

Glycosylated sterols enhance cold tolerance in tomato via membrane stabilization and jasmonate signaling

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Abstract

Free and glycosylated sterols play a central role in maintaining the structural integrity and proper functioning of the plasma membrane, which serves as the primary sensor of cold and initiates signaling cascades to mitigate chilling-induced damage. Here, we characterize the cold-response of tomato (*Solanum lycopersicum* cv. Micro-Tom) mutants with higher and lower ratios of glycosylated to free sterols than wild-type plants, resulting from the overexpression and silencing of the STEROL GLYCOSYLTRANSFERASES 1 and 2 (SLSGT2 and SLSGT1), respectively. The SLSGT2 overexpressing mutants show increased cold tolerance, membrane stability, and oxidative stress responses, while silencing of the SLSGT1 gene causes the opposite phenotypes. Furthermore, changes in the glycosylated to free sterols ratio activate distinct transcriptional programs that establish a preconditioned stress-responsive state under basal conditions and trigger a more efficient response to cold in the SLSGT2 overexpressing mutants, as well as compromise the capacity to withstand the effects of cold stress in the SLSGT1-silenced mutants. The SLSGT2 overexpressing mutants also contain higher levels of jasmonates under basal conditions and display enhanced biosynthesis of these hormones under cold stress compared to SLSGT1-silenced and control plants. The facilitating effect of elevated glycosylated to free sterols ratio on jasmonic acid (JA) biosynthesis and signaling leads to the activation of cold-responsive genes, including those of the CBF-COR pathway, antioxidant defenses, and polyamine biosynthesis. Our findings provide key insights into the mechanisms by which glycosylated sterols help improve cold tolerance in tomato.

Introduction

The plasma membrane (PM) protects cells from external challenges and mediates their communication with the environment by controlling the exchange of metabolites and information between the cytoplasm and the extracellular space. Sterols, in addition to sphingolipids and phospholipids, play a central role in maintaining the structural integrity and proper functioning of this key cellular structure (Mamode Cassim et al. 2019). Plants contain complex mixtures of sterols (phytosterols) that can be present in free form (FS) and conjugated with sugars and/or fatty acid groups, yielding steryl glycosides (SG), acylated steryl glycosides (ASG), and steryl esters (SE). SG contain a sugar moiety, usually a single glucose residue, linked through a glycosidic bond to the hydroxyl group at C3 of the FS backbone, ASG have a long-chain fatty acid group esterified to the hydroxyl group at C6 of the sugar unit, and SE carry a fatty acid group esterified directly to the free hydroxyl group of FS. The different plant species have specific sterol profiles including a variety of minor biosynthetic intermediates and a few major end-products, being β -sitosterol, stigmasterol, and campesterol and their conjugated forms the most common and

abundant ones, although in some plant families, including the *Solanaceae*, substantial amounts of free and conjugated cholesterol are also found. In most plants, FS are the predominant form of sterols, but in some *Solanaceae* species, such as tomato (*Solanum lycopersicum*), glycosylated sterols (GS), the sum of SG and ASG, accumulate to very high levels, representing the predominant form of sterols. SG and SE are synthesized from FS by UDP-GLUCOSE:STEROL GLYCOSYLTRANSFERASES (SGTs) and the acyltransferases PHOSPHOLIPID:STEROL ACYLTRANSFERASE (PSAT) and ACYL-COA:STEROL ACYLTRANSFERASE (ASAT), respectively, while the acyltransferase(s) that synthesize ASG have not been identified yet (Ferrer et al. 2017).

FS and GS localize primarily to the PM, clustering along with sphingolipids and proteins in the so-called lipid rafts. These dynamic and highly ordered nanoscale assemblies of lipids and proteins play important roles in membrane-based signaling processes (Mongrand et al. 2010). There is convincing evidence that the major phytosterols show different capacities to modulate PM organization and physical properties, such as thickness, fluidity, flexibility, and permeability, due to their different chemical

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structures and whether they are glycosylated or not (Grosjean et al. 2015). Changes in the total content and/or the relative proportions of GS to FS (GS/FS ratio) alter PM functioning and, ultimately, affect cellular processes involved in controlling plant growth and development (Ferrer et al. 2017; Chávez et al. 2023), and responses to biotic (Mishra et al. 2017; Castillo et al. 2019) and abiotic stress, including temperature stress (Rogowska and Szakiel 2020). Reduced SGT activity in *Arabidopsis* and *Withania somnifera* increases sensitivity to heat and cold stress, while overexpression of SGT genes in these species improves tolerance to various abiotic stresses, including cold (Ferrer et al. 2017). On the other hand, thermal stress-induced expression of SGT genes has also been reported, along with cold-associated increases in GS in several species (Palta et al. 1993; Takahashi et al. 2016; Khan et al. 2024).

Cold stress, including chilling (0–12 °C) and freezing (<0 °C) temperatures, inhibits plant growth and development, reduces crop productivity, and limits geographical distribution, particularly in plant species from tropical or sub-tropical origin, such as tomato (Chinnusamy et al. 2007). Cold stress reduces membrane fluidity, induces oxidative stress via reactive oxygen species (ROS) accumulation, and leads to photoinhibition due to imbalanced photosynthetic electron flow (Ruelland et al. 2009). Plants mitigate these effects through complex protective strategies, including large-scale transcriptional reprogramming of COLD-RESPONSIVE (COR) regulatory and functional genes (Ding et al. 2024). Among the best-characterized cold-regulatory pathways is the CBF–COR cascade. C-REPEAT BINDING FACTORS (CBFs), also known as DEHYDRATION RESPONSIVE ELEMENT BINDING FACTOR 1 proteins (DREB1), have been identified in several plant species, including tomato (Shi et al. 2018). These genes are rapidly induced by low temperatures and activate a set of downstream COR genes, collectively known as the CBF regulon, which promotes cold acclimation and tolerance (Shi et al. 2018). Dehydrins are well-characterized COR proteins that stabilize cellular structures during stress; in tomato, *DEHYDRIN C17 HOMOLOG* (*SlDRC17*) is one of the best-known dehydrin genes within the CBF regulon (Weiss and Egea-Cortines 2009).

Phytohormones also modulate col-stress responses. Low temperatures modify the levels of abscisic acid (ABA), ethylene (ET), gibberellins (GA), and jasmonates (JAs), all of which have been associated with cold acclimation (Shi et al. 2015). JAs, including jasmonic acid (JA), methyl jasmonate (MeJA), JA-isoleucine (JA-Ile), and 12-OHJA, have emerged as key regulators of cold tolerance. Cold stress increases JA levels, and exogenous JA application improves chilling tolerance in *Arabidopsis*, tomato, and other species (Ding et al. 2024). In JA signaling, JASMONATE-ZIM-DOMAIN (JAZ) proteins, the SKP1–CULLIN–F-BOX COMPLEX CONTAINING CO11 (SCF^{CO11}) receptor complex, and MYELOCYTOMATOSIS 2 (MYC2) transcription factor form a core module (Chini et al. 2007; Thines et al. 2007; Fonseca et al. 2009; Fernández-Calvo et al. 2011). In the absence of JA-Ile, JAZ repress MYC2 activity, but upon JA-Ile accumulation, JAZ are targeted for degradation, releasing MYC2 and activating downstream genes, including JAZ family members, in a negative feedback loop (Pauwels and Goossens 2011; Chini et al. 2016). JA-mediated cold protection has been reported in *Arabidopsis* and other species, and may occur through direct MYC2-regulated protective gene expression or through indirect interaction of JAZ with the INDUCER OF CBF EXPRESSION 1 (ICE1) (Zhao et al. 2013; An et al. 2021). In tomato, JA also helps to mitigate oxidative damage by activating putrescine biosynthesis, glutathione S-transferase (GST) activity, and through interaction with melatonin and ET to modulate cold responses (Ding et al. 2021, 2022a, 2022b, 2022c).

Despite extensive knowledge of different cold-response pathways, the role of PM sterol composition in regulating transcriptional and hormonal cold responses remains largely unexplored. In this study, we investigate the effects of changes in the PM sterol profile, namely contrasting GS/FS ratios, on cold tolerance and the molecular mechanisms behind the observed phenotypes, using tomato (cv Micro-Tom) mutants generated by overexpressing and silencing of *SlSGT2* and *SlSGT1*, respectively. Our findings demonstrate that a high GS/FS ratio promotes JA biosynthesis and JAZ gene expression, which in turn activates the CBF regulon. This signaling cascade facilitates key physiological and metabolic adaptations, including stabilization of the photosynthetic machinery, increased antioxidant enzyme activity, and polyamine accumulation, that collectively result in enhancing chilling tolerance.

Results

Differential impact of contrasting membrane sterol profiles on tomato cold tolerance

Due to its tropical origin, tomato is a chilling-sensitive plant species. To investigate the effects of contrasting profiles of membrane sterols on the tolerance of tomato plants to low temperature, we used the *SlSGT1*-silenced (*SGT1^{ami}*) lines previously reported (Chávez et al. 2023) and two newly generated transgenic lines constitutively expressing the *SlSGT2* isozyme (*SGT2^{ox}-1* and *SGT2^{ox}-4*). These two lines accumulate higher levels of *SlSGT2* mRNA than the WT (5.3- and 7.5-fold, respectively) (Supplementary Fig. S1), which leads to higher levels of SG and ASG (~1.6- and ~1.3-fold, respectively) and lower levels of FS (~0.75-fold) compared to WT under basal (non-stress) conditions (Fig. 1A). This contrasts with the consistent lower levels of SG (~0.65-fold) and higher levels of FS (~1.6 to 2-fold) found in *SGT1^{ami}* lines (Fig. 1B) (Chávez et al. 2023). Interestingly, neither *SGT1^{ami}* nor *SGT2^{ox}* plants showed remarkable differences in SE levels compared to WT (Fig. 1). Despite the imbalance in the relative proportions of membrane sterols caused by *SlSGT2* overexpression (Supplementary Table S1), under standard greenhouse conditions, these plants were indistinguishable from WT plants in contrast to the dwarfism displayed by *SGT1^{ami}* plants (Fig. 2A) (Chávez et al. 2023).

To investigate the contribution of GS to cold-stress tolerance, we exposed 4-week-old *SGT1^{ami}*, *SGT2^{ox}*, and WT tomato plants to 4 °C for 4 days. Under control conditions (25 °C), no obvious differences in wilting or relative electrolyte leakage (REL), an indicator of membrane damage, were observed between genotypes. However, after chilling stress, *SGT2^{ox}* plants showed fewer symptoms of wilting and presented lower REL than WT plants, while *SGT1^{ami}* plants displayed stronger wilting and higher REL than the WT (Fig. 2, A and B). The differences in cold-induced injury symptoms between WT and mutant plants became even more evident after recovery from cold treatment (4 days at 25 °C) (Supplementary Fig. S2B). These results demonstrated that *SGT2^{ox}* plants are more cold-tolerant than WT, unlike *SGT1^{ami}* plants that show an enhanced cold-sensitive phenotype. Similar contrasting effects on chlorophyll content and the maximum quantum efficiency of photosystem II (PSII) (Fv/Fm), a parameter for determining the inhibition of PSII induced by cold, were observed in the mutant genotypes. Indeed, the chlorophyll content reduction in response to cold treatment was less pronounced in *SGT2^{ox}* lines than in WT plants, but higher in *SGT1^{ami}* lines (Supplementary Fig. S2A), and a similar trend was observed for the loss of Fv/Fm (Fig. 2, C and D). These observations support

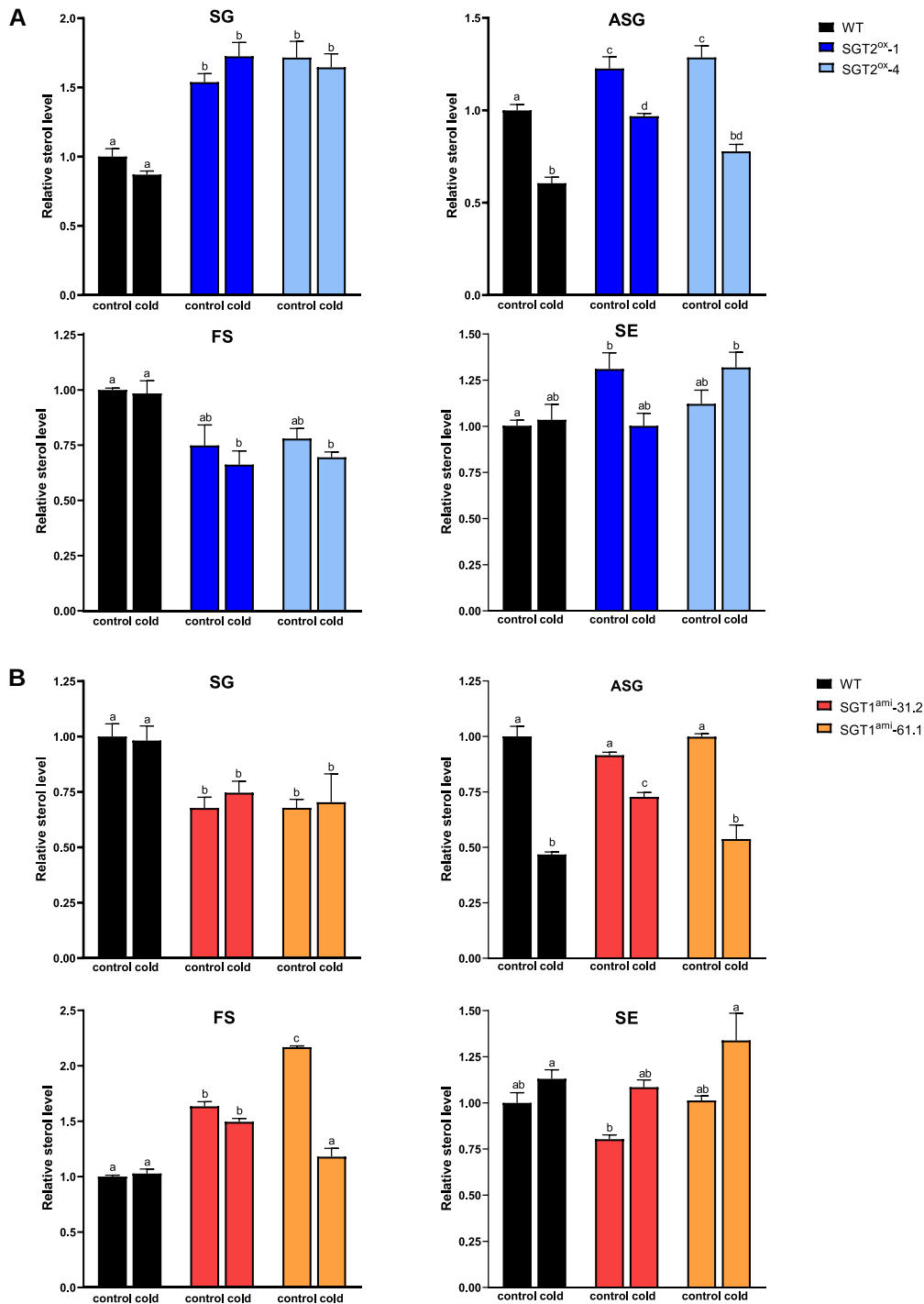


Figure 1. Free and conjugated sterol levels in leaves of SGT2^{ox}, SGT1^{ami}, and WT. The total contents of steryl glycosides (SG), acylated steryl glycosides (ASG), free sterols (FS) and steryl esters (SE), including cholesterol, campesterol, stigmasterol and β -sitosterol, were measured in leaves of WT, SGT2^{ox} (A) and SGT1^{ami} (B) plants grown at 25 °C (control) or after 48 h at 4 °C (cold). Values are expressed relative to the levels in WT plants grown under control conditions, which were assigned a value of 1. Data are presented as mean $\pm$ standard error of the mean (SEM) from 3 independent experiments per genotype, with each experiment consisting of 3 technical replicates. Different letters (A to D) indicate significant differences (adjusted $P < 0.05$) according one-way ANOVA with Tukey's post hoc test.

the idea that changes in the content of GS modulate the reduction of photosynthetic capacity induced by cold stress. The expression profile during chilling stress of the two well-known tomato cold-responsive genes *SlCBF1* and *SlDRC7* is consistent with the cold-sensitive and cold-tolerant phenotypes of SGT1^{ami} and SGT2^{ox} plants, respectively. As expected, the expression of both genes was upregulated by cold in both WT and mutant plants

but at different time points. *SlCBF1* transcript levels reached a maximum 6 h after cold exposure, except in SGT1^{ami} 61.1 line where maximal induction was observed after 12 h (Fig. 3, A and B), while the highest expression of *SlDRC7* was detected 24 h after cold treatment (Fig. 3, C and D). Interestingly, the mRNA levels of both genes were substantially higher in SGT2^{ox} plants than in the WT (Fig. 3, A and C), while in SGT1^{ami} plants the *SlCBF1*

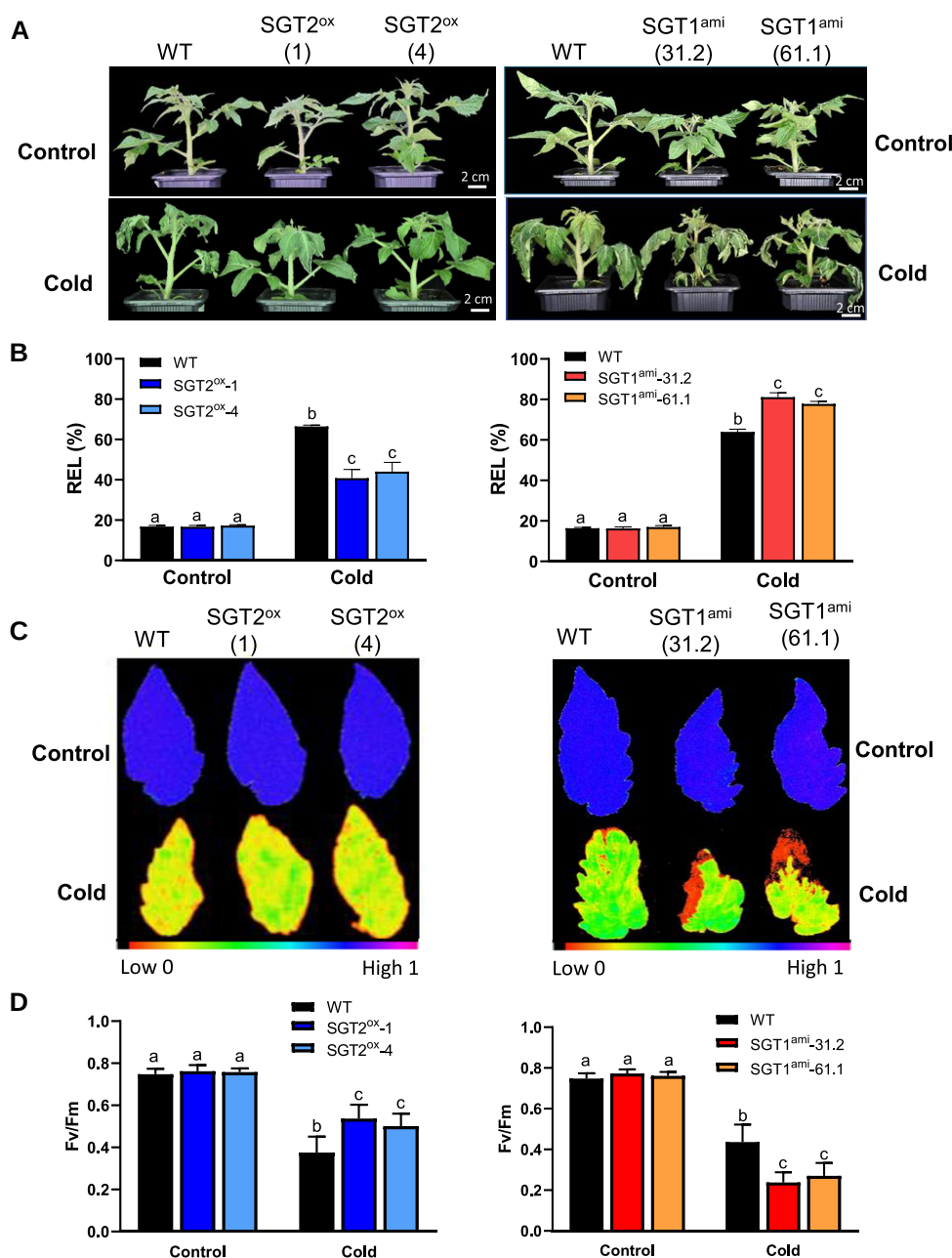


Figure 2. Glycosylated sterols regulate the tomato cold-stress tolerance. Phenotypes (A), changes in relative electrolyte leakage (REL) (B), and maximum photochemical efficiency of PSII (Fv/Fm) (C, D) in 4-week-old WT, SGT2^{ox} (lines 1 and 4) and SGT1^{ami} (lines 31.2 and 61.1) plants grown at 25 °C (control) or at 4 °C (cold) for 4 days. The false color code at the bottom of images in (C) ranging from 0 (black) to 1 (purple), represents the damage in the leaves. In panels A and C, images were digitally extracted for comparison. REL, data are presented as the mean $\pm$ SD from 4 biological replicates per genotype. Fv/Fm values are the mean of 20 leaves from independent plants for each genotype. Different letters (A to C) indicate significant differences (adjusted $P < 0.05$) according to one-way ANOVA with Tukey's post hoc test.

transcript levels were clearly reduced compared to WT (Fig. 3B), and almost no differences were observed in *SlDRC7* transcript levels (Fig. 3D).

To elucidate the reasons underlying the differential response of SGT1^{ami} and SGT2^{ox} plants to cold stress, we quantified the levels of free and conjugated sterols in leaves of SGT1^{ami}, SGT2^{ox}, and WT plants after 48 h of exposure to 4 °C. As shown in Fig. 1, cold treatment has no significant effects on SG levels, neither in WT nor in the transgenic plants, compared to the corresponding control plants. This is in contrast with the marked reduction in ASG levels observed in cold-treated SGT2^{ox}, SGT1^{ami}, and WT plants compared to their non-treated counterparts. No cold-dependent

changes in FS levels were observed either, except for line SGT1^{ami}-61.1. Taken together, these results strongly suggest that the different sensitivity of SGT2^{ox} and SGT1^{ami} plants to cold is due to the specific changes in the profile of membrane sterols caused by the altered levels of *SlSGT2* and *SlSGT1*, rather than to a different capacity of these plants to adjust their sterol levels during cold acclimation. In fact, the relative proportions between GS and FS remain quite stable after cold treatment in both transgenic genotypes, in contrast to the reduction observed in the WT (Supplementary Table S1). Furthermore, the sterol profiling data indicate that increased levels of GS at the expense of FS protect plants against cold stress, and therefore, that proper sterol

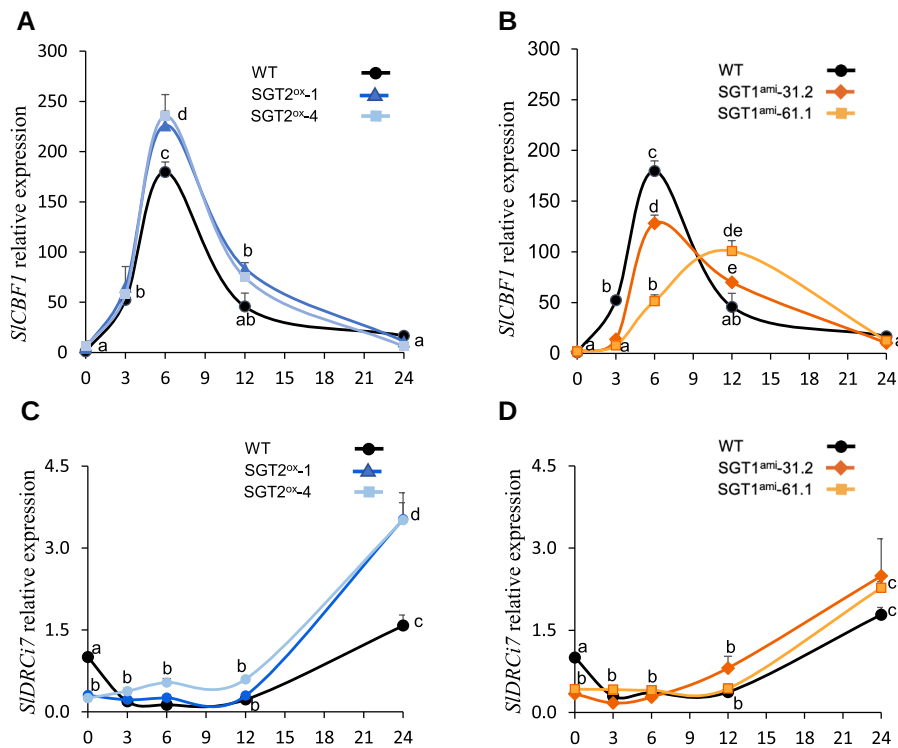


Figure 3. Time-course expression analysis of cold-response marker genes. Expression of *SLCBF1* (A, B) and *SIDRCi7* (C, D) was determined by RT-qPCR in WT, *SGT2^{ox}* (A, C), and *SGT1^{ami}* (B, D) plants exposed to cold. RT-qPCR was performed using RNA samples from leaves of 4-week-old plants collected after cold exposure (4 °C) for 0, 3, 6, 12, and 24 h, and the primer pairs shown in [Supplementary Table S6](#). The mRNA levels of the *ACTIN* gene were used to normalize transcript levels. Data are presented as mean $\pm$ SEM from 3 independent experiments per genotype, with each experiment consisting of 3 technical replicates. Different letters (A to E) indicate significant differences (adjusted $P < 0.05$) according to one-way ANOVA with Tukey's post hoc test.

composition of cell membranes is critical to acclimate tomato plants to cold conditions.

The imbalance of membrane sterols alters the response to cold-induced oxidative damage

Cold stress causes oxidative damage by generating reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide radicals (O_2^-). To investigate whether differences in sterol composition between *SGT2^{ox}* and *SGT1^{ami}* transgenic lines affect their redox status and ultimately their susceptibility or resilience to low temperature, we analyzed the contents of H_2O_2 and O_2^- in the leaves of WT and mutant plants after 4 d of cold treatment using 3,3'-diaminobenzidine (DAB) (Fig. 4A) and nitroblue tetrazolium (NBT) (Fig. 4B) staining, respectively. Under control conditions, WT and mutant plants exhibited similar basal levels of H_2O_2 and O_2^- . However, accumulation of both ROS species induced by cold was more evident in *SGT1^{ami}* plants than in WT, while the opposite pattern was observed in *SGT2^{ox}* plants (Fig. 4, A and B). Given that membrane lipid peroxidation is one of the most evident symptoms of oxidative stress in plants, we also determined the levels of malondialdehyde (MDA), the final product of membrane lipid peroxidation. After cold treatment, MDA content increased ~2-fold in WT plants while no significant differences in MDA levels were observed in the *SGT2^{ox}* lines. This is in sharp contrast to the 3.5- to 4.0-fold increase detected in the *SGT1^{ami}* lines, which almost doubled that in cold-treated WT plants (Fig. 4C). These results confirmed that cold stress causes much more severe oxidative damage in plants with reduced levels of GS (*SGT1^{ami}*) than in

WT plants, while plants with higher GS levels (*SGT2^{ox}*) exhibit reduced oxidative damage.

The complex antioxidant system evolved by plants to scavenge excessive ROS accumulation includes the activation of ROS-detoxifying enzymes such as GST, catalase (CAT), and peroxidase (POD). After cold treatment for 48 h, the activity of these enzymes, particularly CAT and POD, increased to a higher extent in *SGT2^{ox}* than in WT plants compared to plants growing under control conditions (Fig. 5). However, *SGT1^{ami}* lines showed no significant increase in GST and CAT activities after cold treatment, being their levels similar to those measured in WT plants under control conditions (Fig. 5, A and B), and although POD activity increased slightly in response to cold, the activities of these three antioxidant enzymes remained clearly below those in *SGT2^{ox}* lines (Fig. 5C). Thus, the results of antioxidant enzyme activity measurements are fully consistent with the levels of ROS, since in both mutant genotypes there is a clear negative correspondence between these two biochemical parameters after cold treatment. Taken together, these findings indicate that changes in tomato membrane sterol composition modulate redox status under cold conditions, which may explain, at least in part, the contrasting response of *SGT1^{ami}* and *SGT2^{ox}* plants when exposed to this abiotic stress.

Transcriptome-wide analysis of *SGT1^{ami}* and *SGT2^{ox}* plants uncovers antagonistic acclimation states to cold treatment

To gain deeper insight into the molecular mechanisms underlying the effect of altered levels of GS on the response to low temperature in tomato, an RNA-seq profiling was conducted using RNA

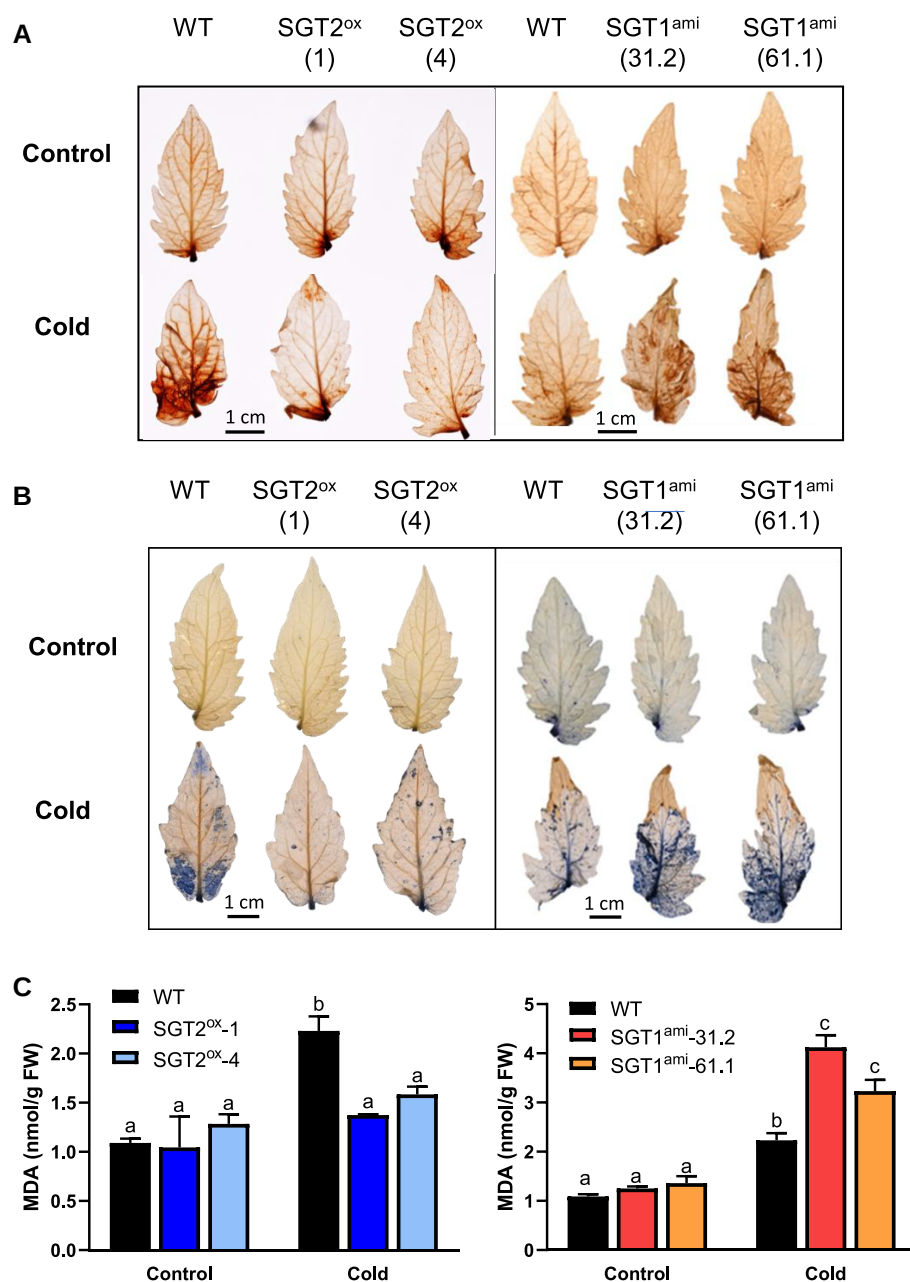


Figure 4. Oxidative damage induced by cold in SGT2^{ox}, SGT1^{ami}, and WT plants. Accumulation of H₂O₂ (A) and O₂⁻ (B) detected by staining with 3,3'-diaminobenzidine (DAB) or nitroblue tetrazolium (NBT), respectively, in leaves from 4-week-old WT, SGT2^{ox}, and SGT1^{ami} plants grown at 25 °C (control) or at 4 °C (cold) for 4 days. Images were digitally extracted for comparison. Malondialdehyde (MDA) content was determined in leaves of the same plants (C). Data are presented as the mean ± SD from 4 biological replicates. Different letters (A to C) indicate significant differences (adjusted $P < 0.05$) according to one-way ANOVA with Tukey's post hoc test.

from leaves of WT and one representative line of each mutant genotype, namely SGT2^{ox}-1 and SGT1^{ami}-61.1, harvested prior (0 h) and at 3 and 24 h of cold-stress treatment. First, the basal impact of the different sterol profiles on the transcriptome expression was evaluated. To this end, RNA-seq datasets of plants before cold exposure were filtered for transcripts exhibiting a significant absolute log₂ fold-change (FC) ≥ 1 relative to WT (adjusted $P \leq 0.05$, Wald's test) to identify differentially expressed genes (DEGs). SGT2^{ox} plants displayed approximately two-fold more DEGs than SGT1^{ami} plants (820 vs. 423), with 142 of these genes common to both genotypes (Fig. 6A, Supplementary Table S2). Functional annotation based on Gene Ontology (GO) term enrichment analysis (fold-enrichment ≥ 2 , adjusted $P \leq 0.05$, Fisher's

exact test) linked the SGT2^{ox}-DEGs to general stress-related processes, including "JA signaling," "response to wounding," "defense response," and "glutathione metabolism." Conversely, DEGs in SGT1^{ami} plants did not show any significant enrichment in GO terms (Fig. 6A, Supplementary Table S3). All these observations indicate that SGT2^{ox} plants exhibit a preconditioned stress-responsive state in the absence of external stimuli, whereas SGT1^{ami} plants do not.

Next, to capture the major gene expression trajectories during cold exposure, transcripts exhibiting significant changes between genotypes during the time-course (adjusted $P \leq 0.05$, likelihood ratio test) were used to generate heatmaps with hierarchical clustering (Fig. 6, B and C). Unsupervised k -means clustering was

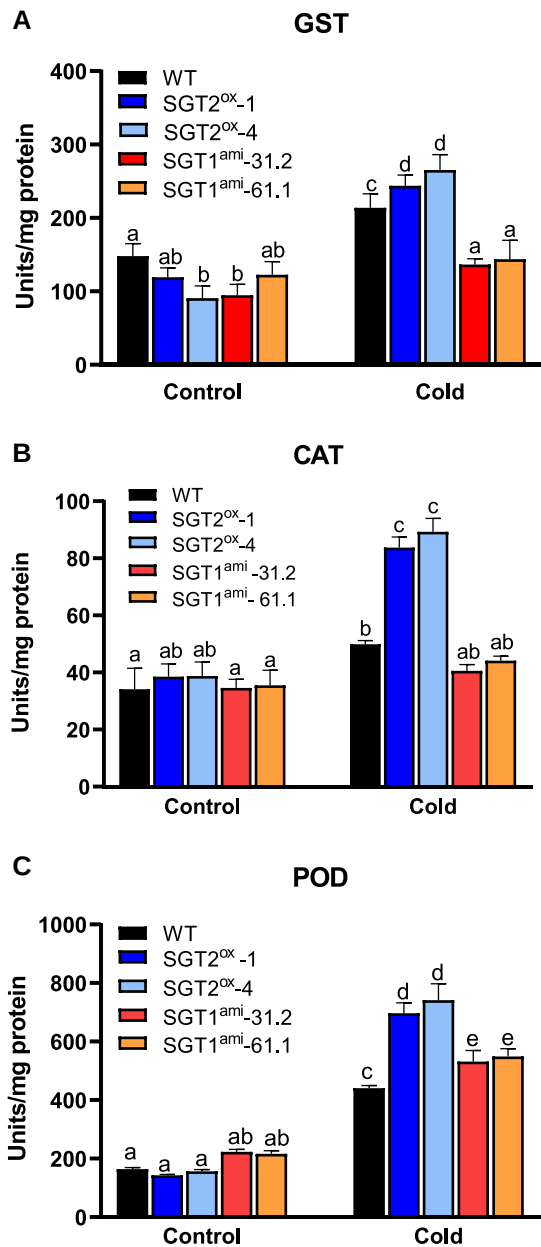


Figure 5. Activity of ROS scavenging enzymes in SGT2^{ox}, SGT1^{ami}, and WT plants under cold stress. The activity of antioxidant enzymes glutathione S-transferase (GST) (A), peroxidase (POD) (B) and catalase (CAT) (C) was measured in leaves from 4-week-old WT, SGT2^{ox}, and SGT1^{ami} plants grown at 25 °C (control) or at 4 °C (cold) for 48 h. Data are presented as the mean $\pm$ SD from 4 biological replicates per genotype ($n = 4$). Different letters (a to e) indicate significant differences (adjusted $P < 0.05$) according to one-way ANOVA with Tukey's post hoc test.

employed to identify core-sets of transcripts displaying similar expression patterns over time (Supplementary Fig. S3 and Supplementary Table S4), which were subsequently annotated for functionality according to GO terms (Table 1 and Supplementary Table S5). Eight transcript clusters exhibited decreasing steady-state levels in both WT and the transgenic lines after 24 h of cold exposure, albeit with different dynamics (Supplementary Fig. S3). Clusters 4 and 6 in SGT2^{ox} plants, and 1 and 4 in SGT1^{ami} plants accumulated lower levels of transcripts in the WT compared to the transgenic lines, contrary to clusters 5 and 7 in SGT2^{ox} plants, and 5 and 6 in SGT1^{ami} plants, in which

WT plants accumulated higher levels of transcripts compared to the transgenic lines. Conversely, we identified four clusters of transcripts, namely clusters 1 and 2 in SGT2^{ox} plants, and 2 and 3 in SGT1^{ami} plants, whose steady-state levels tended to increase at 24 h in both transgenic lines, also with different dynamics and more pronounced accumulation in WT plants (Supplementary Fig. S3). Interestingly, cluster 3 in the SGT2^{ox} mutant includes the largest number of DEGs, which, in addition, accumulate at higher levels in the mutant compared to WT after 24 h of cold treatment. Moreover, transcripts in this cluster are enriched in GO terms for biological processes linked somehow to the cold-tolerant response exhibited by the SGT2^{ox} mutant, namely “response to wounding,” “JA signaling,” and “defense response” (Table 1 and Supplementary Table S5).

A more detailed analysis of DEGs upon cold treatment revealed changes in the expression of several genes closely related to the phenotypes displayed by the SGT2^{ox} and SGT1^{ami} mutants. Higher expression levels of Cu-Zn SUPEROXIDE DISMUTASE (SISODCC1) and PEROXIDASE 21 (SIPOD21) genes were observed in SGT2^{ox} plants compared to WT, whereas no changes in the expression of these genes were detected in SGT1^{ami} plants (Supplementary Fig. S4). This observation is fully consistent with the contrasting levels of oxidative damage observed in the leaves of the SGT2^{ox} and SGT1^{ami} mutants (Fig. 4). Similarly, other bona fide cold-responsive genes such as those coding for DEHYDRINS (DHNs) (Weiss and Egea-Cortines 2009), HEAT SHOCK PROTEIN 20 (HSP20) (Yao et al. 2020), and GIBBERELLIN 2-BETA-DIOXYGENASE (GA2ox) (Pu et al. 2024) were upregulated in SGT2^{ox} plants upon cold treatment, but were either downregulated or remained unchanged in SGT1^{ami} plants (Supplementary Fig. S4). Genes encoding transcription factors linked to stress responses were also upregulated in SGT2^{ox} plants but not in SGT1^{ami} plants following cold exposure. These include a HEAT SHOCK TRANSCRIPTION FACTOR (HSF) (Yang et al. 2016), ABSCISIC ACID-RESPONSIVE ELEMENT BINDING PROTEIN 1 (AREB1), a key component of the ABA signaling pathway known to be cold-inducible in tomato seedlings (Xu et al. 2023), and different members of the APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEIN (AP2/EREBP) and NO APICAL MERISTEM (NAM) transcription factor families (Ma et al. 2013) (Supplementary Fig. S4). Interestingly, cold-treated SGT2^{ox} plants also accumulated higher transcript levels of several genes involved in jasmonate signaling (JAZ2, JAZ4, JAZ7, JAZ9, and JAZ11) and biosynthesis, such as ALLENE OXIDE SYNTHASE 1 (AOS1), OXOPHYTODIENOATE REDUCTASE 3 (OPR3) and METHYL JASMONATE ESTERASE (MJE), compared to WT, while an almost antagonistic trend was observed in SGT1^{ami} plants (Supplementary Fig. S5). Furthermore, several DEGs involved in polyamine metabolism, which undergoes significant adjustments during cold acclimation (Alcázar et al. 2011), were identified in SGT2^{ox} RNA-seq data. Particularly, cold treatment led to lower levels of transcripts encoding SPERMIDINE SYNTHETASES 1 and 2 (SISPDS1 and SISPDS2), and higher levels of transcripts coding for SPERMINE SYNTHETASE (SISPMs) and POLYAMINE OXIDASE 2 (SIPAO2), which, together with SIPAO4, catalyze the complete back-conversion of spermine to putrescine (Hao et al. 2018). Transcript levels of these genes were barely altered in SGT1^{ami} plants compared to WT (Supplementary Fig. S6). Collectively, the results of the transcriptome-wide expression analysis revealed that opposite changes in membrane sterol composition leads to different transcriptional responses that help to implement a cold resistance phenotype in the case of SGT2^{ox} plants and hinder the capacity to withstand the consequences of cold stress in the SGT1^{ami} plants.

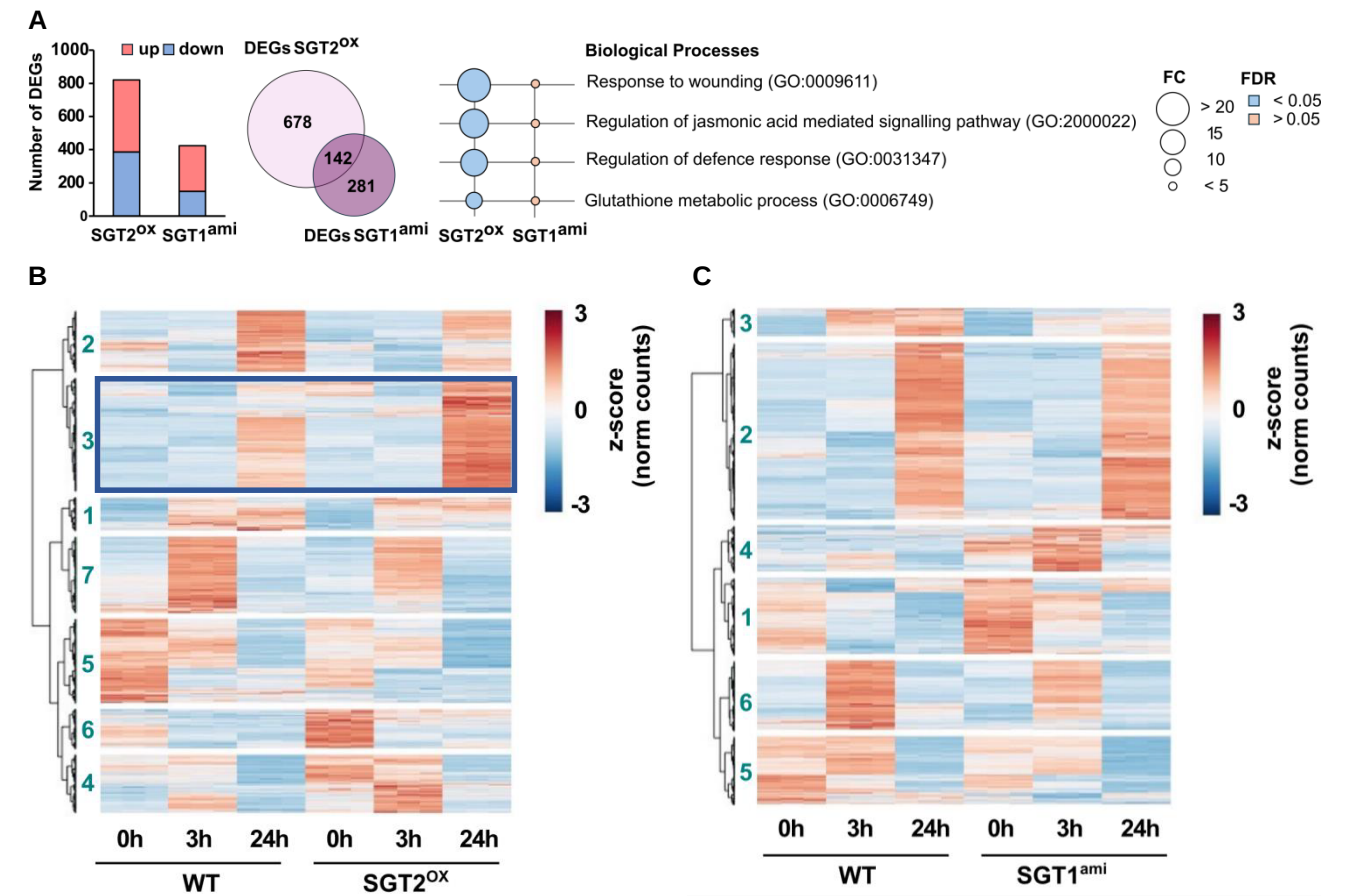


Figure 6. Functional annotation of cold-responsive transcripts in SGT2^{ox} and SGT1^{ami} lines. **A)** Transcripts that displayed log₂ fold-change ≥ 1 to WT (adjusted $P \leq 0.05$, Wald's test) were declared Differentially Expressed Genes (DEGs) and plotted according to the trend (up- or downregulated). Functional annotation of DEGs in SGT1^{ami} and SGT2^{ox} was conducted based on significant enrichment in Gene Ontology (GO) terms for Biological Processes (fold-enrichment ≥ 2 , adjusted $P \leq 0.05$, Fisher's exact test). Circles are proportional to fold-enrichment, and colors are indicative of significance (adjusted P). **B** and **C)** Heatmaps with hierarchical clustering according to the ward D2 algorithm were drawn using z-scores of transcripts significantly changing across cold treatments, depending on the genotype (adjusted $P \leq 0.05$, Likelihood ratio test) in the SGT2^{ox} (**B**) and SGT1^{ami} (**C**) time-courses. Cluster 3 of DEGs in the SGT2^{ox} heatmap is boxed.

Table 1. Functional annotation of cold-responsive transcripts

SGT2 ^{ox} vs WT			SGT1 ^{ami} vs WT		
Patterns		Biological processes	Patterns		Biological processes
Increased	1, 2	Polyamine metabolism Glycolysis Protein maturation and phosphorylation Carbohydrate biosynthesis Response to abiotic stressors	Increased	2, 3	UDP-L-arabinose metabolism Aminoglycan and amino sugar metabolism Endoplasmic reticulum associated protein degradation pathway Amino acid catabolism DNA repair
	3	Response to wounding Jasmonic acid signaling Defense response Response to salt Lipid catabolism	Decreased	1	Protein glycosylation and folding Proteasomal catabolism Amino acid biosynthesis
Decreased	4, 6	Branched-chain amino acid metabolism		4	-
	5	Deoxyribonucleotide biosynthesis Transmembrane transport		5	Protein stabilization Lipid catabolism Transmembrane transport Carboxylic acid metabolism
	7	Glycolysis Transmembrane transport Lipid biosynthesis		6	Inositol biosynthesis Phosphate ion transport Cell wall biogenesis

Core-sets of transcripts displaying similar trajectories in the time-course for SGT2^{ox} and SGT1^{ami} lines were analyzed for significant enrichment in GO terms for biological processes (fold-enrichment ≥ 2 , adjusted $P \leq 0.05$, Fisher's exact test). Summarized are the terms identified in [Supplementary Table S5](#) per cluster with the overall trend.

Membrane sterol imbalance alters polyamines and jasmonates levels

Polyamines and jasmonates are known to play pivotal roles in modulating plant response to cold and, accordingly, their metabolism experiences significant adjustments during cold acclimation (Alcázar et al. 2011; An et al. 2021; Ding et al. 2022a). The transcriptional changes of several genes related to the metabolism and signaling of these compounds pointed to a possible over-accumulation of putrescine and jasmonates in SGT2^{ox} plants under cold conditions compared to WT and SGT1^{ami} plants. Targeted metabolomic analysis demonstrated that, prior to cold treatment, putrescine levels in SGT2^{ox} plants were already ~1.5- and 2.3-fold higher than in the SGT1^{ami} and WT plants, respectively (Fig. 7A). This trend continued after the general increase in putrescine levels caused by cold exposure, as the putrescine content was still ~1.4-fold higher in SGT2^{ox} plants compared to WT and SGT1^{ami} plants (Fig. 7A). The basal levels of spermine were very similar in SGT2^{ox} and WT plants, and a little lower in SGT1^{ami} plants, but after cold treatment there was a clear trend to decrease in SGT1^{ami} and WT plants that was not observed in the SGT2^{ox} plants (Fig. 7A). No noticeable differences were detected in spermidine levels (Fig. 7A). Interestingly, striking differences were observed in the levels of JA, since SGT2^{ox} plants accumulated ~3.5-fold more JA than SGT1^{ami} and WT plants under control conditions and after 24 h of cold exposure (Fig. 7B). A similar effect was observed in the case of JA-Ile, whose levels under control conditions were ~3.7- and ~2–2-fold higher in SGT2^{ox} plants than in WT and SGT1^{ami} plants, respectively, while after cold exposure they were ~4.7- and ~6.6-fold higher, respectively (Fig. 7B). Altogether, these results support the view that polyamines and jasmonates contribution to the cold tolerance phenotype of SGT2^{ox} plants is due to both pre-established higher basal levels and enhanced biosynthesis of these metabolites in response to cold. Our findings demonstrate that increased levels of GS in SGT2^{ox} plants lead to significantly higher jasmonates and putrescine levels in tomato plants under basal and cold-stress conditions, and result in enhanced antioxidant enzyme activity, reduced oxidative damage, and improved cold tolerance, which are not observed in SGT1^{ami} plants with reduced levels of GS.

Discussion

Cold exposure leads to a general disruption of cellular functions, including extensive transcriptional and metabolic reprogramming, and important changes in the composition, structure, and organization of the PM to maintain its integrity, stability, and functionality. In fact, the PM functions as the primary sensor of low temperatures, allowing early detection of its onset and intensity, and the activation of downstream signaling cascades to mitigate chilling-induced damage (Shomo et al. 2024). Sterols, acting in concert with sphingolipids and glycerolipids, play a central role in the cold-induced membrane remodeling process that results in higher levels of ordered phase, reduced fluidity, and increased stiffness. In addition to modulating the membrane global order level, sterols promote lateral phase separation, which facilitates the redistribution of lipids to repair damaged regions in the membrane, thus preserving its integrity, and the formation of the sterol- and sphingolipid-enriched microdomains known as lipid rafts. These dynamic structures play a crucial role in signal transduction and the cellular response to external stimuli (Grosjean et al. 2015; Mamode Cassim et al. 2019).

Changes in membrane sterol composition in response to chilling and the effects of altered sterol levels on cold-stress response

have been mainly investigated in plant species where FS are the predominant class of sterols (Rogowska and Szakiel 2020; Khan et al. 2024). This contrasts with the situation in tomato, where SG and ASG are the major classes of membrane sterols (Palta et al. 1993; Chávez et al. 2023). This, along with the availability of sterol biosynthetic mutants with altered profiles of free and conjugated sterols (Chávez et al. 2023; Lopez-Tubau et al. 2025), makes tomato a particularly suitable model for investigating the contribution of GS to the adaptive responses to cold and the elucidation of the underlying mechanisms. To this end, we analyzed the cold-stress response of tomato (cv. Micro-Tom) SG biosynthetic mutants displaying altered profiles of PM sterols that result in highly contrasting GS-to-FS ratios. Plants overexpressing SLSGT2 (Supplementary Fig. S1) have higher levels of GS and reduced levels of FS compared to WT (Fig. 1), which translates into ~1.5-fold increase in the GS/FS ratio (Supplementary Table S1). On the contrary, SLSGT1-silenced lines (Chávez et al. 2023) (Fig. 1) display a GS-to-FS ratio ~2-fold lower than WT due to decreased GS and increased FS levels (Supplementary Table S1, Fig. 1). The results revealed that these mutants show clearly antagonistic phenotypes related to cold tolerance, membrane stability, and oxidative stress responses. Indeed, increased levels of GS, and therefore of the GS/FS ratio, in SGT2^{ox} plants lead to enhanced cold tolerance (Fig. 2A) and less membrane damage relative to WT, as demonstrated by the reduced levels of REL (Fig. 2B) and MDA (Fig. 4C) in the leaves of these plants. Conversely, lower levels of GS and the resulting reduced GS/FS ratio (Supplementary Table S1, Fig. 1) lead to the opposite phenotype, as the SGT1^{ami} plants are more sensitive to chilling (Fig. 2A) and show higher values of membrane damage indicators than the WT (Figs. 2B, 4C). Chilling also affects photosynthesis, causing PSII photoinhibition that leads to reduced quantum efficiency, PSI overexcitation, and increased production of ROS such as H₂O₂ and O₂⁻ (Peltzer et al. 2002). Under cold stress, SGT2^{ox} plants maintained higher chlorophyll content and Fv/Fm than WT plants, while SGT1^{ami} plants showed the opposite trend (Supplementary Fig. S2, Fig. 2, C and D), suggesting that high GS levels mitigate PSII photoinhibition. Consistent with this, leaves of SGT2^{ox} plants exhibited weaker DAB and NBT staining for H₂O₂ and O₂⁻, whereas SGT1^{ami} leaves showed increased levels of these ROS (Fig. 4) in good correlation with the MDA levels measured in the mutants. To scavenge ROS, plants activate both enzymatic and non-enzymatic antioxidant defenses. Activities of key antioxidant enzymes, such as GST, CAT, and POD (Choudhury et al. 2017), were higher in SGT2^{ox} plants than in WT and SGT1^{ami} under cold stress (Fig. 5). These findings suggest that elevated GS levels enhance antioxidant capacity and confer increased cold tolerance. The direct correlation between the GS-to-FS ratio and the response to cold exposure demonstrates the protective role of GS against cold-induced damage and highlights the importance of maintaining a properly balanced GS-to-FS ratio in the PM to preserve its integrity during chilling stress. It is also worth noting that the phenotypic differences between SGT2^{ox} and SGT1^{ami} plants are attributable to changes in sterol composition caused by the altered levels of SLSGT2 and SLSGT1 rather than to differences in cold-induced sterol remodeling capacity, as the GS/FS ratio declined in WT under cold exposure but remained stable in both mutant genotypes (Supplementary Table S1). Our results show that in tomato, after 48 h of cold treatment only ASG levels are significantly reduced in contrast to FS and SG, whose contents remain essentially unaltered (Fig. 1). This suggests that ASG plays a central role in the tomato PM cold adaptive response, which cannot be considered

facilitating degradation of the JAZ proteins, which act as CBF transcriptional repressors and, consequently, induce COR gene expression (Ding et al. 2024). All these data suggest that GS enhance cold tolerance through the activation of the CBF–COR pathway mediated by JA, although other CBF-dependent and/or CBF-independent cold signaling pathways could also be activated in plants with a high GS/FS ratio, as other cold-responsive transcription factors were also up-regulated only in SGT2^{ox} plants (Supplementary Fig. S5). This cannot be considered unexpected, since CBFs only regulate directly about 20% of COR genes (Zhao et al. 2016).

Collectively, our findings support a model (Fig. 8) in which increased GS levels enhance cold tolerance by stabilizing the PM and activating key stress response pathways. A high GS/FS ratio is expected to promote membrane order and induce the formation and rearrangement of lipid rafts, supporting efficient cold signal transduction. This membrane state would promote JA biosynthesis and signaling, via the COI1–JAZ–MYC2 module, leading to the activation of cold-responsive genes, including those of the CBF–COR pathway, as well as antioxidant defenses and polyamine biosynthesis. All these responses acting in concert reduce chilling damage. In contrast, a low GS/FS ratio is expected to impair membrane structure and compromise stress signaling, thus increasing cold sensitivity. The proposed connection between changes in lipid raft sterol composition and the cold-response phenotypes of the SGT2^{ox} and SGT1^{ami} mutants is consistent with proteomic and lipidomic analyses of lipid rafts with changed sterol composition and glycosylation status, indicating that the functioning of different signaling pathways that initiate in the PM in response to

environmental stimuli is altered (Zauber et al. 2014). Indeed, these PM microdomains are particularly enriched in proteins involved in the perception of external stimuli, signal transduction cascades, and maintenance of redox status (Mongrand et al. 2010). The proposed model highlights the central role of GS in coordinating membrane and signaling-based defenses to improve cold tolerance in tomato.

Finally, the results presented in this study provide direct evidence that increased levels of GS make tomato (cv. Micro-Tom) plants more cold-tolerant without any apparent impact on plant growth in the absence of stress. Whether the same phenotype is observed in non-model tomato varieties remains to be investigated, but in any case, our results highlight the biotechnological potential of manipulating glycosylated sterol metabolism to make plants more tolerant to cold and set the basis for further studies aimed at unveiling the molecular mechanisms underlying this promising phenotype.

Materials and methods

Plant growth conditions and cold treatment

Tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were grown in pots containing a substrate mixture of peat (Klasmann TS2), perlite, and vermiculite (3:1:1) under controlled environmental conditions. The growth chamber was set to a 16-h photoperiod (light intensity: 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and an 8-h dark period, with a day/night temperature of 25/22 °C. When plants were 4-week-old, half of them were transferred to a growth chamber maintained at 4 °C while retaining the same light/dark regime, whereas the remaining plants continued under control conditions. Leaf samples (third and fourth leaves) were collected at different time points from both control and cold-treated plants, and either immediately analyzed or frozen in liquid nitrogen and stored at –80 °C for further processing. For cold recovery experiments, plants exposed to 4 °C for 4 days were transferred back to control conditions and allowed to recover for an additional 4 days.

Generation of transgenic lines overexpressing SlSGT2

The SlSGT2 coding sequence was amplified by PCR using primers SlSGT2-Ox fw and SlSGT2-Ox rv (Supplementary Table S6) and red fruit pericarp cDNA, cloned into the pDONR221 vector using the Gateway BP Clonase II enzyme mix (Invitrogen), and transferred into the pK2GW7 binary vector via the Gateway LR Clonase II enzyme mix (Invitrogen). The resulting construct was introduced into the tomato cv. Micro-Tom following the transformation protocol described by (Cardenas et al. 2019). Homozygous lines carrying a single-copy insertion of the 35SCaMV::SGT2 transgene were identified through quantitative PCR (qPCR) as described by Chávez et al. (2023). Transgenic lines were screened by reverse transcription-quantitative PCR (RT-qPCR) to confirm SlSGT2 overexpression.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from tomato leaves using the Maxwell RSV Plant RNA Kit and the Maxwell 16 Instrument (Promega), following the manufacturer's instructions. First-strand cDNA was synthesized from 2 μg of total RNA using the NZY First-Strand cDNA Synthesis Kit (Nzytech). Gene expression levels were quantified by RT-qPCR using the LightCycler 480 SYBR Green I Master mix (Roche). Relative mRNA levels were calculated using the $2^{-\Delta T}$ Ct method (Livak and Schmittgen 2001), with

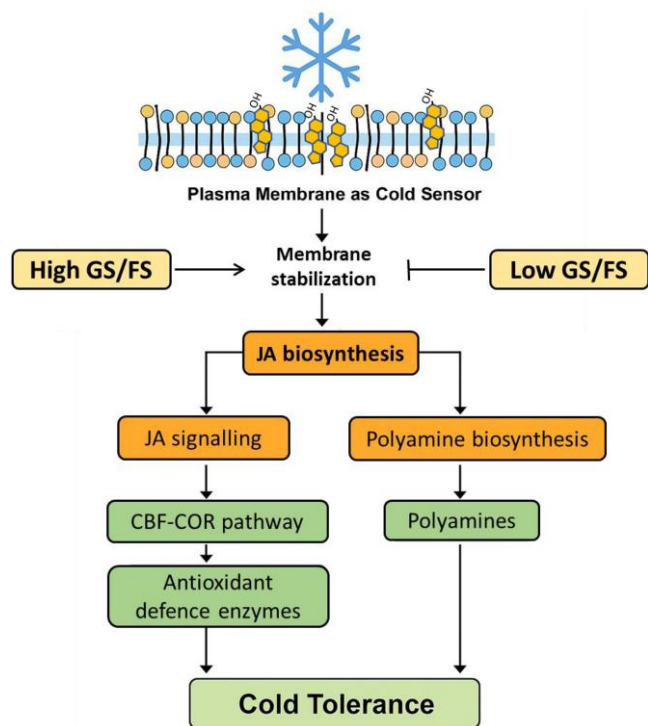


Figure 8. Proposed model illustrating how plasma membrane glycosylated sterols (GS) modulate cold sensitivity in tomato. An increased ratio of glycosylated to free sterols (GS/FS) enhances membrane structural integrity and functionality, promoting jasmonic JA biosynthesis and signaling, which activates the CBF–COR cold-response pathway, antioxidant defenses, and polyamine biosynthesis. Collectively, these responses mitigate chilling injury. In contrast, a low GS/FS ratio impairs membrane structure and stress signaling, resulting in elevated cold sensitivity.

Solyc03g078400 (ACTIN) as the reference gene for normalization. The primers used for RT-qPCR analysis are listed in [Supplementary Table S6](#).

Sterol analysis

Free and conjugated sterols were quantified by gas chromatography-mass spectrometry (GC-MS) following the protocol described by [Chávez et al. \(2023\)](#) in leaf extracts obtained from WT and transgenic plants grown under control conditions (25 °C) and after 48 h of cold (4 °C) exposure. Leaves were collected from 10 plants per replicate and time point.

Electrolyte leakage assay

Leaf discs (14 mm diameter) were excised using a cork borer, placed in tubes containing 30 mL of deionized water, and shaken at 320 rpm for 1 h. Four biological replicates were conducted per genotype, with each replicate consisting of 10 leaf discs. The initial conductivity of deionized water (C_0) and the conductivity of samples containing leaf discs (C_1) were measured using an EC-Meter Basic 30 conductivity meter (Crison Instruments). After boiling the samples for 1 h, the tubes were shaken for an additional hour, cooled to room temperature, and the final conductivity (C_2) was recorded. REL was calculated using the equation: $REL = (C_1 - C_0 / C_2 - C_0) \times 100$.

Chlorophyll content determination

Chlorophyll content was measured following [Porra 2002](#). Leaf samples (300 mg) from the third and fourth nodes were extracted in 7 mL of 80% aqueous acetone and incubated overnight in the dark with shaking. After centrifugation, absorbance was recorded at 645 and 663 nm, and chlorophyll concentrations were calculated using the equations described by [Porra 2002](#).

Maximum quantum efficiency of PSII (Fv/Fm)

Twenty leaves per genotype and treatment were sampled and dark-adapted for 30 min. The maximum quantum efficiency of PSII (Fv/Fm) was determined using an Imaging-PAM fluorometer (Pulse-Amplitude-Modulation, Heinz Walz, Germany).

Histochemical detection of reactive oxygen species (ROS)

Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) accumulation in leaves was detected as described by [Lee et al. 2002](#), using nitro-blue tetrazolium (NBT) staining and DAB staining, respectively.

MDA quantification

MDA levels were determined as described by [Chen and Zhang 2016](#). Leaf samples were frozen in liquid nitrogen and ground to a fine powder. Ground tissue (0.15 g) was homogenized in 1.5 mL of pre-chilled 100 mM phosphate-buffered saline (PBS, pH 7.8) and centrifuged at $16,000 \times g$ for 20 min at 4 °C. The supernatant (100 μ L) was mixed with 1 mL of a 10% (w/v) trichloroacetic acid (TCA) and 0.25% (w/v) thiobarbituric acid (TBA) solution. A blank sample with PBS instead of crude extract was prepared. Reaction mixtures were boiled for 15 min, cooled on ice for 5 min, and absorbance was measured at 532 and 600 nm using a SpectraMax M3 spectrophotometer (Molecular Devices). The concentration of the MDA-TBA complex was calculated as: $MDA (mmol g^{-1} FW) = (A_{532} - A_{600}) / \epsilon \times m \times g^{-1}$, where $\epsilon = 155 \text{ mm}^{-1} \text{ cm}^{-1}$ (extinction coefficient of the MDA-TBA complex at 532 nm).

Enzyme activity assays

Leaf samples were frozen in liquid nitrogen and ground to a fine powder. Ground tissue (0.1 g) was homogenized in 1 mL of pre-chilled 100 mM PBS (pH 7.8) and centrifuged at $16,000 \times g$ for 20 min at 4 °C. The supernatant was used for enzymatic assays. Protein concentration was determined using the Bradford method with bovine serum albumin as the standard.

Enzyme activities were measured as described by [Chen and Zhang \(2016\)](#), with minor modifications. Glutathione S-Transferase (GST) activity was determined in a reaction mixture containing 30 μ L of crude enzyme extract and 970 μ L of reaction buffer (1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 1 mM glutathione in 100 mM PBS, pH 6.5). A blank sample with PBS instead of the enzyme extract was also prepared. Absorbance at 340 nm was recorded every 15 s for 5 min at 25 °C. GST specific activity (units mg^{-1} protein) was calculated as: $(\Delta A_{340} / \text{min sample} - \Delta A_{340} / \text{min blank}) / \epsilon \times V_e \times P$, where $\epsilon = 0.0096 \text{ } \mu\text{M}^{-1} \text{ cm}^{-1}$ (extinction coefficient for the glutathione (CDNB-GSH) conjugate at 340 nm), V_e = enzyme volume (mL), and P = protein concentration (mg mL^{-1}). One unit of GST activity is defined as the amount of enzyme catalyzing the formation of 1 μ mol of CDNB-GSH conjugate per minute at 25 °C.

Peroxidase (POD) activity was measured by incubating 50 μ L of crude enzyme extract in 950 μ L of a reaction mixture containing 0.1% (w/v) guaiacol and 0.03% (v/v) H_2O_2 in 100 mM PBS (pH 7.0). A blank sample with PBS instead of the enzyme extract was included. Absorbance at 470 nm was recorded every 15 s for 4 min at 25 °C. POD specific activity (units mg^{-1} protein) was calculated as $(\Delta A_{470} / \text{min sample} - \Delta A_{470} / \text{min blank}) / V_e \times 0.01 \times P$, where V_e = enzyme volume (mL), and P = protein concentration (mg mL^{-1}). One unit of POD activity is defined as the amount of enzyme increasing absorbance at 470 nm by 0.01 units per minute at 25 °C.

Catalase (CAT) activity was assayed in a reaction mixture containing 50 μ L of crude enzyme extract and 950 μ L of 0.05% (w/v) H_2O_2 in 100 mM PBS (pH 7.0). A blank sample with PBS instead of the enzyme extract was also included. Absorbance at 240 nm was recorded every 15 s for 4 min at 25 °C. CAT specific activity (units mg^{-1} protein) was calculated as $(\Delta A_{240} / \text{min sample} - \Delta A_{240} / \text{min blank}) / V_e \times 0.1 \times P$, where V_e = enzyme volume (mL), and P = protein concentration (mg mL^{-1}). One unit of CAT activity is defined as the amount of enzyme that decreases absorbance at 240 nm by 0.1 units per minute at 25 °C.

Transcriptome profiling and analysis

Leaf samples from control and mutant plants were collected in triplicate at 0, 3, and 24 h of cold treatment. Samples were rapidly frozen in liquid nitrogen, ground to a fine powder, and total RNA was extracted as described above. RNA-Seq libraries were generated and sequenced by BGI (Shenzhen, China) using DNBSEQ technology. RNA-Seq datasets were analyzed as described by [Garcia-Molina et al. 2025](#) using the tomato reference genome ITAG4.0 (https://solgenomics.net/organism/Solanum_lycopersicum/genome). Genes were considered differentially expressed if they exhibited an absolute $\log_2$ fold-change (FC) ≥ 1 relative to WT plants with an adjusted $P \leq 0.05$ (Wald's test).

Hierarchical clustering and heatmap visualization of differentially expressed genes ($P \leq 0.05$, likelihood ratio test) were performed using the heatmap package in RStudio, employing the Ward D2 clustering algorithm. Transcript trajectory analysis was conducted using k-means clustering, with the optimal k determined based on the minimum total within-cluster sum of squares. Functional annotation of transcripts was performed

through Gene Ontology (GO) enrichment analysis for biological processes using Panther (<https://www.pantherdb.org>), considering significantly enriched terms (fold-enrichment ≥ 2 , adjusted $P \leq 0.05$, Fisher's exact test).

The transcriptome datasets are available in the Gene Expression Omnibus repository (GEO) (<http://www.ncbi.nlm.nih.gov/geo>) under accession number GSE292957.

Quantification of jasmonates

Phytohormone extraction was performed as described by (Vives-Peris et al. 2023), with modifications. Briefly, ~100 mg of fresh leaf tissue was homogenized in ultrapure water using a mill ball homogenizer (MillMix 20, Domel, Železniki, Slovenia) at 17 rps for 10 min, with dihydro-jasmonic acid (DHJA) added as an internal standard. After centrifugation at 14,000 rpm for 10 min at 4 °C, the supernatant was transferred to new tubes, and the pH was adjusted to 3.0 ± 0.2 . A double liquid-liquid extraction was performed with diethyl ether, and the organic phase was collected and evaporated using a vacuum centrifuge evaporator. The resulting dry pellet was resuspended in 10% methanol via sonication for 10 min (Elma S30, Elmasonic, Elma Schmidbauer GmbH, Singen, Germany), filtered through 0.22 μm polytetrafluoroethylene syringe filters, diluted (1:3 v/v) with 10% methanol, and transferred to liquid chromatography vials.

Jasmonates were quantified using an ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) system (Xevo TQ-S, Waters, Milford, MA, USA) following a modified protocol from (Durgbanshi et al. 2005). Analyte separation was performed using a C18 reverse-phase column (50 $\times$ 2.1 mm, 1.6 μm particle size; Luna Omega, Phenomenex, Torrance, CA, USA) at a flow rate of 0.3 mL min⁻¹. The mobile phase consisted of a gradient of ultrapure water and acetonitrile, both supplemented with 0.1% formic acid. The triple quadrupole detector was configured at a gas flow rate of 250 L h⁻¹, nitrogen flow of 1,200 L h⁻¹, and a temperature of 650 °C. Data were processed using MassLynx v4.2 software, with quantification performed by interpolation against calibration curves generated from commercial JA and JA-Ile standards.

Polyamine analysis

Free polyamines were quantified by high-performance liquid chromatography (HPLC) following dansyl chloride derivatization. The extraction and determination methods have been described previously (Marcé et al. 1995).

Statistical analysis

Quantitative experimental data were analyzed with GraphPad Prism v8.0.2 software (La Jolla, California, USA). Statistically significant differences were determined by subjecting the data to one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test at $\alpha < 0.05$.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: SLSGT1 (Solyc06g007980), SLSGT2 (Solyc09g061860), SICBF1 (Solyc03g026280), SLDRCi7 (Solyc04g082200), JAZ2 (Solyc12g009220), JAZ4 (Solyc12g049400), JAZ7 (Solyc11g011030), JAZ9 (Solyc08g036640), JAZ11 (Solyc08g036660), SIAOS1 (Solyc11g069800) SIOPR3 (Solyc07g00787), MJE (Solyc03g044820), SISPDs1 (Solyc04g026030), SISPDs2 (Solyc05g005710), SISPMs (Solyc03g007240), SIPAO2 (Solyc07g043590), SIPAO4 (Solyc02g081390).

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Author contributions

T.A. and A.F. conceived, managed, and supervised the research. C.D. performed research and analyzed data related to sterol profiling, as well as molecular, biochemical, and phenotypical characterization of plants. A.G-M. analyzed the transcriptomic data. A.G-C., V.V-P., and R.A. performed research and analyzed data related to jasmonates and polyamines analyses. T.A., A.F., and A.G-M. wrote the manuscript. All authors discussed and interpreted the data and edited and commented on the manuscript.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Figure S1. SLSGT2 mRNA levels in leaves of SGT2^{ox} plants.

Supplementary Figure S2. Differential impact of cold stress on chlorophyll content and post-stress recovery phenotypes in tomato SGT1^{ami} and SGT2^{ox} plants.

Supplementary Figure S3. Transcriptome profiling of SGT1^{ami} and SGT2^{ox} plants.

Supplementary Figure S4. Overview of steady-state transcript levels for selected genes involved in stress.

Supplementary Figure S5. Overview of steady-state transcript levels for genes involved in jasmonate biosynthesis and signaling.

Supplementary Figure S6. Overview of steady-state transcript levels for selected genes involved in polyamine metabolism.

Supplementary Table S1. Ratio of membrane sterols in SGT2ox, SGT1ami, and WT tomato plants grown under control conditions and after cold exposure for 48 h.

Supplementary Table S2. Differentially expressed genes detected in SGT1^{ami} and SGT2^{ox}.

Supplementary Table S3. Functional annotation of GO terms significantly enriched in SLSGT2ox plants growing on basal conditions (25 °C).

Supplementary Table S4. Clusters of transcripts during cold treatment.

Supplementary Table S5. Functional annotation of transcripts describing different trajectories under cold treatments.

Supplementary Table S6. Primers used for cloning and qRT-PCR.

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Conflict of interest statement. None declared.

Data availability

The data underlying this article are available in the Gene Expression Omnibus repository (GEO, <http://www.ncbi.nlm.nih.gov/geo>), and can be accessed with accession number GSE292957.

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