Three R2R3-MYB transcription factors from banana (*Musa* spp.) activate structural anthocyanin biosynthesis genes as part of an MBW complex

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Abstract

Bananas are among the most popular fruits in the world and provide food security and employment opportunities in several developing countries. An increased anthocyanin content could enhance the health promoting properties of banana fruits. The biosynthesis of anthocyanins is largely regulated at the transcriptional level. However, little is known about transcriptional activation of anthocyanin biosynthesis in banana. We analysed the regulatory activity of three *Musa*MYBs predicted by bioinformatic analysis to transcriptionally regulate anthocyanin biosynthesis in banana. *MusaMYBA1, MusaMYBA2* and *MusaMYBPA2* did not complement the anthocyanin deficiency phenotype of the *A. thaliana pap1/pap2* mutant. However, co-transfection experiments in *A. thaliana* protoplasts showed that *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 function as components of a transcription factor complex with a bHLH and WD40 protein, called MBW complex, resulting in the activation of the *anthocyanin synthase* and *dihydroflavonol 4-reductase* promoters from *A. thaliana*. The activation potential of *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 increased when combined with the monocot bHLH *Zm*R instead of the dicot *At*EGL3. This work paves the path towards decoding the MBW complex-mediated transcriptional activation of anthocyanin biosynthesis in banana. Moreover, it facilitates research towards an elevated anthocyanin content in banana and other monocot crops.

Keywords: *Musa acuminata*, banana, specialised metabolites, transcription factor, flavonoid biosynthesis, R2R3-MYB

36 **Introduction**

37 Bananas (*Musa*) are monocotyledonous, perennial plants which are grown in many tropical
38 and subtropical countries. They are one of the most important food crops, particularly in the
39 developing world (1). While the sweet fruits of dessert bananas are popular in Europe and
40 Northern America, plantains or cooking bananas are commonly eaten as a staple food in Africa
41 and Latin America where they provide food security, as well as employment opportunities (2).
42 Furthermore, banana fruits are rich in several health promoting minerals and beneficial
43 phytochemicals such as vitamins and flavonoids (3).

44 Flavonoids are a major group of plant specialised metabolites which share a basic structure of
45 two aromatic C6-rings connected by a heterocyclic ring (4). Reorganisations and modifications
46 e.g. oxidation, glycosylation, acylation, and methylation of the carbon skeleton create a
47 versatile group comprising more than 9,000 different flavonoid derivatives (5). Consequently,
48 flavonoids do not only contribute to the nutritional value of fruits, but also play important roles
49 in manifold processes. While the group of red coloured anthocyanin pigments attracts animals
50 for pollination and dispersal of seeds by colouring flowers and fruits, other flavonoids protect
51 plants against UV-B irradiation or increase plant fertility (6-10). Flavonoids from many species
52 have been reported to have anti-pathogenic properties, this includes flavonoids from *Dianthus*
53 *caryophyllus* which exhibit antifungal activity against the plant's major pest *Fusarium*
54 *oxysporum* f.sp. *dianthi* (11, 12). The tropical race 4 (TR4) of the banana Fusarium wilt
55 (commonly known as 'panama disease') is caused by another *Fusarium* subspecies called
56 *Fusarium oxysporum* f. sp. *cubense* (Foc) and threatens the global banana production (13).
57 Transcriptome analysis of susceptible and resistant banana cultivars infected by Foc TR4
58 revealed an increased transcription of flavonoid biosynthesis related genes in the resistant
59 cultivar, suggesting an involvement of flavonoids in the defence against Foc TR4 (14).

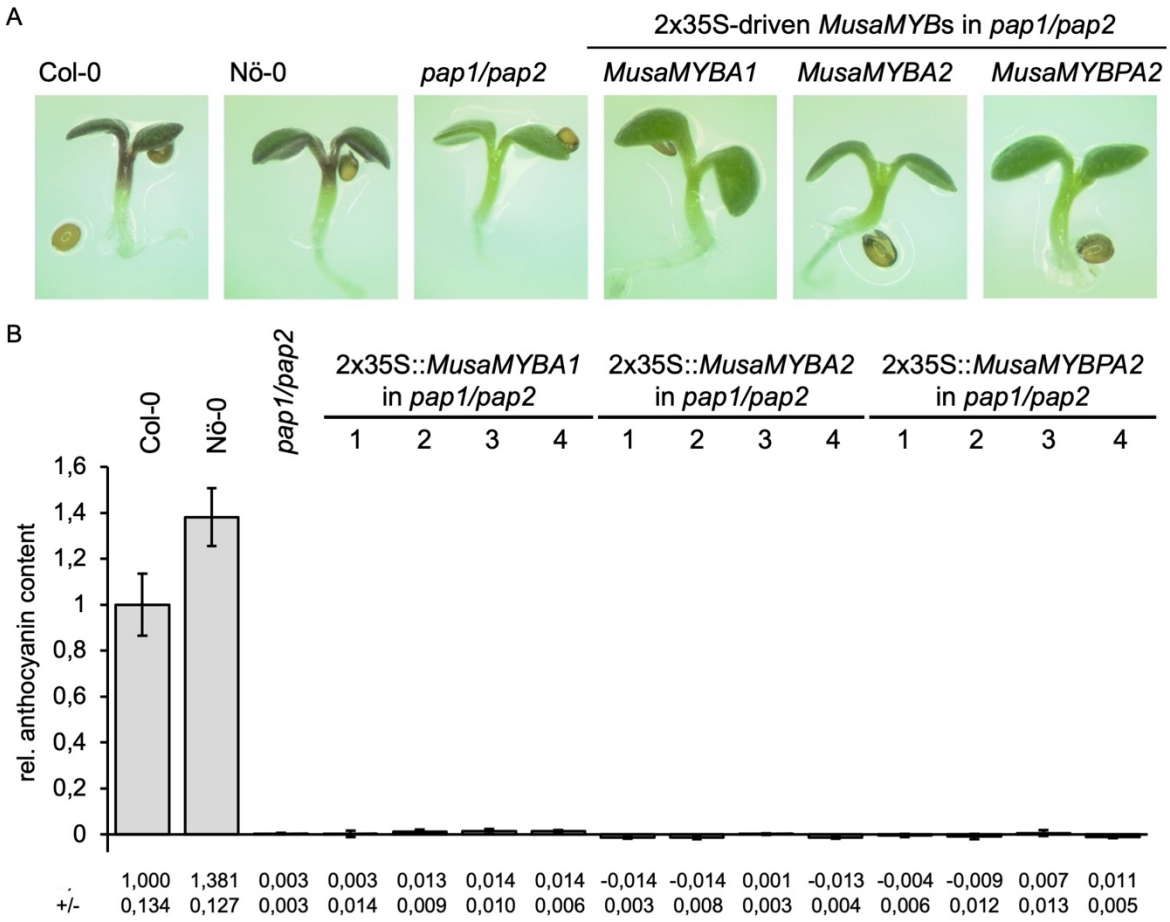
60 Flavonoid biosynthesis is one of the best characterised pathways of the secondary metabolism
61 and has been extensively studied in many plant species (15). In banana, several flavonoid
62 biosynthesis related enzymes have been identified and characterised (16, 17). Regulation of
63 structural genes on a transcriptional level allows a specific response to environmental

64 influences as well as development and organ specific expression (18-20). MYB transcription
65 factors are common transcriptional regulators of flavonoid biosynthesis. While some MYBs act
66 independently, others interact with basic helix-loop-helix (bHLH) and WD40 proteins to form a
67 protein complex called MBW complex (21). MYB proteins are present in all eukaryotes and
68 characterised by highly conserved DNA-binding domains (22). These MYB domains consist of
69 up to three imperfect amino acid repeat sequences, based on which they are classified. R2R3-
70 MYBs are the most abundant class of plant MYBs and reveal versatile functions in plant-
71 specific processes (23). Besides core- and specialised metabolism they are also involved in
72 cell fate and -identity definition, developmental processes and the response to biotic and
73 abiotic stresses (23). Well-known R2R3-MYBs which act as activators of anthocyanin
74 biosynthesis include C1 (COLOURED ALEURONE1) from *Zea mays* (*Z. mays*), as well as
75 PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT1/MYB75) and PAP2 (MYB90) from
76 *Arabidopsis thaliana* (*A. thaliana*) (24, 25). They act as part of an MBW complex and control
77 the promoters of the anthocyanin biosynthesis related structural genes as for example
78 *ANTHOCYANIDIN SYNTHASE* (*ANS*) and *DIHYDROFLAVONOL 4-REDUCTASE* (*DFR*) (26-
79 29).
80 In banana (*Musa acuminata, M. acuminata*), 285 R2R3-MYB proteins, including several
81 putative regulators of flavonoid biosynthesis, have been identified in a genome-wide study
82 (30). Furthermore, MYB31, MYB4 and MYBPR1 - MYBPR4 have been identified as negative
83 regulators of flavonoid biosynthesis in banana (31, 32). Despite the recent identification of two
84 proanthocyanidin biosynthesis activating R2R3-MYBs (33), little functional data is available on
85 positive regulators (activators) of flavonoid and in particular anthocyanin biosynthesis in
86 *M. acuminata*.
87 Here, we describe the regulatory properties of three *Musa*MYBs, named *Musa*MYBA1,
88 *Musa*MYBA2 and *Musa*MYBPA2, with a possible role in the regulation of anthocyanin
89 biosynthesis. As one of these *Musa*MYBs was very recently published under the term
90 *Musa*MYBPA2 (33), we used this name to avoid confusion due to multiple protein naming.
91 Regulatory activity was investigated by *in planta* complementation experiments of the

92 anthocyanin deficient *A. thaliana* regulatory *pap1/pap2* mutant and co-transfection
93 experiments in *A. thaliana* protoplasts (for detailed methods refer to Supplementary File 2).
94 Our results show that *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 are able to activate the
95 promoters of *AtANS* and *AtDFR* as part of an MBW complex. Moreover, we show that the
96 activation potential of *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 is increased when
97 combined with the monocot bHLH *Zm*R instead of the dicot bHLH protein ENHANCER OF
98 GLABRA 3 (*At*EGL3).
99
100 **Main text**
101 We aimed to analyse the regulatory properties of three *Musa*MYBs which have been previously
102 assigned with a possible role in positive regulation of anthocyanin biosynthesis (Ma06_g05960
103 or *MusaMYBA1*, Ma09_g27990 or *MusaMYBA2*, Ma10_g17650 or *MusaMYBPA2*). Since all
104 three *MusaMYB* genes were detected in the haploid *M. acuminata* reference genome
105 sequence DH (doubled-haploid) Phang v2 (34, 35), these *MYBs* appear to be present in the
106 same sub-genome, suggesting that they are different genes and not haplo-copies. We tried to
107 amplify the corresponding coding sequences (CDSs) on a template collection containing cDNA
108 from different banana samples. The CDSs of all three *MusaMYB*s were successfully amplified
109 on cDNA derived from peel tissue.
110 In a first approach, we performed a complementation assay with the regulatory *A. thaliana*
111 *pap1/pap2* double mutant which cannot produce anthocyanins in the seedling (Figure 1).
112 Seedlings were grown on anthocyanin synthesis-inducing media to analyse the ability of
113 2x35S-driven *MusaMYB*s to complement the *pap1/pap2* anthocyanin deficiency. While wild-
114 type seedlings accumulated high amounts of red anthocyanin pigments, *pap1/pap2* seedlings
115 did not. Even though *MusaMYBA1*, *MusaMYBA2* or *MusaMYBPA2* were successfully
116 expressed in the transgenic seedlings (Supplementary Figure S1), the anthocyanin level in
117 *pap1/pap2* plants expressing *MusaMYBA1*, *MusaMYBA2* or *MusaMYBPA2* did not differ from
118 the double mutant. Accordingly, *MusaMYBA1*, *MusaMYBA2* and *MusaMYBPA2* do not seem
119 to be able to complement the mutant phenotype and thus to regulate anthocyanin biosynthesis

120 in *A. thaliana* in combination with the *bHLH* and *WD40* genes expressed in *A. thaliana*

121 seedlings.



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123 **Figure 1: *MusaMYBA1, MusaMYBA2* and *MusaMYBPA2* cannot complement the**
124 **anthocyanin deficient phenotype of *A. thaliana pap1/pap2* mutant seedlings**. (A)
125 Representative pictures of anthocyanin accumulation in 6-day-old *MusaMYB*-expressing
126 *pap1/pap2* seedlings. A representative plant per construct is displayed. (B) Photometrical
127 measurement of the sucrose induced anthocyanin content in *pap1/pap2* seedlings expressing
128 2x35S-driven *MusaMYB*s. Col-0, Nö-0 (wildtypes) and *pap1/pap2* were used as controls. Error
129 bars indicate the standard deviation of three biological replicates. The different numbers
130 represent individual, independent, transgenic lines.

131 Lloyd *et al.* (28) analysed the *Z. mays* anthocyanin regulator C1 by generation of transgenic

132 *A. thaliana* overexpression lines. In their study, Lloyd *et al.* generated three independent

133 *ZmC1*-expressing *A. thaliana* lines that did not show an increased anthocyanin content

134 compared to wildtype. Similar results were obtained in transgenic tobacco. However, further

135 experiments suggested that *Zm*C1 needs to interact with the maize bHLH *Zm*R to activate

136 anthocyanin biosynthesis in the heterologous *A. thaliana* system (28). Phylogenetic analysis

137 (36) showed that the anthocyanin biosynthesis regulating R2R3-MYBs from several monocots
138 such as *Z. mays* and *Oryza sativa* (*O. sativa*) are part of a different phylogenetic clade than
139 the anthocyanin regulators from several dicots, such as *A. thaliana* or *Vitis vinifera* and further
140 monocots, including *Allium cepa* and *Lilium hybrida*. Experiments in snapdragon also showed
141 that the monocot *Ac*MYB1 can activate anthocyanin production in dicots (36). The phylogenetic
142 differences between anthocyanin biosynthesis activating R2R3-MYBs and the dependency of
143 *Zm*C1 on *Zm*R in dicots hint at an explanation for our observations. For example, the
144 *Musa*MYBs could depend on their endogenous or at least monocot bHLH for effective
145 activation of structural anthocyanin biosynthesis genes. To investigate the phylogenetic
146 differences between anthocyanin biosynthesis regulating R2R3-MYBs from different plant
147 species, an approximately maximum-likelihood tree was built (Figure 2A). The resulting tree,
148 which also contained R2R3-MYBs which activate other branches of flavonoid biosynthesis,
149 revealed two distinct clades of anthocyanin-related R2R3-MYBs (highlighted in red).
150 *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 form a clade with MYB10 from *Triticum*
151 *aestivum* and exhibit a close evolutionary relationship with *Zm*C1, *Os*C1 and anthocyanin
152 regulating MYBs from further monocots. In contrast, *At*PAP1 and *At*PAP2 fall into a second
153 clade of anthocyanin-related MYBs. Furthermore, differences between anthocyanin
154 biosynthesis regulating bHLHs were analysed in a second approximately maximum-likelihood
155 tree (Figure 2B). The tree showed that monocot bHLH proteins involved in the regulation of
156 anthocyanin biosynthesis form a separate clade (highlighted in red). Both, *Zm*R and several
157 putative anthocyanin biosynthesis regulating bHLHs from banana fall into this clade. These
158 phylogenetic analyses show that anthocyanin biosynthesis regulating R2R3-MYB and bHLH
159 proteins from several monocot species seem to be distinct from other R2R3-MYB and bHLH
160 proteins regulating anthocyanin biosynthesis, for example from *A. thaliana*. These differences
161 could imply a dependency of *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 on a banana or
162 other monocot bHLH.
163 To follow up this idea, we further investigated the regulatory properties of the three *Musa*MYBs
164 performing co-transfection assays (Figure 3) in At7 protoplasts with different bHLH proteins

165 from *A. thaliana* (*At*EGL3) or *Z. mays* (*Zm*R) and the *A. thaliana* WD40 protein
166 TRANSPARENT TESTA GLABRA 1 (*At*TTG1). Thereby, their potential to activate the
167 promoters of
168

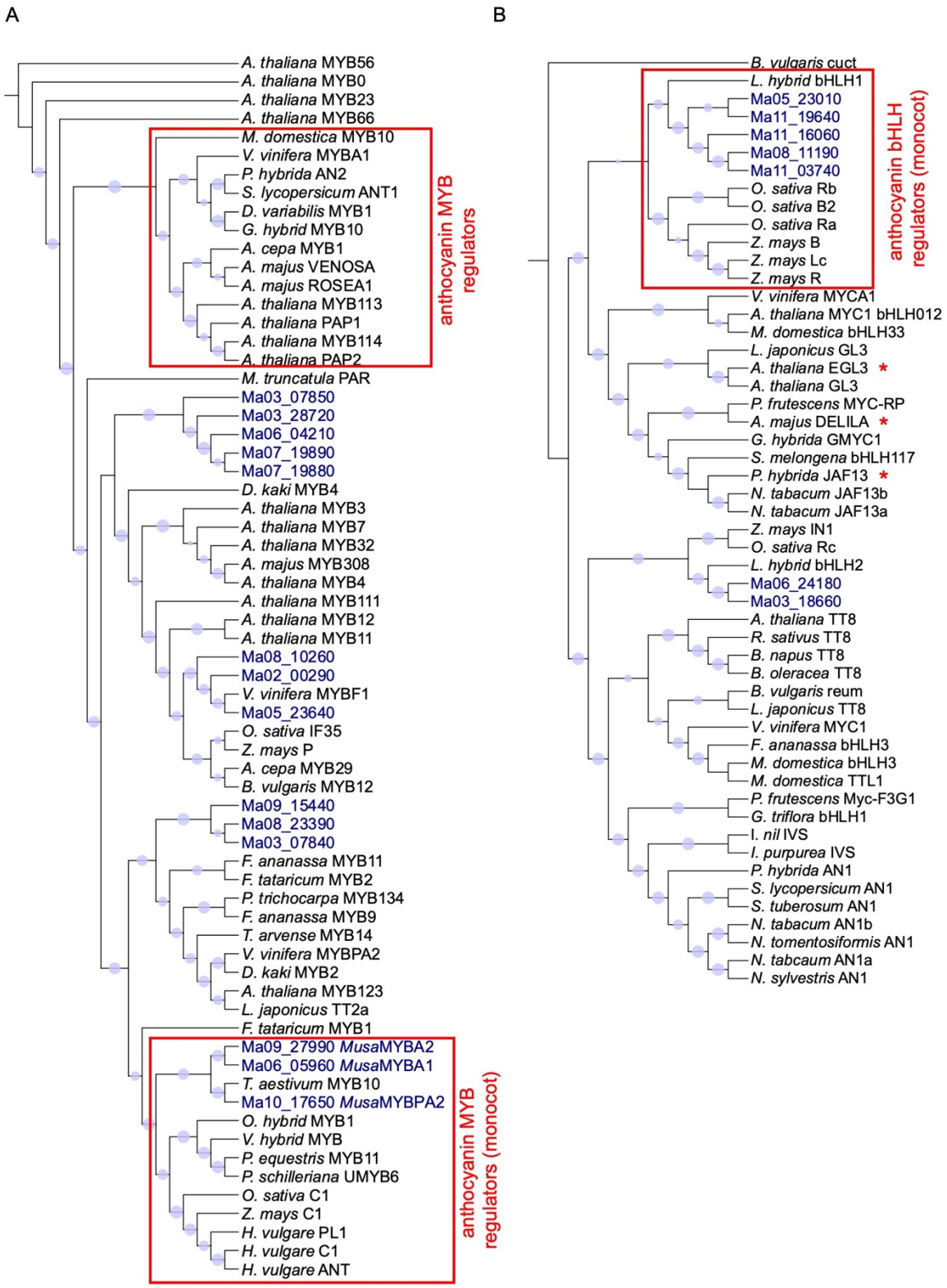


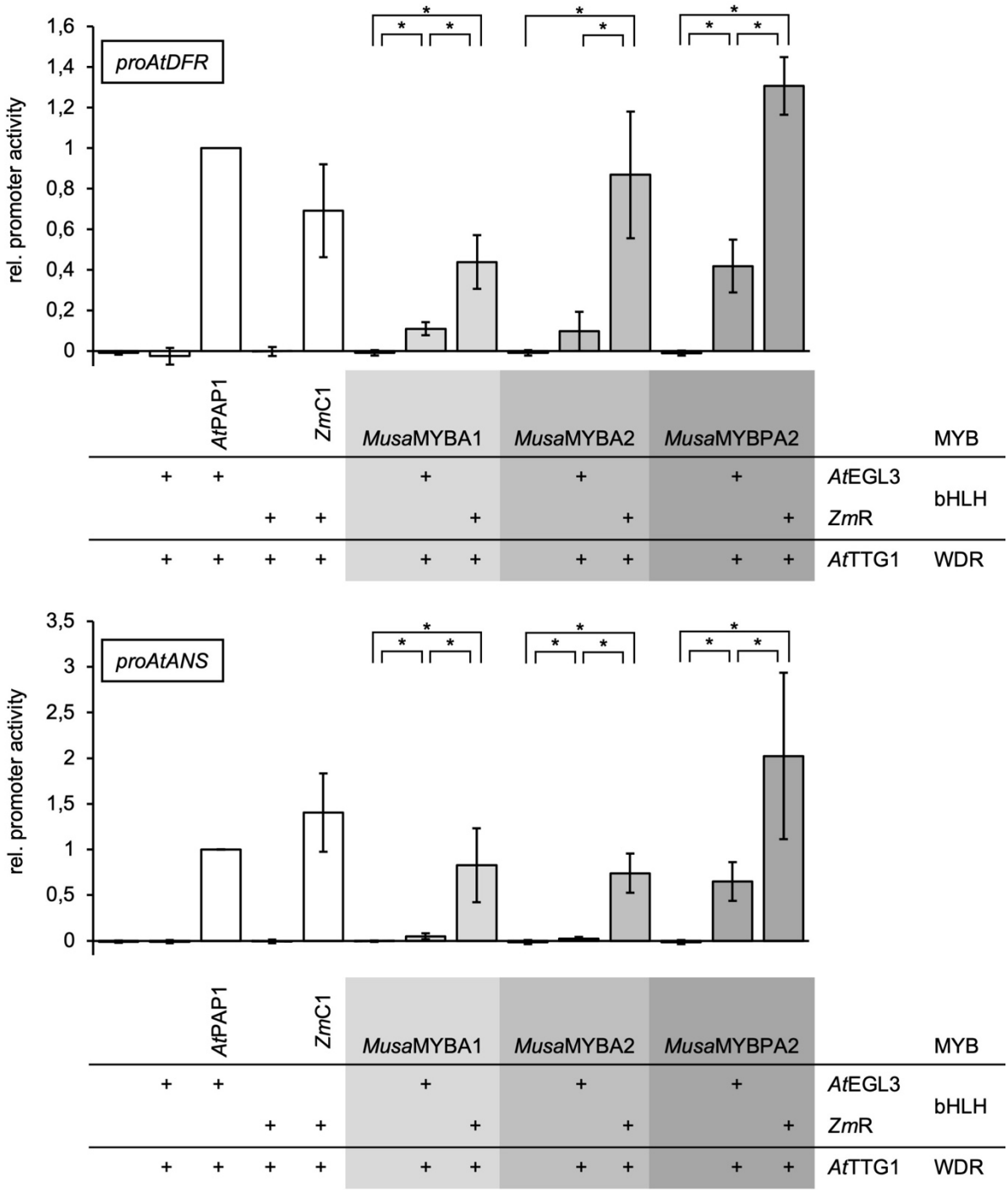
Figure 2: Rooted approximately maximum-likelihood trees of MYB (A) and bHLH (B) transcription factors. Circle sizes represent bootstrap values. Banana proteins are highlighted in blue.

AtDFR and *AtANS*, which are important structural genes of anthocyanin biosynthesis (37), was analysed. While none of the *Musa*MYBs was able to independently activate *proAtANS* or

175 *proAtDFR*, which both contain conserved *cis*-regulatory elements, a slight activation of
176 *proAtDFR* was detectable when *Musa*MYBA1 or *Musa*MYBPA2 were combined with *At*EGL3
177 and *At*TTG1. In combination with *At*EGL3 and *At*TTG1, *Musa*MYBPA2 showed the strongest
178 activation of *proAtDFR* and was also able to activate *proAtANS*. When combined with *Zm*R
179 and *At*TTG1, the three MYBs *Musa*MYBA1, *Musa*MYBA2, and *Musa*MYBPA2 exhibited a
180 significant activation potential on *proAtDFR* and *proAtANS*. All three *Musa*MYBs showed a
181 higher activation potential in an MBW complex with *Zm*R and *At*TTG1 than in combination with
182 *At*EGL3 and *At*TTG1.
183 These results display that *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 are able to activate
184 *proAtANS* and *proAtDFR* as part of an MBW complex and *Musa*MYBPA2 shows the strongest
185 activation potential. Furthermore, *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 show a
186 higher activation potential when combined with the monocot bHLH *Zm*R instead of the dicot
187 bHLH *At*EGL3.
188 The previously mentioned phylogenetic differences could be an explanation for the observed
189 higher activation potentials of the analysed *Musa*MYBs in combination with *Zm*R and *At*TTG1
190 instead of *At*EGL3 and *At*TTG1. It is conceivable that these differences impede the interaction
191 between the tested *Musa*MYBs and the dicot bHLH *At*EGL3. As *Musa*MYBPA2 shows the
192 highest activation potential and is also able to activate *proAtANS* in combination with *At*EGL3
193 or *Zm*R, differences between the three *Musa*MYBs that affect the interaction with the *At*EGL3
194 are likely and should be further studied. Interestingly, *Musa*MYBPA2 did not complement the
195 anthocyanin deficient phenotype of *A. thaliana pap1/pap2* mutant seedlings, but was able to
196 activate *proAtANS* and *proAtDFR* when combined with *At*EGL3 in co-transfection experiments
197 in *A. thaliana* protoplasts. Even though previous studies showed *AtEGL3* expression in
198 *A. thaliana* seedlings (21), it is possible that the level was too low to activate anthocyanin
199 biosynthesis in combination with *Musa*MYBPA2. Moreover, the At7 cell line used for co-
200 transfection experiments was established more than 25 years ago. This long time of
201 propagation in suspension cell culture caused a variety of genomic and transcriptomic changes

202 (38). These changes could also explain differences between the analysis in seedlings and cell

203 culture.



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205 **Figure 3: *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 can activate *proAtDFR* and**
206 ***proAtANS* as part of an MBW complex**. The ability of *Musa*MYBs to activate *proAtDFR-GUS*
207 and *proAtANS-GUS* reporter constructs in combination with different bHLH proteins (*At*EGL3,
208 *Zm*R) and a WDR (*At*TTG1) was analysed by co-transfection in *A. thaliana* At7 protoplasts.
209 The relative promoter activity refers to the measured GUS reporter enzyme activity. Promoter
210 activity is given relative to the values obtained for the *A. thaliana* MBW complex (*At*PAP1,
211 *At*EGL3, *At*TTG1). Error bars indicate the standard deviation of five independent biological
212 replicates. Statistical significance is indicated by asterisks which mark p-values < 0.05.

213 The expression profiles of *MusaMYBA1*, *MusaMYBA2* and *MusaMYBPA2* have previously
214 been analysed in embryogenic cell suspension, seedling, root and different developmental
215 stages of leaf, pulp and peel (30). The expression of *MusaMYBA1* was strongest in pulp
216 (developmental stage S2-S3), *MusaMYBA2* expression was relatively low in all samples and
217 *MusaMYBPA2* showed highest expression in seedlings and early developmental stages of
218 pulp (S1). This gene expression pattern could give a hint at organ specific *MusaMYBA1*,
219 *MusaMYBA2* and *MusaMYBPA2* activity in banana. Since flavonoid biosynthesis is largely
220 regulated at the transcriptional level, it would be particularly interesting to analyse the
221 expression levels of *MusaMYBA1*, *MusaMYBA2,* and *MusaMYBPA2* in anthocyanin rich
222 organs such as bract or pseudostem to relate anthocyanin content to *MusaMYB* transcript
223 abundance.
224 Deng *et al*. (32) performed an expression analysis of flavonoid biosynthesis related genes
225 using leaves of banana plants overexpressing the anthocyanin repressor *MusaMYB4*. They
226 found that the expression of *MusaMYBA1* and *MusaMYBPA2* is decreased along with
227 *MusaDFR* and *MusaANS* in plants overexpressing *Musa*MYB4, compared to wildtype. This
228 data supports a proposed functionality of *Musa*MYBA1 and *Musa*MYBPA2 as transcriptional
229 activators of anthocyanin biosynthesis in banana, as *MusaMYB4* could cause a feedback
230 regulation of the positive regulators of anthocyanin biosynthesis, of *MusaMYBA1* and
231 *MusaMYBPA2*.
232 Very recently, Rajput *et al*. (33) showed that *Musa*MYBPA2 can activate the banana *ANS,*
233 *ANR* and LAR promoters. Moreover, they showed that *Musa*MYBPA2 can partially rescue the
234 proanthocyanin deficiency of the *A. thaliana tt2-1* mutant. The reported ability of *Musa*MYBPA2
235 to activate *proMusaANS* supports the proposed role of *Musa*MYBPA2 in the regulation of
236 anthocyanin biosynthesis. However, the partial complementation of the proanthocyanin
237 deficient phenotype of the *A. thaliana tt2-1* R2R3-MYB mutant, as well as the ability to activate
238 the banana *ANR* and *LAR* promoters, shows an additional role in the regulation of
239 proanthocyanidin biosynthesis. Such dual roles in the regulation of flavonoid biosynthesis have
240 previously been suggested for R2R3-MYB transcription factors from grape, blueberry and

241 apple (39-42). In several species, including *A. thaliana, Z. mays* and *Petunia hybrida*,
242 anthocyanin biosynthesis is regulated by an MBW complex (21, 28, 43-45). Our co-transfection
243 assays showed that *Musa*MYBA1, *Musa*MYBA2 and *Musa*MYBPA2 are able to activate
244 *proAtANS* and *proAtDFR* as part of an MBW complex with *Zm*R and *At*TTG1 *in planta*. As
245 presented in a previous study by Pucker *et al.* (30), it is known that *Musa*MYBA1, *Musa*MYBA2,
246 and *Musa*MYBPA2 all contain a bHLH-binding consensus motif (27). Thus, the regulation of
247 anthocyanin biosynthesis by *Musa*MYBA1, *Musa*MYBA2, and *Musa*MYBPA2 likely depends
248 on bHLH and WD40 proteins. Based on our tree (Figure 2B), the bHLH encoding genes
249 Ma05_g23010, Ma11_g19640, Ma11_g16060, Ma08_g11190 and Ma11_g03740 could be
250 suitable candidates for further studies on the transcriptional activation of anthocyanin
251 biosynthesis by MBW complexes. Moreover, future analyses should additionally include
252 *Musa*TTG1 to identify a whole functional MBW complex in banana and to find out if the use of
253 endogenous WD40 protein further improves the activation potential of the MBW complex.
254 The presented results show that *Musa*MYBA1, *Musa*MYBA2, and *Musa*MYBPA2 have the
255 capacity to transcriptionally activate structural anthocyanin biosynthesis genes in an MBW
256 complex with a suitable bHLH partner. This is a step towards decoding the MBW complex-
257 mediated transcriptional activation of flavonoid biosynthesis in banana. Moreover, it is a basis
258 for further research towards increased anthocyanin production in banana which could promote
259 fruit quality and disease resistance.
260
261 **Limitations**
262 The co-transfection assays revealed that *Musa*MYBA1, *Musa*MYBA2, and *Musa*MYBPA2 can
263 activate two structural genes of anthocyanin biosynthesis from *A. thaliana* as part of an MBW
264 complex. In the future this analysis could be expanded to further genes. Moreover, co-
265 transfection experiments with the promoters of structural anthocyanin biosynthesis genes and
266 bHLH and WDR candidates from banana should be performed. In order to elucidate the
267 regulatory role in banana and confirm possible target genes, future studies should include an
268 overexpression of the three *Musa*MYBs in banana.

Declarations

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

MB, BP and RS planned the experiments. MB performed the experiments and analysed the data. RS and BW supervised the project. MB wrote the initial draft. RS and BP revised the manuscript.

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Abbreviations

ANS	anthocyanidin synthase
A. thaliana	*Arabidopsis thaliana*
bHLH	basic helix-loop-helix
C1	COLOURED ALEURONE1
DFR	dihydroflavonol 4-reductase

297 EGL3 ENHANCER OF GLABRA 3

298 Foc *Fusarium oxysporum* f. sp. *cubense*

299 *M. acuminata* *Musa acuminata*

300 *O. sativa* *Oryza sativa*

301 PAP production of anthocyanin pigment

302 TR4 tropical race 4

303 TTG1 TRANSPARENT TESTA GLABRA 1

304 *Z. mays* *Zea mays*

305

306 **Supplementary information**

307 Figure S1: Expression analysis of *MusaMYBA1, MusaMYBA2 and MusaMYBPA2* in *A.*
308 *thaliana pap1/pap2* seedlings.

309 Figure S2: Full-length gels from Figure S1.

310 Table S1: Oligonucleotide primers used in this work.

311 Table S2: IDs of protein sequences used for the construction of the phylogenetic tree.

312 File S2: Methods

313

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