The bHLH transcription factor AcB2 regulates anthocyanin biosynthesis in onion (Allium cepa L.)

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Abstract
Onion bulb color is a key breeding trait. The red bulb color is caused by the presence of anthocyanins, which are products of the flavonoid biosynthetic pathway. Research on flavonoid regulation in onion is lagging compared with that in other crops. AcB2 encodes a basic helix–loop–helix (bHLH) transcription factor, and its transcription is positively associated with anthocyanin accumulation and correlated with the expression of AcMYB1, which is an activator in the flavonoid biosynthetic pathway in onion. Phylogenetic analysis showed that AcB2 was grouped into the TRANSPARENT TESTA 8 (TT8) clade of the bHLH III subgroup. The AcB2 protein contained an MYB-interacting region and physically interacted with AcMYB1 in yeast and tobacco leaves. AcMYB1 directly bound to the promoters of anthocyanin synthase (AcANS) and flavonoid 3-hydroxylase 1 (AcF3H1) and activated their expression. The coexpression of AcB2 with AcMYB1 in Arabidopsis thaliana protoplasts dramatically increased the expression of AcANS and AcF3H1 compared with that under the expression of AcMYB1 alone. Transient co-overexpression of AcB2 with AcMYB1 induced anthocyanin accumulation in the epithelial cells of onion bulbs. Complementation of the Arabidopsis tt8-1 mutant with AcB2 restored pigmentation defects in tt8-1. In addition, AcB2 physically interacted with AtTT2 in yeast cells and tobacco leaves, indicating that the functions of AcB2 were similar to those of AtTT8. Together, these results demonstrated that AcB2 enhanced the function of AcMYB1 in upregulating anthocyanin biosynthesis in onion, which provides a theoretical basis for breeding onions with higher anthocyanin contents.

Introduction
Flavonols, anthocyanins, and proanthocyanidins (PAs) are all metabolites of the flavonoid biosynthetic pathway and accumulate in the seed coats [1–3], flowers [4–6], fruits [7–9], and leaves [10, 11] of many plants. The flavonoid biosynthetic pathway has been well characterized, and the major biosynthetic steps and the corresponding structural genes have been identified in several model crop plants. Onion bulbs range in color from white and pink to red, and these colors result from many different variations and contents of flavonoid compounds [12, 13]. A comparative transcriptional analysis in onion showed that the expression levels of flavonoid structural genes were positively associated with anthocyanin accumulation [14].

Regulation of the flavonoid biosynthetic pathway commonly occurs at the transcriptional level by the MYB-bHLH-WD40 transcription factor (TF) complex [15–17]. The basic helix–loop–helix (bHLH) protein family is the second-largest class of plant TFs and is divided into 26 subgroups [18]. Flavonoid-related bHLHs belong to subgroup IIIf, which contain an MYB-interacting region (MIR) at the amino terminus, a WD40 repeat domain (WDR)-interacting region, and a conserved bHLH domain [17]. The MIR domain interacts with a conserved motif [(D/E)X2(R-K)X3LX6LX3R] in the R3 repeat of MYBs [19]. Flavonoid-related MYBs usually bind to the promoters of target structural genes to activate their expression, and this binding process depends on the interaction with bHLHs. MBW complexes, such as transparent testa2 (TT2)-TT8-transparent testa glabra1 (TTG1) in Arabidopsis [15, 20–22], anthocyanin2 AN2-JAF13-AN11 in Petunia hybrida [4, 23], and legume anthocyanin production1 (MtLAP1)-MtTT8-MtWD40-1 in Medicago truncatula [24, 25], are necessary for anthocyanin and PA biosynthesis in plants. In Arabidopsis, the MBW complex TT2-TT8-TTG1 regulates flavonoid biosynthesis [15], and the tt8-1 mutant is deficient in flavonoid and PA biosynthesis, resulting in a yellow seed coat [21].

In contrast to those in model plants, studies on the gene regulatory network of anthocyanin biosynthesis in onion are very limited. A previous study showed
that AcMYB1 activated anthocyanin synthesis [26]. The transcript abundance of AcMYB1 correlated with anthocyanin production. Both transient and stable heterologous coexpression of AcMYB1 and the anthocyanin bHLH regulator Zm-Lc in onion and garlic induced the accumulation of anthocyanins [26]. These results suggested that AcMYB1 controlled anthocyanin accumulation together with its regulatory partner bHLH. However, the bHLH partners of AcMYB1 in onion have not been reported. The identification of onion anthocyanin regulatory factors will provide a basis for the breeding of highly nutritious and high-anthocyanin onions.

In addition, at least five major loci (I, C, G, R, and I) controlling bulb color in onion have been identified by inheritance research [27–30]. The I and C loci were found to determine the white bulb color [30, 31]. Recently, the bHLH transcription factor AcB2 was verified as a causative gene for the C locus [32]. A non-autonomous DNA transposon, AcWHITE, was found in the promoter of AcB2 in white onion and linked perfectly to the C locus. However, not all white onion lines harbor the AcWHITE transposon and, more importantly, there are still knowledge gaps regarding the function of AcB2 in regulating anthocyanin biosynthesis. Clarifying the molecular function of AcB2 is necessary to further reveal the regulatory pathway of onion anthocyanin synthesis and to breed high-anthocyanin onions.

This study reports the functional characterization of AcB2. The expression level of AcB2 was drastically reduced in white Ringmaster bulbs compared with red Xiu-Qiu bulbs. However, the AcWHITE insertion was not identified in the AcB2 promoter of Ringmaster onion. Significant transcription reduction of AcB2 was found in the inner non-red layers of Xiu-Qiu onion in this study, suggesting that the transcription pattern of AcB2 was independent of the genotype but correlated with the accumulation of anthocyanins. The bHLH TF AcB2 was grouped with AtTT8 and was located in the MYB-interacting region of the deduced amino acid sequence of AcB2 with bHLH conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain and an MIR domain.

AcB2 encoded a bHLH protein containing an MYB-interacting region

The 2006-bp full-length cDNA of AcB2 was isolated and sequenced to verify the open reading frame (ORF). This gene contained a putative ORF of 1848 bp that encoded a peptide of 615 amino acids (aa), including a conserved bHLH domain and an MIR domain. Alignment of the deduced amino acid sequence of AcB2 with bHLH proteins in other plant species (AtTT8 in Arabidopsis thaliana, MdbHLH3 in Malus domestica, MtTT8 in M. truncatula, PhAN1 in P. hybrida, and VvMYC1 in Vitis vinifera) revealed an N-terminal MIR at 11–194 aa and a conserved bHLH domain at 436–484 aa (Supplementary Data Fig. S1 and Fig. S4A). The conserved MIR domain was a prerequisite for the formation of MBW activation complexes. A phylogenetic tree was constructed using full-length amino acid sequences of AcB2 and 23 other bHLH proteins (Fig. 2). AcB2 belonged to subgroup IIIf clade B (Fig. 2), clustering with AtTT8 (37.52% identity), MtTT8 (47.36% identity), VvMYC1 (46.18% identity), and MdbHLH3 (43.94% identity), which are all direct regulators of anthocyanin genes. Structure and phylogenetic analyses indicated that AcB2 was a candidate regulatory gene with a function similar to that of AtTT8 in the anthocyanin biosynthetic pathway.

Localization of AcB2 in the nucleus and cytoplasm

AcB2 must enter the nucleus of plant cells to function as a TF. A nuclear localization signal peptide (NLS, 444–472 aa) was identified in the predicted AcB2 protein (Supplementary Data Fig. S1) but with a low probability (score = 5.1, using the nLS Mapper, http://nls-mapper.r.iab.keio.ac.jp/cgi-bin/NLS_Mapper). A transient expression experiment in Nicotiana benthamiana leaves was performed to verify the subcellular localization of AcB2. The green fluorescent signal of AcB2–green fluorescent protein (GFP) was detected using a fluorescence microscope (Fig. 3), showing both cytoplasmic and nuclear localization. In contrast, the GFP control could be found throughout the cells of tobacco leaves. The green fluorescent signal of AcMYB1–GFP was mostly observed in the nucleus.

Results

The expression level of AcB2 was correlated with anthocyanin biosynthesis

A comparative transcriptional analysis was performed using Xiu-Qiu (red) and Ringmaster (white) onion bulbs [14]. In addition to the previously identified structural genes, the AcMYB1 and AcB2 genes showed drastic expression reduction in the Ringmaster onion bulbs, and the results were verified by quantitative real-time PCR (qRT–PCR; Fig. 1A). Insertion of the DNA transposon AcWHITE into the AcB2 promoter resulted in white-colored onion bulbs. However, the white Ringmaster onion bulbs did not contain an AcWHITE insertion, and no polymorphism in this gene was identified between red (Xiu-Qiu) and white (Ringmaster) onion bulbs.

The expression levels of AcMYB1 and AcB2 were analyzed in different parts of Xiu-Qiu onion bulbs. The anthocyanin content was reduced dramatically in the inner non-red layers of Xiu-Qiu onion bulbs (Fig. 1B). The qRT–PCR results showed significant transcription reduction of AcMYB1 and AcB2 in the inner non-red layers compared with the red layer of Xiu-Qiu onion bulbs (Fig. 1C), indicating that the expression patterns of AcMYB1 and AcB2 correlated with anthocyanin biosynthesis.
Figure 1. Expression pattern of AcB2. qRT–PCR analysis of AcMYB1 and AcB2 transcript abundances in (A) Xiu-Qiu red onions and Ringmaster white onions and (C) red and inner non-red layers of Xiu-Qiu red onions. Transcript abundance was normalized to β-ACTIN transcript abundance. (B) Pigmentation and anthocyanin content in the red and non-red layers of Xiu-Qiu red onions. All data are from three biological replicates and expressed as mean ± standard deviation. **P < .01 using Student’s t-test (n = 3).

and a weak GFP signal was observed in the cytoplasm. The results indicated that AcB2 and AcMYB1 could be transported to the nucleus.

The N-terminus of AcB2 physically interacted with AcMYB1

As putative transcriptional regulators, bHLH proteins interact with MYB TFs to compose the MBW complex. Yeast two-hybrid assays were performed to investigate whether AcB2 could interact with AcMYB1. A full-length AcB2 protein was found to interact with AcMYB1 directly (Fig. 4B). To verify which domain of AcB2 was responsible for interacting with AcMYB1, truncated fragments of B2, B2-1 (1–194 aa), B2-2 (151–615 aa), B2-3 (1–436 aa), and B2-4 (435–615 aa) (Fig. 4A) were used as bait to probe putative interactions with AcMYB1. The results showed that the truncated N-terminus (1–436 aa) of B2 interacted strongly with AcMYB1, while the MIR domain fragment (1–194 aa) did not (Fig. 4B). All these results implied that, in addition to the MIR domain, the middle region of the peptide (194–436 aa) was also required for AcB2 binding to AcMYB1.

Furthermore, a luciferase complementation imaging (LCI) assay was performed in tobacco leaves by transient coexpression of AcB2 fused with the N-terminus of luciferase (nLUC) and AcMYB1 fused with the C-terminus of luciferase (cLUC). The results in Figure 4C provide further proof of the interaction between AcB2 and AcMYB1 in vivo.

AcMYB1 bound to AcAcF3H1 and AcANS promoters

The transcription levels of the structural genes AcF3H1, AcANS, and dihydroflavonol 4-reductase-A (AcDFR-A) in the anthocyanin biosynthetic pathway were significantly reduced in white onion compared with red onion [14]. To investigate whether AcMYB1 and AcB2 were involved in the transcriptional regulation of AcF3H1, AcANS, and AcDFR-A, yeast one-hybrid assays were performed. The promoters of these three genes were isolated from Xiu-Qiu and Ringmaster onion bulbs, and the predicted MYB-recognizing element (MRE) ANCNNNC and the bHLH-recognizing element (BRE) CANNTG or CACN(A/C/T)(G/T) [33] were identified (Supplementary Data Figs S2–S4). The number and order of candidate MYB and bHLH sites exhibited marked differences in each gene promoter between Xiu-Qiu and Ringmaster onion bulbs (Fig. 5A and B; Supplementary Data Fig.
recognition of AcMYB1 (Fig. 5C). Sequence analysis of another seven red and four white onion inbred lines showed that the red onion lines R784 and R791 had the same AcF3H1 sequence as Ringmaster, and the white onion line W559 had the same AcF3H1 sequence as Xiu-Qiu (Supplementary Data Fig. S6A). These results suggested that the difference in the AcF3H1 promoter was not linked to onion color and that AcF3H1 might have been activated by TFs other than AcMYB1. Truncated fragments of the AcF3H1 promoter (Supplementary Data Fig. S6B) were used in a yeast one-hybrid assay to elucidate which region was responsible for binding to the AcF3H1 promoter. AcMYB1 failed to bind to any truncated fragments, indicating that the full-length AcF3H1 promoter contained the major AcMYB1 binding sites.

Yeast one-hybrid assays showed that AcB2 could not bind to the promoter regions of AcANS and AcF3H1 (Fig. 5C). In addition, the promoter of the AcDFR-A gene showed self-activation in this yeast one-hybrid system (Supplementary Data Fig. S5B). Thus, more experiments are needed to verify whether AcMYB1 and AcB2 can directly bind to the promoter of AcDFR-A.

AcB2 interacted with AcMYB1 to activate AcANS and AcF3H1

Promoter transactivation assays were performed to examine whether the AcMYB1-B2 interaction could activate structural genes. Firefly luciferase reporter constructs were cotransfected with AcMYB1 or AcB2 effector constructs into Arabidopsis leaf protoplasts (Fig. 6A). AcANS was strongly induced by AcMYB1, whereas AcDFR-A and AcF3H1 were moderately upregulated by AcMYB1 (Fig. 6). AcB2 alone could not activate any of these three structural genes. Coexpression of AcB2 and AcMYB1 increased AcANS or AcF3H1 promoter-luciferase reporter activation by >2.5-fold compared with the expression of AcMYB1 alone. However, AcB2 could not increase the activation of the AcDFR-A promoter induced by AcMYB1. These data suggested that AcB2 interacted with AcMYB1 to activate the anthocyanin structural genes AcANS and AcF3H1.

Co-overexpression of AcB2 with AcMYB1 induced anthocyanin accumulation in onion

To verify the ability of AcB2 to upregulate anthocyanin biosynthesis in onion, biolistic transformation was carried out. When AcMYB1-AcB2-GFP was transiently introduced into intraepidermal cells of red onion bulbs, anthocyanin accumulation was observed (Fig. 7), while AcMYB1 and AcB2 alone did not activate anthocyanin biosynthesis. In this assay, anthocyanin accumulation was detected within 24 hours after bombardment to prevent autonomous activation of the pigment. The results indicated that AcB2 coupled with AcMYB1 to activate anthocyanin biosynthesis in onion.

AcB2 interacted with AtTT2 and complemented the Arabidopsis tt8 mutant phenotype

AcB2 was overexpressed in the Arabidopsis tt8-1 mutant (SALK_030966) to test the function of AcB2 in
regulating the biosynthesis of flavonoids. The T2 seed progeny harvested from 11 independent T1 hygromycin B-resistant transgenic lines were isolated, and AcB2-GFP expression was detected (Supplementary Data Fig. S7). The tt8-1OEAcB2-2 transformants had the highest level of expression of AcB2-GFP and exhibited brown seeds similar to those of wild-type Arabidopsis (Fig. 8A). Furthermore, analysis of flavonoid accumulation in the wild-type and T2 seeds was performed. The data showed that flavonoid deposition in the seeds of the complemented tt8-1 was similar to that of the wild type (Fig. 8B), suggesting that the flavonoid production
defect and yellow seed color of tt8-1 were restored by AcB2.

Furthermore, yeast two-hybrid assays (Fig. 8C) and LCI assays (Fig. 8D) demonstrated that AcB2 physically interacted with AtTT2. These data demonstrated that AcB2 had a function similar to that of Arabidopsis TT8.

**Discussion**

**Regulation of AcB2 expression by cis elements and trans factors**

Previous studies showed that the transcription patterns of TFs involved in the anthocyanin biosynthetic pathway correlated with anthocyanin accumulation [3, 26]. Baek et al. reported that the transcription level of AcB2 was significantly reduced in white bulbs compared with yellow bulbs [31]. Subsequent research indicated that the insertion of the DNA transposon AcWHITE into the AcB2 promoter resulted in the white color of onion bulbs [32]. AcWHITE was not found in the AcB2 promoter of any yellow or red onion inbred lines. In our study, Ringmaster was a white onion line with a recessive trait [14] that does not possess AcWHITE in the AcB2 promoter. No other polymorphisms were detected in the AcB2 gene between the Ringmaster and Xiu-Qiu varieties. In addition, the expression of AcB2 in the inner non-red layer showed a significant reduction compared with that in the red layer of Xiu-Qiu onions, suggesting that the transcription pattern of AcB2 was independent of the genotype but correlated with the accumulation of anthocyanins and that other genes might regulate the expression of AcB2. All the data indicated that the transcription of AcB2 was regulated by both cis-elements and trans-factors.

**AcB2 coupled with AcMYB1 to induce the expression of AcF3H1 and AcANS**

Previous studies reported that anthocyanin accumulation was spatiotemporally regulated by MYB and bHLH TFs in a variety of plants [34–36]. AcMYB1 is a positive regulator of anthocyanin biosynthesis in onion [26]. AcB2 was identified as a causal gene for the C locus in controlling onion coloration [32]. However, it is still not clear which structural genes are the target genes of AcB2 and AcMYB1. In this study, the transcription patterns of AcB2 and AcMYB1 and the structural genes AcF3H1, AcDFR-A, and AcANS were positively correlated with the patterns of anthocyanin biosynthesis in onion. Metabolite profiling and transcriptomic analysis indicated that AcB2 regulated a subset of flavonoid structural genes.
Figure 6. AcB2 interacted with AcMYB1 to activate the AcANS and AcF3H1 promoters. (A) Schematic of effector and reporter constructs used in transient expression assays. AcMYB1 and AcB2 driven by the CaMV 35S promoter were cloned into the effector construct, and the reporter construct contained the firefly luciferase (F-LUC) reporter gene driven by the AcDFR-A, AcANS, or AcF3H1 promoter and the Renilla luciferase (R-LUC) gene driven by the CaMV 35S promoter as a control for normalization. (B) Promoters of AcDFR-A, AcANS, and AcF3H1 were activated by coexpression of AcB2 and AcMYB1 in Arabidopsis protoplasts. Promoter activities were calculated as the ratio of firefly luciferase activity to Renilla luciferase activity. Data are from three independent replicates and expressed as mean ± standard deviation.

Figure 7. AcB2 coupled with AcMYB1 to activate anthocyanin accumulation in onion. 35S:AcMYB1-GFP, 35S:AcB2-GFP, and 35S:AcMYB-AcB2-GFP were transferred into intraepidermal cells of red onion bulbs using the particle bombardment method. Bright (left), GFP (middle), and merged images were collected. Anthocyanin accumulation was observed with the transient expression of AcMYB-AcB2, while AcMYB1 or AcB2 alone did not induce a pigment response. Scale bars = 200 μm.
In summary, our results showed that AcMYB1 alone could directly bind the promoters of anthocyanin structural genes and activate the expression of these genes. AcB2 alone could not directly bind the promoters of structural genes and activate their expression. When both AcMYB1 and AcB2 were present, AcB2 promoted the transcriptional activation of structural genes through its interaction with AcMYB1, and the expression of structural genes was increased to higher degrees. For example, in the presence of both AcB2 and AcMYB1, the expression of AcANS and AcF3H1 increased >2.5-fold compared with that under AcMYB1 alone. The increased expression of structural genes ultimately promoted the accumulation of anthocyanins. Our research elucidated the less-studied molecular regulatory mechanism of AcMYB1-AcB2 in anthocyanin accumulation in onion. This study provides a basis for molecular biology research on anthocyanin biosynthesis in onion and provides new opportunities for breeding onions with higher anthocyanin contents.

Materials and methods

Plant materials

Two onion cultivars were used in this research: the white onion Ringmaster and the dark-red onion Xiu-Qiu. Hybridization analysis from a previous study showed that Ringmaster had the recessive white onion trait [14]. The onion cultivars were grown in the experimental field of the Beijing Academy of Agriculture and Forestry Vegetable Research Center under natural conditions. Outer layers of mature Xiu-Qiu and Ringmaster bulbs and outer red layers and inner non-red layers of mature Xiu-Qiu bulbs were used in qRT–PCR and anthocyanin content analyses.

The A. thaliana Columbia-0 ecotype and the mutant line tt8-1 (SALK_030966) were obtained from the Arabidopsis Biological Resource Center. Homozygote identification for the tt8-1 mutant was performed by PCR using the primers tt8-1-1LP, tt8-1-RP and LBb1.3 (Supplementary Data Table S1). Arabidopsis seeds were sterilized in 1.5% (v/v) sodium hypochlorite for 20 minutes and cold-treated at 4°C for 3 days before sowing. Young seedlings were planted in Murashige and Skoog (MS) medium or in soil in an incubator with a light:dark photoperiod of 16 hours/8 hours, 25–22°C.

Gene expression analysis and RNA sequencing

Total RNA was isolated from onions using an RNA extraction kit (Tiangen, DP441). Subsequently, cDNAs were reverse-transcribed using PrimeScript IV First-strand cDNA Synthesis Mix (Takara, 6215A). The ORFs of AcMYB1 and AcB2 were amplified from cDNAs using the primers AcMYB1-RT-F, AcMYB1-RT-R, AcB2-RT-F, and AcB2-RT-R (Supplementary Data Table S1). qRT–PCR was performed using Power SYBR™ Green PCR Master Mix (Thermo Scientific, CAT# 4368577) on an ABI 7500 instrument. qRT–PCR was performed using β-ACTIN

Figure 8. AcB2 complemented the Arabidopsis mutant tt8-1 phenotype and physically interacted with AtTT2. (A) Phenotypic complementation in tt8-1 mutants expressing AcB2. Seeds of wild-type Col-0, tt8-1, and tt8-1OEAcB2-2 (T2 progeny of tt8-1 homozygotes overexpressing AcB2). (B) Flavonoid contents in the seeds of wild-type Col-0, tt8-1 mutant, and tt8-1OEAcB2-2. **P < .01 by Student’s t-test (n = 5). (C) AcB2 and AtTT2 physically interacted in yeast cells. AD, activation domain; BD, binding domain; SD, minimal medium; W, tryptophan; L, leucine; H, histidine; A, adenine. Images are representative of three independently repeated experiments. (D) Luciferase complementation imaging confirming the interaction of AcB2 and AtTT2.
Table S1. Pairs of constructs with bait and used for plasmid construction are listed in Supplementary Data Table S1. The promoters of AcANS and AcDFR-A from Xi-Qiu and Ringmaster onions were amplified using PrimeSTAR Max DNA Polymerase. The primers are listed in Supplementary Data Table S1. The promoters of AcDFR-A, AcANS, and AcF3H1 were cloned into pLacZi, and cDNAs of AcMYB1 and AcB2 were introduced into pB42AD. The pairs of constructs were cotransformed into the yeast strain EGY48. Positive strains were screened for the development of blue color on a medium lacking Trp and Ura but containing X-gal. The primers used for the construction of pLacZi and pB42AD are listed in Supplementary Data Table S1.

Luciferase complementation imaging assay
The full-length cDNAs of AcMYB1 and AtTT2 were inserted into the plasmid 35S::cLuc and the full-length cDNA of AcB2 was inserted into the plasmid 35S::nLuc. The constructs were transferred into Agrobacterium tumefaciens strain GV3101. Various pairs of constructs were co-infiltrated into N. benthamiana leaves as described in a previous study [40]. Images were acquired using a charge-coupled device camera. The primers used for plasmid construction are listed in Supplementary Data Table S1.

Subcellular localization of AcB2
Full-length cDNAs of AcMYB1 and AcB2 were amplified by reverse transcription RT–PCR and introduced into the modified vector pCAMBA1300 [41]. The binary construct containing fused AcMYB1-GFP and AcB2-GFP under the control of a super-promoter was transferred into A. tumefaciens strain GV3101. Agrobacterium-mediated transformation of N. benthamiana leaves was performed as part of an LCI assay. GFP signals were detected 3 days after transformation using a ZEISS LSM710 confocal microscope with excitation at 488 nm and a 522–572 nm filter.

Transactivation assays
Transfection of Arabidopsis protoplasts and promoter transactivation assays were performed as described previously [42]. The promoters of AcANS, AcF3H1, and AcDFR-A were introduced into the reporter plasmid pGreenII 0800 LUC, and cDNAs of AcMYB1 and AcB2 were introduced into the effector plasmid pGreenII 62-SK. A Renilla luciferase gene in pGreenII 0800 LUC was used as the control. The effector and reporter construct pairs were cotransfected into Arabidopsis protoplasts. The luciferase activities were quantified by the Dual-Luciferase Reporter Assay Kit (Vazyme DL101–01). The primers are listed in Supplementary Data Table S1.

Particle bombardment experiment
AcMYB1-GFP, AcB2-GFP, and AcMYB-AcB2-GFP were cloned into the vector pYBA1132 under the control of the CaMV 35S promoter. Particle bombardment using Biolistic PDC-1000/He was carried out as described previously [43]. Intraepidermal skin of red onion bulbs was laid on MS plates for 8 h. The concentration of the constructs in the particles was 0.35 μg/mg gold particles, and 0.6 mg of gold particles was added for each construct. Images
of the brightness and GFP signals were detected using an EVOS FL Auto fluorescence microscope 16 hours after bombardment. Each experiment was replicated three times.

Transformation of attt8-1
Full-length cDNA of AcB2 fused with GFP under the control of a super-promoter was introduced into a modified pCAMBIA1300 vector and transferred into the A. tumefaciens strain GV3101. The positive strain was transformed into attt8-1 using the protocol described in a previous study [43]. The T2 progenies originating from 12 independent T1 hygromycin B–resistant transformants were confirmed by reverse transcription RT–PCR for AcB2 transcription. The primers are listed in Supplementary Data Table S1.

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Author contributions
X.L. designed the project and planned the experiments. X.L., L.C., and B.J. performed the experiments and analyzed the results. H.Y. and C.M. provided the onion materials. X.L. and Y.L. wrote the manuscript.

Data availability
The authors confirm that all data that are needed to replicate this study and to draw conclusions are within the paper.

Conflict of interest
The authors declare no competing interests.

Supplementary data
Supplementary data is available at Horticulture Research online.

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