The HSF–DREB–MYB transcriptional regulatory module regulates flavonol biosynthesis and flavonoid B-ring hydroxylation in banana (*Musa acuminata*)

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Short title: HSF–DREB–MYB module regulates flavonol biosynthesis in banana

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Abstract

Plant flavonols act primarily as ultraviolet radiation absorbers, reactive oxygen species scavengers, and phytoalexins, and they contribute to biotic and abiotic stress tolerance in plants. Banana (*Musa acuminata*), an herbaceous monocot and important fruit crop, accumulates flavonol derivatives in different organs, including the edible fruit pulp. Although flavonol content varies greatly in different organs, the molecular mechanisms involving transcriptional regulation of flavonol synthesis in banana are not known. Here, we characterized three SG7-R2R3 MYB transcription factors MaMYBFA1, MaMYBFA2, and MaMYBFA3) and their upstream regulators, heat shock transcription factor (MaHSF11) and dehydration responsive element binding factor (MaDREB1), to elucidate the molecular mechanism involved in transcriptional regulation of flavonol biosynthesis in banana. MaMYBFA positively regulate *flavonol synthase* 2 (*MaFLS2)* and downregulates *MaFLS1*. We show these transcription factors to be weak regulators of flavonol synthesis.

Overexpression of *MaHSF11* enhances flavonol contents, particularly that of myricetin, and promotes flavonol B-ring hydroxylation, which contributes to the diversity of flavonol derivatives. MaHSF11 directly interacts with the *MaFLS1* and *flavonoid 3', 5'-hydroxylase*1 (*MaF3'5'H1)* promoters, both *in vitro* and *in vivo*. MaHSF11 activates the expression of *MaDREB1* directly, which in turn regulates the expression of *MaMYBFA3*. Overall, our study elucidates a key regulatory mechanism for flavonol synthesis in banana and suggests possible targets for genetic optimization to enhance nutritional value and stress responses in this globally important fruit crop.

Keywords: Flavonol biosynthesis, MYB transcription factors, ABA, banana, HSF transcription factors, transcriptional regulatory module, metabolon

Introduction

Plants produce a multitude of chemical compounds in response and adaptation to external and environmental stimuli, many of which are specialized metabolites (Oh *et al.,* 2009; Mikkelsen *et al.,* 2015; Murcia *et al.,* 2017). Among these, flavonoids are ubiquitous and play vital roles in both regulation of developmental processes and defense against pathogens (Halbwirth *et al.,* 2010; Maloney *et al.,* 2014; Chen *et al.,* 2019; Yang *et al.,* 2021). Based on their structures, flavonoids are classified into flavonols, flavones, flavanones, isoflavones, anthocyanins, and proanthocyanidins (Lago *et al.,* 2014). Flavonols have a basic 16-carbon, triple-ring structure containing a 3-hydroxyflavone backbone, and the different modifications and substituents in this ring give rise to their diversity (Ko *et al.,* 2014). Besides acting as co-pigments with anthocyanins in flower petals and as reactive oxygen species (ROS) scavengers, flavonols have numerous other important functions in plants (Kitamura *et al.,* 2016; Watkins *et al.,* 2017). They absorb ultraviolet radiation (UV), protecting plants from UV-induced oxidative damage (Liang *et al.,* 2020) and also regulate root growth and lateral root development by inhibiting polar auxin transport via stabilization of PIN-FORMED (PIN) protein dimers (Tan *et al.,* 2019; Chapman *et al.,* 2021; Teale *et al.,* 2021). In addition, flavonols regulate pollen development and germination, nodule development, and symbiosis (Zhang *et al.,* 2009; Muhlemann *et al.,* 2018). Flavonols are synthesized in early steps of the flavonoid biosynthesis pathway involving the enzymes chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′ hydroxylase (F3′H), flavonoid 3′5′ hydroxylase (F3′5′H), and flavonol

62 synthase (FLS), which are well characterized in many plant species (Nagamatsu *et al.,* 2007;
63 Yu *et al.,* 2015; Guo *et al.,* 2019; Jiang *et al.,* 2020).

64 The flavonol biosynthesis pathway is regulated at several levels, and its transcriptional
65 regulation has been extensively studied (Matus *et al.,* 2009). A group of MYB transcription
66 factors (TFs), known as subgroup 7 (SG7) R2R3-MYBs, play a major role in the regulation of
67 flavonol biosynthesis (Schwinn *et al.,* 2016); R2 and R3 are conserved regions. MYB12,
68 MYB111, and MYB11, also known as PRODUCTION OF FLAVONOL GLYCOSIDES1–3
69 (PFG1–3), respectively, modulate flavonol biosynthesis in a tissue-specific manner in
70 Arabidopsis (*Arabidopsis thaliana*) (Mehrtens *et al.,* 2005; Stracke *et al*., 2007; Stracke *et al.,*
71 2010), tobacco (*Nicotiana tabacum*) (Misra *et al.,* 2010; Pandey *et al.,* 2014a, b; Pandey *et al.,*
72 2015a), and tomato (*Solanum lycopersicum*) (Pandey *et al.,* 2015b). They regulate the
73 expression of several rate-limiting biosynthesis genes, including *CHS*, *F3H*, and *FLS*. *FLS* is
74 also coordinately regulated by MYB99, MYB21, and MYB24 in Arabidopsis stamens and
75 anthers independently of the PFGs (Battat *et al.,* 2019; Zhang *et al.,* 2021).

76 Because plants are exposed to numerous biotic and abiotic stresses, such as excessive
77 light and UV radiation, suboptimal water conditions (drought and waterlogging), high salt,
78 extreme temperatures, nutrient deficiencies, and pathogen infection (López *et al.,* 2008; Cramer
79 *et al.,* 2011; Kalaji *et al.,* 2017; Choudhury *et al.,* 2017), they have evolved various defensive
80 mechanisms that are activated after exposure to stress and involve a complex interplay of
81 signaling pathways. Other classes of TFs, such as heat shock factors (HSFs) and dehydration-
82 responsive element-binding (DREB) factors, modulate the abscisic acid (ABA)–dependent
83 stress response to drought and high heat (Zhao *et al.,* 2010; Lee *et al.,* 2010; Huang *et al.,* 2016;
84 Zinsmeister *et al.,* 2020). HSFs, in their dimeric form, regulate several stress-responsive genes
85 by binding to heat shock elements (HSEs; nGAAnnTTCn/nTTCnnGAAn) via their N-terminal
86 DNA-binding domains (Liu *et al.,* 2008; Wang *et al.,* 2020). HSFs can be classified into three
87 major classes, A, B, and C, depending upon the type of amino acid residues and the length of
88 flexible linker length between DBD (DNA-binding domain) and OD (oligomerization domain)
89 (Scharf *et al.*, 2012; Manna *et al.,* 2021). In addition to heat stress tolerance, HSFs regulate
90 salt, drought, and cold stress responses in Arabidopsis (Yoshida *et al.,* 2011). MdHSFA8a is a
91 positive regulator of drought stress tolerance and flavonoid synthesis in apple (*Malus*
92 *domestica*) (Wang *et al.,* 2020). The HSFA2 and HSFA3 heterodimeric complex confers
93 transcriptional thermal memory by promoting epigenetic H3K4 hypermethylation (Friedrich *et*
94 *al.,* 2021). DREBs, which belong to the AP2/ERF family of TFs, are involved in several stress

95 signaling pathways, in particular drought, salt, and cold stress (Dubouzet *et al.,* 2003; Chen *et*
96 *al.,* 2016). CBF/DREB TFs bind to the cold and dehydration-responsive CRT/DREB motif
97 core element (CCGAC) present in the promoters of their target genes via the AP2/ERF DNA-
98 binding domain (Li *et al.,* 2020; Wang *et al.,* 2021). In addition, DREBs regulate fruit ripening
99 via a complex transcriptional regulatory pathway in banana (*Musa acuminata*) (Kuang *et al.,*
100 2017).

101 In terms of global production and monetary value, banana is the fourth most important food
102 crop in the world, after rice (*Oryza sativa*), wheat (*Triticum aestivum*), and maize (*Zea mays*)
103 (Wang *et al.,* 2021). Banana is a monocarpic herbaceous crop with climacteric fruits, which is
104 characterized by postharvest ripening because of cellular respiration and increased ethylene
105 production. However, global gross production is often affected by stresses including drought,
106 cold, salinity, and, most importantly, *Fusarium oxysporum* f. sp. *cubense* 4 (Foc4), which
107 causes a devastating wilt disease (Xu *et al.,* 2010; Dong *et al.,* 2020). We identified and
108 functionally characterized three SG7 R2R3-MYB TFs (MaMYBFA1, MaMYBFA2, and
109 MaMYBFA3) as regulators of flavonol synthesis in banana. These MaMYBFAs positively
110 regulate *MaFLS2* and downregulate *MaFLS1*. MaHSF11 directly regulates the expression of
111 *MaFLS1*, *MaF3'5'H1*, and *MaDREB1*. MaDREB1, in turn, regulates the expression of
112 *MaMYBFA3*. We also found that MaFLS1 transiently interacts with MaF3′5′H2 to form a
113 metabolon that promotes the synthesis of the hydroxylated flavonol myricetin, which has strong
114 antioxidant, anticancer, antidiabetic, and anti-inflammatory activities. Additionally, we
115 identified MaHSF11 as a regulator of cytochrome P_{450} (CYP450) B-ring hydroxylase in
116 banana, where it binds directly to the promoter of *MaF3'5'H1.* Taken together, our results
117 elucidate a key regulatory cascade of flavonol synthesis in the important fruit crop banana.

118 **Results**

119 **An MaMYBFA1–3 triad modulates flavonol biosynthesis in banana**

120 Our previous *in silico* study (Pucker *et al.,* 2022) determined that the banana genome contains
121 285 *R2R3-MYB* genes. Three of the encoded MaMYBs form a clade with previously
122 characterized flavonol regulators in a phylogenetic tree (Fig. 1a). We selected these three
123 candidates for further study and named them *MaMYBFA1* (Ma02_g00290), *MaMYBFA2*
124 (Ma05_g23640), and *MaMYBFA3* (Ma08_g10260), where the abbreviation FA stands for
125 flavonol activator. A multiple protein sequence alignment identified the conserved R2R3-MYB
126 domain and the two SG7-specific, conserved motifs: SG7-1 and SG7-2 (Fig. 1b).

127 We used RT-qPCR to analyze the expression of these three *MaMYBFA* genes in
128 different organs/tissues of banana variety 'Grand Naine' (Supplemental Fig. S1). All three
129 *MaMYBFA* genes were differentially expressed in the analyzed organs. *MaMYBFA1* was most
130 highly expressed in bract tissue, followed by unripe peel and root. *MaMYBFA2* transcript levels
131 were highest in unripe peel, followed by bract and unripe pulp, and *MaMYBFA3* was expressed
132 most highly in bract followed by pseudostem, young leaf, and unripe pulp. Transcript
133 accumulation of the three genes was lowest in ripe peel and ripe pulp. Overall, *MaMYBFA3*
134 showed the highest levels of expression in all tissues. These expression patterns suggest
135 functional differences between the three MaMYBFAs in terms of upstream regulation and
136 downstream regulatory potential toward biosynthesis genes.

137 The full-length coding sequences (CDSs) of *MaMYBFA1*, *MaMYBFA2*, and
138 *MaMYBFA3* were PCR-amplified from banana cDNA and used to express the encoded
139 proteins. The subcellular localization of MaMYBFA-YFP fusion proteins was analyzed in
140 *Nicotiana benthamiana* epidermal cells and was restricted to the nucleus, co-localizing with
141 the nuclear marker protein NLS-RFP (Fig. 1c), indicating that MaMYBFA1–3 are nuclear
142 proteins. To analyze the regulatory role of MaMYBFA1–3, we transiently overexpressed the
143 *MaMYBFA1–3* CDSs under the control of the *Zea mays ubiquitin1* promoter (*proZmUBI1*) in
144 immature banana fruit discs (Fig. 2a-b). The activity of a GUS reporter simultaneously
145 transformed on the same T-DNA indicated successful transformation of the fruit tissues (Fig.
146 1b). RT-qPCR analysis revealed upregulation of the respective *MaMYBFA* transcripts in
147 transgenic tissue samples (Fig. 2c). The expression of selected flavonoid biosynthesis genes
148 was analyzed in MaMYBFA-overexpressing (OE) fruit discs. The expression of *CHALCONE*
149 *SYNTHASE* (*MaCHS1–6*), *CHALCONE ISOMERASE* (*MaCHI1–2*), *FLAVANONE 3-*
150 *HYDROXYLASE* (*MaF3H1–2*), and *FLAVONOL SYNTHASE* (*MaFLS1–2*) was modulated to
151 different degrees in the OE fruit discs (Fig. 2d, Supplemental Figure S2). *MaMYBFA1*
152 overexpression enhanced the expression of *MaCHS1*, whereas *MaMYBFA2* and *MaMYBFA3*
153 overexpression did not (Supplemental Fig. S2). *MaCHS4* expression increased significantly
154 with overexpression of all three *MaMYBFA*s. However, *MaCHS3* and *MaCHS5* showed
155 enhanced expression only with overexpression of *MaMYBFA1* and *MaMYBFA3* (Supplemental
156 Fig. S2), and only *MaMYBFA1* and *MaMYBFA2* overexpression increased the *MaCHS6*
157 transcript level. *MaCHI* and *MaF3H* transcripts were also modulated in the transgenic OE
158 fruits: The highest activation of *MaCHI1* resulted from *MaMYBFA1* overexpression, whereas
159 *MaCHI2* transcripts were increased 2-fold by *MaMYBFA3* overexpression (Supplemental Fig.

160 S2). For *MaF3H1*, a significant increase in the transcript level was observed with *MaMYBFA1*
161 and *MaMYBFA3* overexpression (Supplemental Fig. S2). Genes encoding CYP450 proteins
162 also showed differential modulation; only *MaF3'5'H3* and *MaF3'5'H4* transcripts were
163 upregulated in OE fruits, whereas *MaF3'H* and other *MaF3'5'H* paralogs showed a marked
164 decrease in expression (Supplemental Fig. S2). Finally, we analyzed the expression of *MaFLS1*
165 and *MaFLS2*, the putative primary targets of MaMYBFAs, in transgenic *MaMYBFA* OE fruit
166 discs (Fig. 2d). *MaFLS1* was significantly downregulated, whereas *MaFLS2* transcript levels
167 increased in the *MaMYBFA* OE tissues (Fig. 2d). Overall, we found that overexpression of
168 *MaMYBFA1* and *MaMYBFA3* significantly increased the expression of most structural
169 flavonoid biosynthesis genes, whereas *MaMYBFA2* overexpression either decreased or
170 negligibly affected the transcript levels of most of the genes studied.

171 To analyze the modulation of flavonol metabolite contents in transgenic *MaMYBFA*
172 OE fruit slices, we performed LC-MS-based, targeted flavonol quantification (Fig. 2e). We
173 found decreased levels of kaempferol and quercetin in all the *MaMYBFA* OE tissues and an
174 increase in myricetin content in *MaMYBFA2* OE compared to the empty vector (EV) control.
175 Although the observed changes were significant for kaempferol, the contents of quercetin and
176 myricetin were not altered significantly by *MaMYBFA1* and *MaMYBFA3* OE. Only
177 *MaMYBFA2* OE significantly increased the accumulation of myricetin (Fig. 2e). Taken
178 together, these results indicate that MaMYBFA1, MaMYBFA2, and MaMYBFA3 act as a
179 MYB regulatory triad for modulating flavonol synthesis in banana.

180 To test the opposite regulation of *MaFLS1* and *MaFLS2* expression by MaMYBFAs,
181 we examined the promoters of the *MaFLS* genes to identify possible MYB binding sites (MYB
182 core elements, CNGTTR). Structural analyses of a 2556-bp *MaFLS1* (*proMaFLS1–2556*)
183 promoter fragment and a 1500-bp *MaFLS2* (*proMaFLS2–1500*) promoter fragment revealed
184 several MYB core elements (Fig. 3a). To analyze the transactivation capacity of the
185 MaMYBFAs on these *MaFLS* promoter fragments, we performed dual-luciferase assays in *N.*
186 *benthamiana* leaves. The reporter constructs in the p635nRRF vector used for this assay
187 contain the promoters to be analyzed positioned upstream of the LUC gene encoding firefly
188 luciferase, with simultaneous expression of REN (Renilla luciferase) driven by the constitutive
189 cauliflower mosaic virus (CaMV) 35S promoter. The LUC/REN ratio therefore represents the
190 level of activation by the particular promoter analyzed. The effectors in the pBTdest vector
191 contain the *MaMYBBFA* CDS under the control of the CaMV35S promoter (Fig. 3b). The
192 results of these dual-luciferase assays indicated a decreased activity of *proMaFLS1–2556* in

193 response to all three MaMYBFAs, with the largest effect on MaMYBFA1 and MaMYBFA2;
194 the activity of *proMaFLS2–1500* was mildly, but not significantly, increased in response to
195 MaMYBFA1–3 (Fig. 3c). These results suggest that all three MaMYBFAs repress *MaFLS1*
196 expression and that MaMYBFA1 slightly activates *MaFLS2* expression.

197 **MaHSF11 promotes flavonol synthesis in banana by transactivating *MaFLS1* and**
198 **regulating several other biosynthesis genes**

199 None of the banana SG7 R2R3-MYBs we examined upregulated the expression of *MaFLS1*
200 but instead repressed it. Because *MaFLS1* is highly expressed in leaves and is the major enzyme
201 for flavonol synthesis in banana (Pandey *et al.,* 2016; Busche *et al.,* 2021), we attempted to
202 identify a putative regulator of *MaFLS1*. An ~2-kb promoter fragment was searched for
203 possible TF binding sites, including ACGT-motif, E-box, LTRE (DRE/CRT), HSE, and W-
204 box elements, which are recognized by bZIP, MYC, DREB/CBF, HSF, and WRKY factors,
205 respectively (Supplemental File S1). Among these, the HSF TFs are poorly characterized in
206 the context of flavonoids. The *MaFLS1* promoter contained two HSEs (nTTCnnGAAn) at
207 positions −310 (HSE1) and −1154 (HSE2) upstream of the transcription start site (TSS). To
208 identify a HSF as a possible HSE-binding candidate, we searched the publicly available banana
209 transcriptome datasets related to abiotic stresses because both *HSF* and *FLS* are well-known
210 stress-responsive genes. We found a transcriptome dataset of the drought-stressed banana
211 cultivars Saba (ABB, drought tolerant) and Grand Naine (AAA, drought susceptible) to be the
212 most suitable for this analysis (Muthusamy *et al.*, 2016). From the transcriptome deep
213 sequencing data, we found five *HSF* genes, *MaHSF9* (*GSMUA_Achr8G27750_001*),
214 *MaHSF11* (*GSMUA_Achr6G31750_001*), *MaHSF16* (*GSMUA_Achr3G15640_001*),
215 *MaHSF25* (*GSMUA_Achr1G07880_001*), and *MaHSF26* (*GSMUA_Achr1G16240_001*), that
216 were differentially expressed before and after drought treatment in the cultivar Grand Naine
217 (Fig. 4a). *MaHSF25* and *MaHSF26* are class B HSFs, which are known to repress gene
218 expression (Scharf *et al*., 2012). However, *MaHSF9*, *MaHSF10*, and *MaHSF11* are class A
219 HSFs, which are known to act activate gene expression due to the AHA activator motif (Scharf
220 *et al*., 2012).

221 Using a dual-luciferase assay in *N. benthamiana* leaves, we examined whether
222 MaHSF9, MaHSF10, and MaHSF11 could regulate the expression of *MaFLS1* by testing their
223 ability to regulate *proMaFLS1–2556*. We observed a significant increase in the activity of
224 *proMaFLS1–2556* when co-transfected with either *MaHSF10* or *MaHSF11* compared to the

225 EV (Fig. 4b). Between these two, MaHSF11 showed the higher *in vivo* transactivation ability
226 (>3.6-fold, $P < 0.0001$). Therefore, we focused on MaHSF11 in further studies and tested
227 whether MaHSF11 could directly interact with *proMaFLS1*. To verify direct binding of
228 MaHSF11 to the HSEs in the *MaFLS1* promoter, we performed a ChIP-qPCR assay in which
229 *proMaFLS1* was co-infiltrated with a *pro35S:YFP-MaHSF11* construct into *N. benthamiana*
230 leaves and expressed for 3 days. An anti-GFP antibody was used to immunoprecipitate YFP-
231 MaHSF11 and the associated chromatin. A qPCR assay with purified DNA from the
232 precipitated chromatin showed an approximate 5-fold enrichment of fragment F1 containing
233 HSE1; fragment F2, containing HSE2, was not enriched (Fig. 4c). We then performed Y1H
234 assays to validate the direct binding of MaHSF11 to HSEs of *proMaFLS1* using 100-bp, HSE-
235 containing promoter fragments F1 and F2 to test for interaction with the MaHSF11-AD fusion
236 protein. The Y1H growth assay showed strong binding of MaHSF11 to F1 and a weak
237 interaction with F2 (Fig. 4d). Taken together, our results show that MaHSF11 binds directly to
238 the HSEs in the promoter of *MaFLS1* and activates its expression. Phylogenetic analysis
239 revealed clustering of MaHSF11 with AtHSFA4a, which confers salt tolerance in the MPK3/6-
240 dependent pathway in Arabidopsis, and other A-class HSF proteins (Supplemental Fig. S3).
241 Furthermore, a subcellular localization assay in *N. benthamiana* leaves using a *pro35S:YFP-*
242 *MaHSF11* construct confirmed co-localization with a nuclear NLS-RFP marker (Fig. 5a). In
243 addition to nuclear localization, we also observed fluorescence in plasma membrane,
244 suggesting probable post-translational modification.

245 To further test the regulatory role of MaHSF11, we transiently overexpressed
246 *proZmUBI:MaHSF11* in banana fruit discs (Fig. 5b). RT-qPCR analysis confirmed that the
247 expression of *MaHSF11* was significantly higher (8-fold, $P \leq 0.001$) in *proZmUBI:MaHSF11*
248 OE banana fruits than in the EV controls (Fig. 5c). Furthermore, the expression of *MaFLS1*
249 was significantly upregulated (3-fold, $P \leq 0.01$), and the expression of *MaFLS2* was
250 downregulated about 2-fold (Fig. 5c). We also observed differential expression of other
251 structural flavonol biosynthesis genes in *MaHSF11*-OE banana fruit discs (Supplemental Fig.
252 S4-d) as well as ~3-fold upregulation in MaMYBFA3 expression, but no significant
253 upregulation of the other two MYB genes (Supplemental Fig. S4e).

254 We quantified the levels of several different flavonol compounds in *MaHSF11*-OE fruit
255 tissues. LC-MS-based quantification showed increased levels of kaempferol, quercetin, and
256 myricetin in transgenic fruit discs (Fig. 5d). Taken together, these results suggest that

257 MaHSF11 positively regulates flavonol synthesis via regulation of several key biosynthesis
258 genes, including direct regulation of *MaFLS1*.

259 **MaHSF11 directly regulates *MaF3'5'H1* to promote flavonoid B-ring hydroxylation and**
260 **enhances myricetin biosynthesis**

261 MaHSF11 overexpression modulated the expression of *MaF3'H* (Supplemental Fig. S4d) and
262 all the *MaF3'5'H1–7* gene family members (Fig. 6a). *MaF3'5'H1* was the most upregulated
263 gene (a 7-fold increase), followed by *MaF3'5'H*2 and other homologs; *MaF3'H* expression
264 decreased negligibly (Supplemental Fig. S4d). Analysis of *cis* motifs in the promoter of
265 *MaF3'5'H1* revealed a single HSE (nGAAnnTTCn) at the –720-bp position relative to the TSS
266 (Supplemental File S1). We performed Y1H assays to test the interaction of MaHSF11 with
267 the HSE of *proMaF3'5'H1–1335* using a 100-bp, HSE-containing promoter fragment (F1),
268 which showed a strong interaction with MaHSF11, as indicated by yeast growth (Fig. 6b). The
269 ability of MaHSF11 to transactivate *proMaF3'5'H1–1335* was tested in a dual-luciferase assay
270 in *N. benthamiana* leaves. The increased LUC activity with MaHSF11, compared to the EV
271 control, indicated that MaHSF11 activated the *MaF3'5'H1* promoter (Fig. 6c). To further test
272 the direct binding of MaHSF11 via the HSE, we co-infiltrated a *pro35S:YFP-MaHSF11*
273 construct and the *proMaF3'5'H1–1335* fragment into *N. benthamiana* leaves and performed a
274 ChIP assay using an anti-GFP antibody. This ChIP-qPCR assay revealed enrichment of the
275 HSE-containing 100-bp promoter fragment F1 (Fig. 6d). Overall, these results show that
276 MaHSF11 directly regulates the expression of *MaF3'5'H1*.

277 **MaHSF11 regulates *MaMYBFA3* through MaDREB1**

278 Overexpression of *MaHSF11* in banana fruit discs resulted in higher expression of several other
279 structural genes involved in flavonol biosynthesis (Supplemental Fig. S4a-d). Therefore, we
280 investigated the expression of regulatory *MaMYBFA1–3* genes. We found a significant
281 upregulation of only *MaMYBFA3* in banana fruit discs overexpressing *MaHSF11*
282 (Supplemental Fig. S4e). A promoter analysis of the ~2-kb region upstream of the *MaMYBFA3*
283 TSS revealed the absence of any HSEs (Supplemental File S1). Therefore, we hypothesized
284 that MaHSF11 might indirectly regulate the expression of *MaMYBFA3* via an unknown factor.
285 Furthermore, promoter analysis of *MaMYBFA3* revealed the presence of several DRE/CRT
286 (CCGAC) elements in the ~2.5-kb region upstream of the TSS (Supplemental File S1). Because
287 HSFs bind HSEs in the promoter of *DREB* genes (Yoshida *et al.,* 2011; Huang *et al.,* 2016),
288 we hypothesized that DREB TFs may act as mediators between *MaHSF11* and *MaMYBFA3*.

289 To investigate the likelihood that MaHSF11–MaDREB–MaMYBFA3 is a regulatory module
290 in banana, we measured the expression levels of *DREB* genes using the RNA-Seq data from
291 drought-treated bananas of the Grand Naine cultivar (Muthusamy *et al.*, 2016). We found the
292 expression of a single *DREB* gene (*GSMUA_Achr4G19660_001*), which was also upregulated
293 after drought stress (Fig. 7a). This gene was previously characterized as MaCBF1 (C-repeat
294 binding factor1) and described as a critical regulator of cold stress tolerance in banana (Zhao
295 *et al*., 2013; Shan *et al*., 2014). We isolated the 777-bp full-length CDS of this gene and named
296 it *MaDREB1* due to nucleotide sequence dissimilarity with MaCBF1 (Shan *et al.*, 2014).
297 Phylogenetic analysis revealed that MaDREB1 formed a clade with rice OsDREB3 and maize
298 ZmDREB (Supplemental Fig. S5). To examine whether the promoter of *MaDREB1* was
299 regulated by MaHSF11, we analyzed the region ~2 kb upstream of the TSS (*proMaDREB1–*
300 *998*) and identified two HSEs at positions –223 and –576 bp upstream of the TSS
301 (Supplemental File S1). We measured the expression of *MaDREB1* in *MaHSF11* OE fruit discs
302 and determined that it was significantly upregulated (>6-fold) (Fig. 7b).

303 To verify the direct regulation of MaDREB1, we conducted a Y1H assay. We cloned
304 two 100-bp fragments (F1 and F2) harboring HSEs (HSE1 and HSE2, respectively) of the
305 *proMaDREB1* and conducted a Y1H assay with MaHSF11 as the effector. Promoter fragments
306 carrying the HSEs were cloned into the pAbAi vector and transformed into yeast
307 (*Saccharomyces cerevisiae*) Y1H Gold strain together with pGADT7 as a negative control.
308 MaHSF11 in pGADT7 was transformed with both fragments, and the growth assay was
309 performed on media supplemented with aureobasidin A (AbA). MaHSF11 could bind the
310 HSE1-containing fragment F1 of *proMaDREB1 in vitro* (Fig. 7c). To test the transactivation
311 activity of MaHSF11 on *proMaDREB1*, we cloned a 998-bp promoter fragment of *MaDREB1*
312 into the p635nRRF vector and performed dual-luciferase assays. The EV as a negative control
313 and *MaHSF11* as the effector were individually infiltrated together with the reporter construct
314 carrying a particular promoter fragment into *N. benthamiana* leaves, and the assay was
315 performed 3 days post infiltration. LUC activity increased significantly when MaHSF11 was
316 co-transfected compared to the EV control (Fig. 7d). Furthermore, *pro35S:YFP-MaHSF11* and
317 the promoter fragment were also transformed into *N. benthamiana* leaves, and a ChIP assay
318 was conducted. An anti-GFP antibody was used to precipitate MaHSF11-bound chromatin, and
319 anti-IgG was used as a negative control. ChIP-qPCR showed enrichment of the F1 fragment in
320 the immunoprecipitated chromatin, demonstrating that MaHSF11 directly bound the F1
321 fragment and regulated *MaDREB1* (Fig. 7e).

322 To test whether MaDREB1 acted as a direct transcriptional activator of *MaMYBFA3*,
323 we isolated *proMaMYBFA3–1892*, which contains all three DREs (CCGAC), and cloned it into
324 pAbAi. We performed a Y1H assay with MaDREB1 as the effector in pGADT7 and EV as a
325 control. Promoter fragments cloned into the pAbAi vector were transformed into the Y1H
326 strain, and a growth assay was performed on AbA-supplemented media. Growth was observed
327 in the MaDREB1 transformed strain, suggesting that it bound to the promoter fragment (Fig.
328 8a). To study the transactivation activity of MaDREB1, we cloned a 2508-bp fragment of
329 *proMaMYBFA3* into p635nRRF to perform a dual-luciferase assay with MaDREB1 in
330 pBTDest as the effector and the EV as a negative control. LUC activity increased when
331 MaDREB1 was used as the effector compared to the control, indicating its ability to
332 transactivate *proMaMYBFA3* (Fig. 8b). Overall, these findings show direct regulation of
333 *MaMYBFA3* by MaDREB1.

334 To verify the function of *MaDREB1 in planta*, we transiently overexpressed it in banana
335 fruit discs. GUS staining indicated successful transformation of the GUS expression cassette
336 (Fig. 8c). RT-qPCR analysis of the transformed fruit tissue showed high expression (~375-fold,
337 $P < 0.0001$) of *MaDREB1* relative to the EV control, demonstrating successful overexpression
338 (Fig. 8d). Expression analysis of *MaMYBFA3* in the *MaDREB1* OE tissue showed an ~10-fold
339 increase in the expression level ($P < 0.05$). We also found higher expression (~21-fold, $P <$
340 0.001) of *MaMYBFA2*, while *MaMYBFA1* expression was unaltered (Fig. 8d). *MaFLS1* and
341 *MaFLS2* also showed opposite trends in expression in the *proZmUBI:MaDREB1* OE fruit
342 tissues: *MaFLS1* was downregulated, and *MaFLS2* was upregulated (Fig. 8d). Differential
343 expression of other structural flavonol biosynthesis–related genes was also observed in
344 *proZmUBI:MaDREB1* OE banana fruit tissues (Supplemental Fig. S6). To study the
345 modulation of flavonol content resulting from *MaDREB1* overexpression, we used LC-MS to
346 quantify the levels of different metabolites and found significantly higher accumulation of
347 kaempferol, quercetin, and myricetin (Fig. 8e). These results indicate that MaDREB1
348 positively regulates flavonol biosynthesis in banana.

349 **Discussion**

350 Flavonoid biosynthesis pathways developed during the evolution of land plants. As they
351 became terrestrial, plants developed response mechanisms to adapt to and cope with several
352 new environmental conditions. Flavonoid biosynthesis is highly responsive to drought stress
353 hormones like ABA (Loreti *et al.,* 2008; Medina-Puche *et al.,* 2014; Song *et al.,* 2022). The

354 role of ABA in the context of flavonoid biosynthesis has been studied recently, strengthening
355 our knowledge regarding transcriptional regulation of flavonoid biosynthesis. Although
356 flavonol biosynthesis, in particular, is positively modulated by ABA, a conserved response
357 seen throughout the plant kingdom (Xu *et al.,* 2012; Song *et al.,* 2022), the molecular
358 mechanism controlling this response has remained obscure. In this study, we identified a key
359 transcriptional regulatory network involving different classes of TFs and showed their
360 interaction in regulating SG7 *MaMYBFA* and flavonol biosynthesis genes in the herbaceous
361 fruit crop banana. We deciphered important details of the molecular mechanisms involving the
362 transcriptional regulatory module of how HSFs, DREB, and MYB proteins orchestrate flavonol
363 accumulation.

364 Initially, three candidate SG7 R2R3-MYB TFs, *MaMYBFA1–3*, were identified as
365 having high sequence conservation in their R2R3 domains and partial conservation in the SG7-
366 1 (GRTxRSxMK, Stracke *et al.*, 2001) and SG7-2 motifs ([W/x] [L/x]LS, Czemmel *et al.,*
367 2009). Furthermore, the absence of the bHLH-interacting motif ([DE]Lx2[RK]x3Lx6Lx3R,
368 Zimmermann *et al.,* 2004) in the R3 domain suggested that these three candidate genes were
369 independent regulators of flavonol synthesis in banana. Expression analysis of the three
370 *MaMYBFA* genes revealed the highest level of transcript accumulation in bract tissue, a result
371 that correlated well with expression of *MaFLS2*, their probable target. It also showed a positive
372 correlation with *MaFLS2* in other tissues. By contrast, expression of *MaFLS1* showed an
373 inverse correlation regarding the three candidate genes in young leaves, where its transcript
374 level was highest. This finding indicates the presence of a regulator other than SG7 MYB in
375 banana, acting upstream of *MaFLS1*. All these results prompted us to functionally characterize
376 the candidate *MaMYBFA* genes in banana to determine their possible roles as flavonol-specific
377 TFs. To do this, we transiently overexpressed the *MaMYBFA* genes in banana fruit. Although
378 *MaFLS2* expression was activated by MaMYBFA, the expression of *MaFLS1* was
379 downregulated. This finding could be an exception, because none of the SG7 MYB activators
380 downregulate flavonol synthase genes, the key genes in the flavonol biosynthesis pathway.
381 Flavonol content mainly remained unaltered with MaMYBFA overexpression, a finding that
382 can be attributed to the downregulation of *MaFLS1* and *MaF3'H* and increased expression of
383 *MaFLS2*, which could compensate for it. Thus, although we concluded that MaMYBFAs are
384 weak regulators of flavonol synthesis in banana, it is difficult to ascertain their positive
385 regulatory role on flavonol synthesis. However, our results show the division of labor among
386 the SG7 MYB transcriptional regulators within the regulatory cascade. Such a mechanism has

387 been observed in the dicot freesia (*Freesia hybrida*), in which four SG7 MYB proteins,
388 FhMYBF1–4, differentially regulate the expression of *FhCHS* and *FhCHI* paralogs, as well as
389 *FhFLS1*, but not *FhFLS2* (Shan *et al.,* 2020). SG7 MYB TFs regulate flavonol synthesis by
390 directly binding to MYB binding sites (MBSs, CNGTTR) present in the promoters of flavonol
391 biosynthesis genes, activating their expression. In apple, MdMYB22 binds to the MBS in the
392 promoters of *MdCHS* and *MdFLS* to regulate their expression (Wang *et al.,* 2017). In freesia,
393 FhMYB1–4 bind to the MYB core (CNGTTR) and MYBPLANT motifs (MACCWAMC),
394 which are essential for promoter binding and transactivation (Shan *et al.,* 2022). Overall, these
395 results suggest that MaMYBFA1–3 are the key functional flavonol-specific transcriptional
396 regulators in banana.

397 HSFs are critical components of the signaling cascades involved in responses to salt,
398 cold, drought, and heat stress (Chauhan *et al.,* 2011; Bian *et al.,* 2020; Wang *et al.,* 2020).
399 Several HSFs and their regulatory networks have been discovered in Arabidopsis, rice, tomato,
400 soybean (*Glycine max*), wheat, and grape (*Vitis vinifera*) (Guo *et al.,* 2016; Wu *et al.,* 2022).
401 In this study, MaHSF11 was identified as a regulator of *MaFLS1*. MdHSFA8a was previously
402 identified as a regulator of *MdFLS* in apple, promoting flavonol biosynthesis and the drought
403 stress response by regulating *MdFLS* expression (Wang *et al.,* 2020). GmHSFB2B confers salt
404 stress tolerance and flavonol synthesis in soybean by repressing GmNAC2, a negative regulator
405 of *GmFLS1* and *GmFLS2* (Bian *et al.,* 2020). Apart from these, other non-SG7 MYB regulators
406 from other species include AtMYB21, AtMYB24, and FhMYB21L2. The presence of three
407 HSEs in the promoter of *MaFLS1* suggested it might be regulated by a HSF. We determined
408 the role of MaHSF11 to be a regulator of CYP450 B-ring hydroxylase in banana, where it
409 directly bound to the promoter of *MaF3'5'H1* (Fig. 6b). Very few regulators of *F3'5'H* have
410 been identified in plants. In poplar (*Populus tremula×tremuloides*), MYB117 specifically
411 regulates *F3'5'H* expression to promote anthocyanin synthesis and flavonoid B-ring
412 hydroxylation (Ma *et al.,* 2021). MYBC1 and WRKY44 both independently and cooperatively
413 regulate *F3'5''H* expression to regulate the anthocyanin level in kiwifruit (*Actinidia purpurea*)
414 (Peng *et al.,* 2020).

415 In our study, *MaHSF11* overexpression upregulated *MaFLS1* but downregulated
416 *MaFLS2*, indicating the presence of a complex regulatory cascade leading to preference of one
417 paralog over the other. MaHSF11 also promoted the expression of several *MaF3'5'H* gene
418 homologs, while slightly repressing *MaF3'H* (Supplemental Fig. S4d). Since the enzymes
419 compete with each other for the common substrate dihydrokaempferol, MaHSF11 might be

420 specifically promoting the myricetin synthesis branch and tries to suppresses the quercetin
421 synthesis branch catalyzed by MaF3′H, either directly or indirectly. *MaFLS1* was highly
422 expressed in young leaves, and this finding can be correlated with higher *MaHSF11* expression
423 in leaves compared to fruits and roots (Wei *et al.,* 2016). Fruit tissue, particularly peel, had
424 higher expression of *MaF3'H*, leading to a greater accumulation of rutin and quercetin in peel
425 compared to other tissues (Pandey *et al.,* 2016). Of the seven paralogs of *MaF3'5'H* in banana,
426 five were upregulated 2-fold with *MaHSF11* overexpression, suggesting a role for *MaHSF11*
427 as a master regulator of this gene family (Fig. 6a). These results further suggest that MaHSF11
428 promotes flavonol biosynthesis via a distinct regulatory module to regulate stress-responsive
429 genes such as flavonol biosynthesis–related genes involved in drought, cold, and salinity stress
430 responses in banana.

431 Preference of MaHSF11 for the *MaFLS1* and *MaF3'5'H* homologs (relative to *MaFLS2*
432 and *MaF3'H*) suggested a specificity of MaFLS1 for dihydromyricetin as a substrate. In a
433 recent study, MaFLS1 also showed specificities for dihydrokaempferol and dihydroquercetin.
434 In future studies, it will be important to determine whether MaFLS1 has a substrate specificity
435 for dihydromyricetin as well. CYP450 enzymes are involved in the flavonoid metabolon in
436 both rice and soybean (Shih *et al.,* 2008; Waki *et al.,* 2016), where they act as ER membrane–
437 bound nucleation sites for metabolon formation. Interaction among enzymes varies greatly in
438 terms of their stability and complexity. Preferential upregulation of MaF3′5′H and MaFLS1
439 and downregulation of MaF3′H and MaFLS2 suggested probable metabolon formation in the
440 ER. In snapdragon (*Antirrhinum majus*), F3′H associates with CHI to form a metabolon (Fujino
441 *et al.,* 2018). In Arabidopsis, FLS1 interacts with CHS via competition with DFR
442 (dihydroflavonol 4-reductase), which can also bind to CHS (Crosby *et al.,* 2011).

443 We also observed that MaHSF11 upregulated *MaMYBFA3* indirectly via MaDREB1.
444 Interactions of DREB and HSF TFs have been reported previously (Yoshida *et al.,* 2011; Ya-
445 Chen *et al.,* 2016). In Arabidopsis, DREB2A is transcriptionally regulated by HSFA1a,
446 HSFA1b, and HSFA1d (Yoshida *et al.,* 2011), and DREB2A also acts upstream of HSFA3 to
447 regulate its expression (Schramm *et al.,* 2008). In our study, we found ~2-fold increased
448 expression of MaHSF11 when *MaDREB1* was overexpressed in banana, suggesting
449 conservation of an interconnected regulatory cascade (Supplemental Fig. S7). We also
450 determined that MaHSF11 directly bound to the HSE in *proMaDREB1* to regulate its
451 expression. Although overexpression of *MaDREB1* upregulated several biosynthesis genes
452 related to flavonol biosynthesis, leading to increased flavonol accumulation, *MaCHS2* and

453 *MaFLS1* were downregulated (Supplemental Fig. S6a–e). In future work, it will be intriguing
454 to determine whether MaDREB1 directly represses these genes. Recently, *MaCBF1* was
455 reported to be a positive regulator of chlorophyll catabolism: It possesses strong transactivation
456 activity and is localized to the nucleus (Xiao *et al*., 2023). Prior to this study, several DREB
457 proteins were reported to regulate different branches of flavonoid biosynthesis. CitERF32 and
458 CitERF33 act as transcriptional inducers of *CitCHIL1* to promote flavone and flavanone
459 biosynthesis in citrus (*Citrus reticulata cv. Suavissima*) (Zhao *et al.,* 2021). In eggplant
460 (*Solanum melongena*), SmCBF1, SmCBF2, and SmCBF3 interact with SmMYB113 to
461 regulate *SmCHS* and *SmDFR* expression and enhance anthocyanin synthesis (Zhou *et al.,*
462 2020). In our study, we discovered MaDREB1 to be a key regulator of flavonol synthesis,
463 modulating the expression of regulatory SG7 MYB genes. In eggplant as well, SmCBF1,
464 SmCBF2, and SmCBF1 are induced by drought, salt, and ABA, showing that MaDREB1 might
465 also be involved in drought stress signaling as a transcriptional regulator of stress-responsive
466 genes such as flavonoid biosynthesis genes. Our study provides insights into a key regulatory
467 cascade that promotes flavonol biosynthesis in banana under different stress conditions, as
468 shown in our proposed model (Fig. 9).

469 The banana cultivar Grand Naine (Cavendish, AAA) is highly susceptible to different
470 abiotic stresses as well as pathogen infection. Flavonols acting as ROS scavengers and
471 pathogen defense molecules are synthesized in increased amounts under a variety of stresses.
472 Here, we identified the HSF–DREB-MYB transcriptional regulatory module and characterized
473 how it works independently or cooperatively to regulate flavonol synthesis in an ABA-
474 responsive manner. The TFs in this regulatory module can be used for effective engineering of
475 stress resistance in crop plants like banana. These candidate genes could be utilized for
476 metabolic engineering of rutin and myricetin synthesis in banana fruit.

477 **Materials and methods**

478 **Plant material**

479 Banana (cultivar subgroup Cavendish [AAA] variety 'Grand Naine') plants were grown in the
480 fields of National Institute of Plant Genome Research (NIPGR), New Delhi, India. Various
481 tissues, including young leaf, bract, pseudostem, root, peel, and pulp, of different fruit
482 developmental stages were sampled during winter season, frozen in liquid nitrogen, and stored
483 at −80°C until further use. *Nicotiana benthamiana* plants were grown in a Percival AR-41L3

484 growth cabinet at 100 µmol m^{-2} s^{-1} light intensity and 22°C under a 16-h-light/8-h-dark
485 photoperiod.

486 **Identification of candidate flavonol-specific TFs from banana**

487 To identify candidate SG7 R2R3-MYB TFs, which are known activators of flavonol
488 biosynthesis, we retrieved sequences of previously characterized flavonol-specific TFs from
489 various plants from NCBI (https://www.ncbi.nlm.nih.gov). These sequences were used to
490 perform a BLASTp search against the banana genome sequence (DH-Pahang version 2)
491 (https://banana-genome-hub.southgreen.fr). For phylogenetic studies, MEGA X (Kumar *et al.,*
492 2018) was used to construct a maximum-likelihood phylogenetic tree with 1000 bootstrapping
493 rounds. For multiple sequence alignment, MaMYBFA1–3 peptide sequences were aligned with
494 a set of similar MYBs (Stracke *et al.,* 2014) using MAFFT v.7.299b (Katoh and Standley,
495 2013). Different domains and motifs were identified manually (Naik *et al.,* 2021).

496 **Cloning of flavonol-specific transcriptional regulators**

497 The full-length CDSs (without the stop codons) of the MaMYB-encoding genes *MaMYBFA1*
498 (*Ma02_g00290*), *MaMYBFA2* (*Ma05_g23640*), and *MaMYBFA3* (*Ma08_g10260*) were
499 amplified using first-strand cDNA of banana fruit tissue and a set of primers containing
500 Gateway attB sites that were designed based on information from the Banana Genome Hub
501 (Supplemental Table S1). The resulting amplicons were recombined into the Gateway vector
502 pDONR^{TM}zeo (Invitrogen) and transformed into *Escherichia coli* TOP10 (Invitrogen) cells.
503 Sanger sequencing of the resulting entry plasmids was performed by the NIPGR sequencing
504 core facility (New Delhi, India).

505 **Subcellular localization and co-localization assays**

506 CDSs of *MaMYBFA1–3* and *MaHSF11* were cloned into the pSITE-3CA binary vector
507 (Chakrabarty *et al*., 2007) via LR recombination, producing yellow fluorescent protein (YFP)
508 fusion proteins. Empty vectors were used as negative controls. The binary plasmids were
509 transformed into *Agrobacterium tumefaciens* strain GV3101-pMP90 (Koncz & Schell, 1986).
510 Equal volumes of *A. tumefaciens* cultures having the desired gene and the marker were mixed
511 for agro-infiltration. *N. benthamiana* leaves were infiltrated on the abaxial side using a syringe,
512 according to the protocol described by Walter *et al.* (2004). Infiltrated plants were kept in the
513 dark at 25°C for 2 days prior to analysis. Leaf discs were then cut from the infiltrated sites and
514 observed under an argon laser confocal laser-scanning microscope (TCS SP5; Leica

515 Microsystems, Wetzlar, Germany) with YFP and RFP filters. YFP and RFP fluorescence was
516 observed at 514-nm excitation/527-nm emission and 558-nm excitation/583-nm emission
517 wavelengths, respectively. YFP/RFP gains (intensities %) for MaMYBFA1, MaMYBFA2,
518 MaMYBFA3, and MaHSF11 were 760 (39)/158 (39), 818 (39)/158 (39), 818 (30)/158 (39),
519 and 718 (42)/154 (39), respectively.

520 **RT-qPCR analysis**

521 Total RNA was extracted from different banana organs according to a previously described
522 protocol (Asif *et al.,* 2006) and subsequently treated with RNase-free DNase (TURBO DNA-
523 freeTM Kit, Invitrogen). Total RNA was reverse transcribed to generate first-strand cDNA
524 using a RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher) and oligo(dT)
525 primers. RT-qPCR-based gene expression analyses were performed on a 7500 Fast Real-time
526 PCR System (Applied Biosystems) using the 2x SYBR Green PCR Master mix (Applied
527 Biosystems) and diluted cDNA corresponding to 10 ng total RNA in a final volume of 10 µL.
528 *MaACTIN1* (GenBank AF246288) was used to normalize transcript abundance. Relative
529 transcript levels were calculated using the cycle threshold (C_t) $2^{-\Delta\Delta CT}$ method (Livak &
530 Schmittgen, 2001) and are presented as fold changes relative either to the tissue having the
531 lowest expression or to the control condition, depending on the requirement. Three biological
532 replicates were analyzed in each RT-qPCR analysis.

533 **Metabolite analysis**

534 Targeted metabolite analysis was performed to quantify flavonols according to a protocol
535 described previously (Pandey *et al.,* 2016; Naik *et al.,* 2021; Rajput *et al.,* 2022a). Plant
536 metabolites were extracted from shredded plant material in 80% methanol at room temperature.
537 Finely ground tissues were dissolved in 80% methanol, kept at 70°C for 15 minutes, and
538 subjected to centrifugation for 10 minutes at 12,000 rpm (18514 g). After centrifugation,
539 supernatants were mixed with three volumes of 1 N HCl for acid hydrolysis and incubated at
540 94°C for 2 h. After incubation, an equal volume of ethyl acetate was added, and the upper phase
541 was removed for further purification of the desired metabolites. The ethyl acetate was
542 evaporated at 37°C under lower pressure (90–100 mbar) on a Buchi rotavapor R-300 (BUCHI,
543 Switzerland), and samples were finally dissolved in 80% methanol. Liquid chromatography–
544 mass spectrometry (LC-MS) analysis was performed using an ultra-high-performance liquid
545 chromatography (UHPLC) system (Exion LC Sciex, Framingham) coupled to a triple
546 quadrupole system (QTRAP6500+; ABSciex) with electrospray ionization. The voltage was

547 set to 5500 V for positive ionization. Detailed UHPLC and LC-MS parameters have been
548 described by Naik *et al.* (2021). Identification and quantitative analyses were done via Analyst
549 software (version 1.5.2).

550 **Cloning of reporter constructs and identification of putative *cis* elements in promoters**

551 Total genomic DNA was extracted from banana leaves using the DNeasy Plant Mini Kit
552 (Qiagen). Sequences of the putative target gene promoter fragments were retrieved from the
553 banana genome (https://banana-genome-hub.southgreen.fr) (D'Hont *et al.,* 2016) and analyzed
554 for the presence of *cis*-regulatory motifs via New PLACE software
555 (https://www.dna.affrc.go.jp) supplemented by manual curation. Promoter fragments were
556 amplified from banana genomic DNA using designed primer sequences and Phusion™ High-
557 Fidelity DNA Polymerase (Thermo Scientific). The amplified fragments were cloned into the
558 pENTR™/D-TOPO™ vector (ThermoFisher) and sequenced to confirm their integrity. The
559 entry clones of promoters (*proMaFLS1–2508*, *proMaFLS2–1500*, *proMaF3'5'H1–1335*,
560 *proMaDREB1–998*, and *proMaMYBFA3–2508*) were recombined into the vector p635nRRF
561 containing *pro35S:REN* (Kumar *et al.,* 2018) to make the reporter constructs.

562 **Cloning of cDNA from MaHSF and MaDREB transcriptional regulators**

563 Full-length CDSs of selected homologs of MaHSF and MaDREB were amplified by first-
564 strand cDNA synthesis of banana leaves using Phusion™ High-Fidelity DNA Polymerase
565 (Thermo Scientific) and a set of primers containing Gateway attB sites designed on the basis
566 of information from the Banana Genome Hub (Supplemental Table S1). The resulting
567 amplicons were cloned into the pDONR^{TM} zeo vector (Invitrogen) and transformed into *E. coli*
568 TOP10 cells, and integrity was confirmed by Sanger sequencing (Sanger *et al.*, 1977).

569 **Dual-luciferase reporter assay**

570 To test the transactivation potential of the different transcriptional regulators, MaMYBFA1-
571 MaMYBFA3, MaHSF11, and MaDREB1 CDSs were recombined into the destination vector
572 pBTdest (Baudry *et al.,* 2004) to generate CaMV *pro35S*-driven effector constructs. For dual-
573 luciferase reporter assays, reporter and effector constructs were transformed into *A.*
574 *tumefaciens* strain GV3101 (pMP90) (GoldBio) and subsequently infiltrated using a syringe
575 into the abaxial side of *N. benthamiana* leaves according to a previously described protocol
576 (Walter *et al.,* 2004). LUC and REN activities were measured 48 h later in extracts from
577 infiltrated leaf discs using the Dual-Luciferase Reporter Assay System (Promega) according to

578 the manufacturer's protocol. Luminescence in the samples was quantified using the
579 POLARstar Omega multimode plate reader (BMG Labtech), and LUC activity was normalized
580 to REN activity. Values are given as means ± SD of the LUC/REN ratio.

581 **Y1H assays**

582 The Matchmaker Gold Yeast 1-hybrid system (Clontech) with the yeast one-hybrid (Y1H) gold
583 yeast strain was used. Promoter fragments were cloned into the pABAi vector (Takara Bio),
584 and the pABAi plasmids carrying the promoter fragments were linearized and transformed into
585 the Y1H Gold strain according to the Matchmaker protocol, whereby they integrated into the
586 yeast genome by homologous recombination. Effector plasmids and empty pGADT7-GW
587 vector (Gal4-AD fusion, Ura selection) controls were transformed into the Y1H strains
588 previously transformed with AurR-reporter constructs (*proMaFLS1*-AurR, *proMaF3'5'H1*-
589 AurR, *proMaDREB1*-AurR, and *proMaMYBFA3*-AurR). To test for interaction, the yeast
590 colonies containing effector and reporter constructs were spotted onto medium containing AbA
591 per the manufacturer's protocol. Growth of different Ura^- yeast colonies was checked using
592 different concentrations of AbA to determine the minimum inhibitory concentration (MIC)
593 values and to check for possible interactions. The MIC values for *proMaFLS1*, *proMaF3'5'H1*,
594 *proMaDREB1*, and *proMaMYBFA3* were 100 ng mL^{-1} AbA, 1000 ng mL^{-1} AbA, 100 ng mL^{-1},
595 and 900 ng mL^{-1}, respectively.

596 **ChIP-qPCR assay**

597 The ChIP-qPCR assay was performed using the ChIP Kit - Plants kit (Abcam) according to the
598 manufacturer's instructions (Shi *et al.,* 2019). Promoter fragments containing the HSE *cis* motif
599 in p635nRRF that was used for dual-luciferase assays were co-infiltrated with YFP-MaHSF11
600 fusion proteins into *N. benthamiana* leaves. Three days after infiltration, equally sized leaf
601 discs were used for ChIP. Next, 2 µL anti-GFP antibody (Catalog #ab290, Abcam) was used
602 for chromatin precipitation in two biological replicates, and 2 µL of anti-IgG antibody (Catalog
603 #ab48386, Abcam) was used as a negative control. qPCR was performed with 5 µL 2x SYBR
604 Green PCR Master mix (Applied Biosystems), 1 µL ChIP DNA, and 0.5 µL each forward and
605 reverse primer, with 40 cycles of amplification as described above. The relative fold
606 enrichment of the target promoter fragments was calculated using the $2^{-(-\Delta CT)}$ method (Livak
607 & Schmittgen, 2001). The primers used in ChIP-qPCR are listed in Supplemental Table S1.

608

609 **Transient overexpression in banana fruit**

610 For transient overexpression studies, the CDSs of *MaMYBFA1*, *MaMYBFA2*, *MaMYBFA3*,
611 *MaHSF11*, and *MaDREB1* were cloned into pANIC6b (Mann *et. al*., 2012), a Gateway-
612 compatible destination vector suitable for overexpression of genes of interest using *A.*
613 *tumefaciens*-mediated transformation, particularly useful for monocot species. pANIC6b
614 encodes hygromycin resistance under the control of a constitutive rice (*Oryza sativa*) *actin1*
615 promoter (*proOsACT1*) (LOC4338914), a constitutive *Panicum virgatum ubiquitin1+3*
616 promoter (*proPvUBI*)-driven GUSPlus visual marker, and a Gateway cassette driven by a *Zea*
617 *mays ubiquitin1* promoter and intron for overexpression of target genes. The resulting
618 overexpression plasmid and the empty vector control were transformed into *A. tumefaciens*
619 strain EHA105 (Mehra *et al.*, 2019). Resulting *A. tumefaciens* suspensions were then separately
620 introduced into slices of immature green banana fruits by vacuum infiltration as previously
621 described (Rajput *et al.,* 2022a). After 3 days of co-cultivation on MS media (MS basal salt
622 mixture, HiMedia), transformation of banana fruit slices was confirmed by GUS staining.
623 Tissues were harvested and frozen in liquid nitrogen for subsequent expression (RT-qPCR) and
624 metabolite (LC-MS) analyses.

625 **Statistical tests**

626 One-way and two-way analysis of variance (ANOVA) with Tukey's multiple comparison were
627 performed for statistical analysis. Unpaired Student's *t*-tests were used for the specific
628 comparisons. Asterisks indicate the level of significance: $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$,
629 $****P \leq 0.0001$; ns, not significant.

630 **Data availability**

631 All data supporting the findings of this study are available within the paper and within the
632 supplemental data published online.

633 **Supplemental data**

634 **Figure S1.** Tissue-specific expression of *MaMYBFA1-FA3* genes**.**

635 **Figure S2.** Relative expression of structural flavonol biosynthesis gene transcripts in EV and
636 *MaMYBFA1-FA3* transiently overexpressing lines.

637 **Figure S3.** Phylogenetic analysis of MaHSF11 with previously characterized ABF gens from
638 other plant species.

639 **Figure S4.** Relative expression of structural and regulatory flavonol biosynthesis genes in
640 *MaHSF11*-overexpressing (OE) banana fruit discs.

641 **Figure S5.** Phylogenetic analysis of MaDREB1 with previously characterized ABF genes from
642 other plant species.

643 **Figure S6.** Relative expression of structural flavonol biosynthesis genes in *MaDREB1*-
644 overexpressing banana fruit tissues.

645 **Figure S7.** Relative expression analysis of *MaHSF11* in *MaDREB1*-overexpressing banana
646 fruits.

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654

655 **Declarations**

656 The authors declare no conflicts of interest.

657

658 **Figure legends**

659 **Figure 1.** Identification of candidate R2R3-MYB flavonol regulators from the banana genome
660 and their subcellular localization. (a) Various R2R3-MYB proteins were classified into three
661 major groups as flavonol-, anthocyanin-, or proanthocyanidin biosynthesis–specific regulators
662 using landmark flavonoid-specific R2R3-MYBs from different plant species. Numbers on each
663 branch show branch length, and solid dark blue circles represent bootstrap values. (b) Amino
664 acid sequence alignment of candidate SG7 R2R3-MYB transcription factors (MaMYBFA1,

665 MaMYBFA2, and MaMYBFA3, where FA stands for flavonol activator) and selected flavonol
666 regulators from other plant species. The R2 and R3 domains are highlighted in grey. The SG7-
667 1 and SG7-2 motifs, found in subgroup 7 R2R3-MYBs, are highlighted in dark green and
668 yellow, respectively. Accession numbers and two-letter species abbreviations are provided in
669 Supplemental File S2. (c) Subcellular localization of MaMYBFAs. MaMYBFA-YFP fusion
670 proteins were expressed together with the nuclear marker NLS-RFP in *N. benthamiana* leaf
671 epidermal cells. Confocal imaging shows co-localization of YFP and RFP fluorescence,
672 indicating nuclear localization of the three MaMYBFA proteins. Representative images are
673 shown with scale bar of 50 μm.

674 **Figure 2.** MaMYBFA1, MaMYBFA2, and MaMYBFA3 differentially regulate the expression
675 of *MaFLS1* and *MaFLS2* and modulate flavonol biosynthesis in banana. (a) Diagram of the
676 plant expression cassette used for transient overexpression (OE) of *MaMYBFA* in banana fruit
677 discs. The *Zea mays ubiquitin1* promoter (*proZmUBI1*) drives the expression of *MaMYBFA*.
678 The expression of the *GUSPlus* marker gene is driven by the *Phaseolus vulgaris ubiquitin1*
679 promoter (*proPvUBI*). (b) Representative images showing GUS staining of transiently
680 transformed banana fruit discs indicating successful transformation. Images were taken 3 days
681 after transformation. (c-d) Relative expression of *MaMYBFA1–3* (c) and *MaFLS1* and *MaFLS2*
682 (d) OE lines in transiently transformed *MaMYBFA* OE banana fruit tissues as determined by
683 RT-qPCR. EV, empty vector control. (e) Flavonol quantification showing differential
684 alteration in the accumulation of kaempferol, quercetin, and myricetin aglycones in the
685 *MaMYBFA* OE banana fruit discs. Values are given in μg per 100 g of dry weight (DW) of
686 fruit discs of three replicates. One-way ANOVA with Tukey's multiple comparison was used
687 for statistical analyses. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$; ns, not
688 significant.

689 **Figure 3.** MaMYBFAs regulate the expression of *MaFLS1* and *MaFLS2 in vivo*. (a) Schematic
690 representation of the 2556- and 1500-bp promoter fragments of *MaFLS1* and *MaFLS2.* The
691 locations of the different MYB core (CNGTTR) sequences and their positions upstream of the
692 transcription start sites (−1) are indicated by red ovals. A plus (+) sign with a number indicates
693 the length of the 5′ UTR. (b) Diagram of the pBTdest effector and the p635nRRF reporter
694 constructs. Constitutive cauliflower mosaic virus (CaMV) promoter drives the expression of
695 transcription factor encoding genes and renilla *luciferase* (REN). Desired amplified promoter
696 fragments were placed upstream of firefly *luciferase* (LUC) (c) Dual-luciferase assay showing
697 the altered activity of the promoters *proMaFLS1–2508* and *proMaFLS2–1500* when transiently

698 co-infiltrated into *N. benthamiana* leaves with the effectors MaMYBFA1, MaMYBFA2, and
699 MaMYBFA3. Promoter activity is represented as the LUC/REN ratio in four biological
700 replicates ($n = 4$).

701 **Figure 4.** MaHSF11 directly binds to the *MaFLS1* promoter and regulates the expression of
702 *MaFLS1* to modulate flavonol biosynthesis in banana. (a) Heatmap showing expression of five
703 banana candidate *HSF* genes before and after drought treatment (Grand Naine cultivar, AAA
704 genome). FPKM values for the indicated genes were extracted from publicly available RNA-
705 Seq datasets and are represented according to the 0–7 color scale shown. (b) Dual-luciferase
706 assay showing transactivation of *proMaFLS1–2556* by MaHSF. Values are shown as means ±
707 SD of the LUC/REN ratios ($n = 4$). (c) ChIP-qPCR assay analyzing the binding of MaHSF11
708 to fragments F1 and F2 of *proMaFLS1*, both of which contain the HSE consensus sequence
709 (nTTCnnGAAn). Fold enrichment values are given as means ± SD of two biological replicates.
710 AB, antibody. (d) Yeast one-hybrid assay showing interaction of MaHSF11 with the two HSE-
711 containing *proMaFLS1* fragments F1 and F2. The empty pGADT7 vector (EV) was used as a
712 negative control. Interaction was indicated by yeast growth on synthetic Ura– dropout media
713 supplemented with aureobasidin (AbA). AD, activation domain.

714 **Figure 5.** MaHSF11 promotes flavonol synthesis in banana fruit. (a) Subcellular localization
715 of MaHSF11 in *N. benthamiana* leaves. The YFP-MaHSF11 fusion protein localizes to both
716 the nucleus and the cytoplasm and coincides with the signal from the nuclear localization
717 signal-red fluorescent protein (NLS-RFP) fusion in *N. benthamiana* leaves as observed in
718 confocal microscopy. Scale bar, 20 μm. (b) GUS staining of banana fruit slices indicating
719 successful transformation with the empty vector (EV) and the *proZmUBI:MaHSF11* construct.
720 (c) Relative expression of *MaHSF11*, *MaFLS1*, and *MaFLS2* in transiently transformed banana
721 fruit slices overexpressing (OE) *proZmUBI:MaHSF11* as determined by RT-qPCR. The empty
722 vector was transformed as a control (expression level set to 1). Values shown are means ± SD
723 of three technical replicates. (d) Quantification of the flavonols kaempferol, quercetin, and
724 myricetin in transformed banana fruit slices. Values are represented as means ± SD of three
725 replicates. Unpaired student's *t*-tests were used for statistical analyses. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$;
726 $^{***}P \leq 0.001$.

727 **Figure 6.** MaHSF11 promotes flavonoid B-ring hydroxylation by modulating the expression
728 of *MaF3'5'H1*. (a) Relative expression of *MaF3'5'H1* genes in transformed banana fruit slices
729 transiently overexpressing *proZmUBI:MaHSF11*, as determined by RT-qPCR (EV, empty

730 vector control). Values shown are means ± SD of three technical replicates. (b) Yeast one-
731 hybrid assay showing the interaction of MaHSF11 with *proMaF3'5'H1–100* fragment F1
732 carrying HSE (GGAATATTCT). pGADT7 was used as a negative control. Growth was
733 checked on synthetic dropout medium supplemented with aureobasidin. (c) Dual-luciferase
734 assay showing transactivation of *proMaF3'5'H1–1335* by MaHSF11. The promoter sequence
735 driving the expression of luciferase (LUC) was co-infiltrated with the EV or *MaHSF11*
736 constructs in *N. benthamiana* leaves. Values are shown as means ± SD of the LUC/REN ratio
737 ($n = 4$). (d) ChIP-qPCR assay showing binding of MaHSF11 to fragment F1 of *proMaF3'5'H1*
738 carrying an HSE (nGAAnnTTCn). The promoter fragment carrying HSE was co-infiltrated
739 into *N. benthamiana* leaves with the *pro35S:YFP-MaHSF11* construct. Fold enrichment values
740 are represented as means ± SD of two biological replicates. An anti-IgG antibody was used as
741 a negative control, and an anti-GFP antibody was used to precipitate MaHSF11-enriched
742 chromatin. Unpaired Student's *t*-tests were used for statistical analyses. $^{*}P \leq 0.05$.

743 **Figure 7.** MaHSF11 acts upstream of *MaDREB1* to positively regulate its expression in
744 banana. (a) Heatmap showing expression of candidate DREB genes before and after drought
745 treatment in banana (Grand Naine cultivar, AAA genome). FPKM values for the indicated
746 genes were extracted from publicly available RNA-Seq datasets and plotted according to the
747 0–7 color scale shown. (b) Relative expression of *MaDREB1* in banana fruit tissues transiently
748 transformed with EV or *proZmUBI:MaHSF11* as determined by RT-qPCR. (c) Yeast one-
749 hybrid assay showing interaction of MaHSF11 with the F1 and F2 fragments of *proMaDREB1–*
750 *100* carrying an HSE (nGAAnnTTCn). pGADT7 was used as a negative control. Growth was
751 checked on synthetic dropout medium supplemented with aureobasidin. (d) Dual-luciferase
752 assay showing transactivation of *proMaDREB1–998* by MaHSF11. Promoter sequence driving
753 the expression of LUC was co-infiltrated with empty vector and *MaHSF11* construct in *N.*
754 *benthamiana* leaves. Values shown are means ± SD of LUC/REN ratio ($n = 4$). (e) ChIP-qPCR
755 assay showing binding of MaHSF11 to *proMaDREB1*. A promoter fragment carrying an HSE
756 was co-infiltrated into *N. benthamiana* leaf cells with the *pro35S:MaHSF11-YFP* construct.
757 Fold enrichment values are represented as means ± SD of two biological replicates. An anti-
758 IgG antibody was used as a negative control, and an anti-GFP antibody was used to precipitate
759 MaHSF11 enriched chromatin. Unpaired Student's t-tests were used for statistical analyses.
760 $^{*}P \leq 0.05$; $^{***}P \leq 0.001$; ns, not significant.

761 **Figure 8.** MaDREB1 directly binds to the promoter of *MaMYBFA3* to regulate flavonol
762 biosynthesis. (a) Yeast one-hybrid assay showing interaction of MaDREB1 with the

763 *proMaMYBFA3–1892* fragment carrying three DREs (CCGAC). pGADT7 was used as a
764 negative control. Growth was checked on synthetic dropout medium supplemented with
765 aureobasidin. (b) Dual-luciferase assay showing transactivation of *proMaMYBFA3–2508* by
766 MaDREB1. The *proMaMYBFA3–2508* sequence driving the expression of LUC was co-
767 infiltrated with the empty vector or *MaDREB1* construct into *N. benthamiana* leaves. Values
768 are shown as means ± SD of the LUC/REN ratio ($n = 4$). (c) GUS staining of transformed fruit.
769 (d) RT-qPCR-based relative expression of *MaDREB1*, *MaMYBFAs*, *MaFLS1*, and *MaFLS2* in
770 transformed banana fruit tissues transiently expressing EV and *proZmUBI:MaDREB1*. EV was
771 transformed as a control. Values shown are means ± SD of three technical replicates. (e)
772 Quantification of the flavonols kaempferol, quercetin, and myricetin in transformed fruit
773 tissues. Values are represented as means ± SD of three replicates. Unpaired Student's *t*-tests
774 were used for statistical analyses. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; $^{****}P \leq 0.0001$.

775 **Figure 9.** A working model showing the modulation of hydroxylated flavonol synthesis by the
776 MaHSF11–MaDREB1–MaMYBFA regulatory module. The banana SG7 R2R3 MYB,
777 MaMYBFA, targets *MaFLS2* to promote its expression and promote flavonol synthesis.
778 Drought stress increases the transcript level of *MaHSF11*, enhancing the expression of *MaFLS1*
779 and *MaF3′5′H1*. MaFLS1 and MaF3′5′H might interact to form a metabolon at the ER
780 membrane to promote myricetin biosynthesis. MaHSF11 also directly activates the expression
781 of *MaDREB1*. MaDREB1 binds to the *MaMYBFA3* promoter to regulate its expression.
782 MaMYBFA1, MaMYBFA2, and MaMYBFA3 differentially regulate the expression of
783 *MaFLS1* and *MaFLS2*. MRE, MYB recognition element; DRE, dehydration response element;
784 ABRE, ABA response element; HSE, heat shock element. CHS (chalcone synthase) catalyzes
785 the condensation of one molecule of *p*-coumaryl-CoA and three molecules of malonyl-CoA
786 into naringenin chalcone, which is further converted into naringenin by CHI (chalcone
787 isomerase). F3H (flavanone 3-hydroxylase) converts naringenin into DHK
788 (dihydrokaempferol). F3′H (flavonoid 3′-hydroxylase) and F3′5′H (flavonoid 3′5′-
789 hydroxylase) compete for the substrate DHK to synthesize DHQ (dihydroquercetin) and DHM
790 (dihydromyricetin), respectively. FLS (flavonol synthase) converts DHK, DHQ, and DHM into
791 kaempferol, quercetin, and myricetin, respectively. F3′5′H also physically interacts with FLS1
792 to form a metabolon to accelerate myricetin synthesis. Flavonols are likely transported into the
793 vacuole by MATE transporters (multidrug and toxic compound extrusion).

794

795 **Supplemental Figures**

796 **Supplemental Figure S1. Tissue-specific expression of *MaMYBFA1–3* genes.** Relative
797 expression of *MaMYBFA1–3* genes in different tissues of banana as determined by RT-qPCR.
798 Tissues from three independent biological replicates were pooled and used for expression
799 analysis. YL, young leaf; BRT, bract; P.STEM, pseudostem; UR.PL, unripe peel; UR.PP,
800 unripe pulp; R.PL, ripe peel; R.PP, ripe pulp. Values shown are means ± SD of three technical
801 replicates.

802 **Supplemental Figure S2. Relative expression of structural flavonol biosynthesis gene**
803 **transcripts in banana fruit discs transiently overexpressing *MaMYBFA1-FA3*.** RT-qPCR-
804 based transcript profiling of structural flavonol biosynthesis genes *MaCHS1–6*, *MaCHI1–2*,
805 *MaF3H1–2*, *MaF3'5'H1–7*, and *MaF3'H* in banana fruit tissues of *MaMYBFA1–3* OE tissues.
806 EV, empty vector control. Values shown are means ± SD of three technical replicates. One-
807 way ANOVA was used for statistical analysis with Tukey's multiple comparison. *$P \leq 0.05$;
808 **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; ns, not significant.

809 **Supplemental Figure S3. Phylogenetic analysis of MaHSF11 and previously**
810 **characterized HSF genes from other plant species.** The phylogenetic tree was constructed
811 in MEGA-X32 software using the maximum-likelihood method with 1000 bootstrap values
812 and was visualized with iTOL v.6.4 (http://itol.embl.de/). Accessions used are MdHSFA8a
813 (NP_001281294.1), GmHSFB2b (XP_014619265.1), AtHSFA2 (AT2G26150), AtHSF1
814 (AT4G17750), AtHSF3 (AT5G16820), AtHSFA8 (AT1G67970), AtHSFC1 (AT3G24520),
815 AtHSFA3 (AT5G03720), AtHSF4 (AT4G36990), AtHSFB2A (AT5G62020), AtHSFB2B
816 (AT4G11660), and AtHSFA4A (AT4G18880). Md, *Malus domestica*; Gm, *Glycine max*; At,
817 *Arabidopsis thaliana.*

818 **Supplemental Figure S4. Relative expression of structural and regulatory flavonol**
819 **biosynthesis genes in banana fruit discs overexpressing *MaHSF11*.** RT-qPCR-based
820 expression analysis of (a) *MaCHS1–6*, (b) *MaCHI1–2*, (c) *MaF3H1–2*, (d) *MaF3'H*, and (e)
821 *MaMYBFA1–3* in *MaHSF11* OE banana fruit slices. Values shown are means ± SD of three
822 technical replicates. EV, empty vector control. Two-way ANOVA was used with Tukey's
823 multiple comparison. $P \leq 0.05$; **$P \leq 0.01$; ns, not significant. N.D., not determined.

824 **Supplemental Figure S5. Phylogenetic analysis of MaDREB1 with previously**
825 **characterized DREB genes from other plant species.** The phylogenetic tree was constructed

826 in MEGA-X32 software using the maximum-likelihood method with 1000 bootstrap values
827 and visualized with iTOL v.6.4 (http://itol.embl.de/). Accessions used were AtDREB1B
828 (AAV80413.1), AtDREB1C (NP_567719.1), AtDREB1C (AAV80414.1), GmDREB3
829 (AAZ03388.1), GmDREB2 (NP_001237254.2), GmDREB2A2 (NP_001240942.3),
830 OsDREB1 (AAL40870.1), OsDREB1B (AAN85707.1), OsDREB3 (ADO79620.1), SlDREB3
831 (NP_001234707.1), HvDREB2 (AFH75401.1), and ZmDREB (NP_001105081.1). At,
832 *Arabidopsis thaliana*; Gm, *Glycine max*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; Hv,
833 *Hordeum vulgare*; Zm, *Zea mays*)

834 **Supplemental Figure S6. Relative expression of structural flavonol biosynthesis genes in**
835 **banana fruit tissues overexpressing *MaDREB1*.** RT-qPCR-based expression analysis of (a)
836 *MaCHS1–6*, (b) *MaCHI1–2*, (c) *MaF3H1–2*, (d) *MaF3'5'H1–7*, and (e) *MaF3'H* in *MaDREB1*-
837 overexpressing tissues. Values shown are means ± SD of three technical replicates. Two-way
838 ANOVA was used with Tukey's multiple comparison. $P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; ns,
839 not significant.

840 **Supplemental Figure S7. Relative expression analysis of *MaHSF11* in banana fruits**
841 **overexpressing *MaDREB*.** Values shown are means ± SD of three technical replicates.
842 Unpaired Student's *t*-tests were used with Tukey's multiple comparison for statistical analysis.
843 $^{***}P \leq 0.001$

844 **Supplemental File S1.** Promoter sequences with highlighted *cis* motifs used in this study.

845 **Supplemental File S2.** Accession numbers and two-letter species abbreviations used in
846 phylogenetic tree figures.

847 **Supplemental Table S1.** List of primers used in this study.

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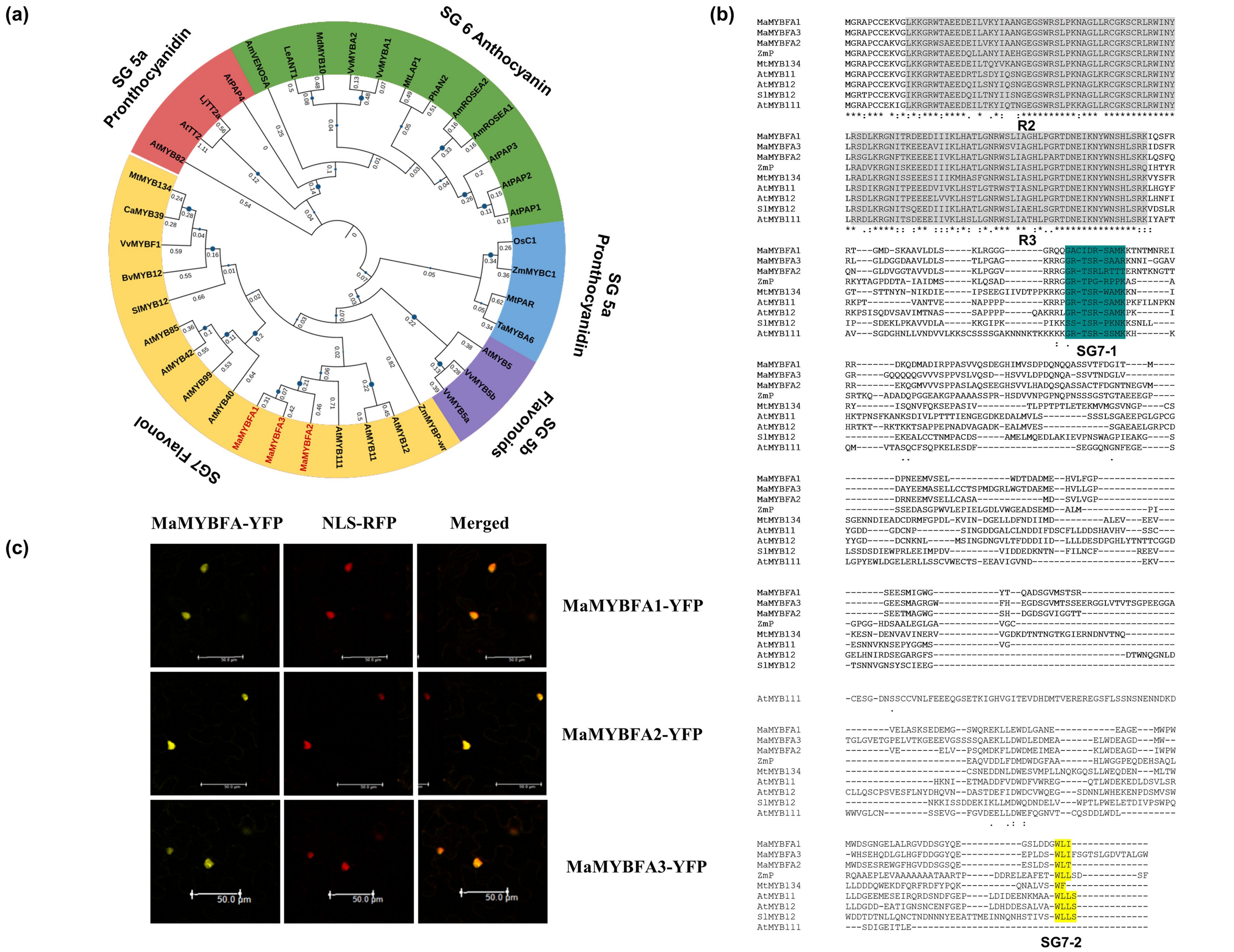


Figure 1. Identification of candidate R2R3-MYB flavonol regulators from the banana genome and their subcellular localization. (a) Various R2R3-MYB proteins were classified into three major groups as flavonol-, anthocyanin-, or proanthocyanidin biosynthesis–specific regulators using landmark flavonoid-specific R2R3-MYBs from different plant species. Numbers on each branch show branch length, and solid dark blue circles represent bootstrap values. (b) Amino acid sequence alignment of candidate SG7 R2R3-MYB transcription factors (MaMYBFA1, MaMYBFA2, and MaMYBFA3, where FA stands for flavonol activator) and selected flavonol regulators from other plant species. The R2 and R3 domains are highlighted in grey. The SG7-1 and SG7-2 motifs, found in subgroup 7 R2R3-MYBs, are highlighted in dark green and yellow, respectively. Accession numbers and two-letter species abbreviations are provided in Supplemental File S2. (c) Subcellular localization of MaMYBFAs. MaMYBFA-YFP fusion proteins were expressed together with the nuclear marker NLS-RFP in *N. benthamiana* leaf epidermal cells. Confocal imaging shows co-localization of YFP and RFP fluorescence, indicating nuclear localization of the three MaMYBFA proteins. Representative images are shown with scale bar of 50 μm.

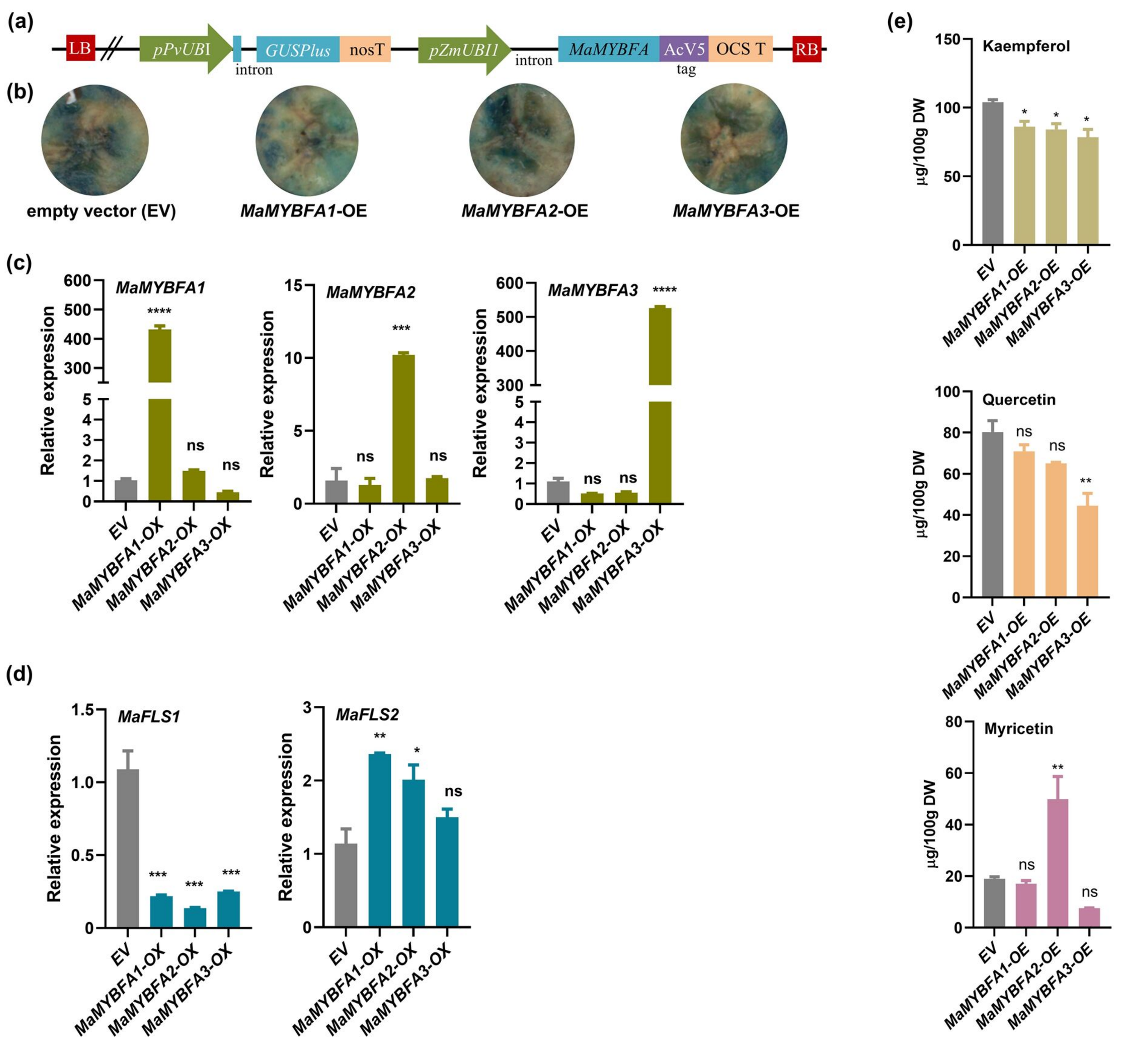


Figure 2. MaMYBFA1, MaMYBFA2, and MaMYBFA3 differentially regulate the expression of *MaFLS1* and *MaFLS2* and modulate flavonol biosynthesis in banana. (a) Diagram of the plant expression cassette used for transient overexpression (OE) of *MaMYBFA* in banana fruit discs. The *Zea mays ubiquitin1* promoter (*proZmUBI1*) drives the expression of *MaMYBFA*. The expression of the *GUSPlus* marker gene is driven by the *Phaseolus vulgaris ubiquitin1* promoter (*proPvUBI*). (b) Representative images showing GUS staining of transiently transformed banana fruit discs indicating successful transformation. Images were taken 3 days after transformation. (c-d) Relative expression of *MaMYBFA1–3* (c) and *MaFLS1* and *MaFLS2* (d) OE lines in transiently transformed *MaMYBFA* OE banana fruit tissues as determined by RT-qPCR. EV, empty vector control. (e) Flavonol quantification showing differential alteration in the accumulation of kaempferol, quercetin, and myricetin aglycones in the *MaMYBFA* OE banana fruit discs. Values are given in μg per 100 g of dry weight (DW) of fruit discs of three replicates. One-way ANOVA with Tukey's multiple comparison was used for statistical analyses. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$; ns, not significant.

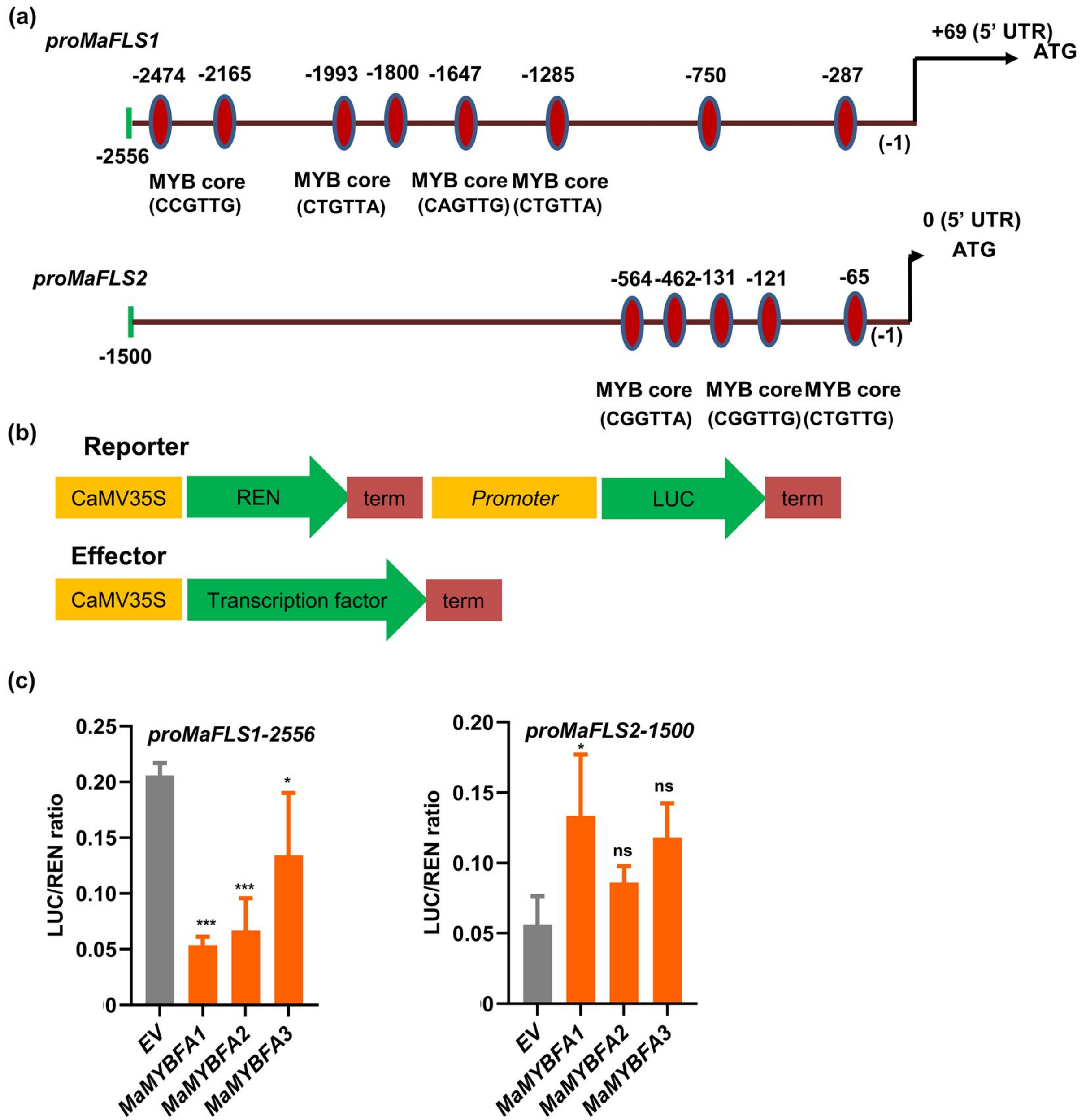


Figure 3. MaMYBFAs regulate the expression of *MaFLS1* and *MaFLS2 in vivo*. (a) Schematic representation of the 2556- and 1500-bp promoter fragments of *MaFLS1* and *MaFLS2*. The locations of the different MYB core (CNGTTR) sequences and their positions upstream of the transcription start sites (−1) are indicated by red ovals. A plus (+) sign with a number indicates the length of the 5′ UTR. (b) Diagram of the pBTdest effector and the p635nRRF reporter constructs. Constitutive cauliflower mosaic virus (CaMV) promoter drives the expression of transcription factor encoding genes and renilla *luciferase* (REN). Desired amplified promoter fragments were placed upstream of firefly *luciferase* (LUC) (c) Dual-luciferase assay showing the altered activity of the promoters *proMaFLS1–2508* and *proMaFLS2–1500* when transiently co-infiltrated into *N. benthamiana* leaves with the effectors MaMYBFA1, MaMYBFA2, and MaMYBFA3. Promoter activity is represented as the LUC/REN ratio in four biological replicates ($n = 4$).

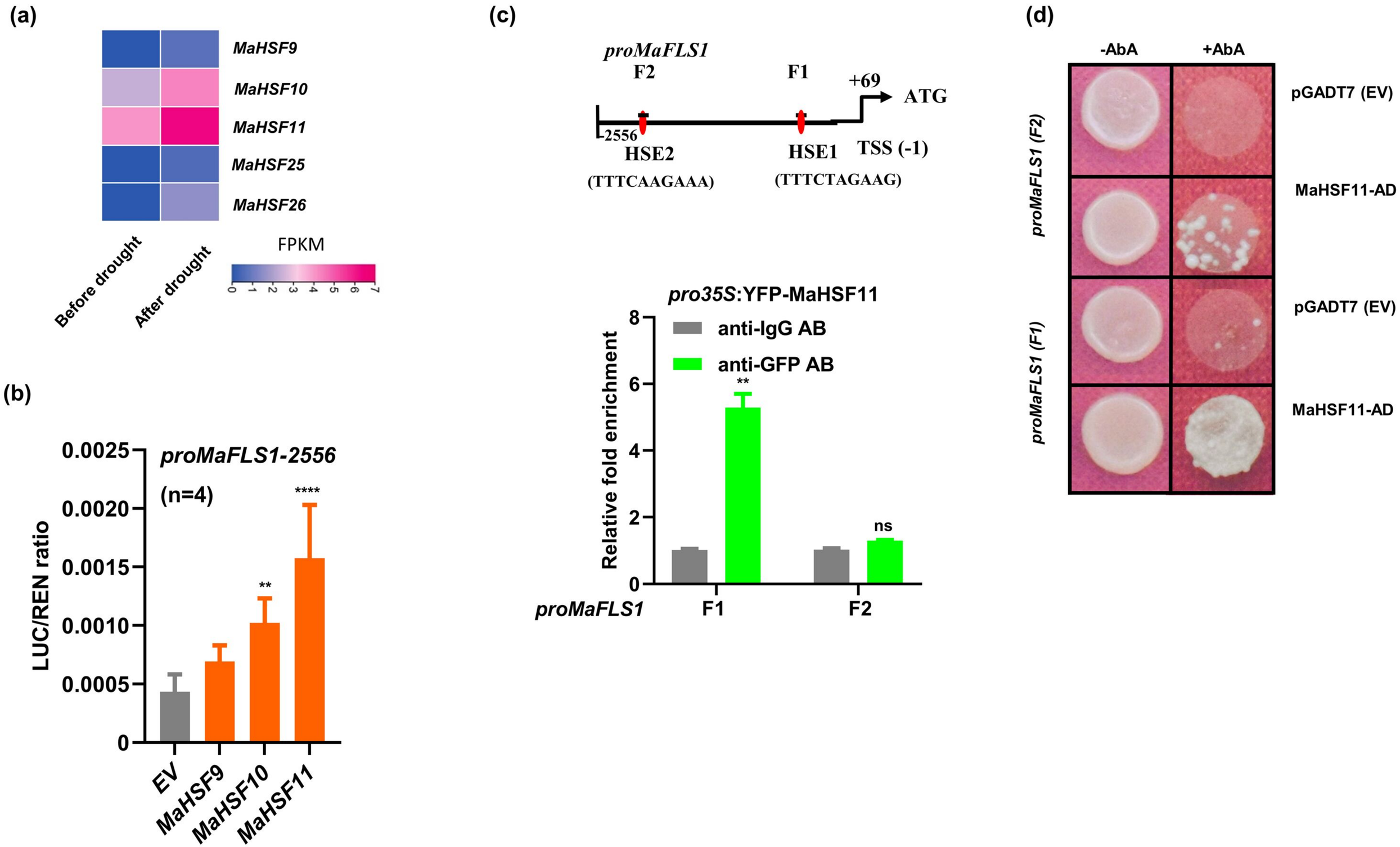


Figure 4. MaHSF11 directly binds to the *MaFLS1* promoter and regulates the expression of *MaFLS1* to modulate flavonol biosynthesis in banana. (a) Heatmap showing expression of five banana candidate *HSF* genes before and after drought treatment (Grand Naine cultivar, AAA genome). FPKM values for the indicated genes were extracted from publicly available RNA-Seq datasets and are represented according to the 0–7 color scale shown. (b) Dual-luciferase assay showing transactivation of *proMaFLS1–2556* by MaHSF. Values are shown as means ± SD of the LUC/REN ratios (n = 4). (c) ChIP-qPCR assay analyzing the binding of MaHSF11 to fragments F1 and F2 of *proMaFLS1*, both of which contain the HSE consensus sequence (nTTCnnGAAn). Fold enrichment values are given as means ± SD of two biological replicates. AB, antibody. (d) Yeast one-hybrid assay showing interaction of MaHSF11 with the two HSE-containing *proMaFLS1* fragments F1 and F2. The empty pGADT7 vector (EV) was used as a negative control. Interaction was indicated by yeast growth on synthetic Ura– dropout media supplemented with aureobasidin (AbA). AD, activation domain.

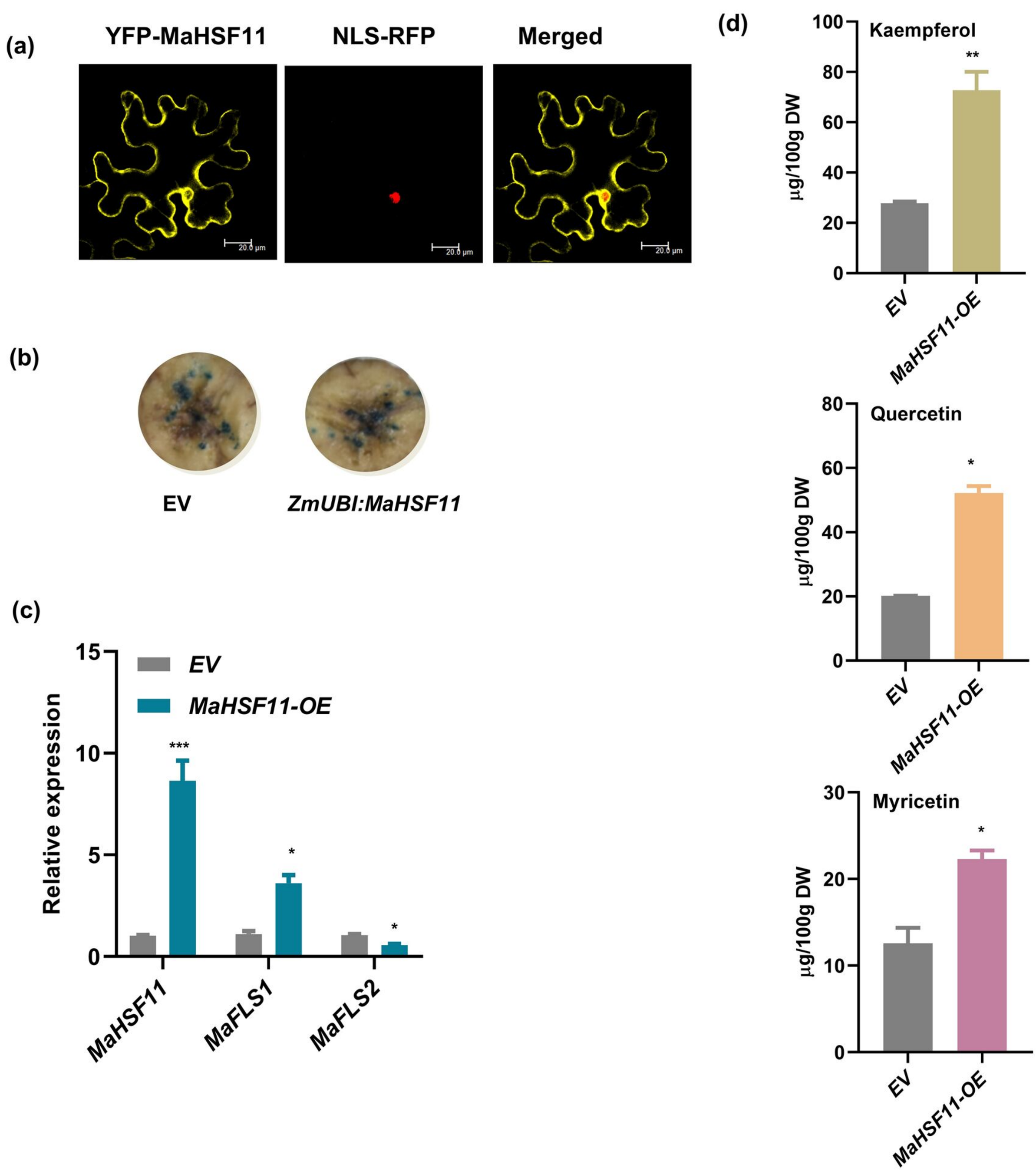
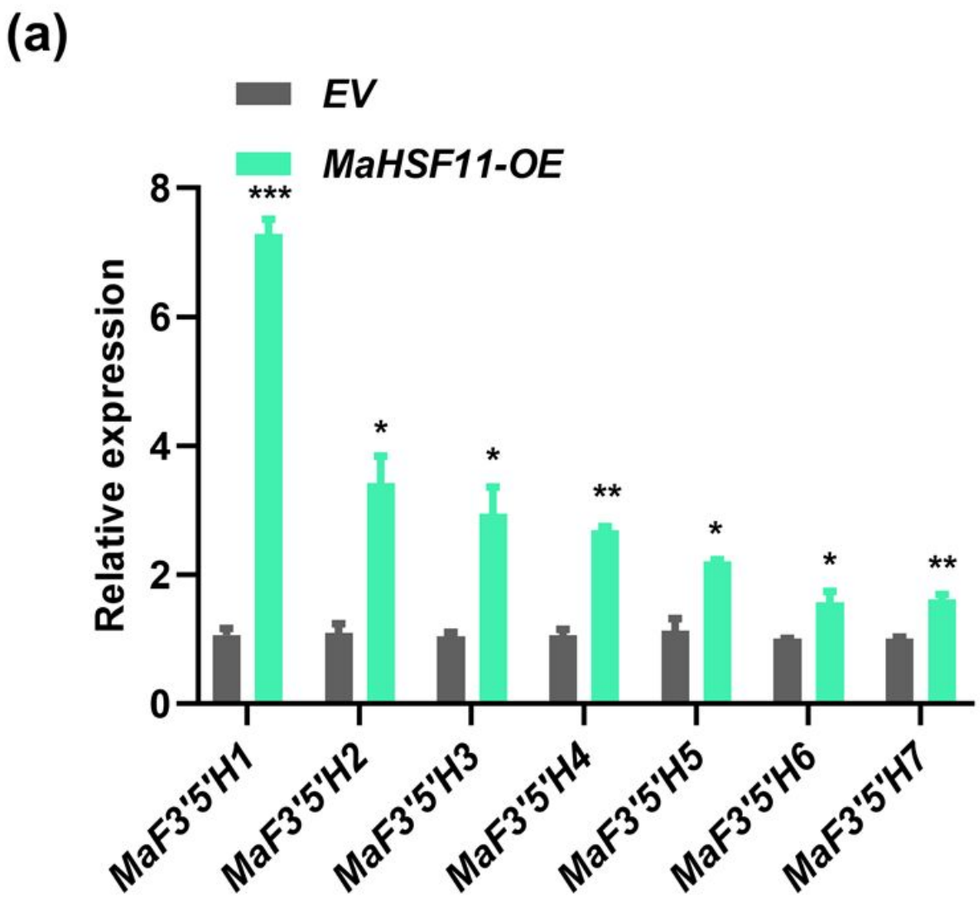
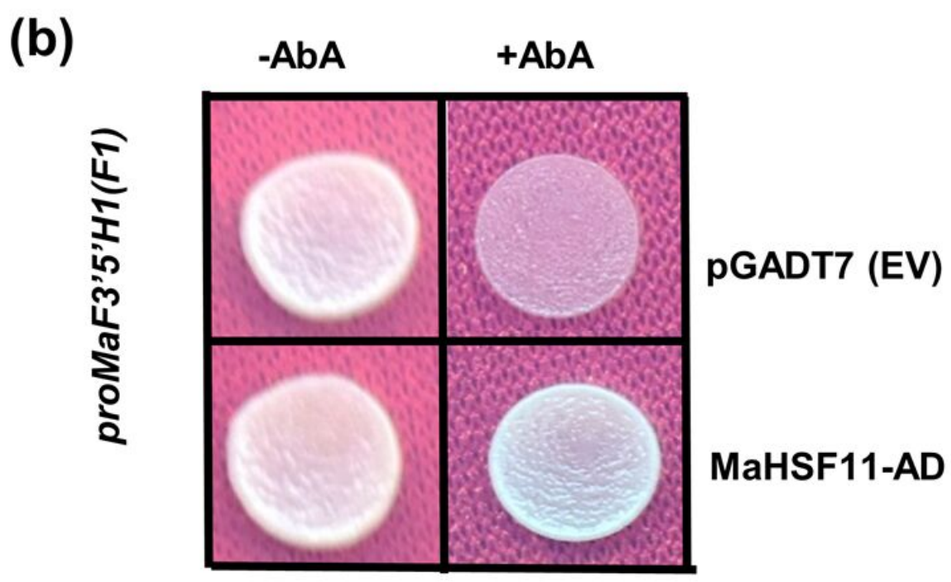
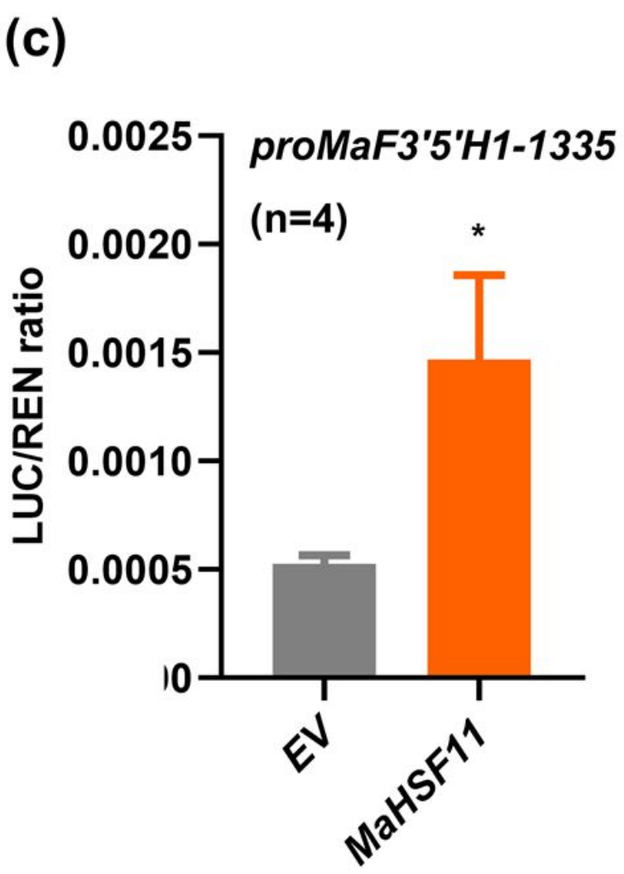
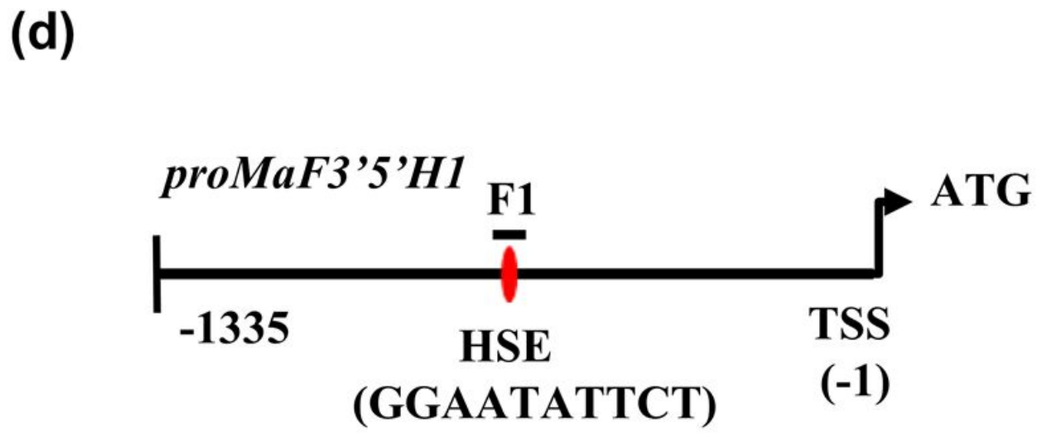


Figure 5. MaHSF11 promotes flavonol synthesis in banana fruit. (a) Subcellular localization of MaHSF11 in *N. benthamiana* leaves. The YFP-MaHSF11 fusion protein localizes to both the nucleus and the cytoplasm and coincides with the signal from the nuclear localization signal-red fluorescent protein (NLS-RFP) fusion in *N. benthamiana* leaves as observed in confocal microscopy. Scale bar, 20 μm. (b) GUS staining of banana fruit slices indicating successful transformation with the empty vector (EV) and the *proZmUBI:MaHSF11* construct. (c) Relative expression of *MaHSF11*, *MaFLS1*, and *MaFLS2* in transiently transformed banana fruit slices overexpressing (OE) *proZmUBI:MaHSF11* as determined by RT-qPCR. The empty vector was transformed as a control (expression level set to 1). Values shown are means ± SD of three technical replicates. (d) Quantification of the flavonols kaempferol, quercetin, and myricetin in transformed banana fruit slices. Values are represented as means ± SD of three replicates. Unpaired student's *t*-tests were used for statistical analyses. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.









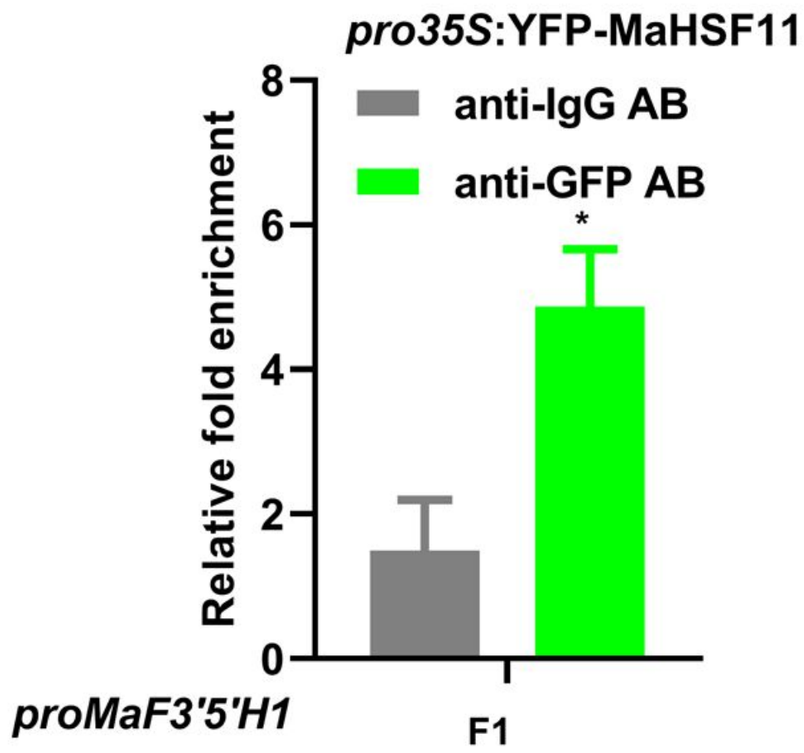


Figure 6. MaHSF11 promotes flavonoid B-ring hydroxylation by modulating the expression of *MaF3'5'H1*. (a) Relative expression of *MaF3'5'H1* genes in transformed banana fruit slices transiently overexpressing *proZmUBI:MaHSF11*, as determined by RT-qPCR (EV, empty vector control). Values shown are means ± SD of three technical replicates. (b) Yeast one-hybrid assay showing the interaction of MaHSF11 with *proMaF3'5'H1–100* fragment F1 carrying HSE (GGAATATTCT). pGADT7 was used as a negative control. Growth was checked on synthetic dropout medium supplemented with aureobasidin. (c) Dual-luciferase assay showing transactivation of *proMaF3'5'H1–1335* by MaHSF11. The promoter sequence driving the expression of luciferase (LUC) was co-infiltrated with the EV or *MaHSF11* constructs in *N. benthamiana* leaves. Values are shown as means ± SD of the LUC/REN ratio (n = 4). (d) ChIP-qPCR assay showing binding of MaHSF11 to fragment F1 of *proMaF3'5'H1* carrying an HSE (nGAAnnTTCn). The promoter fragment carrying HSE was co-infiltrated into *N. benthamiana* leaves with the *pro35S:YFP-MaHSF11* construct. Fold enrichment values are represented as means ± SD of two biological replicates. An anti-IgG antibody was used as a negative control, and an anti-GFP antibody was used to precipitate MaHSF11-enriched chromatin. Unpaired Student's *t*-tests were used for statistical analyses. *$P \leq 0.05$.

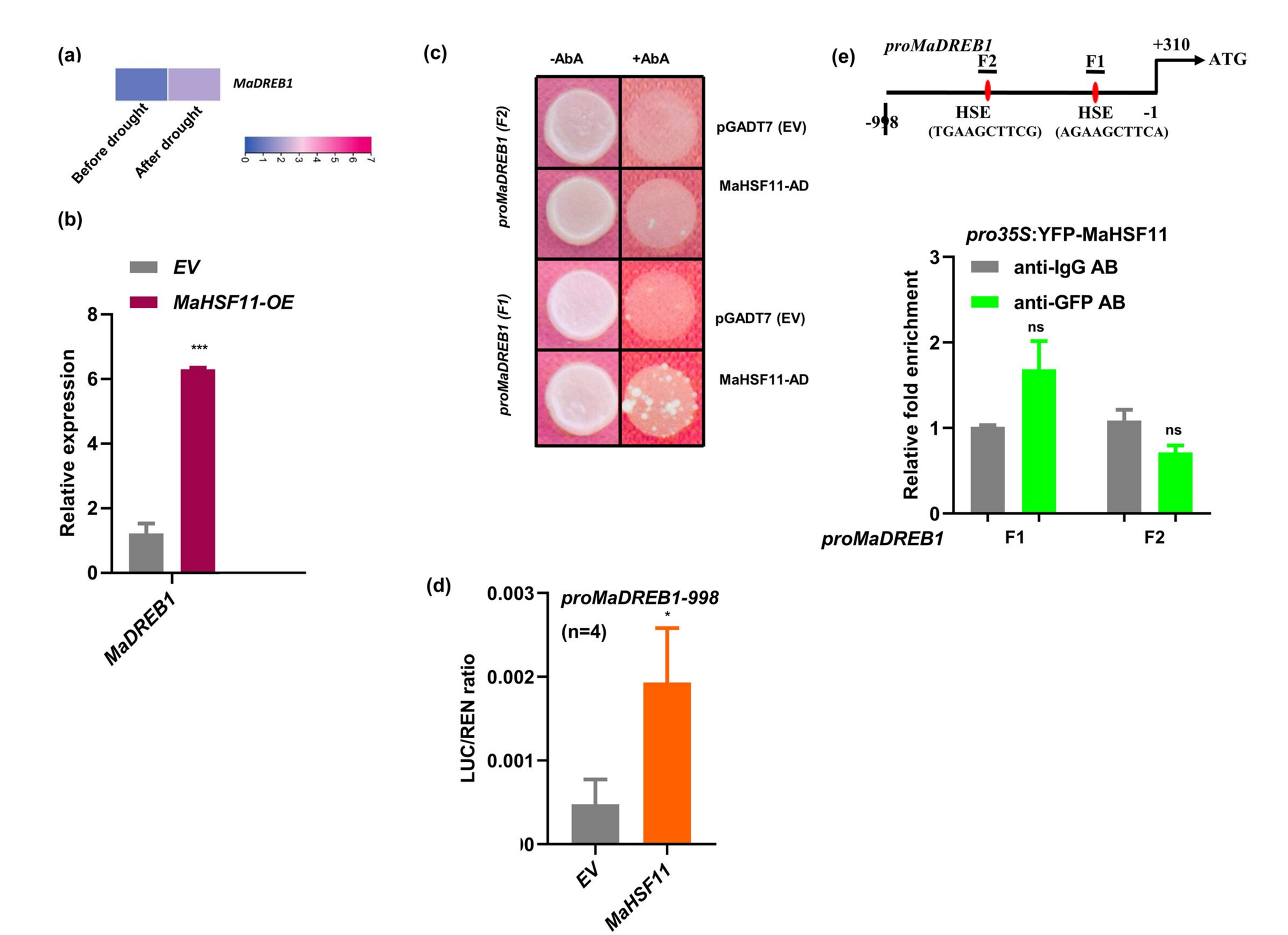


Figure 7. MaHSF11 acts upstream of *MaDREB1* to positively regulate its expression in banana. (a) Heatmap showing expression of candidate DREB genes before and after drought treatment in banana (Grand Naine cultivar, AAA genome). FPKM values for the indicated genes were extracted from publicly available RNA-Seq datasets and plotted according to the 0–7 color scale shown. (b) Relative expression of *MaDREB1* in banana fruit tissues transiently transformed with EV or *proZmUBI:MaHSF11* as determined by RT-qPCR. (c) Yeast one-hybrid assay showing interaction of MaHSF11 with the F1 and F2 fragments of *proMaDREB1–100* carrying an HSE (nGAAnnTTCn). pGADT7 was used as a negative control. Growth was checked on synthetic dropout medium supplemented with aureobasidin. (d) Dual-luciferase assay showing transactivation of *proMaDREB1–998* by MaHSF11. Promoter sequence driving the expression of LUC was co-infiltrated with empty vector and *MaHSF11* construct in *N. benthamiana* leaves. Values shown are means ± SD of LUC/REN ratio (n = 4). (e) ChIP-qPCR assay showing binding of MaHSF11 to *proMaDREB1*. A promoter fragment carrying an HSE was co-infiltrated into *N. benthamiana* leaf cells with the *pro35S:MaHSF11-YFP* construct. Fold enrichment values are represented as means ± SD of two biological replicates. An anti-IgG antibody was used as a negative control, and an anti-GFP antibody was used to precipitate MaHSF11 enriched chromatin. Unpaired Student's t-tests were used for statistical analyses. $^{*}P \leq 0.05$; $^{***}P \leq 0.001$; ns, not significant.

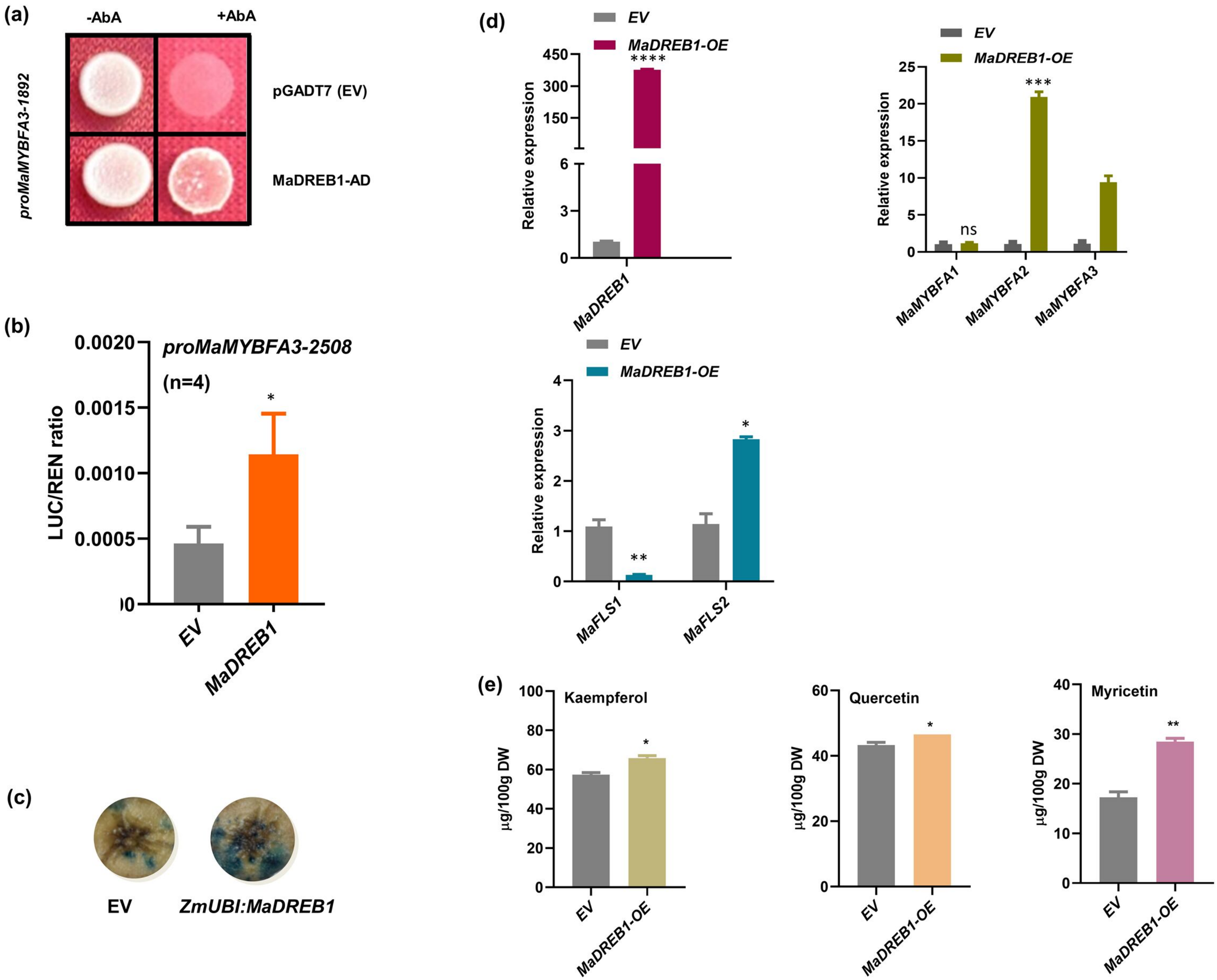


Figure 8. MaDREB1 directly binds to the promoter of *MaMYBFA3* to regulate flavonol biosynthesis. (a) Yeast one-hybrid assay showing interaction of MaDREB1 with the *proMaMYBFA3–1892* fragment carrying three DREs (CCGAC). pGADT7 was used as a negative control. Growth was checked on synthetic dropout medium supplemented with aureobasidin. (b) Dual-luciferase assay showing transactivation of *proMaMYBFA3–2508* by MaDREB1. The *proMaMYBFA3–2508* sequence driving the expression of LUC was co-infiltrated with the empty vector or *MaDREB1* construct into *N. benthamiana* leaves. Values are shown as means ± SD of the LUC/REN ratio (n = 4). (c) GUS staining of transformed fruit. (d) RT-qPCR-based relative expression of *MaDREB1*, *MaMYBFAs*, *MaFLS1*, and *MaFLS2* in transformed banana fruit tissues transiently expressing EV and *proZmUBI:MaDREB1*. EV was transformed as a control. Values shown are means ± SD of three technical replicates. (e) Quantification of the flavonols kaempferol, quercetin, and myricetin in transformed fruit tissues. Values are represented as means ± SD of three replicates. Unpaired Student's t-tests were used for statistical analyses. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$.

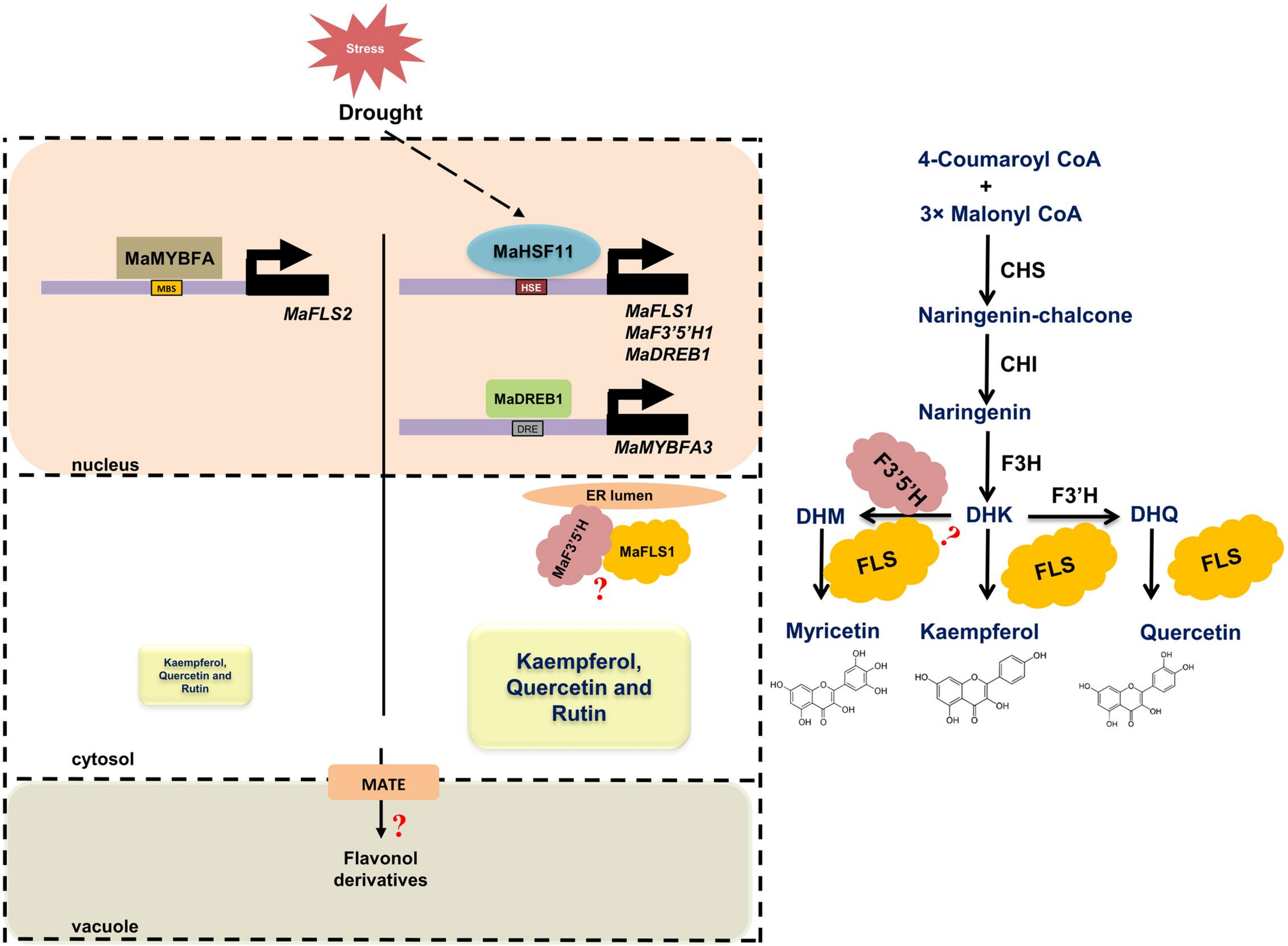


Figure 9. A working model showing the modulation of hydroxylated flavonol synthesis by the MaHSF11–MaDREB1–MaMYBFA regulatory module. The banana SG7 R2R3 MYB, MaMYBFA, targets *MaFLS2* to promote its expression and promote flavonol synthesis. Drought stress increases the transcript level of *MaHSF11*, enhancing the expression of *MaFLS1* and *MaF3'5'H1*. MaFLS1 and MaF3′5′H might interact to form a metabolon at the ER membrane to promote myricetin biosynthesis. MaHSF11 also directly activates the expression of *MaDREB1*. MaDREB1 binds to the *MaMYBFA3* promoter to regulate its expression. MaMYBFA1, MaMYBFA2, and MaMYBFA3 differentially regulate the expression of *MaFLS1* and *MaFLS2*. MRE, MYB recognition element; DRE, dehydration response element; ABRE, ABA response element; HSE, heat shock element. CHS (chalcone synthase) catalyzes the condensation of one molecule of *p*-coumaryl-CoA and three molecules of malonyl-CoA into naringenin chalcone, which is further converted into naringenin by CHI (chalcone isomerase). F3H (flavanone 3-hydroxylase) converts naringenin into DHK (dihydrokaempferol). F3′H (flavonoid 3′-hydroxylase) and F3′5′H (flavonoid 3′5′-hydroxylase) compete for the substrate DHK to synthesize DHQ (dihydroquercetin) and DHM (dihydromyricetin), respectively. FLS (flavonol synthase) converts DHK, DHQ, and DHM into kaempferol, quercetin, and myricetin, respectively. F3′5′H also physically interacts with FLS1 to form a metabolon to accelerate myricetin synthesis. Flavonols are likely transported into the vacuole by MATE transporters (multidrug and toxic compound extrusion).

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