Assessing the redundancy of MADS-box genes during carpel and ovule development

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Carpels are essential for sexual plant reproduction because they house the ovules and subsequently develop into fruits that protect, nourish and ultimately disperse the seeds. The *AGAMOUS* (*AG*) gene is necessary for plant sexual reproduction because stamens and carpels are absent from *ag* mutant flowers[1,2]. However, the fact that sepals are converted into carpelloid organs in certain mutant backgrounds even in the absence of *AG* activity indicates that an *AG*-independent carpel-development pathway exists[2]. *AG* is a member of a monophyletic clade of MADS-box genes that includes *SHATTERPROOF1* (*SHP1*), *SHP2* and *SEEDSTICK* (*STK*)[3], indicating that these four genes might share partly redundant activities. Here we show that the *SHP* genes are responsible for *AG*-independent carpel development. We also show that the *STK* gene is required for normal development of the funiculus, an umbilical-cord-like structure that connects the developing seed to the fruit, and for dispersal of the seeds when the fruit matures. We further show that all four members of the *AG* clade are required for specifying the identity of ovules, the landmark invention during the course of vascular plant evolution that enabled seed plants to become the most successful group of land plants[4].

The fact that *ag* mutants completely lack carpels demonstrates the primary role of *AG* in specifying carpel identity[1,2]. Mutations in the *APETALA2* (*AP2*) gene, which encodes a transcription factor for a putative protein of the same type as ethylene-responsive element-binding proteins, lead to the ectopic expression of *AG* throughout the flower[5] and the replacement of sepals (Fig. 1a) by organs that closely resemble carpels (Fig. 1b). The observation that ectopic carpelloid organs develop in place of sepals in *ag ap2* double mutants indicates that many features of carpels can form in the absence of *AG* activity[6]. These features include the formation of stigmatic papillae, style, replum and placental tissues with ovules (Fig. 1c). Because *ag* single mutants lack carpels, we used *ap2 ag* double mutants to identify the genes that might be responsible for *ag*-independent carpel development.

The carpel- and fruit-specific *SHP1* and *SHP2* genes are good candidates for genes that act redundantly with *AG* because they share extensive sequence similarity and partly overlapping expression patterns with *AG*. In addition, the *SHP* genes are expressed in the ectopic carpels of *ap2* mutants[7,8]. To determine whether the *SHP* genes are involved in the formation of ectopic carpelloid organs in *ag ap2* mutants, we constructed *ap2 ag shp1 shp2* quadruple mutants. Remarkably, all features of carpels, including ovules, were completely absent from the first-whorl organs of the quadruple mutants (Fig. 1d, e). Although a small number of outgrowths along the margins of these organs were observed in the quadruple mutants, these outgrowths had no ovule-like characteristics and contained cuticular ridges and guard cells that are never observed on ovules (Fig. 1f). These data demonstrate that the *SHP* genes are responsible for *AG*-independent carpel and ovule development observed in *ap2 ag* mutants. Although the carpel-specific expression of the *SHP* genes is normally dependent on *AG* activity[7], these data also indicate that the activation of the *SHP* genes in the first-whorl organs of *ap2* mutants is independent of *AG*. Previous studies indicated that the SPATULA (SPT) basic helix–loop–helix transcription factor has a function in *AG*-independent carpel

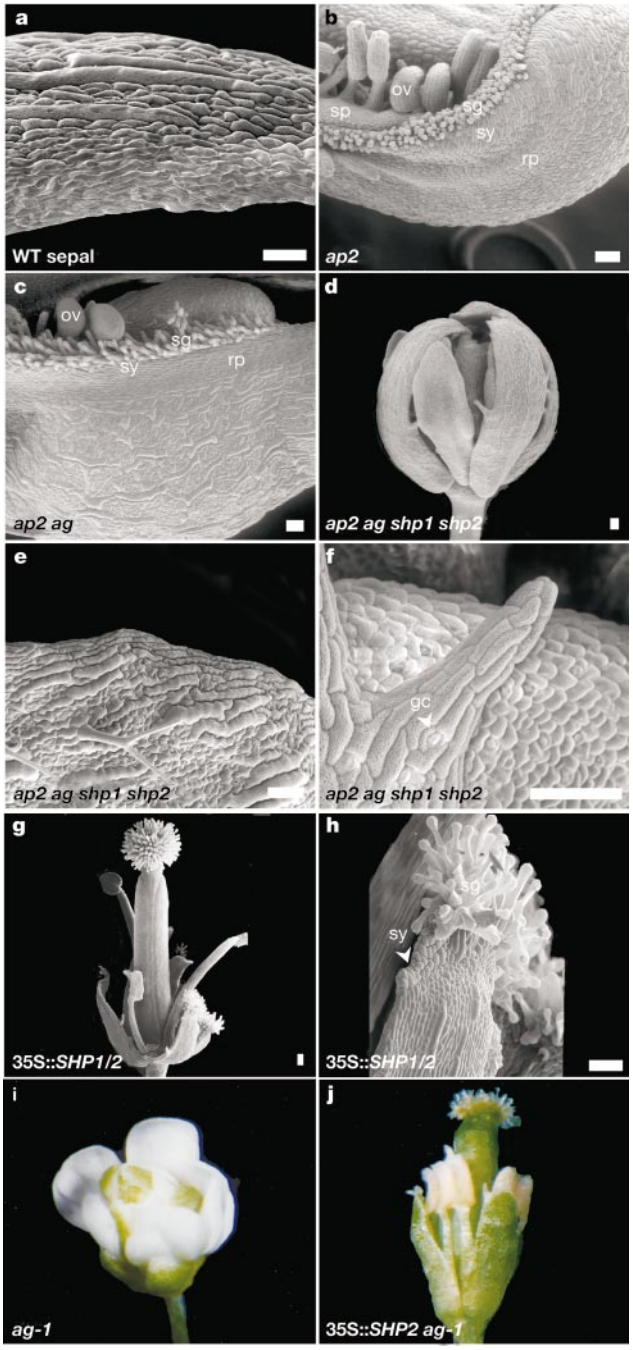

Figure 1 *AG* acts redundantly with *SHP* genes to specify carpel identity. **a**, Margin of wild-type (WT) sepal. **b**, **c** Several carpel cell types: stigma (sg), style (sy), ovule (ov), septum (sp) and replum (rp) are observed at the margin of first-whorl organ of *ap2* (**b**) and *ap2 ag* mutants (**c**). **d**–**f**, First-whorl organs of *ap2 ag shp1 shp2* mutants lack all carpelloid features and have a few small outgrowths with cuticular ridges and guard cells (gc) on the epidermal surface (**f**) that are never observed on wild-type ovules. The *ap2 ag shp1 shp2* mutant is indistinguishable from the *ap2 ag shp1 shp2 stk* mutant (data not shown). **g**, **h**, Constitutive expression of *SHP1/2* genes causes the conversion of sepals toward carpels. Stigmatic tissue (sg) and style-like cells (sy) were observed at the margin of first-whorl organs. **i**, **j**, Overexpression of *SHP2* can rescue stamen and carpel development (**j**) in *ag* mutants (**i**). The rescued carpel produced viable seeds when pollinated with wild-type pollen (see Supplementary Information). Scale bars, 50 μm.

development because mutations in *SPT* largely eliminate the formation of ectopic carpelloid organs[6]. These genetic data, together with the observation that *SPT* expression at the margin of carpelloid organs in *ap2* mutants is reduced in the *ap2 ag* double-mutant background[9], support the suggestion that *AG* and *SHP* might redundantly regulate *SPT* expression.

To determine whether the *SHP* genes are sufficient for promoting carpel development in ectopic positions, we generated transgenic plants in which the *SHP* genes were 'constitutively' expressed from the cauliflower mosaic virus 35S promoter[10]. 35S::*SHP1/2* plants showed a partial conversion of first-whorl sepals toward carpels, as demonstrated by the extensive proliferation of stigmatic papillae as well as cells resembling those normally found on the style of wild-type carpels (Fig. 1g, h). These and other phenotypes, such as the transformation of petals toward stamens (Fig. 1g), early flowering, curly leaves and prematurely open flower buds, are similar to those previously described for 35S::*AG* plants[11], indicating that ectopic expression of the *SHP* genes is sufficient to confer many of the same phenotypes caused by ectopic *AG* expression (see Supplementary Information).

What is the underlying mechanism that led to the evolution of partly overlapping, yet distinct, functions for the *SHP* and *AG* genes? One possibility is that these genes, which are the apparent result of relatively recent gene duplication events, have diverged in their expression patterns even though the proteins themselves have retained similar activities. To test this idea, we introduced the 35S::*SHP2* transgene into *ag* mutants. Remarkably, constitutive expression of the *SHP2* gene was sufficient to largely rescue stamen and carpel development in *ag* mutants (Fig. 1i, j). The fact that the *SHP2* gene can substitute for *AG* in promoting stamen development is particularly striking because *SHP2* is normally not expressed in stamens. These data demonstrate that the *SHP* genes have retained the ability to substitute for *AG* activity and that the differences between the activities of the *SHP* and *AG* genes can largely be attributed to their different patterns of expression.

In addition to their roles in promoting *AG*-independent carpel and ovule development, the *SHP* genes are required for the differentiation of the dehiscence zone in mature fruit[10]. Separation of the dehiscence-zone cells is required for the normal seed-dispersal process. The *SHP* genes, which are expressed in the dehiscence zones of developing fruit, are also expressed in ovules[7,8]. However, *shp1 shp2* mutant ovules seem normal, indicating that a redundant factor might hide their possible roles during ovule development. One candidate for a redundant factor is the *STK* gene (formerly known as *AGL11*; ref. 12), because it shares extensive sequence similarity with the *SHP* genes[13] and because its expression domain overlaps that of the *SHP* genes during ovule development[7,8,12].

Initially, *STK* RNA is uniformly expressed in placental tissues and ovule primordia[12]. STK::*GUS* expression mirrors the pattern of *STK* RNA accumulation (Fig. 2a–c) and GUS activity is strongest in the funiculus, a stalk-like structure that connects the ovule and seed to the remainder of the fruit (Fig. 2d). In mature ovules, STK::*GUS* is expressed strongly in the funiculus and weakly in integuments that will later form the seed coat (Fig. 2e). This expression pattern continues after fertilization until stage 15, when GUS activity is no longer detectable.

To uncover the possible roles of the *STK* gene during ovule and seed development, we first identified *stk* loss-of-function mutants (see Methods). One of the most striking phenotypes observed in *stk* single mutants is a failure of the seeds to be released from the mature fruits (Fig. 2g). Seed detachment normally occurs by separation of the seed body from the funiculus at a site referred to as the seed abscission zone. In wild-type fruit, the seed abscission zone is located immediately adjacent to the seed body and seems constricted owing to the smaller size of cells in this region than in

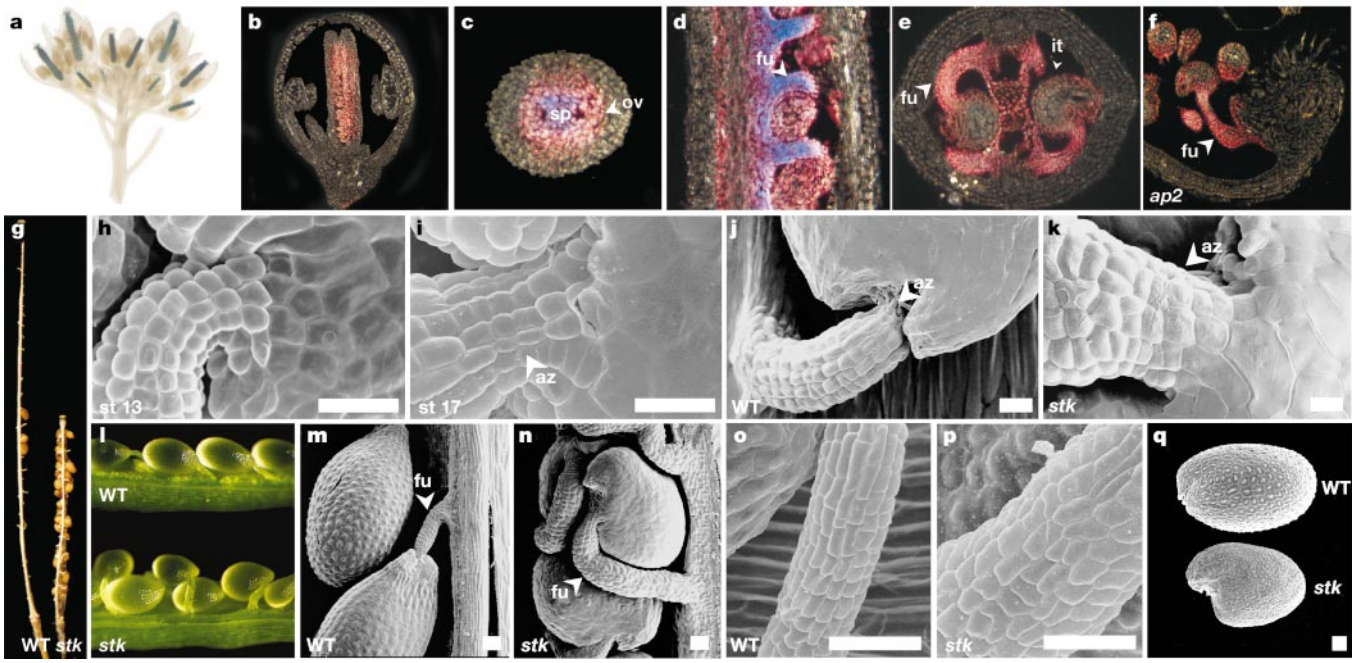

Figure 2 Characterization of *STK*. **a**, Staining of inflorescence for GUS reveals strong expression in carpels but not in other organs. **b**, GUS activity is first detected in placental tissue and young ovule primordia within carpels (stage 8). **c**, Transverse section of a carpel, showing uniform GUS expression in ovule primordia (ov) and septum (sp) (stage 9). **d**, During stage 11–12, expression is strong (blue) in the funiculus (fu) and weaker in the rest of the ovule (pink). **e**, At stage 13, GUS staining is restricted to funiculus and integuments (it). **f**, GUS activity is present in ectopic ovules on first-whorl organs of *ap2* mutants. **g**, Wild-type (WT) fruit (left) and *stk* fruit at stage 19–20. Most seeds stay attached to the fruit in *stk* mutants. **h**, **i**, Differentiation of seed abscission zone (az) is observed at stage 17 (**i**) but not at stage 13 (**h**). **j**, **k**, Seed abscission (**j**) fails to occur in *stk* mutants (**k**). **l**, Arrangement of the developing seeds is irregular in *stk* mutants. **m**, **n**, At stage 17, wild-type funiculus (**m**) is much smaller than *stk* mutant funiculus (**n**). **o**, **p**, Differences in funicular cell size of wild-type cells (**o**) and mutant cells (**p**). **q**, Mature *stk* mutant seeds are smaller than wild-type seeds. Scale bars, 20 μm (**h**–**k**); 50 μm (**m**–**q**).

neighbouring tissue (Fig. 2i). Differentiation of abscission-zone cells occurs after fertilization, because no constriction is observed at the same region of mature wild-type ovules (Fig. 2h). Proper differentiation of these cells seems to be disrupted in *stk* mutants, because the separation of abscission zone cells fails to occur (Fig. 2j, k).

The *stk* mutant fruits are shorter than those of the wild type, typically reaching only 60% of the wild-type length. Inside the developing fruit, the regular positioning of seeds that occurs in the wild type is perturbed in *stk* mutants (Fig. 2l). This irregular seed spacing is apparently caused by a drastically enlarged funiculus in the *stk* mutants (Fig. 2m, n). Close inspection by scanning electron microscopy revealed that mutant funicular cells are larger than in the wild type (Fig. 2o, p) and that the cell number is increased. These results show that *STK* is required to prevent the abnormal growth of the funiculus by controlling cell expansion and cell division. In addition, whereas wild-type seeds adopt an oblong shape, *stk* seeds are rounder and smaller (Fig. 2q).

To determine whether *STK* acts redundantly with the *SHP* genes to control ovule development, we constructed *stk shp1 shp2* triple mutants. Remarkably, normal ovule and seed development was completely disrupted in these mutants. Although some viable but abnormal seeds were obtained, many of the ovules and seeds were arrested during their development. The most striking phenotype is that some of the ovules were converted into either leaf-like or carpel-like structures (Fig. 3a, b). Close inspection of cells on the surface of these converted ovules revealed style-like and valve-like cells characteristic of carpels that are never observed on wild-type ovules (Fig. 3c, d). Occasionally, stigmatic tissue was also observed among the converted ovules. Conversion of ovules into carpelloid structures has also been reported in *Petunia* when the activities of the two putative *STK* gene orthologues *Floral Binding Protein7* (*FBP7*) and *FBP11* were removed simultaneously[14]. Moreover, ectopic expression of *FBP11* in petunia[15] as well as *STK* in *Arabidopsis* (L. Colombo, personal communication) was sufficient to induce the formation of ectopic ovules. Taken together, these results demonstrate that the *STK* gene is both necessary and sufficient to promote ovule development.

Because the *STK* (Fig. 2f), *SHP* and *AG* genes are all expressed in the ectopic ovules that form in the first-whorl organs of *ap2* mutants[5,7,8], we investigated their roles in promoting ectopic ovule development. Although many of the ectopic ovules that form in *ap2* mutants develop normally, about 40% of them develop into carpelloid structures[16]. More ovules (55%) are converted in *ap2 ag* double mutants, confirming previously published results of the role for *AG* in promoting ovule identity[16]. The conversion of ovules into carpelloid structures occurs even more frequently (81%) in *ap2 stk ag* triple mutants (Fig. 3e), indicating that *STK* and *AG* might have redundant roles in promoting ovule identity. Loss of ovule identity is strongest in *ap2 stk shp1 shp2* quadruple mutants (Fig. 3f), because nearly all (95%) ectopic ovules were converted into carpelloid structures. Although the remaining ovules have integument-like characteristics, they did not develop into mature ovules. Taken together, these data demonstrate that all four members of the *AG* clade contribute to proper ovule development.

These studies have revealed the redundant roles of *AG* and the *SHP* genes in promoting carpel development, as well as the redundant roles of *AG*, *STK* and the *SHP* genes in promoting ovule identity (Fig. 4). Although these four genes clearly evolved from a common ancestral gene and retain redundant activities, they have now evolved specific functions, with *AG* promoting reproductive organ identity, the *SHP* genes promoting fruit dehiscence, and the *STK* gene controlling funiculus development and seed detachment. It is interesting that both fruit dehiscence and seed abscission, which are two distinct steps in seed dispersal process, are controlled by closely related MADS-box genes.

The occurrence of large multigene families has emerged as a common theme now that the genome sequences of *Arabidopsis* and other organisms are known. This fact has highlighted the genetic redundancy that probably occurs in closely related family members. The MADS-box gene family in *Arabidopsis* serves as a good example of this redundancy, because it contains dozens of members, many of

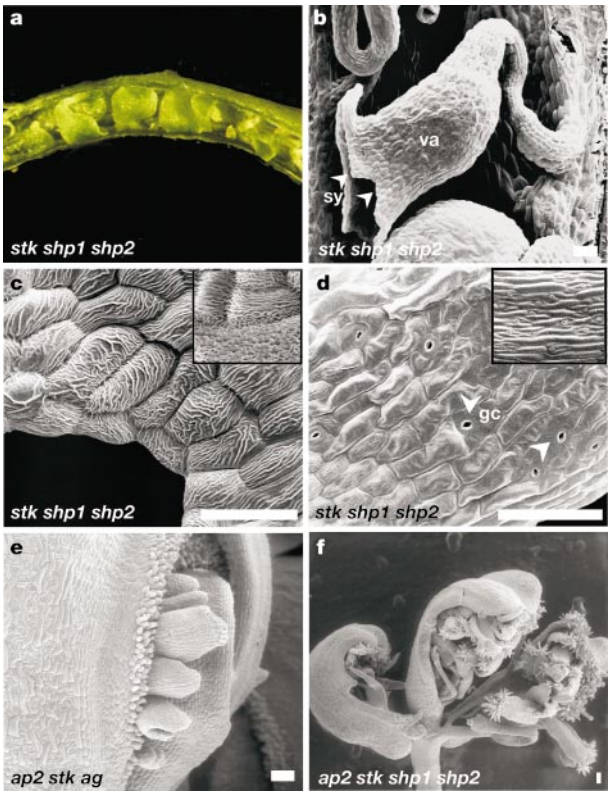

Figure 3 All members of the *AG* clade redundantly specify ovule identity. **a**, **b**, Stage-17 *stk shp1 shp2* mutant fruit reveals conversion of many ovules into carpel-like structures (**b**) that have style-like (sy) regions located at the edges and valve-like (va) regions in the middle. **c**, Close-up of the tip of the carpel-like structure in (**b**) showing style-like cells with cuticular thickening, a characteristic of wild-type stylar cells (inset). **d**, Cells in the middle part of the carpel-like structure in (**b**) resemble valve cells of wild-type carpel (inset). Several guard cells (gc) are indicated. **e**, Most of the ectopic ovules are converted into carpel-like structures in *ap2 stk ag* mutants. **f**, Ectopic ovules are largely absent from first-whorl organs of *ap2 stk shp1 shp2* mutants. Scale bars, 50 μm (except **c**, 20 μm).

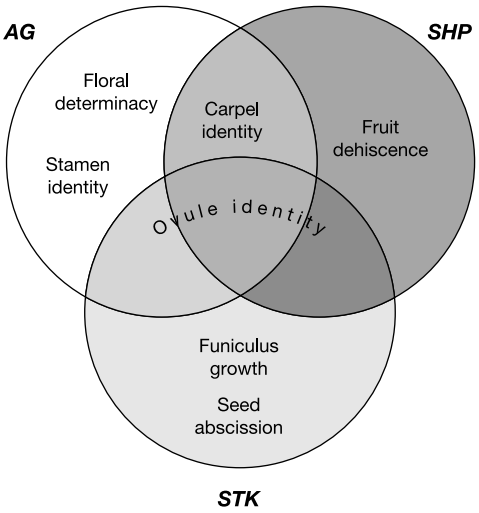

Figure 4 Unique and overlapping functions of genes in *AGAMOUS* clade.

which are known to control aspects of plant development. The *AG* MADS-box gene has a critical function in plant sexual reproduction, because *ag* mutants completely lack reproductive organs. However, because *AG* is only one of four members of a monophyletic clade, it has been necessary to characterize all possible combinations of single, double, triple and quadruple mutants. Now that the individual and redundant roles of the *AG* clade of MADS-box genes are known, we can begin to explore their roles in setting up the patterns of downstream gene expression required for carpel and ovule development. □

Methods

Reporter construct and histology

The STK::*GUS* reporter construct is a translational fusion of sequence 2 kilobases (kb) upstream of the *STK* translation start codon with the GUS (uidA) gene in pDW137 (ref. 17). The sequence was amplified by primer P1 (5′-CCTGCAGCCGTGATTGCAATT GCAAAATTTGAG-3′) and P5 (5′-CTCTAGACCCATCCTTCATTTTAAACA-3′) then cloned into pCRII vector, yielding pPOP163. This 2-kb sequence contains 1.3 kb of the first intron and 0.7 kb of the promoter region. The sequence was then cloned into the *Pst*I and *Xba*I sites of pDW137, resulting in pPOP166. GUS staining was performed as described previously[18] except that the concentrations of potassium ferrocyanide and potassium ferricyanide were 5 mM.

Plant materials

The *stk-1* allele was identified by screening for an *En-1* insertion among a collection of plants carrying about 50,000 independent insertions by using the *STK*-specific primer 11-X5 (5′-CCACTAACCATTTGATGATGGTGTTGT-3′) and the *En-1*-specific primer En8130 (5′-GAGCGTCGGTCCCCACACTTCTATAC-3′)[19]. *En-1* is inserted near the 3′ end of exon 3 (2 base pairs (bp) from the splice site). The *stk-2* and *stk-3* stable alleles were obtained by screening for germinal excision of the *stk-1* allele. The s*tk-2* allele contains a 74-bp insertion near the splice site of third intron. The *stk-3* allele has a 47-bp deletion that removes the splice site. Analysis by polymerase chain reaction (PCR) with reverse transcription showed that mutations in both alleles affected RNA splicing (data not shown). Two additional mutant alleles, *stk-4* and *stk-5*, were obtained from the Madison Alpha collection by using the gene-specific primers 11-X15 (5′-TTCTTGCAGTCTGCC ACTAGTTTGTGTGT-3′) and 11-X18 (5′-AACTGCTTCGTTACGCACCGAACTCA ACA-3′), respectively, together with the T-DNA-specific primer JL202 (5′-CATTTTATA ATAACGCTGCGGACATCTAC-3′). The *stk shp1 shp2* triple mutants were generated by crossing *shp1-1 shp2-1* double-mutant plants with plants carrying either the *stk-2* or the *stk-3* allele. The *shp* alleles[10,20] *ap2-6* (ref. 21) and *ag-2* (ref. 22) used for generating the mutants were described previously. The 35S:*SHP1/2* transgenic plants were described previously[10]. The 35S:*SHP2* transgene was introduced into *ag-1* mutants.

Mutant genotyping

The *stk-2* and *stk-3* alleles were genotyped by PCR with the primers 11-x4 (5′-GCTTGTTCTGATAGCACCAACACTAGCA-3′) and 11-x5 (5′-CCACTAACCATTTGAT GATGGTGTTGT-3′). The *stk shp1 shp2* triple mutants were identified by screening for mutant phenotypes and later confirmed by PCR genotyping all three genes. Genotyping of *shp1-1* and *shp2-1* allele was as described previously[10]. The *ap2-6* allele was genotyped by PCR amplification with the primers G1021 (5′-GCAGCAGCTCGGTATTTTTC-3′) and G1022 (5′-ATCAAACCGGTGGTTCTCAG-3′) followed by digestion with *Hin*dIII. The *Hin*dIII site is present only in the *ap2-6* allele.

Scanning electron microscopy

Flowers and fruits were fixed overnight in 1.6% osmium tetroxide in 50 mM sodium cacodylate buffer at 4 °C. Tissues were washed twice in the same buffer and dehydrated in an acetone series. After critical-point drying, pistils and fruits were dissected to expose ovules or seeds inside. Tissues were coated with gold/palladium. Samples were examined in either a Cambridge S360 scanning electron microscope or a Quanta 600 microscope using an acceleration voltage of 5–20 kV.

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Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells

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Whereas naive T cells migrate only to secondary lymphoid organs[1,2], activation by antigen confers to T cells the ability to home to non-lymphoid sites[3,4]. Activated effector/memory T cells migrate preferentially to tissues that are connected to the secondary lymphoid organs where antigen was first encountered[5–7]. Thus, oral antigens induce effector/memory cells that express essential receptors for intestinal homing, namely the integrin α4β7 and CCR9, the receptor for the gut-associated chemokine TECK/CCL25 (refs 6, 8, 9). Here we show that this imprinting of gut tropism is mediated by dendritic cells from Peyer's patches. Stimulation of CD8-expressing T cells by dendritic cells from Peyer's patches, peripheral lymph nodes and spleen induced equivalent activation markers and effector activity in T cells, but only Peyer's patch dendritic cells induced high levels of α4β7, responsiveness to TECK and the ability to home to the small