MdNup62 interactions with MdHSFs involved in flowering and heat-stress tolerance in apple

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Abstract

Because of global warming, the apple flowering period is occurring significantly earlier, increasing the probability and degree of freezing injury. Moreover, extreme hot weather has also seriously affected the development of apple industry. Nuclear pore complexes (NPCs) are main channels controlling nucleocytoplasmic transport, but their roles in regulating plant development and stress responses are still unknown. Here, we analysed the components of the apple NPC and found that MdNup62 interacts with MdNup54, forming the central NPC channel. MdNup62 was localized to the nuclear pore, and its expression was significantly up-regulated in 'Nagafu No. 2' tissue-cultured seedlings subjected to heat treatments. To determine MdNup62's function, we obtained MdNup62-overexpressed (OE) Arabidopsis and tomato lines that showed significantly reduced high-temperature resistance. Additionally, OE-MdNup62 Arabidopsis lines showed significantly earlier flowering compared with wild-type. Furthermore, we identified 62 putative MdNup62-interacting proteins and confirmed MdNup62 interactions with multiple MdHSFs. The OE-MdHSFA1d and OE-MdHSFA9b Arabidopsis lines also showed significantly earlier flowering phenotypes than wild-type, but had enhanced high-temperature resistance levels. Thus, MdNUP62 interacts with multiple MdHSFs during nucleocytoplasmic transport to regulate flowering and heat resistance in apple. The data provide a new theoretical reference for managing the impact of global warming on the apple industry.

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Run title: MdNup62 interactions with MdHSFs regulate flowering and heat-stress tolerance

Abstract

Because of global warming, the apple flowering period is occurring significantly earlier, increasing the probability and degree of freezing injury. Moreover, extreme hot weather has also seriously affected the development of apple industry. Nuclear pore complexes (NPCs) are main channels controlling nucleocytoplasmic transport, but their roles in regulating plant development and stress responses are still unknown. Here, we analysed the components of the apple NPC and found that MdNup62 interacts with MdNup54, forming the central NPC channel. MdNup62 was localized to the nuclear pore, and its expression was significantly upregulated in 'Nagafu No. 2' tissue-cultured seedlings subjected to heat treatments. To determine *MdNup62* 's function, we obtained *MdNup62* -overexpressed (OE) Arabidopsis and tomato lines that showed significantly reduced high-temperature resistance. Additionally, OE-*MdNup62* Arabidopsis lines showed significantly earlier flowering compared with wild-type. Furthermore, we identified 62 putative *MdNup62* -interacting proteins and confirmed *MdNup62* interactions with multiple*MdHSFs* . The OE-*MdHSFA1d* and OE-*MdHSFA9b* Arabidopsis lines also showed significantly earlier flowering phenotypes than wild-type, but had enhanced high-temperature resistance levels. Thus,*MdNUP62* interacts with multiple *MdHSFs* during nucleocytoplasmic transport to regulate flowering and heat resistance in apple. The data provide a new theoretical reference for managing the impact of global warming on the apple industry.

Keywords

Apple, Flowering, heat stress, nuclear pore complex,*MdNup62* , *MdHSFs*

Introduction

Apple (*Malus* × *domestica* Borkh.) is a widely cultivated and economically important fruit crop in temperate regions worldwide owing to its high nutritional value, good storage, and lengthy supply period. Fuji apple is the main cultivar in China, but there are cultivation and production problems, including flowering difficulties and severe alternate bearing (Fan, Zhang, Lei, Chen, Xing, Ma, Zhao & Han, 2016, Guitton, Kelner, Velasco, Gardiner, Chagné & Costes, 2012). However, with global warming, an increase in the average temperature in winter will result in earlier apple flowering (Romanovskaja & Bakšiene, 2009, 刘璐, 郭梁, 李曼华, 傅玮东 & 栾青, 2020), and if there is cold weather in early spring, then significant flower and fruit losses will result. Additionally, at present, extreme hot weather occurs frequently in summer, causing other problems, such as growth impairment and production decline (Yao, Song, Wang, Song, Jiao, Wang & Zheng, 2020, ZHOU, SUN, LIU, JIN, ZHANG & WEI, 2016), which have seriously affected the development of the apple industry in China.

Floral induction pathways have been extensively studied, and six signalling pathways, photoperiodic, vernalization, autonomic, gibberellin, temperature-sensitive, and age, regulate flowering in the model plant *Arabidopsis thaliana* (Bäurle & Dean, 2006, Komeda, 2004, Teotia & Tang, 2015). In apple, the functions of some key flowering-related genes have been well studied in recent years, such as*APETALA1* (*AP1*), *LEAFY (LFY*), *FLOWERING LOCUS T* (*FT)* , and *TERMINAL FLOWER 1* (*TFL1*). For instance, overexpression of *MdMADS5* , a putative homolog of*AP1* , leads to significant early flowering in Arabidopsis (Kotoda, Wada, Kusaba, Kano-Murakami, Masuda & Soejima, 2002). *AFL1* and*AFL2* , two orthologues of *LFY* , have been isolated from apple buds, and their overexpression lines in Arabidopsis flower significantly earlier than wild type (WT), with the overexpression of*AFL2* leading to a more obvious early-flowering phenotype than the overexpression of *AFL1* (Wada, Cao, Kotoda, Soejima & Masuda, 2002). Apple anti-*TERMINAL FLOWER 1* transgenic lines flower significantly earlier than the WT, with the earliest flowering at 8 months, while the WT did not flower for 6 years (Kotoda, Iwanami, Takahashi & Abe, 2006). The overexpression of the apple *FT* gene in Arabidopsis, poplar, and apple results in significantly earlier flowering in these plants, and transgenic poplar and apple flower during in vitro cultivation (Trankner, Lehmann, Hoenicka, Hanke, Fladung, Lenhardt, Dunemann, Gau, Schlangen, Malnoy & Flachowsky, 2010). The overexpression of

MdFT1 and *MdFT2* independently in Arabidopsis results in significantly earlier flowering under both long- and short-day conditions (Li, Tao, Yao, Hao & You, 2010). Through transcriptome analyses, the induction of apple flower buds was found to be regulated by sugar and hormone signalling pathways (Xing, Zhang, Li, Shen, Zhao, Ma, An & Han, 2015). Other omics studies have revealed the molecular mechanisms involved in responses to exogenous treatments, such as sugar (Liu, Feng, Pan, Zhong, Chen, Yuan & Li, 2016), 6-benzylaminopurine (Li, Zhang, An, Fan, Zuo, Zhang, Zhang, Gao, Han & Xing, 2019), and gibberellins (Zhang, Gottschalk & van Nocker, 2019), and their effects on the flowering of apples. However, research on apple flowering is still relatively limited.

A nuclear pore complex (NPC) is composed of a class of nucleoporins (*Nups*) located in the nuclear pore (Tamura, Fukao, Iwamoto, Haraguchi & Hara-Nishimura, 2010). More than 30 *Nups* have been identified in Arabidopsis and 38 members have been identified in apple (Tamura*et al.* , 2010, Zhang, An, Jia, Zhang, Liang, Zhang, Zhou, Ma, Han, Xing & Ren, 2020). Some Nups interact and form three subcomplexes:*Nup62* , *Nup93* , and *Nup107–160* (Tamura *et al.* , 2010, Zhu, Wang, Tang, Hsu, Xie, Du H, Yang, Tao & Zhu, 2017).*Nups* control the transport of substances, such as RNA and proteins, between the nucleus and cytoplasm (Parry, 2013, Zhang, Wang, Kim, Yan, Yan, Pang & Hua, 2020). Proteins rely on importin α, importin β, and Ran-GTP complexes to pass through the NPC and enter the nucleus (Gorlich, Seewald & Ribbeck, 2003, Hill, 2009, Takizawa, Weis & Morgan, 1999). Importin β1 interacts with importin α, Ran-GTP, and*Nup62* directly in Arabidopsis, which further illustrates that plants and humans share a similar nuclear transport mechanism (Luo, Wang, Ji, Fang, Wang, Tian & Li, 2013). *Nups* play important roles in regulating plant growth and development, as well as biotic and abiotic stresses (Parry, 2013, Xu & Meier, 2008, Yang, Wang, Chu, Zhu & Zhang, 2017). For example, *HOS1* , *Nup96* , *Nup54* ,*Nup58* , *Nup62* , *Nup136* , and *Nup160* are important for plant flowering (Cheng, Zhang, Huang, Huang, Zhu, Chen, Miao, Liu, Fu & Wang, 2020, Jung, Park, Lee, To, Kim, Seki & Park, 2013, Lazaro, Mouriz, Piñeiro & Jarillo, 2015, Parry, 2014, Tamura*et al.* , 2010). *HOS1* , *Nup85* , *Nup96* , and*Nup133* participate in abiotic stress pathways (Dong, Agarwal, Zhang, Xie & Zhu, 2006, Dong, Hu, Tang, Zheng, Kim, Lee & Zhu, 2006, Ishitani, Xiong, Lee, Stevenson & Zhu, 1998, Zhang *et al.* , 2020, Zhu *et al.* , 2017). *MOS7* , *Nup96* , *Nup160* , and*Sec1* play important roles in plant immunity (Cheng, Germain, Wiermer, Bi, Xu, García, Wirthmueller, Després, Parker, Zhang & Li, 2009, Roth & Wiermer, 2012, Zhang & Li, 2005), and *Nup96* ,*Nup160* , and *TPR* affect hormone signalling pathways (Jacob, Mongkolsiriwatana, Veley, Kim & Michaels, 2007, Parry, Ward, Cernac, Dharmasiri & Estelle, 2006, Robles, Deslauriers, Alvarez & Larsen, 2012, Wiermer, Cheng, Imkampe, Li, Wang, Lipka & Li, 2012, Xu, Rose, Muthuswamy, Jeong, Venkatakrishnan, Zhao & Meier, 2007).

Heat shock factors (HSFs) are important components of signal transduction and play important roles in diverse stress pathways (Scharfa, Berberich, Ebersberger & Nover, 2012). The HSF family in plants has more members (21 *HSFs* in Arabidopsis) and more complex regulatory mechanisms (Nover, Bharti, Doring, Mishra, Ganguli & Scharf, 2001, Wang, Liu, Yu, Guo, Chen, Jiang, Xu, Fang, Wang, Zhang & Chen, 2020) than in vertebrates (4 *HSFs*) or Drosophila (only 1*HSF*). The structures of plant HSFs are relatively consistent from the N- to C-termini. The N-terminus has a DNA-binding domain, followed by heptad hydrophobic repeats involved in oligomerization, and a nuclear localization signal. The C-terminus contains a nuclear export signal and short peptide motifs. HSFs may be divided into three classes, A, B, and C, on the basis of their structural differences (Nover *et al.* , 2001). Class A has the C-terminal short peptide AHA domain, which has an activator function, while the B and C classes lack this domain (Kotak, Port, Ganguli, Bicker & von Koskull-Döring, 2004). HSFs specifically identify and bind heat shock elements (HSEs), which contain nGAAnnTTCn or nTTCnnGAAn in the downstream target genes' promoters (Littlefield & Nelson, 1999). Class A members (*HSFA1a* , *HSFA1b* ,*HSFA1d* , *HSFA1e* , *HSFA2* , and *HSFA3*) positively regulate plant heat tolerance (Charng, Liu, Liu, Chi, Wang, Chang & Wang, 2007, Nishizawa-Yokoi, Nosaka, Hayashi, Tainaka, Maruta, Tamoi, Ikeda, Ohme-Takagi, Yoshimura, Yabuta & Shigeoka, 2011, Qian, Chen, Liu, Yang, Li & Zhang, 2014, Schramm, Larkindale, Kiehlmann, Ganguli, Englich, Vierling & Von Koskull-Döring, 2008, Tian, Wang, Zhao, Lan, Yu, Zhang, Qin, Hu, Yao, Ni, Sun, Rossi, Peng & Xin, 2020), while, in contrast, Class B HSFs (*HSFB1* and *HSFB2b*) negatively regulate heat-induced HSFs and plant heat tolerance (Ikeda, Mitsuda & Ohme-Takagi, 2011). In addition to responding to heat stress, plant HSFs also participate in other stress

pathways. For instance,*HSFA1a* -overexpression (OE) plants have enhanced tolerance under low/high pH levels and to hydrogen peroxide treatments (Qian *et al.* , 2014). *HsfA2* knockout (KO) mutants (*hsfa2*) and double mutants (*hsfa1a/hsfa1b*) both lose their heat-dependent adaptability to hypoxia (Banti, Mafessoni, Loreti, Alpi & Perata, 2010). Under high-light stress, the photosystem II activity of KO-*hsfa1d/a1e* mutants decreases, while that of WT plants remains high (Nishizawa-Yokoi *et al.* , 2011). The drought and salt tolerance levels of *Oryza sativa* OE-*HSFBb* transgenic lines significantly decrease, but they are significantly enhanced in*OsHSFBb* -RNAi lines (Xiang, Ran, Zou, Zhou, Liu, Zhang, Peng, Tang, Luo & Chen, 2013). Apple *HSFA8a* regulates the synthesis of flavonoids, thus enhancing the drought tolerance of apple (Wang *et al.* , 2020). In addition, some HSFs (*HSFA2* , *HSFA1E* , and*HSFA4C*) appear to be involved in plant flowering pathways (Chen, Zhao, Ge, Han, Ning, Luo, Wang, Liu, Zhang & Wang, 2018, Liu, Feng, Gu, Deng, Qiu, Li, Zhang, Wang, Deng, Wang, He, Bäurle, Li, Cao & He, 2019). Thus, HSFs may play important roles in plant development and stress tolerance.

Currently, there are no reported functional studies of Nups in apple.*Nup62* is a member of the *Nup62* subcomplex in the central core of the nuclear pore (Tamura *et al.* , 2010, Zhang *et al.* , 2020), and *nup62 A. thaliana* mutants have been reported to flower early, indicating *Nup62* 's involvement in flowering pathways (Parry, 2014). In this study, we characterized apple*Nup62* , which showed a high transcription level at the flower bud developmental stage and was responed to high temperature. The overexpression of *MdNup62* in Arabidopsis resulted in earlier flowering compared with WT. Moreover, The overexpression of*MdNup62* in Arabidopsis and tomato both reduced heat resistance. Further, we performed a yeast two-hybrid (Y2H) sieve library experiment to screen for proteins that interact with *MdNup62* , and the interactions between *MdNup62* and the *MdHSFs* were confirmed. And the overexpression of *MdHSFA1d* and *MdHSFA9b*independently in Arabidopsis resulted in earlier flowering and enhancing heat resistance. Thus, *MdNup62* and the *MdHSFs* regulate flowering and respond to temperature changes. These results provide a theoretical reference for managing the impact of global warming on the apple industry.

Materials and methods

Plant materials and growth conditions

The plant materials were 6-year-old apple trees ('Fuji' /T337/*Malus robusta* Rehd.) growing in the experimental orchard of the Horticulture College of Northwest A & F University (108°04' E, 34deg16' N). We collected new shoots (2–3 mm in diameter) near the tips, fully expanded leaves near buds, flower buds, blooming flowers, and young fruit, which were immediately frozen in liquid nitrogen and stored at -80degC for later use.

The 'Fuji' plants were grown on MS medium containing 0.1 mg*L^{-1} indolebutyric acid and 0.6 mg*L^{-1} 6-benzylaminopurine under long-day conditions (16h-light/8h-dark) at 24degC and were subcultured every 45 days. Arabidopsis plants were grown under long-day conditions (16h-light/8h-dark) at 22degC. Tomato plants ('Ailsa Craig') were grown under long-day conditions (16h-light/8h-dark) at 25degC.

Heat map, protein alignment, and phylogenetic analysis

Based on RNA-seq data of our laboratory, the heat map of apple different tissues was constructed using MeV (Multiple Experiment Viewer) software. A protein sequence alignment of *Nup62* from six Rosaceae plants was performed using DNAMAN software. The *Nup62* protein sequences were obtained from the GDR database (https://www.rosaceae.org/). The phylogenetic tree was constructed using MEGA-X software.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from apple trees, Arabidopsis seedlings, tomato seedlings, and apple seedlings using an RNA Plant Plus Reagent Kit (TIANGEN, Beijing, China). The RNA was used as the template to synthesize cDNA with a PrimeScript RT Reagent Kit (Takara, Shiga, Japan). The qRT-PCR analysis was conducted on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA). The reaction solution contained 10 μL SYBR Green I Master Mix (CWBIO, Beijing, China), 0.5 μmol·L^{-1} primers (SANGON BIOTECH, Shanghai, China), and 1 μL each template in a total volume of 20 μL. The PCR program was

as follows: 95°C for 3 min; 40 cycles of 94°C for 15 s, 60°C for 20 s, and 72°C for 15 s. All the samples were analysed with three biological replicates, each comprising three technical replicates. Relative gene expression levels were calculated in accordance with the 2^{-Ct} method (Livak & Schmittgen, 2001). The primers used for qRT-PCR (Table S4)

were synthesized by the Sangon Biotechnology Co. Ltd. (Shanghai, China).

Subcellular localization

The open reading frames (ORFs) of the *MdNup62* , *MdHSFA1d* , and *MdHSFA9b* genes were inserted independently into the pCAMBIA2300-EGFP vector to generate the 35S::*MdNup62* -EGFP, 35S::*MdHSFA1d* -EGFP, and 35S::*MdHSFA9b* -EGFP recombinant plasmids, respectively. These recombinant plasmids were inserted independently into *Agrobacterium tumefaciens* strain GV3101 cells. The GV3101 cells containing these recombinant plasmids were then infiltrated into tobacco leaves. GV3101 cells containing the pCAMBIA2300-EGFP vector (35S::EGFP) served as the control. After an additional 3 days of growth in the dark, green fluorescent protein (GFP) signals in transformed tobacco leaves were detected using a Leica TCS SP8 SR Laser Scanning Confocal Microscope (Leica, Germany).The primers used are listed in Table S5.

Genetic transformation

The genetic transformations were performed in accordance with published methods for Arabidopsis (Clough & Bent, 1998) and tomato ('Ailsa Craig') (Liu, Sun, Liu, Shi, Chen & Zhao, 2019) plants. The transgenic Arabidopsis and tomato lines were selected on MS plates supplemented with 50 $mg{\cdot}L^{-1}$ and 100 $mg{*}L^{-1}$kanamycin, respectively.

Yeast two-hybrid (Y2H) assay

The *MdNup62* $^{508–613}$ truncated sequence was cloned into the pGBKT7 vector to generate the*MdNup62* $^{508–613}$-pGBKT7 recombinant plasmid. The MdHSFAs' ORFs were inserted individually into the pGADT7 vector to generate the MdHSFAs-pGADT7 recombinant plasmids. The recombinant plasmids were inserted into Gold Yeast Two-Hybrid cells, which were then grown on a selective medium. The primers used are listed in Table S5.

Split luciferase (LUC) complementation

The full-length*MdHSFA1d*and *MdHSFA9b* coding sequences were cloned independently into the CLUC vector, while *MdNup62* was cloned into the NLUC vector. The split-LUC complementation assay was performed with tobacco leaves. The reconstituted LUC activity was detected in the dark using a Princeton Lumazone Pylon 2048B cooling camera (Princeton, USA). The LUC activity was quantified using the Dual-Luciferase Reporter Assay System (Promega, USA). The primers used are listed in Table S5.

Pull-down assays

The ORFs of *MdNup62* and *MdHSFA9b* were cloned into the pET-28a and pGEX-6p-1 vectors, respectively, and subsequently overexpressed independently in *Escherichia coli* BL21(DE3) (Transgene). The pull-down assays were conducted using the His-Tagged Protein Purification Kit (Clontech) in accordance with the manufacturer's instructions. The primers used are listed in Table S5.

Heat-tolerance assays

The 'Fuji' plants at 30 days after propagation were used for the 45degC heat treatment. We collected leaf samples before and at 1, 3, and 6 h after the treatment. The samples were immediately frozen in liquid nitrogen and stored at -80degC for later use.

Two-week-old transgenic Arabidopsis and 3-week-old transgenic tomato were used for the heat treatment in an artificial climate chamber. OE-*MdNup62 A. thaliana* lines were subjected to 45degC for 12 h, and

OE-*MdHSFA9b* and OE-*MdHSFA1d* *A. thaliana*lines were subjected to 45degC for 16 h. OE-*MdNup62* tomato lines were subjected to 45degC for 14 h.

Evaluation of stress tolerance

The superoxide dismutase, peroxidase, and catalase activities and the malondialdehyde and H_2O_2 levels were detected using the corresponding Suzhou Comin Biotechnology test kits (Suzhou Comin Biotechnology Co., Ltd, Suzhou, China). The presence of O^{2-} in leaf samples was determined by staining with nitro blue tetrazolium.

Statistical analyses

Statistical analyses were performed using SPSS software. Data are reported as means +- SDs. Asterisks (*) indicate significant differences between treatments as assessed by Student's t-test at $P < 0.05$ (*) and $P < 0.01$ (**). Different lowercase letters above the bars indicate significant differences ($P < 0.05$, Tukey's test).

Results

Apple NPC structure and composition, and its expression patterns

Compared with vertebrate, apple NPC consists of 38 NUP proteins, but missing some Nups, such as Nup153, Nup358, Pom121, etc. Refer to the structure of vertebrate NPC (Tamura *et al.* , 2010), we devided apple NPC into five parts: Cytoplasmic filaments (Nup214 and Nup88), Cytoplasmic and Nuclear ring (Nup98, RAE1, and Nup107-160 Subcompex), Scaffold and central channal (GP210, NDC1, Nup62 Subcompex, and Nup93 Subcompex), Nuclear basket (Nup50 and Nup136), and Distal ring (Tpr/NUA), as well as GLE1, ALADIN, CG1, and HOS1 also participate in NPC constitution (Figure 1a). Additionally, MdNup62 interacts with MdNup54, forming the central apple NPC channel involving in nucleocytoplasmic transport (Figure S1) (Zhang *et al.* , 2020).

We examined the expression patterns of NPC components in different tissues of several apple varieties (Figure 1b; Table S1). The expression levels of *MdNup62* as central channel component showed significantly higher in buds, stem, roots than in fruit of apples, but other channel component *MdNup54* showed significantly low expression levels in all tissues compared with *MdNup62* , indicating that *MdNup62* play a key role in regulation of growth and stress response by controlling nucleocytoplasmic transport in apple.

Feature, expression, and subcellular localization analyses of*MdNup62*

We initially performed a simple bioinformatics analysis of*MdNup62* . A phylogenetic tree of *Nup62* from six Rosaceae plants (*Rosa chinensis* , *Pyrus communis* ,*Prunus persica* , *M. domestica* , *Rubus occidentalis* , and *Fragaria vesca*) was constructed using MEGA-X. *MdNup62*was most closely related to the *Nup62* of pear (Figure 2a). The aligned protein sequences revealed a conserved Nsp1_C domain (Figure 2b). The subcellular localization of *MdNup62* was determined by introducing 35S::*MdNup62* -GFP into tobacco leaves (Figure 2c). Tobacco leaves transformed with the empty vector 35S::GFP were used as controls. In the tobacco leaves expressing 35S::*MdNup62* -GFP, the GFP signal was observed only in the nuclear pore, while the GFP signal was detected throughout the control tobacco leaf cells, indicating that*MdNup62* localized to the nuclear pore.

The transcript levels of *MdNup62* in different tissues were determined using qRT-PCR (Figure 2d). The highest expression level was in flower buds. An *MdNup62* expression analysis during the flower bud developmental stages revealed that the expression level was stable at 30 to 60 days after flowering and reached its highest level at 70 days after flowering (Figure 2e). Thus, *MdNup62* maintained a high expression level during flower bud induction, indicating that it may be related to bud differentiation in apple.

We exposed apple tissue-cultured seedlings to a heat treatment. The reactive oxygen species (ROS) accumulation in leaves increased from 0 to 6 h under heat-treatment conditions (Figure 2f). Moreover, the expression level of *MdNup62* was determined at different times during the high-temperature treatment (Figure 2g). *MdNup62* was significantly induced by high temperature, and its expression level was highest at 1

h after exposure to the high temperature. Thus,*MdNup62* may be involved in the heat-resistance pathway of apple.

Overexpression of *MdNup62* promotes flowering

To confirm *MdNup62* 's role in flowering, we performed an Agrobacterium-mediated genetic transformation of *MdNup62* into*A. thaliana* . We found that OE-*MdNup62* lines flowered significantly earlier than WT (Figure 3a). Additionally, OE-*MdNup62* lines had significantly fewer rosette leaves than WT during bolting (Figure 3b). The presence of the transgene in OE-*MdNup62* lines was confirmed using genomic PCR (Figure S2a), semi-quantitative RT-PCR (Figure 3c), and qRT-PCR (Figure 3d). The transcript levels of flowering-related genes were analysed by qRT-PCR (Figure 3e). The expression levels of *AtFT* , *AtLFY* , and*AtAP1* significantly increased in OE-*MdNup62* lines compared with WT. This demonstrated that the overexpression of *MdNup62*promoted flowering in Arabidopsis.

Overexpression of *MdNup62* reduces high-temperature resistance

Because *MdNup62* was induced by high temperature, we investigated the high-temperature resistance function of *MdNup62* . OE-*MdNup62* Arabidopsis lines were subjected to a high-temperature (45degC) treatment (Figure 4a). Additionally, the survival rate of transgenic Arabidopsis was significantly lower than that of WT (Figure 4b). We also performed a qRT-PCR analysis of *A. thaliana* HSPs (*AtHSP101* , *AtHSP22-ER* , *AtHSP21.0* , and*AtHSP70T-2*) (Figure 4c). Their expression levels in transgenic Arabidopsis were reduced under high-temperature conditions. Consistently, after the heat treatment, the ROS accumulation in leaves was clear greater in OE-*MdNup62* lines compared with WT (Figure 5a). In addition, the malondialdehyde and H_2O_2 levels were significantly greater than in WT (Figure 5b, c). Moreover, the superoxide dismutase, peroxidase, and catalase activities were lower in OE-*MdNup62*lines than in WT (Figure 5d–f). High-temperature resistance assays were carried out in transgenic tomato plants (Figure 6a). As in transgenic*A. thaliana* , the survival rate of transgenic tomato was significantly reduced compared with WT (Figure 6b). The presence of the transgene in OE-*MdNup62*lines was confirmed by genomic PCR, and qRT-PCR (Figure 6c,d). The expression levels of *HSPs* (*HSP101* , *HSP22-ER* ,*HSP21.0* , and *HSP70T-2*) in transgenic tomato were significantly reduced under high-temperature conditions compared with under normal growth conditions (Figure 6e). These results indicate that*MdNup62* reduces plant high-temperature resistance.

MdNup62-interacting protein screening

To further reveal the function of *MdNup62* , we conducted a Y2H sieve library experiment using a *MdNup62* truncated body (*MdNup62*$^{508-613}$*-pGBKT7*) that is not self-activated. We identified 62 putative *MdNup62* -interacting proteins (Table S3). Some transcription factors were identified, such as HSFs (*MdHSFA1d* , *MdHSFA1e* , *MdHSFA9* , *MdHSF30* ,*MdHSF1* , and *MdHSF8*), as well as *MdMYB21* ,*MdMYC2* , *MdGATA11* , and *MdBAK1* . In addition, some enzymes and other functional genes were found. Because transcription factors that have transcriptional regulatory functions must be transported into the nucleus, and because *MdNup62* has regulatory effects on the transport of the proteins, we hypothesized that*MdNup62* interacts with these *MdHSF* s and controls their transport.

MdNup62 interacts with MdHSFs

We cloned parts of the *MdHSF* s (*MdHSFA1a/b/d/e* and*MdHSFA9a/b*) independently into the pGADT7 vector and then cotransformed each with *MdNUP62*$^{508-613}$*-pGBKT7* .*MdNup62* interacted with these *MdHSF* s (Figure 7a).Additionally, we used *MdHSFA9b* in pull-down assays. The recombinant *MdNup62-HIS* fusion protein was purified with*MdHSFA9b-GST* , but not with GST alone (Figure 7b). The split-LUC complementation assay revealed that the co-expression of*MdNup62-NLUC* with *MdHSFA1d* -CLUC or *MdHSFA9b* -CLUC resulted in a higher LUC activity than the other combinations (Figure 7c–e). These results confirmed the interaction between *MdNup62*and both *MdHSFA1D* and *MdHSFA9b* .

Feature, expression, and subcellular localization analyses of*MdHSFA9b* and *MdHSFA1d*

Phylogenetic tree analysis showed that Apple and Arabidopsis HSFs were divided into four groups(I,II,III,IV), with *MdHSFA1a/b/d/e* in groupII, and *MdHSFA9a/b* in groupI(Figure 8a). We also examined the expression patterns of*MdHSFs* in different tissues of several apple varieties (Figure 8b; Table S2). And the expression levels of MdHSFA1a/b/d/e

and MdHSFA9a/b showed significantly higher in buds.

The subcellular localizations of *MdHSFA9b* and *MdHSFA1d*were studied by independently introducing 35S::*MdHSFA9b* -EGFP and 35S::*MdHSFA1d* -EGFP, respectively, into tobacco leaves (Figure 8c). Tobacco leaves transformed with the empty vector 35S::EGFP served as controls. In the tobacco leaves expressing 35S::*MdHSFA9b* -EGFP and 35S::*MdHSFA1d* -EGFP, the GFP signals were observed in both the nucleus and cytoplasm, while the GFP signal was detected throughout the control tobacco leaf cells, indicating that *MdHSFA9b* and*MdHSFA1d* localized to both the nucleus and cytoplasm. The co-localization of *MdNup62* with both *MdHSFA9b* and*MdHSFA1d* further verified their interactions.

A tissue-specific expression analysis revealed that *MdHSFA1d* was expressed highest in flower buds and stems. The highest expression level of *MdHSFA9b* was in stems, but the expression levels in the other tissues were also high. Subsequently, the expression levels of MdHSFA9b and MdHSFA1d remained high during the flower bud developmental stages, while the highest was at 70 days after flowering (Figure 8d). These results indicated that *MdHSFA9b* and *MdHSFA1d* maintained high expression levels during flower bud induction, suggesting that they may be involved in the bud differentiation of apple.

Overexpression of *MdHSFA9b* and *MdHSFA1d* promotes flowering

To verify the flowering phenotype of HSFs, we performed Agrobacterium-mediated genetic transformations of *MdHSFA9b* and*MdHSFA1d* into *A. thaliana* . Like OE-*MdNup62* , OE-*MdHSFA9b* and OE-*MdHSFA1d* lines flowered significantly earlier than WT (Figures 9a and S3a). Additionally, they also had significantly fewer rosette leaves than WT during bolting (Figures 9b and S3b). We also performed genomic PCR (Figure S2b, c), semi-quantitative RT-PCR (Figures 9c and S3c), and qRT-PCR (Figures 9d and S3d) to confirm the presence of the transgene in the OE-*MdHSFA9b* and OE-*MdHSFA1d* lines. The transcript levels of *AtFT* , *AtLFY* , and *AtSOC1* were significantly increased in OE-*MdHSFA9b* and OE-*MdHSFA1d* lines compared with WT (Figures 9e and S3e).

Overexpression of *MdHSFA9b* and *MdHSFA1d* enhances high-temperature resistance

To study the high-temperature resistance phenotypes of *MdHSFA9b*and *MdHSFA1d* , we also exposed OE-*MdHSFA9b* and OE-*MdHSFA1d* transgenic plants, respectively, to high-temperature (45degC) conditions (Figures 10a and S4a). The survival rates of OE-*MdHSFA9b* and OE-*MdHSFA1d* lines were significantly greater than that of WT (Figures 10b and S4b). Consistently, the ROS accumulation in leaves decreased in transgenic plants after the high-temperature treatment (Figure S5a, b). We also performed a qRT-PCR analysis of *A. thaliana HSPs* (*AtHSP101* ,*AtHSP22-ER* , *AtHSP21.0* , and *AtHSP70T-2*) (Figures 10c and S4c), and their expression levels in transgenic *A. thaliana*increased under high-temperature conditions compared with under normal growth conditions. These results indicated that *MdHSFA9b* and*MdHSFA1d* enhance plant high-temperature resistance.

Discussion

Plant flowering has always been an important topic in crop and horticultural sciences, and issues with apple flowering have long hindered the development of the apple industry in China (Fan *et al.* , 2016, Guitton *et al.* , 2012). The Nups control protein transport between the nucleus and cytoplasm, and they participate in a variety of biological processes, including flowering (Parry, 2013, Zhang *et al.* , 2020).*HOS1* regulates the binding of some nuclear genes to*FLOWERING LOCUS C* (*FLC*) chromatin at low temperatures and weakens the transcriptional inhibition of *FLC* by HDA6 (Jung*et al.* , 2013). In *A. thaliana* , *Nup96* promotes the stability of *HOS1* , and *HOS1* conjugates and degrades*CO* , then promotes *FLC* expression, leading to delayed flowering. In addition, *HOS1* increases the stability of*Nup96* and thus maintains this regulatory pathway to control the flowering time (Cheng *et al.* , 2020, Lazaro *et al.* , 2015). Mutations in *Nup54* ,

Nup58 , *Nup62* , *Nup136* , and *Nup160* have resulted in a prominent earlier flowering phenotype compared with WT (Parry, 2014, Tamura *et al.* , 2010). In the present study, *MdNup62* maintained a high expression level during flower development. To verify the flowering function of*MdNup62* , we determined the flowering phenotypes of OE-*MdNup62 A. thaliana* lines. Interestingly, the phenotypes of the overexpression lines were consistent with Arabidopsis deletion mutants and showed obvious early flowering. Previous studies found that both *Nup62* deletion mutants and overexpression strains of Arabidopsis have increased the sensitivities to auxin, indicating that the overexpression does not result in a functional gain, but rather a functional loss, like the mutant (Boeglin, Fuglsang, Luu, Sentenac, Gaillard & Cherel, 2016). Therefore, the overexpression of*MdNup62* in this study may also result in a functional loss. However, *MdNup62* is involved in the flowering pathway.

With global warming, extreme high-temperatures will occur more frequently, which will seriously affect the normal growth and development of plants (Yao *et al.* , 2020, ZHOU *et al.* , 2016). And Nups are involved in temperature-stress responses. HOS1 is an important negative regulator of cold-signal transduction in plant cells, and *hos1-1* has poor cold resistance compared with WT (Ishitani*et al.* , 1998). *HOS1* specifically binds to and degrades*ICE1* , thereby reducing cold resistance (Dong *et al.* , 2006). The expression levels of cold-resistance-related genes, such as*CBF,* in *atnup160* mutants were impaired and seedlings grew slowly, especially at low temperatures (Dong *et al.* , 2006). *Nup85* and *Nup133* control mRNA output only under warm conditions and are more sensitive to transcription factor localization at warm temperatures (Zhang *et al.* , 2020). In this study, *MdNup62* responded to high-temperature stress in apple. However, OE-*MdNup62* lines had reduced high-temperature resistance in both Arabidopsis and tomato. By analysing the relative expression levels of *HSPs* (*HSP101* , *HSP22-ER* , *HSP21.0* , and *HSP70T-2*) in transgenic plants, we found no obvious correlations between OE-*MdNup62* lines and WT at a normal growth temperature, but OE-*MdNup62* lines had significantly lower HSP expression levels than WT under high-temperature conditions.

In plants, Nup-interacting proteins have been studied (Cheng *et al.* , 2020, Zhang *et al.* , 2020, Zhu *et al.* , 2017), and some potential *Nup85* -interacting proteins have been identified by immunoprecipitation and subsequent mass spectrometry in Arabidopsis, such as the *Nup107–160* subcomplex (*Nup160* , *Nup133* ,*Nup43* , *Nup96* , *Nup107* , *Seh1* , and*Sec13*), several mediator subunits (*MED16* , *MED14* , and *MED18*), *HOS1* , and *Sec13A* . The interactions between *Nup85* and three proteins, *HOS1* , *Sec13A* , and*MED18* , have been confirmed (Zhu *et al.* , 2017). Additionally, a direct interaction between *Nup96* and *HOS1*in Arabidopsis has also been reported (Cheng *et al.* , 2020). In our previous study, the interaction between *MdNup54* and*MdNup62* was confirmed in apple (Zhang *et al.* , 2020). However, there are no reports of direct interactions between transcription factors and Nups in plants. We previously identified an interaction between apple *MdNup54* and *MdKNAT4/6* using a yeast double-hybridization test, but further verification is needed (Zhang *et al.* , 2020). In this study, we verified direct interactions between *MdNup62* and *MdHSF* s, indicating that the *Nups* may directly recognize related transcription factors and thus regulate their transport. This provides a new direction of study for Nups.

Because of the early flowering of OE-*MdNup62* Arabidopsis lines, *MdHSF* s that interact with*MdNup62* may be also involved in the flowering pathway. Consistent with this conjecture, some HSFs are associated with flowering (Chen*et al.* , 2018, Liu *et al.* , 2019). *HSFA1E* and*HSFA4C* directly target and positively regulate the flowering gene*SOC1* in lettuce (Chen *et al.* , 2018). Arabidopsis*HSFA2* directly targets and promotes the expression of*REF6* , and the *REF6–HSFA2* loop directly targets and activates *HTT5* , which coordinates early flowering (Liu *et al.* , 2019). In this study, we found that *MdHSFA9b* and*MdHSFA1d* maintained high expression levels during flower bud induction. Additionally, OE-*MdHSFA9b* and OE-*MdHSFA1d*Arabidopsis lines flower significantly earlier than WT. This suggests that *MdHSFA9b* and *MdHSFA1d* promote plant flowering.*MdNup62* , *MdHSFA9b* , and *MdHSFA1d* share the same flowering phenotype, possibly because the overexpression of*MdNup62* fosters HSF accumulation in the nucleus, promoting the expression of downstream flowering-related genes and advancing flowering.

HSFs play important roles in regulating plant resistance to high temperatures. *HSFA1* positively regulates

the heat tolerance of tomato, the expression of *HSFA2* is dependent on *HsfA1* , and the thermotolerance of the posttranscriptional silencing of the*HsfA1* gene in protoplasts can be restored by plasmid-borne*HsfA2* (Mishra, 2002). In Arabidopsis, *HSFA3* directly up-regulates the expression of *Hsp18.1-Cl* and *Hsp26.5-MII* , and both *HSFA3* mutants and RNAi significantly reduce high temperature resistance (Schramm *et al.* , 2008). The overexpression of *HsfA1a* increases *Hsp18.2* and *Hsp70* expression levels, as well as heat-shock tolerance (Qian *et al.* , 2014).*HSFA1b* regulates high-temperature resistance through *OPR3*and the jasmonate signalling pathway (Tian *et al.* , 2020).*HSFA1d* and *HSFA1e* activate *HsfA2* transcription, and a double knockout of *HSFA1d* and *HSFA1e* impairs tolerance to heat-shock stress (Nishizawa-Yokoi *et al.* , 2011). In Arabidopsis, *HsfA2* acts as a heat-induced transactivator to maintain the expression levels of *HSPs* and prolong the duration of acquired thermotolerance (Charng *et al.* , 2007). In*Medicago truncatula* , *HSFA9* plays important roles in thermotolerance (Zinsmeister, Berriri, Basso, Ly Vu, Dang, Lalanne, Da Silva, Leprince & Buitink, 2020). In the current study, we obtained similar results for *MdHSFA9b* and *MdHSFA1d* . The expression levels of *HSPs* in the two overexpression Arabidopsis lines were significantly greater than in WT, and both lines had enhanced high-temperature resistance levels. Like the flowering and auxin phenotypes (Boeglin *et al.* , 2016), the opposite phenotypes between OE-*MdNup62* and OE-*MdHSFA9b,* OE-*MdHSFA1d*indicates that the overexpression of *MdNup62* may also result in a lack of function under heat-stress conditions. Similar to the results of this study, Zhang et al. (2020) found that *nup85* and*nup133* increase the ubiquitous protoplast (nucleus and cytosol) signals of *IAA17* and *PIF4* at 28degC compared with at 22degC. Furthermore, the *nup96* and *hos1* mutants show significant increases in the ubiquitous localizations of *IAA17* and*PIF4* signals at 28degC (72% and 66%, respectively) compared with 22degC (40% and 49%, respectively)(Zhang *et al.* , 2020, 北京爱琴海乐之技术有限公司, 2005)(北京爱琴海乐之技术有限公司, 2005; Zhang *et al.* , 2020)(Zhang *et al.* , 2020). Thus, the nuclear accumulations of the *IAA17* and *PIF4* proteins in*nup85* , *nup96* , *nup133* , and *hos1* are reduced compared with WT, and the defects are more severe at 28°C. Therefore, we hypothesized that the transport of *MdHSFA9b* , *MdHSFA1d* , and other *MdHSF* s is inhibited in OE-*MdNup62* lines at high temperatures, resulting in the inhibition of the transcription of downstream *HSP* s, which further reduces high-temperature resistance.

On the basis of these findings, we constructed a hypothetical model of*MdNup62*- related pathways involved in high-temperature resistance (Figure 11). At normal temperature, apple *MdHSFs* were not induced, and not much transported into nucleus that cannot lead to up-regulate expression of *MdHSPs* in WT and OE-*MdNup62* . However, at high temperature, apple *MdHSFs* were significantly induced, and then transported into the nucleus through NPC channels to promote the expression of *MdHSPs* in WT, in which enhanced high-temperature resistance. But for OE-*MdNup62* lines, the structure of the apple NPC changed, and blocked the transport of high temperature induced *MdHSFs* into the nucleus that cannot induce much *MdHSPs* expression causing heat injuring (Figure 11). Additionally, OE-*MdNup62* , OE-*MdHSFA9b* and OE-*MdHSFA1d* lines showed significant early flowering phenotype compared with WT (Figure 3, 9; Figure S3).

In conclusion, temperature is an important factor affecting flowering. With global warming, apple flowering will occur earlier, increasing the risk of chilling-related injury. Moreover, extreme hot weather is also occurring frequently. Both climatic conditions seriously affect the development of the apple industry. *MdNup62* interacts with*MdHSFs* to regulate flowering and heat-resistance pathways in plants. Thus, both *MdNup62* and the *MdHSFs* regulate flowering and respond to temperature changes. This research provides a theoretical reference for managing the impact of global warming on the apple industry.

Author contributions

Libo Xing and Chenguang Zhang conceived and designed the experiment. Chenguang Zhang, Peng Jia, Na An, Wei Zhang, Jiayan Liang, Hua Zhou performed the experiment. Chenguang Zhang and Peng Jia analyzed the data. Chenguang Zhang and Libo Xing wrote the manuscript.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Figure legends

Figure 1. The nuclear pore complex (NPC) structure and composition in *Vertebrate* and *Malus*.

(a) A schematic of the nuclear pore with the cytoplasmic side at the top and the nuclear basket at the bottom for Vertebrate (left) and Malus (right).

(b) Tissue specific expression patterns of apple NPC components by RNA-seq. The full names of the different abbreviations are as follows, 'Nagafu No.2' long branches flower buds (FLB), 'Nagafu No.2' short branches flower buds (FSB), 'Nagafu No.2' axillary buds (FYB), 'Qinguan' axillary buds (QYB), 'Nagafu No.2' fruit (FR), 'Yanfu No.3' stem tip (YF3J), 'Yanfu No.6' stem tip (YF6J), and 'M9-T337' root (T337R). Each number after the abbreviation represents a biological repetition.

Figure 2. Identification and analysis of *MdNup62*.

(a) Phylogenetic analysis of Rosaceae *Nup62* . (b) The conservative domain of Rosaceae *Nup62* . (c) Subcellular localization of*MdNup62* . The upper panel shows 35S::EGFP, and the lower panel shows 35S::MdNup62-EGFP. (d) and (e) Analyses of *MdNup62*expression levels in diverse 'Nagafu No. 2' apple tissues (d) and in different flower bud developmental stages of 'Nagafu No. 2' (e). (f) The phenotype of 'Nagafu No. 2' tissue-cultured seedlings (upper panels) and the in situ accumulation of superoxide radical (O^{2-}) at 0, 1, 3, and 6 h under heat treatment conditions (lower panels). Bar = 1 cm. (g)*MdNup62* expression levels in 'Nagafu No. 2' tissue-cultured seedling leaves at 0, 1, 3, and 6 h under heat-treatment conditions. Each sample was analysed with three biological replicates, each comprising three technical replicates. Means followed by different lowercase letters are significantly different at the 0.05 level.

Figure 3. *MdNUP62* promotes flowering in Arabidopsis.

(a) Phenotype of the *MdNUP62* -overexpression Arabidopsis line for flowering time. Bar = 2 cm. (b) Statistical analysis of rosette leaves of *Arabidopsis thaliana* during bolting. Asterisks denote significant differences as determined by a t-test (*P $<$ 0.05). (c) Semi-quantitative RT-PCR analysis of *MdNup62* expression in Arabidopsis samples. (d) qRT-PCR analysis of *MdNup62* expression in Arabidopsis samples. Asterisks denote significant differences as determined by a t-test (*P $<$ 0.01). (e) Relative expression levels of flowering genes (*AtFT* , *AtLFY* , *AtSOC1* , and*AtAP1*) in WT and *MdNup62* -overexpression lines. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (*P $<$ 0.05).

Figure 4. *MdNup62*reduced high-temperature resistance in Arabidopsis.

(a) Phenotype of the *MdNup62* -overexpression Arabidopsis line for high-temperature resistance. Bar = 1cm. (b) Survival rates of WT and*MdNup62* -overexpression Arabidopsis lines after the high-temperature treatment. Asterisks denote significant differences as determined by a t-test (**P $<$ 0.01). (c) Relative expression levels of high-temperature resistance-related genes (*AtHSP101* ,*AtHSP22.0-ER* , *AtHSP21* , and

AtHSP70T-2) in WT and*MdNup62* -overexpression lines at the normal (22degC) temperature and 1 h after exposure to the high-temperature (37degC) treatment. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (**P < 0.01).

Figure 5. Changes in the level of accumulated ROS and activities of ROS-scavenging enzymes in *OE-MdNup62* and WT Arabidopsis leaves under heat-stress conditions.

(a) In situ accumulations of superoxide radicals (O^{2-}) before (upper panels) and after (lower panels) heat treatment as revealed by nitro blue tetrazolium staining. (b) and (c) Quantitative measurement of H_2O_2 and malondialdehyde concentrations in Arabidopsis leaves treated with and without the high temperature. (d)–(f) Activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) at 6 h after the heat treatment. Each sample was analysed with three biological replicates, each comprising three technical replicates. Means followed by different lowercase letters are significantly different at the 0.05 level.

Figure 6. *MdNup62* reduced high-temperature resistance in tomato.

(a) Phenotype of the *MdNup62* -overexpression tomato line for high-temperature resistance. Bar = 5 cm. (b) Survival rates of WT and*MdNup62* -overexpression tomato lines after the high-temperature treatment. Asterisks denote significant differences as determined by a t-test (**P < 0.01). (c) qRT-PCR analysis of *MdNup62*expression levels in tomato samples. Asterisks denote significant differences as determined by a t-test (**P < 0.01). (d) Genomic PCR analysis of *MdNup62* transgenic tomato lines. (e) Relative expression levels of high-temperature resistance-related genes (*SlHSP101* , *SlHSP22.0-ER* , *SlHSP21* , and*SlHSP70T-2*) in WT and *MdNup62* -overexpression lines at the normal temperature (22degC) and 1 h after exposure to the high-temperature (45degC) treatment. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (*P < 0.05).

Figure 7. *MdNup62* interacts with *MdHSF*s.

(a) Interactions between $MdNup62^{508\text{-}613}$ and*MdHSF* s (*MdHSFA9a/b* and *MdHSFA1a/b/d/e*) in Y2H assays. The $MdNup62^{508\text{-}613}$ truncated sequence was cloned into pGBKT7, whereas *MdHSFA* s (*MdHSFA9a/b* and*MdHSFA1Da/b/d/e*) were cloned independently into the pGADT7 vector. Empty pGADT7 plus $MdNup62^{508\text{-}613}$ -pGBKT7 was used as the control. (b) Interactions between *MdNup62* and*MdHSFA9b* in the pull-down assay. Western blotting with a GST antibody revealed that *MdNup62-HIS* was pulled down by*MdHSFA9b-GST* . (c)–(e) Interactions between *MdNup62* and both *MdHSFA9b* and *MdHSFA1d* in a luciferase (LUC) complementation experiment. Empty NLUC and empty CLUC,*MdNup62* -NLUC plus empty CLUC, empty NLUC plus *MdHSFA9b* , and *MdHSFA1d* -CLUC were used as controls. The LUC complementation experiment was repeated three times, with consistent results. Asterisks denote significant differences as determined by t-tests (** P < 0.01).

Figure 8. Subcellular localization and expression analyses of*MdHSFA9b* and *MdHSFA1d*.

(a) Phylogenetic analysis of *HSFs* in Malus and Arabidopsis.(b) Tissue specific expression patterns of apple *MdHSFs* by RNA-seq. The full names of the different abbreviations are as follows, 'Nagafu No.2' long branches flower buds (FLB), 'Nagafu No.2' short branches flower buds (FSB), 'Nagafu No.2' axillary buds (FYB), 'Qinguan' axillary buds (QYB), 'Nagafu No.2' fruit (FR), 'Yanfu No.3' stem tip (YF3J), 'Yanfu No.6' stem tip (YF6J), and 'M9-T337' root (T337R). Each number after the abbreviation represents a biological repetition. (c) Subcellular localizations of *MdHSFA9b* and *MdHSFA1d* . The upper panel shows 35S::EGFP, the middle panel shows 35S::*MdHSFA9b* -EGFP, and the lower panel shows 35S::*MdHSFA1d* -EGFP. (d) Analyses of *MdHSFA9b* and*MdHSFA1d* expression levels in diverse apple 'Nagafu No. 2' tissues and in different flower bud developmental stages of 'Nagafu No. 2'. Each sample was analysed with three biological replicates, each comprising three technical replicates. Means followed by different lowercase letters are significantly different at the 0.05 level.

Figure 9. *MdHSFA9b* promotes flowering in Arabidopsis.

(a) Phenotype of the *MdHSFA9b* -overexpression Arabidopsis line for flowering time. Bar = 2 cm. (b) Statistical analysis of rosette leaves of *Arabidopsis thaliana* during bolting. Asterisks denote significant differences as determined by a t-test (*P < 0.05). (c) Semi-quantitative RT-PCR analysis of *MdHSFA9b* expression in Arabidopsis samples. (d) qRT-PCR analysis of *MdHSFA9b* expression in Arabidopsis samples. Asterisks denote significant differences as determined by a t-test (**P < 0.01). (e) Relative expression levels of flowering genes (*AtFT* , *AtLFY* , *AtSOC1* , and*AtAP1*) in WT and *MdHSFA9b* -overexpression lines. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (*P < 0.05).

Figure 10. ***MdHSFA9b*** **enhanced high-temperature resistance in Arabidopsis.**

(a) Phenotype of the *MdHSFA9b* -overexpression Arabidopsis line for high-temperature resistance. Bar = 2 cm. (b) Survival rates of WT and*MdHSFA9b* -overexpression Arabidopsis lines after the high-temperature treatment. Asterisks denote significant differences as determined by a t-test (**P < 0.01). (c) Relative expression levels of high-temperature resistance-related genes (*AtHSP101* ,*AtHSP22.0-ER* , *AtHSP21* , and *AtHSP70T-2*) in WT and*MdHSFA9b* -overexpression lines at the normal temperature (22degC) and 1 h after exposure to the high-temperature (45degC) treatment. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (*P < 0.05).

Figure 11. Model of MdNup62 interactions with MdHSFs involved in flowering and heat-stress tolerance in apple.

Supporting Information

Table S1. Expression of NPC components in different tissues of several apple varieties.

The full names of the different abbreviations are as follows, 'Nagafu No.2' long branches flower buds (FLB), 'Nagafu No.2' short branches flower buds (FSB), 'Nagafu No.2' axillary buds (FYB), 'Qinguan' axillary buds (QYB), 'Nagafu No.2' fruit (FR), 'Yanfu No.3' stem tip (YF3J), 'Yanfu No.6' stem tip (YF6J), and 'M9-T337' root (T337R). Each number after the abbreviation represents a biological repetition.

Table S2. Expression of MdHSFs in different tissues of several apple varieties.

Table S3. ***MdNup62*** **yeast double-hybridization screening results.**

Table S4. Primers used for qRT-PCR.

Table S5. Primers used for plasmid construction.

Figure S1. Interactions between MdNup62 and MdNup54 in a luciferase (LUC) complementation experiment.

(a) A schematic of the nuclear pore with the cytoplasmic side at the top and the nuclear basket at the bottom for Vertebrate (left) and Malus (right). MdNup62 interacts with MdNup54 forming the central NPC channel. (b) Interactions between MdNup62 and MdNup54 in a luciferase (LUC) complementation experiment, as well as in our previous study (Zhang et al., 2020). Empty NLUC and empty CLUC, MdNup62-NLUC plus empty CLUC, empty NLUC plus MdNup54-CLUC were used as controls. The LUC complementation experiment was repeated three times, with consistent results.

Figure S2. Genomic PCR analyses of ***MdNup62*** **(a),*MdHSFA9b* (b), and** ***MdHSFA1d*** **(c) in transgenic Arabidopsis lines.**

Figure S3. ***MdHSFA1d*** **promotes flowering in Arabidopsis.**

(a) Phenotype of the *MdHSFA1d* -overexpression Arabidopsis line for flowering time. Bar = 2 cm. (b) Statistical analysis of rosette leaves of *Arabidopsis thaliana* during bolting. Asterisks denote significant

differences as determined by a t-test (*P < 0.05). (c) Semi-quantitative RT-PCR analysis of *MdHSFA1d* expression in Arabidopsis samples. (d) qRT-PCR analysis of *MdHSFA1d* expression in Arabidopsis samples. Asterisks denote significant differences as determined by a t-test (*P < 0.01). (e) Relative expression levels of flowering genes (*AtFT* , *AtLFY* , *AtSOC1* , and*AtAP1*) in WT and *MdHSFA1d* -overexpression lines. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (*P < 0.05).

Figure S4. *MdHSFA1d* enhanced high-temperature resistance in Arabidopsis.

(a) Phenotype of the *MdHSFA1d* -overexpression Arabidopsis line for high-temperature resistance. Bar = 2 cm. (b) Survival rates of WT and*MdHSFA1d* -overexpression Arabidopsis lines after the high-temperature treatment. Asterisks denote significant differences as determined by a t-test (**P < 0.01). (c) Relative expression levels of high-temperature resistance-related genes (*AtHSP101* ,*AtHSP22.0-ER* , *AtHSP21* , and *AtHSP70T-2*) in WT and*MdHSFA1d* -overexpression lines at the normal temperature (22degC) and 1 h after exposure to the high-temperature (37degC) treatment. Each sample was analysed with three biological replicates, each comprising three technical replicates. Asterisks denote significant differences as determined by a t-test (**P < 0.01).

Figure S5. Changes in the levels of accumulated ROS in Arabidopsis leaves under heat-stress conditions . (a) and (b) In situ accumulations of MdHSFA9b (a), MdHSFA1d (b), and superoxide radicals (O^{2-}) before (upper panels) and after (lower panels) the heat treatment as revealed by nitro blue tetrazolium staining. Bar = 1 cm.

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