Functional characterisation of banana (*Musa* spp.) 2-oxoglutarate-dependent dioxygenases involved in flavonoid biosynthesis

Short title: banana flavonoid 2-ODDs

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Abstract

Bananas (*Musa*) are monocotyledonous, perennial plants that are well-known for their edible fruits. Their cultivation provides food security and employment opportunities in many countries. Banana fruits contain high levels of minerals and phytochemicals, including flavonoids, which are beneficial for human nutrition. To broaden the knowledge on flavonoid biosynthesis in this major crop plant, we aimed to identify and functionally characterise selected structural genes encoding 2-oxoglutarate-dependent dioxygenases, involved in the formation of the flavonoid aglycon.

Musa candidates genes predicted to encode flavanone 3-hydroxylase (F3H), flavonol synthase (FLS) and anthocyanidin synthase (ANS) were assayed. Enzymatic functionalities of the recombinant proteins were confirmed *in vivo* using bioconversion assays. Moreover, transgenic analyses in corresponding *Arabidopsis thaliana* mutants showed that *Musa*F3H, *Musa*FLS and *Musa*ANS were able to complement the respective loss-of-function phenotypes, thus verifying functionality of the enzymes *in planta*.

Knowledge gained from this work provides a new aspect for further research towards genetic engineering of flavonoid biosynthesis in banana fruits to increase their antioxidant activity and nutritional value.

Introduction

Banana (*Musa* spp.) plants are well-known for their edible fruit and serve as a staple food crop in Africa, Central and South America (Arias et al., 2003). With more than 112 million tons produced in 2016, bananas are among the most popular fruits in the world and provide many employment opportunities (FAO, 2019). Furthermore banana fruits are rich in health promoting minerals and phytochemicals, including flavonoids, a class of plant specialised metabolites which contribute to the beneficial effects through their antioxidant characteristics (Forster et al., 2003; Wall, 2006; Singh et al., 2016). Flavonoid molecules share a C6-C3-C6 aglycon core, which can be reorganised or modified, e.g. by oxidation or glycosylation (Tanaka et al., 2008; Le Roy et al., 2016). Modifications at the central ring structure divide flavonoids into ten major subgroups (i.e. chalcones, aurones, flavanones, flavones, isoflavones, dihydroflavonols, flavonols, leucoanthocyanidins, anthocyanidins, and flavan-3-ols). The diversity in chemical structure is closely related to diverse bioactivities of flavonoids in plant biology and human nutrition (Falcone Ferreyra et al., 2012). For example, anthocyanins are in many cases well known to colour flowers and fruits to attract animals and thus promoting pollination and dispersion of seeds (Ishikura and Yoshitama, 1984; Gronquist et al., 2001; Grotewold, 2006). Flavonols can interact with anthocyanins to modify the colour of fruits (Andersen and Jordheim,

2010) and play a prominent role in protection against UV-B irradiation (Li et al., 1993) and also in plant fertility (Mo et al., 1992).

Many researchers have attributed positive effects on human health to flavonoids: e.g. antigenotoxic (Dauer et al., 2003), anticarcinogenic and antioxidative (Kandil et al., 2002) effects, as well as the prevention of cardiovascular diseases have been suggested (summarised inPerez-Vizcaino and Duarte, 2010). Additionally, Sun *et al.* (2019) suggest an involvement of flavonoids in the plant defence against the tropical race 4 (TR4) of the *Musa* Fusarium wilt (commonly known as 'panama disease') pathogen *Fusarium oxysporum* f. sp. *cubense* (Foc), which is a threat to the global banana production. It is certainly interesting to take a closer look at the biosynthesis of flavonoids in *Musa*.

Flavonoids are derived from the amino acid L-phenylalanine and malonyl-coenzyme A. Their biosynthesis (Figure 1) has been analysed in many different species including the dicotyledonous model plant *Arabidopsis thaliana* (*A. thaliana*) (Hahlbrock and Scheel, 1989; Lepiniec et al., 2006) and the monocotyledonous crop plants *Zea mays* (*Z. mays*) (summarised in Tohge et al., 2017) and *Oryza sativa* (*O. sativa*) (summarised in Goufo and Trindade, 2014). The first committed step, catalysed by the enzyme chalcone synthase (CHS), is the formation of naringenin chalcone from p-coumaroyl CoA and malonyl CoA (Kreuzaler and Hahlbrock, 1972). A heterocyclic ring is introduced during the formation of naringenin (a flavanone) from naringenin chalcone, which can occur spontaneously or catalysed by chalcone isomerase (CHI) (Bednar and Hadcock, 1988). The enzyme flavanone 3-hydroxylase (F3H or FHT) converts flavanones to dihydroflavonols by hydroxylation at the C-3 position (Forkmann et al., 1980). Alternatively, flavanones can be converted to flavones by flavone synthase I or II (FNS I or II) (Britsch, 1990). Flavonol synthase (FLS) catalyses the conversion of dihydroflavonols to the corresponding flavonols by introducing a double bond between C-2 and C-3 (Forkmann et al., 1986; Holton et al., 1993). Moreover, dihydroflavonols can be converted to leucoanthocyanidins by dihydroflavonol 4-reductase (DFR) (Heller et al., 1985), which competes with FLS for substrates (Luo et al., 2016). Anthocyanidin synthase (ANS, also termed leucoanthocyanidin dioxygenase, LDOX) converts leucoanthocyanidins to anthocyanidins (Saito et al., 1999).

The flavonoid biosynthesis enzymes belong to different functional classes (summarised in Winkel, 2006): polyketide synthases (e.g. CHS), 2-oxoglutarate-dependent dioxygenases (2-ODD, e.g. F3H, ANS, FLS, FNS I), short-chain dehydrogenases/reductases (e.g. DFR), aldo-keto reductases (e.g. chalcone reductase, CHR) and cytochrome P450 monooxygenases (e.g. FNS II, F3′H). Flavonoid biosynthesis is evolutionary old in plants (Wen et al., 2020) and, although differences exist, quite similar in dicots like *A. thaliana* and monocots like *Musa* .

In the present study, we focus on the functional class of flavonoid aglycon forming enzymes, namely the 2-ODDs. 2-ODDs occur throughout the kingdom of life and play important roles in

many biological processes including oxygen sensing and DNA repair (Jaakkola et al., 2001; Trewick et al., 2002). They are a class of non-heme iron-containing enzymes, which require 2-oxoglutarate, Fe^{2+} and ascorbate for substrate conversion (summarised in Prescott and John, 1996).

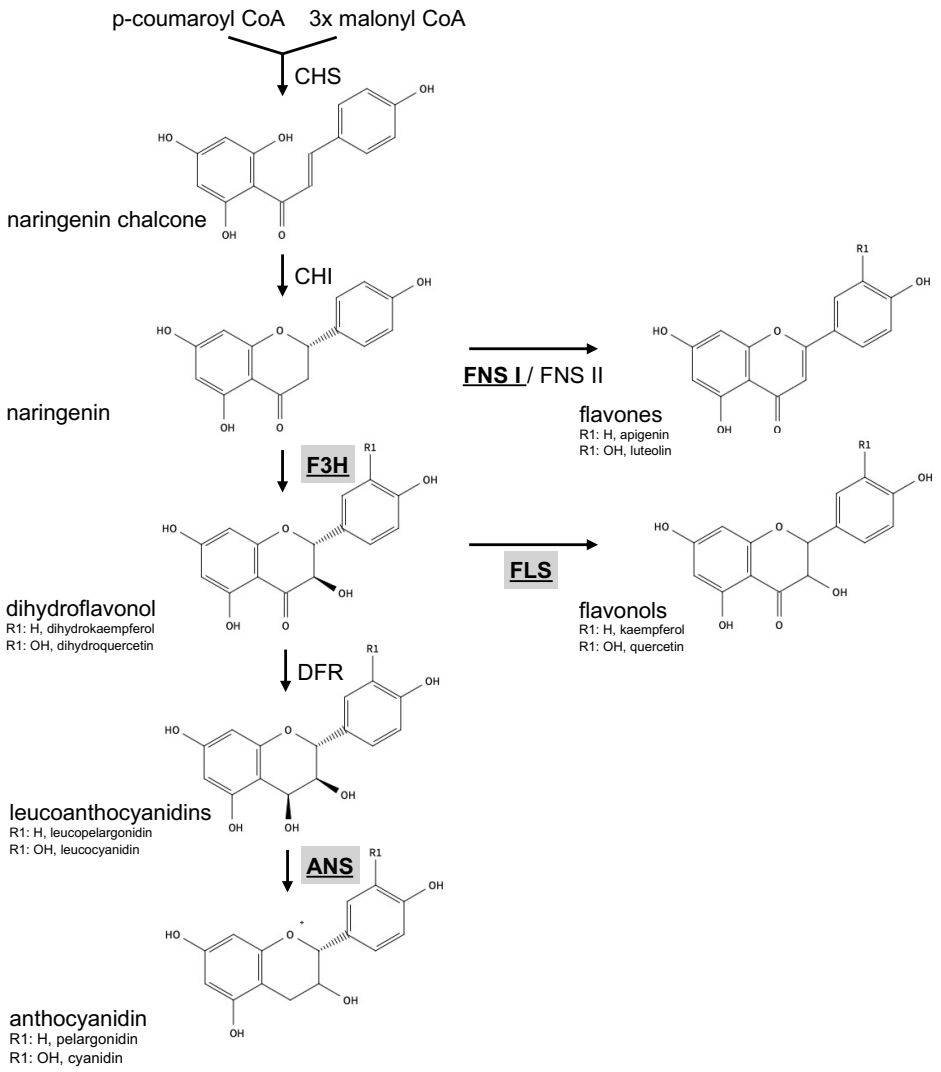


Figure 1: Schematic, simplified illustration of the core flavonoid aglycon biosynthesis pathway in plants.
Chalcone synthase (CHS), the first committed enzyme in flavonoid biosynthesis, connects p-coumaroyl-CoA and malonyl-CoA, forming naringenin chalcone. Chalcone isomerase (CHI) introduces the heterocyclic ring. The resulting naringenin is converted to dihydroflavonol by flavanone 3-hydroxylase (F3H). Dihydroflavonols are converted to flavonols by flavonol synthase (FLS). Alternatively, dihydroflavonol 4-reductase (DFR) can reduce dihydroflavonols to the corresponding leucoanthocyanidin, which is converted to anthocyanidins by the activity of anthocyanidin synthase (ANS). 2-oxoglutarate-dependant oxygenases are given in bold underlined. Enzymes in the focus of this work are highlighted by grey boxes.

Plant 2-ODDs share conserved amino acid residues which coordinate ferrous iron binding (HxDxnH) and binding of 2-oxoglutarate (RxS) (Cheng et al., 2014). They can be divided into three distinct evolutionary classes, DOXA, DOXB and DOXC, based on amino acid similarity

114 (Kawai et al., 2014). The 2-ODDs involved in specialised metabolism were classified into the
115 DOXC class. Yet, all flavonoid biosynthesis related 2-ODDs were found in the DOXC
116 subclades 28 (F3H and FNS I) or 47 (FLS and ANS) (Kawai et al., 2014). In some cases, the
117 high amino acid similarity between the different enzymes leads to overlapping functions. For
118 example, FLSs from *Ginkgo biloba* (*G. biloba*) (Xu et al., 2012) and *O. sativa* (Park et al., 2019)
119 can accept flavanones and dihydroflavonols as substrates. Also, ANSs from *A. thaliana*
120 (Stracke et al., 2009) and *Malus domestica* (*M. domestica*) (Yan et al., 2005) can catalyse the
121 formation of flavonol glycosides.
122 Based on the *Musa acuminata* (*M. acuminata*) genome sequence annotation (DHont et al.,
123 2012), Pandey *et al.* (2016) identified 28 putative *Musa* flavonoid biosynthesis enzymes. This
124 includes seven 2-ODD-type enzymes, namely two F3H, four FLS and one ANS, while no
125 suitable FNS I candidate was identified.. While the expression of the respective genes and
126 correlations of expression to flavonoid metabolite accumulation have been studied, the
127 functionality of these enzymes has not been analysed until now.
128 Here, we describe the sequence-based and functional characterisation of 2-ODDs from *Musa*.
129 Functionalities of recombinant *Musa* enzymes were analysed using *in vivo* bioconversion
130 assays and by *in planta* complementation of corresponding *A. thaliana* loss-of-function
131 mutants. This resulted in the experimental confirmation that the *Musa* flavonoid 2-ODDs
132 studied have the predicted functions. The presented results contribute to the understanding of
133 flavonoid biosynthesis in *Musa*. They provide a strong basis for further research to enhance
134 the efficiency of flavonoid production in banana, in order to increase the fruits' health promoting
135 effects.
136
137
138 **Materials and Methods**
139
140 **Plant material**
141 Banana plants (Grand Naine, plantain) for RNA extraction were grown in the field in Lucknow,
142 India. *Musa* gene annotation identifiers refer to the study of Martin et al. (2016). Columbia-0
143 (Col-0, NASC ID N1092) and Nössen-0 (Nö-0, NASC ID N3081) were used as wildtype
144 controls. The *A. thaliana* mutants *tt6-2* (*f3h*, GK-292E08, Col-0 background) (Appelhagen et
145 al., 2014) and *ans/fls1-2* (synonym *ldox/fls1-2, ldox: SALK_028793,* Col-0 background; *fls1-2:*
146 RIKEN_PST16145, Nö-0 background) (Stracke et al., 2009) were used for complementation
147 experiments.
148
149 **Phylogenetic analysis**

150 Multiple protein sequence alignments were created with MAFFT v7 (Katoh and Standley, 2013)
151 using default settings. The Approximately-Maximum-Likelihood phylogenetic tree of 33 plant
152 2-ODDs with proven F3H, FNS I, FLS and ANS functionality (Supplementary Table S1) and
153 seven *Musa* 2-ODDs was constructed as described by Pucker *et al*. (2020b): MAFFT
154 alignments were cleaned with pxclsq (Brown et al., 2017) and the tree was constructed with
155 FastTree v2.1.10 using the WAG+CAT model (Price et al., 2010). The tree was visualised with
156 Interactive Tree Of Life (Letunic and Bork, 2019), branch lengths were neglected.
157
158 **Expression analysis**
159 Expression data for *Musa* 2-ODD genes was extracted from previous analyses (Pucker et al.,
160 2020a). Short Read Archive IDs can be found in Supplementary Table S2.
161
162 **Total RNA extraction, cDNA synthesis and molecular cloning**
163 Isolation of RNA from different *Musa* plant organs (leaf, pseudostem, bract, fruit peel, fruit pulp)
164 was performed according to a protocol from Asif et al. (2000). cDNA synthesis was performed
165 from 1 µg total RNA using the ProtoScript® First Strand cDNA Synthesis Kit (New England
166 Biolabs, NEB) with the provided random primer mix according to the suppliers' instructions.
167 Amplification of predicted full length CDSs (Pandey et al., 2016) were done using Q5® High-
168 Fidelity DNA polymerase (NEB) and gene-specific primers (Supplementary Table S3)
169 according to standard protocols. Creation of full-length coding sequence constructs (CDS) was
170 performed using the GATEWAY® Technology (Invitrogen). *MusaF3H1* (Ma02_t04650),
171 *MusaF3H2* (Ma07_t17200), *MusaFLS1* (Ma03_t06970), *MusaFLS3* (Ma10_t25110) and
172 *MusaANS* (Ma05_t03850) CDSs were successfully amplified on different cDNA pools. The
173 resulting PCR products were recombined into pDONR™/Zeo (Invitrogen) with BP clonase
174 (Invitrogen) resulting in Entry plasmids, which were sequenced by Sanger technology (Sanger
175 et al., 1977) on 3730XL sequencers using BigDye terminator v3.1 chemistry (Thermo Fisher).
176 Entry plasmids for *AtF3H*, *AtFLS1* and *AtMYB12* were available from previous studies (Preuss
177 et al., 2009; Stracke et al., 2017). The full-length CDSs were introduced from the Entry
178 plasmids into the inducible *E. coli* expression vector pDEST17 (Invitrogen) and the binary
179 expression vector pLEELA (Jakoby et al., 2004) using GATEWAY LR-reaction (Invitrogen).
180
181 **Heterologous expression in *E. coli***
182 pDEST17-based plasmids containing *proT7*-RBS-6xHis-CDS-*T7term* expression cassettes
183 (*proT7: T7 promoter*, RBS: ribosome binding site, 6xHis: polyhistidine tag, *T7term: T7*
184 *transcription terminator*) were transformed into BL21-AI cells (Invitrogen). Cultures were grown
185 in LB to an OD_{600} of about 0.4 and expression was induced with 0.2 % L-arabinose.
186

187 **F3H and FLS bioconversion assay in *E. coli***

188 The enzyme assay was performed using 20 mL *E. coli* cultures expressing the respective
189 constructs right after induction with L-arabinose. 100 µL Naringenin (10 mg/mL), 50 µL 2-
190 oxoglutaric acid, 50 µL $FeSo_4$ and 50 µL 1 M sodium ascorbate were added. The cultures were
191 incubated at 28 °C overnight. To extract flavonoids, 1 mL was removed from each culture and
192 mixed with 200 µL ethyl acetate by vortexing for 30 s. After centrifugation for 2 min. at 14,000 g,
193 the organic phase was transferred into a fresh reaction tube. Samples were taken after 0 h,
194 1 h, 2 h, 3 h, 4 h and 24 h. Flavonoid content was analysed by high-performance thin-layer
195 chromatography (HPTLC). Naringenin (Sigma), dihydrokaempferol (Sigma) and kaempferol
196 (Roth) were dissolved in methanol and used as standards. 3 µL of each methanolic extract
197 were spotted on a HPTLC Silica Gel 60 plate (Merck). The mobile phase was composed of
198 50 % chloroform, 45 % acetic acid and 5 % water. Flavonoid compounds were detected as
199 described previously (Stracke et al., 2007), using diphenylboric acid 2-aminoethyl ester
200 (DPBA) and UV-light (Sheahan and Rechnitz, 1992).

201

202 ***Agrobacterium*-mediated transformation of *A. thaliana***

203 T-DNA from pLEELA-based plasmids containing *2xpro35S*-driven *Musa2-ODDs*, were
204 transformed into *A. thaliana* plants via *Agrobacterium tumefaciens* (Agrobacterium,
205 GV101::pMP90RK, (Koncz and Schell, 1986)) mediated gene transfer using the floral dip
206 method (Clough and Bent, 1998). Successful T-DNA integration was verified by BASTA
207 selection and PCR-based genotyping.

208

209 **Flavonoid staining of *A. thaliana* seedlings**

210 *In situ* visualisation of flavonoids in norflurazon-bleached *A. thaliana* seedlings was performed
211 according to Stracke *et al.* (2007) using DPBA/Triton X-100 and epifuorescence microscopy.

212

213 **Analysis of flavonols in methanolic extracts**

214 Flavonol glycosides were extracted and analysed as previously described (Stracke et al.,
215 2009). *A. thaliana* rosette leaves were homogenised in 80 % methanol, incubated for 15 min.
216 at 70 °C and centrifuged for 10 min. at 14,000 g. Supernatants were vacuum-dried. The dried
217 pellets were dissolved in 80 % methanol and analysed by HPTLC on a Silica Gel 60 plate
218 (Merck). Ethyl acetate, formic acid, acetic acid, water (100:26:12:12) were used as a mobile
219 phase. Flavonoid compounds were detected as described above.

220

221 **Determination of anthocyanin content**

222 To induce anthocyanin production, *A. thaliana* seedlings were grown on 0.5 MS plates with
223 4 % sucrose and 16 h of light illumination per day at 22 °C. Six day old seedlings were used

224 to photometrically quantify anthocyanins as described by Mehrtens et al. (2005). All samples
225 were measured in three independent biological replicates. Error bars indicate the standard
226 error of the average anthocyanin content.
227
228
229 **Results**
230 **Creation of cDNA constructs and sequence based characterisation of *Musa* flavonoid**
231 **2-ODDs**
232 Previously described putative *Musa* flavonoid biosynthesis enzymes encoded in the
233 *M. acuminata* Pahang DH reference genome sequence were used. This included two F3Hs,
234 four FLSs and one ANS, which were classified as 2-ODDs. To functionally characterise these
235 *Musa* enzymes, we amplified the corresponding CDSs from a cDNA template collection
236 derived from different *Musa* organs, using primers designed on the Pahang DH reference
237 genome sequence. The successfully amplified cDNAs of *MusaF3H1* and *MusaF3H2* were
238 derived from plantain pulp, *MusaFLS1* from Grand Naine young leaf, *MusaFLS3* on Grand
239 Naine bract, and *MusaANS* on Grand Naine peel. Unfortunately, we were not able to amplify
240 *MusaFLS2* and *MusaFLS4* from our template collection.
241 Comparison of the resulting 2-ODD cDNA sequences with the reference sequence revealed
242 several single-nucleotide polymorphisms (SNPs) (Supplementary File S1). The derived amino
243 acid sequences show close similarity to other plant 2-ODD proteins known to be involved in
244 flavonoid biosynthesis (Supplementary File S2). The amino acids well known to coordinate
245 ferrous iron binding (HxDxnH) and binding of 2-oxoglutarate (RxS) are also conserved in the
246 *Musa* 2-ODDs. Additionally, the *At*FLS1 residues which have been shown to be involved in
247 flavonoid substrate binding (Supplementary File S2) are conserved. F293 is conserved in all
248 *Musa* 2-ODD proteins, F134 and K202 are found in the *Musa*FLSs and *Musa*ANS and E295
249 is conserved in *Musa*ANS.
250 To analyse the evolutionary relationship between *Musa* 2-ODDs and 33 known flavonoid
251 biosynthesis related 2-ODDs, a phylogenetic tree was built (Figure 2). The phylogenetic tree
252 revealed two distinct clades, which correspond to the DOXC28 and DOXC47 classes of the
253 2-ODD superfamily. In the F3H and FNS I-containing DOXC28 class, *Musa*F3H1 and
254 *Musa*F3H2 cluster with other F3Hs from monocotyledonous plants. In the FLS and ANS-
255 containing DOXC47 class, the *Musa*ANS clusters with ANSs from monocotyledonous plants,
256 while the FLSs from monocotyledonous plants do not form a distinct group, although the
257 *Mus*aFLSs are in proximity to *Zm*FLS and *Os*FLS.
258 Our analyses clearly show that the CDSs identified for *Musa*F3H1, *Musa*F3H2, *Musa*FLS1,
259 *Musa*FLS3 and *Musa*ANS have the potential to encode functional flavonoid 2-ODDs.
260

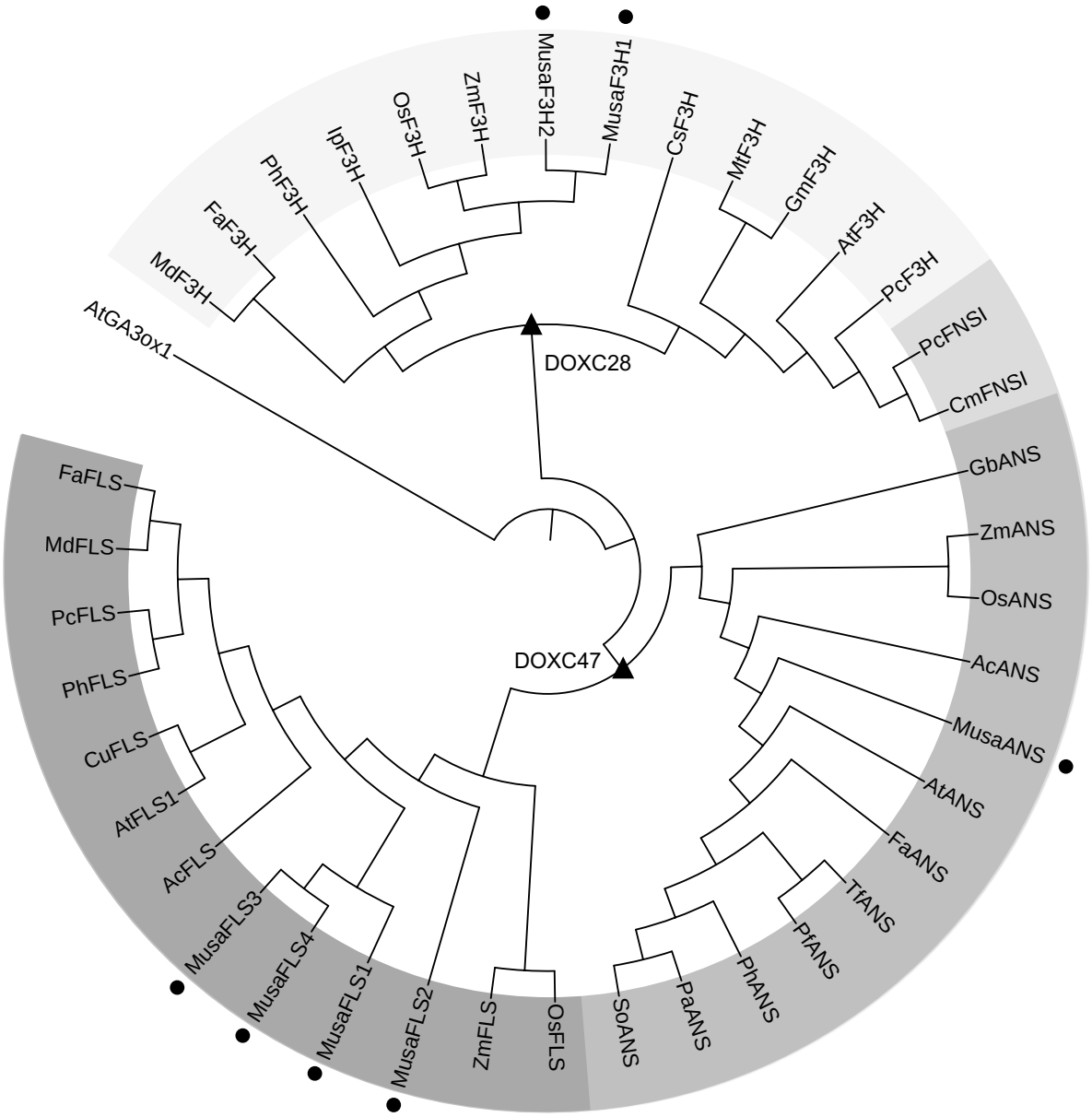


Figure 2: Rooted Approximately-Maximum-Likelihood (ML) phylogenetic tree of 2-ODDs involved in flavonoid biosynthesis.
Additional to the ODDs from *Musa* (marked with black dots), 33 enzymes with proven F3H, FNS I, FLS or ANS activity were included. Different grey scales indicate the F3H, FNS I, FLS and ANS clades. *At*GA3ox1 (gibberellin 3 beta-hydroxylase 1) was used as an outgroup. DOXC28 and DOXC47 classes are marked with black triangles.

Expression profiles of *Musa 2-ODD* genes

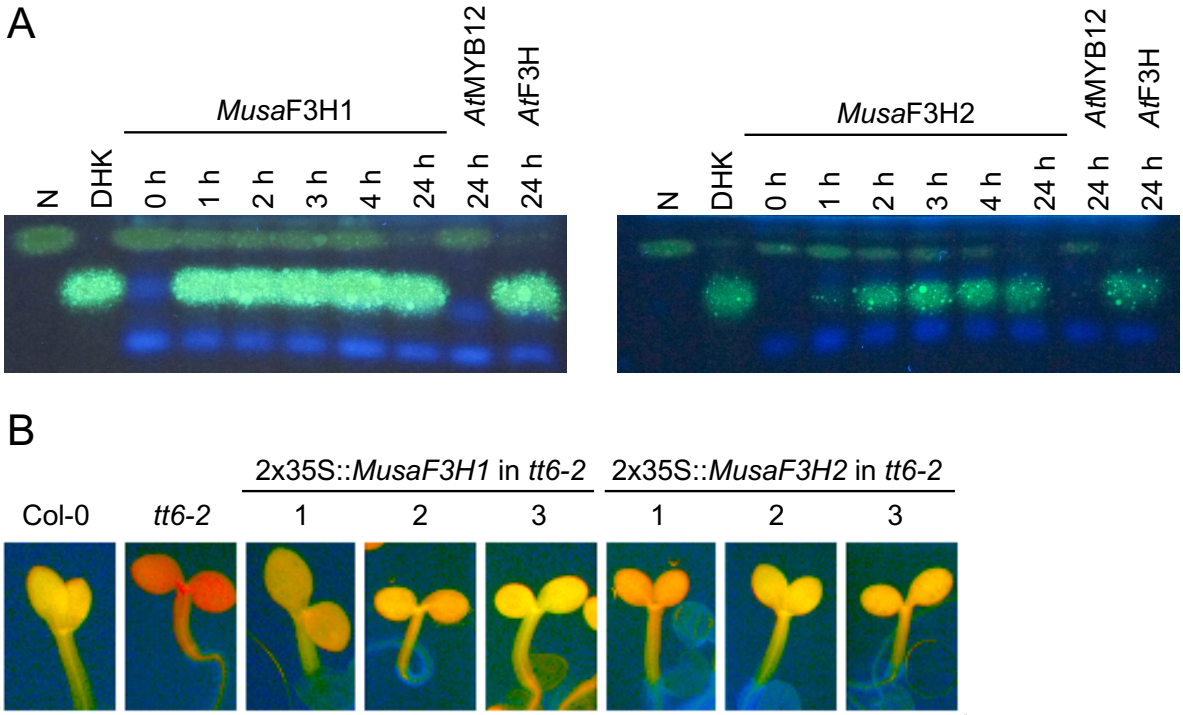
Analysis of the expression patterns of the genes studied was performed using published RNA-Seq data. We obtained normalised RNA-Seq read values for *Musa* 2-ODD genes from several organs and developmental stages (Table 1). *MusaF3H1* and *MusaF3H2* are expressed in almost all analysed organs and developmental stages. *MusaF3H1* expression is highest in early developmental stages of pulp (S1, S2), followed by intermediate developmental stages of peel (S2, S3). *MusaF3H2* shows highest expression in adult leaves.

276 *MusaFLS2* was not expressed in any of the analysed samples. All four *MusaFLS* genes show
277 low or no expression in seedlings and embryonic cell suspension. *MusaFLS1* shows variance
278 in transcript abundance with particularly high levels in peel (S2) and in young and adult leaves.
279 *MusaFLS3* and *MusaFLS4* transcript levels are comparatively constant and low, with highest
280 transcript abundance in root and peel (S1 and S3 respectively). While *MusaFLS1* transcript
281 abundance is highest in adult leaf, *MusaFLS3* and *MusaFLS4* lack expression in this organ.
282 *MusaANS* shows highest transcript abundance in pulp (S1, S2, S4) and peel (S3) and very
283 low expression in embryogenic cell suspension, seedlings and leaves.
284
285 **Table 1: Expression profiles of *Musa2*-ODD genes in different organs and**
286 **developmental stages based on RNA-Seq data.** Expression levels are given in fragments
287 per kilo base per million mapped reads (FPKM). Different grey scales indicate expression
288 levels with higher levels given in darker grey. S1-S4 indicate stages of pulp and peel
289 developments from immature to ripe (Lu et al., 2018).

				leaf				pulp					peel				
gene	embryogenic	seedling	root	leaf	young leaf	adult leaf	old leaf	pulp	pulp S1	pulp S2	pulp S3	pulp S4	peel	peel S1	peel S2	peel S3	peel S4
MusaF3H1	3	9	80	44	73	27	3	170	385	191	43	60	82	46	140	104	36
MusaF3H2	21	0	23	57	39	171	7	32	96	14	3	14	19	7	27	33	9
MusaFLS1	6	0	4	189	91	643	16	2	2	1	2	2	29	3	99	7	7
MusaFLS2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
MusaFLS3	0	0	32	16	17	0	0	16	15	22	10	17	22	27	16	20	25
MusaFLS4	0	0	19	4	0	0	0	10	11	4	12	13	15	16	11	19	14
MusaANS	1	3	90	41	79	3	1	255	559	298	26	137	73	47	50	163	30

290
291 ***Musa*F3H1 and *Musa*F3H2 are functional flavanone 3-hydroxylases**
292 To confirm F3H activity *in vivo*, we used a bioconversion assay. *MusaF3H1* and *MusaF3H2*
293 were heterologously expressed in *E. coli* and the bacterial cultures were fed with naringenin
294 as substrate of F3H (Figure 1). Both recombinant proteins, *Musa*F3H1 and *Musa*F3H2, were
295 able to convert naringenin to dihydrokaempferol (DHK) in the presence of 2-oxoglutarate and
296 ferrous iron (Figure 3A). After 24 h of incubation, only small amounts of naringenin remained
297 un-converted. Conversion of DHK to kaempferol by *Musa*F3H1 or *Musa*F3H2 was not
298 observed. For further *in planta* analysis we chose a complementation assay with an *A. thaliana*
299 *f3h* single mutant *(tt6-2)* which expresses FLS and ANS but no F3H. *MusaF3H1* or *MusaF3H2*
300 were expressed in *tt6-2* plants under the control of the constitutive 2x35S promoter. The
301 accumulation of flavonol glycosides was analysed in herbizide-bleached seedlings using

302 DPBA-staining (Figure 3B). While Col-0 wildtype seedlings appeared orange under UV light,
303 indicating the accumulation of flavonol glycosides, *tt6-2* seedlings showed a red fluorescence.
304 *tt6-2* mutants expressing *MusaF3H1* or *MusaF3H2* were able to complement the mutant
305 phenotype, showing the characteristic orange flavonol glycoside fluorescence of the wild type.
306 These results demonstrate that *Musa*F3H1 and *Musa*F3H2 are functional enzymes with F3H
307 activity and are able to catalyse the conversion of naringenin to dihydrokaempferol.
308



309
310 **Figure 3: *Musa*F3H1 and *Musa*F3H2 are functional flavanone 3-hydroxylases.**
311 (A) HPTLC analysis of F3H bioconversion assays using extracts from *E. coli* expressing
312 recombinant *Musa*F3H1 or *Musa*F3H2. The substrate naringenin (N) and the product
313 dihydrokaempferol (DHK) were used as standards. *At*FLS1 was used as a positive control.
314 *At*MYB12 was used as a negative control. (B) Analysis of *tt6-2* mutant seedlings
315 complemented with *Musa*F3H1 and *Musa*F3H2. Col-0 wildtype and *tt6-2* were used as
316 controls. Representative pictures of DPBA-stained seedlings under UV light are given. Yellow
317 fluorescence indicates flavonol glycoside accumulation. The different numbers indicate
318 individual transgenic lines.
319

320 ***Musa*FLS1 and *Musa*FLS3 are functional flavonol synthases**

321 To confirm FLS activity *in vivo*, the enzymes were assayed in a coupled bioconversion
322 experiment (Figure 4A). Two *E. coli* cultures expressing *MusaF3H1* and a *MusaFLS* were
323 mixed and fed with naringenin in the presence of 2-oxoglutarate and ferrous irons. Here,
324 *Musa*F3H1 converts naringenin to DHK, thus providing the substrate for the FLS enzyme to
325 be tested. While *Musa*FLS1 was found to be able to convert DHK to kaempferol under the
326 assay conditions, *Musa*FLS3 was not. We checked the expression of recombinant *Musa*FLS1
327 and *Musa*FLS3 in the *E. coli* cultures by SDS-PAGE and found both *Musa*FLS proteins being

328 expressed at similar levels (Supplementary File S3). To further analyse the enzymatic
329 properties *in planta*, *MusaFLS1* or *MusaFLS3* were expressed in the flavonol and anthocyanin
330 deficient *A. thaliana ans/fls1-2* double mutant. HPTLC analyses of methanolic extracts from
331 rosette leaves from greenhouse-grown plants (Figure 4B) showed that wildtype plants (Col-0,
332 Nö-0) contained flavonol glycosides, including the prominent derivatives K-3R-7R, K-3G-7R
333 and K-3[G-R]-7R. *ans/fls1-2* plants accumulated several dihydroflavonols but did not show
334 flavonol derivatives. *ans/fls1-2* mutants transformed with 2x35S::*MusaFLS1* or
335 2x35S::*MusaFLS3* constructs were able to form several flavonol derivatives. Nevertheless,
336 intensities and accumulation patterns of flavonol glycosides varied. We also analysed if
337 *Musa*FLS1 and *Musa*FLS3 are able to complement the anthocyanin-deficiency of the *ans/fls1-*
338 *2* double mutant. For this, seedlings were grown on 4 % sucrose to induce anthocyanin
339 accumulation. As shown in Figure 4C, 6-day-old Col-0 and Nö-0 seedlings were able to
340 accumulate red anthocyanin pigments, while *ans/fls1-2* transformed with *Musa*FLS1 or
341 *Musa*FLS3 did not show visible anthocyanins. These results confirm that *Musa*FLS1 and
342 *Musa*FLS3 are functional proteins with FLS activity and are enzymes able to catalyse the
343 conversion of dihydroflavonol to flavonol.

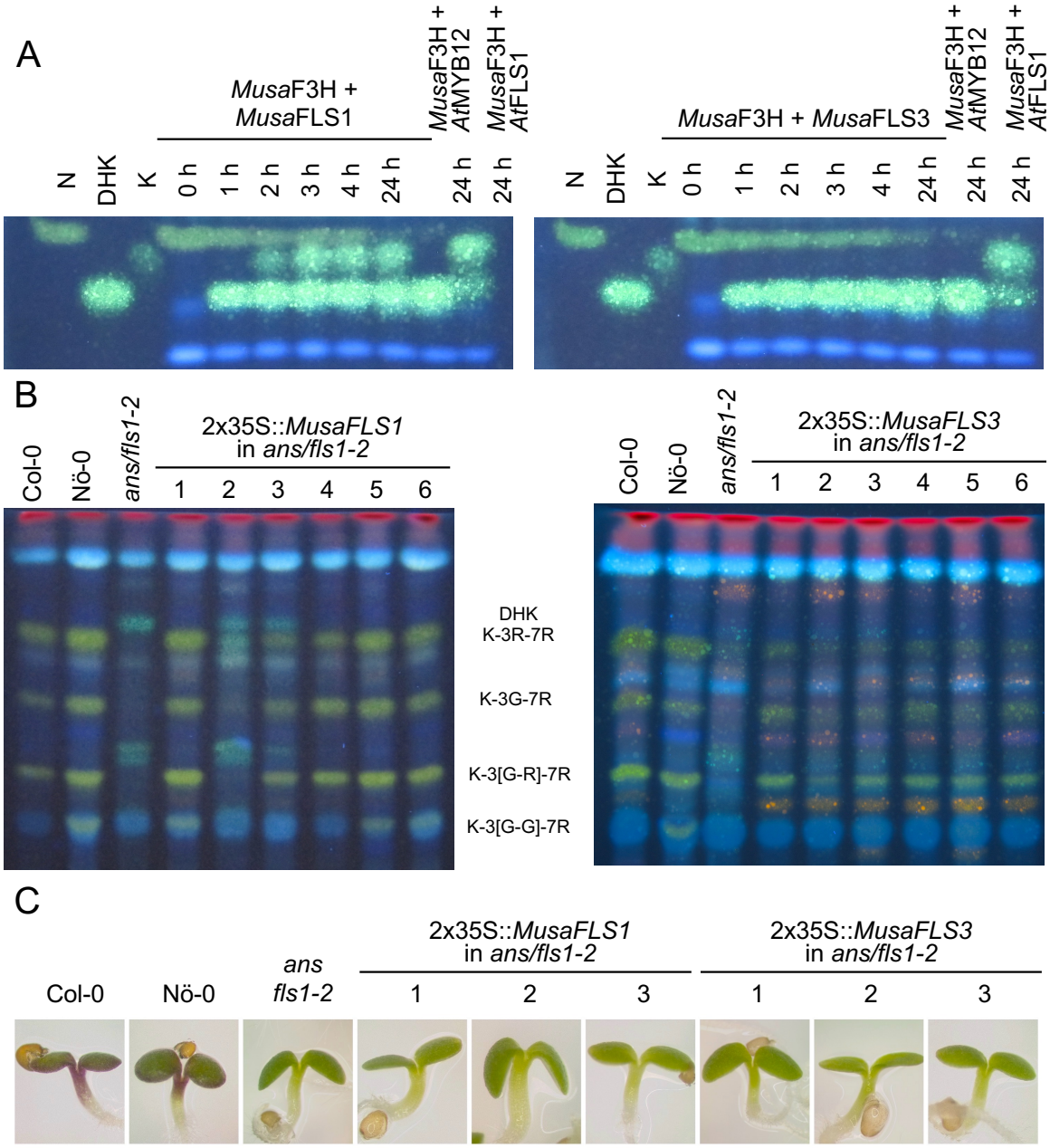


Figure 4: *Musa*FLS1 and *Musa*FLS3 are functional flavonol synthases.
(A) HPTLC analysis of a FLS bioconversion assays using extracts from *E. coli* expressing recombinant *Musa*FLS1 or *Musa*FLS3. The F3H substrate naringenin (N), the FLS substrate dihydrokaempferol (DHK) and the product kaempferol (K) were used as standards. *At*FLS1 served as a positive control and *At*MYB12 was used as a negative control. (B) Flavonol glycoside accumulation in *Musa*FLS-complemented *ans/fls1-2* seedlings, analysed by HPTLC analysis. Col-0, Nö-0 (both wildtype) and *ans/fls1-2* were used as controls. Bright green spots belong to derivates of kaempferol, orange spots are derivates of quercetin and faint blue shows sinapate derivatives. Dark green and yellow spots indicate DHK and DHQ, respectively. G, glucose; K, kaempferol; Q, quercetin; R, rhamnose (C) Representative pictures of anthocyanin accumulation in 6-day-old *Musa*FLS-complemented *ans/fls1-2* seedlings growing on 4 % sucrose.

357 ***Musa*ANS is a functional anthocyanidin synthase**

358 We analysed *Musa*ANS functionality in a complementation assay with the *ans/fls1-2*
359 *A. thaliana* double mutant. To examine the ability of *Musa*ANS to complement the *ans/fls1-2*
360 anthocyanin deficiency phenotype, seedlings were grown on anthocyanin-inducing media.
361 Anthocyanin accumulation was analysed visually and quantified photometrically (Figure 5A-B).
362 While wildtype seedlings showed red pigmentation, the *ans/fls1-2* seedlings did not. *ans/fls1-*
363 *2* seedlings expressing *Musa*ANS showed accumulation of anthocyanins. The anthocyanin
364 content in the complemented seedlings was strongly increased compared to *ans/fls1-2*
365 knockout plants, indicating ANS activity. Furthermore, we analysed the ability of *Musa*ANS to
366 complement the flavonol deficiency phenotype of *ans/fls1-2* plants. For this, methanolic
367 extracts of seedlings were analysed by HPTLC followed by DPBA-staining (Figure 5C). While
368 wildtype seedlings accumulated several kaempferol and quercetin glycosides, *ans/fls1-2*
369 mutants expressing *Musa*ANS showed a flavonoid pattern identical to the *ans/fls1-2* mutant,
370 accumulating dihydroflavonol derivatives, but no flavonol derivatives. These results indicate,
371 that *Musa*ANS is a functional enzyme with ANS activity and is able to catalyse the conversion
372 of leucoanthocyanidin to anthocyanidin.

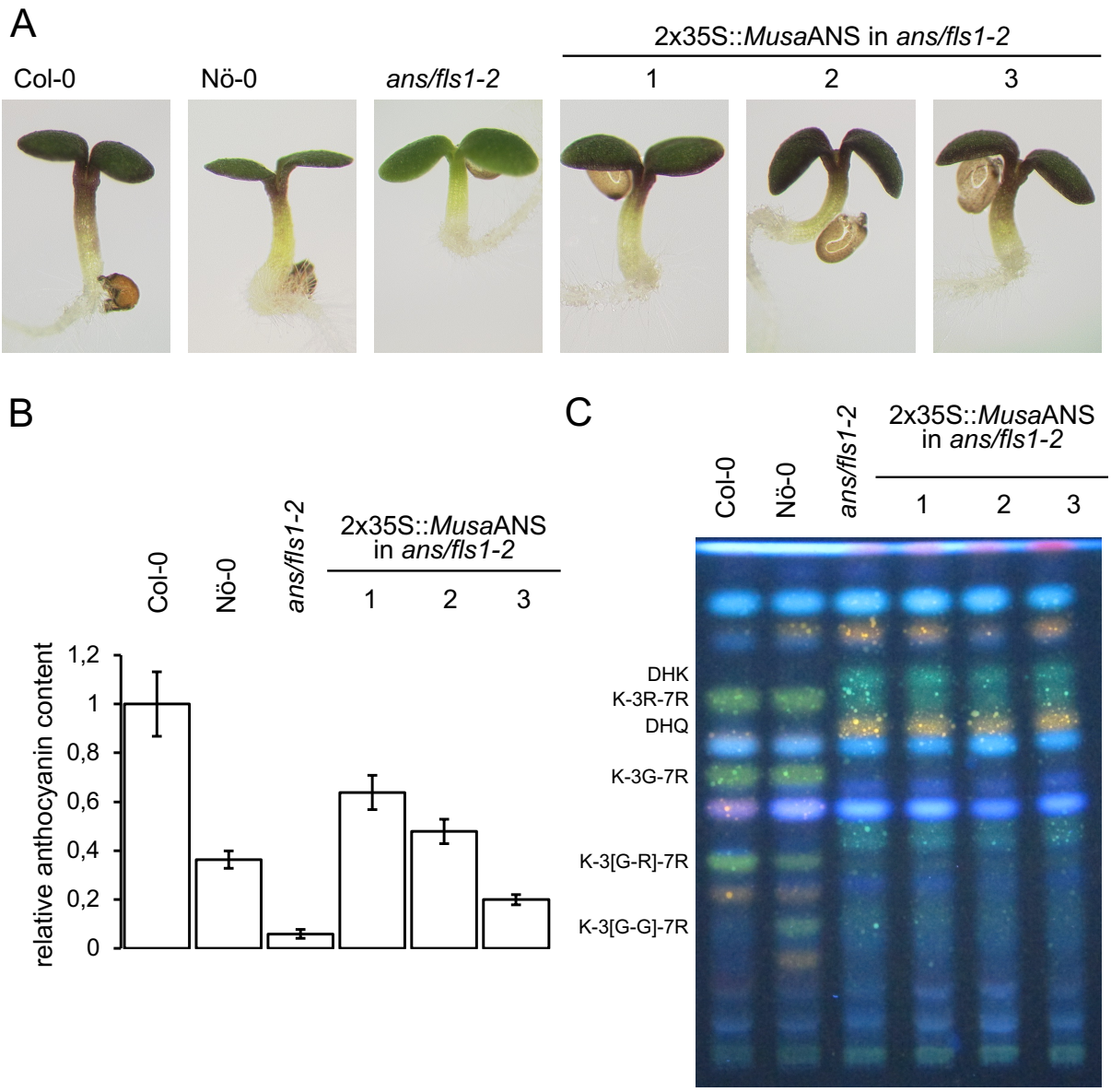


Figure 5: *Musa*ANS is a functional anthocyanin synthase.
Analysis of *ans/fls1-2* double mutant seedlings complemented with 2x35S driven *MusaANS* demonstrate *in planta* ANS functionality of *Musa*ANS by anthocyanin accumulation. The different numbers indicate individual transgenic lines. (A-B) Sucrose induced anthocyanin accumulation in 6-day-old *A. thaliana* seedlings. (A) Representative pictures of seedlings and (B) corresponding relative anthocyanin content. Error bars indicate the standard deviation for three independent measurements. (C) *Musa*ANS does not show *in planta* FLS activity. Flavonol glycoside accumulation in *Musa*ANS-complemented *ans/fls1-2* seedlings, analysed by HPTLC analysis. Col-0, Nö-0 (both wildtype) and *ans/fls1-2* were used as controls. Bright green spots belong to derivates of kaempferol, orange spots are derivates of quercetin and faint blue shows sinapate derivatives. Dark green and yellow spots indicate DHK and DHQ, respectively. G, glucose; K, kaempferol; Q, quercetin; R, rhamnose

387 **Discussion**

388 In this study, we isolated several cDNAs of *in silico* annotated 2-ODD-type flavonoid
389 biosynthesis enzyme coding genes from *Musa* and tested the *in vivo* functionality of the
390 encoded proteins in *E. coli* and *A. thaliana*.

391

392 ***Musa*F3H1 and *Musa*F3H2**

393 *In vivo E. coli* bioconversion assays revealed that *Musa*F3H1 and *Musa*F3H2 can covert
394 naringenin to dihydrokaempferol. Moreover, *Musa*F3H1 and *Musa*F3H2 are able to
395 complement the loss-of-function phenotype of *A. thaliana tt6-2* seedlings, showing *in planta*
396 F3H-activity. Therefore we conclude that *Musa*F3H1 and *Musa*F3H2 are functional flavanone
397 3-hydroxylases.

398 Previous studies annotated *Ma02_g04650* and *Ma07_g17200* as genes encoding flavanone
399 3-hydroxylases (Martin et al., 2016; Pandey et al., 2016; Pucker et al., 2020b). However, the
400 automatic approach developed by Pucker *et al*. (2020b) also considered *Ma07_g17200* as a
401 candidate to encode a FNS I enzyme. For a parsley F3H protein it has been found that a
402 replacement of three amino acids was sufficient to cause FNS I side activity and seven amino
403 acid exchanges almost lead to a complete change in enzyme activity towards FNS I
404 functionality (Gebhardt et al., 2007). These findings underline the particular high similarity
405 between FNS I and F3H. A closer inspection of the deduced peptide sequence of
406 *Ma07_g17200* gene revealed a lack of conservation of amino acid residues known to be
407 relevant for FNS I function (i.e. T106M, T115I, I116V, F131I, E195D, I200V, V215L, R216K).
408 Together with the confirmed F3H activity this indicates that the classification of *Ma07_g17200*
409 as a FNS I encoding gene was inaccurate. Even though Ma07_p17200 is most likely no
410 functional FNS I, flavone derivatives have been identified in *Musa* (Fu et al., 2018). In *Gerbera*
411 and *Glycine max*, FNS II is responsible for the formation of flavones (Martens and Forkmann,
412 1999; Fliegmann et al., 2010). A candidate *Musa*FNS II, encoded by *Ma08_g26160*, has been
413 identified (Pucker et al., 2020b) and is probably responsible for the accumulation of flavones
414 in *Musa*. Since FNS II enzymes are NADPH-dependent cytochrome P450 monooxygenases
415 (Jiang et al., 2016), the putative *Musa*FNS II (encoded by *Ma08_g26160*) was not studied in
416 this work.

417 Our expression study that was based on published RNA-Seq data (Pucker et al., 2020a),
418 revealed highest *MusaF3H1* transcript abundance in early developmental stages of pulp and
419 intermediate stages of peel development, while highest *MusaF3H2* expression was found in
420 adult leaves (Table 1). This is, at first glance, in contrast to the quantitative real-time PCR
421 (qRT-PCR)-derived expression data presented by Pandey et al. (2016), reporting high
422 transcript levels of *MusaF3H1* and *MusaF3H2* in young leaves and bract. In this study, the
423 authors also show increased *MusaF3H1* expression in pseudostem, root and ripe pulp,

424 compared to *MusaF3H2*. These discrepancies are probably due to the different growth
425 conditions, germplasms/cultivars and sampling timepoints in the generation of the expression
426 data and cannot be reasonably analysed further at this point. It should be noted, however, that
427 *MusaF3H2* expression in leaves of plantlets was found to be increased following treatment
428 with the phytohormone methyl jasmonate (MJ), while *MusaF3H1* expression was not (Pandey
429 et al., 2016). As MJ is involved in various regulatory processes, including the response against
430 biotic and abiotic stresses (summarised inCheong and Choi, 2003), the MJ-dependent
431 induction of *MusaF3H2* expression could imply that the *Musa*F3H2 enzyme plays a specialised
432 role in the formation of flavonoids in response to stresses. Such stress response induction of
433 F3H encoding genes was previously shown for a *F3H* gene from the dessert plant *Reaumuria*
434 *soongorica*, which is induced by UV-light (Liu et al., 2013) and two *F3H* genes from *Camellia*
435 *sinensis* (*C. sinensis*), which are induced by UV light and by treatment with abscisic acid (ABA)
436 or sucrose (Han et al., 2017). In addition, overexpression of *F3H* from *C. sinensis* (Mahajan
437 and Yadav, 2014) and from *Lycium chinense* (Song et al., 2016) in tobacco improved the
438 tolerance to salt stress and fungal pathogens in the first case and to drought stress in the latter
439 case. In conclusion, our results clearly show that *Musa*F3H1 and *Musa*F3H2 are functional
440 flavanone 3-hydroxylases and that *Musa*F3H2 might play a role in stress response.
441
442 ***Musa*FLS1 and *Musa*FLS3**
443 *Musa*FLS1 was found to be able to convert dihydrokaempferol to kaempferol in the *E. coli*
444 bioconversion assays. This ability was validated *in planta* by successful complementation of
445 the flavonol deficiency of *A. thaliana ans/fls1-2* double mutant seedlings, while the anthocyanin
446 deficiency phenotype was not restored. These observations could hint to an exclusive FLS
447 activity of *Musa*FLS1. FLS and DFR, the first enzymes of the flavonol and anthocyanin
448 branches of flavonoid biosynthesis, compete for dihydroflavonol substrates (Figure 1). This is
449 demonstrated by several *A. thaliana fls1* single mutants which accumulate higher levels of
450 anthocyanin pigments (Owens et al., 2008; Stracke et al., 2009). Falcone Ferreyra et al. (2010)
451 found that overexpression of *ZmFLS1* in an *A. thaliana fls1* mutant decreases the
452 accumulation of anthocyanins to wildtype level, again indicating that the anthocyanin and
453 flavonol branches of flavonoid biosynthesis can compete for dihydroflavonol substrates in the
454 same cell or tissue. Accordingly, the overlapping substrate usage of FLS and ANS is difficult
455 to analyse in a *fls1* single mutant. Here, the use of an *A. thaliana ans/fls1-2* double mutant is
456 a simple way to analyse the FLS and a possible ANS side activity of flavonol synthases and
457 vice versa *in planta*. To analyse further side activities, a *f3h fls ans* mutant would be beneficial.
458 *A. thaliana ans/fls1-2* plants expressing *MusaFLS3* showed the accumulation of several
459 kaempferol derivatives. In contrast, plants complemented with *MusaFLS1* also accumulate
460 quercetin derivatives. This is most likely an effect of different environmental conditions in plant

461 growth. One set of plants was grown in the greenhouse (*Musa*FLS1), another set was grown
462 in a growth chamber (*Musa*FLS3). In the plants used for *Musa*FLS3 experiments these
463 conditions promoted the accumulation of dihydroquercetin, while the other growth conditions
464 did not in the plants used for *Musa*FLS1 experiments. Accordingly, dihydroquercetin was
465 probably not available as a substrate for *Musa*FLS1 and could not be converted to quercetin.
466 A possible explanation for the varying dihydroquercetin amounts could be the light induced
467 expression of F3'H, which converts dihydrokaempferol to kaempferol. The expression of *F3'H*
468 in cultured *A. thaliana* cells can be induced by UV-light (Schoenbohm et al., 2000) and the
469 activity of the *F3'H* promoter from *Vitis vinifera* is increased under light exposure (Sun et al.,
470 2015). Consequently, a different light quality or higher light intensity could be responsible for
471 increased *F3'H* expression, causing the accumulation of dihydroquercetin in the plants grown
472 in the growth chamber. While *Musa*FLS3 was able to complement the flavonol deficiency in
473 the *ans/fls1-2* mutant, it did not lead to an accumulation of anthocyanins. In contrast to the *in*
474 *planta* results, *Musa*FLS3 did not convert dihydrokaempferol to kaempferol in the *in vivo*
475 bioconversion assays, even though the protein was successfully expressed in the *E. coli*
476 culture. We used the bioconversion assays as a simple and versatile tool to analyse enzymatic
477 activities. Nevertheless, heterologous expression of eucaryotic proteins in *E. coli* is an artificial
478 system. It can cause a divergent pattern of posttranslational modifications or production of high
479 amounts of protein in inclusion bodies, leading to inactive protein (Sahdev et al., 2008). Preuss
480 et al. (2009) reported that *At*FLS3 did not show FLS activity in *E. coli*, but did convert
481 dihydroflavonols to the corresponding flavonols upon expression in yeast, indicating that the
482 stability of *At*FLS3 was improved under the latter assay conditions. The approach carried out
483 in this work might have similar limitations. Further limitations can be caused by different
484 substrate preferences, as reported for *Os*FLS (Park et al., 2019). Nevertheless, the *in planta*
485 complementation of the flavonol deficiency in the *A. thaliana ans/fls1-2* mutant and the lacking
486 complementation of the anthocyanin deficiency phenotype in the *ans/fls1-2* mutant indicate
487 that *Musa*FLS3 is a functional flavonol synthase, which does not exhibit significant ANS
488 activity.
489 *Musa*FLS1 transcript abundance was found to be high in adult leaves and low in roots.
490 *Musa*FLS3 and *Musa*FLS4 revealed opposed transcript levels. Such divergent expression
491 patterns have previously been observed for *FLS1* and *FLS2* from *Freesia hybrida* and *FLS1*
492 and *FLS2* from *Cyclamen purpurascens* (Akita et al., 2018; Shan et al., 2020). These opposed
493 expression patterns could point to differential activity in distinct organs. Furthermore, the
494 tandemly arranged *MusaFLS3* and *MusaFLS4* genes show very similar expression patterns
495 (Table 1), possibly indicating functional redundancy. While we could not find expression of
496 *Musa*FLS2 in our expression analyses, Pandey et al. (2016) observed *Musa*FLS2 expression
497 in several organs (including bract, pseudostem and root), as well as in different developmental

498 stages of peel and pulp. Again, these results show that different cultivars, growth conditions,
499 sampling timepoints and analysed organs can have a strong influence on the resulting data.
500 Accordingly, data from different studies should be evaluated carefully. *MusaFLS1* and
501 *MusaFLS2* expression in leaves of plantlets does not significantly increase after MJ treatment
502 (Pandey et al., 2016). However, UV radiation induces the expression of FLS from *Z. mays*
503 (Falcone Ferreyra et al., 2010) and *M. domestica* (Henry-Kirk et al., 2018) and the relative
504 expression of FLS from *Triticum aestivum* increases during drought stress (Ma et al., 2014).
505 Together with the knowledge that flavonols act as antioxidants (Wang et al., 2006), an
506 involvement of *Musa*FLSs in stress response seems feasible and should be further analysed
507 under a broader range of conditions. In summary, our results show that *Musa*FLS1 and
508 *Musa*FLS3 are functional flavonol synthase enzymes and hint at possible differential organ
509 specific activities of *Musa*FLS1 and *Musa*FLS3 / *Musa*FLS4.
510
511 ***Musa*ANS**
512 *A. thaliana ans/fls1-2* seedlings expressing *Musa*ANS show a strong, red pigmentation,
513 revealing that *Musa*ANS can complement the anthocyanin deficiency caused by mutation of
514 *AtANS*. However, the seedlings did not display flavonol derivatives in HPTLC analyses, which
515 have been reported to detect flavonol glycosides at levels of 50 pMol (Stracke, 2010). These
516 results indicate that *Musa*ANS is a functional anthocyanidin synthase, but does not have FLS
517 activity. The RNA-Seq data-derived expression profiles revealed high *Musa*ANS transcript
518 abundance in pulp and peel. Additionally, *MusaANS* expression has been reported to be high
519 in bract tissue (Pandey et al., 2016), the specialised leaves surrounding the flowers and usually
520 coloured red or purple due to anthocyanin accumulation (Pazmiño-Durána et al., 2001). This
521 spatial correlation of *MusaANS* transcripts and anthocyanin metabolites in bract tissue
522 supports the proposed biological functionality of this enzyme, catalysing the conversion of
523 leucoanthocyanidins to anthocyanidins in *Musa*. *MusaANS* expression increases 24 hours
524 after MJ treatment and decreases following dark treatment (Pandey et al., 2016). As supposed
525 for *MusaF3H2*, the increased expression of *MusaANS* after MJ treatment could imply an
526 involvement in the formation of anthocyanins as a consequence of stress response. ABA,
527 salicylic acid (SA), UV-B and cold treatments have been shown to enhance *ANS* transcript
528 abundance in *G. biloba* (Xu et al., 2008) and overexpression of *OsANS* raises the antioxidant
529 potential in *O. sativa* (Reddy et al., 2007). The accumulation of anthocyanins has often been
530 shown to be induced by (UV-) light (Takahashi et al., 1991; Stapleton and Walbot, 1994; Meng
531 et al., 2004). We therefore assume that also *Musa*ANS could be involved in such stress
532 response.
533 Very recently, a R2R3-MYB-type transcription factor (MaMYB4) has been identified as a
534 negative regulator of anthocyanin biosynthesis in *Musa* acting as repressor on the *Musa*ANS

promoter (Deng et al., 2021). These results give a first insight into the transcriptional regulation of *Musa*ANS expression and confirm the role of *Musa*ANS in the anthocyanin biosynthesis in *Musa*. Taking all available evidence into account, *MusaANS* encodes a functional anthocyanidin synthase with a possible involvement in the plants' stress response.

To deepen the knowledge about 2-ODDs and other enzymes involved in *Musa* flavonoid biosynthesis, it would be beneficial to acquire even more spatially and timely highly resolved transcriptome and in particular metabolite data. This data could serve as a starting point for the analysis of organ-, stress- or development-specific enzyme activities and functions, as well as possible substrate preferences. It could also be used to elucidate the regulatory network of *Musa* flavonoid biosynthesis. Furthermore, knowledge about the influence of different stresses (e.g. pathogens, light, temperature) on specific transcriptomes and metabolomes of the *Musa* plant could help to widen the knowledge of flavonoid biosynthesis and particular the functionality of the *Musa* 2-ODD enzymes. This could also lead to the detection of possible restricted side activities or overlapping functionalities as described for 2-OODs in some other plant species (Falcone Ferreyra et al., 2010; Park et al., 2019) and to further decode the cause of these multifunctionalities in 2-ODDs.

To conclude, in this study the functionality of five 2-ODDs involved in flavonoid biosynthesis in *Musa* was demonstrated *in planta*. Knowledge gained about the structural genes *MusaF3H1*, *MusaF3H2*, *MusaFLS1*, *MusaFLS3* and *MusaANS* in a major crop plant provides a basis for further research towards engineered, increased flavonoid production in banana, which could contribute to the fruits' antioxidant activity and nutritional value, and possibly even enhance the plants' defence against *Foc*-TR4.

Conflict of Interest

The authors declare that there is no conflict of interest.

Author Contributions

MB and RS planned the experiments. MB and CA performed the experiments and analysed the data. RS and BW supervised the project. MB wrote the initial draft. RS and BW revised the manuscript. All authors read and approved the final manuscript version.

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