MdMYB88 and MdMYB124 Enhance Drought Tolerance by Modulating Root Vessels and Cell Walls in Apple[1][OPEN]

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Water deficit is one of the main limiting factors in apple (*Malus × domestica* Borkh.) cultivation. Root architecture plays an important role in the drought tolerance of plants; however, research efforts to improve drought tolerance of apple trees have focused on aboveground targets. Due to the difficulties associated with visualization and data analysis, there is currently a poor understanding of the genetic players and molecular mechanisms involved in the root architecture of apple trees under drought conditions. We previously observed that MdMYB88 and its paralog MdMYB124 regulate apple tree root morphology. In this study, we found that MdMYB88 and MdMYB124 play important roles in maintaining root hydraulic conductivity under long-term drought conditions and therefore contribute toward adaptive drought tolerance. Further investigation revealed that MdMYB88 and MdMYB124 regulate root xylem development by directly binding *MdVND6* and *MdMYB46* promoters and thus influence expression of their target genes under drought conditions. In addition, MdMYB88 and MdMYB124 were shown to regulate the deposition of cellulose and lignin root cell walls in response to drought. Taken together, our results provide novel insights into the importance of MdMYB88 and MdMYB124 in root architecture, root xylem development, and secondary cell wall deposition in response to drought in apple trees.

Water scarcity is a threat to agriculture and human societies. Roots have historically been considered the primary option for plants, including fruit trees, to adapt to water deficits (Vadez et al., 2014). Since roots are the primary interface between plants and surrounding soil to facilitate water uptake, logically roots are the answer to solve issues that arise with water deficits. Despite this knowledge, roots, especially fruit tree root systems, are poorly characterized due to a lack of phenotyping methods. Hence, the molecular and cellular mechanisms underlying root responses to drought stress are poorly understood.

Roots are likely to respond to environmental stresses by co-opting root development. Therefore, the genetic control of root development and root architecture under environmental stress conditions will facilitate our understanding of root system responses to stress (Taylor-Teeples et al., 2015). Under water-limited conditions, shoot growth is inhibited, but roots have the ability to continue to elongate, resulting in an increased root-to-shoot ratio aiding in the adaption to water deficits (Sharp et al., 2004; Yamaguchi and Sharp, 2010). In wheat (*Triticum aestivum*) , a 50% increase in root-to-shoot ratio is observed under drought stress (Rauf et al., 2007). Currently, a number of genes have been identified as root architecture modulators in response to drought stress. *DEEPER ROOTING1* (*DRO1*) is considered a major quantitative trait locus for deep rooting in rice (*Oryza sativa*; Uga et al., 2011). Encoding for a membrane-associated protein, *DRO1* improves drought avoidance by controlling root angle (Uga et al., 2013). In addition, overexpression of *DRO1* homologs in Arabidopsis promotes steeper lateral root angles, whereas in peach it results in deeper-rooting phenotype (Guseman et al., 2017). Other genes and quantitative trait loci responsible for root architecture under drought conditions have been identified in various plant species including rice, wheat, soybean (*Glycine max*), and Arabidopsis (*Arabidopsis thaliana*; Yue et al., 2006; Koevoets et al., 2016; Kulkarni et al., 2017; Ye et al., 2018).

Secondary cell walls, such as those found in xylem, fibers, and anther cells, consist of cellulose, hemicellulose, and lignin. Secondary cell walls provide mechanical

Figure 1. Root morphology of transgenic plants with altered *MdMYB88* and *MdMYB124* expression and *MsMYB88* and *MsMYB124* expression level changes in response to drought. A, Root morphology of nontransgenic plants (GL-3), *MdMYB88* or *MdM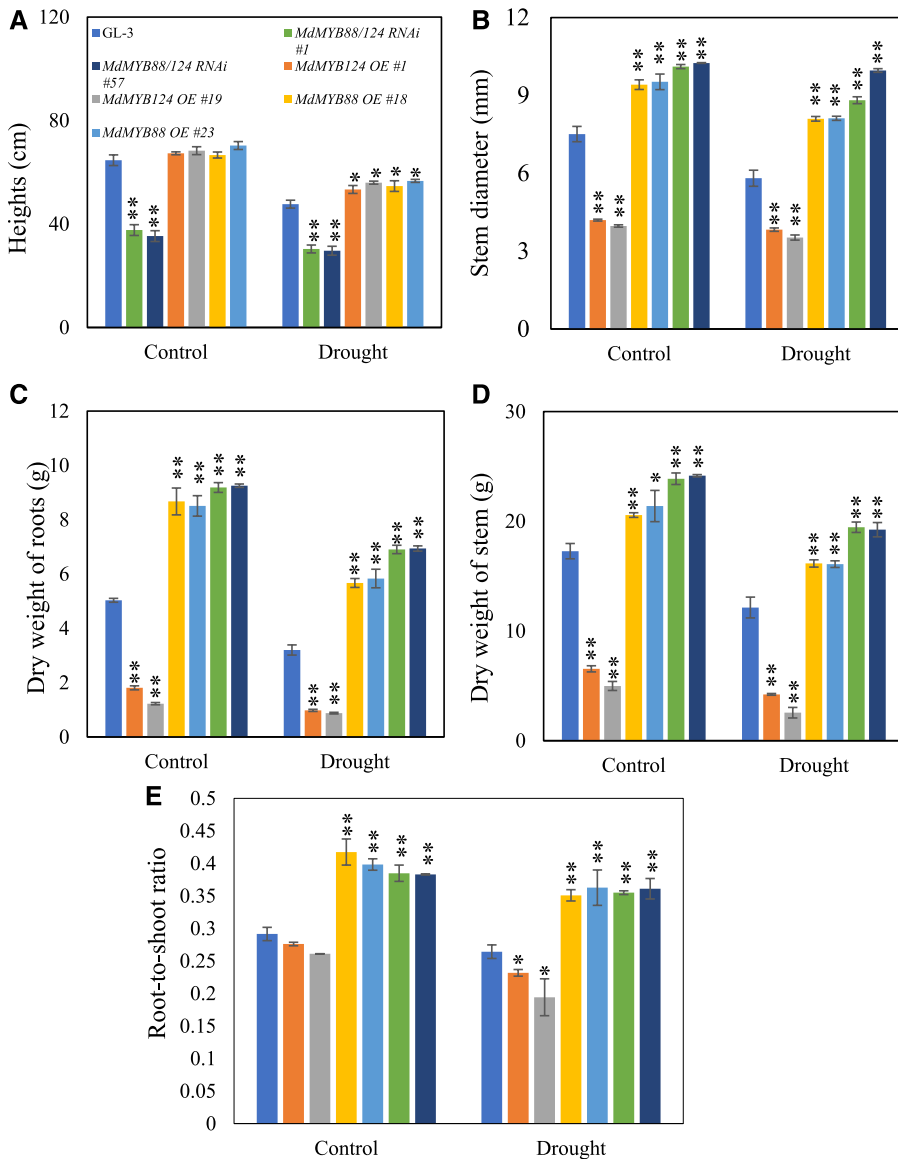YB124* overexpression plants (OE), and *MdMYB88/124* RNAi plants. B, Quantitation of adventitious root length of the plants shown in A. Data are means ± SD (n = 5). One-way ANOVA (Tukey test) was performed, and statistically significant differences are indicated by *$P < 0.05$. C, Relative expression level of *MsMYB88* and *MsMYB124* in *M. sieversii* roots under 20% PEG8000 treatment for 0 or 6 h. Data are means ± SD (n = 3).

support for plant growing bodies and are responsible for long-distance transportation of water and nutrients. Over the years, great progress has been made in understanding the impact of drought stress on secondary cell wall structure and dynamics. In response to drought stress, cellulose biosynthesis is shown to decrease in Arabidopsis, tobacco (*Nicotiana benthamiana*) suspension cells, grape leaves (*Vitis vinifera*), and wheat roots, but increase in cotton (*Gossypium hirsutum*; Le Gall et al., 2015). Increased lignification is a common response to biotic and abiotic stress (Moura et al., 2010), as observed in ryegrass *(Lolium perenne)* (Lee et al., 2012), watermelon roots (*Citrullis vulgaris*; Yoshimura et al., 2008), white clover leaf (*Trifolium repens*; Lee et al., 2007), and *Leucaena leucocephala* (Srivastava et al., 2015). Xyloglycan typically develops a pattern similar to lignin in response to drought (Le Gall et al., 2015).

Moreover, modification of the cell wall architecture can enhance plant growth under drought conditions. For example, overexpression of the drought-responsive AP2/ERF transcription factor *OsERF71* elevates expression of lignin biosynthetic genes, as well as lignification in rice roots, thus increasing rice tolerance to water deficiency (Lee et al., 2016). Mutation of *IRX14* and *IRX14-LIKE*, two closely related glycosyltransferases, causes a decrease in xylem levels and an increase in the drought tolerance of Arabidopsis (Keppler and Showalter, 2010).

Adjustments of the xylem conducting system are important for plants to maximize their water uptake capability and adapt to drought stress (Sperry et al., 2002; Maseda and Fernández, 2006). The xylem-conducting system is composed of a vessel network spanning from roots to leaves, supplying water and nutrients to the
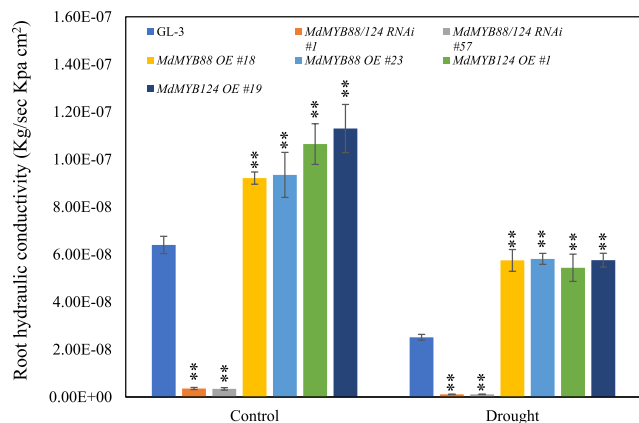
Figure 2. Quantitation of morphological traits of GL-3, *MdMYB88*, or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought conditions. A, Plant height. B, Stem diameter. C, Dry weight of roots. D, Dry weight of stem. E, Root-to-shoot ratio. Plants were subjected to long-term drought stress for 2 months in a greenhouse. Data are means ± SD ($n = 9$). One-way ANOVA (Tukey test) was performed, and statistically significant differences are indicated by $*P < 0.05$ or $**P < 0.01$.

aboveground. The number and diameter of the xylem vessels within the network determine the overall conductivity. Xylem diameter is a major factor determining hydraulic conductivity because of the fourth-power relationship described by the Hagen-Poiseuille law (Tyree et al., 1994). Therefore, even a minor difference in the mean diameter of vessels will lead to a significant difference in hydraulic conductivity. Previous research showed that increasing the number of metaxylem vessels in roots can enhance the drought resistance and seed yield in soybean (Prince et al., 2017). Moreover, xylem cavitation resistance can be used as a relevant criterion for screening drought resist species in *Prunus*, indicating that xylem cavitation, which is related to xylem structure (Guet et al., 2015), is highly related to the drought resistance of *Prunus* (Cochard et al., 2008).

Formation of secondary cell walls is a complicated process, which requires the coordinated regulation of genes involved in secondary cell wall biosynthesis.

In Arabidopsis, biosynthesis of secondary cell walls is mediated by a transcriptional network encompassing a number of NAC and MYB transcription factors, including NST3/ANAC012/SND1, VND6, VND7, PHB, MYB46, MYB83, MYB103, and others (Ko et al., 2014). Of these transcription factors, MYB46 and its paralog MYB83 function as a master switch (Ko et al., 2014). Direct upstream regulators of *MYB46/MYB83* expression include SND1, VND6, and VND7 (Zhong et al., 2007a; Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). In addition, MYB46/MYB83 directly regulates genes associated with biosynthesis of secondary cell wall components (Zhong and Ye, 2012; Kim et al., 2013; Ko et al., 2014).

MYB88 and its paralog FOUR LIPS (FLP/MYB124) are known to regulate the development of guard mother cell proliferation, drought stress tolerance, lateral root development, root gravitropism, and female reproductive development in Arabidopsis (Xie et al.,
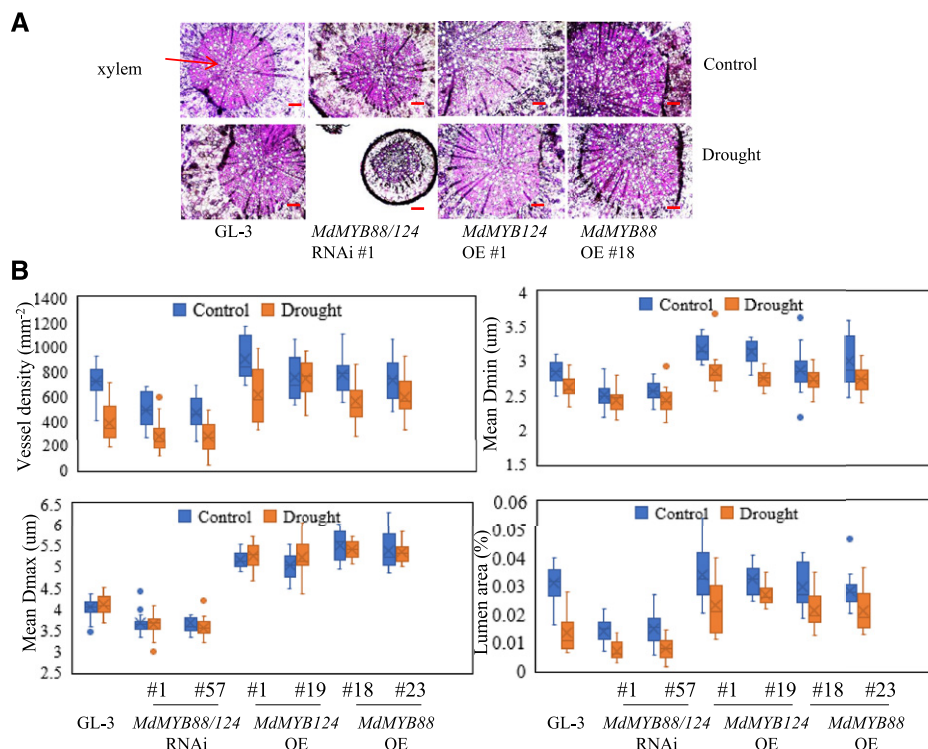
Figure 3. Root hydraulic conductivity of GL-3, *MdMYB88*, or *Md-MYB124* overexpression plants and *MdMYB88/124* RNAi plants under long-term drought conditions. Plants were subjected to long-term drought stress for 2 months in a greenhouse. Data are means ± sD (*n* = 9). One-way ANOVA (Tukey test) was performed, and statistically significant differences are indicated by **$P < 0.01$.

2010a, 2010b; Lai et al., 2005; Makkena et al., 2012; Chen et al., 2015; Wang et al., 2015). We previously demonstrated that MdMYB88 and MdMYB124 are two positive regulators for apple (*Malus × domestica* Borkh.) freezing tolerance (Xie et al., 2018). In this study, we characterized their roles in modulating root architecture, root hydraulic conductivity, root xylem development, and secondary cell wall deposition under long-term drought conditions in apple trees.

RESULTS

MdMYB88 and MdMYB124 Positively Regulate Root Architecture under Long-Term Drought Stress

We previously found that *MdMYB88* and its paralog *MdMYB124* are dominantly expressed in roots of apple trees (Xie et al., 2018). To further investigate their roles in root development, roots of 7-month-old non-transgenic and transgenic apple plants we generated before (Xie et al., 2018) were examined. *MdMYB88* and *MdMYB124* were simultaneously silenced because the sequences of *MdMYB88* and *MdMYB124* are so similar that we cannot silence only one of them by RNAi approach. As shown in Figure 1, A and B, plants overexpressing *MdMYB88* or *MdMYB124* showed vigorous adventitious roots, as determined by adventitious root length. *MdMYB88/124* RNAi plants had weak adventitious root systems, as compared with that of nontransgenic GL-3 plants, indicating potential roles for MdMYB88 and MdMYB124 in apple root development. Considering the important roles of roots in drought tolerance, we examined expression of both genes in apple roots in response to drought. Gene expression analysis revealed that *MdMYB88* and *MdMYB124* were induced slightly in the roots of *Malus sieversii* under simulated drought conditions, indicating their potential participation in drought tolerance (Fig. 1C). We also tested expression of other *MdMYBs*, which displayed higher sequence similarity with *MdMYB88* and *MdMYB124*, in *MdMYB88/124* RNAi plants and found none of these genes were disrupted in their expression

Figure 4. Xylem development in roots of GL-3, *MdMYB88*, or *MdMYB124* over-expression plants and *MdMYB88/124* RNAi plants under long-term drought conditions. A, Cross sections of roots from GL-3 and transgenic plants stained with Safranin O. Bars, 100 μm. B, Quantification of root xylem of plants shown in A. Mean D_{max}, average length of major axis of vessels; mean D_{min}, average length of minor axis of vessels; lumen area, total lumen area, relative to xylem area. $n = 10$.
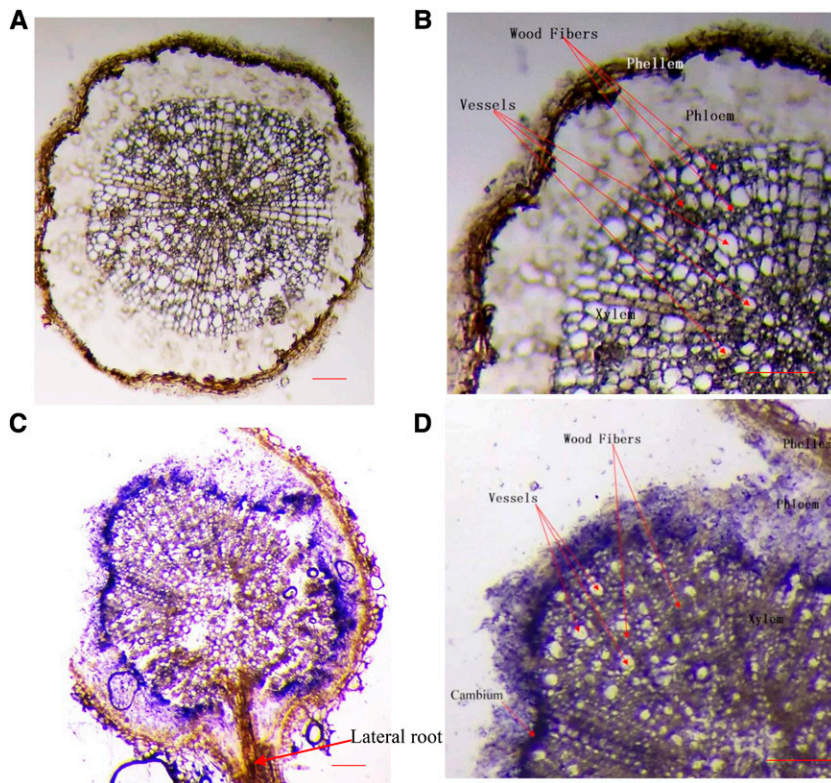
Figure 5. Localization of *MdMYB88* transcripts in roots of GL-3. A, In situ hybridization of *MdMYB88* transcripts using sense probe. B, Enlarged image of A. C, In situ hybridization of *MdMYB88* transcripts using antisense probe. D, Enlarged image of C. *MdMYB88* transcript in roots is indicated by purple coloring. Bars, 100 µm.

(Supplemental Fig. S1A). These results suggest that weak adventitious roots in *MdMYB88/124* RNAi plants are due to disrupted expression of *MdMYB88* and *MdMYB124*, but not other *MdMYBs*.

To further explore the roles of MdMYB88 and MdMYB124 in root development under drought, we applied long-term drought treatment on transgenic and nontransgenic plants (Supplemental Fig. S1B). As shown in Figure 2, drought treatment significantly affected plant height, stem diameter, dry weight of shoots, dry weight of roots, and root-to-shoot ratio. After 2 months of drought stress, *MdMYB88/124* RNAi plants were much shorter, whereas *MdMYB88* or *MdMYB124* overexpression plants were taller, when compared to the height of GL-3 plants (Fig. 2A). The stems of *MdMYB88/124* RNAi plants were much thinner than those of GL-3 plants under drought. Overexpression of *MdMYB88* or *MdMYB124* increased stem diameter compared to that in the control after drought (Fig. 2B). Dry weight of shoots and roots in *MdMYB88/124* RNAi plants were clearly lower than that of GL-3 plants, resulting in a lower root-to-shoot ratio in *MdMYB88/124* RNAi plants under drought stress (Fig. 2, C–E). Consistently, *MdMYB88* or *MdMYB124* overexpression plants had a higher root-to-shoot ratio than that of GL-3 plants in response to long-term drought stress, proportional to the relatively higher dry weight of shoots and roots under drought (Fig. 2, C–E). These data suggest that MdMYB88 and MdMYB124 positively regulate the drought tolerance of apple roots, at least in part, by mediating root architecture.

MdMYB88 and MdMYB124 Regulate Hydraulic Conductivity of Apple Roots under Long-Term Drought Conditions

Two fundamental capabilities of roots are supporting shoot components and transporting water and mineral elements to shoots (Warren et al., 2015). Under drought stress, hydraulic conductivity, an indicator of the ability to transport water, decreases in both roots and shoots (Moshelion et al., 2015). Changed root morphology of transgenic plants under drought stress prompted us to examine their root hydraulic conductivity in response to drought. After 2-month exposure to drought conditions, root hydraulic conductivity as measured by high pressure flow matter (HPFM) was reduced remarkably (Fig. 3). Compared with GL-3 plants, roots of *MdMYB88/124* RNAi plants had a much lower hydraulic conductivity, whereas *MdMYB88* or *MdMYB124* overexpression plants had a clearly higher root hydraulic conductivity (Fig. 3; Supplemental Fig. S2). These data are suggestive of a stronger water transportation ability with *MdMYB88* or *MdMYB124* overexpression under long-term drought stress. We also measured the shoot hydraulic conductivity of the plants tested above and found that, similar to root hydraulic conductivity, shoot hydraulic conductivity of *MdMYB88/124* RNAi plants was much lower than that of GL-3 plants under drought stress (Supplemental Fig. S3). Consistently, *MdMYB88* or *MdMYB124* overexpression plants had a higher shoot hydraulic conductivity than that of GL-3
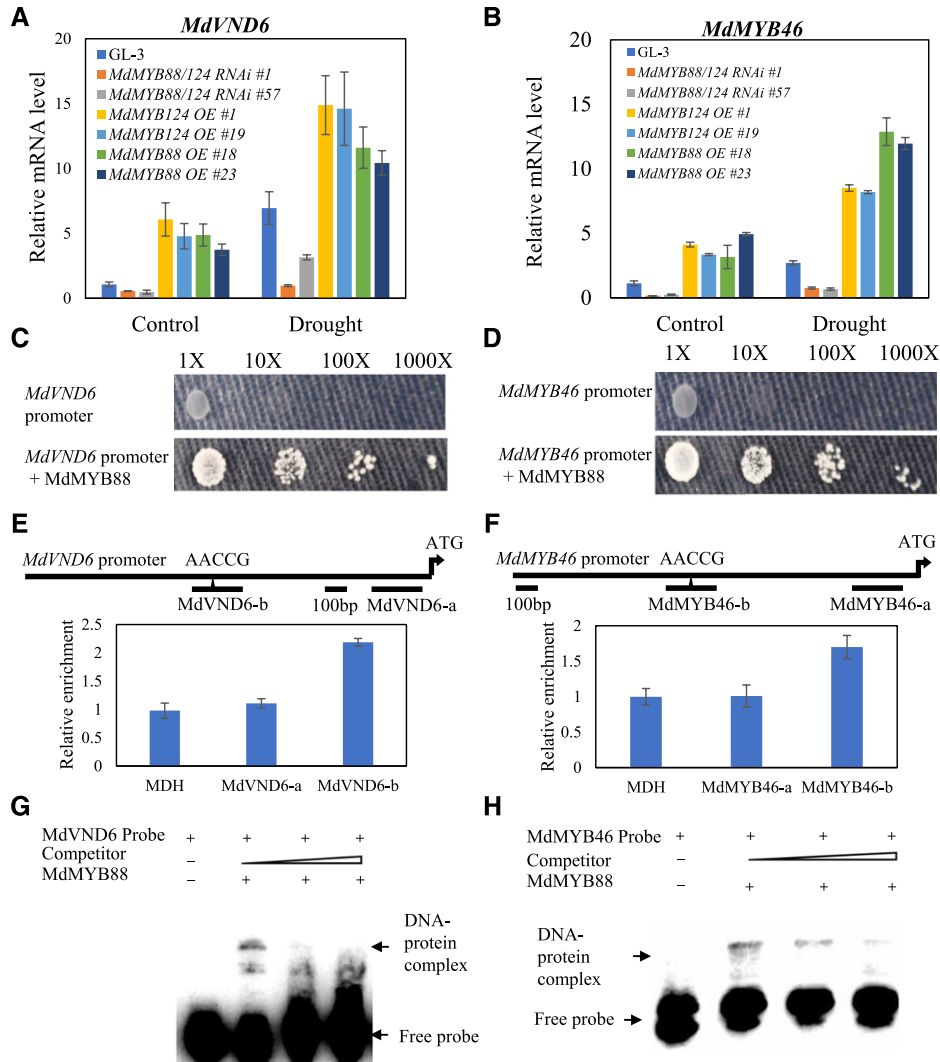
Figure 6. *MdMYB88* and *MdMYB124* regulate *MdMYB46* and *MdVND6* expression by directly targeting their promoters. A and B, Expression level of *MdVND6* and *MdMYB46* in roots of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants in response to drought stress. Plants were subjected to 20% PEG8000 for 0 or 6 h. Data are means ± SD (*n* = 3). C and D, Yeast one-hybrid analysis of interaction between *MdMYB88* and *MdVND6* (C) and *MdMYB46* (D) promoters. AbA concentration is 500 ng/mL. E and F, ChIP-qPCR analysis of *MdVND6* (E) and *MdMYB46* (F) binding by *MdMYB88* and *MdMYB124*. MDH is the negative control, also serves as the reference gene. Fragments *MdVND6*-a and *MdMYB46*-a serve as negative controls in E and F, respectively. Fragments *MdVND6*-b and *MdMYB46*-b both contain cis-element of AACCG. Data are means ± SD (*n* = 3). G and H, EMSA analysis of MdMYB88-His binding to the promoter region of *MdVND6* (G) and *MdMYB46* (H). Arrowheads indicate protein-DNA complex or free probe.

plants in response to drought stress (Supplemental Fig. S3).

MdMYB88 and MdMYB124 Mediate Root Xylem Development under Long-Term Drought Conditions

Water is transported from roots to shoots by vessels; vessel embolism and development therefore significantly affect hydraulic conductivity (Olson et al., 2014). We next asked whether MdMYB88 and MdMYB124 were regulators of root xylem development in response to long-term drought treatment (Fig. 3). We first

stained roots of transgenic plants and GL-3 plants with Safranin O under control and drought treatments (Fig. 4A; Supplemental Fig. S4). Obviously, *MdMYB88/124* RNAi plants had decreased vessel density in response to drought treatment. In comparison with that in GL-3 plants, *MdMYB88* or *MdMYB124* overexpression plants had higher vessel density under drought conditions (Fig. 4A; Supplemental Fig. S4). We quantified vessel density, vessel diameter (average length of major axis of vessels [mean D_{min}], average length of minor axis of vessels [mean D_{max}]), and lumen area (Fig. 4B). As shown in Figure 4B, compared to that in GL-3
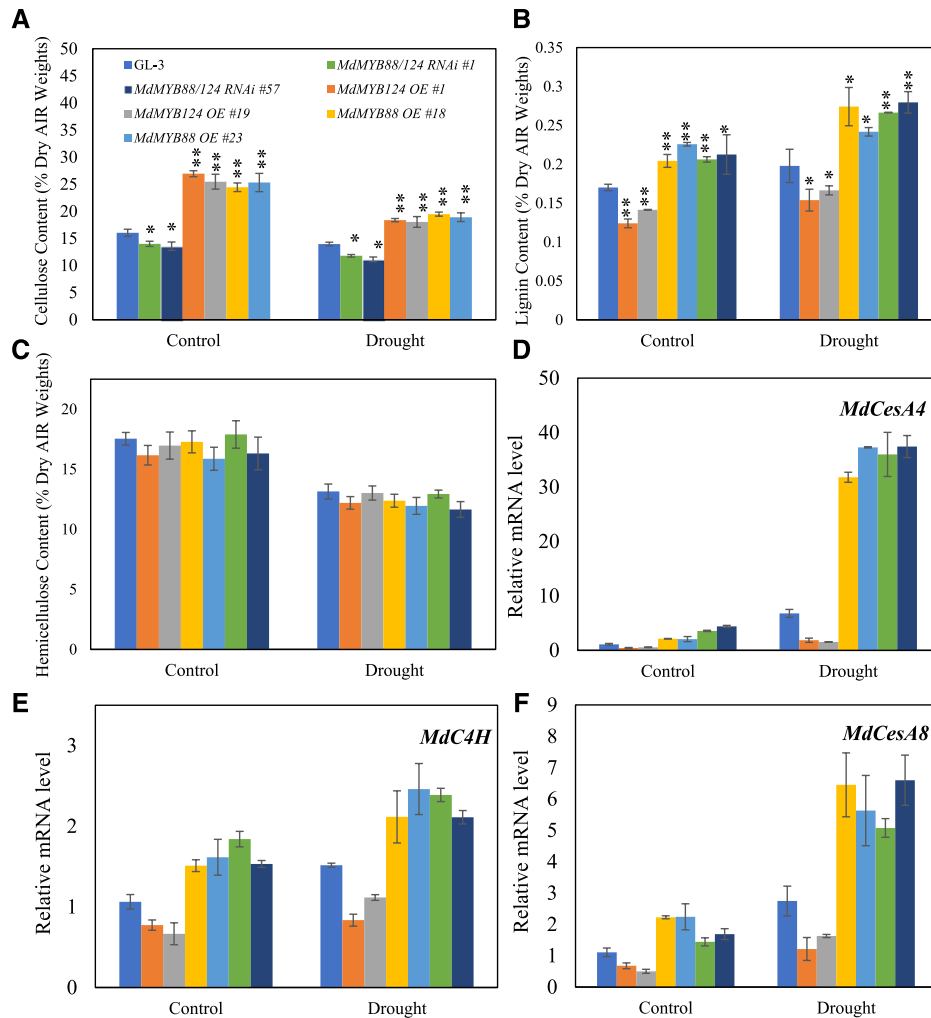
Figure 7. Content of cellulose, lignin, hemicellulose, and expression level of genes associated with secondary cell wall biosynthesis in roots of GL-3, *MdMYB88*, or *MdMYB124* overexpression plants and *MdMYB88/124* RNAi plants under drought conditions. A to C, Contents of cellulose (A), lignin (B), and hemicellulose (C). Plants were subjected to long-term drought stress for 2 months in a greenhouse. Data are means ± sd (*n* = 9). One-way ANOVA (Tukey test) was performed, and statistically significant differences are indicated by **P* < 0.05 or ***P* < 0.01. D to F, Relative expression levels of *MdCesA4* (D), *MdCesA8* (E), and *MdC4H* (F). Plants were subjected to 20% PEG8000 for 0 or 6 h. Data are means ± sd (*n* = 3).

plants, vessel density and vessel diameter were lower in *MdMYB88/124* RNAi plants under drought conditions, whereas those of *MdMYB88* or *MdMYB124* overexpression plants displayed greater vessel density and diameter (Fig. 4B). Lumen area was quantified as the ratio of total vessel area compared to xylem area. In response to long-term drought stress, the lumen area was decreased in *MdMYB88/124* RNAi plants but increased in *MdMYB88* or *MdMYB124* overexpression plants when compared to nontransgenic GL-3 plants (Fig. 4B). We also noticed that root phloem thickness was significantly decreased in *MdMYB88/124* RNAi plants under drought treatment compared with that in GL-3 plants, indicating that MdMYB88 and MdMYB124 might also regulate phloem development in response to drought (Supplemental Fig. S4B).

MdMYB88 and *MdMYB124* Are Predominantly Expressed in Xylem Vessels and Cambium in Apple Roots

Previously, we found that *MdMYB88* and *MdMYB124* are predominantly expressed in the roots of apple plants (Xie et al., 2018). To specifically investigate the localization of *MdMYB88* and *MdMYB124* transcripts in roots of apple, we performed an in situ hybridization (Fig. 5). When using a sense probe, only background was detectable (Fig. 5, A and B); however, strong signals were observed in the vessels and cambium of apple roots when using an antisense probe (Fig. 5C). Enlarged images showed that transcripts of *MdMYB88* and *MdMYB124* were visualized in xylem vessels but not in xylem fiber cells (Fig. 5D). In addition, weak signals were detected in the phloem of apple roots (Fig. 5D).

MdMYB88 and MdMYB124 Mediate Expression of *MdVND6* and *MdMYB46* in Apple Roots under Simulated Drought Conditions

We next asked how MdMYB88 and MdMYB124 regulate xylem vessel development in apple roots. In Arabidopsis, a battery of NAC and MYB genes, including *MYB46*, *VND6*, *VND7*, and *SND1*, are known to mediate xylem vessel development (Zhong et al., 2007b; Ohashi-Ito et al., 2010; Kim et al., 2013). We then investigated expression of some of these genes in the roots of nontransgenic or transgenic plants under control or simulated drought conditions (Fig. 6, A and B; Supplemental Fig. S5). Reverse transcription quantitative PCR (RT-qPCR) analysis suggested a positive relationship between MdMYB88 and MdMYB124 presence and expression of both *MdVND6* and *MdMYB46* in the roots of apple under control or drought conditions (Fig. 6, A and B). In contrast, no such relationship was found with *MdVND7* and *MdSND1* (Supplemental Fig. S5). These data suggest that MdMYB88 and MdMYB124 may regulate root xylem vessel development by mediating expression of *MdVND6* and *MdMYB46*.

MdMYB88 and MdMYB124 Directly Target *MdVND6* and *MdMYB46* Promoters

Previously, we identified one binding site of MdMYB88 and MdMYB124 using chromatin immunoprecipitation qPCR (ChIP-qPCR) and EMSA analyses: AACCG (Xie et al., 2018). Regulation of *MdVND6* and *MdMYB46* expression by MdMYB88 and MdMYB124 under control and drought conditions prompted us to analyze *MdVND6* and *MdMYB46* promoter sequences. As expected, a cis-element of AACCG in the promoter region of *MdVND6* and *MdMYB46* was discovered (Supplemental Fig. S6). By performing yeast one-hybrid (Y1H) analysis, direct binding of MdMYB88 to both promoters was detected (Fig. 6, C and D). ChIP-qPCR analysis was then completed to further determine this direct binding in planta. Our results demonstrated MdMYB88 and MdMYB124 to be capable of binding to the AACCG site in promoters of *MdVND6* and *MdMYB46* (Fig. 6, E and F). EMSA analysis further confirmed MdMYB88 to directly target *MdVND6* and *MdMYB46* promoters (Fig. 6, G and H).

MdMYB88 and MdMYB124 Regulate Cellulose and Lignin Deposition in the Roots of Apple in Response to Long-Term Drought Conditions

In Arabidopsis, MYB46 is a master regulator for secondary wall-associated cellulose accumulation (Kim et al., 2013). Furthermore, VND6 is a key regulator for xylem vessel differentiation, programmed cell death, and secondary wall formation (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010). Direct regulation of *MdVND6* and *MdMYB46* by MdMYB88 and MdMYB124 suggests that, in response to long-term drought stress, MdMYB88 and MdMYB124 may participate in the biosynthesis of secondary cell wall components. We then first examined contents of cellulose, lignin, and hemicellulose in roots of transgenic and nontransgenic plants under control or drought conditions. After 2-month drought treatment, *MdMYB88/124* RNAi plants accumulated less cellulose and lignin compared with that in GL-3 plants. Under control conditions, *MdMYB88* and *MdMYB124* expression was positively associated with cellulose and lignin accumulation (Fig. 7, A and B). Consistently, roots of plants overexpressing *MdMYB88* or *MdMYB124* contained more cellulose and lignin content under control or drought conditions than that of nontransgenic GL-3 plants (Fig. 7, A and B). MdMYB88 and MdMYB124 did not regulate accumulation of hemicellulose in the roots under control or long-term drought conditions (Fig. 7C).

In Arabidopsis, *CELLULOSE SYNTHASE A4, A7,* and *A8* (*CesA4, CesA7, CesA8*), *CINNAMATE 4-HYDROXYLASE* (*C4H*), *PHE AMMONIA LYASE 1* (*PAL1*), *4-COUMARATE:COA LIGASE 1* (*4CL*), *ACAULIS 5* (*ACL5*), *XYLEM CYSTEINE PEPTIDASE 1* (*XCP1*), and IRREGULAR XYLEM 9 (*IRX9*)) are responsible for the biosynthesis of cellulose, lignin, and hemicellulose. We thus examined expression levels of these genes in roots of transgenic and nontransgenic plants under control or drought conditions. We found that expression levels of *MdCesA4, MdCesA8,* and *MdC4H* were decreased in *MdMYB88/124* RNAi plants as compared with that in nontransgenic GL-3 plants under control and drought conditions (Fig. 7, D–F). Consistently, the expression levels of these three genes were significantly elevated in plants overexpressing *MdMYB88* or *MdMYB124* under drought and control conditions. No variation in expression of *MdIRX9, MdPAL1, Md4CL1, MdACL5,* or *MdXCP1* was detected under any conditions (Supplemental Fig. S7).

DISCUSSION

In this study, transgenic apple plants were used to characterize the roles of MdMYB88 and MdMYB124 in drought tolerance of apple trees through the modulation of root xylem development under long-term drought stress. Previously, Xie et al. (2010b) found that Arabidopsis MYB88 and FLP positively regulate plant tolerance to drought stress, as determined by water loss of Arabidopsis leaves and plant survival rate. However, the mechanisms behind the responses in perennial trees to long-term drought stress, specifically changes to tree roots, remains unclear.

In this research, MdMYB88 and MdMYB124 were found to be positive regulators of drought tolerance in apple roots. First, when compared to that in nontransgenic GL-3 control plants, transgenic plants overexpressing *MdMYB88* or *MdMYB124* had higher root-to-shoot ratios under long-term drought stress. In contrast, *MdMYB88/124* RNAi plants had lower root-to-shoot ratios in response to long-term drought stress compared to that in control plants (Fig. 2). Root-to-shoot

ratio is often considered an indicative measurement of plant tolerance to drought stress (Xu et al., 2016). Second, root hydraulic conductivity in *MdMYB88* or *MdMYB124* overexpression plants was higher than that of GL-3 plants, whereas *MdMYB88/124* RNAi plants had lower root hydraulic conductivity compared with that in GL-3 plants under long-term drought stress (Fig. 3). Root hydraulic conductivity represents the capability to transport water from the surrounding soil under drought stress; thus, higher root hydraulic conductivity often indicates greater potential water transport from the soil through the root (Melchior and Steudle, 1993; Gambetta et al., 2013; Olaetxea et al., 2015). Third, preliminary data collected from *MdMYB88/124* RNAi plants, performed in 2016, also obtained similar results (Supplemental Figs. S8–S11). The long-term consistency of these data are indicative of the reproducibility of these findings. Fourth, shoot hydraulic conductivity also plays critical roles in plant drought tolerance (Faustino et al., 2015; Zhang et al., 2018). MdMYB88 and MdMYB124 were found to be positively associated with shoot hydraulic conductivity, in response to long-term drought conditions, further supporting the conclusion that MdMYB88 and MdMYB124 positively regulate apple root drought tolerance (Supplemental Fig. S3). The positive association of MdMYB88 and MdMYB124 in apple root adaptations under drought stress is consistent with previous findings by Xie et al. (2010b), suggesting MYB88 and MYB124 may have conserved roles across plants species.

MdMYB88 and *MdMYB124* were slightly induced by simulated drought in roots of *M. sieversii*; however, this should not indicate a weak role of them in apple drought tolerance. Genome-wide expression profile of *MdMYBs* by Cao et al. (2013) showed lower expression levels of all inducible *MdMYBs* (~2- to 6-fold) by simulated drought, indicating that *MdMYBs* are likely to be expressed at a lower level under simulated drought conditions. We selected 10 *MdMYB* genes, which showed relatively higher expression level in study of Cao et al. (2013), to examine expression of them in *M. sieversii* roots under simulated drought conditions. Consistent with the results by Cao et al. (2013), we found that all these 10 up-regulated *MdMYB* genes were not highly induced by simulated drought stress (Supplemental Fig. S12), indicating that *MdMYB* transcription factors cannot be expressed at higher levels in response to simulated drought treatment. Thus, it is not surprised that expression level of *MdMYB88* and *MdMYB124* was not high in *M. sieversii* roots under simulated drought treatment. In addition, Cao et al. (2013) found that MdMYB121, which was also slightly induced by simulated drought (~2 fold), plays a positive role in tomato drought tolerance.

Many factors affect root hydraulic conductivity, and one of the important factors is vessel (Bramley et al., 2009; Hajek et al., 2014; Brunner et al., 2015). Regulation of root hydraulic conductivity during long-term drought conditions by MdMYB88 and MdMYB124 is primarily the result of root xylem developmental

modulation by expression level of *MdMYB88* and *MdMYB124*. Although in seriously suberized roots water is predominantly absorbed by unsuberized fine roots (Kramer and Boyer, 1995) and radio water flow in roots is regulated mainly by aquaporin (Steudle, 2000), xylem vessels are still an important participator of axial hydraulic conductivity (Melchior and Steudle, 1993; Schuldt et al., 2013; Hajek et al., 2014). Through these relationships, additional relationships between vessel density, vessel diameter, and hydraulic conductivity were discovered. It is often believed that larger vessel density and diameter indicate higher root hydraulic conductivity (Syvertsen and Graham, 1985; Vasconcellos and Castle, 1994; Zhang et al., 2018). In *MdMYB88/124* RNAi plants, xylem vessel development of roots was disrupted; as a result, plants displayed lower vessel density and vessel diameter under long-term drought stress, compared with GL-3 plants (Fig. 4). Lower vessel density and vessel diameter contributed to a lower root hydraulic conductivity in *MdMYB88/124* RNAi plants under drought conditions. In contrast, plants overexpressing *MdMYB88* or *MdMYB124* had higher vessel density and vessel diameter, resulting in a higher root hydraulic conductivity under drought conditions. In addition, less developed roots of *MdMYB88/124* RNAi lines led to lower root dry weight (Fig. 2) and root surface area, potentially resulting in a decreased area of unsuberized fine roots and lower root hydraulic conductivity under drought conditions. Cellulose and lignin are also responsible for xylem conductivity. In poplar (*Populus* spp.), reduced lignin content impairs xylem conductivity and growth efficiency (Voelker et al., 2011). Mutation of *ESK1* in Arabidopsis results in the reduced cellulose content, leading to the collapsed xylem vessels and thus lower xylem hydraulic conductivity (Lefebvre et al., 2011). However, xylem conductivity is not directly regulated by cellulose or lignin but instead by vessel development (Lefebvre et al., 2011; Voelker et al., 2011). In our results, lower hydraulic conductivity in *MdMYB88/124* RNAi plants should not be a direct result of lower content of cellulose and lignin but through disrupted vessel development. Moreover, *MdMYB88* and *MdMYB124* were predominantly expressed in root xylem vessels, but not in root xylem fibers, as determined by in situ hybridization (Fig. 5), further supporting MdMYB88 and MdMYB124 as two positive regulators of root hydraulic conductivity through the modulation of root xylem vessel development.

Root cross section analysis and in situ hybridization also suggested that MdMYB88 and MdMYB124 regulate phloem development (Fig. 5; Supplemental Fig. S4). Phloem does not participate in water conduction directly but can regulate primary root growth as a source of water in maize (Wiegers et al., 2009). Phloem also functions as a capacitance to buffer the pulse of xylem water conduction under drought stress (Pfautsch and Adams, 2013; Pfautsch et al., 2015). Under drought stress, the ability of developing secondary phloem will increase in wooden plants (Robert et al., 2011). Thus,

Figure 8. A model for drought adaptation mediated by *MdMYB88* and *MdMYB124* in apple roots. In apple roots, drought stress activates *MdMYB88* and *MdMYB124*, which then directly target the promoters of *MdVND6* and *MdMYB46* and induce their expression. Up-regulated *MdMYB46* expression enhances drought tolerance by regulating root xylem vessel formation, which results in a greater hydraulic conductivity and thus drought adaptation. Increased expression of *MdMYB46* also activates downstream genes associated with cellulose and lignin biosynthesis, resulting in cellulose and lignin deposition as well as drought adaptation.
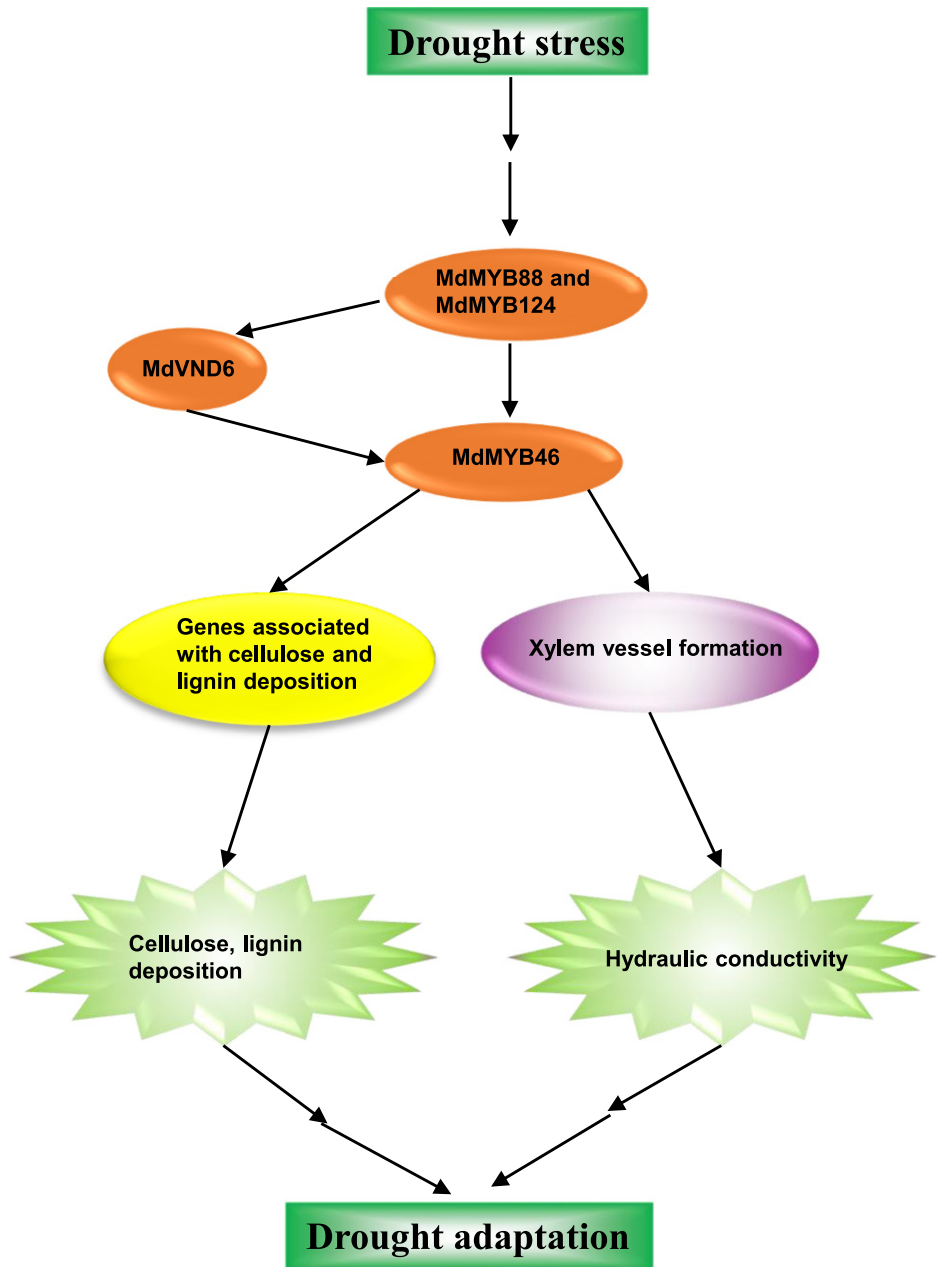
the difference in phloem between *MdMYB88/124* RNAi plants, *MdMYB88* and *MdMYB124* overexpression plants, and GL-3 plants may modulate hydraulic conductivity by regulating root growth.

In Arabidopsis, MYB88 and FLP also participate in the regulation of root gravitropism (Wang et al., 2015) and lateral root development (Lei et al., 2015). Strong expression of *MYB88* or *FLP* has been detected in developing xylem cells (Lei et al., 2015), consistent with our in situ hybridization results as seen in Figure 5. Predominant expression of *MdMYB88* and *MdMYB124* in root xylem vessels, but not in fibers, also explained why *MdSND1* required for secondary cell wall deposition in fiber cells was not modulated by MdMYB88 and MdMYB124 under control or drought conditions (Supplemental Fig. S5).

Root xylem development is regulated by genes including *MYB46*, *VND6*, and *VND7* on a molecular level in Arabidopsis (Zhong et al., 2007a; Yamaguchi et al., 2010; Kim et al., 2013). Among these, MYB46, an R2R3 MYB transcription factor, is the hub for the regulation of xylem vessel development (Kim et al., 2013). VND6 and VND7, two NAC-domain-containing proteins in Arabidopsis, regulate root xylem vessel differentiation by direct regulation of *MYB46* (Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). Furthermore, an Y1H approach revealed key upstream factors of *VND6*, *VND7*, *MYB46*, cellulose-, hemicellulose-, and lignin-associated genes in Arabidopsis: ARABIDOPSIS THALIANA HOMOLOG OF E2F C (E2Fc) (Taylor-Teeples et al., 2015). In this study,

MdMYB88 and MdMYB124 were found to regulate root xylem vessel development by directly modulating expression levels of *MdVND6* and *MdMYB46* in response to drought conditions. First, we found that MdMYB88 and MdMYB124 positively controlled the expression of *MdVND6* and *MdMYB46* in response to drought stress (Fig. 6, A and B). Secondly, EMSA, ChIP-qPCR, and Y1H results supported evidence of direct binding of MdMYB88 (EMSA, Y1H, ChIP-qPCR) and MdMYB124 (ChIP-qPCR) to the promoter regions of *MdVND6* and *MdMYB46* (Fig. 6, C–H). However, we did not find any recognition sites of MdMYB88 and MdMYB124 in the *MdVND7* promoter, indicating that MdMYB88 and MdMYB124 may not directly regulate *MdVND7* in apple roots. This was also consistent with our observation that MdMYB88 and MdMYB124 did not regulate expression of *MdVND7* in apple roots under control or drought conditions (Supplemental Fig. S5).

MdMYB88 and MdMYB124 were found to be two positive regulators of genes responsible for deposition of cellulose and lignin, including *MdCesA4*, *MdCesA8*, and *MdC4H*, in response to drought stress and thus cellulose and lignin accumulation under long-term drought (Fig. 7). Cellulose is the most abundant polysaccharide in plants. Previous research has suggested that modulation of the architecture of secondary cell walls might be one of the mechanisms of plant adaptation to drought stress (Lee et al., 2016). Therefore, modulation of drought tolerance through MdMYB88 and MdMYB124 might be due to regulation of cellulose content in apple roots under drought conditions. Increased lignification is a common response to drought stress (Moura et al., 2010). Hence, elevated lignification in plant roots overexpressing *MdMYB88* or *MdMYB124* while under drought conditions may reflect specific adaptation strategies to drought stress. Furthermore, decreased lignin levels in the roots of *MdMYB88/124* RNAi plants under drought conditions may explain why these plants were more sensitive to drought.

In summary, drought stress in apple roots activates MdMYB88 and MdMYB124, which then directly target and induce the expression of *MdVND6* and *MdMYB46*. Up-regulated *MdMYB46* enhances drought tolerance by regulating root xylem vessel formation, which results in a greater hydraulic conductivity and thus increases drought tolerance. Increased expression of *MdMYB46* also activates its downstream genes associated with cellulose and lignin biosynthesis, resulting in cellulose and lignin deposition, as well as drought adaptation (Fig. 8).

MATERIALS AND METHODS

Plant Materials, Growth Conditions, and Stress Treatment

For long-term drought treatment, 18 tissue-cultured GL-3 (from Royal Gala [*Malus × domestica*] seedlings with high regeneration capacities [Dai et al., 2013]) or transgenic *MdMYB88/124* RNAi or overexpression plants (Xie et al., 2018), were rooted and transferred to pots (30 cm × 18 cm) filled with equal parts of local loess sand and wormcast medium. Pots were placed in a greenhouse under natural illumination, with a temperature of 20°C–35°C and humidity of 35%–55%. In July, seedlings of each line were divided into a well-watered group (*n* = 9) and long-term drought group (*n* = 9). Seedlings of the well-watered group were irrigated daily to maintain field capacity of 75%–85%; seedlings of the long-term drought group were daily irrigated to maintain a field capacity of 45%–55%. Both treatments lasted for 2 months. At the end of treatment, roots were harvested for morphology and vessel analysis.

For RT-qPCR, 3-month-old *Malus sieversii* seedlings, transgenic plants, and nontransgenic GL-3 plants, were transferred into plastic containers containing 20 L of Hoagland solution for an additional month. *M. sieversii* is a drought-tolerant wild species (Liu et al., 2012). All plants were hydroponically cultured in a growth chamber with a temperature of 25°C, illuminance of 4,000 lx, and humidity of 50%–75%. Plants were then treated with 20% (w/v) PEG6000 (Sigma) for 0 h or 6 h. At the end of each treatment, roots were washed and snap frozen with liquid nitrogen. Samples were stored at −80°C until RT-qPCR analysis. Primers used are listed in Supplemental Table S1.

Root Morphology Analysis

Shoot height, diameter of the stem, dry weight of roots, and dry weight of shoots were measured directly after harvesting. Total root length, root surface area, root volume, and average diameter were measured using a Winrhizo 2002 (Regent Corporation, Canada). Five biological replicates were performed for each measurement.

Measurement of Root or Shoot Hydraulic Conductivity

Hydraulic conductivity of roots and shoots of both transgenic and nontransgenic plants was performed with an HPFM (Dynamax, Houston) as described by Tyree et al. (1994) and Wei et al. (1999) with modifications. In brief, after drought treatment, plants were cut into two sections at 2 cm above ground. Sections were then soaked in de-gassed water and connected to HPFM. Root hydraulic conductivity was measured using a transient method, whereas shoot hydraulic conductivity was measured with a quasi steady-state method in accordance with the HPFM manual.

Root Xylem Vessel Analysis

Roots with diameters of 0.5 to 2 mm were selected for vessel analysis. Five root segments of each plant were fixed in FAA stationary solution (5% [v/v] formalin, 5% [v/v] acetic acid, and 90% [v/v] ethyl alcohol) for 24 h, then transferred into 18% (v/v) ammonia at 65°C for 90 min for dissociation. The root segments were subsequently dehydrated in a graded ethyl alcohol series for 3 h (30%, 50%, 75%, 85%, 95%, and 100% twice, v/v). Transparent roots obtained from sequential xylene treatment were embedded in paraffin; embedded blocks were sectioned with a rotary microtome (RM2125RTS, Leica, Germany) and observed with a light microscope (80i, Nikko, Japan). Photos were taken with a digital camera (CFI60, Nikko, Japan) mounted on the microscope and analyzed with Image J software .

The theoretical maximum hydraulic conductivity was calculated with the equation described by Hagen-Poiseuille's law (Eq. 1). Since the cross section of the vessel was ellipse, a modified Equation 2 was used for the calculation of hydraulic conductivity of apple roots (Nobel, 2005).

$$K_s^{theo} = \Sigma \left(\frac{\pi \rho}{8 \eta} \right) r_{ves}^4 / A_{xyl} \; [\text{Kg m}^{-1} \text{ S}^{-1} \text{ Mpa}^{-1}]$$

$$r_{ves}^4 = d_{max}^3 \, d_{min}^3 / 8 \left(d_{max}^2 + d_{min}^2 \right)$$

where ρ is the density of water at 20°C (998.205kg/m^3), η is the viscosity of water at 20°C (1.002 × 10^{-9} Mpa s), r_{ves} is the vessel radius, A_{xyl} is the area of specific root xylem, and D_{max} and D_{min} are the major and minor axis of vessel, respectively.

In situ Hybridization in Apple Roots

Harvested roots of GL-3 with a diameter of 0.5 mm were cut into 1-mm segments and fixed immediately in 4% (w/v) paraformaldehyde in 0.1 M phosphate-buffered saline buffer (pH = 7) for 4 h at room temperature and

then overnight at 4°C. Root segments were then rinsed three times with water, dehydrated in a graded ethanol series (75%, 85%, 95%, and 100%, v/v), embedded in paraffin, and then processed into 10-μm sections using a microtome (RM2125RTS, Leica, Germany). Finally, sections were collected onto po-Lys slides, dried on a hot plate at 45°C for 3 h, and then overnight at 37°C, for complete drying. Prepared slides were store at −80°C until used.

cDNA fragment of *MdMYB88* and *MdMYB124* was cloned into a pST19 vector with the primers listed in Supplemental Table S1, resulting in MdMYB88/124-pST19. MdMYB88/124-pST19 plasmid DNA was transcribed in vitro and labeled with DIG (Digoxin) using a DIG labeling kit (Roche, Switzerland). For hybridization, the probes were hydrolyzed in carbonate buffer (0.04 mM NaHCO$_3$ and 0.06 mM Na$_2$CO$_3$) to 100- to 150-bp fragments, precipitated in 70% (v/v) ethanol, and dissolved in DEPC (Diethylpyrocarbonate)-treated water to a final concentration of 1 ng/μL. In situ hybridization was performed as described by Omori et al. (2009).

Yeast One-Hybrid Analysis

Yeast one-hybrid was performed using MATCHMAKER One-Hybrid System (Clonetech, USA). MdMYB46 and MdVND6 promoters were individually cloned into pABAi vectors, resulting in MdMYB46-pABAi and MdVND6-pABAi plasmids. Plasmids were then transformed into yeast strain Y1H Gold after linearization. Positive clones were used to determine the Aureobasidin A (AbA) concentration due to growth restraints of positive clones on SD (Synthetic Dropout) medium without uracil.

Full-length CDS (coding sequence) of MdMYB88 was cloned into pGADT7 vector to form MdMYB88-pGADT7, which was then transformed into Y1H Gold competent cells carrying MdMYB46-PABAi or MdVND6-PABAi. Cell growth was observed on SD medium without Leu supplemented with AbA.

EMSA and ChIP-qPCR

EMSA and ChIP-qPCR were performed as described by Xie et al. (2018). Probes used for EMSA are listed in Supplemental Table S1.

Quantification of Cellulose, Lignin, and Hemicellulose in Apple Roots

Following root morphology analysis, dried roots from all plants were smashed with a pulverizer. Alcohol was added to prepare alcohol insoluble residues (AIR) of all roots as described by Merali et al. (2013). The cellulose content was measured with AIR, utilizing the anthrone method described by Ondiaka et al. (2015). The lignin content was determined with AIR using the acetyl bromide method described by Brinkmann et al. (2002).

Hemicellulose extraction was performed as described by Mortimer et al. (2015) with modifications. Five to fifteen milligrams AIR was transferred to a 1.5-mL tube and dissolved with 400 μL 4 M NaOH at room temperature for 1 h. After centrifugation at 5,000*g* for 10 min, pellets were discarded, the supernatant was transferred to a new tube, and the sample was neutralized with 4 M HCl. Hemicellulose was precipitated by adding ethanol to a final concentration of 90% (v/v). After centrifugation at 5,000*g* for 10 min, pellets were washed three times with 70% (v/v) ethanol and once with absolute ethanol. The pellets were then dried overnight at 60°C before assessing the hemicellulose content using the previously described anthrone method (Ondiaka et al., 2015).

RNA Extraction and RT-qPCR Analysis

RNA extraction was carried out as described by Xie et al. (2018). The RT-qPCR analysis was performed according to Guan et al. (2013). Primers used are listed in Supplemental Table S1.

Statistical Analysis

Unless noted otherwise, data are reported as the mean ± SD. Statistical significance was determined though one-way ANOVA (Tukey's test) analysis using SPSS (version 21.0, USA). Variations were considered significant if $P < 0.05$ or 0.01.

Accession Numbers

Sequence data used in this article can be found in the GenBankwith the following accession numbers KY569647 (MdMYB88), KY569648(MdMYB124), XP_008376439.1 (MdVND6), XP_008363629.1 (MdMYB46), XP_008380992.1 (MdVND7), NP_001280877.1 MdSND1), XP_008348984.1 (MdCesA4), XP_008383611.(1MdCesA8), NP_001281035.1 (MdC4H), KY359347 (Md4CL1), XP_008387584.1 (MdPAL1), XP_008382503.1 (MdXCP1), and XP_008393603.1 (MdACL5).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Expression of *MdMYB86-like*, *MdMYB40*, and *MdMYB6* in GL-3 control and *MdMYB88/124* RNAi plants and plant morphology of GL-3 control, *MdMYB88*, or *MdMYB124* overexpression, and *MdMYB88/124* RNAi plants under control or long-term drought conditions.

Supplemental Figure S2. The relationship between flow rate and pressure of roots of GL-3 control, *MdMYB88* or *MdMYB124* overexpression, and *MdMYB88/124* RNAi plants under control or long-term drought conditions.

Supplemental Figure S3. Shoot hydraulic conductivity of GL-3 control, *MdMYB88* or *MdMYB124* overexpression, and *MdMYB88/124* RNAi plants under control or long-term drought conditions.

Supplemental Figure S4. Cross section analysis of roots from GL-3 control and transgenic plants after drought stress.

Supplemental Figure S5. Expression level of *MdVND7* and *MdSND1* in roots of GL-3 control, *MdMYB88* or *MdMYB124* overexpression, and *MdMYB88/124* RNAi plants in response to drought stress.

Supplemental Figure S6. Analysis of *MdMYB46* and *MdVND6* promoter sequences.

Supplemental Figure S7. Expression level of *MdPAL1*, *Md4CL1*, *MdIRX9*, *MdXCP1*, and *MdACL5* in roots of GL-3 control, *MdMYB88* or *Md-MYB124* overexpression, and *MdMYB88/124* RNAi plants in response to drought.

Supplemental Figure S8. Dry weight of shoots, shoot height, dry weight of roots, and root-to-shoot ratio in roots of GL-3 control and *MdMYB88/124* RNAi plants under control and long-term drought conditions.

Supplemental Figure S9. Root hydraulic conductivity of GL-3 control and *MdMYB88/124* RNAi lines in response to long-term drought stress.

Supplemental Figure S10. Cross sections of roots from GL-3 control and *MdMYB88/124* RNAi roots under control and long-term drought conditions.

Supplemental Figure S11. Root vessel development of GL-3 control and *MdMYB88/124* RNAi plants under control and long-term drought conditions.

Supplemental Figure S12. Expression of *MdMYB* genes in *M. sieversii* roots under 20% PEG8000 treatment for 0 or 6 h.

Supplemental Table S1. Primers used in this study.

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