



# The Onion (*Allium cepa* L.) *R2R3-MYB* Gene *MYB1* Regulates Anthocyanin Biosynthesis

Kathy E. Schwinn<sup>1</sup>, Hanh Ngo<sup>1</sup>, Fernand Kenel<sup>2</sup>, David A. Brummell<sup>1</sup>, Nick W. Albert<sup>1</sup>, John A. McCallum<sup>2</sup>, Meeghan Pither-Joyce<sup>2</sup>, Ross N. Crowhurst<sup>3</sup>, Colin Eady<sup>2†</sup> and Kevin M. Davies<sup>1\*</sup>

<sup>1</sup> The New Zealand Institute for Plant & Food Research Limited, Palmerston North, New Zealand, <sup>2</sup> The New Zealand Institute for Plant & Food Research Limited, Christchurch, New Zealand, <sup>3</sup> The New Zealand Institute for Plant & Food Research Limited, Auckland, New Zealand

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### \*Correspondence:

Kevin M. Davies  
kevin.davies@plantandfood.co.nz

### † Present address:

Colin Eady,  
New Zealand Agriseeds Ltd,  
2547 Old West Coast Road RD1,  
Christchurch, New Zealand

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Bulb color is an important consumer trait for onion (*Allium cepa* L., Alliioideae, Asparagales). The bulbs accumulate a range of flavonoid compounds, including anthocyanins (red), flavonols (pale yellow), and chalcones (bright yellow). Flavonoid regulation is poorly characterized in onion and in other plants belonging to the Asparagales, despite being a major plant order containing many important crop and ornamental species. *R2R3-MYB* transcription factors associated with the regulation of distinct branches of the flavonoid pathway were isolated from onion. These belonged to sub-groups (SGs) that commonly activate anthocyanin (SG6, *MYB1*) or flavonol (SG7, *MYB29*) production, or repress phenylpropanoid/flavonoid synthesis (SG4, *MYB4*, *MYB5*). *MYB1* was demonstrated to be a positive regulator of anthocyanin biosynthesis by the induction of anthocyanin production in onion tissue when transiently overexpressed and by reduction of pigmentation when transiently repressed via RNAi. Furthermore, ectopic red pigmentation was observed in garlic (*Allium sativum* L.) plants stably transformed with a construct for co-overexpression of *MYB1* and a *bHLH* partner. *MYB1* also was able to complement the acyanic petal phenotype of a defined *R2R3-MYB* anthocyanin mutant in *Antirrhinum majus* of the asterid clade of eudicots. The availability of sequence information for flavonoid-related MYBs from onion enabled phylogenetic groupings to be determined across monocotyledonous and dicotyledonous species, including the identification of characteristic amino acid motifs. This analysis suggests that divergent evolution of the *R2R3-MYB* family has occurred between Poaceae/Orchidaceae and Alliioideae species. The DNA sequences identified will be valuable for future analysis of classical flavonoid genetic loci in *Allium* crops and will assist the breeding of these important crop species.

**Keywords:** onion, garlic, *R2R3-MYB*, anthocyanin, regulation, Asparagales, transgenic

## INTRODUCTION

Alliums have been used for culinary purposes for over 5000 years, and bulb onion (*Allium cepa* L.) is currently one of the most widely cultivated of all crop plants. It belongs to the Asparagales, a major plant order accounting for around 29,000 of the approximately 60,000 known monocotyledonous species, including those of the families Amaryllidaceae, Alliaceae (now

the Alliioideae), Asparagaceae, Hemerocallidaceae, and Orchidaceae. The genus *Allium* is in the Alliioideae sub-family within in the family Amaryllidaceae, which is part of the 'core' Asparagales (Angiosperm Phylogeny Group III, 2009). Despite containing many economically important species, the Asparagales are poorly characterized at the molecular genetic level compared with most leading crop plants. One reason for this is the frequent occurrence of large genome sizes in these plant families (Leitch et al., 2009). Indeed, onion has one of the largest genomes among crop plants, with its 17 pg (16 Gb) haploid nuclear genome being more than 100-fold larger than that of *Arabidopsis*.

Color is a key breeding trait in onion, both for the visual appeal to consumers and the increasing association between plant pigments and human health benefits (Martin et al., 2011; Davies and Espley, 2013). Bulbs may be white, yellow, gold, pink, or red, because of variation in the production of different flavonoid compounds: red anthocyanins, pale yellow flavonols, and bright yellow chalcones. There is also a rare 'chartreuse' color containing unidentified green compounds (Green et al., 1997; Khar et al., 2008). Onion bulb color is the result of the interaction of at least five major loci – *I*, *C*, *G*, *L*, and *R*. Colouration requires recessive alleles for the *I* locus, which has a dominant white action, and at least one functional allele for the *C* locus (Kim et al., 2005b). The other loci have modifying effects on the yellow, red, or chartreuse colouration. The basis of the recessive *g* character, for chartreuse, is not known, although the possible involvement of chalcones has been suggested (Kim et al., 2004c). DNA sequences for several of the flavonoid biosynthetic genes have been identified in onion, and some of these have been assigned to specific chromosomes (Masuzaki et al., 2006). This has enabled some of the classical genetic loci affecting bulb color to be defined. The *L* locus has been shown to correspond to an anthocyanidin synthase (*ANS*) gene (Kim et al., 2005a). A non-functional mutant allele (*ANS-l*) accounts for the difference between yellow and red Brazilian-type onions (Kim et al., 2005a), and a second allele with reduced transcription (*ANS-p*, sometimes referred to as the *P* locus) produces pink colouration (Kim et al., 2004b). Mutations within the *R* locus, which encodes a dihydroflavonol 4-reductase (*DFR*) gene, account for the difference between some yellow and red 'USA-type' onions (Kim et al., 2004a). In addition, loss of chalcone isomerase (*CHI*) function results in production of gold bulbs, presumably because of the accumulation of chalcones (Kim et al., 2004c).

Anthocyanins, flavonols, and chalcones are all products of the flavonoid biosynthetic pathway. The flavonoid pathway is one of the best-characterized secondary metabolite pathways in plants, with all the major biosynthetic steps and many of the pathway regulatory genes defined for a range of model and crop species (Grotewold, 2006; Hichri et al., 2011; Davies et al., 2012a). The primary point of regulation for flavonoid biosynthesis commonly occurs at the transcriptional level. In all species studied to date, anthocyanin synthesis is regulated by a 'MBW' transcriptional activation complex consisting of R2R3-MYB and bHLH transcription factors (TFs) and a WD-repeat (WDR) protein, which acts directly upon the promoters of flavonoid biosynthetic genes (Ramsay and Glover, 2005; Feller et al.,

2011; Hichri et al., 2011; Davies et al., 2012a). Distinct R2R3-MYBs regulate different branches of the flavonoid pathway, forming sub-groups (SG) within the large MYB family, such as anthocyanins (SG 5 and 6), proanthocyanidins (PAs, condensed tannins, SG5), flavonols (SG7), and isoflavonoids (SG2; Stracke et al., 2001; Feller et al., 2011; Shelton et al., 2012). Furthermore, the genes encoding the R2R3-MYB activators (which contain two repeats of a MYB DNA-binding domain) are thought to be key in determining the spatial and temporal occurrence of flavonoids in plants (Schwinn et al., 2006; Stracke et al., 2007; Allan et al., 2008; Feller et al., 2011; Davies et al., 2012a). Other TFs can have a repressive effect on phenylpropanoid production, either by interfering with the formation of the MBW activation complex or through formation of MBW complexes with an active repressive action (Davies et al., 2012a; Gray et al., 2012; Albert et al., 2014a).

The main models for studies on regulation of anthocyanin biosynthesis have been the monocot maize (*Zea mays*) and the dicots *Arabidopsis* (*Arabidopsis thaliana*), petunia (*Petunia hybrida*), and snapdragon (*Antirrhinum majus*), with crops such as apple (*Malus domestica*), grape (*Vitis vinifera*), and rice (*Oryza sativa*) being extensively studied more recently (Allan et al., 2008; Feller et al., 2011; Hichri et al., 2011; Gray et al., 2012). The regulatory system appears to be well conserved across the dicots and the monocots studied to date, but with divergence within the regulatory gene clades. While the separation into SG5 (PA-related) and SG6 (anthocyanin-related) is conserved in dicot species studied, the anthocyanin R2R3-MYB regulators identified from the Poaceae and Orchidaceae cluster in SG5 (Davies et al., 2012a). To date, anthocyanin-related SG6 R2R3-MYB genes from monocot species have been characterized only in Asiatic lily (*Lilium* spp., belonging to the order Liliales; Yamagishi et al., 2010). Sequences for other flavonoid-related MYB SG have not so far been characterized from non-Poaceae monocot species. In particular, genes that regulate flavonoid synthesis have not yet been identified in onion, although it has been suggested that the classical *C* locus may correspond to a regulatory gene (Kim et al., 2005b). In this investigation, we identify a range of R2R3-MYB factors that putatively regulate flavonoid production in onion. One, MYB1 is characterized as a key positive regulator of anthocyanin production.

## MATERIALS AND METHODS

### Isolation of cDNA and Genomic DNA Sequences

Pieces of acyanic internal sheaths from seedlings of onion 'California Early Red' were cultured on 1/2 MS medium under 20–50  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light from Osram 36 W Grolux fluorescent tubes (16 h photoperiod) at 25°C. Under these conditions, anthocyanin production was sporadically initiated by 24 h and increased over 96 h. *MYB1* was isolated from tissue at 48 h using 3'- and 5'-RACE (Invitrogen 5'-RACE system). Genomic regions corresponding to the *MYB1* cDNA sequence were isolated using PCR with primers designed to the cDNA. A genomic region upstream of the coding sequence was obtained from three overlapping PCR products using the GenomeWalker™ kit

(Clontech, Mountain View, CA, USA). Primer sequences are given in Supplementary Table 1.

SG4 (MYB4, MYB5) gene fragments were first isolated by 3'-RACE as described for *MYB1*, with full-length sequences subsequently identified from a transcriptome database generated using TGICL software as described in Crowhurst et al. (2008) from sequences from the following sources: GenBank NT division mRNA sequences, Onion ESTs (Kuhl et al., 2004), RNASEQ data from NCBI BioProjects PRJNA175446 and PRJNA175449 (Duangjit et al., 2013), and PRJNA238142 and PRJNA60277 (Baldwin et al., 2012). The SG7 (MYB29) sequence also was identified from the transcriptome database. Primer sequences are given in Supplementary Table 1. The onion MYB sequences were assigned numbers to reflect the order in which they were identified, as per the guidelines of Gray et al. (2009).

## Phylogenetic and Promoter Sequence Analysis

Maximum likelihood phylogenetic trees based on full-length sequences were generated using PhyML with 1000 bootstrap replicates (Guindon and Gascuel, 2003) upon MUSCLE (Edgar, 2004) amino acid alignments (with manual correction) within Geneious (v7). Analysis of the putative TF recognition sites in the promoter regions used the PLACE database (Higo et al., 1999). GenBank database accession numbers are *Allium cepa* MYB1 (KX785130), MYB4 (KX785131), MYB5 (KX785132), MYB29 (KX785133); *Antirrhinum majus* ROSEA1 (ABB83826), MIXTA (CAA55725), MYB308 (P81393); *Aquilegia formosa* MYB17 (ACQ82820); *Arabidopsis thaliana*, MYB4 (*At4g38620*), MYB12 (ABB03913), MYB20 (*NP\_176797*), MYB24 (*AF175987*), MYB75/PAP1 (AAG42001), MYB79 (AEE83284), MYB103 (AED96722), MYB106 (AEE73615), MYB123/TT2 (CAC40021); *Dahlia pinnata* (syn. *variabilis*) MYB1 (AB601003); *Gerbera hybrida* MYB1 (CAD87007), GMYB10 (CAD87010); *Glycine max* MYB22 (LOC780553); *Gynura bicolor* MYB1 (BAJ17661); *Ipomoea nil* MYB1 (BAE94391); *Lilium* (hybrid division I) MYB6 (BAJ05399), MYB12 (BAJ05398); *Lotus japonicus* TT2a (AB300033); *Malus x domestica* MYB10 (ACQ45201), MYB16 (ADL36756); *Medicago truncatula* LAP1 (ACN79541); *Oncidium* Gower Ramsey MYB1 (ABS58501); *Oryza sativa* C1 (BAD04024), IF35 (BAB20661), Plw-OSB1 (BAB64301); *Petunia x hybrida* AN2 (AAF66727), PH4 (ADX33331); *Phalaenopsis schilleriana* UMyb6 (FJ039860); *Picea mariana* MBF1 (U39448); *Vanda hybrida* MYB6 (ADQ57817); *Vitis vinifera* MYBA1 (BAD18977), MYB4a (ABL61515), MYBPA1 (AM259485), MYBF1 (FJ948477); *Zea mays* C1 (AAA33482), P1 (AAC49394), MYB38 (P20025).

## Analysis of Transcript Abundance Using qPCR

Quantitative RT-PCR (qPCR) was used to measure transcript abundance for *MYB1*, *CHS-A*, and *DFR-A* in white and red colored regions of onion seedlings. *DFR-A* (AY221249) is the only significantly active DFR gene in the onion bulb (Kim et al., 2004a) and *CHS-A* (AY221244) is one of the two significantly active CHS genes (Kim et al., 2005b). Outer leaves of red

onion seedlings were removed to expose the first fully white sheath beneath. Each seedling was cut in half longitudinally and the white sheath tissue of one half was immediately flash frozen in liquid N<sub>2</sub>. The other half was placed on moistened paper in a Petri dish and left to become pigmented. Pigmented sheath tissue was sampled after approximately 3 days. Two samples were analyzed, each consisting of sheath tissue from three seedlings. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). First strand cDNA was made from 500 ng DNase I-treated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) and priming from the supplied anchored oligo dT<sub>18</sub>. qPCR (on cDNA diluted 50-fold) was performed as described in Albert et al. (2011). Relative transcript abundance was determined by comparative quantification. Primers used are given in Supplementary Table 1.

## Particle Bombardment Experiments

Overexpression vectors for *MYB1* (pKES22) and the maize anthocyanin bHLH factor *Lc* (pNASA3) were generated by cloning the respective cDNAs behind a *CaMV* 35S promoter (35*Spro*) in pART7. The RNAi vector for *MYB1*, pHMN3, was based on pDAH2 (Davies et al., 2012b). The construct, driven by 35*Spro*, had inverted repeats to give hairpin RNA. Primers used in making these constructs are given in Supplementary Table 1. Other constructs used were for a GFP internal transformation control (pPN93; 35*Spro*:GFP-ER; Shang et al., 2007), a snapdragon anthocyanin bHLH factor (pJAM1528; 2x35*Spro*:AmMutabilis in pJIT60; supplied by Cathie Martin) and a maize anthocyanin MYB factor (pLN44; 35*Spro*:ZmC1 in pART7; Albert et al., 2010). Constructs were transformed into dorsal petals of the snapdragon *rosea*<sup>dorsea</sup> mutant line (Schwinn et al., 2006), seedling tissue of onion 'California Red,' and shoot tissue from bulbs of an unidentified commercial cultivar of garlic (*Allium sativum*). Particle bombardment using a helium-driven particle in flow gun was carried out generally as described in Davies et al. (2012b). Tissue was not surface sterilized before bombardment. Each experiment involved the bombardment of at least three pieces of tissue with each piece bombarded once (onion/garlic) or twice (snapdragon). Each experiment was replicated at least twice except for the experiment involving pJAM1528. Bombardment used a 30 ms burst of helium at a pressure of 200 kPa for the RNAi experiments, 300 kPa for the other experiments in onion and garlic, and 400 kPa for the experiments in snapdragon. DNA concentrations in the gold particle preparations were 0.4 μg/1 mg gold for the GFP construct, which was added to all preparations, and either 0.8 or 1.0 μg/1 mg gold for each of the other constructs added to a preparation. Post-bombardment onion tissue was placed on moistened filter paper in a Petri dish. Images were recorded using a Leica M205FA microscope with a Leica DFC550 digital camera or an Olympus SZX fluorescent microscope (Olympus Corp., Tokyo, Japan) with a Leica DC500 digital camera (Leica Camera AG, Solms, Germany). Any post image-capture adjustments of brightness or contrast for clarity of the GFP signal were applied equally to all images in a figure.



## Agrobacterium-Mediated Transformation of *Allium sativum*

Transgenic garlic plants ('Printanor') were produced using the method of Kenel et al. (2010). Cassettes for both 35S:*MYB1* and 35S:*ZmLc* were introduced into pART27H, a modified version of pART27 (Gleave, 1992) that has the *NPTII* selectable marker replaced with an *HPTII* (hygromycin) expression cassette. A schematic of the plasmid is shown in Supplementary Figure 2. Presence of the selection marker *HPTII* was detected using PCR on genomic DNA isolated from tissue culture leaf tissue.

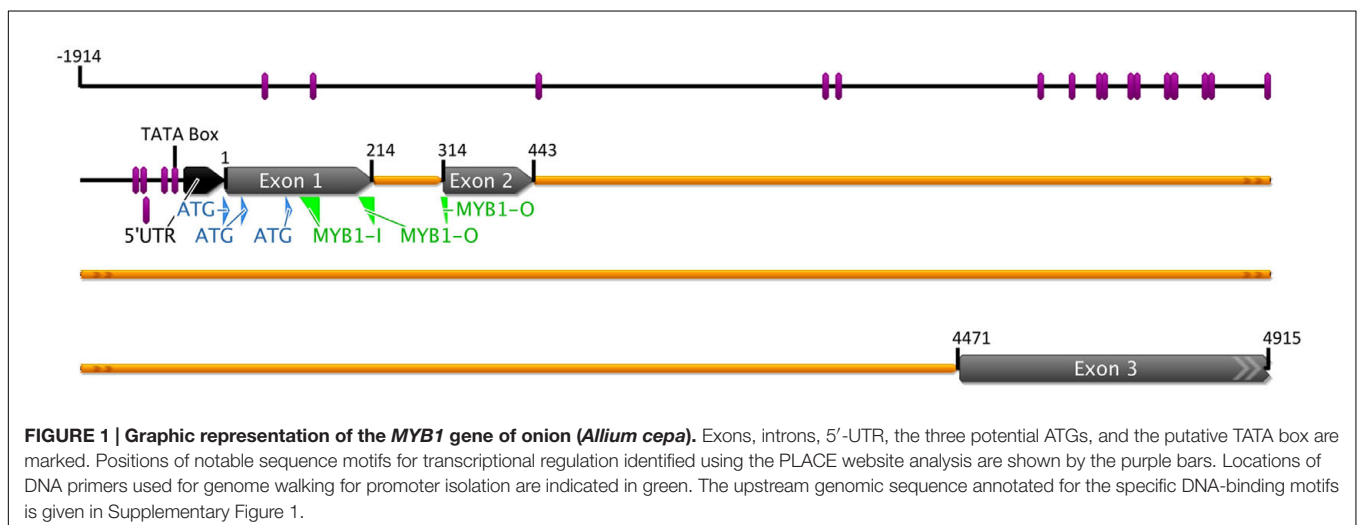
## RESULTS

### Identification of a Candidate Anthocyanin-Related R2R3-MYB Factor

Candidate *R2R3-MYB* genes for anthocyanin regulation were amplified using 3'-RACE PCR from cDNA derived from RNA purified from onion tissue that was induced to produce anthocyanins (see "Materials and Methods"). Degenerate oligonucleotides designed to the conserved MYB DNA-binding domain of R2R3-MYB genes were used as forward primers (Supplementary Table 1). Several *MYB*-related sequences were obtained, including one sequence with similarity to anthocyanin-related SG6 *MYB* genes. The partial cDNA sequence for this candidate gene was extended by 5'-RACE, followed by PCR amplification of a full cDNA coding sequence. This gene was named *MYB1* (GenBank accession KX785130). The *MYB1* cDNA is 1036 bp long with three possible ATG translation initiation codons, all of which are in frame from the first ATG. The second of these has the best fit to the Kozak consensus sequence, and on this basis the deduced polypeptide sequence consisted of 253 amino acids. The closest matches to *MYB1* on a BLASTp search of the NCBI non-redundant protein sequence database were anthocyanin-related R2R3-MYB proteins, but from dicot rather than monocot species (data not shown).

Based on data from other species it was expected that a small multigene family of anthocyanin-related R2R3-MYB activators would be present in onion. A second 3'-RACE sequence was isolated that was identical to *MYB1* except for possessing a 92 bp direct repeat in the coding sequence, and a truncated 3'-UTR (21 bp in length) containing one single nucleotide polymorphism. Compared with *MYB1*, the direct repeat altered the predicted polypeptide sequence, causing 10 sequential variant amino acids followed by a truncation of 75 amino acids in the C-terminus due to a premature stop codon (data not shown). It is not known if this cDNA represents an allele of *MYB1*. To look for further *MYB1* related alleles or genes, a subsequently available in-house transcriptome database was searched (BLASTn and BLASTp) with the *MYB1* sequence. The transcriptome examined contained approximately 296,942 singleton and contig assemblies from sequencing 13 different onion mRNA derived libraries. No additional anthocyanin-related *MYB* genes were detected. However, four partial sequences (between 225 and 615 bp) were identified that differed from *MYB1* by less than 10 nucleotides and which probably represent either allelic differences or sequencing errors (data not shown).

The genomic *MYB1* sequence (from the ATG furthest upstream through the stop codon) was isolated from the same onion cultivar used to isolate the full cDNA. It was 4.915 kb in length and the gene contained three exons and two introns (intron 1 is 0.099 kb and intron 2 is 4.027 kb; **Figure 1**), a common structure in plant *R2R3-MYB* genes. In addition a total of 1.914 kb upstream of the distal start codon was isolated by genome walking. To understand how this gene may be regulated in response to light and other factors, the PLACE database for predicted DNA-binding recognition sites (Higo et al., 1999) was used to analyze the proximal 1.0 kb of this upstream region. A predicted TATA box was located at -25 bp. There was a cluster of predicted recognition motifs of interest within the -500 bp region, including candidate MYB and bHLH sites and a range of sites found in promoters of genes up-regulated by light, such as the T-Box, GT-1, and SORLIP1AT motifs (**Figure 1**; Supplementary Figure 1).

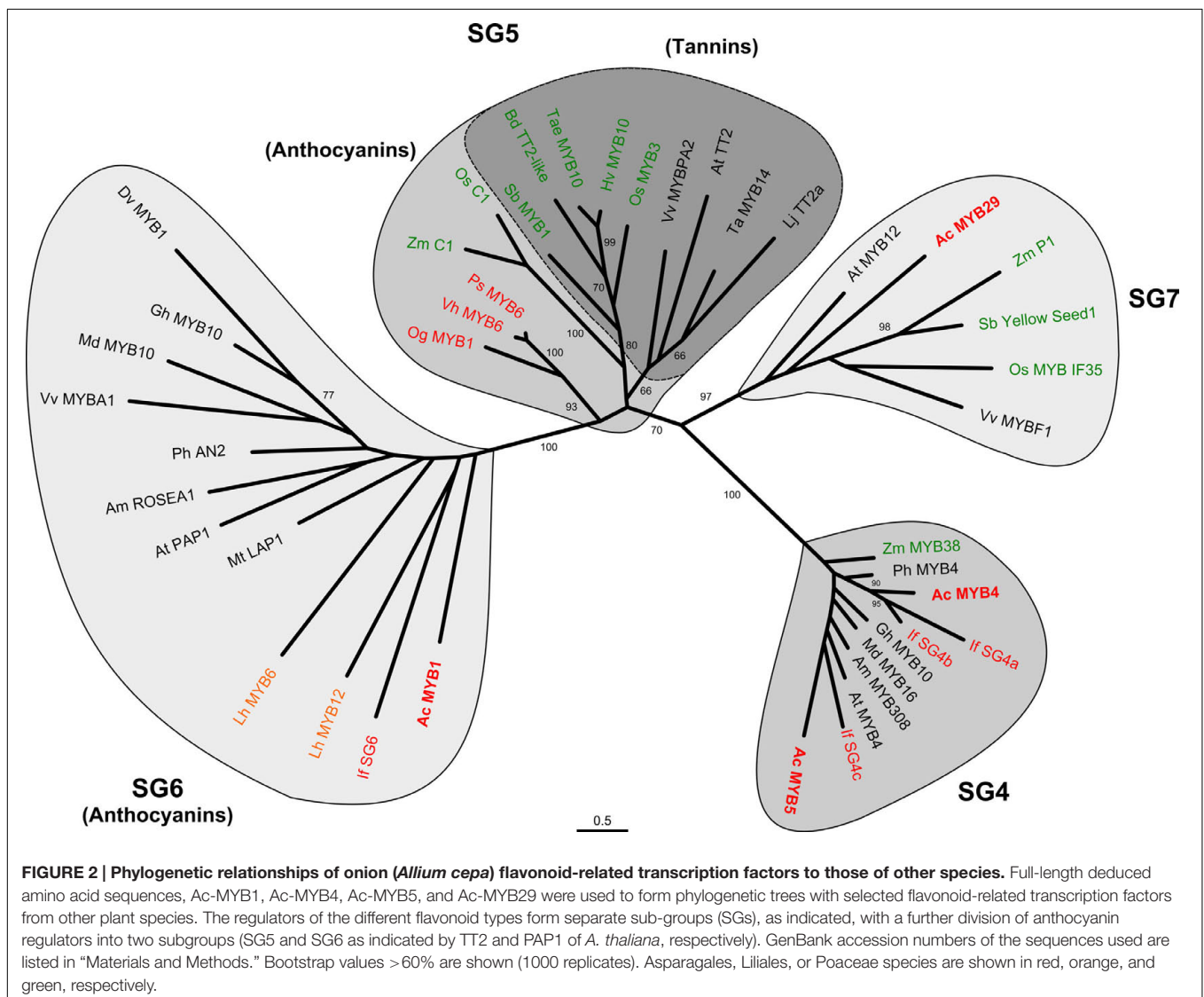


## Identification of Candidates for Other Flavonoid-Related R2R3-MYB Factors

Flavonols are other key flavonoid metabolites in onion bulbs. Given that R2R3-MYB proteins of SG7 have been found to regulate the production of flavonols in a range of dicot species and in the monocot maize, we reasoned that SG7 MYB genes regulate flavonol production in onion. Several highly similar sequences were retrieved from a transcriptome database when searched (BLASTp) with the sequence for the flavonol regulator At-MYB12 from *Arabidopsis*, including a singleton sequence encoding a full-length candidate, *MYB29* (GenBank accession KX785133). The *MYB29* sequence was 1076 bp long, with a putative 5'- and 3'-UTR of 19 and 187 bp, respectively. The EST originated from a mixed tissue mRNA sample of the doubled-haploid red onion genotype 5225. Analysis of related sequences identified one additional SG7 gene family member in the data from the doubled-haploid line, represented by a partial 5'-EST (data not shown).

The deduced amino acid sequence of *MYB29* was used to search (BLASTp) the NCBI non-redundant protein sequence database. The closest matches to *MYB29* were At-MYB12 and another SG7 protein, Zm-P1, a regulator of flavone and phlobaphene production in maize. The motif identified as conserved among flavonol-related SG7 R2R3-MYBs ([K/R][R/x][R/K]xGRT[S/x][R/G]xx[M/x]K; Stracke et al., 2001) was fully conserved in *MYB29* as KKRRKGRTRSRSAMK.

The initial 3'-RACE screen for anthocyanin MYBs predominantly isolated R2R3-MYB sequences belonging to SG4. Characterized SG4 sequences from other species encode proteins with a repressive action on genes of the phenylpropanoid pathway. Full-length cDNAs for two of the genes from the screen, *MYB4* and *MYB5* (GenBank accessions KX785131 and KX785132, respectively), were subsequently identified in the transcriptome database. The *MYB4* sequence was 844 bp long with a deduced peptide of 213 amino acids, and matched a partial EST present on GenBank (CF441129). *MYB5*



was 1144 bp in length with a deduced peptide of 236 amino acids. The closest matches to MYB4 or MYB5 on a BLASTp search of the GenBank Swiss-Prot database were Am-MYB308 from snapdragon and At-MYB4 from Arabidopsis. As with other members of SG4, MYB4, and MYB5 contain in their C-terminals an ERF-associated Amphiphilic Repression (EAR) domain (C2 motif) and the IsrGIDPxT/NHR (C1) motif as defined by Kranz et al. (1998) (Supplementary Figure 2).

## Phylogenetic Analysis of the Candidate Flavonoid Regulators

The relationships of MYB1, MYB4, MYB5, and MYB29 to a range of characterized, flavonoid-related R2R3-MYBs were examined by phylogenetic analysis (Figure 2). The candidate onion sequences grouped with the expected flavonoid regulators. Specifically, MYB29 grouped with SG7 (flavonol/flavone-related) factors, while MYB4 and MYB5 grouped with SG4 (repressors of flavonoid biosynthesis) factors, although in different subclades within SG4. MYB5 showed some amino acid residue changes within the conserved MYB domain as well as some variation in other C-terminal motifs that have been categorized by Cavallini et al. (2015) (Supplementary Figure 2).

There are two clades within the anthocyanin-related R2R3-MYBs, SG5, and S6, based on sequences that are currently available (Yamagishi et al., 2010; Feller et al., 2011; Davies et al., 2012a). These may be defined as whether sequences separate with Arabidopsis At-MYB75 (PAP1) or At-MYB123 (TT2), which regulate anthocyanin and proanthocyanidins (PAs or condensed tannins), respectively. MYB1 grouped with anthocyanin-related factors in SG6, including ones from another Asparagales taxon (*Iris fulva*; If-SG6) and a taxon in the monocot order Liliales (*Lilium hybrida*, Asiatic lily; Lh-MYB6, Lh-MYB12). This contrasts with the anthocyanin-regulating R2R3-MYBs from orchid taxa (Og-MYB1, Vh-MYB6, Ps-MYB6), which despite being members of the Asparagales, are located within SG5 along with representative sequences from the monocot order Poales.

Alignment of a region of 104 amino acid residues containing the MYB domain of SG5 and SG6 sequences (including MYB1) identified five conserved amino acid or motif differences among the clades (Supplementary Figure 3). These were [R] versus [N/H/K], [C/F] versus Y, R versus G, X versus N, and A versus D for the SG6/PAP1 versus SG5/C1-TT2 clade, respectively. These differences are strongly conserved within each clade and are a likely cause of the clade separation. Notably, the A versus D (position 90 in Supplementary Figure 3) was the start of a DNEI motif that is absolutely conserved in the SG5 sequences (and other subgroups like SG4 and SG7) but absent in the SG6 sequences, where the motif was ANDV in all but one example. Both SG5 and SG6 proteins (including MYB1) contain a conserved motif ([D/E]Lx<sub>2</sub>[R/K]x<sub>3</sub>Lx<sub>6</sub>Lx<sub>3</sub>R) within the MYB domain that is necessary to bind bHLH partners (Zimmermann et al., 2004), and is an essential component to the formation of MBW activation complexes.

Within the variable C-terminal region, a motif has been identified as defining the anthocyanin-related SG6 proteins. This was initially identified as KPRPR[S/T]F when SG assignments were made for Arabidopsis (Stracke et al., 2001), and later modified to [K/R]P[Q/R]P[Q/R] based on the Asiatic lily SG6 sequences (Yamagishi et al., 2010). This motif was reasonably conserved within MYB1, as KPQPxxx, but is very poorly conserved in the Orchidaceae and Poaceae sequences that form the C1-TT2 clade (Figure 3). A second motif, closer to the C-terminal, was conserved within the C1-TT2 clade, although it aligned less consistently than the previously defined KPRPR[S/T]F. This is VWAPKAVRCT in C1 and VIRTKAIRCS in TT2, and it was strongly conserved except in Vh-MYB6 (Figure 3). The V[V/I]RTKA sequence is strongly conserved in a range of R2R3-MYBs involved in activation of PA production (Liu et al., 2014).

## MYB1 Transcript Abundance Correlates with Anthocyanin Production

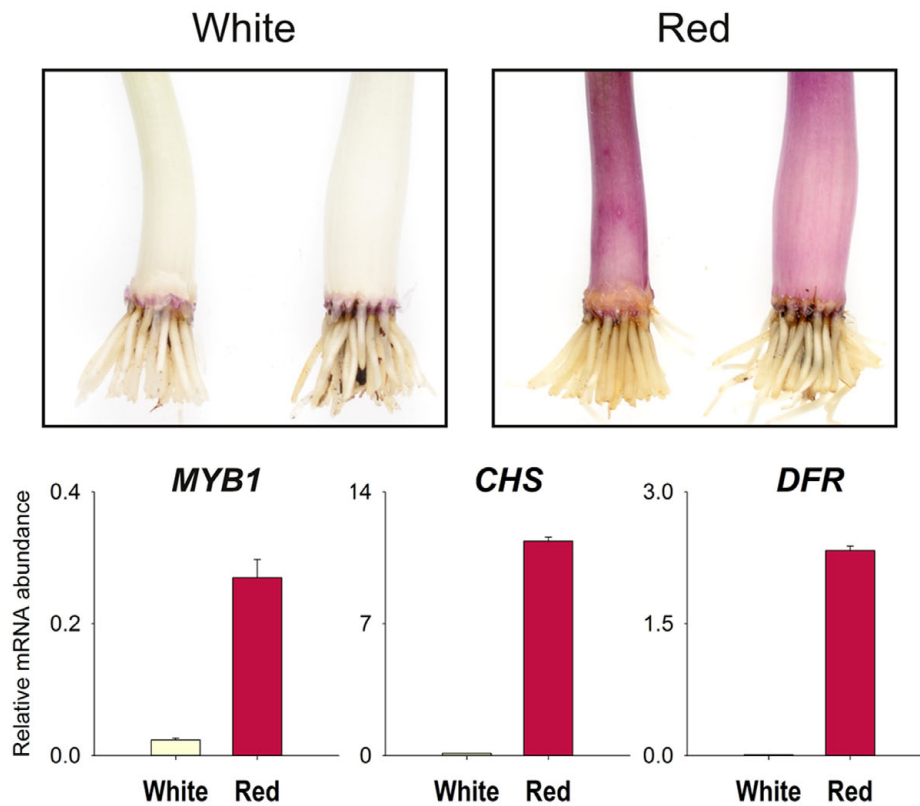
The spatial and temporal expression patterns of the R2R3-MYB genes have been shown to be important in determining anthocyanin phenotypes in a range of species. Transcript abundance for *MYB1* and two anthocyanin biosynthetic genes, *chalcone synthase* (*CHS*) and *DFR*, was compared between pooled samples of white sheaths and sheaths in which anthocyanin production was induced. Transcript amounts for *MYB1*, *CHS*, and *DFR* were much higher in the scales that had become pigmented (Figure 4).

## Transient Overexpression of MYB1 Induces Anthocyanin Production

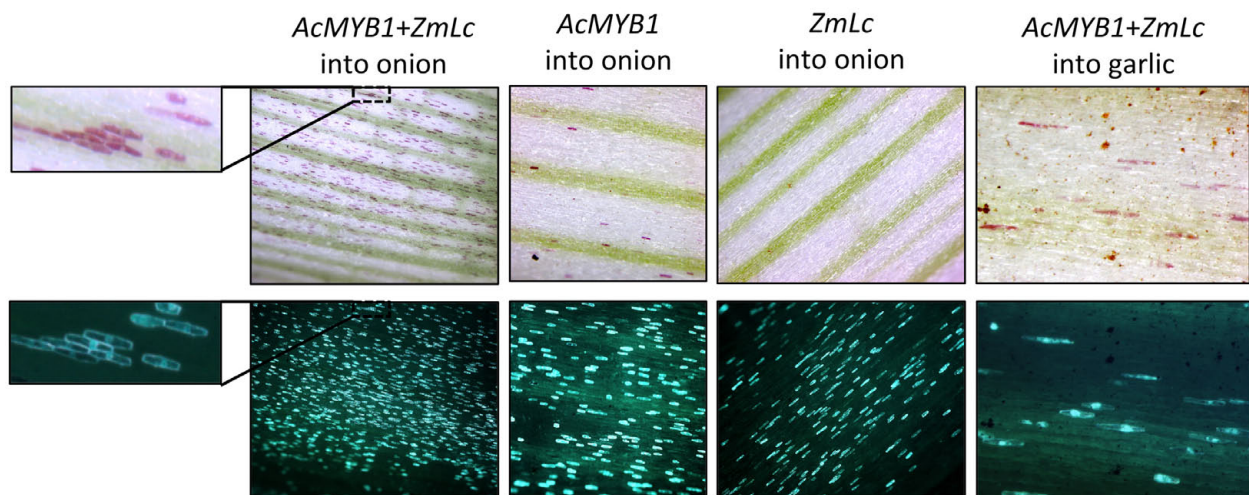
The ability for MYB1 to activate anthocyanin synthesis was tested in both onion and garlic using biolistic transformation (Figure 5; Supplementary Figure 4). Introduction of 35S:*MYB1* into acyanic sheath tissue of inner leaves or inner sprouts of red onion seedlings resulted in single cell red foci at low frequency that were visible by 48 h post-bombardment. However, co-bombardment of 35S:*MYB1* with constructs for heterologous anthocyanin bHLH regulators (*Zm-Lc* and *Am-Mutabilis*) resulted in a greater frequency of red cells (Figure 5; Supplementary Figure 4) and these became visible earlier. Particularly in green sprout tissue, the red cells were visible within 12–24 h post-bombardment. The anthocyanin pigmentation colocalized with GFP fluorescence, further indicating that the pigmentation was due to introduction of the TFs and not from autonomous activation of pigmentation. Autonomous activation does occur, but its delayed timing allowed sufficient time to conduct the experiments. Introduction of the bHLH factor alone did not induce anthocyanin production, nor, interestingly, did the introduction of the SG5 anthocyanin regulator from maize, *ZmC1*, in conjunction with *Zm-Lc* (Supplementary Figure 4). Anthocyanin synthesis was also activated in garlic shoot tissue after co-bombardment with *MYB1* and bHLH (*Zm-Lc*) constructs (Figure 5), but not when *MYB1* alone was used.





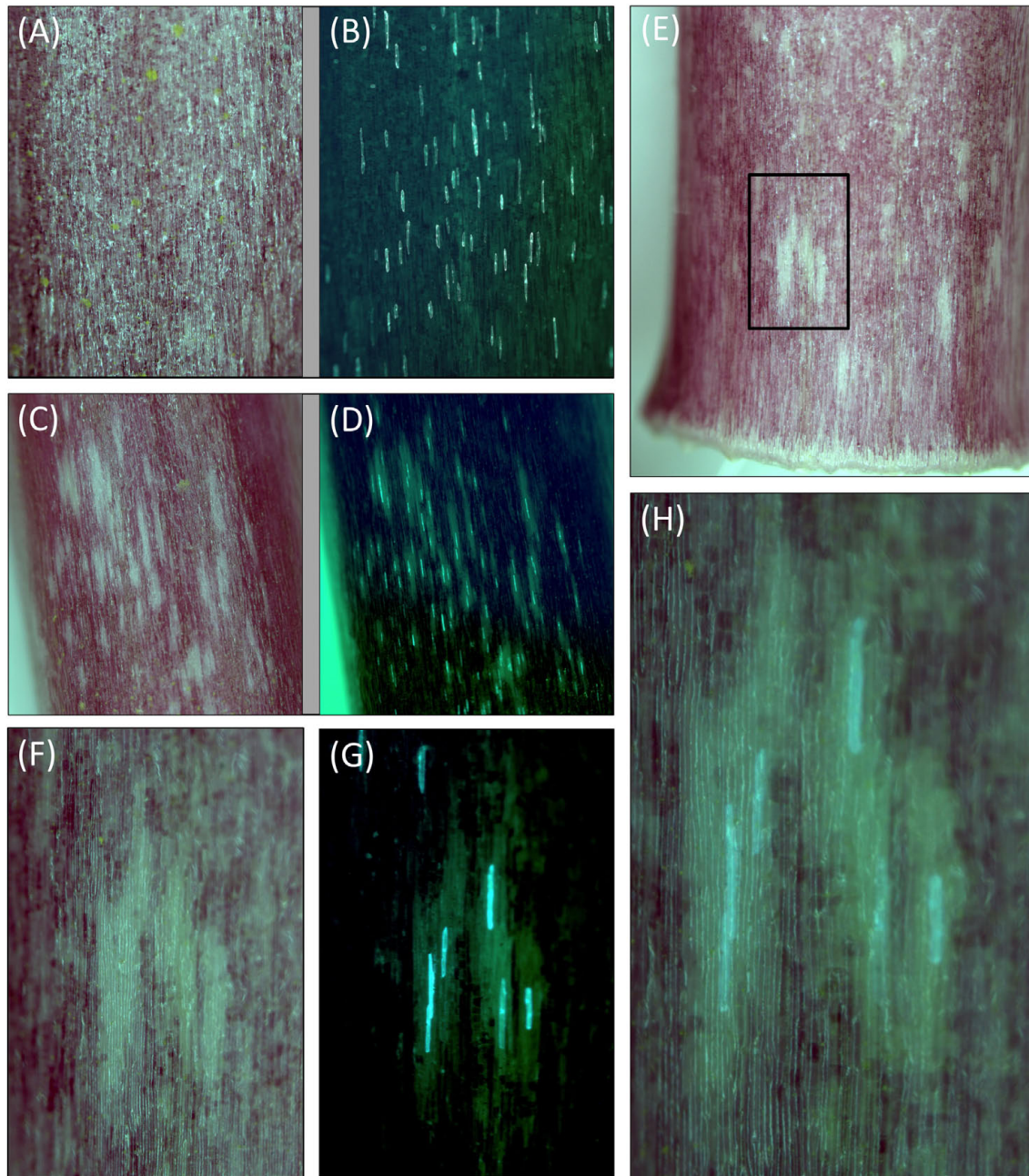


**FIGURE 4 | Increased *MYB1* transcript abundance is associated with anthocyanin production.** Quantitative RT-PCR (qPCR) analysis of red onion (*Allium cepa*) seedling leaf sheath tissue for expression of *MYB1* and two anthocyanin biosynthetic genes, *chalcone synthase-A* (*CHS*) and *dihydroflavonol 4-reductase-A* (*DFR*). Transcript abundance was normalized to the geometric mean of the transcript quantities of *UBQ* and *GAPDH*. The sample consists of pooled tissue from three seedlings. Means  $\pm$  SEM,  $n = 3$  technical replicates are shown. A replicate qPCR experiment gave results consistent with the data presented. Top panels illustrate the phenotype and the type of tissue analyzed rather than an actual sample.



**FIGURE 5 | Transient overexpression of *MYB1* induces anthocyanin pigmentation in onion (*Allium cepa*) and garlic (*Allium sativum*).** Tissue was bombarded with constructs for *35S:MYB1+35S:Zm-Lc+35S:GFP*, *35S:MYB1+35S:GFP*, or *35S:Zm-Lc+35S:GFP*. Tissue from acyanic red onion seedling leaf sheaths and garlic cloves was used. Images are shown under white light (**Top panels**) for anthocyanin pigmentation or blue light (**Lower panels**) for GFP fluorescence. A close up of one region is shown for easier comparison of the co-localisation of GFP signal and red pigmentation. Representative images are shown.

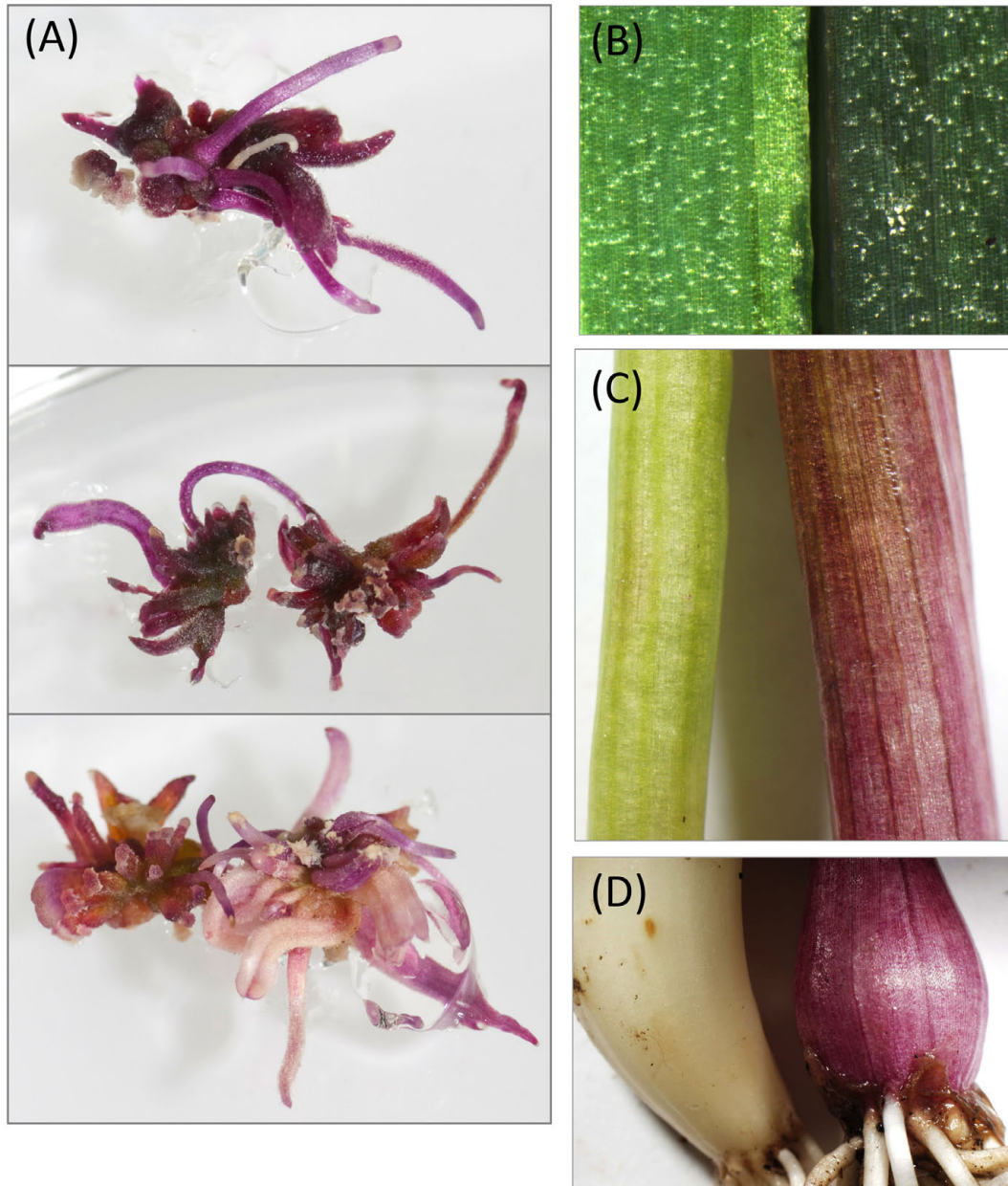




**FIGURE 6 | Transient RNAi inhibition of *MYB1* expression inhibits anthocyanin pigment formation in onion (*Allium cepa*) seedlings.** Acyanic sheath tissue was removed from inner layers of red onion seedlings and biolistically transformed with gene constructs for *35S:GFP* and *35S:MYB1-RNAi* (two different example regions are presented in **C–H**) or *35S:GFP* alone (example region in **A,B**). They were then incubated for around 72 h on moistened filter paper in a Petri dish and exposed to a 16:8 h light:dark cycle. White regions, lacking anthocyanin pigmentation, were observed only when *35S:MYB1-RNAi* was used. (**B,D,G**) Are blue light images for GFP visualization. (**F,G**) Show a close up of one white region (boxed in **E**), and (**H**) shows a further close up in which a partially transparent version of the white light image for anthocyanins (**F**) is overlaid on the GFP image (**H**). Representative images are shown.

from SG5), we tested whether it could substitute for a dicot regulator. Along with a GFP internal control, the *35S:MYB1* construct was biolistically introduced into acyanic petal tissue of the *rosea<sup>dorssea</sup>* anthocyanin *MYB* mutant of snapdragon

(Schwinn et al., 2006). *MYB1* was able to complement the mutant, resulting in cell autonomous production of anthocyanin pigment, which co-localized with GFP fluorescence (**Figure 8**). Introduction of a reporter gene alone does



**FIGURE 7 | Phenotypes of garlic (*Allium sativum*) plants stably transformed with a construct for 35S:MYB1 and 35S:ZmLc. (A)** shows examples of regenerating shoots of independent transgenic events in culture. **(B–D)** show tissue from, respectively, the leaf, stem base, and bulb, with the MYB1+Lc transgenic (line 1111-8A) on the right and a control plant on the left. The control was a transgenic carrying a construct unrelated to pigmentation (an antiviral construct).

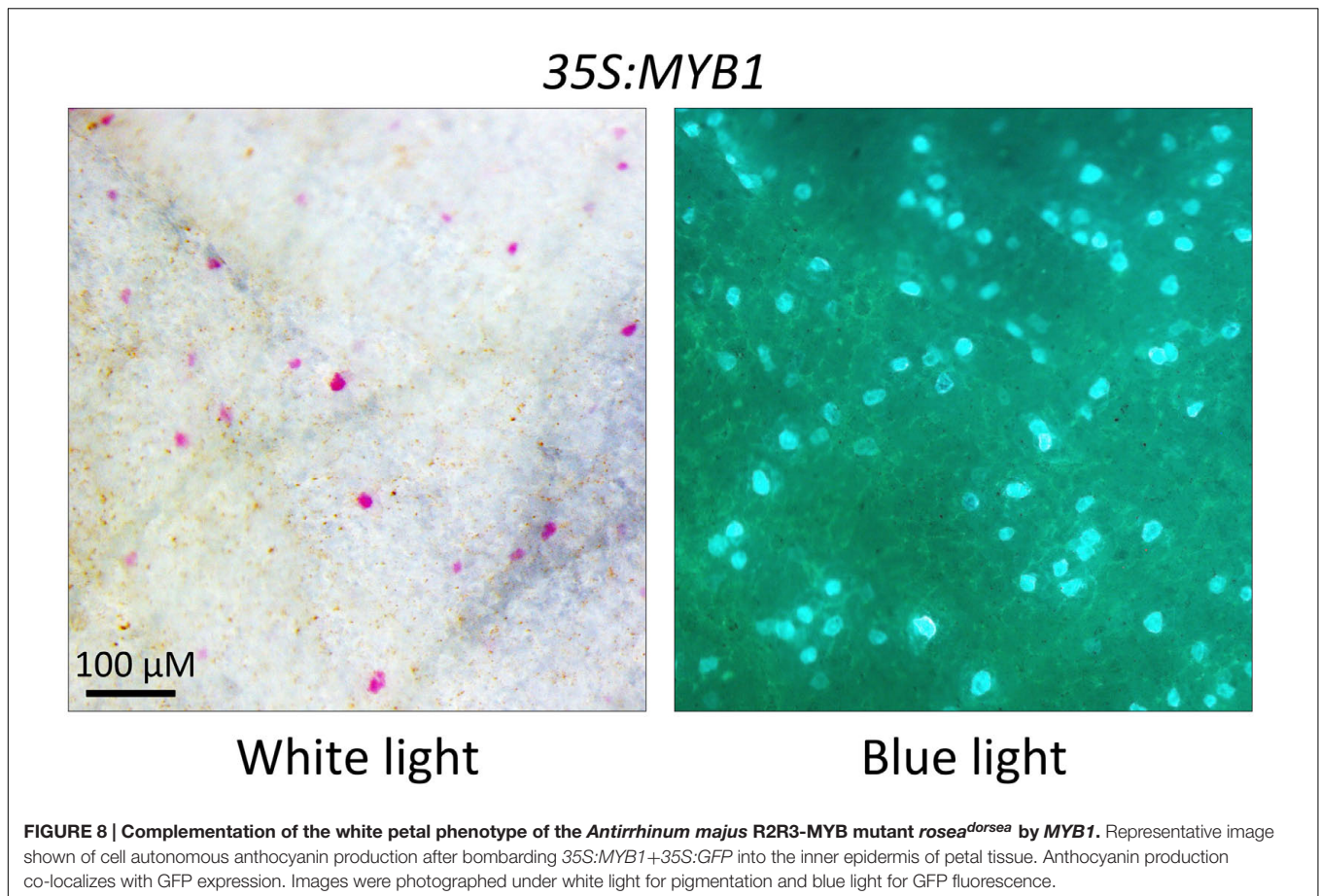
not restore pigmentation in this mutant (Schwinn et al., 2006).

## DISCUSSION

Flavonoids are a key group of secondary metabolites in plants, with roles ranging from attraction of pollinators to defense against abiotic and biotic stresses. Their production is principally

regulated through alterations in the transcription rate of their biosynthetic genes, and the pathway is one of the main model systems for understanding transcriptional regulation in plants. There are several well-established dicot model species, as well as the monocot maize, for the study of the flavonoid pathway. Apart from maize, there is only limited information on the regulation of flavonoid biosynthesis in monocot crop species, and that is mostly from two other Poaceae species – rice and *Sorghum*. Onion is a major crop species from the largest monocot order, the





Asparagales, which contains around 29,000 species. Flavonoids are key to the visual appeal and human health characteristics of onion. However, while there has been much research on the genetic loci and biosynthetic genes underlying flavonoid production in onion, there is little information about the regulatory genes for the pathway. In this study, we have identified five flavonoid-related TF sequences from onion, characterized the importance of the R2R3-MYB factor *MYB1* in controlling anthocyanin biosynthesis, and demonstrated the utility of *MYB1* for application in the molecular breeding of *Allium* crops.

### ***MYB1* Regulates Anthocyanin Synthesis**

A candidate gene approach was taken to identify genes that regulate the production of anthocyanin pigments in onion. *MYB1*, a sequence sharing similarity to characterized anthocyanin-related MYB genes, was identified from red onion. In these onions, anthocyanin pigmentation is first present in the seedlings. It occurs in the swelling, predominantly achlorophyllous sheath tissue of the first few outer layers of the leaves. As the bulb develops and matures, pigmentation extends throughout the inner layers, comprised of the swollen sheaths and scales. *MYB1* expression (along with expression of the investigated biosynthetic genes, *CHS* and *DFR*) was correlated with anthocyanin induction in sheath tissue of the seedlings (Figure 4). Furthermore transient overexpression and

knockdown experiments induced and inhibited, respectively, anthocyanin production in the seedlings (Figures 5 and 6), and ectopic anthocyanin production was observed in garlic co-transformed with transgenes encoding *MYB1* and a heterologous bHLH factor (*ZmLc*; Figure 7). These results demonstrate that *MYB1* positively regulates anthocyanin pigmentation and we conclude that *MYB1* is key for the red bulb phenotype of this crop. As such it is an excellent candidate for underlying a major Quantitative Trait Locus (QTL) controlling anthocyanin concentration and intensity of red bulb color. Duangjit et al. (2014) have identified two such QTLs that are different from the regions controlling red versus yellow bulbs that have been attributed to the *L* locus (which encodes the anthocyanin biosynthetic gene *ANS*) by Kim et al. (2006). They proposed that these newly characterized QTLs may be associated with factors that regulate the pigment pathway.

Vegetative tissues of plants often accumulate anthocyanins in response to light (Gavazzi et al., 1990; Dubos et al., 2008; Albert et al., 2009), particularly in juvenile tissues that may be more sensitive to excess irradiation (Gould, 2004). In onion seedlings only a few of the outer layers of leaves showed anthocyanin pigmentation in the sheaths and when these were peeled away, the exposed acyanic sheath tissue beneath rapidly became pigmented in our experimental conditions. This suggests that light is an important signal in anthocyanin



production in onion, at least early in plant development. This signal is probably integrated through control of *MYB1*. The promoter region of *MYB1* has recognition motifs that have been observed in light regulated genes (Supplementary Figure 1). In addition, the induced accumulation of anthocyanins in acyanic inner leaf sheaths was correlated with the expression of *MYB1* (Figure 4). Juvenile shoots of Asiatic lily produce anthocyanins upon exposure to light because of the expression of the anthocyanin regulators *Lh-MYB6*, *Lh-bHLH1*, and *Lh-bHLH2* (Nakatsuka et al., 2009; Yamagishi et al., 2010). Therefore, light-controlled anthocyanin synthesis is probably regulated by a similar mechanism in both lily and onion. Other signals are expected to be involved in the regulation of anthocyanin production in red onion since by the time the bulbs mature the inner swollen sheaths and scales, all substantially thickened tissues, have become pigmented without being directly exposed to light. It is possible that a light signal is being transduced to the inner leaves; however, pigment regulation during bulb development is likely to involve developmental signals as well.

### MBW Complex Indicated for Regulation of Anthocyanin in *Allium*

A MBW complex regulates anthocyanin synthesis in all species studied to date. While most of the functional data for the MBW complex are from eudicots, the cooperative involvement of MYB, bHLH, and WDR proteins for regulating anthocyanin synthesis is well-established in the monocot model maize (Ramsay and Glover, 2005; Feller et al., 2011). The predicted amino acid sequence of *MYB1* has the motif necessary to bind bHLH TF partners (Supplementary Figure 3), and the co-introduction of a bHLH factor with *MYB1* in the transient assays allowed earlier development of anthocyanin pigmentation along with an increased frequency of pigmented cells (Figure 5). *MYB1* was able also to substitute for the endogenous R2R3-MYB anthocyanin regulator in a dicot species that is a model for MBW regulation of anthocyanin pigmentation (Schwinn et al., 2006) (Figure 8). In the orchid family (also Asparagales), anthocyanin synthesis is also activated when R2R3-MYB and bHLH proteins are co-expressed (Ma et al., 2008; Albert et al., 2010), and endogenous R2R3-MYB genes that regulate anthocyanin production have been identified in the orchid genera *Phalaenopsis* (Ma et al., 2009) and *Oncidium* (Chiou and Yeh, 2008). This suggests that the MBW complex is conserved within Angiosperms, although some aspects of MBW activity have yet to be identified in monocots (Albert et al., 2014b). These include hierarchical activation of bHLH proteins, the involvement of gene-regulation networks, and the action of repressor proteins, which are conserved in eudicots (Albert et al., 2014a). In many species, the IN/AN1/MUT-clade bHLH is essential for regulating anthocyanin synthesis, even if LC/JAF13/DEL-clade bHLH proteins are expressed (Davies et al., 2012a). In Asiatic lilies, the anthocyanin-related R2R3-MYBs (*Lh-MYB6* and *Lh-MYB12*) were able to activate the promoters of the *Lh-CHS*a and *Lh-DFR* genes only when *Lh-bHLH2* (IN/AN1/MUT clade) was co-bombarded (Yamagishi et al., 2010), although *Lh-bHLH1* (LC/JAF13/DEL) was not tested. In maize, the LC/JAF13/DEL

clade bHLH factors regulate anthocyanin synthesis (Ludwig et al., 1989; Tonelli et al., 1991, 1994; Goff et al., 1992), but the IN/AN1/MUT-clade bHLH has lost the ability to activate transcription and acts as a competitive repressor (Burr et al., 1996). Similar patterns of anthocyanin induction were observed in the onion transient assays when bHLH factors of either clade (ZmLC of the LC/JAF13/DEL clade and AmMUT of the IN/AN1/MUT clade) were co-introduced with *MYB1* (Figure 5; Supplementary Figure 4). This indicates either that *MYB1* normally interacts with bHLH factors of both clades in its native environment or that it has retained the ability to function with either type. Identification of the bHLH factors important for pigmentation in *Allium* is required to investigate this further.

### Identification of SG4 and SG7 R2R3-MYB Sequences from Onion

Flavonols accumulate to high concentrations in the bulbs of yellow and red onions, but are only present in trace amounts in white varieties (Patil et al., 1995). *MYB29* was identified that has significant similarity to SG7 R2R3-MYBs, including the characteristic motif present in the C-terminus. While functional data are desirable, the phylogenetic support for Ac-*MYB29* belonging to SG7 is very strong. In Arabidopsis, grape and tomato, the SG7 flavonol regulators coordinately regulate the early flavonoid genes *CHS*, *CHI*, flavanone 3-hydroxylase (*F3H*), and the flavonol-specific gene flavonol synthase (*FLS*; Mehrtens et al., 2005; Luo et al., 2008; Czemplak et al., 2009; Ballester et al., 2010; Stracke et al., 2010). Similarly in maize, P1 directly regulates the *FLS* genes (Ferreira et al., 2010, 2012), although P1 also regulates flavone and phlobaphene synthesis in some tissues (Casati and Walbot, 2005; Grotewold, 2006; Morohashi et al., 2012). However, it is also worth noting that in maize, *FLS* is also regulated by the anthocyanin-related MYB and bHLH factors (Ferreira et al., 2010), suggesting a degree of functional redundancy exists. Taking this into consideration may help to explain the nature of the C locus in onion, which is thought to encode a regulatory gene. C is required for the expression of two genes encoding *CHS* (Kim et al., 2005b), which is the first committed step toward the biosynthesis of flavonoids, including flavonols and anthocyanins. *MYB29* and *MYB1* are excellent candidates for regulating *CHS*, but it is not currently known if they co-localize to the C locus, which is on chromosome 6 (Khar et al., 2008).

Onion R2R3-MYB DNA sequences were also isolated that belong to the phylogenetic SG containing repressors of phenylpropanoid/flavonoid biosynthesis (SG4; *MYB4*, *MYB5*). The identification of *MYB4* and -5 was based on phylogenetic (Figure 2) and amino acid sequence analysis, including the presence of the EAR repression motif in the C-terminus (Supplementary Figure 2). The plant-specific EAR domain occurs in other TF families besides the R2R3-MYBs (Kagale and Rozwadowski, 2010), but alignment of confirmed and candidate At*MYB4*-like R2R3-MYB-EAR repressors for flavonoid biosynthesis from a range of species showed the

(P/L)DLNL(E/D)L version of the motif to be strongly conserved, and the sequence PDLNL(E/D)L is present in MYB4, MYB5 and characterized phenylpropanoid repressors from Arabidopsis, maize, and petunia. There are dominant white bulb phenotypes in onion and a color-inhibiting factor encoded by the *I* locus, which is homozygous dominant and conditions white bulbs (El-Shafie and Davis, 1967). Therefore, it would be interesting to investigate repressive regulatory factors in relation to this.

## Divergence of R2R3-MYB Anthocyanin Regulators within the Asparagales

Flavonoid regulatory genes have been previously characterized from only three monocot families, the Poaceae, Liliaceae, and Orchidaceae. The anthocyanin R2R3-MYBs activators from dicot species separate phylogenetically from those of the Poaceae and Orchidaceae (Figure 2; Supplementary Figure 5). The dicot sequences form the cluster termed SG6 (containing the anthocyanin regulator PAP1) based on the Arabidopsis R2R3-MYB gene family, while the Poaceae and Orchidaceae cluster with Arabidopsis SG5 (containing the PA regulator TT2). The absence of SG6 sequences from the Poaceae and Orchidaceae may suggest divergent evolution of the two MYB clades during the origins of dicots and monocots. However, the *MYB* genes from Asiatic lily (Liliaceae) and onion (Allioideae) cluster in the SG6/PAP1 clade. Thus, based on available sequence data it appears that the R2R3-MYBs that regulate anthocyanins have diverged within Asparagales evolution during formation of the Orchidaceae and Allioideae. Most phylogenetic analysis places the Orchidaceae as the sister group to the rest of the Asparagales, being one of the two initial phylogenetic branches (Angiosperm Phylogeny Group III, 2009), so divergence could well have occurred after this branching. The placement of the onion and Asiatic lily sequences with dicot rather than Orchidaceae/Poaceae sequences is not seen for the target genes of the flavonoid biosynthetic pathway. Rather, the biosynthetic gene sequences form phylogenetic trees close to the current suggested species taxonomic relationships (Supplementary Figure 6 shows a representative tree, for DFR). Additionally, the other flavonoid-related MYBs identified, for SG4 and SG7, group closely with the similar sequences from both dicots and the Poaceae (the only other monocot family for which sequences have been characterized).

SG5/C1-TT2 clade members were not identified in the approximately 250,000 onion Non-Redundant sequences. Nor have they been published for Asiatic lily, even though it may be expected that PA production is regulated by an R2R3-MYB in most monocots, as it is in the Poaceae (Himi and Noda, 2005; Himi et al., 2012) and dicot species (Nesi et al., 2001; Bogs et al., 2007; Hancock et al., 2012; Liu et al., 2014) studied to date. It may be that RNA sequencing for the Liliales and Asparagales species has not been conducted on samples from tissues rich in PAs. Interestingly, seed coats are typically rich in PAs but PAs have been functionally replaced by phytomelans in *Allium* and other taxa of Asparagales,

but not in the Orchidaceae (Boesewinkel and Bouman, 1995; Fritsch and Friesen, 2002; Chase, 2004). Full genome sequences for species such as onion and Asiatic lily (~130 and 900-fold larger, respectively, than that of Arabidopsis) are not yet available but sequencing of the gene rich regions is in progress for onion<sup>1</sup>. Additional DNA sequence resources for monocot species other than the Poaceae may also help to address the lack of reported PAP1-like sequences in some monocot species.

The basis for the absence of identified PAP1/SG6 clade members in the Poaceae and Orchidaceae is unknown. While additional DNA sequencing may identify members of this clade in such species, no PAP1-like R2R3-MYBs have been identified in the rice genome based on analysis of the phylogenies in Katiyar et al. (2012) and nor are any apparent in current transcriptome databases for various orchid species (the authors' unpublished analysis). It seems unlikely that PAP1-like R2R3-MYBs arose independently in dicots, the Liliales and the Asparagales – i.e., convergent evolution after the separation from the Orchidaceae. More likely is that there has been neofunctionalization of the C1-TT2 (SG5) clade in the Poaceae and Orchidaceae with the subsequent loss of the PAP1 clade. The presence of regulators of both anthocyanins and PAs in the same clade in grasses is consistent with this theory. Furthermore in this scenario, both clades would have been retained in the Liliaceae, Allioideae, and dicots, with separation of function developing for anthocyanin and PA regulation. Alternatively, the SG6 clade could have evolved from the SG5 MYBs to regulate anthocyanins, whilst SG5 genes were retained to regulate PA synthesis, reflected by the two subclades of genes associated with anthocyanins and PAs within SG5 in grasses (Figure 2). To address this question fully, further characterisation of the anthocyanin and PA regulators is required from diverse monocot and gymnosperm species.

Finally, it is worth noting that some phylogenetic differences in the anthocyanin regulators were translated into differential activities in the transient assays. In contrast to MYB1, the maize MYB regulator Zm-C1 was not able to activate pigmentation in onion even when its bHLH partner was co-introduced (Supplementary Figure 4). Indeed, the maize anthocyanin regulators have been observed to inhibit pigmentation when bombarded into onion (personal communication, Dr. David Collings). Also, Zm-C1 (-/+ its bHLH partner) was not able to complement the snapdragon anthocyanin *MYB* mutant (the authors' unpublished observation). Whether this reflects evolutionary divergence or different lineages for the regulators awaits further analysis. In the first instance, it would be interesting test the anthocyanin regulators from other lineages within the Asparagales.

## CONCLUSION

We have shown that MYB1 is a positive regulator of anthocyanin production in onion and we have demonstrated its utility as

<sup>1</sup><http://www.oniongenome.net>

a molecular breeding tool. We have also identified a candidate MYB regulator for flavonols, the other key flavonoid metabolites in onion, as well as candidate MYB factors for repressive activities in the regulation of flavonoid or phenylpropanoid metabolism. These genes will be useful to identify classical loci in onion and will help in wider phylogenetic and regulatory studies in monocot lineages.

## AUTHOR CONTRIBUTIONS

KS, HN, FK, CE, DB, KD, and NA conceived of and conducted experiments and/or analyzed and interpreted data; KS, DB, KD, and NA isolated, identified and/or analyzed gene sequences; JM, MP-J, and RC generated transcriptome resources; KD, KS, DB, and JM conceived of the project; KS, NA, and KD prepared the manuscript. All authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01865/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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