Research Article

Apple TIME FOR COFFEE contributes to freezing tolerance by promoting unsaturation of fatty acids

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ABSTRACT

Freezing stress is a major environmental factor that threatens the growth and development of fruit trees. MdMYB88 and its paralogue MdMYB124 have been identified as pivotal regulators in apple (*Malus* × *domestica*) freezing stress tolerance. Here, we demonstrated that a target of MdMYB88 and MdMYB124, *TIME FOR COFFEE* (*TIC*), contributes to freezing tolerance in apple. MdMYB88 and MdMYB124 directly bound the *MdTIC* promoter and positively regulated its expression under cold conditions. *MdTIC* RNAi plants displayed reduced freezing tolerance when *MdTIC* expression was repressed. Moreover, *MdTIC* RNAi plants lowered antioxidant enzyme activity. Transcriptome profiling revealed altered expression of cold-responsive genes in *MdTIC* RNAi plants under cold conditions, including *MdPLC2*, *MdMKK2*, and *MdICE1*. We also discovered that disordered *MdTIC* expression changed the saturation of fatty acids. Taken together, our data suggest that *MdTIC* is required for apple to tolerate freezing by mediating the expression of cold-responsive genes and fatty acid composition.

1. Introduction

Cold stress poses a huge threat to plant growth and development. According to different temperature ranges, cold stress can be divided into chilling (0–15 °C) and freezing (<0 °C) [1]. Chilling damage inhibits plant growth and causes chlorosis and lesions [2]. Under sub-freezing temperatures, plants form ice crystals inside and outside of cells, leading to irreversible damage [3].

The mechanism of the plant response to cold has been widely studied. At present, the CBF (C-repeat binding factor)-dependent signal transduction pathway is currently the most studied, and plays a vital role in plant cold resistance [4]. In Arabidopsis (*Arabidopsis thaliana*), expression of *CBF* genes is quickly induced by cold, thereby activating expression of a set of *COR* (*cold regulated*) genes to alleviate cold damage [5,6]. On the other hand, several transcription factors have been identified to regulate expression of *CBF* genes including positive regulators, such as CAMTAs (calmodulin-binding transcription activators) that induce ICE1/2 (induce CBF expression 1/2) [7,8], and negative regulators, such as MYB15, EIN3 (ethylene insensitive 3), and ZAT12 [9–11]. Moreover, post-transcriptional regulation is also involved in the CBF-dependent signaling pathway. For example, the MEKK-MKK2-MPK4 cascade enhances the stability of ICE1 by suppressing MPK3 and MPK6 activities [12]. As reported previously, CBFs only regulate 12 % of *CORs,* indicating that some CBFs function as independent factors in cold signaling [13].

Cold acclimation is a process that allows plants to increase freezing tolerance after an earlier exposure to low, nonfreezing temperatures. Diverse biochemical and physiological processes are involved, such as metabolism of carbohydrates, alterations in antioxidants, and lipid composition [14]. Plants accumulate a large amount of reactive oxygen species (ROS) when subjected to cold stress, which breaks the electron transport chain, resulting in cell necrosis [15]. Although ROS, including O^{2-}, H_2O_2, and OH· are constantly produced, plants have developed non-enzymatic and enzymatic defense systems to scavenge ROS. Non-enzymatic antioxidants include glutathione, carotenoids, ascorbate, tocopherols, and flavonoids, while the major enzymatic antioxidants include catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and ascorbate peroxidase (APX) [16]. These antioxidant enzymes are distributed in different locations within plant cells and act in concert to alleviate cold damage. SOD functions in converting O_2^- into H_2O_2, which is then removed by CAT and POD [16].

Cold acclimation also promotes stabilization of the plasma

membrane. Membrane fluidity is determined by the variety of lipids and the degree of unsaturation of glycerol lipids [17,18]. Generally, the degree of unsaturated fatty acids in lipid membranes increases with decreasing temperature [19,20]. The contents of unsaturated fatty acids in lipids are higher in cold-tolerant wheat and Arabidopsis than in cold-sensitive plants [21,22]. A plasma membrane with higher unsaturated fatty acid content maintains a lower phase transition temperature; thus, avoiding ion leakage and increasing cold tolerance [18]. The unsaturation of fatty acids is regulated by the variety and quantity of fatty acid desaturases (FADs). *FAD6* is considered the key enzyme for biosynthesis of linoleic acid (C18:2) from oleic acid (C18:1) in the plastidic glycerolipid classes [23]. *FAD7 and FAD8* are highly similar and redundantly catalyze the conversion of C18:2 to linolenic acid (C18:3). Loss of function of *OSFAD8* decreases plant chilling tolerance [24].

MdMYB88 and its paralogue MdMYB124 confer apple cold tolerance by positively regulating CBF-dependent *MdCCA1* and CBF-independent *MdCSP3*, respectively [25]. We identified a number of putative targets related to the circadian clock in a ChIP-seq analysis, including homologs of *CCA1* and Time For Coffee (*TIC*). In Arabidopsis, circadian genes play a vital role in the response to biotic and abiotic stresses [26]. The core component of the biological clock, CCA1/LHY, promotes plant cold tolerance by directly regulating expression of *CBF1/2/3* [30]. TIC is also an important regulator of biological rhythms localized in the nucleus whose function is suggested to reset the core oscillator around the mid to late subjective night [27]. *tic* mutants have defects in morphogenesis, such as shorter leaves, a reduced length-to-width ratio, and increased leaf serrations [28]. In addition, *tic* mutant plants are susceptible to pathogen infection due to disruption in jasmonic acid signaling, but they are tolerant to drought [28,29]. As MdMYB88 and MdMYB124 play positive roles in cold tolerance, and MdTIC might be their direct target, we hypothesized that MdTIC might play important roles in apple cold tolerance.

In the current study, we found that *MdTIC* is a direct target of MdMYB88 and MdMYB124, and is required for survival of apple under freezing stress by modulating the expression of cold-responsive genes and the degree of fatty acid saturation.

2. Results

2.1. *MdMYB88 and MdMYB124 directly bind to the MdTIC promoter region*

In our previous study, MdMYB88 and its paralogue gene, MdMYB124, acted as two atypical MYB transcription factors that bind to specific motifs in the promoters of target genes [25]. To explore the molecular network of MdMYB88 and MdMYB124 under freezing stress, we analyzed the chromatin immunoprecipitation-sequencing (ChIP-seq) results [25], and one of the target genes, *MdTIC,* was selected for further study. We identified three *cis*-elements (AGCCG, AACCG, and CGCGG) in the *MdTIC* promoter region (Supplemental Fig. 1). To confirm binding of MdMYB88 and MdMYB124 to the *MdTIC* promoter, we first conducted a ChIP-quantitative polymerase chain reaction (qPCR) assay using *MdMDH* as a negative control and reference gene. As shown in Fig. 1A, the *MdTIC* promoter was divided into three fragments. Fragment *a* contained *cis*-elements of AGCCG and AACCG, fragment *b* contained CGCGG, and fragment *c* contained AACCG. The ChIP-qPCR assay revealed that MdMYB88 and MdMYB124 were significantly enriched in fragment *a*, but weakly enriched in fragment *c*. However, there was no direct interaction between fragment *b* and MdMYB88 and MdMYB124 (Fig. 1B). Next, direct binding of MdMYB88 to the *MdTIC* promoter was further verified by performing a yeast one-hybrid assay. Yeast cells containing the bait vector harboring the *MdTIC* promoter grew normally on selective media when co-transformed with MdMYB88-AD or MdMYB124-AD (Fig. 1C). We subsequently carried out an electrophoretic mobility shift assay (EMSA). The results showed that the MdMYB88
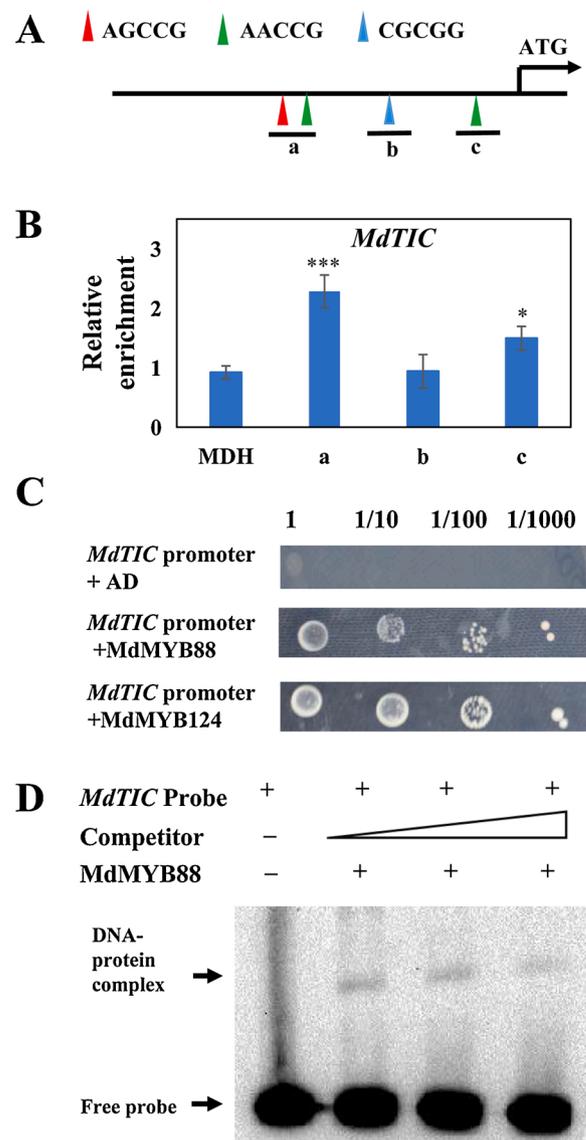
Fig. 1. *MdTIC* is an MdMYB88 and MdMYB124 target. (A) Schematic diagram of the *MdTIC* promoter. The *MdTIC* promoter was divided into three fragments: a, b, and c. (B) ChIP-qPCR analysis of *MdTIC* using cold-treated GL-3 at 4 °C for 8 h. Fragment a (-1580 to -1347 bp) contained AGCCG and AACCG cis-elements, fragment b (-981 to -770 bp) contained a CGCGG cis-element, and fragment c (-519 to −337 bp) contained a AACCG cis-element. MDH was the negative control that also served as the reference gene. (C) Yeast one-hybrid assay. *MdTIC* promoter contained the AGCCG and AACCG cis-elements (−1942 to −1351 bp), Yeast cells were grown on SD-Leu plates with 500 ng/mL aureobasidin A. (D) EMSA analysis. The EMSA probe contained a AACCG motif. Error bars indicate standard deviation (n = 3, from three biological replicates). Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey test) (***P < 0.001, *P < 0.05).

protein bound to *MdTIC* fragment *a,* and the binding decreased by increasing equal amounts of the competitors (Fig. 1D). These results suggest that MdMYB88 and MdMYB124 directly bind to the *MdTIC* promoter.

2.2. *MdTIC expression patterns in response to various treatments*

MdMYB88 and MdMYB124 are critical for apple drought and freezing stress tolerance [25,30]. To understand if *MdTIC* plays a role in apple stress response, we examined expression of *MdTIC* in response to different stresses. When plants were exposed to low temperature (4 °C),

MdTIC expression increased gradually and peaked (~2 times) at 8 h (Fig. 2A). After a 30-min heat (45 °C) treatment, *MdTIC* expression was induced about 2-fold (Fig. 2B). As abscisic acid (ABA) is a hormone involved with various stresses, we sprayed 100 μmol/L ABA on GL-3 plants. As shown in Fig. 2C, *MdTIC* was induced quickly by ABA. To survey the *MdTIC* expression pattern in response to biotic stress, we challenged plants with *Alternaria alternata f.sp mali*, a widespread apple fungus. As a result, the *MdTIC* expression level decreased 36 h post infection (hpi) (Fig. 2D). In addition, tissue-specific expression revealed that *MdTIC* was highly expressed in the root and stem tissues (Fig. 2E).

2.3. Disruption of MdTIC reduces apple freezing tolerance

Because *MdTIC* acts as a downstream target of MdMYB88 and MdMYB124, we determined if *MdTIC* plays a role in apple freezing stress tolerance. We first examined *MdTIC* expression in *MdMYB88* transgenic plants under cold treatment. As shown in Fig. 3A, *MdTIC* expression was downregulated in *MdMYB88/124* RNAi plants, but upregulated in *MdMYB88* overexpressing plants, suggesting positive regulation of *MdTIC* by MdMYB88 and MdMYB124 in response to cold.

We generated transgenic plants with repressed expression of *MdTIC* using an RNA interference method and GL-3 as the genetic background. Using the *Agrobacterium*-mediated transformation approach, we obtained two individual *MdTIC* RNAi lines (#8 and #9) whose expression level decreased to 24–33 % (Supplemental Fig. 2). Electrolyte leakage is an indicator of plasma membrane damage caused by low temperature. No significant difference in electrolyte leakage was found between the GL-3 and *MdTIC* transgenic plants under the control conditions (0 °C). However, when the temperature dropped to −6 °C, *MdTIC* transgenic plants suffered a higher electrolyte leakage rate (Fig. 3B). After 1-week of cold acclimation, electrolyte leakage of both GL-3 and *MdTIC* RNAi plants decreased under freezing conditions; however, the transgenic plants had higher electrolyte leakage, compared with the GL-3 plants under cold stress (Fig. 3B). We also examined freezing tolerance in intact plants. Under non-acclimated conditions, the *MdTIC* RNAi transgenic plants showed more of a wilting and shrinking phenotype than GL-3

after exposure to −10 °C for 30 min. Moreover, the survival rate of *MdTIC* RNAi plants after recovery under normal growth conditions for 3 weeks was lower than that of the GL-3 plants (Fig. 3C). Under the cold-acclimated conditions, the survival rate of *MdTIC* RNAi transgenic plants was still significantly lower than GL-3 after a −10 °C treatment for 45 min (Fig. 3D). These results suggest that disrupting *MdTIC* reduces freezing tolerance in apple plants.

Cold stress also leads to the accumulation of hydrogen peroxide (H_2O_2), which causes leaking of ions by adversely affecting cytomembranes [16]. Therefore, we examined the H_2O_2 content of GL-3 and *MdTIC* RNAi plants at 4 °C for 2 h. As results, H_2O_2 content increased in all plants after the cold treatment, but *MdTIC* RNAi plants accumulated more H_2O_2 in response to cold compared to GL-3 (Fig. 4A). In contrast, cold induced the activities of antioxidant enzymes, including POD and CAT. However, *MdTIC* RNAi transgenic plants displayed lower POD and CAT activities under cold stress compared with that in GL-3 plants (Fig. 4B and C), suggesting a weaker ability of the *MdTIC* RNAi transgenic plants to remove H_2O_2. These results suggest that *MdTIC* RNAi plants exhibit reduced cold hardiness due to lower antioxidant enzyme activities.

2.4. Transcriptome profiling of MdTIC transgenic plants

To elucidate how *MdTIC* is mechanistically linked to plant cold tolerance, we performed whole-genome transcriptome analysis of *MdTIC* RNAi transgenic plants. Three-month-old plants treated with or without low temperature (4 °C) for 8 h were used for total RNA extraction and deep sequencing. After eliminating the low-quality reads, an average of 92.8 % of the filtered reads was mapped to the *Malus × domestica* genome (GDDH13) by Hisat2. RNA-seq analyses revealed that 6074 genes were upregulated and 4902 genes were downregulated (using P < 0.05 and 1.5-fold as a cutoff) in GL-3 after the low temperature (4 °C) treatment for 8 h (Supplemental dataset 1–2).

Sixty genes were upregulated, while only 23 genes were downregulated in *MdTIC* RNAi plants compared to GL-3 under the control conditions (Supplemental dataset 3–4). In response to cold, 755 genes
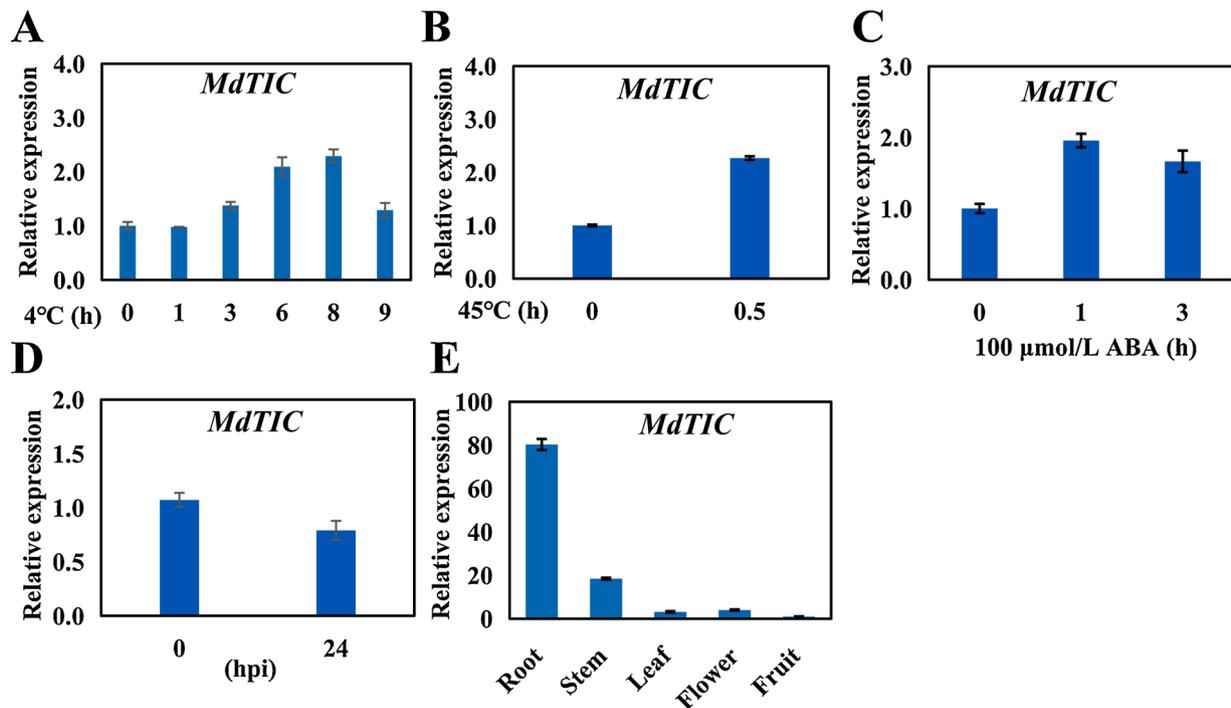

**Fig. 2.** *MdTIC* expression patterns in response to various treatments. (A-B) *MdTIC* expression in response to cold and heat stress. (C) *MdTIC* expression in response to ABA treatment. (D) *MdTIC* expression in response to *Alternaria alternata f.sp mali* infection, post infection (hpi). (E) *MdTIC* expression in different tissues of apple. Data are mean ± SD (n = 3, from three biological replicates).
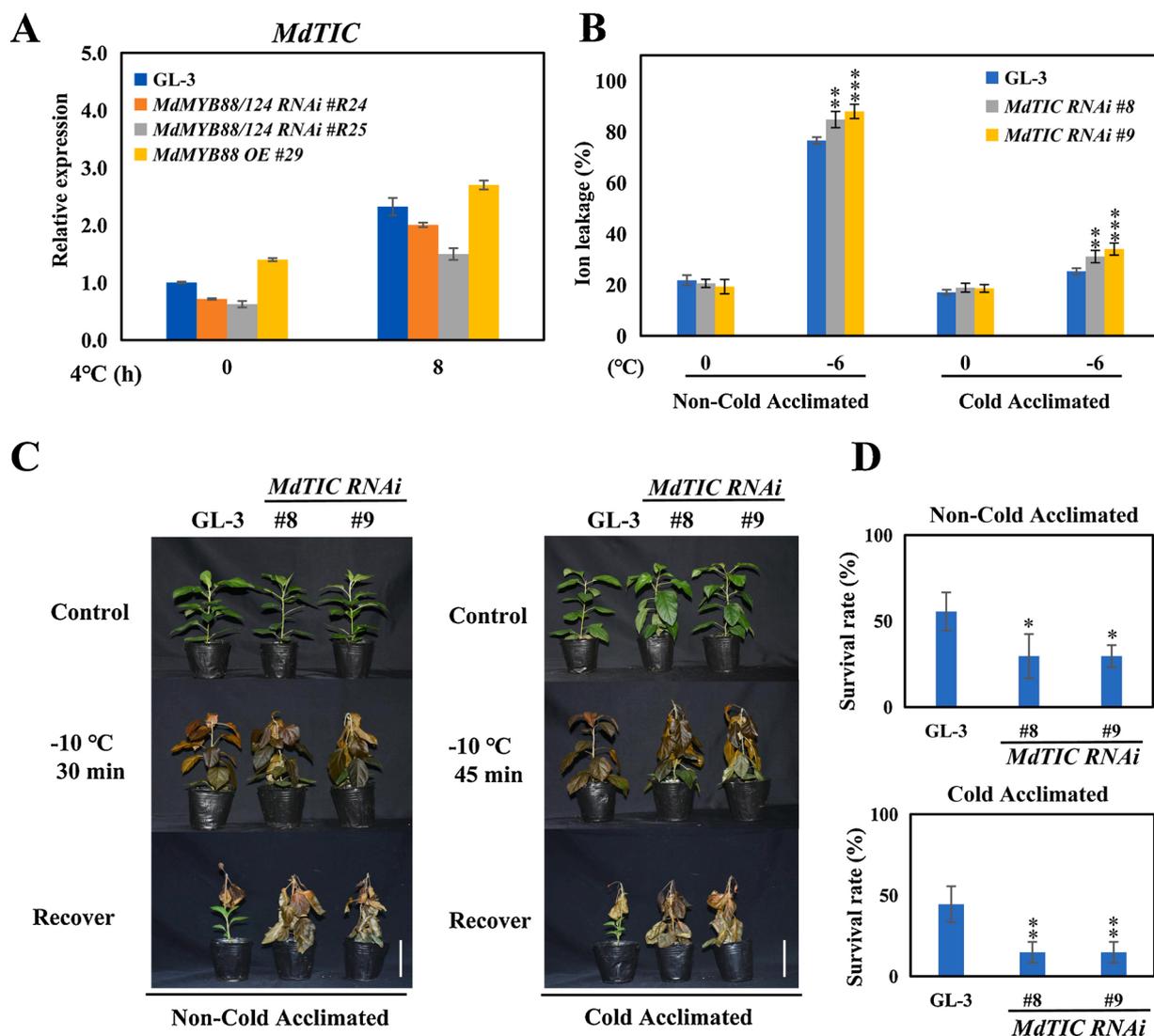
Fig. 3. MdTIC is a positive regulator of freezing tolerance in apple. (A) *MdTIC* expression in MdMYB88 transgenic plants in response to cold. (B) Freezing tolerance of *MdTIC* RNAi plants examined by the leaf electrolyte leakage assay. (C) Intact-plant freezing tolerance of *MdTIC* RNAi plants with or without cold acclimation; bar =10 cm. (D) Survival rates of *MdTIC* RNAi plants (C). Data are mean ± SD (n = 3 in [A], from three biological replicates; n = 6 in [B], six plants and four leaf discs were collected from each plants; n = 27 in [C], 27 plants were used and every nine plants was a biological replication). Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey test) (***P < 0.001, **P < 0.01, *P < 0.05).
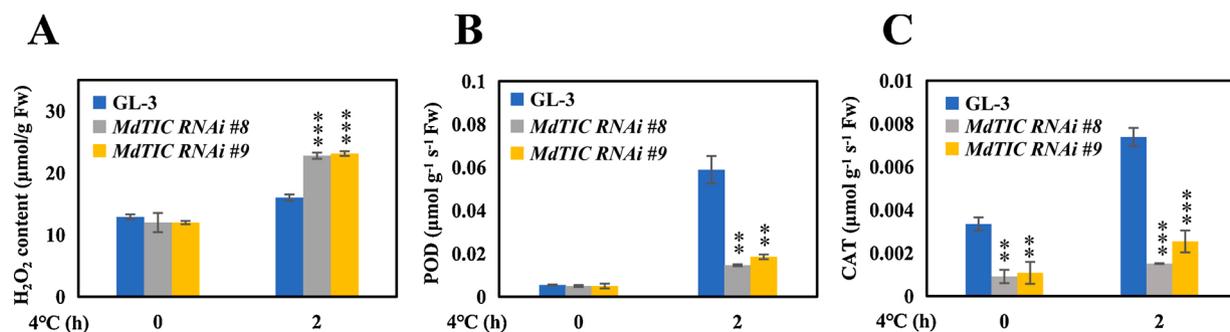
Fig. 4. *MdTIC* RNAi plants lowered the activities of antioxidant enzymes in response to cold stress. Data are mean ± SD (n = 3, from three biological replicates). Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey test) (***P < 0.001, **P < 0.01, *P < 0.05).

were differentially expressed in *MdTIC* RNAi plants compared with GL-3. This result indicated that MdTIC tends to function under cold conditions. Among these 755 differentially expressed genes (DEGs), 331 were upregulated while 424 were downregulated in *MdTIC* RNAi plants

under the cold treatment (Supplemental dataset 5–6). Because we were interested in genes that were affected by *MdTIC* during the cold response, a Venn diagram was used to overlap the cold-responsive genes in GL-3 and the cold-responsive genes in *MdTIC* RNAi versus GL-3. These

overlapped genes were divided into two groups. Group I included 349 genes that were upregulated by the cold treatment but were downregulated in *MdTIC* RNAi plants under cold stress. Group II included 145 genes that were downregulated by the cold treatment but upregulated in *MdTIC* RNAi plants (Fig. 5). A Gene Ontology (GO) enrichment analysis suggested that the genes from the two groups were enriched in biological processes related to stimulus response and metabolic processes, particularly stress and defense (Supplemental dataset 7).

Next, we selected six DEGs to confirm the RNA-seq data by qRT-PCR. The six genes were *MdPLC2*, *MdMKK2*, *MdWRKY31*, *MdICE1*, *MdADF5*, and *MdMYB23*. Homologs of *PLC2*, *MKK2*, *WRKY31* (*WRKY6* in Arabidopsis), and *ICE1* are positive regulators, whereas *ADF5* and *MYB23* (*MYB15* in Arabidopsis) are negative regulators [7,10,31–34]. As shown in Fig. 6A–D, expression of *MdPLC2*, *MdMKK2*, *MdWRKY31*, and *MdICE1* were induced by cold in GL-3 and MdTIC RNAi plants. However, the expression levels of these four genes decreased in *MdTIC* transgenic plants compared with GL-3 plants under cold. In contrast, disrupting *MdTIC* expression increased the expression of *MdADF5* and *MdMYB23* (Fig. 6E and F). These results indicate that altered expression of cold-responsive genes contributes to decreased freezing tolerance in *MdTIC* RNAi plants.

2.5. Changes in fatty acid composition of MdTIC transgenic plants

The close relationship between the fatty acid composition and tolerance to cold is well-established. Previous studies have reported that the level of unsaturated fatty acids in lipid membranes increases under cold stress [19,35]. The content and degree (number of double bonds) of unsaturated fatty acids in lipids are higher in cold-tolerant plants than cold-sensitive plants [21,22]. According to the GO biological process analysis, we were interested in the fatty acid biosynthetic process. Among the enriched genes involved in fatty acid biosynthesis, apple omega-3 fatty desaturase acid (ω-3 FAD), shares 66 % amino acid similarity with FAD8 in Arabidopsis (Supplemental Fig. 3). FAD8 is considered the rate-limiting enzyme catalyzing the conversion of linoleic acid (C18:2) to linolenic acid (C18:3), and its mRNA level is strongly induced under cold conditions [36]. Consistent with the RNA-seq data, the qRT-PCR results also demonstrated that the *ω-3 FAD* expression level increased in GL-3 and *MdTIC* RNAi transgenic plants after the cold treatment. However, a significantly lower *ω-3 FAD* expression level was observed in the *MdTIC* RNAi transgenic plants, compared with GL-3 (Fig. 7A).

The lower *ω-3 FAD* expression level in *MdTIC* RNAi transgenic plants prompted us to examine major fatty acid contents. A gas
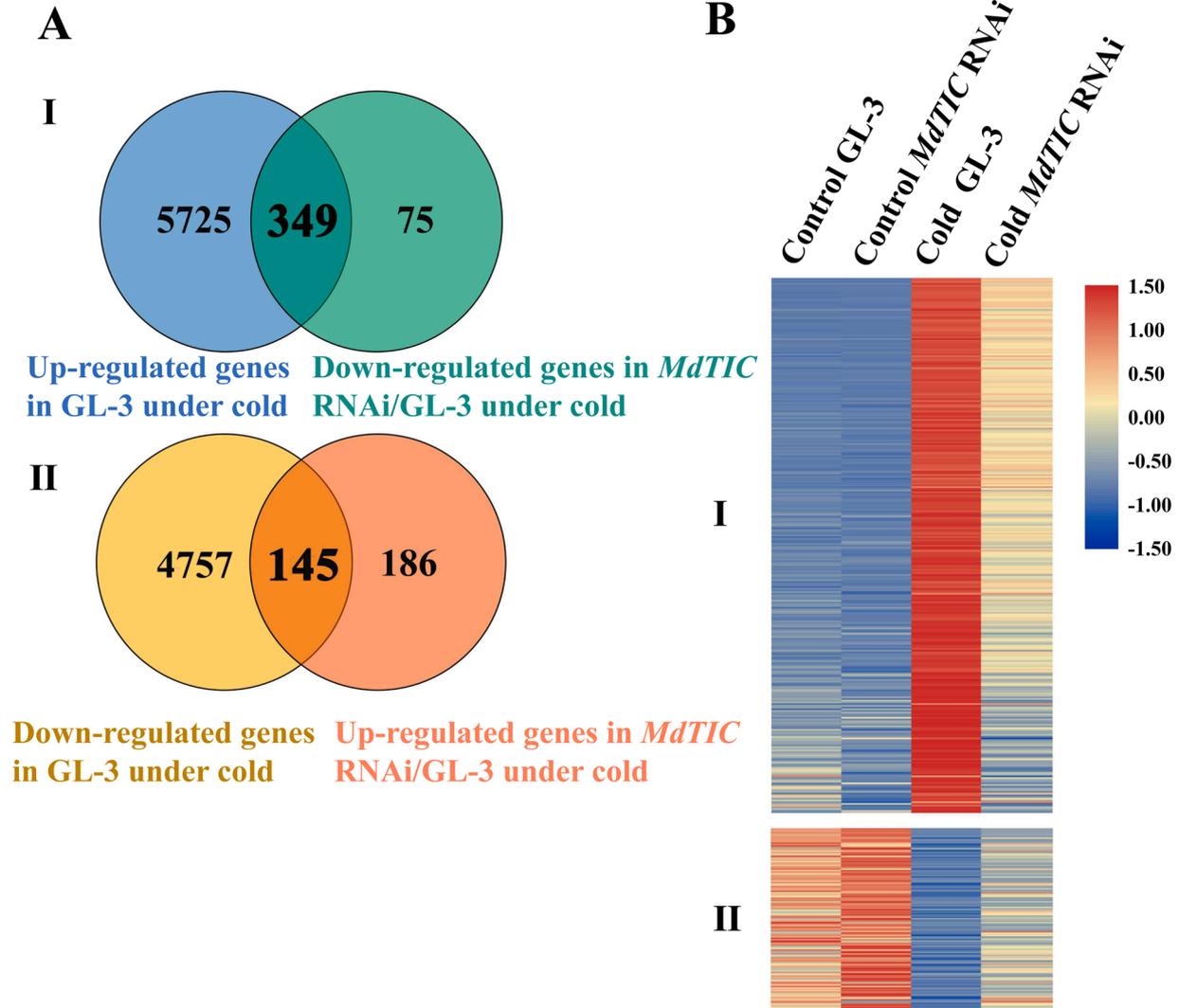
Fig. 5. Transcriptome profiling analysis. (A) Venn diagram analysis showing the number of DEGs upregulated by the cold treatment and the genes that were downregulated in *MdTIC* RNAi plants under the cold treatment (I); the number of DEGs downregulated by cold, and genes that were upregulated in *MdTIC* RNAi plants under the cold treatment (II). (B) Heatmap represents the transcription levels of the DEGs belonging to group I or II as shown in (A). Gene expression level was represented by the FPKM value. DEGs: differentially expressed genes.
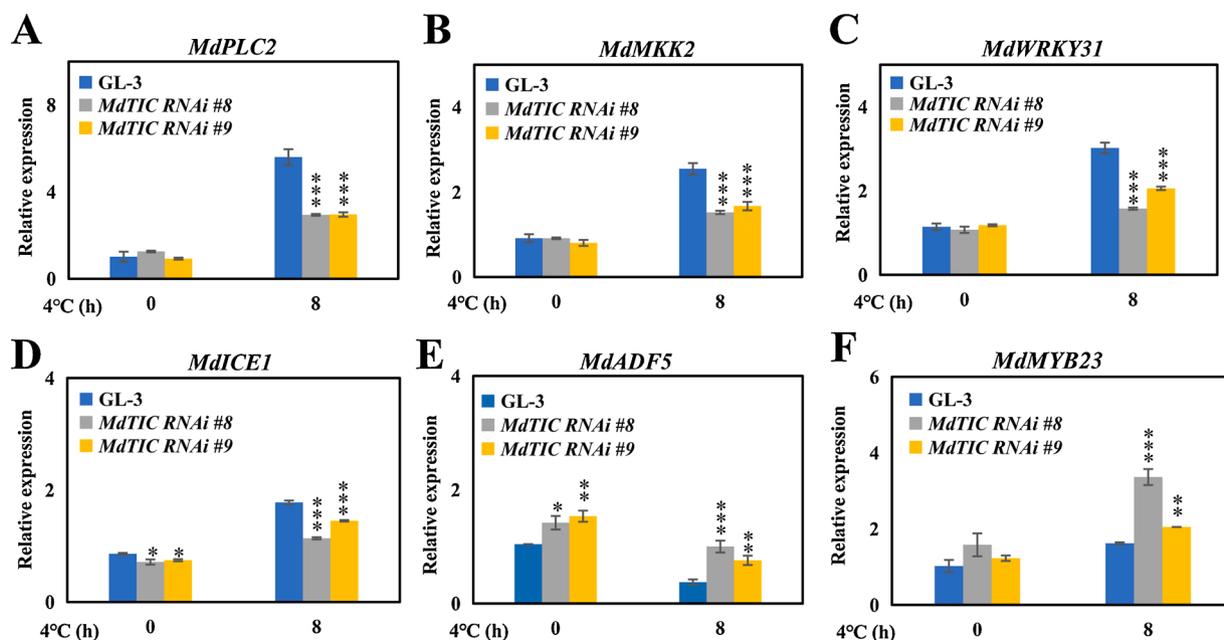
Fig. 6. Verification of RNA-seq data. Data are presented as mean ± SD (n = 3, from three biological replicates). Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey test) (***P < 0.001, **P < 0.01, *P < 0.05).

Fig. 7. Expression of *ω-3 FAD* and the ratio of C16, C18, and C20 unsaturated fatty acids in the GL-3 and *MdTIC* transgenic plants. Plants that were not subjected to the cold treatment were used as a control. Data are mean ± SD (n = 3, from three biological replicates). Asterisks indicate significant differences as assessed by one-way ANOVA (Tukey test) (***P < 0.001, **P < 0.01, *P < 0.05).
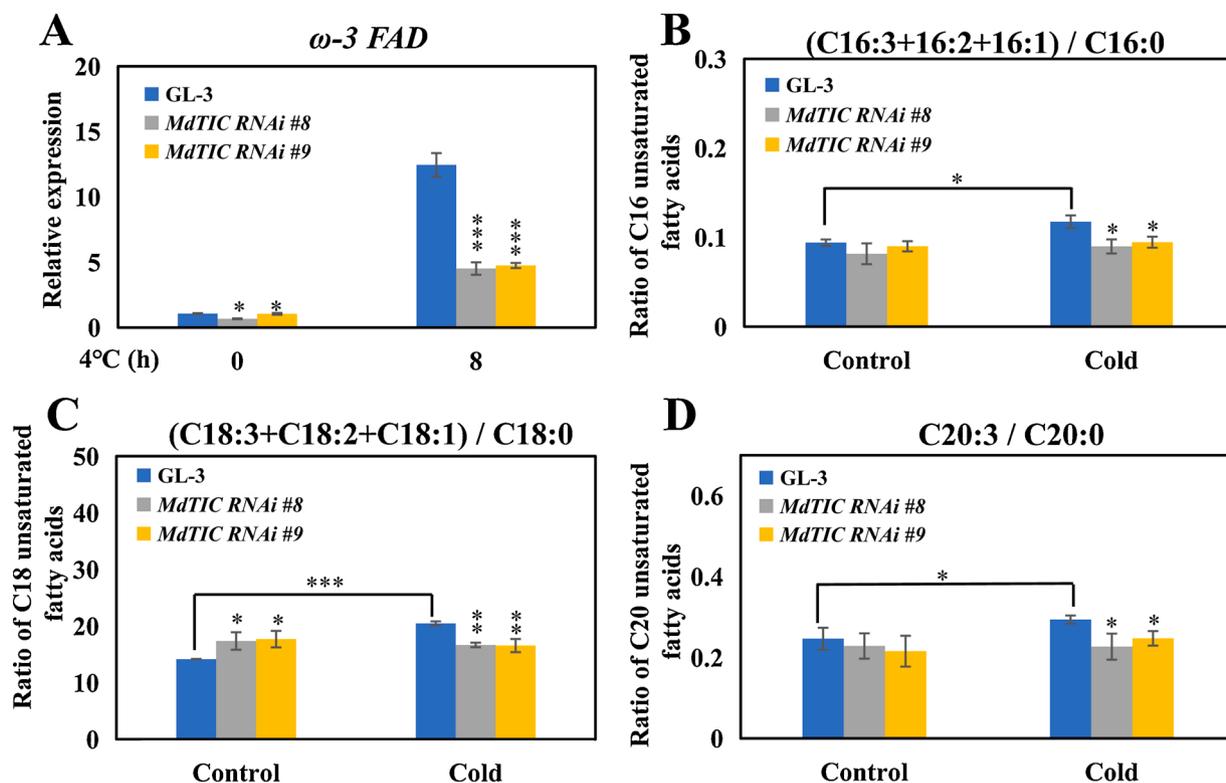
chromatography-mass spectrometry (GC–MS) analysis revealed the relative abundance of the major fatty acid species of the GL-3 and *MdTIC* RNAi transgenic plants after a 1-week treatment or without cold treatment. C18:n and 11,14,17-eicosatrienoic acid (C20:3) content was significantly different between the GL-3 and *MdTIC* RNAi transgenic plants under normal conditions, but not C18:3 (Table 1). Palmitic acid

(C16:0) and stearic acid (C18:0), the two main saturated fatty acids, decreased significantly in GL-3 but increased in *MdTIC* RNAi after a 1-week exposure to the cold (4 °C) (Table 1). Moreover, relative content of C18:3 increased at the expense of C18:2, C18:1, and C18:0 in GL-3 during cold treatment (Fig. 7C). However, a prominent reduction of C18:3 was detected in *MdTIC* RNAi plants exposed to the cold when

Table 1
Relative Fatty acid content (the ratio of each component in the total) in GL-3 and *MdTIC* RNAi plants after a 1-week cold treatment (Cold 4 ℃) or without the cold treatment (Control 25 ℃).

Fatty acid	Control (25 ℃)					Cold (4 ℃)					
	GL-3	MdTIC RNAi #8	P^a	MdTIC RNAi #9	P^a	GL-3	P^a	MdTIC RNAi #8	P^b	MdTIC RNAi #9	P^b
C16:0	0.217 ± 0.004	0.221 ± 0.005		0.215 ± 0.008		0.197 ± 0.001	***	0.214 ± 0.006	**	0.213 ± 0.002	**
C16:1	0.0091 ± 0.0008	0.0072 ± 0.0006		0.008 ± 0.0006		0.0085 ± 0.0011		0.0076 ± 0.0005		0.0076 ± 0.0003	
C16:2	0.0031 ± 0.0003	0.0025 ± 0.0005		0.0026 ± 0.0004		0.0025 ± 0.0003		0.0024 ± 0.0003		0.0027 ± 0.0003	
C16:3	0.0098 ± 0.0014	0.0083 ± 0.0011		0.0087 ± 0.0008		0.0109 ± 0.001		0.0092 ± 0.0006		0.0099 ± 0.0013	
C18:0	0.0499 ± 0.0004	0.0412 ± 0.0035	**	0.0407 ± 0.0034	**	0.036 ± 0.0006	***	0.0433 ± 0.0037	**	0.0434 ± 0.0032	**
C18:1	0.0703 ± 0.0056	0.0446 ± 0.0025	**	0.0545 ± 0.0018	**	0.0527 ± 0.0012	**	0.038 ± 0.0003	***	0.0453 ± 0.0018	**
C18:2	0.105 ± 0.006	0.121 ± 0.007	*	0.119 ± 0.005	*	0.105 ± 0.003		0.118 ± 0.006	*	0.118 ± 0.007	*
C18:3	0.53 ± 0.004	0.535 ± 0.01		0.543 ± 0.011		0.578 ± 0.002	***	0.551 ± 0.002	**	0.552 ± 0.005	**
C20:0	0.00795 ± 0.00043	0.00706 ± 0.00065		0.00716 ± 0.00038		0.00744 ± 0.00048		0.00752 ± 0.0005		0.00724 ± 0.00051	
C20:3	0.00195 ± 0.00012	0.00161 ± 0.0002	*	0.00153 ± 0.00019	*	0.00213 ± 0.00012		0.00169 ± 0.00021	*	0.00178 ± 0.00011	*

Data are presented as the mean ± SD from three biological replicates. Asterisks indicate significance compared with a(CK GL3) and b(Cold GL3) by one-way ANOVA (Tukey test) (***P < 0.001, **P < 0.01, *P < 0.05).

compared to GL-3 (Table 1). Additionally, *MdTIC* RNAi transgenic plants had a slight decrease in relative content of C20:3 (Table 1). Less polyunsaturated fatty acids accumulated in the *MdTIC* RNAi transgenic lines than in GL-3 based on the ratio of unsaturated to saturated fatty acids under cold conditions (Fig. 7B–D). Therefore, these results support that interfering with *MdTIC* expression influences cold tolerance by modulating the synthesis of unsaturated fatty acids.

2.6. Circadian rhythm of MdMYB88 and MdMYB124

TIC is a *MdTIC* homolog that acts as a circadian regulator in Arabidopsis [27,37]. We monitored expression of the *MdTIC* upstream genes, *MdMYB88* and *MdMYB124,* over 24 h in GL-3. As expected, *MdMYB88* and *MdMYB124* were expressed in a circadian rhythm pattern (Supplemental Fig. 4), suggesting that MdMYB88 and MdMYB124 may be involved in circadian regulation.

3. Discussion

The R2R3 transcription factors MdMYB88 and MdMYB124 have been reported to play crucial roles regulating cold and drought resistance in apple [25,30]. Here, we identified the function of one of the targets of MdMYB88 and MdMYB124, *MdTIC*, in response to freezing stress in perennial apple trees. MdTIC is a homologous protein of TIC in Arabidopsis, which encodes a circadian regulator connecting the circadian clock, the stress response, and metabolic homeostasis [28]. Although TIC was found to affect plant developmental and metabolic processes about 20 years ago, the underlying physiological and molecular mechanisms of *MdTIC* in apple in response to cold stress remains elusive.

Several lines of evidence support that MdTIC is a positive regulator of freezing stress tolerance. First, *MdTIC* RNAi plants exhibited higher ion leakage than GL-3 either before or after cold acclimation (Fig. 3B). Second, whole-plant survival assays suggest that *MdTIC* RNAi plants had lower ability to endure freezing stress. Third, *MdTIC* RNAi plants displayed lower antioxidant enzyme activities under cold conditions, compared to GL-3 plants (Fig. 4). Fourth, *MdTIC* RNAi plants accumulated less polyunsaturated fatty acids than GL-3 during the cold treatment (Fig. 7B–D). In addition, the RNA-seq analysis revealed that MdTIC also affected expression of *COR* genes, including *MdPLC2*, *MdMKK2*, and *MdICE1* (Fig. 6). Taken together, these results demonstrate that MdTIC promotes plant resistance to freezing stress.

Plants generate excess ROS, including O^{2-}, H_2O_2, and OH· under cold stress; therefore, activation of the cellular antioxidant machinery is crucial for protection against these toxic oxygen intermediates [16,38]. For example, expression of genes encoding PODs is induced in the cold, which contributes to enhance cold tolerance of *PtrbHLH* overexpressing plants [39]. Ectopic expression of *PbrBAM3* in tobacco plants enhances

cold tolerance by accumulating higher levels of antioxidant enzymes including POD, CAT, and SOD [40]. In our study, CAT and POD activities were lower in *MdTIC* RNAi plants, which contained higher H_2O_2 content (Fig. 4). These data indicate that reduced cold resistance of *MdTIC* RNAi plants was due, in part, to the lower activity of antioxidant enzymes.

We analyzed the RNA-seq profile to investigate global gene expression changes affected by *MdTIC*. Under control conditions, MdTIC regulated expression of 83 DEGs, whereas this number was 755 when plants were treated with cold, indicating that MdTIC has more function in transcriptional regulation under cold conditions. Among the DEGs expressed under cold conditions, expression of a number of cold-positive genes was repressed in *MdTIC* RNAi plants, but the expression levels of cold-negative genes increased (Fig. 5), suggesting that the reduced tolerance of *MdTIC* RNAi plants might be due to decreased expression of cold-positive genes and increased expression of cold-negative genes. Generally, disruption of circadian gene expression will misregulate other genes controlling the clock [41]. For example, the *elf4* mutation causes abnormal expression of *CCA1*, *GI*, and *PRR7* [41]. TIC is reported to reset the clock just prior to dawn [27]; however, we did not find any other circadian genes among the DEGs. It is possible that the transcriptional regulation of these circadian genes by MdTIC might be very minor.

Our previous study reported that the circadian gene *MdCCA1* is directly regulated by MdMYB88 and MdMYB124 [25]. In the current study, we demonstrated that *MdTIC* was another direct target of MdMYB88 and MdMYB124. Considering that both genes are circadian clock genes [27,42], we monitored expression of *MdMYB88* and *MdMYB124* over 24 h. The results showed that expression of *MdMYB88* and *MdMYB124* was regulated by the clock (Supplemental Fig. 4). As *MdTIC* and *MdCCA1* are two direct target genes of MdMYB88 and MdMYB124, the clock-regulating pattern of both MYB transcription factors might be attributed to these two targets. In addition, transcript levels of *CBF* genes are clock regulated, and CCA1/LHY promotes *CBF* expression by binding to the *CBF1/2/3* promoter [43]. Interestingly, *MdTIC* was involved in clock-regulated *CBF* expression (Supplemental Fig. 5). MdMYB88 and MdMYB124 positively regulate expression of *MdCBF1/3* genes under cold stress [25], suggesting the possibility that MdMYB88 and MdMYB124 displayed a clock-regulating pattern by regulating expression of *MdTIC* and *MdCCA1*.

During cold acclimation, the degree (number of double bonds) of unsaturated fatty acid saturation is responsible for remodeling membrane fluidity in plants [17,18]; therefore, this process is responsible for cold tolerance [22]. When plants suffer from cold stress, the synthesis of polyunsaturated (predominantly trienoic) fatty acids is induced [44]. Additionally, the content of trienoic fatty acids in *OsFAD8* RNAi lines is reduced by 40 %, which decreases plant chilling tolerance [24]. In the current study, *ω-3 FAD* was repressed in *MdTIC* RNAi plants under the cold conditions, compared with GL-3 plants. Consistently, less

polyunsaturated fatty acids accumulated in *MdTIC* RNAi plants than in the GL-3 during the cold treatment (Fig. 7B–D). In agreement with the less polyunsaturated fatty acids in *MdTIC* RNAi plants under the cold conditions, greater membrane damage in *MdTIC* RNAi plants was observed as revealed by the electrolyte leakage assay. Therefore, the hypersensitivity of *MdTIC* RNAi plants to freezing stress should be partially attributed to less polyunsaturated fatty acids.

We also observed a slight change in C16:0 fatty acids in *MdTIC* RNAi plants compared to GL-3 plants under cold, but not C16 unsaturated fatty acids. Therefore, *MdTIC* affected C16:0 biosynthesis genes under cold stress. FAD genes are responsible for synthesis of unsaturated fatty acids [35]. As we only identified *MdFAD8* in our RNA-seq data in response to cold, how MdTIC mediated C16:0 fatty acid biosynthesis needs further study.

Soluble sugars, such as glucose, maltose, sucrose, and fructose, increase freezing tolerance by osmotic regulation and protecting the membrane [40]. We measured the content of the soluble sugar in the GL-3 and *MdTIC* RNAi plants under control and cold conditions, and did not find any significant difference between the two genotypes (Supplemental Fig. 6). However, based on the RNA-seq data, chloroplastic β-amylase (At4g17090), which produces maltose, was upregulated about 3.4-fold in GL-3 plants under the cold conditions but was downregulated about 36 % in *MdTIC* RNAi plants compared to GL-3 under cold conditions. The β-amylase 3 (MD04G1056200) sequence in apple shares 73.64 % similarity with its counterpart in Arabidopsis. Therefore, we speculate that the maltose of GL-3 and *MdTIC* RNAi plants might be different, but this requires further study.

Taken together, our findings explored the molecular mechanism of MdTIC in the resistance of apple to freezing stress, enhanced our understanding of MdTIC function, and provided a candidate gene for improving stress resistance through apple breeding.

4. Materials and methods

4.1. Plant materials and growth conditions

The plant material used for genetic transformation was selected from progenies of 'RoyaL Gala' apple (*Malus × domestica Borkh.* cv. Royal Gala) and named GL-3 which has high regeneration capacity. Transgenic plants and GL-3 were grown on MS medium (4.43 g/L MS salts, 30 g/L sucrose, 0.2 mg/L 6-BA, 0.2 mg/L IAA, and 7.5 g/L agar, pH 5.8) under long-day conditions (16 h light/ 8 h dark) at 22 °C. Transgenic *MdMYB88/124* RNAi and overexpression plants were generated previously [25].

4.2. Generation of transgenic apple plants

A 297-bp fragment of *MdTIC* was cloned into the RNA silencing vector pK7GWIWG2D by a gateway recombination technology (Invitrogen, USA), and the resulting plasmid (*MdTIC*-pK7GWIWG2D) was transformed into agrobacterium (strain EHA105). Agrobacterium carrying plasmid of *MdTIC*-pK7GWIWG2D was transformed into leaves of GL-3 through agrobacterium-mediated transformation method as described [45,46]. The primers used for constructing vectors were shown in Supplemental Table 1.

4.3. Yeast one-hybrid

Yeast one-hybrid was performed as previously described [30]. Briefly, *MdTIC* promoter religion (−1942 to −1351 bp) was cloned into pAbAi vector, and full-length CDS of MdMYB88 was cloned into pGADT7 vector, resulting in MdMYB88-pGADT7. Yeast cells carried *MdTIC* promoter-pAbAi were selected on SD-Ura medium and used to determine the AbA concentration. The MdMYB88- pGADT7 vector then was transformed into *MdTIC*-pAbAi Y1H Gold competent cells, and cell growth was observed on SD–Leu/AbA (500 ng/mL).

4.4. EMSA and ChIP-qPCR assays

EMSA assays were carried out using the LightShift Chemiluminescent EMSA Kit (Thermo Scientific, USA) according to the manufacturer's protocol. In brief, 2 µg MdMYB88 protein was incubated with different amount of biotin-labeled or unlabeled probes of *MdTIC* at 25℃ for 20 min. The protein- DNA samples were then separated on 6% native polyacrylamide gels and signal was captured with a ChemiDocTM XRS+ (Bio-Rad). The sequences of probes are listed in in Supplemental Table 1.

ChIP-qPCR was carried out as previously described [25]. In brief, leaves were collected from GL-3 plants grown under cold (4℃) for 8 h before cross-linking in 1 % formaldehyde. Anti-MdMYB88 antibody was used to immunoprecipitate chromatin. The recovered DNA was used for real-time qPCR using the primers listed in Supplemental Table 1.

4.5. Freezing tolerance of apple plants

For electrolyte leakage, non-cold acclimated plants were grown under normal condition, and cold-acclimation plants were treated with 4℃ for one week. The leaves were punched into leaf discs with diameter of 10 mm and placed into a test tube containing 300 µl of deionized H_2O, followed by rapidly adding ice into the tube. The tubes were then placed into Refrigerated and Heated Bath Circulators (Thermo Scientific, USA) with a temperature procedure of described previously [25]. The conductivity was measured by a conductivity meter (Mettler-Toledo, Shanghai). Test tubes were then boiled for 40 min, and conductivity was measured again. Electrolyte leakage was calculated with a ratio of conductivity before to after boiling.

For whole-plant survival assays under freezing conditions, non-cold acclimated and cold acclimated plants were treated with −10 °C for 30 and 40 min, respectively, followed by recovery under normal conditions for 3 weeks. The survival rates were counted after recovery.

4.6. Measurement of hydrogen peroxide (H_2O_2) content, and activity of peroxidase (POD) and catalase (CAT)

GL-3 and *MdTIC RNAi* transgenic plants were treated with 4℃ or 22℃ for 2 h. Then mature leaves were collected. H_2O_2 content was quantified by the detection kits (#H2O2-1-Y, Suzhou Comin Biotechnology Institute, Suzhou, China). Activity of POD and CAT was measured as described by previous study [47].

4.7. Transcriptome profiling analysis

Total RNA were extracted from leaves of three-month-old plants treated with or without cold stress at 4 °C for 8 h, and deep sequencing was performed on an Illumina Hiseq platform. 1.5 Gb clean data for each sample was obtained and mapped to the *Malus × domestica* genome (GDDH13) by Hisat2. The corresponding sample readscount levels were analyzed by Deseq2 to select differentially expressed genes (using both P value <0.05 and 1.5-fold as a cutoff). FPKM (Fragments Per Kilobase of transcript per Million fragments mapped) value was calculated by Cufflinks and used for heatmap analysis. We drew the Venn diagram and heatmap in Toolbox for biologists (TBtools) v0.668374. GO enrichment was analyzed based on the website (http://plantregmap.cbi.pku.edu.cn/).

4.8. RNA extraction and qRT-PCR analysis

Apple plants grown in soil under long-day conditions (16 h light/ 8 h dark) were used for RNA isolation. Total RNA of apple leaves was extracted using an RNApreppure Plant Kit (Omega, USA). RNase-free DNase I (Fermentas) was used to remove residual DNA, followed by reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

Quantitative reverse transcription PCR (qRT-PCR) was performed on

a Bio-Rad CFX96™ instrument (Bio-Rad) using SYBR Green PCR Master Mix kit (Takara, Japan). The malate dehydrogenase (*MdMDH*) gene of apple was used as the reference gene and relative expression was calculated as described previously [25].

4.9. Measurement of fatty acids

Three-month-old plants treated with or without cold stress at 4 °C for one week were used for fatty acid extraction. Mature leaves were collected and fully grinded into powder before were placed into freeze dryer at -80℃ at vacuum condition (Labconco, USA, Freezone). Fatty acid was extracted as previously described with slight modification [48]. 0.2000 g frozen dried leaf powder (three duplications) was accurately weighed by 1/10,000 balance and ultrasonically extracted with 5 ml methanol: chloroform (2:1, v/v, HPLC grade) for 20 min. 50 μl hepta-decanoic acid (1 mg/mL, Alfa Aesar) was used as an internal standard. This process was repeated twice. Then, crude extraction was collected and filtered by 0.45 μm organic membrane (New Asia, Shanghai) and blown into grease by a pressure blowing concentrator (ZhiXin, Shanghai, MD200-2) with nitrogen. Next, 1 ml potassium hydroxide-methanol (22.4 mg/mL) was added to the grease and incubated in a 65 °C water bath for 15 min for saponification. Then 2 ml of boron trifluoride-methanol solution (14 %, Shanghai Anpu) was added and incubated in a 65 °C water bath for 30 min. Finally, 2 ml n-hexane (HPLC grade) was added to the above extracts and supernatant was pipetted out and filtered by 0.22 μm organic membrane (New Asia, Shanghai) into sample vial for GC–MS analysis (Gas Chromatography-Tandem Mass Spectrometry) coupled with the Trace GC ULTRA/ISQ MS detector (Thermo Scientific, USA). Mixed standards of fatty acid methyl ester were from the NU-CHEK company.

For GC–MS, 1 μl extraction sample was injected by 10-μl syringe (Shanghai Anpu) and analyzed by TRACE GC Ultra GC coupled with an ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, USA). The GC was equipped with a DB-5 MS column (60 m × 0.25 mm ×0.25 μm film thickness, Agilent, Palo Alto, USA). Helium was used as the carrier gas, with a split ratio of 20:1, at a flow rate of 1 ml/min. The temperatures of the injection port, ion source, and MS transfer line were 250 °C, 240 °C, and 240 °C, respectively. The temperature increasing procedure of the column chamber was as follows: initial temperature was 80 °C for 2.5 min, followed by a ramp of 15 °C/min to 210 °C, then was increased to 230 °C at a rate of 2 °C/min, and finally kept at 230 °C for 10 min. The MS was operated in positive electron ionization mode at 70 eV, obtaining spectra with a range of 40–460 m/z.

4.10. Statistical analysis

Statistical analysis was carried out by one-way ANOVA (Tukey's test) analysis (SPSS version 21.0, USA). Variations significance level was represented by asterisks: $*P < 0.05$, $**P < 0.01$, $***P < 000.1$.

Accession numbers

Sequence data used in this study can be obtained in NCBI with following accession numbers: XM_008368962.3 (*MdTIC*), XM_008371033.3 (*MdPLC2*), XM_008365867.3 (*MdWRKY31*), XM_008339518.3 (*MdMKK2*), XM_008380831.3 (*MdICE1*), XM_008374682.3 (*MdADF5*), NM_001294040.1 (*MdMYB23*), XM_017331060.2 (ω-3 FAD).

Author contributions

Q.G. and C.Z. designed the project. C.Z., X.L. Y.Xie. and Y.Xu performed the experiments. C.Z., J.H. and F.M. analyzed the data. C.Z. and Q.G. wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.plantsci.2020.110695.

References

[1] M.Q. Zhou, C. Shen, L.H. Wu, K.X. Tang, J. Lin, CBF-dependent signaling pathway: a key responder to low temperature stress in plants, Crit. Rev. Biotechnol. 31 (2011) 186–192.

[2] S. Mahajan, N. Tuteja, Cold, salinity and drought stresses: an overview, Arch. Biochem. Biophys. 444 (2005) 139–158.

[3] M. Uemura, Y. Tominaga, C. Nakagawara, S. Shigematsu, A. Minami, Y. Kawamura, Responses of the plasma membrane to low temperatures, Physiol. Plant. 126 (2006) 81–89.

[4] Y. Shi, Y. Ding, S. Yang, Cold signal transduction and its interplay with phytohormones during cold acclimation, Plant Cell Physiol. 56 (2015) 7–15.

[5] V. Chinnusamy, J.K. Zhu, R. Sunkar, Gene regulation during cold stress acclimation in plants, Methods Mol. Biol. 639 (2010) 39–55.

[6] F. Novillo, J.M. Alonso, J.R. Ecker, J. Salinas, CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 3985–3990.

[7] V. Chinnusamy, M. Ohta, S. Kanrar, B.H. Lee, X. Hong, M. Agarwal, J.K. Zhu, ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis, Genes Dev. 17 (2003) 1043–1054.

[8] C.J. Doherty, H.A. Van Buskirk, S.J. Myers, M.F. Thomashow, Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance, Plant Cell 21 (2009) 972–984.

[9] Y.T. Shi, S.W. Tian, X.Z. Huang, X.Y. Zhang, H.W. Guo, S.H. Yang, Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and Type-A ARR genes in Arabidopsis, Plant Cell 24 (2012) 2578–2595.

[10] M. Agarwal, Y.J. Hao, A. Kapoor, C.H. Dong, H. Fujii, X.W. Zheng, J.K. Zhu, A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance, J. Biol. Chem. 281 (2006) 37636–37645.

[11] J.T. Vogel, D.G. Zarka, H.A. Van Buskirk, S.G. Fowler, M.F. Thomashow, Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis, Plant J. 41 (2005) 195–211.

[12] C. Zhao, P. Wang, T. Si, C.C. Hsu, L. Wang, O. Zayed, Z. Yu, Y. Zhu, J. Dong, W. A. Tao, J.K. Zhu, MAP kinase cascades regulate the cold response by modulating ICE1 protein stability, Dev. Cell 43 (2017) 618–629.

[13] S. Fowler, M.F. Thomashow, Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway, Plant Cell 14 (2002) 1675–1690.

[14] M.F. Thomashow, PLANT COLD ACCLIMATION: freezing tolerance genes and regulatory mechanisms, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 571–599.

[15] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930.

[16] J. You, Z. Chan, ROS regulation during abiotic stress responses in crop plants, Front. Plant Sci. 6 (2015) 1092.

[17] C. Barrero-Sicilia, S. Silvestre, R.P. Haslam, L.V. Michaelson, Lipid remodelling: unravelling the response to cold stress in Arabidopsis and its extremophile relative Eutrema salsugineum, Plant Sci. 263 (2017) 194–200.

[18] R.G. Upchurch, Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress, Biotechnol. Lett. 30 (2008) 967–977.

[19] S. Penfield, Temperature perception and signal transduction in plants, New Phytol. 179 (2008) 615–628.

[20] A. Tovuu, I.S. Zulfugarov, G. Wu, I.S. Kang, C. Kim, B.Y. Moon, G. An, C.H. Lee, Rice mutants deficient in omega-3 fatty acid desaturase (FAD8) fail to acclimate to cold temperatures, Plant Physiol. Biochem. 109 (2016) 525–535.

[21] D.L. Falcone, J.P. Ogas, C.R. Somerville, Regulation of membrane fatty acid composition by temperature in mutants of Arabidopsis with alterations in membrane lipid composition, BMC Plant Biol. 4 (2004) 17.

[22] L. Nejadsadeghi, R. Maali-Amiri, H. Zeinali, S. Ramezanpour, B. Sadeghzade, Membrane fatty acid compositions and cold-induced responses in tetraploid and hexaploid wheats, Mol. Biol. Rep. 42 (2015) 363–372.

[23] D.L. Falcone, S. Gibson, B. Lemieux, C. Somerville, Identification of a gene that complements an Arabidopsis mutant deficient in chloroplast Omega-6 desaturase activity, Plant Physiol. 106 (1994) 1453–1459.

[24] P.M.G. Nair, I.-S. Kang, B.-Y. Moon, C.-H. Lee, Effects of low temperature stress on rice (Oryza sativa L.) plastid ω-3 desaturase gene, OsFAD8 and its functional analysis using T-DNA mutants, Plant Cell Tissue Organ Cult. 98 (2009) 87–96.

[25] Y. Xie, P. Chen, Y. Yan, C. Bao, X. Li, L. Wang, X. Shen, H. Li, X. Liu, C. Niu, C. Zhu, N. Fang, Y. Shao, T. Zhao, J. Yu, J. Zhu, L. Xu, S. van Nocker, F. Ma, Q. Guan, An atypical R2R3 MYB transcription factor increases cold hardiness by CBF-dependent and CBF-independent pathways in apple, New Phytol. 218 (2018) 201–218.

[26] M.F. Covington, J.N. Maloof, M. Straume, S.A. Kay, S.L. Harmer, Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development, Genome Biol. 9 (2008) 130.

[27] Z. Ding, A.J. Millar, A.M. Davis, S.J. Davis, TIME FOR COFFEE encodes a nuclear regulator in the Arabidopsis thaliana circadian clock, Plant Cell 19 (2007) 1522–1536.

[28] A. Sanchez-Villarreal, J. Shin, N. Bujdoso, T. Obata, U. Neumann, S.X. Du, Z. Ding, A.M. Davis, T. Shindo, E. Schmelzer, R. Sulpice, A. Nunes-Nesi, M. Stitt, A.R. Fernie, S.J. Davis, TIME FOR COFFEE is an essential component in the maintenance of metabolic homeostasis in Arabidopsis thaliana, Plant J. 76 (2013) 188–200.

[29] J. Shin, K. Heidrich, A. Sanchez-Villarreal, J.E. Parker, S.J. Davis, TIME FOR COFFEE represses accumulation of the MYC2 transcription factor to provide time-of-day regulation of jasmonate signaling in Arabidopsis, Plant Cell 24 (2012) 2470–2482.

[30] D. Geng, P. Chen, X. Shen, Y. Zhang, X. Li, L. Jiang, Y. Xie, C. Niu, J. Zhang, X. Huang, F. Ma, Q. Guan, MdMYB88 and MdMYB124 enhance drought tolerance by modulating root vessels and cell walls in apple, Plant Physiol. 178 (2018) 1296–1309.

[31] M. Teige, E. Scheikl, T. Eulgem, R. Doczi, K. Ichimura, K. Shinozaki, J.L. Dangl, H. Hirt, The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis, Mol. Cell 15 (2004) 141–152.

[32] D.R. Ruzicka, M.K. Kandasamy, E.C. McKinney, R.B. Meagher, Arabidopsis Actin depolymerizing Factor5 functions in multicellular development and is a novel repressor of the CBF cold response transcription factors, Dev. Biol. 319 (2008) 565.

[33] K. Nokhrina, H. Ray, C. Bock, F. Georges, Metabolomic shifts in Brassica napus lines with enhanced BnPLC2 expression impact their response to low temperature stress and plant pathogens, GM Crops Food 5 (2014) 120–131.

[34] Y. Huang, C.Z. Feng, Q. Ye, W.H. Wu, Y.F. Chen, Arabidopsis WRKY6 transcription factor acts as a positive regulator of Abscisic Acid signaling during seed germination and early seedling development, PLoS Genet. 12 (2016), e1005833.

[35] H. Zhang, J. Dong, X. Zhao, Y. Zhang, J. Ren, L. Xing, C. Jiang, X. Wang, J. Wang, S. Zhao, H. Yu, Research progress in membrane lipid metabolism and molecular mechanism in peanut cold tolerance, Front. Plant Sci. 10 (2019) 838.

[36] S. Gibson, V. Arondel, K. Iba, C. Somerville, Cloning of a temperature-regulated gene encoding a chloroplast omega-3 desaturase from Arabidopsis thaliana, Plant Physiol. 106 (1994) 1615–1621.

[37] A. Hall, R.M. Bastow, S.J. Davis, S. Hanano, H.G. McWatters, V. Hibberd, M.R. Doyle, S. Sung, K.J. Halliday, R.M. Amasino, A.J. Millar, The TIME FOR COFFEE gene maintains the amplitude and timing of Arabidopsis circadian clocks, Plant Cell 15 (2003) 2719–2729.

[38] S.S. Gill, N. Tuteja, Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants, Plant Physiol. Biochem. 48 (2010) 909–930.

[39] X.S. Huang, W. Wang, Q. Zhang, J.H. Liu, A basic helix-loop-helix transcription factor, PtrbHLH, of Poncirus trifoliata confers cold tolerance and modulates peroxidase-mediated scavenging of hydrogen peroxide, Plant Physiol. 162 (2013) 1178–1194.

[40] L. Zhao, T. Yang, C. Xing, H. Dong, K. Qi, J. Gao, S. Tao, J. Wu, J. Wu, S. Zhang, X. Huang, The beta-amylase PbrBAM3 from pear (Pyrus betulaefolia) regulates soluble sugar accumulation and ROS homeostasis in response to cold stress, Plant Sci. 287 (2019), 110184.

[41] W.W. Chen, N. Takahashi, Y. Hirata, J. Ronald, S. Porco, S.J. Davis, D.A. Nusinow, S.A. Kay, P. Mas, A mobile ELF4 delivers circadian temperature information from shoots to roots, Nat. Plants 6 (2020) 416–426.

[42] P.A. Salome, D. Weigel, C.R. McClung, The role of the Arabidopsis morning loop components CCA1, LHY, PRR7, and PRR9 in temperature compensation, Plant Cell 22 (2010) 3650–3661.

[43] M.A. Dong, E.M. Farre, M.F. Thomashow, Circadian clock-associated 1 and late elongated hypocotyl regulate expression of the C-repeat binding factor (CBF) pathway in Arabidopsis, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 7241–7246.

[44] J.G. Wallis, J. Browse, Mutants of Arabidopsis reveal many roles for membrane lipids, Prog. Lipid Res. 41 (2002) 254–278.

[45] H.Y. Dai, W.R. Li, G.F. Han, Y. Yang, Y. Ma, H. Li, Z.H. Zhang, Development of a seedling clone with high regeneration capacity and susceptibility to Agrobacterium in apple, Sci Hortic-Amsterdam 164 (2013) 202–208.

[46] A. Holefors, Z.T. Xue, M. Welander, Transformation of the apple rootstock M26 with the rolA gene and its influence on growth, Plant Sci. 136 (1998) 69–78.

[47] S. Wang, D. Liang, C. Li, Y. Hao, F. Ma, H. Shu, Influence of drought stress on the cellular ultrastructure and antioxidant system in leaves of drought-tolerant and drought-sensitive apple rootstocks, Plant Physiol. Biochem. 51 (2012) 81–89.

[48] C. Contreras, H. Tjellstrom, R.M. Beaudry, Relationships between free and esterified fatty acids and LOX-derived volatiles during ripening in apple, Postharvest Biol Tec 112 (2016) 105–113.