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Differential impact of impaired steryl ester biosynthesis on the metabolome of tomato fruits and seeds

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Abstract

Steryl esters (SE) are a storage pool of sterols that accumulates in cytoplasmic lipid droplets and helps to maintain plasma membrane sterol homeostasis throughout plant growth and development. Ester formation in plant SE is catalyzed by phospholipid:sterol acyltransferase (PSAT) and acyl-CoA:sterol acyltransferase (ASAT), which transfer long-chain fatty acid groups to free sterols from phospholipids and acyl-CoA, respectively. Comparative mass spectrometry-based metabolomic analysis between ripe fruits and seeds of a tomato (*Solanum lycopersicum* cv Micro-Tom) mutant lacking functional PSAT and ASAT enzymes (*slasat1xslpsat1*) shows that disruption of SE biosynthesis has a differential impact on the metabolome of these organs, including changes in the composition of free and glycosylated sterols. Significant perturbations were observed in the fruit lipidome in contrast to the mild effect detected in the lipidome of seeds. A contrasting response was also observed in phenylpropanoid metabolism, which is down-regulated in fruits and appears to be stimulated in seeds. Comparison of global metabolic changes using volcano plot analysis suggests that disruption of SE biosynthesis favours a general state of metabolic activation that is more evident in seeds than fruits. Interestingly, there is an induction of autophagy in both tissues, which may contribute along with other metabolic changes to the phenotypes of early seed germination and enhanced fruit tolerance to *Botrytis cinerea* displayed by the *slasat1xslpsat1* mutant. The results of this study reveal unreported connections between SE metabolism and the metabolic status of plant cells and lay the basis for further studies aimed at elucidating the mechanisms underlying the observed effects.

1 | INTRODUCTION

Sterols can exist in free form (FS) and conjugated with sugars and/or fatty acids (FA), resulting in the formation of steryl glycosides (SG), acyl steryl glycosides (ASG), and steryl esters (SE) (Ferrer et al., 2017). In plants, the relative proportions of free and conjugated sterols are highly variable depending on the species, organs and developmental and environmental conditions, but usually, glycosylated sterols (SG + ASG) represent between 10 and 30% of total sterols (Moreau et al., 2002; Furt et al., 2010; Nyström et al., 2012). However, in some

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Solanaceae like tomato, glycosylated sterols account for up to 80% of total sterols (Duperon et al., 1984; Palta et al., 1993; Chávez et al., 2023). Conjugated sterols are synthesized from FS through two different pathways. Sterol glycosyltransferases (SGTs) catalyze the formation of SG from FS and UDP-activated sugars (Ramírez-Estrada et al., 2017), which in turn can be converted into ASG by one or several as yet unidentified SG acyltransferases (SGAT) (Ferrer et al., 2017). Alternatively, FS can be converted into SE by two different sterol acyltransferases, namely phospholipid:sterol acyltransferase (PSAT) and acyl-CoA:sterol acyltransferase (ASAT), that catalyze the transfer of a long-chain FA group to the free hydroxyl group of FS units from phospholipids and acyl-CoA, respectively (Banas et al., 2005; Chen et al., 2007; Lara et al., 2018).

Free and glycosylated sterols are essential structural and functional components of the plasma membrane (PM). These sterols are unevenly distributed in the membrane bilayer, being particularly enriched along with sphingolipids and selected proteins in ordered membrane microdomains, known as detergent-resistant membrane domains, that are involved in a variety of biological processes (Mongrand et al., 2010; Yu et al., 2020). On the contrary, SE accumulates together with triacylglycerols (TAG) in cytoplasmic lipid droplets (LD) (Bouchnak et al., 2023) and serve as a dynamic reservoir of sterols that helps to maintain proper PM sterol levels throughout plant growth and development (Korber et al., 2017). This is a crucial role since both excess and deficiency of sterols are highly detrimental to plant cells (Manzano et al., 2016; Shimada et al., 2019; Shimada et al., 2020). To maintain PM sterol homeostasis, the SE pool can be mobilized by hydrolysis to meet the high demand for sterols in fast-growing tissues such as young seedlings, pollen tubes and expanding leaves (Dyas and Goad, 1993; Zhou et al., 2019), or resynthesized and stored in LDs to prevent the cytotoxic overaccumulation of sterols associated to enhanced flux through the sterol biosynthesis pathway (Wilkinson et al., 1994; Gondet et al., 1994; Schaller et al., 1995) or to PM disassembly in senescent tissues (Bouvier-Navé et al., 2010).

The characterization of *Arabidopsis thaliana* and tomato (*Solanum lycopersicum*) *SlPSAT1* and *SlASAT1* single and double knock-out mutants has greatly contributed to our knowledge of the biological function of SE and the acyltransferases responsible for their formation. Sterol profiling in these mutants revealed that PSAT1 is a major contributor to the synthesis of SE, while ASAT1 would mainly modulate the flux of the post-squalene segment of the sterol pathway by controlling the amount of sterol precursors available for end-product biosynthesis (Bouvier-Navé et al., 2010; Burciaga-Monge et al., 2022). The strong reduction of SE levels in the leaves and seeds caused by the inactivation of PSAT1 and ASAT1 has clearly detectable physiological consequences in both species. The leaves of the Arabidopsis and tomato *psat1* knock-out mutants show a phenotype of early senescence consistent with the proposed role of sterol esterification in ageing (Bouvier-Navé et al., 2010; Burciaga-Monge et al., 2022). Furthermore, both the single *slpsat1* and double *slasat1xslpsat1* tomato mutants show moderate dwarfism and a reduction in the number of LDs in the leaves, while seeds of *slasat1xslpsat1* display a phenotype of early germination that is also detectable in the *slpsat1* mutant albeit to a much lesser extent (Burciaga-Monge et al., 2022). The characterization of these tomato mutants also revealed that impaired SE biosynthesis leads to altered levels of FS in leaves but not in seeds (Burciaga-Monge et al., 2022).

The fruit of the tomato plant is the edible part of this valuable worldwide horticultural crop species that provides a wealth of important nutrients to the human diet. Furthermore, tomato is a model plant commonly used to investigate the biology of fleshy fruit development (Klee & Giovannoni, 2011; Liu et al., 2022), including seed formation, as the fruits provide a suitable environment for seed development. In fact, seeds and fruits are the key yield components in most crop species. Previous studies have revealed a strong increase in SE content during tomato fruit development (Whitaker, 1988; Chávez et al., 2023), and also the importance of maintaining properly balanced levels of free and conjugated sterols for normal fruit growth and development, as reduced levels of glycosylated sterols induce a transcriptional downregulatory response of genes involved in seed filling, cell wall extension and auxin signalling that leads to smaller fruits with reduced seed yield (Chávez et al., 2023). However, the effects of sterol homeostasis perturbations on tomato fruit and seed metabolism are largely unknown. Thus, to further expand the knowledge of the biological role of ES metabolism in plants, we undertook this study aimed at establishing the overall profile of free and conjugated sterols in fruits and seeds of the *slasat1xslpsat1* mutant and the impact of the observed changes in sterol composition on the metabolome of these organs. The metabolomic results have also been associated with the previously reported phenotype of early seed germination (Burciaga-Monge et al., 2022) and the enhanced tolerance of fruits to *Botrytis cinerea* infection described in this work.

2 | MATERIALS AND METHODS

2.1 | Plant material

Tomato (*S. lycopersicum*, cv Micro-Tom) plants were grown in a greenhouse set for long day conditions (16/8 h light/dark), at 26–28°C (day) and 22–24°C (night), 60–70% ambient humidity, and a minimum light intensity of 200 μmol m^{-2} s^{-1}. Red fruits were collected 36 days after anthesis (DAA). Seeds were imbibed for 12 h at 24°C on filter paper discs moistened with water and placed in Petri dishes to create a humid environment.

2.2 | Sterol analysis

Sterols were quantified as previously described in Chávez et al. (2023). In brief, seed and fruit pericarp tissue samples were ground to a fine powder in liquid nitrogen and lyophilized. Sterol levels were determined in three biological replicates per genotype using aliquots (25–30 mg) of lyophilized tissue, and cholestanol, cholestanyl palmitate, cholestanyl-β-D-glucoside and palmitoyl-β-D-glucosylcholestanol as internal standards. The four sterol fractions were fractionated by thin layer chromatography (TLC) using precoated silica gel PLC 60 F254 plates (20 x 20 cm) (Merck), and the different fractions identified and scraped from the silica plates. The sterol moieties of SG and

ASG were released by acid hydrolysis, while those of SE were obtained by alkaline hydrolysis. After derivatization with Bis(trimethylsilyl) trifluoroacetamide (BSTFA) (Regis technologies), sterols were analyzed by GC–MS using an Agilent 7890A gas chromatograph equipped with a TEKNOKROMA TR-450232 capillary column (30 m x 0.25 mm x 0.25 mm) coupled with a 5975C mass spectrometer (Agilent). Sterol quantification was based on the relative peak area of cholestanol.

2.3 | Metabolite extraction and quantification

The whole metabolite extraction and quantification pipeline from seeds, both dry and imbibed and ripe fruit pericarp (Figure S1) followed the guidelines as described in Giavalisco et al. (2011), Salem et al. (2020) and Bulut et al. (2023), also including a quality control sample for each extra group. Briefly, fruit pericarp and both dry and imbibed seeds were harvested, immediately frozen in liquid nitrogen, ground to a fine powder, and lyophilized. Next, 10–30 mg of lyophilized tissue was extracted in 1 mL of pre-cooled (20°C) methyl tert-butyl ether:methanol (3:1 v/v) by vortexing, shaking and sonicating. Subsequently, 500 µL of water:methanol (3:1 v/v) solution was added, and samples were centrifuged for 10 min at 14000 *g* (4°C). The lower semi-polar phase was used for the analysis of semi-polar secondary and primary metabolites, and the upper hydrophobic phase for lipid analysis. Each phase was transferred to a new tube and dried. For secondary semi-polar metabolites analysis, dried extracts were resuspended in water:methanol (1:1 v/v) and metabolites were analyzed using a Thermo Q Exactive Focus (Thermo Fisher Scientific) coupled to a reverse-phase C18 column. The column was maintained at 40°C with a flow rate of 400 µL min^{-1}, and the eluent system consisted of water (eluent A) and acetonitrile (eluent B), both containing 0.1% (v/v) formic acid. Mass spectra were acquired in full scan mode over a range of 100–1500 m/z in negative mode. For primary semi-polar metabolites analysis, dried extracts were derivatized as described in Lisec et al. (2016). Derivatization was carried out at 37°C for 120 min using 40 µL of 20 mg ml^{-1} methoxyamine hydrochloride in pyridine, followed by a 30-min treatment at 37°C with 70 µL of trimethylsilyl-*N*-methyl trifluoroacetamide (MSTFA). The derivatized samples (1 µL) were injected in splitless mode into a gas chromatograph coupled to a time-of-flight mass spectrometer (Leco Pegasus HT TOF-MS; LECO Corporation). Gas chromatography was performed on a 30-m DB-35 column using helium as the carrier gas. The initial temperature of the oven was 85°C, and it was ramped up at a rate of 15°C min^{-1} to a final temperature of 360°C. Mass spectra were recorded in the range of 70–600 m/z at a rate of 20 scans/s. For the analysis of lipids, dried extracts were resuspended in a solvent mixture of acetonitrile and isopropanol (7:3 v/v), and lipids were separated and identified using an Orbitrap LC–MS system (Exactive, ThermoScientific) coupled to a reverse-phase C8 column. The mobile phase consisted of water and acetonitrile:2-propanol (7:3 v/v), with both solvents containing 0.1% acetic acid. The column temperature was maintained at 60°C, and the flow rate of the mobile phase was 400 µL/ min^{-1}. Mass spectra were acquired in full scan mode over a range of 150 to 1500 m/z in positive mode.

2.4 | Data processing and compound annotation and analysis

LC–MS full scan data were processed using MS-Refiner (Expressionist 14.0). Processing of chromatograms, peak detection, and integration were performed using RefinerMS (version 5.3; GeneData). Metabolite identification and annotation were performed using in-house reference compound library, tandem MS (MS/MS) fragmentation, and metabolomics databases (Alseekh et al., 2021). For the annotation of metabolites measured by GC–MS, the Golm Metabolome Database was used (Kopka et al., 2005). The m/z values and retention times can be found in the supplementary dataset (Data S1). Raw intensities of annotated spectra were normalized to the metabolite abundance previously normalized to the internal standard (ribitol, isovitexin and phosphatidylcholine for primary metabolites, semi-polar secondary metabolites and lipids, respectively) and tissue weight before statistical analysis. Data analysis, including statistics and volcano plotting, was performed using the R packages tidyverse (Wickham et al. 2019) and rstatix (Kassambara 2023) using the t-test function and Holm-Bonferroni Method to account for multiple testing. All 5 or 4 biological replicates for fruits and seeds, respectively, were included in the analysis, including those with zero raw intensities. Volcano plots used log2-transformed foldchanges compared to medians of the wt or dry seeds and adjusted $p < 0.05$ and foldchanges <-0.6 or >0.6 for indication. Heatmaps based on the intensity values were generated and visualized with pheatmap (Kolde, 2019), where hierarchical clustering was based on Euclidian distance as a similarity measure and the 'complete' agglomeration method was used, and row cluster aggregation was not limited. The pheatmap argument "cutree_rows" was set to 40 clusters for the large unannotated heatmap shown in Figure 2 and to 2 for all others. Principal component analysis (PCA) was visualized with the help of the plotly package (Sievert, 2020), displaying PC1 and PC2. Metabolic pathway analysis was computed with the help of MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) based on differentially accumulated metabolites (adjusted $p < 0.05$) respective to the wt median in dry seed, imbibed seed and red fruit tissues.

2.5 | Botrytis cinerea infection

Botrytis cinerea (CECT2100) was obtained from the Spanish collection of type cultures (Universidad de Valencia, Spain) and cultured in potato dextrose agar (PDA) medium for 15–20 days at 25°C in the dark. Conidia were isolated with distilled water, filtered, and collected by centrifugation at 4000 rpm for 10 min. After resuspension in B5 medium supplemented with sucrose 2% (w/v), conidia were quantified with a hemacytometer and the final concentration adjusted to $4x10^6$ conidia mL^{-1}. Red fruits were harvested at 36 days after anthesis (DAA) and surface-cleaned with 10% (v/v) bleach. A small puncture was made on each fruit, and 10 µL of the *B. cinerea* spores suspension were placed in the wound sites. Ten µl of sucrose solution was placed in the wound sites of negative control fruits (mock treatment). Fruits were kept in a sealed glass

container to maintain high humidity necessary for fungal infection progression. Lesion areas were calculated using ImageJ software.

3 | RESULTS AND DISCUSSION

3.1 | Differential effect of impaired sterol ester biosynthesis on the profile of free and glycosylated sterols in tomato seeds and fruits

The simultaneous inactivation of *SlPSAT1* and *SlASAT1* genes in tomato (cv Micro-Tom) causes an SE content reduction of more than 90% compared to wt levels both in seeds (Burciaga-Monge et al., 2022) and fruits (Figure 1 and Table S1). As previously reported in seeds (Burciaga-Monge et al., 2022), the esterified fraction of all four major sterol species is significantly depleted in *slasat1xslpsat1* fruits, with the esterified cholesterol fraction being the least affected (Table S1). Interestingly, the inability to synthesize SE has different qualitative and quantitative effects on the composition of free and glycosylated sterols. Fruits accumulate higher levels of FS and SG (1.6- and 1.8-fold, respectively) and have lower levels of ASG (0.5-fold) compared to wt samples (Figure 1A), while no changes are observed in seed FS levels and those of SG and ASG increase drastically, reaching 3.3- and 2.6-fold the wt levels, respectively (Figure 1C). The impact of these changes on the total amount of sterols (free and conjugated forms) and PM-localized sterols (free and glycosylated forms) is also different in seeds and fruits. The total amount of sterols in fruits remains virtually unchanged, and membrane sterols increase to about 1.2-fold the wt levels (Figure 1B), while in seeds, both groups of sterols increase significantly to 1.7- and 1.2-fold the wt levels, respectively (Figure 1D). These differences translate into a moderate reduction of the glycosylated sterols fraction in fruits (72.6% compared to 79.0% in the wt) (Table S1), in sharp contrast to the strong increase observed in the seeds, where the fraction of glycosylated sterols almost double that in the wt (57.9% *vs* 30.6%) (Table S2). It is also worth noting that in both cases the four major glycosylated sterol species are affected in a similar manner, with reductions ranging from 0.85- to 0.94-fold in mutant fruits (Table S1) and increases ranging from 1.8- to 2.0-fold the wt levels in mutant seeds (Table S2). All these results support the view that different regulatory mechanisms operate in the *slasat1xslpsat1* seeds and fruits to counterbalance the strong perturbation of sterol homeostasis caused by impaired sterol esterification, and reveals that the inability to esterify sterols in the mutant organs leads to a moderate enhancement of sterol biosynthesis along with an extensive organ-specific reallocation of the total sterol pool. The fact that changes in the metabolic flux of one of the branches of the conjugated sterols pathway alters the flux through the other and *vice versa* cannot be considered unexpected since FS serve as common biosynthetic precursors of both glycosylated and esterified sterols. These altered sterols profiles lead to the previously reported early germination phenotype of *slasat1xslpsat1* seeds (Burciaga-Monge et al., 2022) and cause a reduction of about 50% in seed yield per fruit, in contrast to the lack of significant effects on other fruit parameters like hardness and weight (Figure S2), fruit setting, shape and ripening.

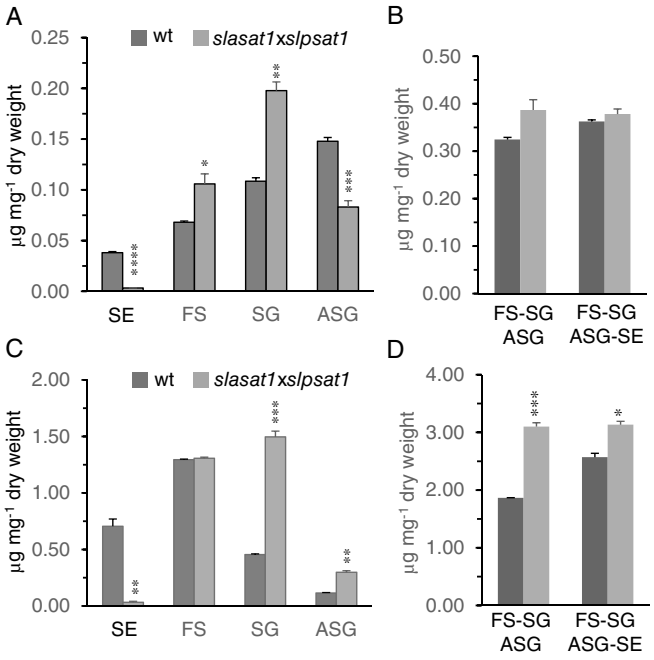

FIGURE 1 Total levels of free and conjugated sterols in wild type (wt) and *slasat1xslpsat1* fruits and seeds. Quantification of steryl esters (SE), free sterols (FS), steryl glycosides (SG) and acyl steryl glycosides (ASG) in red fruit pericarp (A and B) and seeds (C and D) of wt and *slasat1xslpsat1* plants. Data are presented as mean ± SEM from three biological replicates per genotype. Asterisks indicate values that are significantly different compared to those in the control plants determined by t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$).

3.2 | Altered sterol levels cause organ-specific metabolic perturbations in fruits and seeds

The chemical composition of tomato fruits determines its quality and thus its commercial value but also influences its stability under different environmental conditions. It is, therefore, important to establish the metabolic shifts occurring after the introduction of genetic perturbations in order to understand both the resulting phenotypic alterations and the impact on fruit quality (Tohge & Fernie, 2015; Zhu et al., 2022). In fact, a considerable amount of studies tackled this issue in recent years (Alseekh et al. 2015; Zhu et al., 2022; Otify et al. 2023). The metabolome of tomato seeds has also been analyzed regarding changes upon imbibition (Kazmi et al. 2017) or with respect to their genetic diversity in different varieties (Alseekh et al. 2020a), providing insights into important metabolism changes for development and adaptation. However, the impact of sterol biosynthesis perturbations on the plant metabolome remains largely unexplored. As a first step towards filling this gap, we have established the metabolic fingerprints in wt and *slasat1xslpast1* tomato fruits (five samples), and

dry and imbibed seeds (four samples). In total, 730 (350 positive and 380 negatively regulated compared to wt), 656 (467 positive and 184 negative) and 676 (488 positive and 188 negative) differentially regulated metabolites were identified in fruit pericarp, dry seeds and imbibed seeds, respectively (Data S1). PCA shows a clear segregation between wt and mutant fruit samples (Figure 2A). In seeds, wt and mutant samples segregate in different groups according to both the genotype and the imbibition treatment (Figure 2B). Notably, the difference between mutant seeds upon imbibition is not as pronounced as in the wt seeds, which can also be observed by comparing significant changes upon imbibition in mutant and wt seeds, respectively (Figure S3, Data S1). Compounds significantly altered in *slsat1xslpast1* seeds upon imbibition that are not significant in wt, as well as compounds differentially accumulating in different directions, are shown in Table S3. Significant changes in wt seeds can be found in Data S1. A hierarchical clustering heatmap confirmed the results of the PCA, as it shows several clearly contrasting patterns of primary and specialized metabolites between mutant and wt ripe fruits (Figure 2C) and seeds (Figure 2D).

3.3 | Effects on the lipidome

A detailed analysis of changes in the metabolite composition of *slsat1xslpsat1* red fruits revealed a strong perturbation of the lipidome compared to the wt fruits, affecting storage lipids (TAG) as well as major lipid constituents of PM (phospholipids, lysophospholipids and sphingolipids) and plastid photosynthetic membranes (galactolipids) (Figure 3). The general reduction of TAG (Figure 3A) is consistent with the very low levels of SE in the *slsat1xslpsat1* mutant (Figure 1A), since sterols are required for the assembly of LD, and both types of neutral lipids are the main constituents of the hydrophobic core of these storage organelles (Yu et al., 2021). The reduced abundance of LD in the *slsat1xslpsat1* mutant leaves (Burciaga-Monge et al., 2022) is in agreement with this observation. Among the four

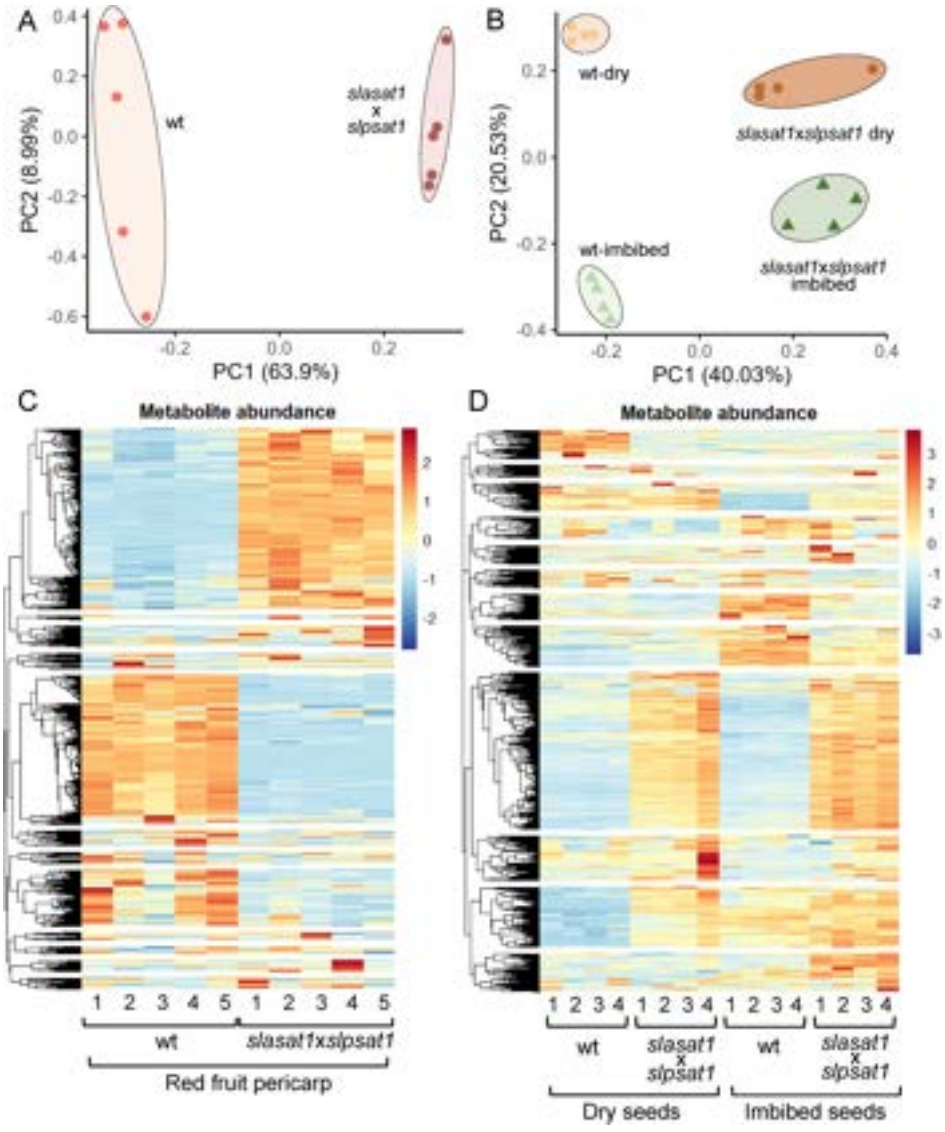

FIGURE 2 Metabolic profile distribution in wild type (wt) and *slsat1xslpsat1* fruits and seeds. (A-B) Principal component analysis (PCA) of the complete annotated metabolite dataset including primary, secondary and lipid metabolic profiles of red fruit pericarp (A) and dry and imbibed seeds (B). The values represent the metabolite abundance previously normalized to the internal standard (ribitol, isovitexin and phosphatidylcholine, respectively) and tissue weight. (C-D) Clustered heatmap analysis displaying the overall metabolite abundance of red fruit (C) and seed tissue (D). Blue and red colors indicate low and high intensity, respectively. The scale is row-based.

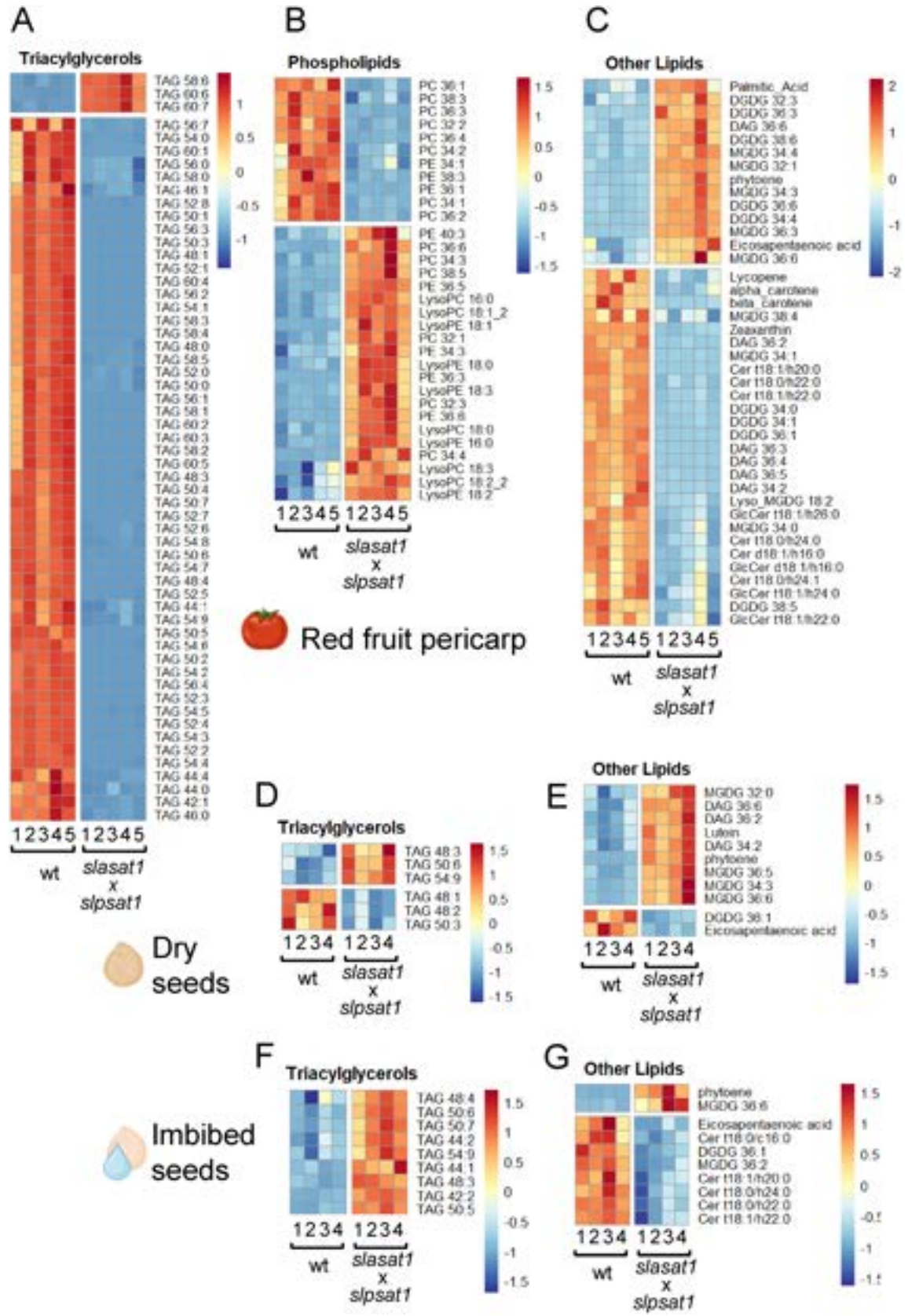

FIGURE 3 Heatmap analysis displaying changes in the profile of lipids in fruits of the *slasat1xslpsat1* mutant. Heatmaps show significantly differentially accumulated (adjusted $p < 0.05$, Holm–Bonferroni method) triacylglycerols (A), phospholipids (B) and other lipids (C) in wild type (wt) and *slasat1xslpsat1* red fruit pericarp, and triacylglycerols (D, F) and other lipids (E, G) in wt and *slasat1xslpsat1* dry seeds (D, E) and imbibed seeds (F, G). Blue and red colors indicate low and high intensity, respectively. The scale is row-based.

major classes of sphingolipids (Michaelson et al., 2016), the levels of free long-chain bases and glycosylinositolphosphoceramides are not altered, while those of some ceramides and glycosylceramides are significantly reduced (Figure 3C). Conversely, mutant fruits display higher levels of lysophosphatydilcholine and lysophosphatydilethanolamine species than wt fruits (Figure 3B). The effect on the amounts of phosphatidylcholine and phosphatidylethanolamine, the major plant glycerophospholipids, is uneven since there is an equal number of compounds that increase and decrease their levels, although those with more unsaturated fatty acids tend to increase at the expense of those having a lower degree of unsaturation (Figure 3B). A trend towards lower levels of diacylglycerols (DAG), the common precursor of glycerophospholipids and galactolipids, is also observed (Figure 3C). It is reasonable to assume that changes in the levels of these essential PM lipid components are not a direct consequence of SE depletion but an adaptive response to cope with the altered profile of free and glycosylated sterols resulting from the inability to esterify sterols. A similarly mixed response with the same trend towards species with more unsaturated fatty acids is observed in the case of mono and digalactosyldiacylglycerols (MGDG and DGDG, respectively), the major galactolipids present in plastid membranes (Figure 3C). The analysis of the plastid lipidome also revealed significant changes in the levels of carotenoids (Figure 3C), a class of lipids that play fundamental roles in plants and are important to human nutrition and health. Interestingly, the content of phytoene, the first committed intermediate of the carotenoid pathway, increases significantly in the *slsat1xslpsat1* fruits in contrast to carotenoids downstream of phytoene, such as lycopene, β-carotene, α-carotene and zeaxanthin, which decrease in the mutant fruits compared to wt (Figure 3C and S4A). The synthesis of phytoene catalyzed by phytoene synthase (PSY) is considered the main rate-limiting step that determines the flux of the pathway and, therefore, total carotenoid levels. PSY activity is regulated at multiple levels, from transcriptional to post-translational, including direct interaction with several auxiliary proteins, although other enzymes located downstream of phytoene also appear to be important in controlling the flux through the carotenoid pathway. The biosynthesis of carotenoids is tightly associated with plastoglobules and/or other membranous structures derived from thylakoid disassembly when chloroplasts differentiate into chromoplasts (Rodríguez-Concepción et al., 2018). Thus, it might be hypothesized that changes in the lipid composition of *slsat1xslpsat1* fruit chromoplast membranes alter the activity of certain enzymes of the carotenoid pathway that are directly and/or indirectly sensitive to changes in their membrane microenvironment, thus leading to the observed imbalance in the carotenoid profile. Downregulation of the flux of the pathway at some step in the post-phytoene segment of the pathway without a concomitant downregulation of PSY activity would lead to the accumulation of carotenoid precursors like phytoene and lower levels of downstream intermediates in the pathway. It is worth highlighting that such a misregulation of the carotenoid pathway makes the *slsat1xslpsat1* fruits a valuable tool to investigate the regulatory mechanisms controlling tomato fruit carotenoid biosynthesis. A more detailed analysis of the carotenoid profile will allow us to determine the impact of the observed changes in the nutritional quality of the *slsat1xslpsat1* fruits and whether the positive effect of higher levels of phytoene may compensate for the reduction of other health-promoting carotenoids such as lycopene and β-carotene (Meléndez-Martínez and Mapelli-Brahm, 2021).

Interestingly, despite the severe effects of impaired SE formation on seed sterol levels, the effects of the *slsat1xslpsat1* mutations on the lipidome of dry and imbibed seeds is far lower than in fruits, as it only affects the concentration of a few TAG, DAG and galactolipid species in dry seeds (Figure 3D and E), and some TAG, ceramide and galactolipid species in imbibed seeds (Figure 3F and G). In the *slsat1xslpsat1* dry seeds, phytoene and lutein contents increase compared to the wt (Figure 3E), while only phytoene levels increase in imbibed mutant seeds (Figure 3G), though this effect is slightly less pronounced upon imbibition and is not observed in wt seeds (Figure S4B, Table S3, Data S1). Overall, the progression of *slsat1xslpsat1* mutant seeds from the dry to the imbibed stage was associated with higher levels of TAGs and lower levels of DAGs, ceramides, MGDGs and DGDGs, compared to wt seeds. Thus, in contrast to the drastic changes caused in fruits, the impact of the altered sterol profile on the tomato seed lipidome is very limited regardless of the rapid metabolic switches associated with seed imbibition (Kazmi et al., 2017), and reflects the obvious differences in primary and specialized metabolism between fruits and seeds, as well as the different contribution of SE in the LDs of these two organs. In fact, the protein and lipid composition of LDs varies according to the type of tissue, with TAGs being by far the largest component in seed LDs (Guzha et al., 2023).

3.4 | Effects on the profile of phenylpropanoids

Phenylpropanoids are a diverse group of compounds that are important for tomato fruit quality (Zhu et al., 2022) and are known to respond to and protect plants from environmental stress signals (Brunetti et al., 2015; Dong & Lin, 2021; Ramaroson et al., 2022). The majority of significantly altered phenylpropanoids in the *slsat1xslpsat1* fruit pericarp decrease their levels compared to the wt (Figure 4A). These include hydroxycinnamic acids, particularly mono-, di- and tri-caffeoylquinic acid species, and flavonoids such as luteolin, neohesperidin, catechin and several quercetin and kaempferol derivatives. Chalcone naringenin and naringenin, the precursors of flavonoids, are also slightly reduced. Conversely, *p*-coumaric acid, an intermediate in the central phenylpropanoid pathway and its precursor phenylalanine are both increased. Thus, it appears that the flavonoid branch of the phenylpropanoid pathway (Ramaroson et al., 2022) is preferentially downregulated in the *slsat1xslpsat1* mutant. The impact of these changes on tomato fruit quality is difficult to assess since, despite a general decrease in phenylpropanoid contents, some of them significantly increase their levels in response to altered sterol levels (Figure 4A). This is the case of apigenin-7-glucoside and hesperitin-7-glucoside, both known for their antioxidant properties and therapeutic applications (Hojati et al., 2011; Falcone Ferreyra et al., 2012), and quercetin-glucoside-arabinoside-rhamnosyl-glucosyl-ferulate (QGRAG). This is the only measured quercetin derivative accumulating to higher levels in the mutant, suggesting that this form is more stable in the mutant fruits and/or is synthesized in greater

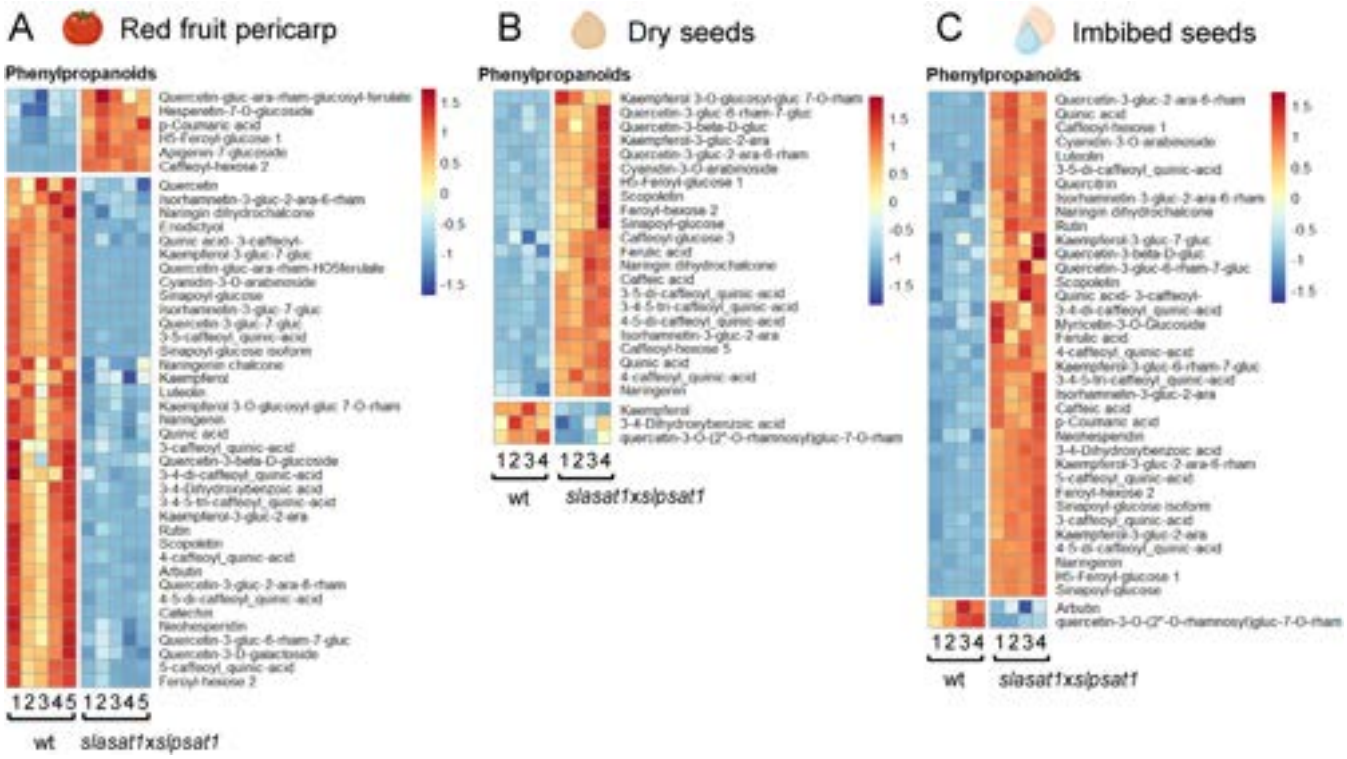

FIGURE 4 Heatmap analysis displaying changes in phenylpropanoid abundance in fruits and seeds of the *slasat1xslpsat1* mutant. Heatmaps show significantly differentially accumulated (adjusted $p < 0.05$, Holm–Bonferroni method) phenylpropanoids in red fruit pericarp (A), dry seeds (B) and imbibed seeds (C) of wild type (wt) and *slasat1xslpsat1* plants. Blue and red colors indicate low and high intensity, respectively. The scale is row-based.

quantities. Highly glycosylated phenylacylated flavonoids are more UV stable and are protected against losing sugar decoration, and might play a role in biotic interactions (Alseekh et al. 2020b).

Contrasting the fruit pericarp, dry and imbibed *slasat1xslpsat1* seeds exhibit higher levels of hydroxycinnamic acids, namely caffeic, ferulic, quinic, cafeoylquininc, their precursor p-coumaric acid, and a number of sugar conjugated forms. The levels of flavonoids, such as luteolin, neohesperidin, kaempferol, rutin, and quercetin derivatives, are also significantly enhanced compared to wt seeds (Figure 4B and C). Unique changes in mutant seeds upon imbibition support this trend (Table S3). All these changes suggest a general activation of the phenylpropanoid pathway (Rasmaroson et al., 2022), being this effect quantitatively and qualitatively more pronounced in imbibed seeds, which tend to accumulate higher levels of phenylpropanoids that are significantly altered in both dry and imbibed seeds and show a higher number of compounds that increase in abundance compared to dry seeds (Figure 4B and C).

3.5 | Impairment of sterol ester biosynthesis induces autophagy in fruits and seeds

In contrast to the differential response of the fruit and seed lipid and phenylpropanoid metabolism to the impairment of SE biosynthesis, both *slasat1xslpsat1* organs show an important accumulation of dipeptides compared to the wt (Figure 5), and to a lesser extent of single amino acids (Figure S5B-S7B), with only a few dipeptides displaying lower levels in the mutant organs compared to the wt. This effect was even more pronounced in seeds (Figure 5B and C) than in fruits (Figure 5A). Dipeptides, with a few exceptions, are proteolytic products that accumulate in response to the induction of autophagy (Thirumalaikumar et al., 2021), a catabolic process responsible for degrading unwanted or damaged cytoplasmic components, including proteins, for recycling their basic building blocks (Iglesias-Fernández and Vicente-Carbajosa, 2022). Autophagy helps to maintain cell homeostasis in different organs both under normal conditions and in response to a variety of developmental and environmental cues (Hofius et al., 2017; Thirumalaikumar et al., 2021; Alseekh et al., 2022; Ince et al., 2022; Wang et al., 2022). Thus, our results suggest that the strong disruption of the free and conjugated sterol balance in fruits and seeds is perceived as a stress signal (Manzano et al., 2016) that induces autophagy. The early leaf senescence shown by the *slasat1xslpsat1* mutant (Burciaga-Monge et al., 2022) further supports this notion, given the close correlation established between autophagy and both normal and stress-induced leaf senescence (Avila-Ospina et al., 2014).

3.6 | The pre-germinative metabolism of *slasat1xslpsat1* seeds is up-regulated

In addition to the altered profiles of lipids, phenylpropanoids and dipeptides observed in the *slasat1xslpsat1* fruits and seeds (Figure 3-5), a

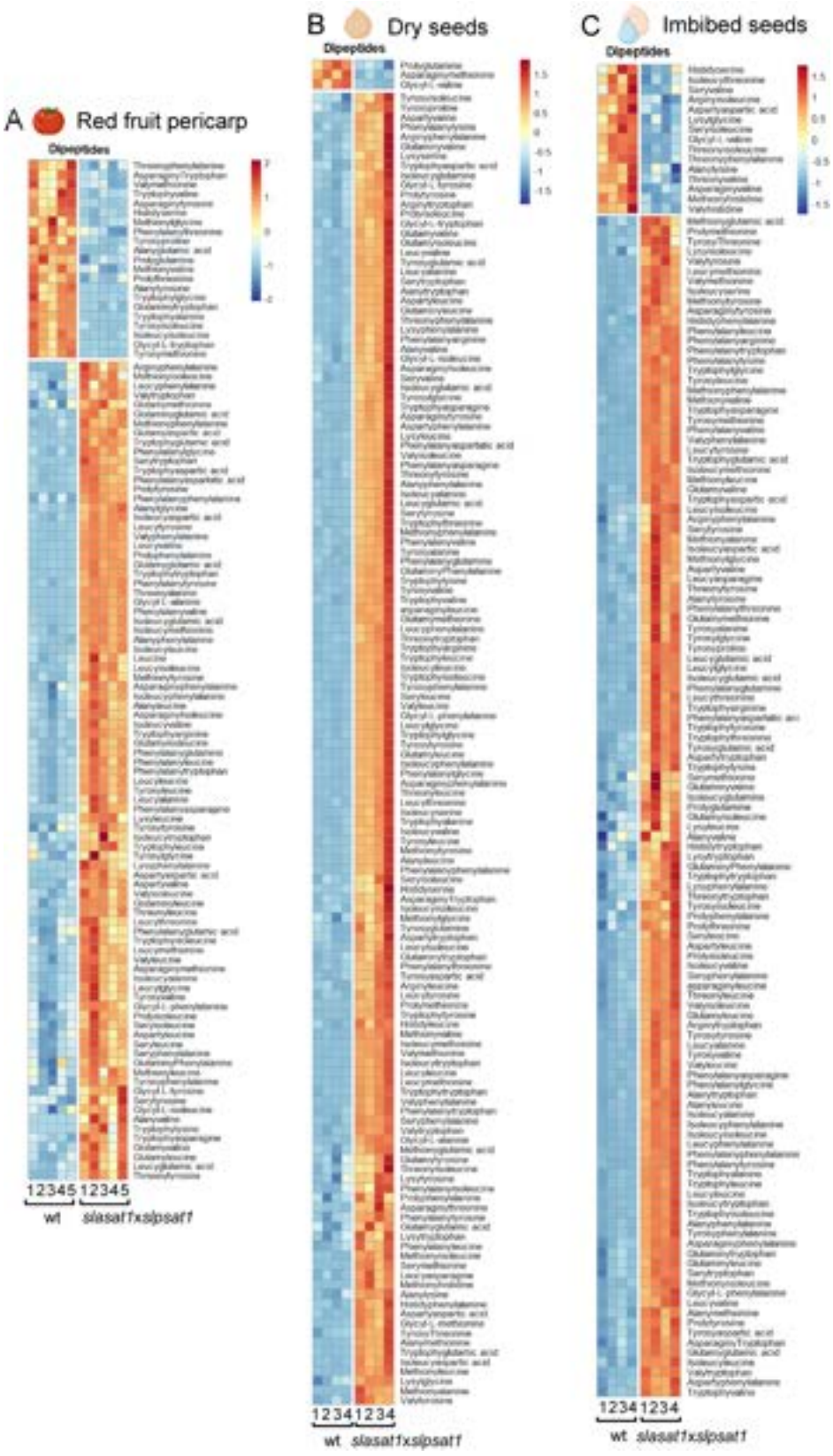

FIGURE 5 Heatmap analysis displaying changes in dipeptide abundance in fruits and seeds of the *slasat1xslpsat1* mutant. Heatmaps show significantly differentially accumulated (adjusted $p < 0.05$, Holm–Bonferroni method) dipeptides in red fruit pericarp (A), dry seeds (B) and imbibed seeds (C) of wild type (wt) and *slasat1xslpsat1* plants. Blue and red colors indicate low and high intensity, respectively. The scale is row-based.

variety of other metabolites also change their levels in these organs, including nucleic acid components, hormones, cofactors, sugars, and organic acids (Figure S5-S7). In all these groups, there are more upregulated metabolites in mutant fruits than in mutant seeds, and both organs show more upregulated than downregulated compounds (Figure S5-S7). This agrees with the results of volcano plot analysis, which show a slight trend towards upregulation in fruits of the *slasat1xslpsat1* mutant (Figure 6A), which is much more evident in both dry and imbibed seeds

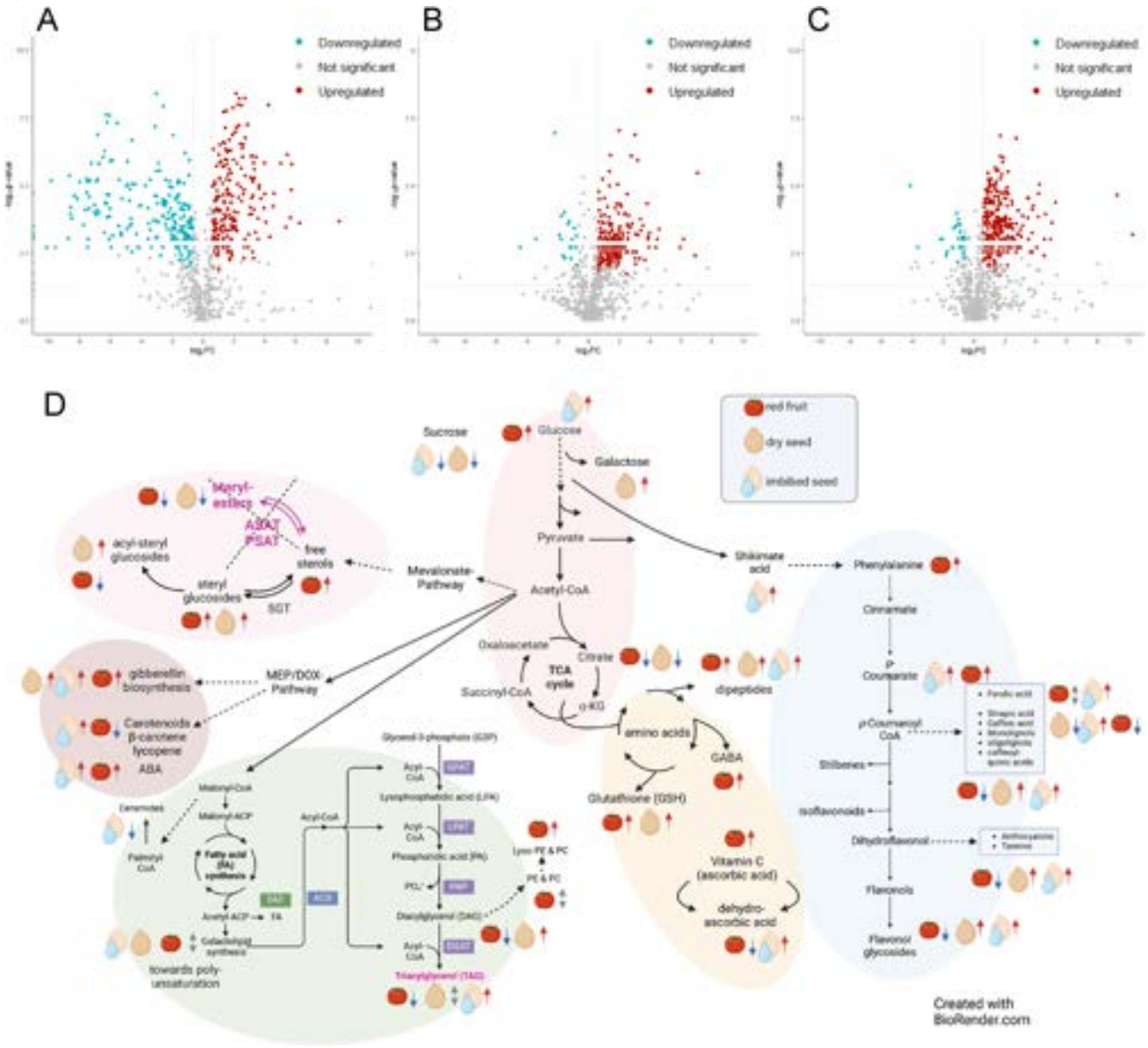

FIGURE 6 Differential metabolite abundance and affected metabolic pathways in fruits and seeds of *slsat1xslpsat1* versus wild type (wt). Volcano plot analysis of metabolites differentially accumulated in red fruit pericarp (A), dry seeds (B) and imbibed seeds (C) of *slsat1xslpsat1* plants normalized against the corresponding wt tissues (log2FoldChange >0.6 and *p*-value <0.05). Metabolites showing non-significant changes are depicted in grey. (D) Schematic representation pathways showing changes in the *slsat1xslpsat1* fruits and seeds compared to the wt. Pathways are based on KEGG.

(Figure 6B and C). This suggests that impaired ES formation favours a certain state of general metabolic activation, particularly in seeds. The metabolic fluxes that are arrested in the dry seeds resume activity upon imbibition (Kazmi et al., 2017), because germinating seeds require the formation of a variety of metabolites needed for cellular growth and differentiation, including the products of the extensive protein breakdown associated with basal autophagy (Iglesias-Fernández et al., 2022). It is thus tempting to speculate that the proposed state of metabolic activation in *slsat1xslpsat1* seeds prior to imbibition is involved in their early germination phenotype (Burciaga-Monge et al., 2022). All in all, the results of this study demonstrate that the disruption of SE biosynthesis leads to a profound rewiring of the tomato primary and specialized metabolome of fruits and seeds, which is moreover qualitatively and quantitatively different (Figure 6D) with the exception of a common induction of autophagy (Figure 5).

3.7 | Fruits of the *slsat1xslpsat1* mutant show enhanced tolerance to *B. cinerea* infection

Ripe fleshy fruits show increased susceptibility to infections by opportunistic pathogens like *B. cinerea* (Cantu et al., 2009). To investigate

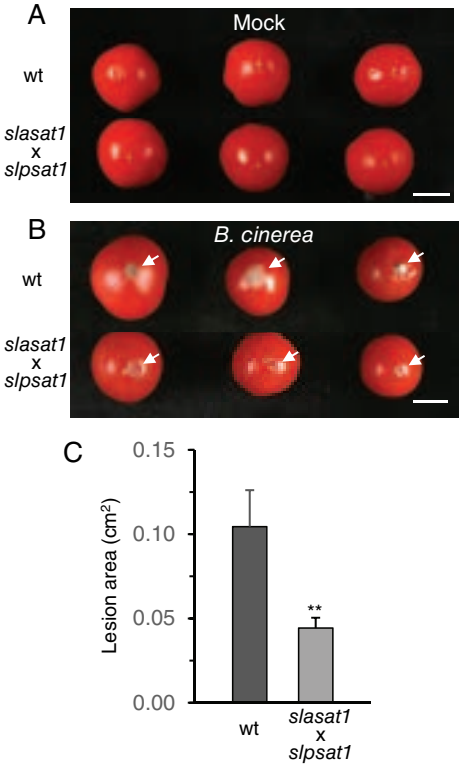

FIGURE 7 Enhanced tolerance of *slasat1xslpsat1* fruits to *Botrytis cinerea* infection. Representative images showing symptoms of infection in fruits of wild type (wt) and *slasat1xslpsat1* fruits that were mock-treated (A) or inoculated with *B. cinerea* spores (B). The images were taken 7 days after treatment. Scale bar = 1 cm. (C) Area of the resulting lesions. Data represent the mean ± SEM of lesion area (white arrows) in 20 fruits. The experiment was carried out twice with similar results. Asterisks indicate values that are significantly different compared to those in the control plants determined by t-test (**$p < 0.01$).

the effect of the *slasat1xslpsat1* mutation on the response to this fungal pathogen, wt and mutant red fruits were inoculated with *B. cinerea* spores, and the size of the resulting lesions was measured 7 days later. The results from two independent experiments showed that the average lesion diameter in the *slpsat1xslasat1* fruits is slightly less than half that in the wt fruits (Figure 7), indicating that the profound metabolic rewiring caused by the impairment of ES biosynthesis in fruits results in reduced fungal growth in comparison to wt. The large and complex metabolic reprogramming in the mutant fruits makes it difficult to attribute this phenotype to changes in a specific group of metabolites, although it is reasonable to speculate that induction of autophagy plays a main role in this response, as there is compelling evidence that autophagy positively modulates plant resistance to necrotrophic fungal pathogens like *B. cinerea* (Hofius et al., 2017; Veloso and van Kan, 2018; Wang et al., 2022). Furthermore, changes in the levels of other metabolites may also contribute to this phenotype. The accumulation of higher levels of glutathione and ascorbic acid and lower levels of dehydroascorbic acid (Figure S5C) points towards enhanced antioxidant capacities that may help to improve pathogen tolerance of the *slasat1xslpsat1* fruits. *B. cinerea* infection leads to an increase of ROS concomitant to a depletion of the antioxidant pool that promotes disease progress (Williamson et al., 2007). Increased levels of antioxidants may limit the oxidative stress associated with infection, thus promoting resistance (van Baarlen et al., 2007). The increase of soluble sugars like sucrose (Figure S5D) may also be involved in the *B. cinerea* tolerance phenotype despite the possible role of these metabolites as carbon nutrients for the pathogen. It is becoming increasingly accepted that sucrose can stimulate immune responses against pathogens, probably by acting as a priming agent for plant innate immunity (Bolouri Moghaddam and Van den Ende, 2012; Morkunas and Ratajczak, 2014). In fact, the accumulation of soluble sugars in tomato fruits does not seem to promote susceptibility to *B. cinerea* infection (Blanco-Ulate et al., 2015). The potential contribution of other metabolites cannot be overlooked either, such as, for instance that of some flavonoids that are among the few with increased levels in the *slasat1xslpsat1* mutant (Figure 4A). Among them, apigenin-7-glucoside was recently identified as a biomarker molecule for pathogen infection in tomato (Singh et al. 2023). Also, the indole alkaloids loganin, secologanin, and tetrahydroalstonine showed increased abundance in the mutant fruits (Figure S5E). The enhanced levels of abscisic acid (ABA) (Figure S5C), a key player along with other hormones in necrotrophic plant-pathogen interactions (Mauch-Mani and Mauch, 2005), would not correlate with the increased tolerance of *slasat1xslpsat1* fruits to *B. cinerea*, as it is generally accepted that ABA promotes susceptibility rather than resistance to fungal infection. However, there is also evidence that ABA can promote basal resistance against *B. cinerea* in tomato (Abuqamar et al., 2009). These contrasting effects may depend on its interaction with other defence signals (Ton et al., 2009) that might be activated in the new metabolic scenario in the *slasat1xslpsat1* mutant fruits. Finally, perturbation of PM lipid levels may alter their geometry and physicochemical properties, as well as the function of membrane proteins whose structure and activity are modulated by the lipid environment (Levental & Lyman, 2023), not to mention the signalling role of many of the altered lipid types (He et al., 2003; Dong et al., 2012; Janda et al., 2013; Michaelson et al., 2016). All the above observations clearly point towards a phenotype that results from a combination of multiple factors acting together.

4 | CONCLUSION

The simultaneous inactivation of the sterol acyltransferases SlPSAT1 and SlASAT1 responsible for tomato ES biosynthesis impacts differentially on the profile of free and glycosylated sterols in fruits and seeds and triggers a number of significant organ-specific qualitative and quantitative shifts in their primary and specialized metabolomes. This is in contrast to the common induction of autophagy observed in both fruits and seeds. The extensive metabolic rewiring due to impaired SE biosynthesis makes it difficult to attribute the phenotypes of early seed germination and enhanced tolerance of ripe fruits to *B. cinerea* infection to changes in specific metabolites or groups of metabolites in these organs. Rather it seems that they are the consequence of the

combined action of multiple metabolic perturbations acting in concert. Overall, the results of this study highlight the crucial role of ES biosynthesis and levels in the maintenance of cellular homeostasis.

AUTHOR CONTRIBUTIONS

Teresa Altabella and Albert Ferrer conceived, managed and supervised the research project. Joan Manel López-Tubau and Alma Burciaga-Monge performed the sterol analyses. Natalie Laibach, Joan Manel López-Tubau and Saleh Alseekh conducted the metabolomics analysis. Cuiyun Deng carried out the *B. cinerea* infection studies. Alisdair R. Fernie supervised the metabolomics analysis. Natalie Laibach, Teresa Altabella and Albert Ferrer wrote the manuscript. All the authors reviewed and approved the manuscript.

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DATA AVAILABILITY STATEMENT

The raw data from metabolomics analysis are stored under the DOI https://doi.org/10.5281/zenodo.13750796. Annotated and processed metabolites can be found in the supplementary dataset. Other relevant data supporting the results of this study are available in the manuscript or can be obtained upon request from the corresponding authors.

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REFERENCES

Abuqamar, S., Luo, H., Laluk, K., Mickelbart, M.V., & Mengiste, T. (2009) Crosstalk between biotic and abiotic stress responses in tomato is mediated by the AIM1 transcription factor. *Plant Journal*, 58, 347–360.

Alseekh, S., Aharoni, A., Brotman, Y., Contrepois, K., D'Auria, J., Ewald, J., Fraser, P.D., Giavalisco, P., Hall, R.D., Heinemann, M., Link, H., Luo, J., Neumann, S., Nielsen, J., Perez de Souza, L., Saito, K., Sauer, U., Schroeder, F.C., Schuster, S., Siuzdak, G., Skirycz, A., Sumner, L.W., Snyder, M.P., Tang, H., Tohge, T., Wang, Y., Wen, W., Wu, S., Xu, G., Zamboni, N. & Fernie, A. R. (2021) Mass spectrometry-based metabolomics: a guide for annotation, quantification and best reporting practices. *Nature Methods*, 18, 747–756.

Alseekh, S., Ofner, I., Liu, Z., Osorio, S., Vallarino, J., Last, R.L., Zamir, D., Tohge, T. & Fernie, A.R. (2020a) Quantitative trait loci analysis of seed-specialized metabolites reveals seed-specific flavonols and differential regulation of glycoalkaloid content in tomato. *Plant Journal*, 103, 2007–2024.

Alseekh, S., Perez de Souza, L., Benina, M., & Fernie, A.R. (2020b) The style and substance of plant flavonoid decoration; towards defining both structure and function. *Phytochemistry* 174: 112347.

Alseekh, S., Tohge, T., Wendenberg, R., Scossa, F., Omranian, N., Li, J., Kleessen, S., Giavalisco, G., Pleban, T., Mueller-Roeber, B., Zamir, D., Nikoloski, Z., & Fernie, A.R. (2015) Identification and mode of inheritance of quantitative trait loci for secondary metabolite abundance in tomato. *The Plant Cell*, 27, 485–512.

Alseekh, S., Zhu, F., Vallarino, J.G., Sokolowska, E.M., Yoshida, T., Bergmann, S., Wendenburg, R., Bolze, A., Skirycz, A., Avin-Wittenberg, T., & Fernie, A.R. (2022) Autophagy modulates the metabolism and growth of tomato fruit during development. *Horticulture Research*. 13, 9:uhac129.

Avila-Ospina, L., Moison, M., Yoshimoto, K., & Masclaux-Daubresse, C. (2014) Autophagy, plant senescence, and nutrient recycling. *Journal of Experimental Botanny*, 65, 3799–3811.

Banas, A., Carlsson, A.S., Huang, B., Lenman, M., Banas, W., Lee, M., Noiriel, A., Benveniste, P., Schaller, H., Bouvier-Navé, P., & Stymne, S. (2005) Cellular sterol ester synthesis in plants is performed by an enzyme (phospholipid:sterol acyltransferase) different from the yeast and mammalian acyl-CoA:sterol acyltransferases. *Journal of Biological Chemistry*, 280, 34626–34634.

Blanco-Ulate, B., Vincenti, E., Cantu, D., & Powell, A.L.T. (2015) Ripening of tomato fruit and susceptibility to *Botrytis cinerea*. In: Fillinger, S., Elad, Y. (eds.) Botrytis–*the Fungus, the Pathogen and its Management in Agricultural Systems*. Springer, Dordrecht, pp. 387–412.

Bolouri Moghaddam, M.R., & Van den Ende, W. (2012) Sugars and plant innate immunity. *Journal of Experimental Botany*, 63, 3989–3998.

Bouchnak, I., Coulon, D., Salis, V., D'Andre'a, S., ' Br'h'lin, C. (2023) Lipid droplets are versatile organelles involved in plant development and plant response to environmental changes. *Frontiers in Plant Science*, 14, 1193905.

Bouvier-Navé, P., Berna, A., Noiriel, A., Compagnon, V., Carlsson, A.S., Banas, A., Stymne, S., & Schaller, H. (2010) Involvement of the phospholipid sterol acyltransferase1 in plant sterol homeostasis and leaf senescence. *Plant Physiology*, 152, 107–119.

Brunetti, C., Guidi, L., Sebastiani, F., & Tattini, M. (2015) Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress. *Environmental and Experimental Botany*, 119, 54–62.

Bulut, M., Wendenburg, R., Bitocchi, E., Bellucci, E., Kroc, M., Gioia, T., Susek, K., Papa, R., Fernie, A.R. & Alseekh, S. (2023) A comprehensive metabolomics and lipidomics atlas for the legumes common bean, chickpea, lentil and lupin. *Plant Journal*, 116, 1152–1171.

Burciaga-Monge, A., López-Tubau, J.M., Laibach, N., Deng, C., Ferrer, A., & Altabella, T. (2022) Effects of impaired steryl ester biosnthesis on tomato growth and developmental processes. *Frontiers in Plant Science*, 13, 984100.

Cantu D., Blanco-Ulate B., Yang L., Labavitch J.M., Bennett A.B., & Powell A.L. (2009) Ripening-regulated susceptibility of tomato fruit to *Botrytis cinerea* requires NOR but not RIN or ethylene. *Plant Physiology*, 150, 1434–1449.

Chávez, A., Castillo, N., López-Tubau, J.M., Atanasov, K.E., Fernández-Crespo, E., Camañes, G., Altabella, T., & Ferrer, A. (2023) Tomato STEROL

GLYCOSYLTRANSFERASE 1 silencing unveils a major role of steryl glycosides in plant and fruit development. *Environmental and Experimental Botany*, 206, 105181.

Chen, Q., Steinhauer, L., Hammerlindl, J., Keller, W., & Zou, J. (2007) Biosynthesis of phytosterol esters: identification of a sterol O-acyltransferase in Arabidopsis. *Plant Physiology*, 145, 974–984.

Dong, W., Lv, H., Xia, G., & Wang, M. (2012) Does diacylglycerol serve as a signaling molecule in plants? *Plant Signaling & Behavior*, 7, 472–475.

Dong, N-Q., & Lin, H-X. (2021) Contribution of phenylpropanoid metabolism to plant development and plant–environment interactions. *Journal of Integrative Plant Biology*, 63, 180–209.

Dyas, L., & Goad, L.J. (1993) Steryl fatty acyl esters in plants. *Phytochemistry*, 34, 17–29.

Duperon, R., Thiersault, M., & Duperon, P. (1984) High level of glycosylated sterols in species of solanum and sterol changes during the development of the tomato. *Phytochemistry* 23, 743–746.

Falcone Ferreyra, M.L., Rius, S.P., & Casati, P. (2012) Flavonoids: Biosynthesis, biological functions, and biotechnological applications. *Frontiers in Plant Science*, 3, 222.

Ferrer, A., Altabella, T., Arró, M., Boronat, A. (2017) Emerging roles for conjugated sterols in plants. *Progress in Lipid Research*. 67, 27–37.

Furt, F., Simon-Plas, F., Mongrand, S. (2010) Lipids of the plant plasma membrane. In: Murphy, A., Schulz, B., Peer, W. (eds) *The Plant Plasma Membrane. Plant Cell Monographs*, vol 19. Springer, Berlin, Heidelberg.

Giavalisco, P., Li, Y., Matthes, A., Eckhardt, A., Hubberten, H. M., Hesse, H., Segu, S., Hummel, J., Köhl, K. & Willmitzer, L. (2011) Elemental formula annotation of polar and lipophilic metabolites using 13C, 15 N and 34S isotope labelling, in combination with high-resolution mass spectrometry. *Plant Journal*, 68, 364–376.

Gondet, L., Bronner, R., & Benveniste, P. (1994) Regulation of sterol content in membranes by subcellular compartmentation of steryl-esters accumulating in a sterol-overproducing tobacco mutant. *Plant Physiology*, 105, 509–518.

Guzha, A., Whitehead, P., Ischebeck, T., & Chapman, K.D. (2023) Lipid droplets: Packing hydrophobic molecules within the aqueous cytoplasm. *Annual Review of Plant Biology*, 74, 195–223.

He, J-X., Fujioka, S., Li, T-C., Kang, S.G., Seto, H., Takatsuto, S., Yoshida, S., & Jang, J-C. (2003) Sterols regulate development and gene expression in Arabidopsis. *Plant Physiology*, 27, 1258–1269.

Hofius, D., Li, L., Hafrén, A., Coll, N.S. (2017) Autophagy as an emerging arena for plant-pathogen interactions. *Current Opinion in Plant Biology*, 38, 117–123.

Hojati, M., Modarres-Sanavy, S.A.M., Ghanati, F., & Panahi, M. (2011) Hexaconazole induces antioxidant protection and apigenin-7-glucoside accumulation in Matricaria chamomilla plants subjected to drought stress. *Journal of Plant Physiology*, 168, 782–791.

Iglesias-Fernández, R., & Vicente-Carbajosa, J.A. (2022) View into seed autophagy: From development to environmental responses. *Plants*, 11, 3247.

Ince, Y.Ç., Krahmer, J., Fiorucci, AS., Fiorucci, A-S., Trevisan, M., Galvão, V.C., Wigger, L., Pradervand, S., Fouillen, L., Van Delft, P., Genva, M., Mongrand, S., Gallart-Ayala, H., Ivanisevic, J., & Fankhauser, C., (2022) A combination of plasma membrane sterol biosynthesis and autophagy is required for shade-induced hypocotyl elongation. *Nature Communications*, 13, 5659.

Janda, M., Planchais, S., Djafi, N., Martinec, J., Burketova, L., Valentova, O., Zachowski, A., & Ruelland, E. (2013) Phosphoglycerolipids are master players in plant hormone signal transduction. *Plant Cell Reports*, 32, 839–851.

Kassambara A (2023) rstatix: Pipe-Friendly Framework for Basic Statistical Tests. *R package version*, 0, 7, 2, https://rpkgs.datanovia.com/rstatix/.

Kazmi, R.H., Willems, L.A.J., Joosen, R.V.L., Khan, N., Ligterink, W. & Hilhorst, H.W.M (2017) Metabolomic analysis of tomato seed germination. *Metabolomics*, 13, 145.

Klee, H.J., & Giovannoni, J.J. (2011) Genetics and control of tomato fruit ripening and quality attributes. *Annual Review of Genetics*, 45, 41–59.

Kolde, R. (2019). Pheatmap: pretty heatmaps. *R package version*, 1, 726.

Kopka, J., Schauer, N., Krueger, S., Birkemeyer, C., Usadel, B., Bergmüller, E., Dörmann, P., Weckwerth, W., Gibon, Y., Stitt, M., Willmitzer, L., Fernie, A.R. & Steinhauser, D. (2005) GMD@CSB. DB: the Golm metabolome database. *Bioinformatics*, 21, 1635–1638.

Korber, M., Klein I., & Daum G. (2017) Steryl ester synthesis, storage and hydrolysis: A contribution to sterol homeostasis. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1862, 1534–1545.

Lara, J.A., Burciaga-Monge, A., Chávez, A., Revés, M., Lavilla, R., Arró, M., Boronat, A., Altabella, T., & Ferrer, A. (2018) Identification and characterization of sterol acyltransferases responsible for steryl ester biosynthesis in tomato. *Frontiers in Plant Science*, 9, 588.

Levental, I., & Lyman, E. (2023) Regulation of membrane protein structure and function by their paralipidomes. *Nature Reviews Molecular Cell Biology*, 24, 107–122.

Lisec, J., Hoffmann, F., Schmitt, C., & Jaeger, C. (2016) Extending the dynamic range in metabolomics experiments by automatic correction of peaks exceeding the detection limit. *Analytical Chemistry*, 88, 7487–7492.

Liu, W., Liu, K., Chen, D., Zhang, Z., Li, B., El-Mogy, M.M., Tian, S., & Chen, T. (2022) *Solanum lycopersicum*, a model plant for the studies in developmental biology, stress biology and food science. *Foods*, 11, 2402.

Manzano, D., Andrade, P., Caudepón, D., Altabella, T., Arró, M., & Ferrer, A. (2016) Suppressing farnesyl diphosphate synthase alters chloroplast development and triggers sterol-dependent induction of jasmonate-and Fe-related responses. *Plant Physiology*, 172, 93–117.

Mauch-Mani, B., & Mauch, F. (2005) The role of abscisic acid in plant-pathogen interactions. *Current Opinion in Plant Biology*, 8, 409–414.

Meléndez-Martínez, A. J., & Mapelli-Brahm, P. (2021) The undercover colorless carotenoids phytoene and phytofluene: Importance in agro-food and health in the Green Deal era and possibilities for innovation. *Trends in Food Science and Technology*, 116, 255–263.

Michaelson, L.V., Napier, J.A., Molino, D., & Faure, J-D. (2016) Plant sphingolipids: Their importance in cellular organization and adaption. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1861, 1329–1335.

Mongrand, S., Stanislas, T., Bayer, E.M.F., Lherminier, J., & Simon-Plas, F. (2010) Membrane rafts in plant cells. *Trends in Plant Science*, 15, 656–663.

Moreau, R.A., Whitaker, B.D., & Hicks, K.B. (2002) Phytosterols, phytostanols, and their conjugates in foods: structural diversity, quantitative analysis, and health-promoting uses. *Progress in Lipid Research*. 41, 457–500.

Morkunas, I., & Ratajczak, L. (2014) The role of sugar signaling in plant defense responses against fungal pathogens. *Acta Physiologiae Plantarum*, 36, 1607–1619.

Nyström, L., Schär, A., & Lampi, A-M. (2012) Steryl glycosides and acylated steryl glycosides in plant foods reflect unique sterol patterns. *European Journal of Lipid Science & Technology*, 114, 656–669.

Otify, A. M., Ibrahim, R. M., Abib, B., Laub, A., Wessjohann, L. A., Jiang, Y., & Farag, M.A. (2023) Unveiling metabolome heterogeneity and new chemicals in 7 tomato varieties via multiplex approach of UHPLC–MS/MS, GC–MS, and UV–Vis in relation to antioxidant effects as analyzed using molecular networking and chemometrics. *Food Chemistry*, 417, 135866.

Palta, J.P., Whitaker, B.D., & Weiss, L.S. (1993) Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of Solanum species. *Plant Physiology*, 103, 793–803.

Ramaroson, M.-L., Koutouan, C., Helesbeux, J.-J., LeClerc, V., Hamama, L., Geoffriau, E.; & Briard, M. (2022) Role of phenylpropanoids and flavonoids in plant resistance to pests and diseases. *Molecules*, 27, 8371.

Ramírez-Estrada, K., Castillo, N., Lara, J. A., Arró, M., Boronat, A., Ferrer, A. & Altabella, T. (2017) Tomato UDP-glucose sterol glycosyltransferases: A family of developmental and stress regulated genes

that encode cytosolic and membrane-associated forms of the enzyme. *Frontiers in Plant Science*, 8, 984.

Rodríguez-Concepción, M., Avalos, J., Bonet, M.L., Boronat, A., Gómez-Gómez, L., Hornero-Mendez, D., Limón, M.C., Meléndez-Martínez, A.J., Olmedilla-Alonso, B., Palou, A., Ribot, J., Rodrigo, M.J., Zacarías, L., and Zhu, C.A. (2018) global perspective on carotenoids: Metabolism, biotechnology, and benefits for nutrition and health. *Progress in Lipid Research*, 70, 62–93.

Salem, M.A., Perez de Souza, L., Serag, A., Fernie, A.R., Farag, M.A., Ezzat, S.M., & Alseekh, S. (2020) Metabolomics in the context of plant natural products research: From sample preparation to metabolite analysis. *Metabolites*, 10, 37.

Schaller, H., Crausem, B., Benveniste, P., Chye, M., Tan, C., Song, Y. & Chua, N.H. (1995) Expression of the *Hevea brasiliensis* (H. B. K.) Müll. Arg. tobacco results in sterol overproduction. *Plant Physiology*, 109, 761–770.

Sievert, C. (2020) *Interactive Web-based data visualization with R, plotly, and shiny*. Chapman and Hall/CRC. ISBN 9781138331457, https://plotly-r.com.

Singh, D.P., Maurya, S., Yerasu, S.R., Bisen, M.S., Farag, M.A., Prabha, R., Shukla, R., Chaturvedi, K.K., Farooqi, M.S., Srivastava, S., Rai, A., Sarma, B.K., Rai, N., & Behera, T.K. (2023) Metabolomics of early blight (*Alternaria solani*) susceptible tomato (*Solanum lycopersicum*) unfolds key biomarker metabolites and involved metabolic pathways. *Scientific Reports*, 13, 21023.

Shimada, T.L., Shimada, T., Okazaki, Y., Higashi, Y., Saito, K., Kuwata, K., Oyama, K., Kato, M., Ueda, H., Nakano, A., Ueda, T., Takano, Y & Hara-Nishimura, I. (2019) HIGH STEROL ESTER 1 is a key factor in plant sterol homeostasis. *Nature Plants*, 5, 1154–1166.

Shimada, T.L., Yamaguchi, K., Shigenobu, S., Takahashi, H., Murase, M., Fukuyoshi, S., & Hara-Nishimura, I. (2020) Excess sterols disrupt plant cellular activity by inducing stress-responsive gene expression. *Journal of Plant Research*, 13, 383–392.

Thirumalaikumar, V.P., Wagner, M., Balazadeh, S., & Skirycz, A. (2021) Autophagy is responsible for the accumulation of proteogenic dipeptides in response to heat stress in Arabidopsis thaliana. *FEBS Journal*, 288, 281–292.

Tohge, T., & Fernie, A.R. (2015) Metabolomics-inspired insight into developmental, environmental and genetic aspects of tomato fruit chemical composition and quality. *Plant and Cell Physioly*, 56, 1681–1696.

Ton, J., Flors, V., & Mauch-Mani, B. (2009) The multifaceted role of ABA in disease resistance. *Trends in Plant Science*, 14, 310–317.

van Baarlen, P., Legendre, L., & Kan, J.L. (2007) Plant defence compounds against *Botrytis* infection. In: Elad, Y., Williamson, B., Tudzynski, P., Delen, N. (eds.) Botrytis*: Biology, Pathology and Control*. Springer, Dordrecht, pp. 143–161.

Veloso, J., & van Kan, J.A.L. (2018) Many shades of grey in *Botrytis*-host plant interactions. *Trends in Plan Science*, 23, 613–622.

Wang, S., Hu, W., & Liu, F. (2022) Autophagy in the lifetime of plants: from seed to seed. *International Journal of Molecular Sciences*, 23, 11410.

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T.L., Miller, E., Bache, S.M., Müller, K., Ooms, J., Robinson, D., Seidel, D.P., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., & Yutani, H. (2019) "Welcome to the tidyverse." *Journal of Open Source Software*, 4, 1686.

Wilkinson, S.C., Powls, R., & Goad, L.J. (1994) The effects of excess exogenous mevalonic acid on sterol and steryl ester biosynthesis in celery (*Apium graveolens*) cell suspension cultures. *Phytochemistry*, 37, 1031–1035.

Williamson, B., Tudzynski, B., Tudzynski, P., & van Kan J.A.L. (2007) *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, 8, 561–580.

Whitaker, B.D. (1988) Changes in the steryl lipid content and composition of tomato fruit during ripening. *Phytochemistry*, 27, 3411–3416.

Yu, M., Cui, Y., Zhang, X., Li, R., & Lin J. (2020) Organization and dynamics of functional plant membrane microdomains. *Cellular and Molecular Life Sciences*, 77, 275–287.

Yu, L., Fan, J., Zhou, C., & Xu, C., (2021) Sterols are required for the coordinated assembly of lipid droplets in developing seeds. *Nature Communications*, 12, 5598.

Zhou, X., Chen, X., Du, Z., Zhang, Y., Zhang, W., Kong, X., Thelen, J.J., Chen, C., & Chen, M. (2019). Terpenoid esters are the major constituents from leaf lipid droplets of camellia sinensis. *Frontiers in Plant Science*, 10, 179.

Zhu, F., Wen, W., Cheng, Y., & Fernie, A.R, (2022) The metabolic changes that effect fruit quality during tomato fruit ripening. *Molecular Horticulture*, 2, 2.

SUPPORTING INFORMATION

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

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