Automatic identification and annotation of MYB gene family members in plants

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Abstract

Background: MYBs are among the largest transcription factor families in plants. Consequently, members of this family are involved in a plethora of processes including development and specialized metabolism. The MYB families of many plant species were investigated in the last two decades since the first investigation looked at *Arabidopsis thaliana*. This body of knowledge and characterized sequences provide the basis for the identification, classification, and functional annotation of candidate sequences in new genome and transcriptome assemblies.

Results: A pipeline for the automatic identification and functional annotation of MYBs in a given sequence data set was implemented in Python. MYB candidates are identified, screened for the presence of a MYB domain and other motifs, and finally placed in a phylogenetic context with well characterized sequences. In addition to technical benchmarking based on existing annotation, the transcriptome assembly of *Croton tiglium* and the annotated genome sequence of *Castanea crenata* were screened for MYBs. Results of both analyses are presented in this study to illustrate the potential of this application. The analysis of one species takes only a few minutes depending on the number of predicted sequences and the size of the MYB gene family. This pipeline, the required bait sequences, and reference sequences for a classification are freely available on github: https://github.com/bpucker/MYB_annotator.

Conclusions: This automatic annotation of the MYB gene family in novel assemblies makes genome-wide investigations consistent and paves the way for comparative studies in the future. Candidate genes for in-depth analyses are presented based on their orthology to previously characterized sequences which allows the functional annotation of the newly identified MYBs with high confidence. The identification of orthologs can also be harnessed to detect duplication and deletion events.

Keywords: MYBs, Croton tiglium, *Castanea crenata*, genome-wide, R2R3 MYBs, orthology, phylogeny

Introduction

MYB transcription factors are named after an Avian myeloblastosis virus protein (v-Myb) which is a modified version of the cellular c-Myb and causes the activation of oncogenes [1]. While MYBs were first discovered in animals, they appear in substantially larger numbers in plants and form one of the largest transcription factor families [2–6]. A characteristic MYB feature is the presence of a conserved DNA-binding domain at the N-terminus [7]. Up to four imperfect amino acid repeats (50-53 amino acids) form three alpha-helices each [7]. Helix two and three of each repeat are arranged to a helix-turn-helix structure [8]. Three regularly spaced tryptophan or other hydrophobic amino acid residues form the core of this structure [8]. The third alpha helix is responsible for the direct DNA interaction [9]. The repeats are classified into R1, R2, and R3 based on similarity to the respective repeats of the first characterized MYB, c-Myb [1]. MYB proteins are classified based on the presence of these repeats. For example, R2R3-MYBs harbor the R2 and R3 repeat while 3R-MYBs have one copy of each of the repeats (R1R2R3). Further classification into subgroups can be achieved based on the phylogenetic relationships and characteristic sequence motifs in the C-terminal region [2, 5, 10]. Different MYB classification systems were proposed in previous studies [2, 5, 10].

R1R2R3-MYBs have been proposed to be regulators of the cell cycle with conserved functions between animals and plants [11, 12]. R2R3-MYBs account for the large MYB family size in plants [2]. The evolutionary origin of R2R3-MYBs and 3R-MYBs is still debated. The loss model proposes that R2R3-MYBs diverged from 3R-MYBs through loss of the R1 repeat [13–15], while the gain model proposes that the 3R-MYBs evolved from the R2R3-MYB through duplication of a repeat [10, 16]. R2R3-MYBs are involved in the regulation of numerous processes including the regulation of developmental processes, response to environmental stresses, and specialized metabolism [17–19]. WEREWOLF/MYB66 is a negative regulator of the root hair formation that determines the pattern of root hairs on the root epidermis of *Arabidopsis thaliana* [17]. An investigation of this MYB based on its crystal structure revealed the DNA binding site AACNGC and also suggests that this MYB is able to differentiate between DNA methylation states [20]. DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION/MYB35 determines the sex of *Asparagus officinalis*, but not in all other species of this genus [21]. The *A. thaliana* PFG1/MYB12 and the paralogs PFG2/MYB11 and PFG3/MYB111 are responsible for the regulation of the flavonol biosynthesis in most tissues [18]. This process appears highly conserved as orthologs of the AtMYB11/AtMYB12/AtMYB111 clade in *Beta vulgaris* [22] and *Medicago truncatula* [19] are also regulators of the flavonol biosynthesis. MYB21 and MYB24 were identified as additional flavonol regulators in the stamen of *A. thaliana* [23]. Some processes like the regulation of the flavonol biosynthesis depend only on MYB regulators [18]. Other specialized metabolite biosynthesis pathways are regulated by the interaction of multiple proteins. The MBW complex, named after the three components MYB, bHLH, and WD40, is one of the best studied transcriptional regulation systems [24–27]. Two branches of the flavonoid biosynthesis, the anthocyanin and proanthocyanidin biosynthesis, are controlled by the MBW complex [24, 25, 28]. Since anthocyanins are responsible for the pigmentation of flowers and other plant structures, mutants in their regulation can be identified based on a visible phenotype. Proanthocyanidins are responsible for the coloration of seed coats thus mutants in their biosynthesis can be identified based on a yellow seed color. Mutations in the regulating MYBs

and other transcription factors [29–32] are often the reason for loss of anthocyanins and/or proanthocyanins. Transcriptional regulation was studied based on these pathways due to their visually detectable phenotypes.

Since MYBs are controlling many processes in plants, there is also a substantial interest to understand their functions in crop species. In Brassicaceae, the glucosinolate content controlled by several MYBs is an economically relevant trait [33]. ATR1/MYB34, HIG1/MYB51, and MYB122 increase the indolic glucosinolates and HAG1/MYB28, PMG2/RAO7/MYB29, and MYB76 the aliphatic glucosinolates in *Arabidopsis thaliana* [34–36]. The red coloration of sugar beets is controlled by BvMYB1 which activates two betalain biosynthesis genes [37]. *AmMYB1* in amaranth was identified as best candidate gene to explain the seed coloration variation between accessions [38]. A MYB appears to be the underlying factor of post-harvest hardening that renders a specific yam accession inedible within a day [39, 40]. MYB duplications between different apple cultivars appear responsible for differences in the red fruit flesh coloration [41]. Consumers prefer apricots with a red blush which is controlled by an anthocyanin biosynthesis activating MYB [42]. The identification of MYB candidate genes and the regulated processes is the first step towards modification through SMART breeding or genome editing [43, 44]. This interest in MYBs sparked numerous genome-wide investigations in species with a new genome or transcriptome assembly [3, 4, 22, 31, 45–48]. The identification of MYBs is repeatedly performed on many different data sets with strong variation in the quality of the analyses. Well described *A. thaliana* MYB sequences [2] are often used as baits to find new MYBs based on sequence similarity. Like all routine tasks with clearly defined steps, the identification of MYBs is a promising target for an automatic approach. We previously developed an automatic workflow, called KIPEs, for the annotation of core flavonoid biosynthesis genes which could also be expanded to the annotation of transcription factor gene families [4]. However, KIPEs is optimized for the identification and assessment of enzymes based on conserved amino acids in the active center. One underlying assumption is a small number of gene copies per species, which is violated by the very large MYB gene family. Also it is technically possible to run KIPEs for the identification of a gene family, the performance decreases with gene family size. Many previous studies relied only on BLAST or added additional filters for the presence of conserved R2R3-MYB domains in candidate sequences [4, 22, 49]. The inspection of MYB domains is laborious when performed manually, but suitable to define a set of fully functional R2R3-MYBs. While specificity of this filtering approach is high, it suffers from a low sensitivity i.e. neglects degenerated copies which might have experienced neofunctionalization. There are other solutions to identify orthologous sequences in a large number of species independent of the presence of specific sequence patterns [50, 51], but these approaches would require a substantial amount of manual cleaning to narrow down a final set of MYB sequences. Particular challenges are analyses based on transcriptome assemblies, because transcriptome assemblies show often a large number of isoforms resulting from alternative splicing or artifacts [52, 53].

Here, we present a Python-based pipeline to provide a high quality annotation of all MYBs that needs a set of peptide or coding sequences as input. Additionally, MYB candidates are checked for conserved domains and assigned to orthologs in other plant species. Genome sequencing and the construction is becoming a routine task and the generation of high quality structural annotations is also advancing

quickly with the aid of massive RNA-Seq data sets and full length transcript sequencing. Therefore, a huge number of data sets will be available to study MYBs in an unprecedented number of different plant species. Our automatic identification of MYBs in a large number of species facilitates pan-MYB analyses to better understand the evolution of the MYBome and to transfer functional insights acquired in one species effectively to orthologs in other species.

Implementation

MYB data sets

The identification of MYBs in novel genome or transcriptome sequences requires broad phylogenetic coverage of bait sequences. The MYB domain sequences of *Arabidopsis thaliana* [2], *Vitis vinifera* [3], *Beta vulgaris* [22], *Musa acuminata* [4], *Vitis vinifera* [3], *Medicago truncatula*, *Populus trichocarpa*, *Citrus sinensis*, *Solanum lycopersicum*, *Solanum tuberosum*, *Aquilegia coerulea*, *Oryza sativa*, *Zea mays* [47], *Amborella trichopoda*, *Picea abies*, *Selaginella moellendorfii*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, *Volvox carteri*, *Micromonas pusilla*, *Ostreococcus lucimarinus*, and *Cyanidioschyzon merolae* [10] were merged to generate a bait sequence collection. Closely related non-MYB sequences including CDC5 were identified by running a BLASTp search with the bait MYB sequences against the Araport11 peptide sequences [54]. Hits with a minimum BLAST score of 100 were collected and stripped of any *bona fide* MYBs. This step allowed the identification of MYB-like sequences, but excludes spurious hits that would slow down the following analysis steps. While the previously described MYBs form a collection of 1889 ingroup sequences, these 26 non-MYB sequences represent the outgroup sequences for down-stream analysis.

Pipeline

The automatic annotation pipeline is summarized in **Fig. 1**. Required inputs are (1) the MYB bait sequences (described above), (2) a classification of the MYB bait sequences into ingroup and outgroup, and (3) a set of coding sequences or peptide sequences that will be analyzed. Step 0: All parameters, tool versions, and the input files are logged in a report file for reproducibility. If the Python module hashlib is available, md5sums are calculated for all input files to ensure an accurate documentation. However, calculation of this checksum is optional and file names (including their paths) will be documented in any case. Cleaning of the input sequences removes any characters from the sequence names that would interfere with the phylogenetic analysis. Step 1: Initial candidates are identified based on local sequence similarity. Default parameters accept BLASTp hits with 50 amino acid length, 80% alignment similarity, and a maximum of 100 hits per bait sequence. This is a very sensitive setting given the large number of bait sequences and an expected MYB gene family size below 300 in most species. If a collection of coding sequences is provided, these will be translated and then compared based on BLASTp to harness the stronger conservation of amino acid sequences compared to nucleotide sequences. Step 3: A phylogenetic tree is constructed with these initial candidates and all bait sequences. Alignments are constructed with MAFFT [55]. Tree construction via RAxML [56] and

FastTree2 [57] is supported. FastTree2 (-wag -nopr -nosupport) is recommended due to substantially higher speed when large data sets are analyzed. Step 3: Calculation of distances between different leaves of the tree is used to identify ortholog relationships between bait and candidate sequences. The Python package dendropy [58] is applied using the patristic distance method and counting edges. To exclude outliers caused by fragmented sequences or annotation artifacts, candidates are excluded if the distance to the next bait sequences exceeds three times the average distance of nearest neighbours. This cutoff was optimized by manually inspecting distributions of this value in context with the corresponding phylogenetic trees, but it is possible to modify this value as well as most other parameters. The bait sequences with the shortest distance are identified for each selected candidate in the tree. If most of these bait sequences are ingroup MYBs, the candidate is classified as MYB (Additional file 1). If most of these bait sequences are outgroup MYBs, the candidate is classified as a MYB-like sequence (aka non-MYB). All sequences passing this filter are considered clean candidates. Step 4: A check for the presence of MYB repeats is performed based on regular expressions (see documentation for details) derived from previously reported alignments [10, 59]. A repeat-based MYB classification is widely used and also supported here. However, it is important to note that these groups do not represent monophyletic groups. Step 5: A new phylogenetic tree is constructed with the clean MYB candidates and all bait sequences. Step 6: An optional step assigns all newly discovered MYBs to a group of reference MYBs e.g. the well characterized *A. thaliana* MYBs. Based on the assumptions that orthologs are likely to have the same functions, this generates hypotheses about the function of the newly discovered MYBs. Additionally, it is possible to identify the expansion and contraction of specific MYB lineages compared to this reference. Step 7: It is possible to collapse large groups of very similar sequences in the analyzed data set and to represent these by only the longest sequence. This option is intended for transcriptome assemblies which can include large numbers of isoforms caused by alternative splicing and artifacts. Step 8: A new phylogentic tree of the representative sequences identified in step 7 and the reference sequence set (e.g. *A. thaliana* MYBs) is constructed.

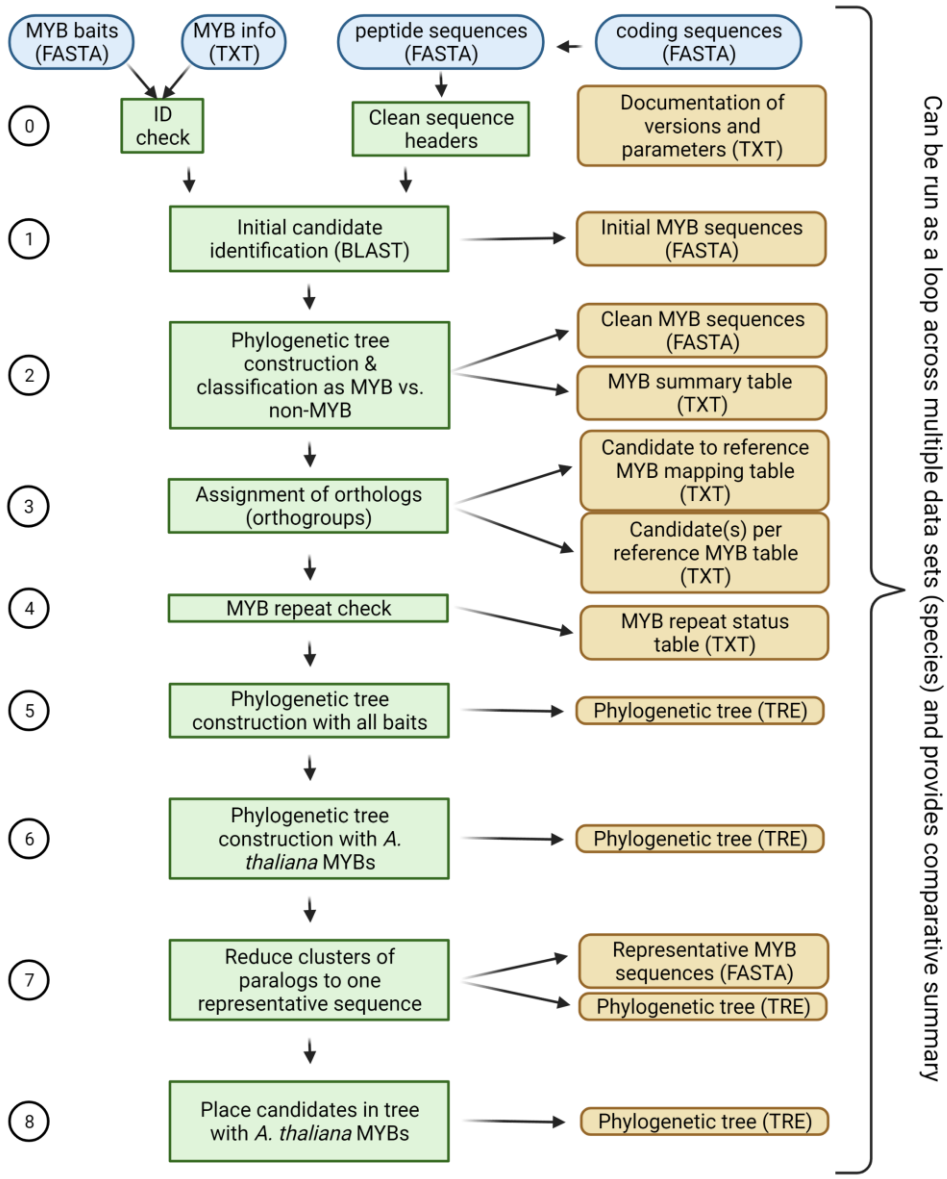

Fig.1: Simplified pipeline for the automatic annotation of MYBs. Please refer to the text and the documentation on github for additional details about the pipeline. There is an option to run this pipeline across all provided input files in a folder. This enables the generation of summary files that compare the MYB gene families between the analyzed species.

Results and Discussion

Proof of concept and benchmarking

185 Several benchmarking data sets were analyzed to ensure that the pipeline performs well for a range of
186 different plant species. Araport11 sequences of the *A. thaliana* accession Col-0 [54] were analyzed and
187 the well characterized MYBs were recovered (example output files on github). This demonstrates that
188 the pipeline works as expected. The annotated sequences of the *A. thaliana* accession Nd-1 [60] were
189 screened for MYBs. As expected, there is a 1:1 relationship between the MYBs of Col-0 and Nd-1
190 (Additional file 2). This demonstrates that not just identical, but also slightly different sequences are
191 accurately identified.

192

193 **Performance**

194 Benchmarking and performance tests were performed on a compute cluster without control over other
195 jobs running on the same machine. This prevented a precise and informative calculation of run times,
196 but also represents realistic conditions. A total of 121 coding sequence sets were downloaded from
197 Phytozome [61] and screened for MYBs. The average run time per species using default parameters, 4
198 CPUs, FastTree, and v0.153 of the pipeline was about 8 minutes (**Fig. 2**, Additional file 3). The memory
199 requirements of all steps in the pipeline are very low (<1GB). The major factor contributing to the run
200 time is the construction of a phylogenetic tree. However, the use of RAxML takes substantially longer. If
201 a job is canceled, the analysis can continue at the last completed check point or at the last analyzed data
202 set (species), respectively. The required hard disk space is minimal (63.5 MB for *A. thaliana*). Changes in
203 the parameters and especially in the number of supplied bait sequences can alter the computational
204 costs substantially. While there are differences with respect to the run time depending on the number
205 of predicted species per family and the size of the MYB gene family, this analysis indicates that large
206 data sets can be processed effectively.

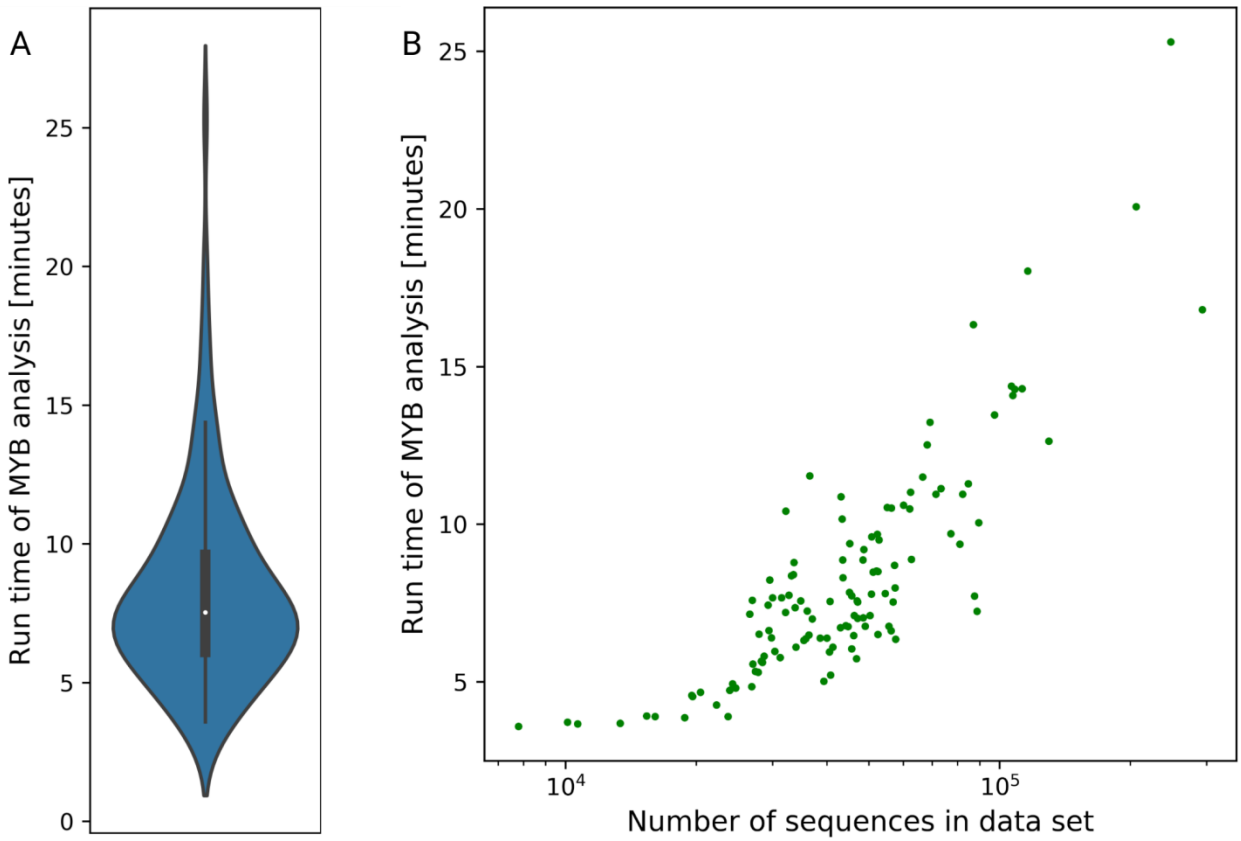

Fig. 2: Average run time per data set (species) with default parameters based on coding sequences (A). Positive correlation of run time with the number of sequences in the data set (B).

Discovery of MYBs in the *Castanea crenata* genome sequence

As a proof of concept, MYBs encoded in the recently sequenced *Castanea crenata* genome [62] were investigated. The predicted peptide sequences were screened with default parameters and resulted in the identification of 136 MYBs (**Fig. 3**, Additional file 4). A R2R3-MYB domain was detected in 112 of them. No orthologs of the *Cruciferae*-specific glucosinolate biosynthesis regulating MYBs AtMYB028, AtMYB029, AtMYB034, AtMYB051, AtMYB076, and AtMYB122 were detected in *C. crenata*. This is not surprising, because *C. crenata* belongs to the *Fagaceae,* and also in line with previous reports about the absence of this MYB linage from non-Cruciferae [3]. Regulators of the flavonoid biosynthesis (AtMYB011/AtMYB012/AtMYB111, Ccr1.0Bg1101.1-S7/Ccr1.0Jg2696.1-S7), anthocyanin biosynthesis (AtMYB075/AtMYB090/AtMYB113/AtMYB114, Ccr1.0Ag5288.1-S6), and proanthocyanidin biosynthesis (AtMYB123, Ccr1.0Ag1758.1 / Ccr1.0Ag1766.1 / Ccr1.0Ag1768.1 / Ccr1.0Ag1770.1 / Ccr1.0Ag1773.1 / Ccr1.0Ag5531.1 / Ccr1.0Ag5542.1 / Ccr1.0Ag5543.1 / Ccr1.0Eg0443.1 / Ccr1.0Eg0444.1 / Ccr1.0Gg0097.1 / Ccr1.0Gg0098.1 / Ccr1.0Hg2677.1 / Ccr1.0Jg0953.1 / Ccr1.0Jg2532.1 / Ccr1.0Lg0385.1 / Ccr1.0Lg3370.1) were detected. In general, copy number differences can be explained by lineage-specific duplication events. Therefore, it is not possible to establish 1:1 relationships between *A.*

thaliana and *C. crenata*. Interestingly, there are multiple homologs of AtMYB123 in *C. crenata* which could indicate a high importance of proanthocyanidins in this species. Further investigations could analyze the transcription of these genes to exclude unexpressed copies. The reduced number of PAP homologs could suggest a lower importance of anthoycanins. While the copies of the anthocyanin regulator show different functions in *A. thaliana* [63], loss of MYB114 in several *A. thaliana* accessions including Col-0 [64] and Nd-1 [65] suggest that there is functional redundancy between them.

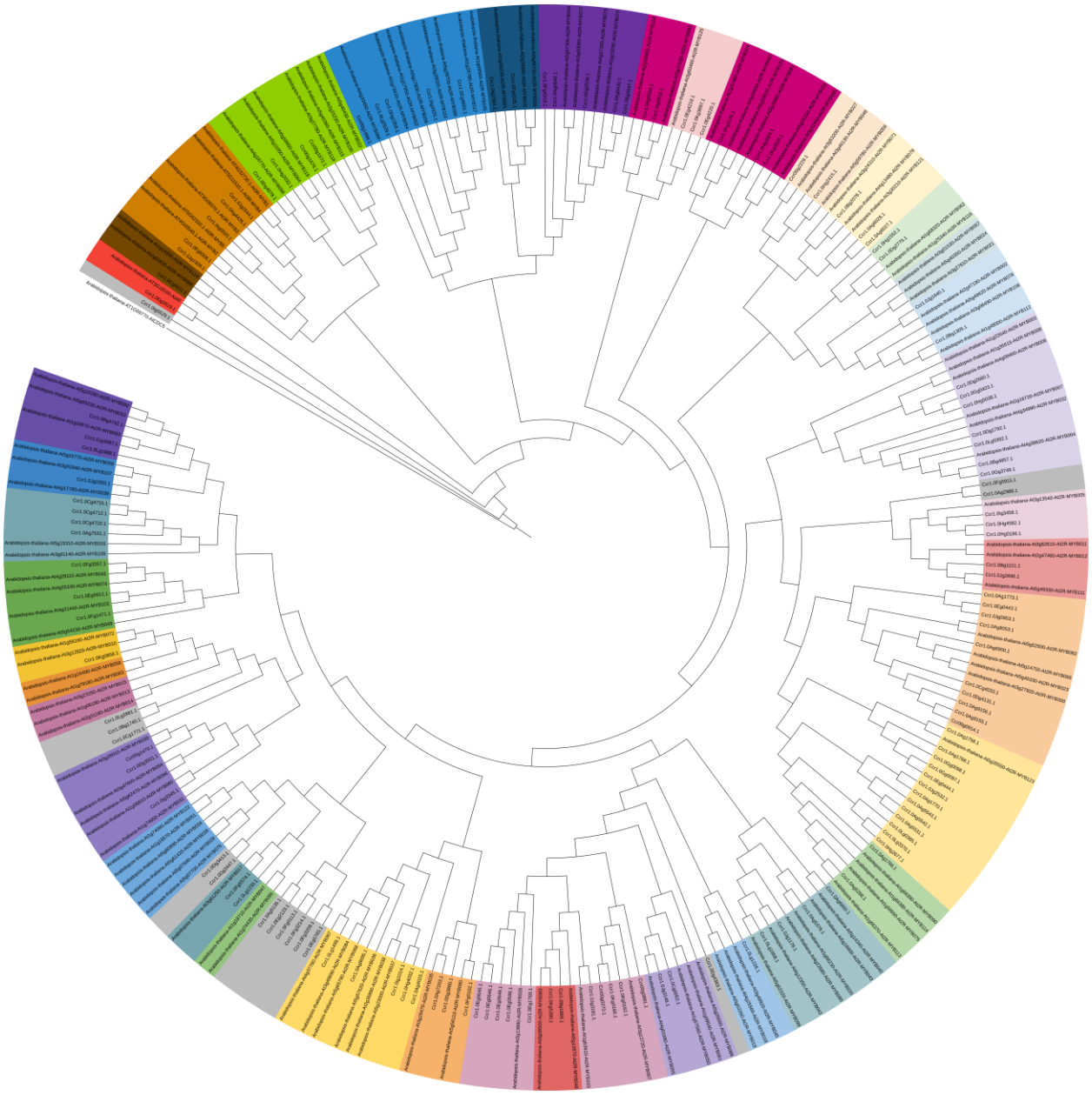

Fig. 3: Phylogenetic tree of *Castanea crenata* MYB candidates and well characterized *Arabidopsis thaliana* MYBs. This figure was constructed with iTOL [66].

236

237 **Discovery of MYBs in the *Croton tiglium* transcriptome assembly**

238 To demonstrate that the pipeline also works for inherently incomplete transcriptome assemblies, MYBs
239 were investigated in the transcriptome assembly of *Croton tiglium* [67]. An analysis with default
240 parameters revealed 140 MYBs (**Fig. 4**, Additional file 5). This includes 103 candidates with a complete
241 R2R3-MYB domain. Transcriptome assemblies are known to be rich in isoforms of the same genes, which
242 can be due to alternative splicing or artifacts. Clusters of these isoforms were represented by the
243 longest sequence among them. This reduced the number of MYB candidates to 79 including 69 with a
244 R2R3 domain. Not all *A. thaliana* MYBs are matched by orthologs in *C. tiglium*. Although this
245 transcriptome assembly is based on paired-end RNA-seq data sets representing leaf, root, stem, seed,
246 and inflorescence, some not or lowly expressed MYBs might not be represented in the assembly.
247 Therefore, they cannot be identified in this analysis. Again, the absence of orthologs of the glucosinolate
248 regulating MYBs aligns well with previous reports [3], because *C. tiglium* belongs to the Euphorbiaceae.
249 The conserved regulators of the flavonoid biosynthesis (AtMYB011/AtMYB012/AtMYB111, TRINITY-
250 DN21046-c0-g1-i2-S7) and proanthocyanidin biosynthesis (AtMYB123, TRINITY-DN31260-c4-g2-i2-S5)
251 were detected. These findings are in line with previous reports of the flavonol regulators and
252 proanthocyanidin regulator being detectable in this transcriptome assembly [68]. The absence of a PAP
253 ortholog from the assembly is not surprising, because none of the sampled tissues showed a
254 pigmentation by anthocyanins [67]. Anthocyanin regulators are well known to be lowly expressed in
255 tissues without anthocyanin pigmentation [69, 70] thus a lack of expression is a likely explanation of this
256 result.

257

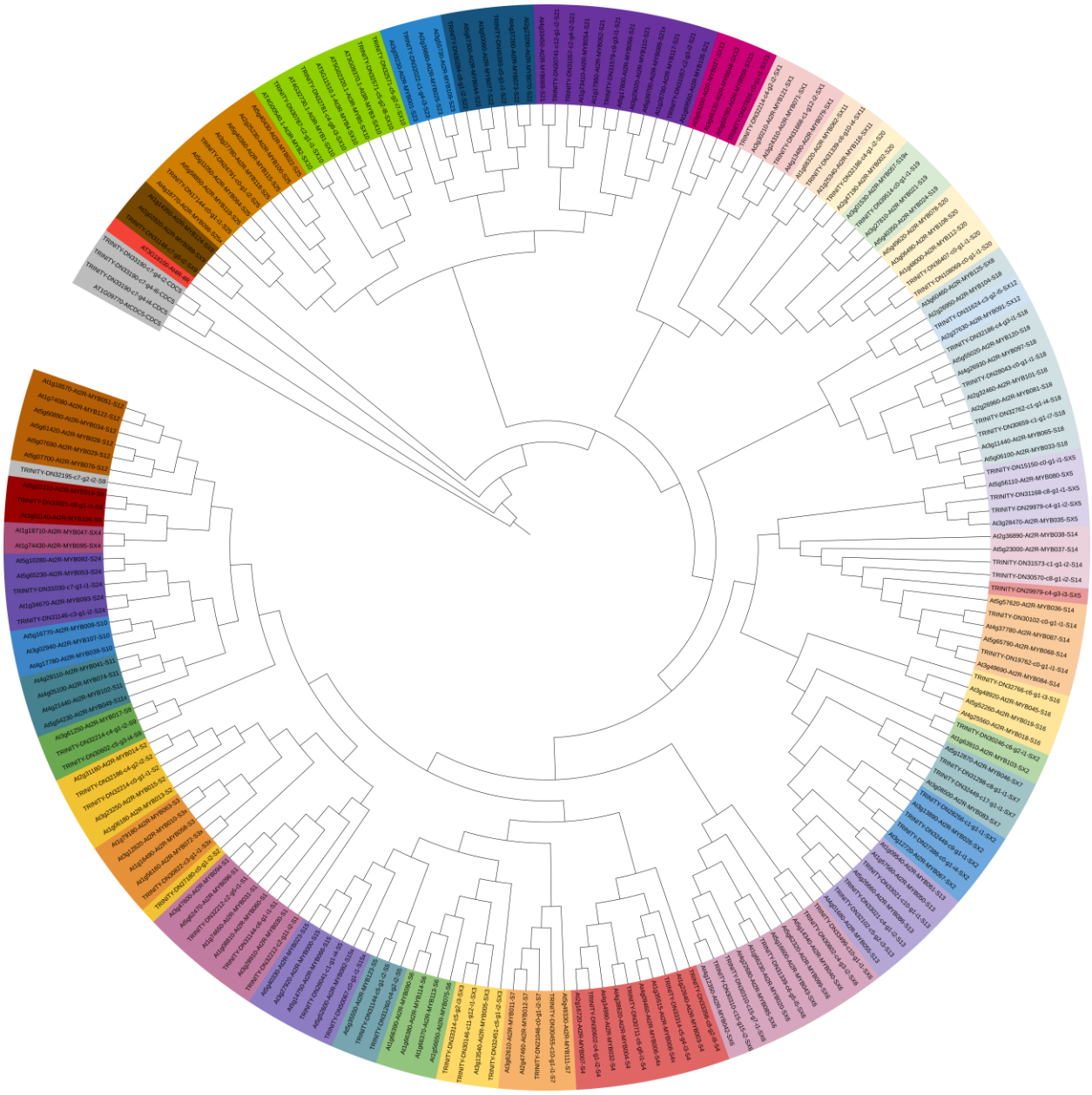

Fig. 4: Phylogenetic tree of *Croton tiglium* and well characterized *Arabidopsis thaliana* MYBs. This figure was constructed with iTOL [66].

Limitations and next steps

The collection of bait sequences distributed with the tool should be appropriate for most applications. However, a narrower set of sequences could reduce the run time if a comprehensive investigation of numerous genome sequences of one taxonomic group is planned. An analysis with a more comprehensive set of MYB sequences could improve the quality of the results, but would also

substantially increase the run time of the analysis. The generation of an ideal bait sequence set which represents the complete phylogenetic diversity of MYBs with a minimal number of sequences is a task for future studies.

Most steps are deterministic, but minor variations might occur as part of the tree building. However, no biologically relevant differences were observed during the analyses of benchmarking datasets.

Conclusions

This approach only relies on standard tools which should be installed on most systems and are also easy to install if not available already. Technical checks on *A. thaliana* datasets indicate that the pipeline is accurately identifying MYBs. The performance allows the investigation of one species within minutes on ubiquitously available hardware. An investigation of the MYB gene families in *Castanea crenata* and *Croton tiglium* revealed expected patterns and demonstrated the potential to analyze transcriptome and genome sequence assemblies. While this approach is dedicated to the analysis of MYBs, it could be adjusted to investigate other transcription factor gene families.

Availability and requirements

Project name: MYB_annotator
Project home page: https://github.com/bpucker/MYB_annotator
Operating system(s): Linux
Programming language: Python3
Other requirements: dendropy, BLAST, MAFFT, RAxML or FastTree2
License: GNU General Public License v3.0
Any restrictions to use by non-academics: none

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

298

299 **Availability of data and materials**

300 The pipeline and the required input data sets are freely available via github:
301 https://github.com/bpucker/MYB_annotator. Additionally, the released version was archived via zenodo
302 (10.5281/zenodo.5573902). The analyzed transcriptomic and genomic resources are publicly available:
303 *Castanea crenata* (BPMU01000001-BPMU01000781) and *Croton tiglium* (PRJNA416498).

304

305 **Competing interests**

306 The authors declare that they have no competing interests.

307

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310

311 **Authors' contributions**

312 BP planned the study, wrote the software, performed the bioinformatic analysis, interpreted the results,
313 and wrote the manuscript.

314

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320 bioRender.com was used to construct some of the figures.

321

322 ## References

323 1. Klempnauer KH, Gonda TJ, Bishop JM. Nucleotide sequence of the retroviral leukemia gene v-myb and
324 its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell. 1982;31 2 Pt 1:453–63.

325 2. Stracke R, Werber M, Weisshaar B. The R2R3-MYB gene family in Arabidopsis thaliana. Current
326 Opinion in Plant Biology. 2001;4:447–56.

327 3. Matus JT, Aquea F, Arce-Johnson P. Analysis of the grape MYB R2R3 subfamily reveals expanded wine
328 quality-related clades and conserved gene structure organization across Vitis and Arabidopsis genomes.
329 BMC Plant Biology. 2008;8:83.

330 4. Pucker B, Pandey A, Weisshaar B, Stracke R. The R2R3-MYB gene family in banana (Musa acuminata):
331 Genome-wide identification, classification and expression patterns. PLOS ONE. 2020;15:e0239275.

332 5. Jiang C-K, Rao G-Y. Insights into the Diversification and Evolution of R2R3-MYB Transcription Factors in
333 Plants1. Plant Physiology. 2020;183:637–55.

334 6. Yuan Y, Yang X, Feng M, Ding H, Khan MT, Zhang J, et al. Genome-wide analysis of R2R3-MYB
335 transcription factors family in the autopolyploid Saccharum spontaneum: an exploration of dominance
336 expression and stress response. BMC Genomics. 2021;22:622.

337 7. Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. MYB transcription factors in
338 Arabidopsis. Trends in Plant Science. 2010;15:573–81.

339 8. Ogata K, Kanei-Ishii C, Sasaki M, Hatanaka H, Nagadoi A, Enari M, et al. The cavity in the hydrophobic
340 core of Myb DNA-binding domain is reserved for DNA recognition and trans-activation. Nat Struct Mol
341 Biol. 1996;3:178–87.

342 9. Jia L, Clegg MT, Jiang T. Evolutionary dynamics of the DNA-binding domains in putative R2R3-MYB
343 genes identified from rice subspecies indica and japonica genomes. Plant Physiol. 2004;134:575–85.

344 10. Du H, Liang Z, Zhao S, Nan M-G, Tran L-SP, Lu K, et al. The Evolutionary History of R2R3-MYB Proteins
345 Across 50 Eukaryotes: New Insights Into Subfamily Classification and Expansion. Sci Rep. 2015;5:11037.

346 11. Ito M. Conservation and diversification of three-repeat Myb transcription factors in plants. J Plant
347 Res. 2005;118:61–9.

348 12. Haga N, Kato K, Murase M, Araki S, Kubo M, Demura T, et al. R1R2R3-Myb proteins positively
349 regulate cytokinesis through activation of KNOLLE transcription in Arabidopsis thaliana. Development.
350 2007;134:1101–10.

351 13. Rosinski JA, Atchley WR. Molecular evolution of the Myb family of transcription factors: evidence for
352 polyphyletic origin. J Mol Evol. 1998;46:74–83.

353 14. Braun EL, Grotewold E. Newly discovered plant c-myb-like genes rewrite the evolution of the plant
354 myb gene family. Plant Physiol. 1999;121:21–4.

355 15. Kranz H, Scholz K, Weisshaar B. c-MYB oncogene-like genes encoding three MYB repeats occur in all
356 major plant lineages. Plant J. 2000;21:231–5.

357 16. Jiang C, Gu J, Chopra S, Gu X, Peterson T. Ordered origin of the typical two- and three-repeat Myb
358 genes. Gene. 2004;326:13–22.

359 17. Lee MM, Schiefelbein J. WEREWOLF, a MYB-Related Protein in Arabidopsis, Is a Position-Dependent
360 Regulator of Epidermal Cell Patterning. Cell. 1999;99:473–83.

361 18. Stracke R, Ishihara H, Huep G, Barsch A, Mehrtens F, Niehaus K, et al. Differential regulation of
362 closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the
363 Arabidopsis thaliana seedling. Plant J. 2007;50:660–77.

364 19. Naik J, Rajput R, Pucker B, Stracke R, Pandey A. The R2R3-MYB transcription factor MtMYB134
365 orchestrates flavonol biosynthesis in Medicago truncatula. Plant Mol Biol. 2021;106:157–72.

366 20. Wang B, Luo Q, Li Y, Yin L, Zhou N, Li X, et al. Structural insights into target DNA recognition by R2R3-
367 MYB transcription factors. Nucleic Acids Research. 2020;48:460–71.

368 21. Harkess A, Huang K, van der Hulst R, Tissen B, Caplan JL, Koppula A, et al. Sex Determination by Two
369 Y-Linked Genes in Garden Asparagus[OPEN]. The Plant Cell. 2020;32:1790–6.

370 22. Stracke R, Holtgräwe D, Schneider J, Pucker B, Rosleff Sörensen T, Weisshaar B. Genome-wide
371 identification and characterisation of R2R3-MYB genes in sugar beet (Beta vulgaris). BMC Plant Biology.
372 2014;14:249.

373 23. Zhang X, He Y, Li L, Liu H, Hong G. Involvement of the R2R3-MYB transcription factor MYB21 and its
374 homologs in regulating flavonol accumulation in Arabidopsis stamen. Journal of Experimental Botany.
375 2021;72:4319–32.

376 24. Walker AR, Davison PA, Bolognesi-Winfield AC, James CM, Srinivasan N, Blundell TL, et al. The
377 TRANSPARENT TESTA GLABRA1 locus, which regulates trichome differentiation and anthocyanin
378 biosynthesis in Arabidopsis, encodes a WD40 repeat protein. Plant Cell. 1999;11:1337–50.

379 25. Nesi N, Debeaujon I, Jond C, Pelletier G, Caboche M, Lepiniec L. The TT8 Gene Encodes a Basic Helix-
380 Loop-Helix Domain Protein Required for Expression of DFR and BAN Genes in Arabidopsis Siliques. Plant
381 Cell. 2000;12:1863–78.

382 26. Ramsay NA, Glover BJ. MYB-bHLH-WD40 protein complex and the evolution of cellular diversity.
383 Trends Plant Sci. 2005;10:63–70.

384 27. Lloyd A, Brockman A, Aguirre L, Campbell A, Bean A, Cantero A, et al. Advances in the MYB–bHLH–
385 WD Repeat (MBW) Pigment Regulatory Model: Addition of a WRKY Factor and Co-option of an
386 Anthocyanin MYB for Betalain Regulation. Plant and Cell Physiology. 2017;58:1431–41.

387 28. Baudry A, Heim MA, Dubreucq B, Caboche M, Weisshaar B, Lepiniec L. TT2, TT8, and TTG1
388 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis
389 thaliana. Plant J. 2004;39:366–80.

390 29. Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L. The Arabidopsis TT2 Gene Encodes an R2R3 MYB
391 Domain Protein That Acts as a Key Determinant for Proanthocyanidin Accumulation in Developing Seed.
392 Plant Cell. 2001;13:2099–114.

393 30. Jin W, Wang H, Li M, Wang J, Yang Y, Zhang X, et al. The R2R3 MYB transcription factor PavMYB10.1
394 involves in anthocyanin biosynthesis and determines fruit skin colour in sweet cherry (Prunus avium L.).
395 Plant Biotechnology Journal. 2016;14:2120–33.

396 31. Wang H, Zhang H, Yang Y, Li M, Zhang Y, Liu J, et al. The control of red colour by a family of MYB
397 transcription factors in octoploid strawberry (Fragaria × ananassa) fruits. Plant Biotechnology Journal.
398 2020;18:1169–84.

399 32. Zheng X, Om K, Stanton KA, Thomas D, Cheng PA, Eggert A, et al. The regulatory network for petal
400 anthocyanin pigmentation is shaped by the MYB5a/NEGAN transcription factor in Mimulus. Genetics.
401 2021;217. doi:10.1093/genetics/iyaa036.

402 33. Tan Z, Xie Z, Dai L, Zhang Y, Hu Z, Tang S, et al. Genome- and transcriptome-wide association studies
403 reveal the genetic basis and the breeding history of seed glucosinolate content in Brassica napus. Plant
404 Biotechnol J. 2021.

405 34. Gigolashvili T, Berger B, Mock H-P, Müller C, Weisshaar B, Flügge U-I. The transcription factor
406 HIG1/MYB51 regulates indolic glucosinolate biosynthesis in Arabidopsis thaliana. The Plant Journal.
407 2007;50:886–901.

408 35. Hirai MY, Sugiyama K, Sawada Y, Tohge T, Obayashi T, Suzuki A, et al. Omics-based identification of
409 Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. PNAS.
410 2007;104:6478–83.

411 36. Frerigmann H, Gigolashvili T. Update on the role of R2R3-MYBs in the regulation of glucosinolates
412 upon sulfur deficiency. Frontiers in Plant Science. 2014;5:626.

413 37. Hatlestad GJ, Akhavan NA, Sunnadeniya RM, Elam L, Cargile S, Hembd A, et al. The beet Y locus
414 encodes an anthocyanin MYB-like protein that activates the betalain red pigment pathway. Nat Genet.
415 2015;47:92–6.

416 38. Stetter MG, Vidal-Villarejo M, Schmid KJ. Parallel Seed Color Adaptation during Multiple
417 Domestication Attempts of an Ancient New World Grain. Molecular Biology and Evolution.
418 2020;37:1407–19.

419 39. Siadjeu C, Pucker B, Viehöver P, Albach DC, Weisshaar B. High Contiguity de novo Genome Sequence
420 Assembly of Trifoliate Yam (Dioscorea dumetorum) Using Long Read Sequencing. Genes. 2020;11:274.

421 40. Siadjeu C, Mayland-Quellhorst E, Pande S, Laubinger S, Albach DC. Transcriptome Sequence Reveals
422 Candidate Genes Involving in the Post-Harvest Hardening of Trifoliate Yam Dioscorea dumetorum.
423 Plants. 2021;10:787.

424 41. Chagné D, Lin-Wang K, Espley RV, Volz RK, How NM, Rouse S, et al. An Ancient Duplication of Apple
425 MYB Transcription Factors Is Responsible for Novel Red Fruit-Flesh Phenotypes1[C][W]. Plant Physiol.
426 2013;161:225–39.

427 42. Xi W, Feng J, Liu Y, Zhang S, Zhao G. The R2R3-MYB transcription factor PaMYB10 is involved in
428 anthocyanin biosynthesis in apricots and determines red blushed skin. BMC Plant Biology. 2019;19:287.

429 43. Mikhaylova EV, Shein MY, Artyukhin AY, Sukhareva AS, Panfilova MA, Kuluev BR. Editing of the MYB
430 genes in Brassica napus as a method to increase anthocyanin pigmentation and stress tolerance. E3S
431 Web Conf. 2020;224:04022.

432 44. Khusnutdinov E, Sukhareva A, Panfilova M, Mikhaylova E. Anthocyanin Biosynthesis Genes as Model
433 Genes for Genome Editing in Plants. International Journal of Molecular Sciences. 2021;22:8752.

434 45. Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, et al. The MYB Transcription Factor
435 Superfamily of Arabidopsis: Expression Analysis and Phylogenetic Comparison with the Rice MYB Family.
436 Plant Mol Biol. 2006;60:107–24.

437 46. Wilkins O, Nahal H, Foong J, Provart NJ, Campbell MM. Expansion and Diversification of the Populus
438 R2R3-MYB Family of Transcription Factors. Plant Physiology. 2009;149:981–93.

439 47. Du H, Feng B-R, Yang S-S, Huang Y-B, Tang Y-X. The R2R3-MYB Transcription Factor Gene Family in
440 Maize. PLOS ONE. 2012;7:e37463.

441 48. Cao Y, Jia H, Xing M, Jin R, Grierson D, Gao Z, et al. Genome-Wide Analysis of MYB Gene Family in
442 Chinese Bayberry (Morella rubra) and Identification of Members Regulating Flavonoid Biosynthesis.
443 Frontiers in Plant Science. 2021;12:1244.

444 49. Li Z, Peng R, Tian Y, Han H, Xu J, Yao Q. Genome-Wide Identification and Analysis of the MYB
445 Transcription Factor Superfamily in Solanum lycopersicum. Plant and Cell Physiology. 2016;57:1657–77.

446 50. Yang Y, Moore MJ, Brockington SF, Soltis DE, Wong GK-S, Carpenter EJ, et al. Dissecting Molecular
447 Evolution in the Highly Diverse Plant Clade Caryophyllales Using Transcriptome Sequencing. Mol Biol
448 Evol. 2015;32:2001–14.

449 51. Emms DM, Kelly S. OrthoFinder: phylogenetic orthology inference for comparative genomics.
450 Genome Biology. 2019;20:238.

451 52. Zhang R, Kuo R, Coulter M, Calixto CPG, Entizne JC, Guo W, et al. A high resolution single molecule
452 sequencing-based Arabidopsis transcriptome using novel methods of Iso-seq analysis. 2021.
453 doi:10.1101/2021.09.02.458763.

454 53. Guang A, Howison M, Zapata F, Lawrence C, Dunn CW. Revising transcriptome assemblies with
455 phylogenetic information. PLOS ONE. 2021;16:e0244202.

456 54. Cheng C-Y, Krishnakumar V, Chan AP, Thibaud-Nissen F, Schobel S, Town CD. Araport11: a complete
457 reannotation of the Arabidopsis thaliana reference genome. The Plant Journal. 2017;89:789–804.

458 55. Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in
459 Performance and Usability. Mol Biol Evol. 2013;30:772–80.

460 56. Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. RAxML-NG: a fast, scalable and user-friendly
461 tool for maximum likelihood phylogenetic inference. Bioinformatics. 2019;35:4453–5.

462 57. Price MN, Dehal PS, Arkin AP. FastTree 2 – Approximately Maximum-Likelihood Trees for Large
463 Alignments. PLOS ONE. 2010;5:e9490.

464 58. Sukumaran J, Holder MT. DendroPy: a Python library for phylogenetic computing. Bioinformatics.
465 2010;26:1569–71.

466 59. Feng G, Burleigh JG, Braun EL, Mei W, Barbazuk WB. Evolution of the 3R-MYB Gene Family in Plants.
467 Genome Biology and Evolution. 2017;9:1013–29.

468 60. Pucker B, Holtgräwe D, Stadermann KB, Frey K, Huettel B, Reinhardt R, et al. A chromosome-level
469 sequence assembly reveals the structure of the Arabidopsis thaliana Nd-1 genome and its gene set.
470 PLOS ONE. 2019;14:e0216233.

471 61. Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, et al. Phytozome: a comparative
472 platform for green plant genomics. Nucleic Acids Res. 2012;40 Database issue:D1178–86.

473 62. Shirasawa K, Nishio S, Terakami S, Botta R, Marinoni DT, Isobe S. Chromosome-level genome
474 assembly of Japanese chestnut (Castanea crenata Sieb. et Zucc.) reveals conserved chromosomal
475 segments in woody rosids. 2021. doi:10.1101/2021.07.29.454274.

476 63. Koo Y, Poethig RS. Expression pattern analysis of three R2R3-MYB transcription factors for the
477 production of anthocyanin in different vegetative stages of Arabidopsis leaves. Applied Biological
478 Chemistry. 2021;64:5.

479 64. Gonzalez A, Zhao M, Leavitt JM, Lloyd AM. Regulation of the anthocyanin biosynthetic pathway by
480 the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. The Plant Journal. 2008;53:814–
481 27.

482 65. Pucker B, Holtgräwe D, Sörensen TR, Stracke R, Viehöver P, Weisshaar B. A De Novo Genome
483 Sequence Assembly of the Arabidopsis thaliana Accession Niederzenz-1 Displays Presence/Absence
484 Variation and Strong Synteny. PLOS ONE. 2016;11:e0164321.

485 66. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and
486 annotation. Nucleic Acids Research. 2021;49:W293–6.

487 67. Haak M, Vinke S, Keller W, Droste J, Rückert C, Kalinowski J, et al. High Quality de Novo
488 Transcriptome Assembly of Croton tiglium. Front Mol Biosci. 2018;5. doi:10.3389/fmolb.2018.00062.

489 68. Pucker B, Reiher F, Schilbert HM. Automatic Identification of Players in the Flavonoid Biosynthesis
490 with Application on the Biomedicinal Plant Croton tiglium. Plants. 2020;9:1103.

491 69. Takos AM, Jaffé FW, Jacob SR, Bogs J, Robinson SP, Walker AR. Light-Induced Expression of a MYB
492 Gene Regulates Anthocyanin Biosynthesis in Red Apples. Plant Physiol. 2006;142:1216–32.

493 70. Guo N, Han S, Zong M, Wang G, Zheng S, Liu F. Identification and differential expression analysis of
494 anthocyanin biosynthetic genes in leaf color variants of ornamental kale. BMC Genomics. 2019;20:564.

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