Identification of *Arabidopsis* MYB56 as a Novel Substrate for CRL3\(^{BPM}\) E3 Ligases

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ABSTRACT

Controlled stability of proteins is a highly efficient mechanism to direct diverse processes in living cells. A key regulatory system for protein stability is given by the ubiquitin proteasome pathway, which uses E3 ligases to mark specific proteins for degradation. In this work, MYB56 is identified as a novel target of a CULLIN3 (CUL3)-based E3 ligase. Its stability depends on the presence of MATH-BTB/POZ (BPM) proteins, which function as substrate adaptors to the E3 ligase. Genetic studies have indicated that MYB56 is a negative regulator of flowering, while BPMs positively affect this developmental program. The interaction between BPMs and MYB56 occurs at the promoter of *FLOWERING LOCUS T* (*FT*), a key regulator in initiating flowering in *Arabidopsis*, and results in instability of MYB56. Overall the work establishes MYB transcription factors as substrates of BPM proteins, and provides novel information on components that participate in controlling flowering time in plants.

Key words: CUL3, BPM, MYB, transcription factor, E3 ligase, flowering


INTRODUCTION

One of the central regulatory mechanisms in plant cells controlling a broad diversity of processes is represented by the ubiquitin (UBQ) proteasome pathway (Chen and Hellmann, 2013). The pathway is highly conserved among eukaryotes, in which the E1 enzyme activates UBQ in an ATP-dependent manner. E1 transfers the activated UBQ to an UBQ-conjugating E2, which in turn can interact with an UBQ E3 ligase. The E3 ligase facilitates the transfer of UBQ to a substrate; upon building up a chain of UBQs the substrate becomes a target for the 26S proteasome, and may undergo proteolytic degradation (Chen and Hellmann, 2013). E3 ligases are by far the most diverse group of enzymes within the UBQ proteasome pathways, with many different classes present in plants (Chen and Hellmann, 2013). These ligases provide specificity to the pathway and allow for the selective control of protein activity through ubiquitylation and, often, subsequent degradation of their targets.

One class of these E3 ligases is composed of a CUL3, a RING-FINGER BINDING PROTEIN 1 (RBX1), and a BTB/POZ protein (hereafter referred to as CRL3 E3 ligase) (Figueroa et al., 2005; Gingerich et al., 2005; Weber et al., 2005). BTB/POZ proteins serve as substrate adaptors to the CRL3 ligase. They use their BTB/POZ fold for interaction with the cullin while a second domain is employed to assemble with a substrate protein (Gingerich et al., 2005; Weber et al., 2005; Gingerich et al., 2007; Weber and Hellmann, 2009; Lechner et al., 2011; Roberts et al., 2011; Fu et al., 2012; Chen et al., 2013).

*Arabidopsis* encodes for a small family of six BPM proteins that contain an N-terminal MATH domain in addition to their C-terminal BTB/POZ fold (Weber et al., 2005). BPM proteins have recently been confirmed as substrate adaptors to a CRL3\(^{BPM}\) E3 ligase (Weber et al., 2005; Weber and Hellmann, 2009; Lechner et al., 2011; Chen et al., 2013), and plants with reduced expression in multiple BPM members are broadly affected in development, including root and leaf growth, flowering, and seed development (Lechner et al., 2011; Chen et al., 2013). Members of the BPM family have also been implicated in abscisic acid (ABA) signaling by controlling the stability of homeobox (ATHB) transcription factors, and in this context they may affect stress tolerance in plants (Lechner et al., 2011). Besides affecting ATHB stability, recent work has shown that CRL3\(^{BPM}\) E3 ligases also target ERF/AP2 transcription factors for proteasomal degradation, and that they target these transcription factors while being associated with DNA (Weber and Hellmann, 2009; Chen et al.,...
2013). Work on ERF/AP2 proteins has mainly focused on WRINKLED1 (WRI1), a key protein controlling fatty acid biosynthesis in seeds of monocots and eudicots (Cernac and Benning, 2004; Baud et al., 2009; Pouvreau et al., 2011; To et al., 2012; Ma et al., 2013), and RELATED TO APETALA 2.4 (RAP2.4), which is known in context with abiotic stress tolerance (Lin et al., 2008; Xu et al., 2010; Iwase et al., 2011). Studies from our group have indicated that a broad range of ERF/AP2 family members are likely targets of a CRL3BPM E3 ligase (Weber and Hellmann, 2009; Chen et al., 2013). Here we show that CRL3BPM E3 ligases assemble with members of a third transcription factor family that are represented by R2R3-MYB proteins. Like ERF/AP2 proteins, R2R3-MYBs constitute one of the major transcription factor families in plants, with 126 annotated members in Arabidopsis (Dubos et al., 2010). Our results show for one R2R3-MYB family member, MYB56, that the assembly with BPMs causes its instability. We have further identified that CRL3BPM E3 ligases assemble with MYB56 at the DNA level. The work also establishes MYB56 as a novel negative regulator of flowering by controlling expression of the FLOWERING LOCUS T (FT), and that CRL3BPM and MYB56 have antagonistic functions on FT expression. Overall this study provides information that broadens the spectrum of CRL3BPM substrates, and also deepens our understanding of how this E3 ligase family affects cellular and developmental processes in plants.

RESULTS

Characterizing Homomeric and Heteromeric Assemblies of MYB56

In an effort to understand how diverse BPM proteins interact with substrates, we previously performed extensive yeast two-hybrid (Y2H) screenings (Weber and Hellmann, 2009; Chen et al., 2013). Besides identifying several ERF/AP2 proteins as substrates, we also found with a high frequency (13 of 83 sequenced clones) MYB56/At5g17800. More detailed Y2H studies showed that MYB56 can assemble with all six BPMs and MYB69 (Figure 1A and 1B). MYB56 is a protein 332 amino acids long with a predicted mass of approximately 37 kDa. It belongs to the MYB class of transcription factors and contains two conserved DNA-binding motifs, R2 and R3, which stretch from amino acid residues 92–139 and 145–190, respectively (Supplemental Figure 1A). According to Dubos et al. (2010), the Arabidopsis R2R3-MYB superfamily encompasses 126 members that can be grouped into 24 subfamilies. According to this grouping, MYB56 belongs to the subfamily 21 (S21), which consists of six additional members, with MYB110 and MYB69 being among the closest relatives (Dubos et al., 2010)( Supplemental Figure 1C and 1D). Based on this relationship, we also tested MYB69 for self-assembly, as well as assembly with BPMs and MYB56. In all cases, robust assembly was detected among the different proteins (Figure 1B–1E). The assembly with BPMs was further confirmed through pull-down assays in which GST-tagged MYB56 and MYB69 proteins successfully precipitated in vitro translated BPM1 (Figure 1F). Through immunoprecipitation (IP) using a specific anti-MYB56 antibody (Supplemental Figure 1B), we also corroborated in planta assembly of MYB56 with CUL3 in wild-type (WT) Col-0 plants (Figure 1G). As expected in 6xamiBPM plants, which are downregulated in all six BPM genes (Chen et al., 2013), this assembly was not detectable (Figure 1G).

Overall these data indicate that MYB56 and MYB69 are potential targets of a CRL3BPM E3 ligase in plants.

Analysis of MYB56 Expression Pattern in Different Tissues

The gene and protein expression patterns in different organs of Arabidopsis were analyzed. Quantitative (q) RT–PCR and
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Figure 2. Expression Level and Subcellular Localization of MYB56.
(A) qRT–PCR analysis on expression of MYB56 in root (R), rosette leaf (RL), cauline leaf (CL), stem (St), siliques (Si), and flower (Fl). Error bars represent the value of the standard error. (B) Tissue-specific Western blot analysis shows highest MYB56 expression in flowers (60 μg of total protein extract loaded per lane). (C) MYB56 promoter:GUS analysis in developing flowers shows strongest staining in anthers. (D) Transient expression analysis in N. benthamiana leaves show that a GFP:MYB56 fusion protein localizes in the nucleus and cytosol. Left-hand side, GFP fluorescence; right-hand side, fluorescence from DAPI staining. Arrows indicate nuclei. Experiments were independently repeated at least three times.

Western blot results showed a consistent pattern in which MYB56 is expressed highest in flowers, followed by siliques (Figure 2A and 2B). Analysis of plants carrying an MYB56 promoter:GUS construct showed a strong staining in anthers of developing flowers as well as at the base of flower primordials (Figure 2C). Investigation of MYB56 subcellular localization through transient expression of a 35S;GFP:MYB56 construct in Nicotiana benthamiana leaves revealed that the protein can be found in the cytosol as well as in the nucleus (Figure 2D).

MYB56 is Unstable in a CRL3BPM E3 Ligase-Dependent Manner

The interaction of MYB56 with BPM proteins made it possible that this transcription factor is degraded by the 26S proteasome in a CRL3BPM-dependent manner. To verify this we exposed plant tissues to either the specific 26S proteasome inhibitor MG132 (Figure 3A), or a combination of the transcriptional inhibitor actinomycin D2 (ActD2) and the translational inhibitor cycloheximide (CHX) (Figure 3B). Seedlings treated with MG132 resulted in a significant increase in MYB56 protein content in WT, which was not observed in the myb56-1 null mutant (Figure 3A). For ActD2 and CHX treatments we chose flowers, wherein MYB56 is expressed at the highest levels, making a reduction in protein content clearly detectable. As shown in Figure 2B, MYB56 was not detectable 6 h after treatment with the ActD2/CHX inhibitors, but did accumulate in the presence of MG132. These findings demonstrate that MYB56 is an unstable protein that is degraded by the 26S proteasome. We further tested whether stability also depends on a functional CUL3BPM E3 ligase. For this purpose we took advantage of a previously described CUL3 double mutant (cul32pp; Thomann et al., 2009), and of plants that are downregulated through an artificial microRNA in all six BPM members (6xamiBPM; Chen et al., 2013). We tested seedlings and flowers, wherein wild-type MYB56 was undetectable after ActD2/CHX treatments, and observed in both 6xamiBPM and cul32pp backgrounds stabilization of MYB56 protein (Figure 3C). These results indicated that MYB56 may be a substrate of a CRL3BPM E3 ligase.

Loss of MYB56 Results in an Early-Flowering Phenotype

Based on the strong expression patterns in flowers and the lack of information available for MYB56 function in Arabidopsis, we investigated the development of two different alleles of myb56 (myb56-1 and myb56-2) null mutants under long-day (LD) growth conditions. Compared with WT, both mutants showed early flowering (Figure 4A). Previously we had observed a late-flowering phenotype in 6xamiBPM plants with reduced BPM expression (Chen et al., 2013; Figure 4A). This was intriguing because the opposing flowering behaviors indicate that BPMs positively affect flowering, whereas MYB56 is a negative regulator of this developmental process. These genetic-based findings were in agreement with the potential interplay of MYB56 proteins and the CUL3BPM complex, potentially offering a good model system for further investigation into flowering regulation.

CRL3BPM and MYB56 Participate in Controlling FT Expression

One of the major pathways controlling the time point of flower development depends on the expression of FT, which is also referred to as the florigen (Song et al., 2013). This protein is expressed in leaves and transported through the phloem to the apical meristem, where it participates in inducing a developmental switch from the vegetative to the reproductive stage (Andres and Coupland, 2012). The FT gene expression pattern depends on various environmental and developmental cues, and changes significantly over the course of the day. For example, while expression is comparably low in the middle of the day, a significant increase is seen at the end of the photoperiod (Andres and Coupland, 2012; Song et al., 2013). This complex expression pattern is tightly regulated through the
activities of a multitude of different transcription factors (Song et al., 2013), some of which belong to the ERF/AP2 transcription factor family (Schmid et al., 2003; Yant et al., 2010).

We tested FT expression in leaves of WT, two independent 6xamiBPM lines, and the two myb56 null mutants over a time course of 4 weeks. Sampling started with 3-week-old plants, and all samples were harvested in the late afternoon. We observed a significant increase in FT expression during the third and fourth weeks in myb56-1 and myb56-2 mutants when compared with WT, and no change in expression in the two 6xamiBPM lines over the course of the 4-week period (Figure 4C; Supplemental Table 1).

We further tested how FT and MYB56 expression varies in rosette leaves of 4-week-old plants across a time period of 10 h, from 8 AM until 6 PM. qRT–PCR analysis showed that FT expression is low at the beginning of the photoperiod, increases till 10 AM, drops slightly between 10 AM and 2 PM, and rises again until 6 PM (Figure 4D; Supplemental Table 2). The observed expression was in agreement with earlier work on FT (Suarez-Lopez et al., 2001). MYB56 showed an opposing expression pattern with highest levels at 8 AM and lowest at 6 PM (Figure 4D). Western blot analysis for MYB56 content within the same time frame indicated that MYB56 protein amounts are also higher at the beginning of the day than in late afternoon (Figure 4D).

Flowering and expression data across the different genetic backgrounds indicated that it is possible that FT is directly controlled through CRL3BPM and MYB56 activities.

To investigate this further, anti-CUL3 and anti-MYB56 ChIP analyses were done comparing WT with two 6xamiBPM lines and myb56-1 null mutant plants, respectively (Figure 5). Since it was not known where MYB56 and CRL3BPM may bind to the FT promoter, we decided to analyze a region around 1400 bp upstream of the ATG. The region was divided into six sections with each part covering roughly 230 bp (Figure 5A).

Based on the FT qRT–PCR results, we decided to do ChIP experiments on 3-week-old leaves, wherein FT expression was comparably low. This time point could reflect a relatively high level of negative regulation and thereby yield a significant enrichment if either CUL3 or MYB56 are associated with the FT promoter. ChIP analysis using the anti-CUL3 antibody showed that the cullin is bound to the FT promoter primarily within the first 400 bp upstream of the ATG (Figure 5B, regions 5 and 6; Supplemental Table 3). We also found significant enrichment in a range of 1200–1400 bp upstream (Figure 5B, region 1; Supplemental Table 3). Both of these peaks were not detectable in either 6xamiBPM background, indicating that the presence of CUL3 at these loci depends on BPM proteins (Figure 5B; Supplemental Table 3). ChIP experiments using the anti-MYB56 antibody identified three enriched sites: first, within the region of the first 400 bp upstream of the ATG (Figure 5C, regions 5 and 6), which appears to be overlapping with, but not identical to, the pattern seen in the anti-CUL3 ChIPs; second, within a region of ~600–770 bp upstream (Figure 5C, region 3; Supplemental Table 3); and third, within the region ~1200–1400 bp upstream (Figure 5B, region 1; Supplemental Table 3), similar to the enrichment observed for anti-CUL3 ChIP. As a control myb56-1 null mutants were used, and no enrichment was detected in leaf material from these plants, showing that enriched peaks are specific for MYB56. In addition, we were not able to detect any enrichment in a region within a coding region of FT used as a negative control (Figure 5B and 5C; Supplemental Table 3). Overall these results indicate that both CRL3BPM and MYB56 participate in the direct control of FT expression.

**DISCUSSION**

The current data provide new insights into the function of CRL3BPM E3 ligases in plants. We have established that members of the R2R3 MYB family interact with BPM proteins. Specifically for MYB56, we showed that it is a substrate of the 26S proteasome and that it is recognized by a CRL3BPM E3 ligase at the DNA level. The work further identified FT, a central regulator of flowering time, as a direct target of MYB56 and CRL3BPM.
activities, and provides novel insights into how expression of this central gene is controlled.

MYB56 and MYB69 belong to the S21 subfamily of MYB family. This group of transcription factors contains five other members, MYB52, MYB54, MYB105, MYB110, and MYB117 (Dubos et al., 2010). MYB56 and MYB69 have been reported in context with seed and endosperm development (McKeown et al., 2011; Zhang et al., 2013). Overexpression of MYB56 leads to larger seeds, similar to what we observed in 6xamiBPM plants (Chen et al., 2013; Zhang et al., 2013). Given that LOF proteins are also substrates of the CRL3BPM complex, one may expect that these E3 ligases are widely involved in reproductive processes ranging from the initiation of flowering to seed development.

Work in rice on a close relative to MYB56, CONSTITUTIVE SHADE AVOIDANCE (CSA), has shown that it is directly involved in controlling expression of the MONOSACCHARIDE TRANSPORTER 8 (MST8) (Zhang et al., 2010). MST8 is strongly
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Figure 5. ChIP-qPCR Analysis Shows Enrichment of FT Promoter Regions.
(A) Schematic drawing of six different FT promoter regions analyzed via qPCR after α-CUL3 ChIP experiments. “0” indicates location of the start codon.
(B) α-CUL3 ChIP samples show that significant enrichments were detectable in regions 1, 5, and 6 in WT, but not in a 6xamiBPM control.
(C) α-MYB56 ChIP samples show significant enrichments in regions 1, 3, 5, and 6 in WT, but not in a myb56-1 null mutant. “NC” indicates negative control primer set afterward of FT gene’s start codon. (B) and (C) both show the average value of a representative three technical replicates of one biological replicate. Two biological replicates were done in corresponding backgrounds, and ChIP-qPCR data are summarized in Supplemental Table 3. Asterisks indicate significant enrichment of DNA compared with individual control (t-test; \( P < 0.05 \)). Error bars indicate the value of the standard deviation.

work from multiple groups has established a highly complex regulatory network of different transcription factors that positively or negatively affect the expression of FT in leaves (Song et al., 2013). Not surprisingly, a variety of environmental and developmental cues, such as temperature and photoperiod, regulate activity to precisely time FT expression to induce flower development (Song et al., 2013). Our current work adds another important facet to this regulatory network. The observed opposing expression of MYB56 in comparison with FT, together with upregulated FT expression in myb56 null mutants, and their early-flowering phenotype, assign the role of a negative regulator in the control of flowering time and FT expression to MYB56, with CRL3BPM E3 ligases acting antagonistically to MYB56.

It must be emphasized that the work presented here was done under LD conditions. We did some analyses under short-day (SD) conditions, and observed similar flowering in the myb56 null mutants compared with WT plants (Supplemental Figure 5). These findings indicate that the photoperiod and light intensity are critical parameters for the integration of MYB56 activities into flowering time control. 6xamiBPM plants, in contrast, still maintained their late-flowering phenotype under SD conditions (Supplemental Figure 5), which may point to additional functions that CRL3BPM E3 ligases have in flowering control that could be independent of FT expression. In addition, under LD conditions average leaf numbers of early-flowering myb56 mutants were slightly but not significantly changed, while late-flowering 6xamiBPM plants had significantly fewer rosette leaves at the time of flowering (Figure 4B). This was surprising, since many flowering mutants have either fewer or more rosette leaves corresponding to whether they are early or late flowering, respectively (for an overview see Koornneef et al., 1998). We saw better correlation of leaf number and flowering time point under SD conditions with the increased leaf numbers in late-flowering 6xamiBPM plants (Supplemental Figure 5). The exact reasons for these differences are not clear. For the myb56 mutants, this may be related to the relatively mild early-flowering phenotype we saw in these plants, and expanding growth conditions to incorporate additional environmental cues will be of interest in future studies on MYB56-dependent FT regulation. The BPM family, by contrast, participates broadly in development and ABA signal transduction (Lechner et al., 2011; Chen et al., 2013), accounting for the wide range of regulatory pathways disturbed in 6xamiBPM plants, and the aberrant FT expression likely represents only one facet of BPM activities.

Several ERF/AP2 transcription factors, SCHLAFMÜTZE (SMZ), SCHNARCHZAPFEN (SNZ), TEMPRANILLO (TEM) 1 and 2, and TARGET OF EAT (TOE) 1, 2, and 3, are also described to function as negative regulators of FT expression (Schmid et al., 2003; Jung et al., 2007; Castillejo and Pelaz, 2008; Glazinska et al., 2009; Mathieu et al., 2009). Another interesting question for future research will be to explore if any of these can be targets of CRL3BPM E3 ligases as well. In the context of our partial overlapping α-CUL3 and α-MYB56 ChIP results, the CRL3BPM complex might also be found to be associated with these ERF/AP2 transcription factors. A likely candidate here would be, for example, TEM1, which binds shortly upstream of the start codon of FT (Castillejo and Pelaz, 2008). Consequently, the observed consistent repression of FT expression over the
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in *Escherichia coli*; pK7FWG2 was used for expression in plants and subcellular localization studies as described by Karimi et al. (2002). Generation of artificial microRNA construct was as described by Chen et al. (2013). For the promoter of MYB56, a 1648 bp fragment upstream of the ATG was amplified from genomic DNA, and after sequence verification cloned into the binary vector pCB308 using SpeI and HindIII restriction sites, respectively (Xiang et al., 1999). Primers used for cloning are listed in Supplemental Table 1.

**GUS Staining and Subcellular Localization Studies**

Detection of GUS expression was done as described before, following the procedure described by Jefferson (1989). In brief, flower tissues were incubated in GUS staining solution for up to 24 h. Clearing of tissue was done overnight in 100% ethanol. Pictures were taken with an Olympus Digital camera C-4040Zoom mounted on a stereo microscope SZX12 (Olympus, Hamburg, Germany). Fluorescent fusion proteins of the GFP:MYB56 were transiently expressed in tobacco epidermal cells, following the method described by Sparkes et al. (2006). GFP expression was detected and documented with a Zeiss LSM 510 Meta confocal microscope (Jena, Germany).

**Y2H Studies, Complex Assembly Analysis, and Stability Assays**

Y2H studies were followed as described by Weber et al. (2005). IP analysis was done as described by Chen et al. (2013). For stability assays, Arabidopsis seedlings were cultured on solid ATS medium for 2 weeks before transfer to 5 ml of ATS liquid media following the protocol of Chen et al. (2013). Western blot analysis and protein detection was followed using standard procedures as described by Chen et al. (2013). *In vitro* transcription/translation studies were done as described by Weber and Hellmann (2009). The antibodies against MYB56 and CUL3 were generated in rabbits, and designed and produced by GenScript (Piscataway, NJ, USA).

**RNA Isolation and Expression Analysis**

Total Arabidopsis RNA was extracted following the protocol of the Isolate RNA Kit from Bioline (Taunton, MA, USA); reverse transcription was done according to the manual of the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT–PCR analysis was done as described by Bernhardt et al. (2006). qRT–PCR reactions (95°C, 7 min; 96°C, 15 s; 60°C, 1 min; 40 cycles) were done on a 7500 Fast Real-Time PCR System (Applied Biosystems). ACTIN2 was selected as reference gene in qRT–PCRs for data modification. Fourteen 2-week-old seedlings were pooled for each biological replicate. Primers used for qRT–PCR are listed in Supplemental Table 4.

**ChIP Assays**

ChIP assays were followed according to the protocols described by Morohashi et al. (2009) and Chen et al. (2013), with the modification that for all samples 60 mg (fresh weight) of seedlings or rosette leaf tissue, respectively, were collected for each ChIP experiment and crosslinked for 20 min under vacuum in crosslink buffer containing 1% formaldehyde. As input control for data normalization, a portion of flower tissues was recognized by MYB56, specific primers were designed (Supplemental Table 4). EF1α was selected as reference gene for internal control. qPCR reactions (95°C, 10 min; 95°C, 15 s; 60°C, 1 min; 50 cycles) were done in WT and different mutant backgrounds.

**ACCESSION NUMBERS**

ACTIN2: At3g18780; BPM1: At5g19000; BPM2: At3g06190; BPM3: At2g39760; BPM4: At3g03740; BPM5: At5g21010; BPM6: At3g43700;
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CUL3a: At1g26830; CUL3b: At1g66390; FT: At1g65480; MYB56: At1g73410; MYB56: At1g73410; MYB69: At4g33450; STP1: At1g11260.

SUPPLEMENTAL INFORMATION
Supplemental Information is available at Molecular Plant Online.

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