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Comprehensive physiological and transcriptomic analysis of seed germination in two tomato inbred lines under low-temperature stress

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Abstract

Low-temperatures severely limit tomato seed germination, leading to reduced crop yields. Elucidating the cold resistance regulatory mechanisms is crucial for cold-resistant breeding. This study used the cold-tolerant tomato inbred line F266 and the cold-sensitive inbred line M196 as experimental materials. Phenotypic analysis revealed that under normal-temperature conditions of 25°C, there was no significant difference in germination rates between the two inbred lines. However, under low-temperature treatment of 16°C, F266 germinated earlier and exhibited a more pronounced advantage in hypocotyl elongation. Comparative transcriptome analysis results indicate that the two lines exhibit genotype-specific differences in oxidative stress response, carbohydrate metabolism, and plant hormone signaling pathways. F266 has a stronger antioxidant response capability, with higher accumulation of galactinol, raffinose, and sucrose. At the same time, F266 has higher endogenous auxin (IAA) and gibberellin (GA_3) content, and genes related to auxin and gibberellin show differential expression. Integrative analysis further identified candidate genes from the ANN p35、GATA、CAMTA、MAPK、WRKY58、WRKY33 and bHLH150, which may mediate the interactions between the aforementioned response modules. This result provides a new theoretical basis for subsequent gene function validation, improvement of tomato cold germination traits, and the development of cold-resistant tomato breeding.

Keywords: Tomato; Low-temperature stress; Seed germination; RNA-Seq; Inbred lines

1. Introduction

Low-temperature stress disrupts the growth and development of crops and further reduces crop yield and quality [1,2]. For early-sown tomatoes, low-temperature stress occurring during seed germination and seedling emergence severely limits stand uniformity and subsequent yield potential. To survive the pressure caused by low-temperature stress, tomato has evolved a sophisticated cold-response regulatory network through long-term adaptive evolution [3,4]. Therefore, unraveling the molecular mechanisms underlying cold signaling pathways is of great significance for breeding new cold-tolerant tomato varieties and improving cold resistance in crops.

The imbibition of mature dry seeds occurs in three sequential stages: water absorption, reactivation of metabolic activity, and initiation of embryonic growth [5]. As the most pivotal developmental event in the seed life cycle, seed germination is highly susceptible to temperature fluctuations. Successful seed germination relies on the coordination of intricate cellular and biochemical processes, including cell cycle activation, hormonal signaling regulation, energy metabolism, and biological repair mechanisms [6]. Under low-temperature conditions, elevated

indole-3-acetic acid (IAA) levels maintain normal starch hydrolysis and sugar metabolism, thereby facilitating seed germination [7]. Comparative transcriptome profiling between the cold-tolerant cotton cultivar KN27-3 and the cold-sensitive cultivar XLZ38 (*Gossypium hirsutum* L.) has demonstrated that auxin (IAA), cytokinin (CTK), and gibberellin (GA) positively regulate seed germination in cold-tolerant genotypes, whereas abscisic acid (ABA) exerts an inhibitory effect [6].

Accumulation of ROS can inflict varying degrees of damage to proteins, lipids, and nucleic acids. Such damage may manifest as altered membrane fluidity, loss of enzyme activity, disruption of protein synthesis and turnover, and DNA strand breaks [8]. ROS buildup is also a common feature during embryogenesis, and efficient antioxidant machinery is widely viewed as a prerequisite for producing high-vigor seeds [9]. Under cold stress, antioxidants have been demonstrated to play important roles in enhancing cold tolerance [10]. Specifically, enzymes including peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) have been reported to reinforce cold resistance and the antioxidant defense system in plants under low-temperature conditions [11-13]. Therefore, the contents and activities of these three enzymes are often adopted as physiological indicators of plant stress resistance to evaluate plant capacity to withstand adverse environments.

Soluble sugars are essential for sustaining seed energy supply and stabilizing cell membranes during seed germination under low-temperature stress. As the predominant free sugar in plant cells, sucrose accumulates substantially under low-temperature, which is critical for maintaining cell membrane stability and turgor pressure [14]. Raffinose family oligosaccharides (RFOs) confer potent plant tolerance to oxidative stress and freezing damage [15]. As a typical RFO-transporting crop, cucumber (*Cucumis sativus* L.) exhibits seed vitality closely associated with raffinose accumulation [16]. Consistently, transgenic apple callus and tobacco lines overexpressing *MdGolS5* display enhanced cold tolerance [17]. In potato (*Solanum tuberosum*), overexpression of *ScGolS1* remodels ethylene and CBF signaling pathways, alters endogenous sugar composition, and ultimately improves frost resistance [18]. Furthermore, key sugar signaling molecules, including arabinose and trehalose, have been verified to modulate seed germination and seedling development [19,20].

TFs recognize and initiate anti-reversal responses by binding to upstream cis-acting elements in the promoter regions of stress response genes [21]. As a bHLH-type transcription factor, *ICE1* acts as a master regulator governing core signal cascades associated with cold acclimation. *ICE1* functions as a positive regulator to activate CBF gene expression, which further improves plant low-temperature resistance [22,23]. Two independent T-DNA knockout mutants of *WRKY2* display excessive sensitivity to abscisic acid (ABA) throughout seed germination and the subsequent seedling early developmental phase. *WRKY2* participates in ABA-mediated germination regulation and restricts the vegetative growth of young seedlings post-germination [24]. In rice, *OsMYB3R-2* confers strengthened chilling tolerance by upregulating *OsDREB1* and the cell cycle-related gene *OsCycB1* [25].

Numerous signaling pathways and intricate gene regulation networks are involved in how plants react to cold stress. Thus, identifying the mechanisms behind plant cold responses is aided by assessing gene expression under cold stress. In this study, transcriptome analysis and physiological tests were conducted using two tomato inbred lines with different levels of cold tolerance: the cold-sensitive line M196 and the cold-tolerant line F266. The findings demonstrated

that coordinated alterations in reactive oxygen species (ROS) homeostasis, raffinose-family oligosaccharide (RFO) metabolism, hormone signaling pathways, and transcription factor expression are linked to F266's enhanced low-temperature germination ability. In addition to laying the foundation for future breeding or genetic engineering efforts to increase tomato seed germination and cultivation performance in cold environments, our results offer important insights into the genetic basis of seed germination under cold conditions.

2. Materials and methods

2.1. Plant materials and growth conditions

Two tomato (*Solanum lycopersicum*) inbred lines with contrasting low-temperature germination responses, M196 (cold-sensitive) and F266 (cold-tolerant), were selected from a collection of 92 inbred lines based on preliminary screening of seed germination performance at 16 °C (screening data not shown). Fifty seeds were selected and placed evenly on two layers of filter paper within each Petri dish. Seeds were incubated in darkness at 25 °C and 16 °C, respectively. A seed was regarded as germinated when its hypocotyl elongated to more than half of the seed length, and the germination rate was recorded every 24 h. Three biological replicates were set for each treatment.

2.2. RNA extraction and quantitative real-time PCR (qPCR) analysis

Total RNAs were extracted with Ultrapure RNA Kit Ultrapure RNA Kit (CWBIO, Jiangsu, China) from seeds, and 1 μg was transcribed into complementary DNA (cDNA) using PrimeScript ™ RT Master Mix (TaKaRa). The reaction products were diluted 3 times. Then, The expression levels of target genes were detected by an Applied Biosystems 7,500 Real-Time PCR system (qTower3G, Jena, Germany) with SYBR Green PCR Master Mix Kit (TaKara Bio Inc., Kusatsu, Japan) [26]. All primers used are listed in Supplementary Table S1.

2.3. Application of transcriptomic technology

Total RNA was extracted from seeds that germinated for 3 days under normal-temperature conditions at 25°C (M196-N and F266-N) and for 6 days under low-temperature conditions at 16° C (M196-L and F266-L). After RNA extraction, purification, and library construction, the samples were subjected to paired-end (PE) sequencing using second-generation sequencing technology (Next-Generation Sequencing, NGS) based on the Illumina sequencing platform. This project was carried out by Shanghai Personalbio Biotechnology Co., Ltd. (Shanghai, China).

2.4. Measurement of POD, SOD, and CAT contents

Following the manufacturer's instructions and using the appropriate assay kits, the contents of POD, SOD, and CAT were measured from seeds that germinated for three days at a normal-temperature of 25°C (M196-N and F266-N) and for six days at a low-temperature of 16°C (M196-L and F266-L). In particular, the POD test kit (Solarbio, Cat. No. BC0900) was used to detect POD content, the SOD assay kit (Solarbio, Cat. No. BC0170) was used to measure SOD content, and the CAT assay kit (Solarbio, Cat. No. BC0200) was used to quantify CAT content.

2.5. Extraction and determination of soluble carbohydrates

Soluble carbohydrates were extracted and determined according to Wang et al. [27] with minor modifications. Seeds of M196 and F266 were collected after 8 h of imbibition under normal-temperature conditions (25 °C; M196-N and F266-N) and after 6 d of germination under low-temperature conditions (16 °C; M196-L and F266-L) for soluble carbohydrate analysis. The seed samples were thoroughly ground in liquid nitrogen, and 0.3 g of each sample was accurately weighed and completely transferred into a 15-mL centrifuge tube containing 3 mL of 80% ethanol.

After thorough vortex mixing, the samples were extracted in a water bath at 80 °C for 40 min, with inversion 3–5 times during the extraction. After extraction, the samples were cooled on ice for 5 min and centrifuged at 12,000 × *g* for 5 min at 4 °C. The supernatant was transferred to a new 15-mL centrifuge tube. Subsequently, the pellet was extracted with 3 mL of 50% ethanol following the same procedure described above, and then extracted for a third time with 3 mL of ultrapure water using the same procedure. The supernatants obtained from the three extractions were combined to obtain the crude soluble carbohydrate extract. The combined supernatant was purified using a C18 solid-phase extraction cartridge to remove pigments, proteins, lipids, and other interfering substances as much as possible. The purified extract was then dried using a vacuum freeze dryer until the water and ethanol were completely evaporated. Finally, the dried sample was reconstituted in ultrapure water and subjected to ion chromatography for soluble carbohydrate analysis.

2.6. Determination of IAA and GA_3 contents

IAA and GA_3 were extracted from seeds germinated for 3 days under normal-temperature conditions at 25 °C (M196-N and F266-N) or for 6 days under low-temperature conditions at 16 °C (M196-L and F266-L).

For IAA determination, fresh seed samples were immediately frozen in liquid nitrogen. Approximately 0.3 g of each sample was weighed and mixed with 1.5 mL of pre-cooled 80% (v/v) aqueous methanol. The samples were ground thoroughly into a homogenate and extracted overnight at 4 °C. After centrifugation at 8,000 × g for 10 min, the supernatant was collected, and the remaining residue was re-extracted with 0.5 mL of 80% (v/v) aqueous methanol for 2 h. Following centrifugation, the two supernatants were combined and evaporated under a stream of nitrogen at 35 °C until the organic solvent was completely removed. The resulting aqueous phase was mixed with 2 mL of petroleum ether and extracted three times for decolorization. The pH of the lower aqueous phase was subsequently adjusted to 2.0–3.0 with 1 mol L^{-1} citric acid, followed by two extractions with 2 mL of ethyl acetate. The upper organic phases were collected, combined, and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.2 mL of methanol, thoroughly mixed, and passed through a syringe filter prior to analysis. IAA was quantified using a Waters 2695 high-performance liquid chromatography (HPLC) system equipped with a Compass C18(2) reversed-phase column (250 mm × 4.6 mm, 5 μm). IAA measurements were performed by Wuhan Pronets Biotechnology Co., Ltd. (Wuhan, China).

For GA_3 determination, fresh seed samples were immediately frozen in liquid nitrogen, and approximately 0.3 g of each sample was accurately weighed. The samples were extracted overnight at 4 °C with a 10-fold volume of acetonitrile. After centrifugation at 12,000 × g for 5 min, the supernatant was collected, and the pellet was re-extracted twice with a fivefold volume of acetonitrile. All supernatants were combined, and 50 mg of C18 sorbent was added. The mixture was vortexed vigorously for 30 s and centrifuged at 10,000 × g for 5 min. The resulting supernatant was collected and evaporated to dryness using a vacuum centrifugal concentrator. The residue was reconstituted in 200 μL of methanol, passed through a 0.22-μm organic-phase membrane filter, and stored at −20 °C until instrumental analysis. GA_3 was quantified by high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) using a Shimadzu LC-30AD high-performance liquid chromatography system coupled to a SCIEX QTRAP 6500 mass spectrometer.

2.7. Statistical analysis

Experiments were conducted in a completely randomized design. Data analyses were performed using Student's t-test with SPSS software (IBM Corp., Armonk, NY, USA). Differences were considered significant at a P-value < 0.05.

3. Results

3.1. Phenotypic differences between M196 and F266 during seed germination under low-temperature conditions

The morphological characteristics of dry seeds from M196 and F266 were first evaluated (Fig. 1A). Significant differences in seed size and weight were observed between the two inbred lines. F266 had a significantly lower 100-seed weight than M196 (Fig. 1B), together with shorter seed length and smaller seed width (Fig. 1C, D). Under normal-temperature conditions (25 °C), M196 and F266 exhibited similar germination dynamics, with no significant difference in germination percentage throughout the experimental period (Fig. 1E, F). However, F266 developed longer hypocotyls than M196 at the fourth day of germination (Fig. 1G). In contrast, pronounced differences in germination performance emerged under low-temperature conditions (16 °C) (Fig. 1H, I). Under cold stress, F266 initiated germination sporadically on the fifth day and displayed a substantially higher germination percentage by the seventh day. Additionally, F266 maintained significantly longer hypocotyls than M196 during the entire cold-induced germination period (Fig. 1J). Collectively, these results indicate that F266 exhibits a greater capacity for seed germination and early seedling growth under low-temperature conditions than M196.

3.2. Differentially expressed genes (DEGs) analysis of the M196 and F266 at the germination stage under cold stress

To investigate the reasons for the differing cold stress in the two tomato inbred lines, mixed samples were prepared for RNA extraction and RNA-Seq analysis under normal-temperature conditions of 25°C (M196-N and F266-N) for 3 days, and low-temperature conditions of 16°C (M196-L and F266-L) for 6 days. Three biological replicates were designed for each variety. The cDNA libraries from the 12 samples were prepared for RNA-Seq analysis (Supplementary File S1). The sample PCA and correlation analysis results indicated that the data were highly repeatable and high-quality (Fig. S1).

Under normal-temperature conditions (25℃), M196-N_VS_F266-N exhibited a total of 1,542 DEGs, comprising 525 upregulated genes and 1,017 downregulated genes (Fig. 2A). Under low-temperature conditions (16℃), M196-L_VS_F266-L showed a total of 1,431 DEGs, including 727 upregulated genes and 704 downregulated genes (Fig. 2A). In the comparison groups M196-N_VS_M196-L, there were a total of 5,756 DEGs, comprising 1,847 upregulated genes and 3,909 downregulated genes (Fig. 2A). In contrast, in the comparison groups F266-N_VS_F266-L, there were a total of 3,789 DEGs, including 1,253 upregulated genes and 2,536 downregulated genes (Fig. 2A). We randomly selected 10 DEGs from the M196-N_VS_F266-N and M196-L_VS_F266-L groups for RT-qPCR analysis to validate the RNA-seq gene expression results (Supplementary File S2). As shown in Figure S2, the results confirmed the reliability of the RNA-seq data, and all these DEGs were subjected to further analysis. To further analyze the DEGs between the M196 and F266 tomato varieties with differing cold stress responses, Venn diagram analyses were performed. A total of 225 DEGs were shared under normal- and low-temperature conditions (Fig. 2B). Based on functional annotations and their potential involvement in seed germination and abiotic stress responses, eight representative candidate

genes were selected for further expression-pattern analysis (Fig. 2C). These candidate genes are involved in several biological processes potentially associated with low-temperature responses, including transcriptional regulation, membrane-associated processes, glycosylation, and stress signaling.

3.3. Functional classification using Gene Ontology (GO) terms

GO functional classification annotation was performed on the DEGs of M196 and F266 (Fig. 3A-D; Supplementary File S3-6). The top 20 enriched terms in the four comparison groups were mainly concentrated in pathways related to 'oxidoreductase activity', 'response to oxidative stress', 'peroxidase activity and antioxidant activity', 'oxidoreductase activity, acting on peroxide as acceptor'. These results preliminarily indicated that the two tomato inbred lines exhibited distinct transcriptional differences in redox metabolism pathways under cold stress, which might participate in regulating the stress response of tomato seeds during germination under low-temperature conditions. To further verify the physiological characteristics corresponding to GO enrichment results, the activities of three key antioxidant enzymes were determined in the two tomato lines. Higher POD activity was maintained in cold-tolerant line F266 under both normal-temperature and low-temperature conditions, suggesting that F266 possessed stronger basal antioxidant capacity (Fig. 3E). By contrast, no significant differences in SOD and CAT activities were observed between the two lines under normal-temperature, while their activities were obviously upregulated only after cold stress, indicating that SOD and CAT mainly functioned as emergency defensive enzymes in response to low-temperature stress (Fig. 3F and G). Collectively, changes in antioxidant enzyme activities could alter cellular redox homeostasis, which partially contribute to the difference in cold tolerance between the two tomato lines during seed germination. Notably, in the M196-L_VS_F266-L and F266-N_VS_F266-L groups (Fig. 3C and D), pathways related to 'glycosyltransferase activity', 'carbohydrate metabolic processes', 'hexosyltransferase activity', 'inositol-3-phosphate synthase activity' and 'inositol biosynthetic processes' were enriched. These pathways were not enriched in the M196-N_VS_F266-N group at normal-temperature and in the M196-N_VS_M196-L (Fig. 3A and B) group without F266. We speculate that the enrichment of these pathways is an important factor that enables the F266 variety to germinate earlier under low-temperature conditions.

3.4. KEGG enrichment classification of DEGs

To further clarify the roles of the DEGs in response to cold stress, the KEGG databases were employed to categorize DEGs into their respective pathways (Supplementary File S7-10). In these four comparisons, notable enrichment was found in significant enrichment of "Galactose metabolism" and "Starch and sucrose metabolism" pathways was detected in the comparisons M196-L_VS_F266-L and F266-N_VS_F266-L groups (Fig. 4C and D). In contrast, none of these pathways exhibited significant enrichment in M196-N_VS_F266-N and M196-N_VS_M196-L groups (Fig. 4A and B). Collectively, starch and sucrose metabolism pathways serve as the core pathways for F266 to cope with low-temperature stress. Therefore, we measured the soluble sugar content in dry seeds soaked in clean water for 8 h and seeds germinated at low-temperature for 6 days. The results showed that the contents of galactinol, fructose, glucose, and sucrose were higher in the F266 than in the M196. whereas myo-inositol showed no significant differences in dry seeds (Fig. 4E). In seeds germinating for 6 days at low-temperature conditions, there were no significant differences in myo-inositol, glucose, and fructose levels. However, in the F266 variety, the levels of galactinol, raffinose, and sucrose were significantly higher than those in

the M196 variety (Fig. 4F). This result indicates that the specific accumulation of RFOs in the F266 inbred line is critical for it to withstand cold stress and ensure low-temperature germination.

KEGG enrichment analysis also revealed that the "plant hormone signal transduction" pathway was enriched in the M196-L_VS_F266-L comparison. A total of 255 genes were enriched in this pathway, including 20 DEGs, of which 13 genes belong to the auxin pathway. Therefore, we measured the auxin content in seeds. The results indicated that under normal-temperature conditions, the IAA content in F266 was lower than that in M196. However, under low-temperature conditions, the IAA level in F266 was significantly higher than that in M196 (Fig. 5A). To further analyze the transcript levels of IAA family genes in the auxin signaling pathway, we found that *IAA2*, *IAA19*, *IAA15*, *IAA32*, *IAA17*, *IAA22*, and *IAA29* genes had a higher FPKM value in F266 than in M196 (Fig. 5C). This result suggests that the cold tolerance of F266 is closely associated with the accumulation of IAA under low-temperature conditions. Meanwhile, the GA_3 content in F266 was slightly higher than that in M196 under normal-temperature conditions. After low-temperature stress, GA_3 contents increased in both inbred lines, and the GA_3 accumulation level in F266 was significantly higher than that in M196 (Fig. 5B). In the GA pathway, the GA receptor gene *GID1B* was significantly highly FPKM value in F266 under low-temperature conditions, whereas *GAST1* exhibited lower FPKM value in F266 at normal-temperature and low-temperature induction. The FPKM values of GA catabolic genes *GA2ox1*, *GA2ox2-like*, and *GA2ox2* in F266 were lower than those in M196 under low-temperature conditions, while the FPKM value of *GA2ox8* was higher than that in M196 (Fig. 5D). These results indicate that low-temperature induces the accumulation of IAA and GA_3 in the F266 inbred line and is accompanied by increased transcript levels of auxin signaling-related genes and the GA receptor gene *GID1B*. The distinct expression patterns of *GA2ox* family genes further suggest that GA metabolism may be differentially regulated between F266 and M196 under low-temperature conditions, which may contribute to their contrasting germination responses.

3.5. Analysis of significant expression pathway of the GO-GSEA and KEGG-GSEA

Standard enrichment analyses built upon the hypergeometric distribution only screen genes with DEGs, which frequently omit genes with non-significant differential expression yet prominent biological values. Gene Set Enrichment Analysis (GSEA) effectively addresses this limitation [28]. Accordingly, gene sets with significant enrichment were identified using the thresholds of $|NES| > 1$, NOM p-value < 0.05 and FDR q-value < 0.25. According to the GO-GSEA results (Table 1). except for the M196-N_VS_F266-N group, the other three groups all showed enrichment in the top ten GO-GSEA pathways enriched for 'peroxidase activity' (GO:0004601), 'antioxidant activity' (GO:0016209), 'oxidoreductase activity', 'acting on peroxide as acceptor' (GO:0016684), or 'response to oxidative stress' (GO:0006979). In the two groups of M196-L_VS_F266-L and M266-N_VS_F266-L, 'glycosyltransferase activity' (GO:0016757), 'acyltransferase activity' (GO:0016746), and 'acyltransferase activity', 'transferring groups other than amino-acyl groups' (GO:0016747) are significantly enriched, Synergistic activation of low-temperature triggered antioxidant homeostasis regulation and sugar-modified secondary metabolic pathways constitutes a vital factor conferring strong cold tolerance to F266 (Supplementary File S11-14). According to the KEGG-GSEA results (Table 2). The top 10 enriched pathways included 'plant hormone signal transduction' (sly04075) and 'starch and sucrose metabolism' (sly00500) in the M196-L_VS_F266-L group, ,which were not present in the other groups. This indicates that soluble sugars and plant hormones play an important role in seed

germination at low-temperature conditions, a result that is generally consistent with the KEGG analysis (Supplementary File S15-18).

3.6. Different TFs regulation in inbred lines M196 and F266

TFs play core regulatory roles in plant cold acclimation. Previous studies have demonstrated that several TFs families, including HD-Zip [29], MYB [30], and bHLH [31], are involved in the regulation of seed germination under low temperatures conditions. From the transcriptomic data, we identified multiple TF families involved in this process, namely WRKY, bHLH, MYB, GATA, AP2/ERF, and NAC (Fig. S3A–D; Supplementary Files S19–22). A Venn diagram of the differentially expressed TFs revealed that six genes were co-expressed across all four groups (Fig. S3E). To further dissect the differences in seed germination between M196 and F266 under low-temperature conditions, we performed a targeted analysis of differentially expressed TFs in the M196-N_VS_F266-N and M196-L_VS_F266-L comparisons and identified 12 genes that were co-expressed in both groups (Fig. 6A and S3F), namely MYB (Solyc03g121740.3), MYB-related (Solyc06g076270.3), bHLH (Solyc12g088130.3), TCP (Solyc03g116320.3), WRKY58 (Solyc05g050340.4), WRKY33 (Solyc09g014990.4), GATA (Solyc01g090760.4), ERF (Solyc01g091760.3), bHLH (Solyc02g091800.4), MYB-related (Solyc04g005100.3), bHLH (Solyc09g065100.3), and CAMTA (Solyc12g035520.3). On this basis, we further focused on the M196_L_VS_F266_L comparison and examined the expression of genes belonging to three major TF families, HD-Zip, MYB and bHLH (Fig. 5B–D). The numbers of differentially expressed genes in these three families were 5, 12 and 9, respectively. The specific expression patterns of these genes may exert unique regulatory effects on the differences in seed germination between the two inbred lines.

4. Discussion

4.1. Coordinated antioxidant defense and carbohydrate metabolic responses contribute to low-temperature germination in the tomato inbred line F266

Genetically homozygous inbred lines provide valuable experimental materials for dissecting the molecular regulatory networks underlying crop stress tolerance and identifying key genes associated with agronomically important traits [32]. In the present study, the tomato inbred lines M196 and F266 showed no significant difference in seed germination under normal-temperature conditions (Fig. 1E). Under low-temperature conditions, however, the two lines exhibited markedly different germination responses. Compared with M196, F266 initiated germination earlier and maintained greater hypocotyl elongation (Fig. 1I and J), indicating a greater capacity of F266 to maintain germination and early seedling growth under low temperature.

Transcriptomic and physiological analyses further suggested that the contrasting low-temperature responses of the two inbred lines were closely associated with differences in their antioxidant defense systems. GO enrichment analysis showed that the DEGs were predominantly enriched in functional categories related to “oxidoreductase activity,” “response to oxidative stress,” “peroxidase activity,” “oxidoreductase activity, acting on peroxide as acceptor,” “antioxidant activity,” and “oxidoreductase complex” (Fig. 3A–D). Consistent with these transcriptomic responses, measurements of the antioxidant enzymes SOD, POD, and CAT indicated a stronger antioxidant response in F266 under low-temperature conditions (Fig. 3E–G). These findings are consistent with the importance of maintaining reactive oxygen species (ROS) homeostasis during seed germination. At appropriate levels, ROS can function as signaling molecules involved in the regulation of germination, whereas excessive ROS accumulation may

cause oxidative damage and impair normal germination [33]. A recent study in rice further demonstrated that the *OsNAC3–OsITPK4–OsMT2b* regulatory module promotes seed germination by maintaining ROS homeostasis and preventing excessive ROS accumulation [34]. This finding further highlights the importance of tightly regulated ROS homeostasis for successful seed germination. Moreover, GO-GSEA revealed significant enrichment of gene sets associated with redox metabolism and oxidative stress responses in the low-temperature-related comparisons (Table 1). The consistency between transcriptomic enrichment and antioxidant enzyme responses suggests that an enhanced antioxidant defense capacity may help F266 maintain cellular redox homeostasis during germination at low-temperature, thereby alleviating potential cold-induced oxidative stress and supporting seed germination and hypocotyl growth.

Carbohydrates accumulated in seeds not only serve as major energy sources for embryo growth and development during germination but also function as signaling molecules that regulate diverse biochemical and physiological processes, thereby influencing seed germination and early seedling establishment [35-39]. In the present study, F266 dry seeds contained higher basal levels of galactinol, fructose, glucose, and sucrose than M196 seeds (Fig. 4E). Despite these higher basal sugar levels, no significant difference in germination percentage was observed between M196 and F266 under normal-temperature conditions, although F266 developed longer hypocotyls. In contrast, after 6 days of germination under low-temperature conditions, F266 accumulated substantially higher levels of galactinol, raffinose, and sucrose than M196, whereas fructose and glucose levels remained relatively stable (Fig. 4F). This distinct pattern suggests that galactinol–raffinose metabolism and sucrose accumulation, rather than changes in monosaccharides alone, may be more closely associated with the enhanced low-temperature germination of F266. Galactinol and raffinose are key components of RFOs metabolism. Previous studies have shown that the accumulation of RFOs during seed maturation is closely associated with desiccation tolerance [40]. Moreover, overexpression of the chickpea galactinol synthase genes *CaGolS1* and *CaGolS2* in *Arabidopsis* improved seed vigor and longevity [41]. Therefore, the greater accumulation of galactinol and raffinose observed in F266 under low-temperature conditions suggests that RFO-associated metabolic remodeling may contribute to its low-temperature germination response.

This interpretation was further supported by transcriptome-based pathway analyses. KEGG enrichment and KEGG-GSEA analyses revealed significant enrichment of carbohydrate metabolism-related pathways, including “galactose metabolism,” “starch and sucrose metabolism,” “fructose and mannose metabolism,” and “pentose and glucuronate interconversions,” in the low-temperature response of F266, whereas the same enrichment pattern was not observed in M196 (Fig. 4A–D, Table 2). The convergence between metabolite accumulation and transcriptomic pathway enrichment therefore suggests that the enhanced low-temperature adaptation of F266 cannot be explained simply by an increase in total soluble sugar content. Instead, it may involve a broader reprogramming of carbohydrate metabolism, particularly RFO- and sucrose-associated metabolic processes. Taken together, the enhanced germination performance of F266 under low-temperature conditions appears to be associated with the coordinated responses of antioxidant defense and carbohydrate metabolism. On the one hand, enhanced antioxidant capacity may contribute to the maintenance of cellular redox homeostasis; on the other hand, metabolic remodeling involving galactinol, raffinose, and sucrose may support seed germination and early seedling growth through energy supply, osmotic adjustment, and cellular protection. The

coordinated regulation of these processes may therefore constitute an important physiological and metabolic basis for the superior germination performance of F266 under low-temperature conditions.

4.2. Endogenous hormone contents regulate seed germination of F266 in response to cold stress

Plant hormones play pivotal roles in regulating diverse aspects of plant growth and development, including secondary metabolism [42]. Among them, auxin establishes concentration gradients and local concentration differences through its biosynthesis, metabolism, polar transport, and signal transduction, thereby regulating organogenesis, polarity establishment, and plant adaptation to environmental changes [43]. Previous studies have shown that genes associated with auxin, gibberellin (GA), abscisic acid (ABA), and brassinosteroid biosynthesis and signaling undergo substantial changes during seed germination, highlighting the importance of coordinated hormonal regulation in radicle protrusion and seed germination [44]. Differences in endogenous hormone levels are also closely associated with genotypic variation in seed responses to low temperature. For example, during chilling-induced germination in upland cotton, the chilling-tolerant genotype KN27-3 exhibited higher levels of IAA, cytokinins (CTKs), and GA_3 than the chilling-sensitive genotype XLZ38, whereas ABA showed the opposite trend, suggesting that differences in endogenous hormone balance may contribute to seed germination under low-temperature conditions [6]. Similarly, transcriptomic analysis of waxy corn showed that low-temperature germination was associated with significant changes in auxin-related responses, with "cellular response to auxin stimulus" being highly enriched in the cold-tolerant inbred line N28 [45]. Low-temperature treatment has also been reported to alter endogenous IAA levels and the expression of auxin biosynthesis-related genes, further supporting the involvement of auxin in seed responses to low temperature [46].

In this study, KEGG enrichment analysis revealed significant enrichment of the "plant hormone signal transduction" pathway in the M196-L_VS_F266-L comparison. A total of 255 genes were assigned to this pathway, including 20 DEGs, 13 of which were associated with auxin signaling, suggesting that auxin signaling may represent an important hormonal pathway associated with the contrasting germination responses of M196 and F266 under low-temperature conditions. Consistent with this observation, endogenous IAA measurements revealed that F266 contained less IAA than M196 under normal-temperature conditions, whereas under low-temperature conditions, the IAA content was significantly higher in F266 than in M196 (Fig. 5A). This result indicates that F266 does not maintain constitutively high basal IAA levels but instead exhibits greater IAA accumulation under low-temperature conditions. In *Arabidopsis*, cold exposure promotes the degradation of Aux/IAA repressors, thereby releasing *ARF7* and *ARF19* and subsequently activating *CRF3* expression, revealing a mechanism through which Aux/IAA–ARF signaling participates in the plant response to low temperature [47]. Consistent with the potential involvement of this signaling pathway, several Aux/IAA family genes, including *IAA2*, *IAA19*, *IAA15*, *IAA32*, *IAA17*, *IAA22*, and *IAA29*, exhibited higher FPKM values in F266 than in M196 in the present study (Fig. 5C). These findings suggest that greater IAA accumulation and differential expression of auxin signaling-related genes under low-temperature conditions may be closely associated with the enhanced germination performance of F266.

GA is a major phytohormone regulating seed dormancy and germination, and changes in GA metabolism and signaling are closely associated with seed responses to low-temperature. Previous studies have shown that low-temperature can influence seed germination and dormancy by

altering GA and ABA metabolism and signaling. In rice, for example, low-temperature treatment at 15 °C suppresses GA signaling while enhancing ABA biosynthesis, thereby delaying seed germination [48]. In wheat, the *TaDof-2D–miR1832–TaP450-7A* module participates in low-temperature induced seed dormancy release by affecting α-amylase activity and ABA and GA metabolism [49]. Similarly, exogenous GA_3 markedly promotes seed dormancy release in *Magnolia sieboldii*, accompanied by changes in the ABA/GA balance and starch and sucrose metabolism [50]. Together, these findings indicate that GA-associated regulation of seed germination under low-temperature conditions involves not only hormone metabolism and signaling but may also be closely linked to reserve mobilization and carbohydrate utilization.

Although fewer GA-related DEGs than auxin-related DEGs were represented in the "plant hormone signal transduction" pathway in the present study, several genes involved in GA perception and metabolism exhibited clear genotype-dependent expression differences. We therefore further examined GA_3 accumulation and the expression patterns of GA-related genes in the two tomato inbred lines. Under normal-temperature conditions, GA_3 content was slightly higher in F266 than in M196. Following low-temperature treatment, GA_3 levels increased in both inbred lines, but the accumulation of GA_3 was significantly greater in F266 (Fig. 5B). Consistently, the GA receptor gene *GID1B* exhibited a higher FPKM value in F266 under low-temperature conditions, whereas *GAST1* showed relatively lower expression in F266 (Fig. 5D). Genes associated with GA metabolism also exhibited distinct expression patterns: *GA2ox1*, *GA2ox2*-like, and *GA2ox2* showed lower FPKM values in F266 than in M196 under low-temperature conditions, whereas *GA2ox8* displayed the opposite pattern (Fig. 5D). These contrasting expression patterns indicate that individual *GA2ox* family members respond differently to low temperature and therefore do not support a simple interpretation of uniformly enhanced or reduced GA catabolism. Together with the higher GA_3 content and *GID1B* expression in F266, these results suggest that GA_3 accumulation and differential expression of genes associated with GA perception and metabolism may contribute to the maintenance of seed germination and hypocotyl growth in F266 under low-temperature conditions.

4.3. Identification of candidate genes potentially associated with low-temperature seed germination in F266

Transcriptome analysis facilitates the elucidation of gene expression patterns and regulatory mechanisms. By comparing DEGs among different genotypes or treatment conditions and integrating functional annotation and expression pattern analyses, transcriptome analysis provides a basis for the further identification and functional validation of key genes [51]. In the present study, comparison of the M196-N_VS_F266-N and M196-L_VS_F266-L groups identified 12 TF genes that were differentially expressed in both comparisons (Fig. 6A and Fig. S3F). These genes belonged to several TF families, including MYB, MYB-related, bHLH, TCP, WRKY, GATA, ERF, and CAMTA, suggesting that different types of transcriptional regulators may be involved in the contrasting low-temperature germination responses of M196 and F266.

Among these candidate TFs, *SlGATA* (Solyc01g090760.4) is of particular interest because the GATA family has been closely associated with seed germination, dormancy, and low-temperature responses. In *Arabidopsis*, a GATA zinc-finger protein acts as a positive regulator of seed germination, and seeds of the corresponding knockout mutant exhibit enhanced dormancy and reduced sensitivity to cold stratification [52]. The GA signaling repressor *RGL2* can form a complex with *DOF6* and regulate *GATA12*, thereby contributing to the maintenance of primary

seed dormancy [53]. In wheat, *TaGATA1* promotes seed dormancy by regulating *TaABI5* expression [54]. In addition to their roles in seed dormancy and germination, GATA TFs are also involved in plant responses to low temperature. For example, overexpression of *OsGATA16* enhances cold tolerance at the seedling stage in rice [55], whereas heterologous overexpression of *PvGATA28* prolongs the dormancy period of transgenic tobacco seeds [56]. These studies indicate that different members of the GATA family can participate in the regulation of seed dormancy, germination, and low-temperature responses. Therefore, Solyc01g090760.4 identified in the present study may represent an important candidate TF linking low-temperature responses with seed germination, although its specific function requires further validation. In addition to GATA, the other candidate TFs may also have potential biological significance. The ERF family represents an important group of regulators in ethylene signaling, and ethylene can interact with other phytohormones and ROS to jointly regulate seed dormancy and germination [57]. The bHLH and MYB families are also widely involved in plant growth, development, and abiotic stress responses. Protein kinase-mediated phosphorylation can affect the stability, subcellular localization, and transcriptional activity of bHLH proteins [58]. In rice, *OsMAPK3*-mediated phosphorylation of *OsbHLH002* enhances its function in cold signal transduction [59]. Members of the MAPKK/MAPKKK families in tomato also exhibit pronounced transcriptional responses to various environmental stresses [60], further suggesting that coordinated regulation between protein kinases and TFs may represent an important component of plant stress responses. MYB TFs are likewise involved in seed germination and low-temperature responses. *MYB70* increases seed sensitivity to ABA-mediated inhibition of germination by interacting with ABI5 and enhancing its stability [61], whereas tomato *SlMYB15* positively regulates cold tolerance through the CBF pathway [62]. These studies provide functional support for the potential involvement of the MYB, bHLH, and ERF candidate genes identified in this study in low-temperature seed germination. In addition, CAMTA (Solyc12g035520.3) and TCP (Solyc03g116320.3) were identified as candidate TFs in the present study. CAMTA proteins respond to Ca^{2+}/calmodulin signaling and participate in plant responses to environmental stresses. For example, GmCAMTA12 is associated with drought tolerance in soybean [63], whereas several CAMTA family members in tobacco show significant changes in expression under cold stress [64]. Moreover, overexpression of *CnTCP4* increases the sensitivity of Arabidopsis to low temperature, suggesting that members of the TCP family may also participate in the regulation of cold responses [65]. Therefore, the CAMTA and TCP genes identified in this study further expand the range of candidate TFs potentially involved in the regulation of low-temperature seed germination in tomato. However, because these candidate genes were identified mainly on the basis of differential expression and functional annotation, their specific regulatory roles in F266 require further genetic and molecular validation.

In addition to TFs, we further screened a group of potentially biologically relevant candidate genes from the 225 shared DEGs identified in the comparisons between the two inbred lines. These genes are involved in membrane-related processes, glycosylation, signal transduction, and stress responses, some of which are highly consistent with previously reported mechanisms associated with seed germination and cold tolerance. For example, the maize annexin genes *ZmANN33* and *ZmANN35* participate in plasma membrane recovery during seed germination, and heterologous expression of these genes improves membrane integrity during recovery from chilling, increases POD activity, and reduces lipid peroxidation [66]. These findings suggest that

membrane stability and recovery from membrane damage may be important for protecting seeds against chilling injury and maintaining germination. Therefore, the membrane-associated candidate genes identified in the present study may provide important clues for further investigating the cellular protective mechanisms that enable F266 to maintain germination under low-temperature conditions. UDP-glycosyltransferases (UGTs) can affect the stability, activity, and transport of various plant metabolites and signaling molecules through glycosylation. Previous studies have shown that the rice UDP-glycosyltransferase gene *OsUGT706D2* is induced by low temperature and positively regulates cold and submergence tolerance in rice; gain of function enhances stress tolerance, whereas loss of function reduces cold tolerance [67]. Therefore, the UGT candidate gene(s) identified among the shared DEGs in the present study may represent potentially important genes associated with low-temperature responses. Considering the differential IAA/GA_3 responses and the changes in the levels of carbohydrates such as galactinol, raffinose, and sucrose observed in F266, these UGT candidates provide potential targets for further investigating the relationship between glycosylation processes and seed germination under low-temperature conditions.

The identification of these candidate genes not only provides new molecular clues for explaining the contrasting low-temperature germination phenotypes of M196 and F266, but also provides prioritized targets for further elucidating the mechanisms underlying low-temperature seed germination in tomato through RT-qPCR, gene editing, overexpression, and genetic transformation, as well as potential genetic resources for improving low-temperature germination tolerance in tomato germplasm.

5. Conclusions

In this study, the cold-sensitive tomato inbred line M196 and the cold-tolerant inbred line F266 were used to systematically compare their seed germination phenotypes under normal- and low-temperature conditions. Under normal-temperature conditions, M196 and F266 showed generally similar seed germination performance; however, under low-temperature conditions, F266 initiated germination earlier and exhibited stronger germination capacity and hypocotyl elongation, indicating that the differences in germination phenotype between the two inbred lines were mainly manifested under low-temperature stress.

Based on the transcriptomic and physiological and biochemical results, a model for low-temperature seed germination in F266 was proposed in this study (Fig. 7). Under low-temperature conditions, the germination advantage of F266 may be associated with three interconnected processes. First, stronger SOD, POD, and CAT antioxidant responses, together with the differential expression of redox-related genes, may contribute to maintaining ROS homeostasis, thereby alleviating potential oxidative damage. Second, the accumulation of galactinol, raffinose, and sucrose, together with changes in related carbohydrate metabolic pathways, may support low-temperature germination through osmoprotection, maintenance of membrane stability, and energy supply. Third, the higher accumulation of IAA and GA_3, together with the differential expression of Aux/IAA, GID1B, and several GA2ox family genes, suggests that auxin- and GA-related responses may be beneficial for embryo growth and cell elongation under low-temperature conditions. Meanwhile, transcriptome analysis identified several genes associated with low-temperature responses, including GATA, CAMTA, WRKY58, bHLH150, and UGT, which may participate in the low-temperature germination response of F266 at different levels. In contrast, the above physiological and molecular responses were generally weaker in M196 under low-

temperature conditions, consistent with its delayed germination and inhibited hypocotyl growth. Therefore, the low-temperature germination advantage of F266 may not be determined by a single gene or metabolic pathway, but may instead be closely associated with the coordinated effects of antioxidant defense, RFO/sucrose-related carbohydrate metabolism, and IAA/GA_3 hormone responses, and may be jointly regulated by multiple transcription factors. This study provides new evidence for understanding the physiological and molecular basis of low-temperature seed germination in tomato and identifies a group of candidate genes worthy of further functional validation, providing potential genetic resources for functional analysis of genes associated with low-temperature germination and for the improvement of tomato germplasm with enhanced low-temperature tolerance.

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Author contributions

H.X. contributed to the investigation, data analysis, interpretation of results, and manuscript preparation; Z.G. contributed to the experimental investigation, data collection, and interpretation of results; Y.W. contributed to the experimental work, sample preparation, data collection, and organization of experimental results; L.J. contributed to the experimental investigation, data analysis, and manuscript review; S.W. supervised the research, contributed to the interpretation of results, and critically revised the manuscript for important intellectual content. All authors contributed critically to the drafts and gave final approval for publication.

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Availability of data and material

The data supporting the findings of this study are included in the article and its supplementary materials. The processed RNA-seq datasets, including gene expression matrices for all samples, have been deposited in the OMIX repository of the National Genomics Data Center (NGDC), China National Center for Bioinformation, under accession number OMIX022509 (https://ngdc.cncb.ac.cn/omix/). Additional information is available from the corresponding author upon reasonable request.

Declaration

Ethics approval and consent to participate Not applicable.

Statement of ethical approval in compliance with institutional, national, or international guidelines for the use of animals All local, national or international guidelines and legislation were adhered to for the use of plants in this study.

Consent for publication Not applicable.

Declaration of Generative AI and AI-assisted technologies in the writing process During the preparation of this manuscript, the author(s) used Chat GPT and Grammarly to improve the technical and English language of the paper. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

Competing interests The authors declare no competing interests.

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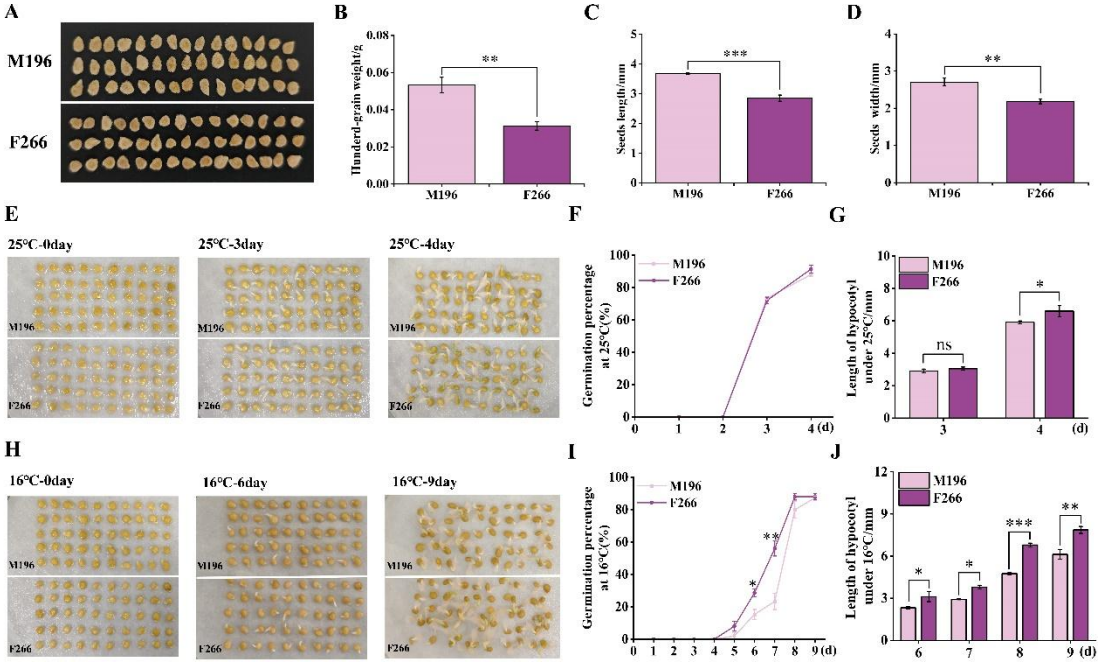


Fig. 1. Phenotypic characteristics and statistical analysis of seed germination under NT and LT conditions for M196 and F266.

A. Phenotypic characteristics of dry seeds for M196 and F266.

B. Hundred-grain weight of M196 and F266.

C. Seeds length of M196 and F266.

D. Seeds width of M196 and F266.

E. Phenotypic characteristics of seed germination of M196 and F266 under NT.

F. Germination rates of M196 and F266 seeds after treatments under NT.

G. Length of hypocotyl of M196 and F266 seeds after germination under NT.

H. Phenotypic characteristics of seed germination of M196 and F266 under LT.

I. Germination rates of M196 and F266 seeds after treatments under LT.

J. Length of hypocotyl of M196 and F266 seeds after germination under LT.

The error bars indicate the mean ± SD of three replicates. Asterisks indicate significant differences: *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, Student's t-test.

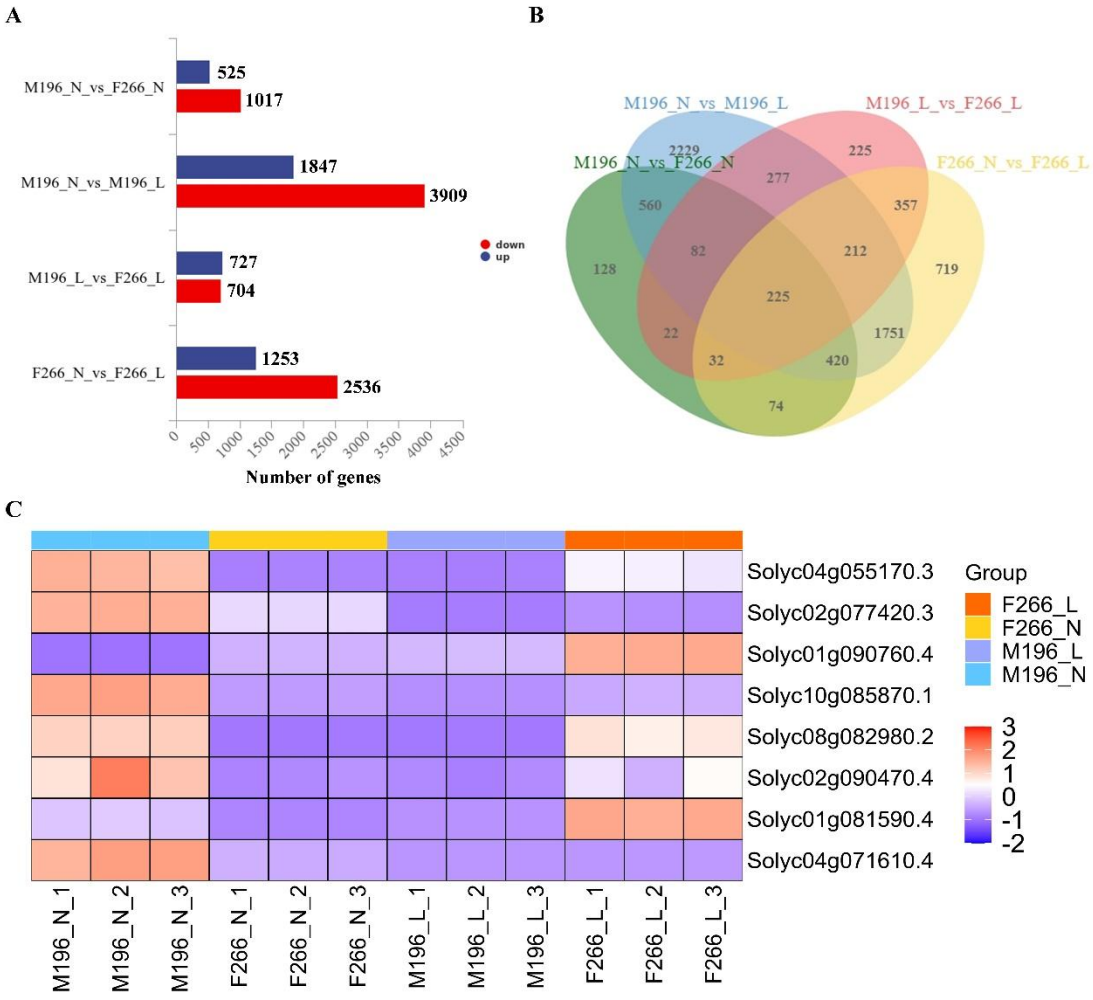


Fig. 2. The statistics of DEGs and Venn diagram between the M196 and F266.

A. The bar chart displays the number of DEGs from four comparison groups: M196_N_VS_F266_N, M196_L_VS_F266_L, M196_N_VS_M196_L, and F266_N_VS_F266_L. DEGs were screened based on the criteria of $|\log_2 FoldChange| > 1$ and P-value < 0.05.

B. The Venn diagram shows the intersection of DEGs identified in four groups of the M196_N_VS_F266_N, M196_L_VS_F266_L, M196_N_VS_M196_L, and F266_N_VS_F266_L.

C. Heatmap showing the expression patterns of eight candidate genes selected from the 225 shared DEGs between M196 and F266 under normal-temperature and low-temperature conditions. The heatmap was generated using Z-score-normalized FPKM values. Red and blue indicate relatively high and low expression levels, respectively.

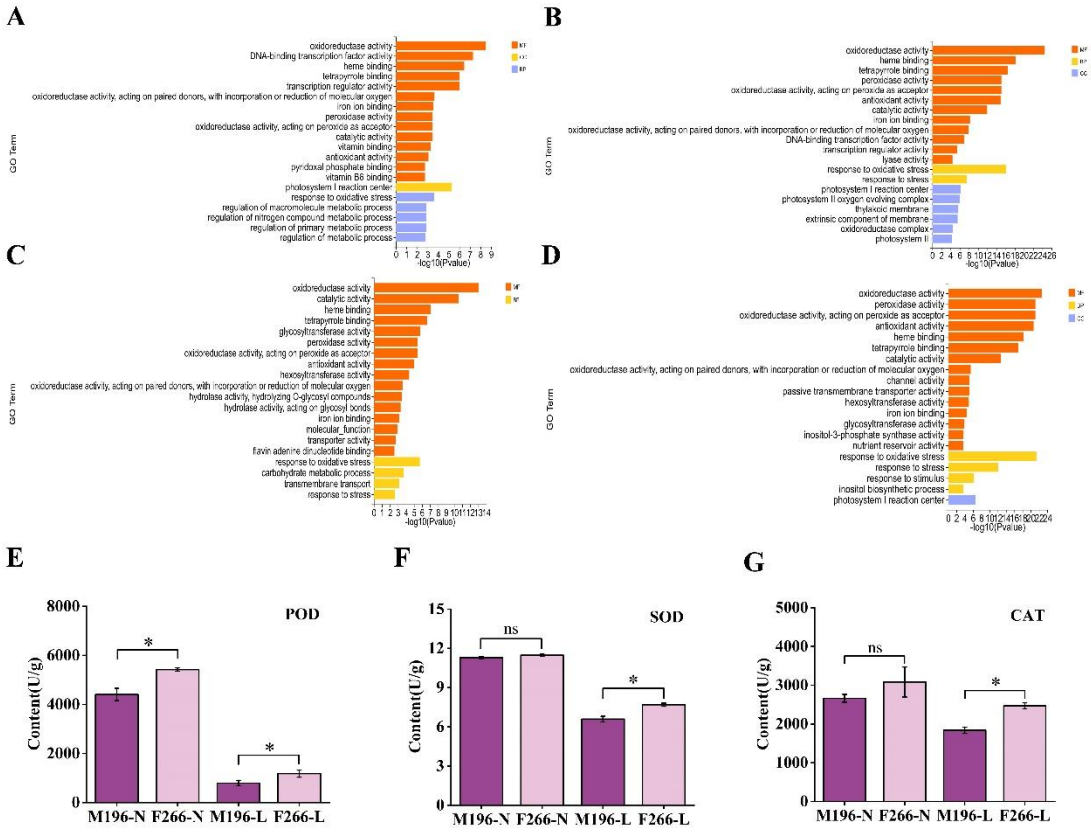


Fig. 3. GO functional annotation and POD, SOD, CAT content.

A. Differentially expressed genes GO annotation classification statistics chart in M196_N_VS_F266_N.

B. Differentially expressed genes GO annotation classification statistics chart in M196_N_VS_M196_L.

C. Differentially expressed genes GO annotation classification statistics chart in M196_L_VS_F266_L.

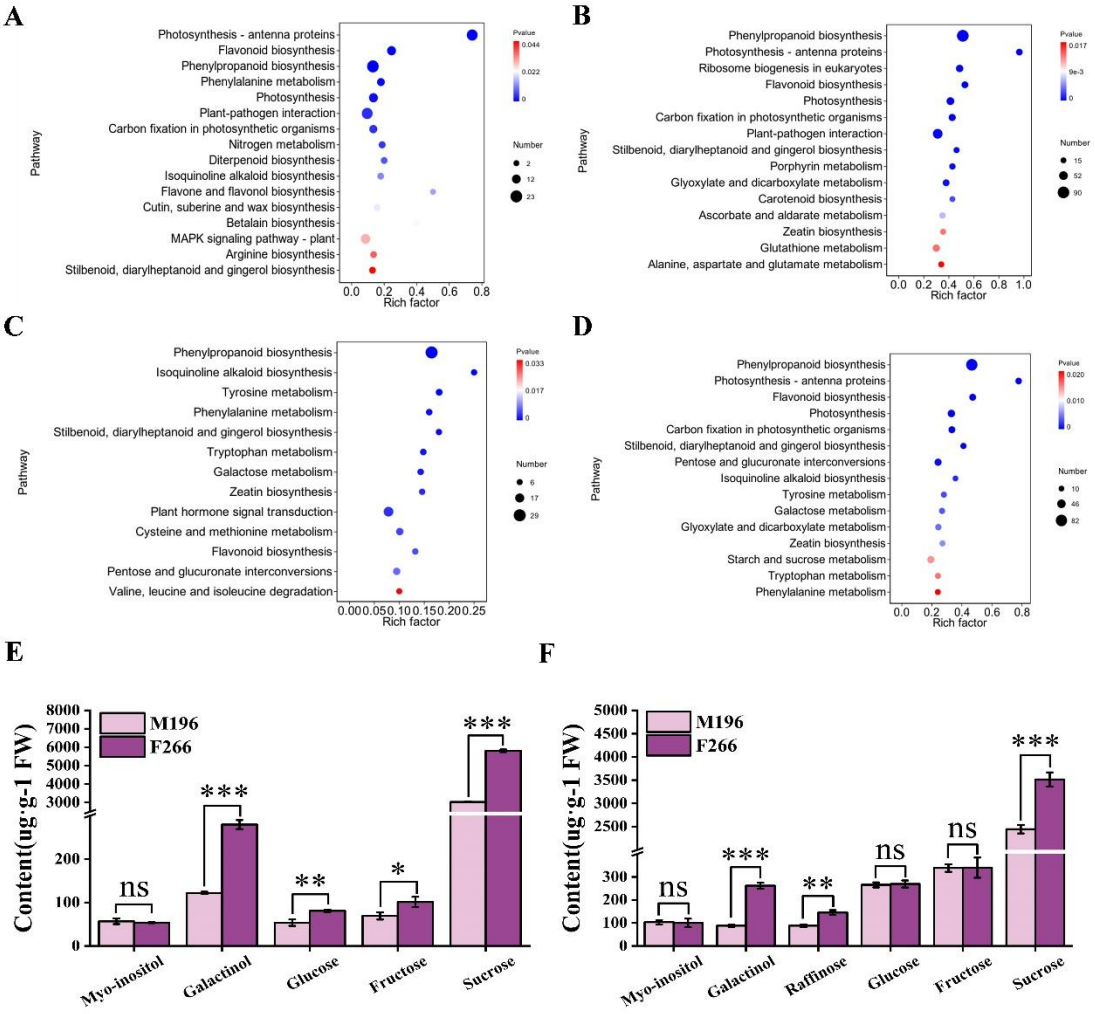
D. Differentially expressed genes GO annotation classification statistics chart in F266_N_VS_F266_L.

E. POD content under normal-temperature and low-temperature conditions in M196 and F266 of two tomato inbred lines.

F. SOD content under normal-temperature and low-temperature conditions in M196 and F266 of two tomato inbred lines.

G.CAT content under normal-temperature and low-temperature conditions in M196 and F266 of two tomato inbred lines.

The error bars indicate the mean ± SD of three replicates. Asterisks indicate significant differences: *P < 0.05, ns, not significant, Student's t-test.



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Fig. 4. Classification map of the KEGG and determination of soluble sugar content.

A. Differentially expressed genes KEGG annotation classification statistics chart in M196_N_VS_F266_N.

B. Differentially expressed genes KEGG annotation classification statistics chart in M196_N_VS_M196_L.

C. Differentially expressed genes KEGG annotation classification statistics chart in M196_L_VS_F266_L.

D. Differentially expressed genes KEGG annotation classification statistics chart in F266_N_VS_F266_L.

E. The content of myo-inositol, galactinol, glucose, fructose, and sucrose in dry seeds soaked in clean water for 8 h in M196 and F266 of two tomato inbred lines.

F. The content of myo-inositol, galactinol, raffinose, glucose, fructose, and sucrose after 6 days of germination under low-temperature conditions in M196 and F266 of two tomato inbred lines.

The error bars indicate the mean ± SD of three replicates. Asterisks indicate significant differences: *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, Student's t-test.

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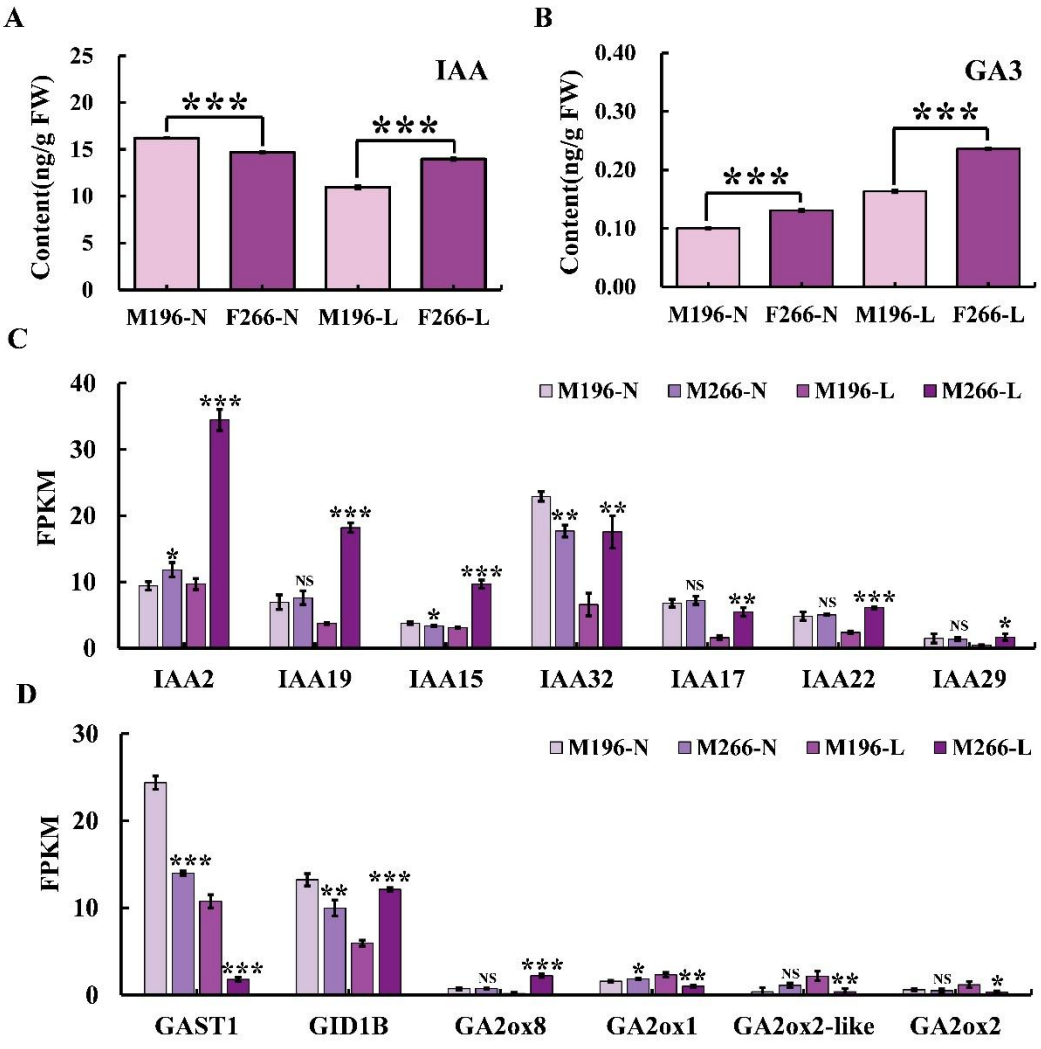


Fig. 5. Endogenous hormone contents in M196 and M266 and the expression of the genes related to hormone metabolism.

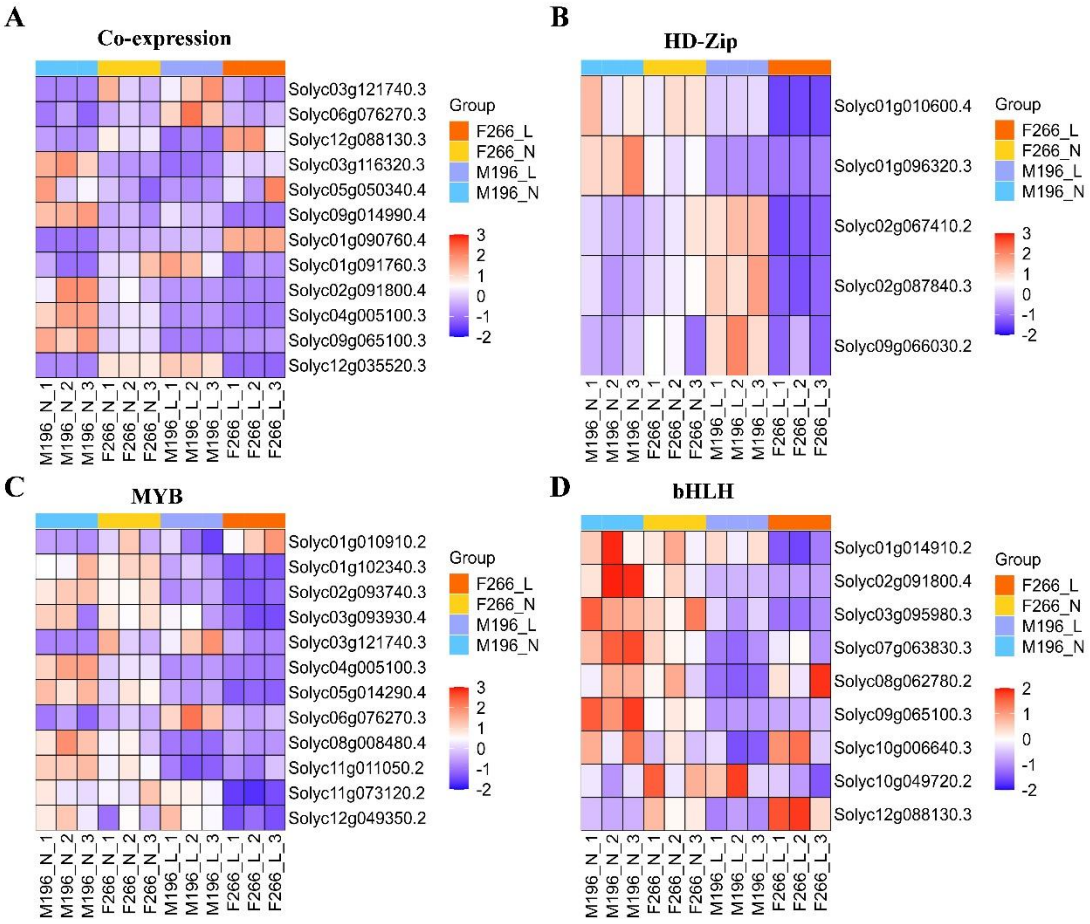
A. The content of IAA after 3 days of germination under NT and after 6 days of germination under LT for M196 and F266.

B. The content of GA3 after 3 days of germination under NT and after 6 days of germination under LT for M196 and F266.

C. FPKM values of genes involved in the auxin signaling pathway after 3 days of germination under NT and after 6 days of germination under LT for M196 and F266.

D. FPKM values of genes involved in the gibberellin signaling pathway after 3 days of germination under NT and after 6 days of germination under LT for M196 and F266.

The error bars indicate the mean ± SD of three replicates. Asterisks indicate significant differences: *P < 0.05, **P < 0.01, ***P < 0.001, ns, not significant, Student's t-test.



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Fig. 6. Expression dynamics of co-expressed genes and three transcription factor families HD-Zip, MYB, and bHLH visualized as heatmaps under normal-temperature and low-temperature conditions.

A. Heatmap showing the expression patterns of co-expressed genes selected from the TFs shared DEGs between M196 and F266.

B. Heatmap showing the expression patterns of HD-Zip transcription factor family selected from the TFs shared DEGs between M196 and F266.

C. Heatmap showing the expression patterns of MYB transcription factor family selected from the TFs shared DEGs between M196 and F266.

D. Heatmap showing the expression patterns of bHLH transcription factor family selected from the TFs shared DEGs between M196 and F266.

The heatmap was generated using Z-score-normalized FPKM values. Red and blue indicate relatively high and low expression levels, respectively.

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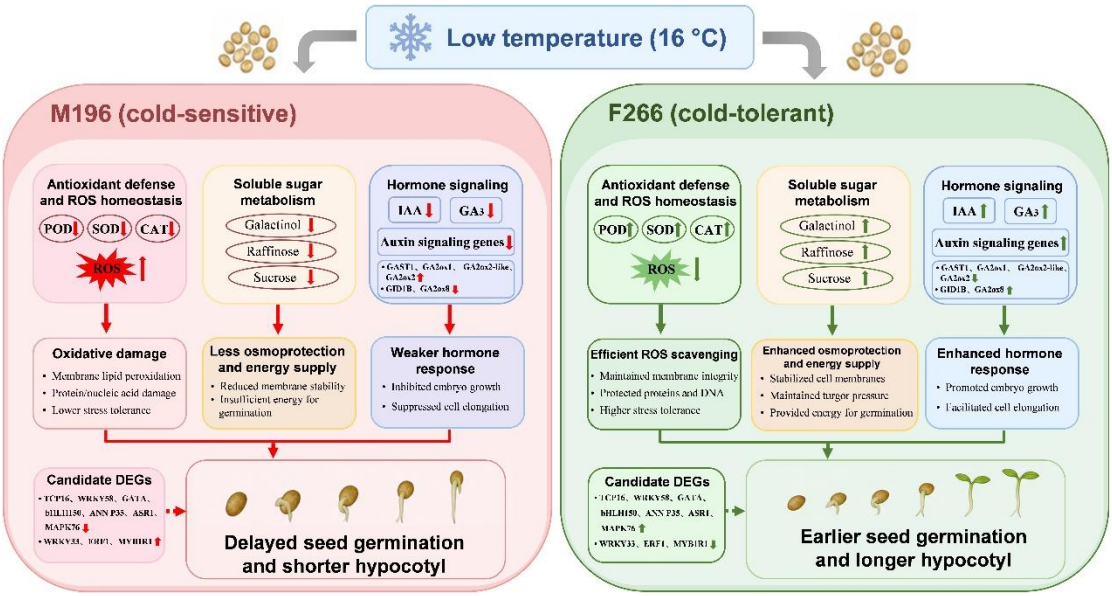


Fig. 7. Proposed model for the differential low-temperature germination responses of tomato inbred lines M196 and F266.

Under low-temperature (16 °C), F266 exhibits stronger antioxidant defense, enhanced accumulation of galactinol, raffinose, and sucrose, and stronger IAA- and GA_3-related responses, which are associated with earlier germination and greater hypocotyl elongation. In contrast, the relatively weaker responses in M196 are associated with delayed germination and shorter hypocotyls. Candidate DEGs potentially associated with low-temperature germination were identified by transcriptome analysis. Solid arrows represent measured or associated responses supported by this study, whereas dashed arrows indicate potential associations that have not been functionally validated. Red and green arrows indicate changes in M196 and F266, respectively, with upward and downward arrows representing relatively higher and lower levels or expression, respectively.

Table 1

The top 10 pathways were analyzed by GO-GSEA across four groups of M196 and F266 varieties.

Group	ID	Description	ID	Description
	GO:0008270	zinc ion binding	GO:0003700	dna-binding transcription factor activity
M196-N	GO:0043232	intracellular non-membrane-bounded organelle	GO:0005198	structural molecule activity
vs	GO:0043228	non-membrane-bounded organelle	GO:0140110	transcription regulator activity
F266-N	GO:0005840	ribosome	GO:0044260	cellular macromolecule metabolic process
	GO:0003735	structural constituent of ribosome	GO:0050896	response to stimulus
	GO:0006950	response to stress	GO:0016684	oxidoreductase activity, acting on peroxide as acceptor
M196-N	GO:0020037	heme binding	GO:0004601	peroxidase activity
vs	GO:0050896	response to stimulus	GO:0016209	antioxidant activity
M196-L	GO:0046906	tetrapyrrole binding	GO:0005507	copper ion binding
	GO:0006979	response to oxidative stress	GO:0006396	rna processing
	GO:0006979	response to oxidative stress	GO:0016757	glycosyltransferase activity
M196-L	GO:0016209	antioxidant activity	GO:0000786	nucleosome
vs	GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	GO:0020037	heme binding
F266-L	GO:0004601	peroxidase activity	GO:0044815	dna packaging complex
	GO:0046906	tetrapyrrole binding	GO:0043531	adp binding
	GO:0006979	response to oxidative stress	GO:0004601	peroxidase activity
F266-N	GO:0020037	heme binding	GO:0016209	antioxidant activity
vs	GO:0006950	response to stress	GO:0050896	response to stimulus
F266-L	GO:0046906	tetrapyrrole binding	GO:0016746	acyltransferase activity
	GO:0016684	oxidoreductase activity, acting on peroxide as acceptor	GO:0016747	acyltransferase activity, transferring groups other than amino-acyl groups

Table 2

The top 10 pathways were analyzed by KEGG-GSEA across four groups of M196 and F266 varieties.

Group	ID	Description	ID	Description
	sly03010	ribosome	sly00195	photosynthesis
M196-N	sly03008	ribosome biogenesis in eukaryotes	sly00909	sesquiterpenoid and triterpenoid biosynthesis
vs	sly00196	photosynthesis - antenna proteins	sly00940	phenylpropanoid biosynthesis
F266-N	sly03040	spliceosome	sly03018	rna degradation
	sly00941	flavonoid biosynthesis	sly03015	mrna surveillance pathway
	sly03008	ribosome biogenesis in eukaryotes	sly03013	nucleocytoplasmic transport
M196-N	sly00940	phenylpropanoid biosynthesis	sly00909	sesquiterpenoid and triterpenoid biosynthesis
vs	sly00196	photosynthesis - antenna proteins	sly03015	mrna surveillance pathway
M196-L	sly00195	photosynthesis	sly00710	carbon fixation in photosynthetic organisms
	sly03018	rna degradation	sly03020	rna polymerase
	sly00940	phenylpropanoid biosynthesis	sly00195	photosynthesis
M196-L	sly04075	plant hormone signal transduction	sly00280	valine, leucine and isoleucine degradation
vs	sly00410	beta-alanine metabolism	sly04626	plant-pathogen interaction
F266-L	sly00710	carbon fixation in photosynthetic organisms	sly00500	starch and sucrose metabolism
	sly00100	steroid biosynthesis	sly03008	ribosome biogenesis in eukaryotes
	sly00940	phenylpropanoid biosynthesis	sly00941	flavonoid biosynthesis
F266-N	sly03008	ribosome biogenesis in eukaryotes	sly00195	photosynthesis
vs	sly00196	photosynthesis - antenna proteins	sly00945	stilbenoid, diarylheptanoid and gingerol biosynthesis
F266-L	sly03013	nucleocytoplasmic transport	sly03018	rna degradation
	sly03040	spliceosome	sly03015	mrna surveillance pathway