Dual roles of the bZIP transcription factor PERIANTHIA in the control of floral architecture and homeotic gene expression

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Flowers develop from floral meristems, which harbor stem cells that support the growth of floral organs. The MADS domain transcription factor AGAMOUS (AG) plays a central role in floral patterning and is required not only for the specification of the two reproductive organ types, but also for termination of stem cell fate. Using a highly conserved cis-regulatory motif as bait, we identified the bZIP transcription factor PERIANTHIA (PAN) as a direct regulator of *AG* in *Arabidopsis. PAN* and *AG* expression domains overlap, and mutations in either the PAN-binding site or *PAN* itself abolish the activity of a reporter devoid of redundant elements. Whereas under long-day conditions *pan* mutants have merely altered floral organ number, they display in addition typical *AG* loss-of-function phenotypes when grown under short days. Consistently, we found reduced *AG* RNA levels in these flowers. Finally, we show that *PAN* expression persists in *ag* mutant flowers, suggesting that *PAN* and *AG* are engaged in a negative-feedback loop, which might be mediated by the stem-cell-inducing transcription factor WUSCHEL (WUS).

KEY WORDS: PERIANTHIA, AGAMOUS, Stem cells, *Arabidopsis*

INTRODUCTION

Flowers have attracted the attention of humanity for millennia, not only because of their beauty, but also because they give rise to fruits and seeds. As flowers are the sites of sexual reproduction of flowering plants, their proper development is essential for survival of the species. Evolution has brought about a dazzling array of floral phenotypes as flowering plants have found their way into virtually every ecological niche on land. Still, the molecular machinery that controls the identity and stereotypic arrangement of floral organs has been well conserved (Theissen et al., 1996). Flowers of the reference plant *Arabidopsis thaliana* contain four major organ types: sepals, petals, stamens and carpels, which are arranged in four concentric rings or whorls. Organ identity is specified by the overlapping activities of three classes of homeotic genes, termed A, B and C, as predicted in the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991). The activity of a floral homeotic gene is typically confined to two adjacent whorls, with the A class represented by *APETALA1* (*AP1*) and *APETALA2* (*AP2*) acting in whorls one and two, the B class genes *APETALA3* (*AP3*) and *PISTILATA* (*PI*) in whorls two and three, and the C class gene *AGAMOUS* (*AG*) in whorls three and four (reviewed by Lohmann and Weigel, 2002). With the exception of *AP2*, all floral homeotic genes code for MADS domain transcription factors. Essential co-regulators in this process are the MADS domain transcription factors of the SEPALLATA class (SEP1, SEP2, SEP3 and SEP4), which form tetrameric complexes with different combinations of homeotic ABC factors (Ditta et al., 2004; Honma and Goto, 2001; Melzer et al., 2009; Pelaz et al., 2000).

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While the primary function of floral homeotic genes appears to be the specification of floral organ identity, several of them have additional roles. The most prominent example is the C class gene *AG*. In addition to specifying reproductive organs, stamens and carpels, it has a key role in limiting stem cell proliferation in the center of emerging flowers (Bowman et al., 1989; Bowman et al., 1991; Mizukami and Ma, 1995; Sieburth et al., 1995).

Although the molecular nature of *AG* was uncovered almost two decades ago (Yanofsky et al., 1990) our understanding of *AG* regulation is far from complete. The proper spatiotemporal expression of *AG* RNA is dependent on sequences located in the second intron, which is one of the longest found in the *A. thaliana* genome (Sieburth and Meyerowitz, 1997). Functional characterization of this intragenic enhancer demonstrated that multiple redundant regulatory modules mediate the response to competing activating and repressing inputs (Bomblies et al., 1999; Busch et al., 1999; Deyholos and Sieburth, 2000). One of the most prominent direct regulators is the meristem identity factor LEAFY (LFY) (Busch et al., 1999; Parcy et al., 2002; Parcy et al., 1998), which acts in concert with the homeodomain transcription factor WUSCHEL (WUS) to activate *AG* in the center of developing flowers (Lohmann et al., 2001). Additional inputs are provided by SEP3, which can act as activator (Castillejo et al., 2005) or repressor of *AG* transcription (Sridhar et al., 2006), depending on the regulatory environment. Once *AG* transcription has been activated, an autoregulatory mechanism is in place to ensure stable expression throughout flower development (Gomez-Mena et al., 2005).

Important repressors of *AG* expression are LEUNIG (LUG) and SEUSS (SEU), which act as co-repressors in higher order complexes with DNA-binding transcription factors, such as AP1, SEP3 and AGAMOUS-LIKE 24 (Franks et al., 2002; Gregis et al., 2006; Liu and Meyerowitz, 1995; Sridhar et al., 2006). In addition, *AG* expression is repressed in the outer whorls by the AP2 transcription factor (Drews et al., 1991), which in turn is under negative regulation by the microRNA miR172 (Aukerman and Sakai, 2003; Chen, 2004).

An additional layer of regulatory complexity is introduced by epigenetic silencing of the *AG* locus, which is mediated by trimethylation of lysine 27 on histone H3 proteins. It has been shown

that these modifications are dependent on a complex containing CURLY LEAF (CLF) and EMBRYONIC FLOWER 2 (EMF2), two members of the polycomb group protein family, as well as the plant-specific protein EMBRYONIC FLOWER 1 (EMF1) (Calonje et al., 2008). Consequently, mutations in the corresponding genes cause ectopic *AG* expression (Goodrich et al., 1997). The activity of the repressor complex is antagonized by ARABIDOPSIS HOMOLOG OF THRITHORAX 1 (ATX1) (Alvarez-Venegas et al., 2003).

As the proper spatiotemporal expression of *AG* involves a plethora of regulators, which act through redundant modules in the large intragenic enhancer, direct identification of important cis-regulatory elements has been difficult. Candidates for cis-regulatory motifs have instead been identified using phylogenetic footprinting and shadowing (Hong et al., 2003), although many of the corresponding trans-factors have remained unknown. Recent studies have shown that regulatory elements have been a driving force for the evolution of floral diversity (Causier et al., 2009). Here, we identify a new activator of *AG*, the bZIP factor PERIANTHIA (PAN), which was previously known to affect floral organ number (Chuang et al., 1999; Running and Meyerowitz, 1996), but not homeotic gene expression.

MATERIALS AND METHODS
Plant material
Descriptions of all plant lines used are given in Table 1. All experiments were carried out in the *KB14 AG::GUS* reporter in Col-0 background under long-day conditions, unless otherwise noted.

Expression analyses
GUS staining was performed as described (Lohmann et al., 2001). In situ hybridizations were performed in accordance with standard protocols (Weigel and Glazebrook, 2002) with the addition of 10% PVA to the staining solution. RNA probes were prepared from full-length cDNAs. Real-time RT-PCR analyses were performed on three replicates of independently grown plant material. RNA from a pool of primary inflorescences was prepared using RNeasy Plant Mini Kit (Qiagen), followed by cDNA synthesis with RevertAid H Minus First Strand cDNA

Synthesis Kit (Fermentas). PCR was carried out in the presence of SYBR Green using *BETA-TUBULIN-2* (At5G62690) for normalization. Primer sequences can be obtained upon request.

Plasmids
A list of all constructs is given in Table 2.

Cloning of plant binary vectors
For construction of RH146 the AAGAAT box was deleted from KB8 (Busch et al., 1999) and the 5′AG enhancer was cloned in forward orientation into pDW294 (Busch et al., 1999) by *Bam*HI/*Hin*dIII restriction sites. pSST210 was obtained using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) on KB8 following the manufacturer's protocol and subcloning of the 5′ enhancer into pDW294 using *Bam*HI/*Hin*dIII. Primer sequences can be obtained upon request.

Yeast one-hybrid screen
As bait for the yeast one-hybrid screen, the 33 bp AAGAAT box was trimerized using *Xho*I and *Sal*I sites, inserted in the *Xho*I site of pEMBLYi22 (Baldari and Cesareni, 1985) to yield pSST90, linearized and integrated into the *URA3* locus of the W303-A1a yeast strain (for detailed information see: http://wiki.yeastgenome.org/index.php/CommunityW303.html). Using standard protocols the screen was performed with a cDNA library constructed of RNA from young floral tissue in pEXPAD-502 (Wigge et al., 2005). Double transformants were selected on CSM-TRP/-HIS + 15 mM 3AT plates. Plasmids of positive clones were rescued, re-transformed and confirmed by X-Gal filter-lift-assay before sequencing. For independent confirmation, full-length cDNAs of candidate factors were cloned into pGEM-T easy (Promega) and subcloned into pEXPAD-502 (*TRP1*, Invitrogen). All were transformed stably into the yeast strain EGY48 (Golemis et al., 1996) that contained wild-type or mutated versions of the trimerized AAGAAT box upstream of the *lacZ* reporter in the vector KF1, a derivative of pLG718 (Guarente and Mason, 1983). pSST093 contained the wild-type AAGAAT box sequence, for pSST197 two nucleotides of the putative binding sites for bZIP were mutated [using the QuikChange Site-

Table 1. Plant lines used in this study

Plant line	Identity
ag-2	Mutation of *AG* (At4g18960) in Ws background
KB14	5′*AG::GUS* reporter (WT)
N510040	T-DNA insertion line for At4g13640 *UNE16*
N514183	T-DNA insertion line for At1g79430 *APL*
N518154	T-DNA insertion line for At3g12250 *TGA6*
N524632	T-DNA insertion line for At4g37180
N530112	T-DNA insertion line for At3g24120
N557570	T-DNA insertion line for At1g08320 *AtbZIP21*
N557609	T-DNA insertion line for At1g08320 *AtbZIP21*
N568444	T-DNA insertion line for At1g79430 *APL*
N577802	T-DNA insertion line for At4g37180
N591349	T-DNA insertion line for At1g08320 *AtbZIP21*
N613627	T-DNA insertion line for At4g13640 *UNE16*
N614420	T-DNA insertion line for At3g24120
N630355	T-DNA insertion line for At5g06960 *TGA5*
N641618	T-DNA insertion line for At1g08320 *AtbZIP21*
N642682	T-DNA insertion line for At3g24120
pan	T-DNA insertion line N557190
RH146	5′*AG::GUS* reporter (AAG box deleted in KB14)
SH57	*CLV3::WUS*
sSST196	5′*AG::GUS* reporter (mutated bZIP binding site in KB14)
sSST262	*pan KB14*
sSST36	*LFY::LFY:VP16* in *KB14*
sSST41	*LFY::LFY:VP16* in *RH146*

All lines are in a Columbia (Col-0) background (except where indicated).

Table 2. Plasmids used for yeast assays and preparation of RNA probes

| Plasmid	Identity
pMD105	pGEM-T easy *WUS*
pSH57	pFK317 *CLV3::WUS*
pSST090	pEMBLYi22 AAGAAT box trimer
pSST093	pKF1 AAGAAT box trimer
pSST094	pGEM-T easy At3g24120
pSST095	pGEM-T easy At3g12250 *TGA6*
pSST096	pGEM-T easy At4g37180
pSST097	pGEM-T easy At5g06960 *TGA5*
pSST099	p424_Gal1 At3g12250 *TGA6*
pSST100	p423_Gal1 At3g24120
pSST106	pEXPAD-502 At5g06960 *TGA5*
pSST108	pGEM-T easy At1g68640 *PAN*
pSST109	pEXPAD-502 At3g12250 *TGA6*
pSST110	p424Gal1 At5g06960 *TGA5*
pSST114	p423Gal1 At4g37180
pSST126	p424Gal1 At1g68640 *PAN*
pSST130	pGEM-T easy At1g79430 *APL*
pSST131	pGEM-T easy At1g08320 *AtbZIP21*
pSST137	p424Gal1 At1g08320 *AtbZIP21*
pSST152	pGEM-T easy At4g13640 *UNE16*
pSST155	p423Gal1 At4g13640 *UNE16*
pSST160	pEXPAD-502 At1g08320 *AtbZIP21*
pSST162	pEXPAD-502 At1g68640 *PAN*
pSST167	pEXPAD-502 At4g3718
pSST168	pEXPAD-502 At4g13640 *UNE16*
pSST169	pEXPAD-502 At3g24120
pSST170	pEXPAD-502 At1g79430 *APL*
pSST197	pKF1 mutated bZIP site in AAGAAT box
pSST209	pKF1 mutated GARP sites in AAGAAT box
pSST210	pART 5′*AG::GUS* mutated bZIP site in AAGAAT box
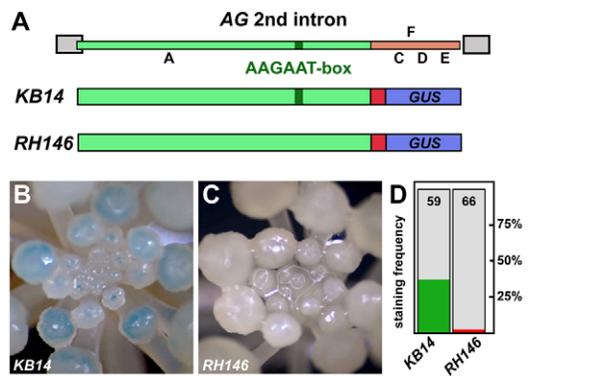
Fig. 1. Requirement of AAGAAT box for *AG* enhancer activity.
(**A**) Second *AG* intron. Flanking exons are indicated in gray. Conserved
DNA elements are annotated according to Hong et al. (Hong et al.,
2003). The 5′ enhancer (green) contains a potential LFY-binding site (A)
and the 33 bp AAGAAT box (dark green). The 3′ enhancer (orange)
contains two CArG boxes (C and E), and two linked CCAAT boxes (D).
In addition, the 3′ enhancer harbors two pairs of LFY/WUS-binding sites
(F). For the reporters, regulatory sequences were linked to a minimal
CaMV 35S promoter (red) and the coding region for bacterial β-
glucuronidase (GUS; blue). RH146 has a 33 bp deletion of the sequence
shown in Fig. 2A. (**B**) GUS activity in a primary inflorescence of a *KB14*
plant. (**C**) GUS expression was lost in *RH146*. (**D**) The distribution of
GUS-positive plants among primary transformants (*KB14*, *n*=59;
RH146, *n*=66).

Directed Mutagenesis Kit (Stratagene) following the manufacturer's
protocol] and the resulting ΔbZIP AAGAAT box trimer was inserted using
*Spe*I/*Xba*I sites into KF1. For pSST209 the same procedure was followed
by mutating two nucleotides in each of the putative GARP-binding sites as
described in Fig. 2A and inserting the fragment into KF1.

Transactivation assays
GARP and bZIPs transcription factor cDNAs were cloned in p423Gal1 and
p424Gal1 (Mumberg et al., 1994), respectively, and co-transformed into
yeast. Liquid cultures were assayed for β-galactosidase activity to quantify
transcriptional activation of the AAGAAT box reporter gene. Color reactions
were carried out using ONPG (o-nitrophenyl-beta-D-galactoside) as a
substrate and an ELISA reader. Relative reporter gene activity was
calculated according to Miller (Miller, 1972).

RESULTS
A highly conserved cis-regulatory element is essential for *AG* enhancer function
Phylogenetic footprinting and shadowing had previously been used
to identify conserved motifs in the second *AG* intron, which is
essential for proper transcriptional regulation of *AG* (Busch et al.,
1999; Deyholos and Sieburth, 2000; Hong et al., 2003). One of the
least variable sequences was the 33-bp-long AAGAAT box, which
is not an obvious candidate for a motif bound by known regulators
such as LFY, WUS or MADS domain factors (Fig. 1A). Recently,
Causier et al. (Causier et al., 2009) have shown that the AAGAAT
box has been conserved in sequence and relative position in
monocots and dicots, suggesting that it arose before the split of the
two lineages roughly 140 million years ago. The enhancer in the
second *AG* intron is composed of two redundant regions (Bomblies
et al., 1999; Busch et al., 1999; Deyholos and Sieburth, 2000), and
the AAGAAT box is located in the 5′ fragment defined by the *KB14*
reporter (Fig. 1A), which drives *GUS* reporter expression in the *AG*
domain (Fig. 1B) (Busch et al., 1999). The deletion of the AAGAAT
box in this context (*RH146*) caused a severe reduction of reporter
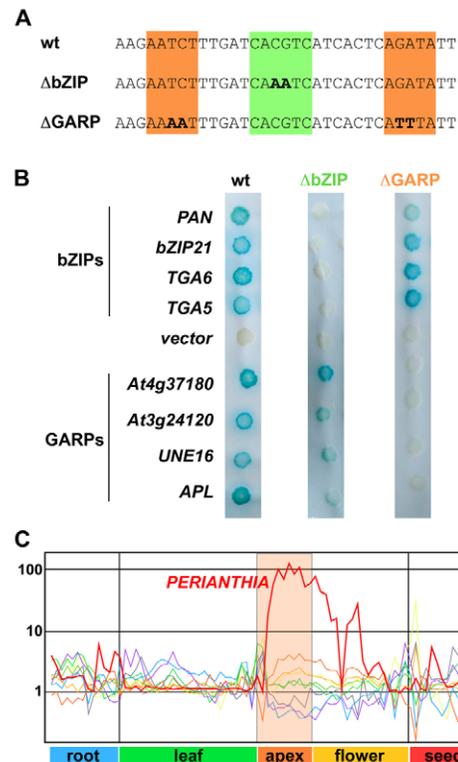

Fig. 2. Characterization of AAGAAT-interacting factors.
(**A**) Sequence of wild-type (wt) and mutated AAGAAT boxes. bZIP and
GARP consensus motifs are indicated in green and orange, respectively.
Mutated bases are in bold. (**B**) Yeast one-hybrid assays. The empty
pEXPAD-502 vector was used as control. (**C**) Global expression profiles
of candidate genes for AAGAAT-interacting factors. Mean normalized
expression data are from the AtGenExpress developmental series
(Schmid et al., 2005). Red, *PAN*; purple, *bZIP21*; light green, *TGA5*;
yellow, *TGA6*; light blue, *APL*; light brown, *UNE16*; orange,
At3g24120; dark blue, *At4g37180*; olive, *ARR2*.

gene expression (Fig. 1B-D), indicating that this element is essential
for activity of the 5′*AG* enhancer. The *AG* activator LFY acts
through DNA motifs contained in *KB14*, which are distinct from the
AAGAAT box. Thus, we crossed *KB14* and *RH146* to plants
expressing an activated form of LFY, *LFY:VP16* (Busch et al., 1999;
Parcy et al., 1998), to test the functionality of the *RH146* reporter.
We observed strong and ectopic GUS expression, confirming that
the regulatory input of other factors binding to the 5′*AG* enhancer is
not impaired in the *RH146* lines (see Fig. S1 in the supplementary
material).

Transcription factors of two classes bind to the AAGAAT box
To identify factors that act through the AAGAAT box, we performed
a yeast one-hybrid screen, using a cDNA fusion library prepared
from RNA of microscopically dissected inflorescence apices (Wigge
et al., 2005) and an AAGAAT box trimer as bait (pSST90). Two
independent screens of more than 2.5 million transformants
identified 50 positive clones, which contained inserts coding for
transcription factors of the GARP or bZIP families. We found four
different bZIPs, At1g08320/ATBZIP21, At1g68640/PERIANTHIA
(PAN), At3g12250/TGA6, and At5g06960/TGA5, and five GARP
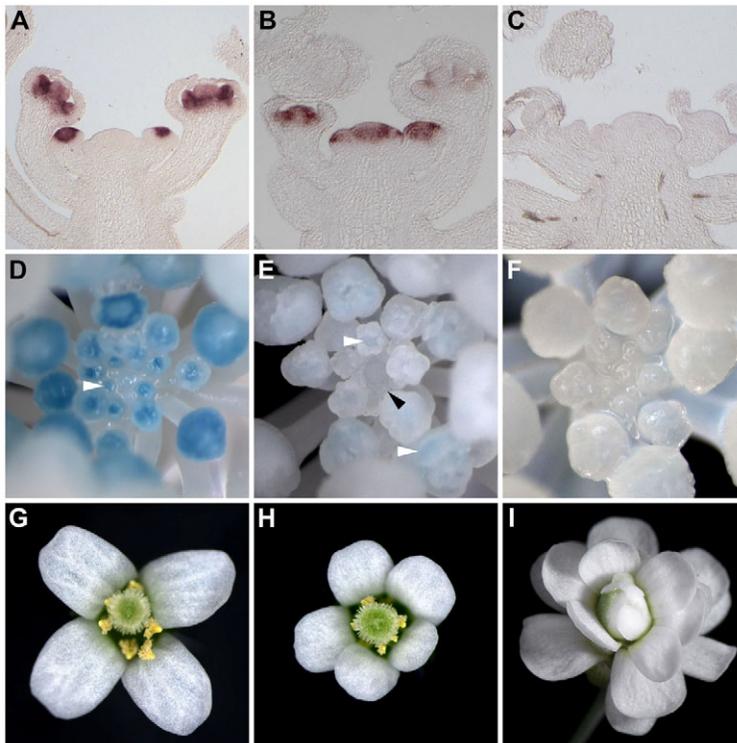factors, At1g79430/ALTERED PHLOEM DEVELOPMENT

Fig. 3. Expression, activity and phenotypes of *PAN* and *AG*. *AG* (**A**) and *PAN* (**B**) RNA expression in wild-type flowers. (**C**) *PAN* RNA was not detected in the *pan* mutant (Salk_057190 T-DNA insertion line). KB14 GUS expression in primary inflorescences of wild-type (**D**) and *pan* mutant (**E**) apices. White arrowheads point to flowers with AG::GUS activity; the black arrowhead indicates lack of staining in early *pan* flowers. (**F**) Point mutations in the bZIP-binding site abolished 5′*AG* enhancer activity. Floral morphologies of wild-type (**G**), *pan* mutant (**H**) and *ag* mutant (**I**) flowers grown in long-day conditions.

(APL), At3g24120, At4g13640/UNFERTILIZED EMBRYO SAC 16 (UNE16), At4g16110/ARABIDOPSIS RESPONSE REGULATOR 2 (ARR2), and At4g37180. Full-length cDNAs of all but ARR2 were fused with the GAL4 activation domain and interaction with the AAGAAT box was confirmed.

The AAGAAT box contains one consensus binding motif for bZIP factors (CACGTC) and two potential GARP-binding motifs (AATCT and AGATA) (Fig. 2A) (Foster et al., 1994; Hosoda et al., 2002). Within the AAGAAT box, these elements were almost invariant, even outside the Brassicacae (Hong et al., 2003; Causier et al., 2009), supporting the conserved functional importance of these sequences. To determine if the consensus motifs are indeed bona fide binding sites for the identified transcription factors, we selectively mutated them in the context of the yeast reporter construct. Mutations in the putative bZIP-binding site (ΔbZIP) (Fig. 2A,B) resulted specifically in the loss of response to the four bZIP factors. Conversely, mutations in the GARP motifs selectively interfered with reporter gene expression in response to the GARP transcription factors (ΔGARP) (Fig. 2A,B). These results demonstrated that the bZIP and GARP transcription factors identified in the one-hybrid screen bind in a sequence-specific manner to distinct motifs within the AAGAAT box.

The bZIP transcription factor PERIANTHIA acts on *AG* enhancer sequences in vivo

If any of the identified transcription factors were to play a role in the activation of *AG* transcription, one would expect overlapping expression in early flowers, where *AG* mRNA is first detected. To address this issue, we queried the AtGenExpress database (Schmid et al., 2005). From the nine candidates, only the bZIP transcription factor gene *PAN* showed strong and specific expression in apices and flowers (Fig. 2C) (Chuang et al., 1999), while the other mRNAs are expressed more uniformly across the various tissues. *pan* mutants have prominent defects in floral organ number (Fig. 3G,H) (Chuang

et al., 1999; Running and Meyerowitz, 1996), whereas T-DNA insertion mutants for the other factors identified in the one-hybrid screen did not have any floral phenotypes (see Table 1), suggesting that among the one-hybrid candidates only *PAN* has important roles in flower development, or that genetic redundancy is masking the function of the other regulators.

In situ hybridization confirmed a large overlap of *PAN* and *AG* expression domains in flowers (Fig. 3A,B). To investigate the importance of *PAN* for the activity of *AG* regulatory sequences, we introduced the *KB14* reporter into a *pan* T-DNA insertion mutant, in which *PAN* RNA expression was reduced below the levels detectable by in situ hybridization (Fig. 3B,C). 5′*AG::GUS* reporter activity was drastically reduced in early *pan* flowers (Fig. 3D,E; see Fig. S4 in the supplementary material), with some residual GUS activity at later stages of flower development (Fig. 3E, white arrowheads). These results demonstrated that *PAN* is required for activity of *AG* regulatory elements and were in agreement with *PAN* not being expressed beyond floral stage 7 (Fig. 3B).

A similar effect on *AG::GUS* expression as in the *pan* mutant background was observed when the bZIP-binding site was mutated in the context of the *KB14* reporter (Fig. 3F; see Fig. S4 in the supplementary material). In 18 of 25 primary inflorescences from T1 plants, GUS activity could not be detected at all, while the remaining seven apices showed only weak staining in young flowers. This was consistent with results of transactivation assays in yeast, which showed that PAN can synergistically activate transcription from the AAGAAT box in concert with a GARP transcription factor, At4g37180 (see Fig. S2 in the supplementary material), which is expressed in an overlapping domain with *PAN* mRNA (see Fig. S3 in the supplementary material). Interestingly, the closely related TGA proteins showed repressive activity in the same assay (see Fig. S2 in the supplementary material). Taken together, these results confirmed the relevance of *PAN* and the bZIP-binding motif for transcriptional activation mediated by *AG* enhancer sequences.
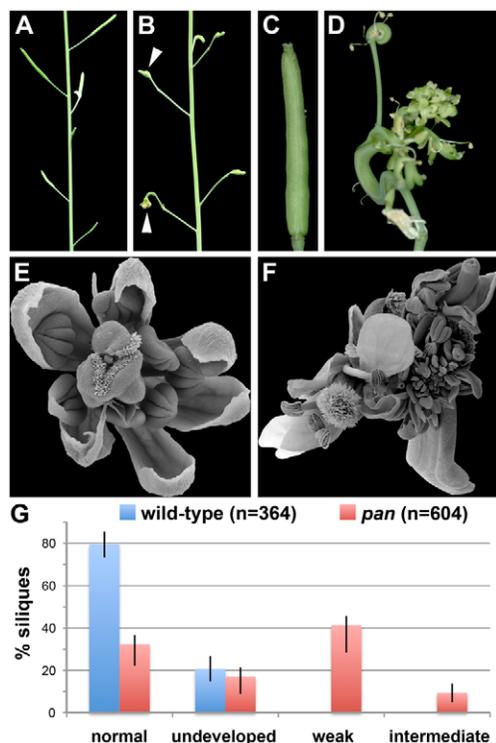
Fig. 4. Defects in *pan* mutant flowers from plants grown in short-day conditions. (**A-F**) Phenotypes of wild-type (A,C) and *pan* (B,D-F) flowers. Note determinacy defects in *pan* mutant (D and arrowheads in B). Scanning electron micrographs of weak (E) and intermediate (F) floral phenotypes of *pan* mutants. (**G**) Quantification of silique phenotypes (weak phenotype as in Fig. 4E and Fig. S5A in the supplementary material, intermediate as in Fig. 4F; see Figs S5B and S8 in the supplementary material). Fruits of the 'undeveloped' category were patterned normally, but did not set seeds.

Fig. 5. Expression of *AG* and *WUS* in *pan* mutant flowers grown in short-day conditions. (**A**,**B**) *AG* RNA in early-stage (stage 5) wild-type (A) and *pan* (B) flowers. (**C**,**D**) *AG* RNA in wild-type (C) and *pan* (D) flowers in intermediate stages (stage 11-12) of development. *AG* RNA accumulated ectopically in flowers of *pan* mutants (white arrowheads in D). The black arrowhead in D highlights the meristem-like structure. (**E**,**F**) Expression of *WUS* RNA in wild-type (E) and *pan* (F) flowers in intermediate stages of development. The arrowhead in F highlights the meristem-like structure with *WUS* expression.

Quantitative analyses by RT-PCR, however, did not indicate any reduction of endogenous *AG* RNA levels in *pan* mutants (data not shown), suggesting that other factors act redundantly with PAN. This is consistent both with *pan* mutants not having *ag*-like defects (Fig. 3G-I) (Running and Meyerowitz, 1996), and with the 5′ enhancer fragment (*KB14*) from the second *AG* intron acting redundantly with the non-overlapping 3′ enhancer fragment (Fig. 1A) (Busch et al., 1999; Deyholos and Sieburth, 2000).

PAN is essential for *AG* activation in early flowers of short-day-grown plants

In contrast to the *pan* mutant defects, which are restricted to flowers, PAN protein is much more widely expressed, indicating that PAN has redundant functions in several different regulatory networks (Chuang et al., 1999; Running and Meyerowitz, 1996). We speculated that genetic perturbations have so far not been able to fully expose *PAN* function, and we therefore tested whether variations in environmental conditions could be a means to elucidate the role of *PAN* in *AG* activation. Photoperiod has been shown before to affect the phenotypes of plants that are homozygous for a mutation in *AG*, or heterozygous for a mutation in the *AG* activator *LFY* (Okamuro et al., 1996).

Thus, we compared *pan* mutants and wild-type plants grown under short days (SD; 8 hours light), long days (LD; 16 hours light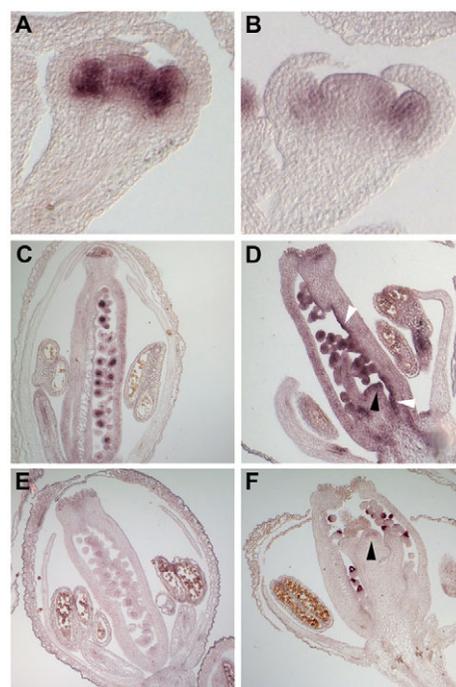) and continuous light (CL), all at 23°C. Whereas the floral defects of *pan* mutants grown under CL and LD were limited to the floral organ number defects known before, SD caused a loss of determinacy, reminiscent of *ag* loss-of-function defects (Fig. 4A-F). The phenotypes ranged from partially unfused carpels, which developed into deformed and bulged siliques, to fully unfused central organs that had carpeloid features and on which new floral meristems arose. These meristems in turn produced mostly petaloid, stamenoid and carpeloid tissues (Fig. 4D,F; see Fig. S5 in the supplementary material). These strong phenotypes were most apparent among the first ten flowers of the primary inflorescence. Later-arising flowers showed less severe defects consisting mainly of bulged carpels (Fig. 4B; see Fig. S5A in the supplementary material), which after dissection showed growth of floral organs in an aberrant fifth whorl. Together, these defects were detectable in roughly half of all fruits on the primary inflorescence (Fig. 4G). Similar defects were found in plants carrying other *pan* mutant alleles grown in SD (not shown) and plants expressing dominant-negative alleles of *PAN* in LD (Das et al., 2009). Whereas the reduction of KB14 activity in *pan* mutant inflorescences (see Fig. S6 in the supplementary material) was similar to that observed in LD-grown plants, SD-grown *pan* mutants showed in addition a marked reduction in *AG* mRNA expression in early flowers (Fig. 5A,B; see Fig. S7 in the supplementary material), which was in agreement with the phenotypic defects.

DEVELOPMENT

Fig. 6. Negative feedback between *PAN*, *AG* and *WUS*.
(**A**,**B**) Expression of *PAN* RNA in wild-type (A) and *ag* mutant (B) flowers.
The black arrowhead indicates declining *PAN* RNA levels in intermediate
floral stages of wild type. The white arrowhead in B points to strong,
persisting *PAN* expression in an *ag* mutant flower. (**C**) PAN RNA
CLV3::WUS plants (*SH57*). (**D**) Distribution of *AG* RNA in the same
CLV3::WUS flower as shown in C.

At later stages of flower development in SD-grown *pan* mutants
we detected ectopic expression of *AG* (Fig. 5C,D, white arrowheads)
and activation of the *KB14* reporter (see Fig. S8 in the supplementary
material), consistent with the ectopic formation of reproductive floral
organs (Fig. 4A-F; see Fig. S5C in the supplementary material). This
could be regarded as a consequence of losing proper early *AG*
activation in *pan* mutants and thus floral meristem determinacy.
Consistent with such a scenario, we found tissue outgrowths that
resembled floral meristems in shape and tissue layer organization
(Fig. 5D, black arrowhead). To determine the identity of these
structures more directly, we assayed expression of the stem-cell
regulator *WUS* (Mayer et al., 1998). In wild-type flowers, *WUS*
expression is terminated in an *AG*-dependent fashion around stage 6,
when patterning of the flower is accomplished (Lenhard et al., 2001;
Lohmann et al., 2001). In line with the observation of ectopic
meristems initiating inside the developing gynoecium of SD-grown
pan mutants, we observed *WUS* expression in these tissues (Fig. 5F,
black arrowhead; see Fig. S9A in the supplementary material).
Although during early stages *WUS* expression was rather diffuse in
the emerging meristems, once these meristems had firmly established
themselves, *WUS* mRNA was confined to the organizing center (see
Fig. S9B in the supplementary material). We conclude that ectopic
expression of *AG* and *WUS* at later stages of flower development
reflects indirect effects of compromised *PAN* activity during earlier
stages, and that redundant activators of *AG* are normally active
throughout flower development.

Taken together, our results show that *PAN* plays an essential role
in the activation of *AG* and that both genes are embedded in a
regulatory network that is sensitive to environmental conditions.

PAN and *AG* are engaged in a negative-feedback loop

The fact that *PAN* mRNA is not restricted to flowers but is also
highly expressed in proliferating cells of the shoot apical meristem
suggested more general functions of *PAN* in meristem maintenance.
A similar case is represented by *WUS*, which has dual roles in stem-

cell induction and floral patterning via *AG* activation. Consequently,
WUS expression and thus stem-cell maintenance is terminated
during flower development to allow for tissue differentiation, and
this process is dependent on *AG* activity (Lenhard et al., 2001;
Lohmann et al., 2001). Thus, we tested whether a similar feedback
interaction existed between *PAN* and *AG*. Consistent with such a
scenario, we found that strong *PAN* RNA expression persisted much
longer in *ag* mutant flowers than in wild type (Fig. 6A,B),
demonstrating that PAN is not only an essential activator of *AG*
during early floral stages, but that at the same time *PAN* expression
is under control of AG at later stages. As the *PAN* expression domain
in large parts overlaps with *WUS* RNA, and because *PAN* expression
also persists in *clavata* mutants (Chuang et al., 1999), in which *WUS*
is ectopically expressed, we investigated whether WUS activity
might mediate the feedback between AG and *PAN*. To this end, we
analyzed *PAN* RNA distribution in flowers ectopically expressing
WUS from the *CLAVATA3* (*CLV3*) promoter (Brand et al., 2002).
Flowers of plants with intermediate phenotypes develop
meristematic tissues from which stamenoid and carpeloid organs
arise, due to the activation of *AG* in these cells. In agreement with
the hypothesis that WUS mediates at least a good part of the
regulatory interaction between AG and *PAN*, we observed ectopic
PAN expression as well as accumulation of *AG* transcripts in WUS-
positive cells (Fig. 6C,D).

DISCUSSION
In this study, we have used a short, conserved enhancer motif as a
starting point for the identification of a new regulator of *AG*
expression, the bZIP transcription factor PAN. We have shown that
PAN not only has an important role in the control of perianth organ
number specification (Chuang et al., 1999; Running and
Meyerowitz, 1996), but also in the regulation of floral determinacy
through the direct activation of *AG*. The identification of *PAN*, which
is also expressed in shoot meristems (Running and Meyerowitz,
1996), as an *AG* activator supports the model that flower-specific
factors such as LFY interact with factors expressed in similar
patterns in both shoot and floral meristems to control region- and
flower-specific expression of floral homeotic genes (Lee et al., 1997;
Lenhard et al., 2001; Lohmann et al., 2001; Parcy et al., 1998).

PAN belongs to the D-group of bZIP transcription factors, which
share a high degree of sequence similarity in the bZIP region
(Jakoby et al., 2002). Intriguingly, all bZIP transcription factors
isolated in our screen fall into this group. Despite the fact that we
have recovered multiple clones for all factors identified in the
screen, we did not isolate all D-group members in our screen. As at
least *TGA2* and *TGA3* are expressed at substantial levels in
meristematic and floral tissue and therefore should have been
represented in our library, it is tempting to speculate that these
factors might have different DNA-binding specificities.

The findings that PAN can synergize in yeast with the GARP
transcription factor At4g37180 and that both share overlapping
expression domains in the SAM and early flowers suggest that
At4G37180 and related GARP transcription factors are co-factors
of PAN in *AG* regulation. Regulators of diverse molecular nature can
act together in a redundant fashion during flower development and
in particular during the activation of *AG*, as recently demonstrated
by Prunet et al. (Prunet et al., 2008). However, because the family
of GARP transcription factors is rather large (Riechmann et al.,
2000), and because we have found members of both GARP
subgroups (ARR-B and GARP-G2), it will be difficult to identify
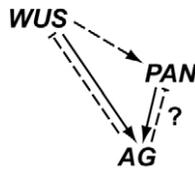and functionally test the most promising candidates for roles in *AG*
regulation.

Fig. 7. Regulatory interactions between *WUS*, *PAN* and *AG*. WUS activates *PAN* and *AG* expression in a feed-forward loop. Once AG protein has accumulated it suppresses *WUS* transcription, which in turn leads to decreased *PAN* activation. In addition, AG might act on *PAN* regulation in a *WUS*-independent manner. Solid lines indicate known direct interactions, and dashed lines denote hypothetical regulatory mechanisms.

Similar to what has been described for the stem-cell regulator *WUS* (Lenhard et al., 2001; Lohmann et al., 2001), PAN not only contributes to *AG* activation but in turn is under negative control by AG during later stages. The finding that WUS is able to ectopically activate *PAN* expression, along with the fact that *PAN* RNA also persists in *clv* mutants (Chuang et al., 1999), suggests that *WUS* could at least partially mediate this feedback regulation. In such a scenario, WUS at the same time contributes to the activation of *PAN* and *AG* in a feed-forward loop at early stages of flower development, while the repression of *WUS* by AG at later stages would also lead to a reduction of *PAN* expression (Fig. 7). The lack of *WUS* repression in *ag* mutants (Lenhard et al., 2001; Lohmann et al., 2001) would in turn cause ectopic activation of *PAN*. However, as only the regulatory interactions between WUS and *AG* and PAN and *AG* have been characterized mechanistically, the true nature of this regulatory module remains to be elucidated.

Finally, our work has revealed that other factors must act redundantly with *PAN* and that this network is sensitive to variation in environmental conditions. This finding shows that the combination of genetic perturbation with environmental variation can be a powerful tool to uncover redundant regulatory mechanisms that are normally not thought to be under environmental control.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/136/10/1613/DC1

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