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**Article**

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# Multi-omics analysis reveals the mechanism of systemin-induced chilling tolerance in tomato fruit

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**Abstract**

Tomato fruit are susceptible to chilling injury (CI) throughout cold storage, leading to tremendous economic losses. Here, the function of the peptide hormone systemin in chilling response was observed in postharvest tomato fruit. Systemin treatment improved fruit chilling tolerance and the expression of transcription factors (TFs), *CBFs*. In contrast, knockout of the systemin precursor *prosystemin* (*prosys*) increased CI symptoms and CI index. Phosphoproteomics analysis revealed that systemin induced the phosphorylation of several kinases, such as MAPK cascade members, as well as the TFs HY5 and ABI5. However, the activity of MAPKs was arrested in *prosys* mutants under chilling stress. Through the transcriptomics assay, several kinases and TFs were identified, including four TFs downregulated in the *prosys* mutants. Combined with the metabolomic analysis, prosystemin modulated the accumulation of numerous shikimates and phenylpropanoids, alkaloids, and terpenoids, along with the expression of genes in their biosynthesis pathways. Besides, the activities and expression of enzymes in flavonoid and capsaicin biosynthesis were affected in *prosys* mutants. Consequently, our findings indicated that systemin improved fruit chilling tolerance through signaling transduction, transcriptional and metabolic networks, highlighting its potential in quality preservation of tomato fruit during cold storage.

**Keywords**: Chilling stress; Flavonoid; MAPKs, Systemin; Tomato

## Introduction

Tomato (*Solanum lycopersicum*) is not only one of the most consumed horticultural crops in the world, but also the model species for fleshy fruits[1]. Tomato is used for both processing and fresh markets and contains large amounts of vitamin C, as well as many other healthful nutrients including antioxidants, especially lycopene, polyphenols, amino acids and fiber. Low temperature is widely used to prolong postharvest storage and transportation of fruits and vegetables, reducing the metabolic rate of cells, ameliorating decay, and maintaining quality[2]. However, tomato is very sensitive to cold and vulnerable to chilling injury (CI). The main CI symptoms are uneven ripening, reduced flavor quality, and increased disease susceptibility, leading to huge economic losses[3]. Therefore, understanding the molecular mechanisms of chilling response in fruit and identifying effective strategies to alleviate CI are critical for the tomato industry.

Chilling stress (0–15°C) is an important environmental factor that inhibits plant growth and development. It reduces the membrane fluidity of plant cells, disturbs protein stability, and affects gene expression, protein biosynthesis, and enzyme activities[4]. For survival, plants have developed a series of physiological, biochemical, and molecular mechanisms to respond to cold stress, such as lipid composition, production of soluble sugars and proline, and synthesis of detoxification enzymes[5]. Chilling stress also impacts the accumulation of secondary metabolites, especially flavonoids and terpenoids[6,7]. Flavonoids have been observed to protect plants against cold stress[8]. Flavonoid deficiency affects the stability of C/N metabolism and photosynthesis in the early chilling acclimation period, and controls stability and folding of proteins and proteasomal degradation in the late stage of chilling acclimation[9]. Besides, chilling stress increases terpenoid content and expression of biosynthesis genes and enzymes of terpenoids.

During cold acclimation, plants initiate a series of regulatory networks, consisting of the dehydration-response element-binding protein 1/C-repeat binding factors (DREB1/CBFs)-dependent module as the most well-understood cold regulatory pathway[10,11]. The CBFs belong to the APETALA2/ethylene-responsive factor

(AP2/ERF) transcription factors (TFs) that bind to the dehydration-responsive element (DRE). In *Arabidopsis*, cold stress rapidly induces the expression of *CBF1*, *CBF2*, and *CBF3* to activate the expression of *COLD-REGULATED* (*COR*) genes, thereby increasing cold tolerance[12]. In tomato, the expression of *SlCBF1* is induced by salicylic acid (SA)- and hydrogen peroxide ($H_2O_2$)-induced chilling tolerance[13]. The *slcbf1* mutant exhibited more severe cold injury symptoms, such as a decrease in proline and protein content and an increase in hydrogen peroxide and activities of antioxidant enzymes[14]. The leucine zipper (bZIP) transcription factor LONG HYPOCOTYL 5 (HY5), critical in the photomorphogenesis of plants, is also important in cold response[15]. The transcriptional and translational levels of HY5 are induced by cold stress, and HY5 regulates several genes in cold response[16,17]. In tomato, HY5 affects many TFs, such as BBX31, MYB15, ABI5, and CBFs, to induce cold tolerance[18-20].

As signaling molecules, peptide hormones are produced from their precursors, secreted into the apoplast, and perceived by specific receptors to manipulate diverse biological pathways, such as self-incompatibility, pollination, fertilization, plant-pathogen and plant-insect interactions, and stress response[21]. In loquat (*Eriobotrya japonica* Lindl. cv Luoyangqing), phytosulfokine 1 (PSK1) is upregulated by cold. Fruits treated with PSK1 peptide increase chilling tolerance by regulating ATP content and energy charge[22]. Besides, PSKα restrains the progression of the chilling injury (CI) index and weight loss of the peach fruits, as well as reduces the firmness and total soluble solids content[23]. The expression of six *PpCBFs* was enhanced by PSKα treatment. The peptide EPIDERMAL PATTERNING FACTOR LIKE 6 (EPFL6) is required for reproductive success at low temperature in *Arabidopsis*, which can enhance cell proliferation of stamen filaments under cold stress[24]. However, research on peptide hormones in chilling response is still limited. The 18-aa systemin was identified to improve the production of two wound-inducible proteinase inhibitor proteins, which is a key regulator in tomato immune response[25]. Application of systemin peptide enhanced postharvest tomato fruit resistance to *Botrytis cinerea*[26]. Upon pathogen attack, systemin is produced from a small protein named prosystemin[27] and sensed by a pair

of leucine-rich repeat receptor-like protein kinases (LRR-RLKs) named SYR1 and SYR2 at the plasma membrane[28]. Moreover, LRR-RLK SOMATIC EMBRYOGENESIS RECEPTOR KINASEs (SERKs) as their co-receptors and the RLK PEPR1/2 ORTHOLOG RECEPTOR-LIKE KINASE1 (PORK1)-receptor-like cytoplasmic kinase (RLCK) TOMATO PROTEIN KINASE1b (TPK1b) complex are important components in systemin-mediated defense responses[29,30]. However, the functions of systemin in other biological pathways are largely unknown[27,31]. Our previous RNA-seq results indicate that systemin induces the expression of several genes associated with freezing, cold, and temperature stimulus responses of tomato fruit, implying its involvement in the cold stress response[26].

In this study, we demonstrated that systemin enhanced tomato fruit resistance to chilling stress by increasing the expression of *CBFs*. Phosphoproteomics analysis indicated that systemin induced the phosphorylation of several kinases such as RLKs and MAKP cascade members, as well as the TFs HY5 and ABI5. In addition, the activity of MAPKs was arrested in *prosys* knockout fruits after chilling stress. The RNA-seq assay identified some kinases and four TFs, whose expression was downregulated in both *prosys* knockout fruits under chilling stress. In addition, prosystemin modulated the accumulation of numerous shikimates and phenylpropanoids, alkaloids, and terpenoids and the expression of their biosynthetic genes, as well as the activities of enzymes in flavonoid and capsaicin biosynthesis. Thus, our findings uncovered the comprehensive mechanism of systemin in tomato fruit resistance to chilling stress and provide the theoretical basis for application of systemin in postharvest quality preservation.

## Results

### Systemin enhanced tomato fruit tolerance to chilling stress

In our previous study, systemin enhanced the expression of genes associated with response to chilling in tomato fruit, suggesting the involvement of systemin in tomato chilling responses[26]. Therefore, to investigate the function of systemin in chilling response, we treated tomato mature fruits with systemin, followed by 14 d cold storage.

As a result, fruits treated with systemin displayed fewer chilling injury (CI) symptoms with smaller denting regions compared with the control (Fig. 1A). The CI index of fruits was decreased significantly by systemin treatment (Fig. 1B). In addition, application of systemin increased proline content, which improves plants tolerance to chilling stress (Fig. 1C)[32]. The expression level of *SlCBFs* was also detected in systemin-treated tomato. The results indicated that systemin enhanced the expression of *SlCBF1*, *SlCBF2*, and *SlCBF3*, and the expression of *SlCBF1* increased the most (Fig. 1D).

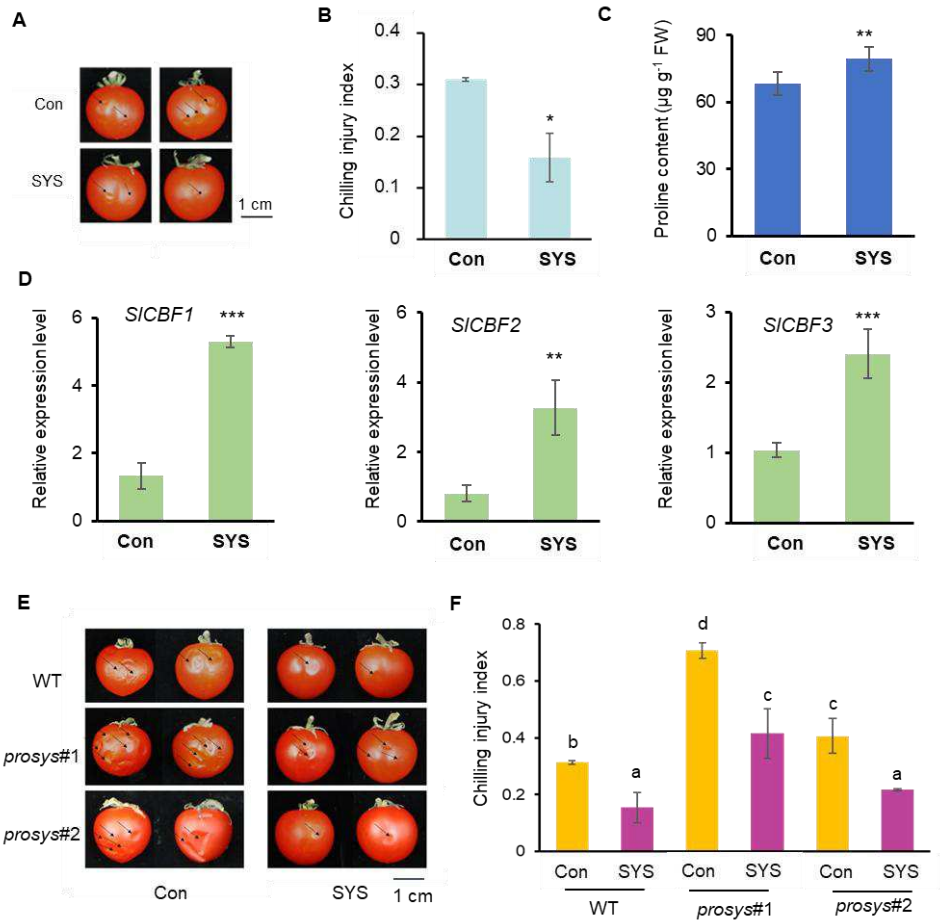



**Fig. 1. Systemin increased tomato fruit resistance to chilling stress.** Surface CI symptoms (A), CI index (B), and proline content (C) of WT tomato fruit treated with 1 μM systemin after 14 d cold storage. (D) Relative expression of *SlCBF1*, *SlCBF2,* and *SlCBF3* in tomato fruits treated with systemin for 24 h. Error bars indicate SD. Statistically significant differences between systemin treatment and control are depicted with asterisks (*$P < 0.05$; **$P < 0.01$, Student's *t*-test). Surface CI symptoms (E) and CI index (F) of WT and *prosys* mutants (#1 and #2) after 14 d cold storage. Con, control; SYS, systemin treatment. Error bars indicate SD. Different letters indicate significant

differences (ANOVA test followed by Tukey's multiple comparisons test, $P < 0.05$).

To further elucidate the function of systemin in chilling tolerance of tomato fruits, we generated two *prosystemin* knockout mutant lines by the CRISPR/Cas9 system, *prosys#1* and *prosys#2* (Fig. S1A and B). A 2-bp deletion in *prosys#1* resulted in a premature stop codon at 5'-20$^{th}$. The *prosys#2* had a 4-bp deletion at the C-terminus, leading to a premature stop codon closely before the systemin mature peptide sequence. After 14 d storage at 4 ℃, both *prosys#1* and *prosys#2* mutants increased the CI symptoms, with *prosys#1* showing more severe symptoms (Fig. 1D). Besides, the CI index of both mutants increased, and the *prosys#1* mutant exhibited the highest CI index (Fig. 1E). Treatment with systemin alleviated the CI symptoms and reduced the CI index of the two mutants, indicating that systemin could restore the cold tolerance of *prosystemin* mutants. Moreover, the CI index of the *prosys#2* mutant treated with systemin showed no obvious difference compared with WT fruits treated with systemin. These results demonstrated that systemin plays an important role in tomato fruit resistance to cold stress.

**Phosphoproteomics analysis of the systemin signaling pathway**

Considering the importance of protein phosphorylation modification in signaling transduction of plants[33], we performed a phosphoproteomics analysis of tomato fruit treated with systemin for 1 h to investigate its downstream responses. The differentially expressed phosphopeptides were defined by a fold change >1.5 or < 0.75 with $p < 0.05$. In total, 447 differentially expressed phosphopeptides belonging to 371 differentially expressed phosphoproteins (DPPs) were identified in tomato fruits treated with systemin, consisting of 156 upregulated proteins and 215 downregulated proteins (Fig. 2A and B, Table S1). In addition, the 371 DPPs were subjected to GO (Gene Ontology) enrichment analysis. The results showed that several DPPs were related to molecular function (MF) terms, 1-phosphatidylinositol 4-kinase activity and kinase activity, and associated with cellular components (CC) items, chloroplast inner membrane, plastid inner membrane, and plastid membrane (Fig. 2C). The KEGG (Kyoto Encyclopedia of Genes and Genomes) revealed that significant enrichment of DPPs involved in the

typical environmental information processing (EIP) pathways, phosphatidylinositol signaling system, plant hormone signal transduction, and MAPK signaling pathway-plant (Fig. 2D).

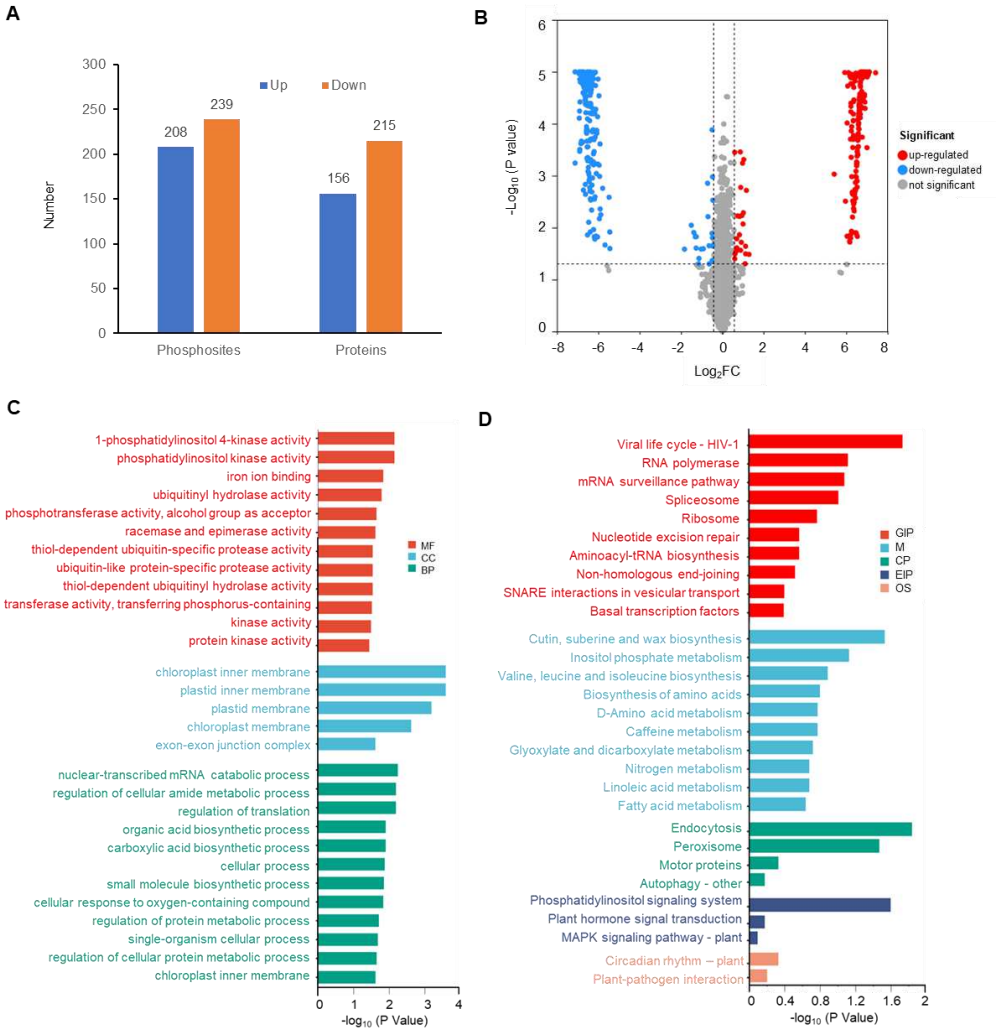



**Fig. 2. Phosphoproteomics analysis of proteins induced by systemin.** (A) The number of identified phosphor-sites and proteins in tomato fruits treated with systemin for 1 h. (B) Volcano plots of fold changes of phosphor-sites induced by systemin. (C) GO enrichment analysis of the differentially expressed phosphoproteins (DPPs) regulated by systemin. CC, cellular component; MF, molecular function; BP, biological process. (D) KEGG enrichment analysis of DPPs regulated by systemin. GIP, genetic information processing; M, metabolism; CP, cellular processes; EIP, environmental information processing; OS, organismal systems.

Further analysis of these DPPs, we found 20 kinase proteins encompassing 23 phosphopeptides were upregulated by systemin, such as calcium-dependent protein

kinases (CPKs), calcineurin B-Like (CBL)-interacting protein kinases (CIPKs), sucrose nonfermenting 1-related protein kinases (SnRKs), mitogen-activated protein kinase kinase kinases (MAP3Ks), and receptor-like kinases (RLKs) (Fig. 3A). Two different phosphopeptides belonging to a MAP4K were identified (Fig. 3B and C). To explore the roles of MAP4Ks in tomato, eight MAP4Ks were characterized in tomato, and this systemin-induced MAP4K was the homolog of *Arabidopsis* serine/threonine kinase 1 (AtSIK1) (Fig. S2). Then, we analyzed the activity of MAPKs in *prosys#1* and *prosys#2* mutants. After 24 h cold treatment, phosphorylation level of MAPKs was obviously decreased in both *prosys* mutants (Fig. 3D). Notably, the key cold tolerance regulator HY5 downstream of MAPKs was detected to be phosphorylated by systemin, as well as HY5-mediated protein ABA INSENSITIVE 5 (ABI5) (Fig. 3E and F). Therefore, these results demonstrated that the MAPK-HY5-ABI5 could be downstream of the systemin signaling pathway.

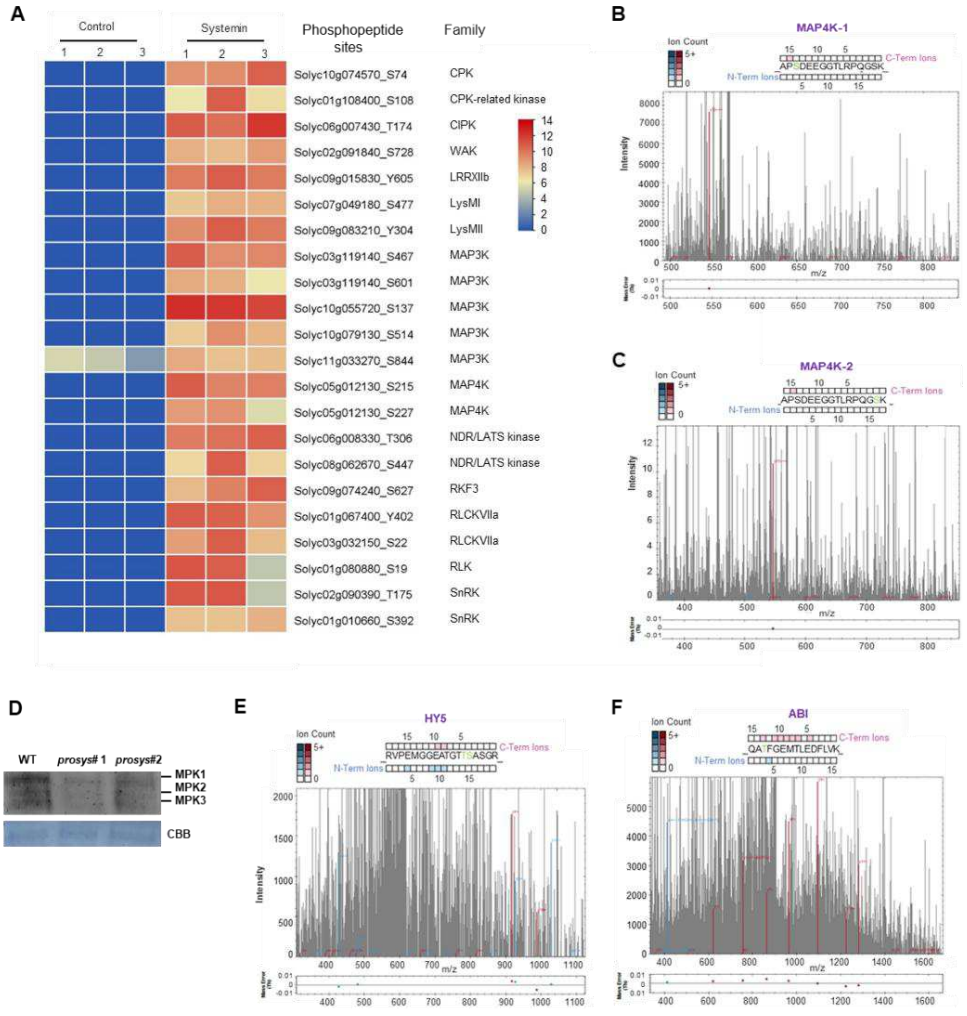


**Fig. 3. Phosphorylation level of proteins regulated by systemin.** (A) Heatmap of the normalized phosphorylation level of kinases in systemin-treated fruits. Systemin induced the phosphorylation of SlSIK1 at Ser215 (B) and Ser227 (C). (D) The phosphorylation level of MAPKs decreased in *prosys* (*#1* and *#2*) tomato fruit exposed to 4℃ for 1 h. Three independent experiments were performed, and similar results were obtained. CBB, Coomassie Brilliant Blue staining. Systemin induced the phosphorylation of HY5 at Thr54 (E) and ABI5 at Thr172 (F).

## Transcriptomics analysis of systemin-mediated responses in chilling stress

To analyze the functional mechanism of systemin in chilling tolerance, an RNA-seq was performed using the *prosys#2* mutant, as systemin treatment could fully recover the CI of *prosys#2*. After 24 h chilling treatment, a total of 963 differentially expressed genes (DEGs) ($q \leq 0.05$ and $|\log2FC| \geq 1$) were detected in *prosys#2*, encompassing 661 upregulated and 303 downregulated genes (Fig. 4A, Table S2). Besides, 19 genes encoding kinases were downregulated in the *prosys#2* mutant after chilling stress, such as MAP3Ks and RLK genes (Fig. 4B). GO enrichment analysis indicated that the majority of DEGs are associated with MF terms, catalytic activity, oxidoreductase activity, and receptor serine/threonine kinase binding (Fig. 4C). Besides, monolayer-surrounded lipid storage body, plasma membrane, lipid droplet, and extracellular space were the most highly enriched CC items. The GO results also suggested that several DEGs were involved in biological process (BP), defense response to fungus, defense response, response to freezing, and diterpenoid metabolic process. On the other hand, KEGG results exhibited that DEGs were related to the metabolism of fatty acid (fatty acid biosynthesis, fatty acid elongation, and fatty acid degradation) and terpenoids (sesquiterpenoid and triterpenoid biosynthesis, and diterpenoid biosynthesis) and EIP pathways (plant hormone signal transduction, ABC transporters, phosphatidylinositol signaling system, MAPK signaling pathway-plant (Fig. 4D).

Furthermore, we focused on transcription factors (TFs) based on the critical roles of TFs in the regulation of gene expression. In total, we identified 24 TFs belonging to six families, such as Zinc finger transcription factors and bHLHs (Table. S2). Further qRT-PCR results indicated that the expression of *ERF.D4*, *C2H2-ZF*, *MYB10*, and

*WRKY75* was decreased in both *prosys#1* and *prosys#2* mutants (Fig. 4E).

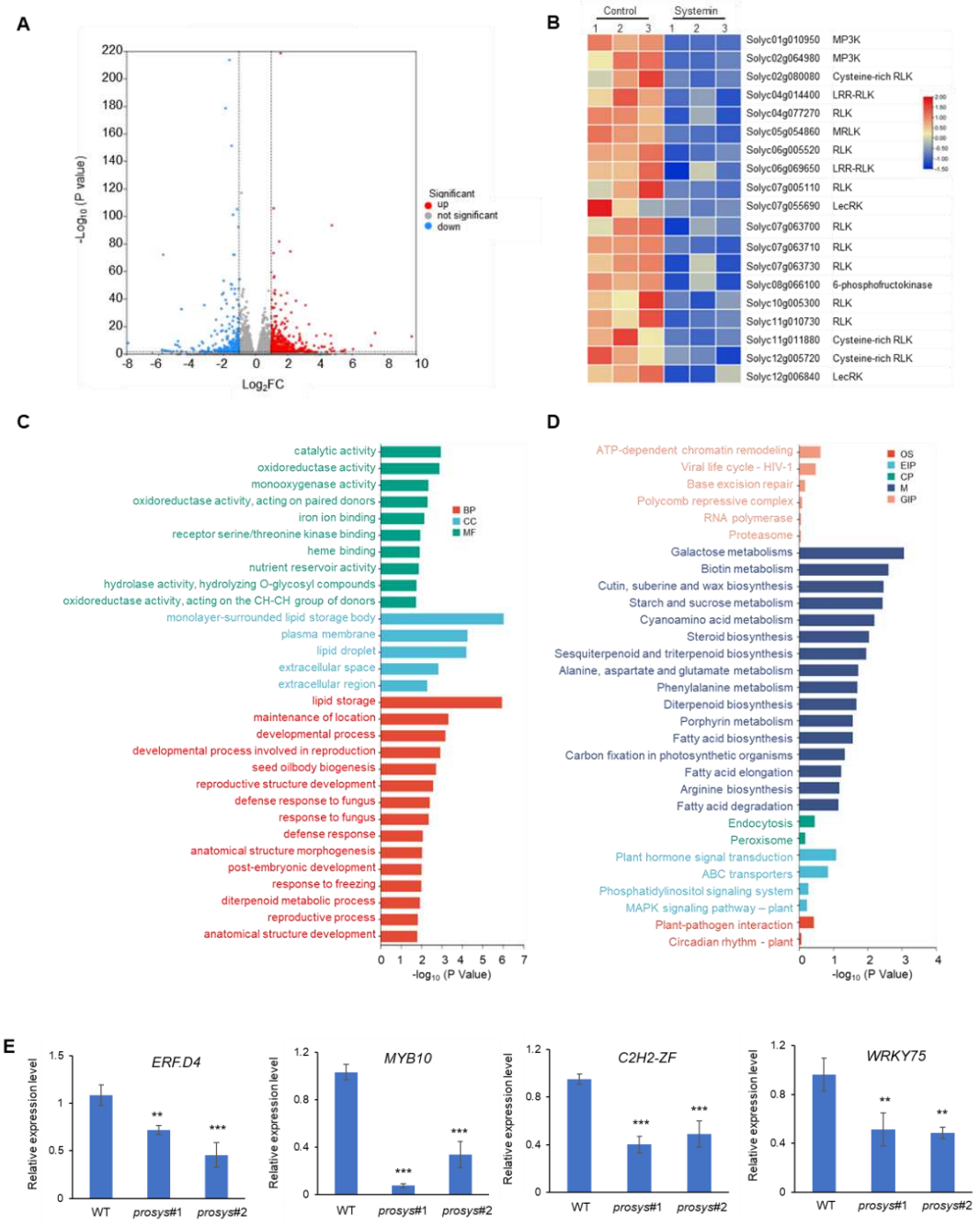



**Fig. 4. Identification and functional analysis of prosystemin-regulated genes by RNA-seq assay.** (A) Volcano plots of fold changes of differentially expressed genes (DEGs) in *prosys#*2 exposed to 4℃ for 24 h. (B) Expression levels of the kinases regulated by prosystemin under chilling stress. (C) GO enrichment analysis of the DEGs regulated by prosystemin. CC, cellular component; MF, molecular function; BP, biological process. (D) KEGG enrichment analysis of DEGs regulated by prosystemin. GIP, genetic information processing; M, metabolism; CP, cellular processes; EIP, environmental information processing; OS, organismal systems. (E) Prosystemin promotes the

expression levels of four TFs. The expression of *ERF.D4*, *MYB10*, *C2H2-ZF*, and *WRKY75* was decreased in *prosys* mutants exposed to 4℃ for 24 h. Error bars indicate SD. Statistically significant differences between mutants and WT are depicted with asterisks (**$P < 0.01$; ***$P < 0.001$, Student's *t*-test).

**Metabolomics analysis of systemin-mediated responses in chilling stress**

In order to dissect the metabolites regulated by systemin in cold responses, a metabolomics assay was conducted using both *prosys#1* and *prosys#2* mutants after 14 d of cold storage through analysis of 9555 metabolite compounds. The differentially expressed metabolites (DEMs) were selected using the thresholds of $p$-value $\leq 0.05$ and VIP $\geq 1$. Consequently, a total of 417 DEMs were identified in *prosys#1*, including 182 upregulated and 235 downregulated metabolites (Fig. 5A, Table S3). Besides, 245 upregulated and 166 downregulated metabolites were detected in *prosys#2* (Fig. 5B, Table S4). In detail, terpenoids were the largest group of the identified metabolites, which occupied 24.94% and 30.86% of DEMs of *prosys#1* and *prosys#2*, respectively (Fig. 5C and E). The second and third largest DEM groups were shikimates and phenylpropanoids (24.46%) and alkaloids (23.03 %) in the *prosys#1* mutant. In the *prosys#2* mutant, the second and third largest DEM groups were alkaloids (20.57%) and shikimates and phenylpropanoids (13.71%). Fatty acids were the fourth-largest DEM group in both *prosys#1* (10.31%) and *prosys#2* (13.71%) mutants. KEGG analysis indicated that 6 and 11 DEMs in *prosys#1* and *prosys#2* mutants were assigned to 5 and 11 KEGG items, respectively (Table S5 and S6). Moreover, 113 DEMs were detected in both mutants, remaining 304 and 298 DEMs specific in *prosys#1* and *prosys#2*, respectively (Fig. 5E). The shikimates and phenylpropaniods (24.78%) and alkaloids (23.01%) were the top two groups of these overlapped DEMs (Fig. 5F).

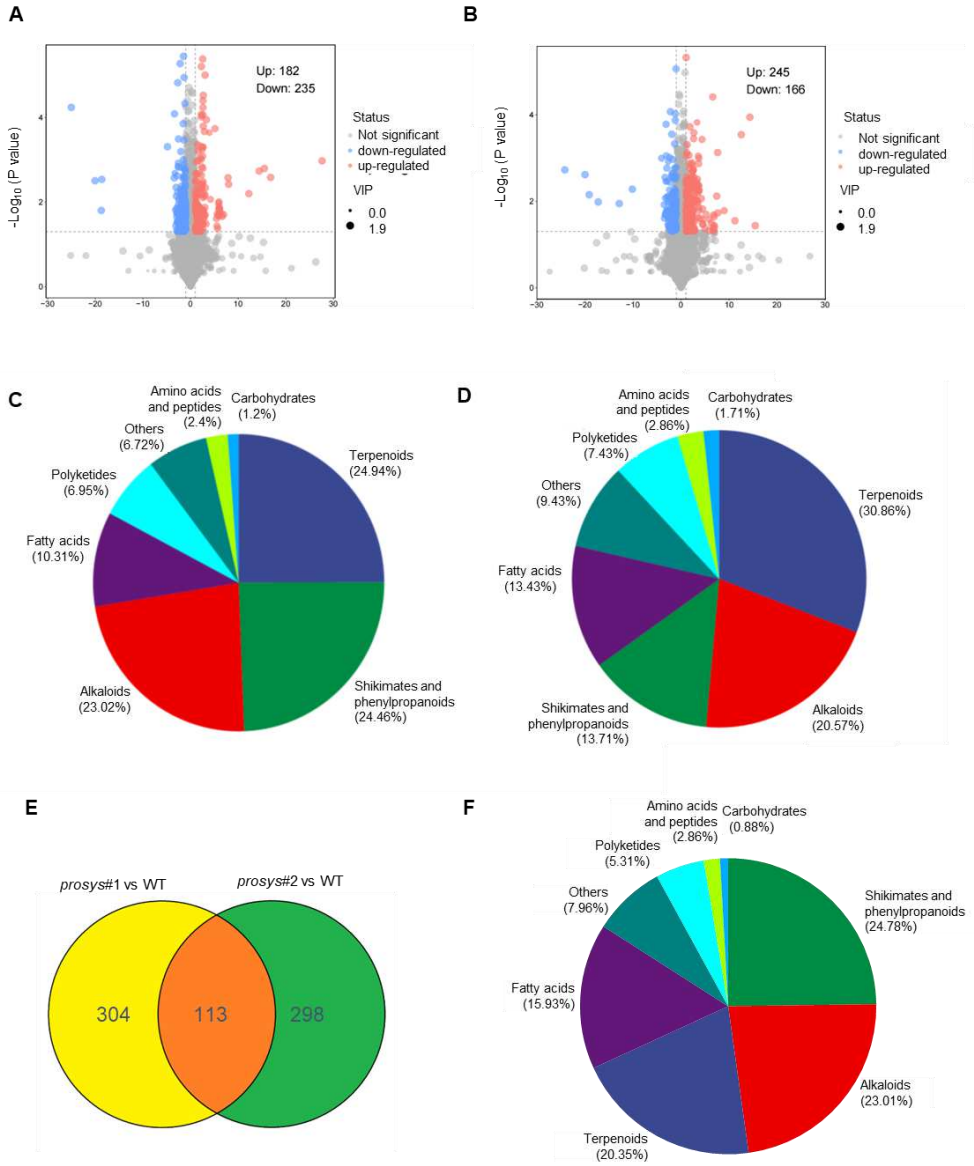



**Fig. 5. Metabolites regulated by prosystemin in chilling stress.** Volcano plots of fold changes of DEMs identified in *prosys#1* (A) and *prosys#2* (B) after 14 d cold storage. Classification and proportions of DEMs in *prosys#1* (C) and *prosys#2* (D), respectively. (E) Venn diagram of overlapped DEMs in *prosys#1* and *prosys#2*. (F) Classification and proportions of the overlapped DEMs.

## Systemin regulates flavonoid biosynthesis

Among the overlapped DEMs, we observed eleven important flavonoids in shikimates and phenylpropaniods group, such as quercetin, peltatoside, kaempferol, jaceosidin, and flavanone (Fig. 6A). Further analysis of the RNA-seq data, nine DEGs were involved in flavonoid biosynthesis pathway (sly00941), consisting of four genes

encoding key enzymes, cinnamic acid 4-hydroxylase (C4H), O-methyl transferases (OMT), flavanone 3-hydroxylase (F3'H), and flavanone 3', 5'-hydroxylase (F3'5'H) (Fig. 6B). The expression of the four genes was checked in *prosys#1* and *prosys#2* mutants (Fig. 6C). The results suggested that the expression of *C4H* and *OMT* was decreased in both mutants. The expression of *F3'H* was upregulated in the two mutants. In comparison, the expression of *F3'5'H* was only increased in the *prosys#2* mutant. We further detected the activity of the four enzymes in the mutants. The activity of C4H in *prosys#1* showed no obvious changes compared with WT. However, C4H activity was increased in the *prosys#2* mutant (Fig. 6D). The activity of OMT was decreased in both mutants. The activities of F3'H and F3'5'H were improved only in the *prosys#2* mutant.

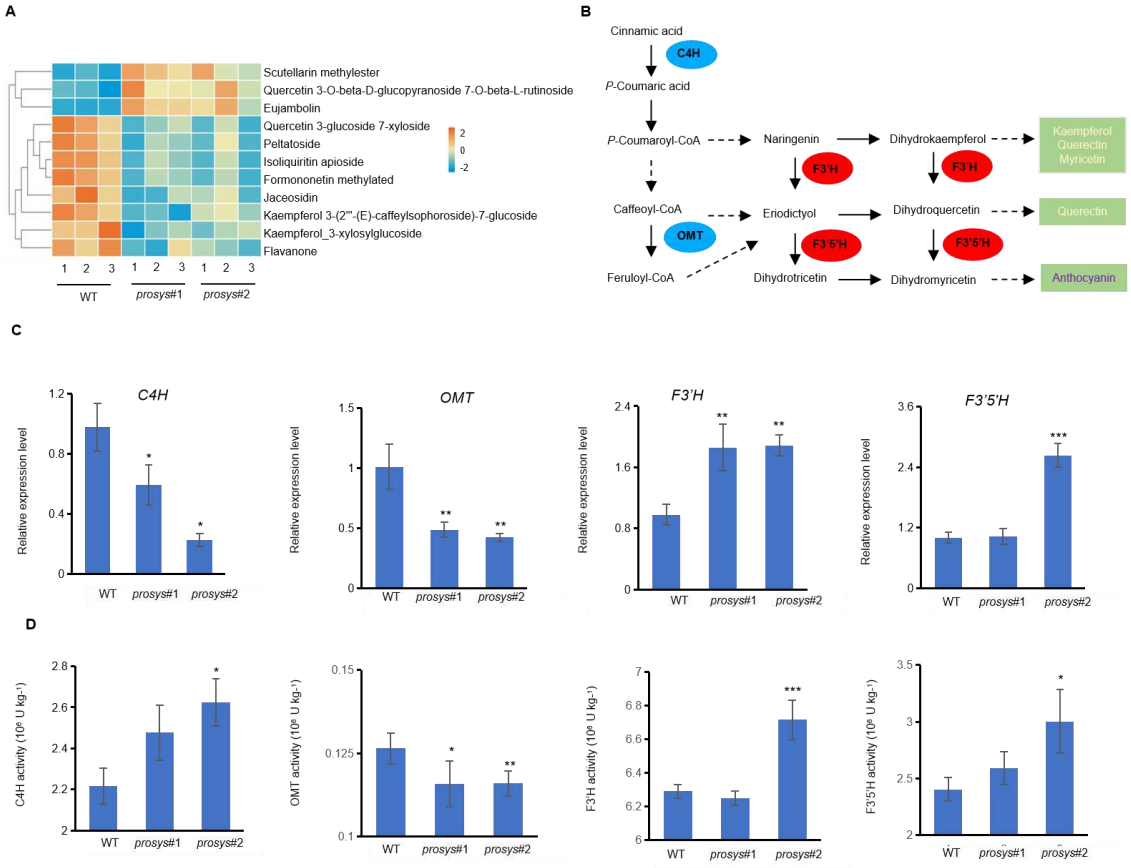



**Fig. 6. Prosystemin-regulated flavonoid biosynthesis during cold storage.** (A) Heatmap of flavonoids in *prosys#1* (A) and *prosys#2*. (B) Process of the flavonoid biosynthesis pathway. The expression (C) and activity (D) of C4H, OMT, F3'H, and F3'5'H.

## Systemin regulates capsaicin biosynthesis

Moreover, nine well-known alkaloid DEMs were identified in *prosys#1* and *prosys#2* mutants, including five upregulated and four downregulated alkaloids (Fig. 7A). The RNA-seq results revealed that three DEGs were related to the biosynthesis of various alkaloids pathway (sly00996). Among them, two genes encode enzymes for capsaicin biosynthesis, a gamma-aminobutyric acid transaminase (GABA-T) and an acyltransferase (AT) (Fig. 7B). We checked the original metabolomics results, and found that the content of vanillin was decreased by 0.16-fold in *prosys#1* and 0.23-fold in *prosys#2*, respectively (Fig. S3). Consistent with the RNA-seq results, the expression of *GABA-T* and *AT* increased and decreased in *prosys#1* and *prosys#2* mutants by qRT-PCR, respectively (Fig. 7C). However, the activity of GABA-T was induced only in the *prosys#2* mutant. The AT activity was downregulated in both mutants (Fig. 7D).

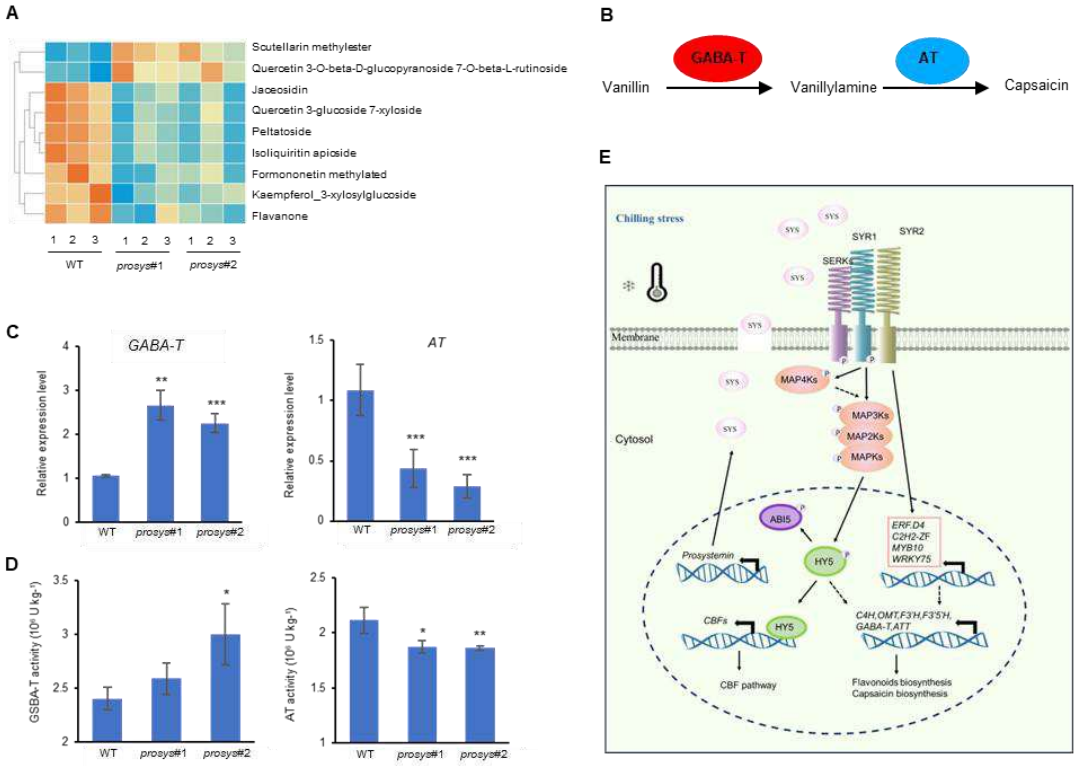



**Fig. 7. Prosystemin-regulated alkaloid biosynthesis after cold storage.** (A) Heatmap of alkaloids in *prosys#1* and *prosys#2*. (B) Process of the capsaicin biosynthesis pathway. The expression (C) and activity (D) of GABA-T and AT. (E) A proposed model for the mechanism of systemin-regulated tomato fruit chilling resistance. The SYR1/2 perceive the chilling-induced systemin peptide and trigger the phosphorylation of kinases (SIK and MA3KPs), leading to the activation of MAPKs. In

addition, the phosphorylated TF HY5 enhances the expression of *CBFs* to induce the CBF-regulated cold responses. Meanwhile, systemin induced the expression of four other TFs and the biosynthesis of flavonoids and capsaicin.

## Discussion

Cold storage is an efficient technology to maintain postharvest quality of fruits and vegetables. However, many cold-sensitive fruits and vegetables are prone to develop chilling injury (CI) at low temperature, which significantly impacts the food industry. Plant hormones are key modulators to activate cold response pathways, leading to the enhancement of plant resistance[34]. Research on the functions of plant hormones, such as jasmonate acid (JA), brassinolide (BR), ethylene (ET), cytokinin (CK), and strigolactone (SL), in the regulation of plant responses to cold stress has increased dramatically[4,35,36]. Though the importance of plant peptide hormones in biotic and abiotic stresses has been revealed recently. Research on peptide hormones responding to cold stress is relatively rare[22]. Our study indicated that systemin increased tomato fruit resistance to chilling stress. Further combination of phosphoproteomics, transcriptomics, and metabolomics analyses revealed potential mechanisms of systemin regulating chilling resistance in tomato (Fig. 7E).

Protein phosphorylation modification is crucial for signaling transduction in plants, which is mainly regulated by protein kinases[33]. Several protein kinases were identified in our phosphoproteomics and transcriptomics assays. In plants, RLKs, one of the largest protein families, are vital for both biotic and abiotic stress responses[37,38]. Some systemin-regulated RLKs and RLCKs could be candidates for co-receptors in the systemin signaling pathway (Fig. 2B). In addition, we identified many MAP kinase kinase kinases (MAP3Ks, MEKKs). The canonical MAPK cascades are composed of MAP3K, MAPK kinase (MAPKK, MAP2K, MEK), and MAPKs (MPKs)[39,40]. MAPK cascades are key signaling modules downstream of RLKs and RLCKs responding to endogenous and exogenous signals[41]. Cold stress activate MPK3/6, which are inhibited by CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE1/2 (CRLK1/2) and the MEKK1-MKK2-MPK4 cascade[5,42]. Notably, phosphorylation of

two peptides derived from a MAP4K was induced by systemin (Fig. 2B and C). The yeast MAP4Ks and the mammalian MAP4Ks directly activate MAPK cascades[43,44]. Though there is no evidence that plant MAP4Ks act on MAP3Ks so far, many MAP4Ks have been identified to function in plant abiotic stresses[40,45,46]. Furthermore, eight MAP4Ks in the tomato genome were isolated in tomato (Fig. S1). The systemin-induced MAP4K is the homolog of AtSIK1, which plays important roles in immunity, as well as cell proliferation and expansion[47,48]. Therefore, it is meaningful to analyze whether SlSIK1 regulates some of these identified MAP3Ks in the systemin signaling pathway. In addition, systemin induced the phosphorylation of MAPKs in tomato fruits[26]. The MAPK activity was decreased in *prosys#1* and *prosys#2* mutants after chilling stress (Fig. 3D). Collectively, these results revealed the systemin signal transduction in response to chilling stress through the MAPK cascades.

Transcriptional regulation plays an essential role in cold acclimation via recruiting many critical transcription factors (TFs)[49]. The CBF pathway is central in plant cold tolerance by directly activating the expression of COLD-RESPONSIVE (COR) genes[8]. Systemin treatment increased the expression of *CBF1*, *CBF2*, and *CBF3*. In addition, systemin induced the phosphorylation of the TF HY5, which regulates CBFs in cold resistance of tomato[20]. The knockout *hy5* mutants decrease cold tolerance and the levels of *CBF* transcripts. In contrast, overexpression of *HY5* improves cold tolerance and the expression of *CBFs*. HY5 activates the expression of *CBF* by binding directly to the promoters of CBF genes. Besides, the phosphorylation activity of a downstream molecule of HY5, ABA INSENSITIVE5 (ABI5), was also enhanced by systemin[19]. The function of HY5 in cold tolerance is related to SlMPK1 and SlMPK2, which physically interact with and phosphorylate the upstream molecule of HY5, SlBBX17[50]. Taken together, it is suggested that systemin regulates chilling tolerance via the MAPKs-HY5-ABI5 module in a CBF-dependent manner. On the other hand, we also identified several TFs in RNA-seq, such as four downregulated TFs (Fig. 4E). These four TFs could be key downstream molecules of systemin-regulated cold resistance.

Flavonoids are one of the most important secondary metabolites responding to

cold. Flavonoids act as antioxidants to scavenge reactive oxygen species (ROS) generated by stresses[7]. Accumulation of flavonoid metabolites combined with gene expression related to the flavonoid biosynthesis pathway is increased in chilling condition of chrysanthemum (*Chrysanthemum morifolium*, ground-cover Chrysanthemums)[51]. Through metabolomics assay, we identified a large number of DEMs, including shikimates and phenylpropanoids as the top metabolite group in both *prosys#1* and *prosys#2* mutants (Fig. 5). Furthermore, the expression of genes encoding key enzymes in flavonoid biosynthesis was observed in the two mutants, as well as the activity of the enzymes. Except for C4H with converse results between expression level and enzyme activity, the expression of *OMT*, *F3'H,* and *F3'5'H* was consistent with the enzyme activity, resulting in changes of flavonoid content in the two mutants (Fig. 6). Possibly, the different results between the C4H expression level and enzyme activity are due to other C4Hs with elevated activity in tomato. In addition, the different results of *F3'H* and *F3'5'H* between the two mutants may reflect the functional distinction between the precursor and mature peptide of systemin. Previous research suggested that HY5 mediated chilling tolerance via directly binding to the promoter regions, and stimulated the transcription of flavonoid biosynthesis genes, such as *chalcone synthase 1* (*CHS1*), *chalcone isomerase 3* (*CHI3*), and *flavanone 3-hydroxylase* (F3H), thereby increasing the biosynthesis of flavonoids[52]. In addition, the TF SlGRAS4 also promotes chilling tolerance through regulating flavonoid content[53]. Consequently, it is possible that systemin employed HY5 or other TFs to regulate the biosynthesis of flavonoids in chilling response, which requires further analysis.

Alkaloids represent a large family of secondary compounds, containing at least one nitrogen atom with diverse structures and bioactivities[54]. The roles of alkaloids have been identified in biotic and abiotic resistance[55]. Abiotic stresses such as drought and salinity affect the accumulation of alkaloids in many plant species. Nevertheless, research on the role of alkaloids in cold response is hardly found. From the metabolomics data, we identified a large number of alkaloids, especially cinnamoylcocaine, scopine, and tyramine. Moreover, the expression and activity of two

enzymes in the capsaicin biosynthesis pathway, gamma-aminobutyric acid transaminase (GABA-T) and acyltransferase (AT), were upregulated and downregulated in both mutants. Besides, the content of vanillin was reduced to a low level in the mutants after cold storage (Fig. S2). The function of capsaicin is widely focused on antidiabetic, anticancer, and body weight control substance, highlighting its anti-inflammatory properties[56]. However, its roles in plants are largely unknown. Therefore, our results demonstrated the novel regulatory pathway of systemin on capsaicin biosynthesis in cold stress. Terpenoids are another important group of secondary metabolites of plants. We observed several metabolites belonging to terpenoids in *prosys#1* and *prosys#2* mutants after chilling stress (Fig. 5). Accordingly, genes involved in terpenoid biosynthesis were identified in the RNA-seq (Fig. 4D). Collectively, our findings suggested that systemin regulated the production of various metabolites to respond to chilling stress.

In conclusion, our combination of multiple omics revealed the overall signaling transduction pathway, gene regulatory network, and metabolite biosynthesis regulated by systemin in responding to chilling stress. Many kinases, such as SlSIK1, and the four downregulated TFs could be important candidates in systemin-regulated chilling tolerance. Notably, it is suggested that systemin regulated MAPKs-HY5-CBFs to modulate flavonoid biosynthesis, which requires further studies such as genetic functional validation. Moreover, these results indicated that systemin could be an efficient molecule to improve tomato fruit quality during cold storage.

## Methods

### Plant materials and treatments

Tomato (*Solanum lycopersicum* L. cv. MicroTom) was used as the wild-type (WT). The knockout mutants of *prosystemin* were constructed via the CRISPR/Cas9 genome editing system. Tomato seeds were sterilized in 10% (v/v) sodium hypochlorite and sown on half-strength Murashige and Skoog (1/2 MS) medium (pH 5.8) agar plates. After germination, the seedlings were grown at 25 °C under a 16 h light and 8 h dark photoperiod.

The mature fruits were harvested, and those of similar size and color without blemishes were selected for the experiments. The WT or mutant fruits were randomly divided into three groups, with at least ten fruits for each group. The mature peptide sequence of systemin (AVQSKPPSKRDPPKMQTD) was synthesized by Shanghai Top-peptide Biochemical Company (China) and dissolved in double-distilled water. The fruits were sterilized and sprayed with 1 μM systemin or double-distilled water as the control. After 24 h, the fruits were stored at a low temperature (4 °C) for 14 d, and then moved to room temperature for 3 d to observe the chilling stress phenotype.

**Chilling injury (CI) index Analysis**

The main symptom of tomato CI is surface lesions, such as shrinking and denting[57]. The chilling symptoms were classified into five classes according to the proportion of damaged surface area: 0 = no CI symptom, 1 = less than 10%, 2 = from 10% to 25%, 3 = from 25% to 50%, 4 = from 50% to 75%, and 5 = more than 75%. CI index = Σ (scale × number of fruit within this scale) / (total fruit number × 5)[58].

**Phosphor-proteomics**

WT fruits were treated with 1 μM systemin for 1 h, put in liquid nitrogen, and stored at −80°C until use. Three biological replicates were performed with five fruits for each. Protein was extracted via the tris-balanced phenol extraction (BPP) method, and protein concentration was determined by the Bradford assay, followed by SDS-PAGE for quality control. A total of 100 μg protein was digested by Trypsin at 37 °C overnight. The concentration of peptide was determined by a peptide quantification kit (Thermo, 23275) and adjusted to 0.25 μg/μL for each sample. The peptides were first dissolved using the wash buffer, loaded onto the column, and incubated for 30 min. After twice elution, the peptides were added an equal volume of 20% TFA acidification, stage-tip desalination, and dried by a vacuum concentrator. The peptides were redissolved in spectrometry loading buffer and analyzed by LC-MS/MS performed on a VanquishNeo liquid chromatography system coupled with an Orbitrap Astral mass spectrometer (Thermo, USA) in data-independent acquisition (DIA) mode at Majorbio Bio-Pharm Technology Co. Ltd. (China). Spectronaut software (Version 19) was used

to search the DIA raw data in the tomato genome (SL4.0), and the MaxLFQ method was used for protein quantification. The peptides with $p$-value < 0.05 and a fold change >1.5 or < 0.75 were identified as differentially expressed. GO annotation analysis of target proteins was conducted using the Gene Ontology database (http://geneontology.org/). Additionally, the KEGG pathway database (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg//) was used to analyze the functional pathways associated with the target proteins.

**MAPK phosphorylation assay**

Fruits of WT and mutants were exposed to 4°C for 24 h, harvested, put in liquid nitrogen, and stored at −80°C until use. Fruits were ground in liquid nitrogen, and 0.1 g of sample was suspended in 1 mL extraction buffer (150 mM Tris-HCl, pH 8.0, 100 mM KCl, 1.4 mM sucrose, 0.2% Nonidet P-40, 5 mM DTT, 1 × complete EDTA-free protease inhibitor cocktail (Roche, Switzerland), 1 × PhosSTOP phosphatase inhibitor cocktail (Roche, Switzerland)). The concentration of protein was measured by the Pierce 660 nm Protein Assay (Thermo Fisher Scientific, USA). Immunoblotting of phosphorylated MAPKs was performed using the anti-phospho p44/42 MAPK antibody (Cell Signaling, USA) and peroxidase-conjugated goat anti-rabbit IgG (Sigma, USA) antibody[59].

**RNA-seq assay**

The fruits of WT and the *prosys#2* were stored at 4°C for 24 h, put in liquid nitrogen, and stored at −80°C until use. Three biological replicates were performed with five fruits for each. Total RNA was extracted using the RNA Extraction Kit (Tiangen Biotech, DP441, China). The transcriptome library was constructed by TruSeqTM RNA Sample Preparation Kit from Illumina (San Diego, USA) and sequenced on an Illumina HiSeq xten/NovaSeq 6000 sequencer. The raw paired end reads were trimmed, and the quality was controlled by SeqPrep and Sickle with default parameters. Then, clean reads were separately aligned to the tomato genome (SL4.0)[26]. Differential expression analysis of *prosys#2* compared with the WT samples was conducted by DESeq2 with q ≤ 0.05 and $|\log_2 FC| \geq 1$. The Gene Ontology (GO) functional enrichment and Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathway analysis were performed by Goatools and KOBAS with *P*-value ≤ 0.05.

**qRT-PCR**

After cold treatment, fruits of WT and mutants were collected, put in liquid nitrogen, and stored at −80°C until use. Three biological replicates were performed with five fruits for each. Total RNA was extracted by using an RNA Extraction Kit (Tiangen Biotech, DP441, China) and then reverse-transcribed by the cDNA synthesis kit (TAKARA, Japan). The qRT-PCR reactions were performed on a 6500 Real-Time PCR System (Applied Biosystems) using SYBR Fast qPCR Mix (Qingke, China). Relative expression level was calculated using the $2^{-\triangle\triangle CT}$ method[60]. The tomato *Slactin* was used as an internal control to normalize gene expression among different samples, and the primers were listed in Table S7.

**Metabolomics**

The fruits of WT, *prosys#1* and *prosys#2* were exposed to 4°C for 14 d, put in liquid nitrogen, and stored at −80°C until use. After being homogenized into fine powder in liquid nitrogen, a total of 25 mg of powder from each sample was used to extract metabolites in 1000 μL solution (methanol/water = 4:1, v/v). 25 μL of each sample was used, and the UPLC separation was carried out using a Vanquish (Thermo Fisher Scientific) with a Kinetex C18 (2.1 mm × 50mm, 2.6 μm). The mobile phase A was 0.01% acetic acid in water, and the mobile phase B was 50% ACN/IPA at a flow rate of 0.3 mL/min. A Stellar (Thermo) mass spectrometer was used for sample detection. Ion source parameters were: Spray Voltage: 3.8 kV/-3.4 V, Sheath Gas: 50 Arb, Aux Gas: 15 Arb, Sweep Gas: 1 Arb, Ion Transfer Tube Temp: 320 ℃, Vaporizer Temp: 400 ℃. The MS data acquisition and quantitative analysis of target compounds were completed using Biobud JZGB automated data processing software by Biotree Biotech Co., Ltd. The Variable importance in the projection (VIP) was obtained from the OPLS-DA model. Metabolites with *p*-value ≤ 0.05 and VIP ≥ 1 were determined as the differentially expressed metabolites (DEMs), and KEGG annotations and enrichment analysis of DEMs were applied.

## Proline content and Enzyme activity assay

Tomato fruit samples were homogenized into fine powder in liquid nitrogen. A total of 100 mg of fruit was dissolved in 1 mL PBS (pH 7.4) buffer and centrifuged at 5000 g for 15 min. The supernatant was collected and used for proline content detection by the Proline (Pro) Content Detection kit (Solarbio, China). The activity of cinnamic acid 4-hydroxylase (C4H), O-methyl transferases (OMT), flavanone 3'-hydroxylase (F3'H), flavanone 3'5'-hydroxylase (F3'5'H), gamma-aminobutyric acid transaminase (GABA-T), and acyltransferase (AAT) was measured by the enzyme-linked immunosorbent assay (ELISA) kits from Mmbio company (China).

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## Author contributions

P. Y. W., Y. G., Z. G. L., and Q. G. G. designed, wrote, and revised the manuscript. P. Y. W., Y. C. H., J. X. F., and Y. W. W. performed the experiments. P. Y. W., and J. X. F. drew the figures and made the tables. All authors read and approved the final manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Data availability

There are no new data associated with this article.

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