplified carotenoid pathway showing the steps catalyzed by GGPPS and PSY enzymes and the carotenoid products that can be detected in tomato tissues. Color boxes mark the carotenoid products shown in the plots. Dotted arrows represent multiple steps. (B) Carotenoid profile of young leaves from 15 DAG seedlings. (C) Carotenoid profile of petals collected from flowers at anthesis. (D) Carotenoid profiles of fruit pericarps at the breaker (B) stage. Error bars correspond to the SD of $n = 3$ independent biological replicates. Statistically significant differences are represented with letters (one-way ANOVA followed by Duncan's multiple range test, $p < 0.05$). See section (A) for color legend.

mutant (Figure 3B). These results suggest that the SlG2 activity present in SlG3-defective mutants might be induced when PSY activity is decreased. Indeed, RT-qPCR analysis of *SlG2* transcript accumulation showed increased levels in *psy1 slg3* and *psy2 slg3* leaves compared to single *slg3* or WT samples (Figure 4A). If higher *SlG2* expression results in enhanced SlG2 activity, it would be expected that an enhanced supply of SlG2-derived GGPP could feed other isoprenoid pathways. In agreement, chlorophylls and tocopherols were more abundant in *psy1 slg3* and *psy2 slg3* compared to *slg3* leaves (Figure 5).

3.3 | Flower carotenoid phenotype of double mutants

Flower petals mostly contain esterified forms of β,β-xanthophylls and some lutein. The levels of these carotenoids in petals of single *psy1* mutants were reduced compared to *psy2* mutants and WT controls, suggesting a major role for PSY1 in the production of carotenoid precursors in petal chromoplasts (Ezquerro et al. 2023a). The carotenoid profile of single *slg2* or *slg3* flowers was not reported before. We detected WT levels of esterified xanthophylls in *slg3* petals whereas those of SlG2-defective plants contained increased levels (Figure 3C). Consistent with the possibility that SlG3-mediated supply of GGPP for carotenoid production is upregulated when SlG2 is absent, petal carotenoid levels were also increased in *psy1 slg2* and *psy2 slg2* compared to their respective single PSY-defective mutant (Figure 3C). However, compared to single *slg2* flowers, xanthophyll levels were reduced in *psy1 slg2* but not so much in *psy2 slg2* mutants (Figure 3C), in agreement with the previous conclusion that PSY1 is the main isoform for carotenoid synthesis in this tissue. Removal of SlG3 in double *psy1 slg3* and *psy2 slg3* mutants did not result in statistically significant changes compared to single *psy1* and *psy2* mutants, respectively (Figure 3C), suggesting that SlG3 is a minor contributor of GGPP for carotenoid biosynthesis in petals when SlG2 is present. When compared to *slg3*, *psy1 slg3* but not *psy2 slg3* flowers showed reduced carotenoid levels, again supporting a major role for PSY1 (Figure 3C).

3.4 | Fruit carotenoid phenotype of double mutants

Single *psy1* and *psy2* mutants showed a WT profile of pericarp carotenoids at the MG stage but it changed during ripening: while *psy1*

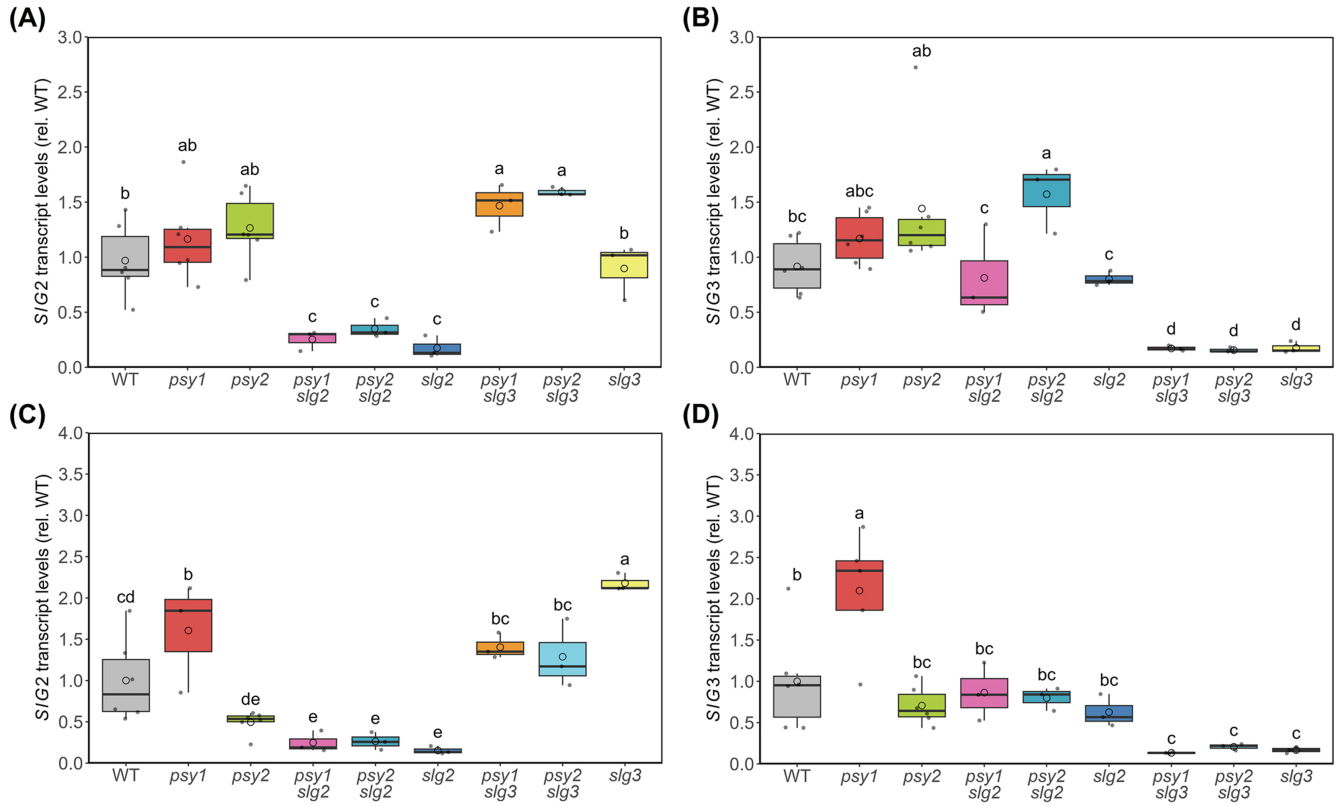

FIGURE 4 Levels of *SlG2* and *SlG3* transcripts in leaves and fruit pericarp tissues. (A) *SlG2* gene expression in young leaves from 15 DAG seedlings. (B) *SlG3* gene expression in young leaves from 15 DAG seedlings. (C) *SlG2* gene expression in the pericarp of B fruits. (D) *SlG3* gene expression in the pericarp of B fruits. The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the hollow circle in the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Dots mark data values and whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Statistically significant differences are represented with letters (one-way ANOVA followed by Duncan's multiple range test, $p < 0.05$).

fruits failed to accumulate carotenoids in R fruits, a WT profile was observed in ripe *psy2* tomatoes (Ezquerro et al. 2023a). In the case of GGPPS-defective mutants, fruit pericarp carotenoid levels were similar in WT, *slg2* and *slg3* lines at the MG stage but they were lower than the WT in R fruit of both mutants, with a stronger effect in the case of *slg3* (Barja et al. 2021). Here we compared the levels of carotenoids in single and double mutants at the B stage, when chlorophylls start to degrade, carotenoid production starts to be activated, and the expression of both *SlG2* and *PSY1* is boosted (Barja et al. 2021). The only single mutant with WT levels of carotenoids in B fruit was *slg3* (Figure 3D). In part, this could be explained by the up-regulation of *SlG2* expression in the pericarp of *slg3* fruits, which might provide the extra GGPP needed to complement the loss of SlG3 function (Figure 4). Mutant *psy1*, *psy2* and *slg2* fruits showed similarly decreased levels of carotenoids, mostly lutein and β-carotene, compared to the WT (Figure 3D). However, only *psy1* fruits showed altered levels of GGPPS-encoding transcripts as both *SlG2* and *SlG3* were up-regulated (Figure 4). This is probably a compensatory response that does not result in higher carotenoid levels because the absence of PSY1 activity cannot be compensated by the remaining PSY2 enzyme. While double mutants of *slg2* and *psy1* or *psy2* showed unchanged carotenoid levels relative to the single mutant parentals, the carotenoid content of *psy1 slg3* and *psy2 slg3* fruits was similar to that of the *psy1* and *slg3* parentals, respectively (Figure 3D). A possible interpretation of these results together is that SlG2 and PSY1 are more relevant than SlG3 and PSY2 for carotenoid synthesis at the B stage, i.e., at the onset of ripening.

3.5 | ABA content and derived phenotypes of double mutant fruits

Decreased carotenoid contents in the pericarps of single mutant fruits has been shown to lead to reduced contents of the carotenoid-derived ABA and a concomitant reduction in fruit weight and volume compared to WT controls (Barja et al. 2021; Ezquerro et al. 2023a). Here, we used our double mutant lines to analyze further the link between carotenoid and ABA levels in fruits (Figure 6). Unlike what we observed in the pericarp of R fruits, in which ABA levels were reduced in *psy1*, *slg3* and *slg2* mutants compared to WT samples (Barja et al. 2021; Ezquerro et al. 2023a), at the B stage only the *psy1* mutant showed a slight, yet not statistically significant, reduction in ABA contents (Figure 6A). This result supports the conclusion that ABA production in B fruits (Figure 6A) is not directly dependent on

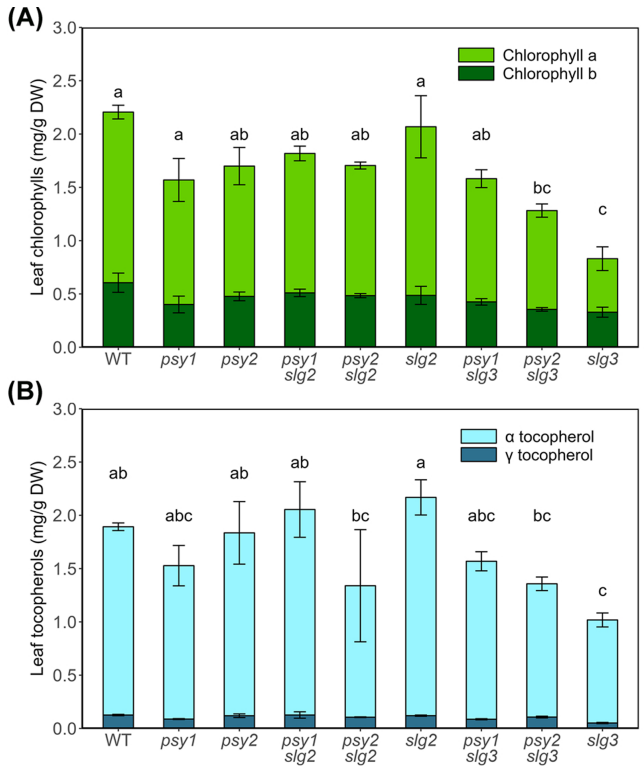

FIGURE 5 Chlorophyll and tocopherol levels in young leaves from 15 DAG seedlings of the indicated tomato genotypes. (A) Chlorophylls. (B) Tocopherols. Error bars correspond to the SD of n = 3 independent biological replicates. Statistically significant differences are represented with letters (one-way ANOVA followed by Duncan's multiple range test, $p < 0.05$).

the levels of their carotenoid precursors (Figure 3D). Then, we analyzed the size, including weight and volume, of the fruits produced by plants of all the genotypes at 190 DAG (Figure 6B). Harvested fruits from single and double mutant plants lacking PSY1 showed reduced weight (Figure 6C) and volume (Figure 6D) compared to WT fruits, with the exception of the *psy1 slg3* double mutant. All lines lacking SlG2 showed reduced fruit sizes, a phenotype that was not connected to the ABA content of B fruits (Figure 6A) but might be the consequence of lower ABA levels in R fruits (Barja et al. 2021). Similarly, *slg3* fruits also showed a phenotype of reduced weight and volume (Figure 6C-D) that was correlated to the lower ABA levels of R fruits (Barja et al. 2021) but not B fruits (Figure 6A). By contrast, double *psy1 slg3* and *psy2 slg3* mutants showed fruits of WT size, suggesting some sort of compensatory mechanism.

4 | DISCUSSION

Carotenoids are formed from GGPP, a hub intermediate for the production of many other plastidial isoprenoids essential for photosynthesis (chlorophylls, tocopherols, plastoquinone, phylloquinones) and plant development (gibberellins). GGPPS paralogs from several plant species physically interact with the enzymes catalyzing the conversion of GGPP into the first committed intermediate of the pathways leading to the production of these isoprenoid end-products (Ruiz-Sola et al., 2016; Wang et al., 2018a; Barja et al., 2021; Barja and Rodriguez-Concepcion, 2021; Dong et al., 2023; Ezquerro et al., 2023b). This observation led us to suggest that protein–protein interactions could facilitate the channeling of GGPP into specific pathways depending on specific cell demands. Indeed, GGPPS-PSY interaction is required for efficient delivery of GGPP to PSY for its conversion into phytoene (Camagna et al., 2019), and a Nudix hydrolase 23 (NUDX23) protein was recently found to interact with both GGPPS and PSY enzymes to promote their stability and assembly in a large protein complex in Arabidopsis (Rao et al., 2024). However, in species with several GGPPS and PSY paralogs, it was observed that such interactions are isoform specific. In tobacco, the only GGPPS paralog contributing to carotenoid biosynthesis was found to interact with PSY1 but not with PSY2 or PSY3 isoforms (Wang et al., 2021; Dong et al., 2022; Dong et al., 2023). In tomato, SlG1 interacts with PSY3, SlG2 with PSY1 and PSY2, and SlG3 with none of the three PSY paralogs (Barja et al., 2021; Ezquerro et al., 2023b). This is striking because the analysis of single mutants suggested that SlG3 is the housekeeping GGPPS isoform, i.e., the one that produces most GGPP in plant cells, whereas SlG2 is a helper isoform. The interaction of tomato SlG3 with PSY paralogs might be mediated by the formation of heterodimers with GGPPS-like SSU-II proteins like those reported in tobacco and pepper (Wang et al., 2018; Zhou and Pichersky, 2020; Barja et al., 2021; Dong et al., 2023). However, the capacity to directly interact with PSY1 and PSY2 suggests that SlG2 might be more efficient than SlG3 in channeling GGPP into the carotenoid pathway in tissues, developmental scenarios or environmental conditions in which a fast and effective production of carotenoids is required. The data reported here support this hypothesis.

In leaves, carotenoids are continuously needed for photoprotection. In this tissue, a constant supply of GGPP is ensured mainly by SlG3 (Barja et al., 2021; Ezquerro et al., 2023b; Figure 3B). The observation that *SlG2* transcripts are increased in *psy1 slg3* and *psy2 slg3* leaves compared to single *slg3* or WT samples (Figure 4) further suggests that leaf PSY activity represses SlG2, maybe to avoid excessive diversion of GGPP to the carotenoid pathway. Both PSY1 and PSY2 appear to participate in carotenoid biosynthesis in leaves under normal growth conditions, and the loss of any of these paralogs can be similarly rescued by the remaining isoform in chloroplasts (Figure 3B). The trend towards decreased carotenoid content in young leaves from single *psy1* and *psy2* mutants appears to have virtually no effect on photosynthesis (Figure 2) and plant growth (Figure 1). By contrast, lines lacking SlG2 or SlG3 grew more slowly that WT plants (Figure 1), a phenotype that only correlates with lower photosynthetic performance in the case of SlG3-defective plants (Figure 2). It is possible that loss of SlG2 reduces the production of gibberellins or other isoprenoids related to growth, hence causing the observed phenotype of slower plant growth. A reduced supply of SlG2-derived GGPP for gibberellin biosynthesis in fruits might also explain their reduced growth in all lines harboring the *slg2* mutation (Figure 6).

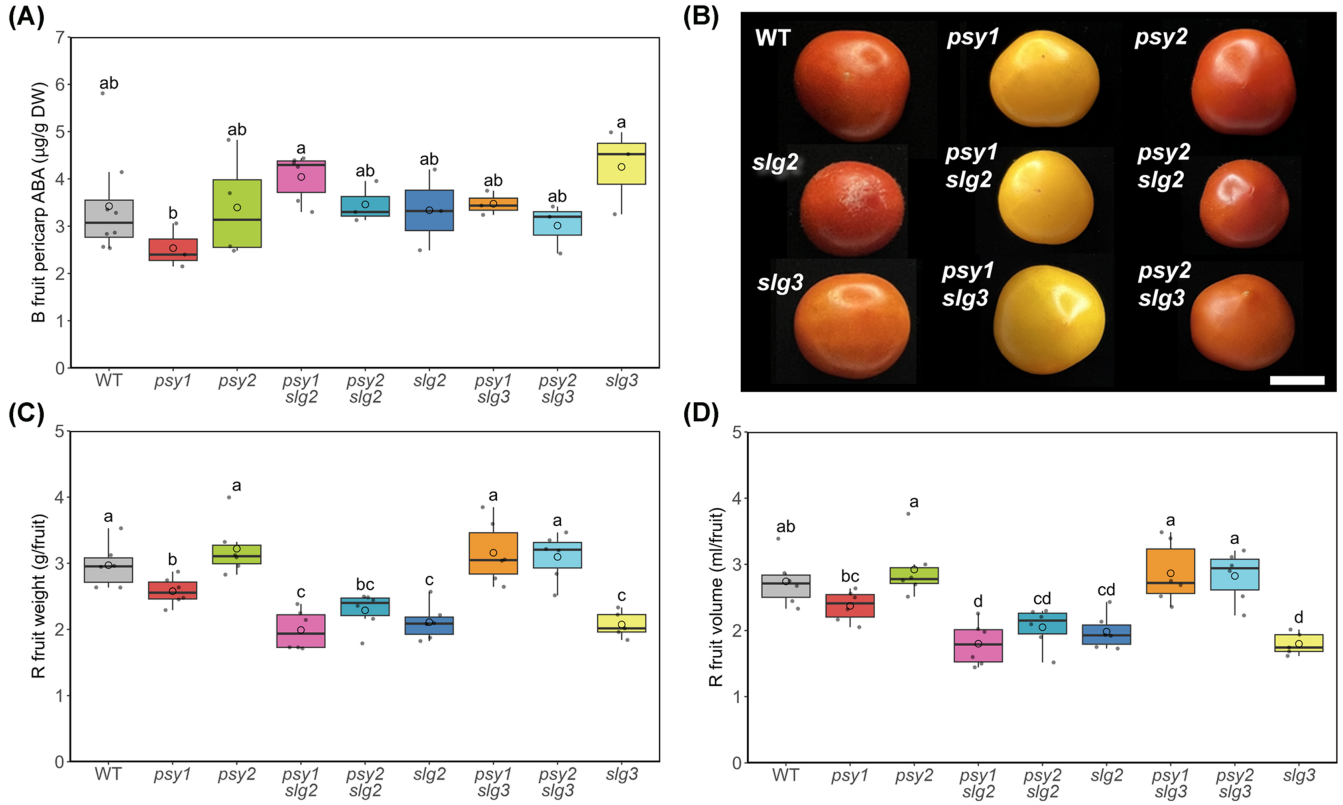

FIGURE 6 ABA levels and fruit sizes in the indicated tomato genotypes. (A) ABA levels in the pericarp of B fruits. (B) Representative fruit of WT and mutant plants at 190 DAG. (C) Fruit weight of harvested fruits. (D) Fruit volume of harvested fruits. The lower boundary of the boxes indicates the 25th percentile, the black line within the boxes marks the median, the hollow circle in the boxes marks the mean, and the upper boundary of the boxes indicates the 75th percentile of the data distribution. Dots mark data values and whiskers above and below the boxes indicate the minimum and maximum values (excluding outliers). Letters represented statistically significant differences (one-way ANOVA and Duncan's multiple range test, $p < 0.05$). Scale bar, 2 cm.

SlG2 was proposed to help SlG3 to supply GGPP when a boost in carotenoid production is needed (Barja et al., 2021). In agreement with this model, SlG2 appears to be the main GGPPS paralog supplying GGPP for the enhanced production of carotenoids for pigmentation that takes place during the development of tomato flowers and fruits (Figure 3). The capacity of SlG2 (but not SlG3) to directly interact with PSY1 and PSY2 provides a mechanistic explanation to the improved performance of SlG2 in providing GGPP when urgently or massively needed (Barja et al., 2021; Barja and Rodriguez-Concepcion, 2021). Genetic evidence provided here with single and double mutants, further supports a major contribution of PSY1 over PSY2 for carotenoid synthesis in petals and pericarp cells. Consistent with the operation of a SlG2-PSY1 tandem, the expression of *SlG2* is up-regulated in the pericarp of *psy1*, but not *psy2* fruits (Figure 4). While the existence of this and other compensatory mechanisms altering the expression of specific genes in certain genetic backgrounds complicates the interpretation of the results, the model arising from our results indicates that SlG3 would predominate over SlG2 in providing GGPP for PSY2 and also PSY1 to produce carotenoids for photoprotection and normal photosynthetic function in leaf chloroplasts whereas SlG2 would be the main supplier of GGPP to produce carotenoid pigments in flowers and fruits mostly via PSY1 activity. This model is consistent with the expression data retrieved from public gene expression databases, which show that genes encoding SlG2, SlG3, PSY1 and PSY2 are simultaneously expressed in leaves, flowers and fruits, although at different levels. Regarding the GGPPS-encoding genes, *SlG3* is typically expressed at higher levels while *SlG2* is more responsive to requirements of enhanced carotenoid production, e.g. during seedling deetiolation or fruit ripening (Barja et al., 2021). Also in agreement with our model, levels of *PSY1* transcripts are higher than those encoding PSY2 in chromoplast-containing flower petals and ripening fruit pericarp tissues, whereas *PSY2* transcripts are more abundant in leaves (Ezquerro et al., 2023a).

Our data further support an important role for PSY1 in the production of ABA in fruit pericarp tissues (Figure 6). Indeed, several mutants showed reduced carotenoid levels in B fruits (Figure 3D) but ABA contents were only reduced in the pericarp of *psy1* fruits at this developmental stage (Figure 6A). The likely existence of compensatory mechanisms might explain why ABA contents are restored to WT levels in double mutants lacking PSY1 and one of the two GGPPS isoforms (Figure 6A). In any case, it can be concluded that ABA production in B fruits is more dependent on PSY1 activity than on the levels of their carotenoid precursors (Figure 3D). At the mechanistic level, it is possible that PSY1 might somehow associate with ABA biosynthetic enzymes in protein scaffolds such as those recently reported to be

involved in seed dormancy (Wang et al., 2018b). We also reasoned that the ABA found in the fruit pericarp at the onset of ripening, i.e., at the B stage, might be more relevant to determine the final size of the fruits than those measured at the end of ripening, i.e., at the R stage. However, our results suggest that the ABA contents of R fruits correlate better with final fruit weight and volume (Figure 6; Barja et al. 2021). The underlying mechanisms connecting PSY1 activity with ABA production in the pericarp and eventually fruit size remain to be further explored.

5 | CONCLUSIONS

Together, the results reported here unveil a complex scenario of different enzyme isoform combinations contributing to carotenoid and ABA synthesis depending on the specific requirements of the tissue or/and developmental stage of tomato plants. This work provides valuable information to more precisely engineer the biofortification of specific tissues with healthy carotenoids with minimal negative impact on related processes such as the production of other GGPP-derived products required for normal plant functions.

AUTHOR CONTRIBUTIONS

E.B.-E., M.E. and M.R.-C. conceived the project and designed the experiments. E.B.-E. and M.E. generated the double mutants. E.B.-E. cultivated and analyzed the mutants. P.S.-B. and V.F. performed the ABA measurements. E.B.-E., P.S.-B., V.F. and M.R.-C. analyzed and discussed the data. E.B.-E. and M.R.-C. wrote the paper. All authors reviewed the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Barja MV, Ezquerro M, Beretta S, Diretto G, Florez-Sarasa I, Feixes E, Fiore A, Karlova R, Fernie AR, Beekwilder J, Rodríguez-Concepción M (2021) Several geranylgeranyl diphosphate synthase isoforms supply metabolic substrates for carotenoid biosynthesis in tomato. *New Phytol.* 231:255–272.

Barja MV, Rodriguez-Concepcion M (2021) Plant geranylgeranyl diphosphate synthases: every (gene) family has a story. *aBIOTECH* 2: 289–298.

Camagna M, Grundmann A, Bär C, Koschmieder J, Beyer P, Welsch R (2019) Enzyme fusion removes competition for geranylgeranyl diphosphate in carotenogenesis. *Plant Physiol.* 179:1013–1027.

Dong C, Qu G, Guo J, Wei F, Gao S, Sun Z, Jin L, Sun X, Rochaix JD, Miao Y, Wang R (2022) Rational design of geranylgeranyl diphosphate synthase enhances carotenoid production and improves photosynthetic efficiency in *Nicotiana tabacum*. *Sci Bull.* 67:315–327.

Dong C, Zhang M, Song S, Wei F, Qin L, Fan P, Shi Y, Wang X, Wang R (2023) A small subunit of geranylgeranyl diphosphate synthase functions as an active regulator of carotenoid synthesis in *Nicotiana tabacum*. *Int J Mol Sci.* 24:992.

Ezquerro M, Burbano-Erazo E, Rodriguez-Concepcion M (2023a) Overlapping and specialized roles of tomato phytoene synthases in carotenoid and abscisic acid production. *Plant Physiol.* 193:2021–2036.

Ezquerro M, Li C, Pérez-Pérez J, Burbano-Erazo E, Barja MV, Wang Y, Dong L, Lisón P, López-Gresa MP, Bouwmeester HJ, Rodríguez-Concepción M (2023b) Tomato geranylgeranyl diphosphate synthase isoform 1 is involved in the stress-triggered production of diterpenes in leaves and strigolactones in roots. *New Phytol.* 239:2292–2306.

Giorio G, Stigliani AL, 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.

González-Aguilera KL, Saad CF, Chávez Montes RA, Alves-Ferreira M, de Folter S (2016) Selection of reference genes for quantitative real-time RT-PCR studies in tomato fruit of the genotype MT-Rg1. *Front Plant Sci.* 7:1386.

Rao S, Cao H, O'Hanna FJ, Zhou X, Lui A, Wrightstone E, Fish T, Yang Y, Thannhauser T, Cheng L, Dudareva N, Li L (2024) Nudix hydrolase 23 post-translationally regulates carotenoid biosynthesis in plants. *Plant Cell.* 36:1868–1891.

Rodriguez-Concepcion M, Avalos J, Bonet ML, Boronat A, Gomez-Gomez L, Hornero-Mendez D, Limon MC, Meléndez-Martínez AJ, Olmedilla-Alonso B, Palou A, Ribot J, Rodrigo MJ, Zacarias L, Zhu C (2018) A global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Prog Lipid Res.* 70:62–93.

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

Ruiz-Sola MÁ, Coman D, Beck G, Barja MV, Colinas M, Graf A, Welsch R, Rütimann P, Bühlmann P, Bigler L, Gruissem W, Rodríguez-Concepción M, Vranová E (2016) Arabidopsis GERANYLGERANYL DIPHOSPHATE SYNTHASE 11 is a hub isozyme required for the production of most photosynthesis-related isoprenoids. *New Phytol.* 209: 252–264.

Sadali NM, Sowden RG, Ling Q, Jarvis RP (2019) Differentiation of chromoplasts and other plastids in plants. *Plant Cell Rep.* 38:803–818.

Sánchez-Bel P, Sanmartín N, Pastor V, Mateu D, Cerezo M, Vidal-Albalat A, Pastor-Fernández J, Pozo MJ, Flors V (2018) Mycorrhizal tomato plants fine tunes the growth-defence balance upon N depleted root environments. *Plant Cell Environ.* 41:406–420.

Song S, Jin R, Chen Y, He S, Li K, Tang Q, Wang Q, Wang L, Kong M, Dudareva N, Smith BJ, Zhou F, Lu S (2023) The functional evolution of architecturally different plant geranyl diphosphate synthases from geranylgeranyl diphosphate synthase. *Plant Cell.* 35:2293–2315.

Stauder R, Welsch R, Camagna M, Kohlen W, Balcke GU, Tissier A, Walter MH (2018) Strigolactone levels in dicot roots are determined by an ancestral symbiosis-regulated clade of the PHYTOENE SYNTHASE gene family. *Front Plant Sci.* 9:255.

Sun T, Yuan H, Cao H, Yazdani M, Tadmor Y, Li L (2018) Carotenoid metabolism in plants: the role of plastids. *Mol Plant*. 11:58–74.

Wang Q, Huang XQ, Cao TJ, Zhuang Z, Wang R, Lu S (2018a) 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.

Wang M, Li W, Fang C, Xu F, Liu Y, Wang Z, Yang R, Zhang M, Liu S, Lu S, Lin T, Tang J, Wang Y, Wang H, Lin H, Zhu B, Chen M, Kong F, Liu B, Zeng D, Jackson SA, Chu C, Tian Z (2018b) Parallel selection on a dormancy gene during domestication of crops from multiple families. *Nat Genet*. 50:1435–1441.

Wang Z, Zhang L, Dong C, Guo J, Jin L, Wei P, Li F, Zhang X, Wang R (2021) Characterization and functional analysis of phytoene synthase gene family in tobacco. *BMC Plant Biol.* 21:32.

Zhou F, Pichersky E (2020) The complete functional characterisation of the terpene synthase family in tomato. *New Phytol.* 226:1341–1360.

Zhou F, Wang CY, Gutensohn M, Jiang L, Zhang P, Zhang D, Dudareva N, 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.

Zhou X, Rao S, Wrightstone E, Sun T, Lui ACW, Welsch R, Li L (2022) Phytoene synthase: the key rate-limiting enzyme of carotenoid biosynthesis in plants. *Front Plant Sci.* 13:884720.

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