Plant Physiology and Biochemistry 139 (2019) 642–650

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Ectopic expression of the *Aechmea fasciata APETALA2* gene *AfAP2-2* reduces seed size and delays flowering in *Arabidopsis*

Ming Lei[a,b,c,d,e], Zhi-ying Li[a,c,d,e], Jia-bin Wang[a,c,d,e], Yun-liu Fu[a,c,d,e], Li Xu[a,c,d,e,]

[a] Institute of Tropical Crop Genetic Resources, Chinese Academy of Tropical Agricultural Sciences, Danzhou, 571737, China
[b] Guangxi Key Laboratory of Medicinal Resources Protection and Genetic Improvement, Guangxi Botanical Garden of Medicinal Plants, Nanning, Guangxi, 530023, China
[c] Key Laboratory of Crop Gene Resources and Germplasm Enhancement in Southern China, Danzhou, 571737, China
[d] Key Laboratory of Tropical Crops Germplasm Resources Genetic Improvement and Innovation, Danzhou, 571737, China
[e] Mid Tropical Crop Gene Bank of National Crop Resources, Danzhou, 571737, China

ARTICLE INFO

Keywords:
Aechmea fasciata
APETALA2
Transcriptional activator
Flowering
Seed size
Bromeliad

ABSTRACT

The Bromeliaceae family, which is distributed pantropically, is one of the most morphologically diverse families. Except for the edible pineapple (*Ananas comosus*), the vast majority of bromeliads cultivated worldwide are appreciated mainly for their ornamental value. As subtropical and tropical flowering plants, these bromeliads, among with *Aechmea fasciata*, have significant economic importance. However, the molecular mechanism of flowering in bromeliads remains unrevealed. In this study, an APETALA2 (AP2) homologue, AfAP2-2, which belongs to the AP2/ethylene response element binding protein (AP2/EREBP) transcription factor superfamily, was identified in *A. fasciata*. AfAP2-2 contains two conserved AP2 domains and is a nuclear-localized transactivator. The expression level of *AfAP2-2* was predominantly higher in vegetative organs of the reproductive phase than in those of the vegetative phase. Ectopic expression of *AfAP2-2* in *Arabidopsis* specifically delayed flowering in short-day (SD) conditions. Furthermore, the size and weight of seeds of AfAP2-2-overexpressing *Arabidopsis* plants were significantly reduced compared to those of the wild type (WT). Our findings suggest that AfAP2-2 might be a negative regulator of flowering and seed size and weight. These results may help facilitate the molecular breeding of bromeliads.

1. Introduction

As components of signal transduction pathways, transcription factors (TFs) modulate the expression of numerous genes (Hernandez-Garcia and Finer, 2014). The APETALA2/ethylene response element binding protein (AP2/EREBP) transcription factor superfamily, which is one of the most well-known TF families, regulates various developmental and stress-responsive pathways (Licausi et al., 2013). Based on their different numbers or structures of AP2 and other conserved domains, AP2/EREBPs could be classified into AP2, Related to Abscisic acid insensitive3/Viviparous1 (RAV), Dehydration Responsive Element Binding Proteins (DREB), Ethylene Responsive Factors (ERF), and other categories (Sakuma et al., 2002; Nakano et al., 2006). The members of the AP2 subfamily are characterized primarily by a tandem repetition of two AP2 domains, each of which comprises approximately 60–70 amino acid residues (Nakano et al., 2006). Previous studies have demonstrated that members of the AP2 subfamily play important roles in floral development. For example, there are six members of the AP2 subfamily in *Arabidopsis*, including AP2, TARGET OF EAT1 (TOE1), TOE2, TOE3, SCHLAFMÜTZE (SMZ), and SCHNARCHZAPFEN (SNZ) (Aukerman and Sakai, 2003), and all are regulated by microRNA172 (miR172) (Chen, 2004). AP2 has been shown to affect the floral meristem (FM) and to determine floral organ identity and development (Jofuku et al., 1994; Chen, 2004; Wollmann et al., 2010; Dinh et al., 2012; Krogan et al., 2012; Liu et al., 2014). TOEs could delay flowering as repressors of variable flowering enhancers or integrators (Jung et al., 2007, 2014; Tao et al., 2012; Zhai et al., 2015; Zhang et al., 2015). Additionally, SMZ and SNZ act redundantly with TOEs to repress flowering (Aukerman and Sakai, 2003; Chen, 2004; Mathieu et al., 2009; Yant et al., 2010). Further investigations showed that at least TOE1 and SMZ were able to bind directly to the promoter sites of the putative florigen *Flowering Locus T* (*FT*) to prevent flowering (Zhang et al., 2015; Mathieu et al., 2009). Furthermore, among these six AP2 members, AP2 was also required for ovule and seed coat development

* Corresponding author. Institute of Tropical Crop Genetic Resources, Chinese Academy of Tropical Agricultural Sciences, Danzhou, 571737, China.
E-mail addresses: leiming_catas@126.com (M. Lei), xllizhiying@vip.163.com (Z.-y. Li), jiabinwangfuhu@sina.com (J.-b. Wang), fyljj_2007@126.com (Y.-l. Fu), xllzy@263.net (L. Xu).

https://doi.org/10.1016/j.plaphy.2019.03.034
Received 15 November 2018; Received in revised form 18 February 2019; Accepted 23 March 2019
Available online 25 March 2019
0981-9428/

Abbreviations

AbA	Aureobasidin A
ACTB	*β-actin* gene
ANT	AINTEGUMENTA
AP2	APETALA2
CaMV	Cauliflower mosaic virus
CDS	coding sequence
CTAB	hexadecyl trimethyl ammonium bromide
DAF	days after flowering
DAG	days after germination
DNMRT	Duncan's new multiple range test
DREB	Dehydration Responsive Element Binding Proteins
EREBP	Ethylene Response Element Binding Proteins
ERF	Ethylene Responsive Factors
FM	floral meristem
FT	*Flowering Locus T*
FUL	*FRUITFULL*
LD	long day
LFY	*LEAFY*
MEME	Multiple EM for Motif Elicitation
miR172	microRNA172
MS	Murashige and Skoog
MW	molecular weight
NLS	nuclear localization signal
ORF	open reading frame
PBD	pGBKT7
PI	isoelectric point
RACE	rapid amplification of cDNA ends
RAV	Related to Abscisic acid insensitive3/Viviparous1
RT-qPCR	reverse transcription followed by quantitative real-time PCR
SD	short day
SMZ	SCHLAFMÜTZE
SNZ	SCHNARCHZAPFEN
SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1*
TOE1	TARGET OF EAT1
TUB	*α-tubulin* gene
UTR	untranslated region
WT	wild type

and subsequently regulated seed size and seed weight (Jofuku et al., 1994, 2005; Léon-Kloosterziel et al., 1994; Western et al., 2001; Ohto et al., 2005, 2009), indicating its potential impact on agronomic traits such as seed yield.

The Bromeliaceae, which comprises 3248 species in 58 genera, is one of the largest vascular plant families with a Neotropical distribution, outstanding ecological range, and rich diversity of life forms (Crayn et al., 2004; Versieux et al., 2012). Among them, numerous bromeliads cultivated worldwide are appreciated for the astonishing flavour and fragrance of their fruits (for example, pineapple, *Ananas comosus*) or for their ornamental value of their vegetative forms and flowers. To date, some efforts have been made to reveal the molecular mechanism of flowering in bromeliads with significant economic importance. Previous studies identified several crucial factors participating in flowering pathways (Lv et al., 2012a, b; Lei et al., 2016, 2018). In particular, genes related to ethylene biosynthesis and ethylene-inducing flowering pathways in bromeliads have been characterized (Trusov and Botella, 2006; Cong et al., 2012; Li et al., 2016a, b). With the release of pineapple genome data (Zhang et al., 2014; Ming et al., 2015), several TFs that might be involved in flowering regulation were identified (Ali et al., 2017; Xie et al., 2018). All these results are valuable for better understanding the various molecular mechanisms of flowering in bromeliads.

In this study, *AfAP2-2*, a gene that encodes an AP2 homologue, was identified in the genome of *Aechmea fasciata*, a popular ornamental flowering bromeliad. The expression level of *AfAP2-2* was predominantly higher in vegetative organs during the reproductive phase than during the vegetative phase. Transactivation assays in yeast demonstrated that AfAP2-2 was a transactivator. In *AfAP2-2*-overexpressing (*AfAP2-2-OX*) *Arabidopsis*, the bolting time exhibited no significant difference from that of the wild type (WT) under long-day (LD) conditions, but flowering was delayed in *AfAP2-2-OX Arabidopsis* in short-day (SD) conditions. Furthermore, the size and weight of seeds of *AfAP2-2-OX Arabidopsis* plants were significantly reduced compared to those of the WT. These results suggested that AfAP2-2 might be a flowering regulator in *A. fasciata*.

2. Materials and methods

2.1. Plant materials

The *A. fasciata* specimens used in this study were planted in a greenhouse (ambient temperature of 30–32 °C) located in the experimental area of the Institute of Tropical Crop Genetic Resources, Chinese Academy of Tropical Agricultural Sciences. Juvenile (6 months), adult (12 months) and 39-day-after-flowering (DAF) (18–20 months) *A. fasciata* plants grown in pots were selected for investigation. Different tissue samples, including mature leaves, central leaves, stems, roots and various flower organs, were collected and immediately frozen in liquid nitrogen for further research.

The seeds of both WT and transgenic *Arabidopsis* plants were surface-sterilized in 0.1% $HgCl_2$ for 10 min and then washed with sterilized distilled water five times before being plated on Murashige and Skoog (MS) medium containing sugar (2%) and agar (0.8%). After being incubated in the dark at 4 °C for 2 days, the plates were moved to a chamber at 23 °C under LD (16 h light) or SD (8 h light) conditions with a photon flux density of 120 μmol m^{-2} s^{-1}. Ten days later, the seedlings were transplanted into turf soil for continuous growth.

2.2. Sequence identification and bioinformatics analysis

Total RNA was extracted from the central leaves of *A. fasciata* using the hexadecyl trimethyl ammonium bromide (CTAB) method (Lei et al., 2016). The cDNA of *AfAP2-2* was characterized based on transcriptome data, and the 5′ and 3′ ends were identified using the SMARTer™ RACE cDNA Amplification Kit (Clontech, Tokyo, Japan). The primers used are listed in Table S1 online.

The online Open Reading Frame (ORF) Finder (https://www.ncbi.nlm.nih.gov/orffinder/) was used to predict the ORF of *AfAP2-2*. The physicochemical properties of the AfAP2-2 protein were predicted and calculated using Expasy ProtParam (http://web.expasy.org/protparam/). MEGA version 6.0 was used to construct the phylogenetic tree (Tamura et al., 2013). The sequence logo was generated by the online WebLogo 3 platform (http://weblogo.threeplusone.com/). Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/index.php) was used to generate the scheme of exon-intron structures. The Multiple EM for Motif Elicitation (MEME) online software (http://meme-suite.org/tools/meme) was used with the default settings to identify putative motifs in the variable AP2s.

2.3. Reverse transcription followed by quantitative real-time PCR (RT-qPCR)

First-strand cDNA was synthesized using the TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing, China), and qPCR was performed using the TransStart Tip Green qPCR

SuperMix Kit (Transgen, Beijing, China). qPCR was conducted in triplicate on a Therma PikoReal 96™ Real-Time PCR System (Thermo Fisher Scientific, New York, NY, USA). The relative expression levels were calculated as previously reported (Livak and Schmittgen, 2001). The *α-tubulin (TUB)* or *β-actin* (*ACTB*) gene of *A. fasciata* or *Arabidopsis* was used as the internal control. All primers used for qPCR are listed in Table S1 online.

2.4. Subcellular localization

The 1480 bp coding sequence (CDS) region of AfAP2-2 was amplified and subcloned into the pEASY-blunt vector (Transgen, Beijing, China) and confirmed by sequencing. Then, the inserts were transferred to the destination vector pBI221. The Agrobacterium-mediated transformation of onion epidermal cells was conducted as previously reported (Sun et al., 2007). GFP fluorescence was observed by confocal scanning microscopy (Olympus FV1000, Olympus, Tokyo, Japan). For GFP, 488 and 505–530 nm were used for excitation and emission, respectively.

2.5. Transactivation analysis of AfAP2-2 in yeast cells

The coding sequence of full-length AfAP2-2, the N terminus of AfAP2-2 (1–305 AA) and the C terminus of AfAP2-2 (296–468 AA) were amplified and cloned into the destination vector pGBKT7 (pBD). Transactivation analysis assays were carried out in the yeast strain Y2HGold using the Yeastmaker Yeast Transformation System 2 (Clontech, Tokyo, Japan). The primers used are listed in Table S1 online.

2.6. Exogenous ethephon treatment of A. fasciata

To test ethylene response, adult (12-month-old) *A. fasciata* plants grown in pots in our greenhouse were treated with 10 mL of ethephon at 0.3 g L^{-1}, 0.6 g L^{-1}, 1.2 g L^{-1}, 2.4 g L^{-1}, 4.8 g L^{-1} for 1, 2, 4, 8, 24 or 48 h or with the same quantity of water as a control. The central leaves were then physically isolated and immediately frozen in liquid nitrogen for further research.

2.7. Plant transformation

To generate the transgenic constructs, the CDS of *AfAP2-2* was cloned into the binary vector Cam35S-gfp under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Transgenic *Arabidopsis* plants were generated by Agrobacterium-mediated transformation using the floral dipping method (Clough and Bent, 1998). The selection of transgenic plants was carried out on MS agar medium supplemented with hygromycin (25 mg/L). Transgenic plants were verified by genomic PCR, RT-PCR and GUS staining. The primers used (*AfAP2-2-OX* F and *AfAP2-2-OX* R) are listed in Table S1 online. T3 transgenic plants were used for the subsequent experiments.

2.8. Data analysis

Values represent the means ± standard deviations of two or three biological replicates. ANOVA was conducted, and means were separated by Duncan's new multiple range test (DNMRT).

3. Results and discussion

3.1. AfAP2-2 is a transcriptional activator that belongs to the AP2 subfamily

The members of the AP2 family, which have two AP2 domains, can be further divided into two clades, AP2 and AINTEGUMENTA (ANT) (Mizukami and Fischer, 1999). In addition to *Arabidopsis*, the AP2 subfamily has also been found in many other plant species (Zumajo-Cardona and Pabón-Mora, 2016), such as petunia (Maes et al., 2001), tomato (Chung et al., 2010), maize (Stephen and Moose, 1994; Chuck et al., 1998), *Picea abies* (Vahala et al., 2001; Nilsson et al., 2007), *Pinus thunbergii* (Shigyo and Ito, 2004), larch (Guillaumot et al., 2008), *Capsicum annuum* (Borovsky et al., 2015), peanut (*Arachis hypogaea*) (Park and Grabau, 2016), and *Brassica* (Zhang et al., 2018).

In the present study, we identified the *AP2-like* gene *AfAP2-2* from *A. fasciata*, an economically valuable, SD ornamental plant. The cDNA of *AfAP2-2* was 2160 bp long and contained a 606-bp 5′ untranslated region (UTR), a 150-bp 3’ UTR and a 1404-bp ORF. An exon-intron organization analysis showed that the structure of *AfAP2-2* was composed of nine exons and eight introns (Fig. 1); this structure was similar to that of *TOE1*, indicating putative evolutionary conservation between these genes.

A phylogenetic tree was constructed with AfAP2-2, AfAP2-1 and the AP2 proteins of several other species using the neighbour-joining method with 1000 bootstrap replicates, confirming that AfAP2-2 is a member of the AP2 subfamily (Fig. S1). Furthermore, the unrooted phylogenetic tree showed that AfAP2-2 was mostly closely related to AfAP2-1, implying that they have similar origins. In addition, AfAP2-1 and AfAP2-2 were both clustered with six other AP2-like proteins of monocots (*Aegilops tauschii*, *Dendrobium crumenatum*, *Hordeum vulgare* ssp. *vulgare*, *Oryza sativa* Japonica Group and *Zea mays*) (Fig. S1), indicating that these proteins diverged prior to the divergence of dicots and monocots.

A sequence comparison of AfAP2-2 with AfAP2-1, and six AP2 family members in *Arabidopsis* showed that the putative nuclear localization signal (NLS) was completely conserved among them (Fig. S2). In addition, both the AP2 domains and the linker between them (called the joint peptide, Jp) were highly similar (Fig. S2).

Further conserved sequence examination between AfAP2-2, AfAP2-1 and six AP2 members in *Arabidopsis* using the online MEME tool showed that all members shared the eight conserved motifs except SMZ and SNZ, and these motifs had similar distributions. Motif 1 belonged to AP1R1, motif 2 belonged to AP1R1 and Jp, motifs 3 and 5 belonged to AP2R2, and motif 4 belonged to NLS (Figs. S3A and B). In addition, motifs 6, 7 and 8 were all conserved in these AP2 members (Figs. S3A

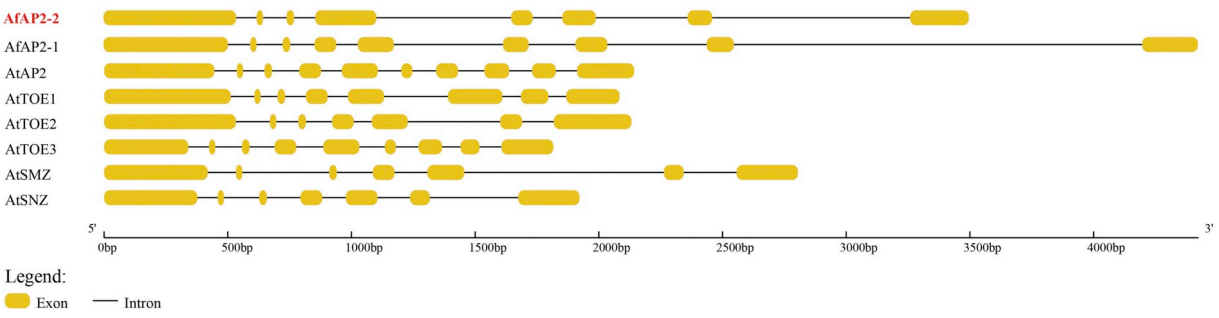

Fig. 1. Exon-intron structures of *AP2* and *AP2-like* genes.

and B), indicating putative functional conservation among them.

A large number of genes can be regulated by TFs by being bound at specific sites or motifs (Krishnaswamy et al., 2011; Krogan et al., 2012). AfAP2-2 was shown to be localized predominantly to the nucleus, indicating that it might be an active TF (Fig. 2A). A previous study demonstrated that two AP2 domains separated by a spacer region were crucial for AP2 subfamily members to bind the promoter regions of target genes (Okamuro et al., 1997). Here, a sequence analysis showed that two AP2 domains and Jp were highly conserved in AfAP-2 (Figs. S2, S3A, B), indicating that it might have a conserved ability to bind target genes. Further assays in yeast cells showed that AfAP2-2 was a transactivator, and the activation domain was located in the C terminus, which does not contain two AP2 domains (Fig. 2B). This result was consistent with that obtained for AfAP2-1, another AP2 member isolated from *A. fasciata* (Lei et al., 2016).

3.2. Ectopic expression of AfAP2-2 in Arabidopsis delayed flowering under SD conditions

Flowering, which is regulated by numerous genes, is a complex developmental process (Nilsson et al., 2007). Among these genes, numerous AP2 members have been demonstrated to repress floral induction. In *Arabidopsis*, all six AP2 family members act redundantly to repress flowering (Aukerman and Sakai, 2003; Jung et al., 2007; Mathieu et al., 2009; Yant et al., 2010; Zhang et al., 2015), and overexpression of *TOE1*, *SMZ* or *SNZ* can directly downregulate the expression of *FT* under LD conditions (Jung et al., 2007; Mathieu et al., 2009). In *Cardamine flexuosa*, CfTOE1 negatively regulates the expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* of *C. flexuosa* (*CfSOC1*), which encodes a floral induction factor, to repress flowering (Zhou et al., 2013). In maize, the expression of *GLOSSY15* (*GL15*), an *AP2*-related gene, is correlated with the juvenile-to-adult phase transition and inhibits flowering (Lauter et al., 2005). The constitutive expression of the conifer *Picea abies* (Norway spruce) *APETALA2 LIKE2* (*PaAP2L2*) gene in *Arabidopsis* conferred a late-flowering phenotype compared with that of WT *Arabidopsis* (Nilsson et al., 2007). The APETALA2 transcription factor homologue CaAP2 in *Capsicum annuum* was demonstrated to be a candidate flowering repressor (Borovsky et al., 2015). In *Ipomoea nil,* InAP2-like was involved in photoperiodic flower induction (Glazinska et al., 2009).

To gain insights into the role of *AfAP2-2* in *A. fasciata*, RT-qPCR was performed to analyse its expression patterns. *AfAP2-2* transcripts could be detected in all tested vegetative and reproductive tissues (Fig. 3A and B). Notably, the expression level of *AfAP2-2* was relatively lower in a variety of reproductive tissues, especially in most flowering organs, than in the vegetative tissues (Fig. 3B). In addition, the quantity of

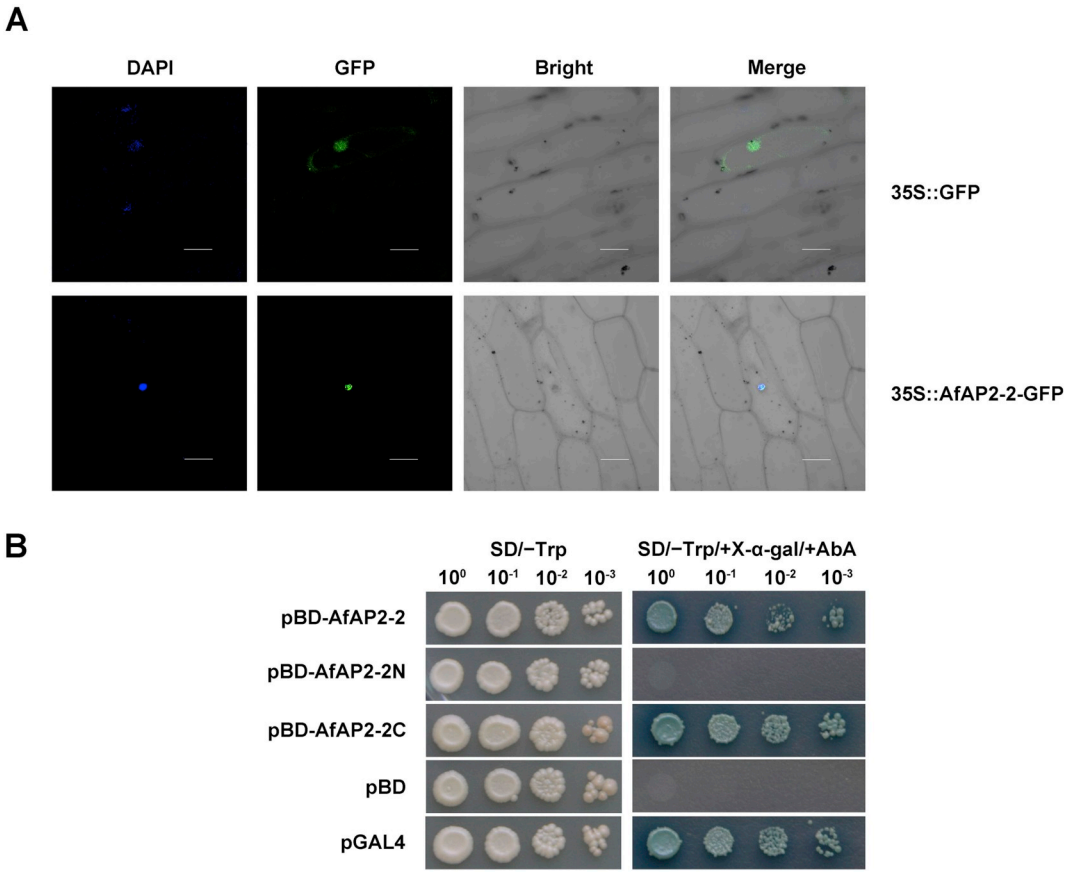

Fig. 2. Subcellular localization and transactivation activity assay of AfAP2-2. (**A**) Subcellular localization of AfAP2-2-GFP fusion protein. Plasmids harbouring a C-terminal GFP driven by the CaMV 35S promoter (35S:GFP) were used as a control. Recombinant plasmids harboured a C-terminal GFP fusion with AfAP2-2 driven by the CaMV 35S promoter (35S:AfAP2-2-GFP). Green fluorescence indicates GFP, and blue fluorescence indicates nuclear staining by 4′,6-diamidino-2-phenylindole (DAPI). Bars = 50 μm. (**B**) Transactivation activity assay of AfAP2-2 in yeast cells. The constructs of pBD vectors fused with the full-length AfAP2-2 (pBD-AfAP2-2), the N terminus of AfAP2-2 (pBD-AfAP2-2-N) and the C terminus of AfAP2-2 (pBD-AfAP2-2-C) were transformed into Y2HGold cells, which contain the reporter genes AUR1-C and MEL1. pBD and pGAL4 plasmids were transformed into Y2HGold cells and used as negative and positive controls, respectively. Yeast clones containing the confirmed constructs grew on SD/-Trp medium at dilutions of 1, 1/10, 1100 and 1/1000 for three to five days and then were transferred onto SD/-Trp/ + AbA/ + X-α-Gal medium for continuous growth for three days to test their transactivation activities. SD: synthetic dropout; AbA: aureobasidin A; SD/-Trp: SD medium without Trp; SD/-Trp/ + AbA/ + X-α-gal: SD medium without Trp but with 40 mg/L X-α-gal and 200 μg/L AbA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

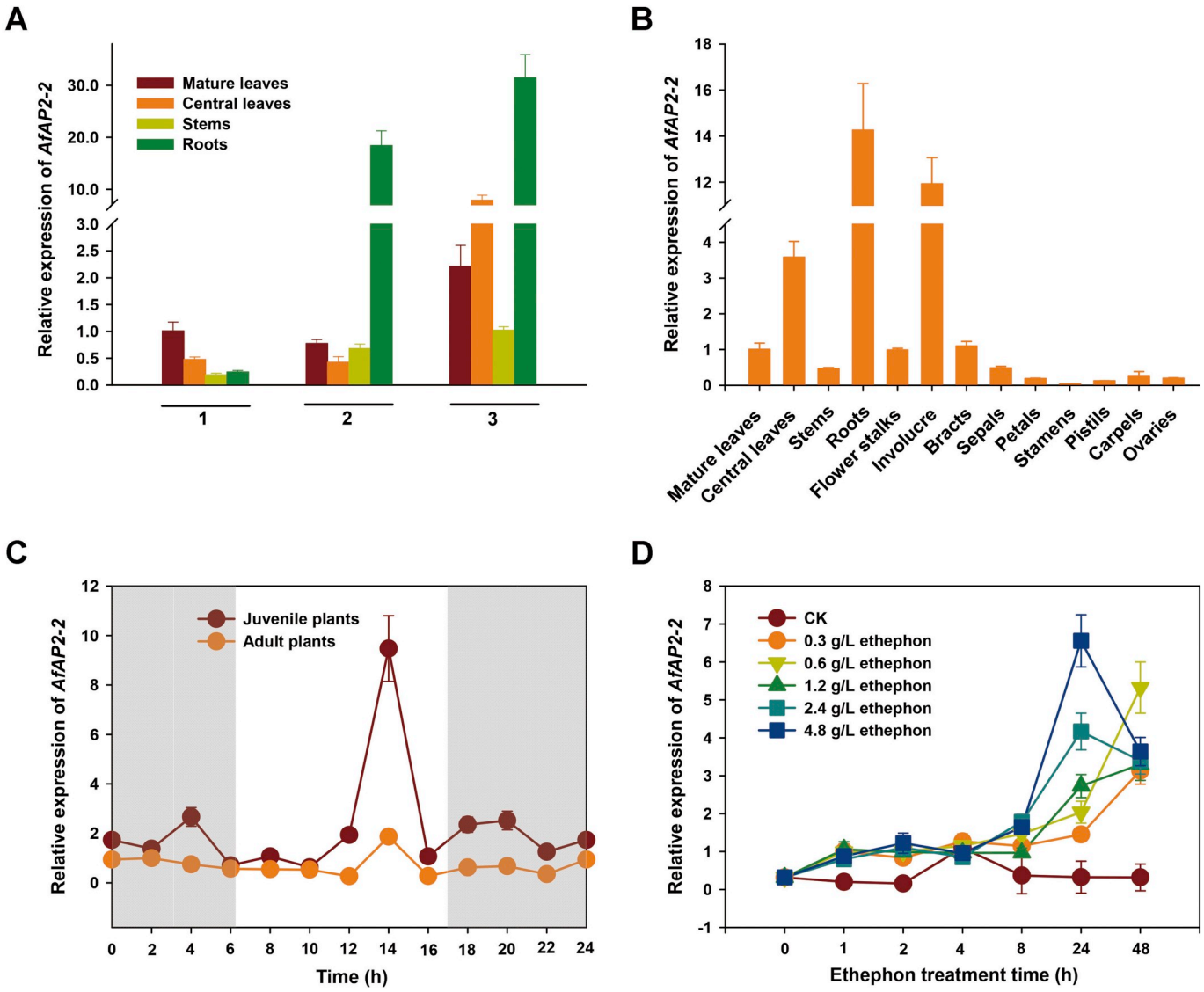

Fig. 3. Expression of *AfAP2-2* transcripts in various tissues of *A. fasciata*. (**A**) Expression levels of *AfAP2-2* transcripts in various tissues of juvenile and adult plants. 1, juvenile plants; 2, adult plants before differentiation; 3, 39-DAF adult plants; (**B**) Expression levels of *AfAP2-2* transcripts in various tissues of 39-DAF adult plants; (**C**) Expression levels of *AfAP2-2* transcripts in the central leaves of *A. fasciata* grown in the greenhouse under a natural daily light rhythm. Samples were collected at 2 h intervals. The grey regions indicate the dark period, and the white region indicates the light period. (**D**) Expression level of *AfAP2-2* transcripts in the central leaves of *A. fasciata* in response to exogenous ethephon treatment at different concentrations for different time. In the panels, 0, 1, 2, 4 and 8 h represent the samples collected at 10:00, 11:00, 12:00, 14:00, and 18:00, respectively, and 24 h and 48 h represent the treated samples collected at 10:00 a.m. on the next day and the next two days, respectively. For CK, 10 mL of distilled deionized H_2O was poured into the leaf cylinder of *A. fasciata*. 0 h represents samples not treated with ethephon or distilled deionized H_2O. Three independent experiments were performed; values are shown as the means, and error bars indicate the standard deviations ($n = 3$).

AfAP2-2 transcripts was higher in the central leaves of juvenile plants than in those of adult plants, indicating that *AfAP2-2* might be involved in the juvenile-to-adult phase transition (Fig. 3C).

To assess the function of *AfAP2-2* in flowering, we induced the ectopic expression of *AfAP2-2* under the CaMV 35S promoter (*Pro35S::AfAP2-2*) in *Arabidopsis* ecotype Columbia (Col-0) (WT) (Fig. S4). *Pro35S::AfAP2-2* transgenic plants showed no significant difference from WT in flowering time under LD conditions (Figs. S5A and B), and no defects in floral morphology were observed in the transgenic plants (Figs. S5C, D, E, F, G). Interestingly, the flowering time of *Pro35S::AfAP2-2* transgenic plants was significantly later than that of WT under SD conditions (Fig. 4A and B). Compared to that of WT, the expression level of the genes *SOC1* and *LEAFY (LFY)*, which encode floral induction factors, was substantially reduced at the shoot apex of *Pro35S::AfAP2-2* transgenic plants (Fig. 5A, C). In addition, the expression of another gene, *FRUITFULL* (*FUL*), which also encodes a floral induction factor, was significantly downregulated at the shoot apex of lines 77 and 157 of *Pro35S::AfAP2-2* transgenic plants but upregulated in line 74 (Fig. 5B) compared to that of WT. The expression of *FT*, an integrator of flowering pathways, and floral organ identity genes, such as *AP1*, *AP2* and *AP3*, was also considerably downregulated (Fig. 5D, E, F, G). These results indicated that AfAP2-2 was a putative flowering repressor involved in flowering induction and might be a potential target of genetically engineered breeding, as a large number of bromeliads, including *A. fasciata*, are SD plants.

The gas hormone ethylene is widely used to induce flowering in bromeliads. Previous studies have demonstrated that exogenous ethylene mediates the flowering of bromeliads by stimulating the synthesis of endogenous ethylene (Wang et al., 2007; Cong et al., 2012). To date, several efforts have been made to identify the functional factors

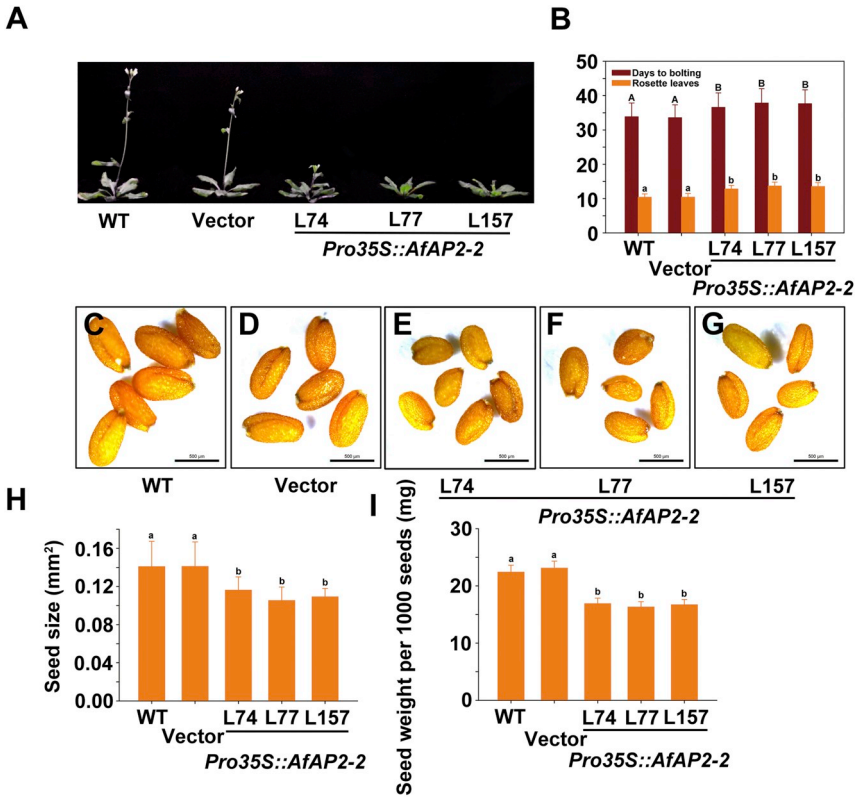

Fig. 4. Phenotype analysis of *Pro35S::AfAP2-2* transgenic plants. (**A**) Phenotypes of WT and transgenic plants grown under SD conditions at 38 days after germination (DAG). Vector indicates WT transformed with the empty vector, and L74, L77, and L157 indicate different lines of *Pro35S::AfAP2-2 Arabidopsis* transgenic plants. (**B**) Days and rosette leaves to bolting of WT and transgenic plants grown under SD conditions. (**C**), (**D**), (**E**), (**F**), and (**G**) indicate mature seeds of WT, empty vector, L74, L77, and L157, respectively. (**H**) Average seed sizes of WT and transgenic plants grown under SD conditions. Seventy-four to seventy-seven seeds of WT, empty vector, or each line of transgenic plants were used for calculation. Values are shown as the means, and error bars indicate the standard deviations. (**I**) Seed masses of WT and transgenic plants grown under SD conditions. Seed mass is given in units of mg per 1000 seeds. Values are shown as the means, and error bars indicate the standard deviations.

participating in ethylene transduction pathways and ethylene-induced-flowering signalling pathways in bromeliads. Li et al. identified four ethylene receptor genes in pineapple and suggested that *AcERS1b*, *AcETR2a*, and *AcETR2b* were involved in pineapple flowering induced by exogenous ethephon (Li et al., 2016b). Our previous studies also identified two ethylene-responsive genes, *AfAP2-1* and *AfSPL14*, which were significantly repressed or induced, respectively, by exogenous ethylene treatment, indicating their important roles in the ethylene-induced flowering of *A. fasciata* (Lei et al., 2016, 2018). Here, the possible response of *AfAP2-2* in adult plants prior to flower bud differentiation in *A. fasciata* after exogenous ethephon treatment was investigated at different concentrations. As shown in Fig. 3D, the expression of *AfAP2-2* was significantly induced by exogenous ethephon treatment, independent of concentration. Interestingly, the expression level reached a maximum after treatment with exogenous ethephon at relatively high concentrations (2.4 g/L and 4.8 g/L) at 24 h, while the expression of *AfAP2-2* rose gradually when treated with exogenous ethephon at lower concentrations (0.3 g/L, 0.6 g/L and 1.2 g/L), even after 24 h (Fig. 3D). These results were in contrast to those of *AfAP2-1*, for which transcript levels were repressed but not induced by exogenous ethylene treatment (Fig. 3D). Furthermore, the expression of *AfAP2-2* induced by exogenous ethylene treatment was dose dependent (Fig. 3D). Interestingly, the expression level of *AfAP2-2* generally appeared to gradually increase in various vegetative organs with age (Fig. 3A). All these results indicated that AfAP2-2 might be involved in the development of vegetative organs and might not be involved in the ethylene-induced flowering of *A. fasciata*.

3.3. Ectopic expression of AfAP2-2 in Arabidopsis altered seed size and weight

The *Arabidopsis* seed consists of the seed coat, embryo, and endosperm. These three components are derived from the ovule integuments, the fusion of the egg cell and one sperm cell, and the fusion of the central cell of the embryo sac with the second pollen sperm cell, respectively (Becker et al., 2014; Figueiredo and Kohler, 2014; Khan et al., 2014; Lafon-Placette and Kohler, 2014). Seed development was influenced by both environmental factors and endogenous genetic signals (Orozco-Arroyo et al., 2015). Among these agronomic traits, seed size and mass are considered important determinants of seedling survival and vigour upon germination (Jofuku et al., 2005). In *Arabidopsis*, seed size is mainly influenced by the rapid proliferation of the endosperm and the seed coat cells and to date, several pathways regulating seed size are known (Orozco-Arroyo et al., 2015). For example, genome imprinting and parent-of-origin effects could influence the developmental process of the endosperm and thereby regulate seed size. In addition, factors controlling integument cell proliferation and/or elongation could influence the developmental process of the seed coat, altering seed size. Furthermore, phytohormones such as brassinosteroids, cytokinins, auxins and abscisic acid have also been proposed to coordinate seed development (Sun et al., 2010). Among these pathways, several key TFs have been characterized, and AP2 was demonstrated to play a crucial role in controlling endosperm cellularization and seed coat development (Jofuku et al., 2005; Ohto et al., 2005, 2009). The endosperm proliferation stage of *ap2* mutant seeds was extended, and cellularization was then delayed (Ohto et al., 2009). In addition, the cell number and cell size of *ap2* mutants were increased in association with enlarged embryo size (Jofuku et al., 2005; Ohto et al., 2005). Our studies showed that floral morphology did not show observable defects in *Pro35S::AfAP2-2* transgenic *Arabidopsis* plants (Figs. S5C, D, E, F, G), in contrast to that in *TOE3*-overexpressed (*TOE3-OX*) *Arabidopsis* plants (Jung et al., 2014). Interestingly, the seeds of *Pro35S::AfAP2-2* transgenic *Arabidopsis* plants were smaller than those of WT (Fig. 4C, D, E, F, G, H), and the average seed weight of *Pro35S::AfAP2-2* transgenic *Arabidopsis* plants was decreased by 24.55%–27.23% compared to that of WT (Fig. 4I), indicating that AfAP2-2 might play crucial roles in the regulation of seed size and weight. *A. fasciata* is an ornamental plant that has only flowers but not fruits or seeds, the fact of which may be the reason that the transcript level of *AfAP2-2* was lower in the reproductive organs than in the

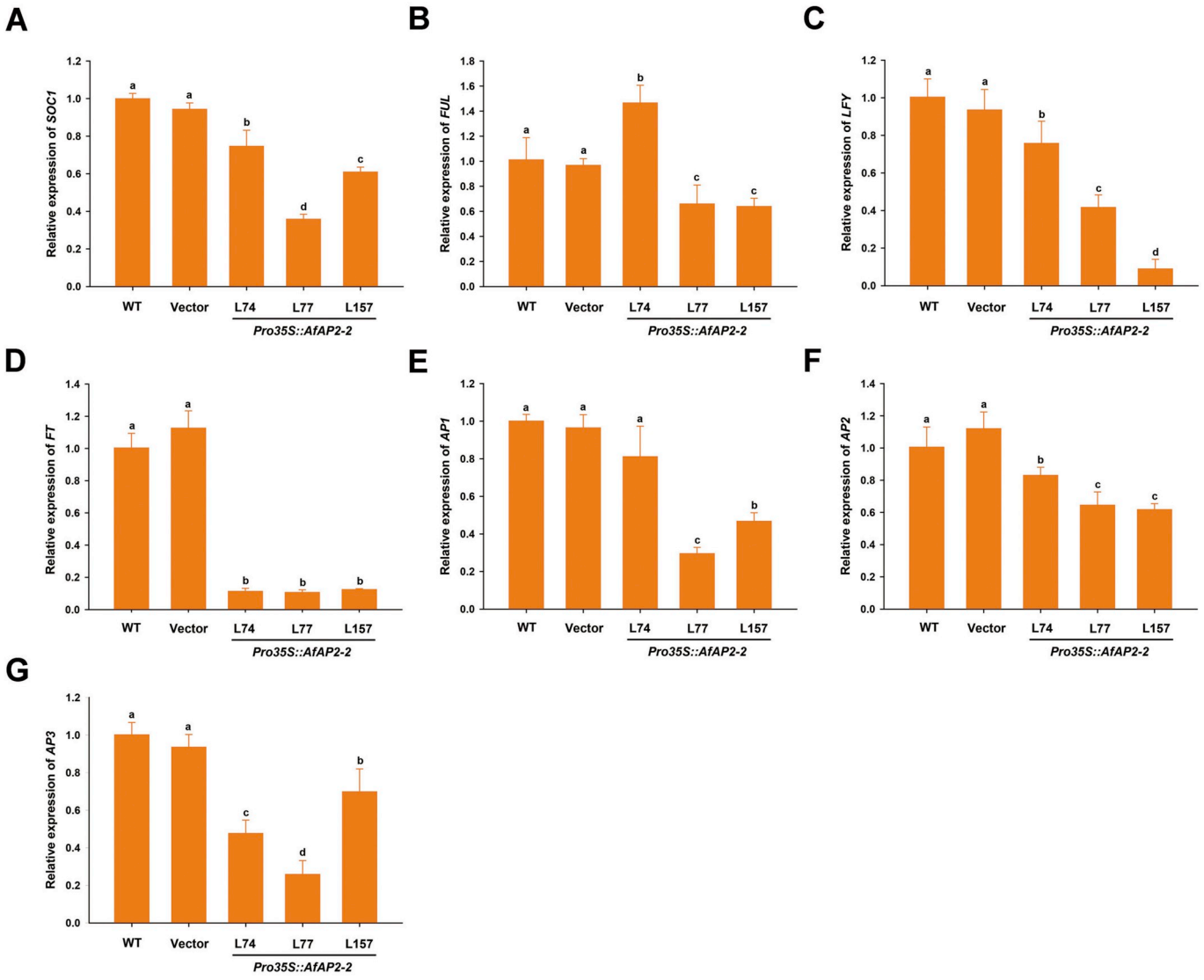

Fig. 5. Relative expression levels of flowering-related genes at the shoot apex of WT, empty vector and *Pro35S::AfAP2-2* transgenic plants determined by RT-qPCR. Three biological replicates and three technical replicates were performed using 14-day-old long-day-grown seedlings. The *AtACTB* gene was used as a reference. All primers used are listed in Table S1 online.

vegetative organs of *A. fasciata* (Fig. 3B). Except for ornamental *A. fasciata*, a variety of bromeliads cultivated worldwid are appreciated for the astonishing flavour and fragrance of their fruits (for example, pineapple, *A. comosus*). Interestingly, an AP2 homologue, which was identified in *A. comosus* and named as AcAP2-2, shares very similar amino acid sequences with AfAP2-2. Here, we speculate that AcAP2-2 might have similar functions with AfAP2-2 in the regulation of flowering time and the size and weight of fruits. Further studies should focus on the characterization of floral morphology in *AfAP2-2-OX A. fasciata* plants and/or the size and weight of fruits of *AcAP2-2-OX A. comosus* plants.

Author contributions

All authors read and commented on the manuscript.

LX, ZYL and ML designed the study and guided the research. ML performed the experiments, prepared all the figures and wrote the draft manuscript text. JBW and YLF performed some of the experiments. LX modified this manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

We thank professor Fei Qiao, professor Yu-wei Hua and assistant professor Su-na Peng for technical support in the subcellular localization experiment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.034.

Funding sources

This work was supported by grants from the National Natural Science Foundation of China (31601793) and the Fundamental Scientific Research Funds for CATAS-TCGRI (1630032018018).

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