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Research Article

Investigation of Galactinol Synthase Genes in Plant Defense Mechanisms against Adverse Environmental Conditions in Hot Pepper (*Capsicum annuum*)

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ABSTRACT

The galactinol synthase (GolS) and raffinose synthase (RS) play crucial roles in the synthesis of raffinose family oligosaccharides, which are involved in stress protection, carbohydrate transport, and fruit development in plants. This study aimed to conduct a genome-wide identification and characterization of *GolS* and *RS* genes in hot pepper (*Capsicum annuum*) and to investigate their responses to environmental and hormonal factors. Six *CaGolS* and seven *CaRS* genes were identified in the pepper genome. Structural analysis showed that *CaGolS* genes have relatively compact exon-intron arrangements, while *CaRS* genes possess more complex structures. Phylogenetic comparison with related species revealed strong evolutionary conservation within the Solanaceae group. Transcriptome analysis showed that *CaGolS1, CaGolS2, CaGolS3, CaRS2,* and *CaRS7* were highly responsive to abiotic stresses such as cold, heat, salinity, and osmotic stress, as well as to hormonal treatments involving abscisic acid, jasmonic acid, salicylic acid, and ethylene. Several genes also showed differential expression during fruit development and ripening, indicating their contribution to sugar metabolism and stress adaptation during maturation. The results provide new insights into the GolS and RS gene families in hot pepper and identify potential gene targets for developing stress-tolerant and high-quality pepper cultivars.

1. Introduction

Hot pepper (*Capsicum annuum*), a member of the Solanaceae family, is one of the most economically and nutritionally important vegetable crops cultivated worldwide (Silvar *et al.* 2025). It originated in Central and South America and later spread across Asia, Africa, and Europe through global trade and domestication (Karim *et al.* 2021). As a rich source of vitamins, carotenoids, and capsaicinoids, chili contributes significantly to human health and the food industry, while also serving as a model species for studying fruit development, secondary metabolism, and stress physiology (Faisal and Mustafa 2025). However, the productivity and quality of chili are often severely affected by various abiotic stresses, such as drought, salinity, and temperature extremes, as well as biotic stresses, including pathogen infection, which collectively disrupt growth, photosynthesis, and reproductive development (Lee and Kim 2018; Bulle *et al.* 2025). These environmental constraints induce oxidative stress and disrupt osmotic balance, altering key metabolic pathways and causing yield losses and a decline in fruit quality. Therefore, understanding the molecular mechanisms underlying stress tolerance in chili is crucial for developing resilient cultivars.

In this context, investigating the role of galactinol synthase (GolS) and raffinose synthase (RS), key enzymes

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in the biosynthesis of raffinose family oligosaccharides involved in osmoprotection and reactive oxygen species detoxification, provides valuable insight into the plant's adaptive defense mechanisms against adverse environmental conditions (Liu *et al.* 2024). In particular, GolS catalyzes the first committed step in raffinose family oligosaccharide biosynthesis by transferring a galactosyl moiety from uridine diphosphate galactose to myo-inositol, forming galactinol, the critical galactosyl donor for subsequent reactions (Sengupta *et al.* 2012). Raffinose synthase then utilizes galactinol to transfer the galactosyl group to sucrose, producing raffinose, which functions as an osmoprotectant and antioxidant under stress conditions (Nishizawa *et al.* 2008). Numerous studies in model and crop plants, including maize (*Zea mays*) (Zhou *et al.* 2012), tomato (*Solanum lycopersicum*) andpurple false brome (*Brachypodium distachyon*) (Filiz *et al.* 2015), rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*) (Fan *et al.* 2017), cassava (*Manihot esculenta*) (Li *et al.* 2018), cotton (*Gossypium arboreum*, *G. raimondii*, *G. hirsutum*, and *G. barbadense*) (Cui *et al.* 2021), kiwifruit (*Actinidia eriantha* and *A. chinensis*) (Yang *et al.* 2022), and rose gum (*Eucalyptus grandis*) (Cao 2013; Jie *et al.* 2025), have demonstrated that the putative functions of *GolS* and *RS* genes enhance tolerance to abiotic stresses by stabilizing membranes, protecting macromolecules, and scavenging reactive oxygen species. However, research on the identification and characterization of *GolS* and *RS* genes in hot pepper remains limited. This knowledge gap hinders a full understanding of the raffinose family oligosaccharides biosynthetic pathway and its regulatory role in the stress response of this important crop.

This study aimed to identify and characterize the GolS and RS gene families in hot pepper, to analyze their phylogenetic relationships and gene structures, and to determine their expression profiles under different abiotic stresses, hormonal treatments, and fruit developmental stages. The study also aimed to compare the GolS and RS gene families in hot pepper with those in other plant species to clarify their evolutionary conservation and functional diversification. In addition, the research sought to define the roles of *GolS* and *RS* genes in stress tolerance, sugar metabolism, and fruit ripening, to provide molecular insights that can support genetic improvement for better stress resilience and fruit quality in hot pepper.

2. Materials and Methods

2.1. Screening of Galactinol Synthase and Raffinose Synthase

Putative *GolS* and *RS* genes in hot pepper were screened from the latest reference genome of the CM334 (ASM51225v2, last update on December 28th, 2022) and proteome (Kim *et al.* 2014) available in the Ensembl Plants (https://plants.ensembl.org/Capsicum_annuum/Tools/Blast) (Bolser *et al.* 2017) using a dual-strategy pipeline. First, hidden Markov model searches were performed using curated profiles of glycosyltransferase families 8 (GolS/GT8) and 36 (RS/GT36); candidates were retained with sequence E-values ≤1.0e-20 and domain E-values ≤1.0e-5. Second, BLASTP searches used verified GolS and RS proteins from Solanaceae relatives (tomato, potato) (Filiz *et al.* 2015; Jing *et al.* 2023) as queries, and hits were kept with amino-acid identity ≥ 40% and query and subject coverage ≥ 70% at E-value ≤1.0e-20. Redundant entries were collapsed with CD-HIT at 95% identity, and proteins lacking the diagnostic GT8/GT36 domains were discarded after InterProScan confirmation. Finally, all pepper GolS and RS sequences were validated by using the genome of the Hugo cultivar (GCA_042919565.1), which was recently released in 2024 via NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_042919565.1/).

2.2. Characterization of Galactinol Synthase and Raffinose Synthase

Characterization of the identified GolS and RS proteins was performed using the ExPASy ProtParam tool (https://web.expasy.org/protparam/) (Gasteiger *et al.* 2003). The protein sequences of all identified GolS and RS proteins were retrieved in FASTA format from the hot pepper proteome database (Kim *et al.* 2014) and submitted to ProtParam (Gasteiger *et al.* 2003) for *in silico* analysis. The parameters evaluated included amino acid composition, protein length (number of residues), molecular weight (kDa), theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), aliphatic index (AI), and instability index (II). Among them, the II predicts the stability of the protein in a test tube, with a value below 40 indicating a stable protein; the GRAVY score represents the average hydropathicity of all amino acids, with negative values indicating hydrophilic proteins and positive values showing hydrophobic proteins.

2.3. Phylogeny Analysis of Galactinol Synthase and Raffinose Synthase

The phylogenetic relationships of the identified GolS and RS proteins in hot pepper were analyzed using the MEGA software version 12 (Kumar *et al.* 2024). The amino acid sequences of all identified GolS and RS proteins were first aligned with their respective homologous *Arabidopsis thaliana* sequences, retrieved from the TAIR database (Berardini *et al.* 2015), which served as reference sequences to infer evolutionary relationships within the GolS and RS gene families. Multiple sequence alignments were performed using the ClustalW algorithm with default parameters (Sievers and Higgins 2021). After alignment, phylogenetic trees were constructed using the Maximum Likelihood method with the Jones-Taylor-Thornton substitution model. The reliability of the tree topology was evaluated using bootstrap analysis with 1,000 replicates, as described in previous studies (Cao 2022; Chu *et al.* 2024; Nguyen *et al.* 2025).

2.4. Gene Structure of Galactinol Synthase and Raffinose Synthase

The gene structures of the identified GolS and RS genes in hot pepper were analyzed using GSDS 2.0 (http://gsds.gao-lab.org/) (Hu *et al.* 2015), as described in previous studies (Cao 2022; Chu *et al.* 2024). The genomic DNA and corresponding coding sequence files for each gene were retrieved from the hot pepper reference genome database (Kim *et al.* 2014) and aligned to determine exon-intron organization. Both genomic and coding sequences were uploaded to GSDS in FASTA format, and the tool automatically aligned the coding sequence to the genomic sequence to visualize gene structures.

2.5. Transcriptomic Analysis of Galactinol Synthase and Raffinose Synthase

The reanalysis of the transcriptome atlas for expression profiling of *GolS* and *RS* genes in hot pepper was conducted using publicly available RNA-Seq datasets representing four biological contexts, including abiotic stress (Kang *et al.* 2020), hormone treatments (Lee *et al.* 2020), and fruit development and ripening (Razo-Mendivil *et al.* 2021). For abiotic stress, transcriptomic data (GSE154622) were retrieved from the study by Kang *et al.* (2020), which profiled hot pepper exposed to heat (40°C), cold (10°C), salt (400 mM NaCl), and osmotic stress (400 mM mannitol) across six time points (Kang *et al.* 2020). For hormone signaling, expression data (GSE149037) from Lee *et al.* (2020) were used, which included 78 RNA-Seq libraries of hot pepper treated with salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA) at 0, 1, 3, 6, 12, and 24 hours post-treatment (Lee *et al.* 2020). For fruit development and ripening, transcriptomic data (GSE171889) from Razo-Mendívil *et al.* (2021) were analyzed, covering immature (green) and mature (red) stages in hot pepper (Razo-Mendívil*et al.* 2021). Expression levels were evaluated based on relative expression (fold-change). Genes with $\log_2$ fold change values ≥ 1 were considered upregulated, whereas those with $\log_2$ fold change values ≤ -1 were regarded as downregulated.

3. Results

3.1. Identification and Characterization of Galactinol Synthase and Raffinose Synthase in Hot Pepper

The comprehensive genome-wide screening of hot pepper led to the identification of six *GolS* and seven *RS* genes, designated as *CaGolS1*-*CaGolS6* and *CaRS1*-*CaRS7* (Table 1). The nomenclature “CaGolS” refers to *C. annuum* galactinol synthase, while “CaRS” denotes *C. annuum* raffinose synthase. These genes encode enzymes that catalyze two key reactions in the raffinose family oligosaccharide biosynthetic pathway. CaGolS catalyzes the formation of galactinol from uridine diphosphate galactose and myo-inositol, and CaRS mediates the subsequent transfer of a galactosyl residue from galactinol to sucrose to produce raffinose.

The identified *CaGolS* genes showed considerable diversity in sequence length, protein size, and biochemical properties (Table 1). Their coding sequences ranged from 1,710 to 2,927 bp, encoding proteins of 271-340 amino acids with molecular masses of 32.17-38.65 kDa. The theoretical pI varied from 5.34 to 6.99. All CaGolS proteins exhibited negative GRAVY values (-0.42 to -0.19), while their AI scores (70.48-86.09) and II values (34.95-48.36) suggested moderate stability. Similarly, the CaRS gene family displayed broader variation in gene structure and physicochemical traits (Table 1). Gene lengths ranged from 1,174 to 19,671 bp, encoding proteins of 217-873 amino acids with predicted molecular masses of 23.90-96.89 kDa. The pI values spanned 5.23-8.86,

Table 1. Physicochemical properties of identified galactinol synthase and raffinose synthase proteins in *Capsicum annuum*

Gene name	Locus name	Protein ID	Gene length (bp)	Protein length (aa)	mW (kDa)	pI	GRAVY	AI	II
CaGolS1	T459_02411	PHT94529	2927	325	37.50	6.60	-0.25	86.09	35.62
CaGolS2	T459_04119	PHT89006	2084	329	37.87	6.40	-0.30	83.22	39.83
CaGolS3	T459_06371	PHT91258	1214	336	38.65	5.74	-0.27	83.24	34.95
CaGolS4	T459_14143	PHT81128	1805	282	32.46	5.34	-0.19	82.94	37.22
CaGolS5	T459_14176	PHT81161	1710	271	32.17	5.66	-0.42	70.48	41.05
CaGolS6	T459_33104	PHT63084	2592	340	38.52	6.99	-0.21	82.03	48.36
CaRS1	T459_02728	PHT94846	3296	873	96.89	5.47	-0.22	79.71	28.75
CaRS2	T459_06047	PHT90934	4143	870	95.78	5.23	-0.18	85.34	37.17
CaRS3	T459_07060	PHT91947	3963	779	85.86	5.41	-0.15	82.37	26.53
CaRS4	T459_09026	PHT86920	19671	783	86.90	5.89	-0.16	85.24	37.62
CaRS5	T459_09422	PHT87316	1174	217	23.90	8.86	0.04	94.38	23.53
CaRS6	T459_10139	PHT88033	2722	780	86.93	5.58	-0.20	79.91	36.22
CaRS7	T459_20967	PHT77445	4382	756	84.09	5.96	-0.24	84.43	31.20

mW: molecular weight, AI: Aliphatic index, pI: Iso-electric point, GRAVY: Grand average of hydropathicity, II: instability index

indicating both acidic and basic members, with mostly negative GRAVY scores (-0.24 to 0.04) that suggest a generally hydrophilic nature. The AI values (79.71-94.38) indicated a high proportion of aliphatic side chains. Their II scores (23.53-37.62) indicated that most CaRS proteins are stable under physiological conditions. These results reveal that hot pepper harbors moderately sized, diverse GolS and RS families with distinct biochemical characteristics.

3.2. Phylogeny Analysis of Galactinol Synthase and Raffinose Synthase in Hot Pepper

To investigate the evolutionary relationships of CaGolS and CaRS proteins in hot pepper, phylogenetic trees were constructed in MEGA12 using the Maximum Likelihood method with 1,000 bootstrap replications, and homologous sequences from *A. thaliana* were included as references.

For the GolS family, the phylogenetic tree grouped the six CaGolS proteins into three distinct clusters (Figure 1A). In particular, CaGolS5, CaGolS4, CaGolS2, and CaGolS3 formed a strongly supported clade (bootstrap value 97-100) closely related to AtGolS1. CaGolS1 clustered with AtGolS1 (bootstrap 54), while CaGolS6 grouped with AtGolS4 and AtGolS7 (bootstrap value 99). These results suggest that *CaGolS* genes underwent lineage-specific diversification after the separation of Solanaceae and Brassicaceae, with possible neofunctionalization among paralogs.

For the RS family, the phylogenetic tree classified the seven CaRS proteins into four major clades, each associated with corresponding *Arabidopsis* homologs (Figure 1B). Among them, CaRS7 and CaRS4 grouped with AtRS1 and AtRS3 (bootstrap value 95-100). CaRS2 clustered with AtRS2 and AtRS6 (bootstrap value 100), while CaRS5 and CaRS1 formed a distinct cluster with AtRS4 and AtRS5, supported by high bootstrap values. CaRS3 and CaRS6 were positioned within a separate subclade (bootstrap value 80-100).

3.3. Gene Organization of Galactinol Synthase and Raffinose Synthase in Hot Pepper

The structural organization of *CaGolS* and *CaRS* genes in hot pepper was analyzed using GSDS 2.0 to visualize the arrangement of exons and introns based on genomic and coding sequence alignments. For the *CaGolS* gene family, the number of exons ranged from 2 to 4, indicating moderate structural diversity among family members (Figure 2A). Among them, *CaGolS4* contained two exons, whereas *CaGolS2* and *CaGolS3* each had three exons. Meanwhile, *CaGolS1, CaGolS5,* and *CaGolS6* exhibited more complex structures with four exons separated by variable intron lengths.

In contrast, the CaRS gene family showed greater structural complexity, with exon numbers ranging from 4 to more than 10 (Figure 2B). Particularly, *CaRS2, CaRS4,* and *CaRS7* contained numerous short exons interspersed with long introns. *CaRS5* also showed a fragmented structure with multiple short exons, while *CaRS1, CaRS3,* and *CaRS6* had relatively compact exon-intron organizations, each containing six to eight exons.

3.4. Expression Patterns of Galactinol Synthase and Raffinose Synthase in Hot Pepper

At 20 days after anthesis, several *CaGolS* and *CaRS* genes showed clear differences in expression during early fruit development (Figure 3). Among the *CaGolS* genes, *CaGolS1* and *CaGolS3* were upregulated with $\log_2$ fold changes of 1.17 and 4.75, which indicates strong transcriptional activity in early fruit growth

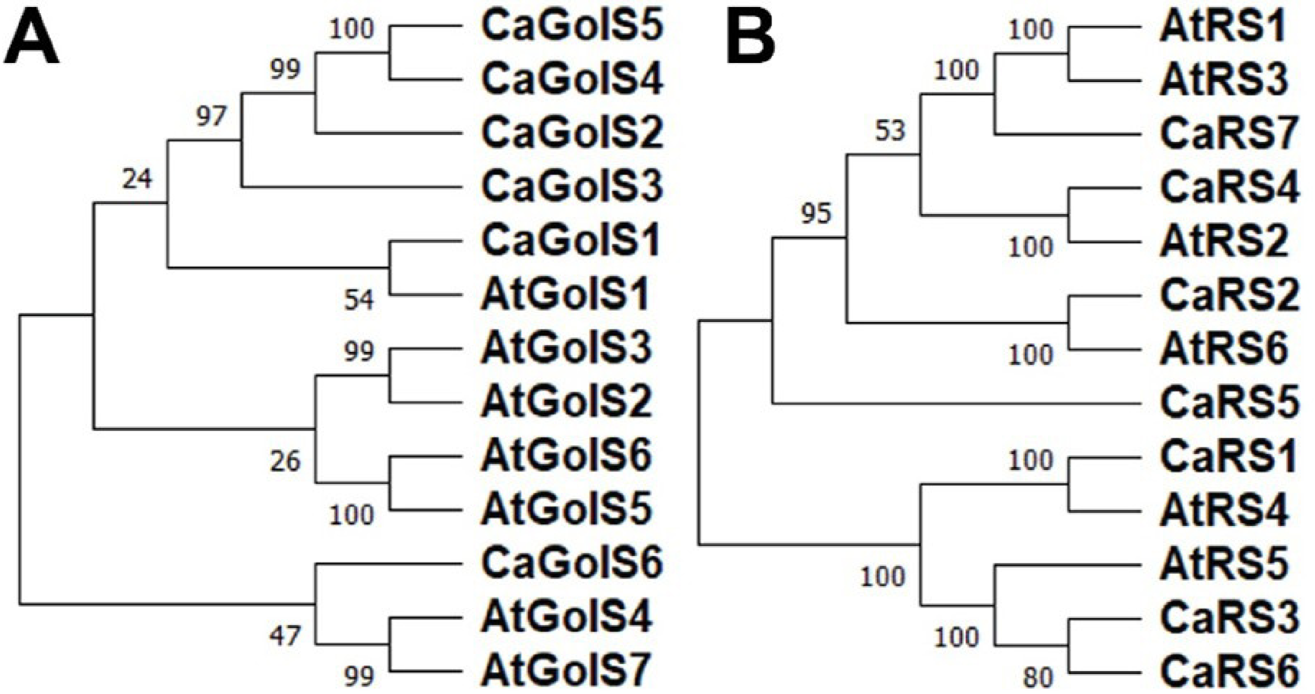

Figure 1. Phylogenetic trees of (A) *galactinol synthase* and (B) *raffinose synthase* proteins from *Capsicum annuum* and *Arabidopsis thaliana*

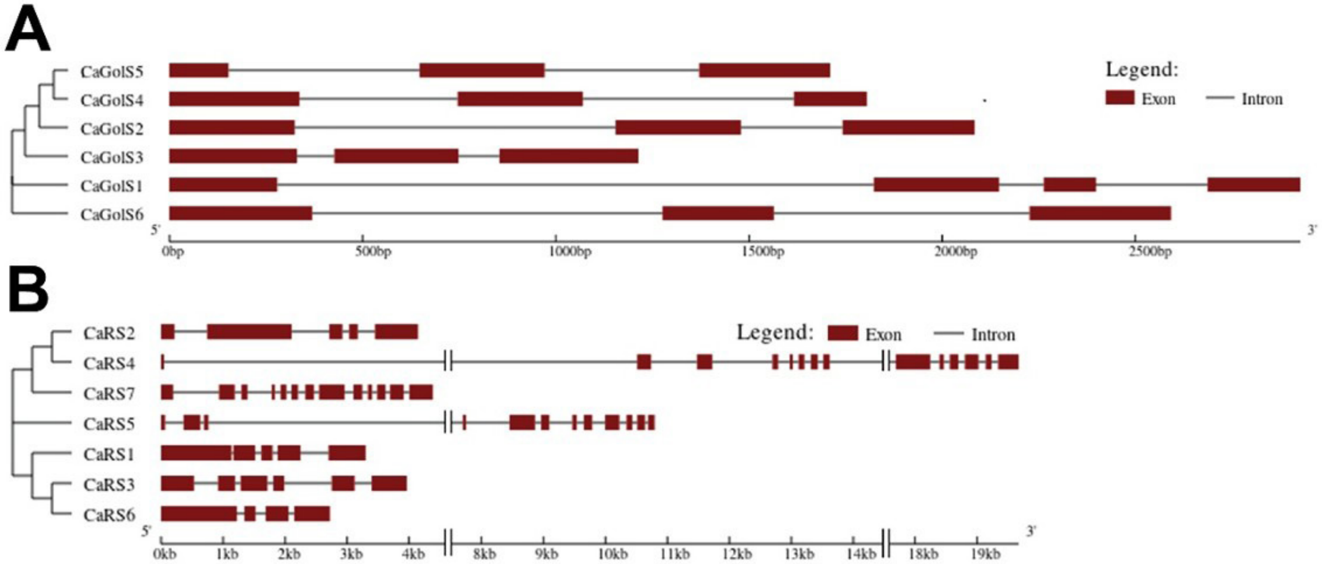

Figure 2. Gene structure of (A) *galactinol synthase* and (B) *raffinose synthase* genes in *Capsicum annuum*

and carbohydrate metabolism. In contrast, *CaGolS2, CaGolS4, CaGolS5,* and *CaGolS6* were downregulated, with values of -2.00 or lower. The strongest repression occurred in *CaGolS4* and *CaGolS5* (-7.87 each). These results suggest that only a few *CaGolS* genes are active during early fruit formation, while the others remain transcriptionally repressed. In the *CaRS* family, *CaRS1, CaRS2,* and *CaRS7* were upregulated, with *CaRS2* showing the highest $\log_2$ fold change (~4.03). Meanwhile, *CaRS4* and *CaRS6* were downregulated, indicating different levels of raffinose biosynthetic activity among the gene members at this stage. At 68 days after anthesis, the expression pattern changed substantially. *CaGolS1, CaGolS2,* and *CaGolS6* were upregulated with $\log_2$ fold changes between 2.04 and 4.90, which exhibited increased transcriptional

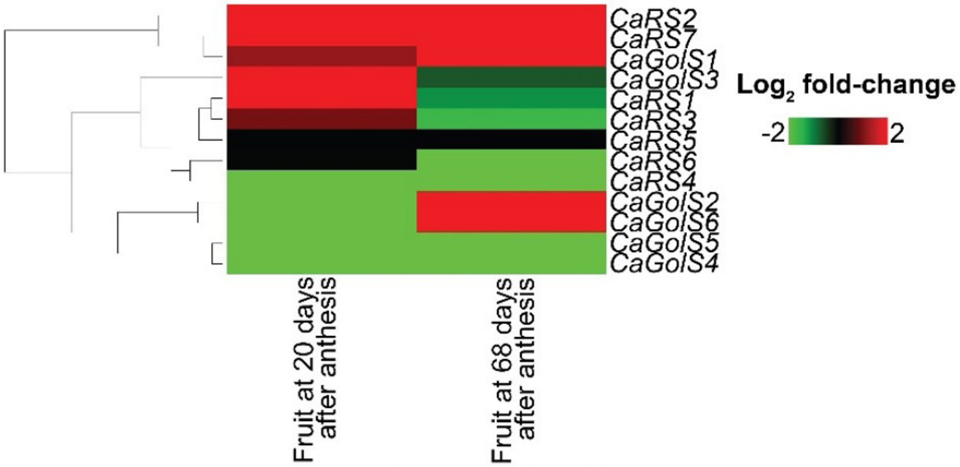

Figure 3. Heatmap showing the relative expression profiles of *galactinol synthase* and *raffinose synthase* genes in hot pepper fruits at 20 and 68 days after anthesis

activity during fruit maturation. *CaGolS3* decreased slightly (log$_2$ fold change ~ -0.66), while *CaGolS4* and *CaGolS5* remained strongly repressed (log$_2$ fold change ranging from -6.10 to -5.01). In the *CaRS* group, *CaRS2* and *CaRS7* were highly upregulated, with log$_2$ fold changes of ~9.75 and ~3.94, respectively, which indicates major activation during ripening. *CaRS4* and *CaRS6* continued to show strong downregulation, whereas *CaRS1* and *CaRS3* showed mild decreases. Our study revealed that *CaGolS1, CaGolS2, CaGolS6, CaRS2,* and *CaRS7* were the main genes activated during ripening, suggesting an important role in sugar metabolism and stress-related functions in mature pepper fruits.

Under salt stress, *CaGolS1, CaGolS2,* and *CaGolS3* showed strong and sustained induction throughout the treatment (Figure 4A). The expression levels reached 4.46, 2.27, and 2.22 at 72 h, suggesting a role in salt-induced osmoprotection. *CaRS4* showed the highest upregulation (log$_2$ fold change ~2.64 at 24 h), while *CaRS2, CaRS3,* and *CaRS6* showed moderate increases. *CaRS1* and *CaRS7* were repressed, which suggests selective transcriptional activation among RS members under salt stress. Under cold stress, *CaGolS3* showed the highest induction with a log$_2$ fold change of ~8.46 at 12 h, followed by *CaGolS2* (log$_2$ fold change ~4.08) and *CaGolS1* (log$_2$ fold change ~3.04) (Figure 4B). These results indicate a strong activation of *GolS* genes under low temperature. In the CaRS family, *CaRS1* and *CaRS3* reached peak values of 3.81 and 6.69 at 12 h, suggesting that raffinose synthesis increases under cold conditions. The other *CaRS* genes remained stable or showed slight decreases. Under heat stress, *CaGolS1* showed strong and continuous upregulation at all time points, with log$_2$ fold changes ranging from 3.70 to 5.84, indicating an important role in heat tolerance (Figure 4C). *CaGolS2* and *CaGolS3* fluctuated, with moderate induction at 6 h and repression at later time points. The strongest suppression occurred in *CaGolS3* at 12 h (log$_2$ fold change ~ -5.10). Among the *CaRS* genes, *CaRS6* showed consistent upregulation (log$_2$ fold change ranging from 2.20 to 2.85) at early and late stages, while *CaRS2* and *CaRS4* showed mild increases. *CaRS3* and *CaRS7* were strongly downregulated, which indicates variation in heat responsiveness within the RS family. Under osmotic stress, *CaGolS1, CaGolS2,* and *CaGolS3* showed strong upregulation at several time points, especially at 12 h and 24 h, with log$_2$ fold changes greater than 3.0 (Figure 4D). *CaGolS6* showed a clear increase at 12 h (log$_2$ fold change ~4.07), indicating coordinated activation of *GolS* genes during dehydration. Within the CaRS family, *CaRS2, CaRS4,* and *CaRS6* showed moderate upregulation, while *CaRS1* remained repressed. *CaRS7* changed from early downregulation to later upregulation at 72 h (log$_2$ fold change ~1.92), which shows delayed activation under prolonged osmotic conditions.

Under SA treatment, *CaGolS1* showed slight upregulation at 1 h (log$_2$ fold change ~1.00) but remained stable afterward (Figure 5A). *CaGolS2* increased strongly at 3 h (2.26) and decreased sharply at 24 h (log$_2$ fold change ~ -3.43). Within the CaRS family, *CaRS2, CaRS6,* and *CaRS7* showed clear induction. *CaRS7* reached the highest level with values between 0.87 and 2.28, while *CaRS2* and *CaRS6* peaked between 6 h and 12 h (log$_2$ fold change ranging from 1.43 to

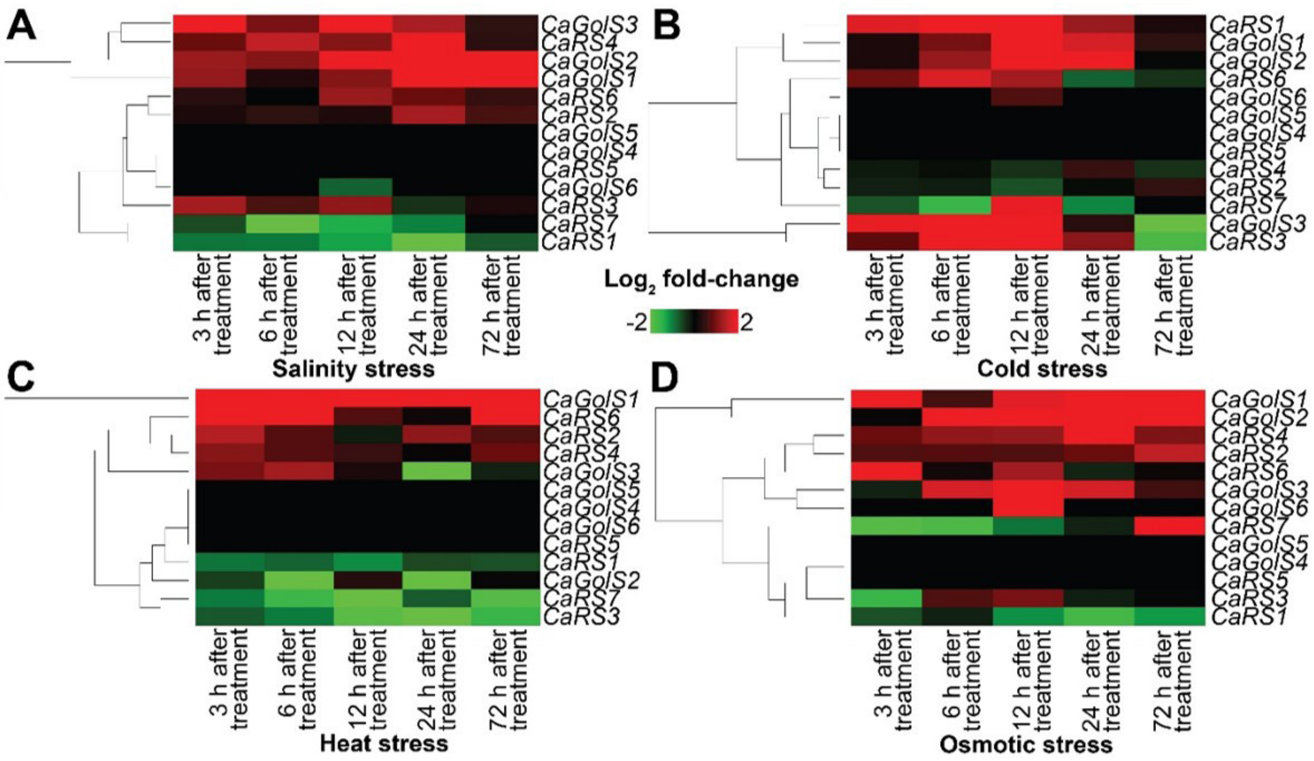

Figure 4. Heatmap representation of the expression profiles of *galactinol synthase* and *raffinose synthase* genes in hot peppers under (A) salinity stress, (B) cold stress, (C) heat stress, and (D) osmotic stress

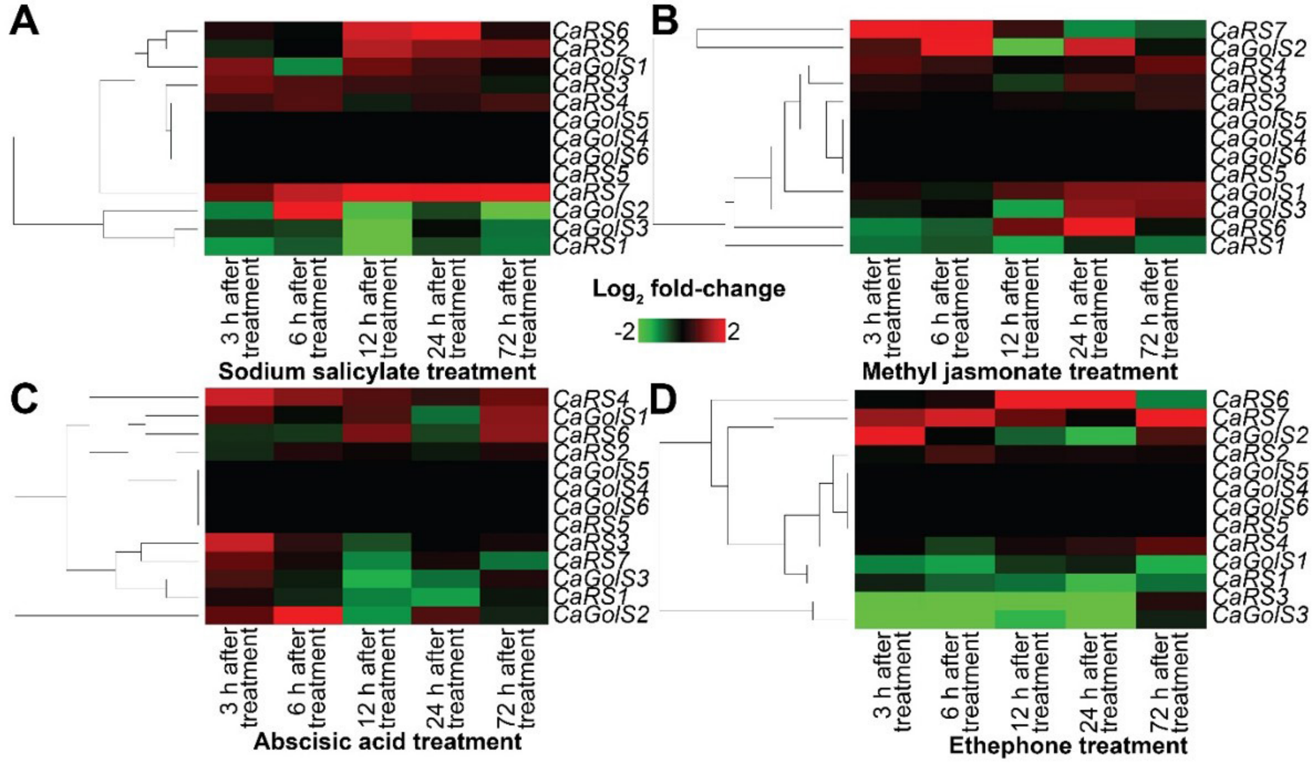

Figure 5. Heatmap representation of the expression profiles of *galactinol synthase* and *raffinose synthase* genes in hot peppers under (A) salicylic acid, (B) jasmonic acid, (C) abscisic acid, and (D) ethylene treatments

1.95). Under JA treatment, *CaGolS1, CaGolS2,* and *CaGolS3* showed increased expression, with *CaGolS2* reaching 2.38 at 3 h and *CaGolS3* reaching 1.10 at 12 h (Figure 5B). These increases indicate that the *CaGolS* genes responded strongly to JA. In the *CaRS* group, *CaRS6* showed consistent induction, peaking at 12 h ($\log_2$ fold change ~1.96), while *CaRS7* increased at 1 h ($\log_2$ fold change ~2.27) and 3 h ($\log_2$ fold change ~1.90) before decreasing later. *CaRS4* showed mild activation across all time points. These data indicate that *CaGolS2, CaGolS3, CaRS6,* and *CaRS7* responded most strongly to JA signaling. Under ABA treatment, *CaGolS1* showed moderate upregulation at 1 h and 24 h, while *CaGolS2* increased sharply at 3 h ($\log_2$ fold change ~2.89) and fluctuated at later stages (Figure 5C). *CaGolS3* rose slightly at 1 h but decreased between 6 h and 12 h, then recovered by 24 h. Among the *CaRS* genes, *CaRS3, CaRS4,* and *CaRS6* showed clear responsiveness. *CaRS3* and *CaRS4* reached their highest expression at 1 h ($\log_2$ fold change ~1.57 and 1.60, respectively), while *CaRS6* increased gradually and peaked at 24 h ($\log_2$ fold change ~1.12). The remaining *CaRS* genes showed minor changes. These results indicate that ABA primarily activated *CaGolS2, CaRS3, CaRS4,* and *CaRS6* in both early and late response phases. Under ET treatment, *CaGolS2* showed transient upregulation at 1 h ($\log_2$ fold change ~1.85), while *CaGolS1* and *CaGolS3* remained repressed across all time points (Figure 5D). In the CaRS family, *CaRS6* reached strong expression at 6 h (3.43) and 12 h (2.06), and *CaRS7* showed continuous activation from 1 h to 24 h, with the highest value at 24 h (2.27). Most other *CaRS* genes, including *CaRS1* and *CaRS3*, showed reduced expression. These patterns suggest that *CaRS6* and *CaRS7* were the major ethylene-responsive members that contributed to raffinose accumulation during ET response.

4. Discussion

The present study identified 6 *CaGolS* and 7 *CaRS* genes in hot pepper, which may expand current knowledge of the raffinose family oligosaccharide pathway in the Solanaceae. Comparative genomic and structural analyses revealed distinct patterns of gene family size, sequence characteristics, phylogenetic relationships, and gene organization relative to other plant species, such as *A. thaliana*, potato (Jing *et al.* 2023), tomato (Filiz *et al.* 2015), rapeseed (Fan *et al.* 2017), maize (Zhou *et al.* 2012), and tobacco (Fan *et al.* 2017).

The number of *GolS* and *RS* genes identified in hot pepper (six and seven, respectively) falls within the range observed in other species, though smaller than *Eucalyptus* (17 GolS and 14 RS members) (Cao 2013) and polyploid crops, such as rapeseed (20 GolS members) and cotton (12-25 RS members) (Fan *et al.* 2017; Cui *et al.* 2021). The modest number of *GolS* genes in hot pepper is comparable to tomato (four *GolS* genes) and potato (four *GolS* and nine *RS* genes) (Filiz *et al.* 2015; Jing *et al.* 2023). This similarity among Solanaceae members suggests that gene duplication events in the GolS-RS pathway stabilized early in this lineage, with later diversification driven more by regulatory or structural divergence than by large-scale gene expansion.

The predicted protein characteristics of CaGolS and CaRS members were consistent with orthologs from other crops. Most CaGolS proteins showed molecular masses between 32-38 kDa and slightly acidic to neutral isoelectric points (pI ≈ 5.3-6.9), similar to tomato SlGolS1-4 (36-39 kDa, pI 5.3-6.4) and potato StGolS1-4 (37-39 kDa, pI 5.4-6.7) (Filiz *et al.* 2015; Jing *et al.* 2023). Negative GRAVY values indicate hydrophilicity, which agrees with the fact that GolS proteins function mainly in the cytoplasm as osmoprotectants under abiotic stress. In contrast, CaRS proteins showed a broader molecular weight range (23-97 kDa) and pI diversity (5.2-8.8), suggesting functional variation among isoforms. Similar heterogeneity was also observed in potato and maize, where *RS* genes encoded proteins from ~80 to 100 kDa, some of which were strongly stress-inducible under drought or salinity (Zhou *et al.* 2012; Jing *et al.* 2023).

The phylogenetic trees constructed using *A. thaliana* reference sequences provided insights into evolutionary conservation. CaGolS proteins clustered into three main clades alongside AtGolS1-4 homologs, consistent with observations in potato and tomato, where orthologous grouping indicates functional conservation across Solanaceae (Filiz *et al.* 2015; Jing *et al.* 2023). The presence of distinct clusters containing CaGolS6 and AtGolS7 suggests lineage-specific diversification. Similarly, CaRS proteins formed four major clades corresponding to AtRS1-6. This pattern mirrors the five-group classification reported in potato, with conserved subfamilies linked to raffinose accumulation during abiotic stress (Jing *et al.* 2023). In contrast, rapeseed *GolS* genes exhibited significant lineage expansion and subfamily-specific duplication (notably GolS6-type), likely due to polyploidization events (Fan *et al.* 2017). Therefore, the limited copy number and clear orthology in hot pepper indicate evolutionary conservation rather than functional redundancy, suggesting that each gene may have distinct regulatory roles under stress.

Analysis with GSDS revealed structural diversity between the two gene families. *CaGolS* genes contained 2-4 exons, which are similar to the compact gene architectures found in tomato (3-4 exons) and potato (3-4 exons) (Filiz *et al.* 2015; Jing *et al.* 2023). In contrast, *CaRS* genes showed more complex exon-intron structures (4-10 exons). This pattern aligns with other species, in which longer *RS* genes have larger coding regions and more intricate catalytic domains. Rapeseed *GolS* genes generally have three exons and two introns (Fan *et al.* 2017), while tobacco homologs display greater variation (Fan *et al.* 2017), which suggests intron loss or gain during Solanaceae evolution. The conserved exon-intron boundaries in *CaGolS* genes exhibit functional stability, whereas the variable structures in *CaRS* genes may result from subfunctionalization or differences in regulation.

The expression analysis of *CaGolS* and *CaRS* genes in hot pepper revealed that both gene families play essential roles in regulating plant responses to abiotic stresses and hormone signaling. The patterns observed in hot pepper correspond closely with those reported in other plant species, including tomato (Filiz *et al.* 2015), cotton (Cui *et al.* 2021), cassava (Li *et al.* 2018), maize (Zhou *et al.* 2012), rose gum (Jie *et al.* 2025), and rapeseed (Fan *et al.* 2017). For example, in *A. thaliana*, *AtGolS1-3* are strongly induced by drought, cold, and ABA, while *AtGolS4-7* remain less responsive (Taji *et al.* 2002). Similar stress-dependent activation has been reported in cotton, where *GhGolS1* and *GhGolS2* increase markedly under heat and salt stress, suggesting conserved regulatory mechanisms for raffinose family oligosaccharide biosynthesis (Cui *et al.* 2021). In cassava and maize, *GolS* genes are also upregulated by dehydration and osmotic treatments, indicating that galactinol production contributes to osmotic adjustment and protection of cell membranes (Zhou *et al.* 2012; Li *et al.* 2018). The present results for hot pepper, particularly the strong induction of *CaGolS1, CaGolS2,* and *CaGolS3* under cold, salt, and mannitol treatments, support the hypothesis that GolS enzymes serve as primary regulators in osmoprotection through raffinose precursor synthesis. For the RS family, the transcriptional profiles of hot pepper share common features with those of other crops. In tomato and potato, *RS* genes are expressed in both vegetative and reproductive tissues, with enhanced expression during fruit ripening and under abiotic stress (Filiz *et al.* 2015; Jing *et al.* 2023). The high expression of *CaRS2* and *CaRS7* during fruit ripening and their induction by ET and SA treatments in the current study align with these findings, which suggests their contribution to sugar accumulation and stress tolerance in developing fruits. In rose gum, *EgRS2* and *EgRS3* are activated under salt and drought conditions (Jie *et al.* 2025), while in maize and rapeseed, *RS* expression correlates with stress-induced raffinose accumulation, which protects chloroplast membranes and maintains photosynthetic stability (Zhou *et al.* 2012; Fan *et al.* 2017). Additionally, the high fold-change values observed for several *CaGolS* genes under stress should be interpreted in the context of metabolic flux regulation rather than as simple linear activation of the entire raffinose biosynthetic pathway. In several stress conditions, strong induction of *CaGolS* genes coincides with stable or reduced expression of specific *CaRS* members, which suggests that galactinol accumulation may be favored over raffinose synthesis at certain stages of stress response. Galactinol has been shown to function as an effective osmoprotectant and antioxidant independently of raffinose, and its accumulation may represent an energetically efficient strategy during early or acute stress. Differential regulation of *GolS* and *RS* genes likely demonstrates a flexible metabolic strategy that allows plants to balance carbon allocation, sucrose availability, and protective metabolite production under fluctuating environmental conditions.

Although the present study is based on genome-wide and transcriptomic analyses, it is important to note that transcript abundance does not always directly translate into metabolite accumulation. Experimental studies in multiple plant species have demonstrated that induction of *GolS* and *RS* genes is commonly associated with increased levels of raffinose family oligosaccharides under abiotic stress, which contribute to osmoprotection and oxidative stress mitigation. The expression patterns observed in hot pepper in this study are highly consistent with these established genotype–phenotype relationships. Nevertheless, direct biochemical quantification of raffinose and related oligosaccharides, for example, through enzymatic assays or thin-layer chromatography, would provide valuable physiological confirmation of the inferred functional roles of these genes. Such validation represents an important direction for future work and would further strengthen the link between transcriptional regulation and metabolic adaptation in hot pepper.

In conclusion, this study presents the first comprehensive identification, characterization, and expression analysis of GolS and RS gene families in hot pepper. Six *CaGolS* and seven *CaRS* genes were identified with moderate diversity in gene length, protein size, and biochemical features. Phylogenetic and structural analyses revealed conserved evolutionary relationships with other Solanaceae species, which indicates that both gene families have maintained essential biological roles while undergoing lineage-specific divergence. The exon–intron organization showed compact structures in *CaGolS* genes and more complex patterns in *CaRS* genes. Transcriptome reanalysis revealed that *CaGolS1, CaGolS2, CaGolS3, CaRS2,* and *CaRS7* showed strong responses to abiotic stresses, including cold, heat, salt, and osmotic stress, as well as to hormonal treatments, including ABA, JA, SA, and ET. Their expression patterns during fruit development and ripening indicate important roles in osmoprotection, sugar metabolism, and stress adaptation. These findings demonstrate both the evolutionary conservation and functional diversification of the GolS-RS pathway in hot pepper and provide useful molecular targets for genetic improvement of stress tolerance and fruit quality in Solanaceae crops.

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