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The *At*MYB12 activation domain maps to a short C-terminal region of the transcription factor

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Abstract: The *Arabidopsis thaliana* R2R3-MYB transcription factor MYB12 is a light-inducible, flavonol-specific activator of flavonoid biosynthesis. The transactivation activity of the *At*MYB12 protein was analyzed using a C-terminal deletion series in a transient *A. thaliana* protoplast assay with the goal of mapping the activation domain (AD). Although the deletion of the last 46 C-terminal amino acids did not affect the activation capacity, the deletion of the last 98 amino acids almost totally abolished transactivation of two different target promoters. A domain swap experiment using the yeast GAL4 DNA-binding domain revealed that the region from positions 282 to 328 of *At*MYB12 was sufficient for transactivation. In contrast to the R2R3-MYB ADs known thus far, that of *At*MYB12 is not located at the rearmost C-terminal end of the protein. The *At*MYB12 AD is conserved in other experimentally proven R2R3-MYB flavonol regulators from different species.

Keywords: activation domain; flavonol regulator; MYB12; production of flavonol glycosides; transcription factor.

Dedication: This article is dedicated to my academic teacher Prof. Dr. Lothar Jaenicke, with whom I had the great pleasure to biochemically study *Volvox carteri* throughout the year 1984.

1 Introduction

Transcription factors (TFs) are proteins that are capable of activating and/or repressing transcription. Generally, TFs contain a sequence-specific DNA-binding domain (DBD) [1]. They are largely responsible for the selectivity in gene regulation, and are often expressed in a tissue-specific, developmental stage-specific, or stimulus-dependent manner. Because many biological processes in metazoans, including plants, are regulated at the level of transcription, the evolution of many traits during the domestication of plants has not surprisingly been associated with changes in TFs or their regulation and/or their expression patterns. Therefore, understanding plant TF function is an important step toward understanding plant development, adaptation, and evolution. An essential component of gene regulation is the transcriptional activation of genes, which takes place at promoters. The process is dependent on the assembly of regulatory multiprotein complexes at promoter sequences, which include TFs responsible for cell type-specific or stimulus-responsive gene expression. These complexes direct the transcriptional machinery, composed of RNA polymerase II and many additional factors, to the start site of gene transcription [2]. Although factors that do not contain a DBD are sometimes regarded as TFs, the standard TF consists of a domain that is responsible for sequence-specific binding to regulatory *cis*-acting elements in promoters, and additional domains with regulatory functions. The identification of distinct regulatory domains allows the investigation of interactions with other proteins present in initiation complexes. A typical modular TF usually contains, in addition to a DBD that is used to sort TFs into TF families, a transcription regulation domain, a nuclear localization signal, and often a dimerization interface as well. The latter two might both be part of the DBD, and the sum of the functional regions defines the characteristics, the localization, and the regulatory role of a given TF. Domains reported to be responsible for transcriptional activation contain acidic, glutamine-rich, or proline-rich stretches of amino acids [3].

Flavonols are products of flavonoid biosynthesis, which forms various C15 molecules that vary in oxidation level and decoration [4], and they accumulate in their glycosylated form in the vacuoles of plant cells [5]. The *Arabidopsis thaliana* protein MYB12, encoded by the locus *At2g47460*, is a member of the R2R3-MYB family of TFs that share the MYB DBD [6, 7]. *At*MYB12 was found to be a flavonol-specific activator of flavonoid biosynthesis, with the two flavonoid biosynthesis genes *CHS* (*At5g13930*, encoding chalcone synthase) and *FLS1* (*At5g08640*, encoding

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flavonol synthase) as its primary targets [8]. Two additional R2R3-MYB TFs were identified in *A. thaliana*, namely, MYB11 (*At3g62610*) and MYB111 (*At5g49330*), which are structurally and functionally related to MYB12 [9]. These three TFs form subgroup 7 (SG7) of the R2R3-MYB family of TFs [6]. Because these factors specifically control flavonol glycoside accumulation, *At*MYB12, *At*MYB11, and *At*MYB111 are also referred to as production of flavonol glycosides 1 to 3 (PFG1, PFG2, and PFG3), respectively. Although the light-dependent factor *At*MYB12 and the light-independent factor *At*MYB111 control flavonol glycoside accumulation in different parts of the seedling (Figure 1), as well as in various organs of adult plants, MYB11 activity is restricted to certain organs of adult plants [10].

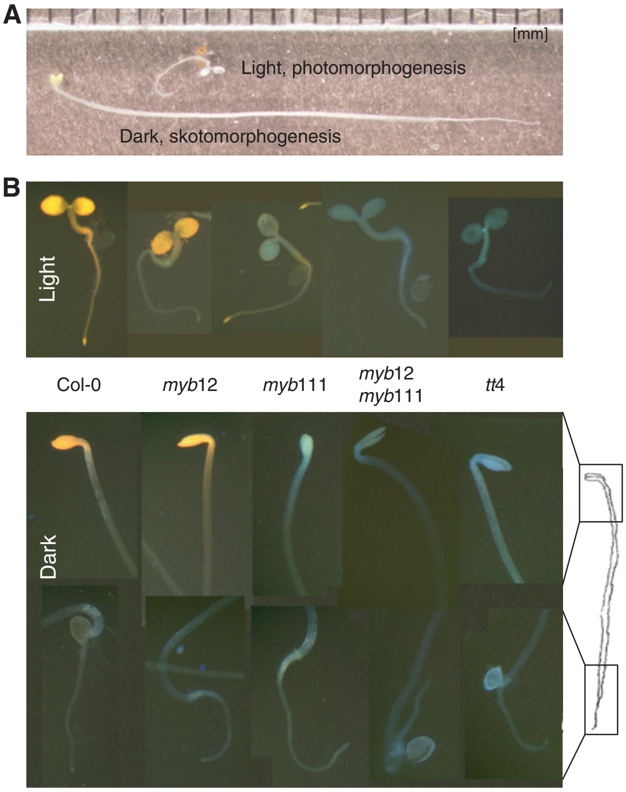

Figure 1: Flavonol accumulation in *A. thaliana* seedlings is dependent on MYB12 and MYB111 activity.
Representative pictures are shown in all panels. (A) Five-day-old, norflurazon-bleached *A. thaliana* seedlings, developed in the light or darkness, displaying normal or etiolated morphology. (B) MYB12-controlled flavonol accumulation in the root is light-dependent, whereas MYB111-controlled flavonol accumulation in cotyledons is light-independent. Norflurazon bleached wild-type and loss-of-function mutant seedlings were stained with DPBA, and flavonol staining (yellow-orange color) was visualized on an epifluorescence microscope using a filter with an excitation wavelength of 340–380 nm (UV-A light) and a 425-nm long-pass splitter. As indicated by the sketch of an etiolated seedling on the right, the pictures of dark-grown seedlings do not show the complete elongated hypocotyl.

Upregulation and downregulation of MYB12 in *A. thaliana* mutants, i.e. the comparison of an *At*MYB12 overexpressor line with a homozygous *myb12* mutant, shows a clear correlation to expression levels of the target genes *CHS* and *FLS1* [8]. Additionally, *myb12* mutant plants display a phenotype, or more exactly, a chemotype that becomes visible upon specific staining, which indicates a changed accumulation of flavonol glycosides in the roots of light-grown *A. thaliana* seedlings (Figure 1A). Finally, transient expression alongside promoter mutation studies showed that a MYB recognition element (MRE) in the promoters is directly bound by *At*MYB12 to cause activation of the *CHS* and the *FLS1* promoter [8]. In the present study, we set out to identify and map the activation domain (AD) of the flavonol regulator *At*MYB12.

2 Materials and methods

2.1 Plant material

The molecular characterization of the *myb12* (Nottingham Arabidopsis Stock Center (NASC, Loughborough, UK) number N9602), *myb111* (NASC number N9813), and *myb12 myb111* (NASC number N9980) TF mutants has been described previously [9]. All three alleles have been shown to encode nonfunctional versions of the genes. The flavonoid-deficient *transparent testa4* (*tt4-11, SALK_020583*) mutant, carrying a loss-of-function chalcone synthase (*chs*) allele [11], is compromised in the first enzymatic step of flavonoid biosynthesis [12]. The seeds used in the present study are from the same batch.

2.2 Flavonoid staining in seedlings

Seedlings were grown in the light or darkness on filter paper soaked with an aqueous solution of 3 ppm of the bleaching herbicide norflurazon (Supelco/Sigma-Aldrich PS-1044, Bellefonte, PA, USA) to avoid background fluorescence from chlorophyll. Flavonol glycosides in 5-day-old bleached seedlings were stained using 0.25% diphenylboric acid β-aminoethyl ester (DPBA, Roth, Karlsruhe, Germany) and 0.01% Triton X-100 for at least 15 min. Fluorescence of DPBA-flavonol glycoside conjugates was visualized on a Leica DM5500 B epifluorescence microscope (Leica, Wetzlar, Germany) using Leica Filtercube A with an excitation wavelength of 340–380 nm and a 425-nm long-pass splitter. Pictures were taken using a JVC KY-F75U 3CCD digital camera (JVC, Yokohama, Japan).

2.3 Plasmids

*At*MYB12 variants were expressed from effector constructs under the control of the cauliflower mosaic virus (CaMV) 2x35S promoter using the Gateway-compatible vector pBTdest (ENA/GenBank AJ551314) [13]. C-terminal *At*MYB12 deletion constructs were prepared from a plasmid containing the full-length CDS of *At*MYB12 (pNTK1). The constructs were created using Gateway recombination technology (Invitrogen, Carlsbad, CA, USA). *At*MYB12 deletion variants (Figure 2) were amplified from pNTK1 by PCR, using a forward primer containing the START codon (MYB12-GWf, 5′-attB1-CCATGGGAAGAGCGCCATGTTG-3′) and eight different reverse primers (Supplementary Table S1), each introducing a Stop codon at the positions indicated in Figure 2. The resulting eight PCR fragments were introduced into pDONR201 through Gateway BP reaction. All resulting plasmids were verified by Sanger sequencing and used in LR reactions with the destination vector pBTdest, and the resulting effector constructs were named pNTK2 to pNTK8. The *proCHS* and *proFLS1* reporter constructs driving β-glucuronidase (GUS) expression from the *uidA* open reading frame have been described previously [8].

The effector construct expressing the GAL4DBD-MYB12AD fusion was created by amplification of the *At*MYB12AD coding sequence using the primers RS1162 5′-CTCACCGGTCTTCTTCAGTCTTGTCCATCGG-3′ and RS1163 5′-TTGACTTAAGTCAATTCTCTTTCTCATGCCAAAG-3′ and subsequent insertion through *Age*I and *Afl*II sites into

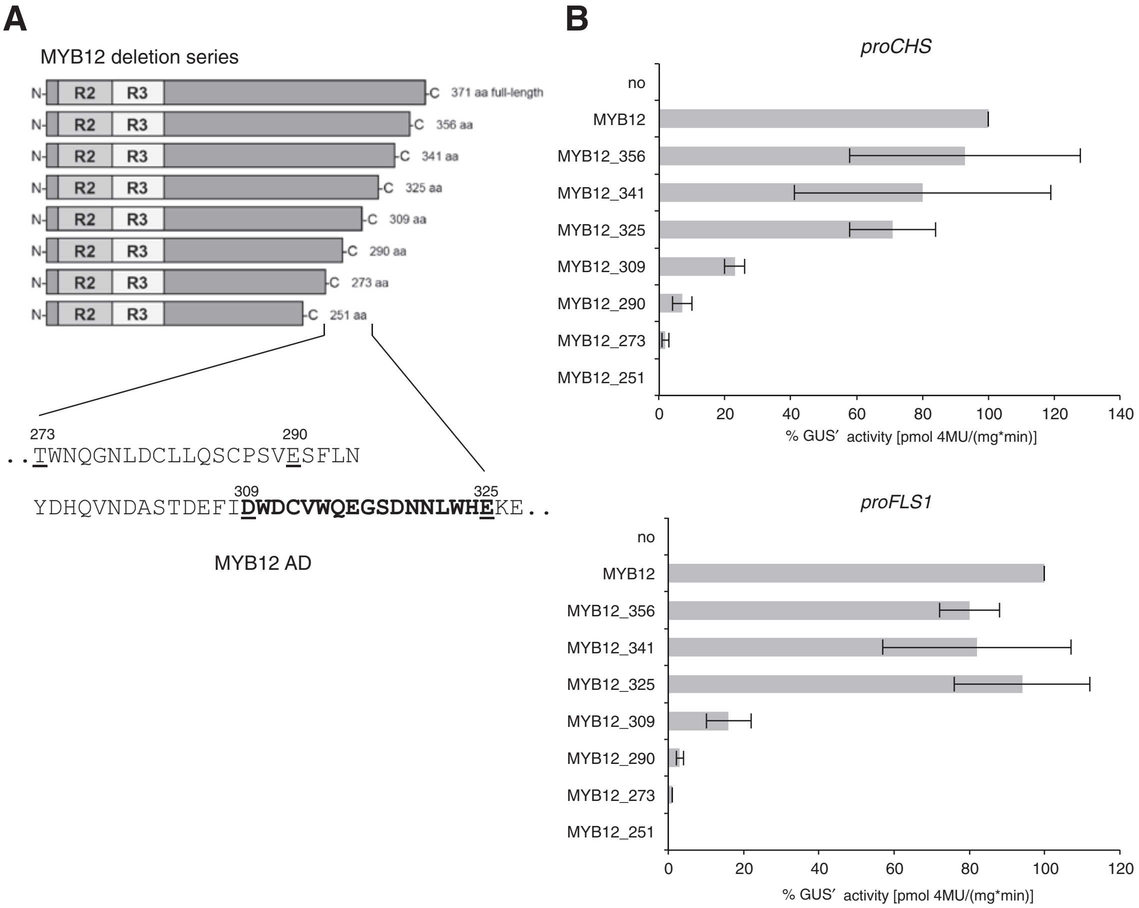

Figure 2: Identification of MYB12 AD.
The MYB12 AD is located between amino acid positions 273 and 325, with the region 309–325 having the most relevant effect on activation capacity. A series of C-terminal MYB12 deletion variants (A) expressed under the control of the CaMV 35S promoter were tested for their capability to activate the chalcone synthase (*CHS*) and flavonol synthase 1 (*FLS1*) promoters in *A. thaliana* At7 protoplasts through cotransfection analysis (B). In this assay, the GUS reporter enzyme activity formed is interpreted as a measure for the transcription activation potential. Error bars give the standard deviation of four independent biological repetitions, wherein each experimental series the GUS′ value obtained with the full-length MYB12 protein was set to 100%. Abbreviations: GUS′, standardized specific β-glucosidase activity; 4MU, 4-methylumbelliferone; R2/3, MYB repeat 2/3.

the 35S-GAL4DBD-containing construct pMS44 [14]. The reporter construct for detecting GAL4DBD binding (construct pBW512) contained four copies of the GAL4 binding site (GAL4-UAS for upstream activating sequence) [15] in front of the – 46 to + 8 TATA fragment of the 35S promoter of pBT10GUS [16].

2.4 Transfection experiments

For use in protoplast cotransfection assays, effector and reporter constructs were transformed into *Escherichia coli* K12 2925 dam$^-$ cells, and unmethylated plasmid DNA of each construct was isolated using the *Qiagen Plasmid plus Midi Kit* (Qiagen, Hilden, Germany). Protoplast isolation and transfection experiments for the detection of transient expression were performed as described [17]. In the cotransfection experiments, a total of 25 µg of premixed plasmid DNA was transfected, containing 10 µg of reporter plasmid, 1 µg of effector plasmid, and 5 µg of the luciferase (LUC) standardization plasmid. A promoter-deleted filling plasmid pBT10-Δ-LUC was added to keep the total amount of transfected plasmid DNA constant. Protoplasts were incubated for 20 h at 26 °C in the dark before LUC and GUS enzyme activities were determined. Specific GUS activity is given in pmol 4-methylumbelliferone (4MU) per milligram of protein per minute. Standardized specific GUS activity (GUS′) was calculated by multiplication of the specific GUS activity value, with a correction factor derived from the ratio of the specific LUC activity in the given sample to the mean specific LUC activity (describing the transformation efficiency) of a set of four complete experiments.

2.5 Multiple sequence alignment

Protein sequences were aligned with the software tool Clustal Omega [18] using the web service of EBI (www.ebi.ac.uk/Tools/msa/clustalo/) with default parameters. The resulting Clustal file was manually revised using Jalview [19].

3 Results

To further examine the phenotype caused by mutation of *MYB12* and/or *MYB111*, we checked flavonol glycoside accumulation in dark-grown *A. thaliana* seedlings. These seedlings display etiolated growth that results from skotomorphogenic development. As shown in Figure 1B, roots of etiolated wild-type (wt) seedlings do not accumulate flavonol glycosides. Under these conditions, there is no obvious difference between wt and *myb12* seedlings. In contrast, *myb111* seedlings lose the flavonol glycosides that are triggered by MYB111 in light-grown as well as in dark-grown seedlings. As expected, *myb111* seedlings grown in the light do display flavonol glycoside accumulation in the root tissue in a similar fashion as the wt. This indicates that light is required for *At*MYB12-dependent flavonol glycoside accumulation in the roots of *A. thaliana* seedlings.

To identify the AD of *At*MYB12, a transient expression system using protoplasts of cultured At7 cells was used. In this system, different combinations of constructs expressing *At*MYB12 C-terminal deletion variants as effectors, with constructs containing promoters targeted by *At*MYB12 as reporters, were cotransfected. This allowed quantification of promoter activity as well as the determination of the transactivation potential of the various TF deletion versions. In these experiments, the amount of GUS reporter enzyme formed is interpreted as a measure for the transcriptional activation potential of the respective *At*MYB12 variant. The promoters of the flavonoid biosynthesis genes *AtCHS* and *AtFLS1* were tested as known targets for *At*MYB12 activation (Figure 2). The first 309 N-terminal amino acids of *At*MYB12, which displays a total wt length of 371 amino acids, were found to be necessary to activate *proCHS* and *proFLS1*. A 290 amino acid fragment could activate only to a very low extent, whereas more extended *At*MYB12 C-terminal deletions were completely inactive (Figure 2). We concluded that the core part of the *At*MYB12 AD is located between amino acid positions 309 to 325, with some contribution from residues between 273 and 308.

To confirm that the *At*MYB12 region identified by deletion analysis is also sufficient for transcriptional activation, we transferred the *At*MYB12 AD to a different DBD in a domain swap experiment. The DBD of the yeast factor GAL4 was used, and tested on reporter constructs containing a synthetic promoter consisting of the 35S core promoter region providing TATA box and transcription start site as well as a tetramer of the GAL4 recognition sequence (GAL4-UAS, upstream activating sequence). GAL4 DBD alone did not significantly activate the GAL4 reporter, but the fusion of GAL4 DBD and *At*MYB12 AD caused strong activation of the GAL4-UAS reporter (Figure 3). This shows that the identified *At*MYB12 domain has transcriptional activation potential.

A multiple alignment of protein sequences from experimentally proven flavonol regulators, including the *At*MYB12 homologs from grapevine [20], sugar beet [21],

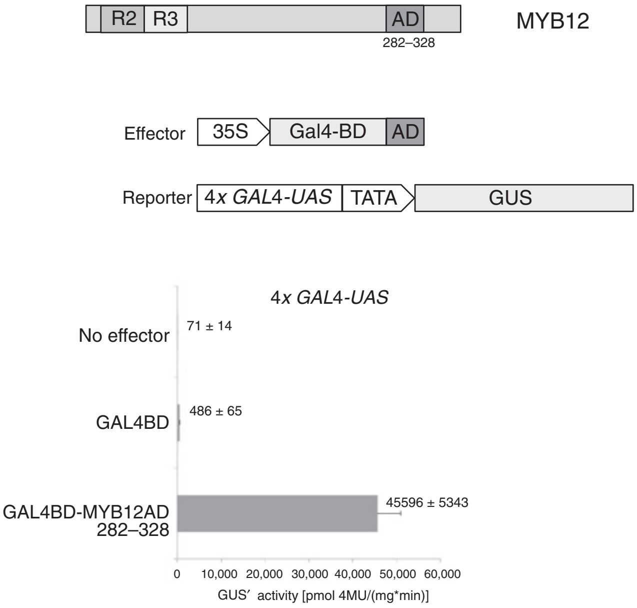

Figure 3: The *At*MYB12 AD stays functional when transferred to a different DBD.
A fusion of GAL4 DBD and MYB12 AD expressed under the control of the CaMV 35S promoter, was able to activate transcription of a GAL4-upstream activating sequence (GAL4-UAS) driven GUS reporter in an At7 protoplasts cotransfection assay. In this assay, the GUS reporter enzyme activity formed is interpreted as a measure of the transcription activation potential. Error bars give the standard deviation of three independent biological repetitions. The mean value of luciferase activity (LUC) was 8991 relative luminescence units RLU/(μg protein × s). Abbreviations: GAL4, galactose-responsive transcription factor 4; GUS′, standardized specific β-glucosidase activity; 4MU, 4-methylumbelliferone.

and tomato [22], led to the identification of putative AD regions in all proteins (Figure 4). These regions differ in length, but show some conserved amino acids, predominantly an aspartic acid/tryptophan (DW) and a leucine/tryptophan (LW) motif.

4 Discussion

Plant roots react very sensitively to light despite growing underground. They show negative phototropism, bending away from a light source [23]. This tropism, which allows roots to escape unfavorable light conditions, is mediated by auxin transport, which is affected by flavonol accumulation [24]. Also, flavonol glycosides will prevent DNA damage caused by UV light because flavonols and other flavonoid pigments serve as photoprotectors [25]. The R2R3-MYB transcription factor *At*MYB12, which is expressed in response to light and which controls flavonol accumulation in seedling roots, is the focus of our study.

The experimentally proven knowledge about transcriptional ADs in plant R2R3-MYB TFs is limited. From studies by Goff et al. [26], it is known that the AD of the *Zea mays* anthocyanin regulator C1 is located in a carboxy-terminal acidic region (amino acids 234–273, of 273). Further studies identified the hydrophobic amino acid residue leucine 253 as being important for activation [27]. Also, the C-terminal acidic region of *At*MYB2 (amino acids 221–274, of 274), a transcriptional activator in abscisic acid signaling [28], was found to be able to activate transcription [29].

In the first description of *At*MYB12 [8], the modular structure of MYB12 was speculated as being similar to that of other R2R3-MYB factors, with the MYB DBD positioned at the N-terminus and the C-terminal part of the protein harboring the transactivation domain. A consideration of the *myb12_f* loss of function allele [8], which has a premature Stop codon after amino acid position 260, suggests that the AD is located somewhere C-terminally of amino acid position 260. This is in good accordance with our findings from both the deletion series and the domain swap experiment.

The *At*MYB12 AD identified in this study is located in the amino acid region 273 to 325 (of 371). This region contains several acidic amino acids, which is in good accordance with the features of the ADs of *Zm*C1 and *At*MYB2. Glutamine-rich or proline-rich amino acid stretches, as reported for ADs of other non-R2R3-MYB-type TFs, were not identified. In contrast to the ADs of the R2R3-MYBs *Zm*C1 and *At*MYB2, the *At*MYB12 AD is not located at the rearmost C-terminal end of the protein but close to the C-terminus. It is possible that this difference reflects the fact that *At*MYB12 is acting independently of bHLH factors in activating the phenylpropanoid biosynthesis genes, whereas *Zm*C1 requires partnering with the bHLH factor *Zm*R or related proteins for productive promoter activation [30]. In this respect, *At*MYB12 behaves similarly to the maize factor *Zm*P controlling phlobaphene biosynthesis [31], whereas *Zm*C1 and *Zm*R behave like *At*PAP1, which is dependent on coaction with bHLH factors like *At*GL3 or *At*EGL3 [32].

Amino acid sequence comparison to other R2R3-MYB TFs from SG7, which have been shown to activate flavonol biosynthesis in a number of different species, indicated that the *At*MYB12 AD is shared as a conserved feature with other members of SG7. In further experiments, the significance for activation capacity of specific amino acids in the SG7 R2R3-MYB AD should be proven, thereby the motifs DW and LW (Figure 4) might be of special interest.

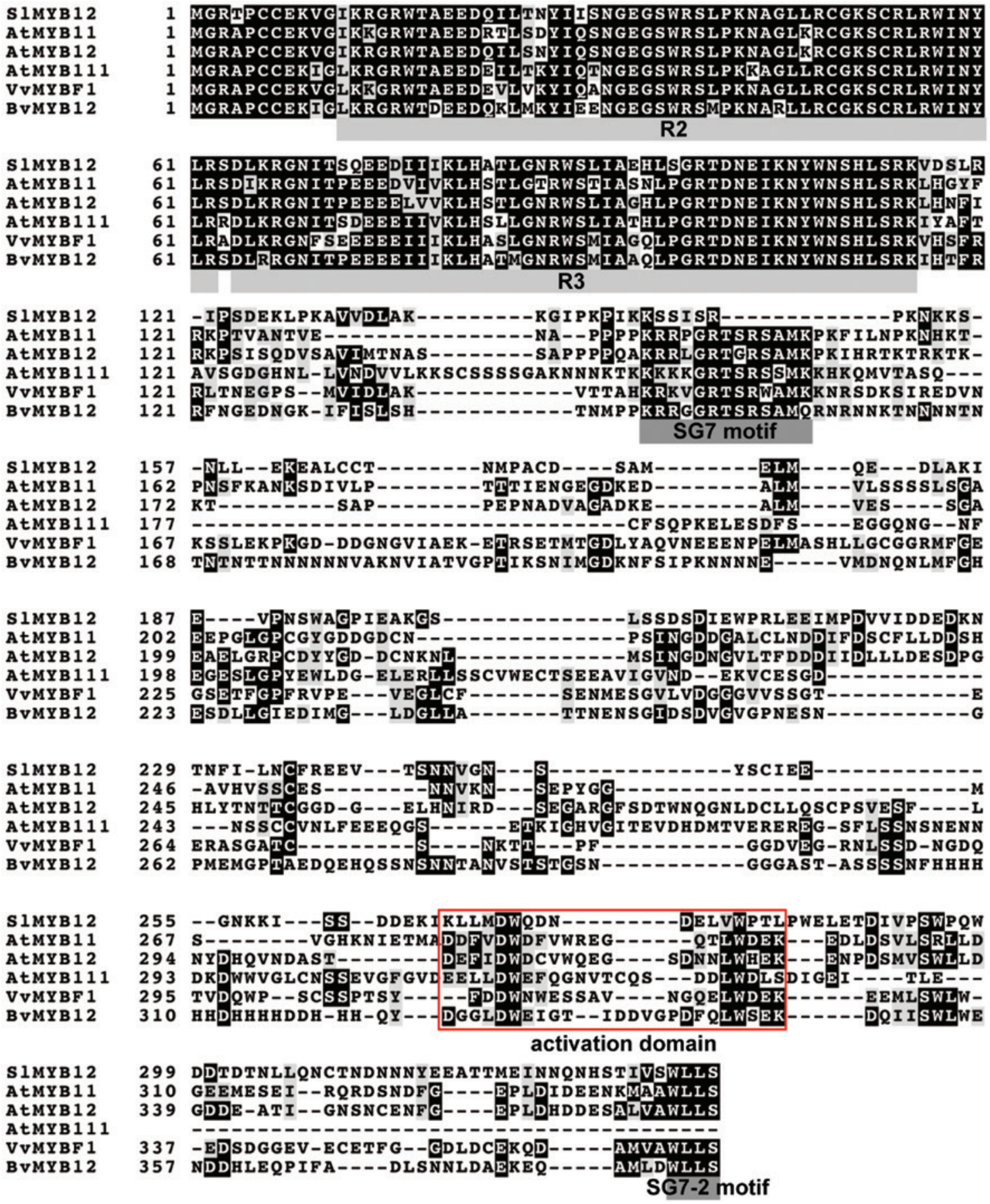

Figure 4: Multiple protein sequence alignment of experimentally proven R2R3-MYB-type flavonol regulators.
The R2 and R3 MYB repeats are indicated as gray bars below the amino acid sequence, as well as the two motifs described for flavonol-regulator clade SG7 R2R3-MYBs [6, 20]. The SG7 AD identified is marked with a red box. Protein sequences were aligned by Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) using the default parameters. The resulting Clustal file was manually revised using Jalview.

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