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Authors

Liu, Xigang
Dinh, Thanh Theresa
Li, Dongming
et al.

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AUXIN RESPONSE FACTOR 3 integrates the functions of *AGAMOUS* and *APETALA2* in floral meristem determinacy

Xigang Liu[1,2,&,*], **Thanh Theresa Dinh**[2,&,§], **Dongming Li**[1,2], **Bihai Shi**[3], **Yongpeng Li**[1], **Xiuwei Cao**[1], **Lin Guo**[1], **Yanyun Pan**[4], **Yuling Jiao**[3], and **Xuemei Chen**[2,5,*]

[1]Key Laboratory of Agricultural Water Resources, Hebei Laboratory of Agricultural, Water-Saving, Center for Agricultural Resources Research, Institute of Genetics and, Developmental Biology, Chinese Academy of Sciences, 286 Huaizhong Rd, Shijiazhuang 050021, China

[2]Department of Botany and Plant Sciences, Institute of Integrative Genome Biology, University of California, Riverside, CA 92521

[3]State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental, Biology, Chinese Academy of Sciences, Beijing 100101, China

[4]College of Life Sciences, Hebei Agricultural University, Baoding 071001, China

[5]Howard Hughes Medical Institute, University of California, Riverside, CA 92521

SUMMARY

In *Arabidopsis*, AUXIN RESPONSE FACTOR 3 (ARF3) belongs to the auxin response factor (ARF) family that regulates the expression of auxin-responsive genes. *ARF3* is known to function in leaf polarity specification and gynoecium patterning. In this study, we discovered a previously unknown role of *ARF3* in floral meristem (FM) determinacy through the isolation and characterization of a mutant of *ARF3* that enhanced the FM determinacy defects of *agamous (ag)-10*, a weak *ag* allele. Central players in FM determinacy include *WUSCHEL* (*WUS*), a gene critical for FM maintenance, and *AG* and *APETALA2* (*AP2*), which regulate FM determinacy by repression and promotion of *WUS* expression, respectively. We showed that *ARF3* confers FM determinacy through repression of *WUS* expression, and associates with the *WUS* locus in part in an AG-dependent manner. We demonstrated that *ARF3* is a direct target of *AP2* and partially mediates *AP2*'s function in FM determinacy. *ARF3* exhibits dynamic and complex expression

*Corresponding authors. xgliu@sjziam.ac.cn, xuemei.chen@ucr.edu, Address: Center for Agricultural Resources Research, 286 Huaizhong Rd, Shijiazhuang, 050021, China. Tel: 86-311-85810502, Fax: 86-311-85815093.
&These authors contributed equally to this work.
§Current address: Stanford University School of Medicine, Department of Pathology, Lane Building, Mail code 5324, Stanford, CA. 94305-5324

SUPPORTING INFORMATION

Figure S1. Molecular characterization of *arf3-29* and the phenotype of *ett-3*.
Figure S2. *WUS, AG* and *ARF3* expression in various genotypes.
Figure S3. ARF3 protein distribution in 35S: :*AP2m3* floral meristems.
Figure S4. *ARF3* transcript and protein distribution in FMs and floral organs.
Figure S5. *ARF3* expression in various transgenic plants.
Table S1. Primers used in the study.
Method S1. EMS mutagenesis and map-based cloning of *ARF3*
Method S2. RNA extraction and real-time PCR
Method S3. Plasmid construction
Method S4. Tissue preparation for fluorescence detection

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patterns in floral organ primordia; altering the patterns spatially compromised FM determinacy. This study uncovered a role for *ARF3* in FM determinacy and revealed relationships among genes in the genetic network governing FM determinacy.

Keywords

AUXIN RESPONSE FACTOR 3; floral determinacy; *AGAMOUS*; *APETALA2*; *WUSCHEL*; *Arabidopsis*

INTRODUCTION

Auxin is a key phytohormone that controls many developmental processes in plants, including embryogenesis, organogenesis, vascular differentiation, tropic growth, root and shoot architecture and senescence (Reinhardt *et al.*, 2000; Vanneste and Friml 2009). Auxin signaling is mediated by two protein families: auxin response factors (ARFs) and Aux/IAA proteins. The 23 ARFs in *Arabidopsis* function as transcription factors and specifically bind TGTCTC auxin response elements (AuxREs) in the promoters of primary or early auxin response genes (Ulmasov *et al.*, 1995; Guilfoyle *et al.*, 1998; Ulmasov *et al.*, 1999b; Ulmasov *et al.*, 1999a; Guilfoyle and Hagen 2007; Boer *et al.*, 2014). All *Arabidopsis* ARFs contain a conserved amino-terminal DNA-binding domain (DBD) responsible for AuxRE binding, and all except ARF23 have a middle region that functions as an activation or repression domain. All of the ARFs except ARF3, ARF13 and ARF17 contain a carboxy-terminal dimerization domain (CTD) that interacts with motifs III and IV of Aux/IAA proteins (Liscum and Reed 2002; Tiwari *et al.*, 2003; Guilfoyle and Hagen 2007), which are short-lived nuclear proteins encoded by primary or early auxin response genes. This interaction between motifs III and IV of Aux/IAA proteins and the CTD of ARFs leads to the inhibition of ARF activity (Reed 2001; Tiwari *et al.*, 2003).

Although its encoded protein lacks a CTD, *ARF3*, which is also known as *ETTIN* (*ETT*), nevertheless functions in some auxin-regulated pathways, such as gynoecium morphogenesis, self-incompatibility and *de novo* organ regeneration (Nemhauser *et al.*, 2000; Tiwari *et al.*, 2003; Tantikanjana and Nasrallah 2012; Cheng *et al.*, 2013). The roles of *ARF3* in leaf polarity and floral meristem (FM) and reproductive organ patterning have also been well characterized. *ARF3* is expressed in vegetative and developing reproductive tissues (Sessions *et al.*, 1997; Pekker *et al.*, 2005) and can be detected in groups of cells that give rise to new FMs in the inflorescence meristem (IM). In stage 1 and 2 FMs (stages according to (Smyth *et al.*, 1990)), *ARF3* is expressed throughout the FM. In stages 3 and 4 FMs, *ARF3* RNA is concentrated on the abaxial side of incipient stamen primordia and present in the gynoecium primordium, but is not found in the sepals. In stages 5 to 7 flowers, *ARF3* exhibits an abaxial expression pattern in the petal, stamen and gynoecium primordia (Sessions *et al.*, 1997). Consistent with the complex expression pattern of *ARF3* and its functions in FM formation and floral organ initiation, *arf3/ett* mutants display floral organogenesis defects, such as increased sepal and petal number, decreased stamen number and reduced anther formation (Sessions *et al.*, 1997; Sessions 1997).

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The establishment of abaxial-adaxial polarity during leaf development involves two small RNA-target pairs (Pulido and Laufs 2010). Although *ARF3* is transcribed throughout the leaf, its transcripts and those of the *ARF3* homolog *ARF4* are restricted to the abaxial side of the leaf by the action of *TAS3*-derived trans-acting small interfering RNAs (ta-siRNAs) known as tasiR-ARFs. tasiR-ARFs are generated at the adaxial side of the leaf and from a gradient towards the abaxial side, and this gradient effectively restricts *ARF3* and *ARF4* to the leaf abaxial domain (Williams *et al.*, 2005; Adenot *et al.*, 2006; Garcia *et al.*, 2006; Chitwood *et al.*, 2009). In a similar manner, transcripts from the class III *HOMEODOMAIN LEUCINEZIPPER* (*HD-ZIPIII*) genes *PHABULOSA* (*PHB*), *PHAVULOTA* (*PHV*) and *REVOLUTA* (*REV*) are restricted to the leaf adaxial domain by the microRNAs miR165/miR166 (McConnell *et al.*, 2001; Kidner and Martienssen 2004).

The SAM continously produces above-ground plant parts throughout the life of a plant (Kaufmann *et al.*, 2010). In contrast, the FM, which develops from the inflorescence meristem (IM), produces a defined number of floral organs and exhibits determinate growth. The termination of floral organ production is known as FM determinacy and serves as an ideal model for the study of meristem maintenance and termination (Liu *et al.*, 2011). FM determinacy is controlled by the coordinated activities of multiple genes in a molecular framework (Sablowski 2007). *WUSCHEL* (*WUS*), which encodes a homeodomain-containing protein critical for SAM establishment and SAM, IM and FM maintenance (Laux *et al.*, 1996; Schoof *et al.*, 2000; Gallois *et al.*, 2004), represents a critical node in this regulatory framework. *WUS* is expressed in the organizing center (OC), a cluster of cells responsible for maintaining the identity of the stem cells above the OC (Laux *et al.*, 1996; Mayer *et al.*, 1998). During floral development, *WUS* expression is turned off at stage 6 once the primordium of the gynoecium is produced, resulting in the termination of the floral stem cells (Lenhard *et al.*, 2001). AGAMOUS (AG), a MADS-domain transcription factor that specifies stamen and carpel identity, is required for the temporally regulated repression of *WUS* expression. In the *ag-1* null mutant, prolonged *WUS* expression beyond stage 6 results in a flowers-in-flower phenotype (Bowman *et al.*, 1989; Lenhard *et al.*, 2001). The delay between the onset of *AG* expression at stage 3 and the termination of *WUS* expression by *AG* at stage 6 suggests that *AG* regulates *WUS* indirectly (Lenhard *et al.*, 2001). However, more recent studies have revealed that *AG* influences *WUS* expression both directly and indirectly: *WUS* is directly repressed by AG through Polycomb Group (PcG) recruitment at the *WUS* locus and indirectly repressed by AG through the AG target gene *KNUCKLES* (*KNU*) (Sun *et al.*, 2009; Liu *et al.*, 2011; Sun *et al.*, 2014). APETALA2 (AP2), an AP2-domain containing transcription factor, promotes SAM and FM maintenance by promoting *WUS* expression and is itself repressed by miR172 (Aukerman and Sakai 2003; Chen 2004; Wurschum *et al.*, 2006; Zhao *et al.*, 2007). The finding that the expression of miR172-resistant *AP2* (*AP2m3*) could dramatically enhance the *ag-1* phenotype (Zhao *et al.*, 2007) indicates that *AP2* acts independently of *AG* in terms of FM determinacy. Others studies have shown that AP2 functions as both a transcriptional activator and repressor by directly binding a TTTGTT/AACAAA motif (Yant *et al.*, 2010; Dinh *et al.*, 2012). However, precisely how *AP2* promotes FM maintenance and how multiple genes are coordinated to regulate FM determinacy have yet to be determined.

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From an ethylmethane sulfonate (EMS) mutagenesis screen of *ag-10*, a weak *ag* allele, we isolated and functionally characterized several genes involved in FM determinacy (Ji *et al.*, 2011; Liu *et al.*, 2011; Yumul *et al.*, 2013; Dinh *et al.*, 2014). The present study describes the identification of a previously unknown role of *ARF3* in FM determinacy. An *arf3* mutant that enhanced the FM determinacy defects of *ag-10* was isolated, and molecular genetic studies revealed that *ARF3* confers FM determinacy by repressing *WUS* expression. A chromatin immunoprecipitation assay revealed that *ARF3* is associated with the *WUS* locus *in vivo* and this association is promoted by *AG*. *ARF3* was also identified as an AP2 target gene and was found to partially mediate the function of *AP2* in FM determinacy. Moreover, the complex patterns of *ARF3* expression and protein distribution in floral organ primordia were found to be important for its FM determinacy function. These studies not only identify a new player in floral determinacy but also reveal the complex molecular interactions that underlie floral determinacy.

RESULTS

ARF3 is required for floral meristem determinacy

An EMS mutagenesis screen was performed in the *ag-10* background to identify FM determinacy regulators (Ji *et al.*, 2011; Liu *et al.*, 2011). While 94% of the siliques in *ag-10* plants are morphologically similar to wild-type siliques, 6% are bulged with additional tissue growing inside, indicating a slight FM determinacy defect in *ag-10* (Figure 1a,b). Given that the occasional presence of additional organs within *ag-10* siliques may be attributable to prolonged stem cell activity, we focused on the isolation of mutants in which all of the siliques were bulged, a phenotype that is reminiscent of, although weaker than, the flowers-in-flower phenotype of the *ag-1* mutant.

One such mutant with conspicuously enhanced *ag-10* FM determinacy defects had bulged and unfused carpels with organs growing inside despite having normal sepals, petals and stamens (Figure 1c). Map-based cloning revealed a G-to-A mutation at the junction of the eighth exon and eighth intron of *ARF3* that disrupted normal splicing and maturation of the mRNA (Supplementary Figure S1a,b). To confirm the effect of the mutation, hereafter referred to as *arf3-29*, in the *ag-10* background, we introduced *ett-3*, an *arf3/ett* allele harboring a nonsense mutation in the eighth exon (Sessions *et al.*, 1997) (Supplemental Figure S1a), into *ag-10*. As with *ag-10 arf3-29*, the *ag-10 ett-3* double mutant also exhibited severe FM determinacy defects characterized by abundant organ growth within the unfused primary carpels (Figure 1d). Introduction of an *ARF3::ARF3-GFP* transgene into *ag-10 arf3-29* completely rescued the mutant phenotype (Figure 1e), further indicating that the *arf3-29* mutation was responsible for the enhanced FM determinacy defects of *ag-10 arf3-29*.

The *ag-10 arf3-29* double mutant was crossed to wild type to obtain the *arf3-29* single mutant. *arf3-29* was found to be an *arf3/ett* allele of intermediate strength, and its gynoecium developmental defects resembled those of *ett-3*, with reduced valves covered by stigma and pronounced medial outgrowths (Figure 1f, g and Supplementary Figure S1c). The extended gynophore of *arf3-29* or *ett-3* (delimited by arrowheads in Figure 1g and Supplementary Figure S1c), which may be attributed to additional cell layers at the base of

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the gynoecium at stage 6 (Sessions and Zambryski 1995; Sessions 1997), may also be indicative of a role of *ARF3* in regulating floral stem cell activity.

ARF3 confers FM determinacy through the repression of *WUS* expression

To investigate the molecular basis of the FM determinacy defects of *ag-10 arf3-29*, we performed *in situ* hybridization to assess the spatial-temporal expression patterns of *WUS* (Laux *et al*., 1996). In wild-type flowers, *WUS* expression was undetectable at stage 6 of floral development (Supplementary Figure S2a) (Mayer *et al*., 1998; Ji *et al*., 2011; Liu *et al*., 2011; Yumul *et al*., 2013). While 90% of the flowers (n=13) from *ag-10* plants had normal *WUS* expression patterns as observed in wild type, *WUS* expression was observed at stage 7 in a small percentage of *ag-10* flowers (2/13), consistent with the slight FM determinacy defects of the *ag-10* mutant (Supplementary Figure S2b) (Liu *et al*., 2011). *WUS* expression beyond stage 6 was not observed in *arf3-29* flowers, indicating that the *arf3-29* mutation alone did not lead to prolonged *WUS* expression (Figure 3a). *WUS* transcript abundance in *ag-10 arf3-29* whole inflorescences was not statistically different from that in *ag-10* and *arf3-29* inflorescences (Supplementary Figure S2c). However, in contrast to *ag-10* flowers, all of the *ag-10 arf3-29* flowers (9/9) examined had prolonged *WUS* expression until or beyond stage 7 (Figure 2b). Together, these findings indicate that *ARF3* is required for the repression of *WUS* expression when AG activity is partially compromised.

To investigate the genetic relationship between *ARF3* and *WUS*, we crossed *ag-10 arf3-29/+* plants with the loss-of-function *wus-1* mutant (Laux *et al*., 1996) and generated the *ag-10 arf3-29 wus-1* triple mutant. *wus-1* plants exhibit premature FM termination, and the incompletely developed flowers of *wus-1* have normal sepals and petals, but terminate in one or two stamens (sepal: 3.7±0.6; petal: 3.5±0.9; stamen: 0.8±0.4; n=11) (Figure 2c). The precocious termination of *ag-10 arf3-29 wus-1* triple mutant flowers was similar to that of *wus-1* flowers, indicating that *wus-1* is epistatic to *ag-10 arf3-29* in terms of FM determinacy (sepal: 3.8±0.7; petal: 3.6±0.8; stamen: 1.1±0.5; n=15) (Figure 2d).

ARF3 functions partially in the *AG* pathway in FM determinacy

AG is a key regulator of FM determinacy, and many genes have been found to promote FM determinacy by maintaining *AG* expression in the center of the meristem (Schultz *et al*., 1991; Alvarez and Smyth 1999; Carles *et al*., 2005; Prunet *et al*., 2008; Das *et al*., 2009; Maier *et al*., 2009). To investigate the relationship between *ARF3* and *AG*, we first compared *AG* transcript levels in early-stage flowers between wild type and *arf3-29* and between *ag-10* and *ag-10 arf3-29*. We found that *arf3-29* had no effect on *AG* transcript levels (Supplementary Figure S2c). Moreover, we did not detect ARF3 binding to the *AG* locus in our chromatin immunoprecipitation (ChIP) assay using *ARF3::ARF3-GFP* transgenic plants. Thus, the data indicate that *ARF3* does not regulate *AG* expression.

We next examined whether *AG* affected *ARF3* expression. In *ag-10, ARF3* transcript levels were decreased (Supplementary Figure S2d). Additionally, we employed *35S::AG-GR*, a widely used rat glucocorticoid receptor (GR)-induction system in which the AG-GR fusion protein localizes to the nucleus and functions on its target genes after dexamethasone (DEX)

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treatment (Sablowski and Meyerowitz 1998, Wagner *et al.* 1999, Ito *et al.* 2004, William *et al.* 2004) to evaluate the effects of *AG* on *ARF3* expression., *ARF3* expression was increased after 4 days of DEX treatment of *35S::AG-GR ag-1* inflorescences (Supplementary Figure S2e). However, no enrichment of AG was observed at the *ARF3* locus in our ChIP assay using *AG::AG-GFP* plants. These results indicate that *AG* modulates *ARF3* expression indirectly, which is consistent with previous reports (Ng *et al.*, 2009).

Next, genetic analysis was performed to determine the molecular pathway in which *ARF3* is involved. We separately introduced the *ag-1* and *knu-1* mutations into *ag-10 arf3-29* by crossing. Flowers of the *ag-1* null mutant are indeterminate and continually produce new organs in the fourth whorl, resulting in a flowers-in-flower phenotype (Figure 2e) (Bowman *et al.*, 1989). *ag-1 arf3-29* flowers appeared morphologically similar to *ag-1* flowers, except that all of the floral organs produced in the former after the first whorl sepals were petals, indicating a role of *ARF3* in specifying sepal identity in the internal flowers (Figure 2e,f). However, longitudinal sections through *ag-1 arf3-29* and *ag-1* flowers at the same developmental stage revealed a domelike meristem in *ag-1 arf3-29* flowers that was statistically larger than that in *ag-1*, indicating that *arf3-29* enhanced the determinacy defects of *ag-1* (Figure 3g–i). *KNU* is an AG target gene that partly mediates *AG*'s function in FM determinacy by repressing *WUS* expression (Sun *et al.* 2009). Compared to *ag-10 arf3-29*, the *ag-10 arf3-29 knu-1* triple mutant exhibited a more severe floral indeterminacy phenotype with several iterations of stamens and carpels growing from the primary, unfused carpel (Figure 3j). Collectively, these results suggest that *ARF3*'s function in FM determinacy in at least partly independent of the *AG* pathway.

ARF3 is an AP2 target gene

AP2 encodes an AP2 domain-containing protein involved in floral organ identity and FM maintenance (Bowman *et al.*, 1991; Zhao *et al.*, 2007). As a bifunctional transcription factor, AP2 can either activate or repress its targets, such as *AGL15* and *SOC1*, respectively (Yant *et al.*, 2010). A previous genome-wide analysis of AP2 binding sites revealed an enrichment of AP2 at the *ARF3* locus, indicating that AP2 may directly bind *ARF3* (Yant *et al.*, 2010). To determine whether *ARF3* is indeed an AP2 target gene, we performed ChIP-qPCR with *AP2::AP2-GFP* transgenic plants. Among the *ARF3* regions tested, high occupancy of AP2 was observed at a region 2.5kb upstream of the *ARF3* transcription start site (TSS), indicating that AP2 binds a specific region of the *ARF3* locus (Figure 3a). To assess whether AP2 directly regulates *ARF3* expression, we quantified the transcript levels of *ARF3* and known AP2 target genes *AGL15* and *SOC1* in *35S::AP2m3-GR* and *35S::GR* plants treated with either DEX or DEX plus cycloheximide (CHX). Daily DEX treatment of *35S::AP2m3-GR* inflorescences for 1 week led to the induction of the *AP2m3* phenotype, thereby demonstrating that the transgene was functional (Zhao *et al.*, 2007; Dinh *et al.*, 2012). After 6 hours of treatment, inflorescences containing stage 8 and younger flowers were micro-dissected, and RT-qPCR was conducted to measure the transcript levels. As expected, *AGL15* and *SOC1* transcript levels were increased and reduced, respectively, in *35S::AP2m3-GR* compared with *35S::GR* in both DEX and DEX plus CHX treatments. *ARF3* transcript levels were reduced by approximately 50% in *35S::AP2m3-GR* relative to

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35S::GR in both treatments (Figure 3b). We also detected higher and lower *ARF3* transcript levels in *ap2-2* and *35S::AP2m3* inflorescences, respectively, relative to wild-type, along with changes in *AG* transcript levels consistent with the repression of *AG* by *AP2* (Figure 3c) (Drews *et al.*, 1991; Zhao *et al.*, 2007; Wollmann *et al.*, 2010). Moreover, we examined ARF3-GFP signals in inflorescences of *ARF3::ARF3-GFP 35S::AP2m3* and *ARF3::ARF3-GFP*. Usually, ARF3-GFP was detected in sepals and the rib zone of floral meristems (see details below and Supplementary Figure S3a). However, reduced GFP signals in sepals and the rib zone of meristems were detected in *ARF3::ARF3-GFP 35S::AP2m3* compared with *ARF3::ARF3-GFP* (compare Supplementary Figure S3a with S3b and Supplementary Figure S3c with Figure 4i). Collectively, these results demonstrated that *ARF3* is a direct target of *AP2*.

We investigated the genetic relationship between *AP2* and *ARF3* in terms of FM maintenance by introducing the *AP2m3* transgene into *ag-10 arf3-29*. Consistent with the presence of an indeterminate FM, *35S::AP2m3* has normal organs in the outer two whorls, numerous stamens produced in a spiral phyllotaxy in the center of the flower and no carpels (Figure 3d) (Chen 2004; Zhao *et al.*, 2007). The floral determinacy phenotypes of *ag-10 arf3-29 35S::AP2m3* resembled those of *35S::AP2m3* (Figure 3e).

ARF3 expression and protein distribution patterns in the FM and floral organs

One of the major functions of *ARF3* is FM and reproductive organ patterning (Sessions *et al.*, 1997). To investigate the molecular basis of this function, we monitored the patterns of *ARF3* expression and protein distribution in the FM and floral organs using *in situ* hybridization and GFP-tagged transgenic plants.

Using an *ARF3* antisense probe, strong *ARF3* mRNA signals were detected in the peripheral zone of the IM, which produces FMs; no specific signals were found when an *ARF3* sense probe was used (Figure 4a and Supplementary Figure 4a). In stage 2 FMs, *ARF3* was ubiquitously expressed with high transcript levels in the rib zone of the meristem (Figure 4b). In stages 3 and 5 FMs, *ARF3* expression was concentrated in the stamen and carpel primordia and absent in the sepals (Figure 4a–c). From stage 5 to later stages (e.g., stage 8), abaxial *ARF3* expression was observed in petals, stamens and the gynoecium (Figure 4d and Supplementary Figure 4b). These results reveal complex patterns of *ARF3* expression during early floral development.

The *ARF3::ARF3-GFP* transgene fully rescued the floral determinacy and gyneocium developmental defects of *ag-10 arf3-29*, indicating that the transgene conferred the whole range of *ARF3* functions. As a transcription factor, ARF3 localizes in the nucleus (Kelley *et al.*, 2012), and consistently, ARF3-GFP signals were also nuclear (Figure 4e–i) in *ARF3::ARF3-GFP* plants. The GFP signal was present in the IM and stages 1 to 8 FMs. The high levels of *ARF3* expression during early floral development is consistent with its function in FM and floral organ patterning (Figure 4e,f). Surprisingly, microscopic three-dimensional (3D) imaging of early-stage flowers showed ubiquitous GFP signals in stages 1 to 3 FMs, which differed from the *ARF3* mRNA patterns described above (Supplementary Figure 4c, compare Figure 4e,f with Figure 4a,b). Additionally, strong GFP signals were observed in the sepals of stage 4 flowers, although *ARF3* mRNA was not detected in sepals

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by *in situ* hybridization (compare Figure 4e,f, j and i with Figure 4c). To further investigate the distribution of ARF3-GFP in the floral organs, we embedded and sectioned the tissue then performed fluorescence detection as previously described (Goldshmidt *et al.*, 2008). GFP signal was detected throughout the IM without a gradient (Figure 4e,f). In stage 1 to early stage 3 FMs, GFP signal was again observed throughout the stem cell population, and was particularly high in the rib zone of the meristem (Figure 4g, h). In late stage 3 FMs, GFP signal was concentrated in groups of cells that would give rise to the sepal primordia (Figure 4i, l). In stage 5 flowers, GFP signal was detected in the center of the FM and the incipient stamen primordia (Figure 4i). In stage 6 flowers, GFP signal was detectable in all organ primordia including sepals and particularly concentrated in the rib zone of the meristem without a gradient (Figure 4j). In stage 6–8 flowers, GFP signal was evenly distributed throughout the gynoecium (Supplementary Figure S4d). After stage 8, ARF3-GFP was restricted to the abaxial side of the gynoecium (Figure 4k). The differences between the patterns of *ARF3* transcript and protein distribution suggest that translational or post-translational mechanisms or protein movement may influence the distribution of the protein.

In light of the abundance of ARF3-GFP in the OC, where *WUS* is expressed, we further investigated whether the expression patterns of *ARF3* and *WUS* overlap by crossing an *ARF3::ARF3-GFP* plant with the *WUS* reporter line *WUS::DsRed-N7* (Gordon *et al.*, 2007). Indeed, a high degree of overlap between GFP and RFP signals was observed, indicating that *ARF3* and *WUS* are coexpressed in early flower development (Figure 4l and Supplementary Figure S4e, f).

The spatial distribution patterns of *ARF3* are important for its FM determinacy function

Although *ARF3* is transcribed throughout the leaf during leaf development, *ARF3* transcripts are restricted to the abaxial leaf domain by tasiR-ARFs, and the resulting spatial pattern of *ARF3* expression is critical for the establishment of leaf polarity (Chitwood *et al.*, 2009). To determine whether the spatial patterns of *ARF3* expression in the flower similarly influence its FM determinacy function, we generated a *WUS::ARF3m-GFP::WUS3'* construct. The construct included 3.2kb of the *WUS* promoter; 1.5kb of the sequence downstream of the *WUS* coding region; and *ARF3m-GFP*, which is resistant to regulation by tasiR-ARFs (Hunter *et al.*, 2006; Liu *et al.*, 2011). *ag-10 arf3-29*/+ plants were transformed with the construct. In the T1 population, all positive transgenic lines were genotyped, and 14 transgenic plants were identified as homozygous for both *ag-10* and *arf3-29* (i.e., *WUS::ARF3m-GFP::WUS3' ag-10 arf3-29*). These plants exhibited FM determinacy defects similar to that of *ag-10 arf3-29* (Figure 5a, b). As expected, ARF3m-GFP fluorescence was found to be restricted to the OC (Figure 5c). Thus, OC-specific expression of *ARF3* failed to rescue the FM determinacy defects of *ag-10 arf3-29*. We also generated an *ARF3::ARF3m-GFP* construct, which is predicted to lead to ubiquitous distribution of *ARF3* RNA and an *ARF3::ARF3m-PHB-GFP* construct, in which tasiR-ARF-resistant *ARF3* is fused to a sequence containing the miR165/166 recognition site from *PHB* to yield abaxial-adaxial inversion of *ARF3* expression. In contrast to *ARF3::ARF3-GFP ag-10 arf3-29* plants (Figure 5d,e), *ag-10 arf3-29* plants harboring a single copy insertion of *ARF3::ARF3m-GFP* or *ARF3::ARF3m-PHB-GFP* displayed only partial

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complementation: while the FM determinacy defects of later flowers were nearly fully rescued, early flowers clearly exhibited FM determinacy defects (Figure 5f–i). To confirm the expected patterns of *ARF3* expression from the transgenes, *in situ* hybridization was performed in *ARF3: :ARF3m-GFP* and *ARF3: :ARF3m-PHB-GFP* plants. As expected, *ARF3* was expressed throughout *ARF3: :ARF3m-GFP* flowers. In contrast, *ARF3: :ARF3m-PHB-GFP* flowers had stronger *ARF3* signal on the adaxial side of floral organs, with the abaxial signal due to endogenous *ARF3* transcripts (Supplementary Figure S5a–c).

AG promotes the binding of ARF3 to the *WUS* locus *in vivo*

ARF3 and *WUS* coexpression in the OC raised the possibility that *WUS* is a target of ARF3. ARF family members specifically bind the AuxRE TGTCTC (Liu *et al.*, 1994; Ulmasov *et al.*, 1995; Liu *et al.*, 1997). *In vitro* binding analysis revealed that substitutions at position +5 of the TGTCTC element being tolerated by ARF3 (Ulmasov *et al.*, 1999b). We examined the *WUS* locus sequence and found one TGTCTC motif in the first intron (*WUSp4* region) and two tandem TGTCCCTT sequences in the promoter region (*WUSp2* region) approximately 500 bp upstream of the TSS (Figure 6a). TGTCCCTT is very similar to the TGTCCCAT *cis*-acting element found in the auxin-responsive region of the pea *PS-IAA4/5* promoter and the soybean *SAUR* gene (Ballas *et al.*, 1993; Li *et al.*, 1994; Ballas *et al.*, 1995). Using ChIP, we found high ARF3 enrichment at the *WUS* promoter region containing the TGTCCCTT tandem sequences but not at the first intron of *WUS* or other regions (Figure 6b).

Next, we investigated whether ARF3 directly binds the *WUS* locus using yeast one-hybrid analysis. The *WUSp2: :LacZ* and *WUSp4: :LacZ* reporters were tested against AD (activation domain)-ARF3 or AD alone in yeast strains. While AD alone was unable to activate either reporter, AD-ARF3 activated the expression of *WUSp2: :LacZ* but not *WUSp4: :LacZ*, as shown by aβ-galactosidase assay (Figure 6c), suggesting that ARF3 can directly bind the *WUS* promoter..

As we previously found that AG also binds the *WUS* promoter region (Liu *et al.*, 2011), we investigated whether AG affects the binding of ARF3 to *WUS*. We performed ChIP analysis with *ARF3: :ARF3-GFP arf3-29* and *ARF3: :ARF3-GFP arf3-29 ag-1* inflorescences. Interestingly, the occupancy of ARF3 at the *WUS* promoter was dramatically reduced in *ARF3: :ARF3-GFP arf3-29 ag-1* compared to *ARF3: :ARF3-GFP arf3-29* (Figure 6b). Given that *AG* promotes *ARF3* expression, the reduced ARF3 occupancy at *WUS* in *ag-1* could be due to reduced *ARF3* expression. If so, we would expect ARF3 occupancy at its other target genes to be similarly reduced. We examined ARF3 occupancy at the promoter of *atIPT5*, which was reported to be bound by ARF3 (Cheng *et al.*, 2013). ARF3 occupancy at *atIPT5* in *ARF3: :ARF3-GFP arf3-29 ag-1* was no different from that in *ARF3: :ARF3-GFP arf3-29*. Therefore, AG promotes the binding of ARF3 to the *WUS* promoter (Figure 6b).

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DISCUSSION

ARF3 is required for FM determinacy

The roles of *ARF3* in FM and floral organ patterning, leaf polarity and gynoecium development have been described (Sessions and Zambryski 1995; Sessions *et al.*, 1997; Fahlgren *et al.*, 2006). *arf3* mutants typically exhibit an elongated gynophore, which has been attributed to additional cell layers at the base of the gynoecium primordium in stage 6 *arf3* flowers (Sessions and Zambryski 1995; Sessions 1997). Given that floral organ primordia are successively produced from the outer whorls to the inner whorls and that the production of the gynoecium represents the last organogenesis event before the termination of the floral stem cells, the elongated gynophore of *arf3* mutants may be indicative of prolonged FM activity. Using forward genetics screening and genetic analysis, we found clear evidence supporting a role of *ARF3* in FM determinacy. Specifically, a mutation in *ARF3, arf3-29*, enhanced the weak FM determinacy defects of *ag-10*, as does the previously described *arf3* mutant *ett-3*. The double mutants have numerous additional organs growing within the primary unfused sepaloid carpels. Collectively, these results support a role of *ARF3* in specifying FM determinacy.

ARF3 partially integrates the *AP2* and *AG* pathways to confer FM determinacy

FM determinacy is a complex process involving the coordination of various regulatory factors during the different floral developmental stages. *WUS* plays a vital role in SAM and FM maintenance and integrates several genetic pathways that either maintain or terminate the FM (Sablowski 2007; Sun *et al.*, 2009; Ji *et al.*, 2011; Liu *et al.*, 2011; Yumul *et al.*, 2013). The present findings indicate that *ARF3*, along with *AG*, promotes FM determinacy by repressing *WUS* expression within the FM.

Although *ARF3* is required for FM determinacy as is *AG*, our results suggest that *ARF3* may largely act independently of the *AG* pathway. First, in early floral developmental stages, *ARF3* was expressed in the IM and FM independently of floral organ identity genes such as *AG*. While AG-GR increased *ARF3* expression, this effect was only observed after hours of DEX treatment, indicating that the induction of *ARF3* by AG was indirect. This finding is consistent with a previous report that *GIANT KILLER* (*GIK*) mediates the effect of AG on *ARF3* expression (Ng *et al.*, 2009). Second, although *ag-1 arf3-29* flowers resembled *ag-1* flowers in terms of FM determinacy defects (Figure 2e,f), longitudinal sections revealed larger FMs in *ag-1 arf3-29* flowers than *ag-1* flowers.. Finally, genetic analysis revealed that *knu-1* enhanced the *ag-10 arf3-29* FM determinacy defects, indicating that *ARF3* acts in parallel to *KNU*, an AG target that represses *WUS* expression (Sun *et al.*, 2009; Sun *et al.*, 2014). However, it is possible that *ARF3* partially mediates the FM determinacy function of *AG* in light of the finding that *AG* promotes *ARF3* binding to the *WUS* locus.

AP2 has been implicated in SAM and FM maintenance by promoting *WUS* expression (Wurschum *et al.*, 2006; Zhao *et al.*, 2007), but the underlying molecular mechanism is unknown. Using ChIP-qPCR and an inducible expression system in the present study, *ARF3* was found to be an AP2 direct target, with AP2 repressing *ARF3* expression. Genetic

analysis indicated that *ARF3* mediates the FM determinacy function of *AP2* insofar as *ag-10 arf3-29* being unable to enhance *35S::AP2m3*. However, comparing the weak FM determinacy defects of *arf3-29* and the strong determinacy defects of *35S::AP2m3*, mediation of *AP2* function by *ARF3* may only be partial.

Collectively, the results of the genetic and gene expression analyses led to a working model of *ARF3* in regulating FM determinacy (Figure 7). First, *ARF3* is probably required for the full function of *AG* in FM determinacy. Once expressed at stage 3, *AG* enhances *ARF3* expression indirectly and promotes ARF3 binding to the *WUS* locus, thereby coordinating or enhancing the function of *ARF3* at *WUS*. Second, as a target gene repressed by AP2, *ARF3* partially mediates the FM maintenance function of *AP2*. Therefore, *ARF3* partially integrates the functions of both *AP2* and *AG* in FM maintenance and determinacy.

ARF3 exhibits dynamic and functionally relevant expression patterns

During leaf development, the abaxial distribution of *ARF3* transcripts is critical for leaf polarity establishment. Altering the spatial distribution of *ARF3* transcripts using tasiR-ARF-resistant *ARF3* impairs leaf and gynoecium development (Fahlgren *et al*., 2006). *in situ* hybridization analysis in the present study revealed complex expression patterns of *ARF3* in the IM and early FMs. *ARF3* is expressed in clusters of cells that give rise to new FMs or floral organ primordia, consistent with its functions in FM patterning and floral organ identity. However, differences were observed between the distribution of *ARF3* RNA and protein. First, while *ARF3* RNA was abaxially distributed in the IM and floral organ primordia of early FMs, ARF3 protein was evenly distributed throughout the IM and gynoecium primordium of early FMs . Second, *ARF3* RNA was not detected in sepals by *in situ* hybridization, but ARF3 protein strongly accumulated there. The latter finding suggests that *ARF3* may be involved in sepal organogenesis and is also consistent with the petaloid sepal phenotype of the internal flowers of *ag-1 arf3-29* (Figure 2f). The differences in the distribution patterns of *ARF3* RNA and protein suggest that the ARF3 protein may be mobile. We note that the patterns of *ARF3* RNA distribution are similar to those of auxin in the IM and early stage FMs (Vernoux *et al*., 2010). This raises the possibility that *ARF3* is induced by auxin, although there is currently no evidence for this hypothesis. In turn, the even distribution of ARF3 protein throughout the IM and stages 1–2 FMs may allow ARF3 to coordinate developmental decisions within the meristems.

The abaxial distribution of *ARF3* RNA in floral organ primordia was found to contribute to the FM determinacy function of *ARF3*, as transgenes that altered the *ARF3* RNA distribution failed to complement the FM determinacy defects of *ag-10 arf3-29*. OC-specific expression of *ARF3* also failed to rescue the FM determinacy defects of *ag-10 arf3-29*, although ARF3 was found to bind the *WUS* promoter region (Figure 6b). These somewhat paradoxical findings suggest that ARF3 exerts both direct and indirect effects on the repression of *WUS* expression. One possibility is that *ARF3* acts via auxin in FM maintenance and determinacy. *ARF3* has been found to be involved in SAM induction during *de novo* organ regeneration through auxin and cytokinin signaling (Cheng *et al*., 2013). Additionally, physical interactions between ARF3 and KANADI 1 (KAN1) were found to contribute to integument development and polarity determination (Kelley *et al*.,

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2012). *KAN1* regulates auxin biosynthesis, transport and signaling by binding a specific *cis*-element as a repressor (Huang *et al.*, 2014). It is possible that ARF3-KAN1 acts outside of the OC to regulate auxin biosynthesis or signaling, which in turn influences FM determinacy.

EXPERIMENTAL PROCEDURES

Plant materials

Plants were grown in soil as previously described by Liu *et al.* (2011). *ag-10, ett-3, ag-1, wus-1, 35S::AP2m3, knu-1, 35S::AP2m3-GR*,,*35S::GR* and *35S::AG-GR ag-1* were previously described (Sessions *et al.*, 1997; Zhao *et al.*, 2007; Ji *et al.*, 2011; Liu *et al.*, 2011; Dinh *et al.*, 2014).

ChIP and *in situ* hybridization

The methods used for ChIP and *in situ* hybridization including information on the *WUS* probe were previously described (Liu *et al.*, 2011). To generate *ARF3* probes for *in situ* hybridization,, *ARF3* sequence was amplified by PCR with *ARF3SP6* and *ARF3T7* primers and the *ARF3::ARF3m* plasmid (see Method S3) as the template. The amplified product was purified and used as the template for *in vitro* transcription with either T7 or SP6 RNA polymerase to generate the antisense and sense probes, respectively. The primers used are listed in Supplementary Table S1.

Yeast one-hybrid analysis

PCR reactions were performed using *WUSp2Y1HF* and *WUSp2Y1HR* or *WUSp4Y1HF* and *WUSp4Y1HR* as primers and L*er* genomic DNA as the template to obtain the fragments of *WUSp2* and *WUSp4*, respectively. The fragments were cloned into the *Sma*I site of *pLacZi* (Clontech), creating *pWUSp2::LacZ* and *pWUSp4::LacZ*. Each plasmid was linearized by *Apa*I digestion prior to transformation of the yeast strain YM4271 (Clontech). The full-length cDNA of *ARF3* was amplified by PCR from the *ARF3::ARF3m* plasmid using primers *ARF3Y1HF* and *ARF3Y1HR* and cloned into the *pGADT7* vector resulting in the *pGADT7-ARF3* plasmid. The *pGADT7-ARF3* or *pGADT7* plasmids were subsequently transformed into the yeast strain containing the *pWUSp2::LacZ* or *pWUSp4::LacZ* construct and transformants were selected for growth on selection medium (SD/−Leu). β-galactosidase assay was performed according to the Yeast Protocols Handbook (Clontech, PT3024-1). The primers used are listed in Supplementary Table S1, Method S1, Method S2, Method S3, Method S4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *ARF3* is required for FM determinacy

(a) Wild-type (L*er*) siliques.

(b) *ag-10* siliques. The one on the right is an example of a bulged silique with internal floral organs.

(c) An *ag-10 arf3-29* flower with additional floral organs growing inside of the unfused sepaloid carpels.

(d) An *ag-10 ett-3* flower with a similar phenotype as *ag-10 arf3-29*.

(e) Siliques from two *ARF3::ARF3-GFP* transgenic lines (middle and right pairs) and *ag-10 arf3-29* siliques as the control (left pair).

(f) An *arf3-29* flower (top view).

(g) An *arf3-29* flower (side view). The arrows mark the ends of the gynophore.

Bars: 1mm in (a–g).

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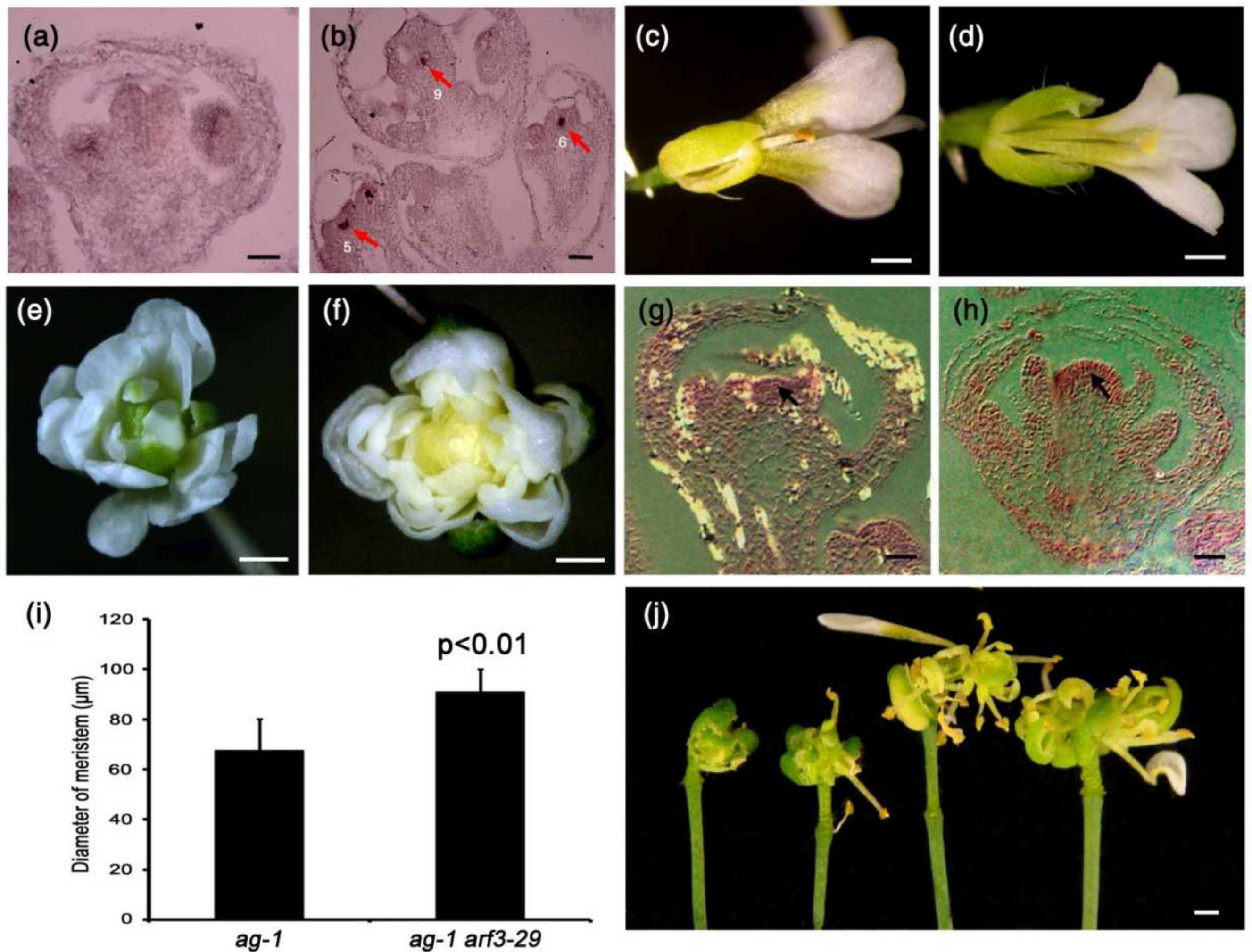

Figure 2. Genetic interactions between *arf3-29* and *wus-1, ag-1* and *knu-1*

(a) *WUS* expression in a stage 6 *arf3-29* flower as examined by *in situ* hybridization. No *WUS* signal was detected.

(b) *WUS* expression in *ag-10 arf3-29* flowers as examined by *in situ* hybridization. Arrows indicate *WUS* signal, and numbers indicate the floral developmental stage.

(c) A *wus-1* flower.

(d) An *ag-10 arf3-29 wus-1* flower.

(e) An *ag-1* flower.

(f) An *ag-1 arf3-29* flower.

(g,h) Longitudinal sections through stage 9 *ag-1* (g) and *ag-1 arf3-29* (h) flowers. Arrows indicate the floral meristem.

(i) FM diameter of *ag-1* and *ag-1 arf3-29* flowers. The values indicate means ± SD (n=10). The mean values for *ag-1* and *ag-1 arf3-29* were significantly different according to a Student's *t*-test (p<0.01).

(j) Siliques of *ag-10 arf3-29* (left pair) and *ag-10 arf3-29 knu-1* (right pair).

Bars: 50μm in (a,b) and (g,h); 1mm in (c–f,j).

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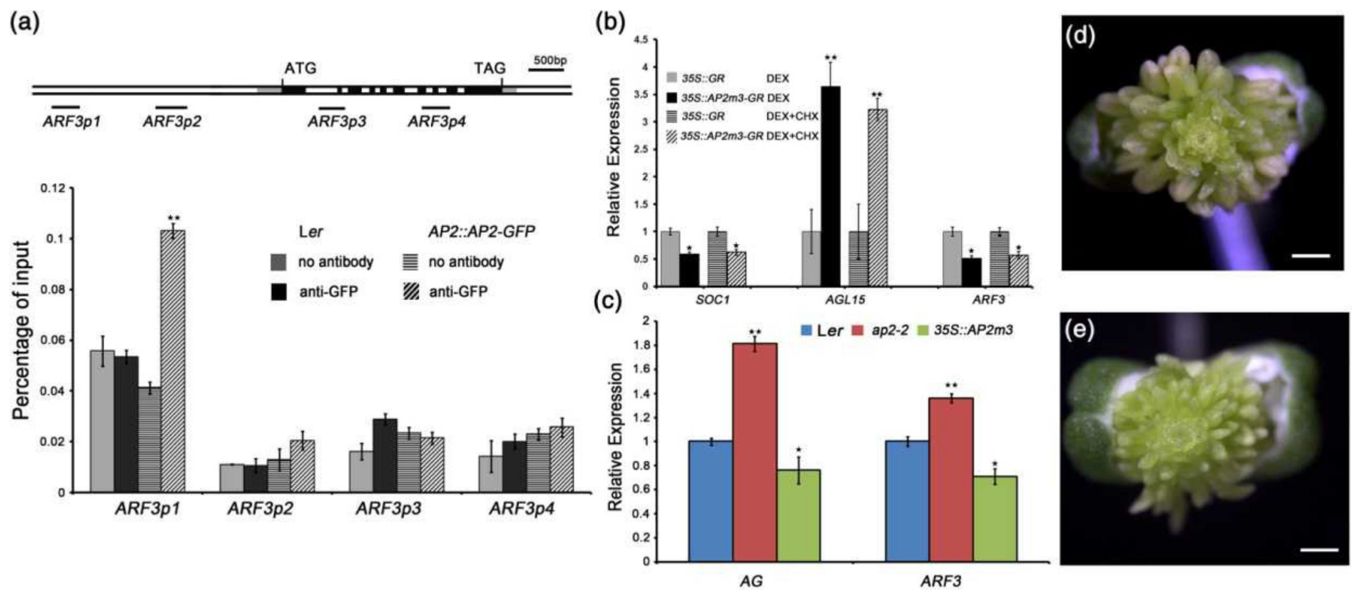

Figure 3. *ARF3* is an AP2 target gene

(a) ChIP-qPCR showing that AP2 binds the *ARF3* locus. The tested regions of *ARF3* are diagramed in the upper panel. ATG and TAG represent the start and stop codons, respectively. The gray, black and white rectangles represent the 5' or 3' untranslated regions, coding regions, and introns or intergenic regions, respectively. The black lines indicate the tested regions. Anti-GFP antibody was used for the analysis, and "no antibody" served as the negative control. Inflorescences containing all unopened flowers were dissected for ChIP assay.

(b) Real-time RT-PCR analysis of *ARF3, SOC1* and *AGL15* in *35S::GR* and *35S::AP2m3-GR* inflorescences treated with dexamethasone (DEX) for 6 hours with or without cyclohexamide (CHX). Inflorescences containing stage 8 and early flowers were used.

(c) *AG* and *ARF3* expression in L*er, ap2-2* and *35S::AP2m3* as determined by RT-qPCR Inflorescences containing stage 8 and early flowers were used.

(d) A *35S::AP2m3* flower. Bars: 0.5mm.

(e) A *35S::AP2m3 ag-10 arf3-29* flower. Bars: 0.5mm.

Error bars in (a–c) represent SD calculated from three biological replicates. Statistically significant changes are indicated by ★ (p-value < 0.05) and ★★ (p-value < 0.01).

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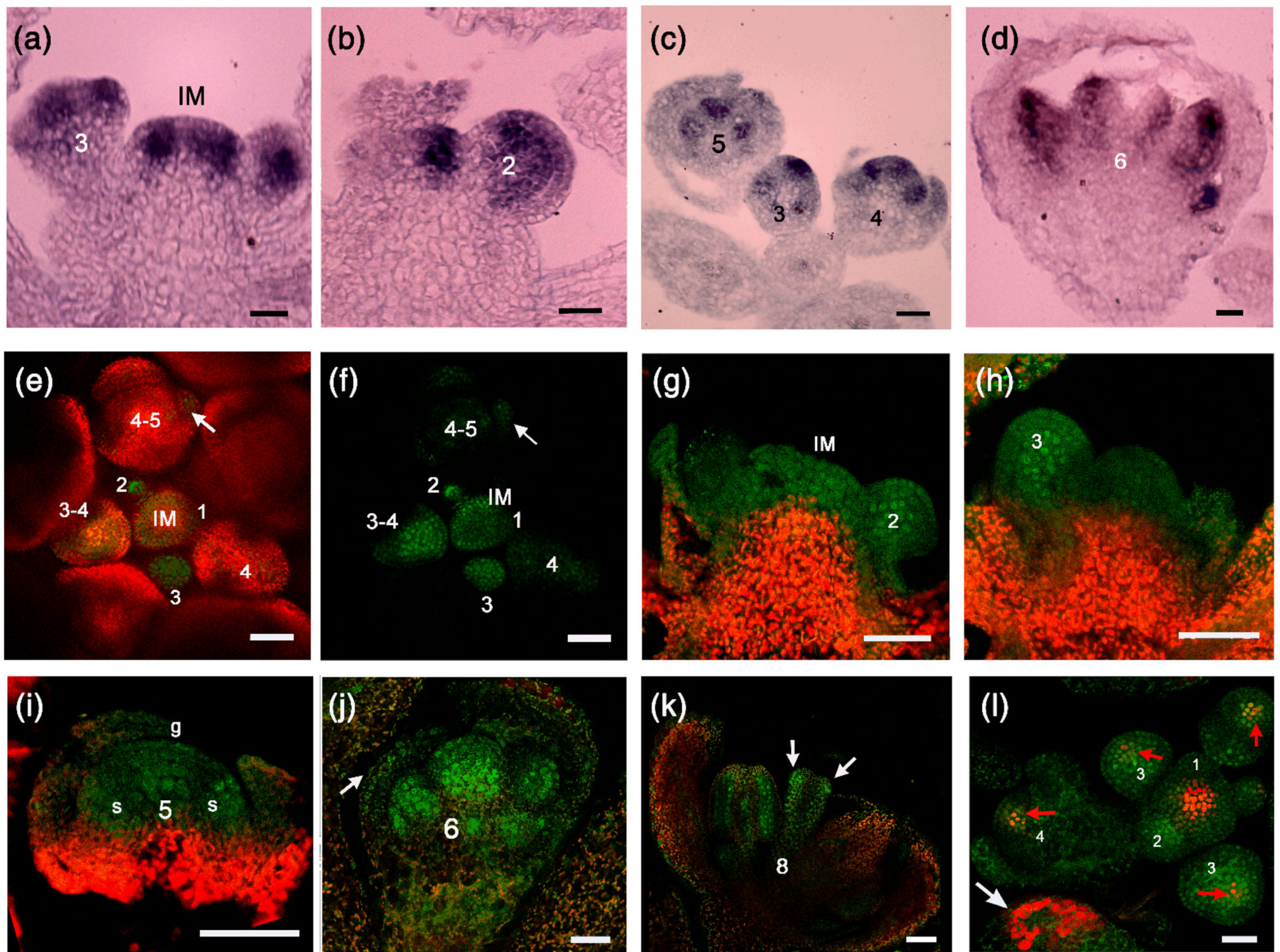

Figure 4. *ARF3* RNA and protein distribution in flowers

(a–d) *in situ* hybridization with an *ARF3* antisense probe. *ARF3* expression patterns in the IM and stage 3 (a), stage 2 (b), stage 5 (c) and stage 6 (d) FMs are shown. Numbers indicate the developmental stage.

(e,f) A global view of ARF3-GFP signal distribution in the FM and floral organ promordia during early floral development as observed with a confocal microscope (e: GFP and chlorophyll fluorescence merged channels; f: GFP channel alone). Numbers indicate the developmental stages of the FMs, and the arrow indicates GFP signal in a sepal primordium. Note that the GFP signal is present in the center of the IM but is masked by the strong chlorophyll fluorescence.

(g–k) ARF3-GFP signal in the IM and stage 2 (g), stage 3 (h), stage 5 (i), stage 6 (j) and stage 8 (k) FMs and flowers. Numbers indicate the floral developmental stage. The arrow in (j) indicates GFP signal in a sepal, and the arrows in (k) indicate the abaxial distribution of ARF3-GFP signals. g: gynoecium; s: stamen. Note that in (g) to (i), the regions showing strong chlorophyll fluorescence (red) also had GFP signals, which were masked by the red fluorescence.

(l) Coexpression of *ARF3* and *WUS* in the FM. *ARF3::ARF3-GFP* (green) and *WUS::DsRed-N7* (red) were detected in an *ARF3::ARF3-GFP WUS::DsRed-N7* inflorescence using a confocal microscope, and the signals were merged. Red arrows indicate coexpression regions, and the white arrow indicates chlorophyll autofluorescence. Numbers indicate the floral developmental stage.

Bars: 50µm in (a,b) and (e–j); 25µm in (c,d) and (k,l).

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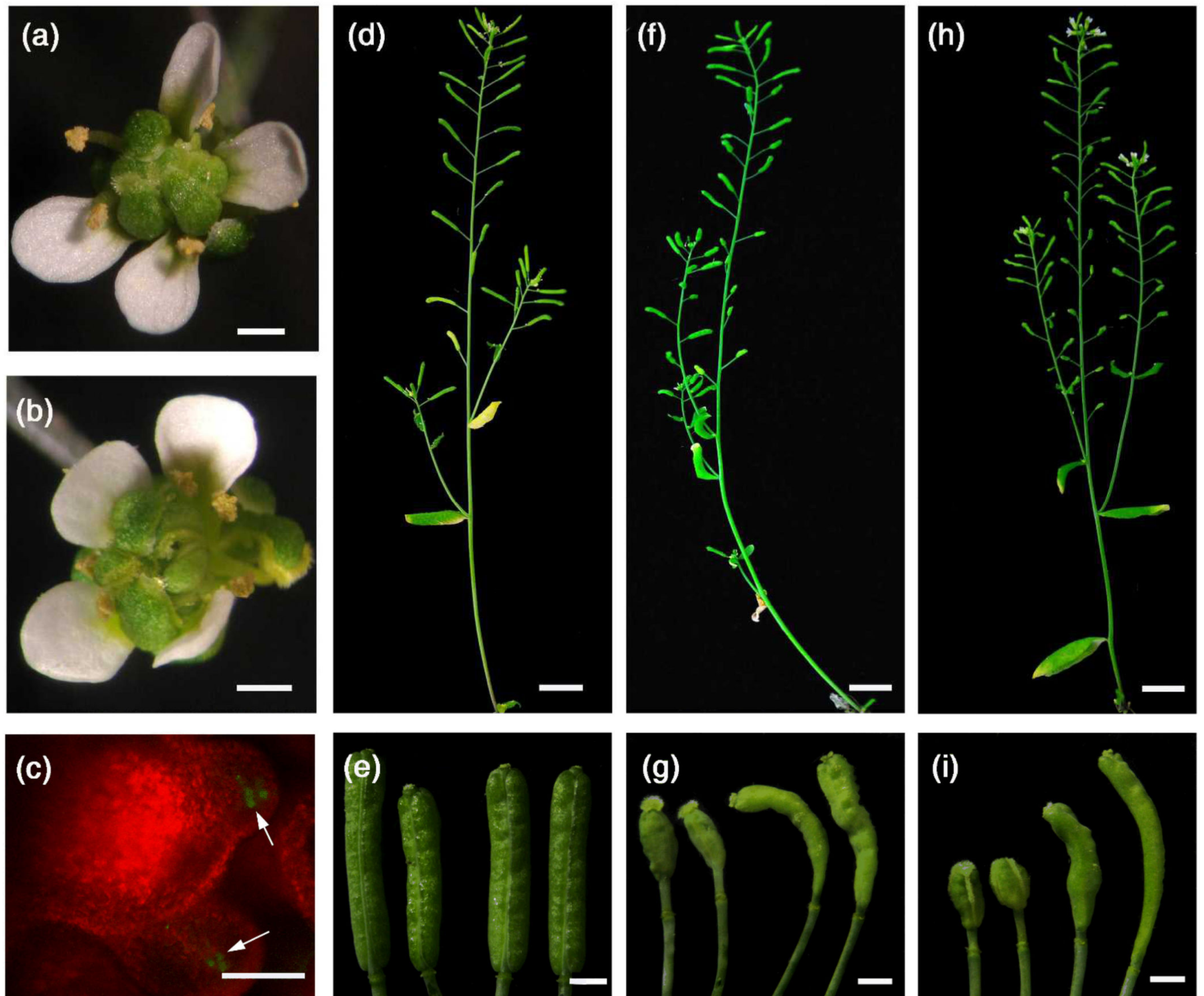

Figure 5. *ARF3*'s proper spatial expression is important for its function in FM determinacy

(a) An *ag-10 arf3-29* flower.

(b) A *WUS::ARF3m-GFP::WUS3' ag-10 arf3-29* flower.

(c) Fluorescence detection of ARF3m-GFP in a *WUS::ARF3m-GFP::WUS3' ag-10 arf3-29* flower. White arrows indicate the GFP signals.

(d,e) An *ARF3::ARF3-GFP ag-10 arf3-29* plant (d) and its siliques (e).

(f,g) An *ARF3::ARF3m-GFP ag-10 arf3-29* plant (f) and its siliques (g).

(h,i) An *ARF3::ARF3m-PHB-GFP ag-10 arf3-29* plant (h) and its siliques (i).

Bars: 1mm in (a,b); 50μm in (c); 1cm in (d,f,h); 1mm in (e,g,i).

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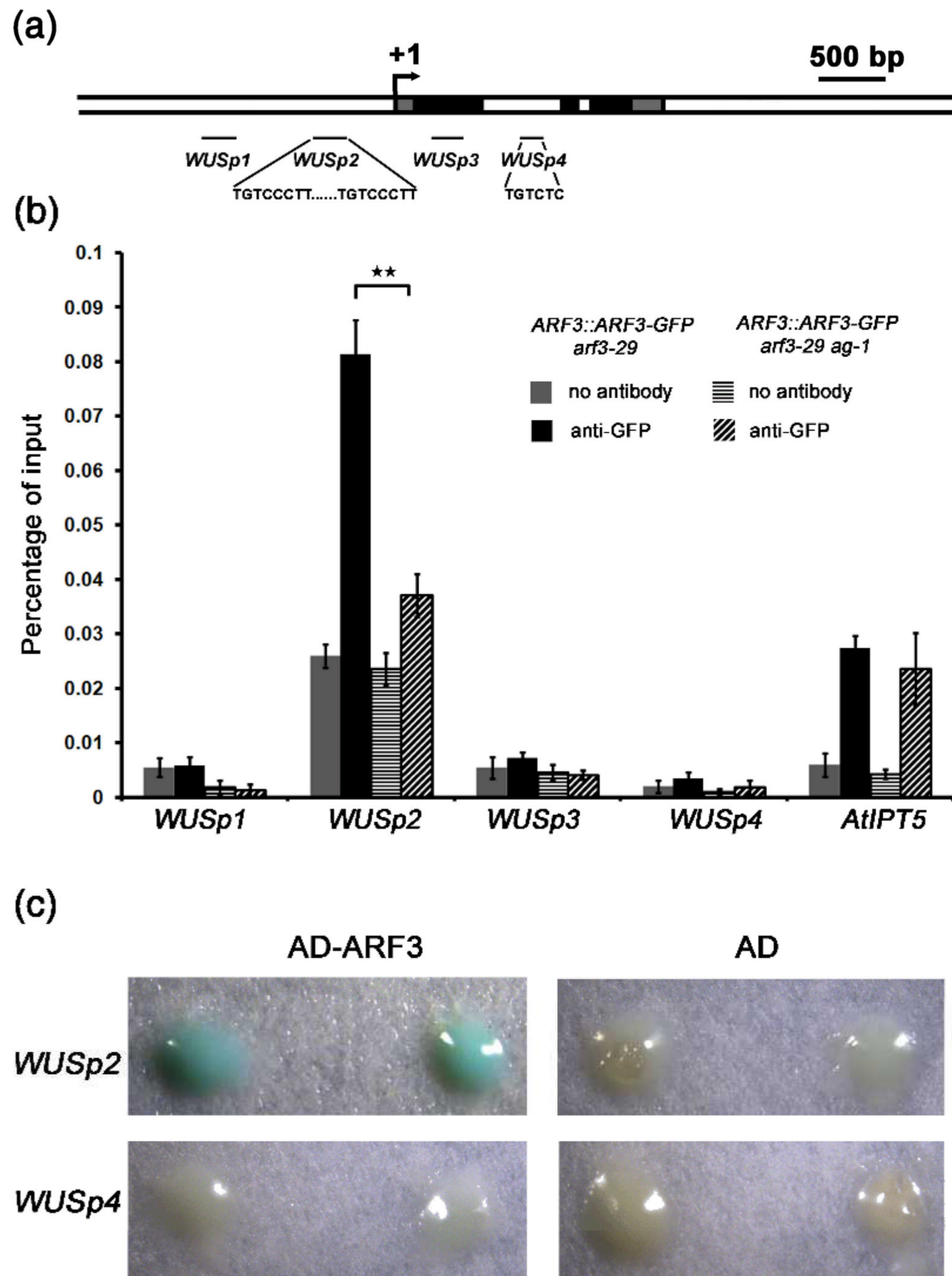

Figure 6. *AG* promotes the binding of ARF3 to the *WUS* locus *in vivo*

(a) A diagram of the *WUS* genomic region with "+1" corresponding to the transcription start site. The gray, black and white rectangles represent the 5' or 3' untranslated regions, coding regions and introns or intergenic regions, respectively. The black lines indicate the regions examined in ChIP. The sequences of the putative ARF3 binding sites are also shown.

(b) ARF3 occupancy at *WUS* and *atIPT5* as determined by ChIP-qPCR. Anti-GFP antibody was used for ChIP, and "no antibody" served as the negative control. Error bars represent

SD calculated from four biological replicates. Statistically significant changes are indicated by ★★ (p-value < 0.01).

(c) Yeast one-hybrid analysis revealing the direct binding of ARF3 to *WUSp2*. Yeast strains containing *WUSP2: :LacZ* or *WUSp4: :LacZ* reporters were transformed with *AD-ARF3* or *AD* vectors, respectively. Two independent transformants growing on selective medium (SD/-Leu) were selected for β-galactosidase assay.

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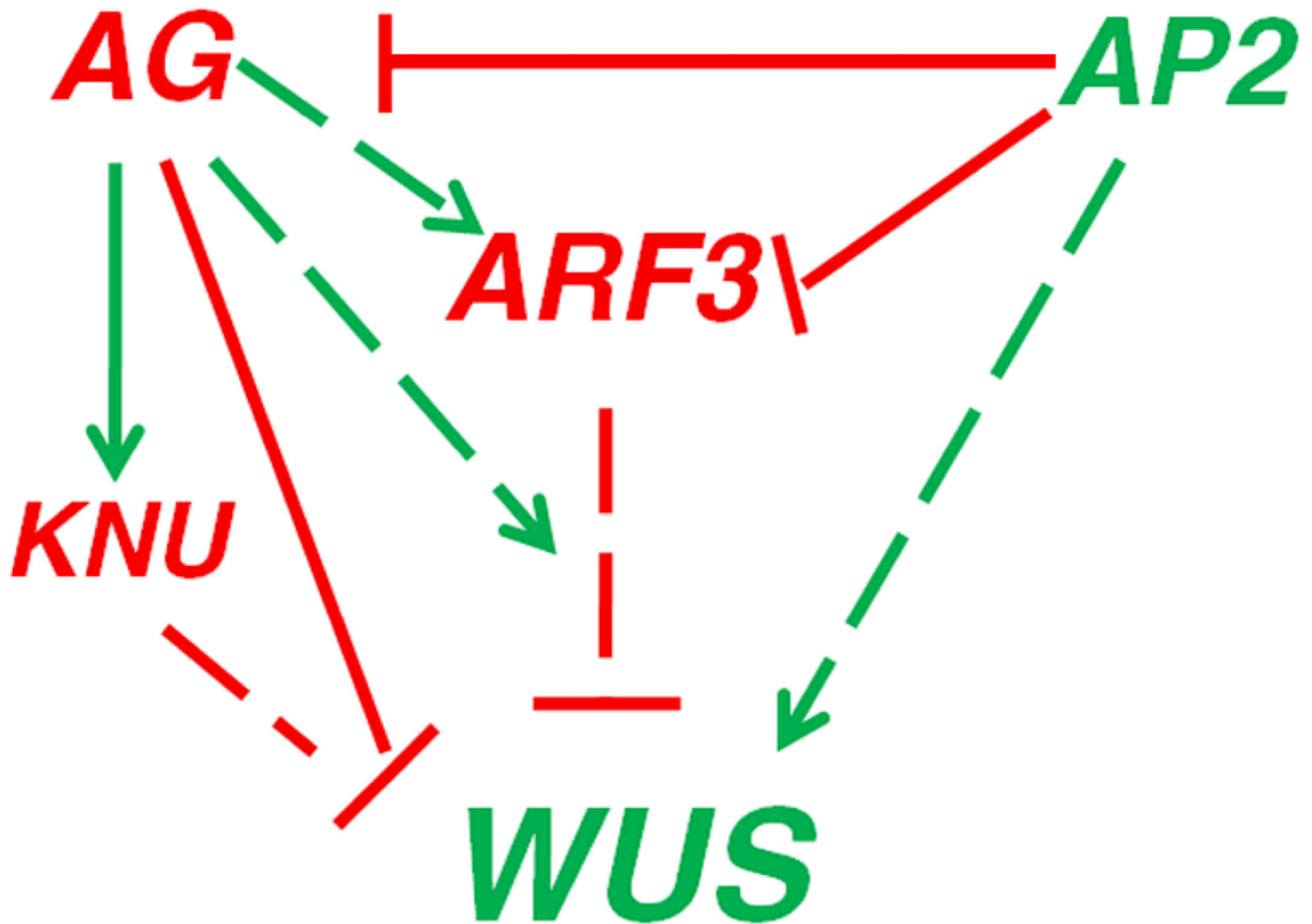

Figure 7. A model of *ARF3* in FM determinacy

ARF3 terminates the floral stem cells by directly or indirectly repressing *WUS* expression. Besides the direct repression of *WUS* by AG through the recruitment of PcG to *WUS* and the indirect repression of *WUS* through the activation of *KNU* expression (Sun *et al.*, 2009; Liu *et al.*, 2011), *AG* indirectly enhances *ARF3* expression and promotes ARF3 binding to the *WUS* locus. As a target gene repressed by AP2, *ARF3* partially mediates the FM maintenance function of *AP2*. Red and green arrows indicate positive and negative effects, respectively. Solid and dotted arrows indicate direct and indirect effects, respectively. *ARF3* is likely to have a direct effect on *WUS* expression, but this has not been definitively proven *in vivo*, thus a dotted arrow is used.