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# The combination of transcriptomics and metabolomics uncovers mepiquat chloride improves the response of Litchi chinensis leaves to low-temperature stress in late spring

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# Abstract

Under low-temperature stress, the application of chemical regulation into litchi leaves remains largely uninvestigated especially during the late spring season. In this study, we came up with transcriptomics- and metabolomics-based strategies to comprehensively survey the response mechanisms of litchi to low temperature under the treatment of mepiquat chloride. After a big fraction of transcripts and metabolites were found differentially expressed, the pathway enrichment analysis showed that carbon metabolism accounted for a large number of DEGs (Differentially Expressed Genes). The effects of mepiquat chloride on carbon metabolism were found up regulated in the enrichment analysis for differential metabolites or transcripts. In addition, the combinational analysis of transcriptomics and metabolomics indicated mepiquat chloride coordinately regulates transcripts and metabolites by forming a tightly linked network. The integration of metabolomics and transcriptomics revealed that mepiquat chloride potentially promotes the expression of transcription factor and drives the upregulation of citrate synthase, and isocitrate dehydrogenase (NAD+) in the pathways of carbon metabolism. Meanwhile, upregulation of antioxidant related genes in the pathway of peroxisome. Considering that carbon metabolism is tightly related to photosynthesis, the physiological measurements of photosynthetic indexes suggested mepiquat chloride presented the potentials to improve the ability of photosynthesis for litchi under low-temperature stress. This comprehensive study combining transcriptomics and metabolomics provided a basis and strategy for understanding how higher plants respond stresses under chemical regulation.


# Introduction

Litchi (*Litchi chinensis* Sonn.) is one of the plant species very sensitive to climate change. With global warming and precipitation variability increasing the frequency and intensity of extreme weather, the global crops have been dramatically influenced by agrometeorological changes(Calleja-Cabrera et al., 2020). Among them, the low temperature of late spring is a severe injury, which often happens in the late stage of spring and is often regarded as an abnormal season than normal years(Zohne et al., 2020).

The low temperature of late spring generally occurs from March to April, during which period litchi blossoms and fruit yield would be greatly disrupted. In addition, if the average temperature of the female flower of litchi is lower than 20℃ for more than 5 days, the pollination and fertilization of litchi would be poorly affected, which could resultantly impact the fruit production(Zhang et al., 2007). Therefore, to reduce the damage caused by the low temperature in late spring, prompt measures should be taken to eliminate the low-temperature stress.

Chemical regulators are very useful agents for releasing crops from a variety of stresses(Nakamura and Asami, 2014). For example, mepiquat chloride is one of the chemical regulators showing a low toxicity(Department, 1992). Also, it could enhance photosynthesis and yield of Siraitia grosvenorii, mulberry leaves and grapes(Zhou et al., 2013; Wu and Du, 1993; Yang et al., 1987; Xu, 1986). It can also improve the chlorophyll content and yield of Sweet Potato(Li et al., 2009). Previous studies have shown that spraying mepiquat chloride with appropriate concentration can improve cotton photosynthesis and

enhance its tolerance to drought(Xing et al., 2018). In addition, Irrigation of sweet pepper seedlings with mepiquat chloride could enhance the resistance to low temperature(Luo et al., 2010). However, although many studies on applying mepiquat chloride as a chemical regulator to release low-temperature stress in a variety of crops, its potential applications in litchi were rarely reported as we know.

With the development of high-throughput sequencing and bioinformatics tools, the gene regulatory networks under low temperature, drought, and oxidative stress on litchi flowers have been established(Lu et al., 2017; Shen et al., 2016; Zhang et al., 2014). However, the studies of litchi leaves under low-temperature stress are very restricted in the physiology field(Zhang et al., 2016; Zhang and Liu, 2015; Yu and Zhang, 2009) and its functional mechanisms are still unclear. Meanwhile, the regulation and adaptation of plants to stresses including low-temperature stress is a very sophisticated response, which involves systematic regulation at many biological levels, such as gene regulation, post-transcriptional regulation, post-translational modification, and metabolite feedback(Wang and Cheng, 2017). Therefore, it becomes a demanding task to find a strategy to understand the responsive mechanisms of litchi leaves under different stresses and treatments.

In this study, we comprehensively surveyed the mechanism of mepiquat chloride on alleviating the low-temperature stress of litchi leaves collected in late spring. Based on the transcriptomics and metabolomics of litchi with/without the treatment of mepiquat chloride, we discovered several critical genes involved in promoting carbon metabolism under low-temperature stress and illustrated these genes were probably driven by specific transcription factors, which results in the upregulation photosynthesis rate. The strategy raised in this study would push forward the understanding of how higher plants respond to specific stresses and the potential application of chemical regulation.

# Results

### Mepiquat chloride alleviates low-temperature stress by globally altering transcriptome and metabolome

To select the optimal concentration of mepiquat chloride for treating litchi leaves collected in late spring , we compared the fresh leaf weight under the treatment of mepiquat chloride with different concentrations. After measuring some physiological indicators in the past, we found that treatment B had the best effect. Thus, we focused on the concentration of B and performed transcriptomic/metabolic analysis to see how mepiquat chloride rescues litchi from low-temperature stress. To ensure the quality of RNA-sequencing, we set triplicates for control (only water, CK1-CK3) or treatment B (mepiquat chloride (ml)/$H_2O$ (kg) = 5/10, B1-B3) and evaluated the overall quality of the reads. The value of FPKM (**F**ragments **P**er **K**ilobase **M**illion) (Supplementary Figure S1a), the transcript number along FPKM (Figure S1b), as well as the correlation among the three replicates (Figure S1c), revealed a good reproduction of the RNA sequencing data. Also, when comparing the global difference between CK and the treatment of mepiquat chloride, there was a difference observed (Figure S1b and Figure S1c).

To investigate how mepiquat chloride affects the litchi under low temperature, we first inspected the global difference caused between mepiquat chloride and CK by performing PCA analysis for transcriptome and metabolome. As a result, it was found that there was evident isolation between CK and mepiquat chloride-treated groups (Figure 1a and Fig. 1b), suggesting the treatment of mepiquat chloride contributes to transcriptional and metabolic change. Furthermore, the cluster analysis for differentially altered transcripts (Figure 1c, blue vs red) and metabolites (Figure 1d, blue vs red) showed apparent discrimination between CK and mepiquat chloride treatment, indicating that mepiquat chloride probably released low-temperature stress by affecting the expression of a group of transcripts and thus downstream metabolites. In addition, by defining the fold change (|log2FC| >0.58) and p-value (p-value < 0.05), we calculated the significantly affected gene number. It was found 7073 genes were displaying differential expression after the treatment with mepiquat chloride, and among which 3947 were upregulated and 3126 were downregulated (Figure S2a).

For metabolome, there were 302 metabolites up-regulated and 312 metabolites down-regulated under the treatment of mepiquat chloride (Figure S2b) compared to CK. Among these metabolites, lipids and lipid-like molecules, organic oxygen compounds, organic acids and derivatives, phenylpropanoids, and polyketides were significantly accumulated (Figure S2c). Statistically, lipids and lipid molecules account for about 50.08% of the total metabolites, organic oxygen compounds metabolites account for about 8.32% of the total metabolites, while carbohydrates and carbohydrate complexes metabolites account for the largest 80.05% of organic oxygen compound metabolites, including carbohydrates and carbohydrate complexes metabolites like D-(+)-Raffinose, D-Sedoheptulose 7-phosphate, galactaric acid, mannan, chronic acid and so on (Figure S2c).

## Mepiquat chloride positively regulates carbon metabolism

Next, we sought to understand how these differential metabolites or genes confer mepiquat chloride to release low-temperature stress of litchi. To do that, we performed KEGG (**K**yoto **E**ncyclopedia of **G**enes and **G**enomes) enrichment analysis for up-/down-regulated genes or metabolites. Interestingly, among the top 20 up-regulated pathways in transcriptome under the treatment of mepiquat chloride (Figure 2a), many of them (6/20) are related to carbon/or energy metabolism such as TCA cycle, starch, sucrose metabolism, amino sugar, and nucleotide sugar metabolism (Figure 2a).

Considering that carbon metabolism is tightly related to metabolic pathways(Serefidou et al., 2019; Annibal et al., 2021), we also analyzed the top 20 KEGG enrichment pathways for metabolome (Figure 2b). Consistently, there were also many carbon-related pathways enriched including carbon fixation in photosynthetic organisms, one carbon pool by folate, as well as TCA cycle. Based on that, we assume mepiquat chloride affects carbon metabolism by coordinately regulating metabolome and transcriptome, and some upstream factors or genes should play a critical role in regulating carbon-related pathways.

Following the enrichment analysis, we aimed to investigate the genes involved in carbon metabolism. As an example, we checked one of the essential pathways for plant growth: carbon fixation in

photosynthetic organisms (Figure 2c). From the network plot, we noticed that sedoheptulose-7P was significantly up-regulated, and some of its related genes like the transketolase (*LcTK*) gene, fructose-1,6-bisphosphatase (*LcFBP*), and malate dehydrogenase (*LcMDH*) were up-regulated. Therefore, we assumed this gene potentially plays one of the essential roles in mepiqut chloride-induced carbon metabolism. Furthermore, we also investigated another important pathway in carbon metabolism, the TCA cycle pathway, which was both enriched in the transcriptome (Figure 3a) and metabolome (Figure 3b) as shown in Figure 3. When comparing the KEGG map of TCA for transcriptome and metabolome, we found two metabolites citrate and 2-Oxo-glutarate was up-regulated (Figure 3b). When we trace back to the upstream genes for these two nodes in the transcriptome (Figure 3a), it was noticed that two genes citrate synthase (2.3.3.1) and isocitrate dehydrogenase (NAD+) (1.1.1.42) were shown up-regulated.

Overall, based on the enrichment analysis in metabolome and transcriptome, we found that mepiquat chloride presents the potential to improve carbon metabolism-related pathways in both transcriptomic and metabolic levels. The joint analysis furtherly suggests that mepiquat chloride could positively increase the expression level of related genes to improve carbon fixation, and citrate synthase (or isocitrate dehydrogenase) to improve the TCA cycle.

In higher plants, photosynthesis is tightly linked to carbon metabolism(Dusenge et al., 2019; Marques et al., 2017). Given that carbon metabolism was highly up-regulated under the treatment of mepiquat chloride. For this reason, photosynthesis may be closely related to transcriptome and metabolome. In our study, we found chloride significantly improves Tr and Pn 16.39% and 14.46% respectively, but does not change Gs and Ci too much, suggesting that mepiquat chloride could release the litchi leaves from low-temperature stress by supplying more resources for photosynthesis(Li et al., 2022). Furthermore, the very consistent result shown from transcriptome, metabolome, and physiological experiments suggested that mepiquat chloride uniformly leads to a tight regulation in litchi, which can be suggested by the condense network from interactive analysis of transcriptome and metabolome (Figure 4).

### Mepiquat chloride alleviates low-temperature stress by up-regulating some physiological related genes

In order to clarify the DEGs (**D**ifferentially **E**xpressed **G**enes) encoding antioxidant enzymes in litchi leaves under low temperature, combined with the literature, the genes encoding SOD and CAT enzymes showed differential expression compared to CK(Table S2). The expression of most of these genes were up-regulated, and the FC values of the three SOD genes *TRINITY_DN10586_c0_g1_i2, TRINITY_DN10612_c0_g1_i1* and *TRINITY_DN14699_c0_g1_i1* were 19.8,17.5 and 15.2 in mepiquat chloride treatment. Similar to the SOD gene, 9 DEGs of CAT were screened from data, of which one was down-regulated after mepiquat chloride treatment, and the other 8 DEGs were up-regulated (including 5 genes with multiple expression greater than 3).

Under low temperature stress, the accumulation of reactive oxygen species led to membrane lipid peroxidation, resulting in the increase of MDA content. In present study, it was found that under low temperature stress, compared with the control, spraying regulators significantly decreased the content of MDA in leaves, and the expression of LOX gene (Table S2**)**was down-regulated.

## DEGs of plant hormone under the treatment of mepiquat chloride

Compared with CK, the expression of indole-3-pyruvate monooxygenase (*LcIDO*) gene was up-regulated in the pathway of auxin synthesis in mepiquat chloride treatment(Table S3). Mean while, in auxin signal transduction pathway, auxin inward transport vector auxin influx carrier *(LcAUX1*) gene and its downstream auxin responsive GH3 gene family (*LcGH3*) gene were up-regulated with treatment of mepiquat chloride. Cytokinin trans-hydroxylase (*LcCYP735A*) gene was up-regulated in cytokinin biosynthesis pathway. Two-component response regulator ARR-A family (*LcARR-A*) gene was up-regulated in cytokinin signal transduction pathway. The expression of abscisic-aldehyde oxidase (*LcAAO3*) gene in abscisic acid biosynthesis pathway were down-regulated, while abscisate beta-glucosyltransferase (*LcAOG*) gene was up-regulated. In abscisic acid signal transduction pathway, abscisic acid receptor PYR/PYL family (*LcPYL*) and protein phosphatase 2C (*LcPP2C*) genes were up-regulated and ABA response element binding factor ABA responsive element binding factor (*LcABF*) genes were down-regulated. There were three differentially expressed genes in gibberellin biosynthesis pathway, among which ent-kaurenoic acid monooxygenase (*LcKAO*) gene and GA2-ox gene were up-regulated. Gibberellin receptor protein (*LcGID1*) gene was down-regulated in GA signal transduction pathway. 1-aminocyclopropane-1-carboxylate synthase (*LcACS*) genes, the key genes in ethylene biosynthesis pathway, were up-regulated. Ethylene-insensitive protein 3 (*LcEIN3*) gene was up-regulated in ethylene signal transduction pathway. Asmonic acid-amino synthetase (*LcJAR1*) gene, jasmonate ZIM domain-containing protein JAZ gene and transcription factor (*LcMYC2*) gene were all down-regulated in methyl jasmonate signal transduction, indicating that the plant growth regulator affected methyl jasmonate signal transduction. The plant growth regulator affects salicylic acid signal transduction pathway and low temperature resistance of litchi leaves by down-regulating regulatory protein NPR1 and transcription factor TGA genes.

## Transcription factor AP2/ERF-ERF is up-regulated under the treatment of mepiquat chloride

Transcription factors ( TFs ) play a key role in the regulation of gene expression under abiotic and biotic stresses in plants. By defining the fold change (|log2FC| >0.58) and p-value (p-value < 0.05), RNA-seq results showed that many TFs were regulated by plant growth regulators under low-temperature stress in litchi(Table S3). In total, 202 TFs genes which were divided into 45 categories were identified from leaves. In particular, families showing especially strong responses to mepiquat chloride treatment under low-temperature stress included *LcC2H2, LcAP2/ERF, LcMYB, LcHSF, LcbHLH, LcbZIP*, and so on(Table S4). Some genes from these families were up-regulated more than 30 times during cold treatment(Table S4). For example, the FC values of the *LcC2H2* gene TRINITY_DN21117_c0_g1_i2 and the *LcAP2/ERF* gene TRINITY_DN16770_c0_g1_i1 were 39.07 and 36.56.

## Validation of DGEs by qPCR

To validate DGEs in transcriptome sequencing, we selected seven genes for qPCR validation, including the fructose-bisphosphate aldolase (*LcFBA*), malate dehydrogenase (*LcMDH2*), citrate synthase (*LcCS*), transketolase (*LctktA*), isocitrate dehydrogenase (*LcIDH1*), catalase (*LcCAT*) and superoxide dismutase

(*LcSOD*) genes. The translation initiation factor 4A (*LcEIF4A*) gene was used as a reference to normalize the expression level. The expression levels of these genes in B treatment were higher than that in CK treatment, respectively (Figure 5).

# Discussion

The low temperature was reported to widely affect plant photosynthesis(Yamori et al*.,* 2014), which consists of light reaction and dark reaction(Li et al*.,* 2009, Takahashi and Badger, 2011). The light reaction is mainly the absorption of sunlight by chlorophyll while the dark reaction mainly includes the Calvin cycle. Previous studies have shown that low temperature stress can inhibit the carboxylation process of Calvin cycle in black cabbage seedlings and reduce the key enzymes (Rubisco, sFBPase, GAPDH and Aldolase) of Calvin cycle(Fang, 2018). The exogenous salicylic acid (SA) and 2 ,4-epibrassinolide (EBR) enhance photosynthesis of cucumber seedlings under low temperature stress and enhance their adaptability to low temperature by increasing the activities of ribulose-1-diphosphate carboxylation/ oxygenase (Rubisco), crassulose-1-heptose-1-bisphosphatase(SBPase), transketolase (TK) and fructose-1-6-diphosphate aldolase (FBA)(Xu et al., 2016). In present study, sedoheptulose-7P was significantly up-regulated and the key genes of photosynthetic carbon fixation were screened from the transcriptome data, the main *LcTK*, *LcFBA,* and *LcMDH* genes were up-regulated. Meanwhile the related photosynthetic parameters showed that the net photosynthetic rate of litchi leaves treated with plant growth regulators increased significantly(Li et al., 2022). This suggests that these genes were up-regulated, which may be related to the improvement of photosynthesis, similar to previous studies. Increased photosynthesis leads to the accumulation of more carbohydrates. As is well known, the TCA cycle is an important energy source pathway for plants. The TCA cycle is the ultimate metabolic pathway of the three major nutrients (sugars, lipids, and amino acids), and it plays a key role in the connection and transformation of sugars, lipids, amino acids, and nucleic acid metabolism(Martinez-Reyes et al., 2016; Zhang et al., 2017). In present study, we found two metabolites citrate and 2-Oxo-glutarate(α-ketone glutaric acid) was up-regulated in the KEGG map of TCA for transcriptome and metabolome (Figure 3b). Meanwhile the upstream genes for these two nodes such as citrate synthase (2.3.3.1) and isocitrate dehydrogenase (NAD+) (1.1.1.42) were shown up-regulated in the transcriptome (Figure 3a). Yu et al. found that under the condition of low phosphorus, the citrate synthase activity, citric acid content and ield traits of transgenic plants with Citrate synthase gene, were superior to non transgenic control plants. Isocitrate dehydrogenase is one of the key enzymes in the citric acid cycle, which relies on the metal ion $Mg^{2+}$(or $Mn^{2+}$) and coenzyme NAD (P) to catalyze the oxidative decarboxylation of isocitric acid α-ketone glutaric acid and CO2 provide energy for cell metabolism and precursor for biosynthesis(Zhang et al., 2009). In this study, mepiquat chlorid can increase the content of citric acid and α-ketone glutaric acid by up-regulating the citric acid synthase gene and isocitrate dehydrogenase gene in the TCA cycle, which provides energy support for litchi's resistance to low temperature.

In addition to the above changes, we were found that mepiquat chlorid could increase the content of carbohydrates in leaves under low temperature stress, such as D-(+)-Raffinose, D-Sedoheptulose7-

phosphate, Mannan and so on. Previous studies have shown that improving osmotic stress related genes can increase the content of osmotic stress resistant substances such as sugar, inositol galactose, and raffinose, thereby enhancing the tolerance of transgenic plants to low temperatures (Wang et al. 2009). Raffinose family oligosaccharides can place themselves on membrane bilayer lipids to protect liposomes and can also be used as free radical scavengers(Hincha et al., 2003; Nishizawa et al., 2008). It can be speculated that mepiquat chloride under low temperature stress can promote photosynthesis and carbon sequestration by increasing the related genes of photosynthetic carbon sequestration in litchi leaves, and then improve photosynthesis. The increase of photosynthesis can promote the accumulation of anti-osmotic stress substances, protect the lipid membrane, eliminate too many free radicals, and alleviate the damage of low temperature to leaves.

Under low temperature stress, it was found that ROS accumulated in plants. At the same time, plant cells increased the activity of antioxidant enzymes and decreased the level of ROS in response to oxidative stress. Previous studies have shown that SA pretreatment can up-regulate the mRNA expression of CAT and increase the activity of CAT enzyme in cucumber leaves, and then improve the low temperature tolerance of cucumber(Wang et al., 2010). Pro can up-regulate the expression of APX, CAT and SOD-related genes to alleviate the damage caused by low temperature to plants(Qimei et al., 2020). Previous studies have found that the higher expression of *LcSOD* gene and the higher activity of SOD regulated by grafted cucumber is the main reason why the cold resistance of grafted cucumber is stronger than that of self-rooted cucumber(Gao et al., 2009). In this study, the transcriptome sequencing analysis of litchi leaves showed that most of the *LcSOD* and *LcCAT* genes were up-regulated, and four genes were highly up-regulated, in which the relative expression of *LcCAT* gene Log2FC was more than 4. At the same time, the plant growth regulators used in present study could increase the activities of antioxidant enzymes SOD and CAT in litchi leaves under low temperature. The results showed that the plant growth regulator could stimulate the expression of SOD and CAT related genes, increase the activities of SOD and CAT, accelerate the clearance of ROS, reduce the damage of ROS to cells and reduce the damage of low temperature to leaves under low temperature stress. However, not all *LcSOD* genes were up-regulated in this study, with all Cu-ZnSOD genes significantly up-regulated and two Fe-MnSOD genes down regulated. Previous studies have shown that there are three SOD enzymes (Mn-SOD, Fe-SOD, and Cu/Zn SOD) present in moss plants, and the increase in SOD enzyme activity is mainly due to the increase in enzyme activity of Cu/Zn SOD (Wu, 2008). It is speculated that the increase in SOD activity in this experiment is mainly due to the up-regulation of Cu/Zn SOD gene expression.

Lipoxygenase (LOX) exists as a polygene family. Members of different gene families play an important role in plant growth and development, maturation and senescence, stress regulation and aroma synthesis(Zhang, 2016). Previous results showed that under low temperature stress, the activity of LOX enzyme in transgenic tobacco plants with *LOX1* interference gene of 'Ruoguang' pear was inhibited by 37.8% compared with non-transgenic control plants, and the content of MDA decreased significantly(Hu 2007). Previous studies have shown that oxalic acid treatment can up-regulate the genes related to active oxygen scavenging pathway, reduce three *LOX* genes in membrane lipid oxidation pathway, and reduce the content of MDA, so as to alleviate postharvest chilling injury(Wang, 2018). This is similar to our study.

Under low temperature stress, plant growth regulator can down-regulate *LcLOX* gene and reduce MDA content in litchi leaves compared with the control. Other studies have shown that low temperature pre-storage treatment can effectively reduce the chilling injury rate and chilling injury index of 'Hongyang' kiwifruit, significantly reduce the accumulation of malondialdehyde (MDA), a product of membrane lipid peroxidation, and inhibit the increase of lipoxygenase (LOX) activity, and maintain a high rate of good fruit(Ma et al., 2014). Under low temperature stress, mepiquat chloride decreased the content of MDA by up-regulating the expression of *LcSOD* and *LcCAT* genes and down-regulating the expression level of *LcLOX* genes. However, these can alleviate the damage of lipid membrane caused by low temperature and enhance the low temperature resistance of litchi leaves.

Gene expression was known to be highly related to the transcription level(Zhang et al., 2019; Brouwer et al., 2019), so over-expression of carbon metabolism was potentially linked to activation of specific transcription factors (TFs). To evaluate the potential TFs involved in mepiquat chloride-induced chemical regulation, we overlooked TFs-related genes that were activated after mepiquat chloride treatment. Through comparing the up/down-regulated TFs in DEGs, we predicted a series of transcription factors showing dramatic change under the treatment of mepiquat chloride.

Specifically, among the up-regulated TFs, it was found that the transcription factor *C2H2* and *AP2/ERF-ERF* was up-regulated almost 40 times. Given that carbon metabolism-related genes were up-regulated under the mepiquat chloride, we hypothesized the transcription factor *C2H2* and *AP2/ERF-ERF* should play an essential in the activation of carbon metabolism-related genes and then carbon metabolism pathways. C2H2 zinc finger protein transcription factor may play a key role in banana response to abiotic stresses such as low temperature (Wang et al. 2019). Modifying the expression of genes encoding C2H2-type transcription can improve plant stress resistance (Huang et al., 2007; Sugano et al., 2003;  Kim et al., 2001). Tyagi et al (2004) found that transferring the rice zinc finger protein gene *OSISAP1* into tobacco can enhance its resistance to low temperature. Similarly, *AP2/ERF-ERF* (APETALA2/Ethylene Responsive Factor) was known to be activated under abiotic stress in *Arabidopsis*(Xie et al*.,* 2019) and it also functions in regulating the metabolism(Shoji and Yuan, 2021) and involves in the plant development (Licausi et al*.,* 2013). However, less was known about its functionality in carbon metabolism. As we know, this is probably the first time to link *AP2/ERF-ERF* specifically to carbon metabolism. In a brief summary, mepiquat chloride activates the transcription factor *AP2/ERF-ERF*, which therefore potentially activates the expression of carbon-metabolism-related genes, and furtherly leads to the alleviation of the low-temperature stress of litchi in late spring.

However, how these transcription factors (TFs) were activated by mepiquat chloride, or how TFs drive the related genes and concentrate the downstream metabolites requires more studies. Furthermore, as a two-way authentication, the physiological experiments regarding photosynthesis validated that mepiquat chloride is a chemical regulator that potentially activates photosynthesis-linked carbon metabolism and escapes the litchi from low-temperature stress. However, as we all know, carbon metabolism is a complicated system in higher plants, so how mepiquat chloride activates the transcription factors,

upgrades carbon metabolism, and what kind of carbon sources are mostly affected require further studies.

In addition, except for the carbon metabolism was found up regulated, we also noticed that the plant hormone signal transduction pathway enriches a lot of DEGs. As important signal molecules in plants, plant hormones are involved in the regulation of important agronomic traits of crops, such as controlling the utilization of crop water and nutrition and regulating crop adaptability to biotic and abiotic stresses through interaction with environmental factors(Lin, 2012; Chen, 2016). As reported before, under low-temperature stress, seed coating agent increased the contents of IAA (auxin) and CTKs (cytokinin) and decreased the contents of GAs and ABA in soybean seedlings, which affected the change process of soybean endogenous hormone system and improved the cold resistance of soybean seedlings(Feng et al., 2005). In our study, the indole-3-pyruvate monooxygenase gene, a key gene for the IAA biosynthesis pathway, was noticed up-regulated as well. Hence, we speculated that mepiquat chloride may also promote IAA-related signal transduction in litchi leaves under low-temperature stress. The change of IAA content in cucumber leaves was related to its low temperature tolerance, in which the materials with strong low-temperature tolerance increased greatly, while those with weak low-temperature tolerance decreased, and those with moderate low temperature tolerance increased slightly(Yang et al., 2009). In our study the *LcCYP735A* genes were up-regulated in the CTK biosynthesis pathway, and the A-ARR gene was up-regulated in the cytokinin signal transduction pathway. The *CYP735A* genes can hydroxylate isopentenyl phosphate into trans zeatin nucleoside (tZRTP / tZRDP / tZRMP) and synthesize trans zeatin nucleoside (tZ riboside, tZR)(Xu et al., 2020). Therefore, we speculate that mepiquat chloride may promote IAA, CTK synthesis and signal transduction in litchi leaves under low-temperature stress. Previous studies have shown that cytokinin receptor Arabidopsis histidine kinase 2 (AHK2) and AHK3 and type an Arabidopsis response regulator (ARR) proteins play a regulatory role in cold stress signal transduction by inhibiting ABA response(Jeon et al., 2010). This is similar to our results. In this study, the expression of *ABF gene* in ABA signal transduction pathway was down-regulated. Also, the expression of *LcGT1* in abscisic acid (ABA) synthesis pathway was up-regulated. GT1 is a key enzyme in the catabolic pathway of ABA and plays an important role in maintaining the normal physiological level of ABA in plants(Wang et al., 2018). And the higher expression levels of *GT1* and *CYP1* make the content of ABA lower(Wang 2011). Previous studies have also shown that 6-BA pretreatment can significantly reduce the content of ABA and improve the low temperature resistance of pepper seedlings under low-temperature (Zhang et al., 2013). Therefore, under low-temperature stress, mepiquat chloride may promote the transformation of free ABA to ABA glucose ester (ABA glucoseester, ABA-GE), thus reduce the content of free ABA and inhibit its signal transduction. Previous studies have shown that overexpression of *GA2ox* gene will reduce the content of GA in plants(Bolduc and Hake, 2009).This was similar to our results, in which the expression of *LcGA2ox* gene was up-regulated in the pathway of GA production and the expression of *LcGID1* protein gene in signal transduction pathway was down-regulated. To sum up, it is suggested that under low temperature stress, mepiquat chloride can promote the synthesis and signal transduction pathway of IAA and CTK in leaves, reduce the content of ABA, jasmonic acid and GA, and inhibit their signal transduction pathway.

The amount of ethylene released from seedling cotyledons after low temperature stress can be used as an identification index of cold tolerance of cucumber, and there is a positive correlation between cold tolerance and ethylene release of cucumber varieties(Liu et al., 1994). Previous studies have shown that under low-temperature stress, *AnGolS1* overexpression can not only enhance the low temperature resistance of tomato plants, but also enhance the expression of genes related to ethylene (ET) signal transduction, such as one ACC synthase (ACS1) and five ACC oxidase (ACO) up-regulated significantly(Liu, 2019). Present study shows that mepiquat chloride could increase the expression level of key enzyme genes *LcACS* in ethylene biosynthesis pathway, and promote the expression of EIN gene in ethylene signal transduction pathway, thus promoting ethylene signal transduction pathway. All these pieces of evidence shown by transcriptomic and transcriptomic analysis revealed a diverse role of mepiquat chloride in releasing low-temperature stress. We believe our study would provide a very basic route to understand the response of plants to stresses from RNA-sequencing and metabolomics data.

# Conclusions

In conclusion, we came up with a strategy to comprehensively understand the mechanism of chemical regulation in crops under stress. Based on the transcriptome and metabolome analysis, we found that mepiquat chloride could lead to a transcriptome- and metabolome-wide change. The chemical regulation driven by mepiquat chloride could induce metabolic- and transcriptomic-wide change, specifically up regulate carbon metabolism-related genes, and furtherly affect TCA cycle and carbon fixation (Figure 6). Since TCA and carbon fixation are very related to photosynthesis, we concluded that mepiquat chloride could alleviate low-temperature stress in litchi by activating photosynthesis-related pathways.

# Materials And Methods

### Sample preparation

The whole experiment was conducted in Yangjiang City of China from March 20 to April 20, 2020. The soil samples were collected at 0 cm to 60 cm soil depth before the experiment. The chemical mepiquat chloride (98%) was kindly provided by the company Zhong Mian Xiao Kang Biotechnology Co., Ltd (Henan, China). The soil pH, alkali-hydrolyzable nitrogen, available phosphorus, available potassium, and organic matter were 4.85, 43.71 mg/kg, 108.81 mg/kg, 27.47 mg/kg, and 10.61g/kg respectively.

Three litchi trees (n=3) with the same age and growth condition were selected for each treatment. On March 26, 2020 we sprayed the conditioner. mepiquat chloride 5mL and 10kg water were evenly sprayed on the leaves of a "feizixiao" litchi tree as treatment B, while the litchi trees sprayed with the same amount of water were used as control.  5 ml of mepiquat chloride mixed evenly with 10kg of water were evenly sprayed on the each litchi tree as treatment B, mean while the litchi trees sprayed with the same amount of water were used as control (CK). On the morning of April 30, 2020, select the second mature functional leaf on the branch closest to the main flower ear of litchi plant and freeze it in liquid nitrogen for at least 30 minutes, then store it in the refrigerator at-80℃ for the determination of transcriptome and metabolic

group. In addition, the average temperature during collection for 10 consecutive days was below 20℃. Before writing this article, we measured leaf photosynthesis, superoxide dismutase activity, peroxidase activity, catalase activity and other indicators, and published them in Journal of Tropical Crops(Li et al., 2022).

### Sample preparation for LC-MS

Before LC-MS, 80 mg of sample was transferred to a 1.5 ml Eppendorf tube containing two small steel balls. 20 μL of 2-chloro-l-phenylalanine (0.3 mg/ml) dissolved in methanol was set as the internal standard, and 1 ml of the mixture of methanol and water (7/3, v/v) was added to each tube. After placing at - 20℃ for 2 min, the sample was milled with a grinder at 60 Hz for 2 minutes, ultrasonic at ambient temperature for 30 minutes, and lastly kept at - 20℃ overnight. On the following day, the samples were centrifuged at 13000 rpm at 4 ℃ for 5 minutes, and 50 μL of the supernatant in each tube was collected by a crystal syringe, passed into a 0.22 μM microfiltration, and transferred into LC vials. The vials were then stored at - 80℃ for LC-MS analysis, and 6 replicates were set for each group (n=6).

An ACQUITY UHPLC system (Waters Corporation  Milford, USA) coupled with an AB SCIEX Triple TOF 5600 System (AB SCIEX, Framingham, MA) was used to analyze the metabolic profiling in both ESI positive and ESI negative ion modes.

### Processing of metabolomics data

Progenesis QI software (Waters Corporation, Milford, USA) was used to analyze the LC-MS raw data with parameters of precursor tolerance 5 ppm, fragment tolerance 10 ppm, and the retention time (RT) tolerance of 0.02 min. The noise elimination level was set to 10.00, and the minimum intensity was set to 15% of the base peak intensity. Metabolites were finally identified by Progenesis QI (Waters Corporation, Milford, USA) data processing software based on public databases: The Human Metabolome Database (hmdb), LIPID MAPS, and self-built databases.

After combining the positive and negative data and importing them into the R ropls package. Principal component analysis (PCA) and orthogonal partial least square discriminant analysis (O PLS-DA) were used to evaluate the metabolic changes. Variable Importance in Projection (VIP) was used to rank the overall contribution of each variable in the OPLS-DA model, and the variables with VIP > 1 were regarded as related to group discrimination. In this study, the default 7-round cross-validation was used to prevent overfitting, with 1/7 sample per round excluded from the mathematical model. The differential metabolites were selected based on the combination of the statistical significance threshold of the variable influence (VIP) values (>1.0) obtained from the OPLS-DA model, as well as the p-value (<0.05) from the two-tailed student’s t-test from the normalized peak area.

### RNA Isolation, cDNA Library Construction, and Analysis

One μg of RNA for each sample was extracted and digested with DNase and enriched with magnetic beads with Oligo (dT). After fragmentation using chemicals, the fragmented mRNA was used as a

template to synthesize single-stranded cDNA with hexamer random primers. Following second-stranded DNA synthesis and purification, the sample was size selected, and PCR amplified. The quality product was checked by Agilent 2100 Bioanalyzer. After that, the constructed library was sequenced on Illumina HiSeq X Ten sequencers to generate pair-ended with the length of 125bp to 150bp. Three replicates (n=3) were set for each group.

After the raw reads with fastq format were generated, Trimmomatic(Bolger et al., 2014) software was used to filter out low-quality bases and N bases. In addition, the Trinity (version:2.4)(Grabherr et al., 2013) software was used to assemble the transcript sequence and the longest one was selected as the Unigene according to the sequence similarity and length. To build the reference, the Unigene was annotated using blast(Altschul et al., 1990), and the threshold lower than $1e^{-5}$ was matched into the NR, COG/KOG, and Swissprot databases, respectively. Based on the result of Swissprot, the Swissprot ID is annotated by GO term and the KEGG(Kanehisa et al*.,* 2008) database in order to analyze the enriched pathway. The reads were mapped by bowtie2(Langmead and Salzberg, 2012), and FPKM was quantified by eXpress software(Roberts and Pachter, 2013). For comparison, the data was normalized by the estimate Size Factors function in DESeq(Anders and Huber, 1998) package of R, and the p-value and fold change values of among different groups were calculated by nbinom Test function. The differential Unigenes with p-value less than 0.05 and log2 (fold change) bigger than 2 were selected.

### Quantitative RT-PCR analysis

Primers for validated genes were designed using Primer Premier 5.0 (Table S1). Translation initiation factor 4A(*EIF4A*) was used as a reference gene because it was stably expressed in B\CK treatments. The RNA samples used for qPCR assays were the same as those used in the DGE experiments, with three replicates per treatment. Real-time fluorescence quantitative PCR was performed by Sangon Biotechnology (Shanghai) Co., Ltd., and the relative expression levels of genes were calculated according to the $2^{-\Delta\Delta Ct}$ method.

# Declarations

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**Author contributions.** All authors contributed to conceptualizing this study. TL, NF and DZ, conceived and designed the experiments.TL, MC, LZ, helped in the data collection. TL, NF, DZ and MC wrote and summarized the manuscript.

**Competing interests.** The authors declare that they have no competing interests.

**Ethics approval and consent to participate.**The permission for collection of *Litchi chinensis* Sonn. was approved by the farm owner. Tianzi Li participated in formally identifying and preparing all related samples of litchi.

**Availability of data and materials.** The RNA-sequencing data of this study were deposited into Gene Expression Omnibus (GEO) database, and the accession number is GSE190304 (Reviewer token: upefcwqsfnydnqd). All generated or processed data of this study are included in this manuscript. The voucher specimen of litchi cultivar used in this study was deposited in South China Botanical Garden, Chinese Academy of Sciences (accession number  available soon)

**Consent for publication.** Not applicable.

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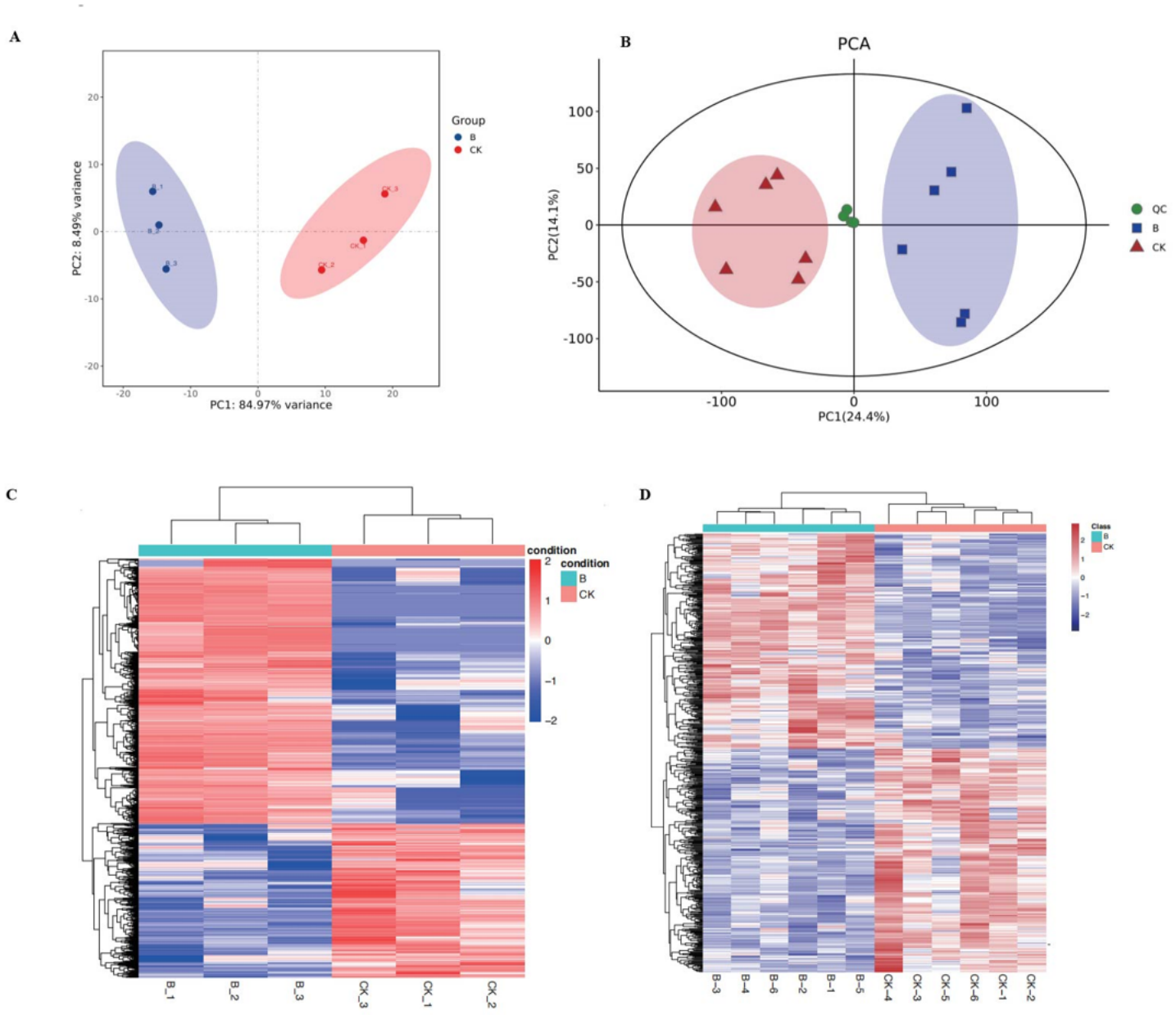
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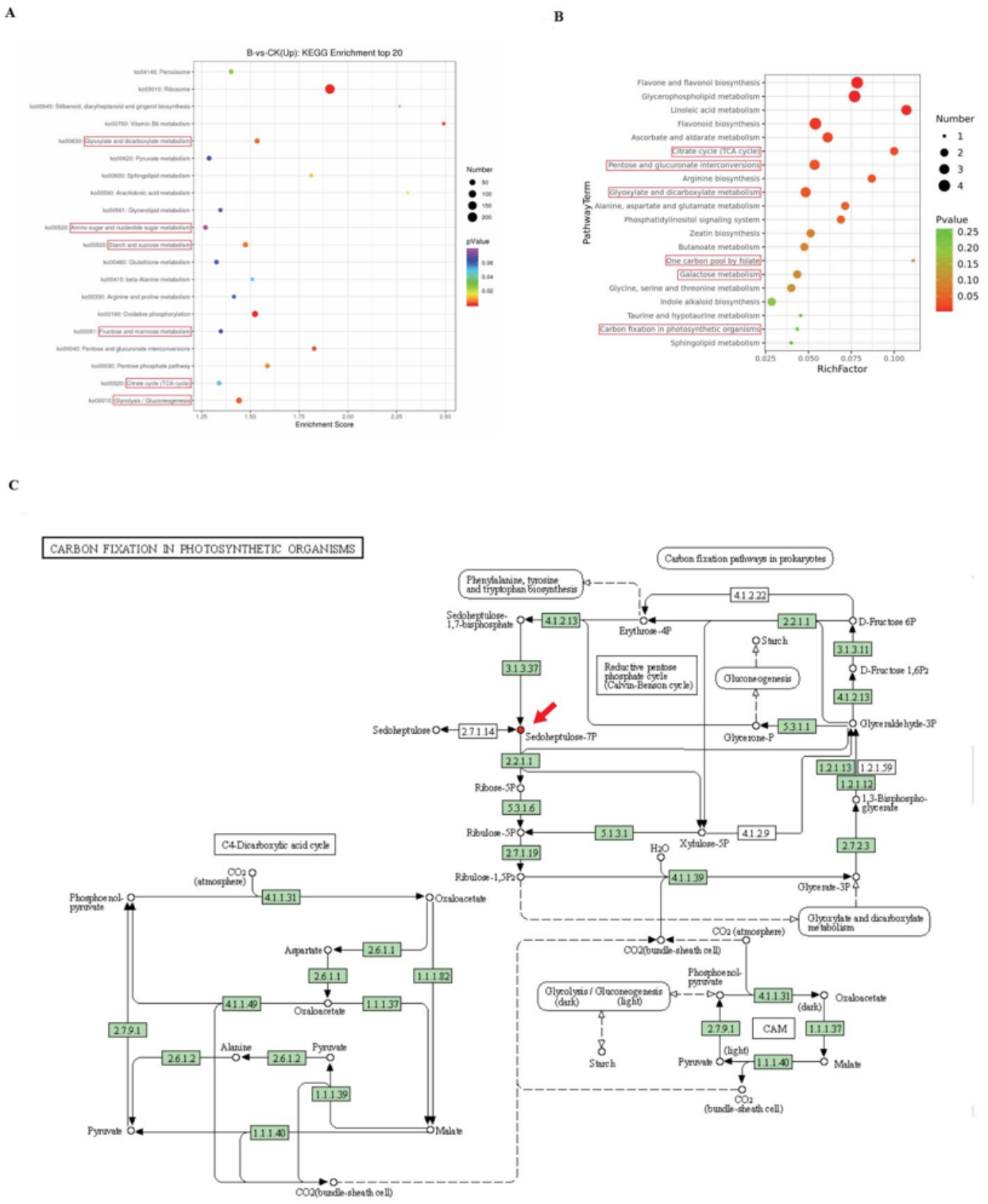
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# Figures




## Figure 1

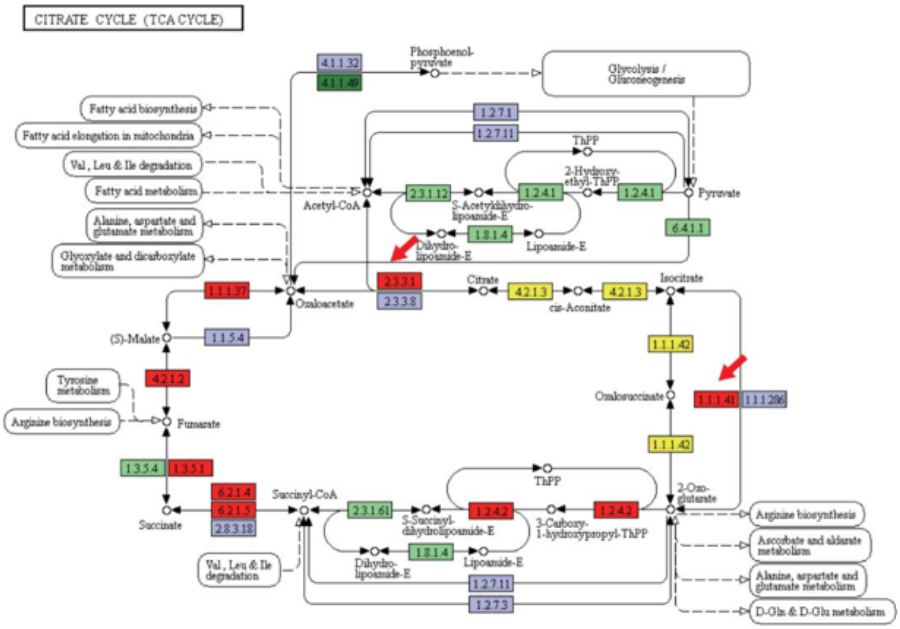
Overall comparison of mepiquat chloride and CK transcriptomically and metabolically. A: PCA analysis for transcriptome change under the treatment of mepiquat chloride (B) and CK. B: PCA analysis of metabolic change under the treatment of CK and mepiquat chloride (B), quality control (QC) was set as a standard. C: Cluster analysis for transcriptome treated with mepiquat chloride (B) or CK. D: Cluster analysis for metabolome treated with mepiquat chloride (B) or CK. The number 1-6 means the biological replicates 1-6.



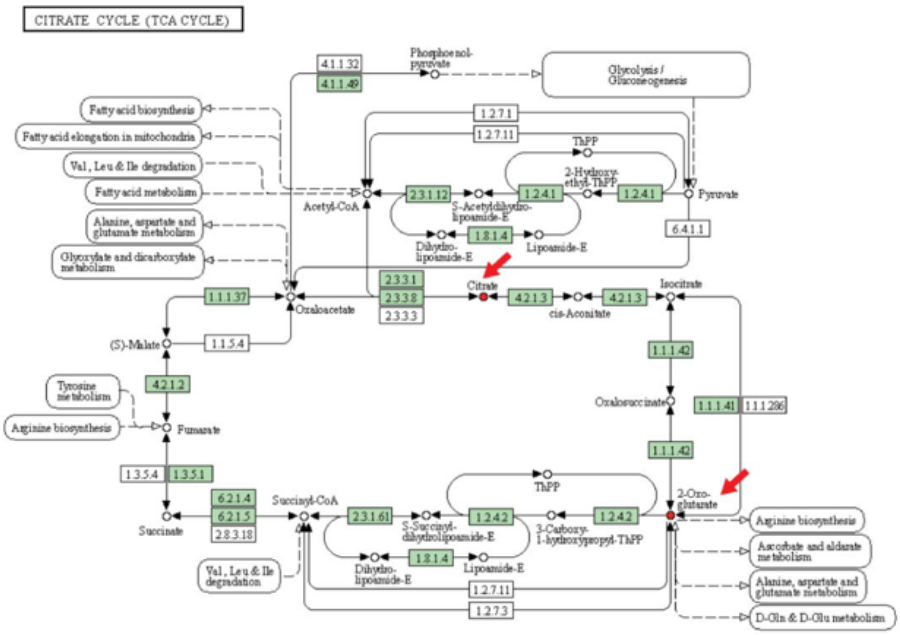

## Figure 2

**Enrichment pathways analysis for transcriptome and metabolome. A: The top 20 upregulated pathways from transcriptome using KEGG. B: The top 20 upregulated pathways from metabolome using KEGG. C: The network analysis for carbon fixation in the photosynthetic pathway. The carbon metabolism-related pathways are circled with red in a and b, and the upregulated metabolite is highlighted in red in c.**

A

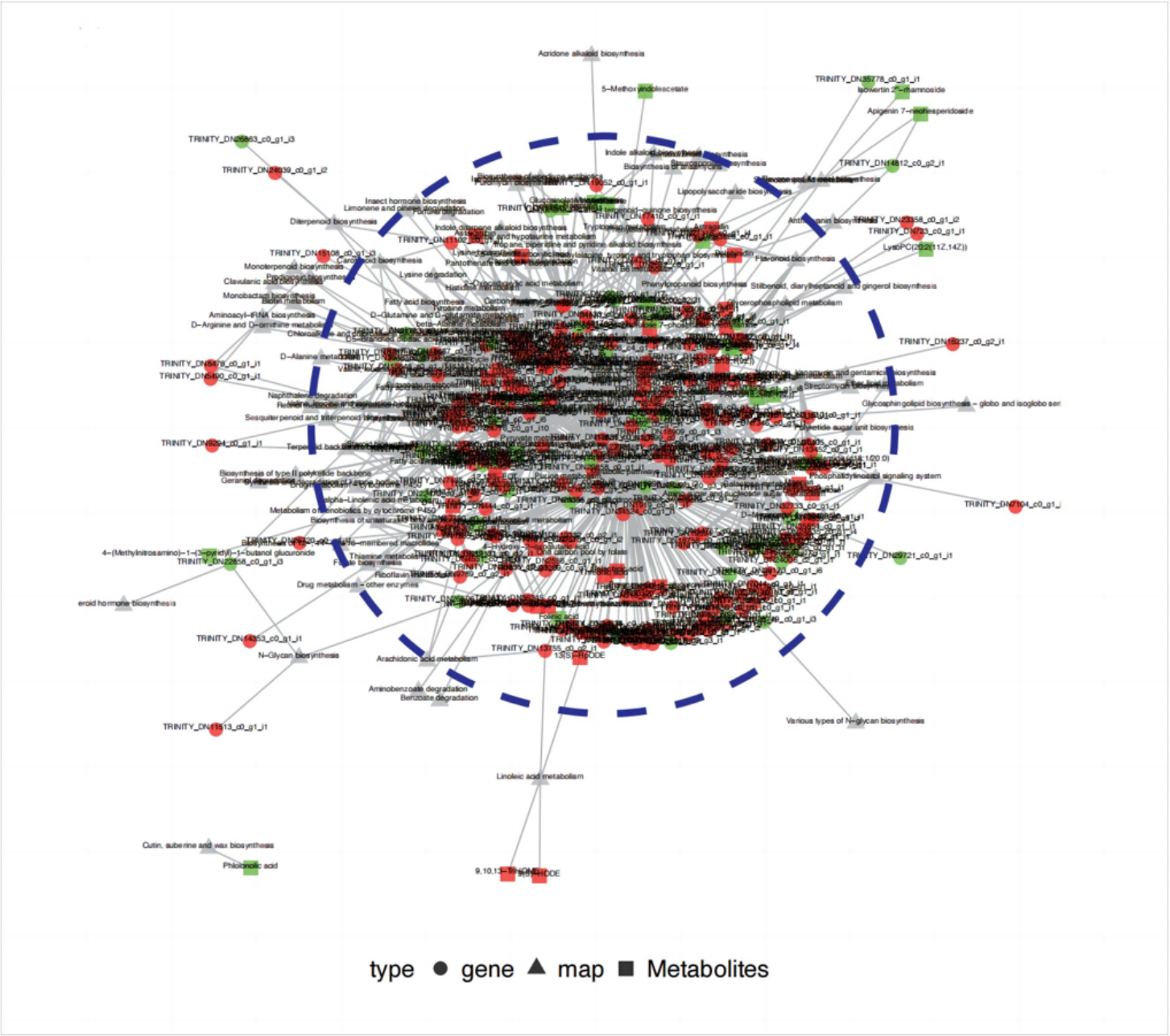



B




## Figure 3

**Comparative analysis of TCA cycle pathway for a) transcriptome and b) metabolome, in which the upregulated metabolites or transcripts are highlighted as red.**




## Figure 4

**Integrative analysis of metabolome and transcritome, in which the upregulated metabolites or genes are highlighted as red, while downregulated ones are labeled as green.**

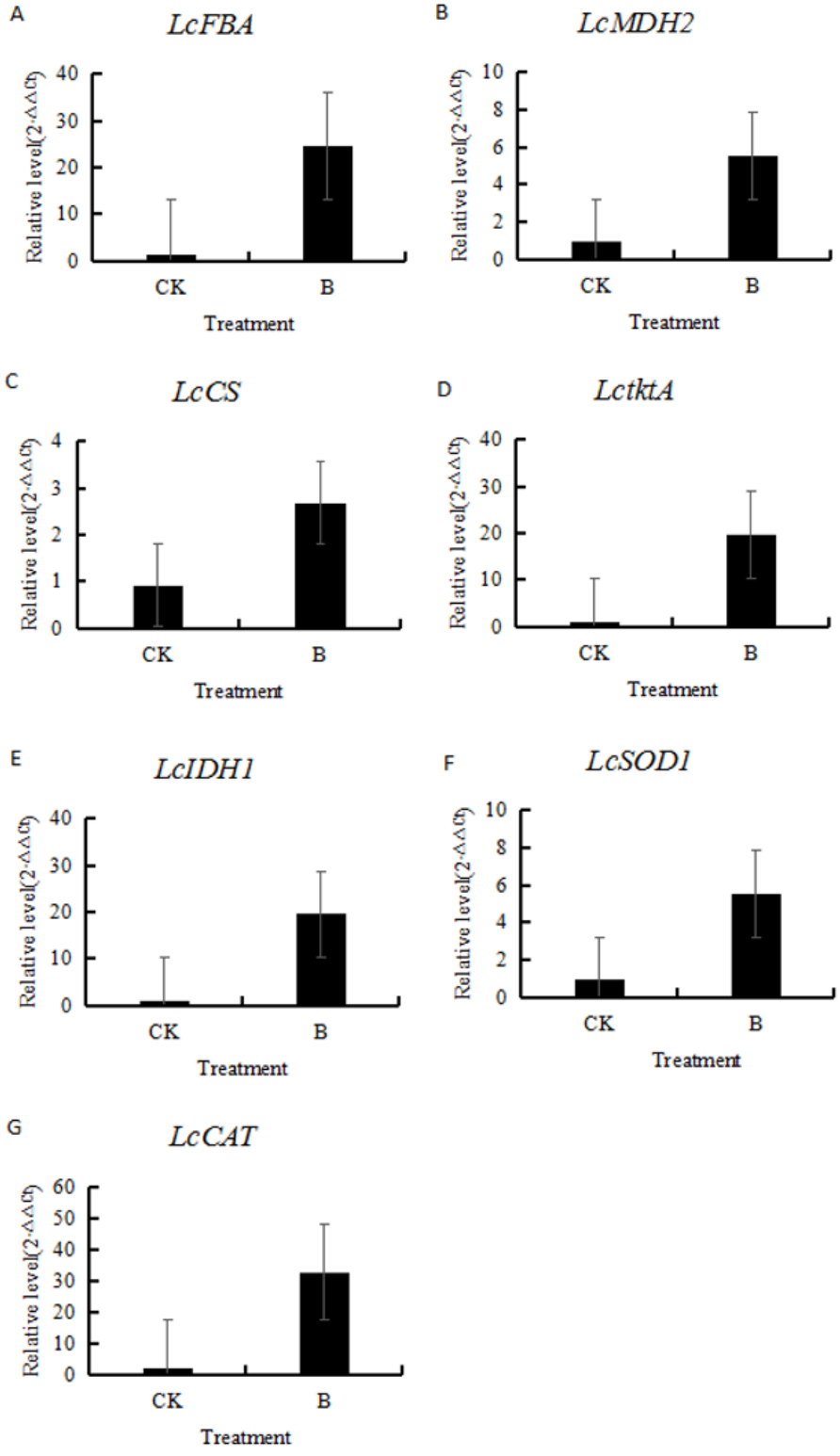



Figure 5

Seven differentially expressed genes have been identified by qRT-PCR in CK and B treatment. A:Relative level of FBA (TRINITY_DN25416_c0_g3_i1). B: Relative level of MDH2 (TRINITY_DN18500_c1_g1_i3). C: Relative level of CS (TRINITY_DN12305_c0_g2_i1). D: Relative level of tktA (TRINITY_DN10963_c0_g1_i1). E: Relative level of IDH1 (TRINITY_DN18451_c0_g1_i6). F: Relative level of

SOD1 (TRINITY_DN10586_c0_g1_i2). G: Relative level of CAT (TRINITY_DN28777_c1_g1_i4). The translation initiation factor 4A (LcEIF4A)gene was used as a reference to normalize the expression level.

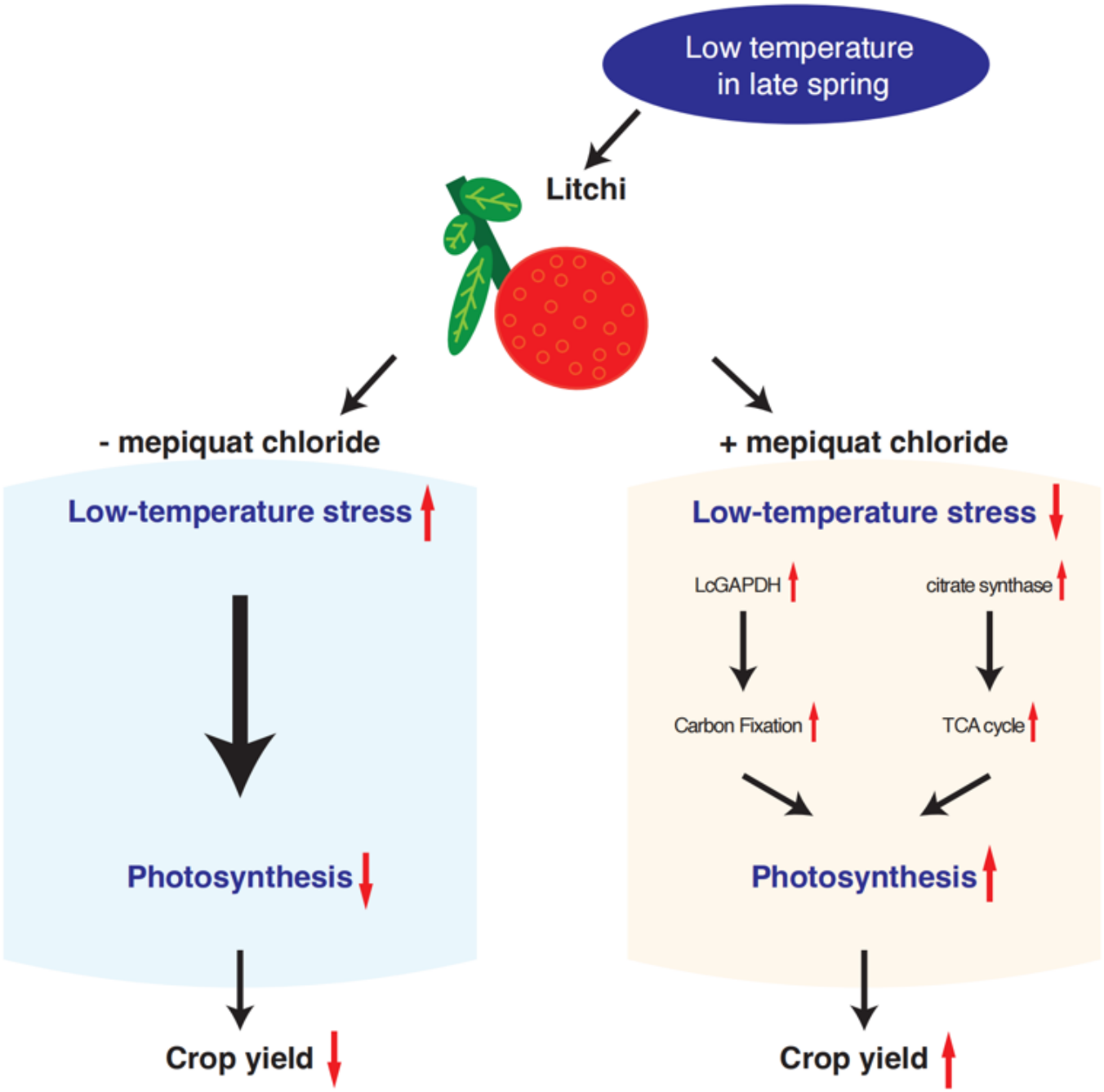



Figure 6

Schematic of the mechanism for mepiquat chloride in releasing low-temperature stress.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials.docx