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A Role for *Arabidopsis PUCHI* in Floral Meristem Identity and Bract Suppression [C][W][OA]

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At the onset of flowering, the *Arabidopsis thaliana* primary inflorescence meristem starts to produce flower meristems on its flank. Determination of floral fate is associated with changes in the growth pattern and expression of meristem identity genes and suppression of a subtending leaf called a bract. Here, we show a role in floral fate determination and bract suppression for the *PUCHI* gene, an AP2/EREBP family gene that has previously been reported to play roles in lateral root morphogenesis. Mutations in *PUCHI* cause partial conversion of flowers to inflorescences, indicating that *PUCHI* is required for flower meristem identity. *PUCHI* is transiently expressed in the early flower meristem and accelerates meristem bulging while it prevents the growth of the bract primordium. The function of *PUCHI* in floral fate determination and bract suppression overlaps that of the *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* genes, which encode a pair of redundant regulatory proteins involved in various developmental processes, including leaf morphogenesis and flower patterning. We also show that *PUCHI* acts together with *BOP1* and *BOP2* to promote expression of *LEAFY* and *APETALA1*, two central regulators of floral meristem identity. Expression patterns of the *PUCHI* and *BOP* genes point to a role in spatial control of flower-specific activation of these meristem identity genes.

INTRODUCTION

Most aerial parts of a plant are generated postembryonically by the activity of the shoot meristem, a group of mitotically active cells that continuously add new structures at the shoot apex throughout the life cycle (Steeves and Sussex, 1989; Poethig, 2003). The shoot meristem initially produces vegetative leaves at its periphery and then produces flowers as it enters the reproductive phase. Plants show a wide variety of inflorescence morphologies, and the pattern of any particular inflorescence form is highly dependent on when and where flower primordia arise in the shoot meristem (Coen and Nugent, 1994; Benlloch et al., 2007; Prusinkiewicz et al., 2007).

In *Arabidopsis thaliana*, the primary inflorescence produces lateral meristems that develop into either secondary inflorescences or flowers (Schultz and Haughn, 1991). Secondary inflorescences, or branches, are produced immediately after the transition from the vegetative to the reproductive phase and show an indeterminate growth pattern that reiterates the pattern of the primary inflorescence. After several rounds of branch production, the primary inflorescence meristem begins to produce determinate floral meristems, which generate a fixed number of floral organs. The conversion of meristem identity from secondary inflorescence to flower is largely dependent on endogenous and environmental cues, which eventually converge on the expression of the floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), both encoding transcription factors (Weigel et al., 1992; Mandel et al., 1992; Weigel and Meyerowitz, 1993; Blázquez et al., 2006; Baurle and Dean, 2006; Kobayashi and Weigel, 2007).

Mutations in *LFY* and *AP1* cause partial conversion of flowers into branch-like structures, whereas constitutive expression of either gene is sufficient to convert branches into flowers, indicating that these genes are critical factors for specifying floral meristem identity (Weigel et al., 1992; Bowman et al., 1993; Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Expression of *LFY* is weak in leaf primordia during the vegetative phase but is strongly activated in floral meristems at the onset of flowering (Weigel et al., 1992; Blázquez et al., 1997; Hempel et al., 1997). The LFY protein directly activates transcription of *AP1* and its redundant homolog *CAULIFLOWER* (*CAL*) in the floral meristem (Parcy et al., 1998; Wagner et al., 1999; William et al., 2004). *AP1* and *CAL* in turn maintain *LFY* expression to ensure correct floral identity (Bowman et al., 1993; Liljegren et al., 1999).

The above results indicate that the attainment of the high level of *LFY* expression is a key step for floral meristem specification. Although many factors besides *AP1* and *CAL* have been reported to promote *LFY* expression, none are expressed specifically in floral meristems but rather in a broader region (Blázquez et al., 1998; Blázquez and Weigel, 2000; Lee et al., 2000; Samach et al., 2000; Yu et al., 2002; Michaels et al., 2003; Smith et al., 2004; Abe et al., 2005; Wigge et al., 2005; Kanrar et al., 2008; Lee et al.,

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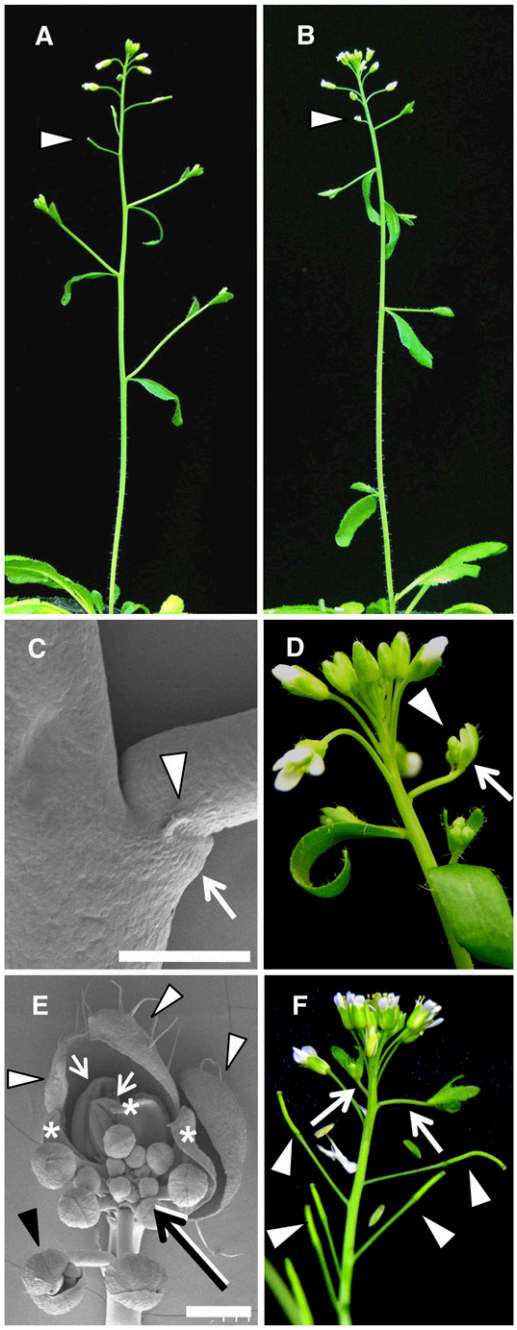

Figure 1. Inflorescence Phenotypes of the Wild Type and *puchi-1*.

(A) to **(E)** Inflorescence of wild type **(A)** and *puchi-1* **([B]** to **[E])** grown under continuous light conditions.

(A) Inflorescence of a wild-type plant.

(B) Inflorescence of a *puchi-1* plant. Compared with the wild type, the number of nodes with secondary inflorescences is increased in *puchi-1*. Arrowheads indicate the first flower formed after the transition from secondary inflorescences to flowers.

(C) Scanning electron micrograph of a *puchi-1* branch that lacks a subtending cauline leaf but instead has a flat leaf-like organ (arrow) flanked by a pair of pin-shaped projections (only one of them is apparent in this image; arrowhead). Bar = 500 μm.

2008), raising the question of how local activation of *LFY* expression is regulated.

In *Arabidopsis*, an important feature that distinguishes a flower from a secondary inflorescence is the absence of subtending leaves or bracts in the flower. Whereas the secondary inflorescence meristem is initiated in the axil of a primordium that develops into a subtending leaf, the floral meristem is initiated as an adaxial subdomain of a flower primordium that also contains the abaxial cryptic bract domain. Subsequent development of the cryptic bract is strongly suppressed by an unidentified signal derived from the floral meristem (Nilsson et al., 1998; Long and Barton, 2000), resulting in the formation of a flower that lacks a visible subtending bract. Both *LFY* and its coregulator *UNUSUAL FLORAL ORGANS* (*UFO*) are involved in this process (Schultz and Haughn, 1991; Hepworth et al., 2006). Besides these, the two paralogous genes *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2*, which encode proteins related to the disease resistance regulatory protein NONEXPRESSOR OF PR1 (NPR1), are redundantly required for suppression of the bract (Hepworth et al., 2005; Norberg et al., 2005). Although *BOP1* and *BOP2* have been suggested to participate in the transition from vegetative to reproductive development, their precise role in flower development is not yet clear.

Arabidopsis PUCHI, which is required for lateral root morphogenesis (Hirota et al., 2007), is another factor that is potentially involved in meristem identity and bract suppression. Mutations in this gene cause ectopic cell proliferation at the base of lateral root primordia, indicating that *PUCHI* is involved in cell division control during lateral root formation. In the shoot, on the other hand, *puchi* mutants produce characteristic ectopic tissue that is reminiscent of a bract although its exact identity remains unclear. The PUCHI protein belongs to the AP2/ethylene-responsive element binding protein family and is highly homologous to the maize (*Zea mays*) protein BRANCHED SILKLESS1 (BD1) and the rice (*Oryza sativa*) protein FRIZZY PANICLE (FZP), both of which affect floral meristem identity (Chuck et al., 2002; Komatsu et al., 2003).

Here, we provide evidence that, in addition to its role in lateral root development, *PUCHI* is involved in the determination of floral meristem identity and suppression of bract growth. *PUCHI* is expressed on the adaxial side of early floral primordium and is required for proper conversion of secondary inflorescences to flowers. The *puchi* mutations cause a prolonged phase of bract primordium growth while they delay the bulging of the floral

(D) Primary inflorescence of *puchi-1*, showing a mosaic branch consisting of a flower (arrow) and an inflorescence (arrowhead).

(E) Scanning electron micrograph of a mosaic branch of *puchi-1*. White arrowheads indicate sepal-like organs in the first whorl. Asterisks and white arrows indicate petal- and stamen-like organs, respectively. The black arrow indicates the inflorescence-like shoot, and the black arrowhead indicates an extra flower produced from the pedicel. Bar = 500 μm.

(F) Primary inflorescence of *puchi-1* grown under short-day conditions, showing ectopic secondary inflorescences (arrows). The ectopic secondary inflorescences are produced after six to nine flowers/siliques (arrowheads) have arisen on the primary inflorescence.

[See online article for color version of this figure.]

meristem. We also show that *PUCHI* has an overlapping function with *BOP1* and *BOP2* in controlling floral meristem identity and that these genes together promote expression of *LFY* and *AP1*. The expression domains of *PUCHI* and *BOP* are restricted to lateral meristems and may provide a positional cue for flower-specific activation of these meristem identity genes.

RESULTS

puchi Mutations Affect Floral Meristem Identity

Floral transition in *Arabidopsis* is regulated by multiple endogenous and environmental factors, including daylength (Baurle and Dean, 2006; Kobayashi and Weigel, 2007). To investigate *PUCHI* gene function in flower development, we characterized two recessive alleles, *puchi-1* and *puchi-2* (Hirota et al., 2007), under continuous-light and short-day conditions. The timing of the meristem identity transition is commonly measured by counting the number of secondary inflorescences produced on the bolting stem prior to flower formation (Ratcliffe et al., 1998). We also counted rosette leaf number, which correlates well with flowering time (Koornneef et al., 1991).

Under continuous-light conditions, both *puchi-1* and *puchi-2* mutants showed a small but significant increase in the number of secondary inflorescences compared with that of the wild type, whereas the number of rosette leaves was unaffected (Figures 1A and 1B, Table 1; see Supplemental Table 1 online). This phenotype was interpreted as very early arising flowers being completely transformed into secondary inflorescences. In addition, 20% (10 of 50) of *puchi* plants lacked a subtending cauline leaf in the uppermost secondary inflorescence and instead formed a flat leaf-like structure flanked by a pair of pin-shaped projections (Figure 1C; see Supplemental Figure 1A online). These solitary branches lacking normal cauline leaves occasionally (4%: 2 of 50 inflorescences) showed a mosaic of inflorescence and flower phenotypes (Figure 1D; see Supplemental Figure 1B online). The apex of the mosaic structures consisted of three sepal-like organs in the first whorl, a few petals and stamens inside them, and an indeterminate shoot at the center (Figure 1E). In addition, an extra flower often formed from the pedicel of these mosaic structures (Figure 1E, black arrowhead). These phenotypes appeared to represent an incomplete conversion of a flower to an inflorescence.

Under short-day conditions, *puchi* mutants clearly possessed more secondary inflorescences than did the wild type (Table 1). In addition, *puchi* plants produced ectopic secondary inflorescences after six to nine flowers had arisen on the primary inflorescence (Figure 1F; see Supplemental Figure 1C online). Typically, in such cases, one to three ectopic inflorescences were produced sequentially; these phases of ectopic inflorescence production could occur up to four times during inflorescence development, with each phase being separated by the formation of 1 to 10 flowers. These ectopic inflorescences reiterated the process of primary inflorescence (see Supplemental Figure 1D online), suggesting that the transformation of flowers to secondary inflorescences was complete.

Taken together, these results show that mutations in *PUCHI* caused partial conversion of flowers into inflorescences in the two photoperiod conditions examined and indicate that *PUCHI* controls the fate of lateral meristems. The *puchi-1* and *puchi-2* mutants gave essentially the same phenotypes in all aspects of shoot development, and we chose the *puchi-1* allele for further analyses.

puchi Mutant Flowers Have Rudimentary Bracts

We next examined flower phenotypes in the *puchi* mutant. All *puchi* flowers had ectopic structures at the base of each pedicel, whereas wild-type flowers showed a smooth surface at the same position (Figures 2A and 2B; Hirota et al., 2007). These ectopic structures consisted of a flat leaf-like part and a pair of pin-shaped projections, similar to those observed at the base of the solitary secondary inflorescences (Figures 1C and 2B). The pin-shaped projections were morphologically similar to stipules formed at the base of the leaves, although their size was somewhat larger than normal stipules. Based on these observations, we speculated that these structures were a rudimentary bract associated with a pair of stipules. To test this prediction, we analyzed expression of green fluorescent protein (GFP) in the

Table 1. Inflorescence Architecture of *puchi*, *bop1 bop2*, and *bop1 bop2 puchi* Mutants Grown under Continuous-Light and Short-Day Conditions

Condition	Genotype	SI with Cauline Leaf	SI without Cauline Leaf	Total SI	Plants Scored
CL	Col	3.33 ± 0.11	0.0 ± 0.0	3.33 ± 0.11	30
	puchi-1	4.60 ± 0.10**	0.20 ± 0.07**	4.80 ± 0.11**	30
	puchi-2	4.43 ± 0.15**	0.20 ± 0.07**	4.63 ± 0.18**	30
	bop1 bop2	4.7 ± 0.14**	0.30 ± 0.09**	5.0 ± 0.18**	30
	bop1 bop2 puchi	4.67 ± 0.11**	19.60 ± 0.45**	24.27 ± 0.46**	30
SD	Col	10.05 ± 0.32	0.0 ± 0.0	10.05 ± 0.0	20
	puchi-1	12.15 ± 0.32**	3.35 ± 0.49**	15.50 ± 0.57**	20
	puchi-2	10.90 ± 0.25*	3.4 ± 0.41**	14.30 ± 0.33**	20
	bop1 bop2	32.44 ± 0.96**	2.67 ± 0.94**	35.11 ± 0.86**	9
	bop1 bop2 puchi	49.55 ± 1.27**	0.0 ± 0.0	49.55 ± 1.27**	9

The number of secondary inflorescences (SI) produced on the primary bolting stem was scored for each genotype. Values are mean ± SE. Differences between wild-type and mutant plants are significant at the $0.05 > P > 0.01$ (*) or the $P < 0.01$ (**) levels. Under short-day (SD) conditions, SI that formed at positions below and above the lowermost flower were both scored. In continuous light (CL), none of the genotypes produced any ectopic branches.

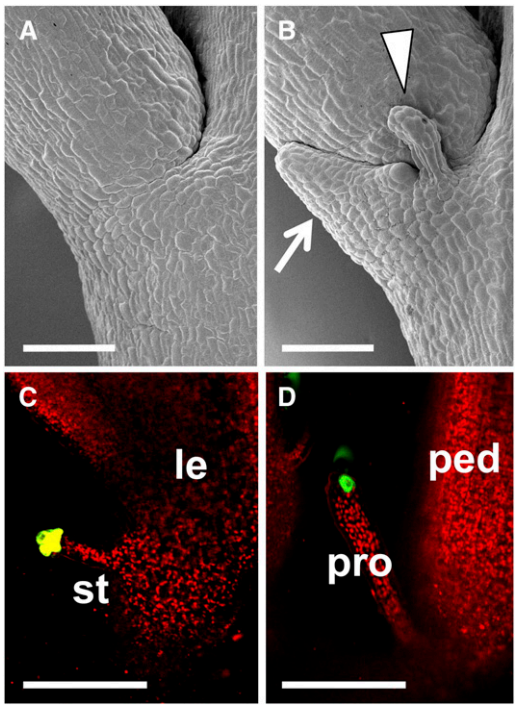

Figure 2. *puchi* Mutant Flowers Have Rudimentary Bracts at the Base of Their Pedicels.

(A) Scanning electron micrograph showing the base of the wild-type pedicel.
(B) Scanning electron micrograph showing the base of the *puchi-1* pedicel. *puchi* produces a flat leaf-like organ (arrow) flanked by a pair of pin-shaped projections (only one is visible in this image; arrowhead).
(C) GFP expression of the enhancer trap line E1238 is detected in the stipule of the wild-type cauline leaf.
(D) GFP expression of the enhancer trap line E1238 is detected in the pin-shaped projection of the *puchi-1* flower.
le, leaf; st, stipule; ped, pedicel; pro, pin-shaped projection. Bars = 100 μm.

enhancer trap line E1238 (http://enhancertraps.bio.upenn.edu/default.html), in which the signal is detected in the stipules of leaves (Figure 2C). The GFP signal was detected in the pin-shaped projections of *puchi* mutant flowers (Figure 2D). Furthermore, mutation of the *PRESSED FLOWER* (*PRS*) gene, which is required for stipule formation in the leaf (Matsumoto and Okada, 2001; Nardmann et al., 2004), resulted in the loss of the pin-shaped projections when combined with the *puchi* mutation (see Supplemental Figures 2A to 2D online). These results indicate that the ectopic structures at the base of *puchi* pedicels comprise a rudimentary bract associated with a pair of stipules and that *PUCHI* is involved in the suppression of bract growth in flowers. Given that the absence of a subtending leaf is one of the characters that discriminate flowers from secondary inflorescences in *Arabidopsis*, the failure of bract suppression in the mutant may be explained as a partial conversion of flowers into secondary inflorescences.

In contrast with the phenotype in the morphology of the flower base, we could not detect any obvious abnormalities in the identity or the number of individual floral organs of *puchi* (see Supplemental Figures 3A to 3D and Supplemental Table 2 online), indicating that *PUCHI* is not involved in the specification or patterning of flower organs.

Early Flower Primordium Development in *puchi*

To investigate how *PUCHI* affects bract growth, we sought to examine early flower development in the *puchi* mutant in detail. To this end, we used a sensitive, noninvasive replica method combined with a three-dimensional reconstruction algorithm, which can reliably detect a cryptic bract in early flower primordia (Kwiatkowska, 2006).

Curvature plots on wild-type inflorescence apices enable the definition of four consecutive stages in flower primordium development (Kwiatkowska, 2006; Szczesny et al., 2009). The first floral stage is an initial bulging that leads to the formation of a shallow crease between the primordium protrusion and the primary inflorescence meristem (Figure 3A). This region is concave in the meridional direction (red curvature cross arms in Figure 3A) and convex in the latitudinal (black arms). The next stage is a second bulging, at which a convex region (marked by curvature crosses with both arms black) appears at the bottom of the shallow crease (Figure 3B; see also Kwiatkowska, 2006; Szczesny et al., 2009). The convex region corresponds to the floral meristem proper, while the concave region at the distal end (arrowhead in Figure 3B) corresponds to the bract primordium. In the third stage of bulge formation (Figure 3C), the temporarily apparent bract primordium disappears. During the final stage, the sepal primordia are formed (P5 in Supplemental Figure 4A online).

In the *puchi* mutant, we also recognized four similar consecutive stages, although both their geometry and their timing were different from those in the wild type. During the first stage of initial bulging, the mutant primordium was indistinguishable from that of the wild type at the beginning (data not shown) but then protruded further from the shoot axis than it did in the wild type (cf. Figures 3A and 3D). The upper surface of the primordium was largely flat or only slightly concave, forming a shelf-like shape (Figure 3D). The duration of initial bulging leading to formation of this shelf-like primordium, measured as the mean number of plastochrons, was longer than the equivalent stage in the wild type (mean of 3.89 plastochrons ± 0.11 SE in *puchi* versus 3.16 ± 0.09 SE in the wild type; n = 9 and 16, respectively). During the stage of the second bulging in *puchi*, a convex region (where both curvature cross arms are depicted in black) appeared within the shelf-like region, similar to the wild type. Unlike the wild type, however, the mutant bract did not disappear; the shelf-like primordium was partitioned into the floral meristem proper and the bract (Figure 3E).

The major difference between *puchi* and the wild type in the third stage was the permanent presence in *puchi* of a bract with stipules (cf. Figure 3C with 3F). Moreover, the duration of this stage was extended in the mutant. The mutant floral meristem proper grew into a finger-like structure devoid of sepals (Figure 3F) until it overgrew the primary inflorescence meristem (P7 in Supplemental Figure 4B online). At this stage, the cells of the floral meristem proper appeared enlarged both in the wild type

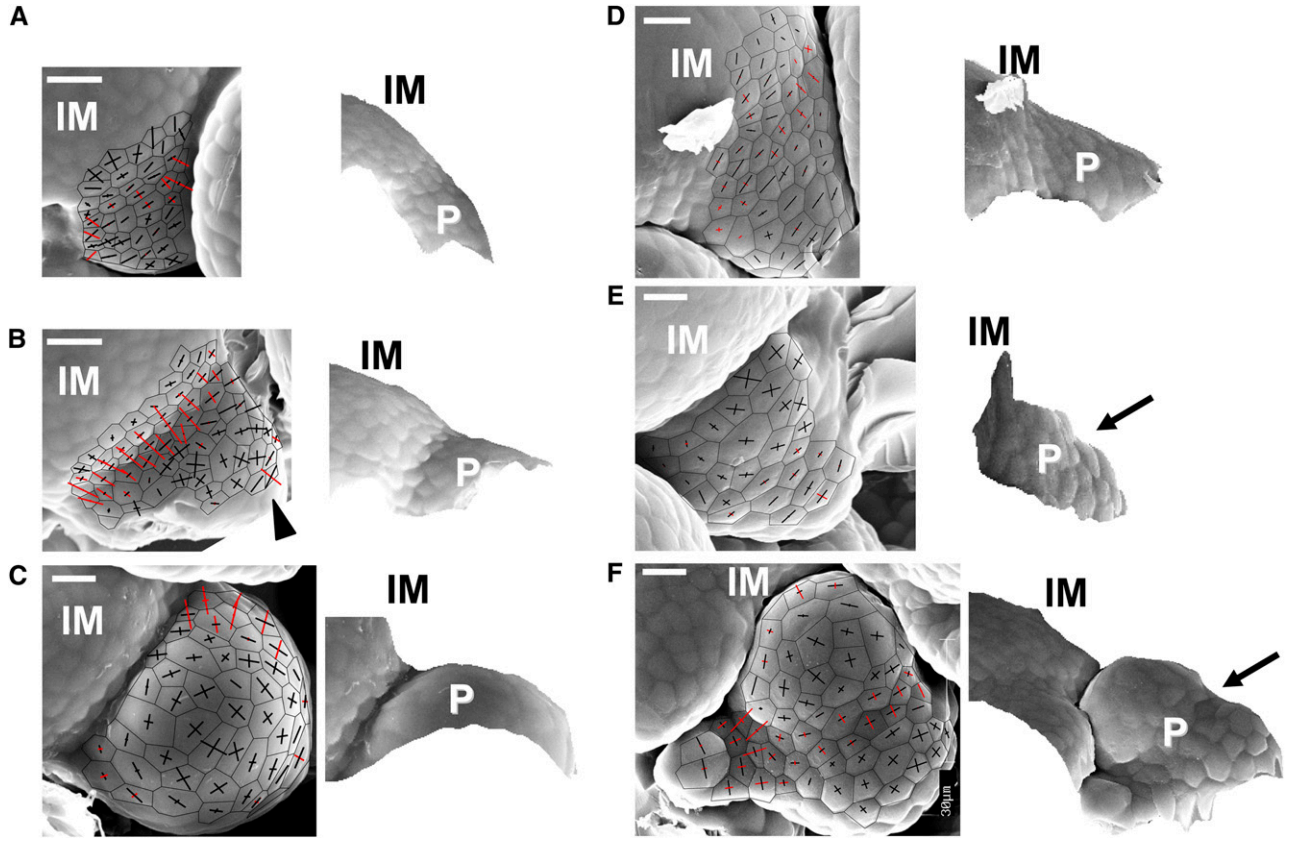

Figure 3. Early Flower Primordium Development in *puchi*.

(A) The wild-type primordium at the initial bulging stage.
(B) The wild-type primordium at the second bulging stage.
(C) The wild-type primordium at the bulge stage.
(D) The *puchi-1* primordium at the initial bulging stage.
(E) The *puchi-1* primordium at the second bulging stage.
(F) The *puchi-1* primordium at the bulge stage.
In each panel, scanning electron micrographs on which curvature crosses are overlaid (left), and side views of the reconstructed surface (right), were obtained from replicas of inflorescence shoot apices. Curvature cross-arms are aligned with the direction of curvature. The length of each cross-arm is proportional to the degree of curvature. Arms appear in red if the surface is concave in this direction and in black if it is convex. Three of the four consecutive stages, initial bulging, second bulging, and bulge, are shown. The arrowhead in **(B)** indicates the concave region at the distal end. Arrows in **(E)** and **(F)** point to the boundary between flower meristem proper and bract. IM, primary inflorescence meristem; P, flower primordium. Bars = 10 μm.

and in *puchi* (cf. Figure 3C with 3A and Figure 3F with 3D). A prolonged duration of the bulge stage in the mutant was manifested in delayed sepal formation (cf. P5 in Supplemental Figure 4A with P9 in Supplemental Figure 4C online, in which the youngest sepal primordia are indicated by asterisks). This stage in the wild type begins at 6.69 plastochrons (mean ± 0.17 SE; n = 16), but in *puchi* at 8.94 plastochrons (± 0.49 SE; n = 9).

In summary, early flower development of *puchi* is characterized by a prolonged period of the initial bulging stage, leading to the formation of a shelf-like bract primordium instead of a shallow crease as in the wild type. Consequently, initiation of the second bulging that forms the floral meristem proper is delayed. These results point to a role for *PUCHI* in regulating the early phase of floral meristem development in the axil of a cryptic bract.

PUCHI Is Expressed in Lateral Meristems Developing at the Periphery of the Primary Meristem

To investigate how *PUCHI* gene expression correlates with the mutant phenotypes, we monitored *PUCHI* mRNA by in situ hybridization. In the inflorescence apex, *PUCHI* expression was first detected in cells that had apparently begun to emerge from the inflorescence meristem as a buttress, which was morphologically equivalent to the stage 1 floral meristem (Figure 4A; Smyth et al., 1990). *PUCHI* expression continued until early stage

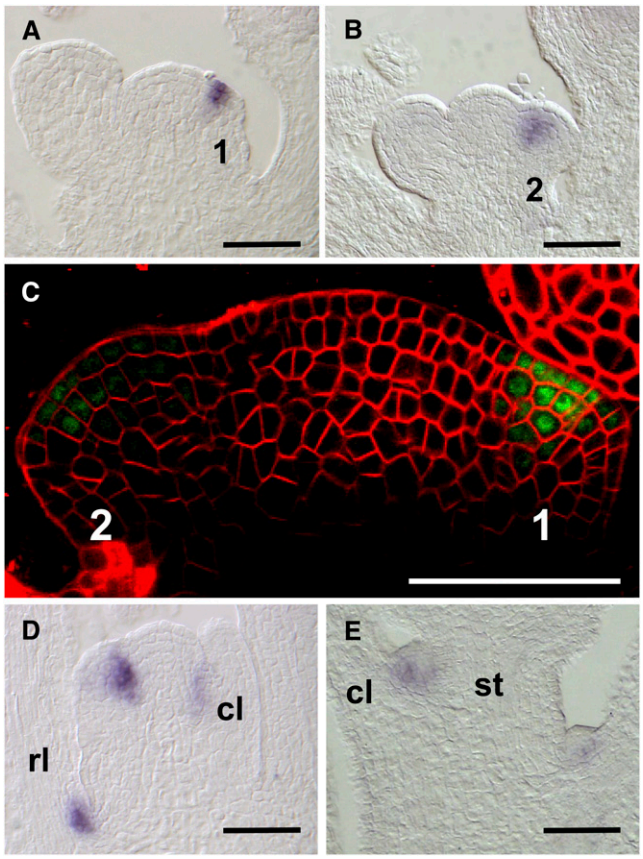

Figure 4. Expression Patterns of *PUCHI*.

(A) to **(C)** Wild-type inflorescence apices.
(A) *PUCHI* mRNA is detected on the adaxial side of the stage 1 flower primordium.
(B) *PUCHI* mRNA is detected on the adaxial side of the stage 2 flower primordum.
(C) Localization of GFP-PUCHI fusion protein expressed under the *cis*-regulatory elements of the *PUCHI* gene. The fusion protein is detected on the adaxial side of stages 1 and 2 floral meristem proper.
(D) Fourteen-day-old wild-type seedling apex that has just begun bolting. *PUCHI* mRNA is detected in the rosette and cauline leaf axils.
(E) *PUCHI* mRNA is detected in the axillary meristems of cauline leaves.
Numbers indicate stages of flower development (Smyth et al., 1990).
rl, rosette leaf; cl, cauline leaf; st, stem. Bars = 50 μm.

2 (Figure 4B) and disappeared before the initiation of sepal primordia. Accumulation of *PUCHI* mRNA was restricted to the adaxial side of floral primordia. The duration of *PUCHI* expression roughly corresponded to the first two stages in the analysis of surface morphology described above (i.e., the initial and second bulging stages).

Although *PUCHI* mRNA was detected on the adaxial side of the floral meristem, the *puchi* mutant displayed ectopic bract formation on the abaxial side. To localize the site of *PUCHI* action more precisely, we examined localization of a GFP-PUCHI fusion protein driven by the regulatory elements of *PUCHI* in the *puchi-1* mutant background (genomic GFP-PUCHI; Hirota et al., 2007). The GFP signal was localized to the adaxial side of floral meristems at stages 1 and 2 (Figure 4C), corresponding well with the pattern found in the in situ hybridization experiments. Furthermore, the genomic GFP-PUCHI construct fully complemented the rudimentary bract phenotype of *puchi*. These results indicate that adaxial localization of PUCHI protein is sufficient to suppress bract outgrowth on the abaxial side.

Because *PUCHI* is involved in the determination of floral meristem identity, we examined whether *PUCHI* expression was restricted to the floral meristem or was also present in other types of lateral meristems. During vegetative development, *PUCHI* transcript accumulation was not detected in the shoot apex (data not shown). Shortly after the onset of flowering, however, *PUCHI* mRNA accumulated in the axillary meristems of rosette and cauline leaves (Figures 4D and 4E). These results suggest that *PUCHI* is expressed in all lateral meristems after the transition from the vegetative to the reproductive phase.

PUCHI and *BOP* Have Overlapping Functions

The paralogous genes *BOP1* and *BOP2* are redundantly required for various processes of shoot organ development, such as leaf formation, flower patterning, and formation of floral organ abscission zone (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008). Notably, *bop1 bop2* mutant flowers are subtended by ectopic bracts (Norberg et al., 2005). We reexamined the *bop1-4 bop2-11* double mutant and found that the bracts that subtended early arising flowers were rudimentary and thus were not readily visible (Figure 5C), whereas those formed in late arising flowers were much larger and showed more complete leaf-like features (Figures 5C, inset, and 5L, left; Norberg et al., 2005). In addition, *bop1 bop2* showed significantly more secondary inflorescences than the wild type (Table 1), raising the possibility that *BOP1* and *BOP2* are involved in the determination of floral meristem identity. Because *PUCHI*, *BOP1*, and *BOP2* affect similar processes of flower development, we tested for a possible interaction between the *PUCHI* and *BOP* genes.

We first generated a *puchi-1 bop1-4 bop2-11* triple mutant, which displayed significantly enhanced phenotypes compared with the parental mutants with regard to both the determination of meristem identity and bract suppression (Figures 5A to 5L). The most striking feature of *puchi bop1 bop2* plants was their altered inflorescence structure, which was characterized by the presence of a much higher number of secondary inflorescences: up to six- to sevenfold more than in either of the parental mutants (Table 1). Each of these secondary branches typically had nodes with associated axillary shoots and showed indeterminate growth (Figure 5I). Such branches were always subtended by well-developed cauline leaves when they were produced at the basal nodes, whereas the upper branches were not (see Supplemental Figure 5 online). Thus, the transition from secondary inflorescence meristems to floral meristems in this triple mutant was more severely impaired that in either of the parent mutants. Scanning electron microscopy of the primary inflorescence apex of this triple mutant confirmed that, during the initial stages of inflorescence development, the primary inflorescence meristem yielded secondary meristems that produced lateral organs in a

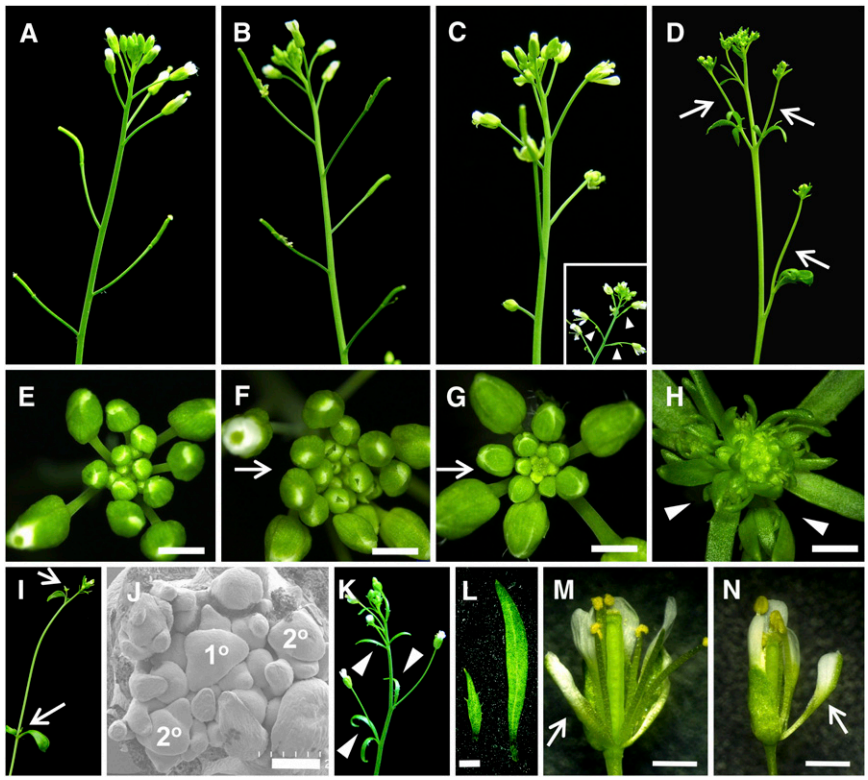

Figure 5. Genetic Interaction between *puchi* and *bop* Mutants.

(A) to **(D)** Forty-day-old primary inflorescences.
(A) The wild type.
(B) *puchi-1*.
(C) *bop1-4 bop2-11*. The inset shows an older (55 to 60 d old) inflorescence, producing visible bracts on the flower pedicels (arrowheads).
(D) *puchi-1 bop1-4 bop2-11*. Flowers are transformed into secondary inflorescence-like structures (arrows).
(E) to **(H)** Close-up view of inflorescence apices photographed when the inflorescences were ~10 mm in length.
(E) The wild type.
(F) *puchi-1*.
(G) *bop1-4 bop2-1*.
(H) *puchi-1 bop1-4 bop2-11*.
Unlike *puchi* and *bop1 bop2* flowers (arrows in **[F]** and **[G]**), the triple mutant produces secondary inflorescences (arrowheads in **[H]**).
(I) A secondary inflorescence of *puchi-1 bop1-4 bop2-11* in a position normally occupied by a flower in the wild type. Arrows indicate the formation of tertiary shoots in the leaf axils.
(J) Scanning electron micrograph showing a *puchi-1 bop1-4 bop2-11* inflorescence apex at a similar stage to **(H)**. Primordia produced by the primary inflorescence meristem (1°) behave like inflorescence meristems rather than like flower meristems (e.g., primordia indicated with 2°).
(K) Sixty-day-old inflorescence of a *puchi-1 bop1-4 bop2-11* triple mutant showing formation of flowers subtended by well-developed bracts (arrowheads).
(L) Typical bracts of *bop1-4 bop2-11* (left) and *puchi-1 bop1-4 bop2-11* (right). Note that *puchi* mutant flowers have rudimentary bracts that are much smaller than those in double and triple mutants (cf. **[L]** with Figure 2B; see Supplemental Figure 2A online).
(M) Flowers of a *bop1-4 bop2-11* double mutant.
(N) Flowers of a *puchi-1 bop1-4 bop2-11* triple mutant.
Arrows in **(M)** and **(N)** indicate sepal-petal hybrid organs in the first whorl.
Bars = 1 mm in **(E)** to **(H)** and **(L)** to **(N)** and 100 μm in **(J)**.
[See online article for color version of this figure.]

spiral arrangement that is typical of a branch, rather than a whorled pattern as in the floral meristem (Figure 5J).

After ~24 branches had appeared (Table 1), the primary shoot of the triple mutant started to produce flowers. However, these flowers were subtended by well-developed bracts that were much larger than those in *puchi* or *bop1 bop2* mutants (Figures 5K and 5L). These results show that the activity of bract formation is also enhanced in the triple mutant. It has been reported that *bop1 bop2* mutants produce flowers with abnormal morphology, such as increased numbers of floral organs and the presence of sepal-petal hybrid organs on the abaxial side of the first whorl (Hepworth et al., 2005; Norberg et al., 2005). The *puchi* mutation,

however, did not exacerbate the floral phenotypes of *bop1 bop2* (Figures 5M and 5N), again suggesting that *PUCHI* does not play a role in floral organ patterning. When *puchi bop1 bop2* plants were grown under short-day conditions, they showed even more extreme phenotypes than when they were grown under continuous light (Table 1), indicating that the effects of these mutations and short-day photoperiod are additive.

We next compared expression patterns of *BOP1* and *BOP2* between wild type and *puchi* mutant backgrounds. In the wild type, the earliest expression of both genes was found in the floral anlagen, from which a flower primordium will arise (Figures 6A and 6C; Hepworth et al., 2005; Norberg et al., 2005). Their expression persisted throughout early stage 1 and 2 floral meristems (Figures 6A and 6C). In the stage 2 primordium, *BOP1* and *BOP2* expression was detected in a central region that roughly corresponded to the zone between the floral meristem and the cryptic bract (Figures 6A and 6C; Long and Barton, 2000; Dinneny et al., 2004). In *puchi*, expression of *BOP1* and *BOP2* was similar to that in the wild type except that the signal was somewhat broader (Figures 6B and 6D). *PUCHI* expression in the *bop1 bop2* mutant inflorescence apex was also analyzed and was generally similar to that in the wild type, although the signal was localized more internally in *puchi* mutant primordia at late stage 2 (Figures 6F and 6F). Collectively, these results suggest that the *PUCHI* and *BOP* genes are not related to each other in a hierarchical order of transcriptional control.

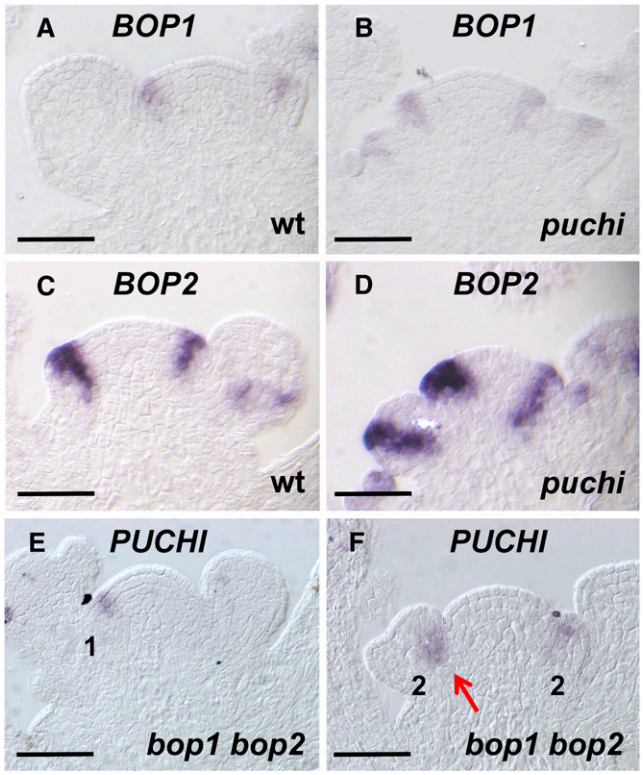

Figure 6. *BOP* and *PUCHI* Are Expressed Independently of Each Other.

(A) *BOP1* mRNA in longitudinal section of the wild-type inflorescence apex.
(B) *BOP1* mRNA in longitudinal section of the *puchi-1* inflorescence apex.
(C) *BOP2* mRNA in longitudinal section of the wild-type inflorescence apex.
(D) *BOP2* mRNA in longitudinal section of the *puchi-1* inflorescence apex.
(E) *PUCHI* mRNA in longitudinal section of *bop1-4 bop2-11* the inflorescence apex, showing expression in the stage 1 primordium.
(F) *PUCHI* mRNA in longitudinal section of the *bop1-4 bop2-11* inflorescence apex, showing expression in the stage 2 primordia. *PUCHI* expression is unaffected (cf. **[E]** with Figure 4A) except in the late stage 2 flower, in which it tends to localize to the inner cells (cf. **[F]**, arrow**,** with Figure 4B).
Bars = 50 μm.

The *PUCHI* and *BOP* Genes Are All Required for *LFY* and *AP1* Expression

The perturbation in floral meristem specification in *puchi bop1 bop2* suggested that other genes responsible for floral meristem specification, such as *LFY* and *AP1*, might be inactive in this triple mutant. We therefore tested whether the *puchi* and *bop* mutations had any effect on *LFY* and *AP1* expression.

In the wild-type inflorescence apex, *LFY* mRNA is first detected at a low level in the floral anlagen (Figure 7A; Weigel et al., 1992; Blázquez et al., 1997). *LFY* was uniformly expressed at a higher level throughout stage 1 and 2 flower primordia (Figure 7A). *LFY* expression was normal in *puchi* and *bop1 bop2* mutant backgrounds (Figures 7B and 7C) but was markedly reduced in the *puchi bop1 bop2* triple mutant inflorescence apex (Figure 7D). These results indicate that the *PUCHI* and *BOP* genes redundantly promote *LFY* expression during inflorescence development.

Next, we examined *AP1* expression patterns. In the wild type, *AP1* mRNA was detected at a high level in the adaxial cells of stage 1 and 2 floral primordia (Figure 7E; Mandel et al., 1992). A small group of abaxial cells in these early floral primordia did not express *AP1* (Figure 7E); these cells correspond to the cryptic bract region of floral primordia. In the *puchi* and *bop1 bop2* mutants, *AP1* expression was detected in a much smaller proportion of the adaxial cells of young floral primordia (Figures 7F and 7G), consistent with ectopic bract formation in these backgrounds, and was almost undetectable in the inflorescence of *puchi bop1 bop2* (Figure 7H). Thus, the severe inflorescence phenotype in the triple mutant correlates with a drastic reduction in the expression of genes involved in floral meristem specification.

DISCUSSION

PUCHI Is Required for Floral Meristem Identity

In this study, we have shown that *puchi* mutations affect inflorescence architecture in two ways. First, the number of secondary inflorescences is increased, indicating a conversion of early arising flowers into branches. Second, mutant flowers are subtended by rudimentary bracts, partially displaying the character of secondary inflorescences, which normally bear a subtending leaf. These results show that *PUCHI* is required for proper conversion of secondary inflorescences to flowers.

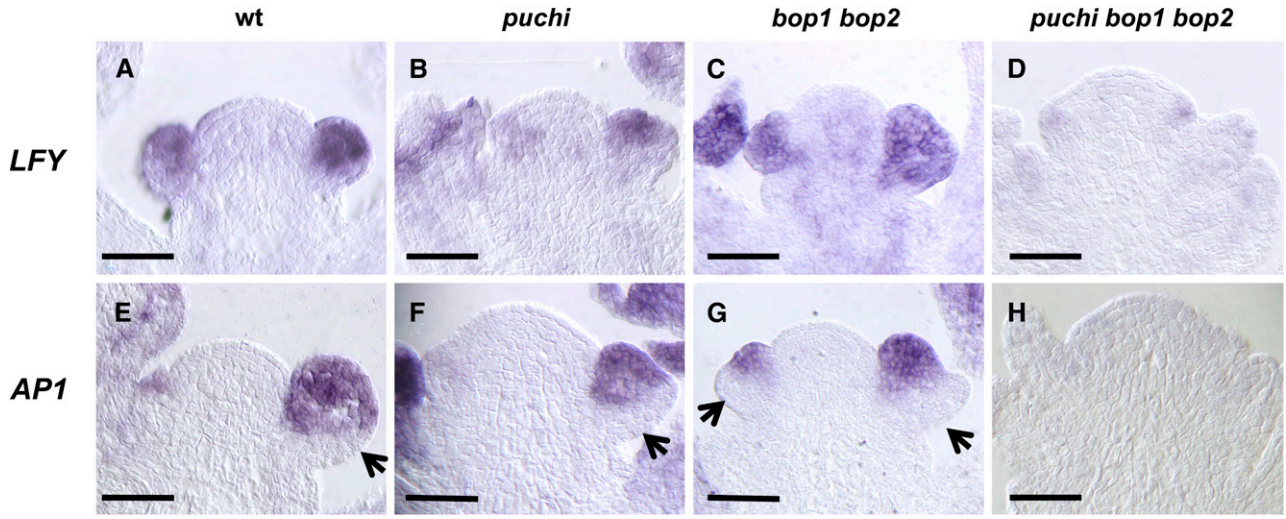

Figure 7. Expression of the Floral Meristem Identity Genes *LFY* and *AP1* in the *puchi bop1 bop2* Mutant.

(A) to **(H)** Longitudinal sections hybridized with either a *LFY* or an *AP1* probe.
(A) The wild-type inflorescence apex hybridized with the *LFY* probe.
(B) The *puchi-1* inflorescence apex hybridized with the *LFY* probe.
(C) The *bop1-4 bop2-11* inflorescence apex hybridized with the *LFY* probe.
(D) The *puchi-1 bop1-4 bop2-11* inflorescence apex hybridized with the *LFY* probe. Note that compared with the wild type **(A)**, *LFY* expression does not change in *puchi* single **(B)** or *bop1 bop2* double **(C)** mutants but is markedly reduced in the *puchi bop1 bop2* triple mutant **(D)**.
(E) The wild-type inflorescence apex hybridized with the *AP1* probe.
(F) The *puchi-1* inflorescence apex hybridized with the *AP1* probe.
(G) The *bop1-4 bop2-11* inflorescence apex hybridized with the *AP1* probe.
(H) The *puchi-1 bop1-4 bop2-11* inflorescence apex hybridized with the *AP1* probe. Note that compared with the wild type **(E)**, *AP1* expression is detected in a much smaller proportion of the adaxial cells of young flower primordia of *puchi* single **(F)** and *bop1 bop2* double **(G)** mutants and is almost undetectable in the *puchi bop1 bop2* triple mutant **(H)**.
Arrows indicate the absence of *AP1* expression on the abaxial side. Bars = 50 μm.

PUCHI is orthologous to maize *BD1* and rice *FZP*, both of which also affect inflorescence architecture (Chuck et al., 2002; Komatsu et al., 2003; Hirota et al., 2007). The inflorescences of grasses show a unique type of lateral meristem called spikelet meristems, from which floral meristems arise (Thompson and Hake, 2009). Spikelet meristems initially produce bract-like organs called glumes. In *bd1* and *fzp* mutants, spikelet meristems are replaced by indeterminate branch-like structures, indicating some functional similarity between the grass genes and *PUCHI* in the control of meristem identity. Several observations, however, point to important differences (this article; Chuck et al., 2002; Komatsu et al., 2003). First, expression of *PUCHI* is detected in the floral meristem proper (Figures 4A to 4C), whereas *BD1* and *FZP* are expressed in the axil of glumes but not in the spikelet meristem itself. Second, both *bd1* and *fzp* mutants display ectopic meristem formation in the axil of glumes, whereas no corresponding phenotype is observed in the *puchi* mutant. Third, the *puchi* mutation affects bract suppression, but neither *bd1* nor *fzp* mutations affect this process. These results together suggest that *Arabidopsis* has adopted this type of gene to its own fate determination process in a different way to the grass species.

Another difference between *PUCHI* and *BD1*/*FZP* lies in the strength of the mutant phenotypes: the inflorescence phenotype of *puchi* is much more subtle than that of *bd1* or *fzp*. It is possible that other *Arabidopsis* proteins function redundantly with PUCHI and partially mask the effects of the *puchi* single mutation. A good candidate is LEAFY PETIOLE, which is most closely related to PUCHI and shares 95% amino acid identity within its AP2 domain (van der Graaff et al., 2000; Hirota et al., 2007).

Relationship between the *PUCHI* and *BOP* Genes

Our analysis demonstrates that the *PUCHI* and *BOP* genes have overlapping functions and indicates that the relationship between these genes does not involve mutual transcriptional control. The strong phenotype in the *puchi bop1 bop2* triple mutant reveals the critical roles played by the *PUCHI* and *BOP* genes in the control of meristem identity and bract suppression, although the molecular mechanism underlying this synergistic phenotype is currently unknown. *BOP1* and *BOP2* encode proteins with a BTP/POZ domain and ankyrin repeats, both of which are involved in protein–protein interactions. Their homolog NPR1 regulates pathogen-inducible gene expression by interacting with TGACG sequence-specific binding transcription factors (TGAs) in the nucleus. BOP1 and BOP2 have also been shown to interact with a TGA protein, PERIANTHIA (PAN), to regulate floral organ patterning (Hepworth et al., 2005). Because our analysis shows that the expression domains of *PUCHI* and *BOP* genes overlap in lateral meristems, at least partially, it will be important to test whether PUCHI interacts directly with BOP proteins.

The *PUCHI* and *BOP* Genes May Provide a Positional Cue for Activation of *LFY* and *AP1* Expression

LFY and *AP1* expression was greatly reduced in lateral meristems of the *puchi bop1 bop2* triple mutant inflorescence apex (Figures 7D and 7H), demonstrating critical roles for the *PUCHI* and *BOP* genes in activating expression of these meristem identity genes. Because expression of *AP1* requires *LFY* (Liljegren et al., 1999; Ratcliffe et al., 1999; Wagner et al., 1999), the loss of *AP1* expression in the triple mutant is most simply explained by the loss of *LFY* activation. A threshold level of *LFY* expression is required to confer flower identity on the lateral primordia during the transition from vegetative to reproductive phase (Blázquez et al., 1997). Expression of *LFY* is regulated by multiple inputs, including SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1), AGAMOUS-LIKE24 (AGL24), FLOWERING LOCUS T (FT), and gibberellins (Blázquez and Weigel, 2000; Yu et al., 2002; Schmid et al., 2003; Moon et al., 2005). Among these, the precise distribution of FT and gibberellins in the shoot apex remains unclear.

On the other hand, the two MADS transcription factors SOC1 and AGL24, which together form a complex and bind directly to the *LFY* promoter, are expressed throughout the shoot apex (Lee et al., 2000; Samach et al., 2000; Yu et al., 2002; Michaels et al., 2003), raising the possibility that other unknown factors are involved in floral meristem–specific activation of *LFY* (Lee et al., 2008). *PUCHI* and *BOP* genes are candidates for this effect because their expression is specific to lateral meristems. *PUCHI* and *BOP* genes are required for specification of floral meristem identity under both continuous-light and short-day conditions, suggesting that their actions are largely independent of these environmental cues. Our analysis thus suggests that *PUCHI* and the two *BOP* genes provide a positional cue for *LFY* and *AP1* to be expressed in lateral meristems and perhaps act in concert with other flower-promoting signals, such as photoperiod.

Interestingly, expression of *PUCHI* is not restricted only to floral meristems but also occurs in secondary inflorescence meristems (Figures 4D and 4E), which normally maintain low levels of *LFY* expression (Ratcliffe et al., 1999). This result suggests that activation of *LFY* by *PUCHI* may require additional factors that are expressed in the floral meristems but not in secondary inflorescence meristems. It is also possible that some negative factor(s), such as TERMINAL FLOWER1, which is known to limit *LFY* expression to the floral meristem, may repress PUCHI function in the secondary inflorescence meristem.

PUCHI Is a Novel Regulator for Shaping the Flower Primordium

Studies using molecular markers (Long and Barton, 2000) and, more recently, a sensitive method for surface morphology (Kwiatkowska, 2006) have indicated that the floral meristem of *Arabidopsis* is initiated in the axil of the cryptic bract, whose development is later suppressed by a signal derived from the floral meristem (Nilsson et al., 1998). We have shown here that *PUCHI* mRNA is transiently detected on the adaxial side of early floral primordia. Moreover, expression of GFP-PUCHI, in the same domain and at the same time, is sufficient to suppress the *puchi* phenotype. We propose that the domain of *PUCHI* expression corresponds to the floral meristem proper in the axil of a cryptic bract (Figure 8). Accumulation of the PUCHI protein in this domain accelerates the second bulging of the floral meristem proper and suppresses the growth of the shelf-like cryptic bract primordium. The expression domain of *PUCHI* does not overlap the cryptic bract, raising the possibility that *PUCHI* acts non-cell-autonomously in bract suppression. *PUCHI* may promote expression of a signaling molecule that can move from the floral meristem proper toward the abaxial cryptic bract region. Another possibility is that promotion of the second bulging of the floral meristem proper by *PUCHI* may indirectly affect growth of the cryptic bract, either by changing the distribution of mechanical stress across the flower primordium (Hamant et al., 2008) or by incorporating cells that would otherwise become a part of the bract into the floral meristem proper. The proposed function for *PUCHI* in flower primordium development is very similar to that suggested for *UFO*, whose mutation causes a delay in the development of the floral meristem proper relative to bract development (Hepworth et al., 2006). It will thus be important

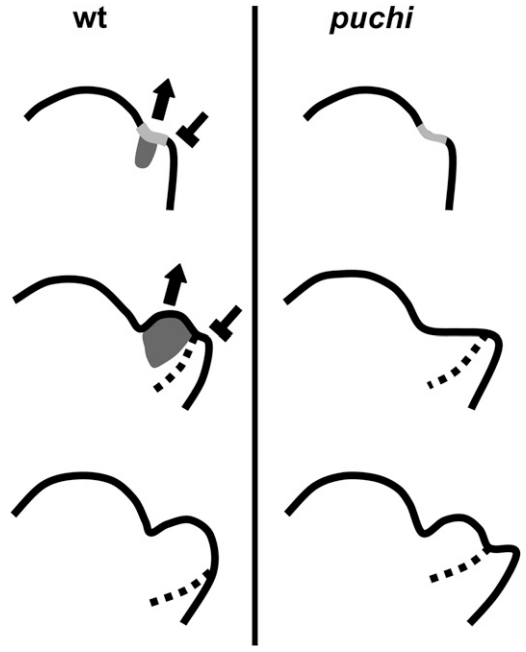

Figure 8. A Model for Control of Morphogenesis by *PUCHI* in the Early Flower Primordium.

Flower primordium formation in the wild type (left) and *puchi* (right). The expression domain of *PUCHI* (dark gray) is deduced from Figures 4A to 4C. Dotted lines represent a putative boundary between the floral meristem proper and the cryptic bract, deduced from the *AP1* expression domain (Figures 7E and 7F). Top: Initial bulging leading to the appearance of a shallow crease (light gray). In the wild type, PUCHI promotes the second bulging of the floral meristem (arrow) and represses the cryptic bract (T bar). Middle: The second bulging occurs from the shallow crease in the wild type primordium (left), whereas *puchi* forms a shelf-like primordium because the second bulging is delayed (right). Bottom: The bulge completes in the wild type and morphological signs of the cryptic bract disappear. In *puchi*, the second bulging now occurs and the cryptic bract remains morphologically apparent.

to determine whether these two genes interact in early flower development.

Previous analysis has shown that *PUCHI* is involved in morphogenesis of early lateral root primordia (Hirota et al., 2007). Initiation of a lateral root begins with anticlinal cell divisions of one or two pericycle cells and subsequent periclinal and anticlinal divisions result in bulging of a primordium with a restricted size along the radial dimension (Malamy and Benfey, 1997; Dubrovsky et al., 2001). Expression of *PUCHI* begins in cells throughout the early lateral root primordium and is later downregulated in the center (Hirota et al., 2007). By affecting the frequency of anticlinal relative to periclinal divisions, the *puchi* mutation causes ectopic cell proliferation in the periphery of the primordium, resulting in the formation of a wider and flatter lateral root primordium with a less prominent central dome (Hirota et al., 2007). These results indicate that *PUCHI* prevents cell proliferation in the periphery through the control of cell divisions. This phenotype in early lateral root formation is reminiscent of the ectopic bract growth observed in early flower formation in the *puchi* mutant. Although flowers and roots are very different in their anatomy, their developmental origins, and the regulatory genes involved in the fate specification process, further detailed analysis of *PUCHI* function may lead to the identification of a common mechanism that regulates morphogenesis of early lateral primordia both in the shoot and in the root.

METHODS

Plant Materials and Growth Conditions

All mutants were in the *Arabidopsis thaliana* cv Columbia (Col) background unless otherwise noted. The *puchi-1* and *puchi-2* mutants, both of which are the Col background, have been described previously (Hirota et al., 2007) and were backcrossed three times to Col before phenotypic analyses. The GAL4-GFP enhancer trap line E1238 (http://enhancertraps.bio.upenn.edu/default.html) was obtained from the ABRC (Ohio State University, Columbus, OH; stock number CS70083). *bop1-4* and *bop2-11* are null alleles (Ha et al., 2004, 2007) and were kindly provided by J.C. Fletcher and C.M. Ha. The *prs* mutant, which is in the Landsberg *erecta* background, was kindly provided by K. Okada. Seeds were imbibed, surface sterilized, and incubated at 4°C for 3 d. They were then sown and germinated on soil and grown at 23°C under continuous-light or short-day (8 h light/16 h dark) conditions unless otherwise noted. Light intensity was 15 μmol m^{-2} s^{-1} and 28 μmol m^{-2} s^{-1} in continuous light and short-day conditions, respectively.

Phenotypic Analyses

The number of rosette leaves was counted at bolting, and the number of secondary inflorescences was counted after formation of the first flower. Ectopic secondary inflorescences were counted shortly before senescence. Leaves on the primary bolting stem were considered as cauline leaves if they bear indeterminate secondary inflorescences. Leaves or rudimentary leaf-like structures subtending flowers were regarded as bracts (Dinneny et al., 2004). To estimate the number of secondary inflorescences, all plants were grown at the same time, in the same growth chamber, and at the same density per pot. These precautions were particularly important when counting the number of secondary inflorescences because the phenotype appeared sensitive to small fluctuations in growing conditions, such as temperature, humidity, or nutrients.

Photography and Microscopy

Photographs were taken with a digital camera (Velbon; Nikon). A Keyence VHX-900 digital microscope was used to take close-up images. Scanning electron microscopy of plant material was performed as described previously (Aida et al., 1999). To detect expression of GFP-PUCHI, inflorescence apices were fixed in 5% agarose (Gibco BRL) and incubated at 4°C for 20 min. Longitudinal sections of 100 μm were made using a vibrating-blade microtome (Microm International). Samples were stained with 50 μg/mL FM4-64 (Invitrogen), and fluorescence images were obtained using an FV1000 confocal laser scanning microscope (Olympus). GFP fluorescence was detected with the spectral settings at 490 to 540 nm for emission and 488 nm for excitation. FM4-64 fluorescence was detected with the spectral settings at 590 to 690 nm for emission and 543 nm for excitation.

Sequential Replicas and Quantitative Analysis of Primordium Shape

Inflorescence shoot apices of nine *puchi* plants were studied with the aid of the nondestructive sequential replica method (Williams and Green, 1988), in which dental polymer molds were taken from individual apices at 12-h intervals. Epoxy resin casts prepared from these molds were observed by scanning electron microscopy (sputter-coated; LEO435VP microscope). Replicas were taken from plants at 7 to 8 weeks after germination (11 h light/13 h dark), when the inflorescence axis length was between 2 and 10 mm and before the oldest flower bud had opened. Sequences of replicas obtained from *puchi* apices were compared with previously studied wild-type Col apices (Kwiatkowska, 2006).

For each cast, two micrographs were taken, one tilted at 10° with respect to the other, and used for stereoscopic reconstruction (Routier-Kierzkowska and Kwiatkowska, 2008) and geometric quantitation (Dumais and Kwiatkowska, 2002). Computer programs used for this analysis were written in Matlab (The Mathworks). Reconstructed surfaces of flower primordia were rotated so that the side views of the primordia could be compared. The shape of each primordium was quantified by means of principal curvature directions (i.e., the directions in which the curvature attained either maximal or minimal values).

In Situ Hybridization

For in situ hybridization, inflorescence apices were collected and fixed shortly after bolting, when the inflorescences were <10 mm in length. In situ hybridization was performed according to Takada et al. (2001). The *BOP1* probe has been described by Ha et al. (2004). The *LFY* probe was transcribed using T3 RNA polymerase (Promega) from pDW124 (a gift from D. Weigel) linearized with *Bam*HI. *AP1* and *BOP2* probes were transcribed using T3 RNA polymerase from RAFL22-60-H11 and RAFL15-22-D12 (provided by RIKEN) linearized with *Eco*RI. To synthesize the *PUCHI* probe, a cDNA fragment was amplified using PUCHI_F (5′-CTCCACAGTTTGTCATCGATC-3′) and PUCHI_R (5′-GACTGAGTAGAAGCCTGTAG-3′) primers, which excluded the AP2 domain to avoid cross-hybridization, and the blunt PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen). The plasmid was linearized with *Spe*I and transcribed using T7 RNA polymerase (Promega). Hybridization was performed at 45°C. Western Blue (Promega) was used as the substrate for signal detection.

Accession Numbers

Sequence data from this article can be found in the GenBank and/or The Arabidopsis Information Resource data libraries under the following accession numbers: *PUCHI* (NP_197357/At5g18560), *PRS* (NP_180429/At2g28610), *BOP1* (NP_191272/At3g57130), *BOP2* (NP_181668/At2g41370),

LFY (NP_200993/At5g61850), *AP1* (NP_177074/At1g69120), *BD1* (NP_001105200), and *FZP* (AB103120).

Supplemental Data

Supplemental Figure 1. Inflorescence Phenotypes of the *puchi-2* Mutant.

Supplemental Figure 2. Formation of Pin-Shaped Projections in *puchi* Is Dependent on the *PRESSED FLOWER* Gene.

Supplemental Figure 3. *puchi* Flower Phenotypes.

Supplemental Figure 4. Sepal Formation Is Delayed in the *puchi* Mutant.

Supplemental Figure 5. Fifty-Day-Old Primary Inflorescence of the *puchi bop1 bop2* Mutant Grown under Continuous-Light Conditions.

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