BRANCHED1 Interacts with FLOWERING LOCUS T to Repress the Floral Transition of the Axillary Meristems in Arabidopsis

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Plant architecture shows a large degree of developmental plasticity. Some of the key determinants are the timing of the floral transition induced by a systemic flowering signal (florigen) and the branching pattern regulated by key factors such as BRANCHED1 (BRC1). Here, we report that BRC1 interacts with the florigen proteins FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF) but not with TERMINAL FLOWER1, a floral repressor. FT protein induced in leaves moves into the subtended bud, suggesting that FT protein also plays a role in promotion of the floral transition in the axillary meristem (AM). The brc1-2 mutant shows an earlier floral transition in the axillary shoots compared with the wild type, suggesting that BRC1 plays a role in delaying the floral transition of the AMs. Genetic and gene expression analyses suggest that BRC1 interferes with florigen (FT and TSF) function in the AMs. Consistent with this, BRC1 ectopically expressed in the shoot apical meristem delays the floral transition in the main shoot. These results taken together suggest that BRC1 protein interacts with FT and TSF proteins and modulates florigen activity in the axillary buds to prevent premature floral transition of the AMs.

INTRODUCTION

There is a large degree of developmental plasticity in plant architecture, which is determined in part by the timing of floral transition and the branching pattern. The floral transition is induced by a systemic flowering signal (florigen), and plant architecture is often remodeled upon the floral transition in the shoot apical meristem (SAM). In the rosette-forming plant Arabidopsis thaliana, floral transition is followed by rapid internode elongation and activation of axillary bud development in inductive long-day (LD) conditions. Since axillary bud development proceeds differently than that of the main shoot, the systemic flowering signal may also be perceived differently in the two locations.

Appropriate regulation of axillary bud development is achieved through integrating internal and environmental signals at each of the three developmental stages: axillary meristem (AM) initiation, bud differentiation, and shoot elongation. Among them, the regulation of AM initiation and shoot elongation has been studied intensively. Molecular genetic approaches have helped identify genes required for initiation of AMs. In Arabidopsis, REVOLUTA and related HD-ZIP genes are required to specify adaxiality of lateral organs and act to form AMs (Talbert et al., 1995; Emery et al., 2003). AM initiation requires the GRAS family gene LATERAL SUPPRESSOR (Schumacher et al., 1999; Greb et al., 2003) and the Myb-like transcription factor (TF) REGULATOR OF AXILLARY MERISTEM1 (Keller et al., 2006; Müller et al., 2006). On the other hand, axillary shoot elongation is regulated by multiple plant hormones. Auxin produced in the main shoot suppresses axillary shoot elongation (Dun et al., 2006). Strigolactones, carotenoid-derived hormones produced in roots, also act as negative regulatory factors (Gomez-Roldan et al., 2008; Umehara et al., 2008; Crawford et al., 2010). Cytokinins are thought to be a secondary messenger and acts more locally in the buds to promote outgrowth.

Compared with initiation and elongation of the axillary bud, regulation of differentiation including floral transition is far less understood. One gene expressed in the axillary bud known to regulate differentiation is BRANCHED1/TEOSINTE BRANCHED1-LIKE1 (BRC1/TBL1; hereafter referred as BRC1). BRC1 encodes a member of the TEOSINTE BRANCHED1, CYCLOIDEA, and PCF family gene

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(TCP) TF family and was identified as an ortholog of maize (Zea mays) Teosinte branched1 (Tb1) (Doebley et al., 1997; Aguilar-Martínez et al., 2007; Finlayson, 2007). It was suggested that Tb1-like genes act downstream of strigolactone signaling to suppress development of axillary shoots in Arabidopsis and pea (Pisum sativum) (Aguilar-Martínez et al., 2007; Finlayson, 2007; Brewer et al., 2009). Since axillary buds of the brc1 mutant show advanced development compared with the wild type, it was suggested that BRC1 acts as a negative regulator of differentiation in axillary buds (Aguilar-Martínez et al., 2007). The brc1 mutant occasionally develops ectopic AMs at cotyledonary axils, suggesting that BRC1 also has an inhibitory role in initiation of AMs (Aguilar-Martínez et al., 2007). It was also suggested that BRC1 suppresses the elongation of axillary shoots (Aguilar-Martínez et al., 2007; Finlayson, 2007). These pleiotropic effects reflect multiple roles of BRC1 in axillary bud development. The expression of BRC1 is regulated by environmental signals, such as planting density (Aguilar-Martínez et al., 2007; Finlayson et al., 2010). Based on these observations, BRC1 was proposed to be an integrator of axillary bud development (Aguilar-Martínez et al., 2007). A BRC1 paralog, BRC2, is thought to have a partially redundant but distinct role in axillary bud development (Aguilar-Martínez et al., 2007; Finlayson et al., 2010).

In Arabidopsis, initiation of axillary bud development is closely linked to floral transition in the SAM under LD conditions (Hempel and Feldman, 1994). The molecular mechanism controlling floral transition is well understood in Arabidopsis and other species. Among the key regulators of floral transition, FT acts as a floral pathway integrator. Both endogenous and environmental signaling pathways are integrated in the transcriptional regulation of FT, which encodes a 20-kD protein of the PEBP/RKIP (for phosphatidylyethanolamine binding protein/Raf kinase inhibitor protein) family that acts as a long-distance flowering signal (Kardailsky et al., 1999; Kobayashi et al., 1999; Lifschitz et al., 2006; Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007; Mathieu et al., 2007; Tamaki et al., 2007; Notaguchi et al., 2008). FT expression is induced in phloem companion cells in cotyledons and leaves under inducive LD conditions (Takada and Goto, 2003). FT protein then moves into the SAM, where it interacts with the bZIP TF FD, to induce the expression of a floral meristem identity gene, APETALA1 (AP1) (Abe et al., 2005; Wigge et al., 2005). A recent study in rice (Oryza sativa) showed that the interaction between Hd3a (an FT ortholog) and FD1 (an FD ortholog) is not direct but is mediated by 14-3-3 proteins (Taoka et al., 2011). Os-FD1 interacts with a 14-3-3 protein through a conventional 14-3-3 recognition motif R-K-X-S-S-X-P, whereas Os-Hd3a has a noncanonical binding site for 14-3-3 proteins (Yaffe et al., 1997; Pnueli et al., 2001; Taoka et al., 2011). Other PEBP/RKIP proteins are also involved in the regulation of the floral transition in Arabidopsis. TWIN SISTER OF FT (TSF), the paralog of FT, also acts as a floral promoter (Michaels et al., 2005; Yamaguchi et al., 2005). Although TSF has a minor role under LD conditions in which FT is robustly expressed, TSF makes a prominent contribution to the floral transition under short-day (SD) conditions (Yamaguchi et al., 2005). By contrast, TERMINAL FLOWER1 (TFL1) delays the floral transition through interaction with FD (Bradley et al., 1997; Hanano and Goto, 2011). TFL1 also plays a key role in maintenance of inflorescence meristems. In the tfl1 mutant under LD conditions, the main inflorescence is terminated with a fused floral structure and the axillary shoots are replaced by single flowers (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992).

In Arabidopsis, when floral transition is induced in the SAM, preexisting primordia develop into cauline leaves and newly formed primordia become floral buds. AMs are initiated and develop basipetally after floral transition of the SAM in LD conditions (Hempel and Feldman, 1994). It was suggested that AMs are at first insensitive to flowering signals since they form vegetative nodes before making flowers even though a sufficient amount of the systemic flowering signal is produced to induce floral transition in the SAM (Hempel and Feldman, 1994; Grbic and Bleecker, 1996). Therefore, florigen activity and/or perception are likely to be differently modulated in AMs than in the SAM.

In this study, we identified BRC1 as an interactor of FT without mediation by a 14-3-3 protein and demonstrated that BRC1 interacts with TSF but not TFL1. Our results indicate that BRC1 modulates the florigen activity in the AMs and confers a specific mode of regulation to the floral transition in AMs.

**RESULTS**

**BRC1 Interacts with FT**

The fact that FT acts with the bZIP protein FD in a transcriptional complex prompted us to seek other TFs as possible interacting proteins. To identify TFs that can interact with FT, we performed yeast two-hybrid screening using two different TF libraries. Robotized screening of the first library (Steve Kay Lab) revealed 64 candidates when the threshold was set to twofold higher than the average (see Supplemental Table 1 online). By manual screening of a second TF library (Mitsuda et al., 2010), 10 TFs were found to show reproducible interaction with FT (see Supplemental Figure 1 online). Interestingly, five candidates in the first screening and four in the second were TFs belonging to the TCP family. Because some TCP family members were not represented in the two TF libraries, we performed yeast two-hybrid assays with the individual TCP transcriptional factors that have been reported to be involved in various developmental processes. Among them, BRC1 reproducibly interacted with FT in yeast two-hybrid assays (Figure 1A). We focused on BRC1 as a good candidate to be a link between the floral transition and concomitant architectural re-modeling through branch development.

We further investigated interaction between BRC1 and two FT homologs, TSF (in the FT clade) and TFL1 (in the TFL1 clade), by yeast two-hybrid assay. TSF also showed interaction with BRC1, while TFL1 did not (Figure 1A). No interactions between the TCP protein BRC2, which is closely related to BRC1 (Aguilar-Martínez et al., 2007), and these PEBP/RKIP proteins were detected in our yeast two-hybrid experiments (Figure 1A).

Protein interaction between FT and BRC1 was further confirmed in plant cells by bimolecular fluorescence complementation (BiFC) assays in Nicotiana benthamiana and in vitro by pull-down assays. A clear BiFC signal was detected in the
nucleus, indicating that FT interacts with BRC1 in plant cells and that the FT-BRC1 complex is localized to the nucleus (Figure 1B). BRC1 was able to pull down FT in the in vitro assay, indicating that their interaction is likely direct (Figure 1C).

Analysis using a series of deletion constructs showed that the C-terminal region of BRC1 including the conserved R domain is dispensable for interaction with FT (Figure 1D). However, removal of the entire C terminus after the TCP domain disrupted the interaction (see Supplemental Figure 2 online). Combinations in which one of the fusion constructs was replaced with a fragment of EYFP (YN or YC) were used as negative controls (central and right columns). Arrowheads indicate complemented EYFP fluorescence in the nucleus. Bar = 200 μm.

In vitro pull-down assay demonstrating the interaction between BRC1 and FT. Closed arrowhead, open arrowhead, and arrow indicate T7-FT, GST-BRC1, and GST, respectively.

**Figure 1.** Protein Interaction between BRC1 and FT.

(A) Yeast two-hybrid analysis of protein interaction between BRC1, BRC2, or GRF3 and FT, TSF, or TFL1. Clones containing each combination of bait and prey vectors were grown on nonselective medium (+His) or on selective medium (−His). For the clones containing BRC1, 3-amino-1,2,4-triazole (3-AT) was added to the medium (left two rows). pCUY and pGAD424 empty vectors were used as a negative control (BD [binding domain] and AD [activation domain], respectively).

(B) BiFC analysis of the interaction between BRC1 and FT in N. benthamiana leaf epidermal cells. The N-terminal fragment of EYFP was fused to BRC1 (YN-BRC1) and the C-terminal fragment of EYFP to FT (FT-YC). The fusion proteins were transiently expressed under the cauliflower mosaic virus 35S promoter through agroinfiltration. EYFP fluorescence (top panels) and EYFP fluorescence merged with bright-field (BF) image (bottom panels) are shown. Combinations in which one of the fusion constructs was replaced with a fragment of EYFP (YN or YC) were used as negative controls (central and right columns). Arrowheads indicate complemented EYFP fluorescence in the nucleus. Bar = 200 μm.

(C) In vitro pull-down assay demonstrating the interaction between BRC1 and FT. Closed arrowhead, open arrowhead, and arrow indicate T7-FT, GST-BRC1, and GST, respectively.

(D) Yeast two-hybrid assay of protein interaction between FT and truncated versions of BRC1. TCP, TCP domain; R, R domain; a.a., amino acids.

14-3-3 Proteins Do Not Mediate the Interaction between BRC1 and FT

In rice, 14-3-3 proteins mediate the interaction between Hd3a (an FT ortholog) and FD1 (Taoka et al., 2011). Since Arabidopsis FT can interact with 14-3-3 proteins, it is likely that they mediate FT–FD interaction in Arabidopsis as well (Pnueli et al., 2001). This raises the question whether 14-3-3 proteins also mediate the interaction between BRC1 and FT. To explore whether 14-3-3 proteins mediate the interaction between BRC1 and FT, two sets of yeast two-hybrid assay were performed. First, we examined whether point mutations in FT that abolish interaction with 14-3-3 proteins also mediate interaction with BRC1. All three mutant FT proteins tested still showed interaction with BRC1, suggesting that 14-3-3 proteins do not affect interaction with BRC1. All three mutant FT proteins tested still showed interaction with BRC1, suggesting that 14-3-3 proteins do not affect interaction with BRC1. In this sequence (S390A) did not abolish interaction with FT, further supporting that 14-3-3 proteins are not required for the interaction (Figure 2A). Second, Ala scanning was performed to identify residues in FT required for interaction with BRC1. Among 46 mutant FT proteins tested, seven did not show interaction with BRC1 but could interact with a 14-3-3 protein.
BRC1 Represses FT Function in the Axillary Meristem

Yeast two-hybrid analysis of interaction between BRC1 and mutant FT proteins defective in interaction with 14-3-3 proteins. T66I, P94L, G102D, and P75L are mutant FT proteins, the former three of which do not interact with 14-3-3 proteins. S390A is a mutated BRC1 in which a Ser residue in a putative 14-3-3 recognition site (S390) was substituted by Ala. GRF3 was chosen as a representative 14-3-3 protein. Yeasts were grown on nonselective medium (+His) or on selective medium (-His + 3-AT). WT, the wild type.

Bacterial expression of FT-GUS (for β-glucuronidase) plants (Notaguchi et al., 2008). Because of its large molecular mass (88.9 kD), FT-GUS fusion protein is a good indicator of expression pattern. It does not seem to move out of the cells where originally expressed (Notaguchi et al., 2008) and did not promote flowering when expressed in the leaf (see Supplemental Figure 4 online). As previously reported, GUS staining was observed in vascular bundles toward the periphery of the rosette leaf blade in gft; GUS plants (Takada and Goto, 2003; Yamaguchi et al., 2005; Notaguchi et al., 2008; Figure 3A). As in the rosette leaves, FT expression was detected in the vasculature in the distal part of cauline leaves but not in the axillary bud (Figure 3B). Further observations of sections of axillary buds did not reveal staining in the buds at the cauline or rosette leaf axils (Figures 3C and 3D; see Supplemental Figure 4 online). We next examined whether FT protein expressed in a leaf blade moves into the axillary bud subtended by the leaf using the ProHSP18.2:FT-EGFP-inducible reporter system (Notaguchi et al., 2008). Induction of FT-EGFP (for enhanced green fluorescent protein) protein in a single leaf blade was confirmed after local transient heat treatment (see Supplemental Figure 5 online). One leaf blade was heated for 2 h to induce FT-EGFP fusion protein, and 24 h after the end of the heat treatment, FT-EGFP fluorescence in the axillary bud subtended by the heat-treated leaf was observed (Figure 3E). When the heat-treated leaf blade was removed by cutting the base of the leaf blade immediately after the heat treatment, fluorescence was not detected in the axillary bud (Figure 3F). These results showed that FT-EGFP fusion protein expressed in the leaf blade can move into the axillary bud. Based on expression in leaves and the ability to move into the axillary bud from the subtending leaf, we concluded that FT protein is likely to be present in axillary buds.
**brc1-2 Mutants Show Accelerated Floral Transition in the AMs**

In order to gain insight into the physiological role of the interaction between FT and BRC1, we looked for relevant phenotypes in the brc1-2 (btl-1) mutant (Alonso et al., 2003; Aguilar-Martínez et al., 2007; Finlayson, 2007). As previously reported, more axillary shoots grew out in the brc1-2 mutant than in the wild type (Aguilar-Martínez et al., 2007; Finlayson, 2007; Figure 4A). We also confirmed that the development of AMs is more advanced in brc1-2 than in the wild type (Aguilar-Martínez et al., 2007; Figure 4B). Further observation indicated that almost all the existing axillary buds proceeded to reproductive phase in brc1-2 mutant shortly after bolting of the main shoot. By contrast, lower axillary buds remained vegetative in the wild type (Figure 4B). Furthermore, the axillary shoots of the uppermost nodes formed no leaves in many brc1-2 plants, whereas two leaves were usually formed in wild-type plants (Figure 4A). Quantification of leaf number showed that brc1-2 has fewer leaves on the axillary shoots at every position compared with the wild type (Figure 4C). A similar phenotype was observed for the lowest cauline and uppermost rosette nodes in a previous report (Finlayson et al., 2010).

Because leaf number is generally considered to reflect the timing of the floral transition, we assumed that the reduction of leaf number in brc1-2 indicates earlier floral transition in the AMs. To confirm this possibility in the context of gene expression, we examined AP1 expression in the AMs using a ProAP1:GUS reporter (Hempel et al., 1997). In the SAM, the timing of the onset of AP1 expression did not differ between brc1-2 and wild-type backgrounds, in accordance with the fact that the leaf number of the main shoot did not significantly differ between the two genotypes. By contrast, the floral transition of the AMs proceeded differently between the brc1-2 mutant and wild-type plants. Eighteen days after germination, almost all the brc1-2 plants (92%; 22/24) had at least one AP1-expressing axillary bud, whereas only about a half of the wild-type plants (53%; 16/30) did. The quantification of AP1-expressing buds indicated that the brc1-2 mutant formed more reproductive axillary buds than did the wild type (Figures 4D and 4E). Introduction of a genomic fragment of BRC1 into brc1-2 complemented the leaf number phenotype (see Supplemental Figure 6 online). Therefore, we concluded that floral transition of the AM is accelerated in the brc1-2 mutant.

**The ft-2 Mutation Is Epistatic to the brc1-2 Mutation**

The observations that the number of leaves on the axillary shoots differed depending on the node positions in the wild type and that floral transition of AMs in the brc1-2 mutant was accelerated imply that floral transition of AMs is a regulated process (Figure 4). Furthermore, the observation that FT protein could move into the axillary buds suggests that FT could be involved in this regulation. To address this, we examined floral transition of AMs in ft-2 single and brc1-2 ft-2 double mutants. Compared with the wild type, the ft-2 mutant formed more leaves on its axillary shoots (Figure 5A), suggesting that floral transition in AMs was delayed in the ft-2 mutant. The brc1-2 ft-2...
Figure 4. Early Floral Transition of the Axillary Shoots in brc1 Mutants.

(A) Axillary shoot phenotype of the wild type (Col) and brc1-2 mutant. Successive axillary shoots with the subtending leaf of 44-d-old plants are shown. Arrowheads and arrows indicate vegetative nodes and the first flowers in the most apical branches. Plus numbers represent cauline-leaf axils in the acropetal direction, and minus numbers represent rosette-leaf axils in the basipetal direction as shown in the right diagram. In the diagram, open circles, arrows, and bars represent flowers, shoots, and leaves, respectively.

(B) Developmental stages of the buds in the axils of cotyledons (c1 and c2) and rosette leaves (L1 to L15) of 10 plants of wild-type Col (left) and brc1-2 (right). Developmental stages were defined according to Aguilar-Martínez et al. (2007). Gray indicates absence of the leaf node such that the position is occupied by a flower. Observations were made on the days when the first floral buds became visible (before bolting), the main shoot became 1-cm long (1 cm bolting), and the main shoot became 5-cm long (5 cm bolting).

(C) Number of leaves formed on axillary shoots at the five axil positions of the wild type (Col) and brc1-2 mutant grown in LD. The axil position is defined in (A). Leaves in the basal part of axillary shoots without internodes are classified as axillary rosette leaves (ARL) and those with elongated internodes as cauline leaves (CL). Error bars indicate the sd (n = 9). The differences significant between Col and brc1-2 in two-tailed multiple t test with Bonferroni correction (P < 0.05) are indicated with asterisks.

(D) and (E) Expression of AP1 in the axillary buds monitored by ProAP1:GUS. Number of GUS-positive axillary buds in ProAP1:GUS plants in wild-type (Col) and brc1-2 background grown under LD (D). Error bars indicate the sd (n = 21). The differences significant in two-tailed multiple t test with Bonferroni correction (P < 0.05) with respect to the wild type (Col) are indicated with asterisks. Representative 22-d-old ProAP1:GUS plants in wild-type (Col) and brc1-2 backgrounds (E). Arrowheads indicate GUS-positive axillary buds at rosette-leaf axils.
double mutant formed almost the same number of leaves as the ft-2 single mutant on axillary shoots of cauline leaves (Figure 5A). Thus, the acceleration of AM floral transition observed in the brc1-2 mutant requires functional FT in cauline axils. In axillary shoots of rosette leaves, the brc1-2 ft-2 mutant formed fewer leaves than the ft-2 mutant (Figure 5A). The elongation of axillary shoots at the rosette axils was also enhanced in brc1-2 ft-2 compared with ft-2 (Figure 5B).

In the ft mutant background, TSF promotes the floral transition during prolonged vegetative phase (Yamaguchi et al., 2005; Hiraoka et al., 2013). Because our yeast two-hybrid assay showed that TSF is also able to interact with BRC1 (Figure 1A), TSF is likely to be responsible for the acceleration of floral transition in the AMs at rosette axils in the brc1-2 ft-2 mutant. To test this hypothesis, the phenotype of the brc1-2 ft-2 tsf-1 triple mutant was examined. As observed in the brc1-2 ft-2 and ft-2
mutants, *brc1-2 ft-2 tsf-1* formed almost the same number of leaves as the *ft-2 tsf-1* double mutant in axillary shoots of cauline leaves (Figure 5C). The *ft-2 tsf-1* double mutant did not form elongated axillary shoots on its rosette axes under our experimental conditions (Hirakoa et al., 2013; Figure 5D). In the *brc1-2 ft-2 tsf-1* triple mutant, rosette leaves did form axillary buds and developed many leaves, but over 100 d after bolting of the main shoot, the buds failed to make flowers and did not elongate (Figure 5D). These results suggest that prompt floral transition and subsequent elongation of the axillary shoots in the *brc1-2* mutant require either *FT* or *TSF* functions.

In contrast with the *ft-2* single and *ft-2 tsf-1* double mutants, both of which were epistatic to the *brc1-2* mutant, the *tfl1-17* mutant showed an additive effect on reduction in leaf number of axillary shoots in the double mutant with *brc1-2* (see Supplemental Figure 7 online). Taking this together with the results of yeast two-hybrid assay (Figure 1A), *BRC1* and *TFL1* are likely to act independently to suppress floral transition in AMs.

**Relationship between BRC1 and Flowering-Time Genes**

*FT* acts together with *FD* to promote floral transition in the SAM. Because *FD* is also expressed in the AMs (Abe et al., 2005; Wigge et al., 2005; Figure 6A), it is likely that *FD* also has a role in floral transition of the AMs. Similar to the *brc1-2 ft-2* mutant, the *brc1-2 ft-1* mutant showed substantial suppression of the *brc1-2* phenotype in terms of the leaf number of axillary shoots (Figure 6B). This indicates that *FD* also acts as a promoter of the floral transition in AMs.

It was proposed that *AP1*, *FRUITFULL* (*FUL*), and *SUPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) are downstream genes of the *FT-FD* floral transition pathway (Abe et al., 2005; Michaels et al., 2005; Tepfer-Barnolker and Samach, 2005; Wigge et al., 2005; Yoo et al., 2005; Searle et al., 2006). To examine the effect of *BRC1* on the expression of these genes, wild-type and *brc1-2* mutant plants were grown under non-inductive SD conditions and then transferred to LD. After bolting of the main shoot, a plant section containing the axils of the rosette leaves was harvested by removing the main shoot, expanded leaves, hypocotyl, and root at three time points (13, 16, and 19 d after the LD shift) and subjected to expression analysis. Quantitative RT-PCR showed that *AP1* and *FUL* were already upregulated 13 d after the LD shift in *brc1-2* compared with the wild type (Figure 6C). These two genes were further upregulated in *brc1-2* during further growth in LD but remained at a low level in the wild type. Although *SOC1* expression was not upregulated in *brc1-2* compared with the wild type at 13 d after the LD shift, a gradual increase was observed during the subsequent growth in LD (Figure 6C). The floral meristem identity gene *LEAFY* (*LFY*) is expressed in an *FT*-independent manner. Although *LFY* expression level was elevated in *brc1-2* 13 d after the LD shift, unlike *AP1* and *FUL*, subsequent upregulation did not occur at later time points (see Supplemental Figure 8 online). These results indicate that *BRC1* represses photoperiod-dependent expression of *FT* downstream genes in axillary buds.

*SOC1* functions in floral transition of AMs together with *FUL* and other *SOC1*-related MADS box genes (Melzer et al., 2008; Dorca-Fornell et al., 2011). In accordance with this, the *soc1-2* mutant formed many more leaves on axillary shoots compared with the wild type (Figure 6D). Nevertheless, the *soc1-2* mutation only partially suppressed the phenotype of the *brc1-2* mutant (Figure 6D). These results suggest that, unlike the cases of *FT* and *TSF*, the *brc1-2* phenotype is not fully dependent on *SOC1* function.

**BRC1 Can Delay Floral Transition When Expressed in the SAM**

The observations described so far indicate that *BRC1* has a role in preventing AMs from undergoing early floral transition. The abilities of *FT* to be transported into the axillary buds and to interact with *BRC1* suggest that *BRC1* is likely to act through inhibition of *FT* function. This raises the possibility that *BRC1* could delay the floral transition of the main shoot when expressed ectopically in the SAM. To test this possibility, the flowering-time phenotype of transgenic plants expressing *BRC1* in the SAM under the control of the *FD* promoter (*ProFD:BRC1*) was examined. As expected, *ProFD:BRC1* plants showed significant delay in floral transition of the SAM under inductive LD (Figure 7; see Supplemental Figure 9 online). Previously, it was shown that overexpression of *BRC1* under the control of cauliflower mosaic virus 35S promoter resulted in pleiotropic developmental defects (Aguilar-Martínez et al., 2007). In this study, *ProFD:BRC1* plants showed mild retarded growth in early developmental stages but no obvious morphological alterations (Figure 7A). In SD, the effect of *ProFD:BRC1* was more subtle (see Supplemental Figure 10 online).

**DISCUSSION**

In this study, we showed that the *BRC1* interacts with *FT*. *BRC1* interacts also with *TSF*, whereas it did not interact with *TFL1*. This establishes *BRC1* as an interactor specific to the *FT* clade of PEBP/RKIP proteins. Our study indicates that *FT* protein expressed in the leaf is transported into the axillary bud and promotes floral transition of the AM. *BRC1* expressed in the axillary bud interacts with *FT* to inhibit its function. Thus, *BRC1*, previously proposed to be an integrator of branching signals within the axillary buds, plays a role in modulating the activity of a long-distance florigen signal to delay floral transition in AMs.

**FT Is Able to Interact with Various TFs**

Yeast two-hybrid screening using TF libraries revealed numerous candidate FT-interacting proteins. Although the functional significances of these interactions are beyond the scope of this study, it is to be noted that some of the candidates obtained here overlapped with recently reported candidates for interactors of *FT* and related proteins. Yeast two-hybrid screening using a cDNA library from inflorescence meristems, floral meristems, and floral buds identified bZIP52, TCP8, TCP15, and TCP22 as candidates to interact with *FT* in *Arabidopsis* (Liu et al., 2012). Yeast two-hybrid screening with Md-FT1, a homolog of *FT* in apple (*Malus × domestica*) showed that Md-VOZ1, Md-TCP2, and Md-TCP4 (belonging to the same clade as At-VOZ1, At-TCP2 and 24, and At-TCP3 and 4, respectively) interact with Md-FT1 (Mimida et al., 2011). Given the repeated identification in separate screenings, these TCP, bZIP, and VOZ proteins are promising candidates for factors acting with *FT* protein.
BRC1 Interacts with FT and TSF Proteins without Mediation by 14-3-3 Proteins

We identified BRC1 as specifically interacting with FT and TSF, but not with TFL1. By contrast, many of the previously reported FT interactors showed interaction with TFL1 as well. For example, FD and FD PARALOG interact with both FT/TSF and TFL1 (Abe et al., 2005; Wigge et al., 2005). Tomato (Solanum lycopersicum) SELF-PRUNING-interacting proteins (SPAK, SIP4, and SPGB) and maize FD homolog DELAYED FLOWERING also interact with FT clade and TFL1 clade proteins (Pnueli et al., 2001; Danilevskaya et al., 2008, 2010). For some FT interactors, such as...
Figure 7. Ectopic Expression of BRC1 Delays Floral Transition in the SAM.

(A) Thirty-day-old plants of wild type (Col) and two independent lines of ProFD:BRC1 (#1 and #2) grown in LD.

(B) Flowering time measured by the number of rosette leaves (RL) and cauline leaves (CL). Error bars indicate the SD (n = 15). The differences significant in two-tailed Dunnett’s test (P < 0.01) in the total leaf numbers with respect to Col are indicated with asterisks. [See online article for color version of this figure.]

FT-interacting protein1 and other factors from the screening mentioned above, it remains to be examined whether they interact with TFL1 clade proteins (Mimida et al., 2011; Liu et al., 2012). FT clade and TFL1 clade proteins seem to share common interactors through the mediation of 14-3-3 proteins. FT and TFL1 share the ability to interact with a common subset of 14-3-3 isoforms (e.g., GRF3) (Figure 1A). In rice, 14-3-3 proteins mediate the formation of the Hd3a-FD1 (corresponding to FT-FD complex (Taoka et al., 2011). In tomato, the SELF-PRUNING-interacting factors mentioned above also interact with 14-3-3 proteins (Pnueli et al., 2001). By contrast, BRC1 does not seem to require 14-3-3 proteins for interaction with FT.

It is likely that FT interacts with a variety of proteins using 14-3-3-dependent FT interactors, whereas other candidates seemed to be independent of 14-3-3 proteins (see Supplemental Figure 1 online). These observations indicate that interaction mediated by 14-3-3 is not the only way in which FT interacts with TFs, raising the possibility that the protein complex containing FT has more components than known at the moment. Another possibility is that FT uses different sets of partners in different cells or for different functions.

**BRC1 Is a Negative Regulator of Floral Transition in AMs**

FT acts as a systemic flowering signal (florigen) and moves from the leaves to the SAM (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lin et al., 2007; Mathieu et al., 2007; Tamaki et al., 2007; Notaguchi et al., 2008). FT protein expressed under the HSP18.2 promoter in a single leaf also moved into the subtending axillary bud (Figure 3). In addition, ft-2 mutants showed delayed floral transition in AMs (Figure 5). Taken together, these findings demonstrate that FT is a key regulator of floral transition in AMs as well as in the SAM. Recent work showed that FT is involved in the regulation of axillary shoot elongation (Hiraoka et al., 2013), indicating that FT has an additional role in axillary buds after the transition.

On the other hand, BRC1 was previously proposed to be an integrating repressor of axillary bud development (Aguilar-Martínez et al., 2007), BRC1 suppresses the initiation, differentiation, and elongation of axillary buds (Aguilar-Martínez et al., 2007; Finlayson, 2007). In this study, the brc1-2 mutant showed a reduction in the number of leaves on axillary shoots, as was, in part, reported previously (Finlayson et al., 2010), and earlier onset of AP1 expression in the axillary buds (Figure 4). Furthermore, when ectopically expressed in the SAM, BRC1 delayed floral transition of the main shoot, with an increase in the number of leaves formed on the main axis (Figure 7). These observations, taken together, strongly suggest that BRC1 acts as a negative regulator of the floral transition in AMs.

Since BRC1 protein interacts with FT and TSF proteins, and brc1 and ft (tsf) mutants showed opposite phenotypes in the AMs, BRC1 and FT play antagonistic roles likely through their protein–protein interaction. Genetic analyses indicated that the ft-2 and tsf-1 mutations are epistatic to brc1-2, suggesting that BRC1 inhibits FT and TSF functions (Figure 5). This is supported by the SD-to-LD shift transcript profiling experiment, where genes downstream of FT were overinduced in the brc1-2 mutant (Figure 6).

TFL1 also antagonizes FT function, but through competitive interaction with the common partner, FD (Bradley et al., 1997; Abe et al., 2005; Wigge et al., 2005; Hanano and Goto, 2011). Under LD conditions, when florigen activity is high, the main (primary) inflorescence of the tfl mutant makes only a few flowers and terminates soon after bolting (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992). In addition, TFL1 is expressed in the AMs as well as in the SAM, and some axillary shoots are replaced by solitary flowers in tfl mutants (Shannon and Meeks-Wagner, 1991; Alvarez et al., 1992; Bradley et al., 1997), indicating TFL1 is also involved in the suppression of phase transition in AMs. However, BRC1 showed no interaction with TFL1, and the tfl1-17 brc1-2 double mutant showed an additive reduction in the leaf number of axillary shoots under SD conditions (see Supplemental Figure 7 online). These results suggest that TFL1 and BRC1 act independently.

Although the mechanism by which BRC1 modulates the activity of FT/TSF remains to be clarified, the observation that FT interacts with BRC1 through a different region than the 14-3-3 protein interaction surface (Figure 2) supports the possibility that BRC1 binds to the FT/14-3-3/FD complex. In the deduced structure of the FT/14-3-3/FD complex based on Taoka et al. (2011), the positioning of the region of FT responsible for interaction with BRC1 would allow binding of BRC1 to the complex (see Supplemental Figure 11 online). If this is the case, BRC1 could inhibit the transcriptional activator function of the
FT/14-3-3/FD complex. However, the possibility that BRC1 traps FT and prevents it from interacting with 14-3-3 proteins cannot be excluded.

Possible Conservation of the Antagonistic Mechanism between BRC1 and FT

The BRC1-like and FT-like genes seem to be conserved among species. BRC1 was first identified as a maize Tb1 ortholog in Arabidopsis (Aguilar-Martínez et al., 2007; Finlayson, 2007). Similar to the phenotype of Arabidopsis brc1 mutants, maize tb1 mutants show increased numbers of elongated axillary shoots (Doebley et al., 1995). Based on comparative genomics and expression analysis, it was suggested that Zea mays CENTRORADIALIS8 (ZCN8) is a maize counterpart of FT (Danilevskaya et al., 2008; Meng et al., 2011; Lazakis et al., 2011). The FD orthologous gene Zm-DLF1 was also identified. Mutation in Zm-DLF1 resulted in a late-flowering phenotype (Muzynski et al., 2006). Zm-ZCN8 interacts with Zm-DLF1, indicating that the FT-DL-FD flowering activator complex is also conserved in maize. TB1/FINE CULM1 from rice is orthologous to Zm-Tb1, and the fc1 mutant shows enhanced tillering (Takeda et al., 2003). Os-Hd3a (an FT ortholog) and Os-FD1 interact with each other and promote floral transition in rice (Taoka et al., 2011).

The tomato BRC1b and SINGLE FLOWER TRUSS genes, BRC1 and FT orthologs, respectively, were also characterized (Lifschitz et al., 2006; Martin-Trillo et al., 2011). These studies suggest that there is functional conservation of the FT-like and BRC1-like genes as florigens and integrators of the axillary bud development, respectively. Our study proposes that these two pathways interact in the AMs of Arabidopsis. It is worth investigating whether BRC1-like genes have conserved function in regulation of floral transition in AMs and whether FT-like and BRC1-like proteins interact with each other in these species.

The Significance of Regulating AM Floral Transition with Florigen and BRC1

In wild-type Arabidopsis, not all axillary buds elongate, and in ProAP1:GUS plants, buds formed at lower axils remain vegetative for longer after bolting of the main shoot (Figure 4). This may contribute to preventing existent buds from elongating simultaneously and preparing for future alterations in environmental and autonomous conditions.

AM-specific modulation of florigen activity by BRC1 might be more important under adverse growth conditions, when flowering is accelerated. For example, an increased number of neighboring plants leads to a change in light quality to a low ratio of red to far-red radiation (reviewed in Smith, 1982). When plants grow under far-red-rich light, FT, as an integrator gene of the flowering pathway, is upregulated and induces premature floral transition (Devlin et al., 2003; Endo et al., 2005; Sessa et al., 2005; Wollenberg et al., 2008). On the other hand, expression of BRC1 is also increased by a high planting density and in phyB mutants (Aguilar-Martínez et al., 2007; Finlayson et al., 2010). Although overproduced FT protein potentially activates the developmental program in undesirable parts of the plant, such as AMs, increased levels of BRC1 will suppress florigen activity specifically in AMs and thus enable the plant to concentrate its limited resources on the main shoot and promote reproductive success. Regulation of FT expression by environmental and internal signals is well studied (reviewed in Simpson and Dean, 2002; Boss et al., 2004; Fomara et al., 2010), and expression of BRC1 is also affected by external and hormonal cues (Aguilar-Martínez et al., 2007; Finlayson, 2007). These signals would converge to create a balance between FT and BRC1 and regulate floral transition in AMs. This additional mode of regulation in AMs enables modulation of the timing of axillary bud differentiation and thus determines plant architecture depending on external and internal conditions.

METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana Columbia-0 (Col) was used as the wild type. The ProAP1:GUS reporter (POP40) was kindly provided by M.F. Yanofsky (University of California at San Diego). The T-DNA insertion mutant brc1-1 (tb1-1) was obtained from the ABRC and corresponds to the line SALK_091920 previously characterized (Aguilar-Martínez et al., 2007; Finlayson, 2007). Plant materials below were previously described: gft:GUS (Notaguchi et al., 2008), ProFD:GUS (Abe et al., 2005), ft-2 (Imura et al., 2012), fd-1 (Abe et al., 2005), and soc1-2 (Lee et al., 2009). ProHSP18.2:FT-EGFP was constructed by fusing a promoter fragment (~533 bp) with a 3′ fragment of the Arabidopsis HSP18.2 gene (Takahashi and Komeda, 1989) with the FT-EGFP open reading frame (ORF). ProFD: BRC1 was constructed based on the previously described ProFD:GUS construct (Abe et al., 2005) by replacing the GUS ORF with the BRC1 ORF. Seeds were stratified by keeping them at 4°C for 2 to 4 d and then transferred to 22°C LD (16 h light/8 h dark) with white fluorescent light (~80 μmol m⁻² s⁻¹) or SD (8 h light/16 h dark) with white fluorescent light (~100 μmol m⁻² s⁻¹) conditions. For analyses of the axillary shoot phenotypes and FT protein movement, plants were grown on vermiculite. Developmental stages of the axillary buds were determined according to Aguilar-Martínez et al. (2007). Observations were made on the days when the individual plant first showed a visible floral bud (before bolting), reached 1 cm in height (1 cm bolting), and reached 5 cm in height (5 cm bolting). The leaf number of the axillary shoot was counted after internode elongation. Leaves at the base of the axillary shoot without a visible elongated stem were counted as axillary rosette leaves and leaves on the elongated shoots as cauline leaves. For expression analyses, plants were grown on half-strength Murashige and Skoog medium supplemented with 0.5% Suc and 0.8% agar.

Plasmid Constructs

Plasmid constructs used in this study and primers used for cloning are described in Supplemental Tables 2 and 3 online.

Yeast Two-Hybrid Assay

For the first yeast two-hybrid screening, a TF library containing 1729 TFs was used (Pruneda-Paz et al., 2009). The ORF of the full-length FT protein was cloned in pENTR/DO-TOPO (Life Technologies) and then transferred to pDEST32 (Life Technologies) using LR recombination reaction. The lacZ activity was measured by β-galactosidase assay. For the second screening and two-hybrid assays, the FT ORF was cloned in pCUY vector (Suzuki et al., 2001). A TF library containing 1503 TFs constructed in pDEST_GAD424 was described previously (Mitsuda et al., 2010). Plasmids isolated from positive colonies were transformed into Escherichia
buffer, pH 7.4, to a
added to the medium for prevention of nonspeci
coli (DH5α) for sequencing. The interaction was confirmed after re-
transformation of yeast, pCUY and pGAD424 (Clontech/TAKARA) were
used for construction of the other yeast two-hybrid constructs. The
appropriate plasmids were transformed into a yeast strain AH109 (Clontech/Takara) using the lithium acetate method and selected on
synthetic dropout medium plates lacking Leu and Trp. After 3 or 4 d of
incubation at 30°C, yeast cells were transferred to liquid synthetic dropout
medium lacking Leu and Trp and incubated for 24 h. After measuring
OD600 1 mL of each culture was centrifuged and resuspended in TE
buffer, pH 7.4, to a final OD600 of 0.33. Five microliters of suspended yeast
was spotted on synthetic dropout medium lacking Leu, Trp, and His for
the His requirement test (−His). 3-Amino-1,2,4-triazole (0.5 mM) was
added to the medium for prevention of nonspecific growth, where
specified (−His+3-AT). As positive controls, yeast cells were also spotted
on synthetic dropout medium lacking Leu and Trp (+His). These plates
were incubated at 30°C and scored for growth of the yeast cells. To
visualize the location of the residues important for protein interaction, an
FT ribbon model was produced using Pymol (http://www.pymol.org) from
the Protein Data Bank file (Ahn et al., 2006; Protein Data Bank ID 1WK). BiFC Assay in N. benthamiana Leaf Epidermal Cells
A split EYFP fragment encoding the N-terminal fragment of EYFP (YN; 1 to
155 amino acids) was cloned into pBI121 vector and then BRC1 ORF was
inserted in frame after the YN to make YN-BRC1. Other constructs (FT-
YC, YN, and YC in pBI121) were described previously (Abe et al., 2005).
Nicotiana benthamiana plants were grown under continuous light con-
ditions at 23°C. Complemented EYFP fluorescence was excited at 515 nm by an argon laser and detected in a range from 500 to 600 nm with confocal laser scanning microscopy (FV1000; Olympus).
In Vitro Pull-Down Assay
The BRC1 full-length ORF was cloned into pET41a (Novagen), and FT full-
length ORF was cloned into pET24d (Novagen). The plasmids were
transformed into E. coli BL21 (DE3) to express glutathione S-transferase
(GST)-BRC1 and T7-FT, respectively. pET41a empty vector was used to
insert BRC1 ltrated into the
ProHSP18.2 (brc1-2) plant was observed with microscopy, and the
number of stained buds was counted.
Single-Leaf Heat Treatment and Fluorescence Microscopy
ProHSP18.2:FT-EGFP plants grown under LD conditions for 22 d were
subjected to single-leaf heat treatment essentially as described previously
(Notaguchi et al., 2008). The leaf blade of the sixth leaf was exposed to
37°C for 2 h by placing it in the water-filled space between a heated
copper plate and a slide glass to ensure good contact. Twenty-four hours
after heat treatment, plants were embedded in 4% agar gel with PBS and
sectioned at a thickness of 80 μm with a vibratome (VIB-1500; Intracel).
GFP fluorescence was excited at 488 nm by an argon laser and detected
in a range from 500 to 600 nm with confocal laser scanning microscopy (FV1000; Olympus).
Quantitative RT-PCR Analysis
Plants grown under SD for 24 d were transferred to LD and harvested after
13, 16, and 19 d. Rosette-axil samples were obtained by removing the
main shoot, leaves, hypocotyl, and root. Total RNA was extracted using
TRizol reagent (Life Technologies) and treated with DNase I Amplification
Grade (Life Technologies) according to the manufacturer’s instructions.
Total RNA (0.5 μg) was reverse transcribed in a 20-μL reaction mixture
using Transcript (Roche Applied Science). The resultant cDNA solution
was diluted with 30 volumes of water, and 3-μL aliquots were analyzed.
Quantitative RT-PCR analyses were performed with the CFX96 system
(Bio-Rad Laboratories) using SYBR green I. The primers used are de-
scribed in Supplemental Table 4 online. Quantitative RT-PCR results
were normalized to the geometric means of ASCORBATE PEROXI-
DASE3 (APX3; Hazen et al., 2005), REGULATORY PARTICLE TRIPLE-A
1A (RPT1a; Michael et al., 2008), and THIOREDOXIN H-TYPE3 (TRX3;
Michael et al., 2008) and shown as the average of nine different reactions
(biological × technical triplicate). Internal control genes used in this study
were selected from nine genes according to Vandesompele et al. (2002).

Supplemental Data
The following materials are available in the online version of this article.
Supplemental Figure 1. Yeast Two-Hybrid Assay between FT and
Putative FT-Interacting Proteins.
Supplemental Figure 2. Yeast Two-Hybrid Assay between FT and
BRC1 Fragments.
Supplemental Figure 3. Yeast Two-Hybrid Assay between Ala-
Scanned Mutant FT Proteins and BRC1 or GRF3.
Supplemental Figure 4. Expression Analysis Using FT-GUS Fusion
Protein.
Supplemental Figure 5. Heat Treatment of Leaf Blades of ProHSP18.2:
FT-EGFP Plants.
Supplemental Figure 6. Complementation Test of the brc1-2 Mutant
Using a Genomic Fragment of BRC1.

Accession Numbers
Arabidopsis Genome Initiative locus identifiers for genes mentioned in this
article are as follows: At3g18550 (BRC1), At1g65480 (FT), At1g68800 (BRC2), At4g20370 (TSF), At5g03840 (TFL1), At5g38480 (GRF3),
At2g21320 (bZIP30), At4g35900 (FD), At1g69120 (AP1), At5g60910
(FUL), At2g45660 (SOC1), At5g61850 (LFY), At4g35000 (APX2), At1g53750
(RPT1a), At5g42980 (TRX3), At5g69720 (HSP18.2), At1g06850 (bZIP2),
At4g18390 (TCP2), At1g52330 (TCP3), At3g15030 (TCP4), At1g58100
(TCP8), At1g69690 (TCP15), At1g72010 (TCP22), and At4g18390 (TCP24).

GUS Staining
Plants were removed at 15 min in 90% acetone on ice and washed in PBS
buffer twice. Fixed samples were incubated at 37°C for ~16 h with
staining solution (0.5 mg/mL X-Gluc, 50 mM sodium phosphate buffer, pH
7.0, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and
0.2% TritonX-100). For resin sectioning, samples were dehydrated
through an ethanol series and embedded in Technovit 7100 (Heraeus
Kulzer) and sectioned at a thickness of 5 μm with a microtome (RM2155;
Supplemental Figure 7. BRC1 and TFL1 Likely Act Independently in the Regulation of Floral Transition of Axillary Shoots.

Supplemental Figure 8. Relative Expression of LFY after a Shift from SD to LD.

Supplemental Figure 9. Relative Expression of BRC1 in ProFD:BRC1 Plants.

Supplemental Figure 10. Flowering-Time Phenotype of ProFD:BRC1 Grown under SD.

Supplemental Figure 11. Amino Acid Residues Essential for the Interaction with BRC1 Shown on a Ribbon Model of FT in the Complex with 14-3-3 Protein and FD.

Supplemental Table 1. List of Candidate FT-Interacting Proteins Obtained from Yeast Two-Hybrid Screening of the Kay Lab Transcription Factor Library.

Supplemental Table 2. Constructs.

Supplemental Table 3. Primers Used in Plasmid Construction.

Supplemental Table 4. Primers Used in qRT-PCR.

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AUTHOR CONTRIBUTIONS


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BRANCHED1 Interacts with FLOWERING LOCUS T to Repress the Floral Transition of the Axillary Meristems in Arabidopsis

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