MOLECULAR GENETICS OF FLORAL PATTERNING IN *ARABIDOPSIS THALIANA*

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Abstract

During development, pattern formation is an important process by which individual cells have their position specified and interpret this positional information by undergoing a particular cell differentiation. To better understand the mechanism specifying position, I have used *Arabidopsis thaliana* to study genes involved in floral patterning.

perianthia (*pan*) was recognized mainly by its extra organ mutant phenotype, in which flowers were transformed from tetramerous to largely pentamerous, a characteristic of flowers of ancestral plants. In addition to its function in establishing floral organ number, *PAN* is also involved in carpel development. *PAN* encodes a member of the basic region/leucine zipper class of transcription factors. In situ hybridization and immunolocalization revealed that its expression pattern is primarily correlated with floral organ initiation. The near absence of a phenotype in transgenic plants overexpressing *PAN* and the contrast between the broad expression of *PAN* and the specificity of its mutant phenotype suggest that its activity may be regulated post-translationally or by the presence of partner proteins. *PAN* was proposed to play a role in the switch from pentamerous to tetramerous flowers during the evolution of flower form in the mustard family.

A small gene family composed of three highly homologous genes, *TOT1*, *TOT2*, and *TOT3*, were isolated as potential targets of *PAN* by using differential display. These genes are up-regulated by *PAN* and encode products with similarity to a putative ABA-responsive protein. One way to elucidate the biological role of these potential target genes is to get their loss-of- function or reduction-of-function mutants. Double-strand RNA (dsRNA)-mediated gene silencing, which has been observed in a number of organisms, was successfully

applied to four *Arabidopsis* genes, *AGAMOUS*, *CLAVATA3*, *APETALA1*, and *PAN*, with well-characterized functions in flower development. Use of dsRNA interference with *TOT* genes demonstrated these target genes are involved in the same pathway as *PAN* to specify floral organ patterning. The discovery of *TOT* genes reveals a possible role of ABA signal transduction in determination of floral organ number and carpel development.

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Chapter 1

Introduction

Wild-type *Arabidopsis thaliana* plants have a small number of leaves and an indeterminate number of flowers. The growing tip of a plant shoot is a population of stem cells, referred to as the shoot apical meristem (SAM), that gives rise to all of the above ground parts (leaves, stems, and flowers) of the plant. The SAM forms leaf primordia during vegetative growth, and after the transition to flowering the SAM forms floral primordia that develop into flowers. *Arabidopsis* flowers initially arise as undifferentiated bulges on the flank of the apical meristem. Soon afterward, whorls of organ primordia arise sequentially from the outermost first whorl to the innermost fourth one. Most wild-type flowers have four sepals, four petals, six stamens, and two fused carpels (Smyth et al., 1990).

Flower development includes numerous events controlling flower initiation, floral organ identity and floral organ number. Multiple genes are involved in these events. Some of these genes, such as floral meristem identity genes and floral homeotic genes, have been implicated in the control of flower initiation and floral organ identity, respectively (Simpson et al., 1999; Weigel and Meyerowitz, 1994; Yanofsky, 1995). However, how appropriate floral organ number is determined is less understood. To address the last issue, my thesis has studied genes involved in this process. In this review, I will briefly introduce some basics concerning floral initiation and determination of floral organ identity. Then, I will introduce genes directly or indirectly affecting floral organ number and focus on studies of *PERIANTHIA* (*PAN*) (Chapter 2) and its targets, *TOTs* (Chapter 4). Finally, I will introduce RNA interference (RNAi) and its possible mechanism (Chapter 3).

Flower initiation and determination of floral organ identity

The first step of flower development is the switch of the meristem fate from vegetative to floral, which is primarily controlled by the floral meristem identity genes *LEAFY* (*LFY*), *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), *FRUITFULL* (*FUL*), *APETALA2* (*AP2*), and *UNUSUAL FLORAL ORGANS* (*UFO*) (Ferrandiz et al., 2000; Levin and Meyerowitz, 1995; Simpson et al., 1999; Wilkinson and Haughn, 1995; Yanofsky, 1995). Mutations in the floral meristem identity genes cause primordia that would normally develop into flowers to form shoot-like structures.

The floral meristem identity genes regulate a second set of genes, floral organ identity genes, which include *AP1*, *AP2*, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AG* (Bowman, 1994; Bowman et al., 1993; Bowman et al., 1989; Bowman et al., 1991; Hill and Lord, 1989; Irish and Sussex, 1990; Jack et al., 1992; Komaki et al., 1988; Kunst et al., 1989; Weigel and Meyerowitz, 1994). Except for *AP2*, the organ identity genes encode transcription factors of the MADS domain family and are expressed in region-specific patterns (Goto and Meyerowitz, 1994; Jack et al., 1992; Mandel et al., 1992; Yanofsky et al., 1990). In contrast, *AP2* encodes a member of the AP2 domain proteins and its expression is not limited to those floral whorls that are primarily affected in *ap2* mutants (Jofuku et al., 1994).

Molecular genetic studies of flower development have led to the ABC model of the determination of organ identity (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). In this model, three classes of genes, A, B, and C, were proposed to function either alone or in combination within three overlapping fields, each comprised of two adjacent whorls. In wild type, A alone determines the development of sepals, A and B that of petals, B and C that of stamens, and C alone that of carpels. Furthermore, A and C are mutually antagonistic, such that A represses the activity of C in whorls 1 and 2, and C represses the activity of A in whorls 3 and 4. Among the floral organ identity

genes identified to date, *AP1* and *AP2* belong to class A genes, *AP3* and *PI* are class B genes, and *AG* represents class C genes.

Determination of floral organ number

While much is known about the establishment of the floral organ identity, less is known about how the proper number of floral organs is determined. There are a number of reports demonstrating the existence of genes directly or indirectly controlling floral organ number. These genes include *CLAVATA1* (*CLV1*) (Clark et al., 1993; Clark et al., 1997), *CLAVATA2* (*CLV2*) (Jeong et al., 1999; Kayes and Clark, 1998), *CLAVATA3* (*CLV3*) (Clark et al., 1995; Fletcher et al., 1999), *ETTIN* (*ETT*) (Sessions et al., 1997; Sessions and Zambryski, 1995), *FASCIATA* (*FAS1*), *FASCIATA2* (*FAS2*) (Leyser and Furner, 1992), *FON1* (Huang and Ma, 1997), *MGOUN1* (*MGO1*), *MGOUN2* (*MGO2*) (Laufs et al., 1998), *PERIANTHIA* (*PAN*) (Chuang et al., 1999; Running and Meyerowitz, 1996), *PINHEAD* (*PNH*) (Lynn et al., 1999), *REVOLUTA* (*REV*) (Talbert et al., 1995), *TOUSLED* (*TSL*) (Roe et al., 1997; Roe et al., 1993), and *WIGGUM* (*WIG*) (Running et al., 1998).

Except for *PAN*, mutations in each of these genes also show phenotypes outside of the flower. For example, plants with mutations in the *CLV* and *WIG* genes have enlarged meristems and produce flowers with extra organs, suggesting that the increase in organ number is correlated with increased cell number in the floral meristem (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998; Running et al., 1998). *CLV1* encodes a leucine-rich repeat transmembrane receptor serine-threonine kinase (Clark et al., 1997); *CLV2*, a receptor-like protein (Jeong et al., 1999); and *CLV3*, a putative ligand for CLV1 (Fletcher et al., 1999). Furthermore, mutations in *CLV*, *FAS*, and *MGO* severely disrupt the apical meristem structure, leading to abnormal phyllotaxy and

bifurcation, flattening, and broadening of the stem (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998; Laufs et al., 1998; Leyser and Furner, 1992). Thus, most of these genes may play an indirect or non-specific role in determining floral organ number, such as by regulating the size or shape of meristems (Running and Meyerowitz, 1996).

Mutations in the *PAN* gene primarily cause extra organ phenotypes, such that most mutant flowers have five sepals, five petals, five stamens, and two carpels. *pan* mutant flowers do not show obvious carpel phenotypes, except for a delay in carpel fusion in some flowers (Running and Meyerowitz, 1996). However, *PAN* functions with other genes, such as *TSL*, *ETT*, *CRABS CLAW* (*CRC*), and *LUG* in gynoecium development (Chuang and Meyerowitz, 2000; Chuang et al., 1999; Roe et al., 1997; Sessions et al., 1997) (C.-F. Chuang and E. M. Meyerowitz unpub.). *PAN* encodes a putative basic region/leucine zipper (bZIP) transcription factor and its expression is mainly correlated with floral organ initiation and carpel development. That transgenic plants overexpressing *PAN* do not show noticeable phenotypes suggests the existence of spatially, or quantitatively limited factors that regulate PAN activity, for example by heterodimerization with PAN (Chuang et al., 1999). As ancestral plants of the mustard family, including *Arabidopsis*, had pentamerous flowers, it has been suggested that *PAN* may have been involved in the switch from pentamerous to tetramerous flowers during the evolution of flower form (Chuang et al., 1999; Running and Meyerowitz, 1996).

TOTs (for *Trio Targets*) were isolated by the differential display approach using *pan-2; 35S::PAN-GR* plants, which are transgenic *pan*-2 mutants containing a gene fusion between *PAN* and the hormone binding domain of a rat glucocorticoid receptor. The *TOT* gene family is composed of three closely related genes, *TOT1*, *TOT2*, and *TOT3*, which form a cluster on chromosome 5.

Each of these genes contains a small intronless coding region and is positively up-regulated by *PAN*. Northern blot analysis of poly(A) RNA from wild-type and *pan-2* flowers suggests that members of the *TOT* gene family are expressed at some levels and in some domains independently of *PAN*. PAN is similar to a class of plant bZIP proteins that bind the TGACGT element (Chuang et al., 1999). Two copies of such an element are found in the promoter regions of *TOT1* and *TOT2*, and one copy is located upstream of *TOT3*, suggesting that *TOTs* might be direct targets of *PAN*. Both up-regulation of *TOT* gene expression by *PAN* and reduction-of–function phenotypes in *crc-1; TOT2 (RNAi)* mutants suggest a role of *TOT* genes in the *PAN* pathway to control floral patterning.

RNAi

RNAi is a process through which double-stranded RNA (dsRNA) mediates gene silencing in a sequence-specific manner. The RNAi phenomenon has been observed in a number of organisms, including worms (Fire et al., 1998; Montgomery et al., 1998; Shi and Mello, 1998; Tabara et al., 1998; Timmons and Fire, 1998), plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998), flies (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999), trypanosomes (Ngo et al., 1998), planaria (Sanchez Alvarado and Newmark, 1999), hydra (Lohmann et al., 1999), zebrafish (Wargelius et al., 1999), and mice (Wianny and Zernicka-Goetz, 2000). In addition, RNAi seems to be related to previously reported posttranscriptional gene silencing (PTGS) phenomena, in which an endogenous gene is silenced by the introduction of a transgenic copy of the gene, such as cosuppression in plants (Baulcombe, 1999; Vaucheret et al., 1998; Voinnet et al., 1998) and quelling in the fungus *Neurospora* (Cogoni et al., 1996; Cogoni and Macino, 1997; Cogoni and Macino, 1999a; Cogoni and Macino,

1999b).

While dsRNAs are injected into most of these organisms to analyze RNAi effects that are unstable and transient, dsRNA-expressing constructs transformed into plants are stable and inheritable, thus, RNAi effects persist to, or recur in, new generations of transgenic plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998). That the severity of phenotypes in RNAi mutant plants varies between individuals is probably because of positional effects of particular DNA insertion events, but not the transgene copy number (Chuang and Meyerowitz, 2000). Furthermore, it had been suggested that RNAi occurs posttranscriptionally and may involve mRNA degradation (Chuang and Meyerowitz, 2000; Fire et al., 1998; Ketting et al., 1999; Montgomery et al., 1998; Ngo et al., 1998; Sanchez Alvarado and Newmark, 1999).

Genetic screens in plants, fungi, and worms have identified mutants deficient for cosuppression, quelling, and RNAi, respectively (Cogoni and Macino, 1997; Cogoni and Macino, 1999a; Cogoni and Macino, 1999b; Elmayan et al., 1998; Ketting et al., 1999; Tabara et al., 1999). *Arabidopsis* plants mutated in *SGS* genes, whose identity and function are still unknown, are specifically affected in transgene-induced posttranscriptional gene silencing (Elmayan et al., 1998). In *Neurospora*, *qde-1* and *qde-3*, which are required for quelling, encode genes related to RNA-dependent RNA polymerase and RecQ DNA helicase, respectively (Cogoni and Macino, 1999a; Cogoni and Macino, 1999b). These results suggest a possible role for RNA synthesis and DNA-DNA interaction in the gene silencing process.

In *C. elegans*, two classes of RNAi-deficient mutants have been recently studied intensively (Grishok et al., 2000; Ketting et al., 1999; Tabara et al., 1999). One class, comprised of *rde-1* and *rde-4*, is strongly resistant to RNAi but with no obvious defects in growth or development (Tabara et al., 1999). The predicted

gene product of *rde-1* is similar to those of *piwi* (Cox et al., 1998) and *sting* (Schmidt et al., 1999) from *Drosophila*, *ARGONAUTE* (Bohmert et al., 1998) and *PINHEAD/ZWILLE* (Lynn et al., 1999; Moussian et al., 1998) from *Arabidopsis*, and *elF2C* (Zou et al., 1998) from rabbit. The second class, which includes *rde-2*, *rde-3*, *mut-2*, and *mut-7*, is resistant to RNAi and exhibits transposon mobilization in the germ line, suggesting that the natural function of RNAi may be to silence multicopy sequences such as transposons, transgenes, and viruses by producing transcripts of each strand via a readthrough mechanism (Ketting et al., 1999). *mut-7* encodes a protein with homology to RNaseD, suggesting that RNAi works by dsRNA-directed, enzymatic RNA degradation.

Further genetic analysis revealed that *rde-1* and *rde-4* genes function at the first step to form the secondary interfering agent, whereas *rde-2* and *mut-7* function at the second step to maintain heritable RNAi, a phenomenon in which RNAi-induced gene silencing is established by injection of dsRNA into a worm, then inherited in the F2 and subsequent generations (Grishok et al., 2000; Sharp and Zamore, 2000). Recent findings strongly suggest that small RNAs are the best candidate for the secondary interfering agent. First, 25 nucleotide RNAs were recently found in silenced transgenic plants (Hamilton and Baulcombe, 1999). Second, a complex, which contains a nuclease activity that degrades a target mRNA *in vitro*, co-fractionates with a discrete, approximately 25-nucleotide RNA species (Hammond et al., 2000). Third, 21-23 nucleotide RNAs are generated from dsRNA added to *in vitro* RNAi reactions and the target mRNA is cleaved at sites 21-23 nucleotides apart (Zamore et al., 2000).

The current body of evidence favors a model in which RNAi begins with cleavage of the dsRNA to 21-23 nucleotide products by a dsRNA-specific nuclease, followed by dissociation of these short dsRNA by an ATP-dependent helicase and targeting of 21-23 nucleotide antisense RNAs to the mRNA for

cleavage (Zamore et al., 2000).

Overview of thesis

I will introduce cloning and expression analysis of the *PAN* gene whose mutations primarily affect floral organ number (Chapter 2), identification of a *TOT* gene family of three homologous genes as potential targets of *PAN* (Chapter 4), application of RNAi to alter expression of specific genes including *TOTs*, (Chapter 3 and 4), and isolation and functional analysis of a *PAN* homolog, *PAL1* (Appendix).

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Chapter 2

The *PERIANTHIA* gene encodes a bZIP protein involved in the determination of floral organ number in *Arabidopsis thaliana*

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Abstract

Mutations in the *PERIANTHIA* (*PAN*) gene of *Arabidopsis thaliana* specifically transform flowers from tetramerous to largely pentamerous, which is a characteristic of flowers of ancestral plants. We have cloned the *PAN* gene and here we show that it encodes a member of the basic region/leucine zipper class of transcription factors. Immunohistochemical analysis shows that the encoded protein is present in the apical meristem, the floral meristem, each whorl of organ primordia, and in ovule primordia during wild-type flower development. PAN expression occurs independently of genes affecting floral meristem identity, floral meristem size, or floral organ number. The near absence of a phenotype in transgenic plants overexpressing *PAN* and the contrast between the broad expression of PAN and the specificity of its mutant phenotype suggest that its activity may be regulated post-translationally or by the presence of partner proteins. Based on these results and on previously reported data, we propose models for the role of *PAN* in the evolution of flower pattern in the mustard family.

Introduction

Arabidopsis flowers initially arise as undifferentiated bulges on the flank of the apical meristem. Soon afterward, whorls of organ primordia arise sequentially. Four sepal primordia develop along the edges of the floral meristem, establishing the first whorl. Then four petal and six stamen primordia initiate in whorls 2 and 3, respectively, followed by development of two carpel primordia in the center of the floral meristem (Smyth et al. 1990).

A proposed mechanism of floral organ primordium initiation and formation must account for how a primordium is formed and also when and where on the floral meristem it develops. The eventual fate of organ primordia is determined by the organ identity genes (Bowman et al. 1989, 1991, 1993; Hill and Lord 1989; Irish and Sussex 1990; Jack et al. 1992; Komaki et al. 1988; Kunst et al. 1989; Weigel and Meyerowitz 1994); however, the position in which they arise (and thus their number) appears to be established independently (Meyerowitz 1997). How proper floral organ number is achieved is not well understood.

One way to understand the molecular mechanisms by which appropriate floral organ number is determined is to find mutations that affect floral organ number but do not affect floral organ identity or floral meristem identity, and then to elucidate the nature and the function of the corresponding wild-type genes. There are a number of reports demonstrating the existence of such mutations, including *clavata1* (*clv1*), *clavata2* (*clv2*), *clavata3* (*clv3*), *ettin* (*ett*), *fasciata1* (*fas1*), *fasciata2* (*fas2*), *mgoun1* (*mgo1*), *mgoun2* (*mgo2*) *perianthia* (*pan*), *revoluta* (*rev*), *tousled* (*tsl*), and *wiggum* (*wig*) (Clark et al. 1993, 1995, 1997; Kayes and Clark 1998; Laufs et al. 1998; Leyser and Furner 1992; Roe et al. 1993, 1997; Running and Meyerowitz 1996; Running et al. 1998; Sessions and Zambryski 1995; Sessions et al. 1997; Talbert et al. 1995). In *clv1*, *clv3*, and *wig* mutants, the

increase in organ number is correlated with increased cell number in the floral meristem (Clark et al. 1993, 1995; Running et al. 1998). This indicates a mechanism by which the positions of floral organs depend on spacing: the organs arise with a fixed distance between them in each whorl, and more organs arise in whorls containing more cells (Meyerowitz 1997).

pan was recognized mainly by its extra organ mutant phenotype and has been well characterized by phenotypic and genetic analysis. Flowers of plants mutant for *pan* are characterized by an increase in the organ number of the first two whorls, and a decrease in the organ number of the third whorl. Mutant flowers usually have five sepals with the position of the adaxial sepal as in wild type, five petals alternate with the sepals, five stamens alternate with the petals, and two carpels. Unlike the other mutations mentioned above, mutations in *PAN* specifically affect floral organ number, without detectable defects in floral meristem cell number or in other parts of plant (Running and Meyerowitz 1996). Thus, *PAN* seems to directly affect the spacing mechanism that determines the relative positions of floral organs (Meyerowitz 1997). Genetic analysis shows that *PAN* acts downstream of the floral meristem identity genes *LEAFY* (*LFY*), *APETALA1* (*AP1*), and *APETALA2* (*AP2*), but independently of the floral meristem size genes *CLV1*, *CLV3*, and *WIG* and the floral organ identity genes *APETALA3* (*AP3*), *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) in specifying early flower patterning (Running and Meyerowitz 1996; Running et al. 1998). In addition, *PAN* functions redundantly with *ETT* (Sessions et al. 1997) and *TSL* (Roe et al. 1997) in both organ primordium initiation and gynoecium development.

To gain insight into the molecular mechanisms underlying the specification of floral organ patterning in *Arabidopsis*, we have cloned the *PAN* gene and determined its pattern of expression in developing flowers. *PAN* encodes a putative basic region/leucine zipper (bZIP) transcription factor. PAN

protein is expressed in the apical meristem, the floral meristem, and each of the floral organ primordia. After each whorl of floral organs has been initiated, PAN expression becomes specific to particular regions in developing petals (the base), stamens (the adaxial surface), and carpels (the ovule primordia and mature ovules). Although *PAN* requires floral meristem identity genes for its activity (Running and Meyerowitz 1996), PAN expression is not dependent on these genes. PAN expression is also independent of the floral meristem size genes and other genes controlling floral organ number.

Results

Molecular cloning of PAN

We previously described the isolation of the *pan-1* and *pan-2* alleles from a screen of T-DNA insertion-mutagenized lines of ecotype Wassilewskija (Ws) (Fig. 1B; Feldmann 1992; Running and Meyerowitz 1996). The T_3 plants of one line (descended from a single transformed seed) segregated both *pan-1* and the Kan^R gene associated with the T-DNA, but tests of T_4 plants showed that the mutant phenotype was unlinked to Kan^R, suggesting that the *pan -1* mutation was not caused by a T-DNA insertion. The T_3 plants of another line segregated *pan-2*, but all plants in this family were also kanamycin resistant. Southern blot hybridization of genomic DNA from T_4 plants, using the T-DNA left and right border sequences as probes, revealed the presence of multiple T-DNA insertions in the genome of *pan-2* plants. Homozygous *pan-2* plants were backcrossed to wild type Landsberg *erecta* 5 times, and F_2 plants from the fifth backcross showed cosegregation of the *pan-2* phenotype with a single Kan^R marker. We made a λ genomic library from a homozygous *pan-2* F_3 family derived from the

fifth backcross, and screened this library with probes derived from the T-DNA left and right border. One clone from this library contained the T-DNA left border and plant genomic DNA, and another clone contained the T-DNA right border and plant genomic DNA. Restriction fragments containing both T-DNA and plant DNA were subcloned and used to probe a flower cDNA library (Weigel et al. 1992). We isolated a single cDNA (of 1324 base pairs in length) out of approximately 1 million clones, the sequence of which corresponded to genomic sequences flanking both sides of the T-DNA insert. A genomic clone was isolated by screening a wild-type *Arabidopsis* genome library, using the cDNA as a probe. The cDNA was mapped to a 7.2 kb *Xba* I fragment and a 12 kb *Pvu* II fragment of the genomic clone (Fig. 2A).

To verify that the cloned sequences encode the *PAN* gene, we transformed these two genomic fragments into *pan-1* mutant plants. Flowers from transgenic plants carrying either of the *PAN* genomic fragments are indistinguishable from those of wild-type plants (Fig. 1A, C). These results (and sequencing results discussed below) demonstrate that the cloned gene corresponds to *PAN*.

PAN is a bZIP protein with C-terminal glutamine-rich regions

The 1.3 kb cDNA, which contained a 3' poly(A)$^+$ tail, identified a 1.7 kb mRNA by RNA blot analysis (data not shown), suggesting that the cDNA is not full length and is missing 5' sequences. The transcriptional start site was identified by rapid amplification of cDNA ends (5' RACE; Frohman et al. 1988). An in-frame stop codon precedes the first ATG in the longest open reading frame, suggesting that the protein coding sequence is complete (Fig. 2B). The full-length *PAN* cDNA is 1686 bp and encodes a protein of 451 amino acids. Comparison of these

sequences to the GenBank database revealed that the deduced amino acid sequences were similar to members of the bZIP (basic region-leucine zipper) class of transcription factors.

Genomic DNA blot analysis suggests the presence of a single copy of the *PAN* gene in the *Arabidopsis* genome (data not shown). Alignment of the cDNA and genomic DNA sequences indicated that the *PAN* gene is composed of 11 exons (Fig. 2A,B). All exon-intron junctions conform to the GT-AG rule. The intron sizes range from 68 to 96 bp except that the fourth intron is 436 bp, the exon sizes range from 47 to 330 bp. The basic region and leucine zipper are encoded by separate small exons, as in other plant bZIP encoding genes (Fig. 2B). The PAN protein also contains two glutamine-rich regions (~30% glutamine residues) near the C terminus: 9/29 glutamine residues at amino acids 333-361 and 6/21 glutamine residues at amino acids 403-423, which may function as transcriptional activation domains (Fig. 2B,3; Courey and Tjian 1988).

Sequence of pan mutations

The *pan-3* to *pan-6* alleles were isolated from a screen for *crc* (*crabs claw*) enhancers in ecotype Landsberg *erecta* (Y. Eshed and J.L. Bowman, unpub.). To identify mutations in the *pan* alleles and to confirm that we had identified the *PAN* gene, we used PCR to amplify genomic DNA or cDNA from each of the six *pan* alleles and directly sequenced these PCR products. The allele *pan-1* contains a single base pair insertion at codon 91, which results in a frameshift at this position (Fig. 2A,B). The *pan-2* allele has a T-DNA insertion in the ninth exon (Fig. 2A,B), resulting in the deletion of a 23 bp fragment immediately after the insertion site. The *pan-3* allele is a G to A substitution at the splice donor site of the ninth intron (Fig. 2A,B). Sequence analysis of RT-PCR products revealed

three improperly spliced forms of *pan-3* mRNA, all encoding truncated proteins lacking the glutamine-rich domains. The point mutation found in the *pan-4* allele results in a premature stop codon within the glutamine-rich domain I and therefore is expected to eliminate the glutamine-rich domain II. In *pan-5*, there is a single nucleotide change (G to A) at the splice acceptor site of the eighth intron and the first nucleotide (G) is used as an alternative splice acceptor site, resulting in a frameshift and a premature stop codon after the basic region and leucine zipper. The allele *pan-6* has a premature stop codon caused by a nucleotide change in the leucine zipper region. Identification of mutations in these *pan* alleles, and complementation of the *pan-1* mutant phenotype by the cloned gene confirm that we have isolated the *PAN* gene.

Structural similarity between PAN and other plant bZIP proteins

The basic region/leucine zipper motif and the glutamine-rich regions are highly conserved between PAN and other plant bZIP proteins, including the *Arabidopsis* protein TGA1 (Schindler et al. 1992a), the wheat protein HBP-1b (Tabata et al. 1991), the maize proteins OBF 3.1 and OBF 3.2 (Foley et al. 1993), and the tobacco protein TGA1a (Katagiri et al. 1989), all of which have been shown to interact with the TGACGT/C motif identified in viral and plant promoters (Fig. 3). These proteins have 100% amino acid identity with PAN within the basic region which is responsible for binding DNA. In contrast, the DNA binding domain of PAN is more divergent from the *Arabidopsis* bZIP proteins GBF1, GBF2, and GBF3 (Schindler et al. 1992b; Fig. 3), all of which interact with the G-box (CCACGTGG). These G-box binding proteins contain a leucine zipper consisting of five or six leucines instead of just three leucines as in PAN and the TGACGT/C binding proteins (Fig. 3). In addition, the G-box

binding proteins do not have the glutamine-rich regions.

Expression of PAN RNA and subcellular localization of PAN protein in the apical meristems and organ primordia

To investigate the spatial and temporal expression pattern, as well as the subcellular localization of the PAN protein, immunohistochemical analysis using a PAN-specific polyclonal antibody was performed. During vegetative growth, PAN protein is first detected in the shoot apical meristem of the mature embryo (Fig. 4A) and is localized to the nucleus (Fig. 4B), consistent with the indication that PAN is a potential transcription factor. PAN expression extends from the apical meristem to young leaf primordia in the seedling (Fig. 4C). After the transition from vegetative growth to flowering, PAN protein is expressed at high levels throughout the apical region of the inflorescence meristem (Fig. 4D,E), and PAN remains strongly expressed in the floral meristem in stage 1 and 2 floral buds (Fig. 4D,E; stages as defined in Smyth et al. 1990). At stage 3, when sepals initiate, PAN protein is confined to regions of the floral meristem interior to the sepals, where further organ initiation events are taking place (Fig. 4D,E). PAN protein is still detected in the inner three whorls of organ primordia in late stage 4 and 5 flowers (Fig. 4D,E).

At stage 6, when each whorl of floral organ primordia has initiated, expression of PAN becomes spatially restricted to specific regions within developing petals, stamens, and carpels. During stages 6-8, PAN is expressed weakly in the bases of the emerging petal primordia, whereas expression is strong in the adaxial half of stamens and the cells lining the central invagination (Fig. 4F). PAN protein is restricted to the ovule primordia and the parietal placental tissue during stages 9-11 (Fig. 4G,H). Late in floral development, at

stage 12, PAN expression becomes weaker in ovules and placenta (Fig. 4I). By contrast, only low background is detected in control experiments using the preimmune serum as the primary antibody (data not shown). PAN is not detected in roots, stems, and leaves (data not shown). The PAN protein is not detected above background in *pan-1* (Fig. 4J) and *pan-2* (data not shown) mutants, suggesting that these are null or severe reduction of function alleles, and demonstrating the specificity of the antibody.

In situ hybridization experiments showed that *PAN* RNA is present in the inflorescence meristem, the floral meristem (Fig. 4K), and in developing petals, stamens, and carpels (data not shown) in the same spatial and temporal pattern as that of PAN protein.

Localization of PAN in lfy, ap1, and ap2 mutants

Double mutant analyses demonstrate that the floral organ identity mutants *lfy*, *ap1*, and *ap2* are epistatic to *pan*, suggesting that *PAN* acts downstream of these genes (Running and Meyerowitz 1996). To determine whether *PAN* expression is regulated by these upstream genes, we analyzed the expression of PAN protein in *lfy*, *ap1*, and *ap2* mutant flowers.

Mutations in the *LFY* gene cause partial transformation of flowers into inflorescence shoots (Weigel et al. 1992). Secondary flowers occasionally arise from the axils of the outer organs in strong *lfy* flowers. In *lfy-6* mutants, PAN protein localization is largely normal in the inflorescence meristem and the floral primordia of primary (Fig. 5A) and secondary flowers (Fig. 5B).

Similarly to *lfy*, mutations in *AP1* result in the development of secondary flowers in the axils of the first whorl organs, indicating a partial conversion of a floral meristem into an inflorescence meristem (Irish and Sussex 1990). In *ap1-1*

mutants, PAN expression is largely normal in the mutant inflorescence meristem, abnormal floral primordia (Fig. 5C), and ovule primordia (Fig. 5D). In addition, PAN is also expressed in the secondary floral buds (Fig. 5E).

Mutations in *AP2* usually cause homeotic transformation of floral organs in the first and second whorls. In strong *ap2* mutants, sepals are transformed into carpels, petal development is suppressed, and stamens are reduced in number (Bowman et al. 1991). In *ap2-2* mutants, PAN expression is largely normal in the inflorescence meristem, young floral primordia (Fig. 5F), and ovule primordia in the fourth whorl carpels (Fig. 5F,G). PAN is also expressed in the ovule-bearing carpels in the first whorl (Fig. 5F,G), indicating that PAN expression in the carpels is cell-type specific, and not whorl specific.

We conclude from these results that the spatial and temporal expression pattern, as well as the expression level of PAN protein are largely normal in *lfy*, *ap1*, and *ap2* mutants, suggesting that *LFY*, *AP1*, and *AP2* protein functions are not required for PAN expression during flower development.

Expression of PAN in clv1-4, clv3-2, ett-3, wig-1 and tsl-1

To determine whether *PAN* expression patterns are altered in other mutants with abnormal floral organ number but with normal floral organ identity, we examined the PAN protein level in mutant flowers. Mutations in *CLV1*, *CLV3*, *ETT* and *WIG* increase organ number within the flower, whereas mutations in *TSL* decrease organ number (Clark et al. 1993, 1995, 1997; Roe et al. 1993, 1997; Running et al. 1998; Sessions and Zambryski 1995; Sessions et al. 1997). Plants carrying a mutation in either of the *CLV1* or *CLV3* genes also produce flowers with additional whorls of carpels in the center of flowers. PAN protein is expressed normally in the inflorescence meristem and the floral meristem in

these mutants (Fig. 6A,C-F). In addition, PAN is also expressed in the fifth whorl of *clv1-4* (Fig. 6B) and *clv3-2* flowers (data not shown).

Ectopic expression of PAN

To examine the effect of ectopic PAN expression on flower development, we constructed transgenic lines that constitutively express *PAN*. To select a functional transgene, the *PAN* cDNA driven by the constitutive 35S promoter from cauliflower mosaic virus was transformed into *pan-1* plants and the rescued plants were crossed to wild-type plants. The resultant F_2 plants which contained the *35S::PAN* transgene, but did not contain the *pan-1* allele, were analyzed.

Western blot analysis of plant proteins from flower tissues showed that the expression of PAN protein was increased in *35::PAN* flowers compared to wild-type (data not shown). Immunolocalization assays on sections of flower tissues further demonstrated that the PAN protein was expressed uniformly throughout the flower in the *35S::PAN* transgenic plants (data not shown). The *35S::PAN* transgene, which can rescue the mutant phenotype in *pan-1* plants, does not cause any noticeable phenotype with respect to floral organ number in wild-type plants (data not shown).

Discussion

Arabidopsis flowers consist of four concentric whorls of organs. Most wild-type flowers have four sepals, four petals, six stamens, and two fused carpels, from the outermost first whorl to the innermost fourth whorl. While much is known about the establishment of floral organ identity, less is known about how the proper number of floral organs is achieved. Recently we identified one mutant,

pan, specifically affecting floral organ number (Running and Meyerowitz 1996). Here we describe the isolation of the *PAN* gene. Two lines of evidence demonstrate that we have cloned the *PAN* gene: 1) Mutations were found in the six *pan* alleles. 2) Two different genome fragments containing the cloned gene can complement the *pan* mutant phenotype.

PAN encodes a bZIP protein

PAN shares homology with members of the bZIP class of transcription factors. The basic region is implicated in DNA binding and the leucine zipper mediates homo- and heterodimerization. Several plant bZIP proteins have been shown to bind the ACGT *cis*-acting element identified in the promoters of bacterial, viral, and plant genes (Mikami et al. 1987; Giuliano et al. 1988; Bouchez et al. 1989; Lam et al. 1989; Schindler et al. 1992a, 1992b). Based on their DNA binding specificity and heterodimerization characteristics, these plant bZIP proteins can be classified into at least two classes: the G-box (CCACGTGG) binding factor (GBF) family and the TGACGT/C binding proteins (Foster et al. 1994; Schindler et al. 1992a). Proteins from each class are also distinct in their overall protein structure. Members of the GBF family are characterized by an N-terminal proline-rich region and a bZIP region at the C-terminus (Schindler et al. 1992b). In contrast, proteins of the second class, including the *Arabidopsis* protein TGA1 (Schindler et al. 1992a), the tobacco protein TGA1a (Katagiri et al. 1989), the wheat protein HBP-1b (Tabata et al. 1991), the maize proteins OBF 3.1 and OBF 3.2 (Foley et al. 1993), contain an N-terminal bZIP domain and a C-terminus enriched in glutamine and acidic amino acids. Sequence alignment analysis revealed that PAN is more similar to members of the second class of bZIP proteins than it is to members of the GBF family (Fig. 3). However, there is a 118 amino acid domain

at the N-terminus of PAN that shows no similarity to previously cloned genes.

PAN expression pattern is correlated with floral organ initiation

Immunohistochemical analysis revealed that PAN protein is localized in both floral tissues and vegetative tissues including the apical meristems during vegetative and reproductive growth, the young leaf primordia, four whorls of floral organ primordia, and developing petals, stamens, and ovules. In situ hybridization showed the same spatial and temporal pattern as that of PAN protein.

Mutations in the *PAN* gene only affect the floral organ number in the first three whorls. However, expression domains of PAN protein in wild-type plants are not restricted to the tissues which are affected in *pan* flowers. There are two possibilities to explain why some tissues expressing PAN are not affected in *pan* plants. PAN may be functionally redundant with other factors in some tissues, or PAN does not participate in vegetative growth and apical meristem development. PAN expression in the gynoecium may explain a delay in carpel fusion in some *pan* flowers (Running and Meyerowitz 1996), and is consistent with the previous findings that *PAN* functions redundantly with *TSL* (Roe et al. 1997), *ETT* (Sessions et al. 1997), and *CRC* (Y. Eshed and J. L. Bowman, unpub.) in gynoecium development.

The PAN protein expression pattern is largely normal in mutants affected in meristem identity (*LFY*, *AP1* and *AP2*), and in organ number without changes in organ identity (*CLV1*, *CLV3*, *ETT*, *WIG*, and *TSL*). We conclude from these results that the activity of these genes is not required for PAN expression.

Implications from PAN ectopic expression

Overexpression of *PAN* can rescue the phenotype of *pan* mutant plants but does not confer any phenotype in wild-type plants. This result suggests the existence of spatially and/or quantitatively limited factors which modulate PAN protein activity, or heterodimerize with PAN; thus, overexpression of *PAN* alone is not enough to cause a dominant phenotype. PAN functions downstream of the meristem identity genes *LFY*, *AP1*, and *AP2*. However, PAN expression is not altered in *lfy*, *ap1*, and *ap2* mutants and PAN is expressed in a wider domain than *LFY* and *AP1*. Therefore, *LFY*, *AP1*, and *AP2* may activate a spatially limited partner of PAN.

It is possible that the activity of PAN is regulated post-translationally. Much evidence suggests that phosphorylation of transcription factors can affect their DNA binding properties (Prywes et al. 1988; Yamamoto et al. 1988), or their ability to activate transcription in animal systems (Cherry et al. 1988; Tanaka and Herr 1990; Weiss et al. 1991). In plants, it has been shown that the phosphorylation of an *Arabidopsis* leucine zipper protein, GBF1, by casein kinase II from broccoli stimulates its binding to the specific DNA site (Klimczak et al. 1992). Western blot analysis revealed the existence of two forms of PAN protein (data not shown). One may represent a phosphorylated form of PAN, supporting this hypothesis. It is also possible that PAN needs to function with other factors in a complex. Protein/DNA binding studies using cotranslated bZIP templates have shown that *Arabidopsis* proteins GBF1,2,3 (Schindler et al. 1992b), maize proteins OPH1 and Opaque2 (Pysh et al. 1993), and parsley proteins CPRF1, 2, 3 (Armstrong et al. 1992) were able to form heterodimers.

Possible models for PAN in regulation of floral organ number

By analogy to a morphogen theory of leaf primordium positioning, the position in which the floral organ primordia arise could be controlled by inhibitory and promoting types of mechanisms. In this model, an inhibitor of adjacent organ inception is produced at the position of initiation of each organ. The inhibitor decreases in effectiveness with increasing age of the primordium, meaning that the inhibitor decays with time. New organ primordia will arise at concentration minima in the inhibitory field. However, the different floral whorls seem to have a promoting rather than an inhibitory effect on each other's positioning (Lyndon, 1990).

Flowers of the family Brassicaceae (mustards), including *Arabidopsis*, are tetramerous. Interestingly, flowers of *pan* mutant plants become pentamerous, which is characteristic of flowers of ancestral plants such as members of the family Capparaceae (Meyerowitz et al. 1997). This suggests that *PAN* may be involved in the switch from pentamerous to tetramerous flowers during the evolution of flower form in the mustard family (Fig. 7A). We propose that the tetramerous flower of the mustard family was established by introducing the *PAN* gene into the pre-existing inhibitory pathway mentioned above.

We propose three possible ways by which *PAN* may participate in the inhibitory pathway to control floral organ patterning. First, *PAN* might facilitate inhibitor diffusion, thereby allowing the inhibitor to spread more widely (Fig. 7B), thus, the second organ primordium in the first or second whorl can form at the position opposite of the first one (Fig. 7A, right). The inhibitory fields created by the first and the second primordia combine to determine the position of lateral primordia. When *PAN* activity is reduced or eliminated, the inhibitor reaches a concentration that allows organ formation at a position closer to the first primordium due to reduced concentration and normal decay (Fig. 7B), resulting in two primordia formed in the first inhibitory field (Fig. 7A, left).

Similarly, the first three inhibitory fields combine to determine the positioning of the remaining organs (Fig. 7A, left). Second, *PAN* could enhance reception of the inhibitor. In this scenario, loss of *PAN* activity decreases the sensitivity of cells to the inhibitor, allowing primordia to form at a higher inhibitor concentration and thus narrower spacing of organ primordia (Fig. 7A,C). Third, *PAN* might activate inhibitor production, indirectly leading to its wider diffusion (Fig. 7A,D).

Expression of *PAN* throughout the floral meristem supports the first and second models in which *PAN* functions in the whole meristem to regulate its response to the inhibitor. However, we cannot rule out the possibility that PAN could interact with spatially limited factors to regulate localized expression of the diffusible inhibitor. In any case, introduction of *PAN* activity to the morphogen model leads to wider spacing of organ primordia and thus fewer organs in the first two whorls. The increase in organ number in the third whorl is perhaps explained by duplication of the medial stamens, resulting in six instead of four third-whorl organs (Bowman 1994). Loss of *PAN* function would cause a flower form similar to that of ancestral plants. Future experiments expressing *PAN* in relatives of ancestral plants should help to test these models.

Materials and methods

Cloning of PAN

Genomic DNA was isolated from the five times-backcrossed *pan-2* line, partially digested with *Sau*3A I, then partially filled in and ligated to *Xho* I half-site arms of λ GEM11 (Promega), and packaged in vitro. The library was then screened using the T-DNA left and right borders as probes.

We screened approximately $1x10^6$ clones of an *Arabidopsis* flower cDNA

library in lambda ZAPII made from wild type Landsberg *erecta* ecotype with mixed probes containing left and right border T-DNA and the flanking plant genomic DNA. We isolated a single clone, which was converted to a plasmid by in vivo excision according to manufacturer's instructions (Stratagene). Remaining sequences (residues 1-556) of the full-length *PAN* cDNA were determined by 5' RACE System (BRL) using total RNA from floral buds of Wassilewskija ecotype. A λ phage genomic library made from wild-type Columbia ecotype of *Arabidopsis* (Hua et al. 1995) was screened at high stringency (65°C hybridization and a final wash with 0.1X SSPE) with a 1353 bp PCR fragment (residues 174-1526) of *PAN* cDNA as a probe.

DNA sequencing

The cDNA and genomic DNA fragments were subcloned into pBluescript SK(+) (Stratagene), pGEM3Zf(+) (Promega) and pUC19 vectors for sequencing. Sequencing was done using the Sequenase Kit (U.S. Biochemical) or the dye terminator cycle sequencing kit (The Perkin-Elmer corporation) according to the protocols provided. Both strands were sequenced.

Growth of plants

Plants were grown in a 4:3:2 or 1:1:1 mixture of soil:vermiculite:perlite in 3x5- in pots placed in plastic trays, imbibed at 4°C for four days, then maintained at 20°C beneath 600 foot-candles of constant cool-white fluorescent light. Plants were fertilized at regular intervals.

Complementation constructs and Agrobacterium-mediated transformation

The 7.2 kb *Xba* I and 12 kb *Pvu* II genomic fragments, which contain the *PAN* gene, were subcloned into the *Bam*H I site of the T-DNA expression vector pDHB321.1. These T-DNA constructs were transformed into the *Agrobacterium* strain C58C1(pMP90). *Agrobacterium* carrying either of the *PAN* genomic fragments was then used to transform plants by vacuum infiltration (Bechtold et al. 1993). Transformants were selected by sowing seeds on sand sub-irrigated with water containing 7.5 mg/liter of Basta herbicide. Basta-resistant plants were then transferred to soil.

Production of PAN-specific polyclonal antiserum

The PAN protein was translationally fused to a series of six histidine residues which allows simple purification of recombinant proteins by immobilized metal affinity chromatography (Hochuli et al. 1987). To make this construct, *PAN* cDNA encoding the full-length PAN protein was cloned into the expression vector pRSETA (Invitrogen) using *Bam*H I and *Kpn* I restriction sites. This plasmid was transformed into *E. coli* strain BLR(DE3), and upon induction with 0.8 mM IPTG for 3 hr at 37°C, significant levels of *PAN* fusion protein were produced. The protein was partially purified using the Ni-NTA resin (QIAGEN), and used to immunize rabbits. The titre of antiserum was determined by Western blot analysis. The specificity of the antibody was demonstrated by control experiments that showed no immunolocalization signal in *pan-1* and *pan-2* mutants.

Immunolocalization

Tissue was fixed in 1xPBS containing 4% paraformaldehyde, 0.1% Triton-X-100, and 0.1% Tween-20 at 4°C overnight. Fixed tissue was dehydrated with ethanol, cleared with histoclear (National Diagnotics), embedded in paraffin (Paraplast Plus), and sectioned at 8 um. Antibody reactions and staining were done using the Blocking kit, the VECTASTAIN Elite ABC kit and the DAB substrate kit (Vector Laboratories) according to the protocols provided.

In situ hybridization

A 306-bp fragment of the *PAN* cDNA (bases 190-495), which contained sequences not closely homologous to previously reported sequences on database searches, was amplified by PCR and cloned into the TA cloning vector (Invitrogen). In vitro transcription and ^{35}S labeling of probes was performed using the Promega Riboprobe kit, with template linearized by *Bam*H I and transcribed by T7 polymerase (sense probe), or linearized by *Eco*R V and transcribed by SP6 polymerase (antisense probe). In situ hybridization was performed as described by Drews et al. (1991), with modifications by Sakai et al. (1995). Tissue was sectioned at 5.5 um thickness. Exposure time was 5-7 weeks.

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Note

The nucleotide sequence data of *PAN* reported in this paper will appear in the EMBL, GenBank, and DDBJ databases under accession number AF111711.

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Figure 1. (A) Wild-type flower. (B) *pan-1* flower. (C) *pan-1* flower with a 7.2-kb *Xba* I genomic fragment containing the *PAN* gene. A, Wild-type flowers have four sepals, four petals, six stamens, and two carpels. B, Most *pan* mutant flowers have five sepals, five petals, five stamens, and two carpels. C. *pan-1* plants carrying the transgenic wild-type *PAN* gene produce flowers indistinguishable from wild type.

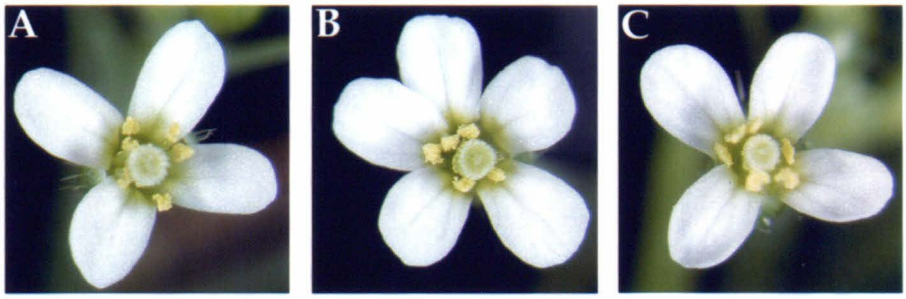
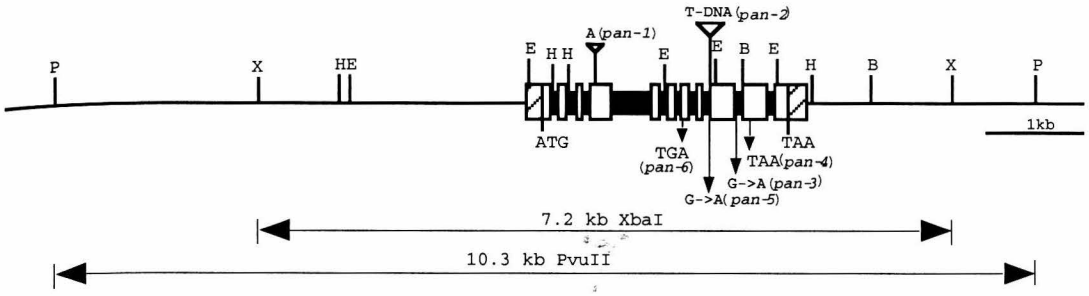

A
B
C

Figure 2. Genomic structure of the *PAN* gene, and sequence of the *PAN* cDNA. (A) Structure and restriction map of the *PAN* genomic region. The open boxes and solid boxes represent exons and introns, respectively. The hatched boxes represent the 5' and 3' untranslated regions. The genomic regions used for complementation of *pan* mutant phenotype are indicated below. ATG and TAA indicate the positions of the putative translational start and stop codons, respectively. Mutations in the six *pan* alleles are also shown. Restriction sites are indicated as follows: B, *Bam*H I; E, *Eco*R I; H, *Hin*d III; P, *Pvu* II; X, *Xba* I.
(B) The cDNA and deduced amino acid sequence of the *PAN* gene. The predicted amino acid sequence for the longest open reading frame is shown directly below the nucleotide sequence. Numbers to the right of the sequence refer to the positions of nucleotide and amino acid residues. Triangles indicate the positions of introns. The basic region is doubly underlined and the leucines in the leucine zipper are in bold and underlined. The glutamine-rich regions are singly underlined. The in-frame stop codon preceding the first methionine is indicated by dots above the nucleotide sequence. One nucleotide insertion in *pan-1* is shown above the nucleotide sequence. The position of the T-DNA insertion in *pan-2* allele is indicated by an arrowhead. The *pan-3* mutation occurs at the splice donor site of the ninth intron. The *pan-5* allele is mutated in the splice acceptor site of the eighth intron. Point mutations resulting in premature translational stop sites in *pan-4* and *pan-6* are shown above the nucleotide sequence.

A



B

```
GATTCGCCATCTTTTCACCTCTTTAATGAATTCTCTCCTCACTCCCTCCTTCCCTTTTAACTTCACTTTCTTCTTCTGATAACCCAACTCCTTAAGAAAG  100

                                                                                                  ...
CTCACTTCTTTTTCCCCTGATTCCCTTTGTGTCATCATCTTTCATCATCTTCTTCTTCTAAGAATCAACAACAATGCAGAGCAGCTTCAAAACCGTTCCT  200
                                                                                    M  Q  S  S  F  K  T  V  P    9

                                                                                                                 v
TTCACTCCTGATTTCTACTCTCAATCCTCTTACTTCTTCAGAGGAGATAGTTGTCTTGAGGAGTTTCATCAACCAGTCAATGGTTTTCACCATGAAGAAG  300
 F  T  P  D  F  Y  S  Q  S  S  Y  F  F  R  G  D  S  C  L  E  E  F  H  Q  P  V  N  G  F  H  H  E  E    42

                                                                                                                 v
CTATCGATTTAAGTCCAAATGTCACTATTGCTTCAGCTAACTTACACTACACGACGTTTGATACGGTTATGGATTGTGGTGGTGGTGGTGGTGGCTTGAG  400
A  I  D  L  S  P  N  V  T  I  A  S  A  N  L  H  Y  T  T  F  D  T  V  M  D  C  G  G  G  G  G  G  L  R   76
                                                                                                                 v
                                                                                                    A (pan-1)
GGAGAGACTTGAAGGAGGAGAAGAGGAGTGTTTGGACACAGGGCAATTAGTGTACCAGAAAGGGACAAGATTAGTAGGAGGAGGAGTAGGAGAAGTGAAC  500
  E  R  L  E  G  G  E  E  E  C  L  D  T  G  Q  L  V  Y  Q  K  G  T  R  L  V  G  G  G  V  G  E  V  N  109

                                                                                                                 v
AGCAGTTGGTGTGATTCGGTTTCAGCTATGGCTGATAACAGTCAACATACTGACACTTCCACAGATATTGATACTGATGACAAGACTCAGTTGAATGGAG  600
 S  S  W  C  D  S  V  S  A  M  A  D  N  S  Q  H  T  D  T  S  T  D  I  D  T  D  D  K  T  Q  L  N  G   142

                                                                                                                 v
GTCATCAAGGGATGCTATTGGCTACAAATTGTTCAGATCAATCCAATGTGAAATCTAGTGATCAAAGGACACTTCGTCGACTTGCTCAGAACCGGGAGGC  700
G  H  Q  G  M  L  L  A  T  N  C  S  D  Q  S  N  V  K  S  S  D  Q  R  T  L  R  R  L  A  Q  N  R  E  A  176
                                                                                                 ==========
                                                                                                                 v
                                                                                                    T (pan-6)
TGCTAGGAAAAGTCGGTTGAGGAAAAAGGCCTATGTTCAGCAACTTGAGAATAGTCGAATCAGGCTTGCACAGCTAGAGGAAGAGCTCAAAAGAGCTCGC  800
  A  R  K  S  R  L  R  K  K  A  Y  V  Q  Q  L  E  N  S  R  I  R  L  A  Q  L  E  E  E  L  K  R  A  R  209
==========

                                                                                                                 v
                                                                                                    3' (pan-5)
CAACAGGGATCTTTGGTTGAAAGAGGAGTTTCAGCGGATCACACGCATTTGGCAGCAGGAAATGGTGTCTTTTCATTTGAATTGGAATATACACGTTGGA  900
 Q  Q  G  S  L  V  E  R  G  V  S  A  D  H  T  H  L  A  A  G  N  G  V  F  S  F  E  L  E  Y  T  R  W   242
  T-DNA (pan-2)
v
AGGAGGAACATCAAAGAATGATCAACGACTTAAGATCGGGTGTGAATTCGCAGTTAGGTGACAACGATCTACGCGTTCTAGTGGATGCTGTGATGAGTCA 1000
K  E  E  H  Q  R  M  I  N  D  L  R  S  G  V  N  S  Q  L  G  D  N  D  L  R  V  L  V  D  A  V  M  S  H  276

CTATGATGAAATATTCAGGCTAAAGGGAATTGGCACTAAAGTTGAAGTCTTTCATATGCTCTCAGGCATGTGGAAGACACCTGCCGAGAGATTTTTCATG 1100
  Y  D  E  I  F  R  L  K  G  I  G  T  K  V  D  V  F  H  M  L  S  G  M  W  K  T  P  A  E  R  F  F  M   309
                                                                                                                 v
                                                                                                    5' (pan-3)
                                                                                                                 T (pan-4)
TGGTTAGGTGGATTTAGATCATCAGAGTTACTTAAGATATTGGGGAACCATGTGGATCCATTGACGGACCAGCAGTTGATAGGCATTTGCAACCTTCAGC 1200
 W  L  G  G  F  R  S  S  E  L  L  K  I  L  G  N  H  V  D  P  L  T  D  Q  Q  L  I  G  I  C  N  L  Q   342
                                                                                 ----------------------

AATCGTCTCAACAAGCAGAGGATGCATTGTCACAAGGCATGGAAGCTCTACAACAATCACTTCTCGAGACGCTTTCTTCTGCTTCTATGGGTCCAAACTC 1300
Q  S  S  Q  Q  A  E  D  A  L  S  Q  G  M  E  A  L  Q  Q  S  L  L  E  T  L  S  S  A  S  M  G  P  N  S  376
----------------------------------------

                                                                                                                 v
TTCAGCAAATGTTGCAGATTATATGGGTCATATGGCTATGGCTATGGGCAAACTTGGCACTCTTGAAAACTTCCTTCGCCAGGCTGATTTATTGAGGCAA 1400
  S  A  N  V  A  D  Y  M  G  H  M  A  M  A  M  G  K  L  G  T  L  E  N  F  L  R  Q  A  D  L  L  R  Q   409
                                                                                 ----------------------

CAAACTCTGCAACAGCTTCACAGAATTCTCACCACACGACAAGCTGCTCGCGCCTTTTTGGTCATCCACGATTATATTTCTCGGCTTAGAGCACTTAGCT 1500
 Q  T  L  Q  Q  L  H  R  I  L  T  T  R  Q  A  A  R  A  F  L  V  I  H  D  Y  I  S  R  L  R  A  L  S   442
----------------------------------------

CTCTATGGTTAGCCAGACCTAGAGACTAAATAAAAAGCCTGCATGAGTCATGATGACCAAAGGAAAAGCATAGTTCTGGTCTTAGGTTTTCATATTTACA 1600
S  L  W  L  A  R  P  R  D stop                                                                          451

TATTTTTAACTAAAAGACCCATAACGCTCTTCTCTTTAGTGTATTAGATCATCTGTTTATTTAGTTGGTAGTGAACGTTACCATTC 1686
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Figure 3. Similarity between PAN and other plant bZIP proteins. (Top) Comparison of the PAN bZIP regions to other plant bZIP proteins: the *Arabidopsis* protein TGA1 (Schindler et al. 1992a), the wheat protein HBP-1b (Tabata et al. 1991), the maize protein OBF 3.1 and OBF 3.2 (Foley et al. 1993), the tobacco protein TGA1a (Katagiri et al. 1989), and the *Arabidopsis* proteins GBF1, GBF2, and GBF3 (Schindler et al. 1992b). Asterisks (*) indicate leucine residues within the leucine zipper domain. (Middle) Schematic presentation of PAN, indicating the positions of the basic region (BR), the leucine zipper domain (LZ), and the two glutamine-rich regions (QI and QII). (Bottom) Sequence alignments within the C-terminal glutamine-rich domains of PAN and the TGACGT/C binding proteins shown in the top panel. Glutamine residues within the glutamine-rich regions of PAN are indicated by dots. Identical amino acids are shown as shaded boxes. Numbers to the left refer to the amino acid positions within individual proteins.

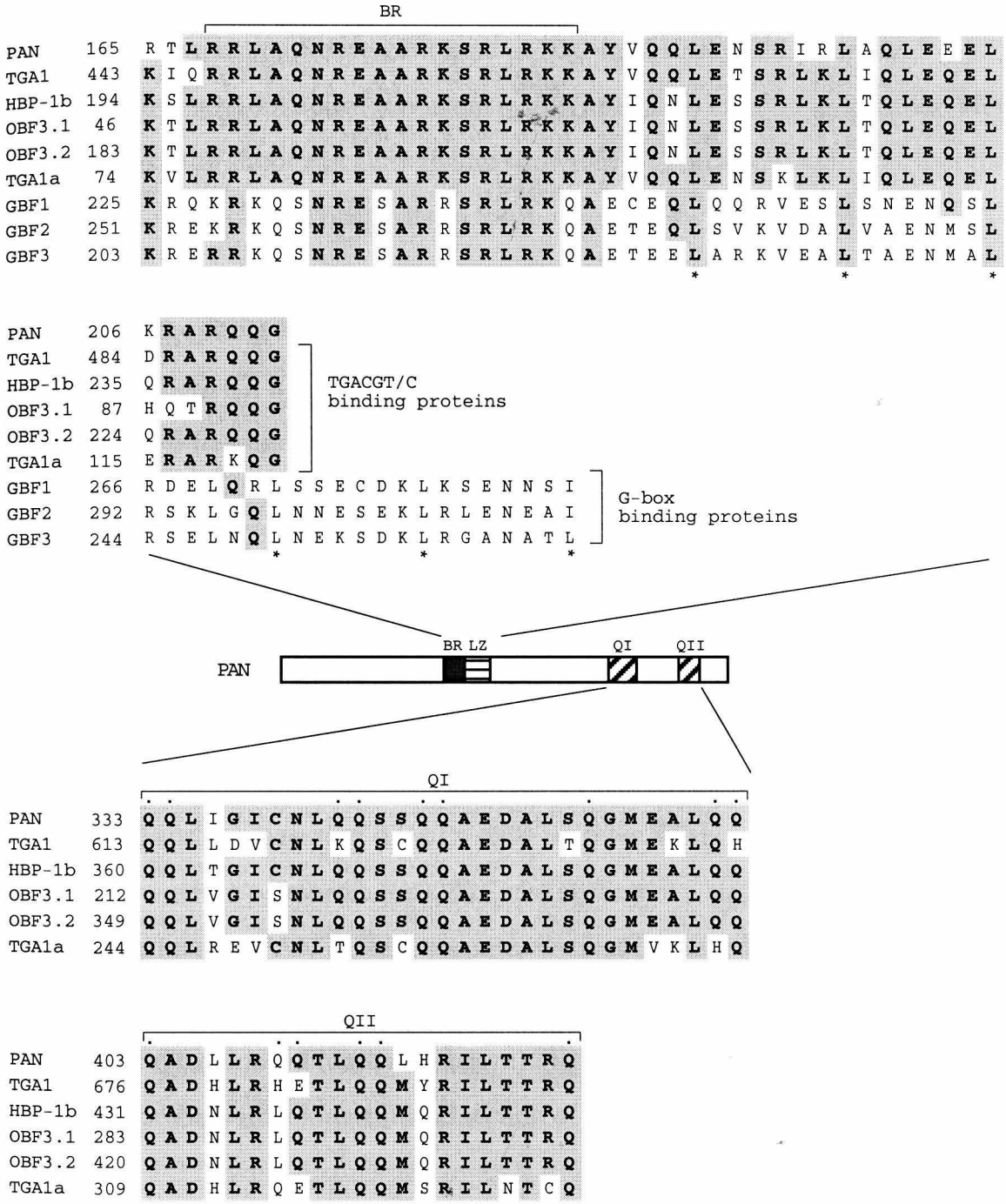
BR
PAN 165 R T L R R L A Q N R E A A R K S R L R K K A Y V Q Q L E N S R I R L A Q L E E E L
TGA1 443 K I Q R R L A Q N R E A A R K S R L R K K A Y V Q Q L E T S R L K L I Q L E Q E L
HBP-1b 194 K S L R R L A Q N R E A A R K S R L R K K A Y I Q N L E S S R L K L T Q L E Q E L
OBF3.1 46 K T L R R L A Q N R E A A R K S R L R K K A Y I Q N L E S S R L K L T Q L E Q E L
OBF3.2 183 K T L R R L A Q N R E A A R K S R L R K K A Y I Q N L E S S R L K L T Q L E Q E L
TGA1a 74 K V L R R L A Q N R E A A R K S R L R K K A Y V Q Q L E N S K L K L I Q L E Q E L
GBF1 225 K R Q K R K Q S N R E S A R R S R L R K Q A E C E Q L Q Q R V E S L S N E N Q S L
GBF2 251 K R E K R K Q S N R E S A R R S R L R K Q A E T E Q L S V K V D A L V A E N M S L
GBF3 203 K R E R R K Q S N R E S A R R S R L R K Q A E T E E L A R K V E A L T A E N M A L
PAN 206 K R A R Q Q G
TGA1 484 D R A R Q Q G
HBP-1b 235 Q R A R Q Q G
OBF3.1 87 H Q T R Q Q G
OBF3.2 224 Q R A R Q Q G
TGA1a 115 E R A R K Q G
TGACGT/C binding proteins
GBF1 266 R D E L Q R L S S E C D K L K S E N N S I
GBF2 292 R S K L G Q L N N E S E K L R L E N E A I
GBF3 244 R S E L N Q L N E K S D K L R G A N A T L
G-box binding proteins
PAN
BR LZ
QI
QII
QI
PAN 333 Q Q L I G I C N L Q Q S S Q Q A E D A L S Q G M E A L Q Q
TGA1 613 Q Q L L D V C N L K Q S C Q Q A E D A L T Q G M E K L Q H
HBP-1b 360 Q Q L T G I C N L Q Q S S Q Q A E D A L S Q G M E A L Q Q
OBF3.1 212 Q Q L V G I S N L Q Q S S Q Q A E D A L S Q G M E A L Q Q
OBF3.2 349 Q Q L V G I S N L Q Q S S Q Q A E D A L S Q G M E A L Q Q
TGA1a 244 Q Q L R E V C N L T Q S C Q Q A E D A L S Q G M V K L H Q
QII
PAN 403 Q A D L L R Q Q T L Q Q L H R I L T T R Q
TGA1 676 Q A D H L R H E T L Q Q M Y R I L T T R Q
HBP-1b 431 Q A D N L R L Q T L Q Q M Q R I L T T R Q
OBF3.1 283 Q A D N L R L Q T L Q Q M Q R I L T T R Q
OBF3.2 420 Q A D N L R L Q T L Q Q M Q R I L T T R Q
TGA1a 309 Q A D H L R Q E T L Q Q M S R I L N T C Q

Figure 4. Localization of PAN protein and distribution of *PAN* RNA in wild-type (A-I, K) and *pan* mutant (J) plants. (A) Section through the shoot apical meristem (sm) in the mature embryo. Seeds were imbibed at 4°C for 2 days and transferred to constant fluorescent light at 20°C for 1 day. PAN is highly expressed in the shoot apical meristem (arrowhead). (B) High magnification view of the cells in the shoot apical meristem shown in A. The arrow indicates that the PAN protein is localized primarily to the nucleus (arrow). (C) Longitudinal section through a shoot apical meristem of 6-day-old seedling. PAN protein is detected in the shoot apical meristem (sm) and leaf primordia (lp), but not in the cotyledons (cot). (D, E) Longitudinal section of an inflorescence meristem (im) with stage 2-5 flowers. PAN protein is detected at high levels throughout the apical region of the inflorescence meristem (arrowhead), throughout the flower meristem of the stage 2 flower, the central dome of the flower meristem of stage 3-4 flowers, and the inner three whorls of organ primordia in the stage 5 flower. se, sepal. (F) Longitudinal section of stage 6-7 flowers. PAN protein becomes restricted in the base of petal (p) primordia, the adaxial side of stamen (st) primordia, and the adaxial side of carpel (c) primordia (arrowhead). Longitudinal section (G) and cross section (H) of a stage 9 flower. PAN protein remains at high levels in the ovule primordia (op). (I) Cross section of a stage 12 flower. Expression level of PAN protein becomes weaker in the ovule (ov). The PAN protein is not expressed at levels above background in sections of inflorescences from *pan-1* (J) and *pan-2* (data not shown). (K) In situ hybridization of a *PAN* antisense probe with a longitudinal section of an inflorescence meristem (im) with stage 1-3 flowers. Expression of *PAN* RNA is detected throughout the apical region of the inflorescence meristem and throughout the flower meristem of stage 1-2 flowers. Expression in the stage 3 flower is limited to regions interior to the sepals (se). In the bright field/dark field double exposures, the silver grains

representing *PAN* expression were made to appear yellow. The number indicated corresponds to the stages of floral development as described by Smyth et al. (1990). All panels shown at the same magnification. Bar, 20 um.

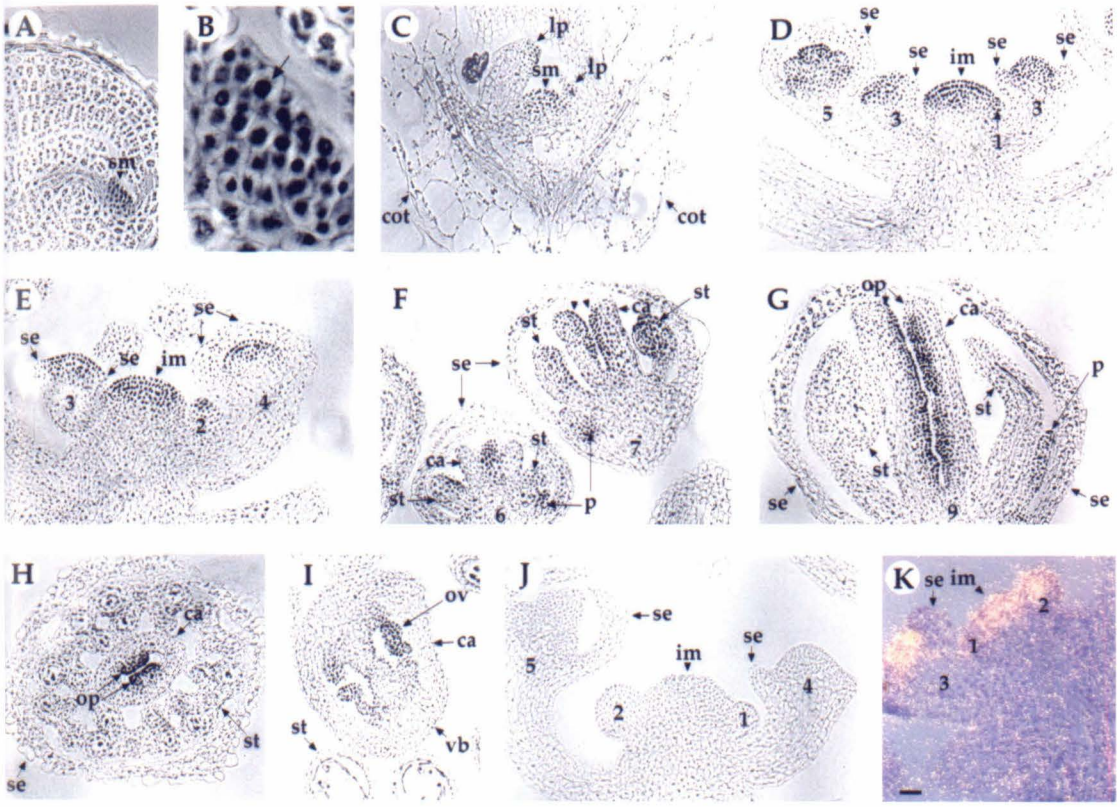
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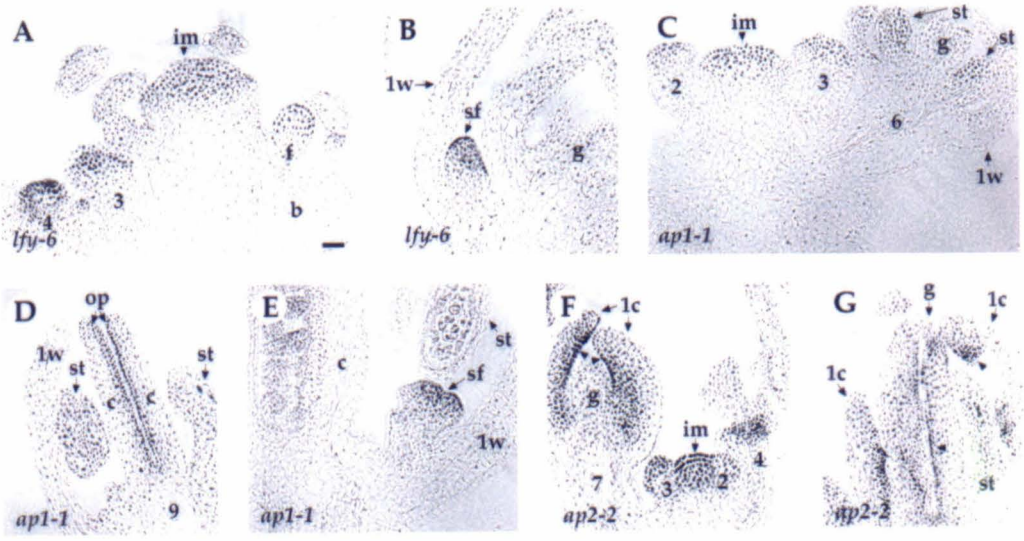
Figure 5. Expression of the PAN protein in *lfy*, *ap1*, and *ap2* mutants. (A-B) *lfy-6*. (A) PAN protein is expressed in the inflorescence meristem (im) and the center of abnormal young flowers. The stages of flowers (3 and 4) and the floral bud (f) in the axil of the bract (b) are indicated. (B) PAN protein is detected in the secondary floral buds (sf) in the axils of the first-whorl organs (1w) of an old *lfy-6* flower. g, gynoecium. (C-E) *ap1-1*. (C) Early expression in the inflorescence meristem (im) and in the stage 2, 3 and 6 floral buds is normal (compared with Figs. 4D-F). st, stamen. (D) PAN protein is expressed normally in the ovule primordia (op) at stage 9 flowers (compare with Fig. 4G). c, carpel. (E) Secondary floral buds (sf) in the axil of the first-whorl organs (1w) show PAN protein expression at levels comparable to the early flower primordia arising on the inflorescence apex (Figs. 4D, E). (F-G) *ap2-2*. PAN protein expression is largely normal in the inflorescence meristem (im) and young floral buds (stage 2-4). At later stages, PAN protein is detected in the ovule primordia (arrowhead) in both of the first-whorl carpels (1c) and the central gyneocium (g). The number indicated corresponds to the developmental stage of each flower (Smyth et al. 1990). Bar, 20 um.


A
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6
1w
ap1-1
D
op
1w
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ap1-1
9
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1c
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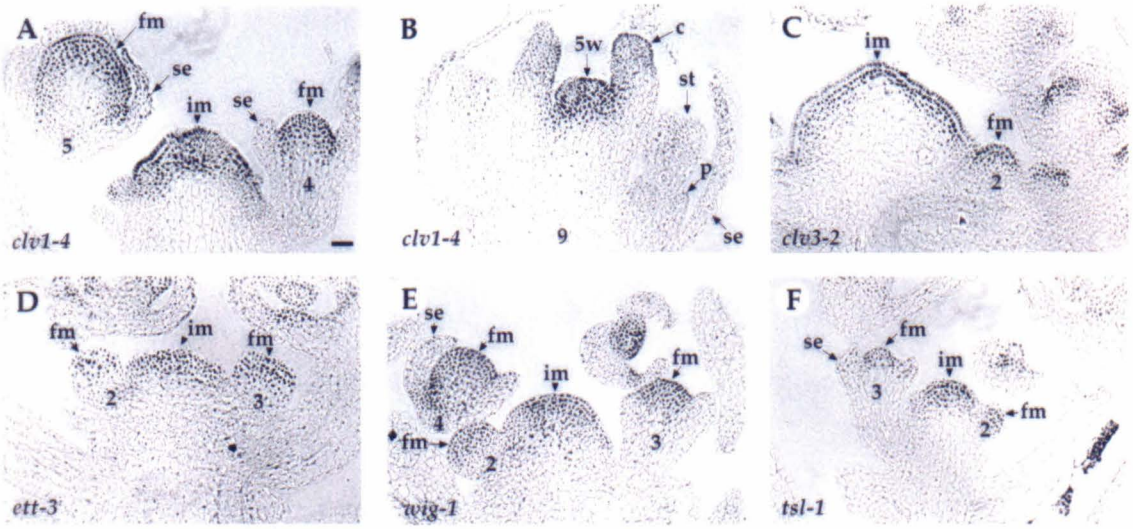
ap2-2

Figure 6. Expression of PAN protein in *clv1-4*, *clv3-2*, *ett-3*, *wig-1* and *tsl-1* mutants. (A-B) *clv1-4*. PAN is expressed in the inflorescence meristem (im), the floral meristem (fm, A), and the fifth-whorl (5w) of *clv1-4* flowers (B). PAN protein expression is normal in the inflorescence meristem and the floral meristem in *clv3-2* (C), *ett-3* (D), *wig-1* (E), and *tsl-1* (F) flowers. se, sepal; p, petal; st, stamen; c, carpel. The number indicated corresponds to the developmental stage of each flower (Smyth et al. 1990). Bar, 20 um.


A
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ett-3
E
se
fm
im
fm
4
fm
2
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wig-1
F
se
fm
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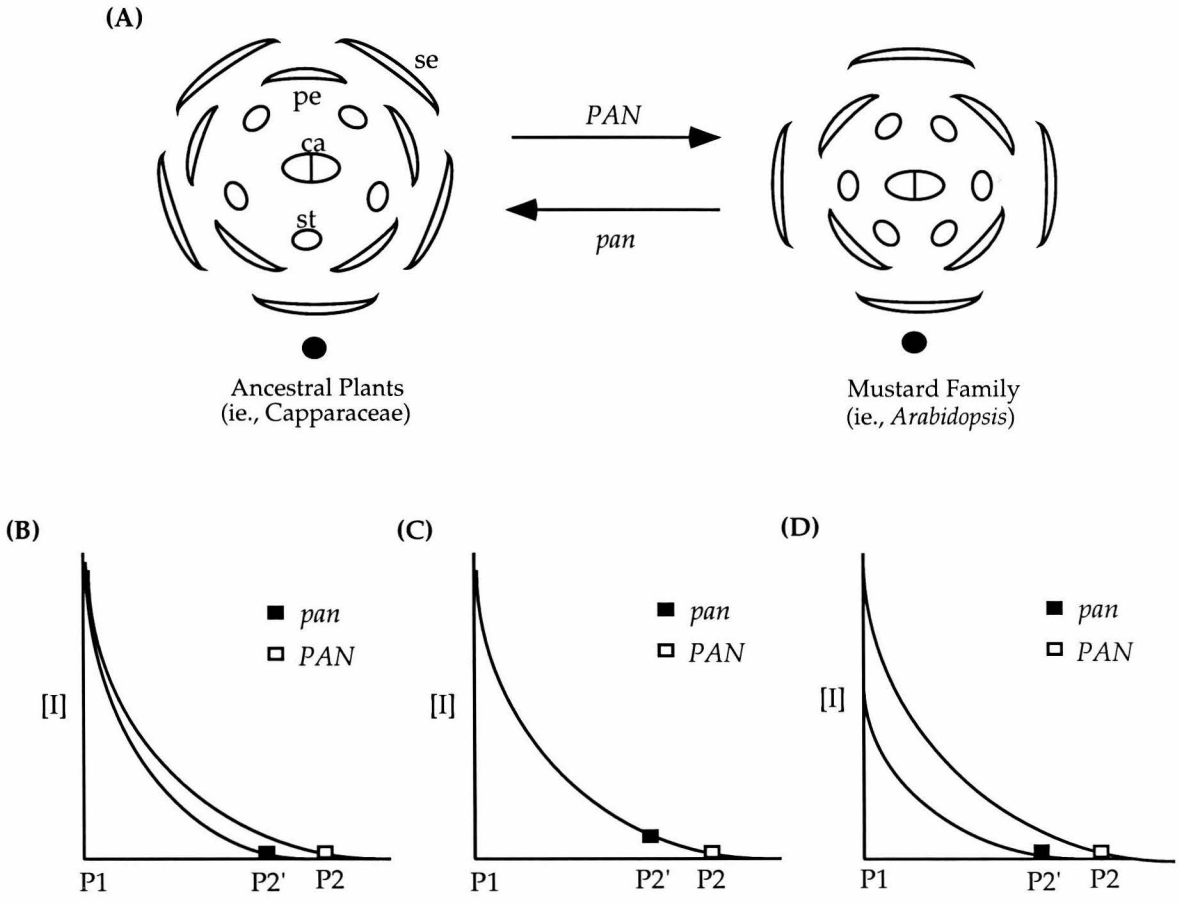
3
2
fm
tsl-1

Figure 7. Possible models for *PAN* in regulating floral organ number. We postulate that there is an inhibitory mechanism controlling the relative positions of first and second whorl floral organ primordia in ancestral plants (i.e., Capparaceae) of the mustard family (i.e., *Arabidopsis*) and *PAN* participates in the pre-existing inhibitory pathway to establish tetramerous flowers in the mustard family. (A) Introduction of *PAN* into the morphogen pathway leads to more distant spacing of organ primordia and thus fewer organs. When *PAN* activity is reduced or eliminated (*pan*), plants produce flowers with a pattern resembling the ancestral flower form. (B-D) The inhibitor concentration [I] is plotted as function of distance. The diffusing inhibitor decays with time and thus distance. P1, the first primordium. (B) *PAN* might facilitate inhibitor diffusion or persistence to allow the inhibitor to spread more widely, resulting in the formation of the second primordium (P2) at a more distant position than in *pan* (P2'). (C) *PAN* might enhance inhibitor reception, leading to formation of the next primordium at a lower inhibitor concentration than in *pan*. (D) *PAN* might increase inhibitor production, indirectly leading to its wider diffusion. se, sepal; pe, petal; st, stamen; ca, carpel. •, inflorescence meristem. ▫ and ■ indicate the lowest concentration in the inhibitory field allowing the new primordium to arise in *PAN* and *pan* floral meristems, respectively.


(A)
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pe
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st
PAN
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Ancestral Plants
(ie., Capparaceae)
Mustard Family
(ie., Arabidopsis)
(B)
pan
PAN
[I]
P1
P2'
P2
(C)
pan
PAN
[I]
P1
P2'
P2
(D)
pan
PAN
[I]
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P2'
P2

Chapter 3

Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*

Chiou-Fen Chuang and Elliot M. Meyerowitz

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Abstract

We investigated the potential of double-stranded RNA interference (RNAi) with gene activity in *Arabidopsis thaliana*. To construct transformation vectors that produce RNAs capable of duplex formation, gene-specific sequences in the sense and antisense orientations were linked and placed under the control of a strong viral promoter. When introduced into the genome of *A. thaliana* by *Agrobacterium*-mediated transformation, double-stranded RNA-expressing constructs corresponding to four genes, *AGAMOUS* (*AG*), *CLAVATA3*, *APETALA1*, and *PERIANTHIA*, caused specific and heritable genetic interference. The severity of phenotypes varied between transgenic lines. *In situ* hybridization revealed a correlation between a declining *AG* mRNA accumulation and increasingly severe phenotypes in *AG (RNAi)* mutants, suggesting that endogenous mRNA is the target of double-stranded RNA-mediated genetic interference. The ability to generate stably heritable RNAi and the resultant specific phenotypes allows us to selectively reduce gene function in *A. thaliana*.

Introduction

In *Arabidopsis thaliana*, reverse genetic techniques for isolating mutants corresponding to known sequences, such as antisense suppression (1-7), co-suppression by overexpression of the target gene (3, 8, 9), targeted gene disruption (10), or the PCR approach of screening for T-DNA insertion libraries (11, 12) have been developed, but are often insufficient and have many unanticipated difficulties. The widespread identification of differentially expressed genes, homologous genes and interacting proteins have created a need for potent and efficient methods for obtaining their loss-of-function or reduction-of-function mutants.

Double-stranded RNA (dsRNA)-mediated interference with expression of specific genes has been observed in a number of organisms including *Caenorhabditis elegans* (13-17), plants (18, 19), *Drosophila* (20, 21), *Trypanosoma brucei* (22) and a planarian (23). Although the mechanism of RNA interference (RNAi) is not well understood, it seems to provide an effective way to discover gene function in many organisms (24-26).

To investigate the potential of dsRNA interference with gene activity in *A. thaliana*, we introduced dsRNA-expressing constructs of selected genes with previously defined functions into plants. Gene constructs delivered into plants with *Agrobacterium*-mediated transformation are stably integrated into the genome of host cells; thus, RNA expression from these constructs in transgenic plants can be persistent and heritable.

In this study, one gene from each of four major categories of genes involved in flower development was chosen, to determine the ability of RNAi to allow functional assessment of genes with diverse developmental functions in flowers. They are the floral organ identity gene *AGAMOUS* (*AG*), the floral meristem size

gene *CLAVATA3* (*CLV3*), the floral meristem identity gene *APETALA1* (*AP1*), and the floral organ number gene *PERIANTHIA* (*PAN*) (27-30). The phenotypes produced by dsRNAs corresponding to these genes are similar to those of their previously identified reduction-of-function or loss-of-function mutants (31-36). The progeny from fertile RNAi mutants, such as *CLV3 (RNAi)* and *AP1 (RNAi)* plants, also showed phenotypes. In addition to high specificity and heritability, a phenotypic series (weak, intermediate and strong) was obtained from dsRNA interference. Furthermore, *in situ* hybridization indicates that endogenous target mRNA is decreased in RNAi mutants. Most constructs that are designed to produce only antisense or only sense RNA do not induce interference. Thus, specific and inheritable dsRNA interference may offer a useful alternative to classical reverse genetic screening of mutants in *A. thaliana*.

Materials and Methods

Constructs. A summary of DNA constructs is shown in Fig. 1. In p35S::A-GUS-S and p35S::A, constructs were ligated to the *Bam*H I and *Xba* I sites of pCGN1547 (37) into which an 842-bp fragment of the cauliflower mosaic virus 35S promoter and a 253-bp fragment of the 3' end of nopaline synthase had previously been inserted in the *Asp*718/*Bam*H I and *Xba* I/*Pst* I sites, respectively (38). Constructs consisting of a 339-bp fragment of the nopaline synthase promoter, gene-specific sequences in the sense orientation and a 253-bp fragment of the 3' end of nopaline synthase were ligated to the *Pst* I and *Hind* III sites of pCGN1547 and p35S::A to make pNOS::S and p35S::A-NOS::S, respectively. In p35S::A-GUS-S, the β-glucuronidase (GUS) fragment containing nucleotides 787-1,809 was used as a linker between gene-specific fragments in the antisense and the sense orientations. *AG*, *CLV3*, *AP1*, and *PAN* cDNA coding sequences used in this

study contain nucleotides 301-855 (27), 3-291 (28), 445-854 (29), and 27-396 (30), respectively.

***Agrobacterium*-Mediated Transformation.** *Agrobacterium* strain ASE carrying DNA constructs in pCGN1547 was used to transform *Arabidopsis* plants (T_0) by vacuum infiltration (39). Transformed *Arabidopsis* lines (T_1) were selected on Murashige/Skoog (Sigma) plates containing kanamycin (50 µg/ml). Kanamycin-resistant seedlings were then transferred to soil. Phenotypic analysis of T_1 and T_2 plants is summarized in Table 1 and Table 2, respectively.

***In Situ* Hybridization.** The *AG* cDNA clone pCIT565 containing nucleotides 9-977 (27) was used to synthesize antisense and sense probes. ^{35}S-labeled RNA probes were synthesized with Riboprobe *in vitro* Transcription Systems (Promega). The template was linearized with *Hind* III and transcribed by T7 RNA polymerase (antisense probe), or linearized with *Xho* I and transcribed by SP6 RNA polymerase (sense probe). Tissue was fixed in 1 X PBS containing 4% paraformaldehyde/0.1% Triton X-100/0.1% Tween 20 at 4°C overnight. Fixed tissue was dehydrated with ethanol, cleared with xylene, embedded in paraffin (Paraplast Plus), and sectioned at 8 µm. *In situ* hybridization was performed as described by Drews et al. (40), with modifications by Sakai et al. (41). Exposure time was 8 -10 days.

Western Blot Analysis. Bud clusters (stage 1-12, including the inflorescence meristem) from one inflorescence were frozen and ground in liquid nitrogen, thawed in 30 µL of lysis buffer (50 mM Tris, pH 7.5/1 mM EDTA/100 mM NaCl/1% Nonidet P-40/0.1% SDS/0.1% Triton X-100/0.7% 2-mercaptoethanol/1 mM PMSF). The extract was mixed with 15 µL of 3 X sample buffer (187 mM Tris,

pH 6.8/6% SDS/30% glycerol/3% 2-mercaptoethanol/0.06% bromophenol blue), boiled for 5 min, and centrifuged (16,000 X *g* for 10 min at room temperature). Twenty microliters of the supernatant was separated on a 12.5% SDS/PAGE. The protein was transferred to nitrocellulose membrane (Schleicher & Schuell), probed with an AG-specific polyclonal antibody (42) and horseradish peroxidase-labeled secondary antibody (Amersham International), and detected with the enhanced chemiluminescence system (Amersham International).

Results

To make constructs that produce dsRNA, gene-specific sequences in the antisense and the sense configurations were either linked with the partial GUS gene and placed under control of the constitutive 35S promoter from cauliflower mosaic virus (p35S::A-GUS-S), or controlled by the 35S promoter and the constitutive nopaline synthase promoter, respectively (p35S::A-NOS::S). A single RNA transcribed from the fusion gene in p35S::A-GUS-S can potentially form a dsRNA stem with a single-stranded loop structure (Fig. 1). Genetic interference effects of sense, antisense and dsRNAs corresponding to *AG*, *CLV3*, *AP1*, and *PAN* are outlined in Table 1. For each of these genes, p35S::A-GUS-S constructs caused potent and specific genetic interference. However, p35S::A-NOS::S, p35S::A and pNOS::S constructs had either no, or weak, genetic interference effects. We will refer to transgenic plants carrying functional p35S::A-GUS-S constructs by listing the gene name followed by RNAi. For unknown reasons, the sense construct corresponding to *CLV3* caused toxicity in *Agrobacterium* and the sense construct of *PAN* resulted in very low transformation efficiency of *crabs claw-1* (*crc-1*) plants. Therefore, interference effects of the *CLV3* sense construct were not determined and only six *crc-1* transgenic plants containing the

PAN sense construct were analyzed.

_AG_ dsRNA-Mediated Genetic Interference. *AG* was chosen for initial characterization of RNAi in developing flowers. *Arabidopsis* flowers consist of four concentric whorls of organs. Most wild-type flowers have four sepals, four petals, six stamens, and two fused carpels, from the outermost first whorl to the innermost fourth whorl (Fig. 2*A*). Mutations in the *AG* gene cause homeotic alterations of the third and fourth whorls of organs in flowers (31). In severe *ag* loss-of-function mutants (Fig. 2*B*), the third whorl primordia develop into petals indistinguishable from those of the second whorl, and the fourth whorl develops into another *ag* flower, resulting in a repetitive pattern of sepals, petals, and petals (32).

The phenotypes produced by *AG* dsRNA are frequent and specific (Fig. 2 *C-I*). All but one of 236 transgenic plants showed *ag* mutant phenotypes. These *AG (RNAi)* mutants can be arranged into a phenotypic series based on the severity of the homeotic transformation in the third whorl and the extent of floral indeterminacy in the fourth whorl. Weak and intermediate *AG (RNAi)* mutant flowers showed partial homeotic transformation in the third whorl organs and slight floral indeterminacy (Fig. 2 *E-H*). However, intermediate/strong and strong *AG (RNAi)* mutant flowers showed complete transformation of the third whorl organs from stamens to petals and severe floral indeterminacy (Fig. 2 *C, D*, and *I*). Particularly, flowers from strong *AG (RNAi)* plants (Fig. 2*C*) are indistinguishable from those of strong *ag* mutant alleles such as *ag-1* (Fig. 2*B*). Weak, intermediate, intermediate/strong, and strong *AG (RNAi)* mutants represent 16, 32, 43, and 9%, respectively, of the transgenic plant populations. In contrast, pNOS::A-GUS-S, in which the nopaline synthase promoter was used to drive the fusion gene, and p35S::A-NOS::S constructs for *AG* caused very weak

genetic interference in 2 out of 32 and 3 out of 124 transgenic plants, respectively (data not shown). Flowers from transgenic plants containing the *AG* antisense (n = 111) or sense (n = 95) construct are indistinguishable from those of wild-type plants (data not shown).

dsRNA Interferes with mRNA Accumulation. *In situ* hybridization was performed to determine the target of dsRNA interference. The earliest expression of *AG* in wild-type flowers is in stage three, in those cells that will give rise to the third- and fourth-whorl organ primordia. Later, *AG* expression is restricted to the stamen and the carpel primordia (Fig. 3*F*) (27). The autoradiogram of the tissue hybridized with an *AG* anti-mRNA probe showed that hybridization signals declined with increasingly severe phenotypes in *AG (RNAi)* mutants (Fig. 3 *A-E*), consistent with the observation that *AG* mRNA accumulation is decreased in *AG (RNAi)* mutants (Fig. 3 *G-J*). These results suggest that endogenous mRNA is a target of dsRNA-mediated genetic interference. When used as a standard control for *in situ* hybridization, an *AG* sense probe hybridized with the tissue from *AG (RNAi)* mutants but not with that from wild-type (Fig. 3 *K-N*), suggesting that *AG* antisense RNA is produced in *AG (RNAi)* mutants. Reverse transcription-PCR analysis with *GUS*- and *AG*-specific primers also showed that expression levels of both strands of *AG* RNA from the fusion gene in p35S::A-GUS-S increase with increasingly severe phenotypes (data not shown).

Furthermore, Western blot analysis of total protein from wild-type and *AG (RNAi)* mutants with an AG-specific polyclonal antibody (42) demonstrated that the severity of phenotypes is correlated with a reduction of the AG protein level in *AG (RNAi)* mutants. AG protein is weakly expressed in weak and intermediate *AG (RNAi)* mutants. In contrast, it is not detected at levels above background in

intermediate/strong and strong *AG (RNAi)* mutants (Fig. 3*O*).

Genetic Interference by *CLV3* and *AP1* dsRNAs. We further assessed the effectiveness and specificity of dsRNA with the *CLV3* and *AP1* genes. Plants with mutations in the *CLV3* gene have enlarged meristems and extra floral organs, especially carpels (Fig. 4 *C*, *F*, and *I*) (33). The majority of *CLV3 (RNAi)* mutants (89%, n = 121) have flowers with extra carpels (Fig. 4*H*); however, only 2% of those plants (n = 108) also have extra sepals, petals, and stamens (Fig. 4*E*). In addition, some *CLV3 (RNAi)* mutant plants (26%, n = 121) have enlarged shoot apical meristems with distortions in phyllotaxy (Fig. 4*B*) and bifurcation, flattening and broadening of the stem (data not shown). In contrast, only 1% (n = 176) of plants transformed with p35S::A-NOS::S for *CLV3* have the extra carpel phenotype. *clv3* mutant phenotypes were not observed in transgenic plants containing the *CLV3* antisense construct (n = 273).

Mutations in the *AP1* gene result in homeotic alterations of the outer two whorls and a partial or complete conversion of a floral meristem into an inflorescence meristem (34, 35). In weak *ap1* mutant alleles, the first and second whorls consist of mosaic sepaloid organs and staminoid petals, respectively (Fig. 5*B*). In intermediate *ap1* mutant alleles, flowers have leaf-like first whorl organs and leaf-like or staminoid second whorl organs (Fig. 5*C*). In strong *ap1* mutant alleles, bract-like organs are produced in the first whorl and petals are usually absent in the second whorl (Fig. 5*D*). In addition, secondary flowers usually arise from the axils of the first whorl organs in flowers of intermediate and strong *ap1* mutant alleles (Fig. 5 *C* and *D*). About 96% of transgenic plants (n = 260) containing the *AP1* dsRNA-expressing construct, p35S::A-GUS-S, produced flowers similar to those of *ap1* mutant alleles (Fig. 5 *E-H*). Weak (Fig. 5*E*), intermediate (Fig. 5*F*), intermediate/strong (Fig. 5*G*), and strong (Fig. 5*H*)

phenotypes were observed in 94, 1, 3, and 2%, respectively, of *AP1 (RNAi)* mutants (n = 249). In contrast, transgenic plants containing the *AP1* construct in the antisense orientation (6%, n = 140) had very weak mutant phenotypes (data not shown). The *AP1* sense construct did not cause mutant phenotypes in transgenic plants (n = 62; data not shown).

One *CLV3 (RNAi)* T_1 plant and 6 *AP1 (RNAi)* T_1 plants of variable severity were selfed to examine the inheritance of genetic interference (Table 2). The progeny from each selected plant showed similar severity of phenotypes to those of the selfed parents, and the severity of phenotypes is constant between mutant siblings of each lineage. In addition, the progeny of *AP1 (RNAi)* plants had 3:1 (mutant/wild-type) segregation ratios, suggesting that each of the 6 *AP1 (RNAi)* T_1 plants contained one copy of the transgene. This result indicates that dsRNA-expressing constructs, which are integrated into the plant genome, are stably inherited in a Mendelian fashion, and that the RNAi effect persists to, or recurs in, new generations of plants.

RNA-Mediated Interference with *PAN*. Flowers of plants mutant for *pan* are characterized by an increase in the organ number in the first two whorls, and a decrease in the organ number in the third whorl. Mutant flowers usually have five sepals, five petals, five stamens, and two carpels (36). When introduced into wild-type plants, the dsRNA-expressing construct of *PAN* caused either no, or weak, extra organ phenotypes. Reverse transcription-PCR analysis showed that *PAN* mRNA was reduced by 30-90% in *PAN (RNAi)* plants compared to wild-type plants (data not shown), suggesting that a small portion of PAN activity is sufficient for its function in wild-type plants. This hypothesis is consistent with results from previous immunohistochemical analysis showing that the mutant *pan-1* and *pan-2* alleles, with high expressivity of the extra organ phenotype, are

likely null alleles (30).

Whereas flowers homozygous for strong mutant alleles show high penetrance of the extra organ phenotype, only the first few flowers from homozygotes of weak mutant alleles show the phenotype. However, both strong and weak *pan* alleles cause high penetrance of unfused carpel phenotypes in a *crc-1* genetic background (Fig. 6 *A*, *B*, *D*, and *E*) (30, 44), suggesting that *crc* mutants provide a more sensitive background than wild type in which to observe phenotypic effects of *PAN* reduction-of-function mutations. Therefore, RNA-expressing constructs corresponding to *PAN* were introduced into a *crc-1* homozygous background to further assess the potential of RNA-mediated interference with *PAN*. Similar to *pan* alleles, *PAN* dsRNA-expressing constructs, p35S::A-GUS-S (87%). Antisense (55%, n = 76) and sense (33%, n = 6) sequences corresponding to *PAN* have similar RNAi effects as well , n = 126) and p35S::A-NOS::S (27%, n = 66) caused extra organ number and unfused carpels in *crc-1* (Fig. 6 *C* and *F*(data not shown), suggesting that low levels of dsRNAs might be produced in such a case and weak interference with *PAN* activity is sufficient to confer an unfused carpel phenotype in *crc-1*.

Discussion

This study shows that dsRNA-mediated genetic interference can operate in *A. thaliana* to efficiently induce sequence-specific inhibition of gene function. Although the technique of RNA microinjection has been widely used in *C. elegans* (13-15, 44), *Drosophila* (20, 21), and planarians (23), methods for RNA injection into zygotes of *A. thaliana* are not available. However, *Agrobacterium*-mediated transformation provides a convenient and efficient method to introduce dsRNA-expressing constructs into the plant genome. Therefore, RNAi in transgenic

plants is persistent and inherited instead of being transient and unstable as in RNA-injected animals (13-15, 20, 21, 23, 45) and transiently transfected cells (22). In addition, inducible and tissue-specific promoters might be used to obtain regulated RNAi.

In this study, two kinds of dsRNA-expressing constructs, p35S::A-GUS-S and p35S::A-NOS::S, were used to investigate RNAi effects. p35S::A-NOS::S is less potent at inducing genetic interference than p35S::A-GUS-S, perhaps because of unequal expression levels of sense and antisense RNAs by two promoters of different strength. The nopaline synthase promoter is much weaker than the 35S promoter, suggested by the observation that pNOS::A-GUS-S has weaker genetic interference than p35S::A-GUS-S. These results suggest that equal and high levels of both strands of each RNA in each cell are essential for inducing potent RNAi. If this is true, use of two strong promoters of similar strength should improve RNAi effects of dsRNA-expressing constructs in which sense and antisense RNAs are produced separately; however, use of two identical promoters in a construct should be avoided to prevent co-suppression (46).

dsRNAs corresponding to four genes selected in this study caused potent and specific genetic interference, suggesting that dsRNA-mediated gene silencing can occur in the tissues where these genes normally function. In addition, a phenotypic series can be obtained from RNAi mutants. The fact that the severity of phenotypes varied between T_1 individuals is possibly because of variable transgene copy number and/or positional effects of particular DNA insertion events. However, our results suggest that severity of phenotypes in *AP1 (RNAi)* T_1 plants is not related to the transgene copy number.

CLV3 dsRNA seems predominantly to block gene function in a subset of the cells where it is normally expressed. *CLV3* is expressed in the inflorescence and the floral meristems (28). Mutations in the *CLV3* gene cause enlarged meristems

and extra floral organs (33). About 89% of *CLV3 (RNAi)* plants have flowers with extra carpels but only 26% of *CLV3 (RNAi)* plants have enlarged inflorescence meristems. This result suggests a strong suppression of the *CLV3* gene function in the center of the floral meristem but less suppression of its function in the inflorescence meristem. It is probably because of differential activity of the 35S promoter which drives expression of dsRNA in these tissues. It is also possible that some tissues could partially resist RNAi (25), or that some phenotypes (such as enlarged inflorescence meristems) are less sensitive to the level of gene activity.

When used as controls for RNAi experiments, the sense and antisense constructs of *PAN* also had the ability to induce genetic interference in a *crc-1* homozygous background; so did the *AP1* antisense construct in wild-type plants. It has been suggested that low levels of dsRNA might be produced from transgenes that are designed to produce only antisense or only sense RNA, via the readthrough transcription from transgenes arranged as an inverted repeat, or transcription from a transgene whose 3' end is adjacent to an endogenous promoter (19, 24, 25, 47). Alternatively, it seems possible that cellular RNA-dependent RNA polymerase could be involved in the conversion of single-stranded RNA to dsRNA in a cell-specific manner, suggested by the cloning and *in vitro* catalytic analysis of an RNA-dependent RNA polymerase from tomato (25, 48).

In situ hybridization revealed a correlation between decreasing levels of *AG* mRNA accumulation and increasing severity of phenotypes in *AG (RNAi)* plants, suggesting that the mechanism blocking mRNA accumulation could be responsible for dsRNA interference. This result is consistent with previous findings that endogenous mRNA is a target of dsRNA-mediated genetic interference (13, 14, 22, 23). In addition, a recent report of isolation of an RNaseD

homolog from *C. elegans* mutants which are resistant to RNAi suggests that RNAi works by dsRNA-directed, enzymatic RNA degradation (49).

Whatever the mechanism by which RNAi acts to reduce specific mRNA levels, the experiments described here show that it is a useful method for determining the loss-of-function phenotypes of genes involved in development and meristem activity in *A. thaliana*.

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Figure 1. Gene constructs used to analyze dsRNA effects. In p35S::A-GUS-S, gene-specific sequences (open boxes with arrows indicating the orientation) in the antisense (A) and the sense (S) orientations were linked with a 1,022-bp fragment of the GUS gene (hatched box) and controlled by the 35S promoter (solid arrow). A schematic structure of the predicted dsRNA stem with a single-stranded loop generated by p35S::A-GUS-S constructs is shown. In p35S::A-NOS::S, gene-specific sequences in the antisense and sense orientations were controlled by the 35S promoter and the nopaline synthase promoter, respectively (open arrow). p35S::A contains gene-specific sequences in the antisense orientation under control of the 35S promoter. pNOS::S contains gene-specific sequences in the sense orientation driven by the nopaline synthase promoter. Solid box, the 3' end of nopaline synthase.

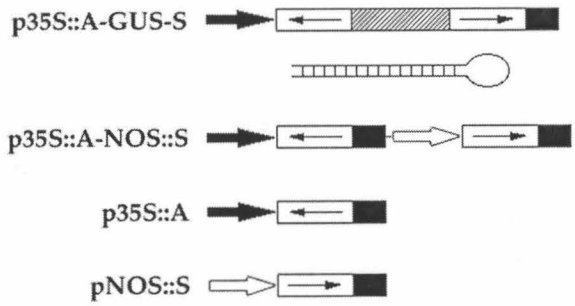

p35S::A-GUS-S
p35S::A-NOS::S
p35S::A
pNOS::S

Table 1. Effects of sense, antisense and dsRNAs on transgenic plants

Gene	Transformed background	Transformed construct	RNAi mutants /total	%
AG	Ws*	p35S::A-GUS-S	235/236	99.6
		pNOS::A-GUS-S	2/32	6
		p35S::A-NOS::S	3/124	2
		p35S::A	0/111	0
		pNOS::S	0/95	0
CLV3	Ws	p35S::A-GUS-S	121/137	88
		p35S::A-NOS::S	2/176	1
		p35S::A	0/273	0
		pNOS::S	ND†	ND
AP1	L-*er*‡	p35S::A-GUS-S	249/260	96
		p35S::A	8/140	6
		pNOS::S	0/62	0
PAN	*crc-1*	p35s::A-GUS-S	110/126	87
		p35S::A-NOS::S	18/66	27
		p35S::A	42/76	55
		pNOS::S	2/6	33

*Ws, Wassilewskija.

†ND, not determined.

‡L-*er*, Landsberg *erecta*.

Table 2. Inheritance of genetic interference in *CLV3 (RNAi)* and *AP1 (RNAi)* mutants

T_1 plants		T_2 plants		Copy no.[†] in T_1 plants
		mutants	WT[*]	
CLV3 (RNAi)	plant 1	14	8	ND[‡]
AP1 (RNAi)	plant 1 (W[§])	25 (W)	8	1
	plant 2 (W)	22 (W)	8	1
	plant 3 (I[¶])	21 (I)	6	1
	plant 4 (I/S[ß])	19 (I/S)	7	1
	plant 5 (S[**])	17 (S)	5	1
	plant 6 (S)	20 (S)	4	1

[*]WT, wild type.

[†]Number of transgene copies estimated from segregation ratios.

[‡]ND, not determined.

[§]W, weak.

[¶]I, intermediate.

[ß]I/S, intermediate/strong.

[*]S, strong.

Figure 2. Flowers of wild-type, *ag-1* and *AG (RNAi)* plants. (*A*) Wild-type flowers have four sepals, four petals, six stamens, and two carpels. (*B*) *ag-1* flowers consist of an indeterminate number of whorls of sepals and petals in the pattern: $(\text{sepals, petals, petals})_n$, with no staminoid or carpeloid tissue. (*C-I*) *AG (RNAi)* flowers with different severity of phenotypes. (*C*) Strong mutant flowers phenocopied *ag-1*. (*D*) Longitudinal section of a strong mutant flower showing a large number of sepals and petals produced by an indeterminate floral meristem (FM). (*E*) Weak mutant flower. The stamens fail to elongate and the anthers are slightly petaloid (arrowhead), with no pollen. (*F*) Intermediate mutant flower with some sepals and petals removed. Anthers are partially transformed into petaloid tissue (arrowheads). The gynoecium is bulged at the top (arrow, F), with inner organs such as carpels (arrowhead, *G*) and/or petals (arrowheads, *H*). (*I*) Intermediate/strong mutant flowers have the repeated pattern of sepals, petals, petals formed in outer whorls and an incomplete flower in the center (arrow). *AG (RNAi)* plants are in the Wassilewskija background; therefore, internode elongation between successive internal flowers are seen in intermediate/strong (*I*) and strong mutant flowers (*C*).

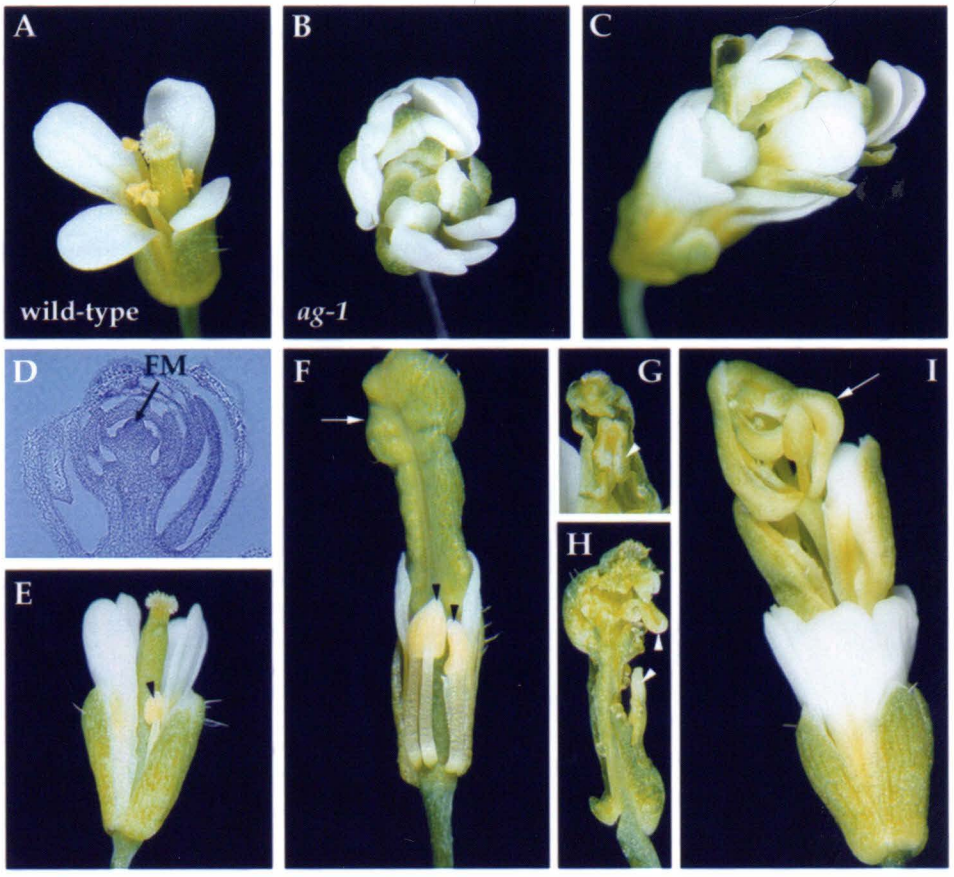

A
wild-type
B
ag-1
C
D
FM
E
F
G
H
I

Figure 3. Effects of *AG* dsRNA on levels of *AG* mRNA and AG protein. (*A-E*) An autoradiogram of the tissue hybridized with an *AG* anti-mRNA probe. The tissue is from wild-type (*A* and *F*), weak (*B* and *G*), intermediate (*C* and *H*), intermediate/strong (*D* and *I*), and strong (*E* and *J*) *AG (RNAi)* mutant plants. (*A-E*) Hybridization signals declined gradually with increasingly severe phenotypes. (*F-J*) The bright field/dark field double exposures of longitudinal section through the inflorescence meristems with stage 2-5 flowers. The silver grains representing *AG* mRNA expression were made to appear yellow with the use of a yellow filter. The number indicated corresponds to the development stage of flowers (43). im, inflorescence meristem. (Bar = 50 μm.) (*K* and *L*) An autoradiogram of the tissue hybridized with an *AG* sense probe. The tissue is from wild-type (*K* and *M*) and intermediate *AG (RNAi)* mutant plants (*L* and *N*). (*O*) Western blot analysis of AG protein. The anti-AG antibody recognizes the carboxyl-terminal part of the AG protein from aa 220-285 which is absent in the AG-1 protein (27, 42); thus, *ag-1* is a control of the specificity of the antibody. Whereas AG protein is weakly expressed in weak (w) and intermediate (i) *AG (RNAi)* mutants compared with wild type (Wt), it is not detected at levels above background in intermediate/strong (i/s) and strong (s) *AG (RNAi)* mutants.

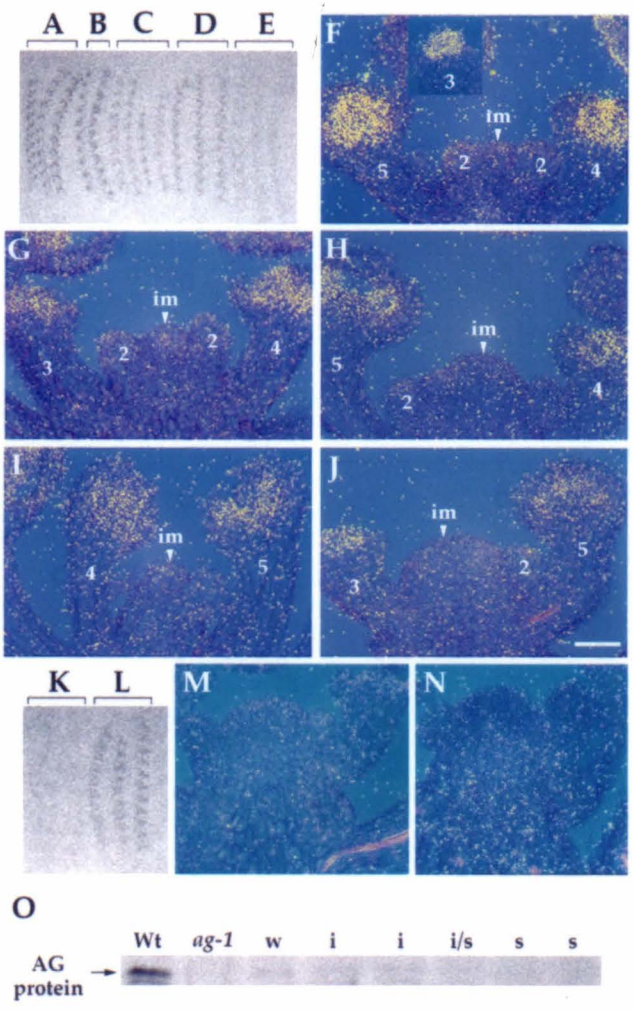

A B C D E
F
3
im
5
2
2
4
G
im
3
2
2
4
H
im
5
2
4
I
im
4
5
J
im
3
2
5
K L
M
N
O
Wt
ag-1
w
i
i
i/s
s
s
AG
protein

Figure 4. Phenotypes of wild-type, *CLV3 (RNAi)* and *clv3-2* plants. Wild-type and *CLV3 (RNAi)* mutants are in the ecotype Wassilewskija, whereas *clv3-2* is in the ecotype Landsberg *erecta* which has reduced internode elongation. The inflorescence meristems are enlarged in *CLV3 (RNAi)* (*B*) and *clv3-2* (*C*) compared with wild type (A). (*D*) Wild-type flower. (*E*) *CLV3 (RNAi)* and (*F*) *clv3-2* flowers have additional organs. (G-I) Cross section of gynoecia showing that the wild-type gynoecium (*G*) consists of two carpels, and gynoecia in *CLV3 (RNAi)* (*H*) and *clv3-2* (*I*) have four carpels.

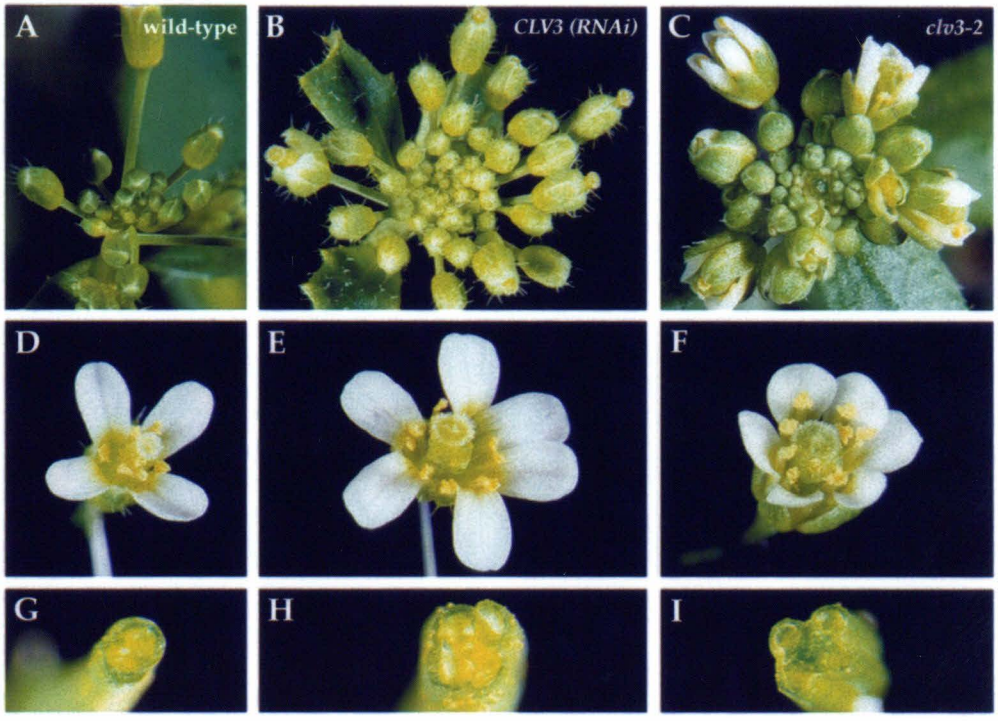

A
wild-type
B
CLV3 (RNAi)
C
clv3-2
D
E
F
G
H
I

Figure 5. Phenotypes of wild-type, *ap1* and *AP1 (RNAi)* flowers. (A) Wild-type flower. (*B*) *ap1-5* flower. (*C*) *ap1-4* flower. (*D*) *ap1-1* flower. (*E-H*) Flowers from weak (*E*), intermediate (*F*), intermediate/strong (*G*) and strong (*H*) *AP1 (RNAi)* plants. Arrowheads indicate leaf- or bract-like first whorl organs. The numbered arrows indicate the primary (1), secondary (2) and tertiary (3) flowers. The black arrows in *C* and *G* indicate leaf-like or staminoid second-whorl organs.

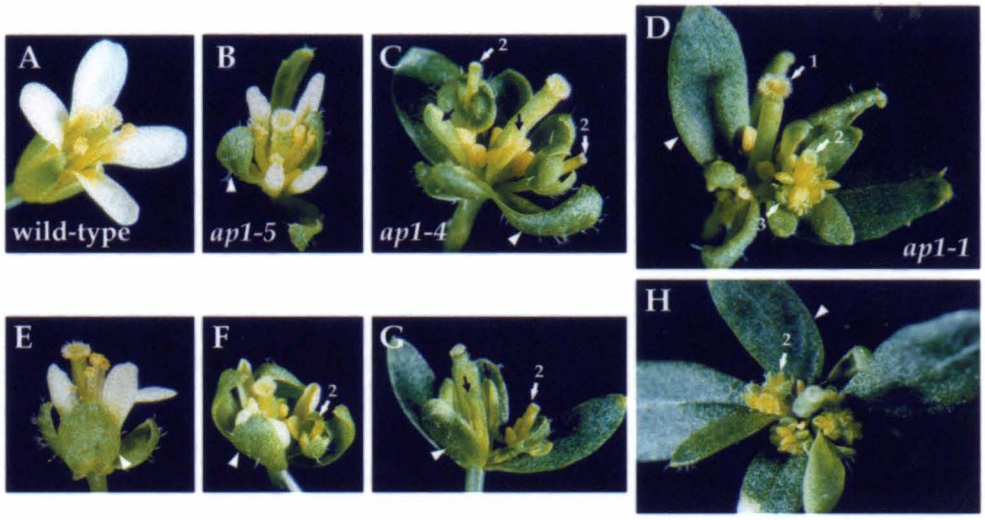
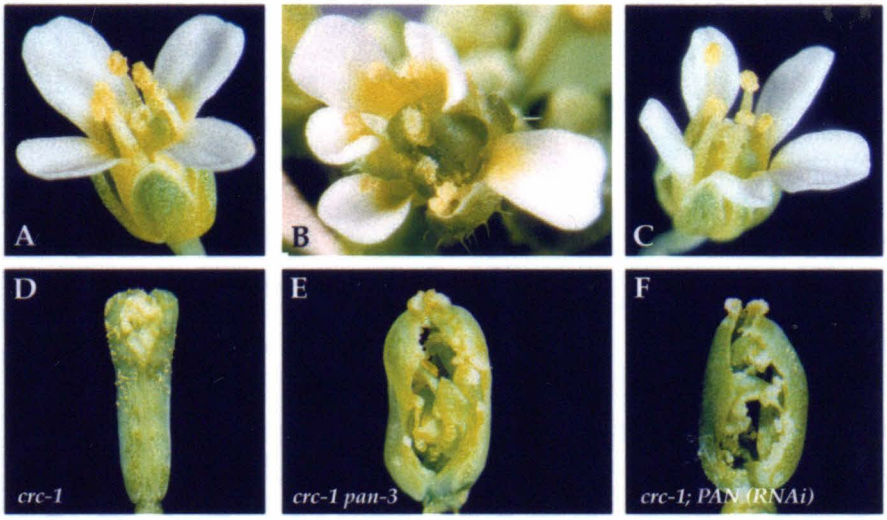

A
wild-type
B
ap1-5
C
ap1-4
D
ap1-1
E
F
G
H

Figure 6. Effects of PAN dsRNA on *crc-1* transgenic plants. (*A* and *D*) *crc-1*. (*B* and *E*) *crc-1 pan-3* and (*C* and *F*) *crc-1; PAN (RNAi)* flowers have extra organs and unfused gynoecia.


A
B
C
D
crc-1
E
crc-1 pan-3
F
crc-1; PAN (RNAi)

Chapter 4

A small gene family, *TOT*, acts in the *PERIANTHIA* pathway patterning *Arabidopsis* flowers

Chiou-Fen Chuang and Elliot M. Meyerowitz

SUMMARY

PERIANTHIA (*PAN*), which encodes a basic region/leucine zipper class of transcription factor, is involved in the determination of organ number, and functions with other genes in carpel development of *Arabidopsis* flowers. Here we isolate three *PAN* target genes, *TOT1*, *TOT2*, and *TOT3*, by using differential display to examine messenger RNAs in transgenic *pan-2* mutant plants carrying a *PAN*-glucocorticoid receptor fusion construct. These genes are closely related and encode products similar to a putative abscisic acid-responsive protein. RT-PCR and RNA blot hybridization analyses show that expression of these genes is up-regulated by *PAN*. Use of dsRNA-mediated interference demonstrates that this gene family is involved in the same pathway as *PAN*, and acts to specify floral patterning.

INTRODUCTION

Arabidopsis plants homozygous for the *perianthia* (*pan*) mutation produce flowers with extra organs in the first two whorls and fewer organs in the third whorl. *pan* mutant flowers usually have five sepals, five petals, five stamens, and two carpels, while wild-type flowers have four sepals, four petals, six stamens, and two carpels. As the ancestors of mustards (such as *Arabidopsis*) had pentamerous flowers, it has been suggested that *PAN*, which encodes a putative basic region/leucine zipper (bZIP) transcription factor, may have been involved in the switch from pentamerous to tetramerous flowers during evolution (Chuang et al., 1999; Running and Meyerowitz, 1996).

In addition to its function in establishing floral organ number, accumulating evidence has indicated that *PAN* is also involved in carpel development. First, mutations in the *PAN* gene cause a delay in carpel fusion in some flowers (Running and Meyerowitz, 1996). Second, *PAN* functions redundantly with *TOUSLED* (*TSL*), *ETTIN* (*ETT*), *CRABS CLAW* (*CRC*), and *LEUNIG* (*LUG*) in gynoecium development (Chuang and Meyerowitz, 2000; Chuang et al., 1999; Roe et al., 1997; Sessions et al., 1997) (C.-F. Chuang and E. M. Meyerowitz unpub.). Lastly, PAN expression in the gynoecium is consistent with a role in the development of carpels (Chuang et al., 1999).

The identification of genes regulated by *PAN* is a step towards understanding the molecular mechanisms by which *PAN* controls floral organ number and carpel development. One way to search for targets of *PAN* is to compare mRNA populations between cells with differential *PAN* activity. Several inducible transcriptional and post-translational activation systems are available in plants (Ainley and Key, 1990; Aoyama and Chau, 1997; Gatz et al., 1992; Lloyd et al., 1994; Mett et al., 1993; Sablowski and Meyerowitz, 1998; Simon et al., 1996;

Wagner et al., 1999; Weinmann et al., 1994). Among these, the glucocorticoid-mediated post-translational activation system provides a highly regulated control of protein activity (Aoyama and Chau, 1997; Lloyd et al., 1994; Sablowski and Meyerowitz, 1998; Simon et al., 1996; Wagner et al., 1999).

In this study, transgenic *pan-2* mutant plants containing a gene fusion of *PAN* and a rat glucocorticoid receptor steroid-binding domain (*PAN-GR*) were constructed to regulate activity of the PAN protein. We applied differential display methods using *pan-2; 35S::PAN-GR* plants to isolate a small gene family of three members, designated *TOT1*, *TOT2*, and *TOT3*. Expression of each of these genes is up-regulated by *PAN*. By using dsRNA interference (RNAi) with the *TOT* genes, we also demonstrate that these genes act in the *PAN* pathway to pattern *Arabidopsis* flowers.

MATERIALS AND METHODS

Construction of *pan-2; 35S::PAN-GR*

The $p\Delta GR_{BX}$ (created by Robert Sablowski) contains the hormone-binding domain of the rat glucocorticoid receptor (GR) inserted in the vector pBluescript II KS (-) (Stratagene). The full-length coding sequences of *PAN* were inserted into the vector $p\Delta GR_{BX}$, creating a translational fusion of PAN and the GR fragment. The resulting protein sequence is PAN (amino acids 1-451)-Ala-Ala-Asp-Pro-GR (amino acids 508-795). PAN-GR was ligated to the *Bam*H I and *Xba* I sites of pCGN1547 (McBride and Summerfelt 1990) into which an 842-bp fragment of the cauliflower mosaic virus 35S promoter and a 253-bp fragment of the 3' end of nopaline synthase (NOS3') had previously been inserted in the *Asp* 718/*Bam*H I and *Xba* I/*Pst* I sites, respectively (Krizek and Meyerowitz, 1996). The 35S::PAN-GR-NOS3' fragment, which was released from the pCGN 1547 with *Kpn* I and

Sph I, was subcloned into the *Bam*H I site of the T-DNA expression vector pDHB321.1. The resulting plasmid was introduced into *pan-2* and transformed plants were selected with Basta herbicide as described previously (Bechtold et al., 1993; Chuang et al., 1999).

Western blot analysis

Total protein was extracted from the inflorescence as described previously (Chuang and Meyerowitz, 2000). After separation on a 10% SDS-polyacrylamide gel, the protein was transferred to nitrocellulose membrane (Schleicher & Schuell), probed with an PAN-specific polyclonal antibody (Chuang et al., 1999) and horseradish peroxidase-labeled secondary antibody (Amersham), and detected with the ECL system (Amersham).

Plant growth and hormone treatment

Transgenic plants were transferred from Basta or Kanamycin selection conditions to a 1:1:1 mixture of soil:vermiculite:perlite and maintained at 22°C beneath 600 foot-candles of constant cool-white fluorescent light. Plants were fertilized at regular intervals.

Dexamethasone (Sigma) was dissolved in ethanol at 5 mM. For complementation tests, 5 μM dilutions made in 0.015% Silwet L-77 (OSI Specialties, Danbury, CT) were applied to the inflorescence every day for 10 days. Dexamethasone was replaced with 0.1% ethanol in the mock treatment. Phenotype was scored three days after the last treatment. For differential display experiments, 10 μM cycloheximide was also added to each treatment.

Differential display and RT-PCR

Total RNA was isolated from the inflorescence with TRI Reagent (Molecular

Research Center) according to the protocols provided. DNase I treatment of RNA, cDNA synthesis from 500 ng of total RNA, PCR amplification, gel analysis, and cloning of differential bands were performed as described previously (Reuber and Ausubel, 1995; Sablowski and Meyerowitz, 1998). The arbitrary primer used to isolate the *TOT2* gene in Figure 2A is LH-AP2 (5'TGCCGAAGCTTAACGAGG3') (Sablowski and Meyerowitz, 1998). In Figure 3A, primers used for PCR amplification are U-N (5'GCTTCCATCAACAACCAC3') and U-3' (5'AGAGAAAACCACTGCGGT3') for *TOT1*, M-N (5'AAAGGTTTCGATGCTTCA3') and M-3' (5'TTAGTAGCTCCTCCGTTT3') for *TOT2*, D-5' (5'AGTAGTCACGGTCGACGG3') and D-C1 (5'TCAATGGTTTCTAGGGCA3') for *TOT3*, and NUBQ (5'GGTGCTAAGAAGAGGAAGAAT3') and CUBQ (5'CTCCTTCTTTCTGGTAAACGT3') for ubiquitin. Conditions for the amplification were as follows: initial denaturation at 94°C for 3 min followed by 28 cycles at 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min. The RT-PCR exponential phase was determined to be from 15 to 30 cycles, to allow quantitative comparisons among cDNAs generated from identical reactions.

Northern blot analysis

Total RNA was isolated as above and 250 µg of total RNA was used to isolate poly(A) RNA with the PolyATract mRNA Isolation Systems (Promega). The RNA was then separated on 1.8% agarose gels containing 1 X MOPS buffer and 2% formaldehyde, blotted to a Biodyne A nylon membrane (Pall BioSupport Div., New York), and hybridized with PCR-labeled probes. Partial *TOT2* cDNA was amplified using primers M-N and M-3'; M-3' was used for probe synthesis. For control hybridization, the blot was also probed with a 250-bp ubiquitin PCR fragment (Sablowski and Meyerowitz, 1998).

Construction of *crc-1; TOT2 (RNAi)* transgenic plants

The p35S::A-GUS-S construct of *TOT2* was made and introduced into *crc-1* plants as described previously (Bechtold et al., 1993; Chuang and Meyerowitz, 2000). *TOT2* cDNA coding sequences used in the construct contain nucleotides 1-630. Transformed plants were selected on Murashige/Skoog (Sigma) plates containing kanamycin (50 μg/ml). Kanamycin-resistant seedlings were then transferred to soil.

RESULTS

Cloning of *TOT* genes by differential display

The transgene *35S::PAN-GR*, in which the *PAN* gene was fused with part of the glucocorticoid receptor and under the control of the viral 35S promoter, was transformed into *pan-2* plants. Western blot analysis using the PAN polyclonal antibody revealed variable expression levels of the PAN-GR fusion protein in flowers of 15 T_1 individuals (data not shown). One line with a high expression level of the fusion protein, as shown in Fig. 1A, was chosen for further characterization. Phenotypic rescue was observed in the dexamethasone-treated T_2 progeny of this line (Fig. 1 B and C), suggesting that the PAN-GR fusion protein is functional. The progeny of one homozygous T_2 plant was used for differential display experiments. The strategy of isolating targets of *PAN* using the differential display approach is outlined in Fig. 1D. The inflorescence of *pan-2; 35S::PAN-GR* (T_3) was treated with cycloheximide alone or cycloheximide combined with dexamethasone. Three parallel treatments were performed for each experiment. After 6 hours of treatment, total RNA was isolated from the treated inflorescence and mRNA populations were compared by RT-PCR analysis in which α^{33}P-dATP was incorporated into PCR products. PCR products

were separated on sequencing gels. The differentially expressed bands that were reproducible in all three treatments of each experiment and in a second independent set of experiments were chosen for sequence analysis.

One of 16 arbitrary primers, with three anchored oligo(dT) primers, produced differentially expressed bands which were reproducible in two independent sets of experiments. These bands were up-regulated by dexamethasone treatment, suggesting that these genes were positively regulated by steroid-activated PAN. Fig. 2A shows an example of the differential bands in three parallel treatments. In contrast, these bands were not differentially expressed in the *pan-2* inflorescence after the dexamethasone treatment, suggesting that these differential bands were dependent on the *35S::PAN-GR* transgene (data not shown).

Sequence analysis showed that the differential bands obtained with the three primer pairs represented the same gene. Therefore, this differential band was observed in 18 independent experiments. Comparison of the cloned sequences to the GenBank database revealed that this gene and another two closely related genes form a cluster on chromosome 5 (Fig. 3A). We named these genes *TOTs* (*T*ri*O* *T*arget*s*). *TOT2* represents the gene isolated by differential display. *TOT1* and *TOT3* are located upstream and downstream, respectively, of *TOT2*. Southern blot analysis with genomic DNA from *Arabidopsis* using a fragment of the *TOT2* gene as a probe demonstrated that the *TOT* gene family is composed of three members (data not shown).

Expression of *TOT* genes is up-regulated by *PAN*

RT-PCR analysis of poly(A) RNA from floral buds (stages 1-12, including the inflorescence meristem) was performed to determine whether *TOT1* and *TOT3* genes are also regulated by *PAN*. Similarly to *TOT2*, *TOT1* and *TOT3* were

positively regulated by steroid-activated PAN (Fig. 2 B). Furthermore, Northern blot analysis showed that expression of the *TOT* gene family was increased in *35S::PAN* plants (by 64%) and decreased in *pan-2* plants (by 31%) compared to wild type (Fig. 2 C and D) (Chuang et al., 1999). These results suggest that each of the *TOT* genes is a potential target of *PAN*. The significant expression level of these genes in *pan-2*, likely a null allele, indicates that members of this gene family are expressed at some levels and in some domains independently of *PAN*.

Promoter regions of *TOTs* contain potential PAN binding sequences

Several plant bZIP proteins have been shown to bind the ACGT *cis*-acting element identified in the promoters of bacterial, viral, and plant genes (Bouchez et al., 1989; Giuliano et al., 1988; Lam et al., 1989; Mikami et al., 1987; Schindler et al., 1992a; Schindler et al., 1992b). Based on their DNA binding specificity and heterodimerization characteristics, these plant bZIP proteins can be classified into at least two classes: the TGACGT/C binding proteins and the G-box (CCACGTGG) binding factor (GBF) family (Foster et al., 1994; Schindler et al., 1992a). Sequence alignment analysis revealed that PAN is more similar to members of the TGACGT/C binding proteins than it is to those of the GBF family (Chuang et al., 1999).

Many potential cis-acting elements with an ACGT core motif were found at the promoter regions of each of the *TOT* genes. Those ACGT-containing motifs that appear at least twice were categorized into three types. There are three, two, and two copies of type 1, 2, and 3, respectively. The type 1, ATGACGTTAG/C, contains the consensus sequences bound by the TGACGT/C binding proteins, which show high degree of similarity to PAN. The type 3, GACACGTGGA, is closely related to the G-box, CCACGTGG. The type 2, GTTACGTAAT, may represent a new class of ACGT element. Two copies of type

1 and one copy of type 2 are located at the promoter region of *TOT1* and *TOT2*. The promoter of *TOT3* contains one copy of type 1 and 2, as well as two copies of type 3 (Fig. 3A).

TOTs encode proteins that are similar to a putative abscisic acid -responsive protein

Each of these *TOT* genes has an intronless coding region, whose size ranges from 630 to 657 bp. The predicted gene products of *TOT1*, *TOT2*, and *TOT3* contain 218, 210, and 219 amino acids, respectively. They are about 79% (174/219) identical and 85% (186/219) similar to one another throughout the whole proteins (Fig. 3B).

TOTs contain two basic regions: one with 32% (9/28) Lys/Arg residues at amino acids 46-74 in TOT2 and 54-82 in TOT1 and TOT3; the other with 35% (6/17) Lys/Arg residues at amino acids 88-105 in TOT2 and 96-113 in TOT1 and TOT3 (Fig. 3B). TOTs also contain one motif, IXXLL (where I is Ile, X is any amino acid, and L is Leu), which is closely related to a consensus motif (LXXLL) that has been shown to mediate binding of transcriptional co-activators to nuclear receptors (Heery et al., 1997; Montminy, 1997; Torchia et al., 1997). The presence of the LXXLL-like motif suggests that TOTs might be targeted to the nucleus, although the basic regions mentioned above do not fit the consensus for nuclear localization signals. In addition, a Src homology 2 (SH2) domain protein profile was found at the C-terminus of each of the predicted TOT proteins using BLOCK Searcher, a program for the detection and verification of protein sequence homology (http://blocks.fhcrc.org/blocks_search.html).

Deduced amino acid sequences encoded by *TOT* genes show similarity to an *Arabidopsis* putative abscisic acid (ABA)-responsive protein (GenBank accession number CAB77730) whose function is still unknown. Their conserved

regions, which are at the C-termini, are 39% (63/160) identical and 67% (107/160) similar (Fig. 3B). ABA responsive elements (ABREs) are present in the promoters of many ABA-inducible genes. The promoter analysis of ABA-responsive genes has revealed a diversity of ABREs, which consist of ACGT-core elements (Guiltinan et al., 1990; Kao et al., 1996; Leung and Giraudat, 1998; Liu et al., 2000; Nelson et al., 1994; Shen and Ho, 1995; Shen et al., 1996; Skriver et al., 1991). Identification of ACGT elements in promoter regions of *TOTs* suggests that *TOTs* might be ABA responsive.

TOT genes function in the _PAN_ pathway

One way to elucidate the biological role of *TOT* genes is to get their loss-of-function or reduction-of-function mutant phenotypes. Previously, we have shown that dsRNA-mediated genetic interference (RNAi) can operate in *A. thaliana* to induce sequence-specific inhibition of gene function. *PAN* RNA-expressing constructs, however, do not show a *pan* phenotype when expressed in a wild-type genetic background. In a *crabs claw* (*crc*) mutant background, however, they cause increases in floral organ number and unfused carpels similar to those in *pan crc* double mutants (Chuang and Meyerowitz, 2000). This result suggests that RNAi can occur in the tissue in which *PAN* normally functions and *crc* mutants provide a more sensitive background than wild type in which to observe phenotypic effects, especially in the carpel, of *PAN* reduction-of-function mutations.

To determine whether *TOT* genes act in the same pathway as *PAN*, the RNA-expressing construct, p35S::A-GUS-S, of *TOT2* was introduced into *crc-1* homozygous plants (Fig. 4B) (Alvarez and Smyth, 1999). The p35S::A-GUS-S construct contains the *TOT2* gene in the antisense and sense orientations linked with the β-glucuronidase (GUS) fragment. A single RNA transcribed from the

fusion gene in p35S::A-GUS-S can potentially form a dsRNA stem with a single-stranded loop structure (Fig. 4A). Since coding regions of *TOT1*, *TOT2*, and *TOT3* share about 83% (543/657) identity in DNA sequences (data not shown), they could be interfered in a similar way. *TOT* genes and the *Arabidopsis* putative ABRP share only 42% identity throughout their whole open reading frames (data not shown); therefore, *TOT2* dsRNA would not be expected to interfere with ABRP.

We referred to *crc-1* plants carrying a functional p35S::A-GUS-S construct of *TOT2* as *crc-1; TOT2 (RNAi)* (Fig. 4D). Three *crc-1; TOT2 (RNAi)* plants, which represent 1.3% of the transgenic plant population (n = 223), showed weak, intermediate, or strong enhancement of the carpel fusion phenotype of *crc-1*. The strong *crc-1; TOT2 (RNAi)* line exhibited moderate expressivity of the extra organ phenotype (data not shown) and high penetrance of drastically unfused carpels in a manner similar to that of *pan-3*, a strong *pan* allele, except that the RNAi plant has smaller floral organs (Fig. 4 C and D). In addition, this strong line had low stature, infertility, and an enlarged shoot apical meristem with distortions in phyllotaxy and bifurcation, flattening and broadening of the stem (data not shown). Most transgenic plants carrying functional antisense (11.4%, n = 70) or sense (4.5%, n = 88) sequences of the *TOT2* gene caused weak interference effects on the carpel fusion phenotype and low, or no, expressivity of the extra organ phenotype in *crc-1* (data not shown). However, one transgenic plant of the antisense construct showed intermediate/strong carpel fusion phenotype (data not shown).

DISCUSSION

We previously showed that *PAN* encodes a bZIP class of transcription factor,

suggesting that *PAN* controls floral organ number and carpel development via transcriptional regulation of downstream genes (Chuang et al., 1999). Here we report a small gene family composed of three closely related members, *TOT1*, *TOT2*, and *TOT3*, which were up-regulated by *PAN*. Based on their high similarity of amino acid sequences and up-regulation by *PAN*, members of the *TOT* gene family might function redundantly in the *PAN* pathway. The fact that these genes were positively regulated by steroid-activated PAN in the presence of cycloheximide, a protein synthesis inhibitor, indicates that *TOT* genes are immediate targets of *PAN*. In addition, identification of putative PAN binding sites in the promoter regions of *TOTs* suggests that these genes might be direct transcriptional targets of *PAN*. *TOT* gene expression levels were reduced by only 31% in *pan*-2, a null-like allele, suggesting that a large proportion of their expression is not related to *PAN*.

Mutations in *PAN* mainly caused the extra organ phenotype in wild-type plants. Whereas flowers homozygous for strong *pan* mutant alleles show high penetrance of the extra organ phenotype, only the first few flowers from homozygotes of weak *pan* mutant alleles show the phenotype. However, both strong and weak *pan* alleles cause high penetrance of unfused carpel phenotypes in a *crc-1* genetic background. Previously, we reported that *PAN (RNAi)* plants displayed very weak, or no, extra organ phenotype in wild type and *crc-1* backgrounds, but high expressivity of unfused carpel phenotypes in *crc-1* (Chuang and Meyerowitz, 2000). These results indicate that *crc* mutants provide a more sensitive background than wild type in which to observe phenotypic effects of *PAN* reduction-of-function mutations and that RNAi under our conditions does not work efficiently to fully reflect the role of *PAN* in the determination of floral organ number. Similar to *crc-1; PAN (RNAi)*, *crc-1; TOT2 (RNAi)* plants have drastic effects on carpel fusion phenotypes, but weak, or no,

effect on the organ number. For unknown reasons, the frequency of *crc-1; TOT2 (RNAi)* plants (1.3%, n = 223) is lower than that of transgenic plants containing functional antisense (11.4%, n = 70) and sense (4.5%, n = 88) constructs of *TOT2*. We propose that *TOT* genes might be also essential for embryonic development and the embryonic lethality phenotype is less sensitive to the level of *TOT* gene activity, such that higher levels of dsRNA would cause embryonic lethality. Therefore, *crc-1; TOT2 (RNAi)* plants may be more potent at inducing embryo lethality than those carrying antisense and sense constructs. Examination of *TOT* gene expression in embryos and use of inducible RNAi would be able to test this possibility.

The *TOT* genes encode products similar to a putative ABA-responsive protein (GenBank accession number CAB77730). ABA is a plant phytohormone, which regulates diverse aspects of developmental processes including abscission, seed development, and germination, as well as responses to environmental stresses (Leung and Giraudat, 1998). One of the ABA-mediated events is the induced expression of a large number of genes that is controlled by cis-regulatory regions known as ABREs, which contain an ACGT core motif. It has been shown that factors that interact with the ABRE are largely, if not entirely, bZIP proteins (Choi et al., 2000; Guiltinan et al., 1990; Kim et al., 1997; Nakagawa et al., 1996; Nantel and Quatrano, 1996; Oeda et al., 1991). The promoter region of each *TOT* gene contains several potential ABREs. This suggests that PAN, which is a bZIP protein, may be involved the ABA regulatory network to modulate expression of the *TOT* genes, which are putative ABA-responsive genes. It is possible that the activity of *PAN* is regulated post-translationally by the ABA signaling pathway. The discovery of *TOT* genes reveals a possible role of ABA signal transduction in determination of floral organ number and carpel development. It is also possible that *TOT* genes are involved in a variety of

developmental processes, suggested by their expression pattern (C.-F. Chuang and E. M. Meyerowitz, unpub.) and broad phenotypes in *crc-1; TOT2 (RNAi),* and their function in the *PAN* pathway is controlled by signals other than ABA.

With the predicted SH2 protein profile, TOT proteins might function as adaptors to couple cell-surface receptors to effector proteins. SH2 is a domain of approximately 100 amino acids containing a binding site for phosphotyrosyl peptides. SH2 domains have been found in a diverse group of signaling molecules and oncogenic proteins (Kuriyan and Cowburn, 1997; Meng et al., 1999). Among these, Cbl is an adaptor protein whose crystal structure revealed a divergent SH2 domain that was not recognizable from the amino acid sequence of the protein (Meng et al., 1999). Although the deduced amino acid sequences of the predicted TOT proteins do not match those of typical SH2 domains, it is still possible that TOT proteins contains a domain with structural similarity to SH2. Structural determination of TOT proteins would be one way to test this possibility.

We conclude that both regulation of *TOT* expression by *PAN* and loss-of-function phenotypes in *crc-1; TOT2 (RNAi)* point to a role of *TOT* genes in the *PAN* pathway to control floral patterning.

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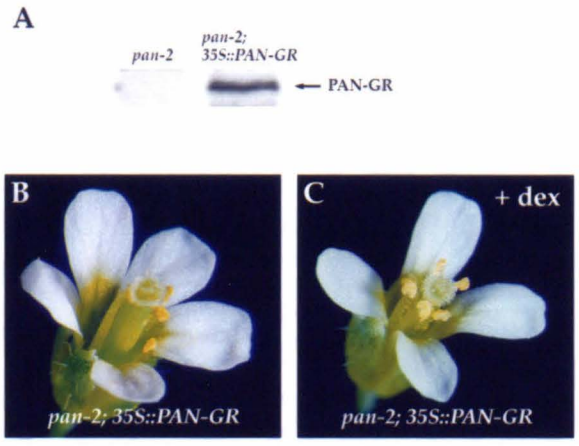
Figure 1. Use of *pan-2; 35S::PAN-GR* to isolate targets of *PAN*. (A) Western blot analysis of PAN-GR protein. Whereas PAN-GR protein is not detectable in *pan-2*, it is highly expressed in *pan-2; 35S::PAN-GR* plants. (B) Flower of *pan-2; 35S::PAN-GR* with mock treatment. (C) Flower of *pan-2; 35S::PAN-GR* treated with dexamethasone (dex). (D) Strategy of isolating targets of *PAN*. The inflorescence of *pan-2; 35S::PAN-GR* was treated with cycloheximide (cyc) alone or cycloheximide combined with dexamethasone. After 6 hours of treatment, the mRNA populations were compared by RT-PCR analysis. In this study, 16 arbitrary primers with three anchored oligo(dT) primers were used. α^{33}P-dATP - labeled PCR products were separated on sequencing gels and differentially expressed bands were sequenced.


A
pan-2
pan-2;
35S::PAN-GR
PAN-GR
B
pan-2; 35S::PAN-GR
C
+ dex
pan-2; 35S::PAN-GR

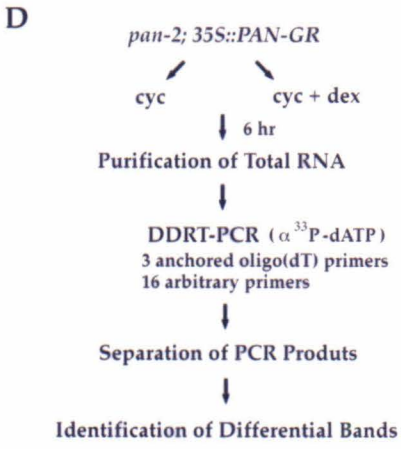

D
pan-2; 35S::PAN-GR
cyc
cyc + dex
6 hr
Purification of Total RNA
DDRT-PCR (α 33P-dATP)
3 anchored oligo(dT) primers
16 arbitrary primers
Separation of PCR Produts
Identification of Differential Bands

Figure 2. Positive regulation of *TOT* gene expression by *PAN*. (A) Identification of differentially expressed bands. The *TOT2* gene was differentially expressed between the *pan-2; 35S::PAN-GR* inflorescence with (+) and without (-) dexamethasone (dex) treatment. Cycloheximide (cyc) was added (+) in each treatment. (B) RT-PCR analysis of each of the *TOT* genes in response to dexamethasone treatment in the *pan-2; 35S::PAN-GR* inflorescence. Expression of the ubiquitin (UBQ) gene was examined as a quantity control. (C) Northern blot analysis of *TOT* gene expression in the inflorescence of *35S::PAN*, Ws and *pan-2* plants. UBQ expression was used as a quantity control. (D) Quantitative analysis of results shown in B. *TOT* expression was normalized by UBQ expression. The relative expression level of *TOT* genes in *35S::PAN*, Ws, and *pan-2* is 1.64, 1 and 0.69.

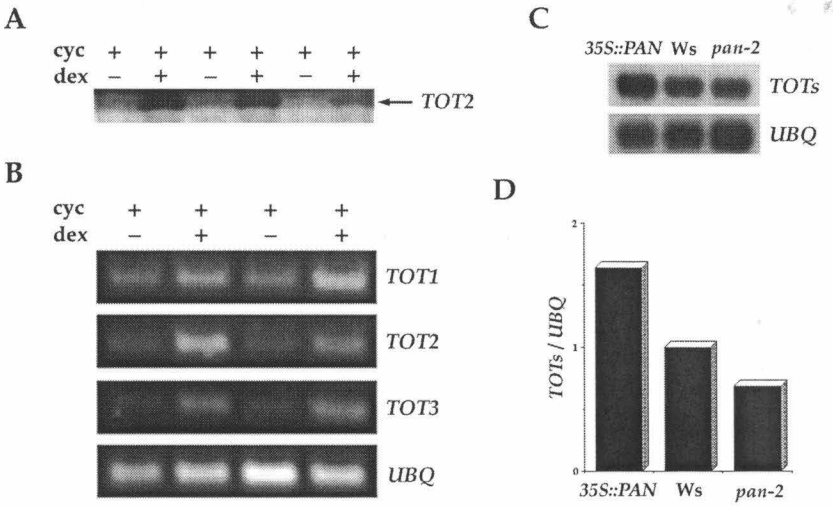
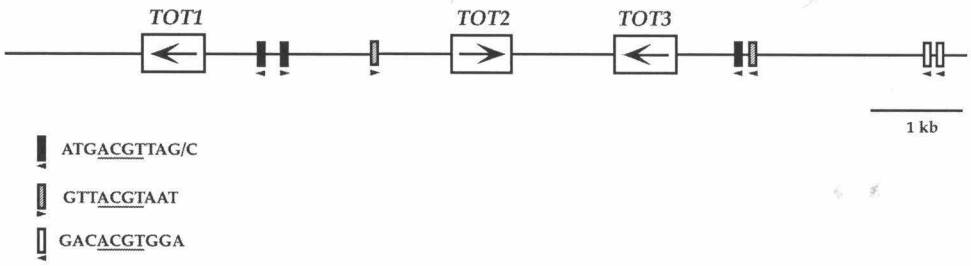

A
cyc + + + + + +
dex – + – + – +
TOT2
B
cyc + + + +
dex – + – +
TOT1
TOT2
TOT3
UBQ
C
35S::PAN Ws pan-2
TOTs
UBQ
D
TOTs / UBQ
2
1
0
35S::PAN
Ws
pan-2

Figure 3. *TOT* is a small gene family. (A) Relative location of *TOT1*, *TOT2*, and *TOT3* on chromosome 5. Arrows indicate the orientation of each open reading frame. Black, striped, and open boxes indicate type 1, 2, and 3, respectively, of ACGT elements. Arrowheads indicate the orientation of each element. (B) Alignments of the deduced amino acid sequences of the predicted TOT1, TOT2, and TOT3 proteins with the putative ABA-responsive protein (ABRP). Sequences were aligned with the program MacVector (Oxford Molecular) using the ClustalW Alignment method. Amino acid residues identical and similar in at least three of the four sequences are displayed in black and gray boxes, respectively. Hyphens indicate gaps introduced to maximize alignment. Indicated regions are: I and II, basic regions; III, LXXLL-like motif; IV, SH2 domain protein profile.

A


TOT1
TOT2
TOT3
1 kb
ATGACGTTAG/C
GTTACGTAAT
GACACGTGGA

B

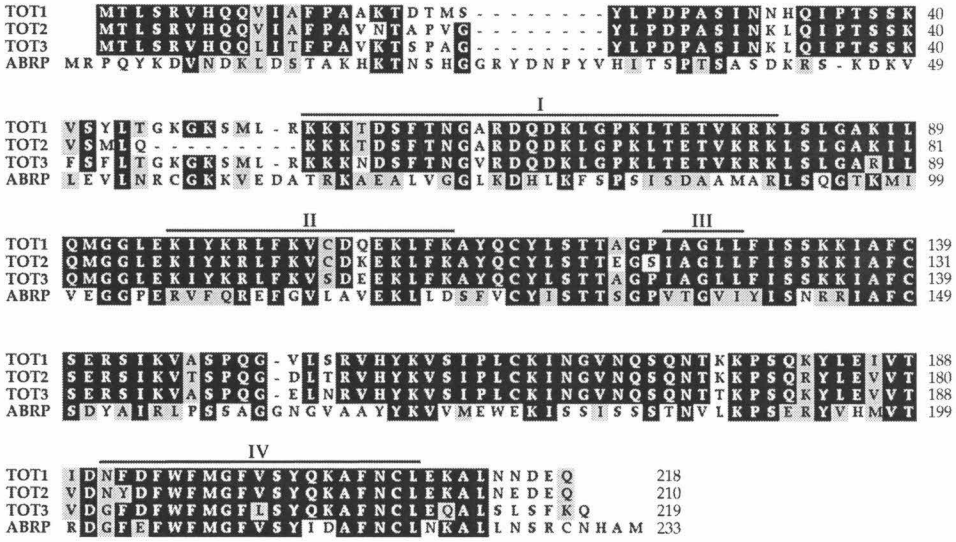
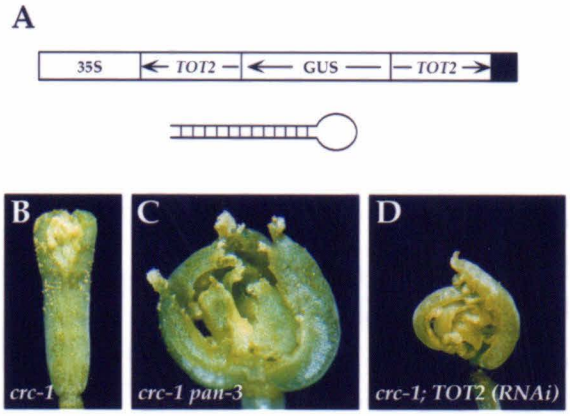

TOT1 MTLSRVHQQVIAFPAAKTDTMS--------YLPDPASINNHQIPTSSK 40
TOT2 MTLSRVHQQVIAFPAVNTAPVG--------YLPDPASINKLQIPTSSK 40
TOT3 MTLSRVHQQLITFPAVKTSPAG--------YLPDPASINKLQIPTSSK 40
ABRP MRPQYKDVNDKLDSTAKHKTNSHGGRYDNPYVHITSPTSASDKRS-KDKV 49
I
TOT1 VSYLTGKGKSML-RKKKTDSFTNGARDQDKLGPKLTETVKRKLSLGAKIL 89
TOT2 VSMLQ--------KKKTDSFTNGARDQDKLGPKLTETVKRKLSLGAKIL 81
TOT3 FSFLTGKGKSML-RKKKNDSFTNGVRDQDKLGPKLTETVKRKLSLGARIL 89
ABRP LEVLNRCGKKVEDATRKAEALVGGLKDHLKFSPSISDAAMARLSQGTKMI 99
II
III
TOT1 QMGGLEKIYKRLFKVCDQEKLFKAYQCYLSTTAGPIAGLLFISSKKIAFC 139
TOT2 QMGGLEKIYKRLFKVCDKEKLFKAYQCYLSTTEGSIAGLLFISSKKIAFC 131
TOT3 QMGGLEKIYKRLFKVSDEEKLFKAYQCYLSTTAGPIAGLLFISSKKIAFC 139
ABRP VEGGPERVFQREFGVLAVEKLLDSFVCYISTTSGPVTGVIYISNRRIAFC 149
TOT1 SERSIKVASPQG-VLSRVHYKVSIPLCKINGVNQSQNTKKPSQKYLEIVT 188
TOT2 SERSIKVTSPQG-DLTRVHYKVSIPLCKINGVNQSQNTKKPSQRYLEVVT 180
TOT3 SERSIKVASPQG-ELNRVHYKVSIPLCKINGVNQSQNTTKPSQKYLEVVT 188
ABRP SDYAIRLPSSAGGNGVAAYYKVVMEWEKISSISSSTNVLKPSERYVHMVT 199
IV
TOT1 IDNFDFWFMGFVSYQKAFNCLEKALNNDEQ 218
TOT2 VDNYDFWFMGFVSYQKAFNCLEKALNEDEQ 210
TOT3 VDGFDFWFMGFLSYQKAFNCLEQALSLSFKQ 219
ABRP RDGFEFWFMGFVSYIDAFNCLNKALLNSRCNHAM 233

Figure 4. Phenotypes of *crc-1*, *crc-1 pan-3*, and *crc-1; TOT2 (RNAi)* carpels. (A) The dsRNA-expressing construct, p35S::A-GUS-S, of *TOT2*. A schematic structure of the predicted dsRNA stem with a single-stranded loop generated by the construct is shown. Arrows indicate the orientation of gene sequences. 35S, the 35S promoter; solid box, the 3' end of nopaline synthase. (B) crc-1. (C) *crc-1 pan-3*. (D) *crc-1; TOT2 (RNAi)*.


A
35S
TOT2
GUS
TOT2
B
crc-1
C
crc-1 pan-3
D
crc-1; TOT2 (RNAi)

Appendix

Dominant negative mutants of a *PAN* homolog, *PAL1*, suppress stamen and carpel development in *Arabidopsis*

Chiou-Fen Chuang and Elliot M. Meyerowitz

Abstract

PERIANTHIA (*PAN*) mutations cause *Arabidopsis* flowers to have a five-fold instead of four-fold symmetry and the *PAN* gene encodes a member of basic region/leucine zipper class of transcription factors. Here we describe the isolation and functional analysis of a *PAN* like gene, *PAL1*. *In situ* hybridization showed that *PAL1* expression in developing flowers is associated with the initiation and development of the stamen and ovule primordia. To investigate the biological role of *PAL1*, we generated transgenic plants overexpressing a dominant negative form of PAL1 in which the DNA binding domain was deleted. The dominant negative mutants of *PAL1* mainly suppress stamen and carpel development, which is consistent with its expression pattern in flowers. We conclude that *PAL1* seems to control stamen and carpel development by regulating downstream genes involved in cell division and cell differentiation.

The class of basic region/leucine zipper (bZIP) proteins are the largest known family of DNA-binding factors in plants. The basic region is implicated in DNA binding and the leucine zipper mediates homo- and hetero-dimerization. Although several plant bZIP proteins have been shown to bind the ACGT *cis*-acting element identified in the promoters of bacterial, viral, and plant genes (Bouchez et al., 1989; Giuliano et al., 1988; Lam et al., 1989; Mikami et al., 1987; Schindler et al., 1992a; Schindler et al., 1992b), the physiological roles of bZIP proteins have been demonstrated only in some cases.

PERIANTHIA (PAN) and HY5 are *Arabidopsis* bZIP proteins whose functions have been deciphered by mutant analyses. *pan* was isolated mainly by its extra organ mutant phenotype (Running and Meyerowitz, 1996). *pan* mutant flowers usually have five sepals, five petals, five stamens, and two carpels. It has been suggested that *PAN* may be involved in the switch from pentamerous to tetramerous flowers during the evolution of flower form in the mustard family (Chuang et al., 1999; Running and Meyerowitz, 1996). The *HY5* gene was shown to be a positive regulator of photomorphogenic development (Ang and Deng, 1994; Chory, 1992). Plants mutated for the *HY5* gene are defective in various aspects of morphogenesis and stimulus responses in the hypocotyl and roots (Oyama et al., 1997). In addition to the bZIP motif, the PAN protein also contains two glutamine-rich regions near the C terminus which may function as transcriptional activation domains (Chuang et al., 1999). However, the HY5 protein has no obvious transcriptional activation domains (Oyama et al., 1997).

Although *PAN* was well characterized by genetical, phenotypical, and molecular analysis, little is known about how *PAN* functions at the cellular level to specify floral organ patterning. Understanding the cellular function of closely related genes of *PAN* may shed lights on this question.

In an attempt to isolate homologues for *PAN*, we used the *PAN* cDNA at

moderate stringency to probe a λ phage genomic library of *Arabidopsis* DNA, yielding many positive clones. Among ten clones analyzed, nine clones represent two previously isolated genes, and only one clone, *PAL1* (PAN-Like), was a previously unidentified gene. The corresponding cDNA of *PAL1* was cloned from a library constructed from floral bud poly(A) RNA. The full-length open reading frame determined by 5'RACE-PCR has the potential to encode a protein of 401 amino acids (Fig. 1A). The predicted proteins of *PAN* and *PAL1* share 85% identity in the basic regions/leucine-zipper motifs and share 61% identity in the C-terminal half containing putative transactivation domains. Differences are most pronounced at the N-terminus (Fig. 1B). *PAL1* was mapped to the top arm of chromosome 1 by RFLP analysis (data not shown).

The pattern of the *PAL1* expression was analyzed by RNA blots as shown in Fig. 2. Unlike the *PAN* transcript detected only in young flowers, significant levels of the 1.9 kb *PAL1* transcript was found in different tissues including the roots, leaves, stems, and young flowers (Fig. 2).

In situ hybridization was performed to determine the precise spatial and temporal expression pattern of *PAL1* in developing flowers. The earliest expression of *PAL1* in wild-type flowers is in the stamen primordia at stage 5 (Fig. 3A) and remains strongly expressed in the developing stamen at stage 6-8 flowers (Fig. 3A-C). During stage 9, *PAL1* expression in the stamen becomes restricted to anther locules (Fig. 3D-F). In addition to the stamen, *PAL1* RNA is also detected in the ovule primordia in stage 9 flowers (Fig. 3D). During stage 12, *PAL1* transcript in anthers is restricted to stromiums and cells comprising the wall of the pollen sac (Fig. 3G). By contrast, only low background is detected in control experiments using a *PAL1* sense probe (data not shown).

It has been suggested that bZIP proteins with mutated basic DNA binding domains can function as *trans*-dominant negative mutants (Ransone et al., 1990;

Rieping et al., 1994; Unger et al., 1993). One dominant mutant form of the *PAL1* gene, *PAL1ΔBR*, was constructed by deleting a region that encodes the basic DNA binding domain (Fig. 4A). The *PAL1ΔBR* driven by the constitutive 35S promoter from cauliflower mosaic virus was transformed into wild-type plants. The T_1 plants which contained the *35S::PAL1ΔBR* transgene were analyzed.

In the most severe *35S::PAL1ΔBR* flowers (early-arising flowers), anthers are about midway below the stigma and most anthers do not produce any pollen (Fig. 4C). The scanning electron microscopy (SEM) was performed to determine the cell number and cell size of stamen filaments from wild type (Fig. 4E) and *35S::PAL1ΔBR* flowers (Fig. 4F). The short filament in *35S::PAL1ΔBR* flowers is caused by reduction in both cell size and cell number (Fig. 4F). In addition, the anther in *35S::PAL1ΔBR* did not show the characteristic morphology found in wild type (Figs. 4H,I). Pollen grains are visible in wild-type anthers (Figs. 4B,H); however, most anthers in *35S::PAL1ΔBR* do not produce any pollen (Figs. 4C,I). In some early flowers of *35S::PAL1ΔBR*, the gynoecium did not close over and did not produce the stigmatic papillae either. Instead, undifferentiated tissue was formed on the top of unfused carpels (Fig. 4C). However, most apical flowers (late-arising flowers) produce long stamens, rare pollens, and carpels bearing the stigmatic papillae (Figs. 4D,G,J). The yeast two-hybrid assay suggests that PAN and PAL1 cannot interact with each other (data not shown), which is consistent with the fact that dominant negative mutants of *PAL1* did not show the *pan* mutant phenotype.

The dominant negative mutants of *PAL1* mainly suppress stamen and carpel development, in agreement with its expression pattern in the reproductive tissue. SEM analysis further suggests that *PAL1* may control stamen and carpel development by regulating downstream genes involved in cell division and cell differentiation. By analogy to the cellular function of *PAL1*, *PAN* might regulate

the relative timing and the relative rate of differentiation between different floral organ primordia in the same whorl.

Materials and methods

Isolation of PAL1

A λ phage genomic library made from wild-type Columbia ecotype of *Arabidopsis* (Hua et al., 1995) was screened at moderate stringency (53°C hybridization and a final wash with 2X SSPE) with the *PAN* cDNA as a probe. The cDNA library constructed from floral bud poly(A) RNA of Landsberg erecta ecotype (Weigel et al., 1992) was screened at high stringency with a 650-bp PCR fragment of *PAL1* genomic DNA as a probe. Remaining sequences (residues 1-396) of the full-length *PAL1* open reading frame were determined by 5' RACE System (BRL) using total RNA from floral buds of Wassilewskija ecotype.

Northern Blot Analysis

Total RNA was isolated from roots (from liquid culture), leaves, stems, bud clusters (stage 1-12, including the inflorescence meristem), and open flowers (after stage 12) using the TRI REAGENT (Molecular Research Center). 700 μg of total RNA was used to isolate poly(A) RNA using the PolyATract mRNA Isolation Systems (Promega). The RNA was then separated on 1% agarose gels, blotted to Hybond-N nylon membrane (Amersham), and hybridized with PCR-labeled probes. Partial *PAL1* cDNA was amplified using N0 (5' CTTCAACCTCATCATAAT 3') and C1 (5' GACTGAACAATCTGCAA 3'); C1 was used for probe synthesis. For control hybridization, the blot was also

probed with a 250-bp ubiquitin PCR fragment (Sablowski and Meyerowitz, 1998).

In Situ Hybridization

To generate *PAL1* probes, a 208-bp fragment of the *PAL1* cDNA was amplified by PCR using -49 (5' GAGACTAATCAAGAAGAG 3') and 5'C1 (5' TGCTTCTTCTAGCTCTCC 3') and cloned into the TA cloning vector (Invitrogen). In vitro transcription and ^{35}S labeling of probes was performed using the Promega Riboprobe kit, with template linearized by *Spe* I and transcribed by T7 polymerase (sense probe), or linearized by *Nco* I and transcribed by SP6 polymerase (antisense probe). Tissue was fixed, dehydrated, cleared, embedded, and sectioned as described (Chuang et al., 1999). *In situ* hybridization was performed as described previously (Drews et al., 1991; Sakai et al., 1995). Exposure time was 4-5 weeks.

Constructs

The *PAL1* cDNA containing 1443 bp of the full-length coding region and 193 bp of the 3' untranslated sequence was cloned into the *Bam*HI and *Kpn* I sites of pGEM7zf(+) (Promega) with the N-terminal sequence oriented toward the *Bam*HI site of the polylinker, resulting in pGEM7-PAL1. To construct pGEM7-PAL1ΔBR, two *PAL1* cDNA fragments encoding amino acid residues 86-180 (PCR amplified by primers: NXbaI + CAflII and cut with *Msc* I + *Afl* II) and 198-258 (PCR amplified by primers: NAflII + C807 and cut with *Afl* II + *Nde* I) were cloned into pGEM7-PAL1 cut with *Msc* I and *Nde* I, which released the fragment encoding amino acids 86-258 of PAL1 protein. The PAL1ΔBR was excised with

*Bam*HI and *Xba* I and placed downstream of the cauliflower mosaic virus 35S promoter in pCGN1547 (Bechtold et al., 1993; Krizek and Meyerowitz, 1996; McBride and Summerfelt, 1990). The resulting plasmid, pCGN-PAL1ΔBR, was used for *Agrobacterium*-mediated transformation as described (Chuang and Meyerowitz, 2000). The sequences of primers used are as follows: NXbaI, 5' ACCTAGTCTAGAATGTTC CCTTCGTGGCCA 3'; CAflI, 5' CTGAGGCTTA AGCGTCTTAGCATCAAGCTG 3'; NAflII, 5' CTGAGGCTTAAGGCGTATGT GCAACAG 3'; C807, 5' AGCCTGAAG ACCGGTTCG 3'. The *Afl* II restriction site used to ligate two *PAL1* cDNA fragments is underlined.

Scanning Electron Microscopy

Samples were fixed, dried, coated, dissected, and photographed as described (Bowman et al., 1989; Bowman et al., 1991).

Acknowledgments

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Figure 1. Comparison of the gene products of *PAN* and *PAL1*. The predicted proteins of *PAN* and *PAL1* share 85% identity in the basic regions/leucine-zipper motifs, and 61% identity in the C-terminal half containing putative transactivation domains. Differences are most pronounced at the N-terminus.

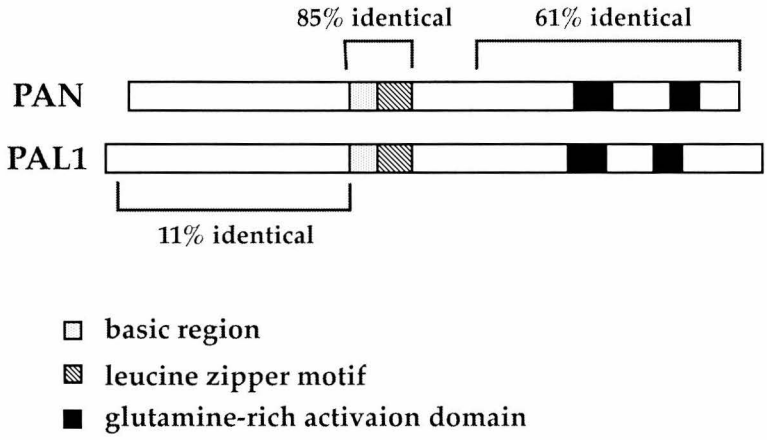

85% identical
61% identical
PAN
PAL1
11% identical
basic region
leucine zipper motif
glutamine-rich activaion domain

Figure 2. Northern analysis of *PAL1* mRNA. Poly(A) RNA isolated from roots (R), leaves (L), stems (S), bud clusters (B, stage 1-12, including the inflorescence meristem), and open flowers (F, after stage 12) were hybridized to the PCR-labeled probe made from *PAL1* cDNA. The blot reprobed with ubiquitin is shown to allow comparison of the total amount of RNA per sample. The *PAL1* transcript is detected in roots, leaves, stems, and bud clusters.

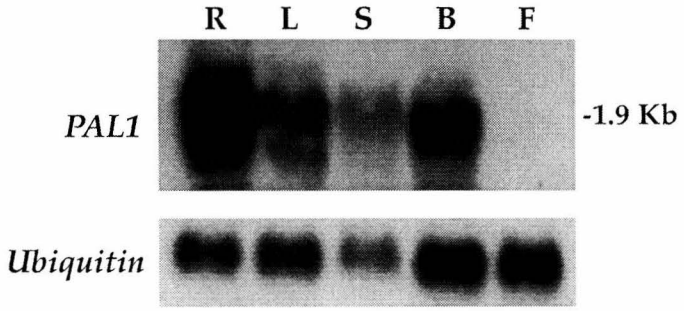
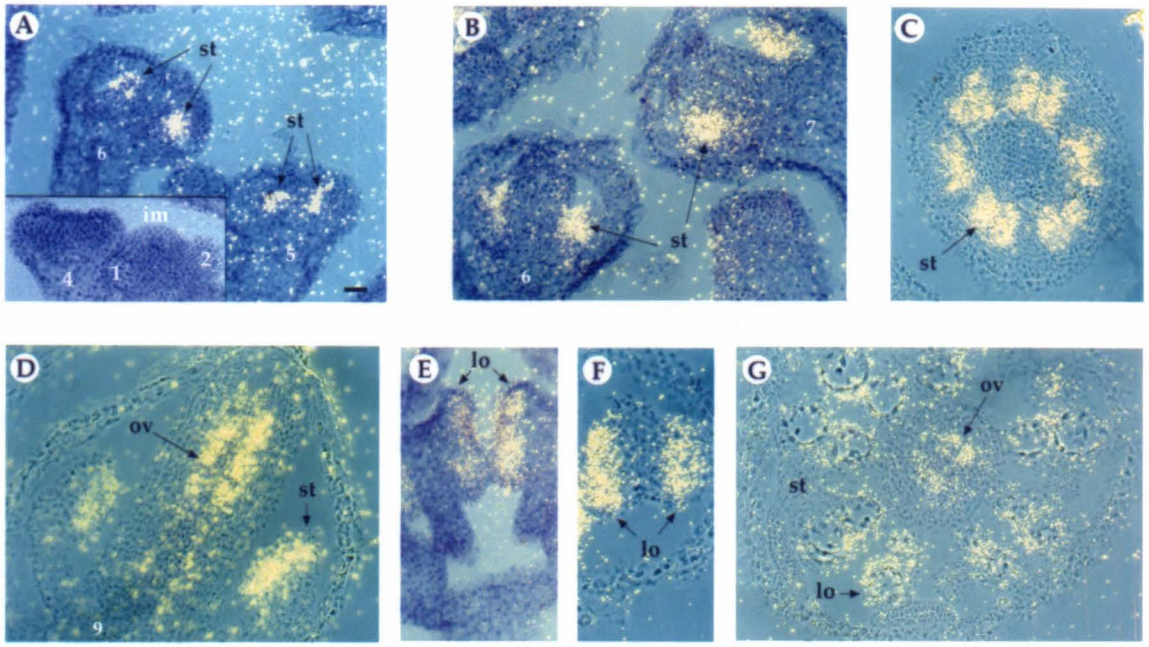

R
L
S
B
F
PAL1
-1.9 Kb
Ubiquitin

Figure 3. Expression of *PAL1* RNA in wild-type flowers. (A) Longitudinal section of an inflorescence meristem (im) with stages 1, 2, 4, 5, and 6 flowers. The number indicated corresponds to the development stage (Smyth et al., 1990). The earliest expression of *PAL1* in wild-type flowers is in the stamen (st) primordia at stage 5 and remains highly expressed in the stamen at stage 6. Bar, 20 μm. (B) Longitudinal section of stage 6, 7 flowers. (C) Cross section of a stage 8 flower. *PAL1* is expressed only in the stamen. (D) Longitudinal section of a stage 9 flower. In addition to the stamen, *PAL1* RNA is also detected in the ovule (ov) primordia. Side view of stamens (E) and adaxial view of the anther (F) in stage 9 flowers. *PAL1* expression in the stamen becomes restricted to anther locules (lo). (G) Cross section of a stage 12 flower. *PAL1* transcript in anthers is restricted to stromiums and cells comprising the wall of the pollen sac.


A
st
st
6
im
4
1
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ov
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9
E
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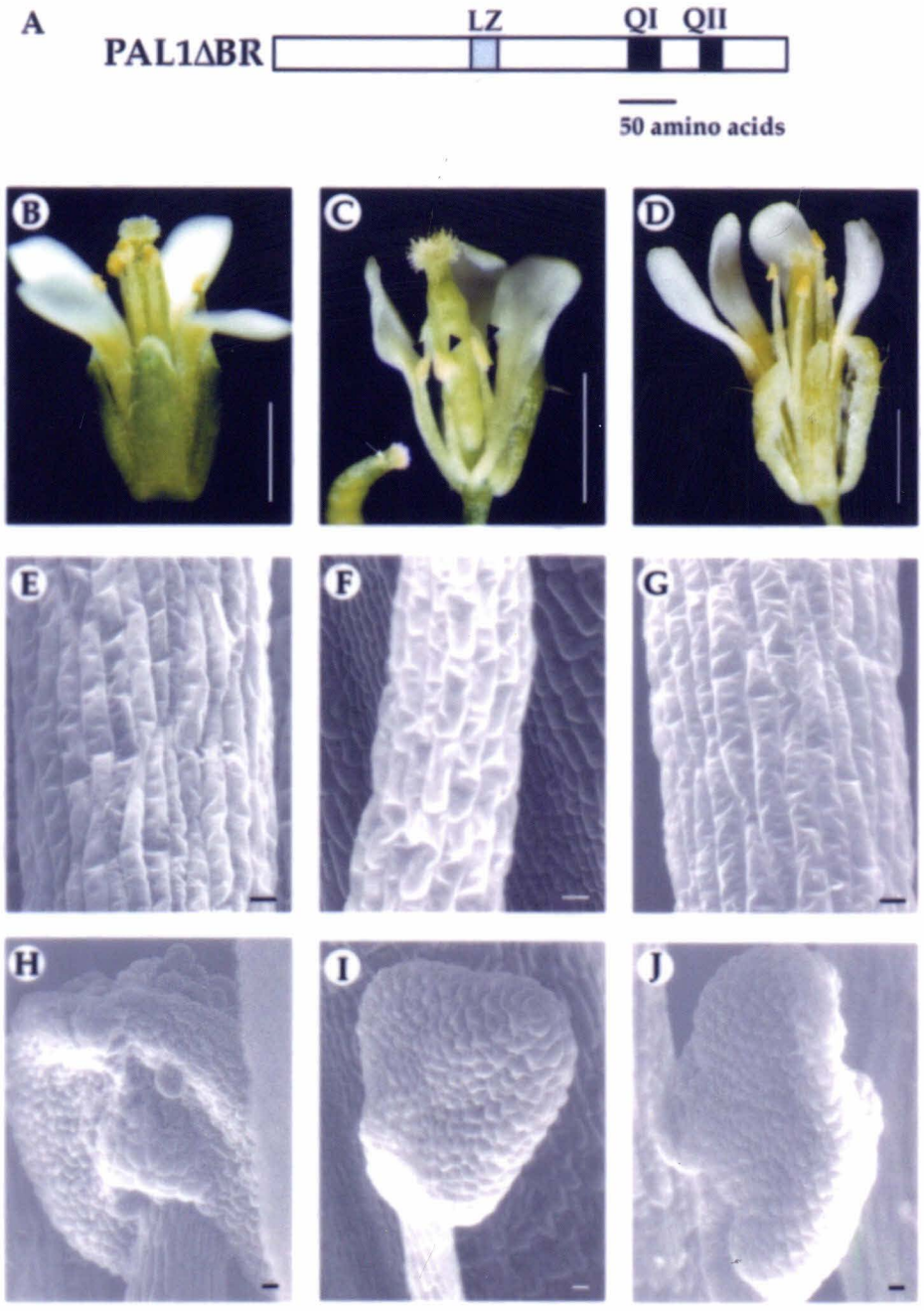
ov
st
lo

Figure 4. Flowers of wild-type and dominant negative mutants of *PAL1* (*35S::PAL1ΔBR*). (A) Mutant form of PAL1. The basic DNA binding domain was deleted, but the leucine zipper dimerization domain was still maintained. (B-D) Bars, 1 mm. (E-G) Scanning electron micrographs of filaments. Bars 10 μm. (H-J) Scanning electron micrographs of abaxial view of anthers. Bars, 10 μm. (B, E, H) Wild-type flowers. (C, F, I) *35S::PAL1ΔBR* basal flowers. In most severe *35S::PAL1ΔBR* flowers, anthers (arrowhead) are about midway below the stigma. In some early flowers, the gynoecium did not close over and did not produce the stigmatic papillae either. Instead, undifferentiated tissue (arrow) was formed on the top of unfused carpels (C). The short filament of the stamen in *35S::PAL1ΔBR* is caused by reduction in both cell size and cell number (F). The anther in *35S::PAL1ΔBR* (I) did not show the characteristic morphology found in wild-type (H). Pollen grains are visible in wild-type anthers (H, arrow); however, anthers in *35S::PAL1ΔBR* basal flowers did not produce any pollen. (D, G, J) *35S::PAL1ΔBR* apical flowers. The elongation of filaments (D), the cell size and the cell number of the filament (G) are quite normal compared to wild-type (B, E). Although the morphology of the anther is not totally normal (J), it did produce rare pollen.


A
PAL1ΔBR
LZ
QI
QII
50 amino acids
B
C
D
E
F
G
H
I
J