1 **RESPONSE TO REVIEWERS**

2

3 **MycoRed: Betalain pigments as in vivo real-time visual markers for arbuscular**

4 **mycorrhizal colonisation of transgenic root systems**

5 Timoneda A, Yunusov Y, Quan C, Gavrin A, Brockington SF, Schornack S

6

7

8 **Editor's comments**

9 The reviews of your manuscript are appended below. You will see that the reviewers find the
10 work potentially interesting. However, based on their specific comments and following
11 discussion with the academic editor, I regret that we cannot accept the current version of the
12 manuscript for publication. We remain interested in your study and we would be willing to
13 consider resubmission of a comprehensively revised version that thoroughly addresses all
14 the reviewers' comments. We cannot make any decision about publication until we have
15 seen the revised manuscript and your response to the reviewers' comments. Your revised
16 manuscript would be sent for further evaluation by the reviewers.

17 As you will see, the majority of reviewers found your work to be interesting and useful to the
18 field. However, all of them raised several important concerns that we would need to see
19 experimentally addressed before considering the manuscript further for publication. Among
20 other issues, the reviewers ask that you confirm that the symbiosis is functional,

21 **Response:** We have now carried out additional experimentation showing that the symbiosis
22 genes *MtSTR* and *MtRAM2* are transcriptionally induced upon colonisation of betalain
23 producing roots with AM fungi.

24 provide evidence that your system works without false positives when other microbes are
25 present

26 **Response:** We performed root infections of the NbPT5b-p3 and BCP1b-p3 lines with
27 *Phytophthora palmivora* and no visible red accumulation of red betalain pigment was
28 observed. Furthermore, in all our colonisation experiments we use non-sterile sand substrate
29 and non-sterile plants. There are other microbes present at all times, yet the color
30 development is specific to the colonisation by AM fungi which have been supplied in
31 addition. This is exemplified in the Medicago *dmi3* mutants which carry the reporters, were
32 exposed to non-sterile substrate for weeks but did not show Betalain pigment accumulation
33 (see Fig. S13).

34 and investigate whether there is a quantitative relationship between colonization and amount
35 of pigment present.

36 **Response:** We never claimed that there is a quantitative relationship between colonisation
37 and the amount of pigment present. Our method and resource is not aimed at quantifying
38 colonisation based on the amount of pigment, it has been specifically established to report
39 the spatial distribution over time. We believe that additional experimentation to correlate
40 pigment amount with colonisation level is beyond the scope of this manuscript.

41 We would ask that you address reviewer #1's concerns about the usefulness of the resource
42 in the text, preferably describing how you see this being used as, indeed, it would seem to
43 be a difficult tool to use in a wide variety of plant genetic backgrounds.

44 **Response:** We apologize that reviewer 1 must have missed our previous lines clearly
45 describing the limitations of the system and that it requires transformation (first submission,
46 line 526). Please note that our comments on natural variation referred to the interaction
47 rather than a variety of plant genetic backgrounds only. Our understanding on natural
48 variation among AM fungal isolates and their root system colonisation dynamics is very
49 limited and this resource will enable such studies. We have now amended the text to make
50 these points more clear.

51 The reviewers would also like the work to include information on how you plan to handle
52 distribution of the resource you have created (seeds and DNA constructs). We suggest using
53 a resource such as the Arabidopsis Biological Resource Center
54 (https://abrc.osu.edu/researchers) or similar for distribution to improve the accessibility of
55 your resource to the scientific community.

56 **Response:** We have deposited all relevant plasmids at Addgene and have added a list of
57 these with their descriptions and Addgene identifiers in Supplementary Table 3. Seed
58 material for *N. benthamiana* MycoRed reporter lines (*NbPT5b-p3* and *NbBCP1b-p3*) are
59 stored at the Sainsbury Laboratory Cambridge University dedicated storage facility in
60 controlled environmental conditions following the International Day Room Standard of 15%
61 relative humidity and a temperature of 15˚C (https://www.slcu.cam.ac.uk/facilities/seedstore).
62 Seed material will be shipped to anyone requesting them. We plan to ship at least 15 seeds
63 per request. N. benthamiana has small dry seeds and we have already bulked-up sufficient
64 amounts for 50 requests. Phytosanitary certificates can be provided where needed. We have
65 now included this information on storage and accessibility of vectors and seeds to our
66 Material and Methods section.

67 We appreciate that these requests represent a great deal of extra work, and we are willing to
68 relax our standard revision time to allow you six months to revise your manuscript. We
69 expect to receive your revised manuscript within 6 months.

70

71 **Reviewer's Responses to Questions**

72 **Reviewer #1:**

73 This manuscript describes the development of betalains produced by transgenes under AM-
74 specific promoters. The biggest constraint is that the construct will have to be introduced into
75 every plant under examination. Whenever a different genetic background is needed, it must

76 be introduced. In most species, such an introduction is a tedious process either by
77 transformation or genetic crosses. So it doesn't easily lend itself to dissecting the genetic
78 mechanism. It only allows for analysis in the same genetic background. In this reviewer's
79 opinion, having to transform a large construct before the AM dynamics can be analyzed
80 hardly counts as "significant outperformance" or offers the "potential to address, for the first
81 time, a pressing biological question".

82 The authors are correct in stating that "The most powerful application of our betalain-based
83 AM reporter lies in its ability to non-invasively document colonisation in fully developed root
84 systems over time".

85 **Response:** Thank you for agreeing with our claim.

86 They listed a number of possibilities. However, they failed to use their system to actually
87 address any of these issues, and to demonstrate how they are pressing issues that cannot
88 be addressed otherwise.

89 **Response:** The guidelines of Plos Biology do not require a method or resource to be applied
90 to address a new biological question. As confirmed by the reviewer we listed a number of
91 possibilities. The most important one is the non-invasive tracking of the dynamic colonisation
92 of a root system over time depending on local or systemic changes in its environment. We
93 (and reviewer 2 - see their comment III) are not aware of another published method which
94 can achieve such tracking non-invasively. We would however appreciate the reviewers'
95 insight to be able to discuss this in our manuscript.

96 Their claim that "A second important application is in the survey of induced or natural genetic
97 variation that impacts on root system colonisation" is unconvincing. Not only one would have
98 to introduce this reporter system into every line of a natural variation collection,
99 transformation by itself will result in positional effect due to the randomness of transgene
100 insertion site, or in their own words "varying degrees of transgene expression that could

101 impact reporter intensity and functionality", which applies to variations between stably
102 transformed lines as well as between hairy roots.

103 **Response:** We understand the limitations pointed by the reviewer, and we already
104 acknowledge these in our statement on line 525 of our initial submission after the mentioned
105 claim: "*Here its use is only limited by the transformability of the species* [...]". We have
106 edited this sentence to be more specific about the type of genetic variation we refer to.
107 Natural variation in this context refers to variation in the interactions such as natural isolates
108 of different AM fungi which may have different colonisation differences within a root system.
109 These aspects can well be addressed with a single transgenic reporter line being a host to a
110 range of AM fungal species and strains.

111 My second concern is whether betalains are a robust reporter at all. Does it have enough
112 resolution? In Medicago truncatula, the resolution seems rather low. The images are much
113 more encouraging in N. benthamiana. It is hard to conclude which species is the outlier.

114 **Response:** Resolution is dictated by the imaging technology rather than the reporter. Root
115 architecture and thickness also significantly differs between Medicago and *N. benthamiana.*
116 We have not carried out high resolution imaging with Medicago roots. We have done this
117 with *N. benthamiana* roots, the main species of interest in this study. The system allows for
118 cell level resolution and labs working with specific plant systems will have optimised their
119 imaging setup.

120 Again, as the authors pointed out, the most promising application may be live imaging at the
121 whole root system level. It is unclear why more conventional reporters, such as luciferase,
122 would not suffice.

123 **Response:** To date there is no conventional published reporter to sequentially study AM
124 fungal colonisation extent within the same root system over time. Luciferase would require
125 the invasive sequential application of luciferin substrate at each imaging step as well as a

126 light-sensitive camera setup. Accessibility of luciferin to the inner root cortex cells colonised
127 by AM fungi would not be homogenous. Furthermore, this would also be a transgenic
128 reporter. I hope this reviewer agrees that a visual reporter which can be imaged using a
129 simple flatbed scanner and without the application of expensive substrate allows for a wider
130 range of research teams to utilise them easily.

131

132 **Reviewer #2:**

133 The manuscript by A. Timoneda et al. is a "method and resources" article that presents
134 MycoRed, a method to visualize the occurrence of arbuscular mycorrhizal symbiosis in roots,
135 in a non-destructive manner. The method is based on the expression of the betalain
136 biosynthesis genes under control of AM symbiosis-specific promoters.

137 The authors tested the systems in two dicots, the legume Medicago truncatula and Nicotiana
138 benthamiana. They isolated AM symbiosis-specific promoters in N. benthamiana. They
139 identified and tested constructs that are not expressed when the plants are not inoculated,
140 which is essential for further use of the system (low false-positive rate). Lastly, they used a
141 rhizotron system to show the potential for real-time monitoring of AM symbiosis.

142 Symbiotic interactions are attracting more and more attention due to their potential as
143 biofertilizer. This makes the development of such a tool particularly relevant and timely. As
144 an expert in AM symbiosis research, I can foresee the potential for forward-genetic screens,
145 which have been almost ignored due to technical difficulties, or to decipher cross interactions
146 with biotic and abiotic factors. It is a much needed system and shows a great potential.

147 **Response:** Thank you for this very positive view on the potential of our resource.

148 I found the manuscript very clearly written and the reported experiments well conducted.

149 As this article is submitted as a resource to be widely used by the community, I have,
150 however, a few additional requests and suggestions.

151 Major requests:

152 1. Confirm that the symbiosis is functional. Although the colonization of the transgenic
153 lines, and the increased colonization rate observed over time, suggests that the
154 association is "normal", it would be important to demonstrate it. As a proxy the
155 authors should provide qRT-PCR data for the expression of marker genes such as
156 RAM2 or STR/STR2 in the engineered lines following colonization by R. irregularis.

157 **Response:** As requested we have performed qRT-PCR for the MtSTR, MtRAM2 marker
158 genes on *MtPT4*-p1 *Medicago truncatula* hairy roots and shown their expression is
159 upregulated in roots which are colonised and have formed red betalain pigment (see revised
160 Fig. S1). This supports the reviewers point that symbiosis is functional in the presence of
161 betalains..

162 2. Demonstrate the specificity. The authors tested the lines in artificial substrate and in
163 vitro. The presented data are very convincing, however I am wondering whether the
164 lines would behave similarly in more natural, microbe-rich, soils. The authors should
165 test for the potential activation of betalain production in the "elite" line (NbPT5b-p3) in
166 presence of microbes other than AM fungi. Including a pathogen that infect the roots
167 would be particularly relevant.

168 **Response:** We have carried out our AM colonisation experiments in non-sterile substrates
169 supplemented with AM fungal spores. Figure S13 shows that in Medicago *dmi3* mutants
170 which have been cultivated in non-sterile substrate for weeks, no betalain activation is
171 observed, this suggests that the reporters cannot be activated by other substrate microbiota
172 present. We have now carried out additional experimentation to address this aspect in *N.*
173 *benthamiana* under controlled infection with a root pathogen. We have carried out root

174 infections of the *NbPT5b*-p3 and *NbBCP1b*-p3 lines with *Phytophthora palmivora*. No visible
175 red accumulation of red betalain pigment was observed (see new Fig. S11). Furthermore,
176 RNAseq data obtained previously show no evidence for expression of *NbPT5b* -
177 Niben101Scf02726g00004.1 and *NbBCP1b* - Niben101Scf07438g04015 in published *P.*
178 *palmivora N. benthamiana* root infection (Evangelisti *et al.*, 2017;
179 https://doi.org/10.1186/s12915-017-0379-1, see supplementary material, both identifiers are
180 absent from additional file 5 where all differentially expressed genes during a 72h infection
181 time course have been reported). This supports our finding that the *N. benthamiana*
182 promoters utilised in these lines are responsive to AM fungal colonisation in a similar fashion
183 to the more widely studied *Medicago truncatula* homologs *MtBCP1* and *MtPT4*.

184 Suggestions:

185 1. The authors could provide a list (with reference numbers) for the L0 and L2 used for
186 the study as well as a clear statement on the procedure to obtain these DNA
187 modules, and .gb files of the vectors.

188 **Response:** We have deposited the L0 and L2 plasmids used in this manuscript in Addgene.
189 A complete list of these and their Addgene reference numbers can now be found in new
190 Supplementary Table 3.

191 2. The authors could explain the procedure to obtain seeds of the stable N.
192 benthamiana NbPT5b-p3, including the amount that can be possibly provided. Again,
193 this is a resource and distribution is an important aspect.

194 **Response:** *N. benthamiana* produces a significant amount of small dry seeds which can be
195 easily harvested, stored and distributed. The authors can provide sufficient seeds to start
196 their own plants to everyone requesting them (including phytosanitary certificates for
197 shipments outside the UK where needed) and researchers can then grow their own stable

198 transgenic plants to harvest plenty more seeds. We have now included information on
199 storage and accessibility of seeds to our Material and Methods section.

200 In addition the possibility to store seeds in the JIC Germplasm resource exists as well
201 (https://www.jic.ac.uk/research-impact/germplasm-resource-unit/). We now state clearly in
202 the manuscript that seeds are available.

203 3. I was particularly impressed by the rhizotron experiment, because following AM
204 symbiosis in real-time is a challenge that has never been solved. I was wondering
205 whether the authors could use the images to quantify a proxy for the level of
206 colonization, on the color-extracted files (red / white for instance?). This is obviously
207 not a requirement, but having a way to quantify colonization with a simple scan, on
208 living material, would be amazing.

209 **Response:** Thank you for your positive words highlighting that our rhizotron experiment may
210 allow us to solve the challenge following AM symbiosis in real-time. We agree with the
211 reviewer developing a fast and easy protocol for color quantification as proxy for colonisation
212 would be very useful. Efforts are already being conducted to develop appropriate imaging for
213 this, but we unfortunately consider they fall outside the scope and time frame of this
214 manuscript.

215 I am not an expert on the biosynthesis of Betalain and I did not evaluate that particular
216 aspect of the manuscript.

217

218 **Reviewer #3:**

219 The manuscript by Timoneda and colleagues offers a nice technical contribution to the field
220 of AM symbiosis. AM fungi develop inside the root tissues and there are only a few (and
221 rare) morphological features that allow identifying the mycorrhizal root segments with the

222 naked eye. For this reason, the development of transgenic plants which allow an easy
223 detection of the arbuscules is surely a useful contribution.

224 The research has been carefully performed with clear experiments and correct controls.

225 **Response:** Thank you for highlighting the usefulness of our contribution and your
226 satisfaction with the way we carried out experiments.

227 There are however some points which deserve attention, and some missing information
228 should be added: all these issues have been listed following the text.

229 **Response:** We have commented on your individual points below.

230 In addition, the text sounds as a bit redundant since the same experiments are described for
231 Medicago and then for Nicotiana, even if the results and the conclusions are very similar. I
232 fully understand that the stable transformation of Nicotiana was done in a second moment,
233 but probably a shortening of the text could lead to a more attractive reading.

234 **Response:** Not the same, but similar experiments using different genetic resources as well
235 as new promoters are described for Medicago and Nicotiana. We consider it important that
236 the details of the experiments are laid out so that they are reproducible.

237 Title: is it clear that the visualisation of betalain pigments requires the introduction of reporter
238 genes in model plants? In my opinion, the authors should state that a genetic transformation
239 is at the basis of the experiment.

240 **Response:** We have renamed the title to '*Betalain pigments as* in vivo *real-time visual*
241 *markers for arbuscular mycorrhizal colonisation of transgenic roots systems*'.

242 Summary line 20: probably the word fungi after arbuscular mycorrhiza is missing. AM are not
243 fungi!

244 **Response**: Yes indeed. Thank you for spotting this. We have corrected it in the manuscript.

245 Introduction

246 Line 48. Glomeromycota. Please note that one of the most accepted taxonomy proposes AM
247 fungi as Glomeromycotina, a subphylum (Spatafora et al 2016). Alternatively, see the
248 taxonomic view as proposed by Leho Tedersoo Line 50 ".... can be formed by 70-90% of
249 extant land plant species.". Please note that the references are not the most updated. It is
250 assumed that only 70-72% of land plants are colonized by AM fungi (see Brundett and
251 Tedersoo, 2018; Genre et al 2020); 90% is the percentage of plants which are associated to
252 mycorrhizal fungi.

253 **Response**: Thank you for clarifying this, we have revised the text.

254 Line 57-60 Please note that also the other intracellular hyphae of AM fungi (those originated
255 from the hyphopodia, the coils ..) are always surrounded by a host membrane. This new
256 membrane is not exclusive of the arbuscules and is more correctly defined as the perifungal
257 membrane.

258 **Response**: We have edited this introduction section and have removed the wording
259 periarbuscular membrane.

260 78: "complex microscopy" not clear

261 **Response**: We have changed this to “light or fluorescence microscopy”

262 85-86 probably the yellow is not exclusive of cereals. Also Liliaceae have yellow mycorrhizal
263 roots.

264 **Response**: Thank you for clarifying this, we have revised and updated the text.

265 96: Is there information on the natural expression of betalains in early diverging fungi?

266 **Response**: As mentioned in our manuscript (line 94-96), Betalains have only been found in
267 the basidiomycetes Amanita and Hygrocybe. We are not aware of any other report or
268 dataset on fungal betalain expression.

269 143--144 ".... Heterologous expression of betalain biosynthesis genes specifically driven by
270 AM-responsive promoters effectively tracked AM colonisation dynamics in both species.."
271 Perfect rationale, however, I would have appreciated to see the use of at least one gene also
272 related to the first steps of colonization (i.e. signalling, transcription factors). These genes
273 could give us more relevant information than the genes which are the markers of the
274 established colonization

275 **Response**: For us the most relevant information on symbiosis is the expression of the
276 phosphate transporter reporting the transfer of nutrients from fungus to plant. We agree that
277 different research groups may have different preferences as to which aspect of colonisation
278 they consider most relevant and there can be many different promoter-reporter fusions
279 tested and generated but our method and resource focused on what in our opinion are two
280 of the most relevant ones.

281 Line 163: Can the authors provide some further information on the UBQ10 promoter? And
282 provide some rational for the use of the constitutive promoters, which indeed have not been
283 the better choice.

284 **Response:** We have added a sentence further explaining the rationale behind this, i.e. to
285 avoid transcriptional silencing due to repeated elements. The Ubi10 promoter was further
286 described in the Methods section of our original submission, and we have now added its
287 length in bp.

288 Fig. 2 it seems that the betalain red is mostly associated to the inner root segments and not
289 to the thinnest root branches (lateral roots). Have the authors done some statistical
290 evaluation of the staining distribution looking at the morphology of the root system?

291 **Response:** We established this reporter system to do exactly this in future research which is
292 beyond the frame of this manuscript. At present we do not have sufficient data to conclude
293 and correlation of root morphology and staining.

294 Fig. 3 The red seems to be more abundant in the endodermis cells, where surely PT4 is not
295 expressed. The authors write that red betacyanin distribution extends to cells beyond those
296 with promoter activity ...Can betacyanin move across membranes, given that it is water-
297 soluble?

298 **Response:** Betalains are produced in the cytoplasm and stored in the vacuoles in native
299 producing species. There is currently no information, however, on the transport mechanisms
300 of betalain pigments. As small water-soluble compounds, betalains have the potential to
301 move symplastically through root plasmodesmata, but this has not been proven.
302 Nevertheless, expression in *N. benthamiana* roots seemed to be much more defined to
303 single cells. It is also possible that these differences could be generated by slightly different
304 sectioning methodologies. These points were covered in the discussion section of our
305 original submission. We have now added a sentence acknowledging the possibility of
306 betalain symplastic movement in the root.

307 172: can the betalain expression change the transcriptomic profile of arbuscule containing
308 cells?

309 **Response:** We saw the formation of normal arbuscules. We have performed further
310 experimentation to analyse expression levels of MtSTR and MtRAM2 and found they are
311 normally induced in colonised Medicago lines producing red pigment. We have not tested
312 whether other specific genes are altered in their expression as this would require a large
313 scale experiment beyond the scope of this manuscript. However, we can conclude that any
314 such potential changes did not impact on the formation of this symbiosis.

315 204-206. Medicago is a model plant for many labs. It is not clear why the authors moved to
316 Nicotiana instead of producing stable Medicago transformants.

317 **Response:** Agreed, this could be done in several plants. We have opted for *N. benthamiana*
318 as it is a model plant for many labs including in mycorrhiza symbiosis (doi:
319 10.1111/tpj.13908, Hause & Fester 2004 https://www.jstor.org/stable/23388865) and we are
320 particularly interested in it. Furthermore it takes ~1.5 years to get stable homozygous
321 Medicago. By contrast *N. benthamiana* takes 3-6 months to generate such a resource. In
322 addition, *N. benthamiana* is more compatible with our rhizotron setup. Medicago roots are
323 too thick and the root system is too big.

324 Fig. 4 Expression of NbPT5b and NbBCP1b increased after two weeks and showed
325 significantly elevated transcript levels 3-4 weeks post inoculation (Fig 4a). In my
326 opinion, this Fig 4 a does not show an original or unexpected result. I would suggest moving
327 the Figure to the supplementary materials. Indeed the GUS constructs reveal a strong
328 diffusion of the staining…

329 **Response:** We respectfully disagree. While the result might not be unexpected for the
330 reviewer, the two *N. benthamiana* promoters have not been characterised previously, and
331 solely concluding from sequence similarity of their downstream ORFs that they respond in a
332 similar fashion to the better characterised Medicago promoters is not appropriate. We
333 provide original and new data showing that two previously uncharacterised genes respond
334 transcriptionally to AM fungal colonisation.

335 The two following paragraphs present many repetitions. Betalains can be used to visualise
336 AM fungus colonisation in living Medicago truncatula roots vs Betalains visualise AM fungus
337 colonisation in living Nicotiana benthamiana roots The authors should go in a more direct
338 way to the solution, shortening the results obtained with the first constructs. It is clear that
339 the constitutive promoters give problems...)

340 **Response:** We disagree that it is clear that the constitutive promoters give problems. Other
341 published stable transgenic lines constitutively expressing betalain biosynthesis genes have
342 not had reports of problems associated with them (Polturak et al., 2016, DOI:
343 10.1111/nph.13796; Polturak et al., 2017,https://doi.org/10.1073/pnas.1707176114).
344 Therefore we consider it essential to report our observations.

345 Fig. 5 a "Schematic of the multi-gene vectors constructed for inducible betalain expression in
346 N. benthamiana roots where only the first gene of the betalain biosynthesis pathway is
347 controlled by AM symbiosis specific promoters". The legend is a bit confusing: the reader
348 looks for differences between fig 5 vs. fig 2. But indeed the only difference is that promoters
349 of CYP76AD1 are from Nicotiana and not from Medicago.

350 **Response:** Given the number of similar constructs used during this manuscript, we actively
351 decided to include a schematic of each of them in their respective figures. We believe this
352 will facilitate the reading process and a more effective acquisition of information. We regret
353 that the reviewer does not agree with our style choice.

354 Fig 5 (e g) seems to be quite poor in quality.

355 **Response:** We consider the quality of the image sufficiently and effectively fulfills the
356 objective of the figure which is to show fungal structure presence in those root regions.
357 Internal hyphae, arbuscules and vesicles are clearly observable both in Fig 5e and 5g.

358 Table 1 the authors used ink staining to visualise the colonisation. But the method is not
359 reported in the text (line 270) as well as the quantification methodology. Lastly, from which
360 transformed plants were the data obtained?

361 **Response:** Data was obtained from N. benthamiana plants expressing NbPT5b-p1 and
362 NbBCP1-p1 4 weeks post-inoculation with *R. irregularis* as stated in the text and the legend
363 of Table 1 in lines 269 and 288 of our initial submission respectively. Methodology is also

364 summarised in line 269 of our initial submission and described in more detail in the“*Staining*

365 *and quantification of fungal structures*” section of Material and Methods.

366 Paragraph "Stable expression of NbPT5b-p1 and NbBCP1b-p1 can cause shoot

367 developmental defects in N. benthamiana". In my opinion this entire paragraph should be

368 summarised, the details could be moved to the supplementary materials, since it is not so

369 strictly relate to the main focus. On the other hand, it seems that the promoter PT5b is active

370 in leaves. This could be an interesting result, even if not strictly related to this research. Is

371 the transporter active in the leaves from Myc and not myc WT plants? It is known that PT is

372 expressed also in root tips, irrespectively of the mycorrhizal colonization....

373 **Response:** All details on the developmental aspect are already exclusively described in

374 supplementary figures. In an RNAseq experiment with N. benthamiana leaves exposed to

375 control or pathogen infection (Carella et al, 2019

376 https://www.sciencedirect.com/science/article/pii/S096098221930692X, we did not obtain

377 any reads for *NbPT5b* [Niben101Scf02726g00004.1] suggesting that this gene is not

378 detectably expressed in leaves. We have discussed that Our *NbPT5b*-p1 construct

379 transformation selection process could have been biased towards T0 plants with a degree of

380 escaped *CYP76AD1* expression, which would explain the presence of betanin in a number

381 of T1 plants descending from *CYP76AD1* shoot-expressing lines (Fig. S6, S8). Further

382 experimentation is required to support this hypothesis, but in any case developmental

383 defects and vegetative betanin expression can be avoided when all three biosynthesis

384 enzymes are driven by AM symbiosis specific promoters.

385 Fig. 6 b-e do not add any relevant information.... it could be fused with Fig. 7. Fig. 8 is much

386 more interesting.

387 **Response:** Figures 6 b-e add relevant information of the tissue and cell level distribution of

388 pigment in colonised tissues. We consider this essential information in this manuscript.

389 In this context, it is not clear why the time course with the PT promoter is not shown, since it

390 works better than the BCP one.

391 **Response:** Please note that in our original submission as well as in this version, both

392 *NbBCP1b* and *NbPT5b* time course images were shown (see Fig. 8 for *NbBCP1b* and Fig.

393 S15 for *NbPT5b*).

394 Line 348-350 - Vegetative? Not clear...do they mean shoot and leaves? Also roots are

395 vegetative organs. And at line 350 "… vegetative betalain production.."

396 **Response:** Thank you for clarifying this, we have revised the text.

397 364 what is the meaning? Why do they come back to medicago? But.... does not make

398 infection does not express PT4...since all the signalling is blocked and as a consequence the

399 following processes…

400 **Response:** We have edited the manuscript for a more clear interpretation. *dmi3* plants were

401 included in this transformation round as further negative controls, where we wouldn't expect

402 any color production regardless of presence or absence of mycorrhizal inoculum. This also

403 serves as proof that any other microbes present in our non-sterile substrate and which do

404 not rely on the symbiosis signalling genes activate the reporter, a question which has been

405 raised by other reviewers.

406 The last part of the discussion (from 451 line) should be deleted since the point has already

407 been largely introduced and discussed in the Results

408 **Response:** We prefer to retain this short summary at the end of the manuscript, but are

409 happy to remove it upon request by the editor.

410 Line 445: " A future solution to document total AM colonisation could involve the

411 Establishment of systems whereby the betalain biosynthesis genes are activated by

412 transactivators, which could be then driven by promoters that are active at early, main and

413 late AM fungal colonisation stages (44-46). I apologize, but for me the sentence is not clear.

414 Transactivators?

415 **Response:** We wanted to highlight the possibility that establishing a betalain reporter line

416 under control of an artificial promoter once will then allow crossing to many other lines which

417 carry the transactivating transcription factor gene under control of one or multiple context

418 specific promoters (e.g. promoter active at early and late stages of colonisation.

419 In conclusion, the authors should better show that the p3 constructs do not change plant and

420 mycorrhizal phenotype, at least showing that the colonization percentage does not change

421 between WT and transformants.

422 **Response:** We concur with the reviewer and have performed a new set of experiments to

423 prove that the p3 constructs do not change colonization between WT and transformants.

424 Specifically, we have colonised WT, pNbPT5b-p3 and pNbBCP1b-p3 transgenic *N.*

425 *benthamiana* plants with *R. irregularis* over several weeks and then have stained and

426 manually counted colonisation structures. No significant differences in root system

427 colonisation as well as in the ratio of arbuscules in colonised sections were observed.

428

429 **Reviewer #4:**

430 The manuscript by Timoneda and coworkers presents the results of using Betalain

431 biosynthetic genes to demonstrate effectiveness of the natural pigments as in vivo visual

432 markers for arbuscular mycorrhizal colonization of both Medicago and tobacco root systems.

433 They used AM-inducible promoters and assembled multi-gene reporter constructs and

434 demonstrated that the innovative method MycoRed could allow for the non-invasive tracing

435 of fungal colonization over time. The presented results fully support the conclusions and the

436 study looks overall very convincing and solid. This research is of broad interest to the

437 audience of plant biologists and timely places among an increasing interest and importance
438 of investigating AM.

439 **Response:** We thank the reviewer for their positive insight of our work.

440 The text reads fluently and the state of the art, results and conclusions are very clear.
441 However, I have the following a few critics to raise.

442 Major points:

443 1. In this study, promoters of late AMS marker genes (MtPT4, MtBCP1, and tobacco
444 homologs) were used to drive expression of betalain biosynthetic genes. The early
445 infection and AM colonization is an essential part of AMS research, I would suggest
446 testing the early AMS marker genes such as AM1 and AM3 and assay if the red
447 pigmentation can be used to trace the infection at early stage of mycorrhizal
448 infection, i.e., before 14 dpi.

449 **Response:** We agree with the reviewer that testing early AMS marker genes would be of
450 great interest, but we unfortunately consider this falls out of the scope of our manuscript,
451 which has been designed as a proof of concept of betalains as markers for colonisation. We
452 chose two promoters that have been extensively studied and used in previous AMS research
453 and which represent hyphal colonisation as well as arbuscule stages.

454 2. Stable transgenic tobacco plants were used to trace fungal colonization. A
455 quantitative study could be performed to compare betalain pigmentation (by imaging
456 or by HPLC) and fungal biomass accumulation (by qRT-PCR of R. irregularis
457 housekeeping genes) and test if linear correlations exist.

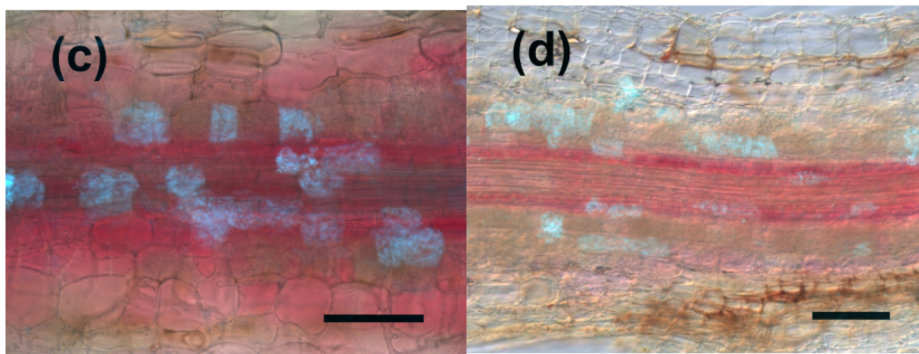
458 **Response:** We also agree with the reviewer that a correlation of pigment intensity and
459 colonisation levels could be of great use and we would really like to see this developed in the
460 future. But we respectfully disagree with the experiment suggested, the purpose of this

461 paper was to show spatial correlation and we do not consider we need to perform such
462 experimentation to support our conclusions.

463 Minors:

464 Figure 3: Panels c and d, overlay images of pigments (red) and WGA-FITC staining (blue)
465 should be provided.

466 **Response:** We provide overlays for the reviewers, however we do not think that overlay
467 images of these panels communicate the data in a more clear way. Therefore we prefer not
468 to include it in the manuscript unless requested by the editor.



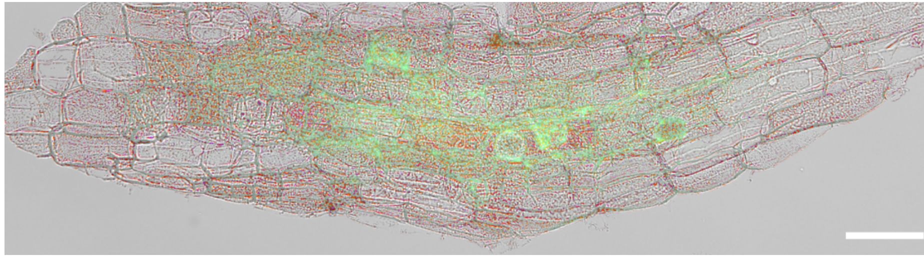
469

470

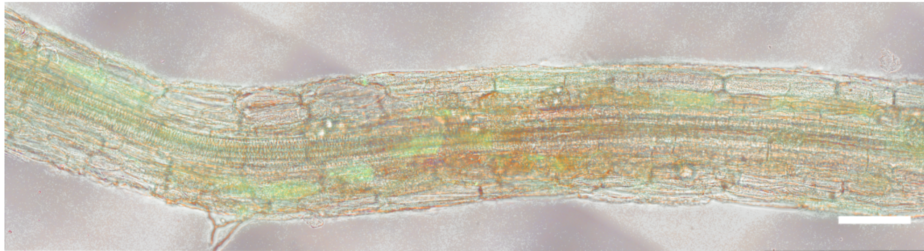
471 Figure 5: Overlay images of panels d&e, f&g are recommended.

472 **Response:** We here provide overlays as suggested by the reviewer. We believe this
473 visualisation does not make the data clearer (especially for color vision impaired readers).
474 Therefore we prefer not to include it in the manuscript unless requested by the editor.

475 Merged d&e) - *NbPt5b*-p1

476 

477 Merged f&g) - *NbBCP1b*-p1

478 

479

480 Figure 8: All dashed squares should be delimited in panel b.

481 **Response:** We initially designed the figure to include dashed squares in all panels, but we
482 realised the repetitiveness of the squares were very obstructing for the proper visualisation
483 of the figure. We hope that by indicating in the legend that this is "*is a magnification of the*
484 *area delimited by the dashed square over time*" we make it more clear to the reader.

485 Figure S9: Panel j, what are the two types of arrows?

486 **Response:** Thank you for spotting this. We have corrected it in the manuscript.

487 Figure S11: Scale bars are missing.

488 **Response:** Thank you for spotting this. We have corrected it in the manuscript.

489 Table 1: 4.8±13? 3.0±8? 1.1±3?

490 Table S1: 8.8±17? 2.4±7?

491 **Response:** The error values shown in Table 1 and Table S1 represented the standard
492 deviation of the data. We have now changed this to represent the standard error. We hope
493 with this we align with the reviewer's request.

494 Line 235: Definition of NbEF is needed.

495 **Response:** Thank you for clarifying this, we have revised and edited the text.

496 Line 263: d,f should be d,e

497 **Response:** Thank you for spotting this. We have corrected it in the manuscript.

498 Line 266: d-d should be d-g.

499 **Response:** Thank you for spotting this. We have corrected it in the manuscript.

500 Line 399: (a, b) should be (a, c)

501 **Response:** Thank you for spotting this. We have corrected it in the manuscript.

502 Lines 571-574: space is need between digits and units, i.e., 40 mM instead of 40mM.

503 **Response:** Thank you for clarifying this, we have revised the text.

504 Line 591&592: NbEF and RiEF are inaccurate. Gene IDs or RefSeq should be provided.

505 **Response:** Reference sequence identifiers for NbEF: Niben101Scf04639g06007.1, RiEF:
506 XM_025321412.1, and RiBtub: XM_025314309.1 are now included the manuscript.