Genetic analysis of the liverwort Marchantia polymorpha reveals that R2R3MYB activation of flavonoid production in response to abiotic stress is an ancient character in land plants


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Summary

- The flavonoid pathway is hypothesized to have evolved during land colonization by plants c. 450 Myr ago for protection against abiotic stresses. In angiosperms, R2R3MYB transcription factors are key for environmental regulation of flavonoid production. However, angiosperm R2R3MYB gene families are larger than those of basal plants, and it is not known whether the regulatory system is conserved across land plants. We examined whether R2R3MYBs regulate the flavonoid pathway in liverworts, one of the earliest diverging land plant lineages.
- We characterized MpMyb14 from the liverwort Marchantia polymorpha using genetic mutagenesis, transgenic overexpression, gene promoter analysis, and transcriptomic and chemical analysis. MpMyb14 is phylogenetically basal to characterized angiosperm R2R3MYB flavonoid regulators.
- Mpmyb14 knockout lines lost all red pigmentation from the flavonoid riccionidin A, whereas overexpression conferred production of large amounts of flavones and riccionidin A, activation of associated biosynthetic genes, and constitutive red pigmentation. MpMyb14 expression and flavonoid pigmentation were induced by light- and nutrient-deprivation stress in M. polymorpha as for anthocyanins in angiosperms.
- MpMyb14 regulates stress-induced flavonoid production in M. polymorpha, and is essential for red pigmentation. This suggests that R2R3MYB regulated flavonoid production is a conserved character across land plants which arose early during land colonization.

Introduction

Flavonoid biosynthesis is a central component of plant secondary metabolism that is thought to have evolved during land colonization, c. 450–500 Myr ago (Ma) (Vogt, 2010; Weng, 2013; de Vries et al., 2017). Derived from the larger phenylpropanoid pathway, the acquisition of flavonoids is hypothesized to have been a key adaptation for coping with the additional abiotic stresses faced with the transition to a nonaquatic lifestyle. These included drought, extreme temperature fluctuations and, in particular, increased exposure to UV-B light (Bjorn et al., 2002). UV-B has severe detrimental effects on plant cells, and was at particularly high intensities during the period of land colonization because the ozone layer was not fully developed. Flavonoids are ubiquitous in extant land plants, including the liverworts, which may be the closest living relatives of the first land plants. However, they are absent from all algae, including Charophytes, which are thought to be most similar to the aquatic ancestors of land plants (de Vries et al., 2017).

Many flowering plants (angiosperms) produce colourless UV-B absorbing flavone and flavonol glycosides in response to UV-B exposure, as well as following other abiotic stresses. They also produce red/purple anthocyanin flavonoid pigments in response to nutrient deprivation, drought, wounding, cold and high light, as well as for signalling to animals for pollination and seed dispersal (Allan et al., 2008; Agati & Tattini, 2010; Davies et al., 2012a; Cheynier et al., 2013). The flavones/flavonols and anthocyanins can directly screen excess UV-B or white light energy, respectively, but also are thought to provide broad stress tolerance through scavenging of reactive oxygen species (ROS; Agati & Tattini, 2010; Agati et al., 2012; Landi et al., 2015).

In all angiosperm species examined to date, R2R3MYB transcription factors are essential for environmental and developmental regulation of flavonoid biosynthesis (Albert et al., 2011; Feller et al., 2011; Cheynier et al., 2013; Xu et al., 2015; Lloyd et al., 2017). For activation of the anthocyanin or proanthocyanidin pigment pathways, R2R3MYBs of subgroups (SGs) 5 or 6 act as part of an R2R3MYB-bHLH-WDR (MBW) transcriptional...
complex. Additionally, members of SG4 can have a repressive role on genes of the phenylpropanoid pathway as part of the MBW complex and contain transcriptional repressor domains in their C-terminal (Matsui et al., 2008; Albert et al., 2014; Cavallini et al., 2015). For activation of flavonol biosynthesis R2R3MYBs of SG7 act independently of the MBW complex. Although the MBW complex is often regarded as universal for anthocyanin regulation, it is actually an open question as to what aspects of the system are conserved across the major land plant groups (Albert et al., 2014; Liu et al., 2015; Xu et al., 2015; Lloyd et al., 2017). The recent finding of a conserved MBW complex able to activate flavonoid biosynthesis in a gymnosperm species (Nemesio-Gorriz et al., 2017) suggests an ancient origin in the plant lineage, as angiosperms and gymnosperms are thought to have diverged c. 300 Ma (Lu et al., 2014). However, genomes of the basal plant groups liverworts (Bowman et al., 2017) and mosses (Rensing et al., 2008) contain markedly fewer MYB and bHLH genes than those of angiosperms and gymnosperms, suggesting a more limited range of regulatory activities.

Liverworts together with mosses and hornworts form the three main lineages of the basal land plant group, the bryophytes. Marchantia polymorpha (hereafter, marchantia) has been developed as a model species for liverwort studies. Marchantia has a dominant haploid gametophytic generation, rapid growth rate, is dioecious and can reproduce asexually in large numbers through single-cell derived clonal gemmae (Bowman et al., 2016). The last common ancestor of liverworts and angiosperms is thought to have existed c. 450 Ma, so comparative studies of the two plant groups can help to inform us about systems that have an early evolutionary origin. Liverworts lack some of the typically flavonoid pigmented tissues of angiosperms, such as flowers, fruits and seed coats, and knowledge about flavonoid production in liverworts is comparatively limited. They produce a range of red flavonoid pigments similar to those of angiosperms (Asakawa et al., 1993). Additionally, although liverworts lack the diversity of anthocyanins reported for angiosperms, they do produce a cell wall-localized red flavonoid pigment that has been identified as the aglycone anthocyanin riccionidin A, including from marchantia (Kunz et al., 1993).

The analysis of Bowman et al. (2017) identified only 21 R2R3MYB genes in the marchantia genome, as compared for example to the estimated 138 in Arabidopsis, 157–188 in maize (Zea mays) and 244–288 in soybean (Glycine max) (Stracke et al., 2001; Feller et al., 2011; Liu et al., 2015). Often the marchantia genome contains one or two genes that are basal to large clades containing multiple angiosperm subgroups with differing but related functions. There are no clear orthologues in marchantia for genes of the phenylpropanoid-related SGs 4, 5, 6 or 7, but MpMyb02 and MpMyb14 are basal to a clade containing 19 Arabidopsis R2R3MYBs comprising SGs 4, 5, 6, 7, 15 and 44 (Bowman et al., 2017). Following the hypothesis that R2R3MYB gene(s) will regulate abiotic stress-responsive flavonoid production in liverworts, we characterized the function of the candidate gene MpMyb14. The findings show that MpMyb14 activates both flavone glycoside and riccionidin A biosynthesis, being essential for riccionidin A production but acting redundantly in regulation of flavone biosynthesis. Riccionidin A production is induced in liverworts by similar abiotic stress factors as previously characterized for anthocyanin production in angiosperms. It is notable that structurally distinct red flavonoid pigments are produced in marchantia and angiosperms, although some of the same flavonoid biosynthetic genes are activated in each plant group. The results suggest that R2R3MYB proteins were co-opted for regulation of the flavonoid pathway from the early stages of land plant evolution, and supports the hypothesis that an inducible flavonoid pathway was an early evolutionary adaption to the abiotic stresses of life on land.

Materials and Methods

Plant lines and growth conditions

Marchantia polymorpha L. accessions Sey-1 (male) and Aud-2 (female) were grown on Jiffy-7 peat pellets (Egmont Seeds, New Plymouth, New Zealand), and were induced to produce sexual structures with supplemental far-red light (Chiyoda et al., 2008) and crossed to generate spores for transformations.

Plant lines were maintained asexually through the propagation of gemmae, either plated directly on agar medium (0.5 × Gamborg’s B5 medium (Duchefa Biochemicals, Haarlem, the Netherlands) 1% (w/v) sucrose, 1.2% (w/v) agar) or onto a sterile filter paper disc covering the medium. Standard culture conditions were 25°C, 16 h photoperiod and 30 µmol m⁻² s⁻¹ light intensity provided by cool fluorescent tubes.

For nutrient deprivation experiments, gemmae (three per tub/biological replicate; three biological replicates per treatment) were plated onto filter papers on complete media (0.5 × B5, 1% sucrose, 1.2% agar) and grown for 4 wk. After this time, plants were transferred on the filter paper to fresh complete media or onto minimal media (1% sucrose, 1.2% agar) and grown for a further 13 d. Tissue was collected and immediately frozen in liquid nitrogen; aliquots were used for RNA extraction or freeze-dried for metabolite analysis.

For light induction experiments, gemmae (three to four per tub/biological replicate; three biological replicates per treatment) were plated onto complete media (0.5 × B5, 1% sucrose, 1.2% agar) and grown for 4 wk under high or low light conditions. Illumination was provided by a combination of warm and cool white LEDs (high light, 200 µmol m⁻² s⁻¹). The low light treatment (50 µmol m⁻² s⁻¹) was performed using the same light source, except that plants were shielded with shade cloth. MYB14-GUS (GUS, β-glucuronidase) plants were grown under the same high/low light conditions, except that they were sampled at either 16 or 20 d.

Light microscopy

Images of marchantia plants and hand sections were taken with a Leica DFC550 digital camera mounted on a Leica M205FA dissecting microscope. Z-stack images were generated with Leica Application Suite X software.
Transformation

Overexpression constructs with the CaMV35S promoter were generated by LR recombination of the MpMyb14 coding sequence (synthesized by Genscript, Piscataway, NJ, USA) into pHARTII, which confers hygromycin resistance (10 mg l⁻¹). CRISPR/Cas9 constructs were generated by annealing oligonucleotides corresponding to the variable regions of the guide RNAs, ligation into pMpU6pro/pENTR and LR recombination into the binary destination vector pMpGE010 (Sugano et al., 2014). Marchantia sporelings were transformed by inoculation with Agrobacterium tumefaciens (GV3101) strains harbouring binary vectors, essentially as described in Tsuboyama & Kodama (2014) with selection on hygromycin (10 mg l⁻¹) and ticarcillin (500 mg l⁻¹).

Screening CRISPR mutations

Genomic DNA was isolated from plants harbouring the CRISPR/Cas9 construct by CTAB/chloroform extraction (Stewart & Via, 1993), followed by PCR amplification of the target region within MpMyb14 (NA322 5′-GCATTCTCTCCGACACCGAGA3′, NA460 5′-GATCCACCTCAGTCTACACGCT3′). PCR reactions were prepared for DNA sequencing by Exonuclease/Shrimp Alkaline Phosphatase treatment, and sequenced directly using BigDye v.3.1 chemistry. Genomic DNA samples that contained multiple MpMyb14 alleles (chimeras) were readily identified by mixed signals on the chromatogram where the sequences differed. Once plants reached a stage where the tissue culture media became exhausted, mutants for MpMyb14 could readily be identified because of their lack of red pigmentation and genetic chimeras were easily detected visually. These mutants were confirmed by DNA sequencing. Gemmae were propagated (G1 generation) from mutant tissue and re-sequenced to ensure that pure mutant lines were obtained. Plants used for experiments were G2 generation or later.

Promoter:GUS

MpMyb14 promoter:GUS fusions were made by amplifying 2 kb of sequence upstream of the initiating ATG (Bowman et al., 2017) and ligating this in front of an intron-containing GUS reporter gene (pDAH2, Davies et al., 2012b). The Nod cassette was ligated into the binary vector pKART, conferring resistance to gentamicin/G418 (7.5 mg l⁻¹). Histochemical localisation of GUS activity was performed as described in Albert et al. (2011).

Flavonoid analysis

Flavonoids were extracted from 10 mg freeze-dried ground thallus tissue with 1 ml methanol : water : formic acid (80 : 19 : 1). Ultra High Performance Liquid Chromatography (UHPLC) was used to separate and quantify the flavonoids present in extracts of thallus tissue. Liquid chromatography – mass spectrometry (LC-HRAM-MS) and LC-HRAM-MS/MS were used to confirm the identity of the compounds.

The UHPLC system used was a Dionex Ultimate® 3000 Rapid Separation LC system equipped with a binary pump (HPR-3400RS), autosampler (WPS-3000RS), column compartment (TCC-3000RS) and a diode array detector (DAD-3000RS). The analytical column used was a Kinetex XB-C18 50 mm × 3 mm, 2.6 μm (Phenomenex, Torrance, CA, USA), maintained at 35°C. A binary solvent program was used with Solvent A (formic acid : MQ water, 1 : 99) and Solvent B (acetonitrile) at a flow of 1 ml min⁻¹. The initial solvent composition was 90%A 10%B until 0.5 min, then changed to 50%A 50%B at 2.5 min, and 5%A 95%B at 3.5 min. After a 1-min hold at 5%A 95%B, the composition was returned to 90%A 10%B ready for the next injection. Total UHPLC analysis time was 6 min per sample. All solvent gradients were linear. The injection volume was 3 μl. Spectral data (260–600 nm) were collected for the entire analysis.

Flavones were quantified by integrating peak areas from chromatograms at 340 nm, and external calibration curves were constructed for luteolin 7-O-glucoside (Extrasyntence, Genay, France). Flavone concentrations were calculated as luteolin 7-O-glucoside equivalents. Individual flavones were identified based on absorption spectra and LC-HRAM-MS/MS fragmentation data (described below), and order of elution reported in Markham et al. (1998). Riccionidin metabolites were quantified from chromatograms extracted at 484 nm and reported as peak areas, because no standard is available for these compounds. Chromatographic data were collected and manipulated using the Chromelone® Chromatography Management System v.7.2 (Dionex Corp., Sunnyvale, CA, USA).

The LC-HRAM-MS/MS system was composed of a Dionex Ultimate® 3000 Rapid Separation LC and a microTOF QII high resolution mass spectrometer (Bruker Daltonics, Bremen, Germany) fitted with an electrospray ion source. The LC column was a Hypersil Gold 200 × 2.1 mm, 1.9 μm (Thermo Scientific, Auckland, New Zealand) and was maintained at 40°C. The flow was 400 μl min⁻¹. The solvents were A = 0.2% formic acid and B = 100% acetonitrile. The solvent gradient was: 10%A 90% B until 0.5 min; linear gradient to 100% A 0.5 to 22 min; composition held at 100% A 22 to 40 min; linear gradient to 10% A 90% B 40 to 40.2 min; to return to the initial conditions before another sample injection at 45 min. The injection volume for samples and standards was 1 μl. The microTOF QII parameters for flavonoid analysis were: temperature 225°C; drying N₂ flow 61 min⁻¹; nebulizer N₂ 1.5 bar, endplate offset –500 V, mass range 100–1500 Da, data were acquired at 2 scans s⁻¹. Negative ion electrospray was used with a capillary voltage of –3500 V. Post-acquisition internal mass calibration used sodium formate clusters with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis. Flavonoids were quantified using QUANTANALYSIS (Bruker Daltonics, Bremen, Germany) software.

RNA-seq and DNA sequence analysis

Total RNA was extracted from control (35Spro::GFP) or 35Spro::MYB14 (line #8) thallus tissue with acid guanidinium/phenol extraction (Chomczynski & Sacchi, 1987) followed by
purification with a Nucleospin® RNA clean-up column (Macherey-Nagel, Düren, Germany). RNA sequencing was on Illumina TruSeq Stranded mRNA libraries by Illumina HiSeq, and was conducted by the Australian Genome Research Facility (Melbourne, Australia). Bioinformatics analysis used DEseq2 (Love et al., 2014) with comparison of reads to a de novo transcript assembly and the published marchantia genome sequence and transcript assemblies (Bowman et al., 2017). The deduced amino acid sequences of the R2R3MYB domain were aligned using CLUSTALW in the GENEIOUS suite of software (Kearse et al., 2012).

Quantitative RT-PCR

Total RNA was isolated from frozen ground thallus tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer’s instructions. First strand cDNA was prepared from DNaseI-treated RNA, and used for quantitative reverse transcription polymerase chain reaction (qRT-PCR) essentially as described in Albert (2015). Unless otherwise stated, reverse transcription polymerase chain reaction (qRT-PCR) was conducted by the Australian Genome Research Facility. First strand cDNA was pre-determined relative to the reference gene ACTIN (Pfaffl, 2001). Primers used were

- **MpACTIN NA336**: 5’GATCTTGCTGAGTGATCTT3’
- **NA337**: 5’GCTTCTCCTTCATGTCTTAC3’
- **MpMyb14 transgene NA534**: 5’GTATCCCGCTGCAATC TG3’
- **NA535**: 5’GATACCTTATTCGTAGACAA3’
- **MpMyb14 NA320**: 5’GTCCGACAGATTCTGTGTA3’
- **NA321**: 5’GCCGACACAAACTCTGTAAGA3’
- **CHS NA515**: 5’GAGTTGGAATCAGAGTGGGTATG3’
- **NA514**: 5’TTCAGGTTGCAGACGTITT3’
- **MpCHI NA378**: 5’AGCGCCTGTGGACAAATTA3’
- **NA379**: 5’TCAAGCTCATCTCCTGTTG3’
- **MpCHI-Like NA515**: 5’ACTCTCCACCTCCAAATTTCT C3’
- **NA516**: 5’TATGTCCCTCAGGCGATCAC3’

Statistical analyses

The data were analysed by ANOVA using GenStat v.18 (VSN International, Hemel Hempstead, UK). The data were log-transformed as necessary, to equalize variances before performing ANOVA. Post hoc comparisons among treatment means were made using Fisher’s Least Significant Difference (LSD) values at \( \alpha = 0.05 \).

Results

Marchantia R2R3MYB gene MpMyb14 is basal to a clade of R2R3MYB phenylpropanoid pathway regulatory genes

The analysis of Bowman et al. (2017) found that MpMyb02 and MpMyb14 were basal to a clade containing Arabidopsis SGs 4, 5, 6 and 7. We generated a phylogenetic tree using the deduced MYB domain amino acid sequences of MpMyb02 and MpMyb14 aligned against the MYB domain sequences of characterized phenylpropanoid regulators from a range of gymnosperm and angiosperm species, BLAST hits from across the land plant lineage, and examplar non-phenylpropanoid R2R3MYBs (Supporting Information Fig. S1). MpMyb14 was basal to a clade containing the angiosperm SG4, 5, 6 and 7 sequences, which also contained representatives from all the major groups of land plants examined. MpMyb02 was basal to the SG5/6/7 sub-clade within this. A further sub-clade of SGs 5 and 6 was present containing lycophyte (Selaginella moellendorffii), gymnosperm and angiosperm sequences but no bryophyte sequences. We chose to functionally characterize MpMyb14, as it may relate to the sister ancestral sequence for the proposed phenylpropanoid clade. Additionally, 35SCaMV:MYPB02 plants showed no change in flavonoid content based on UHPLC/LC-HRAM-MS analysis (present authors’ unpublished data).

MpMYB14 can activate flavonoid production in transgenic marchantia plants

Transgenic marchantia lines were produced by overexpressing MpMyb14 under the control of the 35SCaMV (35S) promoter, which confers moderate constitutive gene expression in marchantia, but is not strongly expressed in meristems (Althoff et al., 2014). The 35SSprov:MYB14 lines showed orange/red colour from when transgenic cell-clusters first began to regenerate, and thalli of fully differentiated plants were pigmented orange/red except for around the meristem. Plants grown from gemmae of these lines had red pigmentation from a young age and adult plants had the same high intensity pigmentation as the clonal parents, including red pigmentation of developing gemmae (Figs 1a, S2). The red pigment was localized within cell walls (Fig. S2b), which matched early observations of red pigmentation in liverwort cell walls (Nagai, 1915; Herzfelder, 1921). Control plants (35Sprov: GFP) grown under the same conditions were predominantly green, with red pigmentation restricted to a small region of the ventral thallus midrib (Fig. 1a). Analysis of the 35SSprov:MYB14 lines in comparison to controls using UHPLC found a five-fold increase in amounts of flavone O-glucuronides (Fig. 1b,d) and a 50-fold increase in amounts of an orange-red pigment (lambda max c. 486 nm, Fig. 1c,e), identified as riccionidin A based on the UV-Vis absorption spectrum and accurate mass data from LC-HRAM-MS. Riccionidin A is a cell wall located anthocyanin comprising four rings rather than the usual three of anthocyanins, based on the MS/NMR defined structure, and is the only red pigment identified from marchantia (Kunz et al., 1993).

The major ion of riccionidin A (compound 2 in Fig. 1) had an accurate mass at m/z 283.0230 (consistent with the published C15H20O6 structure, mass difference 1.8 mDa, isotope ratio 22.8 mSigma), and this was detected in both control and transgenic lines. However, there was a second compound (compound 1 in Fig. 1) that was abundant in the transgenic plants but below the accurate detection limit in control plants. LC-HRAM-MS gave a major ion for compound 1 with an accurate mass at m/z 591.1340, consistent with a formula of C27H20O5 (mass difference 1.6 mDa, isotope ratio 5.4 mSigma). MS/MS fragmentation of this m/z 591.1340 ion produced a fragment ion at m/z 283.0232, suggesting that compound 1 is a previously unidentified glycoside of riccionidin A.
MpMYB14 activates flavonoid biosynthetic gene expression

In order to compare the regulatory action of MpMyb14 with the published data on angiosperm flavonoid-related R2R3MYB activators, RNAseq was used to identify changes in transcript abundance in the 35Spro:MYB14 transgenics. The transgene induced changes in transcript abundance for a large number of genes, with c. 600 genes having a greater than two-fold change and 223 having an adjusted P-value of < 1910−5 (Table S1). These values are in a similar range to the numbers of genes showing at least a two-fold change in response to overexpression of flavonoid-related R2R3MYBs in angiosperm transgenics (Zhang et al., 2015). Transcripts for three phenylpropanoid biosynthetic enzymes – phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and chalcone isomerase-like (CHI-L) – were among the top 25 changes ranked by either log2FoldChange or adjusted P-value (Tables S1–S3). These were the only three enzymes whose genes were upregulated by at least two-fold from among the 61 genes annotated (Bowman et al., 2017) for the shikimate and phenylpropanoid pathways of marchantia (Table S3). This suggests that MpMyb14 specifically promotes the channeling of substrate into the phenylpropanoid section of the shikimate pathway, particularly the flavonoid branch. Moreover, the activation by MpMyb14 was specific to individual genes in the large marchantia PAL and CHS gene families, which contain 10 and 24 members, respectively. The activation of CHS (family member Mapoly0021s0159) and CHI-L by MpMyb14 overexpression was confirmed by qRT-PCR in independent transgenic lines (Fig. S2d).

Biosynthetic routes to flavone O-glucuronides and riccionidin A after the chalcone isomerase/CHI-L steps have not been determined for liverworts, and there could be flavonoid biosynthetic genes upregulated in the 35Spro:MYB14 transgenics that have yet to be functionally annotated. Two ABCG-type transporter genes were significantly upregulated (Mapoly0015s0086 and Mapoly0027s0141). ABC proteins transport anthocyanins, flavones or other flavonoids into the vacuole in species such as grape (Vitis vinifera), and Medicago truncatula (Francisco et al., 2013; Hwang et al., 2016; Biala et al., 2017), and also transport lignin precursors to the cell wall (Alejandro et al., 2012).

There are alternative routes to flavone O-glucuronides and riccionidin A after the chalcone isomerase/CHI-L steps that have not been determined for liverworts, and there could be flavonoid biosynthetic genes upregulated in the 35Spro:MYB14 transgenics that have yet to be functionally annotated. Two ABCG-type transporter genes were significantly upregulated (Mapoly0015s0086 and Mapoly0027s0141). ABC proteins transport anthocyanins, flavones or other flavonoids into the vacuole in species such as grape (Vitis vinifera), and Medicago truncatula (Francisco et al., 2013; Hwang et al., 2016; Biala et al., 2017), and also transport lignin precursors to the cell wall (Alejandro et al., 2012).

There are alternative routes to flavone production in angiosperms involving either cytochrome P450 mono-oxygenase (CypP450) or 2-oxoglutarate dependent-dependent dioxygenase (2OGD) genes, with examples from both enzyme groups for flavone synthase (FNSI and FNSII) and flavanone 2-hydroxylase (F2H) (Bredebach et al., 2011; Farrow & Facchini, 2014; Du et al., 2016). The 2OGD and CypP450 groups are large gene families in plants, including in marchantia (Bowman et al., 2017).
There are five CytP450 genes among the 100 most highly upregulated genes in the RNA-seq data (Table S2), but no 2OGD genes. The only liverwort FNS candidate sequence functionally characterized to date, from Plagiochasma appendiculatum, is a 2OGD with both FNSI and F2H activity (Han et al., 2014). There is one strong marchantia BLAST hit to the P. appendiculatum sequence, Mapoly0002s0224 with 67% amino acid identity, and this is the only marchantia gene that clades in the 2OGD sub-group (DOXC28) that contains FNSI and F2H of angiosperms. However, Mapoly0002s0224 is not significantly upregulated in the 35Spro:MYB14 transgensics, suggesting that CytP450s may be involved in flavone biosynthesis in marchantia.

In addition to activation of the flavonoid biosynthetic pathway, many of the other strongly induced genes in 35Spro:MYB14 plants have predicted roles in abiotic stress or defence responses (Tables S1, S2). Analysis of the Gene Ontology terms (where available from BLAST matches) for transcripts with at least a two-fold change found that the two largest categories for either up- or downregulated genes, were ‘oxidation-reduction process’ (22% and 11% of genes, respectively) and ‘membrane’ (15% and 14% of genes, respectively). MpMyb14 may thus regulate other stress responsive pathways in addition to flavonoids. Alternatively, these changes could reflect downstream responses to the production of flavonoids, as it is suggested that flavonoids can modify general redox signalling pathways (Agati & Tattini, 2010).

MpMYB14 is essential for production of the red flavonoid pigment riccionidin A in marchantia

In order to confirm the functionality of MpMyb14 in activation of the flavonoid pathway and to examine whether there is redundancy of pathway regulation, knockout mutant lines were produced using CRISPR/Cas9 mutagenesis. Agrobacterium-mediated transformation of spores was conducted with constructs encoding two guide RNAs that targeted the MpMyb14 gene, and > 30 independent transgenic events were obtained. These plants were screened for mutations within MpMyb14 by PCR amplification and DNA sequencing; 14 lines were identified with mutations ranging from single nucleotide deletions or insertions to deletions of up to 592 nucleotides (Fig. 2a).

Wild-type (WT) marchantia plants generally lacked red pigmentation in the thallus, but developed red pigmentation in older thallus tissues when tissue culture media become exhausted. The myb14 mutants never developed red pigmentation, even when grown to an age and stage when WT plants developed strong red colour in the central part of the plant (Fig. S3). This was well illustrated by examining plant lines that were chimeric for the myb14 mutation, as somatic events were easily visualized in the haploid background (Fig. 2b). Sharply divided green and red sectors were present, and in some examples presumed myb14 CRISPR events could be seen as green sectors within the red tissues. Genotyping tissue from green or red thallus regions of the same plant confirmed their chimeric nature. Except for loss of red pigmentation, the myb14 mutants were phenotypically like the control lines, indicating a specific action of MpMyb14 (Figs 2b, S3).

Light- or nutrient-deprivation stress induces MpMyb14 expression and flavonoid production in marchantia

Although the physiological roles of the anthocyanins during abiotic stress is uncertain, it is generally accepted that they can provide screening against damaging intensities of white light and it is thought that they also function as antioxidants under...
conditions that promote oxygen free radical stress. Little is known about functions of nonphotosynthetic pigments in bryophytes such as marchantia. However, it has long been noted that red pigmentation in different liverwort species becomes more intense in high light conditions (Kny, 1890; Nagai, 1915; Herzfelder, 1921), and riccionidin A production is induced by UV-B in Antarctic liverworts (Newsham, 2010). We thus examined stress induction of riccionidin A in marchantia and whether this was mediated by MpMyb14, by exposing WT, 35S<em>pro:</em>MYB14, and myb14 mutant lines to a variety of nutrient and light stresses that are known to induce anthocyanin and/or flavonol/flavone production in angiosperms.

Transfer of WT marchantia from nutrient-rich medium to minimal medium induced production of red pigmentation within c. 7 d, which intensified with increasing time (Fig. 3a). The myb14 mutant lines completely lacked red pigmentation, and UHPLC analysis found no detectable riccionidin A but quantities of flavone O-glucuronides that were equivalent to WT (Figs 3b,c, S3). Transcripts for MpMyb14, determined by qRT-PCR, were induced by the nutrient stress treatment in both WT and the myb14-1 mutant (Fig. 3d). These findings confirmed MpMyb14 as a major activator of the flavonoid pathway with no redundant regulation for riccionidin A production. However, the presence of flavones in the myb14 mutant lines showed that there was some MpMyb14 independent activation of the core flavonoid biosynthetic pathway.

Wild-type and myb14 mutants were grown under low or high intensity white light conditions, to determine if light intensity alters flavonoid accumulation in liverworts as it does in angiosperms. Additionally, because in angiosperms the R2R3MYB genes determine the spatial and temporal regulation of flavonoids (Schwinn <em>et al.</em>, 2006; Stracke <em>et al.</em>, 2010a; Davies <em>et al.</em>, 2012a), marchantia plants transformed with a <em>MYB14</em> promoter-GUS reporter construct were used to examine whether...
MpMyb14 also contributes to spatial and temporal regulation patterning. Older WT plants accumulate red riccionidin pigmentation on the ventral midrib, particularly within tissues that contact soil (Fig. 4a). The activity of the MpMyb14 promoter in young plants, as measured by GUS, β-glucuronidase (GUS) activity staining of MYB14pro::GUS line #2. GUS activity was restricted to the ventral midrib in a similar pattern to that in mature plants, although no riccionidin A was apparent at this growth stage. (c) GUS activity pattern in MYB14pro::GUS plants grown under low (50 μmol m⁻² s⁻¹) or high (200 μmol m⁻² s⁻¹) intensity white light. Bars, 2 mm. Quantification of flavones (d) and riccionidin (e) compounds in WT and myb14-1 mutant plants grown under high or low light conditions. (f) Relative transcript abundance of MpMyb14. Means ± SE, n = 3 biological replicates are shown; significant differences (LSD, P = 0.05) between means are indicated where letters above differ.

Fig. 4 MpMyb14 promoter activity and light-responsive riccionidin accumulation. (a) A mature wild-type (WT) Marchantia polymorpha plant showing production of red riccionidin A pigment on the ventral midrib (with tissue cleared of chlorophyll shown in the right panel). (b) Activity of the MpMyb14 promoter in young plants, as measured by GUS, β-glucuronidase (GUS) activity staining of MYB14pro::GUS line #2. GUS activity was restricted to the ventral midrib in a similar pattern to that in mature plants, although no riccionidin A was apparent at this growth stage. (c) GUS activity pattern in MYB14pro::GUS plants grown under low (50 μmol m⁻² s⁻¹) or high (200 μmol m⁻² s⁻¹) intensity white light. Bars, 2 mm. Quantification of flavones (d) and riccionidin (e) compounds in WT and myb14-1 mutant plants grown under high or low light conditions. (f) Relative transcript abundance of MpMyb14. Means ± SE, n = 3 biological replicates are shown; significant differences (LSD, P = 0.05) between means are indicated where letters above differ.

Discussion

The findings on Marchantia (Marchantia polymorpha) MpMyb14 show that R2R3MYBs are conserved as key activators of abiotic stress-responsive flavonoid production between angiosperms and their most distant land plant relatives, the liverworts. Thus, this may be a character trait that has been inherited from their common early land plant ancestor which is conserved across all land plants. All of the major plant groups examined contained sequences that formed a clear phylogenetic clade with MpMyb14 at the base. As the R2R3MYB gene family has undergone less diversification during liverwort evolution than has occurred in angiosperms, the R2R3MYBs activators for anthocyanins (SG5 or SG6) and proanthocyanidins (SG5) act as part of the MBW complex, whereas the R2R3MYB activators for flavones/flavonols (SG7) do not (Feller et al., 2011). The conserved motif [DE]Lx2[RK]x3Lx6Lx3R has been identified as facilitating the MYB-bHLH interaction. Changes in any one of the conserved amino acids can reduce or abolish the bHLH interaction for Arabidopsis proteins, with amino acids L and R that start and end the Lx6Lx3R sequence of particular importance (Zimmermann et al., 2004; Dai et al., 2016). MpMYB14 has only a partially conserved motif (Fig. 5), including lacking two of the conserved residues [DE] and [RK]. MpMYB02 also has only a partial motif. However, greater conservation of the motif is found in R2R3MYBs from the moss Physcomitrella patens, and is fully conserved in those from the gymnosperm Picea.
Research of the R2R3MYB domain were aligned in CLUSTALW. The lycophyte MYBPA1, AM259485; MYBF1, FJ948477; the gymnosperms Malus BAG12893), (ROSEA1, ABB83826), hybrid (MYB12, BAJ05398), Marchantia polymorpha differing between SG5 and SG6 are indicated with asterisks. The Sequence logo of amino acid frequency at each position is shown at the top. In addition to residues of the domain for interaction with bHLH partners are indicated below the sequence. Amino acid positions identified by Schwinn et al. angiosperm R2R3MYB flavonoid regulators (Schwinn et al. 2006; Stracke et al. 2010a), MpMyb14pro:GUS indicates that induce MpMyb14, and which may regulate other branches of the flavonoid pathway and the environmental trig-

![CLUSTALW Alignment](image)

The regulatory role of MpMyb14 in marchantia shows many commonalities with the characterized flavonoid-related R2R3MYBs of angiosperms. This includes both the genes targeted within the flavonoid pathway and the environmental trigg-

and HY5 is central to regulating light-responsive changes in the transcription of AtMYB12 (SG7) and AtMYB75 (SG6) and associated flavonoid biosynthetic gene expression in Arabidopsis (Stracke et al., 2010b; Shin et al., 2013). It is possible that HY5 could contribute to the redundancy for activation of flavone gly-

coside biosynthesis that we found in marchantia. The lack of change in flavone or riccionidi A production in 35S:MYB02 plants indicates that MpMyb02 does not contribute to activation of these flavonoid pathway branches (present authors’ unpublished data).

The lack of a fully conserved bHLH interaction motif in MpMyb14 (Fig. 5) suggests that it is not forming part of an MBW complex. Thus, MpMyb14 may be more like ZmP1 (SG7) from maize, which acts independently of a bHLH partner (ZmP1 lacks three of the conserved motif residues; Hernandez et al., 2004) yet can activate production not only of flavone, but also red phlobaphene flavonoid pigments (Feller et al., 2011). Phlobaphenes are 3-deoxyflavonoids formed from the oxidation of colourless flavan-4-ol monomers or polymers, with the flavan-

4-ols being formed by a variant dihydroflavonol 4-reductase using flavanones as substrates rather than dihydroflavonols (Morohashi et al., 2012). The existence of the R2R3MYB-regulated phlobaphene pathway raises interesting questions regarding riccionidin A biosynthesis. The biosynthetic enzymes that produce riccionidin A are still unknown. It may be that only riccionidin A’s red colour and the structural similarity of the core structure to that of anthocyanins has led to it being classified as an anthocyanidin. It cannot be ruled out that it is produced by a yet to be described branch of the flavonoid pathway.
Angiosperms have at least four separate R2R3MYB subgroups (SGs 4, 5, 6 and 7) known to be involved in regulation of the flavonoid pathway (Feller et al., 2011; Cheynier et al., 2013), and each of these is typically represented as a small multigene family (SG4, 6 and 7 each have four members in Arabidopsis). By contrast, marchantia has only two putative R2R3MYB orthologues to these (MpMyb02 and MpMyb14). For the residues within the MYB domain identified as differing between SG6 and the other subgroups (Schwinn et al., 2016), in most cases MpMYB02 and MpMYB14 are more closely aligned to SG5/7 and not SG6. This is supported by the phylogenetics, as no SG6 sequences have been identified from nonangiosperm species (Du et al., 2015; and Fig. S1). The marchantia genes could reflect the more basal evolutionary state, with the other multigene flavonoid-related R2R3MYB subgroups being more recent developments through gene duplication and sub-functionalization following the acquisition of flowers, fruit and seed. The SG4 of transcriptional repressors may have arisen after the separation of the bryophyte lineage, as no bryophyte sequences corresponding to SG4 were apparent, and SG6 may have arisen after the separation of the angiosperm lineage. Alternatively, the progenitor genes corresponding to these subgroups may have been lost in bryophytes during evolution since the separation from the last common ancestor with other plant groups.

The RNA-seq data from the 35Spro:MYB14 transgenics contained candidate genes for the yet to be identified biosynthetic routes to flavones and riccionidin A in marchantia. This included two ABCG transporter genes. ABCG proteins transport the flavanone liquiritigenin to the vacuole in M. truncatula (Biala et al., 2017) and monolignols to the cell wall in Arabidopsis (Alejandro et al., 2012; Hwang et al., 2016). In grape, an ABC subgroup protein transports anthocyanins to the vacuole (Francisco et al., 2013). Marchantia has 95 annotated ABC transporter genes, with 23 assigned as ABCG and 15 as ABCB (Bowman et al., 2017). Assignment of function based solely on sequence similarity between the marchantia and angiosperm sequences is difficult, and functional studies will be required to determine the roles of the candidate genes. This is also the case for the flavone synthase gene candidates. The liverwort P. appendiculatum FNSI/F2H is a 2OGD (Han et al., 2014), as is the FNSI of the horsetail species Equisetum arvense, which phylogenetically is positioned between bryophytes and angiosperms (Bredebach et al., 2011). However, only CtrpP450 genes were strongly upregulated by MpMyb14.

The major non-carotenoid red pigments of angiosperms are the mutually exclusive anthocyanins and betacyanins. Betacyanins replace anthocyanins in some families of the plant order Caryophyllales (Jain & Gould, 2015; Lloyd et al., 2017). Although structurally distinct and the products of different biosynthetic pathways, betacyanins and anthocyanins are induced by the same set of abiotic stresses. They are both vacuole-located glycosides and it has been suggested that they serve similar functions, by screening excess light but also through scavenging reactive oxygen species (ROS) (Jain & Gould, 2015). The riccionidin pigments of liverworts are another group of structurally distinct red pigments also induced by a similar variety of abiotic stresses. However, they are principally cell wall-bound aglycones, which would limit antioxidant function. Thus, the light screening ability rather than ROS scavenging may be the main function in liverworts, and may have been the principal function in the early land plant ancestor. In this case, there would be an evolutionary drive towards the red colour itself rather than the specific structure generating it. A previously unreported glycoside of riccionidin A was detected in the 35Spro:MYB14 transgenics, and at trace amounts in wild-type (WT) plants. Compared to the amount of cell wall-bound riccionidin, the amount of soluble riccionidin glycoside in WT plants was very low, probably too low to be significant in stress tolerance. It is possible that it could represent a transport intermediate between the site of synthesis and the cell wall.

Anthocyanins and riccionidin A may have arisen independently during evolution since the last common ancestor of angiosperms and liverworts. Yet as the biosynthesis of these structurally distinct pigments is controlled by putatively orthologous genes it raises the question of whether one ancestral compound or both compounds were present in the common ancestor of all land plants. Notably, there is one report of riccionidin A in an angiosperm species, although there is no information on the relationship of the biosynthetic pathway to that of liverworts (Taniguchi et al., 2000). If both types of pigment were present in a common ancestor then retention of anthocyanins in angiosperms may have been favoured as they can generate a wider range of colours and have ROS scavenging activities. Interestingly, it is thought that when the ancestor of the Caryophyllales species Beta vulgaris (beet) switched from producing anthocyanins to betacyanins it retained the SG6 R2R3MYB for pathway activation (Hatlestad et al., 2015; Lloyd et al., 2017). Analysis of the beet betacyanin-related BmMYB1 found that it had amino acid changes that caused it to lose the ability to interact with bHLH partners and that it could activate the betalain biosynthetic genes independent of the MBW complex, thus showing similarities to the action of MpMYB14.

We show here that the R2R3MYB gene MpMyb14 is a key regulator of flavonoid production in the liverwort M. polymorpha. Knockout lines of myb14 lose all red flavonoid pigmentation from riccionidin A, whereas overexpression confers production of large amounts of flavonoids, activation of the associated biosynthetic genes, and constitutive red pigmentation. MpMyb14 expression and riccionidin A production are induced by the same light- and nutrient-deprivation stress triggers in marchantia as for anthocyanins in angiosperms, despite riccionidin A being cell wall-located and anthocyanins vacuolar. The results suggest that R2R3MYB-activation of flavonoid production is a conserved character across land plants that may have arose early during land colonization. The regulatory system in marchantia could reflect a basal state that has subsequently been expanded through duplication and sub-functionalization of R2R3MYB genes during evolution, facilitating the diversity of pigmentation and flavonoid functions observed in angiosperms.

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Author contributions
K.M.D., S.C.D., K.E.S., N.W.A. and B.R.J. conceived the project; N.W.A. performed experiments; T.K.M. conducted metabolite analyses; A.H.T., K.M.D. and J.L.B. conducted bioinformatic analysis; N.W.A., W.C., S.C.D., K.E.S., B.R.J. and K.M.D. contributed to experimental design and interpretation of data; and K.M.D. and N.W.A. wrote the manuscript, and all authors contributed to manuscript editing.

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References


Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Phylogenetic relationships of Marchantia polymorpha MybB02 and MpMyb14 to phenylpropanoid-related R2R3MYBs of other species.

Fig. S2 MpMyb14 overexpression activates flavonoid synthesis in Marchantia polymorpha L.

Fig. S3 MpMyb14 is essential for nutrient stress-induced riccinidin accumulation in Marchantia polymorpha.

Fig. S4 High light induces MpMyb14 transcription and flavonoid accumulation in Marchantia polymorpha.

Table S1 Adjusted P-value, BaseMean, log2FoldChange and annotation for the 223 genes showing an adjusted P-value < 1 × 10⁻⁵ for transcript abundance difference in RNAseq
analysis of *Marchantia polymorpha* 35Spro:MYB14 transgenics vs control plants

**Table S2** BaseMean, log2FoldChange, adjusted *P*-value and annotation for the 100 genes showing the largest fold increase in transcript abundance in RNAseq analysis of *Marchantia polymorpha* 35Spro:MYB14 transgenics vs control plants.

**Table S3** BaseMean and log2FoldChange values for genes of the shikimate and phenylpropanoid pathway from RNAseq analysis of *Marchantia polymorpha* 35Spro:MYB14 transgenics vs control plants.

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