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Analysis of the Determination of Floral Meristem Identity in *Arabidopsis thaliana*

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ABSTRACT

At the onset of flowering, the *Arabidopsis* 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 bract. During the transition from vegetative to reproductive phase, the primary inflorescence produces lateral meristems that develop into either branches or flowers. The conversion of meristem identity from branch to flower is largely dependent on floral meristem identity genes *LEAFY* (*LFY*) and *APETALA1* (*AP1*), both encoding transcription factors. Several studies, however, suggest that the attainment of the high level of *LFY* expression is a key step to confer correct floral identity to lateral meristems. Although many factors have been shown to promote *LFY* expression, none are expressed specifically to lateral meristems, raising the question of how local activation of *LFY* is regulated.

In an attempt to find out new factor involved in the determination of floral meristem identity, I analyzed the function of the *Arabidopsis PUCHI* gene, a putative transcription factor of the AP2/EREBP family which has previously been shown to play roles in lateral root morphogenesis. I characterized two recessive *puchi* alleles, *puchi-1* and *puchi-2*, grown under continuous-light and short-day conditions and showed that both mutants influenced inflorescence architecture in two ways. First, the number of branches is increased due the conversion of early arising flowers into branches. Second, *puchi* mutant flowers are subtended by rudimentary bracts, exhibiting a partial feature of branches. These results indicate that *PUCHI* is required for floral meristem identity and bract suppression. *PUCHI* is transiently expressed in the adaxial side of early flower primordia. Moreover, expression of GFP-PUCHI, in the same domain and at the same time, is sufficient to suppress the *puchi* phenotype. The

expression domain of *PUCHI* does not overlap the cryptic bract, indicating that *PUCHI* acts to suppress bract formation non-cell autonomously.

Next, I examined the interaction of *PUCHI* with *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2*, which encode a pair of redundant regulatory proteins involved in various developmental processes including bract suppression. In addition to the ectopic bract formation that has previously been reported, I found that the *bop1 bop2* double mutant displayed partial conversion of flowers to branches. Furthermore, the *puchi bop1 bop2* triple mutant showed synergistic enhancement of defects in the determination of floral meristem identity and ectopic bract formation. The *BOP1* and *BOP2* expression did not rely on *PUCHI* and vice versa. I also demonstrated that the defect in floral meristem specification in the triple mutant associated with a drastic reduction of the *LFY* and *AP1* expression.

The present study thus suggests that *PUCHI* is required for proper conversion of secondary inflorescences to flowers. Functions of *PUCHI* in the floral fate determination and bract suppression overlap with that of *BOP1* and *BOP2*, and that *PUCHI* acts together with the *BOP* genes to promote expression of *LFY* and *AP1*, 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.

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CHAPTER 1

INTRODUCTION

1.1 General Introduction

Most aerial parts of a plant are generated postembryonically by the activity of the shoot meristem, a group of mitotically active cells that add continuously 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 (Figures 1A to 1D; Coen and Nugent, 1994; Benlloch et al., 2007; Prusinkiewicz et al., 2007). Two types of inflorescences have been classified in plants on the basis of whether the shoot apices end in a terminal flower or not (Weberling, 1989). When the inflorescences do not terminate, the inflorescences are classified as indeterminate. Typical example of an indeterminate inflorescence is the raceme, which is present in *Arabidopsis thaliana* or in *Antirrhinum majus*. The apex of indeterminate inflorescence is able to grow indefinitely, generating a continuous main axis that laterally produces floral organs (Figures 1A and 1B). On the other hand, inflorescences that form terminal flowers called determinate. A classical type of determinate inflorescence is the cyme, found in *Silene latifolia*. Cymose inflorescences lack a main axis: the main shoot terminates in a flower, while growth continues through lateral axes produced below the terminal flower (Figure 1C). Another kind of determinate inflorescence is the panicle. In contrast to cyme, the panicle inflorescence has a main axis but terminated by a flower. Panicle inflorescence is found in rice (*Oryza sativa*) (Figure 1D).

In *Arabidopsis*, the shoot apical meristem undergoes two phases, vegetative and reproductive; both phases are characterized by reiterative and indeterminate pattern of growth and organogenesis. The vegetative meristem produces a compact rosette consisting of a short stem and a variable number of leaves (Figure 2). By contrast, the reproductive meristem 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 vegetative to reproductive phase and show an indeterminate growth pattern that reiterates the pattern of the primary inflorescence (Figure 2). After several rounds of branch production, the primary inflorescence meristem begins to produce determinate floral meristems, which generate a fixed number of floral organs (Figure 2). This implies that, with the floral transition the fate of these lateral meristems has to be reprogrammed so that they acquire the identity of floral meristems. The conversion of meristem identity from secondary inflorescence to flower is largely dependent on endogenous and environmental factors (Baurle and Dean, 2006; Kobayashi and Weigel, 2007).

1.2 Molecular Genetic Analyses of Floral Meristem Identity

The acquisition of floral meristem identity by the lateral meristems is controlled by the interaction of positive and negative regulators. Several factors have been shown to play important roles in regulation of floral meristem identity in *Arabidopsis*. The current understandings about the roles of these factors are reviewed here.

LEAFY (*LFY*)

LEAFY is required for the specification of floral meristem identity, which is clearly deduced from the phenotypes of *lfy* mutant plants (Figure 3A). Mutations in the *LFY* locus cause partial conversion of flowers into branch-like structures (Schultz and Haughn, 1991; Huala

and Sussex, 1992; Weigel et al., 1992). The shoot-like character of the *lfy* flowers is more marked in the early arising positions in the inflorescence, while structure formed in more apical positions acquire a floral identity due to independent activation of other floral identity genes such as *APETALA1* (*AP1*) (Huala and Sussex, 1992; Bowman et al., 1993). Another aspect of the *lfy* phenotype is that, while wild-type flowers are bractless, many of the *lfy* flowers are subtended by bracts, indicating that *LFY* has an additional role in bract suppression (Schultz and Haughn, 1991).

LFY encodes a transcription factor that so far has been found only in the plant kingdom (Maizel et al., 2005). Consistent with the phenotype observed in the mutant, high level of *LFY* expression is observed throughout the young floral meristems from the earliest stage of development (Figure 3B; Weigel et al., 1992). *LFY* expression is also detected at a low level in the leaf primordia during the vegetative phase, and gradually increases at the commencement of flowering (Blázquez et al. 1997; Hempel et al., 1997).

In agreement with its proposed roles in floral meristem specification, ectopic expression of *LFY* under the control of the 35S CaMV promoter (35S:LFY) causes precocious flowering and converts all lateral meristems into flowers, indicating that *LFY* is not only necessary but also sufficient to confer correct floral identity to emerging lateral meristems (Weigel and Nilsson, 1995). It has been reported that 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).

APETALA1 (*AP1*) and *CAULIFLOWER* (*CAL*)

AP1 is one of the important regulators of floral meristem identity. The flowers of *ap1* mutants produce bract-like organs instead of sepals. In the axils of those first-whorl organs, new floral meristems are produced that reiterate this pattern, generating highly branched flowers (Figure 3A; Irish and Sussex, 1990; Bowman et al., 1993). *AP1* encodes a transcription factor of MADS-box gene family (Mandel et al., 1992). It is expressed throughout stage 1 and stage 2 floral meristems (Figure 3B; Mandel et al., 1992). Constitutive expression of *AP1* is also consistent with its role in floral meristem identity: 35S:AP1 plants are early flowering and show shoot to flower conversion, which is similar to 35S:LFY transgenic plants (Mandel and Yanofsky, 1995).

CAL is another member of MADS-box gene family, which is highly related in its sequence to *AP1* and show similar expression pattern with that of *AP1* (Figure 3B). Although mutations in *CAL* alone do not show any phenotype, simultaneous loss of *AP1* and *CAL* causes a complete conversion of floral meristems into inflorescence-like meristems, which give rise to new inflorescence-like meristems; this pattern reiterates an indefinite number of times to form structure similar to the cauliflower head (Bowman et al., 1993; Mandel and Yanofsky, 1995). Furthermore, overexpression of *CAL* shows similar phenotype to that of 35S:AP1 (Liljegren et al., 1999). These results indicate that *AP1* and *CAL* act redundantly to specify floral meristem identity.

TERMINAL FLOWER1 (*TFL1*)

The role of *TFL1* in floral meristem specification is opposite to that of *LFY*, *AP1* and *CAL*. Mutation of *tfl1* causes conversion of shoot meristems to flowers both in the primary and in the secondary meristems, similar to the phenotypes of 35S:LFY (Figure 3A; Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). Therefore, while *LFY*, *AP1* and *CAL* specify floral meristem identity, *TFL1* specifies shoot identity. *tfl1* mutants flower earlier than wild

type, indicating that *TFL1* also acts as a repressor of flowering (Shannon and Meeks-Wagner, 1991; Schultz and Haughn, 1993).

TFL1 is strongly expressed in the centre of the primary and secondary inflorescence meristems but not in the floral meristems (Figure 3B). *TFL1* expression pattern is complementary to that of *LFY* and *AP1*, which are expressed in the floral but not in the inflorescence meristems. Action of *TFL1* in the inflorescence meristem is pivotal to its function, as a major role of *TFL1* is to prevent these meristems from assuming the expression of floral meristem identity genes. Moreover, *LFY* is ectopically expressed in the inflorescence meristem of *tfl1* mutants (Weigel et al., 1992). The role of *TFL1*, therefore, seems to be a repressor of *LFY* and *AP1*. This is further supported by 35S:TFL1 plants, in which flower specification is severely delayed and similar in phenotype to *lfy* mutants (Ratcliffe et al., 1998). Conversely, several pieces of evidence suggest that *LFY* and *AP1* prevent *TFL1* expression in floral meristems (Liljegren et al., 1999; Ratcliffe et al., 1999; Ferrándiz et al., 2000).

TFL1 does not encode a transcription factor but a protein homologous to phosphatidylethanolamine-binding proteins (PEBP; Bradely et al., 1997). The PEBP family proteins are also present in bacteria, yeast and animals, and play diverse roles related to signaling pathways controlling growth and differentiation (Yeung et al., 1999; Hengst et al., 2001). *TFL1* belongs to small gene family (Mimida et al, 2004), one of whose members is *FLOWERING LOCUS T* (*FT*), which regulates flowering time. Mutations in *FT* cause late flowering and 35S:FT plants show precocious flowering, showing that *FT* has an opposite function to that of *TFL1* (Kardailsky et al., 1999; Kobayashi et al., 1999). The mechanism of TFL1 action is not yet clear, but recent studies indicate that its homologue FT promotes flowering by acting in the nucleus, as part of a complex with the bZIP transcription factor *FD* (Abe et al., 2005; Wigge et al., 2005). Structures of the TFL1 and FT proteins have been

resolved and are very similar (Ahn et al., 2006). In addition, swapping of discrete domains between these proteins reveals that the function of TFL1 can be converted to that of FT and vice versa, suggesting that the biochemical function of both protein is very similar and that differences in their functions may be due to differential binding to interacting partners (Hanzawa et al., 2005; Ahn et al., 2006).

1.3 Upstream Regulators of Floral Meristem Identity Genes

The appropriate time at which newly emerging primordia switch from a vegetative to a floral fate is regulated by multiple environmental and endogenous factors. The molecular mechanism that integrates this information and triggers the floral developmental program is primarily based in the initial up-regulation of floral meristem identity genes by all these factors that promote flowering (Figure 4). Among known floral meristem identity genes in *Arabidopsis*, the main integrator is *LFY*. Expression of *LFY* is weak in the leaf primordia during the vegetative phase but is strongly activated in the floral meristems at the onset of flowering (Weigel et al., 1992; Blázquez et al., 1997; Hempel et al., 1997). A threshold level of *LFY* expression is absolutely required to trigger the switch to a floral fate. *LFY* expression has been shown to be downstream of four pathways that promote flowering time, namely the gibberellin (GA) pathway, which is essential for flowering under short days, photoperiod pathway, autonomous pathway, and the vernalization pathway (Figure 4; Blázquez et al., 1998; Nilsson et al., 1998; Aukerman et al., 1999; Blázquez and Weigel, 2000).

Evidence for participation of *LFY* in the promotion of flowering by the GA pathway appeared from the genetic analyses: mutants deficient in GA biosynthesis show a drastic reduction in *LFY* expression under short-day conditions and do not flower. This non-flowering phenotype is rescued by *LFY* overexpression, indicating that endogenous GA promotes flowering, at least, by positively regulating *LFY* expression level (Blázquez et al.,

1998). A region responsible for the response to GA has been identified in the *LFY* promoter and contains a cis-element resembling the binding site for R2R3 MYB transcription factors (Blazquez and Weigel, 2000). Among these factors, *AtMYB33* is a good candidate to upregulate *LFY* in response to GA since its expression increases at the shoot apex upon floral transition and the AtMYB33 protein binds the GA response element in the *LFY* promoter (Gocal et al., 2001).

The regulation of *LFY* expression by day length is controlled through the interaction between the flowering time gene *CONSTANS* (*CO*) and the circadian clock but the precise mechanism remains to be determined. A few studies suggest that, on the other hand, the MADS-box transcription factor SUPPRESSOR OF CONSTANS OVEREXPRESSION1 (SOC1) mediates the regulation of *LFY* expression by CO, but this hypothesis remains to be investigated (Lee et al., 2000; Mouradov et al., 2002; Jack, 2004). Another MADS-box gene, *AGAMOUS-LIKE24*, has been shown to regulate *LFY* expression. *AGL24* expression increases in the shoot apex at floral transition and *agl24* mutants flower late and show reduced *LFY* expression (Yu et al., 2002). A recent study suggests that SOC1 and AGL24 together form a complex and bind directly to the *LFY* promoter (Lee et al., 2008).

Expression of *LFY* is also promoted by the autonomous pathway and the vernalization pathway by reducing the level of floral repressor *FLOWERING LOCUS C* (*FLC*), which eventually converge on the activity of flowering time gene *SOC1* (Reeves and Coupland. 2000; Sheldon et al., 2000; Lee et al., 2000).

Recent reports indicate that the flowering time integrator FLOWERING LOCUS T (FT) might contribute to *LFY* regulation. Global analysis of gene expression before and after floral transition shows reduced *LFY* expression in the *ft* (Schmid et al., 2003). How FT affects *LFY* expression is not yet clear. It might involve interaction between FT and the bZIP transcription factor FLOWERING LOCUS D (FD) at the shoot apex, as recently

demonstrated for *AP1* activation by FT (Figure 4; Abe et al., 2005). Alternatively, since FT has been recently shown to induce SOC1 expression (Yoo et al., 2005), *LFY* upregulation by FT might use SOC1 as an intermediate.

The above discussion indicates that a threshold level of *LFY* expression is required for floral meristem specification to lateral meristems. Although many factors besides *AP1* and *CAL* have been reported to promote *LFY* expression, none are expressed in floral meristem but rather in a broader region (Figure 5; Blázquez and Weigel, 1998; Blázquez and Weigel, 2000; Samach et al., 2000; Lee et al., 2000; Michaels et al., 2003; Yu et al., 2002; Smith et al., 2004; Abe et al., 2005; Wigge et al., 2005; Kanrar et al., 2008; Lee et al., 2008), raising the question of how local activation of *LFY* expression is regulated.

1.4 Molecular Genetic Mechanisms of Bract Formation

In *Arabidopsis*, an important feature that distinguishes a flower from a secondary inflorescence is the absence of subtending leaves or bracts from the flowers. 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 (middle and top panels in Figure 2B). 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 (Figure 2A). Both *LFY* and its co-regulator *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 NON-EXPRESSOR 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.

1.5 Rationales for the Work and Its Inference

The *Arabidopsis* gene *PUCHI*, which is required for lateral root morphogenesis, is another factor that is involved in bract suppression (Hirota et al., 2007). The PUCHI protein belongs to the APETALA2/ethylene-responsive element binding protein (AP2/EREBP) family and is highly homologous to the maize protein BRANCHED SILKLESS1 (BD1) and the rice protein FRIZZY PANICLE (FZP), both of which affect floral meristem identity (Chuck et al., 2002; Komatsu et al., 2003). Mutations in *PUCHI* 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* mutant flowers produce characteristic ectopic tissue that possesses a bract identity (Supplemental text S1 and Figures 1A to 1D; Hirota, 2007; Karim et al., 2009). Although *PUCHI* has been suggested to involve in bract suppression, its potential role in flower development remains to be investigated.

Because the absence of a subtending leaf is one of the characters that discriminate flowers from secondary inflorescence in *Arabidopsis*, the failure in bract suppression in *puchi* mutants may be explained as partial conversion of flowers to secondary inflorescences. Therefore, I hypothesized that *PUCHI* might be required for proper conversion of secondary inflorescences to flowers. To investigate the *PUCHI* gene functions in flower development, I carried out the following experiments:

Part I. Analysis of the *PUCHI* gene functions in floral meristem identity and bract suppression: To explore the *PUCHI* gene functions in flower development, I characterized two *puchi* alleles, *puchi-1* and *puchi-2*, grown under continuous-light and short-day conditions. To determine the meristem identity transition, I counted the number of secondary inflorescences produced on the bolting stem prior to flower formation. I also counted the number of rosette leaf, which associates well with flowering time. Under continuous-light and short-day conditions, both *puchi-1* and *puchi-2* plants showed significant increase in the number of secondary inflorescences compared to that of wild-type. These results show that mutations in *PUCHI* cause partial conversion of flowers into secondary inflorescences in the two photoperiods studied, indicating that *PUCHI* regulates the fate of lateral meristems during inflorescence development. To investigate the role of *PUCHI* functions in detail, I analyzed expression patterns of *PUCHI* by in situ hybridization. *PUCHI* transiently expressed in the adaxial side of early flower primordia. I also examined the localization of GFP-PUCHI fusion protein driven by the cis-regulatory elements of *PUCHI*. Localization of GFP-PUCHI, in the same domain and at the same time, is sufficient to suppress the *puchi* mutant phenotype, indicating that *PUCHI* acts to suppress bract formation non-cell autonomously.

Previous studies have indicated that *JAGGED* (*JAG*) is required for bract formation in *lfy* and *ap1* mutants (Dinneny et al., 2004; Ohno et al., 2004). Because *puchi* mutant produce ectopic bract, I investigated the interaction between *PUCHI* and *JAG* to test whether *JAG* is necessary for bract formation in *puchi*. I could not detect ectopic *JAG* expression in the rudimentary bract of *puchi*. Furthermore, mutation of *jag* does not affect the bract phenotype of *puchi*, suggesting that *JAG* is not required for bract formation in *puchi*.

Part II. Interaction between *PUCHI* and other floral meristem regulators: Because *PUCHI*, *BOP1* and *BOP2* affect similar processes of flower development, I tested for a

possible interaction between *PUCHI* and *BOP* genes. First, I generated *puchi-1 bop1-4 bop2-11* triple mutant and analyzed their phenotypes in regards to floral meristem identity and bract suppression. Next, I compared expression patterns of *BOP1* and *BOP1* in wild type and in *puchi* backgrounds and vice versa. I show that *puchi* enhances *bop1 bop2* double mutant phenotypes, such that the triple mutant show very strong defects in floral meristem specification and bract suppression. Consistent with this genetic interaction, I observed that *BOP1/2* expression do not rely on *PUCHI* and vice versa. I also found that the defect in floral meristem specification in the triple mutant is associated with a drastic reduction of the expression of *LFY* and *AP1*. These results suggest that *PUCHI* and the two *BOP* genes are required to promote expression of these meristem identity genes during inflorescence development.

Here I provide evidence that, in addition to its role in lateral root development, *PUCHI* is involved in the determination of floral meristem identity and bract suppression. *PUCHI* is expressed on the adaxial side of early flower primordium and is required for conversion of secondary inflorescences to flowers. I 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.

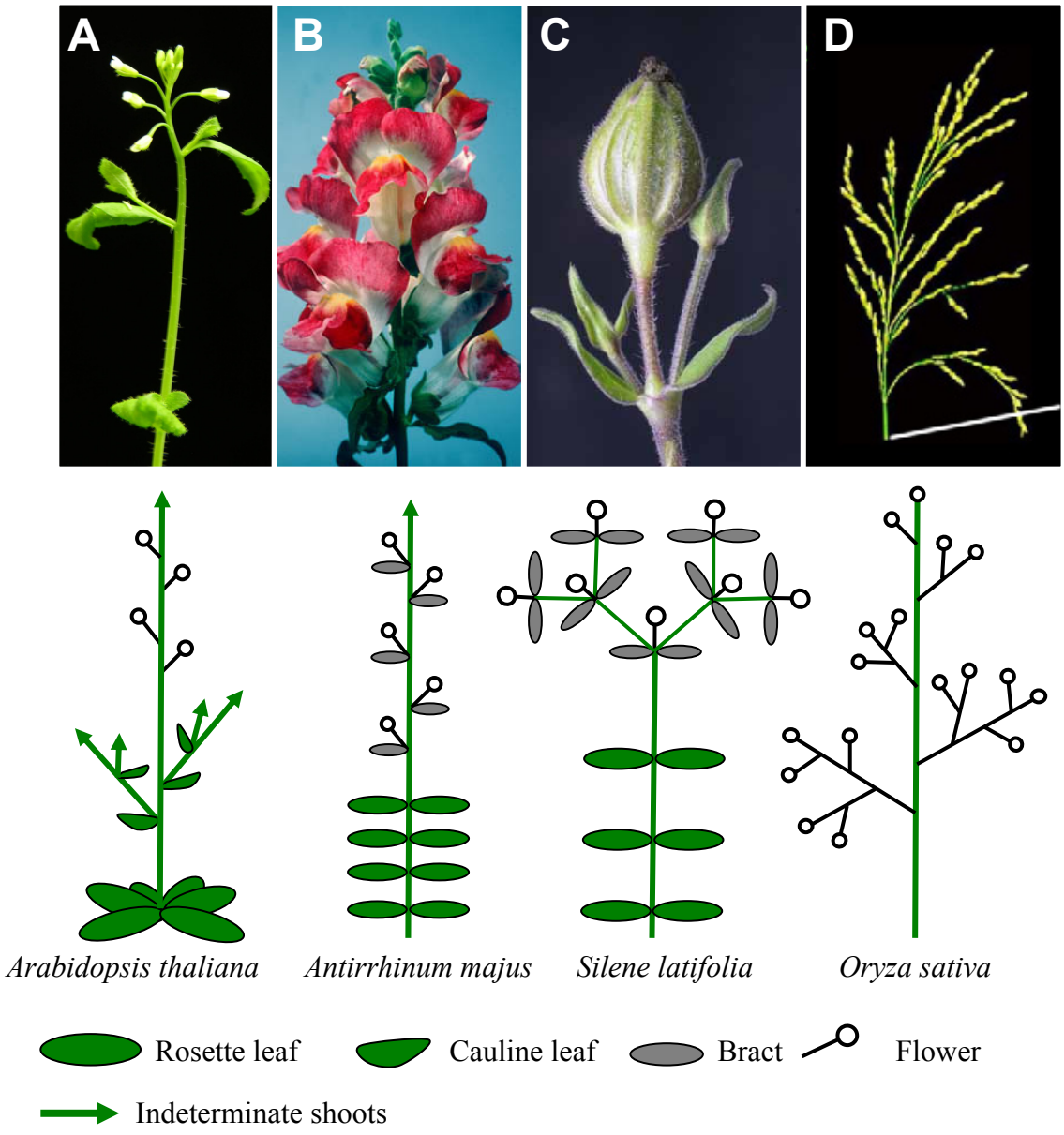

Figure 1. Diagrams of different types of Inflorescences.

([A] to **[B])** Indeterminate, and **([C]** to **[D])** determinate inflorescences.

(A) The simple raceme of *Arabidopsis thaliana*

(B) The simple raceme of *Antirrhinum majus*

(C) Determinate inflorescence of a cyme of *Silene latifolia*

(C) Determinate inflorescence of a panicle of *Oryza sativa*

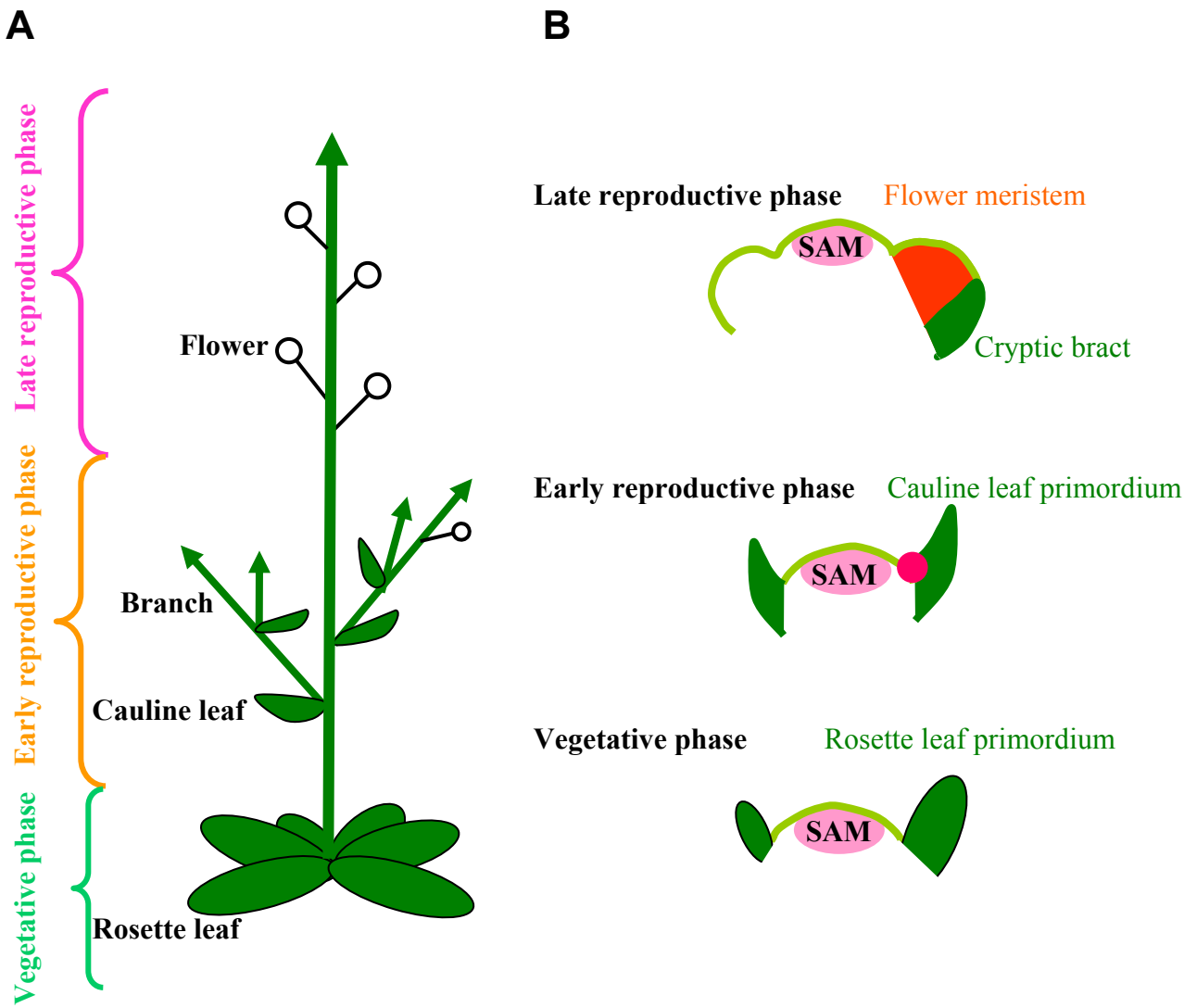

Figure 2. Secondary Inflorescence or Branch and Flower Formation in *Arabidopsis*.

(A) An *Arabidopsis* plant, showing lateral organ production at different stages of development.

(B, bottom) During vegetative phase, the shoot apical meristem (SAM) produces vegetative leaf at its flank.

(B, middle) At the early reproductive phase, the SAM produces branch meristem (red circle) in the axil of a cauline leaf primordium.

(B, top) During the late reproductive phase, the SAM produces floral meristem in the axil of a cryptic bract, whose outgrowth is suppressed in later stage of flower development.

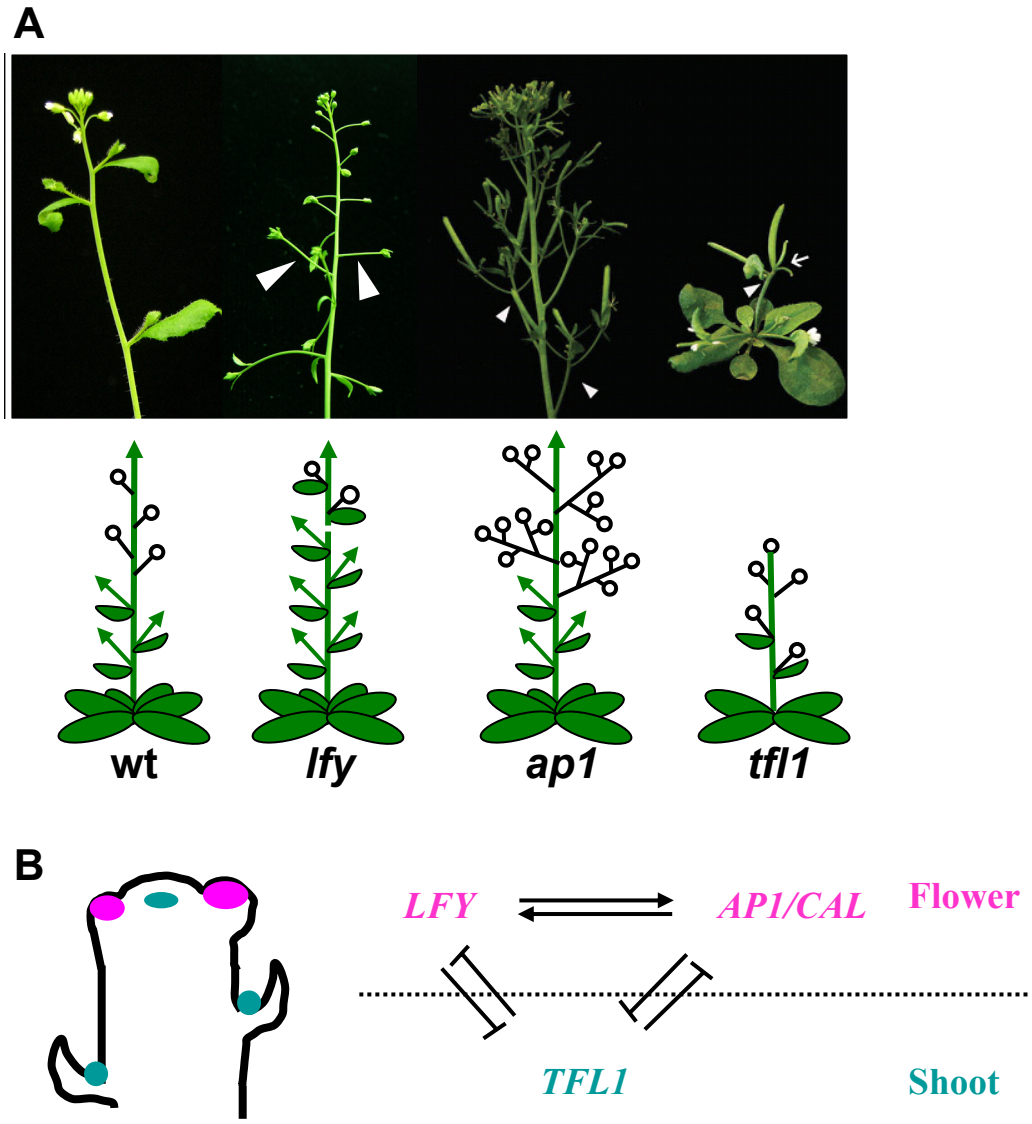

Figure 3. Role of Floral Mersietm Identity Genes.

(A) Inflorescence of the wild type (wt), *lfy*, *ap1* and *tfl1* mutants. In the inflorescences of *lfy* and *ap1*, flowers (open circles) are replaced by shoot-like structures (arrowheads in the photographs), while in the *tfl1* mutant solitary flowers replace shoots in the axils of cauline leaves (arrowheads). The inflorescences of the wild type, *lfy* and *ap1* show indeterminate growth but the inflorescence of *tfl1* is determinate and forms a terminal flower (arrow in the photograph). *ap1* and *tfl1* images are adopted from Benlloch et al. (2007).

(B) Complementary expression of *TFL1* (blue) and *LFY/AP1/CAL* (pink) genes in the *Arabidopsis* inflorescence shoot apex. While *LFY* and *AP1/CAL* specify floral identity, *TFL1* is required to maintain the inflorescence identity of all shoot meristems (left). Relationships among the floral meristem identity genes (right).

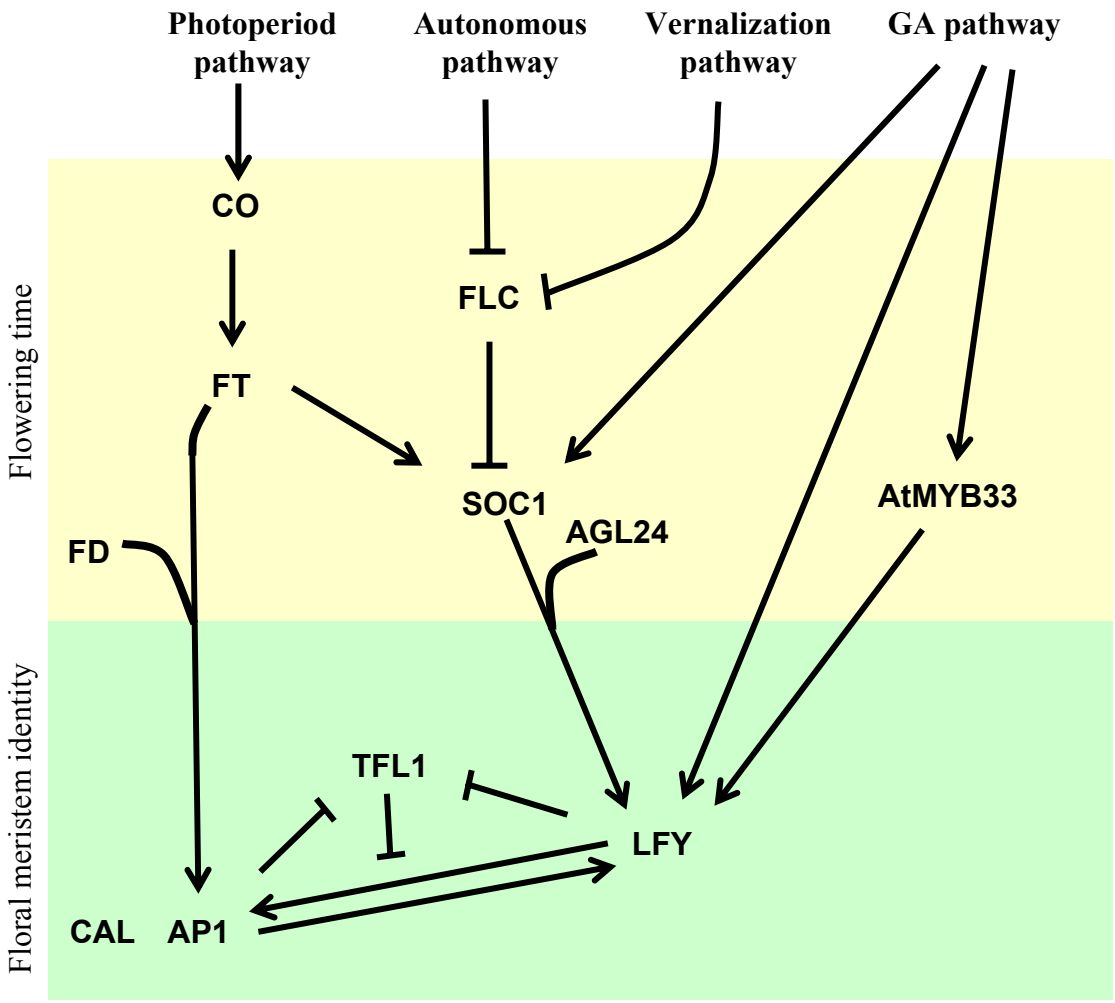

Figure 4. Schematic representation of the interaction involved in the floral meristem identity. See text for details.

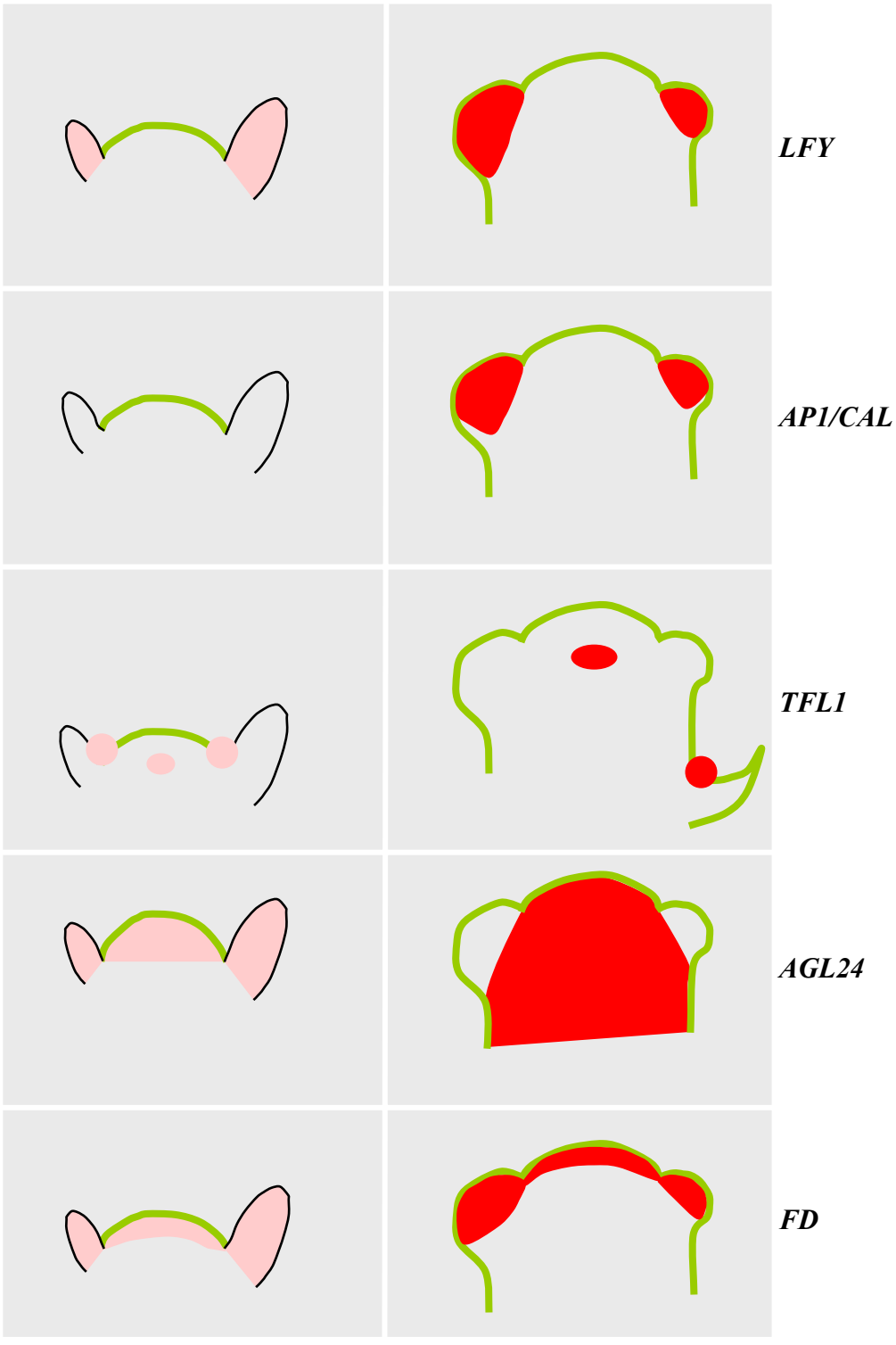

Figure 5. Expression patterns of the meristem identity genes in *Arabidopsis*. Expression domains of mRNA have been shown in the shoot apex before (left column) and after (right column) floral transition. Expression of these meristem identity genes is weak during the vegetative phase but strongly activated at the commencement of flowering.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Materials and Growth Conditions

All mutants were in the *Arabidopsis thaliana* cv. Columbia (Col) background. The *puchi-1* and *puchi-2* mutants have been described previously (Hirota et al., 2007) and were backcrossed three times to Col before phenotypic analyses. *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. Seeds were imbibed, surface sterilized and incubated at 4^{o}C for three days. They were then sown and germinated on soil and grown at 23^{o}C under continuous-light or short-day (8 h light/16 h dark) conditions unless otherwise noted.

2.2 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.

2.3 Photography and Microscopy

Photographs were taken with a digital camera (Velbon, Nikon, Japan). A Keyence VHX-600K digital microscope (Keyence Corporation, Japan) 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^{o}C for 20 minutes. Longitudinal sections of 100 µm were made using a vibrating-blade microtome (Microm Int. GmbH, Walldorf, Germany). 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.

2.4 In Situ Hybridization

For in situ hybridization, inflorescence apices were collected and fixed shortly after bolting, when the inflorescences were less than 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, Tokyo, Japan) 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, Japan) 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 vector (Invitrogen). The plasmid was linearized with *Spe*I and transcribed using T7 RNA polymerase (Promega). The JAG probe has been described by Dinneny et al. (2004). Hybridization was carried out at 45^{o}C. Western Blue (Promega) was used as the substrate for signal detection.

CAHAPTER 3

RESULTS

3.1 Part I: Analysis of the *PUCHI* Gene Functions in Floral Meristem Identity

3.1.1 Mutations in *PUCHI* Affect Floral Meristem Identity

Floral transition in *Arabidopsis* is regulated by multiple endogenous and environmental factors including day length (Baurle and Dean, 2006; Kobayashi and Weigel, 2007). To investigate *PUCHI* gene function in flower development, I 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). I 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 to that of wild type, whereas the number of rosette leaves was unaffected (Figures 6A and 6B; Figure 8B; Tables 1 and 2). 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 (Figures 6C; 7C). These “solitary” branches lacking normal cauline leaves occasionally (4%: 2 of 50 inflorescences) showed a mosaic of inflorescence and flower phenotypes (Figure 6D; Figure 7D). 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 6E). In

addition, an extra flower often formed from the pedicel of these mosaic structures (Figure 6E, 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 (Figure 8D; Table 1). In addition, *puchi* plants produced ectopic secondary inflorescences after 6-9 flowers had arisen on the primary inflorescence (Figures 6F; 5E; 8D). 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 one to ten flowers. These ectopic inflorescences reiterated the process of primary inflorescence (Figure 7F), 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, indicating 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 I chose the *puchi-1* allele for further analyses.

In contrast to the phenotype in the morphology of the flower base, I could not detect any obvious abnormalities in the identity or the number of individual floral organs of *puchi* (Figures 9A to 9D; Table 3), indicating that *PUCHI* is not involved in the specification or patterning of floral organs.

3.1.2 *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, I 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 10A; Smyth et al., 1990). *PUCHI* expression continued until early stage 2 (Figure 10B) and disappeared before the initiation of sepal primordia. Accumulation of *PUCHI* mRNA was restricted to the adaxial side of floral primordia.

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, I 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 10C), 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* (Figures 11A to 11C). 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, I 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 (Figure 10D). Shortly after the onset of flowering, however, *PUCHI* mRNA accumulated in the axillary meristems of rosette and cauline leaves (Figure 10E). These results suggest that *PUCHI* is expressed in all lateral meristems after the transition from the vegetative to the reproductive phase.

3.1.3 *JAG* Is Not Required for Bract Formation in *puchi*

It has previously been shown that *JAG* expression is necessary for bract development in *lfy* and *ap1* mutants (Dinneny et al., 2004; Ohno et al., 2004), which encodes a member of the zinc finger family of plant transcription factors. In addition, ectopic *JAG* expression under the control of 35S CaMV promoter (35S:JAG) causes ectopic bract formation at the base of pedicels, indicating that *JAG* is sufficient for bract formation (Figure 12A; Dinneny et al., 2004; Ohno et al., 2004). To test whether *JAG* is required for bract formation in *puchi,* I introduced the *jag-1* loss of function allele into the *puchi-1* background. The *puchi jag* double mutant displayed an identical phenotype to *puchi* in regards to bract formation, in which the double mutant plants possess a flat leaf-like organ associated with a pair of pin-shaped projections at the base of pedicels (Figure 12B). The only difference could be recognized in the flowers of *puchi jag*, which are similar to *jag* mutant flowers in the sense that they developed sepals and petals that are narrower and shorter than in *puchi*.

Next, I examined *JAG* expression in wild type and *puchi* mutant backgrounds. In wild type, during the flower development *JAG* mRNA was first detected in sepal anlagen (Figure 12C; Dinneny et al., 2004; Ohno et al., 2004). In *puchi*, *JAG* expression was detected in sepal anlagen but not in emerging bract primordium (Figure 12D). Collectively, these results indicate that *JAG* is not necessary for bract formation in *puchi*.

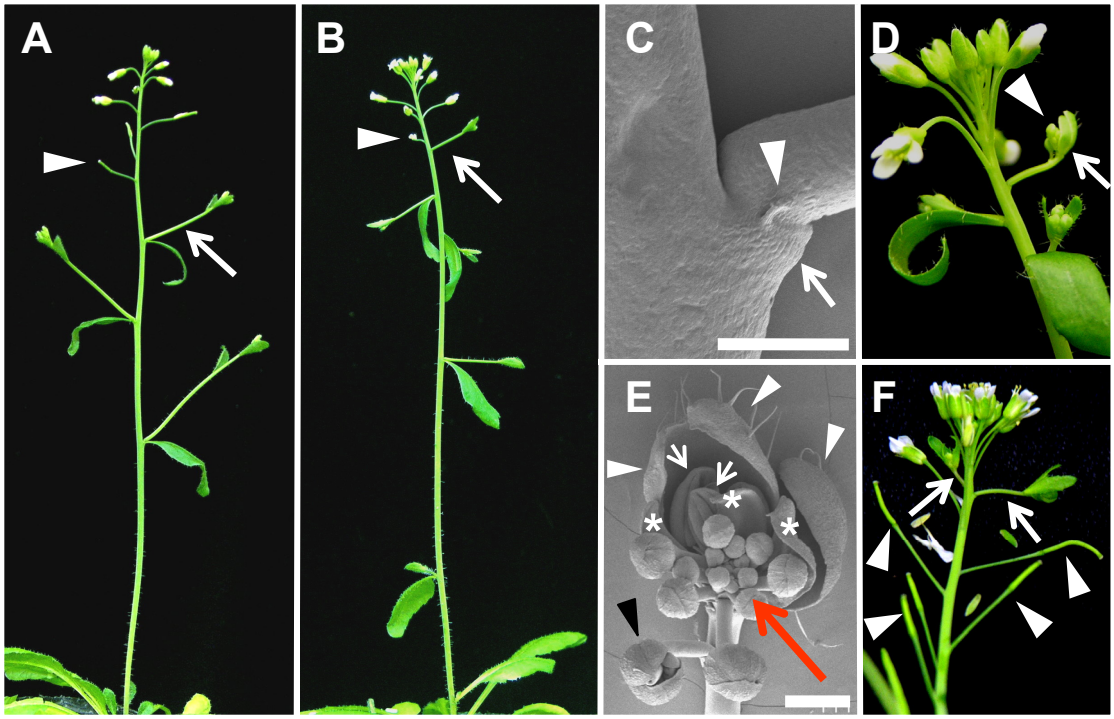

Figure 6. Inflorescence Phenotypes of Wild Type and *puchi-1*.

(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. The arrow indicates the upper most branch subtended by a cauline leaf.

(B) Inflorescence of a *puchi*-1 plant. Compared to wild type, the number of nodes with secondary inflorescences is increased in *puchi-1*. The arrow indicates the upper most secondary inflorescence that lacks a subtending cauline leaf.

Arrowheads in **([A]** and **[B])** indicate the first flower formed after the transition from secondary inflorescences to flowers.

(C) Scanning electron micrograph (SEM) 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.

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

(E) SEM 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 red arrow indicates the inflorescence-like shoot. 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 6-9 flowers/siliques (arrowheads) have arisen on the primary inflorescence.

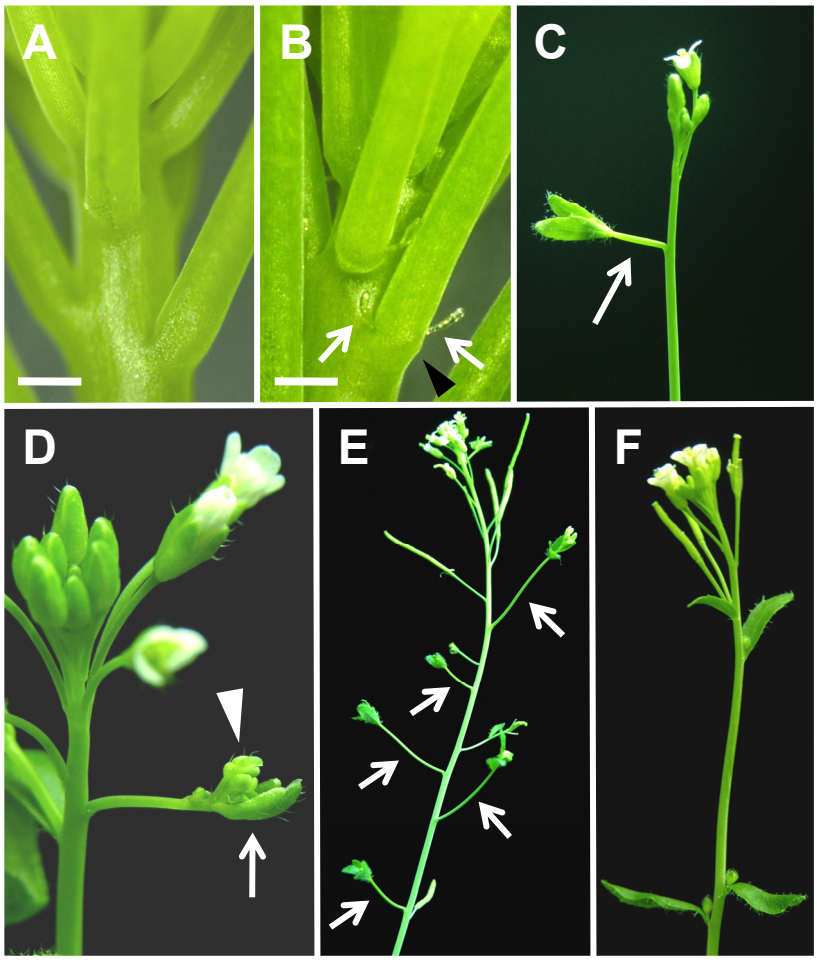

Figure 7. Inflorescence Phenotypes of the *puchi-2* Mutant.

puchi-2 mutant plants grown under continuous-light (**[A]** to **[D]**) or short-day **([E]** and **[F]**) conditions.

(A) and **(B)** Close-up view of primary inflorescences of wild type **(A)** and *puchi-2* **(B)**. *puchi-2* produces a flat leaf-like organ (arrowhead) flanked by a pair of pin-shaped projections (arrows). Bars = 250 μm.

(C) A primary inflorescence. The uppermost secondary inflorescence is not subtended by cauline leaf (arrow).

(D) Primary inflorescence producing a mosaic structure that is intermediate between a flower (arrow) and an inflorescence-like shoot (arrowhead).

(E) Primary inflorescence grown under short-day conditions, showing the production of ectopic branches (arrows).

(F) An ectopic branch, which is appeared from the primary inflorescence in a position normally occupied by a flower in wild type, is indistinguishable from the primary inflorescence.

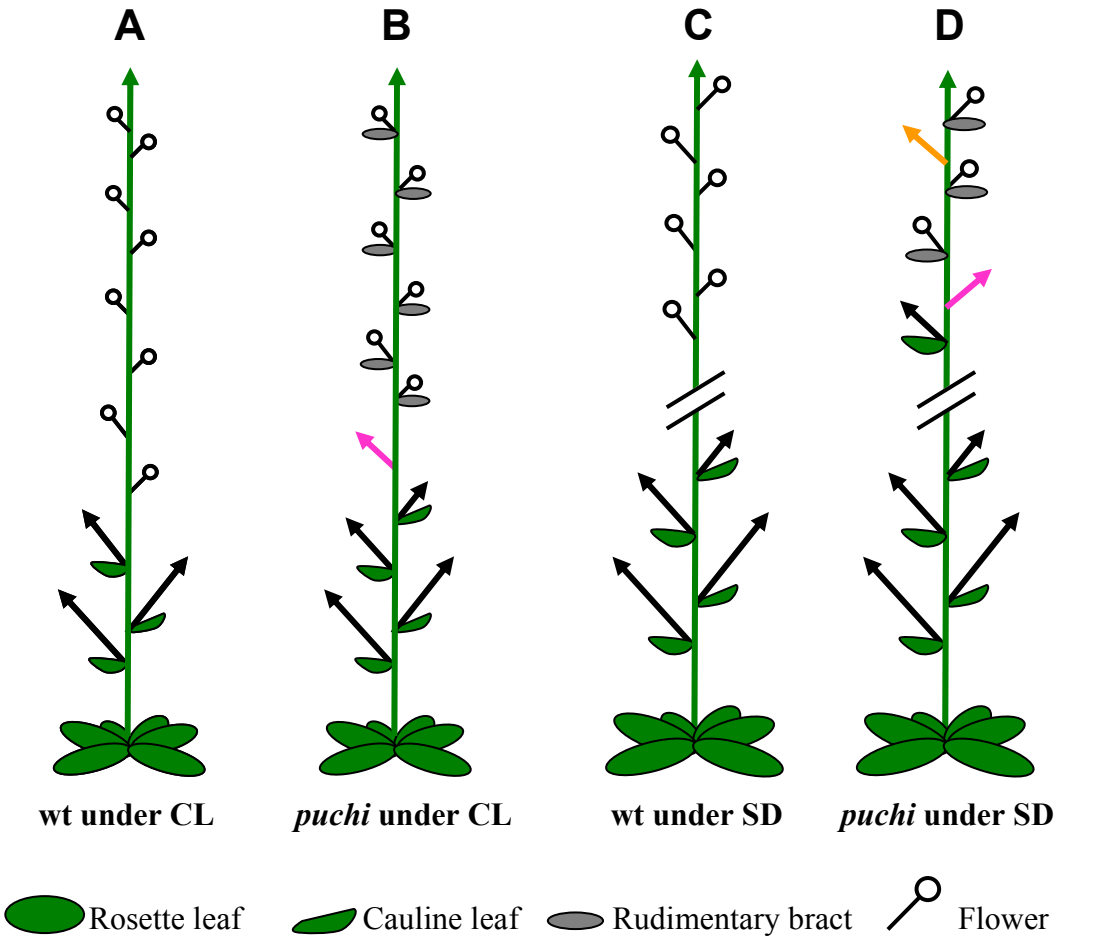

Figure 8. Structure of Wild-Type and *puchi* Plants.

(A) Wild-type plant grown under continuous-light (CL) conditions.

(B) *puchi* plants grown under CL conditions.

(C) Wild-type plant grown under short-day (SD) conditions.

(D) *puchi* plant grown under SD conditions.

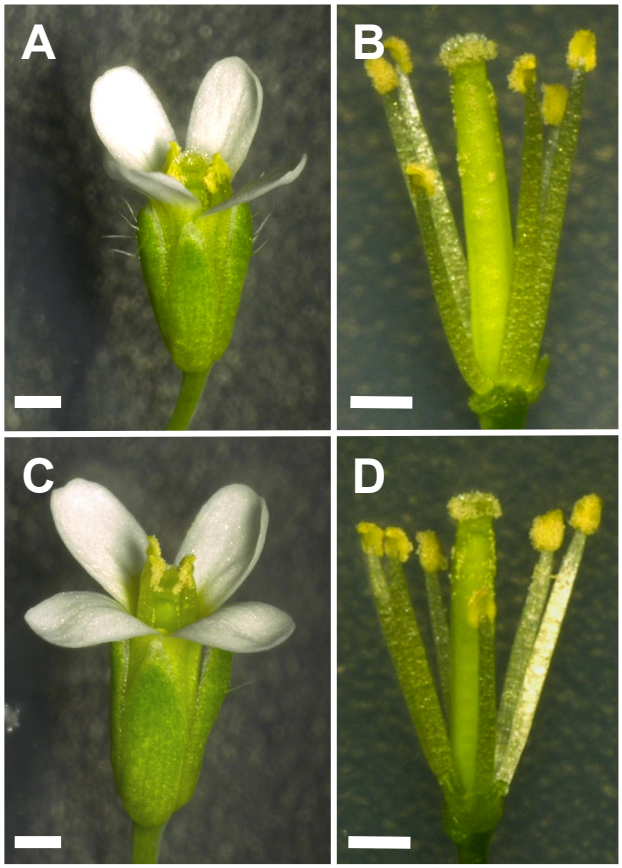

Figure 9. *puchi* Flower Phenotypes.

(A) to **(D)** Mature flowers of wild type (**[A]** and **[B]**) and *puchi-1* (**[C]** and **[D]**). In **(B)** and **(D)** sepals and petals were removed to expose the inner two whorls. *puchi* mutant flowers are indistinguishable from those of wild type. Bars = 500 µm.

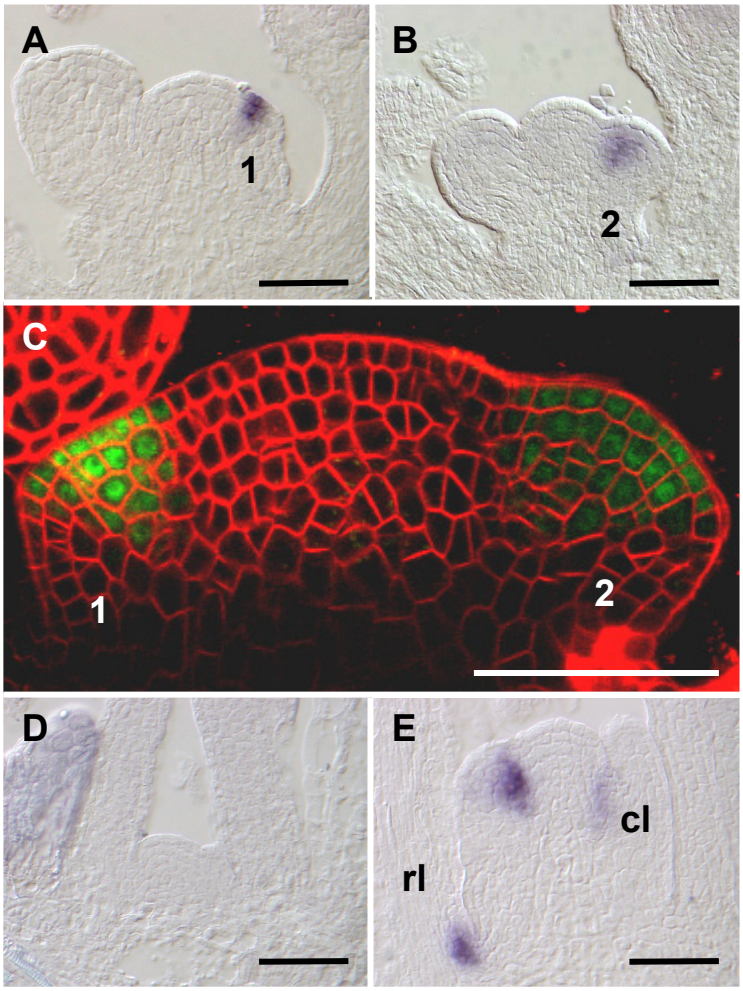

Figure 10. Expression Patterns of *PUCHI*.

(A) and **(B)** Wild-type inflorescence apex.

(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 primordium.

(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) Eight-day-old wild-type seedling. *PUCHI* mRNA is not detected in the vegetative shoot.

(E) *PUCHI* mRNA is detected in the axillary meristems of rosette and cauline leaves.

Numbers indicate stages of flower development (Smyth et al., 1990). rl, rosette leaf; cl, cauline leaf; st, stem.

Bars = 50 μm.

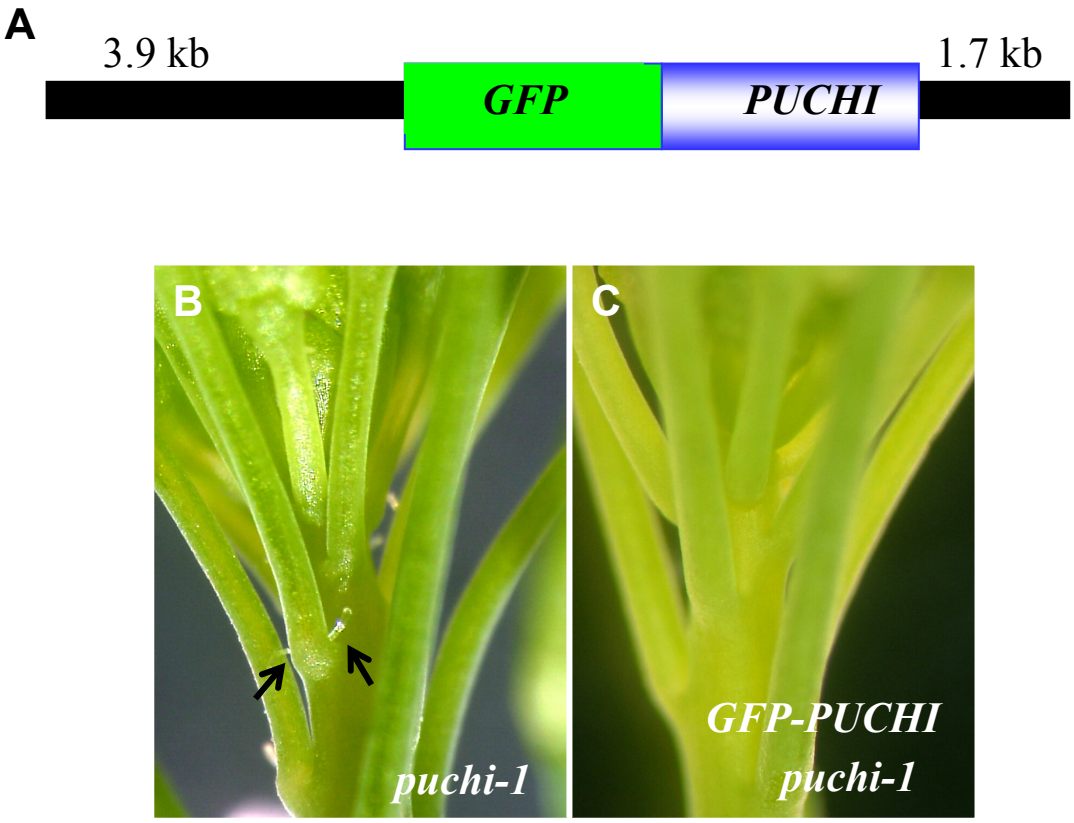

Figure 11. GFP-PUCHI Fusion Protein Complements *puchi-1* Mutant Phenotypes.

(A) Schematic representation of the construction of the GFP-PUCHI fusion protein. Black boxes represent 5' upstream and 3' downstream regions of the *PUCHI* gene. The blue and green boxes indicate the coding region of *PUCHI* and *GFP*, respectively.

(B) Inflorescence of the *puchi* mutant having ectopic stipules (arrows).

(C) Inflorescence of transgenic plant (GFP-gPUCHI), showing rescue the *puchi* mutant phenotypes.

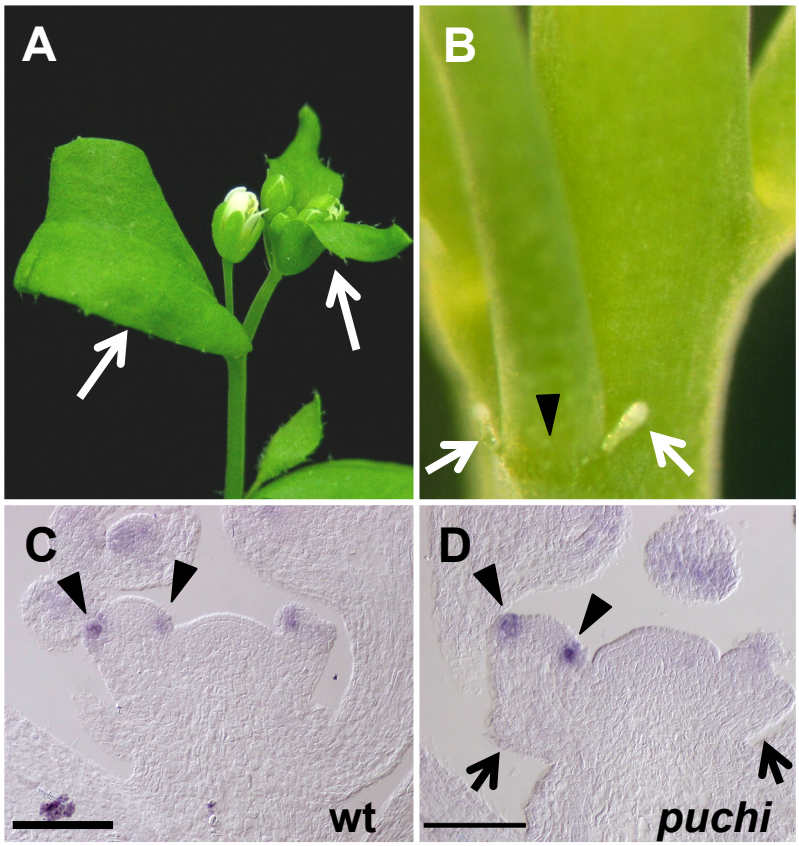

Figure 12. Interaction between *PUCHI* and *JAG*.

(A) Plant homozygous for *jag-5D*, an overexpressor mutant of *JAG* produces inflorescence with ectopic bracts (arrows) subtending flowers.

(B) Close-up view of primary inflorescence of *puchi-1 jag-1* double mutant. The double mutant pedicel comprises a flat leaf-like organ (arrowhead) flanked by a pair of pin-shaped projections (arrows). These ectopic structures are morphologically indistinguishable to those of *puchi*.

(C) and **(D)** *JAG* expression in wild-type **(C)** and *puchi-1* **(D)** inflorescence apices. *JAG* expression is detected in the sepal anlagen (arrowheads) both in the wild-type **(C)** and in the *puchi* **(D)** flowers. Arrows in **(B)** indicate rudimentary bracts of *puchi*, where no ectopic *JAG* expression is observed. Bars = 100 µm.

Table 1. Inflorescence Architecture of *puchi*, *bop1 bop2* and *bop1 bop2 puchi* Mutants Grown Under Continuous-Light (CL) and Short-Day (SD) 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 ± standard error (SE). Differences between wild-type and mutant plants are significant at the $0.05 > P > 0.01$ (*) or the $P < 0.01$ (**) levels. Under SD conditions, SI that formed at positions below and above the lowermost flower were both scored. In CL, none of the genotypes produced any ectopic branches.

Table 2. Number of Rosette Leaves of *puchi* Mutants Grown Under Continuous Light (CL) and Short Day (SD) Conditions.

Conditions	Genotypes	No. of rosette leaves	No. of plants scored
CL	Col	14.47 ± 0.18	30
	puchi-1	14.90 ± 0.24	30
	puchi-2	14.77 ± 0.28	30
SD	Col	59.70 ± 1.13	20
	puchi-1	61.25 ± 0.89	20
	puchi-2	60.1 ± 0.95	20

The number of rosette leaves was counted at bolting. Values are averages ± SE, and errors are standard error of the average.

Table 3. Floral Architectures of *puchi-1* Mutant.

Genotypes	Sepals	Petals	Stamens	Carpels
Col	4.0 ± 0.0	4.0 ± 0.0	5.97 ± 0.02	2.0 ± 0.0
puchi-1	4.0 ± 0.0	4.04 ± 0.03	5.91 ± 0.04	2.0 ± 0.0

Flowers were dissected and individual floral organs were counted under a binocular. Values are means ± SE, and errors are standard error of the mean (n = 45).

3.2 Part II. Interaction between *PUCHI* and Other Floral Meristem Regulators

3.2.1 *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 re-examined 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 13C), whereas those formed in late arising flowers were much larger and showed more complete leaf-like features (Figure 13C, inset; Figure 13L, left; Norberg et al., 2005). In addition, *bop1 bop2* showed significantly more secondary inflorescences than 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, I tested for a possible interaction between the *PUCHI* and *BOP* genes.

I first generated a *puchi-1 bop1-4 bop2-11* triple mutant, which displayed significantly enhanced phenotypes compared to the parental mutants with regard to both the determination of meristem identity and bract suppression (Figures 13A to 13L). 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 seven-fold 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 13I). Such branches were always subtended by well-developed cauline leaves when they were produced at the basal nodes, whereas the upper branches were not (Figure 14). 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 spiral arrangement that is typical of a branch, rather than a whorled pattern as in the floral meristem (Figure 13J).

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 13K and 13L). 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 13M and 13N), 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.

I next compared expression patterns of *BOP1* and *BOP2* between wild type and *puchi* mutant backgrounds. In wild type, the earliest expression of both genes was found in the floral anlagen, from which a flower primordium will arise (Figures 15A and 15C; Hepworth et al., 2005; Norberg et al., 2005). Their expression persisted throughout early stage 1 and 2 floral meristems (Figures 15A and 15C). 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 15A and 15C; Long and Barton, 2000; Dinneny

et al., 2004). In *puchi*, expression of *BOP1* and *BOP2* was similar to that in wild type except that the signal was somewhat broader (Figures 15B and 15D). *PUCHI* expression in the *bop1 bop2* mutant inflorescence apex was also analyzed and was generally similar to that in wild type, although the signal was localized more internally in *puchi* mutant primordia at late stage 2 (Figures 15E and 15F). Collectively, these results suggest that the *PUCHI* and *BOP* genes are not related to each other in a hierarchical order of transcriptional control.

3.2.2 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. I 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 16A; Weigel et al., 1992; Blázquez et al., 1997). *LFY* was uniformly expressed at a higher level throughout stage 1 and 2 flower primordia (Figure 16A). *LFY* expression was normal in *puchi* and *bop1 bop2* mutant backgrounds (Figures 16B and 16C) but was markedly reduced in the *puchi bop1 bop2* triple mutant inflorescence apex (Figure 16D). These results indicate that the *PUCHI* and *BOP* genes redundantly promote *LFY* expression during inflorescence development.

Next, I examined *AP1* expression patterns. In wild type, *AP1* mRNA was detected at a high level in the adaxial cells of stage 1 and 2 floral primordia (Figure 16E; Mandel et al., 1992). A small group of abaxial cells in these early floral primordia did not express *AP1* (Figure 16E); 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 16F and 16G), consistent with ectopic bract formation in these backgrounds, and was almost undetectable in the inflorescence of *puchi bop1 bop2* (Figure 16H). Thus, the severe inflorescence phenotype in the triple mutant correlates with a drastic reduction in the expression of genes involved in floral meristem specification.

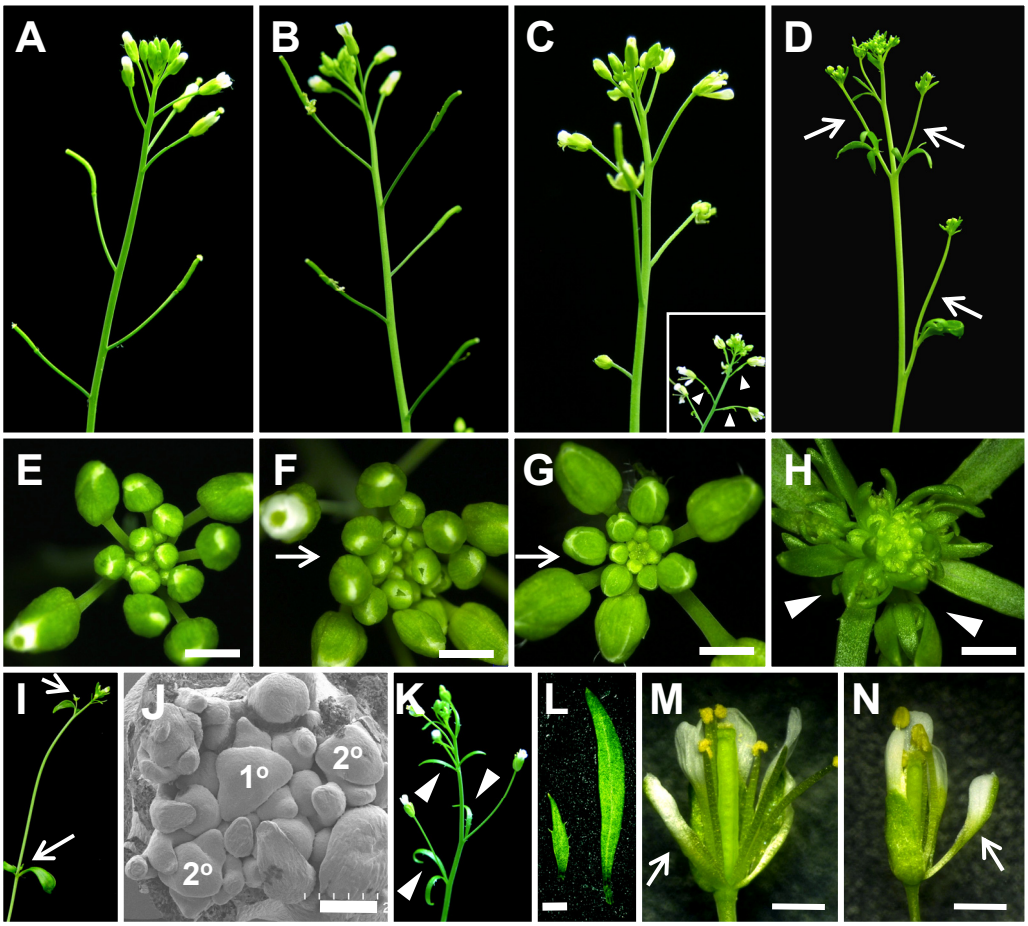

Figure 13. Genetic Interaction between *puchi* and *bop* Mutants.

(A) to **(D)** Forty-day-old primary inflorescences of wild type **(A),** *puchi-1* **(B),** *bop1-4 bop2-11* **(C)** and *puchi-1 bop1-4 bop2-11* **(D).** The inset in **(C)** shows an older (55-60 days old) inflorescence, producing visible bracts on flower pedicels (arrowheads). In **(D),** flowers are transformed into secondary inflorescence-like structures (arrows).

(E) to **(H)** Close-up view of inflorescence apices of wild type **(E**), *puchi-1* **(F),** *bop1-4 bop2-11* **(G)** and *puchi-1 bop1-4 bop2-11* **(H)**, photographed when the inflorescence apices were about 10 mm in length. 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) SEM showing a *puchi-1 bop1-4 bop2-11* inflorescence apex at a similar stage to **(H).** Primordia produced by the primary inflorescence meristem (1º) behave like inflorescence meristems rather than like flower meristems (e.g., primordia indicated with 2º).

(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 (compare **[L]** with Figure 2B; see Supplemental Figure 2A**)**.

(M) and **(N)** Flowers of *bop1-4 bop2-11* double **(M)** and *puchi-1 bop1-4 bop2-11* triple **(N)** mutants. Arrows indicate sepal-petal hybrid organs in the first whorl.

Bars = 1 mm in **(E)** to **(H)** and **(L)** to **(N)**, 100 μm in **(J)**.

Figure 14. Fifty-day-old primary inflorescence of the *puchi bop1 bop2* mutant grown under continuous-light conditions. Basal branches are subtended by cauline leaves (arrows), whereas upper branches are not (arrowheads).

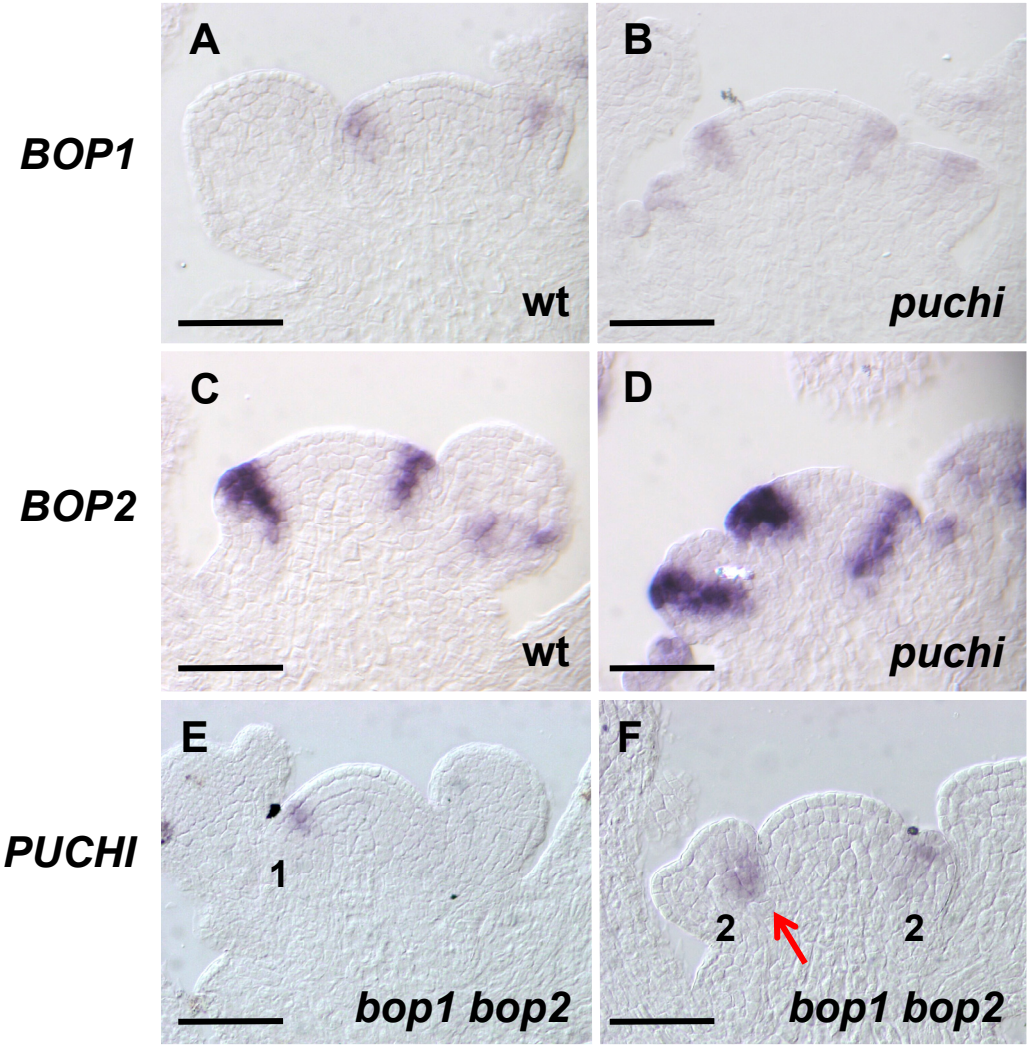

Figure 15. *BOP* and *PUCHI* are Expressed Independently of Each Other.

(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* inflorescence apex, showing expression in the stage 1 primordium.

(F) *PUCHI* mRNA in longitudinal section of *bop1-4 bop2-11* inflorescence apex, showing expression stage 2 primordia. *PUCHI* expression is unaffected (compare **[E]** with Figure 4A), except in the late stage 2 flower, in which it tends to localize to the inner cells (compare **[F**; arrow**]** with Figure 4B).

Bars = 50 μm.

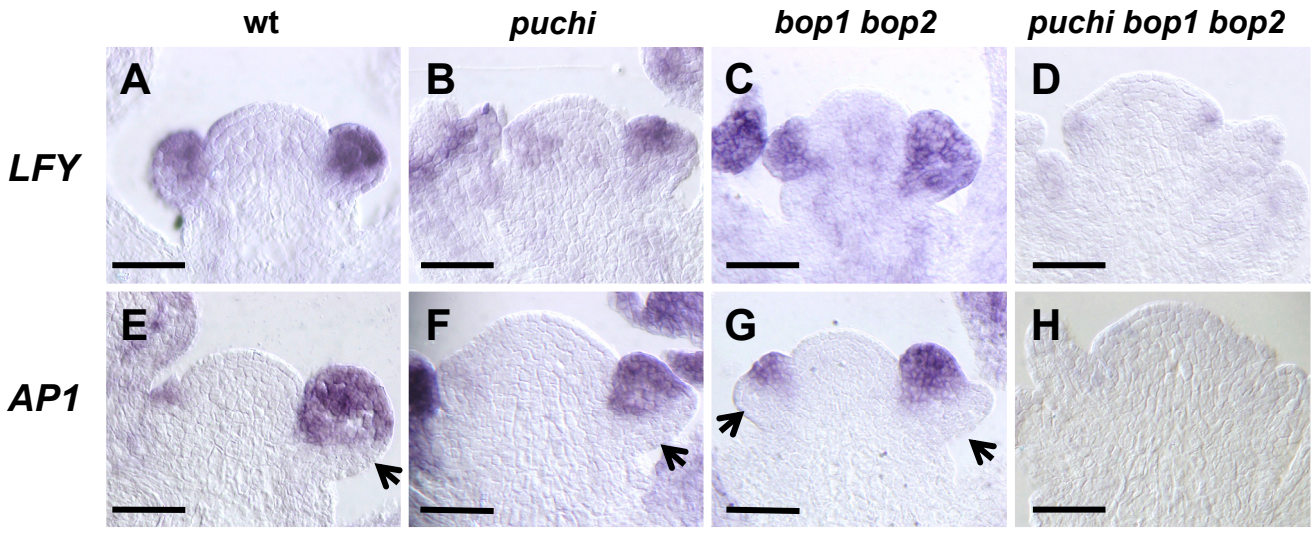

Figure 16. Expression of the Floral Meristem Identity Genes *LFY* and *AP1* in the *puchi bop1 bop2* Mutant.

(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 to 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 to wild type **(E)**, *AP1* expression is detected in a much smaller proportion of the adaxial cells of young flower primordia of *puchi* single **(F)** or *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.

CHAPTER 4

DISCUSSION

4.1 *PUCHI* Is Required for Floral Meristem Identity

In this study, I 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.

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 inflorescence 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 analysis; Chuck et al., 2002; Komatsu et al., 2003). First, expression of *PUCHI* is detected in the floral meristem proper (Figures 10A to 10C), 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 (LEP), 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).

4.2 Relationship between the *PUCHI* and *BOP* Genes

The 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 my 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 to regulate their activity.

4.3 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 16D and 16H), 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., 2000; 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 (Samach et al., 2000; Lee 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. My 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 10E), 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 (TFL1), which is known to limit *LFY* expression to the floral meristem, may repress PUCHI function in the secondary inflorescence meristem.

4.4 *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). Analysis of surface morphology of *puchi* revealed that the early flower development of *puchi* is characterized by a prolonged period of bract primordium formation, leading to a shelf-like bract primordium that is much larger than wild type. Consequently, initiation of second bulging that forms the floral meristem proper is delayed than wild type (Supplemental text and Figures 2A to 2F; 3A to 3C).

I have shown here that *PUCHI* mRNA is transiently detected on the adaxial side of early floral primordia. The duration of *PUCHI* expression roughly corresponds to the first two stags in the analysis of surface morphology (Supplemental text S2; i.e. the initial and second bulging stages). Moreover, expression of GFP-PUCHI, in the same domain and at the same time, is sufficient to suppress the *puchi* phenotype. I propose that the domain of *PUCHI* expression corresponds to the floral meristem proper in the axil of a cryptic bract (Figure 17). 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 towards 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.

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 regulate morphogenesis of early lateral primordia both in the shoot and in the root.

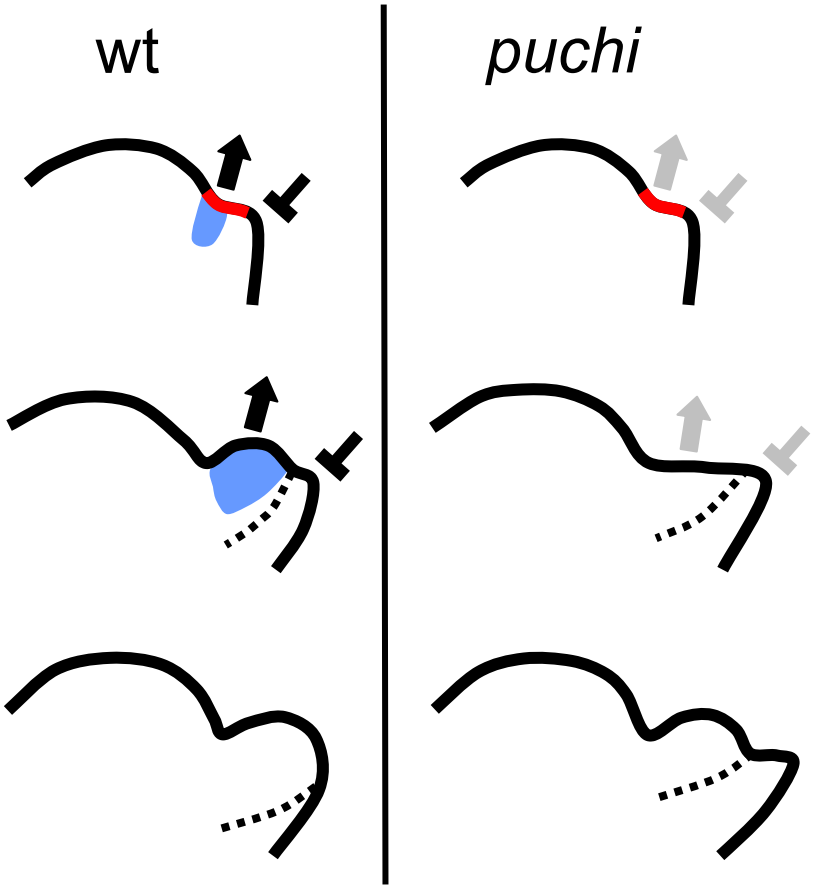

Figure 17. A Model for Control of Morphogenesis by *PUCHI* in the Early Flower Primordium.

Flower primordium formation in wild type (left) and *puchi* (right). The expression domain of *PUCHI* (blue) is deduced from Figures 8A to 8C. Dotted lines represent a putative boundary between the floral meristem proper and the cryptic bract, deduced from the *AP1* expression domain (Figures 14E and 14F).

(top) Initial bulging leading to the appearance of a shallow crease (red). In 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 wild type and morphological signs of the cryptic bract disappear. In *puchi*, the second bulging now occurs and the cryptic bract remains morphologically apparent.

CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

The development of a plant depends on the activities of meristems, i.e. pools of undifferentiated cells that self-perpetuate and provide cells for lateral organs. During the reproductive phase, shoot apical meristem acquires inflorescence meristem identity. The inflorescence meristem initially produces a few numbers of cauline leaves associated with secondary branches and then produces floral meristems. Studies on the phase transition have mainly focused on the transition from vegetative to inflorescence meristem. Numerous genes that control this transition have been identified and their relationships have been described. However, little is known about the molecular mechanisms about the conversion of lateral meristem identity from branches to flowers.

In this study, I have shown that the *Arabidopsis* gene *PUCHI* is required for proper conversion of lateral meristem fate from secondary branches to flowers. I also show that *PUCHI* has an overlapping function with *BOP1* and *BOP2* in controlling of floral meristem identity and bract suppression, and that these factors collectively promote *LFY* and *AP1* expression. Based on the results from the present study, the following experiments will be suggested for future:

i. Molecular mechanisms of *LFY* activation by *PUCHI*: It has been shown that ectopic expression of *LFY* induces early flower in wild type. Therefore, it will be important to examine the effect of forced expression of *LFY* under the control of 35S CaMV promoter (35S:LFY) to test whether the loss of *LFY* expression is involved in the *puchi bop1 bop2* triple mutant phenotypes. In addition, transgenic plants expressing a glucocorticoiod-

inducible version of the PUCHI protein (PUCHI-GR) could be used to analyze the transcriptional response of LFY upon the transient activation of the PUCHI-GR protein.

ii. Analysis of interaction among *PUCHI*, *BOP1* and *BOP2*: The synergistic phenotype in the *puchi bop1 bop2* triple mutant reveals a critical role played by the *PUCHI* and *BOP* genes in the control of floral meristem identity. *BOP1* and *BOP2* encode protein with BTP/POZ domain and ankyrin repeats, both are involved in protein-protein interaction. To investigate the relation between *PUCHI* and *BOP* genes, it will be interesting to test whether PUCHI interacts directly to BOP proteins to regulate their activity by yeast two hybrid assays and immunoprecipitation experiments.

iii. Analysis of interaction among *PUCHI*, *BOP* and *LFY*: I have shown that *PUCHI* and *BOP* genes somehow activate *LFY* expression during inflorescence development. To understand the function of this regulatory mechanism, it is crucial to construct *puchi lfy* double, *bop1 bop2 lfy* triple and *puchi bop1 bop2 lfy* quadruple mutants.

iv. Analysis of interaction between *PUCHI* and *UFO*: 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 to determine whether these two genes interact in early flower development.

Further analysis of the floral meristem identity regulators *PUCHI*, *BOP1*, *BOP2* and *LFY* will reveal the mechanisms of floral meristem development and will provide insights into the regulatory networks responsible for diverse plant architectures.

Plants are our greatest source of renewable resources providing food, medicines, industrial byproducts and biofuels. Fundamental knowledge of plant biology at the molecular level is critical for utilization this resource to its full potential. Variation on inflorescence architecture can affect yield potential, the free-threshing character and liberation of seeds. The genetic selection of these traits has been implicated in the crop improvement program. Phenotypic variation in bract development also contributes to agronomic traits, such as seed size and threshing characters. Therefore, the *puchi* mutant may provide valuable information to explain molecular basis underlying the distinct inflorescence architecture of plants, and the difference between domesticated varieties and their wild-type relatives.

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SUPPLEMENTAL TEXT

S1: *puchi* Mutant Flowers Have Rudimentary Bracts (Hirota, 2007; Karim et al., 2009)

Analysis of *puchi* flowers has indicated that all *puchi* flowers had ectopic structures at the base of each pedicel, whereas wild-type flowers showed a smooth surface at the same position (Supplemental Figures 1A and 1B; Hirota, 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 (compare Figure 4C with Supplemental Figure 1B). 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. These observations a possibility that these structures were a rudimentary bract associated with a pair of stipules. To test this prediction, expression of GREEN FLUORESCENT PROTEIN (GFP) in the enhancer trap line E1238 (http://enhancertraps.bio.upenn.edu/default.html), in which the signal is detected in the stipules of cauline leaves (Supplemental Figure 2C), has been tested. The GFP signal was detected in the pin-shaped projections of *puchi* mutant flowers (Supplemental Figure 2D). 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.

S2: Early Flower Primordium Development in *puchi* (Analyzed by Dorota Kwiatkowska; Karim et al., 2009)

To investigate how *PUCHI* affects bract growth, early flower development in the *puchi* mutant has been examined using a sensitive, non-invasive replica method combined with a

3-D reconstruction algorithm, which can reliably detect a cryptic bract in early flower primordia (Kwiatkowska, 2006).

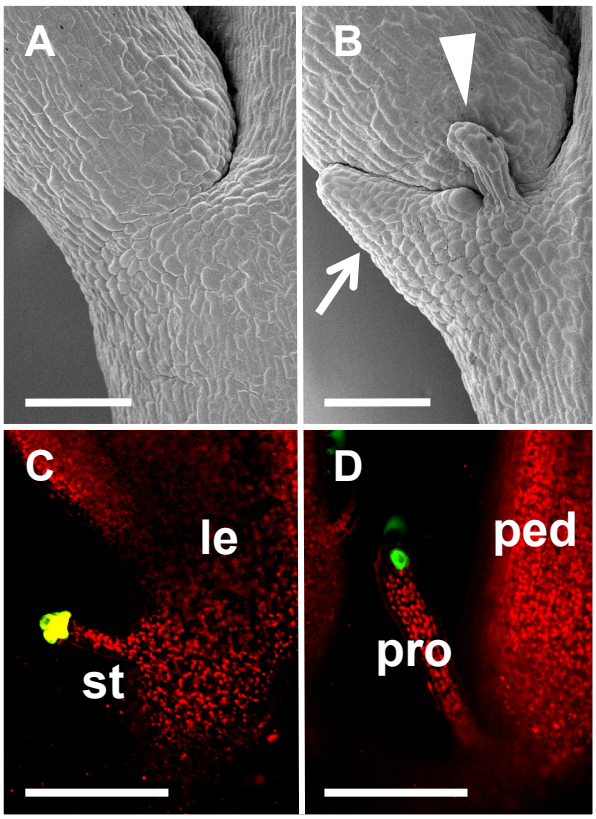
Curvature plots on wild-type inflorescence apices enable the definition of four consecutive stages in flower primordium development (Kwiatkowska, 2006). The first floral stage is an initial bulging that leads to the formation of a shallow crease between the primordium protrusion and the primary inflorescence meristem (Supplemental Figure 2A). This region is concave in the meridional direction (red curvature cross arms in Supplemental Figure 2A) 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 (Supplemental Figure 2B; see also Kwiatkowska 2006). The convex region corresponds to the floral meristem proper, while the concave region at the distal end (arrowhead in Supplemental Figure 2B) corresponds to the bract primordium. In the third stage of bulge formation (Supplemental Figure 2C), the temporarily apparent bract primordium disappears. During the final stage, the sepal primordia are formed (P5 in Supplemental Figure 3A).

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 wild type at the beginning (data not shown) but then protruded further from the shoot axis than it did in the wild type (compare Supplemental Figures 2A and 2D). The upper surface of the primordium was largely flat or only slightly concave, forming a shelf-like shape (Supplemental Figure 2D). 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 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 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 (Supplemental Figure 2E).

The major difference between *puchi* and wild type in the third stage was the permanent presence in *puchi* of a bract with stipules (compare Supplemental Figure 2C with 2F). 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 (Supplemental Figure 2F) until it overgrew the primary inflorescence meristem (P7 in Supplemental Figure 3B). At this stage, the cells of the floral meristem proper appeared enlarged both in wild type and in *puchi* (compare Supplemental Figure 2C with 2A and Figure 2F with 2D). A prolonged duration of the bulge stage in the mutant was manifested in delayed sepal formation (compare P5 in Supplemental Figure 3A with P9 in Supplemental Figure 3C, in which the youngest sepal primordia are indicated by asterisks). This stage in 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 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.



Supplemental Figure 1. *puchi* Mutant Flowers Have Rudimentary Bracts at the Base of Their Pedicels.

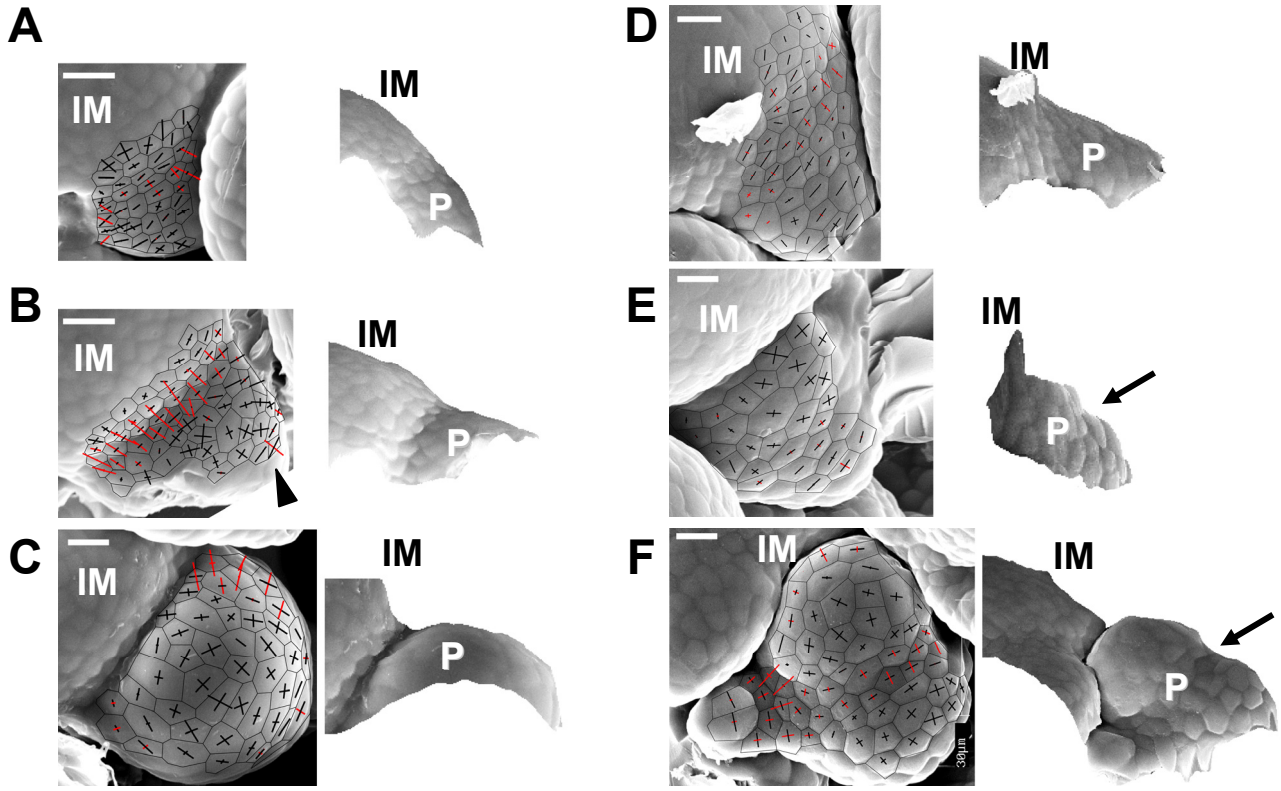
(A) SEM showing the base of wild-type pedicels**.**

(B) SEM showing the base of *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. Adopted from Hirota (2007).



Supplemental Figure 2. 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.

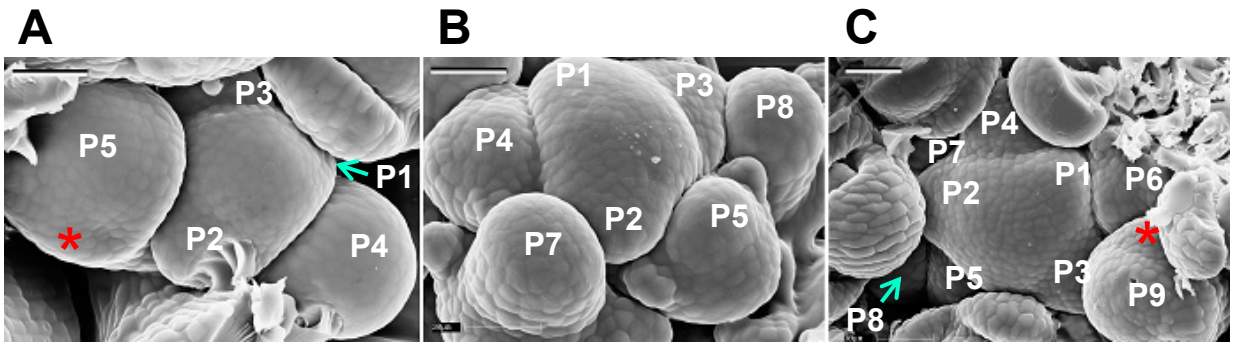
(C) The wild-type primordium at the bulge stage.

(D) The *puchi-1* primordium at the bulging stage.

(E) The *puchi-1* primordium at the second bulging stage.

(F) The *puchi-1* primordium at the bulge stage.

In each panel, SEMs on which curvature crosses are overlaid (left), and side views of the reconstructed surface (right), were obtained from replicas 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, i.e. 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. Adopted from Karim et al. (2009).



Supplemental Figure 3. Sepal Formation Is Delayed in *puchi* Mutant.

(A) to **(C)** Inflorescence shoot apices of wild-type **(A)** and *puchi-1* **([B]** and **[C])** plants are shown to illustrate the delay in sepal (asterisks) formation in the mutant. Numbers indicate the plastochron age of flower primordium. P, flower primordium. Bars = 30 µm. Adopted from Karim et al. (2007).