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1 **MdMYB88 and MdMYB124 Enhance Drought Tolerance by Modulating Root**
2 **Vessels and Cell Walls in Apple**

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15

16 **Short Title:** Role of MdMYB88/MYB124 in apple drought tolerance

17

18 **AUTHOR CONTRIBUTIONS**

19 DG and QG conceived experiments; PX carried out EMSA; XS performed Y1H; DG,
20 XL and LJ performed other experiments; YZ and XZ provided a greenhouse for long-
21 term drought treatment; YX provided transgenic plants; JZ assisted with hydraulic
22 conductivity analysis; FM and CN analyzed data; DG and QG wrote the article.

23

24 **One-sentence Summary:**

25 MdMYB88 and MdMYB124 are critical for apple (*Malus* x *domestica*) tolerance to
26 drought by mediating root architecture, xylem vessel development, and cell wall
27 deposition.

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31 **ABSTRACT**

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

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51 **Key words:** Apple, root architecture, drought stress, MdMYB88/MdMYB124, xylem
52 development, secondary cell wall

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## INTRODUCTION

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, 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 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, a 50% increase in root-to-shoot ratio is observed under drought stress (Rauf et al., 2017). Currently, a number of genes have been identified as root architecture modulators in response to drought stress. *DEEPER ROOTING 1* (*DRO1*) is considered a major quantitative trait locus (QTL) for deep rooting in rice (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 QTLs responsible for root architecture under drought conditions have been identified in various plant species including rice, wheat, soybean, and Arabidopsis (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 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 suspension cells, grape leaves, and wheat roots, but increase in



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88 cotton (Le Gall et al., 2015). Increased lignification is a common response to biotic
89 and abiotic stress (Moura et al., 2010), as observed in ryegrass (Lee et al., 2012),
90 watermelon roots, (Yoshimura et al., 2007), white clover leaf (Lee et al., 2007), and
91 *Leucaena leucocephala* (Srivastava et al., 2015). Xyloglycan typically develops a
92 pattern similar to lignin in response to drought (Le Gall et al., 2015). Moreover,
93 modification of the cell wall architecture can enhance plant growth under drought
94 conditions. For example, overexpression of the drought-responsive AP2/ERF
95 transcription factor *OsERF71* elevates expression of lignin biosynthetic genes, as well
96 as lignification in rice roots, thus increasing rice tolerance to water deficiency (Lee et
97 al., 2016). Mutation of *IRX14* and *IRX14-LIKE*, two closely related
98 glycosyltransferases, causes a decrease in xylose levels and an increase in the drought
99 tolerance of Arabidopsis (Keppler and Showalter, 2010).

100 Adjustments of the xylem conducting system are important for plants to
101 maximize their water uptake capability and adapt to drought stress (Sperry et al.,
102 2002; Maseda and Fernández, 2006). The xylem conducting system is composed of a
103 vessel network spanning from roots to leaves, supplying water and nutrients to the
104 aboveground. The number and diameter of the xylem vessels within the network
105 determine the overall conductivity. Xylem diameter is a major factor determining
106 hydraulic conductivity because of the fourth-power relationship described by the
107 Hagen-Poiseuille law (Tyree and Ewers, 1991). Therefore, even a minor difference in
108 the mean diameter of vessels will lead to a significant difference in hydraulic
109 conductivity. Previous research showed that increasing the number of metaxylem
110 vessels in roots can enhance the drought resistance and seed yield in soybean (Silvas
111 et al., 2017). Moreover, xylem cavitation resistance can be used as a relevant criterion
112 for screening drought resist species in *Prunus*, indicating that xylem cavitation, which
113 is related to xylem structure (Guet et al., 2015), is highly related to the drought
114 resistance of *Prunus* (Cochard et al., 2013).

115 Formation of secondary cell walls is a complicated process, which requires the
116 coordinated regulation of genes involved in secondary cell wall biosynthesis. In
117 Arabidopsis, biosynthesis of secondary cell walls is mediated by a transcriptional
118 network encompassing a number of NAC and MYB transcription factors, including
119 NST3/ANAC012/SND1, VND6, VND7, PHB, MYB46, MYB83, MYB103, and
120 others (Ko et al., 2014). Of these transcription factors, MYB46 and its paralogue
121 MYB83 function as a master switch (Ko et al., 2014). Direct upstream regulators of



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122 *MYB46/MYB83* expression include SND1, VND6, and VND7 (Zhong et al., 2007;
123 Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). In addition, MYB46/MYB83 directly
124 regulates genes associated with biosynthesis of secondary cell wall components
125 (Zhong et al., 2012; Kim et al., 2013; Ko et al., 2014).

126 MYB88, and its paralogue FOUR LIPS (FLP/MYB124), are known to regulate
127 the development of guard mother cell proliferation, drought stress tolerance, lateral
128 root development, root gravitropism, and female reproductive development in
129 Arabidopsis (Lai et al., 2005; Xie et al., 2010a, 2010b; Makkena et al., 2012; Chen et
130 al., 2015; Wang et al., 2015). We previously demonstrated that MdMYB88 and
131 MdMYB124 are two positive regulators for apple (*Malus* x *domestica* Borkh.)
132 freezing tolerance (Xie et al., 2018). In this study, we characterized their roles in
133 modulating root architecture, root hydraulic conductivity, root xylem development,
134 and secondary cell wall deposition under long-term drought conditions in apple trees.

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136 **RESULTS**

137 **MdMYB88 and MdMYB124 Positively Regulate Root Architecture under Long-**
138 **term Drought Stress**

139 We previously found that *MdMYB88* and its paralogue *MdMYB124* are
140 dominantly expressed in roots of apple trees (Xie et al., 2018). To further investigate
141 their roles in root development, roots of seven-month-old non-transgenic and
142 transgenic apple plants we generated before (Xie et al., 2018) were examined.
143 *MdMYB88* and *MdMYB124* were simultaneously silenced because the sequences of
144 *MdMYB88* and *MdMYB124* are so similar that we cannot silence only one of them by
145 RNAi approach. As shown in Figure 1, A and B, plants overexpressing *MdMYB88* or
146 *MdMYB124* showed vigorous adventitious roots, as determined by adventitious root
147 length. *MdMYB88/124* RNAi plants had weak adventitious root systems, as compared
148 with that of non-transgenic GL-3 plants, indicating potential roles for MdMYB88 and
149 MdMYB124 in apple root development. Considering the important roles of roots in
150 drought tolerance, we examined expression of both genes in apple roots in response to
151 drought. Gene expression analysis revealed that *MdMYB88* and *MdMYB124* were
152 induced slightly in the roots of *M. sieversii* under simulated drought conditions,
153 indicating their potential participation in drought tolerance (Fig. 1C). We also tested
154 expression of other *MdMYBs*, which displayed higher sequence similarity with
155 *MdMYB88* and *MdMYB124*, in *MdMYB88/124* RNAi plants, and found none of these
156 genes were disrupted in their expression (Supplemental Fig. S1A). These results
157 suggest that weak adventitious roots in *MdMYB88/124* RNAi plants are due to
158 disrupted expression of *MdMYB88* and *MdMYB124*, but not other *MdMYBs*.

159 To further explore the roles of MdMYB88 and MdMYB124 in root development
160 under drought, we applied long-term drought treatment on transgenic and non-
161 transgenic plants (Supplemental Fig. S1B). As shown in Figure 2, drought treatment
162 significantly affected plant height, stem diameter, dry weight (DW) of shoots, DW of
163 roots, and root-to-shoot ratio. After two months of drought stress, *MdMYB88/124*
164 RNAi plants were much shorter, whereas *MdMYB88* or *MdMYB124* overexpression
165 plants were taller, when compared to the height of GL-3 plants (Fig. 2A). The stems
166 of *MdMYB88/124* RNAi plants were much thinner than those of GL-3 plants under
167 drought. Overexpression of *MdMYB88* or *MdMYB124* increased stem diameter
168 compared to that in the control after drought (Fig. 2B). DW of shoots and roots in



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169 *MdMYB88/124* RNAi plants were clearly lower than that of GL-3 plants, resulting in
170 a lower root-to-shoot ratio in *MdMYB88/124* RNAi plants under drought stress (Fig.
171 2, C–E). Consistently, *MdMYB88* or *MdMYB124* overexpression plants had a higher
172 root-to-shoot ratio than that of GL-3 plants in response to long-term drought stress,
173 proportional to the relatively higher DW of shoots and roots under drought (Fig. 2, C–
174 E). These data suggest that MdMYB88 and MdMYB124 positively regulate the
175 drought tolerance of apple roots, at least in part, by mediating root architecture.

176 **MdMYB88 and MdMYB124 Regulate Hydraulic Conductivity of Apple Roots**
177 **under Long-term Drought Conditions**

178 Two fundamental capabilities of roots are supporting shoot components and
179 transporting water and mineral elements to shoots (Warren et al., 2015). Under
180 drought stress, hydraulic conductivity, an indicator of the ability to transport water,
181 decreases in both roots and shoots (Moshelion et al., 2015). Changed root morphology
182 of transgenic plants under drought stress prompted us to examine their root hydraulic
183 conductivity in response to drought. After two-month exposure to drought conditions,
184 root hydraulic conductivity as measured by high pressure flow matter (HPFM) was
185 reduced remarkably (Fig. 3). Compared with GL-3 plants, roots of *MdMYB88/124*
186 RNAi plants had a much lower hydraulic conductivity, whereas *MdMYB88* or
187 *MdMYB124* overexpression plants had a clearly higher root hydraulic conductivity
188 (Fig. 3, Supplemental Fig. S2). These data are suggestive of a stronger water
189 transportation ability with *MdMYB88* or *MdMYB124* overexpression under long-term
190 drought stress. We also measured the shoot hydraulic conductivity of the plants tested
191 above and found that, similar to root hydraulic conductivity, shoot hydraulic
192 conductivity of *MdMYB88/124* RNAi plants was much lower than that of GL-3 plants
193 under drought stress (Supplemental Fig. S3). Consistently, *MdMYB88* or *MdMYB124*
194 overexpression plants had a higher shoot hydraulic conductivity than that of GL-3
195 plants in response to drought stress (Supplemental Fig. S3).

196 **MdMYB88 and MdMYB124 Mediate Root Xylem Development under Long-**
197 **term Drought Conditions**

198 Water is transported from roots to shoots by vessels; vessel embolism and
199 development therefore significantly affect hydraulic conductivity (Olson et al., 2014).
200 We next asked whether MdMYB88 and MdMYB124 were regulators of root xylem
201 development in response to long-term drought treatment (Fig. 3). We first stained
202 roots of transgenic plants and GL-3 plants with Safranin O under control and drought



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203 treatments (Fig. 4A, Supplemental Fig. S4). Obviously, *MdMYB88/124* RNAi plants
204 had decreased vessel density in response to drought treatment. In comparison with
205 that in GL-3 plants, *MdMYB88* or *MdMYB124* overexpression plants had higher
206 vessel density under drought conditions (Fig. 4A, Supplemental Fig. S4). We
207 quantified vessel density, vessel diameter (average length of major axis of vessels
208 [mean $D_{min}$], average length of minor axis of vessels [mean $D_{max}$]), and lumen area
209 (Fig. 4B). As shown in Figure 4B, compared to that in GL-3 plants, vessel density and
210 vessel diameter were lower in *MdMYB88/124* RNAi plants under drought conditions,
211 whereas those of *MdMYB88* or *MdMYB124* overexpression plants displayed greater
212 vessel density and diameter (Fig. 4B). Lumen area was quantified as the ratio of total
213 vessel area compared to xylem area. In response to long-term drought stress, the
214 lumen area was decreased in *MdMYB88/124* RNAi plants but increased in *MdMYB88*
215 or *MdMYB124* overexpression plants, when compared to non-transgenic GL-3 plants
216 (Fig. 4B). We also noticed that root phloem thickness was significantly decreased in
217 *MdMYB88/124* RNAi plants under drought treatment compared with that in GL-3
218 plants, indicating that MdMYB88 and MdMYB124 might also regulate phloem
219 development in response to drought (Supplemental Fig. S4B).

220 ***MdMYB88* and *MdMYB124* are Predominantly Expressed in Xylem Vessels and**
221 **Cambium in Apple Roots**

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

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

233 We next asked how MdMYB88 and MdMYB124 regulate xylem vessel
234 development in apple roots. In Arabidopsis, a battery of NAC and MYB genes,
235 including *MYB46*, *VND6*, *VND7*, and *SND1*, are known to mediate xylem vessel
236 development (Zhong et al., 2007; Ohashi-Ito et al., 2010; Kim et al., 2013). We then



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237 investigated expression of some of these genes in the roots of non-transgenic or
238 transgenic plants under control or simulated drought conditions (Fig. 6, A and B,
239 Supplemental Fig. S5). Reverse transcription quantitative PCR (RT-qPCR) analysis
240 suggested a positive relationship between MdMYB88 and MdMYB124 presence and
241 expression of both *MdVND6* and *MdMYB46* in the roots of apple under control or
242 drought conditions (Fig. 6, A and B). In contrast, no such relationship was found with
243 *MdVND7* and *MdSND1* (Supplemental Fig. S5). These data suggest that MdMYB88
244 and MdMYB124 may regulate root xylem vessel development by mediating
245 expression of *MdVND6* and *MdMYB46*.

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

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

261 **MdMYB88 and MdMYB124 Regulate Cellulose and Lignin Deposition in the**
262 **Roots of Apple in Response to Long-term Drought Conditions**

263 In Arabidopsis, MYB46 is a master regulator for secondary wall-associated
264 cellulose accumulation (Kim et al., 2013). Furthermore, VND6 is a key regulator for
265 xylem vessel differentiation, programmed cell death, and secondary wall formation
266 (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010). Direct regulation of *MdVND6* and
267 *MdMYB46* by MdMYB88 and MdMYB124 suggests that, in response to long-term
268 drought stress, MdMYB88 and MdMYB124 may participate in the biosynthesis of
269 secondary cell wall components. We then first examined contents of cellulose, lignin,
270 and hemicellulose in roots of transgenic and non-transgenic plants under control or



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271 drought conditions. After two-month drought treatment, *MdMYB88/124* RNAi plants
272 accumulated less cellulose and lignin compared with that in GL-3 plants. Under
273 control conditions, *MdMYB88* and *MdMYB124* expression was positively associated
274 with cellulose and lignin accumulation (Fig. 7, A and B). Consistently, roots of plants
275 overexpressing *MdMYB88* or *MdMYB124* contained more cellulose and lignin content
276 under control or drought conditions than that of non-transgenic GL-3 plants (Fig. 7, A
277 and B). MdMYB88 and MdMYB124 did not regulate accumulation of hemicellulose
278 in the roots under control or long-term drought conditions (Fig. 7C).
279 In Arabidopsis, *CesA4*, *CesA7*, *CesA8*, *C4H*, *PAL1*, *4CL*, *ACL5*, *XCP1*, and *IRX9*
280 are responsible for the biosynthesis of cellulose, lignin, and hemicellulose. We thus
281 examined expression levels of these genes in roots of transgenic and non-transgenic
282 plants under control or drought conditions. We found that expression levels of
283 *MdCesA4*, *MdCesA8*, and *MdC4H* were decreased in *MdMYB88/124* RNAi plants as
284 compared with that in non-transgenic GL-3 plants under control and drought
285 conditions (Fig. 7, D–F). Consistently, the expression levels of these three genes were
286 significantly elevated in plants overexpressing *MdMYB88* or *MdMYB124* under
287 drought and control conditions. No variation in expression of *MdIRX9*, *MdPAL1*,
288 *Md4CL1*, *MdACL5*, or *MdXCP1* was detected under any conditions (Supplemental
289 Fig. S7).
290 **DISCUSSION**
291 In this study, transgenic apple plants were used to characterize the roles of
292 MdMYB88 and MdMYB124 in drought tolerance of apple trees through the
293 modulation of root xylem development under long-term drought stress. Previously,
294 Xie et al. (2010) found that Arabidopsis MYB88 and FLP positively regulate plant
295 tolerance to drought stress, as determined by water loss of Arabidopsis leaves and
296 plant survival rate. However, the mechanisms behind the responses in perennial trees
297 to long-term drought stress, specifically changes to tree roots, remains unclear.
298 In this research, MdMYB88 and MdMYB124 were found to be positive regulators
299 of drought tolerance in apple roots. First, when compared to that in non-transgenic
300 GL-3 control plants, transgenic plants overexpressing *MdMYB88* or *MdMYB124* had
301 higher root-to-shoot ratios under long-term drought stress. In contrast, *MdMYB88/124*
302 RNAi plants had lower root-to-shoot ratios in response to long-term drought stress
303 compared to that in control plants (Fig. 2). Root-to-shoot ratio is often considered an
304 indicative measurement of plant tolerance to drought stress (Xu et al., 2016). Second,



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305 root hydraulic conductivity in *MdMYB88* or *MdMYB124* overexpression plants was
306 higher than that of GL-3 plants, whereas *MdMYB88/124* RNAi plants had lower root
307 hydraulic conductivity compared with that in GL-3 plants under long-term drought
308 stress (Fig. 3). Root hydraulic conductivity represents the capability to transport water
309 from the surrounding soil under drought stress; thus, higher root hydraulic
310 conductivity often indicates greater potential water transport from the soil through the
311 root (Melchior and Steudle, 1993; Gambetta et al., 2013; Olaetxea et al., 2015). Third,
312 preliminary data collected from *MdMYB88/124* RNAi plants, performed in 2016, also
313 obtained similar results (Supplementary Figs. S8–S11). The long-term consistency of
314 these data is indicative of the reproducibility of these findings. Fourth, shoot
315 hydraulic conductivity also plays critical roles in plant drought tolerance (Faustino et
316 al., 2015; Zhang et al., 2018). MdMYB88 and MdMYB124 were found to be
317 positively associated with shoot hydraulic conductivity, in response to long-term
318 drought conditions, further supporting the conclusion that MdMYB88 and
319 MdMYB124 positively regulate apple root drought tolerance (Supplemental Fig. S3).
320 The positive association of MdMYB88 and MdMYB124 in apple root adaptations
321 under drought stress is consistent with previous findings by Xie et al. (2010),
322 suggesting MYB88 and MYB124 may have conserved roles across plants species.
323 *MdMYB88* and *MdMYB124* were slightly induced by simulated drought in roots of
324 *M. sieversii*; however, this should not indicate a weak role of them in apple drought
325 tolerance. Genome-wide expression profile of *MdMYBs* by Cao et al. (2013) showed
326 lower expression levels of all inducible *MdMYBs* (~2–6 fold) by simulated drought,
327 indicating that *MdMYBs* are likely to be expressed at a lower level under simulated
328 drought conditions. We selected 10 *MdMYB* genes, which showed relatively higher
329 expression level in study of Cao et al. (2013), to examine expression of them in *M.*
330 *sieversii* roots under simulated drought conditions. Consistent with the results by Cao
331 et al. (2013), we found that all these 10 up-regulated *MdMYB* genes were not highly
332 induced by simulated drought stress (Supplemental Fig. S12), indicating that *MdMYB*
333 transcription factors cannot be expressed at higher levels in response to simulated
334 drought treatment. Thus, it is not surprised that expression level of *MdMYB88* and
335 *MdMYB124* was not high in *M. sieversii* roots under simulated drought treatment. In
336 addition, Cao et al. (2013) found that MdMYB121, which was also slightly induced
337 by simulated drought (~2 fold), plays a positive role in tomato drought tolerance.
338 Many factors affect root hydraulic conductivity, and one of the important factors is



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339 vessel (Rodríguez-Gamir et al. 2010; Hajek et al. 2014; Kotowska et al. 2015).
340 Regulation of root hydraulic conductivity during long-term drought conditions by
341 MdMYB88 and MdMYB124 is primarily the result of root xylem developmental
342 modulation by expression level of *MdMYB88* and *MdMYB124*. Although in seriously
343 suberized roots, water is predominantly absorbed by unsuberized fine roots (Kramer
344 and Boyer, 1995), and radio water flow in roots is regulated mainly by aquaporin
345 (Steudle, 2000), xylem vessels are still an important participator of axial hydraulic
346 conductivity (Melchior and Steudle, 1993; Schuldt et al., 2013; Hajek et al., 2014).
347 Through these relationships, additional relationships between vessel density, vessel
348 diameter, and hydraulic conductivity were discovered. It is often believed that larger
349 vessel density and diameter indicate higher root hydraulic conductivity (Syvertsen
350 and Graham, 1985; Vasconcellos and Castle, 1994; Zhang et al., 2018). In
351 *MdMYB88/124* RNAi plants, xylem vessel development of roots was disrupted; as a
352 result plants displayed lower vessel density and vessel diameter, under long-term
353 drought stress, compared with GL-3 plants (Fig. 4). Lower vessel density and vessel
354 diameter contributed to a lower root hydraulic conductivity in *MdMYB88/124* RNAi
355 plants under drought conditions. In contrast, plants overexpressing *MdMYB88* or
356 *MdMYB124* had higher vessel density and vessel diameter, resulting in a higher root
357 hydraulic conductivity under drought conditions. In addition, less developed roots of
358 *MdMYB88/124* RNAi lines led to lower root dry weight (Fig. 2) and root surface area;
359 potentially resulting in a decreased area of unsuberized fine roots and lower root
360 hydraulic conductivity under drought conditions. Cellulose and lignin are also
361 responsible for xylem conductivity. In poplar, reduced lignin content impairs xylem
362 conductivity and growth efficiency (Voelker et al., 2011). Mutation of *ESK1* in
363 Arabidopsis results in the reduced cellulose content, leading to the collapsed xylem
364 vessels and thus lower xylem hydraulic conductivity (Lefebvre et al., 2011). However,
365 xylem conductivity is not directly regulated by cellulose or lignin; instead, by vessel
366 development (Voelker et al., 2011; Lefebvre et al., 2011). In our results, lower
367 hydraulic conductivity in *MdMYB88/124* RNAi plants should not be a direct result of
368 lower content of cellulose and lignin, but through disrupted vessel development.
369 Moreover, *MdMYB88* and *MdMYB124* were predominantly expressed in root xylem
370 vessels but not in root xylem fibers as determined by *in situ* hybridization (Fig. 5),
371 further supporting MdMYB88 and MdMYB124 as two positive regulators of root
372 hydraulic conductivity through the modulation of root xylem vessel development.



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373 Root cross section analysis and *in situ* hybridization also suggested that
374 MdMYB88 and MdMYB124 regulate phloem development (Fig. 5, Supplemental
375 Fig. S4). Phloem does not participate in water conduction directly, but can regulate
376 primary root growth as a source of water in maize (Wiegers et al., 2009). Phloem also
377 functions as a capacitance to buffer the pulse of xylem water conduction under
378 drought stress (Pfautsch and Adams, 2013; Pfautsch et al. 2015). Under drought
379 stress, the ability of developing secondary phloem will increase in wooden plants
380 (Robert et al. 2011). Thus, the difference in phloem between *MdMYB88/124* RNAi
381 plants, *MdMYB88* and *MdMYB124* overexpression plants, and GL-3 plants may
382 modulate hydraulic conductivity by regulating root growth.
383 In Arabidopsis, MYB88 and FLP also participate in the regulation of root
384 gravitropism (Wang et al., 2015) and lateral root development (Lei et al., 2015).
385 Strong expression of *MYB88* or *FLP* has been detected in developing xylem cells (Lei
386 et al., 2015), consistent with our *in situ* hybridization results as seen in Figure 5.
387 Predominant expression of *MdMYB88* and *MdMYB124* in root xylem vessels, but not
388 in fibers, also explained why *MdSND1* required for secondary cell wall deposition in
389 fiber cells was not modulated by MdMYB88 and MdMYB124 under control or
390 drought conditions (Supplemental Fig. S5).
391 Root xylem development is regulated by genes including *MYB46*, *VND6*, and
392 *VND7* on a molecular level in Arabidopsis (Zhong et al., 2007; Yamaguchi et al.,
393 2010; Kim et al. 2013). Among these, MYB46, an R2R3 MYB transcription factor, is
394 the hub for the regulation of xylem vessel development (Kim et al., 2013). VND6 and
395 VND7, two NAC-domain containing proteins in Arabidopsis, regulate root xylem
396 vessel differentiation by direct regulation of *MYB46* (Ohashi-Ito et al., 2010;
397 Yamaguchi et al., 2011). Furthermore, an Y1H approach revealed key upstream
398 factors of *VND6*, *VND7*, *MYB46*, cellulose-, hemicellulose-, and lignin-associated
399 genes in Arabidopsis: E2Fc (Taylor-Teeples et al., 2015). In this study, MdMYB88
400 and MdMYB124 were found to regulate root xylem vessel development by directly
401 modulating expression levels of *MdVND6* and *MdMYB46* in response to drought
402 conditions. First, we found that MdMYB88 and MdMYB124 positively controlled the
403 expression of *MdVND6* and *MdMYB46* in response to drought stress (Fig. 6, A and
404 B). Secondly, EMSA, ChIP-qPCR, and Y1H results supported evidence of direct
405 binding of MdMYB88 (EMSA, Y1H, ChIP-qPCR) and MdMYB124 (ChIP-qPCR) to
406 the promoter regions of *MdVND6* and *MdMYB46* (Fig. 6, C–H). However, we did not



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407 find any recognition sites of MdMYB88 and MdMYB124 in the *MdVND7* promoter,
408 indicating that MdMYB88 and MdMYB124 may not directly regulate *MdVND7* in
409 apple roots. This was also consistent with our observation that MdMYB88 and
410 MdMYB124 did not regulate expression of *MdVND7* in apple roots under control or
411 drought conditions (Supplemental Fig. S5).
412 MdMYB88 and MdMYB124 was found to be two positive regulators of genes
413 responsible for deposition of cellulose and lignin, including *MdCesA4*, *MdCesA8*, and
414 *MdC4H*, in response to drought stress, and thus cellulose and lignin accumulation
415 under long-term drought (Fig. 7). Cellulose is the most abundant polysaccharide in
416 plants. Previous research has suggested that modulation of the architecture of
417 secondary cell walls might be one of the mechanisms of plant adaptation to drought
418 stress (Lee et al., 2016). Therefore, modulation of drought tolerance through
419 MdMYB88 and MdMYB124 might be due to regulation of cellulose content in apple
420 roots under drought conditions. Increased lignification is a common response to
421 drought stress (Moura et al., 2010). Hence, elevated lignification in plant roots
422 overexpressing *MdMYB88* or *MdMYB124* while under drought conditions may reflect
423 specific adaptation strategies to drought stress. Furthermore, decreased lignin levels in
424 the roots of *MdMYB88/124* RNAi plants under drought conditions may explain why
425 these plants were more sensitive to drought.
426 In summary, drought stress in apple roots activates MdMYB88 and MdMYB124
427 which then directly target and induce the expression of *MdVND6* and *MdMYB46*. Up-
428 regulated *MdMYB46* enhances drought tolerance by regulating root xylem vessel
429 formation which results in a greater hydraulic conductivity and thus increases drought
430 tolerance. Increased expression of *MdMYB46* also activates its downstream genes
431 associated with cellulose and lignin biosynthesis, resulting in cellulose and lignin
432 deposition, as well as drought adaptation (Fig. 8).
433



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434 **METHODS**

435 **Plant Materials, Growth Conditions, and Stress Treatment**

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

448 For RT-qPCR, three-month-old *M. sieversii* seedlings, transgenic plants, and non-
449 transgenic GL-3 plants, were transferred into plastic containers containing 20 L of
450 Hoagland solution for an additional month. *M. sieversii* is a drought-tolerant wild
451 species (Liu et al., 2012). All plants were hydroponically cultured in a growth
452 chamber with a temperature of 25°C, illuminance of 4,000 lx, and humidity of 50–
453 75%. Plants were then treated with 20% (w/v) PEG6000 (Sigma, USA) for 0 h or 6 h.
454 At the end of each treatment, roots were washed and snap frozen with liquid nitrogen.
455 Samples were stored at −80°C until RT-qPCR analysis. Primers used are listed in
456 Supplemental Table S1.

457 **Root Morphology Analysis**

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

462 **Measurement of Root or Shoot Hydraulic Conductivity**

463 Hydraulic conductivity of roots and shoots of both transgenic and non-transgenic
464 plants was performed with a high-pressure flow meter (HPFM) (Dynamax, Houston)
465 as described by Tyree et al. (1998) and Wei et al. (1999) with modifications. Briefly,
466 after drought treatment, plants were cut into two sections at 2 cm above ground.
467 Sections were then soaked in de-gassed water and connected to HPFM. Root



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468 hydraulic conductivity was measured using a transient method, whereas shoot
469 hydraulic conductivity was measured with a quasi steady state method in accordance
470 with the HPFM manual.

471 **Root Xylem Vessel Analysis**

472 Roots with diameters of 0.5–2 mm were selected for vessel analysis. Five root
473 segments of each plant were fixed in FAA stationary solution [5% (v/v) formalin, 5%
474 (v/v) acetic acid, and 90% (v/v) ethyl alcohol] for 24 h, then transferred into 18%
475 (v/v) ammonia at 65°C for 90 min for dissociation. The root segments were
476 subsequently dehydrated in a graded ethyl alcohol series for 3 h (30%, 50%, 75%,
477 85%, 95%, and 100% twice, v/v). Transparent roots obtained from sequential xylene
478 treatment were embedded in paraffin; embedded blocks were sectioned with a rotary
479 microtome (RM2125RTS, Leica, Germany), and observed with a light microscope
480 (80i, Nikko, Japan). Photos were taken with a digital camera (CFI60, Nikko, Japan)
481 mounted on the microscope and analyzed with Image J software (Collins et al., 2007).

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

486 Equation 1:

487 $$K_s^{theo} = \sum \left(\frac{\pi\rho}{8\eta}\right) r_{ves}^4 / A_{xyl} [\mathrm{Kg\ m^{-1}\ s^{-1}\ Mpa^{-1}}]$$

488 Equation 2:

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

489 where ρ is the density of water at 20°C (998.205kg/m$^3$), η is the viscosity of water at
490 20°C (1.002 × 10$^{-9}$ Mpa s), $r_{ves}$ is the vessel radius, $A_{xyl}$ is the area of specific root
491 xylem, and $D_{max}$ and $D_{min}$ are the major and minor axis of vessel, respectively.

492 ***In situ* Hybridization in Apple Roots**

493 Harvested roots of GL-3 with a diameter of 0.5mm were cut into 1mm segments
494 and fixed immediately in 4% (w/v) paraformaldehyde in 0.1 M PBS buffer (pH=7) for
495 4 h at room temperature and then overnight at 4°C. Root segments were then rinsed
496 three times with water, dehydrated in a graded ethanol series (75%, 85%, 95%, and
497 100%, v/v), embedded in paraffin, and then processed into 10μm sections using a
498 microtome (RM2125RTS, Leica, Germany). Finally, sections were collected onto
499 polysine slides, dried on a hot plate at 45°C for 3 h, and then overnight at 37°C, for



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500 complete drying. Prepared slides were store at -80°C until used.

501 cDNA fragment of *MdMYB88* and *MdMYB124* was cloned into a pST19 vector
502 with the primers listed in Supplemental Table S1, resulting in MdMYB88/124-pST19.
503 MdMYB88/124-pST19 plasmid DNA was transcribed in vitro and labeled with DIG
504 using a DIG labeling kit (Roche, Switzerland). For hybridization, the probes were
505 hydrolyzed in carbonate buffer (0.04 mM $NaHCO_3$ and 0.06 mM $Na_2CO_3$) to 100–150
506 bp fragments, precipitated in 70% (v/v) ethanol, and dissolved in DEPC-treated water
507 to a final concentration of 1 ng/µl. *In situ* hybridization was performed as described
508 by Omori et al. (2009).

509 **Yeast One-Hybrid Analysis**

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

516 Full-length CDS of MdMYB88 was cloned into pGADT7 vector to form
517 MdMYB88-pGADT7, which was then transformed into Y1H Gold competent cells
518 carrying MdMYB46-PAbAi or MdVND6-PAbAi. Cell growth was observed on SD
519 medium without leucine supplemented with AbA.

520 **EMSA and ChIP-qPCR**

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

523 **Quantification of Cellulose, Lignin, and Hemicellulose in Apple Roots**

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

530 Hemicellulose extraction was performed as described by Mortimer et al. (2015)
531 with modifications. Five to fifteen mg AIR was transferred to a 1.5 ml tube, and
532 dissolved with 400 µl 4 M NaOH at room temperature for 1 h. After centrifugation at
533 5,000 x *g* for 10 min, pellets were discarded, the supernatant was transferred to a new



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534 tube, and the sample was neutralized with 4 M HCl. Hemicellulose was precipitated
535 by adding ethanol to a final concentration of 90% (v/v). After centrifugation at 5,000
536 x *g* for 10 min, pellets were washed three times with 70% (v/v) ethanol and once with
537 absolute ethanol. The pellets were then dried overnight at 60°C before assessing the
538 hemicellulose content using the previously described anthrone method (Ondiaka et
539 al., 2015).

540 **RNA Extraction and RT-qPCR Analysis**

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

544 **Statistical Analysis**

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

548 **Accession Numbers**

549 Sequence data from this article can be found in the GenBank/EMBL data
550 libraries under accession numbers: MdMYB88 (KY569647), MdMYB124
551 (KY569648), MdVND6 (XP_008376439.1), MdMYB46 (XP_008363629.1),
552 MdVND7 (XP_008380992.1), MdSND1 (NP_001280877.1), MdCesA4
553 (XP_008348984.1), MdCesA8 (XP_008383611.1), MdC4H (NP_001281035.1),
554 Md4CL1 (KY359347), MdPAL1 (XP_008387584.1), MdXCP1 (XP_008382503.1),
555 and MdACL5 (XP_008393603.1).
556



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565

566 **FOOTNOTES**

567 The author responsible for distribution of materials integral to the findings
568 presented in this article in accordance with the policy described in the Instructions for
569 Authors is: Qingmei Guan (qguan@nwafu.edu.cn).

570

571 **SUPPLEMENTAL DATA**

572 The following supplemental materials are available.

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

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

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

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

585 **Supplemental Figure S5.** Expression level of *MdVND7* and *MdSND1* in roots of GL-
586 3 control, *MdMYB88* or *MdMYB124* overexpression, and *MdMYB88/124* RNAi plants
587 in response to drought stress.

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

589 **Supplemental Figure S7.** Expression level *of MdPAL1*, *Md4CL1*, *MdIRX9*, *MdXCP1*,



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590 and *MdACL5* in roots of GL-3 control, *MdMYB88* or *MdMYB124* overexpression, and
591 *MdMYB88/124* RNAi plants in response to drought.
592 **Supplemental Figure S8**. Dry weight of shoots, shoot height, dry weight of roots,
593 and root-to-shoot ratio in roots of GL-3 control and *MdMYB88/124* RNAi plants
594 under control and long-term drought conditions.
595 **Supplemental Figure S9.** Root hydraulic conductivity of GL-3 control and
596 *MdMYB88/124* RNAi lines in response to long-term drought stress.
597 **Supplemental Figure S10.** Cross sections of roots from GL-3 control and
598 *MdMYB88/124* RNAi roots under control and long-term drought conditions.
599 **Supplemental Figure S11.** Root vessel development of GL-3 control and
600 *MdMYB88/124* RNAi plants under control and long-term drought conditions.
601 **Supplemental Figure S12.** Expression of *MdMYB* genes in *M. sieversii* roots under
602 20% PEG8000 treatment for 0 or 6 h.
603 **Supplemental Table S1.** Primers used in this study.
604

605 **FIGURE LEGENDS**

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

615 **Figure 2. Quantitation of morphological traits of GL-3, *MdMYB88* or**
616 ***MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under long-**
617 **term drought conditions.** A, Plant height. B, Stem diameter. C, Dry weight of roots.
618 D, Dry weight of stem. E, Root-to-shoot ratio. Plants were subjected to long-term
619 drought stress for two months in a greenhouse. Data are means ± SD (*n* = 9). One-
620 way ANOVA (Tukey test) was performed and statistically significant differences are
621 indicated by * (P<0.05) or ** (P<0.01).

622 **Figure 3. Root hydraulic conductivity of GL-3, *MdMYB88* or *MdMYB124***
623 **overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought**



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624 **conditions.** Plants were subjected to long-term drought stress for two months in a
625 greenhouse. Data are means ± SD (*n* = 9). One-way ANOVA (Tukey test) was
626 performed and statistically significant differences are indicated by * (P<0.05) or **
627 (P<0.01).

628 **Figure 4. Xylem development in roots of GL-3, *MdMYB88* or *MdMYB124*
629 overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought
630 conditions.** A, Cross sections of roots from GL-3 and transgenic plants stained with
631 Safranin O. Bars = 100 μm. B, Quantification of root xylem of plants shown in (A).
632 Mean $D_{max}$, average length of major axis of vessels; mean $D_{min}$, average length of
633 minor axis of vessels; lumen area, total lumen area, relative to xylem area. *n* = 10.

634 **Figure 5. Localization of *MdMYB88* transcripts in roots of GL-3.** A, *In situ*
635 hybridization of *MdMYB88* transcripts using sense probe. B, Enlarged image of (A).
636 C, *In situ* hybridization of *MdMYB88* transcripts using antisense probe. D, Enlarged
637 image of (C). *MdMYB88* transcript in roots is indicated by purple coloring. Bars =
638 100 μm.

639 **Figure 6. MdMYB88 and MdMYB124 regulate *MdMYB46* and *MdVND6*
640 expression by directly targeting their promoters.** A and B, Expression level of
641 *MdVND6* and *MdMYB46* in roots of GL-3, *MdMYB88* or *MdMYB124* overexpression
642 plants, and *MdMYB88/124* RNAi plants in response to drought stress. Plants were
643 subjected to 20% PEG8000 for 0 or 6 h. Data are means ± SD (*n* = 3). C and D, Yeast
644 one-hybrid analysis of interaction between MdMYB88 and *MdVND6* (C) and
645 *MdMYB46* (D) promoters. AbA concentration is 500 ng/mL. E and F, ChIP-qPCR
646 analysis of *MdVND6* (E) and *MdMYB46* (F) binding by MdMYB88 and MdMYB124.
647 *MDH* is the negative control, also serves as the reference gene. Fragments MdVND6-
648 a and MdMYB46-a serve as negative controls in (E) and (F), respectively. Fragments
649 MdVND6-b and MdMYB46-b both contain *cis*-element of AACCG. Data are means
650 ± SD (*n* = 3). G and H, EMSA analysis of MdMYB88-His binding to the promoter
651 region of *MdVND6* (G) and *MdMYB46* (H). Arrowheads indicate protein-DNA
652 complex or free probe.

653 **Figure 7. Content of cellulose, lignin, hemicellulose, and expression level of
654 genes associated with secondary cell wall biosynthesis in roots of GL-3,
655 *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi
656 plants under drought conditions.** A to C, Contents of cellulose (A), lignin (B) and
657 hemicellulose (C). Plants were subjected to long-term drought stress for two months



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658 in a greenhouse. Data are means ± SD (*n* = 9). One-way ANOVA (Tukey test) was
659 performed and statistically significant differences are indicated by * (P<0.05) or **
660 (P<0.01). D to F, Relative expression levels of *MdCesA4* (D), *MdCesA8* (E), and
661 *MdC4H* (F). Plants were subjected to 20% PEG8000 for 0 or 6 h. Data are means ±
662 SD (*n* = 3).

663 **Figure 8. A model for drought adaptation mediated by MdMYB88 and**
664 **MdMYB124 in apple roots.** In apple roots, drought stress activates MdMYB88 and
665 MdMYB124, which then directly target the promoters of *MdVND6* and *MdMYB46*
666 and induce their expression. Up-regulated *MdMYB46* expression enhances drought
667 tolerance by regulating root xylem vessel formation which results in a greater
668 hydraulic conductivity and thus drought adaptation. Increased expression of
669 *MdMYB46* also activates downstream genes associated with cellulose and lignin
670 biosynthesis, resulting in cellulose and lignin deposition as well as drought
671 adaptation.
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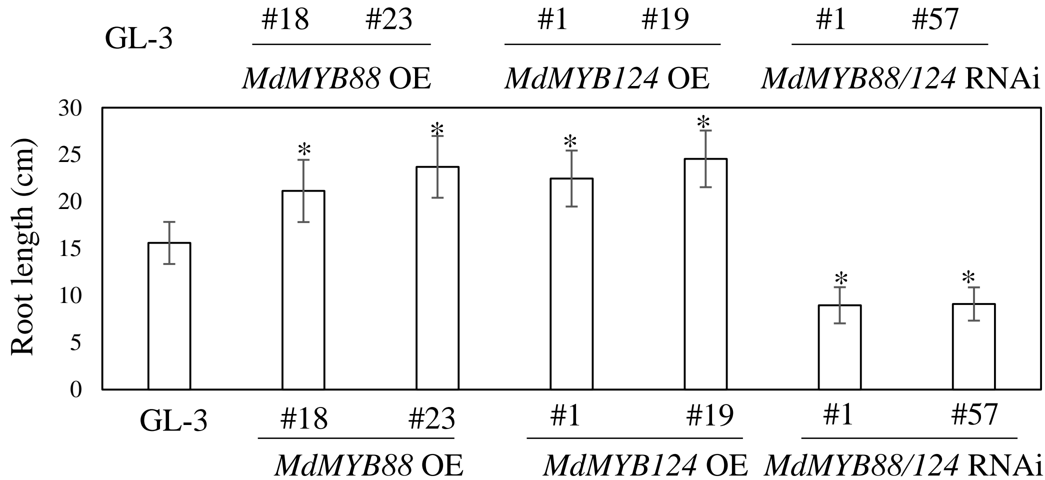


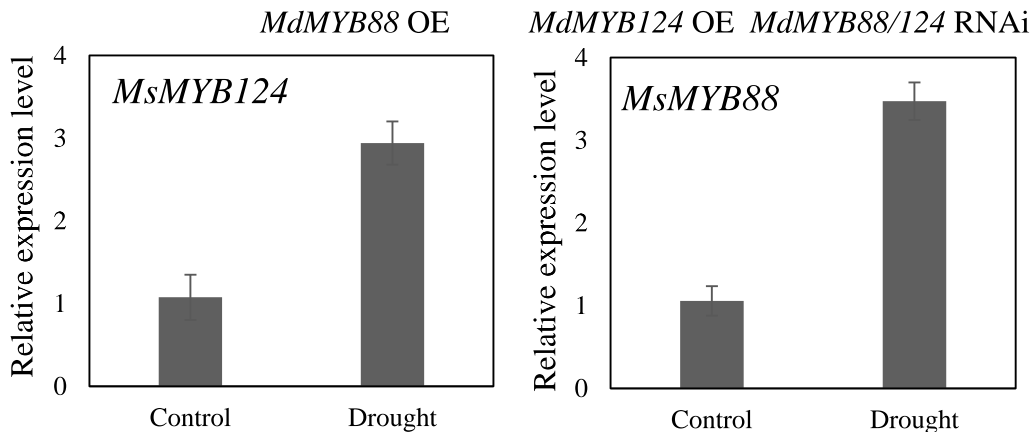


**Figure 1. Root morphology of transgenic plants with altered *MdMYB88* and *MdMYB124* expression, and *MsMYB88* and *MsMYB124* expression level changes in response to drought.** A, Root morphology of non-transgenic plants (GL-3), *MdMYB88* or *MdMYB124* overexpression plants (OE), and *MdMYB88/124* 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).

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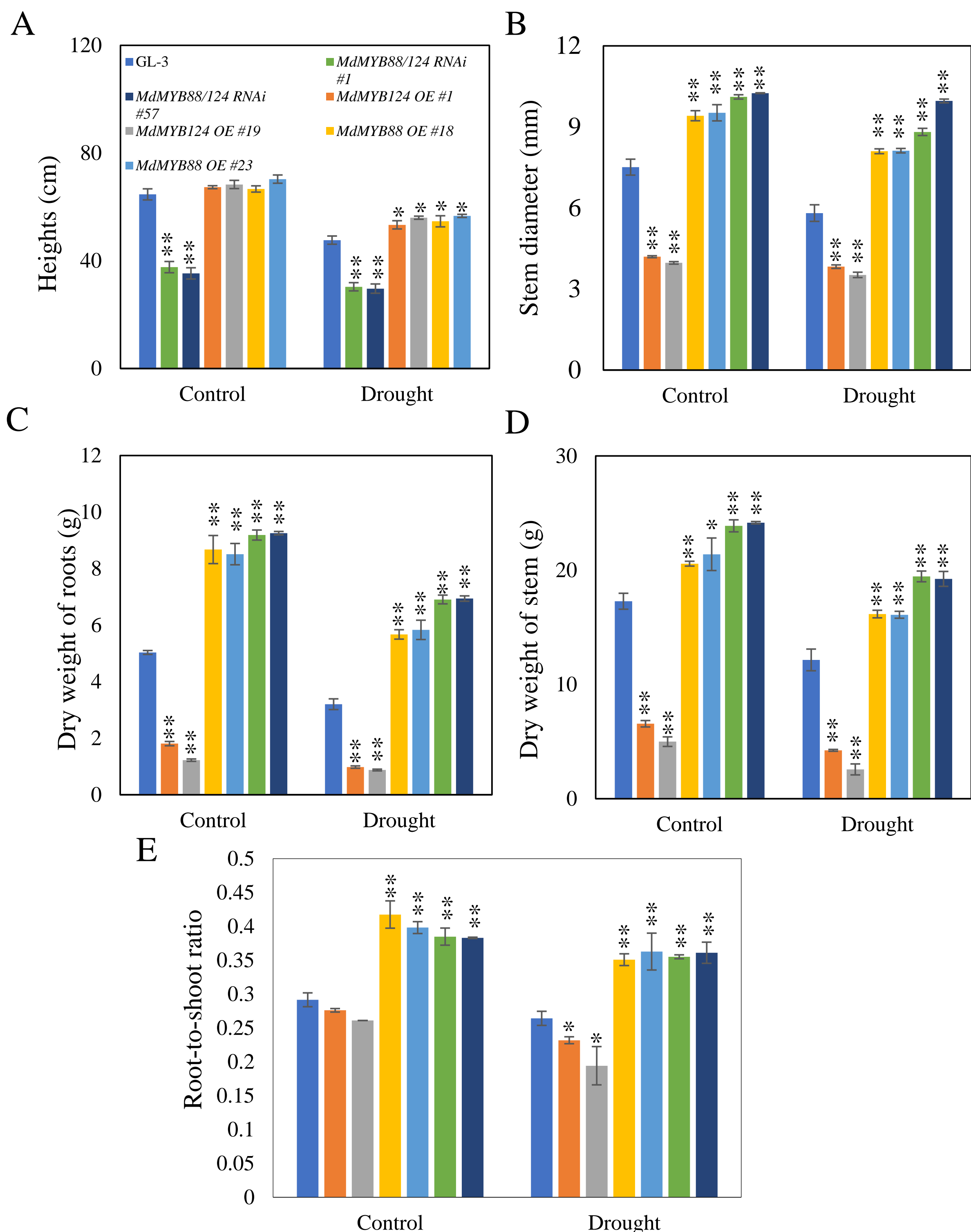


**Figure 2. Quantitation of morphological traits of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought conditions.** A, Plant height. B, Stem diameter. C, Dry weight of roots. D, Dry weight of stem. E, Root-to-shoot ratio. Plants were subjected to long-term drought stress for two 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).

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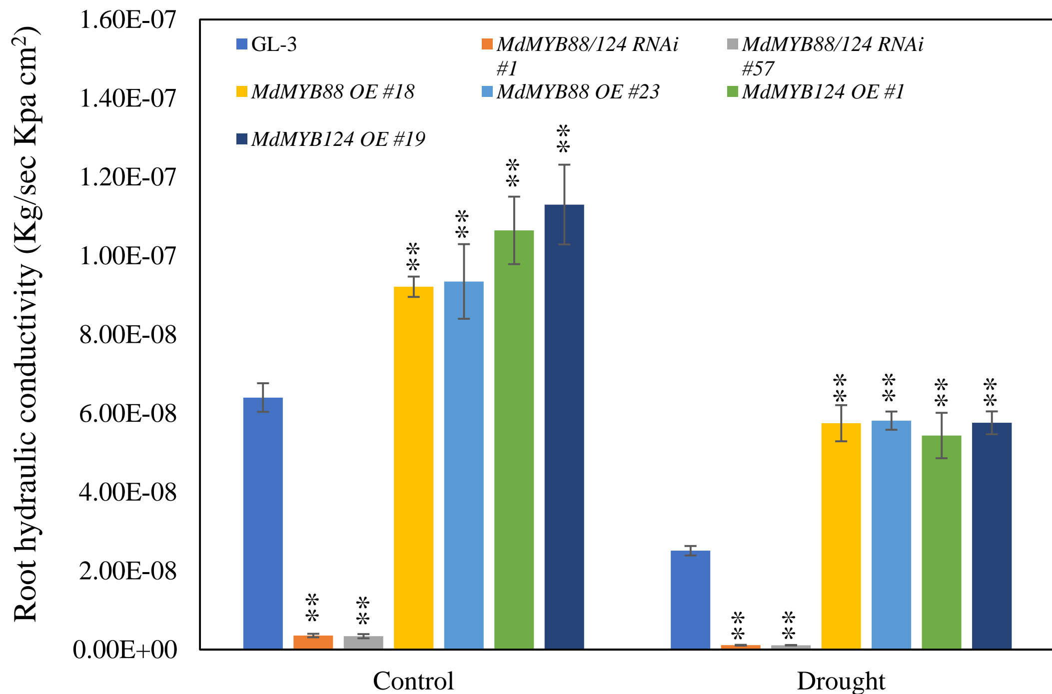


**Figure 3. Root hydraulic conductivity of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought conditions.** Plants were subjected to long-term drought stress for two months in a greenhouse. Data are means ± SD ($n$ = 9). One-way ANOVA (Tukey test) was performed and statistically significant differences are indicated by * (P<0.05) or ** (P<0.01).

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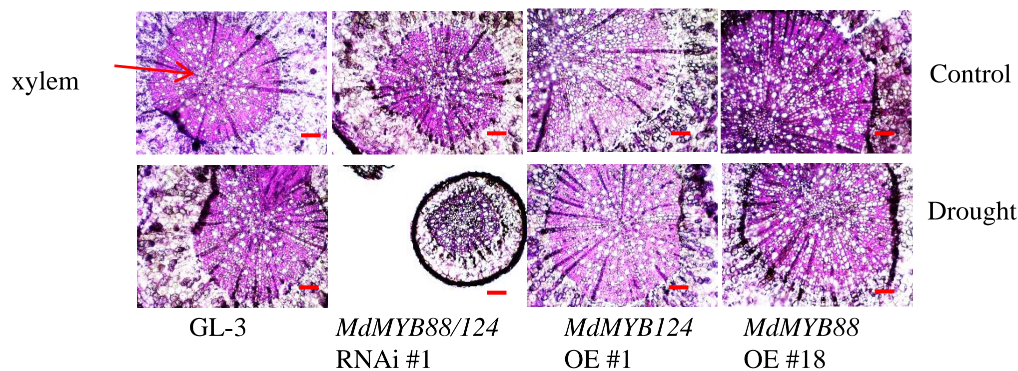




**Figure 4. Xylem development in roots of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under long-term drought conditions.** A, Cross sections of roots from GL-3 and transgenic plants stained with Safranin O. Bars = 100 µm. B, Quantification of root xylem of plants 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.


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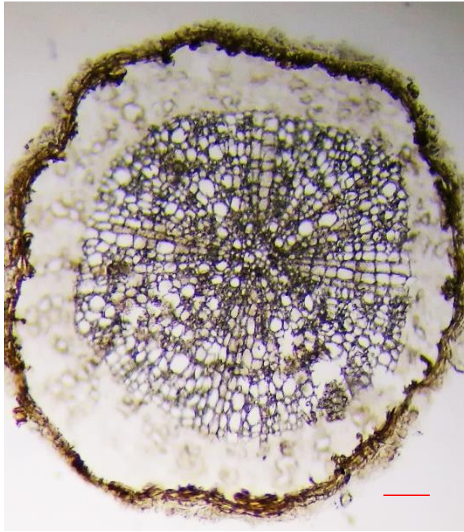

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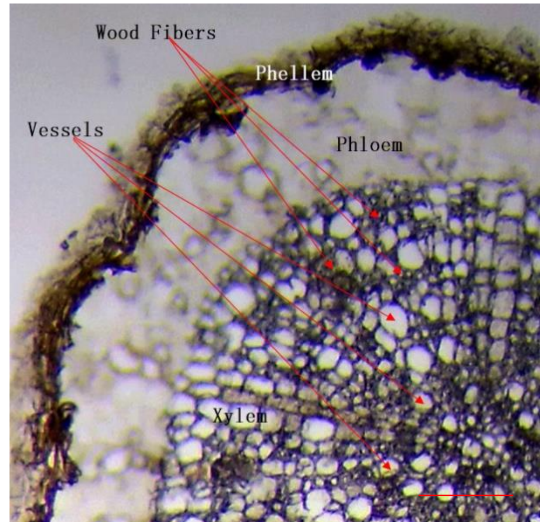


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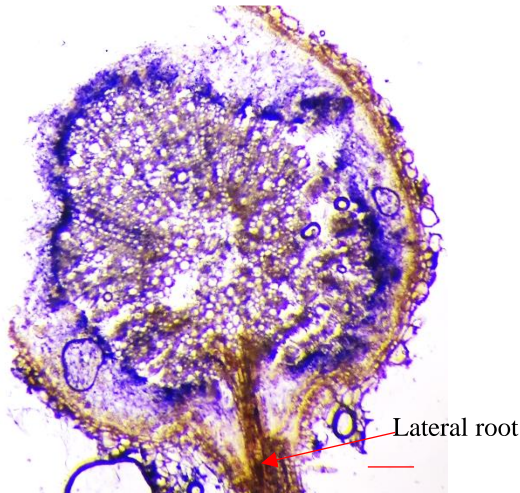


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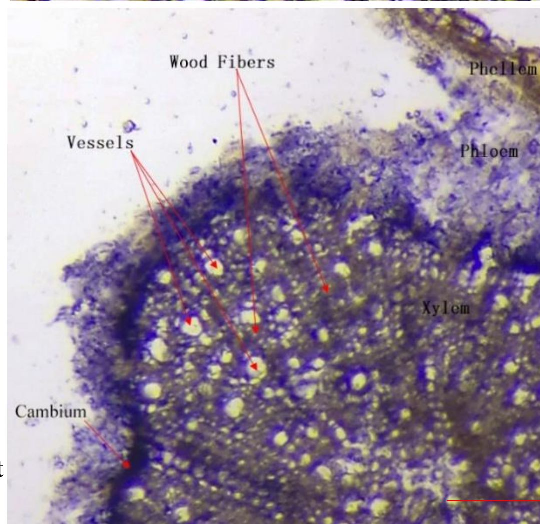


**Figure 5. Localization of *MdMYB88* transcripts in roots of GL-3.** A, *In situ* hybridization of *MdMYB88* transcripts using sense probe. B, Enlarged image of (A). C, *In situ* hybridization of *MdMYB88* transcripts using antisense probe. D, Enlarged image of (C). *MdMYB88* transcript in roots is indicated by purple coloring. Bars = 100 µm.

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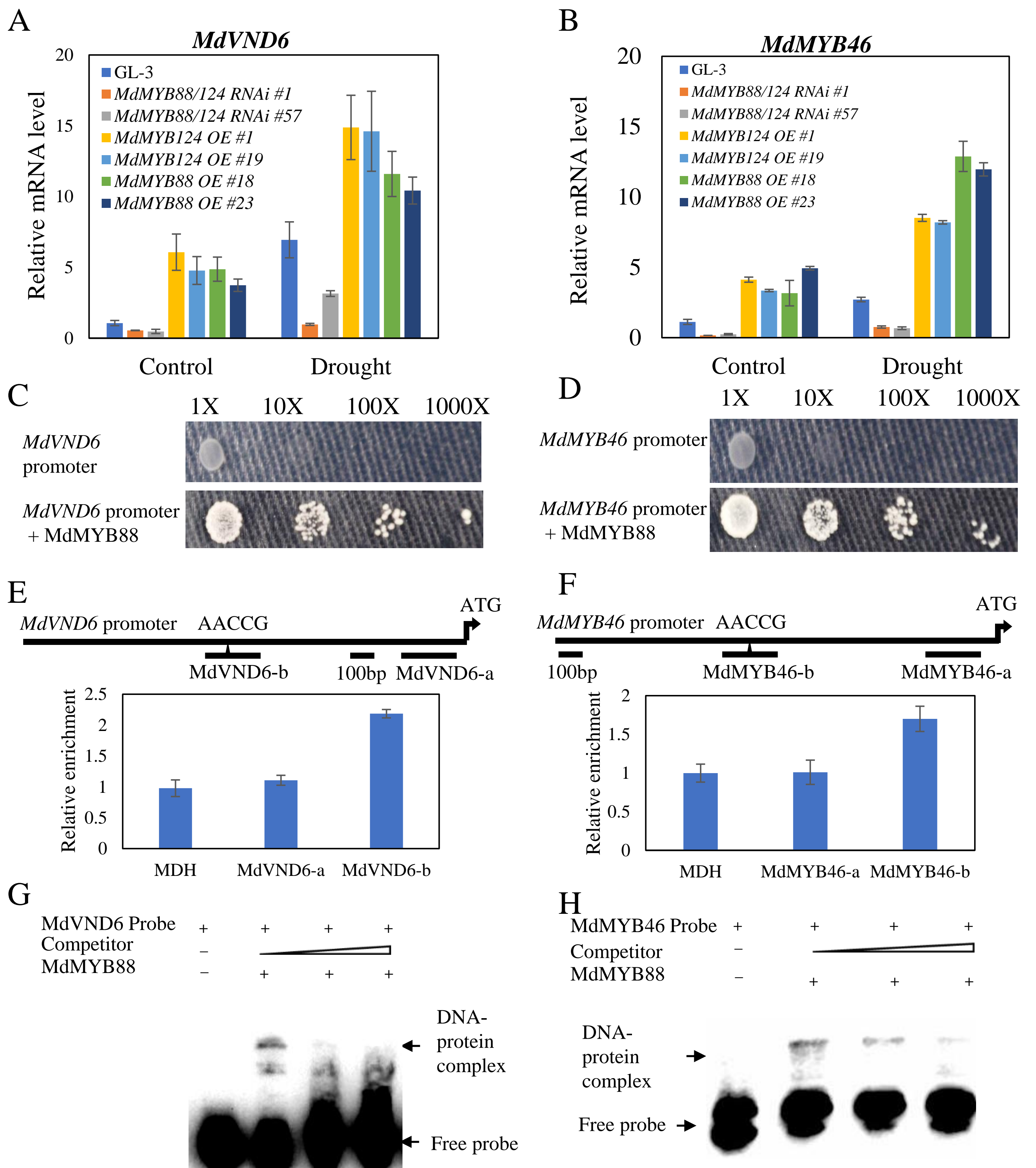


**Figure 6. MdMYB88 and MdMYB124 regulate *MdMYB46* and *MdVND6* expression by directly targeting their promoters.** A and B, Expression level of *MdVND6* and *MdMYB46* in roots of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants in response to drought stress. Plants 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.

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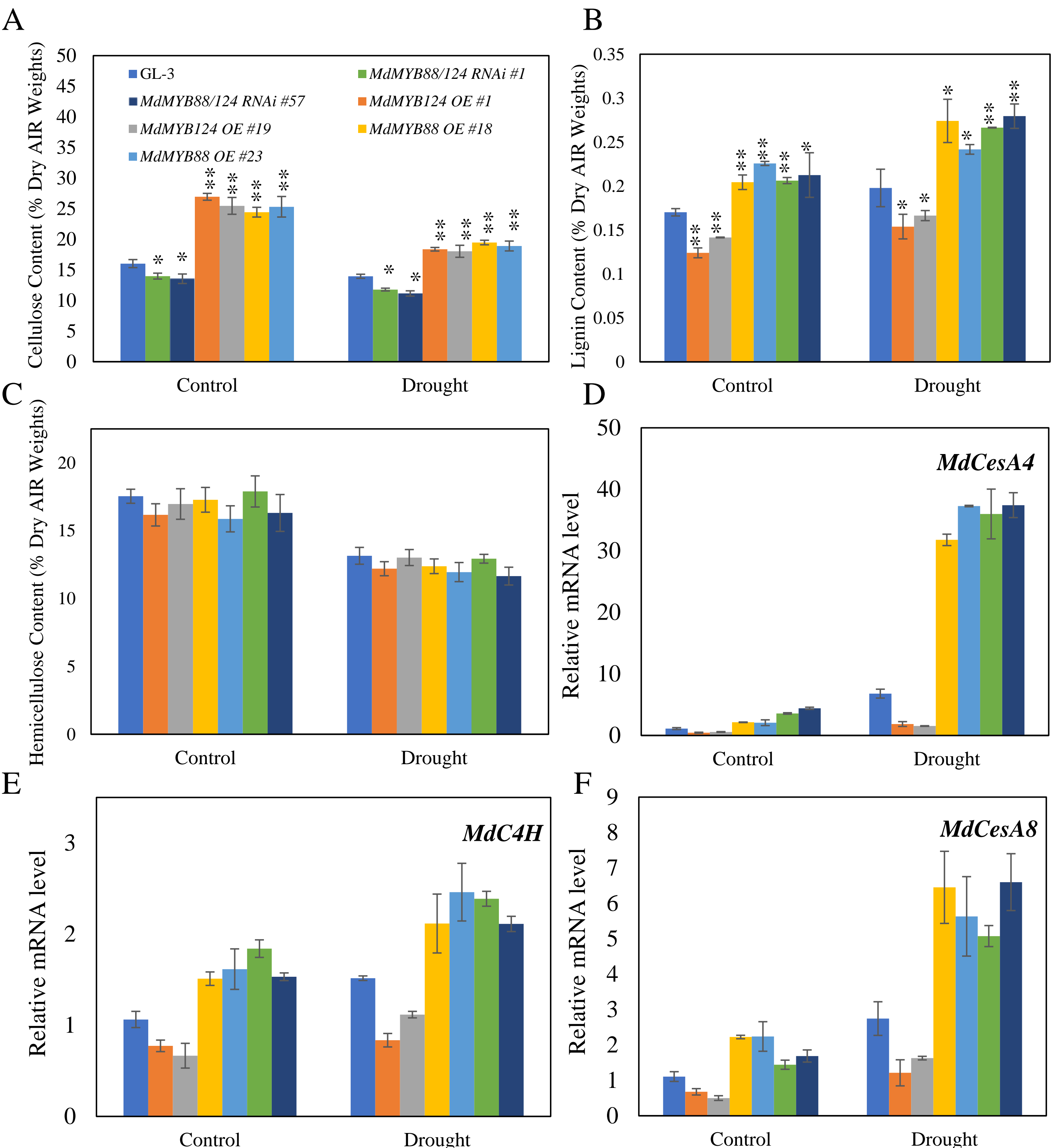


**Figure 7. Content of cellulose, lignin, hemicellulose, and expression level of genes associated with secondary cell wall biosynthesis in roots of GL-3, *MdMYB88* or *MdMYB124* overexpression plants, and *MdMYB88/124* RNAi plants under drought conditions.** A to C, Contents of cellulose (A), lignin (B) and hemicellulose (C). Plants were subjected to long-term drought stress for two 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 ($n = 3$).

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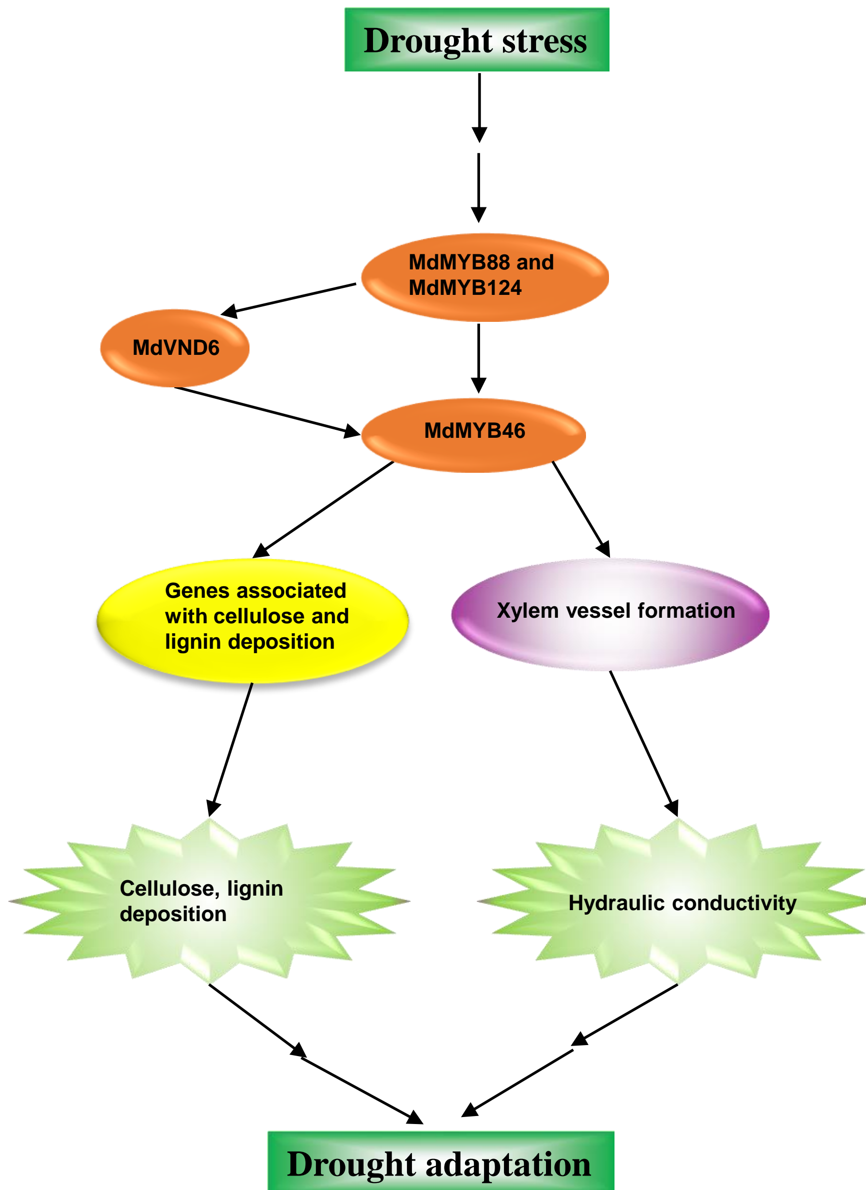


**Figure 8. A model for drought adaptation mediated by MdMYB88 and MdMYB124 in apple roots.** In apple roots, drought stress activates MdMYB88 and MdMYB124, which then directly target the promoters of *MdVND6* and *MdMYB46* and induce their expression. Up-regulated *MdMYB46* expression enhances drought 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.

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