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Arabidopsis BRANCHED1 Acts as an Integrator of Branching Signals within Axillary Buds[W]

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Shoot branching patterns depend on a key developmental decision: whether axillary buds grow out to give a branch or whether they remain dormant in the axils of leaves. This decision is controlled by endogenous and environmental stimuli mediated by hormonal signals. Although genes involved in the long-distance signaling of this process have been identified, the genes responding inside the buds to cause growth arrest remained unknown in *Arabidopsis thaliana*. Here, we describe an *Arabidopsis* gene encoding a TCP transcription factor closely related to *teosinte branched1* (*tb1*) from maize (*Zea mays*), *BRANCHED1* (*BRC1*), which represents a key point at which signals controlling branching are integrated within axillary buds. *BRC1* is expressed in developing buds, where it arrests bud development. *BRC1* downregulation leads to branch outgrowth. *BRC1* responds to developmental and environmental stimuli controlling branching and mediates the response to these stimuli. Mutant and expression analyses suggest that *BRC1* is downstream of the *MORE AXILLARY GROWTH* pathway and that it is required for auxin-induced apical dominance. Therefore, *BRC1* acts inside the buds as an integrator of signals controlling bud outgrowth and translates them into a response of cell growth arrest. The conservation of *BRC1*/*tb1* function among distantly related angiosperm species suggests that a single ancestral mechanism of branching control integration evolved before the radiation of flowering plants.

INTRODUCTION

The vast diversity of plant architectures found in plants today depends largely on the control of branching. Branching patterns determine many aspects of plant form, light interception efficiency, and adaptation to resource availability. Shoot branching patterns are generated during postembryonic development. After germination, the shoot apical meristem (SAM) generates the main shoot, leaf primordia, and new meristems. New shoot meristems formed in the axils of leaves, axillary meristems (AMs), are established at the time of leaf primordia initiation or later in development from groups of cells that retain meristematic potential (Greb et al., 2003; Schmitz and Theres, 2005). After initiation, AMs develop into axillary buds. Branching patterns depend on a key developmental decision: whether axillary buds grow out to give a branch or whether they remain small and dormant in the axils of leaves. This decision is reversibly controlled by developmental and environmental stimuli perceived in different regions of the plant and transduced into the axillary buds to be translated into a local response of growth arrest (Lang et al., 1987; Horvath et al., 2003). This allows the plant to adapt to changing conditions.

In *Arabidopsis thaliana*, axillary bud development is well characterized morphologically (Hempel and Feldman, 1994; Grbic and Bleecker, 2000; Long and Barton, 2000), and some of the genes involved in AM initiation and long-distance signaling have been identified. However, the genes responding to these genetic pathways, acting inside the buds to directly cause cell proliferation arrest, remained unknown.

During prolonged vegetative development, AMs are initiated in an acropetal order, first in the axils of mature leaves distant from the shoot apex and later in younger leaves. After flowering, AMs are initiated in a basipetal order, first in leaf axils closest to the shoot apex (Hempel and Feldman, 1994; Grbic and Bleecker, 2000; Long and Barton, 2000). Genes such as *LATERAL SUPPRESSOR* (*LAS*), encoding a GRAS protein (Greb et al., 2003), and the *REGULATOR OF AXILLARY MERISTEMS* (*RAX*) genes, encoding a group of R2R3 MYB proteins (Keller et al., 2006; Muller et al., 2006), are necessary during AM initiation to maintain the meristematic potential of cells at the base of leaves and to allow the organization of a stem cell niche. *REVOLUTA*/*INTERFASCICULAR FIBERLESS1* (*REV*/*IFL1*) (Talbert et al., 1995; Ratcliffe et al., 2000; Otsuga et al., 2001; Zhong and Ye, 2001), encoding a Homeobox-Leucine-Zipper protein, is also involved in early stages of AM initiation.

Once initiated, AMs go on to form a bud: first, leaf primordia are formed on the periphery of the AM (vegetative phase), and later, flower meristems are initiated (reproductive phase). Axillary buds bearing flowers may then elongate to give a branch, as in the case of cauline leaf buds, or they may become arrested for most of the plant life, as in the case of many rosette leaf buds. Long-range signaling promoting bud arrest is controlled both by auxin produced in the shoot apex and transported basipetally

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and by a novel carotenoid derivative synthesized in the root and transported acropetally (Cline, 1991; Shimizu-Sato and Mori, 2001; Leyser, 2003; Dun et al., 2006). *Arabidopsis* mutants with reduced auxin sensitivity (i.e., *auxin-resistant1* [*axr1*]; Chatfield et al., 2000) have weaker apical dominance, and auxin overproducers (i.e., *yucca1* [*ycc1*]; Zhao et al., 2001) have stronger apical dominance than wild-type plants. Also, mutations in the *MORE AXILLARY GROWTH* (*MAX*) genes, which control the synthesis and activity of the carotenoid-derived hormone (MAX-dependent signal) in *Arabidopsis*, cause an excess of branch outgrowth (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004, 2005). These two hormones, auxin and the MAX-dependent signal, act outside the axillary buds. Additional mechanisms affecting chromatin structure also seem to be involved (Peng et al., 2006). To date, the genes responding to these signals within the buds to directly cause growth arrest have remained uncharacterized in *Arabidopsis*.

Genes promoting bud arrest locally within the bud have been described in monocots. They are *teosinte branched1* (*tb1*) from maize (*Zea mays*) (Doebley et al., 1997) and its homologs from rice (*Oryza sativa*), Os *tb1* (Hu et al., 2003; Takeda et al., 2003), and sorghum (*Sorghum bicolor*), Sb *tb1* (Kebrom et al., 2006). *tb1*-like genes encode transcription factors containing a TCP domain, a 59–amino acid domain that allows nuclear targeting, DNA binding, and protein–protein interactions (Kosugi and Ohashi, 1997; Cubas et al., 1999a; Kosugi and Ohashi, 2002). *tb1* and Os *tb1* are expressed in AMs and buds, where they suppress growth (Hubbard et al., 2002; Takeda et al., 2003). Their mutants, *tb1* and *fine culm1*, respectively, have enhanced shoot branching (Doebley et al., 1997; Wang et al., 1999; Hu et al., 2003; Takeda et al., 2003). However, the general role of *tb1* in the control of shoot branching in angiosperms remained to be established. First, *tb1*-like genes had not been analyzed in wild species, and second, they had not been studied in dicots; therefore, it was unclear whether this function was conserved in this group.

In this study, we have characterized *BRANCHED1* (*BRC1*) and *BRANCHED2* (*BRC2*), two of the three genes most closely related to *tb1* in the wild dicot *Arabidopsis*. We show that both genes, but mainly *BRC1*, play a central role in the control of axillary bud development. *BRC1* expression patterns are restricted mostly to axillary buds, its activity inversely correlates with bud outgrowth, and *brc1* mutant phenotypes are non-pleiotropic and affect exclusively axillary bud development. Moreover, *BRC1* responds to environmental and endogenous signals controlling bud outgrowth, and our genetic analyses indicate that auxin and the *MAX* pathway act through *BRC1* to promote bud arrest. These results indicate that *BRC1* acts as a local integrator of the genetic pathways controlling branch outgrowth.

RESULTS

The *Arabidopsis* TCP Gene Family

To identify the *Arabidopsis* genes closest to *tb1*, the complete *Arabidopsis* TCP gene family was analyzed (Figure 1A). This family comprised 24 genes encoding predicted proteins with a TCP domain (see Supplemental Table 1 and Supplemental Figure 1 online). Phylogenetic analysis of this domain revealed two subfamilies (Cubas et al., 1999a; Cubas, 2002; Kosugi and Ohashi, 2002; Palatnik et al., 2003): class I, formed by 13 predicted proteins related to the PCF rice factors (Kosugi and Ohashi, 1997), and class II, formed by 11 predicted proteins related to the *Antirrhinum CYC* and *CIN* genes and to *tb1* (Luo et al., 1996; Doebley et al., 1997; Nath et al., 2003; Palatnik et al., 2003). Class II could be further subdivided into two groups: the CIN group formed by eight members, some of which are involved in the control of leaf primordia growth (Palatnik et al., 2003), and the tb1/CYC group (called the ECE group by Howarth and Donoghue [2006]), on which we have focused. Genes from this group have an R domain (see Supplemental Figure 1 online) (Cubas et al., 1999a) that is also present in *TCP2* and *TCP24* from the CIN group. Although in monocots only one type of tb1/CYC/ECE gene has been identified (e.g., *tb1*, Os *tb1*, and Sb *tb1*), in eudicots several tb1/CYC/ECE genes are found, and phylogenetic analyses have suggested that duplications within this clade occurred at the base of eudicots (Howarth and Donoghue, 2006). Therefore, no *Arabidopsis* TCP gene is a direct ortholog of *tb1*. *TCP1*, the gene most closely related to *CYC*, has been proposed to be the *CYC* ortholog (Cubas et al., 2001). Therefore, *TCP12* and *TCP18* were the only *Arabidopsis* TCP genes that remained as candidates for having retained a role in branching. Based on their similarity to *tb1* in protein sequence, expression patterns, and mutant phenotypes (see below), they were renamed *BRC1* and *BRC2*, respectively.

Full-length cDNAs of *BRC1* and *BRC2* were isolated. None of them corresponded to the predicted transcripts annotated in the *Arabidopsis* genome databases. The cDNA of *BRC1* (1609 bp, three spliced introns; Figure 1B) contained an open reading frame of 1290 bp encoding a protein of 429 amino acids. The *BRC2* cDNA (1380 bp, one spliced intron; Figure 1C) contained an open reading frame of 1071 bp encoding a protein of 356 amino acids. Both predicted proteins had a TCP domain and an R domain.

It has been proposed that the TCP domain is necessary for nuclear localization (Kosugi and Ohashi, 1997; Cubas et al., 1999a), and some TCP proteins have been shown to be targeted to the nuclei in heterologous systems (Suzuki et al., 2001; Qin et al., 2004). To investigate whether *BRC1* and *BRC2* encode nuclear proteins, the cDNAs of *BRC1* and *BRC2* were fused to green fluorescent protein (GFP), and transgenic *Arabidopsis* lines expressing these proteins under the control of the cauliflower mosaic virus (CaMV) 35S promoter were obtained. GFP:BRC1 and GFP:BRC2 were targeted to the nuclei in all tissues analyzed (Figure 1D for GFP:BRC1; GFP:BRC2 not shown). These data support their proposed role as transcriptional regulators. Plants expressing GFP:BRC1 showed pleiotropic developmental defects and retarded growth (see Supplemental Figure 3 online). Plants expressing GFP:BRC2 did not show any obvious phenotypic effect.

BRC1 and *BRC2* Are Expressed in Axillary Buds

To explore the potential roles of *BRC1* and *BRC2* in the control of plant development, their mRNA levels were analyzed by

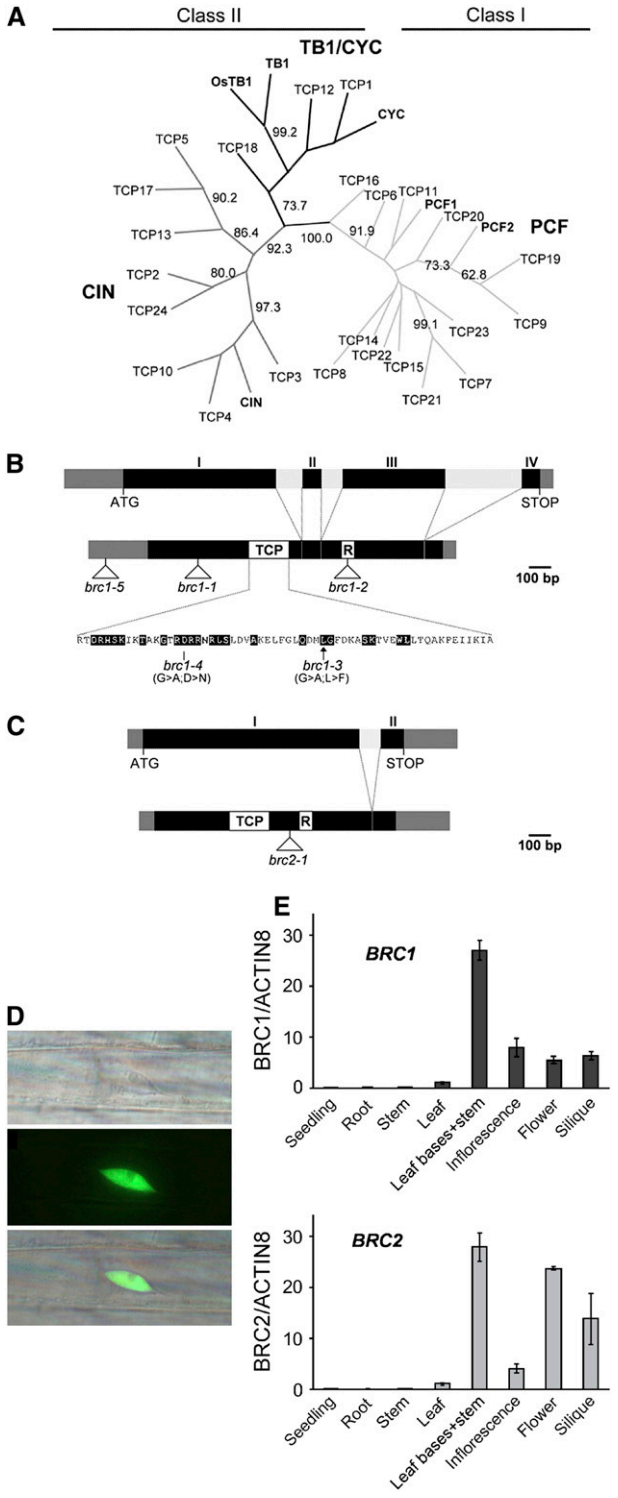

Figure 1. The *BRC1* and *BRC2* Gene Family, Structure, Transcripts, and Proteins.

(A) Unrooted consensus tree showing relationships among the predicted *Arabidopsis* TCP proteins and members of other plant species, CYC, tb1, PCFs, and CIN. The percentage of bootstrap samples in which particular clades were monophyletic is indicated when it is 70% or more. Black lines represent the TB1/CYC clade, dark gray lines represent the CIN clade, and light gray lines represent the PCF clade.

(B) and **(C)** Genomic and cDNA organization of *BRC1* **(B)** and *BRC2* **(C)**. Black boxes represent exons, dark gray boxes represent 5′ and 3′ untranslated regions, light gray boxes represent introns, and white boxes represent conserved domains. Intron sizes were 116, 93, and 337 bp for introns 1, 2, and 3 of *BRC1*, respectively, and 95 bp for *BRC2*. Triangles indicate sites of T-DNA insertion in the mutants. *brc1-5* is located 186 bp upstream of the ATG. Base pair changes resulting in changes in conserved residues of the TCP domain of *brc1-3* and *brc1-4* are indicated. Residues conserved in class II TCP proteins are represented by black boxes.

(D) Nuclear localization of *BRC1*. Top, bright-field image of a transgenic *Arabidopsis* root cell expressing $Pro_{CaMV35S}$:*GFP:BRC1*. Center, UV light view of the same cell; GFP:BRC1 protein accumulates in the nucleus. Bottom, merged image of **(A)** and **(B)**. Plants carrying nonfused GFP do not accumulate the protein in their nuclei (data not shown).

(E) *BRC1* (top) and *BRC2* (bottom) mRNA levels in different tissues analyzed by real-time PCR. Error bars represent SE from three biological replicates. The sample labeled Leaf bases+stem contains dissected rosette tissue highly enriched in axillary buds.

real-time PCR in different tissues (Figure 1E). Both genes were transcribed at high levels in tissue that mainly contained axillary buds, supporting their putative role in the control of bud development. They were also expressed at lower levels (mainly *BRC2*) in other axillary structures such as flowers and siliques.

To define the spatial and temporal patterns of expression of these genes during bud development in more detail, *BRC* mRNAs were detected by in situ hybridization. *BRC1* and *BRC2* expression patterns were dynamic and similar, although *BRC1* expression was much stronger than *BRC2* expression, which was barely detectable in our experiments. Before flowering, when AMs were not yet initiated, *BRC1* and *BRC2* transcripts were not detectable (data not shown). After flowering, AMs became visible in the axils of leaves (Figure 2A) and *BRC1* transcripts accumulated in all cell layers of these meristems (Figure 2D). During bud vegetative development (Figure 2B), *BRC1* was downregulated in the outer layers of the meristem (Figure 2E) and transcripts accumulated in young leaf primordia (Figure 2F). Older expanding leaves did not express *BRC1* (Figure 2G). In buds bearing flowers (Figure 2C), *BRC1* transcripts were detectable in the provascular tissue underlying the bud (Figure 2G). *BRC1* expression was downregulated at the time of bud outgrowth, so mRNA was not detectable in buds showing the first signs of shoot elongation (stem < 0.1 mm; data not shown). *BRC1* expression appeared to be highest in rosette leaf buds that remained arrested for long periods of time and lowest in cauline leaf buds that grew out immediately (Figure 2I). Therefore, *BRC1* is expressed locally in axillary buds in an evolving pattern during bud development and is downregulated at the time of branch elongation. *BRC2* expression levels were much lower and were only clearly detectable in the provascular tissue of buds that had undergone flowering (Figure 2H). Neither gene was expressed in the SAM or in floral or leaf primordia derived from the SAM.

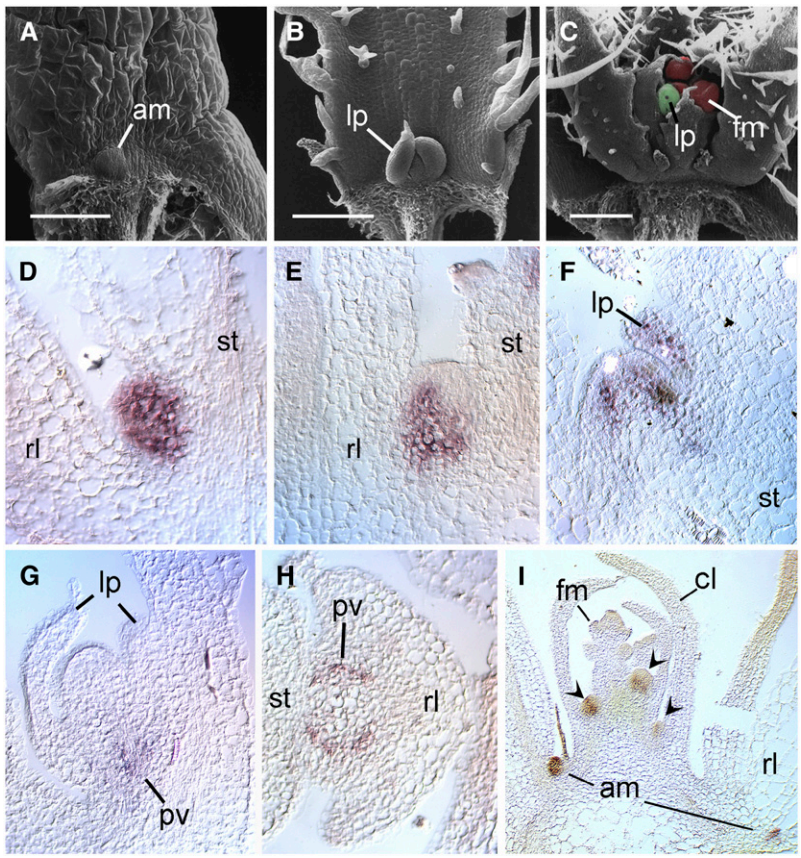

Figure 2. *BRC* Gene Expression during Bud Development.

(A) Scanning electron microscopy image of an AM (meristem stage).
(B) Scanning electron microscopy image of a bud of vegetative 1 stage.
(C) Scanning electron microscopy image of a flowering bud (reproductive stage). Leaf primordia (green) and flower buds (red) are highlighted for clarity.
(D) to **(I)** Sections of *Arabidopsis* rosettes hybridized with digoxigenin-labeled probes complementary to *BRC1* (**[D]** to **[G]** and **[I]**) or *BRC2* **(H)** transcripts.
(D) Detail of an AM comparable to that shown in **(A)**.
(E) Detail of an AM beginning to initiate leaf primordia.
(F) Bud of vegetative 1 stage similar to that shown in **(B)**.
(G) Reproductive stage bud similar to that shown in **(C)**.
(H) *BRC2* mRNA accumulates in the developing vascular tissue of flowering buds.
(I) General view of *BRC1* mRNA distribution in a flowering rosette.
(D) to **(G)** and **(I)** are longitudinal sections, and **(H)** is a transverse section. am, axillary meristem; cl, cauline leaf; fm, flower meristem; lp, leaf primordium; pv, provascular tissue; rl, rosette leaf; st, stem. Bars = 200 μm.

BRC1 and *BRC2* Prevent Axillary Bud Outgrowth

To investigate the function of *BRC1* and *BRC2* in buds, the phenotype of plants with reduced function of these genes was analyzed. RNA interference (RNAi) lines were generated, and mutant collections were screened for insertions and point mutations affecting transcribed regions of *BRC1* and *BRC2* (Figures 1B and 1C; see Supplemental Table 2 online). For *BRC1*, 12 independent RNAi lines, 3 insertional lines, and 2 point mutant lines were analyzed (Figure 1B). *brc1-1*, *brc1-2*, and *brc1-5* carried T-DNA insertions (Alonso et al., 2003; Rosso et al., 2003) located 218 bp downstream of the ATG (predicting a truncated protein of 72 amino acids), within the R domain (giving a protein of 208 residues lacking the R domain), and at the 5′ untranslated regions of the gene, respectively. *brc1-3* and *brc1-4* carried ethyl methanesulfonate–generated point mutations (Till et al., 2003) causing amino acid changes in conserved residues of the TCP domain. For *BRC2*, eight independent RNAi lines and one insertional line (Alonso et al., 2003) were studied (Figure 1C). *brc2-1* predicted a truncated protein of 208 residues lacking the R domain. The double mutant *brc1-2 brc2-1* was also analyzed.

Three weeks after flowering, *brc1* mutants had a significantly higher number of rosette branches (RI and RII) than wild-type plants (Figures 3A, 3C, and 3D; see Supplemental Table 3 online). The phenotype of *brc2* mutants was weaker but consistently affected RI and RII (Figures 3C and 3D; see Supplemental Table 3

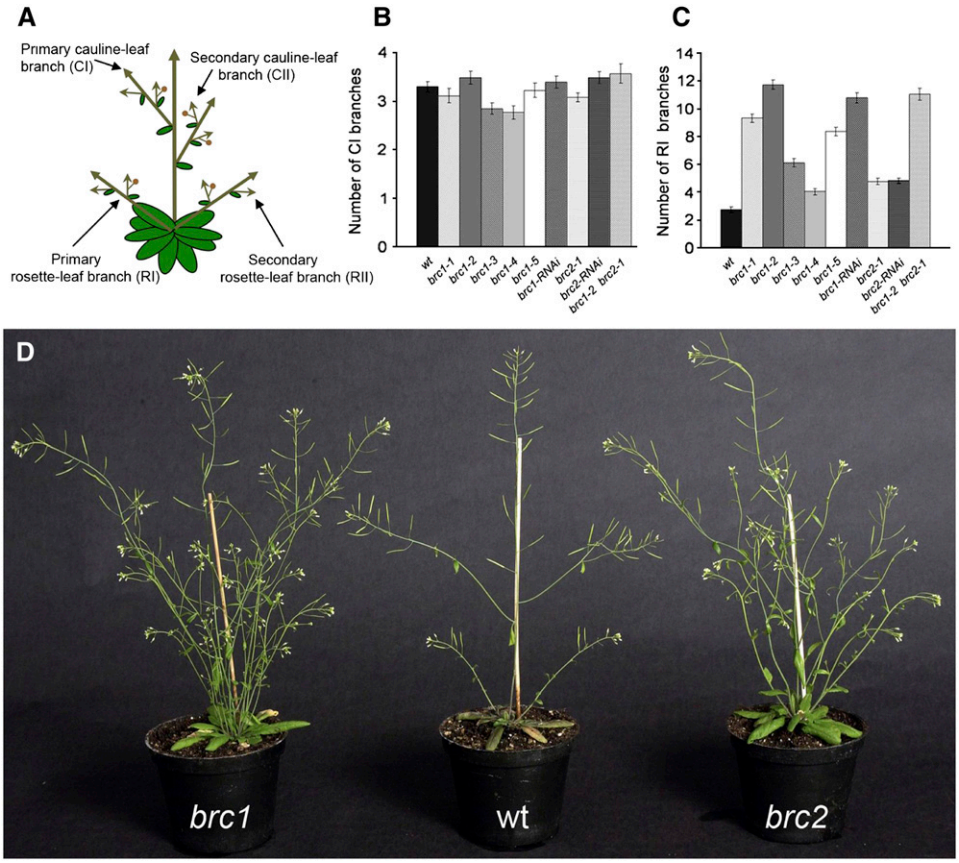

Figure 3. Shoot Branching Phenotype of *brc* Mutants.

(A) *Arabidopsis* branching structure.
(B) Number of primary cauline branches (CI).
(C) Number of primary rosette branches (RI). For **(B)** and **(C)**, one representative RNAi line for each gene was included. Error bars represent SE ($n = 26$ to 27).
(D) Shoot phenotype of mature *brc1-2*, wild-type Columbia, and *brc2-1* plants.

online). Double mutants *brc1-2 brc2-1* had a phenotype similar to that of strong *brc1* mutants (Figure 3C; see Supplemental Table 3 online). The increase in rosette branches of *brc* mutants was not attributable to an increased number of vegetative nodes (see Supplemental Table 3 online) but to an increased frequency of bud outgrowth: for instance, in wild-type individuals, <40% of the RI buds grew out, whereas in *brc1* mutants, almost every RI bud grew. On the other hand, *brc1*, *brc2*, and *brc1 brc2* plants had a similar number of primary and secondary cauline leaf branches (CI and CII) as the wild type (Figures 3A and 3B; see Supplemental Table 3 online). These results indicate that *BRC* genes prevent rosette branch outgrowth.

BRC1 Delays Early Axillary Bud Development

As *BRC1* was expressed at higher levels and had a stronger mutant phenotype than *BRC2*, we focused mainly on this gene for further studies. The phenotype of *brc1* mutants was investigated during early bud development. Axillary buds formed at identical leaf positions (L1 = first-formed leaf; L2 = second-formed leaf, etc.) were compared in *brc1-2* and wild-type plants just before flowering of the main shoot at 14 d after germination (Figure 4) and soon before bolting at 25 d after germination (Figure 5). To visualize AM initiation, these lines were studied in a Pro_{CLV3}:*GUS* background (Brand et al., 2002) that allows the identification of shoot and flower meristems by GUS staining (Figures 4A and 4C). Under long days, AMs are initiated only after flowering, in a basipetal order (Hempel and Feldman, 1994). Consistently, in wild-type plants, vegetative rosettes did not reveal any sign of AM initiation (data not shown). By contrast, 14-d-old vegetative rosettes of *brc1-2* Pro_{CLV3}:*GUS* plants had AMs formed in the axils of cotyledons (c1 and c2) and L2 to L5 (Figures 4B and 4D). This finding indicates that *BRC1* prevents AM initiation.

At 25 d after germination, the SAM of wild-type and mutant plants had undergone flowering. In the wild type, buds nearest to the apex (i.e., L12 buds) were more advanced in development than buds farther from the apex (i.e., L1 buds), so that a gradient of developmental stages was found along the nodes: c1 and c2 never had axillary buds, newly formed meristems or empty axils were found in leaves L1 and L2, leaves L3 to L9 had buds in the

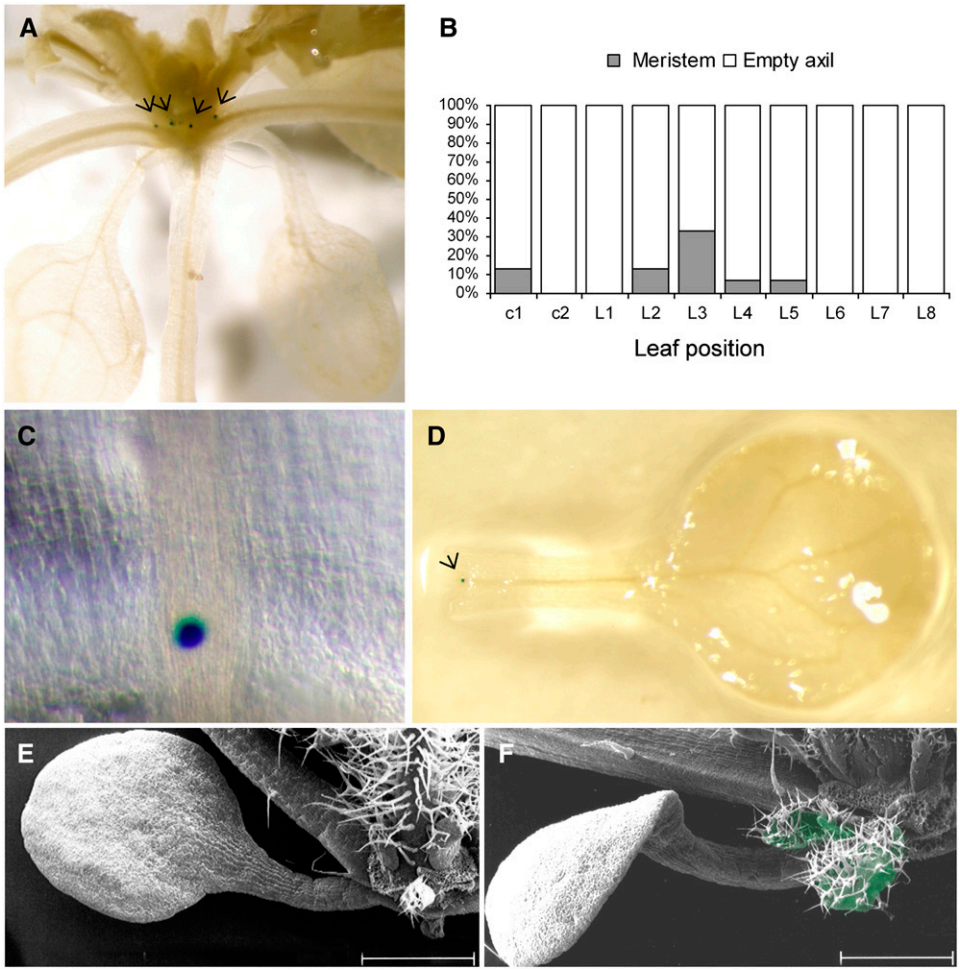

Figure 4. AM Initiation in *brc1* Mutants.

(A) Flowering Pro_{CLV3}:*GUS* rosette stained to visualize GUS activity. Arrows indicate AMs.
(B) Percentage of *brc1-2* CLV3:GUS individuals with GUS-expressing AMs in different leaf positions at 15 d after germination ($n = 16$). All rosettes were vegetative.
(C) Close-up of an AM expressing GUS.
(D) AM in the axil of a cotyledon of a vegetative *brc1-2* plant.
(E) Empty axil of a wild-type cotyledon.
(F) Bud (green) in the axil of a *brc1-2* cotyledon. Bars in **(E)** and **(F)** = 1 mm.

early mid vegetative stage, buds in L10 were mostly in late vegetative stages, and a few flowering buds were found in leaves L11 and L12 (Figures 4E, 5A, 5B, and 5D). In *brc1-2* mutants, the gradient of developmental stages was not so obvious: some c1 and c2 had vegetative or flowering buds, most L1 to L6 had buds in the late vegetative stage, and a large fraction of L7 to L12 had flowering buds (Figures 4F, 5A, 5C, and 5D). Mutant buds were not early flowering (they had a wild-type number of vegetative nodes); therefore, this effect should be attributable to rapid vegetative development. Moreover, leaves of the axillary buds grew faster and were larger than wild-type leaves (Figures 5B and 5C). This effect was more dramatic in plants grown under short days, in which wild-type and mutant AMs were initiated before flowering and bud development was prolonged for many weeks (Figure 5D).

Together, these results indicate that *BRC1* retards all stages of bud development: first, it prevents vegetative AM initiation under long days and AM initiation in cotyledons; second, it delays the progression of bud vegetative development and prevents leaf bud growth and/or expansion; and third, it suppresses lateral shoot elongation. *brc* mutants were not affected in any other developmental trait, indicating that *BRC* genes acted exclusively in axillary buds or that their function was redundant in other developmental pathways.

BRC1 Is Strongly Downregulated in *max* Mutants

The relation of *BRC1* to the genetic pathways controlling axillary bud development was studied. *BRC1* (and *BRC2*) mRNA levels were analyzed in lines with altered AM initiation (*las* and *rev/ifl1*),

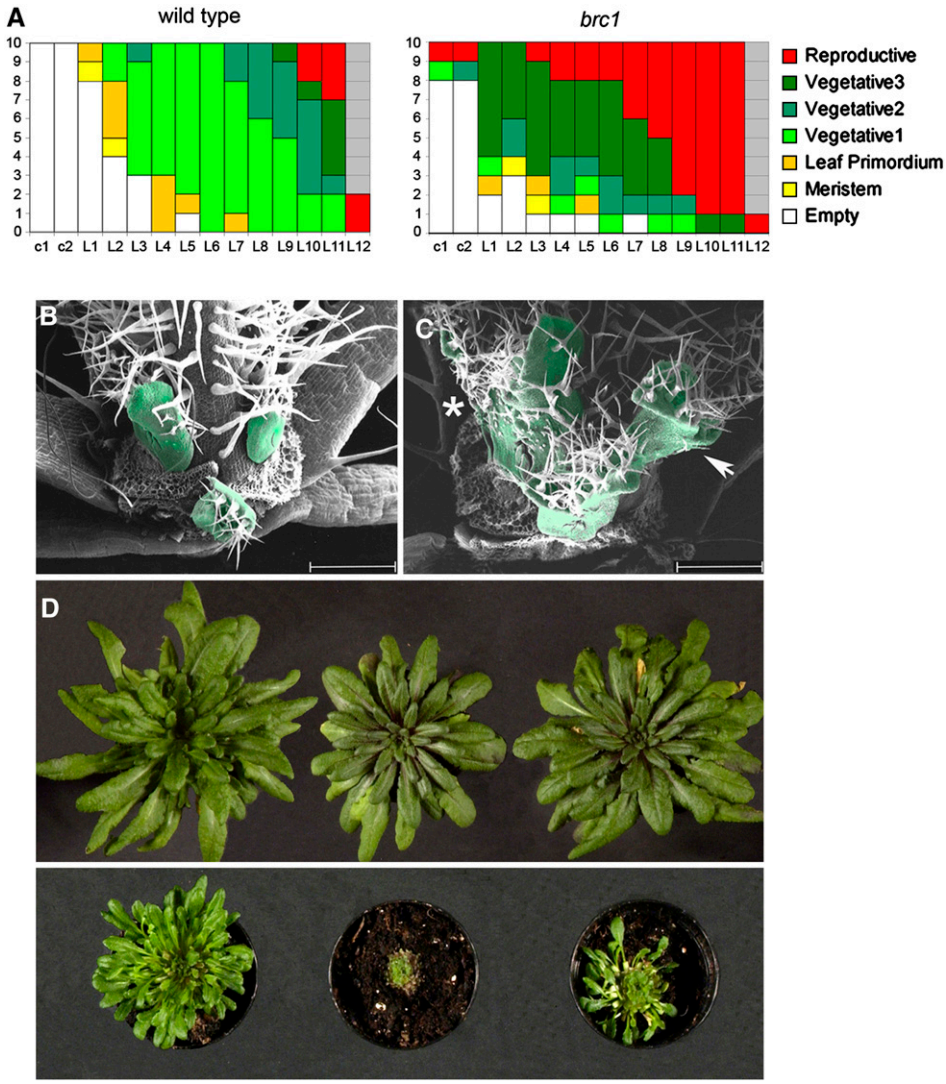

Figure 5. Early Bud Development in *brc* Mutants.

(A) Developmental stages of buds in the axils of cotyledons (c1 and c2) and rosette leaves (L1 to L12) of 10 wild-type (left) and *brc1-2* (right) individuals. Developmental stages are defined in Methods: empty axil (white), meristem (yellow), leaf primordia (orange), vegetative 1 (light green), vegetative 2 (medium green), vegetative 3 (dark green), and reproductive (red).

(B) Wild-type buds in the axils of the youngest rosette leaves (removed) in vegetative 2 stage.

(C) *brc1* buds in the axils of the youngest rosette leaves in vegetative 3 (arrow) and reproductive (asterisk) stages. In **(B)** and **(C)**, the main shoot is <1 mm long, and axillary buds are highlighted in green for clarity. Bars = 500 μm.

(D) Top, vegetative rosettes of plants grown for 50 short days viewed from above. From left to right, *brc1-2*, wild-type Columbia, and *brc2-1*. Bottom, the same plants after removing all of the rosette leaves to display the axillary bud leaves.

bud outgrowth (*ycc1*, *axr1*, and *max1* to *max4*), or both (*amp1*). Moreover, double mutants were obtained between *brc1* mutants and these lines, and their phenotypes were studied. In the mutants *las* (Greb et al., 2003) and *ifl1* (Talbert et al., 1995; Otsuga et al., 2001; Zhong and Ye, 2001), both affected in AM initiation, *BRC1* and *BRC2* levels were reduced, as would be expected if fewer buds were formed compared with the wild type (Figures 6A and 6B). Moreover, *las* and *ifl1* mutations were epistatic to *brc1* (Figures 6C and 6D; see Supplemental Table 4 online), suggesting that *LAS* and *REV/IFL1* are necessary during AM initiation before *BRC1*.

The auxin:cytokinin ratio is a strong determinant of the degree of lateral shoot outgrowth (Sachs and Thimann, 1967; Chatfield et al., 2000). Auxin promotes bud arrest, and cytokinin promotes AM development and shoot outgrowth (Sachs and Thimann, 1964; Turnbull et al., 1997). The *amp1* mutant, for instance, with increased cytokinin levels (Helliwell et al., 2001), has more AMs initiated and more buds that grow out to give a branch

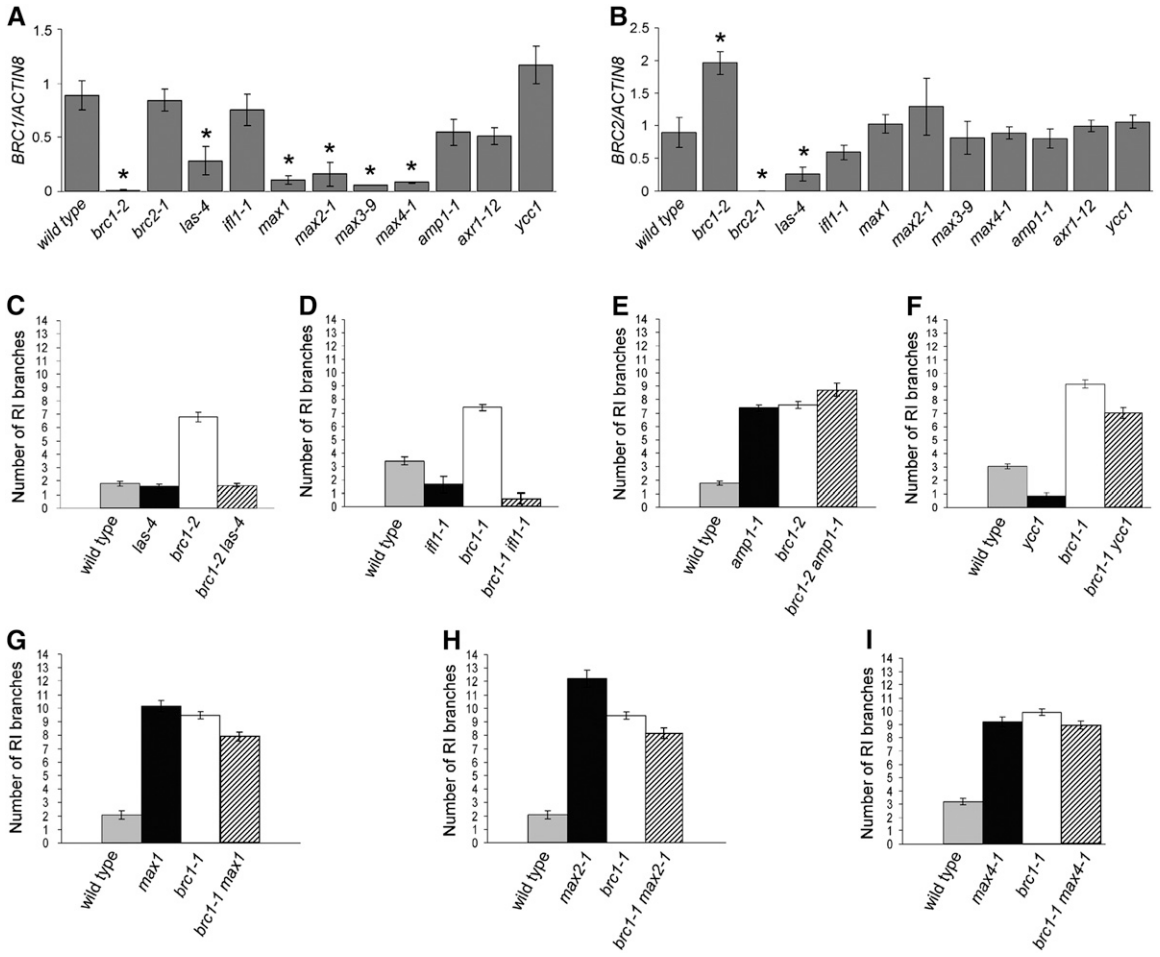

Figure 6. *BRC* Genes and Genetic Pathways of AM Development.

BRC1 **(A)** and *BRC2* **(B)** mRNA levels in different mutant backgrounds analyzed by real-time PCR. Error bars represent the SE from three biological replicate experiments. Differences with respect to the wild type that were found to be significant in a Newman–Keuls test are indicated with asterisks. The other panels show the number of RI branches of double mutants of *brc1* with *las4* **(C)**, *ifl1* **(D)**, *amp1* **(E)**, *ycc1* **(F)**, *max1* **(G)**, *max2* **(H)**, and *max4* **(I)**.

than wild-type plants (Figure 6E; see Supplemental Table 4 online). In *amp1* mutants, *BRC1* levels were reduced slightly (Figure 6A), which could reflect a negative regulation of *BRC1* by cytokinins or simply an effect of more buds elongating at this stage compared with the wild type. *amp1 brc1* double mutants had a higher number of RI branches than the parental lines (Figure 6E), possibly reflecting an additive effect of the extra AMs formed in the *amp1* mutants and the increased outgrowth caused by *brc1*.

The auxin-overproducer *ycc1* mutants (Zhao et al., 2001) had most rosette leaf buds arrested (Figure 6F; see Supplemental Figure 2 and Supplemental Table 4 online), but *BRC1* and *BRC2* mRNA levels were not altered significantly (Figures 6A and 6B). However, *brc1* mutations mostly suppressed the strong apical dominance phenotype of *ycc1* (Figure 6F; see Supplemental Figure 2 online). This finding indicates that loss of *brc1* function can to a great extent overcome the bud arrest caused by an excess of auxin activity. Therefore, although auxin does not seem to control *BRC1* transcriptionally, *BRC1* activity is necessary for the auxin-induced control of apical dominance.

MAX genes promote the synthesis and activity of a carotenoid derivative (Booker et al., 2005) that has been proposed to reduce auxin transport capacity in the stem, thus preventing auxin export from the buds and blocking bud outgrowth (Bennett et al., 2006). The four *max* mutants have an excess of branch outgrowth (Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004). In these mutants, *BRC1* was downregulated much more strongly than in other branching mutants, such as *amp1* and *axr1* (Figure 6A). In addition, the phenotype of *max brc1* double mutants is similar to those of the single *max* and *brc1* mutant parents (Figures 6G to 6I; see Supplemental Table 4 online), indicating that *MAX* and *BRC1* may act in the same pathway and that the *MAX* effect on branching could be attributable mostly to transcriptional control of *BRC1*.

Finally, an upregulation of *BRC2* was observed in *brc1* mutants, which may reflect a negative feedback mechanism to

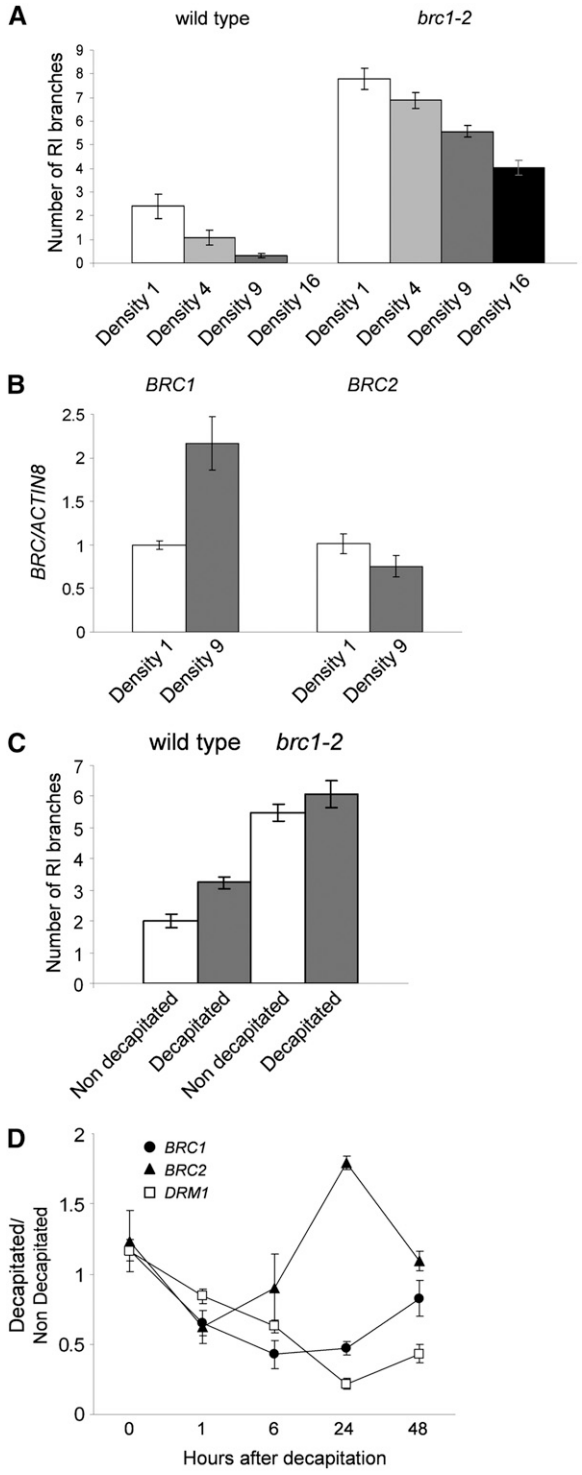

Figure 7. Response of *BRC* Genes to Branch-Suppressing or Branch-Promoting Stimuli.

(A) Number of RI branches of wild-type and *brc1-2* plants grown at different planting densities analyzed at 3 weeks after flowering. Density 1 indicates one plant/pot of 36 cm^2; density 4 indicates four plants/pot; density 9 indicates nine plants/pot; and density 16 indicates 16 plants/pot. All pots were 6 cm deep. Flowering time was not affected. Error bars represent SE ($n = 10$ to 42).

(B) *BRC1* and *BRC2* mRNA levels analyzed by real-time PCR at density 9 related to levels at density 1. Error bars represent the SE from three biological replicate experiments.

(C) Number of RI branches of wild-type Columbia and *brc1-2* plants at 10 d after removal of the main shoot. Error bars represent SE ($n = 14$). Values were subjected to Student's *t* test. Wild-type differences were significant ($P < 0.0003$), whereas *brc1-2* differences were not significant ($P < 0.2782$).

(D) Ratio of mRNA levels between decapitated and nondecapitated plants of *BRC1*, *BRC2*, and *DRM1*, as analyzed by real-time PCR. Error bars represent the SE from four biological replicate experiments.

compensate for the loss of *BRC1* function (Figure 6B). The reverse (*BRC1* upregulation in *brc2* mutants) was not observed.

Together, these results suggest that during AM initiation, *BRC1* acts after *LAS* and *IFL1*. During bud development, auxin-induced apical dominance requires the activity of *BRC1*, and the *MAX*-mediated pathway controls *BRC1* expression. Cytokinins act in an antagonistic pathway independent of *BRC1*.

BRC1 Responds to Signals Controlling Bud Dormancy

The central role of *BRC1* in the control of bud outgrowth raised the possibility that this gene acts as a local switch of axillary bud growth, integrating the responses to different stimuli that control bud dormancy. If that is the case, changes in those stimuli should affect *BRC* mRNA levels or protein activity. To test this hypothesis, *BRC1* and *BRC2* transcript levels were analyzed in plants grown under different environmental and developmental conditions that affected bud arrest.

Planting density is an environmental factor that affects branch outgrowth in many plant species. Plants grown at low density, for example, have more branches than plants grown in crowded conditions as a result of a neighbor-sensing response (Casal et al., 1986). To test whether this is true in *Arabidopsis*, wild-type and *brc1* plants were sown at increasingly higher densities (1, 4, 9, and 16 plants per pot of 36 cm^2) and RI branches were counted at maturity (Figure 7A). Wild-type plants responded to increased planting density with reduced branching such that, at a density of nine plants per pot, branch suppression was almost complete (86% reduction in branch number with respect to plants grown at one plant per pot). By contrast, *brc1* mutants were partly insensitive to this condition (28% reduction with respect to plants at one plant per pot). *brc2* mutants behaved like wild-type plants, and *brc1 brc2* double mutants behaved like *brc1* mutants (data not shown). The levels of *BRC1* and *BRC2* mRNA were then compared in wild-type plants grown at low (one plant per pot) and high (nine plants per pot) density. At high density, *BRC1* mRNA levels were more than double those at low density, whereas *BRC2* levels were similar in both conditions (Figure 7B). These results indicate that the environmentally induced bud dormancy observed in plants grown at high density was partly mediated through transcriptional regulation of *BRC1* but not of *BRC2*.

Apical dominance is the inhibitory effect caused by an actively growing primary shoot apex on lateral shoot outgrowth (Cline, 1991, 1997). Decapitation is a classical assay to study bud reactivation after release from apical dominance (Sachs and Thimann,

1964; Hall and Hillman, 1975; Napoli et al., 1999; Beveridge et al., 2000; Cline, 2000; Tatematsu et al., 2005). In *Arabidopsis*, when the main shoot was removed, one axillary bud elongated prematurely. By contrast, no significant effect of decapitation was detected in *brc1* mutants (Figure 7C). To analyze whether this response correlated with a downregulation of *BRC* genes, *BRC* mRNA levels were analyzed soon after decapitation, before any visible sign of bud outgrowth (Figure 7D). *BRC1* was downregulated significantly in decapitated plants at 1 h after decapitation, reached a minimum at 6 h, and only approached predecapitation levels at 48 h. Downregulation of *DRM1*, an early marker for bud dormancy (Stafstrom et al., 1998; Tatematsu et al., 2005), was delayed with respect to *BRC1* and reached its minimum levels at 24 h after decapitation. *BRC2* was downregulated at 1 h after decapitation but recovered quickly, and at 24 h it was upregulated, possibly as a result of the reduced *BRC1* function (see above). These results suggest that *BRC1* downregulation is an early response to the bud release from apical dominance and is necessary for bud activation.

Together, these results indicate that *BRC1* is transcriptionally regulated by environmental (planting density) and endogenous (apical dominance) stimuli controlling bud dormancy and that this regulation is necessary for the bud response to these signals.

DISCUSSION

We have shown that *BRC1* acts inside developing buds to promote growth arrest. *BRC1* upregulation (i.e., at high planting density) leads to an increase in branch suppression, and *BRC1* negative regulation causes bud outgrowth. Moreover, environmental and developmental stimuli can modulate *BRC1* transcription, and *BRC1* function is necessary for the proper response to these stimuli. This indicates that *BRC1* represents a key point at which signals controlling branching are integrated within axillary buds (Figure 8), allowing plants to tailor their degree of shoot outgrowth to changing conditions. *BRC2*, a closely related gene, seems to play a minor role in this process.

BRC1 Promotes Bud Development Arrest

BRC1 is expressed throughout axillary bud development in different regions of the bud, where it seems to promote growth arrest. Downregulation of *BRC1* leads to a relief of repression that allows the buds to continue their development and generate a branch. *BRC1* may act (downstream of *LAS* and *IFL1*) to antagonize meristem organization or activity (i.e., maintenance of the stem cell niche, cell division, and lateral organ initiation) as the loss of *brc1* function accelerates AM initiation and leads to ectopic AM formation. This would be consistent with the observed downregulation of *BRC1* at the meristem dome before leaf initiation. *BRC1* also controls early stages of bud leaf development, a function reminiscent of that of *CIN*-like genes closely related to *BRC1* (Nath et al., 2003; Palatnik et al., 2003; Crawford et al., 2004). The late expression of *BRC* genes in the provascular tissue underlying mature buds may be necessary to prevent rosette branch outgrowth. This expression may arrest vascular tissue development, isolate buds from shoot growth-promoting signals, or prevent auxin export from the bud. *BRC1* downregulation leads to a relief of growth repression and to lateral shoot outgrowth.

BRC1 Function and Hormone Signaling

Shoot branching is inhibited by hormonal signals that move through the plant. Auxin, moving down the plant in the main stem, and a MAX-dependent carotenoid hormone, moving up the plant, prevent bud outgrowth. Auxin is thought to prevent branching by reducing cytokinin synthesis and import into the bud through an *AXR1*-dependent pathway (Sachs and Thimann, 1967; Li et al., 1995; Chatfield et al., 2000; Leyser, 2003; Nordstrom et al., 2004; Tanaka et al., 2006). Our results suggest

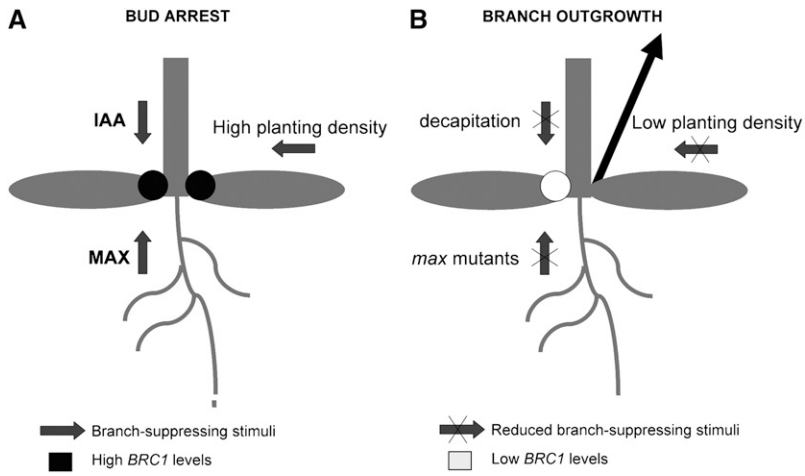

Figure 8. Scheme of *BRC1* Function in the Control of Bud Outgrowth.

Under adverse conditions, branch-suppressing signals are transduced into the bud, resulting in the upregulation of *BRC1* and bud arrest **(A)**. In the absence of these signals, *BRC1* is downregulated and shoots grow out **(B)**. IAA, indole-3-acetic acid.

that *BRC1* is independent of this pathway, as high auxin levels (*ycc1* mutations), *axr1* mutations, or high cytokinin levels (*amp1* mutations) do not affect *BRC1* transcription. Still, we cannot rule out the possibility that this pathway affects BRC1 protein stability. On the other hand, *BRC1* is strongly downregulated in *max* mutants, suggesting that *BRC1* may be downstream of the *MAX* signaling pathway. It has been proposed that the *MAX*-dependent hormone controls shoot branching through a mechanism independent of *AXR1*-mediated auxin signaling, by limiting auxin transport in the main stem (Bennett et al., 2006). As this carotenoid-derived compound acts (is required and sufficient) outside the buds (Turnbull et al., 2002), its control of *BRC1* transcription must be indirect. The direct transcriptional regulators of *BRC1* remain to be identified.

BRC1 Is an Integrator of Signals Controlling Bud Growth Arrest

BRC1 (and to a lesser extent *BRC2*) is, to date, the only gene described in *Arabidopsis* that functions locally within the bud to prevent bud outgrowth and whose downregulation is necessary to allow branches to develop. The central role of *BRC1* in this process raised the possibility that this gene could integrate different pathways controlling branching. Our results confirm that *BRC1* is transcriptionally controlled by both endogenous (apical dominance) and environmental (planting density) stimuli affecting bud dormancy and that *BRC1* function is necessary for proper bud response to both stimuli. Moreover, in the case of decapitation, we have shown that those changes occur very fast (<1 h after decapitation), earlier than changes in markers of bud dormancy such as *DRM1*. This indicates that *BRC1* is an integrator of signaling pathways controlling bud dormancy.

This mechanism of branching control, in which external and internal inputs perceived in different regions of the plant are transduced into the axils of leaves and are translated into local changes of *BRC1* activity, is reminiscent of another key developmental process, the flowering transition (Ausin et al., 2005). During the control of flowering, several genetic pathways, mediated by signals transported through the plant, converge in the activation (transcriptional or posttranslational, respectively) of the integrator genes *SOC1* and *FT* at the shoot apex (Blazquez, 2005; Parcy, 2005), which in turn set off the developmental program of flower initiation. In the case of branching, the integrating response, the promotion/relief of cell proliferation arrest, would depend on the activity of *BRC1*, controlled by the *MAX* signaling pathway.

Conservation of *tb1*/*BRC* Function among Angiosperms

BRC1 is closely related to the maize gene *tb1* in sequence (Howarth and Donoghue, 2006), expression patterns, and mutant phenotypes. However, similarities between *tb1* and *BRC1* are not limited to their shared role in preventing branch outgrowth. *tb1*, like *BRC1*, is expressed in axillary buds as early as AM initiation. It is also expressed in developing husk leaves (the structures homologous with axillary bud leaves), where it suppresses husk leaf blade growth (*tb1* mutants have very long husk leaves) (Hubbard et al., 2002). This function resembles that of *CIN*-like genes that control leaf shape and leaf growth patterns (Nath et al., 2003; Palatnik et al., 2003; Crawford et al., 2004). This similarity may reflect the common evolutionary origin of class II genes and a conservation of regulatory elements and functions. On the other hand, neither *tb1* nor *BRC1* affects branch node number, and neither is transcribed in the main SAM. However, some differences are also evident. Unlike *BRC1*, *tb1* controls branch internode elongation and seems to have a role during maize inflorescence and flower development, functions for which we have not found equivalents in *BRC1*.

Conservation among species has already been described for other genes involved in branching. AM initiation is controlled by *Ls*/*LAS*/*MONOCULM1* (Schumacher et al., 1999; Greb et al., 2003; Li et al., 2003) and *Blind*/*RAX1* (Schmitz et al., 2002; Keller et al., 2006; Muller et al., 2006) in tomato (*Solanum lycopersicum*), *Arabidopsis*, and rice. Bud outgrowth is controlled by *MAX2*/*RMS4*/*D3* (Stirnberg et al., 2002; Ishikawa et al., 2005; Johnson et al., 2006), *MAX3*/*RMS5*/*HTD1* (Booker et al., 2004; Beveridge, 2006; Johnson et al., 2006; Zou et al., 2006), and *MAX4*/*RMS1*/*DAD1* (Sorefan et al., 2003; Snowden et al., 2005) in *Arabidopsis*, pea (*Pisum sativum*), petunia (*Petunia hybrida*), and rice. The newly found functional conservation of *tb1*/*BRC* function between monocots and dicots suggests that the control of axillary bud development, from long-distance signaling to local responses during AM initiation, bud development, bud dormancy, and branch outgrowth, may be controlled by a conserved set of genetic functions throughout angiosperms that may correspond to an ancestral developmental pathway evolved before the radiation of flowering plants. Modulation of the process may be divergent in different species, as revealed by the differential regulation of *MAX*-like genes in pea, *Arabidopsis*, and rice (Beveridge, 2006; Johnson et al., 2006). It remains to be studied whether this conservation extends to more distantly related plant groups.

METHODS

Plant Material

Mutant lines of *Arabidopsis thaliana*, in the Columbia background, were backcrossed twice to wild-type Columbia (*brc1-1*, *brc1-2*, *brc1-5*, and *brc2-1*) or three times (*brc1-3* and *brc1-4*). To confirm the site of T-DNA insertion, genomic DNA of *brc1-1*, *brc1-2*, *brc1-5*, and *brc2-1* was PCR-amplified with primers AB, CD, EF, and GH, respectively (see Supplemental Table 5 online), and the PCR products were sequenced.

Phenotypic Analysis

Arabidopsis seeds were sown on commercial soil and cold-treated (4°C) for 3 d. Then, they were transferred to a growth room at 20°C with a 16-h photoperiod (long days) or an 8-h photoperiod (short days). Branches (shoots > 0.5 cm) were counted 3 weeks after the time when the main inflorescence was visible, except for in the decapitation assay, in which branches were counted at 10 d after decapitation. For the early phenotype analysis, Pro_{CLV3}:*GUS* and *brc1-2* Pro_{CLV3}:*GUS* plants were grown for 14 or 25 d. Fourteen days after germination, 10 plants for each genotype were GUS-stained according to Sessions et al. (1999). Twenty-five days after germination, 10 individuals of each genotype were dissected and the developmental stage of each axillary bud was determined with a stereoscopic microscope. Stages were defined as empty axil: no visible meristem; meristem: meristem with no visible leaf primordia, ~100 μm

(Figure 2A); leaf primordium: incipient first two leaf primordia, bud of ~100 μm; vegetative 1: buds with two or more leaf primordia formed, no trichomes, 150 to 250 μm (Figure 2B); vegetative 2: mid vegetative stage, buds with differentiating trichome-bearing leaf primordia, <400 μm (Figure 5B); vegetative 3: late vegetative stage, buds with expanding trichome-bearing leaf primordia, >400 μm (Figure 5C); and reproductive: flower meristems visible within the bud (Figure 2C).

Phylogenetic Analysis and Sequence Alignment

The predicted amino acid sequences of the TCP and R domains were aligned with ClustalW (Chenna et al., 2003) using the default parameters (Protein Gap Open Penalty = 10.0, Protein Gap Extension Penalty = 0.2, Protein Matrix = Gonnet Protein/DNA, ENDGAP = −1, Protein/DNA GAPDIST = 4) and represented with Genedoc (Nicholas et al., 1997). One thousand bootstrapped data sets were obtained with SEQBOOT, distance matrices were calculated with PROTDIST (Dayhoff PAM matrix algorithm), trees were constructed with NEIGHBOR, and a consensus tree was obtained with CONSENSE. SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSE are from the PHYLIP package (Felsenstein, 1988). Branches with support of ≥70% are indicated.

cDNA Isolation

RNA from dissected flowering rosettes comprising axillary buds but not rosette leaves was obtained with TRIzol (Invitrogen). cDNAs of *BRC1* and *BRC2* were isolated using the BD SMART RACE cDNA amplification kit (Clontech) according to the manufacturer's instructions. For 5′ rapid amplification of cDNA ends (RACE), PCR was performed with a primer anchored to the modified 5′ end and the nested gene-specific primers BRC1-A and BRC1-B for *BRC1* and BRC2-A and BRC2-B for *BRC2* (see Supplemental Table 5 online). For 3′ RACE, the PCR was performed with a primer anchored to the 3′ end and the gene-specific primers BRC1-C and BRC1-D and BRC2-C and BRC2-D, respectively (see Supplemental Table 5 online). Products from two independent experiments were cloned in pGEM-T Easy vector (Promega) and sequenced.

Real-Time PCR

Plant tissue was harvested and RNA was isolated with TRIzol (Invitrogen). Traces of DNA were eliminated with TURBO DNA-free (Ambion). Five micrograms of RNA was used to make cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative PCR was performed with FastStart TaqMan Probe Master-Rox (Roche) probes of the Universal ProbeLibrary Set-*Arabidopsis* (Roche) and the Applied Biosystems 7300 real-time PCR system, according to the manufacturer's instructions. The following pairs of primers were used (see Supplemental Table 5 online): for *BRC1*, RT-PCR-BRC1-A/RT-PCR-BRC1-B; for *BRC2*, RT-PCR-BRC2-A/RT-PCR-BRC2-B; for *ACTIN8*, RT-PCR-actin-A/RT-PCR-actin-B; for *DRM1* (At1g28330), DRM1-L/DRM1-R. Three biological replicates were analyzed in each case. C_T values were obtained with the 7300 Systems SDS software version 1.3 (Applied Biosystems). Relative fold expression changes were calculated by the comparative C_T method: fold change is calculated as $2^{-\Delta\Delta CT}$. The ΔC_T values were calculated as the difference between the C_T value and the C_T value of *ACTIN8*. $\Delta\Delta C_T$ was the difference between ΔC_T and the C_T value of the calibrator. In Figure 1E, the calibrator is the leaf sample; in Figures 6A and 6B, the calibrator is wild-type levels; in Figure 7B, density = 1; and in Figure 7D, time = 0.

Planting Density Test

Wild-type Columbia and *brc1-2* plants were grown under long days at low density (one plant per pot) or high density (nine plants per pot). Five rosettes of each genotype were dissected when bolts were 1 cm long. RNA was extracted, and real-time PCR was performed as described. The experiment was repeated three times.

Apical Dominance Test

Wild-type Columbia and *brc1-2* plants were grown under long days. When the main inflorescence began to bolt (<0.5 cm), plants were labeled. Four days later, in half of them, the main shoot, including the cauline nodes, was removed. Seven to 10 decapitated and nondecapitated rosettes were collected at T0 = 0 h, T1 = 1 h, T2 = 6 h, T3 = 24 h, and T5 = 48 h. RNA was extracted as described, and real-time PCR was performed. The experiment was repeated four times.

$Pro_{CaMV35S}$:RNAi Constructs

BRC1- and *BRC2*-specific PCR products (645 and 499 bp, respectively) were cloned into the binary vector pFGC1008 (http://www.chromdb.org) using restriction sites *Asc*I/*Swa*I and *Bam*HI/*Spe*I for the first and second cloning, respectively. Primers TCP18S5′/TCP18S3′ were used for *BRC1* and TCP12S5′/TCP12S3′ were used for *BRC2* (see Supplemental Table 5 online).

$Pro_{CaMV35S}$:GFP:BRC Constructs

The cDNAs of *BRC1* and *BRC2*, cloned in pGEM, were amplified using Pwo polymerase (Roche) with primers 18B1/18B2 (see Supplemental Table 5 online). The PCR fragment was BP cloned into the entry vector pDONR207 (Gateway, Invitrogen) and then LR cloned into the destination vector pGWB6 (from Tsuyoshi Nakagawa, Shimane University).

Arabidopsis Transgenic Plants

Transgenic plants (Columbia ecotype) were generated by agroinfiltration using the floral dip method (Clough and Bent, 1998). T3 homozygous lines generated from T1 individuals carrying a single insertion of the transgene were analyzed.

In Situ Hybridization

Digoxigenin labeling of RNA probes, tissue preparation, and hybridization were performed as described by Calonje et al. (2004). The templates for *BRC1* and *BRC2* digoxigenin-labeled probes were 1.2- and 1-kb linearized fragments containing the complete coding regions. The hybridized sections were visualized with Nomarski optics in a DMR microscope (Leica).

Scanning Electron Microscopy

Rosettes were dissected and prepared for scanning electron microscopy analysis as described by Carmona et al. (2002).

Accession Numbers

The GenBank accession numbers for *BRC1* cDNA and *BRC2* cDNA are AM408560 and AM408561, respectively. Accession numbers for the complete *Arabidopsis* TCP gene family are given in Supplemental Table 1 online.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Arabidopsis Genome Initiative Numbers for the Complete *Arabidopsis* TCP Gene Family.

Supplemental Table 2. Mutant Alleles and RNAi Lines Used in This Work.

Supplemental Table 3. Shoot Branching Phenotypes of *brc* Mutants.

Supplemental Table 4. Shoot Branching Phenotypes of Double Mutants with *brc1*.

Supplemental Table 5. Oligonucleotides Cited in Methods.

Supplemental Figure 1. Alignment of the TCP Domain for the Predicted *Arabidopsis* TCP Proteins.

Supplemental Figure 2. Shoot Branching Phenotypes of *ycc1*, *ycc1 brc1-1*, and *brc1-1* Mutants.

Supplemental Figure 3. Phenotype of $Pro_{CaMV35S}$:*GFP:BRC1* Plants.

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