Development 138, 3591 (2011) doi:10.1242/dev.072058

RABBIT EARS, encoding a SUPERMAN-like zinc finger protein, regulates petal development in *Arabidopsis thaliana*

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There was an error published in *Development* **131**, 425-434.

On page 428, the description of the *rbe-1* mutation and its position as depicted in Fig. 2B were incorrect. The correct description is as follows. In *rbe-1*, a C to T transversion at nucleotide position 241 in the cDNA sequence results in the replacement of an arginine with a stop codon at amino acid 81 in the RBE protein.

The authors apologise to readers for this mistake.

RABBIT EARS, encoding a SUPERMAN-like zinc finger protein, regulates petal development in *Arabidopsis thaliana*

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Accepted 21 October 2003

Development 131, 425-434
Published by The Company of Biologists 2004
doi:10.1242/dev.00938

Summary

Floral organs usually initiate at fixed positions in concentric whorls within a flower. Although it is understood that floral homeotic genes determine the identity of floral organs, the mechanisms of position determination and the development of each organ have not been clearly explained. We isolated a novel mutant, *rabbit ears* (*rbe*), with defects in petal development. In *rbe*, underdeveloped petals are formed at the correct position in a flower, and the initiation of petal primordia is altered. The *rbe* mutation affects the second whorl organ shapes independently of the organ identity. *RBE* encodes a SUPERMAN-like protein and is located in the nucleus, and thus may be a transcription factor. *RBE* transcripts are expressed in petal primordia and their precursor cells, and disappeared at later stages. When cells that express *RBE* are ablated genetically, no petal primordia arise. *RBE* is not expressed in *ap1-1* and *ptl-1* mutants, indicating that *RBE* acts downstream of *AP1* and *PTL* genes. These characteristics suggest that *RBE* is required for the early development of the organ primordia of the second whorl.

Key words: *Arabidopsis thaliana*, Petal development, *RABBIT EARS* (*RBE*), Zinc finger protein

Introduction

Flowers have four kinds of floral organs: sepals, petals, stamens and carpels. Their number and position are usually genetically determined in a flower. In *Arabidopsis thaliana*, four sepals, four petals, six stamens and two carpels are formed at fixed positions that are arranged in four concentric whorls. The positions of floral organs seem to be determined according to putative axes in the floral meristem. For example, a sepal is formed on the adaxial side, another on the abaxial side, and two on the lateral sides in the outermost whorl.

The identity of floral organs is controlled by three classes of homeotic genes, termed ABC genes, which encode MADS-box transcription factors (Goto and Meyerowitz, 1994; Jack et al., 1992; Mandel et al., 1992; Yanofsky et al., 1990). According to the present model, the ABC genes determine the fate of floral organs, which depends on the whorl the organ primordia are formed in (Bowman et al., 1991; Coen and Meyerowitz, 1991; Meyerowitz et al., 1991; Weigel and Meyerowitz, 1994). This idea is consistent with findings that ABC genes are expressed in a ring-shaped region that corresponds to hypothetical whorls. In addition to the ABC homeotic genes, another class of MADS-box genes, *SEPALLATA1*, *2* and *3* (*SEP1*, *2* and *3*) has been reported (Pelaz et al., 2000). In a *sep1 sep2 sep3* triple mutant, petals and stamens are transformed into sepalloid organs, and the innermost whorl is replaced by a new flower that repeats this same phenotype. Activities of *SEP2* or *SEP3* with other floral homeotic genes can convert vegetative leaves into floral organs (Honma and Goto, 2001; Pelaz et al., 2001a; Pelaz et al., 2001b). It is unlikely, however, the floral homeotic genes define the size and position of whorls, because the mutations in these genes do not generally affect the size and position of floral organ primordia. For example, in *pistillata* (*pi*), one of a class B mutant, early development of organ primordia is indistinguishable from that of wild type (Hill and Lord, 1989). This indicates that the concentric regions have been defined spatially before these genes start to be expressed.

Petals are the most conspicuous organs in a flower because their colors and shapes vary widely among plant species. According to the ABC model, petal identity is established in the second whorl by class A, class B and *SEP* genes. Because these genes are expressed in a ring-like pattern, other factors are required for the proper arrangement of petal primordia in a floral meristem. Floral organ numbers increase in *clavata* (*clv*) mutants because of the enlarged floral meristem (Clark et al., 1993; Clark et al., 1995; Kayes and Clark, 1998). In *perianthia* (*pan*), the number of sepals, petals and stamens changes to five, which suggests that *PAN* is required for the establishment of the tetramerous structure of a flower (Running and Meyerowitz, 1996). Thus, these genes control the number of floral organ primordia.

After petal primordia form, they follow the process of cell proliferation and cell specialization to develop to mature petals. However, the mechanisms remain poorly understood. In a *petal loss* (*ptl*) mutant, the orientation and growth of the second whorl organs are aberrant, even if the identity of the second whorl organs is changed (Griffith et al., 1999). Thus, *PTL* might be one of the regulators involved in second whorl

organ development, which is independent of the organ identity. Some other factors are involved in petal growth because petal primordia initiate and aberrant petals form in *ptl* mutants.

We describe analyses of a novel mutant, *rabbit ears* (*rbe*), which has defects in petal development. A cloning and expression analysis of *RBE* reveal that it is a transcriptional factor responsible for the development of the second whorl organs independently of the organ identity.

Materials and methods

Plant growth conditions

The Landsberg *erecta* (L*er*) and Columbia (Col) ecotypes of *Arabidopsis thaliana* were used as wild types. The *rbe-1* mutant was isolated from an M2 population of L*er* that had been mutagenized by ethyl methanesulphonate. Information on *rbe-2*, a T-DNA insertion mutant, was obtained from the SIGnAL web site (http://signal.salk.edu). Seeds were sown on the surface of vermiculite in small pots and incubated for four days at 4°C. Plants were grown under continuous white light at 22-24°C.

Microscopy

For scanning electron microscopy, samples were prepared as previously described (Matsumoto and Okada, 2001). For histological analysis, inflorescences were fixed in FAA, dehydrated in ethanol and embedded in Paraplast. Serial, 8 μm sections were deparaffinized and stained in 0.1% Toluidine Blue.

Mapping and cloning of the *RBE* gene

F_2 plants, which were obtained by crossing *rbe-1* and Col, were used for mapping. The DNA markers used for positional cloning were based on SSLP (simple sequence length polymorphism) and CAPS (cleaved amplified polymorphic sequence) between ecotypes L*er* and Col. Information about nga225, PAI2, ASA1, 217C, n97067 and nga249 markers was obtained from The *Arabidopsis* Information Resource (http://www.arabidopsis/org/). After *RBE* had been mapped between the PAI2 and 217C markers, we isolated recombinants by using these two markers among about 4000 F_2 plants. Subsequently, we identified the relationship between the *RBE* locus and these markers by analyzing the phenotypes of each recombinant. Based on the sequence data from the Kazusa Arabidopsis Data Opening Site (http://www/kazusa/or.jp/kaos) and on the L*er* genomic sequence, we found several polymorphisms between L*er* and Col, which were then synthesized as sequence markers. The primer sequences were: m1, 5′-AGAGGTGGTTATGTCAGTGC-3′ and 5′-GATACACATCAGGGCCAATC-3′; m2, 5′-CCACAAGGATTGACAGAAAC-3′ and 5′-GGAGATATCTAGCCTCTTCC-3′; m3, 5′-TCGTAGCCTAGCGAAGAAAG-3′ and 5′-GGCAGTTGCTGATATCAGTC-3′ and m4, 5′-CTACTAGAGCTTGAGGATGC-3′ and 5′-GATGCTGACGTTGATATCCC-3′. cDNA cloning was performed by both 5′-RACE and 3′-RACE using the SMART RACE cDNA Amplification Kit (Clontech). Sequencing of the PCR-amplified fragment and subcloned inserts was performed by ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits and ABI Prism 310, 377 and 3100 from Applied Biosystems.

Complementation test

The genomic fragment including the open reading frame (ORF) of *RBE* was amplified by PCR (primer sequences 5′-CCTTTAAAGGCTCTCTCGTCTCTCTGTATT-3′ and 5′-CTCACATCTTCGCTCTTCATCAACAGGTCT-3′), digested by *Eco*T22I and *Sal*I, and subcloned into the pPZP211 binary vector (Hajdukiewicz et al., 1994) to generate pRGEN. The pRGEN was transformed to the *rbe-1* mutant by a vacuum infiltration procedure with the *Agrobacterium* strain C58C1. Transgenic plants were screened on an agar medium containing 30 μg/ml kanamycin and 100 μg/ml carbenicillin.

Transient expression analysis of the RBE-GFP fusion protein

Full length *RBE* ORF without the stop codon was amplified by PCR (primer sequences 5′-ATCTCTAGAATGATGGATAGAGGAGAATG-3′ and 5′-TAAGGATCCACCTCCGTTAACCTTAGGCGGATCAGCTCC-3′), digested by *Xba*I and *Bam*HI and cloned into pBluescriptII SK+ (Stratagene) to generate pRFSK. The fragment of G3GFP (Kawakami and Watanabe, 1997) was amplified by PCR (primer sequences 5′-AGTGGATCCGGTGGAAGTAAAGGAGAAGAAGAACTTTTC-3′ and 5′-CCACCGCGGTTATTTGTATAGTTCATGCATGCC-3′), digested by *Bam*HI and *Sac*II and cloned into pBluescriptII SK+ to generate pG3SK. The *Bam*HI-*Sac*II fragment from pG3SK was subcloned into pRFSK to generate pRFG3SK. The *Xba*I-*Sac*II fragment from pRFG3SK containing the *RBE-G3GFP* fusion gene was subcloned into pGEM-3Zf+/35S-NosT to generate pRFG3GFP. The *Hin*dIII-*Eco*RI fragment from pRFG3GFP, which contains the cauliflower mosaic virus (CaMV) *35S* promoter, the *RBE-G3GFP* fusion gene and the *nopaline synthase* (*NOS*) terminator, was subcloned into pBI121 (Clontech) to generate pRFG3BI. pRFG3BI was transformed into an *Arabidopsis* Col-0 cell suspension and screened as previously described (Mathur et al., 1998). Samples were visualized under an Axiophoto2 microscope (Zeiss) with a FITC filter, and photographed with a Nikon COOLPIX 990 digital camera.

RNA isolation and RT-PCR

Total RNA was isolated with the Isogen reagent (Nippon gene). One microgram of total RNA from each tissue was reverse-transcribed using the SUPERSCRIPTII reverse transcription kit (Invitrogen), and 1.0 μl of cDNA was used as a template for PCR. The products were electrophoresed on an agarose gel, transferred to nylon membranes and hybridized with *RBE* and *ACT8* probes. A part of the *RBE* cDNA (corresponding to the sequence at 152-400) was labeled and used for the *RBE* probe. Specific primers for the detection of *ACT8* mRNA were generated as described previously (Aida et al., 1997).

mRNA in situ hybridization

mRNA in situ hybridization was performed as previously described (Matsumoto and Okada, 2001). A part of *RBE* cDNA (corresponding to the sequence at 272-579) was amplified by PCR and cloned into pBluescriptII SK+ at the *Eco*RV site for the antisense and sense probes. For the probe of the *APETALA3* (*AP3*), a part of *AP3* cDNA (corresponding to the sequence at 535-820, including the 3′ UTR region) was amplified by PCR, and cloned into pBluescriptII SK+ at the *Eco*RV site.

Promoter analyses of the *RBE* gene

The *Xba*I-*Eco*RI fragment from pERGSK, which contained G3GFP with ER target signal sequences, was ligated into pBI101 (Clontech) to generate pERGBI. The *RBE* promoter was amplified by PCR (primer sequences 5′-AAACCGCGGTTTCAAGCAGTCTGATCACG-3′ and 5′-TTTTCTAGACAGTAGAAGAAGTTAA-GGTG-3′), digested by *Sac*II and *Xba*I, and then cloned into pBluescriptII SK+ to generate pRPSK. The *Hin*dIII-*Xba*I fragment from pRPSK was then subcloned into pERGBI and pBI101 to generate pRPERGBI and pRPGUS, respectively. To construct the *RBE promoter::diphthria toxin* (*RBEp::DT-A*), DT-A was amplified by PCR from pRDC4, digested by *Xba*I and *Sac*I, and ligated into pBluescriptII SK+ to generate pDTASK. The *Xba*I-*Sac*I fragment of pDTASK was then subcloned into pRPGUS to generate pRPDTABI. Transformation to plants and screening were performed as described above. GUS staining was performed as described previously (Donnelly et al., 1999).

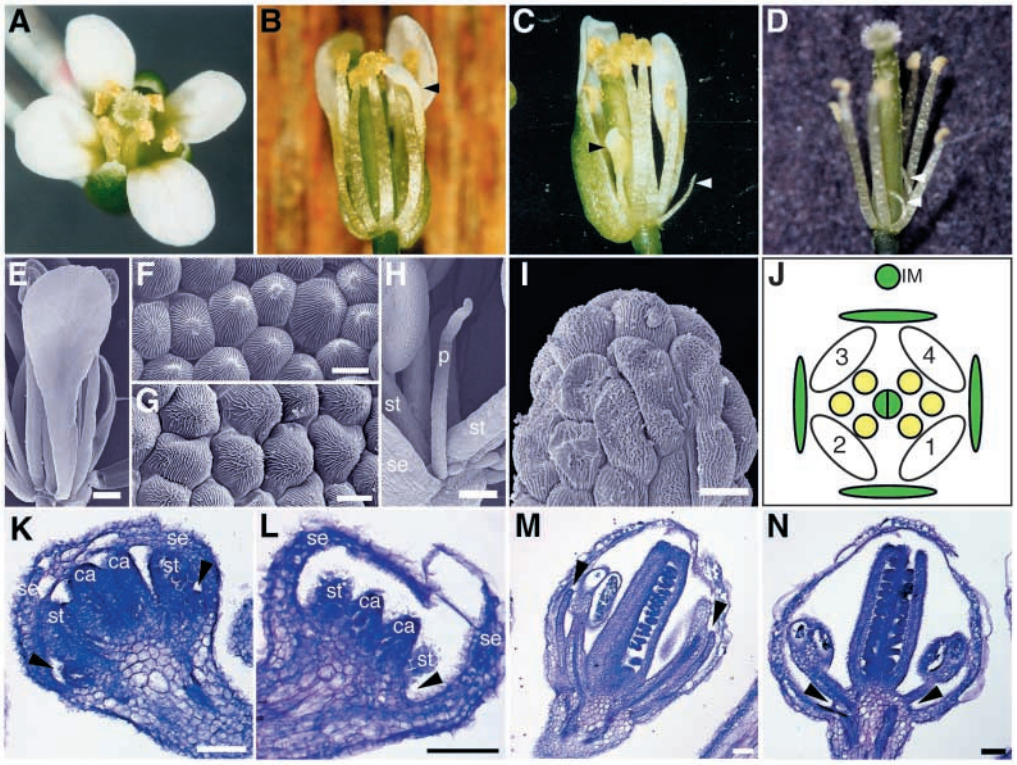

Fig. 1. Structure of wild-type and *rbe* flowers. (A) A wild-type flower. (B,C) *rbe-1* flowers. The arrowhead in B indicates the small petal, and black and white arrowheads in C indicate the spoon-shaped and filamentous petals, respectively. (D) An *rbe-2* flower. Arrowheads indicate filamentous petals. Sepals have been removed in B-D. (E-I) Scanning electron micrographs of petals of wild type (E-G) and *rbe-1* (H,I). (E) A stage 15 wild-type flower. (F,G) Epidermal cells on the upper (adaxial; F) and lower (abaxial; G) surfaces of a wild-type petal. (H) A *rbe-1* filamentous petal. Note that it is formed at the normal position. The sepal on the right side has been removed. (I) A high magnification image of the filamentous petal in H. The epidermal cells are flattened and elliptical with irregular epicuticular ridges. (J) Diagram of a flower. Petals are numbered based on their position relative to the inflorescence meristem (IM). (K-N) Longitudinal sections of wild-type (K,M) and *rbe-2* (L,N) flowers. (K) A wild-type stage 6 flower. Arrowheads indicate the petal primordia. (L) An *rbe-2* stage 6 flower. Arrowhead indicates the position where petal primordia should form. (M) A wild-type stage 10 flower. Arrowheads indicate the elongated petals. (N) An *rbe-2* stage 10 flower. Arrowheads indicate the positions where petal should form. se, sepal; p, petal; st, stamen; ca, carpel. Scale bars: E, 250 μm; F,G,I, 10 μm; H, 200 μm; K,L,M,N, 50 μm.

Results

Mutation of *RBE* affects petal morphology

The most obvious defect of *rbe-1* and *rbe-2* was altered petal morphology. A wild-type flower has four petals of almost the same size and shape (Fig. 1A). A petal is flat from top to bottom, and the bottom part is narrow and greenish (Fig. 1A,E). In *rbe-1*, petals were deformed and their degree of morphological abnormality varied from a mild phenotype of a reduced blade at the top (Fig. 1B), an intermediate phenotype of spoon-shaped petals with a small blade and a short filamentous stalk at the base (Fig. 1C, black arrowhead), to a severe phenotype of filaments only (Fig. 1C, white arrowhead). In some cases, no petals or filaments were observed at the position of petal formation (data not shown). In *rbe-2*, filaments or no petals were formed (Fig. 1D), indicating that *rbe-2* is a more severe allele than *rbe-1*.

To confirm that the deformed organs of the mutant flower have petal characteristics, their epidermal cells were examined by scanning electron microscopy. The epidermal cells of wild-type petals on the upper surface are conical with finely ridged surfaces (Fig. 1F), whereas the cells on the lower surface have a cobblestone-like shape with irregular epicuticular ridges (Fig. 1G). The epidermal cells on the lower surface of the filaments of *rbe-1* had an irregular ridged structure similar to that of the lower surface of the wild type, but the cells were flattened and elliptic (Fig. 1I). The position of the filamentous petals was normal; that is, they were formed in the second whorl at a position between the medial and lateral sepals (Fig. 1H). Furthermore, in the small or spoon-shaped petals of *rbe-1*, the epidermal cells had a ridged structure and were smaller (data not shown). This suggests that the position and identity of the aberrant-shaped petals of *rbe* was not altered.

A histological analysis was performed to investigate the early defects in *rbe*. In wild-type plants, L2 or L3 cells of petal precursor cells divide periclinally (Hill and Lord, 1989) and petal primordia bulges emerge up to stage 6 [Fig. 1K; flower stages as defined by Smyth et al. (Smyth et al., 1990)]. In *rbe-2*, cell division of the petal precursor cells was arrested and no primordia formed where the petal primordia should have emerged (Fig. 1L). In a wild-type stage 10 flower, petals elongate and reach the height of anthers (Fig. 1M). At the same stage of an *rbe-2* flower, no petals formed because of the loss of petal primordia (Fig. 1N). This suggests that the initiation of petal primordia does not occur in *rbe* and that *RBE* is involved in the early development of petal primordia.

We noticed that, in many cases in *rbe-1*, two adjacent petals remained normal, but the other two petals were deformed (Fig. 1B,C; the mutant name is derived from this phenotype). To determine whether this phenotype is position dependent, we scored the phenotype of the four petals based on their positions relative to the inflorescence meristem (Fig. 1J, Table 1). Interestingly, in *rbe-1*, flowers formed immediately after bolting were almost normal (data not shown), but aborted petals became prominent in flowers formed later. When we examined the petal shape of the sixteenth to twentieth flowers on the inflorescence of *rbe* mutants, the two petals on the adaxial side (position 3 and 4) were deformed more severely

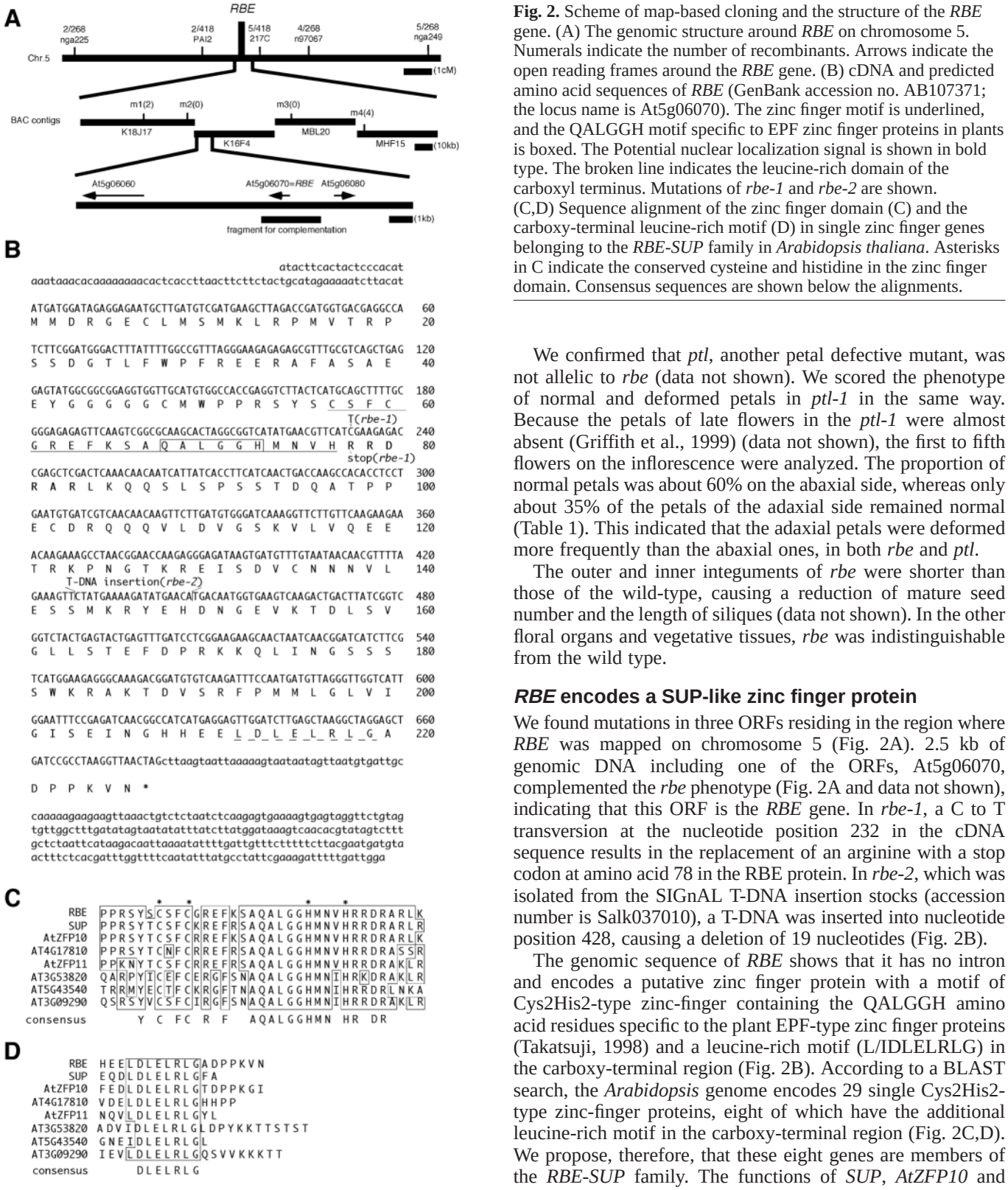

Fig. 2. Scheme of map-based cloning and the structure of the *RBE* gene. (A) The genomic structure around *RBE* on chromosome 5. Numerals indicate the number of recombinants. Arrows indicate the open reading frames around the *RBE* gene. (B) cDNA and predicted amino acid sequences of *RBE* (GenBank accession no. AB107371; the locus name is At5g06070). The zinc finger motif is underlined, and the QALGGH motif specific to EPF zinc finger proteins in plants is boxed. The Potential nuclear localization signal is shown in bold type. The broken line indicates the leucine-rich domain of the carboxyl terminus. Mutations of *rbe-1* and *rbe-2* are shown. (C,D) Sequence alignment of the zinc finger domain (C) and the carboxy-terminal leucine-rich motif (D) in single zinc finger genes belonging to the *RBE-SUP* family in *Arabidopsis thaliana*. Asterisks in C indicate the conserved cysteine and histidine in the zinc finger domain. Consensus sequences are shown below the alignments.

than those on the abaxial side (position 1 and 2) (Table 1). Indeed, in *rbe-1* about 60% of the petals at position 1 and 2 remained normal, whereas only about 30% of the petals at position 3 and 4 were normal. These difference between the adaxial and the abaxial sides was also found in *rbe-2* (Table 1).

We confirmed that *ptl*, another petal defective mutant, was not allelic to *rbe* (data not shown). We scored the phenotype of normal and deformed petals in *ptl-1* in the same way. Because the petals of late flowers in the *ptl-1* were almost absent (Griffith et al., 1999) (data not shown), the first to fifth flowers on the inflorescence were analyzed. The proportion of normal petals was about 60% on the abaxial side, whereas only about 35% of the petals of the adaxial side remained normal (Table 1). This indicated that the adaxial petals were deformed more frequently than the abaxial ones, in both *rbe* and *ptl*.

The outer and inner integuments of *rbe* were shorter than those of the wild-type, causing a reduction of mature seed number and the length of siliques (data not shown). In the other floral organs and vegetative tissues, *rbe* was indistinguishable from the wild type.

RBE encodes a SUP-like zinc finger protein

We found mutations in three ORFs residing in the region where *RBE* was mapped on chromosome 5 (Fig. 2A). 2.5 kb of genomic DNA including one of the ORFs, At5g06070, complemented the *rbe* phenotype (Fig. 2A and data not shown), indicating that this ORF is the *RBE* gene. In *rbe-1*, a C to T transversion at the nucleotide position 232 in the cDNA sequence results in the replacement of an arginine with a stop codon at amino acid 78 in the RBE protein. In *rbe-2*, which was isolated from the SIGnAL T-DNA insertion stocks (accession number is Salk037010), a T-DNA was inserted into nucleotide position 428, causing a deletion of 19 nucleotides (Fig. 2B).

The genomic sequence of *RBE* shows that it has no intron and encodes a putative zinc finger protein with a motif of Cys2His2-type zinc-finger containing the QALGGH amino acid residues specific to the plant EPF-type zinc finger proteins (Takatsuji, 1998) and a leucine-rich motif (L/IDLELRLG) in the carboxy-terminal region (Fig. 2B). According to a BLAST search, the *Arabidopsis* genome encodes 29 single Cys2His2-type zinc-finger proteins, eight of which have the additional leucine-rich motif in the carboxy-terminal region (Fig. 2C,D). We propose, therefore, that these eight genes are members of the *RBE-SUP* family. The functions of *SUP*, *AtZFP10* and *AtZFP11*, which belong to the *RBE-SUP* family, have been analyzed (Dinkins et al., 2002; Dinkins et al., 2003; Sakai et al., 1995; Sakai et al., 2000).

The *RBE* protein is located in the nucleus

Zinc-finger proteins are suggested to function as transcriptional

Table 1. Percentage of petal types in the wild type, *rbe* and *ptl* plants

	Wild type				*rbe-1*				*rbe-2*				*ptl-1*			
Petal phenotype	1*	2*	3*	4*	1*	2*	3*	4*	1*	2*	3*	4*	1*	2*	3*	4*
Normal	100	100	100	100	68	58	24	32	0	0	0	0	60	54	34	34
Small	0	0	0	0	14	24	24	18	2	4	0	0	4	6	6	10
Spoon-shaped	0	0	0	0	8	8	2	18	14	10	0	0	2	10	8	4
Filamentous	0	0	0	0	10	10	48	32	76	74	74	74	10	8	16	18
Absent	0	0	0	0	0	0	2	0	8	12	26	26	24	22	36	34

n=50 for each genotype.
*Position of petal in a flower (see Fig. 1J).

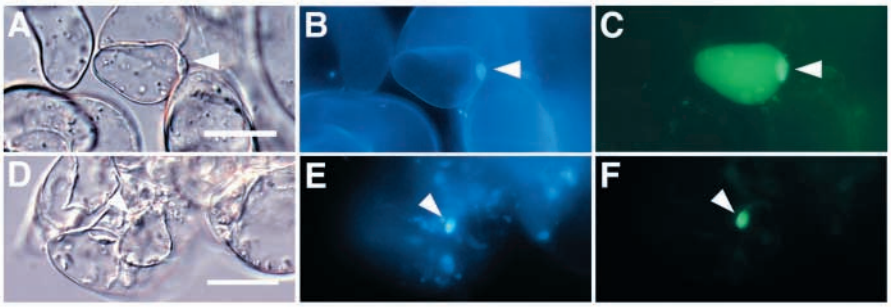

Fig. 3. An *Arabidopsis thaliana* suspension cell transiently expressing GFP protein (A-C) or RBE-GFP fusion protein (D-F). (A,D) Bright-field image; (B,E) DAPI (2 μg/ml) staining; (C,F) GFP fluorescence. Arrowheads mark the location of the nucleus. Scale bars: 100 μm.

factors (Takatsuji, 1998), and the RBE protein contains the potential nuclear localization signal (RRDRAR) just after the zinc finger domain (Fig. 2B) (Dinkins et al., 2003). To confirm that RBE is located in the nucleus, an RBE-GFP fusion protein was expressed transiently in suspension culture cells of *Arabidopsis* under the CaMV *35S* promoter. As shown in Fig. 3, the RBE-GFP fusion protein was located in the nucleus. The same results were obtained when the same construct was introduced to onion epidermal cells (data not shown). These results suggest that RBE is a putative transcriptional factor.

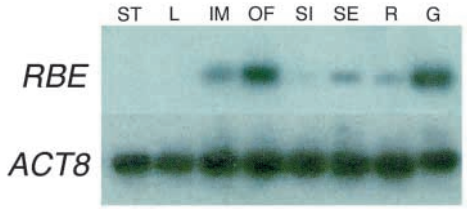

Fig. 4. RT-PCR analysis of *RBE* in tissues of the wild type. *ACT8* was analyzed as a control. ST, stems; L, leaves; IM, inflorescence meristem, including young flower buds; OF, open flowers; SI, siliques; SE, seedlings; R, roots; G, genomic DNA for control.

RBE is expressed in petal primordia and their precursor cells

An RT-PCR analysis revealed that *RBE* was expressed strongly in inflorescences and flowers, and weakly in siliques, seedlings and roots in the wild type (Fig. 4). The expression was detected in both *rbe-1* and *rbe-2* (data not shown), suggesting that *RBE* is not self-regulated transcriptionally. To examine the spatial and temporal expression patterns of the *RBE* transcripts in inflorescences, mRNA in situ hybridization was performed. Fig. 5A,B shows the expression patterns of the *RBE* transcripts in two continuous transverse sections of a wild-type inflorescence. *RBE* transcripts were not detected in the inflorescence meristem or in the flowers at stages 1 and 2. However, *RBE* expression was detected in four restricted regions in the flowers at stages 3 to 6 (shown by arrowheads in Fig. 5A,B), but was not observed in flowers at later stages (data not shown). In a longitudinal section of a stage 3 flower, the *RBE* transcripts were located in regions between the sepal primordia and the large bulge of floral meristem (Fig. 5C). In a flower at stage 6, the *RBE*-expressing cells coincide with the petal primordia (Fig. 5D). These results indicate that *RBE* expression starts in the precursor cells of petal primordia in stage 3 flowers and continues in the developing petal primordia up to stage 6. This expression pattern was confirmed in transgenic plants carrying the GFP or GUS gene connected under the 1.8 kb promoter region of *RBE* (Fig. 5E,F), indicating that this promoter region is sufficient for the spatial and temporal control of *RBE* expression. In flowers, the *RBE* transcripts, GFP signals and GUS stains were detected only in the petal primordia and their precursor cells, not in the other organs. To our surprise, the GFP signals and GUS staining were observed in the lateral root caps and the basal cells of lateral roots, but we did not find an aberrant phenotype in the roots of the mutants (data not shown).

Regions where *RBE* is expressed are necessary for petal initiation

To investigate whether the cells where *RBE* is expressed are necessary for petal formation, the cells were ablated genetically using diphtheria toxin A-chain (DT-A) (Bellen et al., 1992). DT-A is known to kill cells by ribosylating the EF2 translation initiation factor and inhibiting protein synthesis (Pappenheimer, 1977). In *Arabidopsis*, DT-A has been used to selectively ablate tissues and cells with tissue-specific promoters (Day et al., 1995; Nilsson et al., 1998; Tsugeki and Fedoroff, 1999). We constructed transgenic plants, *RBEp::DT-A*, expressing DT-A under the 1.8 kb *RBE* promoter, which was shown to be sufficient for the expression of *RBE* (Fig. 5E,F).

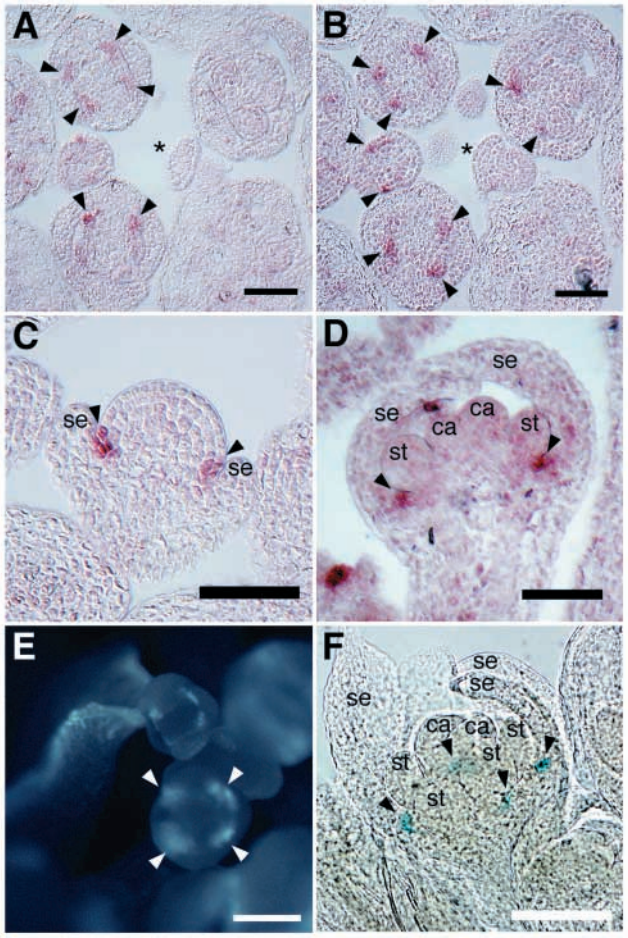

Fig. 5. Spatiotemporal expression patterns of *RBE* in inflorescences and floral buds. (A,B) *RBE* expression patterns in continuous transverse sections of a wild-type inflorescence. Arrowheads indicate *RBE* expression in stage 3 to 6 flowers. Asterisks indicate the position of the inflorescence meristem. (C,D) *RBE* expression patterns in longitudinal sections of a wild-type flower at stage 3 (C) and stage 6 (D). Arrowheads indicate *RBE* expression. (E) Top view of *RBEp::GFP* expression in a transgenic plants. Arrowheads indicate GFP expression. (F) *RBEp::GUS* expression in a transgenic plant. Arrowheads indicate GUS expression. Note that both *RBEp::GFP* and *RBEp::GUS* plants show the same expression patterns as with in situ hybridization. se, sepal; st, stamen; ca, carpel. Scale bars: 50 μm.

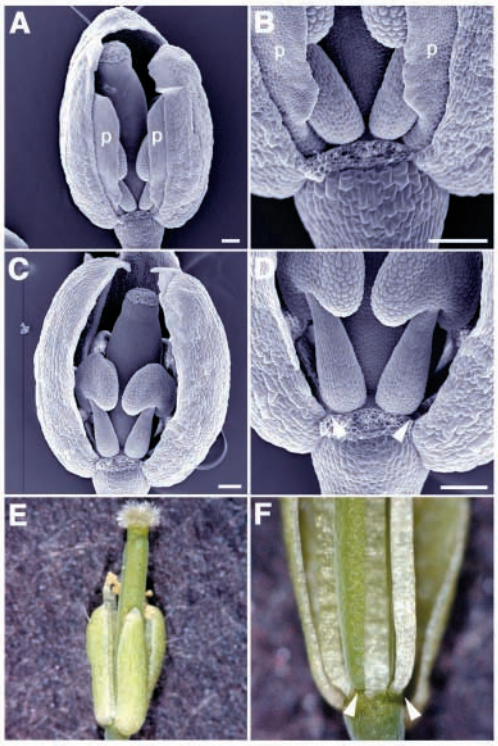

Fig. 6. Structure of *RBEp::DT-A* flowers. (A-D) Scanning electron micrographs of wild-type (A,B) and *RBEp::DT-A* (C,D) flowers. (A) A wild-type stage 12 flower. (B) High magnification image of A. (C) An *RBEp::DT-A* stage 12 flower. (D) High magnification image of C. (E) A mature flower of the *RBEp::DT-A* plant. (F) High magnification image of E. Arrowheads in D and F indicate the position where petals should form. Sepals have been removed in A-D and F. p, petal. Scale bars: 100 μm.

In stage 12 flowers of the wild type, petal length reached the anther of longer stamens (Fig. 6A,B). In contrast, plants carrying the *RBEp::DT-A* transgene lacked petals completely in flowers of the same stage (Fig. 6C,E), and traces of petal primordia were not observed in mature flowers (Fig. 6D,F). These results indicate that *RBE* is expressed in cells that could be involved in, or recruited to, the petal primordia. The other floral organs were not affected, indicating that the ablation of the cells expressing *RBE* did not affect the development of the other floral organs in both the outer and inner whorls.

RBE function depends on the position, not on the organ identity

To examine whether *RBE* is involved in petal identity as well as petal growth, we constructed a double mutant of *rbe-1* and *apetala3-5* (*ap3-5*). *AP3* is a class B homeotic gene and its mutation causes the transition of petals to sepals and stamens to carpelloid or filamentous organs (Fig. 7A,B) (Bowman et al., 1989; Jack et al., 1992). The phenotype of the double mutant flower was additive; the second whorl organs had the identity of sepals because the shape of the epidermal cells was characteristic of sepals, as expected from the *ap3* mutation. However, these organs were small and often converted to filaments, as observed in the second whorl organs of the *rbe-1* single mutant (Fig. 7C,D). This result indicates that *RBE* is not related to the identity of the second whorl organs, and that *RBE* is involved in their development independently of their identity.

We also examined whether the expression of *RBE* and *AP3* was mutually affected. In wild-type plants, *AP3* was expressed in the second and third whorls, thus the expression pattern in transverse sections looks like a ring (Fig. 7E). In longitudinal sections of stage 3 flowers, the *AP3*-expressing regions were larger than, and partially overlapping with, the *RBE*-expressing regions (Fig. 5C, Fig. 7F). The expression pattern of *AP3* in *rbe-1* was the same as in the wild type (Fig. 7G,H), suggesting that *RBE* is not involved in the establishment of concentric whorl regions in the floral meristem. Moreover, *RBE* was expressed in *ap3-5* in the same patterns as in the wild type (Fig.

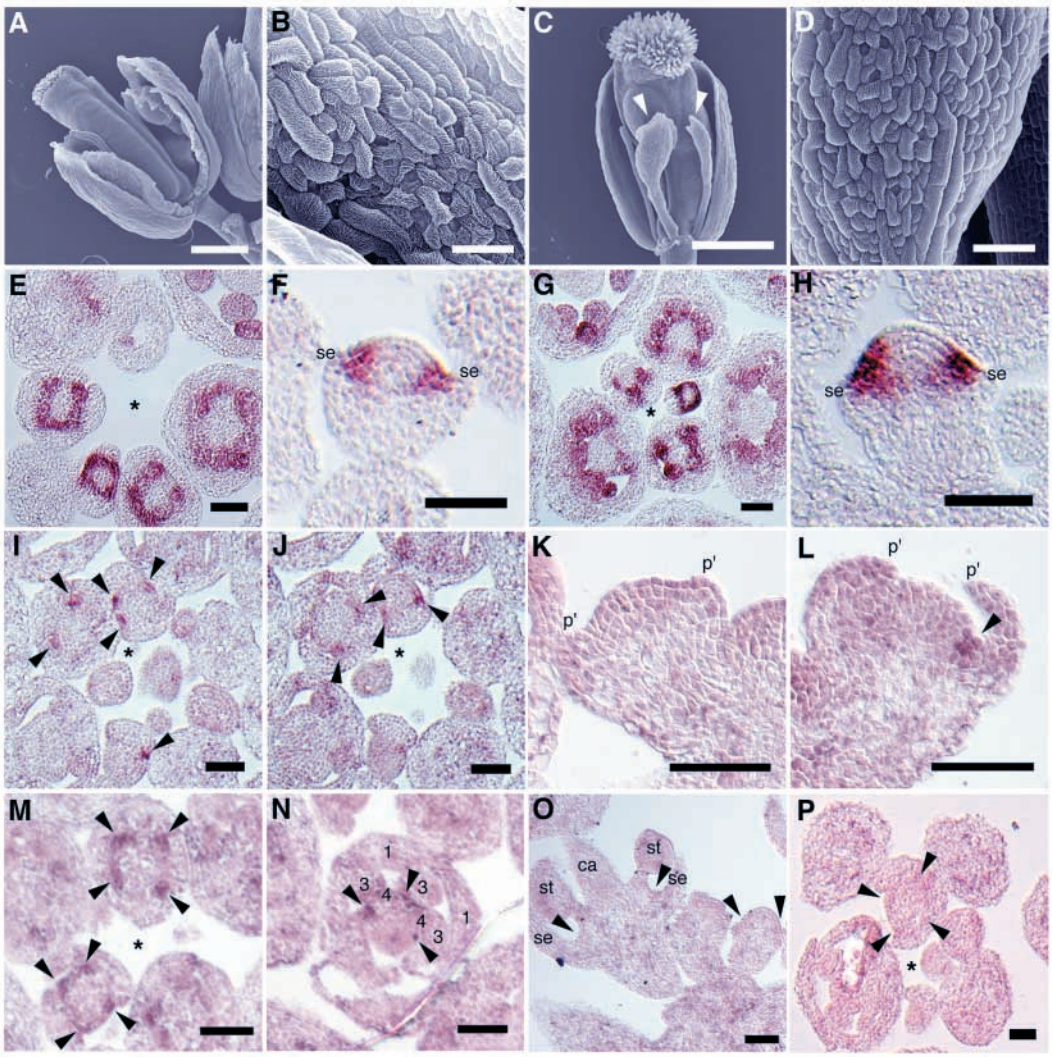

Fig. 7. Flower structure of *rbe-1 ap3-5* double mutant and in situ localization of *RBE* transcripts in floral mutants. (A-D) SEM images of *ap3-5* (A,B) and the *rbe-1 ap3-5* double mutant (C,D). (B) A high magnification image of the second whorl organ in A. The stomata and long cells characteristic of sepal epidermis are present. (C) Arrowheads indicate underdeveloped organs formed in the second whorl. (D) A high magnification image of the second whorl organ in C. Stomata and long cells are present. (E,F) *AP3* expression patterns in a transverse section of a wild-type inflorescence (E) and a longitudinal section of a stage 3 flower (F). (G,H) The *AP3* expression patterns in a transverse section of a *rbe-1* inflorescence (G) and a longitudinal section of a stage 3 flower (H). (I,J) *RBE* expression patterns in continuous transverse sections of an *ap3-5* inflorescence (arrowheads). (K,L) *RBE* expression analysis in longitudinal sections of the early (K) and late (L) stage 3 flowers of *35S::PI 35S::AP3*. *RBE* is not expressed in the organ primordia of the outermost whorl (shown as p′), but is expressed in the precursor cells of the second whorl organ primordia (arrowhead in L). (M,N) *RBE* expression in transverse sections of *ag-1*. Arrowheads indicate *RBE* expression. Numerals in N indicate the whorl number where the organ primordia form. (O,P) *RBE* expression analysis in a longitudinal section of *ap1-1* (O) and a transverse section of *ptl* (P). *RBE* expression is not detected in the region where petal primordia would be expected to form (arrowheads). Asterisks in E, G, I, J, M and P indicate the position of the inflorescence meristem. se, sepal primordia; p′, petal primordia in the outermost whorl; st, stamen primordia; ca, carpel primordia. Scale bars: A, 500 μm; B,D,E-P, 50 μm; C, 200 μm.

7I,J; see Fig. 5A,B). This result indicates again that *RBE* expression is not regulated by *AP3*.

To investigate whether *RBE* is expressed in the petal primordia formed at abnormal positions, *RBE* expression was analyzed in the flowers of mutant or transgenic plants that had ectopic petals. In flowers of *35S::PI 35S::AP3* double transgenic plants that have petals in the first whorl instead of sepals (Krizek and Meyerowitz, 1996), *RBE* expression was not observed in the petal primordia in the first whorl (shown as p′ in Fig. 7K), although normal expression was observed in the petal primordia of the second whorl (Fig. 7L). The flowers of *agamous-1* (*ag-1*) mutants are known to have homeotic conversion of six stamens to petals in the third whorl, and have a meristem of indeterminate character that continuously forms a flower of the three whorls in place of the fourth whorl (Bowman et al., 1989; Bowman et al., 1991). When the expression of *RBE* was examined in an *ag-1* flower, the signal was detected in the cells corresponding to the petal primordia in the fifth whorl (Fig. 7N) in addition to those in the second whorl (Fig. 7M), but not in the primordia of homeotically converted petals in the third whorl (Fig. 7M). These results are consistent with the previous observation that *RBE* expression is restricted to the organ primordia in the second whorl independently of the organ identity.

RBE is not expressed in *ap1-1* and *ptl-1*

To investigate whether *RBE* is expressed in flowers that lack petals, in situ hybridization was performed in the continuous sections of inflorescences of petal-defective mutants, *apetala1-1* (*ap1-1*) and *petal loss* (*ptl*). *ap1-1* has leaf-like organs in place of the sepals, usually no petals and reduced number of stamens (Irish and Sussex, 1990). Although *ap1-1* mutant flowers looked to have a region corresponding to the second whorl, *RBE* was not expressed (Fig. 7O). In flowers of another petal-defective mutant, *ptl*, *RBE* was not expressed in the region corresponding to the primordia of the second whorl

(Fig. 7P). This suggests that *RBE* expression is under control of *AP1* and *PTL*.

Discussion

RBE is responsible for the second whorl organ development independently of the identity

Floral primordia arise from the inflorescence meristem, and floral organs are then formed in the floral meristem. Since the floral organs are formed in a concentric pattern, the concentric region is likely to be determined before the floral organ primordia arise. The floral homeotic genes are known to be expressed in these concentric regions and to determine organ identity. The position of floral organs in each concentric region is fixed relative to the inflorescence meristem, indicating that the organ position is determined after the concentric regions are fixed. *RBE* was expressed in the petal primordia and their precursor cells (Fig. 5), and the expression was decreased and disappeared at later stages. It is suggested that the *RBE*-expressing cells are selected by putative organ position-determinant genes that work prior to *RBE*, and that *RBE* is involved in petal primordia initiation and growth at early stages, but not at later stages.

RBE could function either by promoting precursor cells to form petal primordia or by recruiting cells of other regions to make petal primordia. To investigate these possibilities, we generated transgenic plants carrying *APETALA3 promoter::RBE* (*AP3p::RBE*). In the transgenic plants, organ primordia did not increase in the second and third whorls, and the flowers were indistinguishable from those of the wild type (data not shown). We then generated *35S::RBE* plants that produced flowers in which a change of floral organ number and arrangement was not observed (data not shown). These results indicate that petal precursor cells are susceptible to *RBE* action, but the other cells in the second whorl and other whorls are not.

How are the positions of the primordia determined in floral whorls? *RBE* was not expressed in the *ap1-1* mutant, indicating that *RBE* acts downstream of *AP1*. However, *AP1* does not seem to regulate the position of primordia, because it is expressed in the entire first and second whorls. Furthermore, the mutation of *RBE* did not affect the arrangement of the primordia, and *RBE* was expressed in four-petal-precursor cells, which suggests that *RBE* is not involved in determining the position of petal primordia. We present a model in which an unknown gene, 'X', acts downstream of *AP1*, and is involved in positional determination and regulation of the expression of *RBE* in the petal precursor cells. *RBE* was not expressed in *ptl-1*, implying that *PTL* could be the unknown X gene. Gain-of-function analyses of *PTL* would shed light on the positional determination of petal primordia in a flower.

Abnormal petals in both *rbe-1* and *rbe-2* formed more frequently on the adaxial than abaxial side of the flower (Table 1). This tendency was also observed in *ptl-1* (Table 1), suggesting that petal development is regulated differently on the adaxial and abaxial sides in *Arabidopsis*. It is unlikely, however, that *RBE* and *PTL* promote the development of petals on the adaxial side more than petals on the abaxial sides because (1) *RBE* transcripts were expressed at the same time and intensity in all four petal primordia and their precursor cells, and (2) the abnormality was incomplete in these mutants; that is, normal petals were sometimes formed even on the adaxial side and abnormal petals were frequently formed on the abaxial side (Table 1). We supposed that the growth rate of petals on the two sides is controlled differently in *Arabidopsis*, and that *RBE* and *PTL* have a role in adjusting the rate to keep them even.

It has been shown that the first and fourth whorl organs are not affected when the second and third whorl organs are ablated genetically (Day et al., 1995). We have shown that the genetic ablation of the second whorl organs does not affect the development of other floral organs (Fig. 6). This is consistent with the prediction that some mechanism that defines the position of organ primordia in all four whorls and the timing of the primordia development is settled before stage 3, when the expression of *RBE* and *AP3* starts (Fig. 5C) (Day et al., 1995; Hicks and Sussex, 1971). Further analyses, such as genetic ablation of organ primordia in other whorls, would be required to confirm this hypothesis.

The phenotype of the *rbe-1 ap3-5* double mutants was additive (Fig. 7C,D), which indicates that *RBE* functions independently of organ identity determination. Our in situ analyses also showed that determination of position and identity of floral organs is regulated by independent mechanisms (Fig. 7E-N). In the *ap3* and *pi* mutants, even though the second whorl petals are transformed into sepalloid organs, the size of their primordia is almost the same as that of petals, but not as large as sepal primordia observed in wild-type floral buds (Bowman et al., 1989; Hill and Lord, 1989). This indicates that floral homeotic genes determine organ identity, but they are not involved in the determination of the size of floral primordia or in their growth. *RBE* and *PTL* might be involved in, or regulated by, this pathway.

We also constructed *rbe-1 clv1-4* and *rbe-1 pan* double mutants. In these double mutants, the phenotype of the organs formed in the second whorl was largely additive. The organs were small and under-developed, as shown in *rbe-1*, although their number changed as expected from the second mutation of each double mutant (data not shown). This suggests that the role of *RBE* in petal development is independent of the genes that control organ number.

Molecular function of one-finger type zinc finger proteins

RBE encodes a nuclear-localized one-finger type Cys2His2 zinc finger protein (Figs 2, 3). According to the amino acid sequence of the zinc finger motif, RBE belongs to the EPF family of proteins, which contains the QALGGH amino acid residues within the zinc finger motif (Takatsuji, 1998; Takatsuji, 1999). In this EPF family, *ZPT2-1* (or *EPF1*) of *Petunia* with two Cys2His2 zinc finger motifs has been well analyzed. It interacts with the promoter region of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and is involved in the activation of transcription (Takatsuji et al., 1992). The DNA binding site of two finger proteins is specified by the linker sequence length between the two zinc finger domains (Takatsuji and Matsumoto, 1996). Recently, it has been shown that SUP can bind EPF-binding target sequences, and that amino acid residues around the zinc finger motif are involved in the recognition of the target sequence (Dathan et al., 2002). These data indicate that both one- and two-finger proteins bind DNA, although their mechanisms of binding site

recognition are different. The amino acid residues around the zinc finger motif show a low similarity between RBE and SUP (data not shown). Thus, RBE might bind DNA as well as SUP, but the residues around the zinc finger motif would be involved in the recognition of different binding-target DNA sequences.

Expression patterns of *RBE* and *SUP* in a floral bud are exclusive, suggesting that their functions are divided spatially. In addition, the function of these two genes is likely to be antagonistic in respect to floral development; *SUP* has been suggested to regulate cell proliferation negatively at the boundary of the third and fourth whorls (Sakai et al., 1995; Sakai et al., 2000), and outer integuments of *sup* go through excess elongation (Gaiser et al., 1995). However, *RBE* was expressed in petal primordia and their precursor cells (Fig. 5), and its mutation results in arrested cell division of petal precursor cells and in deformed petals (Fig. 1). In addition, integuments of *rbe* were shorter than those of the wild type (data not shown). These results suggest that *RBE* is involved in cell specification or the activation of cell proliferation, rather than in repression of cell proliferation, like *SUP*. Thus, in the flower, not only the functional region, but also the target genes of *RBE* and *SUP* would be different. We found that another gene in the *RBE-SUP* family was expressed in inflorescences with young floral buds and mature flowers (data not shown), presenting the possibility that this additional gene is also involved in floral organ development. Analysis of this gene would help to understand the function of the single zinc finger genes belonging to the *RBE-SUP* family.

We thank David Smyth for providing *ptl* seeds; Akira Nagatani and Masanobu Nakamura for providing the pGEM-3Zf+/35S-nos vector; Yuichiro Watanabe, Tetsuo Meshi and Atsushi Tamai for providing the G3GFP with ER-retention signal peptide; Hugo Bellen for providing the diphtheria toxin A-chain; Csaba Koncz and Masaaki Umeda for providing *Arabidopsis* suspension cells; Koji Goto for *35S::PI* and *35S::AP3* seeds. Takehide Kato for the idea of gene mapping; Juliette Colinas for reviewing the manuscript; the Salk Institute Genomic Analysis laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutant; the Ohio State University Arabidopsis Biological Resource Center for providing the seeds of the T-DNA insertion mutant; the Arabidopsis Information Resource for providing molecular markers and sequence data for gene cloning; the Kazusa Arabidopsis data Opening Site and especially Yasukazu Nakamura for providing BAC clones and sequence data; and members of the Okada laboratory, especially Ryuji Tsugeki and Sumie Ishiguro for meaningful discussions and technical advice. Funding for the SIGnAL indexed insertion mutant collection was provided by the National Science Foundation. This work was funded by Grants-in-Aid for Scientific Research on Priority Areas (No. 14036220) and for the 21th century COE research (Kyoto University A2) of the Japanese Ministry of Education, Culture, Sports, Science and Technology of Japan, and from the Mitsubishi Foundation.

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