

# Physiological, transcriptomic, and metabolomic analyses of the chilling stress response in two melon (*Cucumis melo* L.) genotypes

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## Research Article

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# Abstract

## Background

Chilling stress is a key abiotic stress that severely restricts the growth and quality of melon (*Cucumis melo* L.). Few studies have investigated the mechanism of response to chilling stress in melon.

## Results

We characterized the physiological, transcriptomic, and metabolomic response of melon to chilling stress using two genotypes with different chilling sensitivity ("162" and "13-5A"). "162" showed higher osmotic regulation ability and antioxidant capacity to withstand chilling stress. Transcriptome analysis identified 4395 and 4957 differentially expressed genes (DEGs) in "162" and "13-5A" under chilling stress, respectively. Metabolome analysis identified 2347 differential enriched metabolites (DEMs), which were divided into 11 classes. Integrated transcriptomic and metabolomic analysis showed enrichment of glutathione metabolism, and arginine and proline metabolism, with differential expression patterns in the two genotypes. Under chilling stress, glutathione metabolism-related DEGs (6-phosphogluconate dehydrogenase, glutathione peroxidase, and glutathione s-transferase) were upregulated in "162," and GSH conjugates (L-gamma-glutamyl-L-amino acid and L-glutamate) were accumulated. Additionally, "162" showed upregulation of DEGs encoding ornithine decarboxylase, proline dehydrogenase, aspartate aminotransferase, pyrroline-5-carboxylate reductase, and spermidine synthase and increased arginine, ornithine, and proline. Furthermore, the transcription factors MYB, ERF, MADS-box, and bZIP were significantly upregulated, suggesting their crucial role in chilling tolerance of melon.

## Conclusions

These findings elucidate the molecular response mechanism to chilling stress in melon and provide insights for breeding chilling-tolerant melon.

## Background

Chilling stress adversely affects plant growth and development, reduces plant biomass and quality, and limits their geographical distribution [1, 2]. Many tropical and subtropical plant species are susceptible to chilling injury. Chilling stress causes various physiological and biochemical changes and alters gene expression patterns. It alters the membrane structure and fluidity, causes excessive accumulation of reactive oxygen species (ROS), limits water availability, inhibits photosynthetic efficiency, and slows down biochemical reactions [3, 4].

Plants respond and adapt to low temperature through a process known as cold acclimation. Cold-responsive genes play a crucial role in cold acclimation [5]. The inducer of C-REPEAT BINDING FACTOR

expression (ICE)-C-repeat binding factor (CBF)-cold-regulated gene (COR) pathway has been extensively studied. As an upstream inducer, ICE induces the expression of *CBF* and a series of COR genes located downstream, thus enhancing plant chilling tolerance [6, 7]. Transcription factors (TFs) such as HSFC1, CZF1, ZAT12, NPR1, ZF, RAV1, and HY5 induce *COR* gene expression in a CBF-independent manner under chilling stress [8, 9]. Many studies have reported that improved cold tolerance in melon is correlated with higher expression levels of *CmCBF1*, *CmCBF2*, *CmCBF3*, *CmCBF4*, and *CmEAF7* [10–13].

Melon, a member of the widespread Cucurbitaceae family, represents an economically important fruit crop. According to FAOSTAT (<http://faostat.fao.org>, 2020), the global melon production was 27.5 million tons. In China, the largest producer of melons worldwide, the melon planting area and output reached  $3.95 \times 10^5$  ha and  $1.38 \times 10^7$  tons, respectively. Melon is sensitive to low temperatures, especially during the germination and seedlings stages. However, melon cultivation in early spring or winter is often affected by the chilling stress, which adversely affects yield and quality [14]. Li et al. (2022) reported that suboptimal low temperatures inhibit melon growth and delay flowering [15].

In recent years, a multi-omics approach has been effectively applied to study the abiotic stress response in plants [16, 17], including cotton [18], wheat [19], maize [20], tomato [21], apple [22], cucumber [23], watermelon [24], and melon [11, 25]. Although the genome of melon has been sequenced [26], few studies have investigated the mechanism of response to chilling stress in melon. In the present study, two melon genotypes with contrasting responses to chilling stress were subjected to physiological, transcriptomic, and metabolomic analyses. The findings are expected to provide novel insights into the molecular mechanism of response to chilling stress in melon.

## Results

### Morphological and Physiological Indicators under Chilling Stress

Growth morphological characteristics differed between the two melon genotypes exposed to chilling stress for 12 h (Fig. 1A). In the 13-5A genotype, the whole plant wilted after 12 h of chilling stress. In contrast, the degree of chilling stress injury in the genotype 162 was lower than that in 13-5A, indicating higher chilling resistance. In the present study, chilling stress caused  $H_2O_2$  accumulation in the leaves of 13-5A genotypes. However, under chilling stress,  $H_2O_2$  content was no significant difference in 162 than in 13-5A (Fig. 1B). Under the chilling stress treatment, the Fv/Fm of the leaves differed between the two melon genotypes. The Fv/Fm of leaves in the genotype 13-5A was significantly lower in comparison with the control, whereas that in the genotype 162 maintained a suitable state (Fig. 1B).

In this study, chilling stress increased the compatible solute content in 162 genotypes under chilling stress (Fig. 1C). At 12 h of chilling stress, the soluble sugar and soluble protein content in 162 was significantly higher than that in 13-5A (Fig. 1C). As shown in Fig. 1C, the leaves of 13-5A showed significantly higher  $H_2O_2$  and MDA content after 12 h of chilling stress than that observed in all other

groups. In the control group (0 h exposure), The SOD and POD activity did not differ between the two melon genotypes. However, in the treatment group (12 h of chilling stress), the SOD and POD activity was significantly higher in the genotype 162 than in 13-5A (Fig. 1D). After chilling stress, the CAT and APX activities of the 162 genotypes showed obviously increased, and it was approximately 32.4-fold to 3.2-fold higher than that in the control group (0 h; Fig. 1D).

Significant physiological differences observed between the two melon genotypes at 12 h of chilling stress treatment suggest that it is an important phase in the chilling response. Therefore, seedlings treated at 6 °C for 0 h and 12 h were selected for the subsequent transcriptomic and metabolomic analyses.

## **Transcriptome Analysis of 162 and 13-5A under Chilling Stress**

RNA-seq was performed using a total of 12 samples of the genotypes 162 and 13-5A under control conditions (28 °C/20 °C, “0h”) and chilling stress treatment at 6 °C for 12 h (“12h”). PCA indicated that the samples showed significant differences among the different treatment groups (Fig. 2B). These findings were consistent with the results of the correlation analysis, which showed high reproducibility among the three biological replicates in each group and showed differences between the two genotypes (Fig. 2A, 2B), indicating that the data were reliable. A total of 4957, 4395, 3320, and 2133 DEGs were identified in the comparisons 5A-12h vs. 5A-0h, 162-12h vs. 162-0h, 162-0h vs. 5A-0h and 162-12h vs. 5A-12h, respectively (Fig. 2D). The Venn diagram in Fig. 2C shows that 215 DEGs were common among all four comparisons, whereas 1209 DEGs were unique in the 5A-12h vs. 5A-0h comparison, and 896 DEGs were unique in the 162-12h vs. 162-0h comparison. The 5A-12h vs. 5A-0h comparison showed the highest number of DEGs, suggesting that the induction effect of chilling stress is more obvious for 13-5A.

For the 5A-12h vs. 5A-0h comparison, GO enrichment analysis indicated that the DEGs were significantly enriched in the cellular component (CC) terms “plasma membrane” (GO: 0005886), “integral component of membrane” (GO: 0016021), and “peroxisome” (GO: 0005777). The DEGs were primarily involved in “circadian rhythm,” “regulation of seed germination,” and “amino acid transport” in the biological process (BP) category and in molecular function (MF) terms such as “DNA-binding transcription factor activity,” “calcium ion binding,” and “UDP-glycosyltransferase activity” (Fig. 2E and S1). KEGG analysis showed that the beta-alanine metabolism (ko00410), plant hormone signal transduction (ko04075), and limonene and pinene degradation (ko00903) pathways were significantly enriched (Fig. 2F).

In the 162-12h vs. 162-0h comparison, the DEGs were found to be enriched in 7 MF terms (e.g., “DNA-binding transcription factor activity,” “carboxylic ester hydrolase activity,” and “ligase activity”), 3 CC terms (“Plasma membrane,” “integral component of membrane,” and “peroxisome”), and 10 BP terms (e.g., “cytokinin biosynthetic process,” “circadian rhythm,” “regulation of jasmonic acid-mediated signaling pathway,” Fig. S2). KEGG analysis showed that the plant hormone signal transduction

(ko04075), alpha-linolenic acid metabolism (ko00592), and beta-alanine metabolism (ko00410; Fig. S3) pathways were significantly enriched.

In the 162-0h vs. 5A-0h comparison, the DEGs were predominantly enriched in “Ribosome” (ko03010), “Sesquiterpenoid and triterpenoid biosynthesis” (ko00909), and “phenylpropanoid biosynthesis” (ko00940; Fig. S3). In the 162-12h vs. 5A-12h comparison, most DEGs were enriched in “phenylpropanoid biosynthesis” (ko00940), “phenylalanine metabolism” (ko00360), “sesquiterpenoid and triterpenoid biosynthesis” (ko00909), “glutathione metabolism” (ko00480) and “tyrosine metabolism” (ko00350; Fig. S3). In all comparison groups, the pathways related to phenylpropanoid biosynthesis (ko00940) and ubiquinone and other terpenoid-quinone biosynthesis (ko00130) were significantly enriched, indicating that they are crucial in the chilling stress response.

## Analysis of Transcription Factors Related to Chilling Stress

In the present study, in comparison with 5A-0h, 216 differentially expressed TF genes (TF-DEGs; 119 upregulated and 97 downregulated) were identified in 5A-12h, and 126 TF-DEGs (70 upregulated and 56 downregulated) were identified in 162-0h. Compared with 162-12h, 75 TF-DEGs were identified in 5A-12h (49 upregulated and 26 downregulated) and 225 TF-DEGs (126 upregulated and 99 downregulated) were identified in 162-0h (Fig. 3A). Remarkably, and the number of upregulated TFs was higher than that of downregulated TFs in all comparison groups. In addition, there were 140 overlapping TF-DEGs regulated by cold stress between the comparisons 5A-12h vs. 5A-0h and 162-12h vs. 162-0h (Fig. 3B). Among the TFs common between the two comparison groups, a total of 25 MYB members, 20 ERF members, 8 WRKY members, and 7 bZIP members were identified (Fig. 3C, Table S2). Members of the MYB, ERF, MADS-box, and bZIP TF families were upregulated under chilling stress treatment, suggesting that these TFs play a vital role in the chilling stress response in melon. The expression levels of ethylene-responsive transcription factor ERF106-like (MELO3C005466.2) were upregulated under cold stress in the genotype 13-5A but downregulated in the genotype 162 (Fig. 3C, Table S2). Members of the MYB family such as MELO3C002090.2, MELO3C013364.2, MELO3C007330.2, MELO3C013925.2, MELO3C032396.2, and MELO3C010893.2 were upregulated in the two genotypes after chilling stress treatment (Fig. 3C, Table S2). These results indicate that TFs play an important role in the cold stress response of melon.

Moreover, six key genes were selected for qRT-PCR analysis. The RNA sequencing and qRT-PCR results showed similar trends under chilling stress (Fig. S4), indicating that these data were reliable.

## Metabolomic Profiles of Two Melon Genotypes under Chilling Stress

Metabolomic analysis performed using LC-MS integrated with GC-MS identified a total of 2347 differentially expressed metabolites (DEMs) in 12 samples. The number of DEMs identified using GC-MS was 362, of which 211 and 151 were downregulated and upregulated, respectively (Table S3, Fig. S5). The DEMs were classified into 11 categories; the top five categories were as follows: lipids and lipid-like molecules, organoheterocyclic compounds, organic oxygen compounds, organic acids and derivatives,

and phenylpropanoids and polyketides (Fig. S6). In addition, 1985 DEMs were identified using LC-MS, including 1158 downregulated and 827 upregulated DEMs (Table S3, Fig. S5). Among them, the top three largest groups of metabolites were organic oxygen compounds (28.48%), organic acids and derivatives (21.84%), lipids and lipid-like molecules (18.04%; Fig. S6). The majority of DEMs belonged to the lipids and lipid-like molecules, organic oxygen compounds, organic acids and derivatives, and phenylpropanoids and polyketides (Table S3).

KEGG enrichment analysis revealed that in the 5A-12h vs. 5A-0h comparison, the most significantly enriched KEGG terms were pentose phosphate pathway, galactose metabolism, TCA cycle, C5-branched dibasic acid metabolism, carbon fixation in photosynthetic organisms. In the 162-12h vs. 162-0h comparison, the top five significantly enriched pathways were the aminoacyl-tRNA biosynthesis; ABC transporters; alanine, aspartate and glutamate metabolism; arginine biosynthesis; and pentose phosphate pathway (Fig. S6).

The Venn diagram in Fig. 4 shows 235 common DEMs between the 5A-12h vs. 5A-0h and 162-12h vs. 162-0h comparisons. The melon leaves exposed to chilling stress showed accumulated a large amount of lipids, organic acids, phenylpropanoids, and polyketides (Fig. S6). Among the significantly differentially expressed lipids, the top upregulated lipids were beta-glycerophosphoric acid, 7Z,9E-dodecadienoic acid, chrysosplenol C 6,4'-diglucoside, and ganoderic acid H,1-(2-methoxy-13-methyl-6Z-tetradecenyl)-sn-glycero-3-phosphoethanolamine. The top downregulated lipids were cannabidiol, quercetin 3-glucuronide-7-glucoside, (25S)-spirostane-3b,5b,6a-triol 3-[4"-rhamnosylglucoside], PGP(a-13:0/18:2(9Z,11Z)), and 1-nonanol. Among the significantly differentially expressed organic acids, allantoic acid, carbonate, S-propyl-L-cysteine, and L-methionine were most significantly upregulated (Table S4). Comparative analysis indicated that most flavonoids, steroids, and steroid derivatives were downregulated in 162 and 13-5A subjected to chilling stress (Table S4).

## Integrated Transcriptomic and Metabolomic Analysis

The KEGG analysis showed that in the 162-0h vs. 5A-0h comparison, the coenriched pathways of DEGs and DEMs included phenylpropanoid biosynthesis and glycine, serine and threonine metabolism. Similarly, glutathione metabolism and glycine, serine and threonine metabolism were coenriched in the 162 – 12 h vs. 5A-12h comparison. There were more coenriched pathways identified in the 5A-12h vs. 5A-0h comparison, including glyoxylate and dicarboxylate metabolism; starch and sucrose metabolism; alanine, aspartate, and glutamate metabolism; ascorbate and aldarate metabolism; ABC transporters; taurine and hypotaurine metabolism; glycine, serine, and threonine metabolism; and arginine biosynthesis. Notably, there were fewer enriched pathways in 162-12h vs. 162-0h comparison, including ABC transporters, glutathione metabolism, glycerolipid metabolism, and arginine and proline metabolism (Fig. 5 and Table 1). Thus, during chilling stress response, the ABC transporters pathway was coenriched in both 162 and 13-5A. In addition, glycine, serine and threonine metabolism (ko00260) and glutathione metabolism (ko00480) were the most common coenriched pathways, suggesting that these pathways play key roles in response to cold stress.

Table 1  
Coenriched pathways of DEGs and DEMs in two melon genotypes under chilling stress.

| Comparison            | KEGG ID | Pathways                                    |
|-----------------------|---------|---------------------------------------------|
| 162-0h vs.13-5A-0h    | ko00940 | Phenylpropanoid biosynthesis                |
|                       | ko00260 | Glycine, serine and threonine metabolism    |
| 162-12h vs.13-5A-12h  | ko00480 | Glutathione metabolism                      |
|                       | ko00260 | Glycine, serine and threonine metabolism    |
| 13-5A-12h vs.13-5A-0h | ko00630 | Glyoxylate and dicarboxylate metabolism     |
|                       | ko00500 | Starch and sucrose metabolism               |
|                       | ko00250 | Alanine, aspartate and glutamate metabolism |
|                       | ko00053 | Ascorbate and aldarate metabolism           |
|                       | ko02010 | ABC transporters                            |
|                       | ko00430 | Taurine and hypotaurine metabolism          |
|                       | ko00260 | Glycine, serine and threonine metabolism    |
|                       | ko00220 | Arginine biosynthesis                       |
| 162-12h vs. 162-0h    | ko02010 | ABC transporters                            |
|                       | ko00480 | Glutathione metabolism                      |
|                       | ko00561 | Glycerolipid metabolism                     |
|                       | ko00330 | Arginine and proline metabolism             |

## Transcriptomic and Metabolic Changes in Glutathione Metabolism under Chilling Stress

Glutathione metabolism was identified as a significantly enriched pathway in the 162-12h vs. 162-0h and 162-12h vs. 13-5A-12h comparisons (Table 1), indicating that this pathway was significantly affected in melon leaves undergoing chilling stress. In this pathway, three metabolites and twenty-six genes were found to be involved in 162 leaves under chilling stress (Fig. 6). 5-oxoproline, L-gamma-glutamyl-L-amino acid, L-glutamate, and L-ornithine were found to be significantly accumulated. In addition, the transcription of most related genes was upregulated. For example, two 6-phosphogluconate dehydrogenase (*G6PDH*) genes, glutathione peroxidase (*GPX*), and 15 glutathione s-transferase (*GST*) genes were upregulated, whereas two *GST* genes were downregulated. Enhanced *GPX* and dehydroascorbate reductase (*DHAR*) expression likely activates the GSSG-GSH cycle. Although the content of GSH did not change substantially, the content of GSH conjugates, L-gamma-glutamyl-L-amino acid and L-glutamate was significantly higher (Fig. 6).

# Transcriptomic and Metabolic Changes in Arginine and Proline Metabolism under Chilling Stress

In the genotype 162 under chilling stress, 5 metabolites and 21 genes were found to be involved in arginine and proline metabolism (Fig. 7). Genes associated with arginine and proline metabolism were significantly upregulated in the genotype 162 in response to chilling stress; these genes included ornithine decarboxylase (MELO3C011335.2), proline dehydrogenase (MELO3C022076.2), aspartate aminotransferase (MELO3C011284.2), pyrroline-5-carboxylate reductase (MELO3C019039.2), and spermidine synthase (MELO3C008477.2). Upregulation of these genes resulted in elevated levels of arginine, ornithine, and proline in 162 subjected to chilling stress, leading to better adaptability to the chilling stress conditions.

## Discussion

Melon is a crop cultivated worldwide but is sensitive to cold stress. Chilling stress restricts the cultivation and production of melon in winter and early spring. In the present study, we conducted physiological, transcriptomic, and metabolomic analyses of two melon genotypes (13-5A, chilling sensitive; 162, chilling tolerant) under chilling stress.

Chilling stress leads to morphological and physiological changes of various plant species [32, 33]. In the present study, in the chilling-sensitive melon genotype 13-5A, the whole plant gradually wilted, and plant growth was severely inhibited (Fig. 1A); moreover, the Fv/Fm of 13-5A decreased under chilling stress (Fig. 1B). However, the chilling-tolerant genotype 162 maintained normal growth after 12h of treatment and was less affected by chilling stress. Furthermore, the accumulation of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> under chilling stress was lower in 162 than in 13-5A (Fig. 1B-C), and the activity of SOD, POD, CAT, and APX was higher in 162 (Fig. 1D), indicating that the genotype 162 had a stronger antioxidant defense response than 13-5A. After exposure to chilling stress, the soluble sugar and soluble protein levels were higher in the genotype 162 than in 13-5A (Fig. 1C), suggesting that 162 is more adaptable to chilling stress than 13-5A owing to the higher levels of osmotic regulatory substances and higher antioxidant enzyme activity.

Furthermore, we analyzed the transcriptome and metabolome of melon leaves exposed to chilling stress to explore the relationship between the expression of cold-responsive genes and metabolite accumulation. Genes related to phenylpropanoid biosynthesis and ubiquinone and other terpenoid-quinone biosynthesis were significantly enriched in all comparison groups. Similarly, several DEGs identified in the 162-12h vs. 5A-12h comparison was found to be involved in pathways such as phenylpropanoid biosynthesis, phenylalanine metabolism, sesquiterpenoid and triterpenoid biosynthesis, glutathione metabolism, and tyrosine metabolism (Fig. 2).

KEGG analysis revealed that most DEMs identified in the present study were associated with amino acid metabolism and sugar metabolism (Fig. 4). Previous studies indicate that lipid, amino acid, and sugar metabolism are highly correlated with stress responses [34–38]. In the present study, the top

upregulated lipids were beta-glycerophosphoric Acid, 7Z,9E-dodecadienoic acid, chrysosplenol C 6,4'-diglucoside, and ganoderic acid H,1-(2-methoxy-13-methyl-6Z-tetradecenyl)-sn-glycero-3-phosphoethanolamine. In contrast, the metabolism of most amino acids (such as D-glutamine, argininic acid, D-proline, L-glutamine, ornithine) was downregulated.

Glutathione plays a vital role in plants exposed to various environmental stresses by alleviating oxidative stress. The glutathione metabolism pathway is crucial in plant response to abiotic stress [37, 39, 40]. Glutathione exists in both oxidized and reduced forms (GSSG and GSH, respectively) [41]. Glutathione reductase is an essential enzyme that catalyzes the reduction of GSSG to GSH via an NADPH-dependent mechanism [42]. As the substrate of GPX and GST, GSH participates in the defense against ROS [43]. In the present study, the increase in the levels of 5-oxoproline, L-gamma-glutamyl-L-amino acid, L-glutamate, and L-ornithine was approximately 1.16-, 1.79-, 0.28-, and 2.08-fold higher in 162 after chilling stress treatment than in 13-5A, respectively. Furthermore, in comparison with control group (0 h exposure), the expression of most phenylpropanoid biosynthetic genes was significantly upregulated. *G6PDH*, *GPX*, and *GST* genes were upregulated in 162 under chilling stress, and the increase in the expression levels of these genes in 162 was significantly higher than that in 13-5A. A previous study indicated that *GST* and *GPX* overexpression promoted tobacco seedling growth under stressful and non-stressful conditions [44]. Moreover, as a hub, glutamate is converted to Pro. Enhanced L-glutamate accumulation may increase the content of proline and its derivatives (Fig. 7). Thus, these results indicate that the above genes together with these metabolites are important for resistance to chilling stress in melon.

Proline (Pro) plays a crucial role in plant development and stress [45, 46]; it triggers or participates in stress defense [47, 48]. The accumulation of proline has been reported to increase under chilling stress in several plant species such as *Elymus nutans*, *Arabidopsis*, and mango [49–51]. Arginine (Arg) is a basic amino acid with the highest nitrogen-to-carbon ratio. Arginine serves as the precursor to synthesize many biologically active metabolites, including nitro oxide (NO), Pro, and polyamines(PAs) [52]. Pro biosynthesis in plants typically occurs through either the glutamate pathway or the ornithine pathway [53]. The glutamate synthesis pathway uses glutamic acid as a substrate, and the ornithine synthesis pathway uses ornithine as a substrate, and the level of Pro depends on the balance between its synthesis and degradation. Arg produces ornithine through a reaction catalyzed by arginase, which is converted to Pro through the ornithine pathway. Arg can be converted to putrescine (Put) under the catalysis of ornithine decarboxylase. Put is in turn converted to spermidine and spermine-two common PAs-by spermidine synthase and spermine synthase. Arg can also generate NO under the catalysis of NO synthase. Thus, Pro, Arg, and PAs in plants can be interconverted, which is crucial in plant stress adaptation [54]. In the present study, arginine, ornithine, urea, and Pro content was increased in 162 plants (Fig. 7). Moreover, most genes involved in Arg and Pro metabolism, such as ornithine decarboxylase, aspartate aminotransferase, and spermidine synthase, were upregulated, which was consistent with the increased accumulation of arginine, ornithine, and proline in leaves of 162 subjected to cold stress. Previous studies suggest that low temperatures induce Pro accumulation in plants by regulating the corresponding genes [11, 45, 55, 56]. The present findings suggest that chilling stress

accelerated the conversion of Glu and ornithine to Pro synchronously and improve the chilling tolerance of melon.

TFs play a vital role in plant growth, development, and stress response [57, 58]. In higher plants, TFs such as *AP2/ERF*, *NAC*, *WRKY*, *MYB*, and *bHLH* participate in the response to chilling stress by regulating downstream stress-responsive genes [59, 60]. In the present study, it was found that the TFs MYB, ERF, MADS-box, and bZIP were induced by chilling stress. In addition, we found that *MYB108* (MELO3C002090.2), *MYB308* (MELO3C013364.2), *MYB34* (MELO3C007330.2), and *MYB44* (MELO3C010893.2) were upregulated in the two genotypes after chilling stress treatment, with 162 being more affected than 13-5A. Similarly, Dong et al. (2021) reported that *RmMYB108* was positively involved in the cold, salt, or drought tolerance responses in *Rosa multiflora* [61]. Furthermore, Li et al. (2019) found that *ZmMYB31* overexpression in maize enhanced plant resistance to chilling stress by reducing ion extravasation, ROS content, and low-temperature photoinhibition [62]. Future studies need to investigate the regulatory network of MYB to provide a basis for the development of melon with enhanced cold tolerance.

## Conclusion

Physiological, transcriptional, and metabolomic analyses of two melon genotypes under chilling stress demonstrated that the genotype 162 has higher chilling tolerance than 13-5A. A comprehensive analysis of transcriptomic and metabolomic datasets highlighted the importance of glutathione metabolism and arginine and proline metabolism in melon leaves exposed to chilling stress. TFs such as MYB, ERF, MADS-box, and bZIP are crucial for enhancing the cold tolerance of melon. Thus, the present findings improve the understanding of the molecular regulatory network associated with the chilling stress response in melon.

## Materials and methods

### Plant Materials

We used the genotypes “162” (chilling tolerant) and “13-5A” (chilling sensitive) as the experimental materials. Melon seeds were harvested from our laboratory at the Shanghai Academy of Agricultural Science (Shanghai, China). The seeds were rinsed thoroughly with distilled water, germinated in an incubator at 30°C, and then transferred into 12 × 12 cm plastic trays containing soil matrix (Tianfeng gardening corporation, Taiwan province, Pingdong, China) and cultivated in a growth chamber (MGC-400H, Shanghai Bluepard Instruments Co., Ltd., Shanghai, China) at 28°C/20°C (day/night), 80% relative humidity, and 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance at Shanghai Academy of Agricultural Sciences. Melon seedlings were exposed to chilling stress (6°C) when they reached the five true-leaf stage. The third fully expanded leaves from 50 uniform seedlings of the two genotypes were sampled at 0 h and 12 h of chilling stress and stored at -80°C for subsequent analysis. Three replicates were used for physiological and transcriptomic analysis, and six replicates were used for metabolomic analysis.

# Determination of Physicochemical Indexes of Melon Leaves

After dark adaptation for 30 min, chlorophyll fluorescence (Fv/Fm) was measured using a plant efficiency analyzer (Hanstech, HandyPEA, UK). Soluble sugar, soluble protein, and malondialdehyde (MDA) content and superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) activity were determined using assay kits (Comin Biotechnology, Suzhou, China). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) content was determined using the method described by Patterson et al. (1984) [27]. The Superoxide radical (O<sub>2</sub><sup>•−</sup>) production rate was determined as described by Elstner and Heupel (1976) [28]. In situ localization of H<sub>2</sub>O<sub>2</sub> was performed by staining the leaves with 3,3-diaminobenzidine (DAB) according to the method described by Xu et al. (2012) [29].

## Transcriptomic Analysis

Leaves from the two melon genotypes in the control and treatment groups (subjected to chilling stress for 0 and 12 h, respectively) were used for transcriptomic analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen). The RNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The RNA libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit.

The libraries were sequenced on the Illumina Novaseq 6000 platform, and 150 bp paired-end reads were generated. Raw sequencing reads were processed to obtain clean reads by filtering out low-quality reads. The clean reads were mapped to the melon reference genome (melonet-db-v1.41P). Transcript abundance of each gene was estimated (in FPKM) [30], and the read count of each gene was obtained using HTSeq-count [31]. Q value < 0.05 and foldchange > 2 or foldchange < 0.5 were set as the threshold for significantly differential expression. Principal component analysis (PCA) analysis was performed using R (v 3.2.0) to evaluate the biological duplication of samples. Gene ontology (GO) analysis and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were performed using R (v 3.2.0).

## Metabolomic Analysis

Liquid chromatography–mass spectrometry (LC-MS) and gas chromatography–mass spectrometry (GC-MS) analysis was performed by Shanghai Lu-Ming Biotech Co., Ltd. (Shanghai, China). A detailed description of the methods is provided in the Supplementary Material.

## Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) and Duncan's multiple range test at the 0.05 level of significance using SPSS 22.0 software package. The figures were prepared using Origin 8.0.

## Abbreviations

APX Ascorbate peroxidase

Arg Arginine

BP Biological process

CAT Catalase

CBF C-repeat binding factor

CC Cellular component

COR Cold-regulated gene

DAB Diaminobenzidine

DEGs Differentially expressed genes

DEMs Differential enriched metabolites

*DHAR* Dehydroascorbate reductase

Fv/Fm Chlorophyll fluorescence

GC-MS Gas chromatography-mass spectrometry

GO Gene ontology

G6PDH 6-phosphogluconate dehydrogenase

GPX Glutathione peroxidase

GST Glutathione s-transferase

H<sub>2</sub>O<sub>2</sub> Hydrogen peroxide

ICE Inducer of C-REPEAT BINDING FACTOR

KEGG Kyoto encyclopedia of genes and genomes

LC-MS Liquid chromatography–mass spectrometry

MDA Malondialdehyde

MF Molecular function

NO Nitro oxide

O<sub>2</sub><sup>-</sup> Superoxide radical

PAs Polyamines

PCA Principal component analysis

POD Peroxidase

Pro Proline

Put Putrescine

ROS Reactive oxygen species

SOD Superoxide dismutase

TFs Transcription factors

## Declarations

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### Authors' contributions

Q.D and S.T designed the research. Q.D., S.T., Y.C. and D.Y., performed the experiments. Q.D., S.T and Y.Z analyzed the data. X.J., H.F. and W.Z collected the plant materials, helped in the experiment and made suggestions. Q.D and S.T wrote the manuscript. All authors have read and approved the manuscript.

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### Data availability

The datasets generated and analysed during the current study are available in the NCBI Gene Expression Omnibus (GEO) repository, under the accession number GSE225921 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE225921>).

### Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare no competing interests.

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Figures

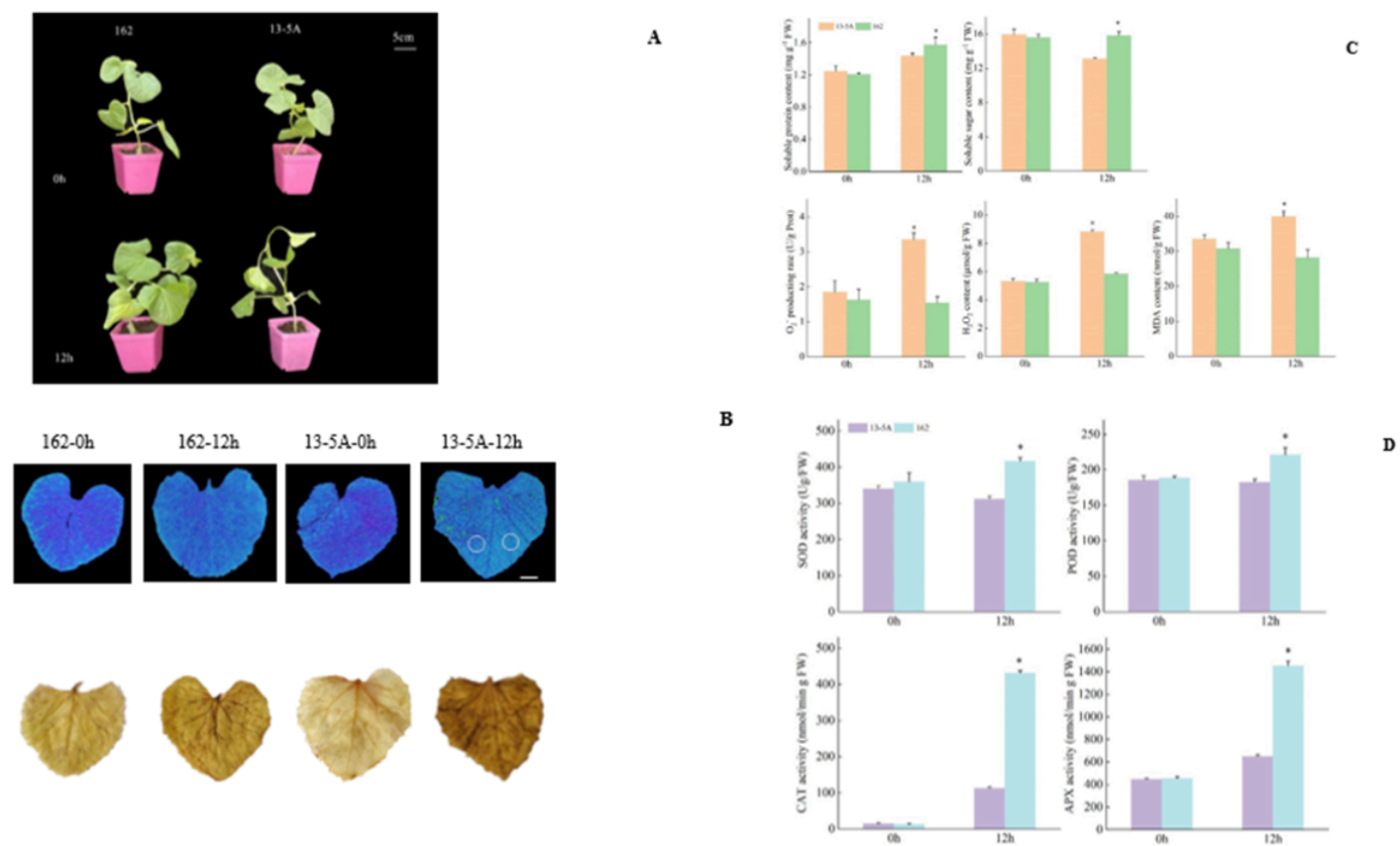


**Figure 1**

Morphological and physiological characteristics of melon seedlings (genotypes 162 and 13-5A) under chilling stress. Melon seedlings exposed to low temperature 6 °C for 12 h. **(A)** Morphological traits; **(B)** Fv/Fm and H<sub>2</sub>O<sub>2</sub> staining; **(C)** soluble sugar, soluble protein content, MDA, O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> contents; and **(D)** SOD, POD, CAT and APX activity of the 162 and 13-5A genotypes in the control group (0 h exposure) and treatment group (12 h exposure). Asterisks indicate significant differences at *P* < 0.05.

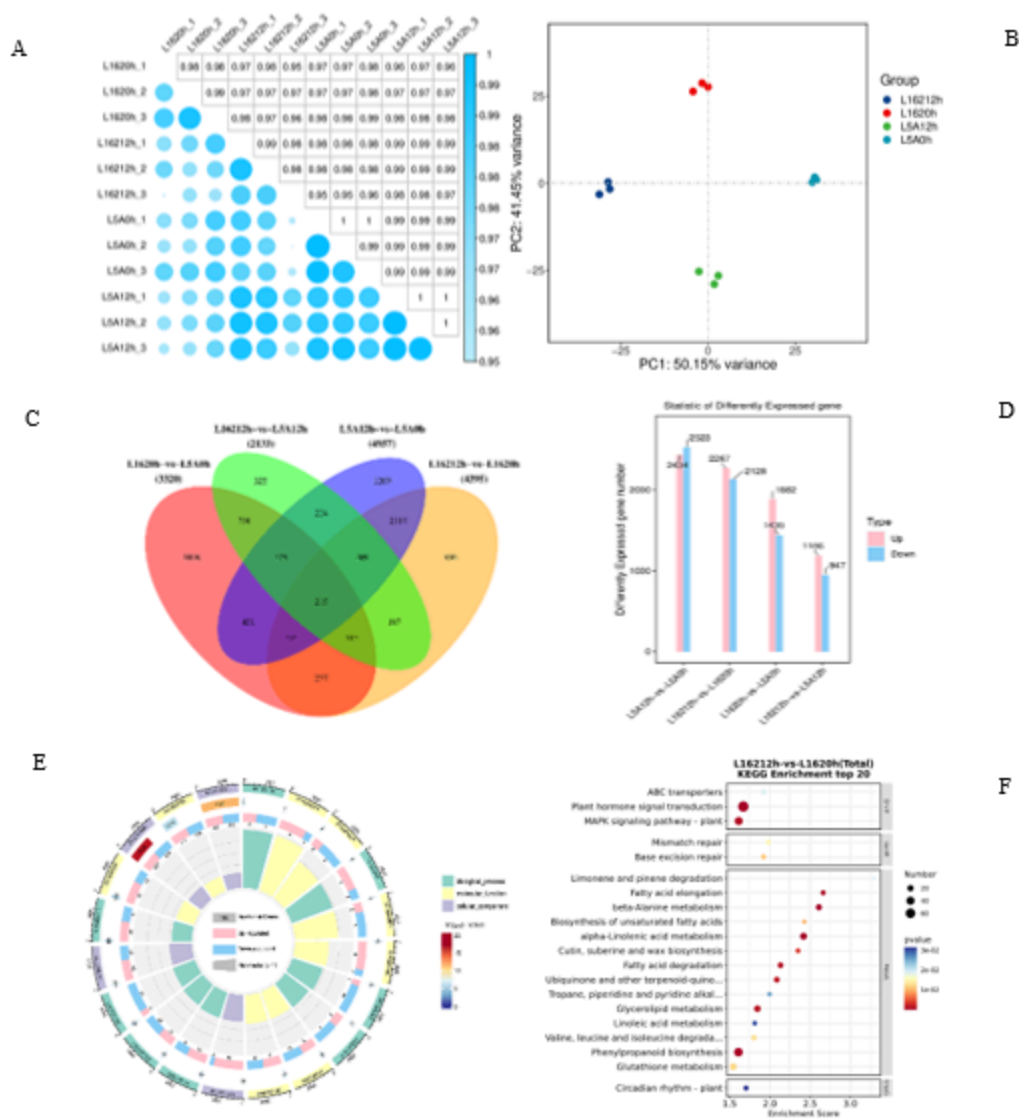


**Figure 2**

Transcriptome analysis of melon genotypes 162 and 13-5A under chilling stress (6 °C) for 0 h and 12 h. **(A)** Correlation analysis ( $n = 12$ ); **(B)** Principal component analysis (PCA;  $n = 12$ ); **(C)** Venn diagram of the number of differentially expressed genes (DEGs) in various comparisons (5A-12h vs. 5A-0h, 162-12h vs. 162-0h, 162-0h vs. 5A-0h and 162-12h vs. 5A-12h); **(D)** Numbers of DEGs in each comparison; **(E)** Gene ontology (GO) analysis of DEGs shown in (D); **(F)** Kyoto encyclopedia of genes and genomes (KEGG) analysis of DEGs shown in (D).

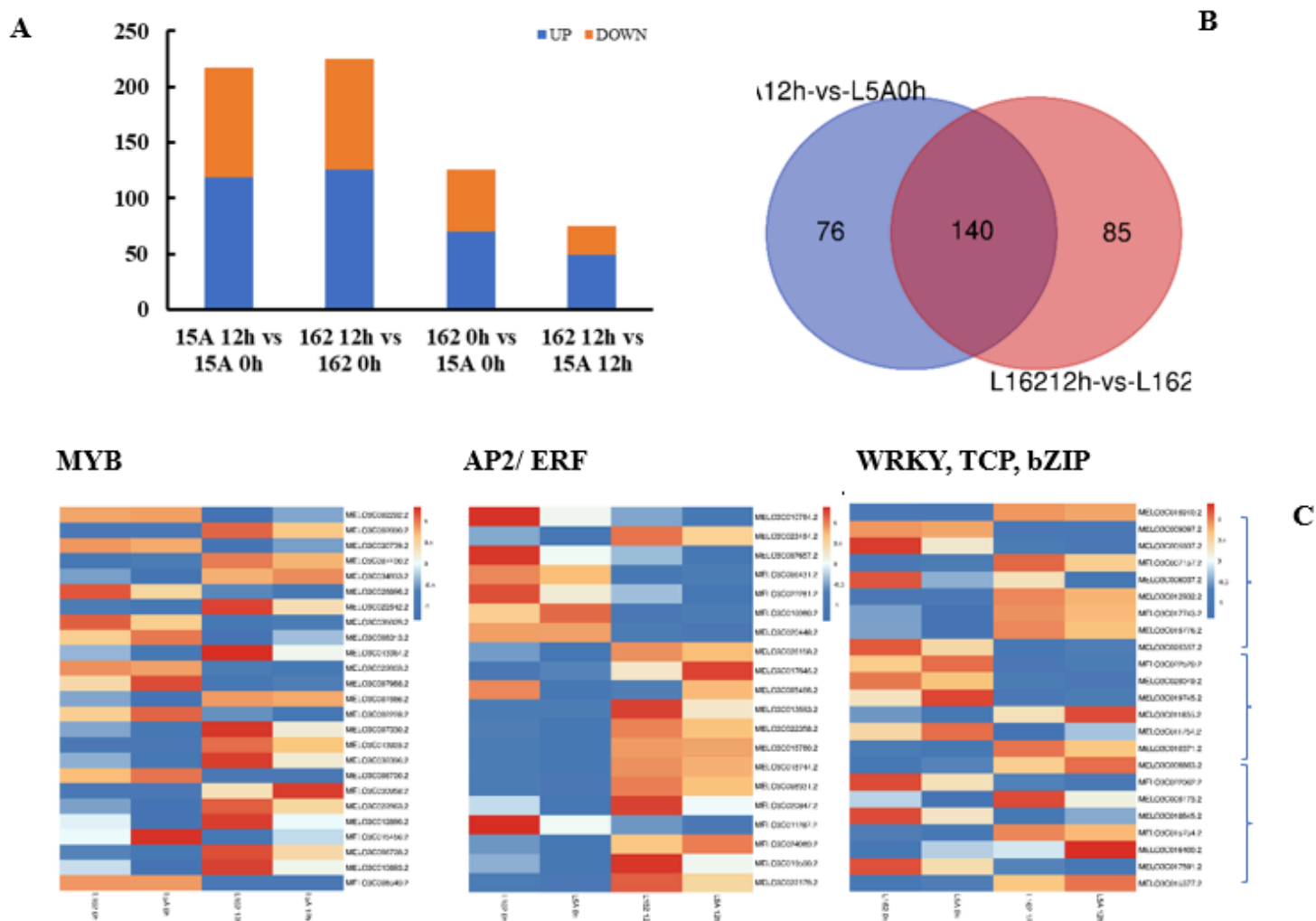


**Figure 3**

Analysis of transcription factors (TFs) in two melon genotypes exposed to chilling stress. **(A)** Number of TFs with differential expression; **(B)** Venn diagram showing the differentially expressed TF genes common between the comparisons 5A-12h vs. 5A-0h and 162-12h vs. 162-0h; **(C)** Expression profiles of the MYB, AP2/ERF, WRKY, TCP, and bZIP genes in the two genotypes.

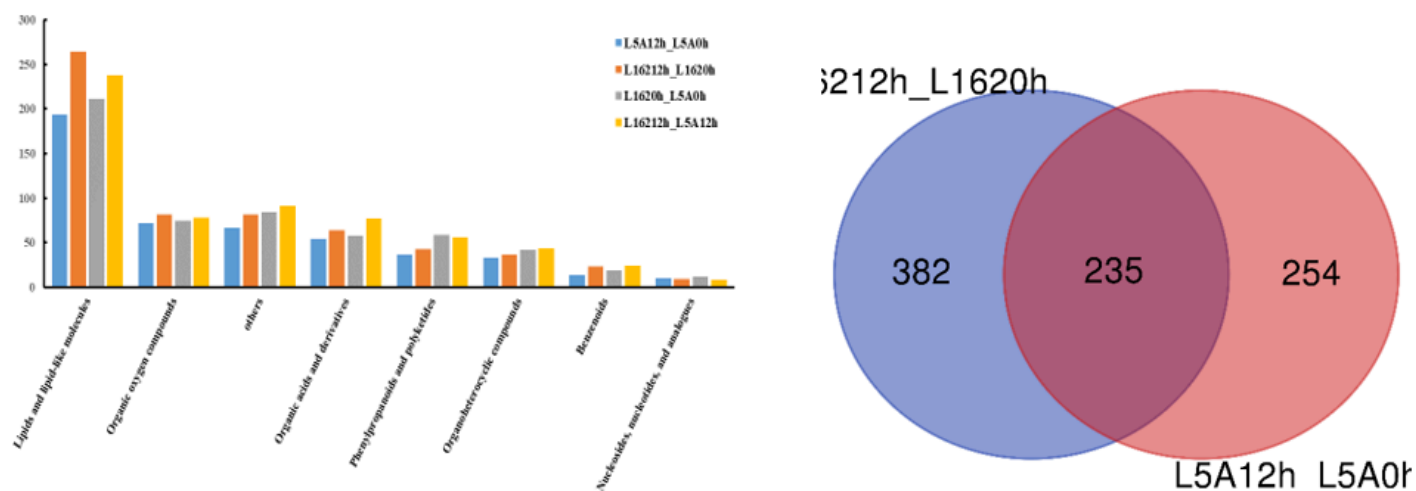


**Figure 4**

Analysis of DEMs in melon leaves under chilling stress. **(A)** The number of DEMs in 5A-12h vs. 5A-0h, 162-12h vs. 162-0h, 162-0h vs. 5A-0h, and 162-12h vs. 5A-12h. **(B)** Venn diagram showing the overlap of DEMs in 162-12h vs. 162-0h and 13-5A-12h vs. 13-5A-0h.



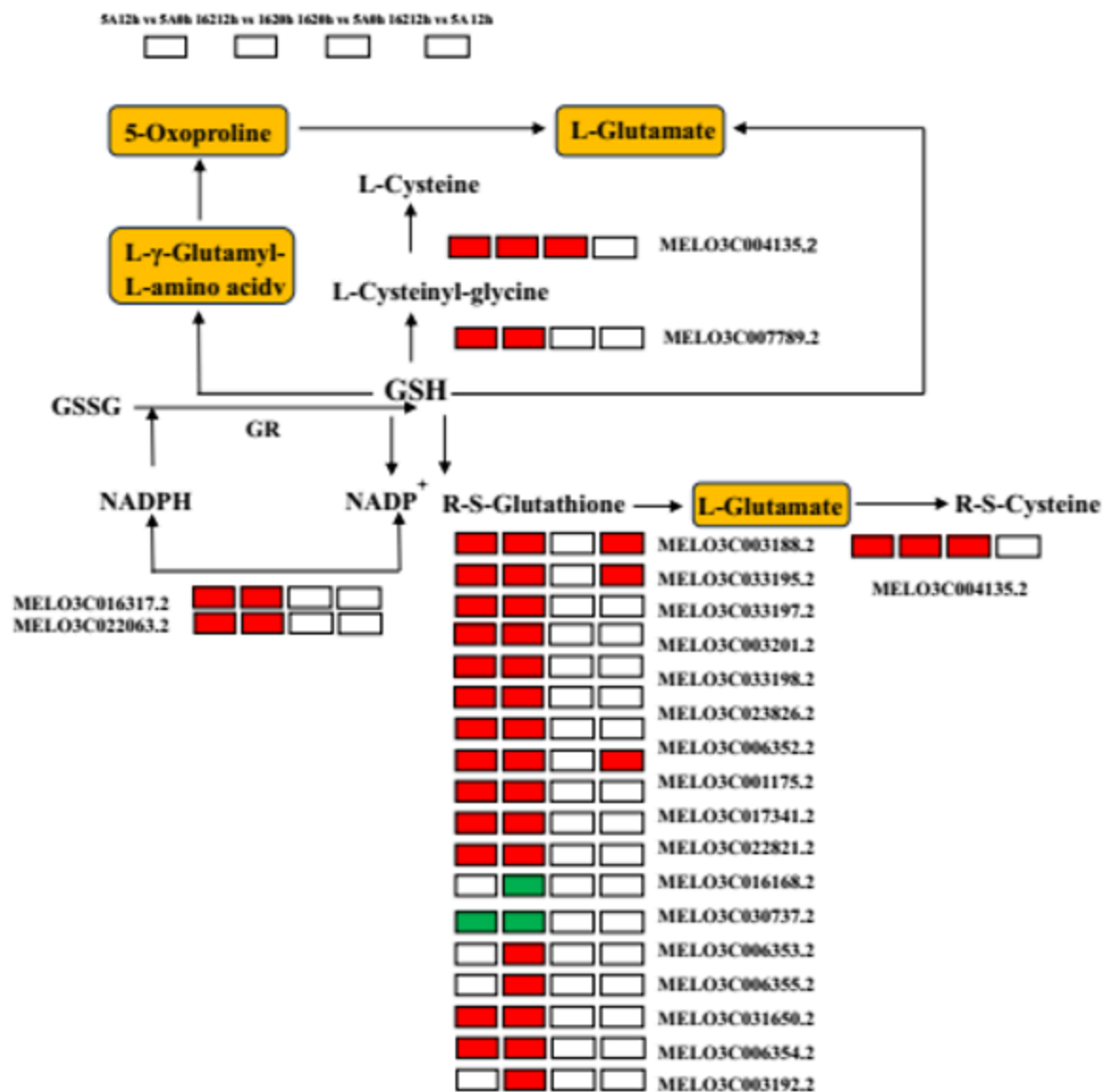


**Figure 6**

Changes in DEGs and DEMs involved in glutathione metabolism in the melon genotypes 162 and 13-5A under chilling stress conditions. The color of the rectangles represents significance.

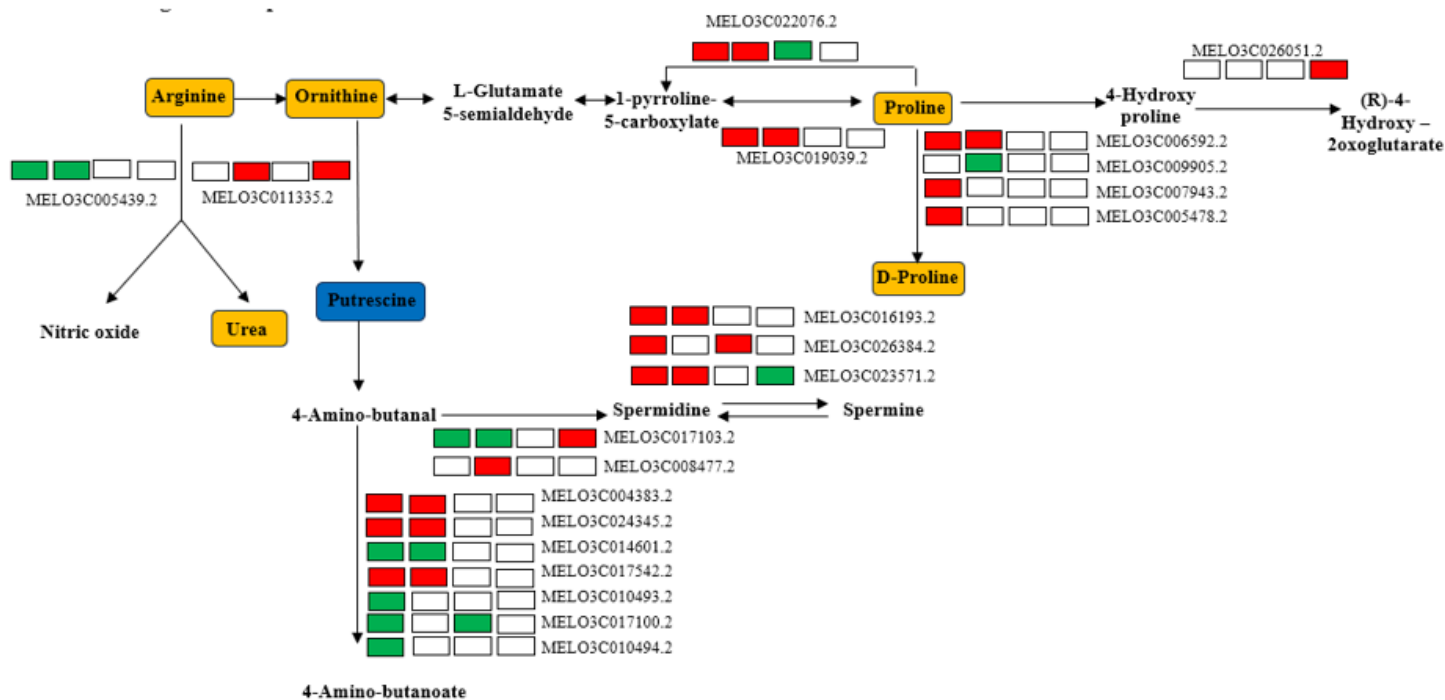


Figure 7

Changes in DEGs and DEMs in arginine and proline metabolism.

## Supplementary Files

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