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miR164g-MsNAC022 acts as a novel module mediating drought response by transcriptional regulation of reactive oxygen species scavenging systems in apple

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Abstract

Under drought stress, reactive oxygen species (ROS) overaccumulate as a secondary stress that impairs plant performance and thus severely reduces crop yields. The mitigation of ROS levels under drought stress is therefore crucial for drought tolerance. MicroRNAs (miRNAs) are critical regulators of plant development and stress responses. However, the complex molecular regulatory mechanism by which they function during drought stress, especially in drought-triggered ROS scavenging, is not fully understood. Here, we report a newly identified drought-responsive miRNA, miR164g, in the wild apple species Malus sieversii and elucidate its role in apple drought tolerance. Our results showed that expression of miR164g is significantly inhibited under drought stress and it can specifically cleave transcripts of the transcription factor MsNAC022 in M. sieversii. The heterologous accumulation of miR164g in Arabidopsis thaliana results in enhanced sensitivity to drought stress, while overexpression of MsNAC022 in Arabidopsis and the cultivated apple line ’GL-3’ (Malus domestica Borkh.) lead to enhanced tolerance to drought stress by raising the ROS scavenging enzymes activity and related genes expression levels, particularly PEROXIDASE (MsPOD). Furthermore, we showed that expression of MsPOD is activated by MsNAC022 in transient assays. Interestingly, Part1 (P1) region is the key region for the positive regulation of MsPOD promoter by MsNAC022, and the different POD expression patterns in M. sieversii and M. domestica is attributed to the specific fragments inserted in P1 region of M. sieversii. Our findings reveal the function of the miR164g-MsNAC022 module in mediating the drought response of M. sieversii and lay a foundation for breeding drought-tolerant apple cultivars.

Introduction

Various abiotic stresses such as drought and high soil salinity inevitably accompany plant growth and development [1–4]. Among them, drought stress severely reduces crop yields by at least 40% worldwide, a proportion that continues to rise due to climate change [5, 6]. Apple (Malus sp.) is one of the most economically important fruit trees and is widely cultivated globally, but apple fruit quality and yield have dramatically declined with the more common occurrence of drought stress [3, 4]. Characterization of the molecular components and signaling pathways related to drought stress represents the first step toward improving drought resistance and developing efficient strategies for breeding drought-tolerant apple cultivars. Malus sieversii Roem., an ancestral species of modern cultivated apples, has become a valuable resource for exploring drought response mechanisms and is the commonly used apple rootstock in some arid and semi-arid regions with exceptional drought stress tolerance [7–9]. However, little is currently known regarding how M. sieversii exhibits such prominent drought tolerance, which severely hampers our ability to breed drought-tolerant apple cultivars.

Drought responses in plants are characterized by reduced leaf water potential and lower turgor pressure, which result in stomatal closure and eventually influence cell growth and elongation [2, 10, 11]. When drought extends beyond a given duration, plant cells start to excessively accumulate reactive oxygen species (ROS), which leads to oxidative damage, including membrane peroxidation, protein denaturation, nucleic acid damage, and ultimately cell death [12, 13]. To counteract the oxidative burst caused by ROS accumulation, plants employ tightly controlled ROS scavenging systems composed of enzymatic and non-enzymatic antioxidants. The enzymatic pathways include superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX), while the non-enzymatic system mainly comprises antioxidants, such as ascorbic acid (AsA), carotenoids, α-tocopherol, and glutathione. Enzyme activity and the contents of these reducing substances are positively correlated with plant resistance to abiotic stress such as drought and salt stress [14, 15]. Therefore, plants with higher drought tolerance are thought to be better equipped at engaging their ROS scavenging systems. For instance, M. sieversii exhibits higher POD and SOD activities under drought stress,
MicroRNAs (miRNAs) are endogenous small single-stranded non-coding RNAs that play vital regulatory roles in plant development. Multiple miRNAs have been reported to cleave transcripts of several drought-responsive genes and hence prevent the accumulation of their encoded proteins [19–23]. For instance, drought stress substantially downregulates the expression of miR169a and miR169c, while the abundance of their target transcript from NUCLEAR FACTOR Y A5 (NF-YAS) accumulates upon drought stress. Transgenic Arabidopsis plants overexpressing NF-YAS display enhanced drought resistance [24]. The abundance of miR165/166 and their target transcript β-1,3-GLUCANASE 1 (BG1) also respond to drought stress. Lower miR165/166 expression leads to a greater accumulation of BG1 transcripts and thus abscisic acid (ABA) contents, ultimately enhancing drought tolerance in Arabidopsis [25].

The miR164 family was first identified as a class of miRNAs that function in plant growth and development in Arabidopsis, various miR164 loci participate in the development of lateral root and shoot apical meristems, the establishment of the cotyledon boundary and floral organs, fruit ripening, and pathogen-induced and age-dependent cell death [26–34]. Roles for miR164 in abiotic stress, especially drought stress, have emerged in recent years. miRNA transcriptome analysis in maize (Zea mays) identified multiple drought-responsive miR164 isoforms [35]. In other plant species such as wheat (Triticum aestivum), populus (Populus trichocarpa) and Medicago (Medicago truncatula), drought stress downregulates the transcription of miR164 genes [36–39]. Such regulation in different species for miR164 family members suggested that they might play crucial roles in drought responses. However, the functional role of miR164 in drought stress is poorly studied.

In the present study, we identified and characterized miR164g and its target transcript MsNAC022 in M. sieversii from our previous high-throughput small-RNA and degradome sequencing [40]. A new member of the apple miR164 family, miR164g, plays a crucial role in the drought response of M. sieversii. miR164g is highly expressed in mature apple fruits and subsequently induces its direct target enzyme activity. The results showed that the activity of ROS scavenging enzymes and the expression of their encoding genes are prominently higher in MsNAC022 overexpression plants. Furthermore, two insertions in the MsPOD promoter resulted in its greater transcriptional activation by MsNAC022, suggesting that improved ROS scavenging activity may contribute to the differences of drought tolerance between M. sieversii and Malus domestica.

**Results**

**Identification of msi-miR164g and different expression patterns for miR164 family members in M. sieversii under drought stress**

M. sieversii is an ancestral species of modern apple cultivars that shows exceptional drought tolerance. To determine the candidate genes responsible for the drought resistance, we previously performed high-throughput small RNA sequencing and identified various small non-coding RNAs (snRNAs) and their targets in drought-exposed M. sieversii [40]. Here, we selected the novel miRNA namely msi-miR11c1, which was differentially expressed under drought conditions, for further study. We conducted specific stem–loop reverse transcription PCR to validate the new predicted msi-miR164g in M. sieversii and M. domestica. The identified free mature miRNA only exists in M. sieversii and aligned with msi-miR11c1 exactly (Fig. 1A, Fig. S1, see online supplementary material). Sequence analysis also revealed that the msi-miR164 mature sequence displays high sequence similarity to the msi-miR164 family, with only one nucleotide difference from msi-miR164a at nucleotide 21 in the 5′ and two nucleotide differences from msi-miR164b/c/d/e/f at positions 17 and 21 in the 5′ end (Fig. S2, see online supplementary material). The difference between msi-miR11c1 and msi-miR164a at position 21 in the 5′ end resulted in better alignment with the recognition sites in the target gene (Fig. 1B). We thus named this novel msi-miR164a as msi-miR164g, as the third mature sequence of msi-miR164 family. We also constructed a phylogenetic tree using the sequences of 126 msi-miR164 precursors from 36 plant species (Fig. S3, see online supplementary material). We determined that miR164s are conserved throughout the plant kingdom and that each plant species has 1–11 miR164 family members (Fig. S3B, see online supplementary material).

Quantitative real-time PCR of msi-miR164a/b/g revealed their high expression levels in roots relative to leaves under normal growth conditions. In addition, msi-miR164 expression was two to three times higher than that of msi-miR164a and msi-miR164b, indicating that msi-miR164g largely contributes to the function of the msi-miR164 family in M. sieversii (Fig. 1C). Promoter analysis of the msi-miR164g locus identified multiple cis-acting elements related to drought, salinity, ABA, and low temperature signaling, indicating that msi-miR164g may accumulate in response to multiple environmental stimuli (Table S1, see online supplementary material). To test whether msi-miR164 responded to drought stress, we mimicked drought conditions using 20% (w/v) PEG 6000 treatment and examined the expression patterns of msi-miR164a/b/g in M. sieversii. The results showed that msi-miR164g expression is much more induced over the course of the treatment than that of msi-miR164a/b. In particular, msi-miR164g expression in roots and leaves were rapidly down-regulated at 2 h of drought treatment, indicating that msi-miR164g response to drought stress (Fig. 1D, E).

**Msi-miR164g cleaves the transcript of target gene MsNAC022**

The miR164 targets identified in other plant species belong to the NAC gene family [27, 30, 32, 39, 41]. To determine which candidate NAC gene msi-miR164g might target in apple, we performed an alignment search using the mature msi-miR164g sequence and the apple transcript database. The transcript for gene MD10G1198400 showed the best alignment with msi-miR164g, making it an excellent candidate target for msi-miR164g.
named this gene MsNAC022 based on the amino acid sequence similarity (Fig. S4, see online supplementary material). Using the msi-miR164g sequence as a query against the Arabidopsis transcript database retrieved the same candidate NAC genes as ath-miR164 (Fig. S5, see online supplementary material), indicating that the miR164g-NAC module is conserved among plant species.

To ascertain the effect of msi-miR164g on the abundance of MsNAC022 transcripts, we performed a dual luciferase-based miRNA sensor assay to qualitatively and quantitatively evaluate the cleavage of MsNAC022 transcripts by msi-miR164g. The putative cleavage sites of msi-miR164g were located in the open reading frame of MsNAC022; we thus constructed a sensor vector LUC-MsNAC022 that expresses the firefly luciferase (LUC) reporter gene cloned in-frame with the MsNAC022 cleavage sites under the control of the cauliflower mosaic virus 35S promoter. We also generated a second sensor, LUC-mMsNAC022, with synonymous mutations in the cleavage sites. The transient infiltration of either sensor construct alone in Nicotiana benthamiana leaves resulted in strong relative LUC activity; by contrast, co-infiltration of the LUC sensors with a construct overexpressing pre-msi-miR164g dramatically reduced LUC activity from LUC-MsNAC022, but not from LUC-mMsNAC022 harboring a mutation in the presumed msi-miR164g target site (Fig. 2A, B). These results were consistent with the notion that msi-miR164g targets the MsNAC022 transcript.

To confirm the cleavage sites, we conducted sequencing of a degradome library with high-throughput 5′ rapid amplification of cDNA ends (RACE). We established that MsNAC022 transcripts are cleaved at the site complementary to msi-miR164g between nucleotides 10 and 11 from the 5′ end of the miRNA (Fig. 2C). Consistently, MsNAC022 transcript levels exhibited a pattern opposite to that of msi-miR164g in response to drought. This feature of MsNAC022 expression was more apparent in leaves, as evidenced by induction after 2 h, followed by repression after 4 h and second rising wave until 24 h (Fig. 2D). Collectively, these findings
**Figure 2.** msi-miR164g targets transcripts encoding the NAC transcription factor MsNAC022. **A, B** Dual luciferase reporter assay to evaluate msi-miR164g effects on its target transcript MsNAC022. *msNAC022*, synonymous mutation of MsNAC022 in the msi-miR164g cleavage sites. Renilla luciferase (REN) was used as a positive control. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences (**P < 0.01, based on Duncan’s multiple range test). **C** msi-miR164g-directed cleavage sites of MsNAC022 transcripts, as identified by degradome analysis with high-throughput 5’ RACE sequencing. The arrow indicates the msi-miR164g cleavage sites in the MsNAC022 transcript. **D** Expression patterns of MsNAC022 in *Malus sieversii* treated with 20% PEG 6000. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences (**P < 0.05, **P < 0.01, based on Duncan’s multiple range test). **E** Functional domains in MsNAC022. **F** Transcriptional activation activity assay of MsNAC022 in yeast strain EGY48. MsDREB6.2 was used as a positive control.

support the notion that msi-miR164g directly cleaves MsNAC022 transcripts to decrease their abundance in response to drought.

Bioinformatics analysis indicated that MsNAC022 carries a NO APICAL MERISTEM (NAM) domain, a canonical domain of the NAC family of transcription factors (Fig. 2E). We determined the transcriptional activity of MsNAC022 using a yeast expression system by separately fusing MsNAC022 and MsDREB6.2 (as positive control) to the DNA binding domain of LexA [42]. Both LexA-MsDREB6.2 and LexA-MsNAC022 activated the transcription of the LacZ reporter gene, indicating that MsNAC022 is a transcriptional activator (Fig 2F). As might be expected for a transcription factor, we colocalized MsNAC022-GFP fusion protein with the nuclear localization transcription factor MsDREB6.2-RFP fusion protein to the nucleus of *N. benthamiana* leaf epidermal cells (Fig. S6, see online supplementary material).

**Heterologous expression of the msi-miR164g-MsNAC022 module in Arabidopsis alters plant resistance to drought, osmotic and salt stresses**

To investigate the potential function of the msi-miR164g-MsNAC022 module, we individually overexpressed msi-miR164g and MsNAC022 in Arabidopsis. We isolated three independent homozygous T3 transgenic lines for each construct with high expression levels for analysis, which msi-miR164g transcripts were increased by 27–30 and 14–16 times in transgenic lines leaves and roots, respectively. As for MsNAC022 transcripts, the expression levels were increased by 29–32 and 17–19 times in transgenic lines leaves and roots, respectively (Fig. S7A–E, see online supplementary material). Consistent with the published phenotypes associated with overexpression of its Arabidopsis counterparts as positive regulators of root development, MsNAC022 overexpression (OE) resulted in longer and more elaborate root systems (Fig. S8C, D, see online supplementary material). By contrast, msi-miR164g OE plants exhibited shorter primary roots compared to wild type (Fig. S8A, B, see online supplementary material). To further investigate the drought response of msi-miR164g-OE and MsNAC022-OE plants, we conducted drought survival assays. After long-term water deprivation, msi-miR164g-OE plants exhibited significantly lower survival rates than the wild type (*P < 0.05, 56% in wild type; 27%, 40%, and 33% in the three msi-miR164g-OE lines), while MsNAC022-OE plants fared better under the same conditions (*P < 0.05, 45% in wild type; 65%, 64%, and 61% in the three MsNAC022-OE lines) (Fig. S8E–H, see online supplementary material).
Figure 3. Overexpression of miR164g-resistant MsNAC022 enhances drought tolerance of transgenic apple plants. A Phenotypes of 60-days-old non-transgenic ‘GL-3’ and MsNAC022-OE apple plants. Scale bar, 5 cm. B, C MsNAC022 transcript levels in wild-type and transgenic apple plants. D, E Average length and number of adventitious roots in the indicated genotypes. F Enhanced drought tolerance in MsNAC022-OE apple plants compared to nontransgenic ‘GL-3’ plants. The size of the pot: 48 cm × 20 cm × 13 cm. Scale bar, 10 cm. G Root system of nontransgenic ‘GL-3’ and transgenicMsNAC022-OE apple lines after 14 d drought treatment. Scale bar, 10 cm. H Average length and diameter of adventitious roots in nontransgenic ‘GL-3’ plants and MsNAC022-OE apple lines after 14 d drought treatment. J, K Relative water content in leaves and basal diameter from nontransgenic and MsNAC022-OE apple lines after 14 d drought stress and 7 d rewatering (R-7 d). Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between the transgenic lines and ‘GL-3’ plants (*P < 0.05, **P < 0.01, based on Duncan’s multiple range test).

In addition, transcript abundance for four miR164-targeted AtNACs, especially Arabidopsis NAC022, was much lower in the three msi-miR164g-OE lines compared to the nontransgenic wild-type control, confirming the targeting of NAC022 transcripts by miR164g (Fig. S8I–L, see online supplementary material). Moreover, as drought and salinity both trigger osmotic stress, we
examine the phenotypes of msi-miR164g-OE and MsNAC022-OE lines in response to mannitol osmotic and salt treatment. Msi-miR164g-OE lines were more sensitive to osmotic and salt stress, while MsNAC022-OE plants showed greater tolerance against these stresses (Fig. S9A, B, see online supplementary material). Collectively, these results suggest that plant resistance to drought, osmotic and salt stresses are negatively and positively modulated by msi-miR164g and MsNAC022, respectively.

Overexpression of mir164g-targeted MsNAC022 enhances drought tolerance in transgenic apple plants

To substantiate the function of the msi-miR164g-MsNAC022 module in M. sieversii, we used Agrobacterium (Agrobacterium tumefaciens)-mediated transformation of the apple cultivar ‘GL-3’ to generate transgenic plants overexpressing mir164g-resistant forms of MsNAC022. We selected three independent transgenic lines (MsNAC022-OE-1, OE-2, OE-4) with high MsNAC022 transcript levels for functional studies (Fig. S7F, G, see online supplementary material). The MsNAC022 transcripts were increased by 5.62–6.35 and 2.36–2.94 times in transgenic lines and roots, respectively (Fig. 3A–C). Phenotypic analysis showed that as in the Arabidopsis assays (Fig. SBC, D, see online supplementary material), overexpression of MsNAC022 also resulted in an enhanced root system in apple, with both longer and more numerous adventitious roots (AR) compared to the non-transgenic ‘GL-3’ apple plants (Fig. 3D, E).

To investigate the role of MsNAC022 on drought stress, we performed a 14-d drought treatment on soil-grown nontransgenic ‘GL-3’ and MsNAC022-OE plants. The decrease in soil moisture caused more leaf curling and wilting in ‘GL-3’ plants than in MsNAC022-OE lines after 7 d and 14 d of drought treatment (Fig. 3F; Fig. S10A, see online supplementary material). In addition, MsNAC022-OE lines recovered from severe drought stress more completely than ‘GL-3’ plants after 7 d rewatering (Fig. 3F). Moreover, MsNAC022-OE lines formed vigorous root systems and exhibited higher relative water content in their leaves relative to nontransgenic control plants (Fig. 3G–J). MsNAC022-OE plants also showed greater tolerance to salinity and mannitol osmotic treatment (Fig. S11A, see online supplementary material), which was in line with the phenotypes observed upon MsNAC022 overexpression in Arabidopsis.

Many studies have reported that drought inhibits photosynthesis [43, 44]. To determine whether MsNAC022 might mitigate such adverse effects, we monitored the photosynthetic capacity of MsNAC022-OE plants during drought stress. Photosynthetic rate ($P_{\text{r}}$), stomatal conductance ($g_{\text{s}}$) and transpiration rate ($T_{\text{r}}$) all decreased as water deprivation was imposed for up to 14 d, but increased upon rewatering. $P_{\text{r}}$ in MsNAC022-OE apple plants was higher than that in ‘GL-3’ across the entire experiment, while $g_{\text{s}}$ and $T_{\text{r}}$ in MsNAC022-OE apple plants were higher than those in ‘GL-3’ at the beginning of the experiment (0 d, before drought), reached the same lower levels as ‘GL-3’ plants after 14 d of drought stress, but then also recovered faster than the wild-type plants upon rewatering (Fig. 4A–C). In addition, instantaneous water-use efficiency (WUE$_{i}$) which refers to the relationship between plant productivity and water use, was also higher in MsNAC022-OE lines compared to ‘GL-3’ under drought stress (Fig. 4D). These results indicate that MsNAC022 enhances drought tolerance by raising both photosynthesis rate and WUE$_{i}$.

Drought and salinity stress are accompanied by excessive ROS that cause oxidative damage [2, 13, 16, 45]. To address the exceptional performance of MsNAC022-OE plants in the face of drought stress, we examined the accumulation of H$_2$O$_2$, using 3,3’-diaminobenzidine (DAB) staining and that of superoxide (O$_2^{-}$)• with nitro blue tetrazolium (NBT) staining. MsNAC022-OE transgenic plants displayed a lighter staining pattern for both DAB and NBT compared to ‘GL-3’, indicating that the activity of ROS scavenging systems is higher in MsNAC022-OE plants (Fig. 4E, F). We then turned to a quantification of the underlying enzymes required for ROS scavenging. SOD, POD, and CAT activity levels were all elevated in the leaves of MsNAC022 overexpression lines compared to nontransgenic ‘GL-3’ plants (Fig. 4G–I). The leaves of MsNAC022 plants accumulated more proline but exhibited lower levels of malondialdehyde (MDA), a marker of cellular oxidative stress (Fig. S11B, C, see online supplementary material). Together, these results suggest that the increased fitness of MsNAC022-OE plants upon drought stress is linked to the higher activity of their ROS scavenging systems.

MsNAC022 promotes the expression of antioxidant genes

We wished to test whether the transcript levels of the drought stress-responsive genes and ROS scavengers also changed in MsNAC022-OE plants. Expression levels of seven typical drought stress-responsive genes and two DREB transcription factor were higher in MsNAC022-OE plants under normal conditions and drought stress. Likewise, the expression levels of six ROS scavenging genes, MdSOD, MdPOD, MdCAT, MdGST (GLUTATHIONE S-TRANSFERASE), MdGPX, and MdAPX, were both significantly increased in MsNAC022-OE apple plants under normal conditions and drought stress (Fig. 5A–L; Fig. S12A–R, see online supplementary material).

Of the six ROS scavenger genes tested here, the expression of MdPOD and MdSOD displayed the strongest increase in MsNAC022-OE apple plants, which prompted us to investigate whether MsNAC022 directly activates their transcription (Fig. S1–I). We performed a transient expression assay using a dual-luciferase system in N. benthamiana leaf cells, in which we placed the LUC reporter gene under the control of the MsPOD or MsSOD promoters, yielding the reporter constructs MsPODpro:LUC and MsSODpro:LUC. LUC activity derived from the MsPODpro:LUC and MsSODpro:LUC reporters increased substantially when co-infiltrated in N. benthamiana leaves with the effector construct 35S:MsNAC022, compared to the control vector (62-SK) (Fig. 5M–O). In addition, we identified several NAC binding sites among the cis-elements in the MsPOD and MsSOD promoters. However, we failed to detect direct binding between MsNAC022 and the MsPOD or MsSOD promoters in a yeast one-hybrid assay (Fig. S13, see online supplementary material), indicating that other transcription factors might be recruited to connect MsNAC022 to the MsPOD and MsSOD promoters and activate their transcription.

Different POD expression levels in M. sieversii and M. domestica were attributed to the specific P1 region within the POD promoter

To further dissect the function of MsNAC022, we selected the MsPOD promoter for detailed analysis. We divided the 2.7-kb MsPOD promoter into three fragments based on the distribution of predicted NAC binding motifs to drive the transcription of the β-GLUCURONIDASE (GUS) reporter gene. We then co-infiltrated N. benthamiana leaves with the 35S:MsNAC022 effector and each MsPOD:GUS construct (Fig. 6A). Staining and relative GUS activity analysis showed that MsNAC022 activates transcription from the
Figure 4. Changes in photosynthetic parameters and ROS scavenging systems of nontransgenic and MsNAC022 transgenic apple plants under abiotic stress. A–D \( P_n, G_s, T_r, \) and \( \text{WUE} \) of nontransgenic and MsNAC022 transgenic apple plants after 14 d drought stress and R-7 d. Data are means ± SD from five biological replicates. Different letters indicate significant differences between the transgenic lines and 'GL-3' plants (\( P < 0.05 \), based on Duncan’s multiple range test).

E, F DAB (E) and NBT (F) staining of nontransgenic and MsNAC022 transgenic apple leaves under salt and osmotic stress. Scale bars, 1 cm.

G–I SOD (G), POD (H), and CAT (I) activity in nontransgenic and MsNAC022 transgenic leaves subjected to salt and osmotic stress. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between the transgenic lines and ‘GL-3’ plants (\( **P < 0.01 \), based on Duncan’s multiple range test).

P1 promoter fragment, but not from P2 or P3 (Fig. 6B–D). We confirmed these results in a dual-luciferase assay with each MsPOD promoter fragment driving the transcription of LUC (Fig. 6E–H).

The P1 region of the MsPOD promoter was 910 bp in length and spanned the region from –1819 bp to –2729 bp relative to the ATG start codon. A comparison of the P1 regions from...
Figure 5. MsNAC022-mediated activation of drought stress-related genes and antioxidant enzyme related genes. A–L. Expression levels of drought stress-related and ROS scavenging systems related genes in nontransgenic and transgenic apple plants under normal conditions and drought stress. A–B, MdRD22; C–D, MdRD29A; E–F, MdDREB2A; G–H, MdDREB6.2; I–J, MdSOD; K–L, MdPOD. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between the transgenic lines and ‘GL-3’ plants (∗P < 0.05, ∗∗P < 0.01, based on Duncan’s multiple range test). M–O. Qualitative and quantitative evaluation of MsNAC022-mediated activation of the transcription of genes encoding antioxidant enzyme in a dual-luciferase reporter system, using the MsSOD and MsPOD promoters to drive LUC transcription as reporters. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between the test group and the control group (∗∗P < 0.01, based on Duncan’s multiple range test).

M. sieversii and M. domestica identified insertion polymorphisms within this region. The MsPOD promoter harbored two fragments (−2143 bp to −2178 bp and −2213 bp to −2592 bp) of 35 bp and 379 bp, respectively, that are absent from the MdPOD promoter (Fig. 7A; Table S2, see online supplementary material). Both insertions were located within the F1 fragment. We thus characterized
MsNAC022 activates MsPOD transcription via the P1 promoter region. **Figure 6**. Schematic diagram of the effector and reporter constructs used in the GUS reporter transient expression system. A Schematic diagram of the effector and reporter constructs used in the GUS reporter transient expression system. B–D GUS reporter gene assays of MsPOD promoter activity in the presence of MsNAC022 in N. benthamiana leaves. B, C GUS staining of N. benthamiana leaves from the transient expression assay. D Relative GUS activity (GUS/LUC) from co-infiltrated N. benthamiana leaves. **Figure 6**. Schematic diagram of the effector and reporter constructs used in the LUC reporter transient expression system. E, F–H Dual-luciferase reporter assay of MsPOD promoter activity in the presence of MsNAC022 in N. benthamiana leaves. F, G Images of LUC activity emitted by N. benthamiana leaves. H Relative LUC activity (LUC/REN) from co-infiltrated N. benthamiana leaves. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between treatments and controls (**P < 0.01, based on Duncan’s multiple range test).
activation of MsPOD by MsNAC022 is greater than that of MdPOD. We noticed that the P1 region from M. sieversii (Ms-P1-GUS) resulted in transcription levels about two-fold higher than the P1 region from M. domestica (Md-P1-GUS), which explaining the difference in promoter strength between the two apple varieties (Fig. 7E–G). We obtained similar results in a dual-luciferase assay (Fig. 7H–J).

Based on these results, the specific P1 region within the MsPOD promoter was attributed to enhanced transcriptional activation of MsPOD by MsNAC022, leading to higher accumulation of ROS scavenging enzymes that partially contribute to the higher drought tolerance observed in M. sieversii.

In light of the different promoter strengths seen for the M. sieversii and M. domestica POD promoters in transient assays, we measured the transcript levels of NAC022 and POD in the two varieties, under drought stress and recovery conditions. When grown under normal conditions, NAC022 and POD expression was higher in roots relative to leaves, similar to miR164g (Fig. 8A, B; Fig. 1C). NAC022 and POD expression levels were higher in M. sieversii compared to M. domestica. Upon drought stress, NAC022 and POD transcript levels increased rapidly in M. sieversii and M. domestica, with M. sieversii displaying a more pronounced rise. Moreover, NAC022 and POD expression declined to a greater extent in M. sieversii after 6 h recovery than in M. domestica, indicating a more flexible and plastic response to changes in moisture conditions (Fig. 8C–F). Collectively, these results indicate that the NAC022-POD module may be partially responsible for the different drought response of M. sieversii and M. domestica.

**Discussion**

**Overexpression of msi-miR164g and its target transcript MsNAC022 alters the drought tolerance of transgenic Arabidopsis and apple plants**

Apples are one of the most valuable fruit crops whose productivity and growth is severely restricted by adverse environmental conditions, of which drought is one of the most severe encountered by apples in arid and semi-arid growing areas [3, 4, 46]. Therefore, it is of great scientific and breeding value to identify drought-related components and their underlying molecular mechanisms in apples. miRNAs and their respective targets have roles in responding to various stresses [24, 25, 47–50]. Among these, miR164 is a versatile miRNA with a vital role in plant growth and development. miR164 mediates responses to various abiotic stresses such as salinity, osmosis and drought [26–31, 36, 37, 51–53]. However, the molecular mechanisms by which miR164 and its targets regulate abiotic stress, particularly drought response, are still poorly understood in apples.

Our study characterized the novel miRNA locus msi-miR164g, which was differentially expressed under short-term drought stress treatments in M. sieversii (Fig. 1D and E). Complementary sequence analysis and cleavage assays determined that msi-miR164g directly regulates MsNAC022 transcript levels in M. sieversii (Fig. 2A–C). Overexpression of msi-miR164g or MsNAC022 in Arabidopsis altered tolerance to drought stress. In contrast to the negative regulation of drought stress by msi-miR164g, overexpression of MsNAC022 conferred enhanced drought tolerance in transgenic Arabidopsis and apple (Fig. 3E and F, see online supplementary material; Fig. 3F). Several antioxidant enzymes required for ROS scavenging exhibited high activity levels in MsNAC022-OE apple lines, and their encoding genes were highly expressed (Fig. 4G–I; Fig. 5I–L; Fig. S12K–L, see online supplementary material). In particular, we showed here that MsNAC022 activates MsPOD transcription (Fig. 5N and O). POD promoter analysis in M. sieversii and M. domestica revealed the presence of specific insertions in M. sieversii that contribute to the greater activation of POD transcription by MsNAC022 in this variety (Fig. 7). Together, these results suggest that, in response to drought stress, elevated MsNAC022 transcript levels lead to an activation of MsPOD transcription to detoxify the accumulated ROS and thus enhance drought tolerance.

We observed that msi-miR164g among all msi-miR164s most prominently responds to osmotic treatment. Furthermore, expression analysis of all msi-miR164s revealed that msi-miR164g has a higher basal expression level than other msi-miR164 family members under either normal conditions or stress treatment (Fig. 1C–E). We also noted that mature msi-miR164g is highly similar to the recognition sites of its target gene (Fig. 1B). Based on these observations, we speculate that msi-miR164g may be the main contributing member of the M. sieversii msi-miR164 family. In our drought treatment assay, msi-miR164g in root and leaf exhibited the declined expression trend at 2 h, indicating that msi-miR164g respond to drought rapidly (Fig. 1D and E). In contrast to its low level in leaf, msi-miR164g mainly expressed in roots (Fig. 1C). Considering that root is the first place to perceive drought stress, thus the effect of drought treatment on msi-miR164g expression is more pronounced in root at 2 h (Fig. 1D).

miRNAs cleave the transcripts of their target gene. Previous studies have proved that miR164 directly target the type of NAC transcription factors [18, 27, 30, 32, 34, 39]. The NAC transcription factors directly regulate an array of stress-related genes expression to control drought response [16, 18, 52]. In this study, we established that MsNAC022 transcripts are targeted by msi-miR164g for cleavage, through dual-luciferase-based miRNA sensor assays and degronome sequencing (Fig. 2A–C). In support of this notion, the transcript levels of MsNAC022 and msi-miR164g showed an opposite relationship under drought stress conditions (Fig. 2D). Furthermore, overexpression of msi-miR164g and MsNAC022 resulted in drought-sensitive and drought-tolerant transgenic plants, respectively (Fig. 3E and F, see online supplementary material; Fig. 4F). Thus, we conclude that msi-miR164g negatively mediates drought tolerance by downregulating MsNAC022 transcript levels. Similarly, NAC transcription factors JUB1 and SNAC3 were also reported to improve the drought resistance by enhancing the ROS scavenging systems in tomato and rice, respectively [17, 18]. In addition, the positive regulation on drought tolerance was also identified in maize NAC transcription factors ZmNAC11, which improves water-use efficiency and upregulates the expression of drought-responsive genes [41]. However, the miR164-targeted NAC genes in rice act negatively on drought tolerance, implying that the function of this pathway in abiotic stress is divergent in different plant species [52].

Plant water uptake is ultimately determined by the size, properties, and distribution of the root system [54, 55]. Thus, water deficit induces changes in root architecture to adapt to the adverse environment. Previous studies have shown that overexpression of miR164-targeted NAC transcription factors induces lateral root development in transgenic Arabidopsis [30]. Similar results were reported in maize and soybean (Glycine max), as overexpression of ZmNAC1 or GmNAC020 resulted in more lateral roots and greater root density [32, 56]. In our study, transgenic Arabidopsis and apple plants overexpressing MsNAC022 also showed enhanced root system, especially in transgenic apple plants, the number and length of adventitious roots were significantly increased (Fig. 3E and F, see online supplementary material; Fig. 3A, D, and E). After 14 days of
Figure 7. Insertion polymorphisms in the P1 region of the MsPOD promoter enhance transcriptional response in *Malus sieversii*. A. Schematic diagram of the two *M. sieversii*-specific insertions in the P1 region of the MsPOD promoter. B–D. Dual-luciferase reporter assay of MdPOD promoter activity in the presence of MsNAC022 in *N. benthamiana* leaves. B, C. Images of LUC activity emitted by *N. benthamiana* leaves from the transient expression assay. D. Relative LUC activity (LUC/REN) from co-infiltrated *N. benthamiana* leaves. E–G. GUS reporter gene assay using the full promoter or the P1 region of the POD promoter from *M. sieversii* and *Malus domestica* in the presence of MsNAC022 in *N. benthamiana* leaves. E, F. GUS staining of *N. benthamiana* leaves from the transient expression assay. G. Relative GUS activity (GUS/LUC) from co-infiltrated *N. benthamiana* leaves. H–J. Dual-luciferase reporter assay in the full-length and P1 region of POD promoter activity between *M. sieversii* and *M. domestica* modulated by MsNAC022 in *N. benthamiana* leaves. H, I. Images of LUC activity emitted by *N. benthamiana* leaves from the transient expression assay. J. Relative LUC activity (LUC/REN) from co-infiltrated *N. benthamiana* leaves. Data are shown as means ± SD from three biological replicates. Asterisks indicate significant differences between treatments and controls (*P < 0.05, **P < 0.01, based on Duncan’s multiple range test).

Drought stress, the root difference of transgenic apple was more significant than that of the control plant, indicating that MsNAC022 significantly affected the root development of the transgenic plant during drought stress, thus conferring the transgenic apple plant excellent adaptability to drought stress (Fig. 3F–J). Similar to our study, miR167a expression was significantly reduced in Arabidopsis under high osmotic stress, which subsequently increased the expression level of target genes and promoted lateral root development, while root structure optimization enhanced the tolerance of transgenic plants under
Figure 8. Expression pattern of NAC022 and POD in Malus sieversii and Malus domestica under normal conditions and short-term osmotic stress and recovery. A–B Tissue expression levels of NAC022 and POD in M. sieversii and M. domestica under normal conditions. C–F Expression levels of NAC022 (C, D) and POD (E, F) in M. sieversii and M. domestica treated with 30% (w/v) PEG 6000 followed by rewatering (R). Data are shown as means ± SD from three biological replicates. Different letters indicate significant differences (P < 0.05, based on Duncan’s multiple range test). G Putative model for the miR164g−MsNAC022 module mediating the transcriptional regulation of ROS scavenger systems contributing to the enhanced drought tolerance of M. sieversii. Under drought stress, a drop in miR164g diminishes cleavage of the downstream MsNAC022 transcripts, whose encoding protein in turn activates the transcription of the downstream gene MsPOD and other ROS scavenger genes. As MsPOD and MdPOD are differentially activated by MsNAC022, enhanced ROS scavenging systems partially contribute to the higher drought resistance of M. sieversii. 

osmotic stress [50]. Our results suggest that the msi-miR164g-MsNAC022 module also affects apple root architecture, thereby conferring apple plants’ response to drought, salt, and osmotic stresses. However, the regulatory mechanisms by which msi-miR164g-NAC022-mediated root traits enhance stress resistance is unknown. Thus, more detailed molecular and biochemical
Two insertions in the P1 region of the MsPOD promoter may contribute to the differences in drought resistance between M. sieversii and M. domestica

M. sieversii is the likely progenitor of modern cultivated apple (M. domestica Borkh.) [64]. Among all cultivated rootstocks, M. sieversii is one of the most drought-resistant species in China [7–9]. However, some excellent drought response genes and regulatory mechanisms of M. sieversii may have become weaker or even eliminated in long-term domestication, resulting in the lower observed drought tolerance of M. domestica. Understanding the molecular genetic mechanisms governing drought responses in M. sieversii will help breeding programs enhance the survival of important commercial apple cultivars during periods of drought. Upon osmotic treatment, MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) expression is rapidly upregulated, and M. sieversii displays the highest MAPK expression levels compared to other apple species [65]. Moreover, our previous study reported that MsDREB6.2 regulates cytokinin metabolism and participates in stomatal regulation, root development and aquaporin gene expression, thereby enhancing drought tolerance [42]. Based on these results, the drought response machinery in M. sieversii is thought to be more flexible and plastic than that in M. domestica. Despite much progress, the molecular mechanisms behind the discrepancy of drought tolerance between M. sieversii and M. domestica are not well understood.

In our study, we showed that upon drought stress, ROS overaccumulation slows down in MsNAC022-OE plants overexpressing a miR164g-resistant form of the gene (Fig. 4E and F). As one of the main ROS scavengers, POD transcription was activated by MsNAC022 specifically through the P1 region (Figs 5N and O, and 6). Furthermore, sequence comparison of the P1 region highlighted two insertions present only in M. sieversii (Fig. 7A; Table S2, see online supplementary material). We further established that MsNAC022 activates transcription from the Ms-P1 promoter fragment to greater levels than with Md-P1, which lacks these two unique insertions (Fig. 7E-J). In addition, NAC022 and POD expression was more strongly induced in response to changes in moisture conditions in M. sieversii than in M. domestica (Fig. 8C–F). Based on these findings, we propose a putative model for the transcriptional regulation of ROS scavenger systems that is mediated by the miR164g-MsNAC022 module, which may contribute to the enhanced drought tolerance of M. sieversii (Fig. 8G). Under drought stress, rapidly declining miR164g levels alleviate the cleavage of MsNAC022 transcripts, whose encoding transcription factor in turn activates the transcription of its downstream gene MsPOD and other ROS scavenger genes. As MsNAC022 differentially activates MsPOD and MdPOD, the enhanced ROS scavenging systems might partially contribute to the higher drought resistance of M. sieversii.

Drought stress is becoming one of the most critical determinants that limit apple production. It is highly desirable to breed new apple varieties with high water uptake efficiency or drought tolerance through biotechnology or molecular marker-assisted breeding, but such efforts have met only limited success thus far. M. sieversii was widely used because of its excellent drought resistance [7–9]. Dissecting the unique molecular mechanism of drought responses in M. sieversii will provide valuable genetic resources for breeding new drought-resistant rootstocks. Our work identified a novel regulatory module acting in M. sieversii drought response, which may have applications in apple breeding to increase plant fitness during drought stress.
through modulating the miR164g-MsNAC022 circuitry, ultimately mitigating ROS damage.

**Materials and methods**

**Plant materials and drought stress treatments**

Tissue-cultured plants of M. sieversii and M. domestica were grown at 23°C and 40% relative humidity under a 16 h/8 h (day/night) photoperiod as described [40]. The apple plant ‘GL-3’ (M. domestica Borkh.) from the laboratory of Dr Zhihong Zhang (Liaoning, China) was also cultured under the conditions described above, which will be used for genetic transformation and abiotic stress treatments as previously described [66]. Rooted M. sieversii and M. domestica plants that were hydroponically precultured in half-strength Hoagland nutrient were subjected to drought stress. The plants were treated as described in previous studies with a solution of 20% (w/v) PEG 6000 or 30% PEG 6000 (Xilong Scientific, Shantou, China) for various periods of time [40, 42]. Samples were then separately harvested at 0, 2, 4, 12, and 24 h after drought stress and 6 and 24 h after rewatering, and were quickly frozen in liquid nitrogen for RNA isolation and expression analysis.

Cloning of the msi-miR164g and its target gene MsNAC022

The msi-miR164g and the cleaving to miR164g-targeted gene MsNAC022 was screened via analyses of miRNAs databases of high-throughput small-RNA sequencing and degradome sequencing of M. sieversii which were obtained in our previous study [40].

> To validate the new predicted miRNA, stem-loop reverse transcription PCR were used to obtain the mature of miR164g from M. sieversii, then were cloned into the pTOPO-blunt sample vector (Aidlab, Beijing, China) and clones were confirmed through sequencing. The coding sequence of MsNAC022 was amplified by reverse transcription PCR (RT-PCR) of M. sieversii RNA, and confirmed through sequencing. Primer sequences were listed in Table S3, see online supplementary material.

**Analysis of cis-acting elements related to abiotic stress in msi-miR164g promoter of M. sieversii**

The M. sieversii genomic DNA was extracted using a Plant Genomic DNA Kit (Tiangen, Beijing, China). We checked the msi-miR164g promoter reference sequences by blast the GDR apple genomic database (https://www.rosaceae.org/) with the msi-miR164g precursor sequences obtained from our miRNAs databases, then we designed a specific primer and cloned the msi-miR164g promoter sequences from M. sieversii. The msi-miR164g promoter sequences were submitted to PLANTCARE (http://bioinformatics.psbi. be/webtools/plantcare/html/) to perform cis-acting elements analysis.

**RNA extraction and analysis of expression levels by quantitative real-time PCR (qRT-PCR)**

The Arabidopsis total RNA was extracted with TRIzol Reagent (CWBio, Beijing, China). Small RNA was extracted using an EASYspin Plant microRNA Extract kit (RN40, Aidlab, Beijing, China). The DNA was removed by treating with RNase-free DNase I (RN34, Aidlab, China). After the detection of RNA samples quality by agarose electrophoresis, the same amount of RNA samples (1 μg) was used to generate cDNA with oligo dT primers and specific stem-loop reverse transcription primers of miRNA164 family members, respectively. The qRT-PCR was performed with SYBR Green Mix (CW0659, CWBIO, China). NCBI primer-blast online tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and qPrimerDB (https://biodb.swu.edu.cn/qprimerdb/) online database tools were used to aid in the design of specific primers. The reactions were incubated in a Rotor-Gene Q Machine (Qiagen, Hilden, Germany). AtActin2 and Histone H3 were used as internal controls for Arabidopsis and apple, respectively. Melting curve assay were performed to detection the specificity of qRT-PCR reactions. Gene amplification efficiencies and relative expression levels were analysed by the previous reported method [67]. All primers were listed in Table S3, see online supplementary material.

**Phylogenetic analysis of miR164s and NAC family members**

A total of 126 miR164 precursors from 36 plant species and the NAC transcription factors amino acid sequences from Arabidopsis and apple were used as queries in BLAST searches of the miRBase 22.1, TAIR, and GDR databases, respectively. The phylogenetic analysis was conducted using MEGA X and the neighbor-joining method with 1000 bootstrap replicates [68]. The phylogenetic trees were modified using the Evolview v3 tool [69].

**Dual luciferase-based miRNA sensor assay in N. benthamiana leaves**

The msi-miR164g potential cleavage sites of MsNAC022 were introduced into the AvrII/AgeI sites of the pGreen-dual-luc-ORF-sensor vector as previously described, also its synonymous mutation sequences which were used for a negative control [70]. The primary of msi-miR164g with 482 bp sequence from M. sieversii was driven by the cauliflower mosaic virus 35S (CaMV35S) promoter in the pGreenII 0029 62-5K vector. The PGreen-dual-luc-ORF-sensor vector also carries the REN gene and was used as a positive control. Transformation and infiltration were performed as described [71].

**Analysis of transcriptional activation activity and subcellular localization for MsNAC022**

To investigate the transcriptional activation activity of MsNAC022, the full-length MsNAC022 sequence lacking the termination codon was introduced into pEG202 vector. Sequencing-confirmed plasmid was transformed into yeast strain EGY48, and MsDREB6.2 was used for positive control [42]. Transcriptional activity assay was performed as described [72]. For the colocalization of MsNAC022, the full-length MsNAC022 sequence without the termination codon was fusion expressed with GFP by inserting into the pCAMBIA1302 vector (https://cambia.org/welcome-to-cambialabs/cambialabs-projects/cambia-labs-materials-and-methods-developed-in-cambialabs/). The nuclear localization transcription factor MsDREB6.2 [42] was fusion expression with RFP by inserted into the pCAMBIA1300 vector modified by us. Vectors were transformed into A. tumefaciens strain GV3101. After Agrobacterium microbial concentration reached OD600 = 1.0, they were equally mixed and then were transiently transformed in N. benthamiana leaves and confocal fluorescence observation was performed as described [73].
Construction of overexpression vectors and transgenic plants

To generate the msi-miR164g overexpression vector, the 482 bp sequence from M. sieversii containing the miR164g stem-loop structure was cloned and inserted into the downstream of the CaMV35S promoter in plant transformation vector pCAMBIA1301. Similarly, the coding sequence of MsNAC022 with synonymous mutations in the miR164g cleavage sites was amplified using overlapping PCR and fused into pCAMBIA1301 vector to construct the MsNAC022 overexpression vector. A. tumefaciens strain EHA105 transformed with these vectors was used for genetic transformation in Arabidopsis and apple ‘GL-3’ plants as previously described [74, 75]. After Hygromycin B selection, we performed PCR analysis to identify the presence of the transgenes and the Hygromycin coding sequences in putative transgenic lines; total RNA was also isolated to check the overexpression of msi-miR164g and MsNAC022 by qRT-PCR. In addition, apple leaves GUS staining assay was performed to identification the reporter gene of MsNAC022-OE transgenic ‘GL-3’ apple lines by a modified method [40, 76].

Drought stress treatments, salinity, and osmotic treatment for transgenic plants

For drought stress treatment, the wild type plants and the overexpression T3 Arabidopsis lines were transplanted to the same pot (48 cm × 20 cm × 13 cm) and grew for 2.5 weeks, then were grown under drought stress for 2 weeks or 3 weeks (water was withheld); 5-month-old nontransgenic ‘GL-3’ apple plants and transgenic plants were also transplanted to the same pot and performed 2 weeks drought stress treatment (water was withheld).

For salinity and osmotic treatment, 150 mM NaCl and 250–300 mM mannitol were added into the half-strength MS medium to treat the 14-d wild-type plants and the overexpression T3 Arabidopsis lines as described [40, 77]; 200 mM NaCl and 300 mM mannitol were also added into the MS subculture medium to treat nontransgenic ‘GL-3’ apple plants and transgenic plants as described [40, 66].

Determination of photosynthetic parameters and leaves relative water content

During drought stress, the photosynthetic capacity of MsNAC022-OE plant was monitored on sunny days between 9 and 11 a.m. with a photosynthetic apparatus (Li-6400; LICOR, Huntington Beach, CA, USA). Five measurements were performed for each group as previously described [58]. The relative water content of leaves was determined as described [3].

Determination of several ROS physiological indicators and staining of DAB, NBT

The measurement of ROS physiological indicators, SOD, POD, and CAT activity, Proline and MDA levels in leaves was performed as previously described [3, 78]. DAB and NBT staining using a modified method as described [79].

Analysis of downstream genes expression and dual-luciferase assay

Expression levels of typical stress-responsive genes RD22, RD29A, RD29B, RD26, ERD1, ERD10, and LEA7 were examined in transgenic apple plants by qRT-PCR, as well as the various ROS scavenging systems related genes which were identified in the previous research, such as MdPOD, MsSOD, MdCAT, MsGST, MdGPX, and MdAPX [40, 80–83]. We also selected two DREB transcription factors MdDERB2A and MdDERB6.2 based on their essential roles in drought stress response [42, 84].

The genomic DNA was extracted from M. sieversii and M. domestica using a Plant Genomic DNA Kit (TIANGEN, China). Then promoters of MsPOD, MdPOD, and MsSOD were cloned and fused to pGreenII 0800-LUC vector, respectively. The full-length CDS of MsNAC022 was driven by the CaMV35S promoter in the pGreenII 0029 62-SK vector, then corresponding vectors were transformed into A. tumefaciens strain GV3101 harboring the pSoup plasmids. LUC promoter activity analysis was examined as described [85], as well as the LUC activities of the full-length and different fragments of MsPOD and MdPOD promoters.

GUS reporter assay in N. benthamiana leaves

The promoter of MsPOD and MdPOD was divided into three fragments according to the distribution of NAC binding element, the full-length promoters and different fragments were inserted into the pCAMBIA1301 vector, which contains the GUS reporter gene vector of 35S:LUC that was used for internal control. Vectors were transformed into A. tumefaciens strain GV3101. After Agrobacterium concentration was shaken and adjusted to OD600 = 1.0, various Agrobacterium that harbour effector, reporter, and internal controls were equally mixed and left to stand in the dark for about 1 h before infiltration. Transient transformation in N. benthamiana leaves was performed as described [70]. The histochemical staining of GUS and GUS activity analysis were performed as described [76].

Statistical analyses

All statistical analyses were performed via the one-way ANOVA followed by Duncan’s multiple range test, using the SPSS22.0 for Windows (SPSS Inc., Chicago, IL, USA). Three independent biological replicates were performed for each determination. Data are shown as mean ± standard deviation (SD), differences asterisks between these results were considered as statistically significant (*P < 0.05, **P < 0.01).

Accession numbers

Sequence data from this article can be found in the TAIR database (https://www.arabidopsis.org) and the Genome Database for Rosaceae website (https://www.rosaceae.org/); MsNAC022 (MD10G1198400); MsDREB6.2 (MD15G1365500); MdDRE22 (MD15G1098800); MdRD29A (MD01G1201000); MdRD29B (MD07G1268800); MdRD26 (MD03G1222700); MdERD1 (MD06G1128400); MdERD10 (MD15G1003900); MdLEA7 (MD03G101800); MdDREB2A (MD01G1158600); MdDREB6.2 (MD15G1365500); MdPOD (MD00G112500); MsSOD (MD00G1051500); MdCAT (MD06G1008600); MdGPX (MD06G1081300); MdGST (MD04G1111600); MdAPX (MD08G1150400); AtNAC022 (AT1G56010); AtNAC100 (AT3G12977); AtNAC080 (AT5G07680); AtNAC100 (AT5G61430).

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Author contributions
X.P., Y.L., and T.-H.L. conceptualized this project and designed all experiments. Y.-T.W., X.P., and C.F. treated plant materials and performed the RNA extraction and gene expression analysis. X.P., C.F., and X.Zha performed the dual luciferase and GUS reporter assay. Y.-Y.W. and X.Zha contributed to the phylogenetic analysis of miR164 and NAC family members. Y.-Y.W. and Y.-T.S. contributed to the transcriptional activity analysis and subcellular localization of MsNAC022. Y.-Q.X., Z.-F.Z., and X.Zho measured the physiological indicators. C.F. monitored the photosynthetic characteristics of MsNAC022-OE plants during drought stress. B.-Y.D. and C.W. contributed to the plasmid construction and Arabidopsis and apple genetic transformation. X.P., Y.L. and T.-H.L. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Data availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflict of interest
The authors declare no competing interests.

Supplementary data
Supplementary data is available at Horticulture Research online.

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