

Abscisic acid signaling is controlled by a *BRANCHED1/HD-ZIP I* cascade in *Arabidopsis* axillary buds

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Shoot-branching patterns determine key aspects of plant life and are important targets for crop breeding. However, we are still largely ignorant of the genetic networks controlling locally the most important decision during branch development: whether the axillary bud, or branch primordium, grows out to give a lateral shoot or remains dormant. Here we show that, inside the buds, the TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) transcription factor BRANCHED1 (BRC1) binds to and positively regulates the transcription of three related Homeodomain leucine zipper protein (HD-ZIP)-encoding genes: HOMEBOX PROTEIN 21 (HB21), HOMEBOX PROTEIN 40 (HB40), and HOMEBOX PROTEIN 53 (HB53). These three genes, together with BRC1, enhance 9-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (NCED3) expression, lead to abscisic acid accumulation, and trigger hormone response, thus causing suppression of bud development. This TCP/HD-ZIP genetic module seems to be conserved in dicot and monocotyledonous species to prevent branching under light-limiting conditions.

abscisic acid | TCP proteins | HD-ZIP proteins | bud dormancy | *Arabidopsis*

In flowering plants, lateral shoots develop from axillary buds formed at the base of leaves. These buds, comprising a meristem, a few leaf primordia, and sometimes flower meristems, can become quiescent at this stage or can continue their development to form branches. Bud growth arrest, or dormancy, is promoted by various environmental and developmental factors including a canopy shade rich in far-red (FR) light or an actively growing shoot apex. When these stimuli are suppressed, growth can resume, and the bud develops into a shoot.

In *Arabidopsis thaliana*, the class II TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) gene *BRANCHED1* (*BRC1*) functions inside axillary buds (1) to prevent constitutive branch outgrowth, and it mediates bud dormancy induced by FR-rich light or apical dominance. In *brc1* mutants most buds complete their development without restraint and have a reduced response to changes in the red (R):FR light ratio or decapitation. Moreover, *BRC1* mRNA levels are increased within hours of treatment with white light (W) highly supplemented with FR (W+FR) and are decreased shortly after decapitation (1–4). *BRC1*'s inhibitory effect on growth and development is spatially restricted to axillary buds, and it can be turned off by appropriate signals. However, when ectopically expressed in seedlings, *BRC1* also can cause a rapid growth cessation in shoot and root apical meristem and leaf primordia (3). Likewise, generalized overexpression of the *Solanum tuberosum* ortholog, *StBRC1a*, produces dwarf plants with very small leaves and short internodes in potato (5). Despite these remarkable effects on growth and development and their well-known, critical role in the suppression of shoot branching, the downstream pathways by which *BRC1*-like genes promote bud dormancy are still largely unknown.

Transcriptomic data of wild-type and *brc1* *Arabidopsis* axillary buds treated with W+FR light revealed two *BRC1*-dependent gene-regulatory networks (GRNs) down-regulated in response to *BRC1*.

One is enriched in DNA synthesis-, cell cycle-, and cytokinesis-related genes, the other in protein synthesis-related genes (3). Gene promoters of both GRNs have a significant overrepresentation of TCP-binding sites, and therefore it was proposed that *BRC1* represses these GRNs directly, or indirectly by competition with other TCP factors (3). The same study revealed a strong induction of abscisic acid (ABA)-specific marker genes in response to *BRC1* (3), indicating that *BRC1* enhances or maintains ABA signaling in buds. Indeed, ABA accumulation is required for bud growth suppression in wild-type plants: plants bearing mutations in genes involved in ABA synthesis [*9-CIS-EPOXYCAROTENOID DIOXYGENASE3* (*NCED3*) and *ABA DEFICIENT2* (*ABA2*)] display enhanced bud outgrowth (4, 6). Furthermore, a meta-analysis of three transcriptomic studies of active vs. dormant buds revealed that a GRN of ABA-related genes is induced in dormant buds regardless the stimuli involved (7).

Here we have investigated further the relationship between *BRC1* activity and ABA signaling. We have found that *BRC1* directly activates a group of phylogenetically related genes, *HB21* (*HOMEBOX PROTEIN 21*, *At2g18550*), *HB40* (*HOMEBOX PROTEIN 40*, *At4g36740*), and *HB53* (*HOMEBOX PROTEIN 53*, *At5g66700*), encoding class I Homeodomain leucine zipper (HD-ZIP) transcription factors (TFs). These genes are necessary and sufficient for enhanced expression of *NCED3*, a key ABA biosynthesis gene, and for normal ABA accumulation inside axillary buds in conditions of low R:FR or short photoperiods. This

Significance

Shoot-branching patterns affect key aspects of plant life and are important targets for crop breeding. However, we are still ignorant of the genetic mechanisms controlling locally an important decision during branch development: whether the axillary bud grows out to give a lateral shoot or remains dormant. Here we show that the TEOSINTE BRANCHED1, CYCLOIDEA, PCF (TCP) transcriptional regulator BRANCHED1 (BRC1), which acts inside axillary buds, binds and activates three genes encoding Homeodomain leucine zipper (HD-ZIP) transcription factors. These factors, together with BRC1, trigger a cascade leading to local abscisic acid (ABA) accumulation and response, essential for bud dormancy under light-limiting conditions. This finding demonstrates a direct relationship between *BRC1* and ABA signaling and places ABA downstream of *BRC1* in the control of axillary bud dormancy.

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pathway has a strong influence on the expression and maintenance of an ABA-related GRN induced in dormant buds, and is essential for negative regulation of bud development and branch outgrowth under limiting light conditions.

Results

HB21, HB40, and HB53 are BRC1-Dependent Genes. In dormant buds, a GRN of ABA-related genes is induced (7). Because *BRC1* promotes the expression of ABA-marker genes in buds (3), we investigated the relationship between *BRC1* and ABA signaling. To do so, we searched for *BRC1*-dependent genes (defined as genes induced in wild-type but not in *brc1* buds treated with W+FR) (3) among the genes of the ABA-related GRN (*SI Appendix, Fig. S1A and Dataset S1*). We found 26 *BRC1*-dependent genes in the ABA-related GRN (*SI Appendix, Fig. S1B*). This list included 12 genes encoding TFs of the HD-ZIP, MYB, basic leucine zipper (bZIP), AP2, and NAM, ATAF1,2, CUC2 (NAC) families. Because TFs can have a strong influence on transcriptional networks, we hypothesized that some of these genes could play an important role in the local response to ABA in axillary buds. We focused on three closely related HD-ZIP protein-encoding genes: *HB21*, *HB40*, and *HB53* (*SI Appendix, Fig. S2*).

HB21, *HB40*, and *HB53* mRNA levels correlated with *BRC1* levels and bud growth arrest. Like *BRC1*, they were up-regulated in wild-type buds treated with W+FR light (R:FR = 0.2) for 8 h relative to plants treated with W light (R:FR = 11.7). This response was abolished in *brc1* mutants (Fig. 1A). Also, they were down-regulated in active buds 24 h after decapitation (*SI Appendix, Fig. S3A*). When decapitated plants were apically treated with auxin, mRNA levels reverted to those of intact plants (*HB40* and *HB53*) or to higher levels (*HB21*) (*SI Appendix, Fig. S3A*). Sucrose treatments on buds caused down-regulation of *BRC1* (*SI Appendix, Fig. S3B*) as described in pea and rose (8, 9). Likewise, *HB21* and *HB53* (but not *HB40*) mRNA levels decreased after a sugar treatment (*SI Appendix, Fig. S3B*). Then we investigated whether the expression of these genes correlated with *BRC1* activity in tissues other than buds. Estradiol-inducible *BRC1* (*BRC1^{ind}*) seedlings displayed a strong induction of *BRC1* mRNA (Fig. 1B) and accumulation of the *BRC1* protein (*SI Appendix, Fig. S4*) 2–4 h after estradiol application. Likewise, *HB21*, *HB40*, and *HB53* mRNA levels rose 4–6 h after estradiol application in these seedlings (Fig. 1B and Fig. S5).

To test their response to *BRC1* further, we introduced a *LUCIFERASE* (*LUC*) reporter fused to either the promoter (*HBp:LUC*) or the genomic sequence (promoter and coding region with introns, *HBp:HB:LUC*) of each gene into *HA:BRC1^{ind}*; *brc1-2* lines (Fig. 1C). We monitored *LUC* activity in 7-d-old seedlings after estradiol induction of *HA:BRC1*. *LUC* activity increased following treatment in all lines (Fig. 1D). These results indicate that *BRC1* is sufficient to cause up-regulation of *HB21*, *HB40*, and *HB53* not only in axillary buds where *BRC1* is expressed (1) but also in tissues where *BRC1* usually is not expressed, such as seedlings.

HB21, HB40, and HB53 Are Direct BRC1 Targets. To elucidate whether *HB21*, *HB40*, and *HB53* were *BRC1* direct targets, we looked for *BRC1*-binding sites in their genomic regions. We first studied *BRC1* DNA-binding specificity using protein-binding microarray (PBM11) assays (10, 11) by incubating *BRC1* fused to the MALTOSE-BINDING PROTEIN (*BRC1:MBP*) with PBM11 microarrays. The consensus binding motif obtained was GGgcCCmc (Fig. 1E). We used the position weight matrix obtained to search for *BRC1*-binding sites in the *HB21/40/53* genomic regions including 1 kb upstream of the ATG start codon of each gene. We found the *BRC1*-binding motif in all three promoters and introns as well as in some exons (Fig. 1F). These sequences and their locations were partially conserved in closely related Brassicaceae species (*SI Appendix, Figs. S6 and S7*). To verify that *BRC1* bound directly to these

regions, we performed ChIP assays using *GFP:BRC1^{ind}* seedlings (*SI Appendix, Fig. S8*). We tested *BRC1* binding to the six potential *BRC1* regions with the highest scores for the *BRC1* position weight matrix (Fig. 1F, sites 1–6). We compared the immunoprecipitated DNA of estradiol-treated *GFP:BRC1^{ind}* vs. untagged *BRC1^{ind}* lines and found a significant enrichment for sites 1, 2, 4, and 6 (Fig. 1G), indicating that *BRC1* bound directly to these genomic regions in vivo.

HB21, HB40, and HB53 Are Expressed in Axillary Buds. If these three genes are bona fide *BRC1* direct targets, they should be expressed in regions at least partially overlapping with *BRC1* expression domains (1). To investigate whether these regions do overlap, we studied *HB21*, *HB40*, and *HB53* expression patterns in developing axillary buds in more than 10 representative *Arabidopsis* transgenic lines carrying *HBp:β-GLUCURONIDASE* (*HBp:GUS*) transcriptional fusions (with 1-, 1-, and 2-kb regions upstream of the ATG start codon of *HB21*, *HB40*, and *HB53*, respectively). All three gene promoters drove *GUS* expression in young axillary buds in overlapping but not identical patterns (Fig. 2). Expression of the three genes often was associated with provascular and vascular tissues. Expression was detectable from very early stages in the leaf vascular tissue at the position where axillary meristems initiate (Fig. 2A and B), in young axillary meristems (Fig. 2D, K, L, and O), and at the base of young axillary buds (Fig. 2, H–J, N, P, and Q). *GUS* usually was excluded from bud leaf primordia (Fig. 2C and E–H). In older buds the signal became restricted to the base of buds (Fig. 2H–J, N, and Q). These expression patterns overlapped with those described for *BRC1* (1) and are in agreement with a potential transcriptional regulation of these genes by *BRC1*. The *HB40p:GUS* and *HB53p:GUS* lines also showed *GUS* activity in stomata of floral tissues (*SI Appendix, Fig. S9A, B, D, and E*), the *HB40p:GUS* line showed *GUS* activity in pollen grains (*SI Appendix, Fig. S9C*), and the *HB53p:GUS* line showed *GUS* activity in developing lateral roots (*SI Appendix, Fig. S9F–H*).

HB21, HB40, and HB53 Redundantly Repress Shoot Branching. To study the role of these HD-ZIP proteins during axillary bud development, we obtained homozygous transfer-DNA (T-DNA) insertion lines for the three genes (*hb21-1*, *hb40-1*, and *hb53-1* and *hb53-2*) (*SI Appendix, Fig. S10A*). These mutants were predicted to generate truncated proteins lacking a putative AHA activation domain (13) identified in the C-terminal end of each protein. In the case of *hb40-1*, the mutant protein also lacked the HD-ZIP domain. Moreover, insertions disrupted the transcription of each gene but not that of the other two or of *BRC1* (*SI Appendix, Fig. S10B*), suggesting that transcriptional cross-regulation did not take place between these HD-zip genes and that they did not affect *BRC1* expression.

We studied the branching phenotype of single, double, and triple mutants bearing these insertions in W and W+FR light. We grew wild-type and mutant plants in W light and long days until flowering. Then we transferred half of the plants to W+FR light and maintained the other half in W light. Two weeks later, we counted the number of primary rosette branches (RI) of each plant set. As described (3), wild-type plants grown in W light had around three RI, whereas wild-type plants grown in W+FR light had one or two RI (a 40–50% reduction in the number of RI relative to the number in plants grown in W light) (Fig. 3A). The number of RI in single mutants and in *hb21 hb40*, *hb21 hb53-1*, and *hb40 hb53-1* double mutants grown in W light was similar to the number of RI in wild-type plants (Fig. 3A and *SI Appendix, Fig. S11A*). However, *hb21 hb40* and *hb40 hb53-1* double mutants had a reduced response to W+FR light (62 and 61%, respectively) (Fig. 3A), and *hb21 hb40 hb53-1* and *hb21 hb40 hb53-2* triple mutants had an even more reduced response (79 and 90%, respectively) (Fig. 3B). Moreover, unlike wild-type plants, the *hb21 hb40 hb53-2* triple mutant had a similar number of secondary cauline (CII) branches

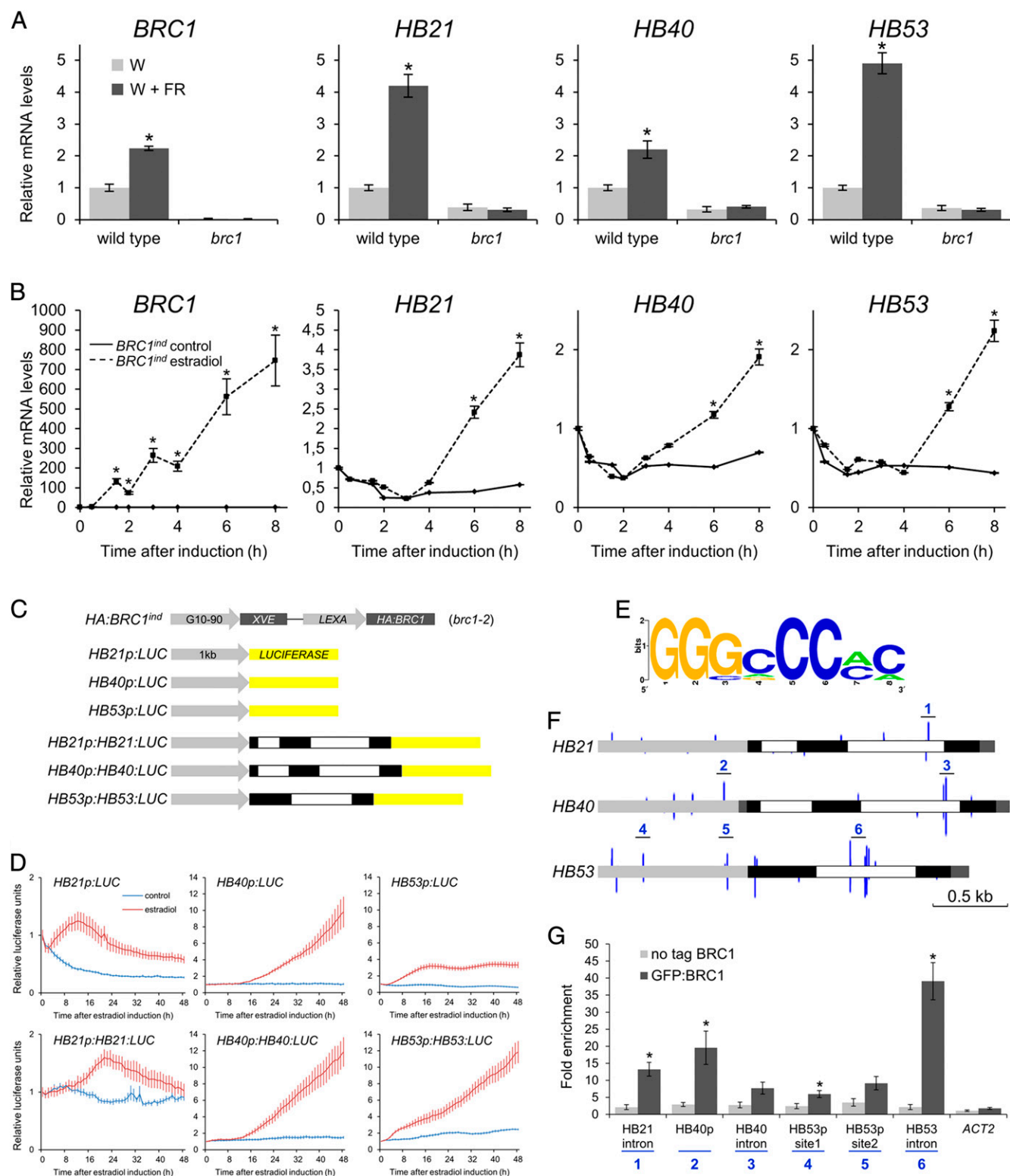


Fig. 1. BRC1 binds HB21, HB40, and HB53 and controls their transcription. (A and B) HB21, HB40, and HB53 mRNA levels correlate with BRC1 levels. mRNA levels of BRC1, HB21, HB40, and HB53 were analyzed by quantitative PCR in wild-type and *brc1* buds treated with W or W+FR light for 8 h (A) and in 7-d-old *BRC1*^{ind} seedlings after treatment with 10 μ M estradiol (B). (C) Schematic representation of reporter constructs transformed into HA:*BRC1*^{ind}; *brc1*-2 lines. (D) LUC activity after BRC1 induction with 10 μ M estradiol. Levels are relative to t = 0 after induction. Error bars show SEM of eight plants per line for each treatment. (E) Logo representing the frequency matrix of the consensus motif obtained from the alignment of the 10 best-scored binding sites in PBM assays. (F) BRC1-binding motifs in a 1-kb region upstream of the ATG start codon (gray) and genomic regions (exons black, introns white) of HB21, HB40, and HB53. Peak height is proportional to the similarity between sequence and consensus. Numbers indicate the peaks with the highest Rsat score (12). (G) Relative enrichment of GFP:BRC1 binding to sites 1–6. ACT2 was used as a negative control. Error bars show the SEM of three biological replicates (A and B), eight biological replicates (D), and three biological replicates with two technical repetitions (G). Asterisks indicate significant differences ($P < 0.05$; student's *t*-test) between control and treated plants (A) and between untagged *BRC1*^{ind} and GFP:*BRC1*^{ind} lines (G).

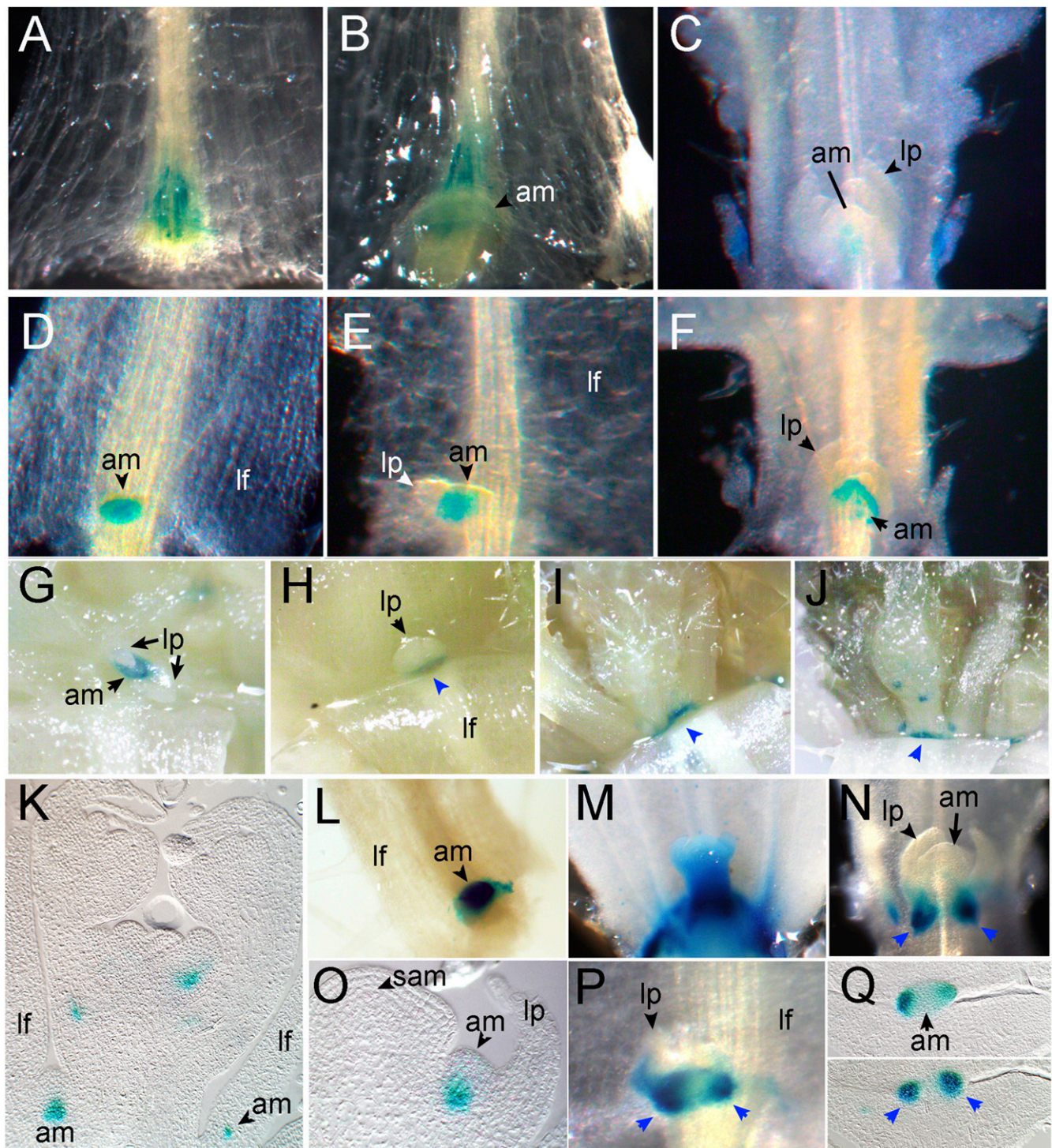


Fig. 2. *HB21*, *HB40*, and *HB53* are expressed in axillary buds. GUS activity in axillary buds of transgenic *HB21p:GUS* (A–C), *HB40p:GUS* (D–J), and *HB53p:GUS* (K–Q) lines. *HB21p:GUS* leaf vascular tissue stained at the position where the axillary meristem will initiate (A), underneath young axillary meristem (B), and at the inner layers of young axillary buds but absent from leaf primordia (C). (D–G) *HB40p:GUS* activity is present in the axillary meristem but is excluded from leaf primordia. (H–J) In *HB40p:GUS* older axillary buds, GUS signal is restricted to the base of the bud (arrowheads). (K–M) *HB53p:GUS* activity in young axillary meristems. (N–Q) *HB53p:GUS* activity at the base of leaf primordia in buds (arrowheads). K, O, and Q are sections of stained material embedded in plastic. am, axillary meristem; lf, leaf; lp, leaf primordia; sam, shoot apical meristem.

in both light regimes (*SI Appendix*, Fig. S11B). In summary, the branch-suppression response to W+FR light was significantly reduced in double and triple mutants of *HB21*, *HB40*, and *HB53*. The reduced response of the triple mutants was even more reduced than that of *brc1* mutants (Fig. 3B) (3).

We then investigated whether these genes affected early (vegetative) bud development, as described for *BRC1* (1). In long days (16 h light/8 h dark) we could not find significant phenotypic differences between the mutant and wild-type plants (*SI Appendix*, Fig. S11C). Then we grew the triple mutants under short-day conditions (8 h

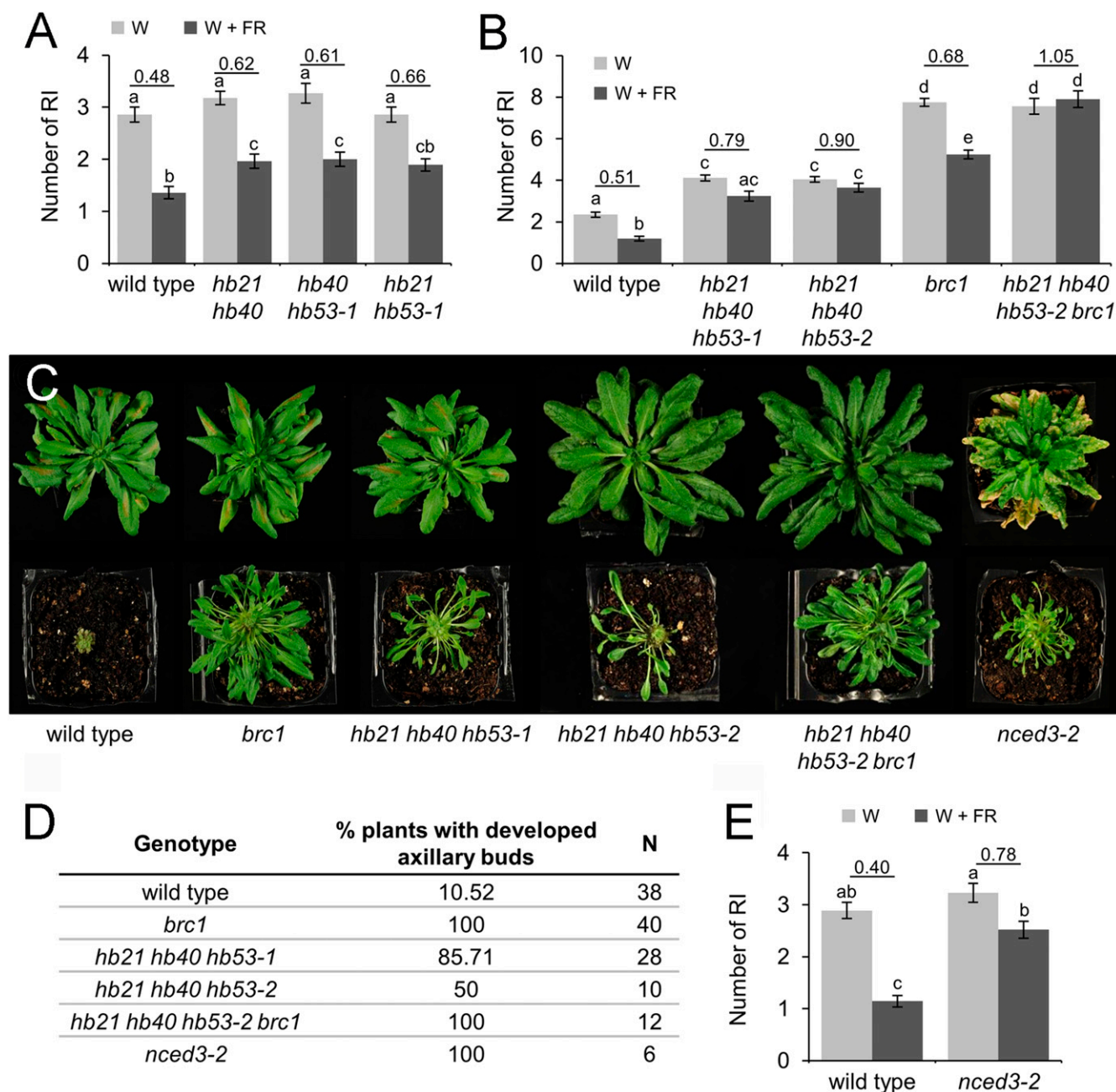


Fig. 3. *HB21*, *HB40*, and *HB53* act redundantly to repress shoot branching. Branching phenotypes of plants grown in W or W+FR light for 2 wk after flowering ($n = 25$ – 52). (A) Wild-type plants and double *hb* mutants. (B) Wild-type plants, *brc1* mutants, *hb* triple mutants, and quadruple *hb21 hb40 hb53-2 brc1* mutants. (C, Upper) Rosettes of plants grown until flowering in short-day conditions viewed from above. (Lower) The same plants after the removal of all of the rosette leaves to display axillary bud leaves. (D) Percentage of plants grown in short-day conditions that display axillary buds with developed leaves. (E) Branching phenotype of wild-type plants and *nced3-2* mutants 2 wk after bolting. $n = 21$ – 28 . Error bars show SEM. Letters denote significant differences among means ($P < 0.05$; one-way ANOVA).

light/16 h dark), in which axillary buds undergo vegetative development for several weeks before flowering of the main shoot, and found that leaves of mutant buds were remarkably more developed than those of wild-type buds (Fig. 3 C and D). This phenotype resembled but was milder than that of the *brc1* mutants (Fig. 3 C and D) (1). Furthermore, the branching phenotype of the quadruple mutant *hb21 hb40 hb53-2 brc1* was not more extreme than that of the parents, either in W+FR light or in short-day photoperiods (Fig. 3 B–D), indicating that *HB21/40/53* and *BRC1* act in the same pathway.

Taken together, these results suggest that *HB21*, *HB40*, and *HB53* act redundantly to slow axillary bud development and

branch outgrowth in the same genetic pathway as *BRC1* and that their functions are essential in low R:FR light conditions and in short-day photoperiods.

***BRC1*, *HB21*, *HB40*, and *HB53* Regulate *NCED3* Expression and ABA Levels in Buds.** Another *BRC1*-dependent gene coregulated with *HB21*, *HB40*, and *HB53* is *NCED3* (*At3g14440*) (SI Appendix, Fig. S1B and Dataset S1) (3, 7). *NCED3* encodes an enzyme that catalyzes the cleavage of 9-*cis*-epoxycarotenoids to xanthoxin, a key regulatory step of ABA biosynthesis (14). *NCED3* also plays a role in the control of branch outgrowth: *nced3-2* mutants have increased

branching in low and high R:FR light (4, 6). In our W conditions, the number of branches in *nced3-2* mutants was similar to that of wild-type plants, but their branch suppression response to W+FR light was reduced (78%) (Fig. 3E), and they displayed accelerated bud development in short days (Fig. 3C and D). These phenotypes resemble those of *hb21 hb40 hb53* triple mutants.

Therefore we investigated whether *HB21*, *HB40*, and *HB53* could influence *NCED3* expression. First we studied whether *NCED3* up-regulation in W+FR light was reduced in *hb21 hb40 hb53* mutants relative to wild-type plants. To do so, we treated wild-type plants and triple mutants with either W or W+FR light and compared *NCED3* mRNA levels in buds. Indeed, *NCED3* induction was reduced in the triple mutants (Fig. 4A), and this reduction resembled the *brc1* mutant response (Fig. 4B) (3). Furthermore, in W light, *NCED3* mRNA levels were significantly lower in the triple *hb21 hb40 hb53* mutants than in the wild-type plants (Fig. 4A). These results indicated that *HB21*, *HB40*, and *HB53* (and *BRC1*) are necessary for the expression of wild-type levels of *NCED3* in buds in W+FR, and to some extent, in W light.

Next, we investigated whether *BRC1*, *HB21*, *HB40*, and *HB53* were not only necessary but also sufficient for *NCED3* up-regulation. To do so, we used seedlings, a stage in which *BRC1*, *HB21*, *HB40*, and *HB53* are hardly or not expressed, using estradiol-inducible lines (*HA:BRC1^{ind}*, *HA:HB21^{ind}*, *HA:HB40^{ind}*, and *HA:HB53^{ind}*) (*SI Appendix*, Figs. S4A and S12). We treated 7-d-old seedlings with estradiol for 8 h and quantified *NCED3* transcripts (Fig. 4C). Induction of *BRC1*, *HB21*, *HB40*, or *HB53* caused a 31-, 48-, nine-, and 31-fold increase, respectively, in *NCED3* mRNA levels. Changes in *NCED3* mRNA levels have been shown to correlate positively with changes in ABA levels (14). We confirmed this correlation by measuring ABA in these

samples. Indeed, induced plants displayed a significant increase in ABA levels after the estradiol treatment (Fig. 4D).

Transcriptional induction of *NCED3* by *BRC1*, *HB21*, *HB40*, and *HB53* may be direct. DNA affinity purification sequencing (Dap-Seq) data of *HB21*, *HB40*, and *HB53* (15) indicates that these proteins directly bind a genomic region 800–900 bp upstream of the *NCED3* transcription start site (*SI Appendix*, Fig. S13A–C). In addition ChIP assays using *GFP:BRC1^{ind}* seedlings indicate that *BRC1* also binds directly to the *NCED3* promoter (*SI Appendix*, Fig. S13D and E).

BRC1, *HB21*, *HB40*, and *HB53* mRNA levels were not affected in axillary buds of *nced3-2* mutants (*SI Appendix*, Fig. S14A), in agreement with *NCED3* acting downstream of this pathway (see Fig. 6). However, *HB21* and, to a lesser extent, *HB40* and *HB53* were responsive to ABA application in buds (*SI Appendix*, Fig. S14B), indicating that although *NCED3* (and ABA) are not essential for the induction of these genes, ABA nevertheless could help maintain their expression. In contrast, *BRC1* mRNA levels did not change in response to ABA (*SI Appendix*, Fig. S14B) and were not significantly affected in the *hb21 hb40 hb53* triple mutants (*SI Appendix*, Fig. S14C), confirming that *BRC1* is the most upstream gene of this pathway (see Fig. 6).

All these results suggest that *BRC1*, *HB21*, *HB40*, and *HB53* are necessary and sufficient to cause *NCED3* induction and ABA accumulation and that *BRC1*-dependent transcriptional activation of *HB21*, *HB40*, and *HB53* could boost local ABA signaling and response in axillary buds.

ABA Rescues the Excess-Branching Phenotype of *hb21 hb40 hb53* Triple Mutants. If the excessive branching phenotype of *hb21 hb40 hb53* triple mutants in W+FR light is caused mostly by a failure to accumulate

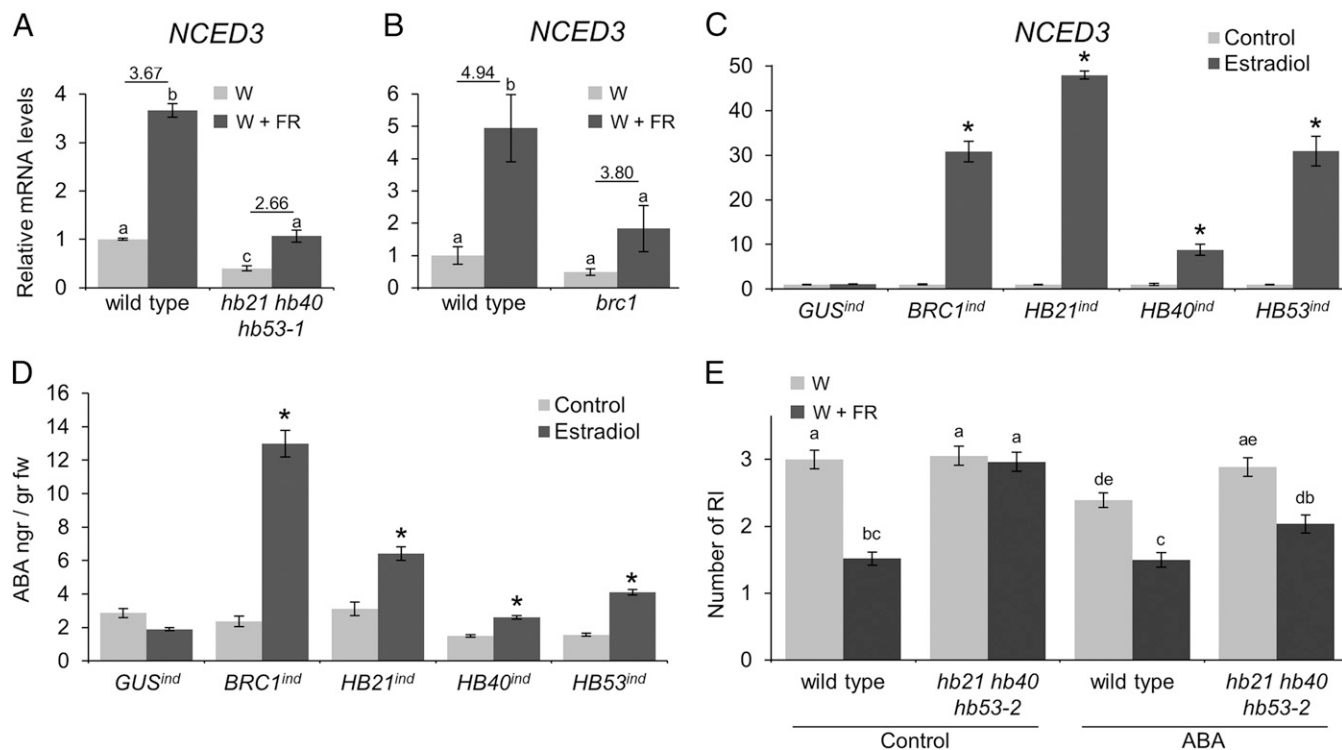


Fig. 4. *BRC1*, *HB21*, *HB40*, and *HB53* promote ABA accumulation via *NCED3*. (A and B) *NCED3* mRNA levels analyzed by quantitative PCR measured in axillary buds of wild-type plants and *hb21 hb40 hb53-1* mutants (A) and *brc1* mutants (B) treated with W or W+FR light for 8 h. (C) *NCED3* mRNA levels in 7-d-old *GUS^{ind}*, *HA:BRC1^{ind}*, *HA:HB21^{ind}*, *HA:HB40^{ind}*, and *HA:HB53^{ind}* seedlings after an 8-h treatment with 10 μ M estradiol. (D) ABA levels measured in estradiol-treated seedlings of the genotypes in C. (E) Branching phenotype of wild-type and *hb21 hb40 hb53-2* plants treated with W or W+FR light and 50 μ M ABA or mock (control) for 2 wk after bolting ($n = 28$). Error bars show the SEM of three biological replicates. Asterisks show significant differences ($P < 0.05$; student's t -test) between control and treated plants. Letters denote significant differences ($P < 0.05$; one-way ANOVA) among means.

ABA in buds, ABA application to buds should rescue the phenotype of the triple mutants. We tested this notion by applying 50 μM ABA directly to buds of wild-type and *hb21 hb40 hb53-2* triple-mutant plants every day for 15 d after bolting and quantified their branching phenotypes in W and W+FR light (Fig. 4E). In the triple mutants ABA restored the wild-type response to W+FR light. In wild-type plants, ABA application led to a further reduction in branch number in W light but had no effect in W+FR light. These results are consistent with the possibility that the increased branching phenotype of *hb21 hb40 hb53* triple mutants in low R:FR light is caused mainly by a failure to accumulate ABA in buds.

HB21, HB40, and HB53 Promote Expression of the ABA-Related GRN. *BRC1*, *HB21*, *HB40*, and *HB53* could promote ABA accumulation, which could in turn enhance the expression of the ABA-

related GRN induced in dormant buds. To test this possibility, we studied the expression of genes of this GRN under conditions of loss/gain of *BRC1* or *HB21/40/53* function. We selected four genes encoding proteins associated with ABA signaling and response: *ABA RESPONSIVE ELEMENTS-BINDING FACTOR 3* (*ABF3*; *At4g34000*), encoding a bZIP master regulator of ABA signaling (16), *ABI FIVE BINDING PROTEIN 3* (*AFP3*; *At3g29575*), *G-BOX BINDING FACTOR 3* (*GBF3*; *At2g46270*), and *NAC-LIKE, ACTIVATED BY AP3/PI* (*NAP*; *At1g69490*) (SI Appendix, Fig. S1B). These factors could modulate and amplify the transcriptional responses of the GRN. We compared induction levels of these genes in wild-type plants and *hb21 hb40 hb53-1* mutants after a W+FR light treatment. The four genes showed a reduced induction in the triple mutants (Fig. 5A). These results resembled those obtained in *brc1* mutants (3) (SI Appendix, Fig. S1A) and suggested that *BRC1* and its direct targets, *HB21*, *HB40*, and

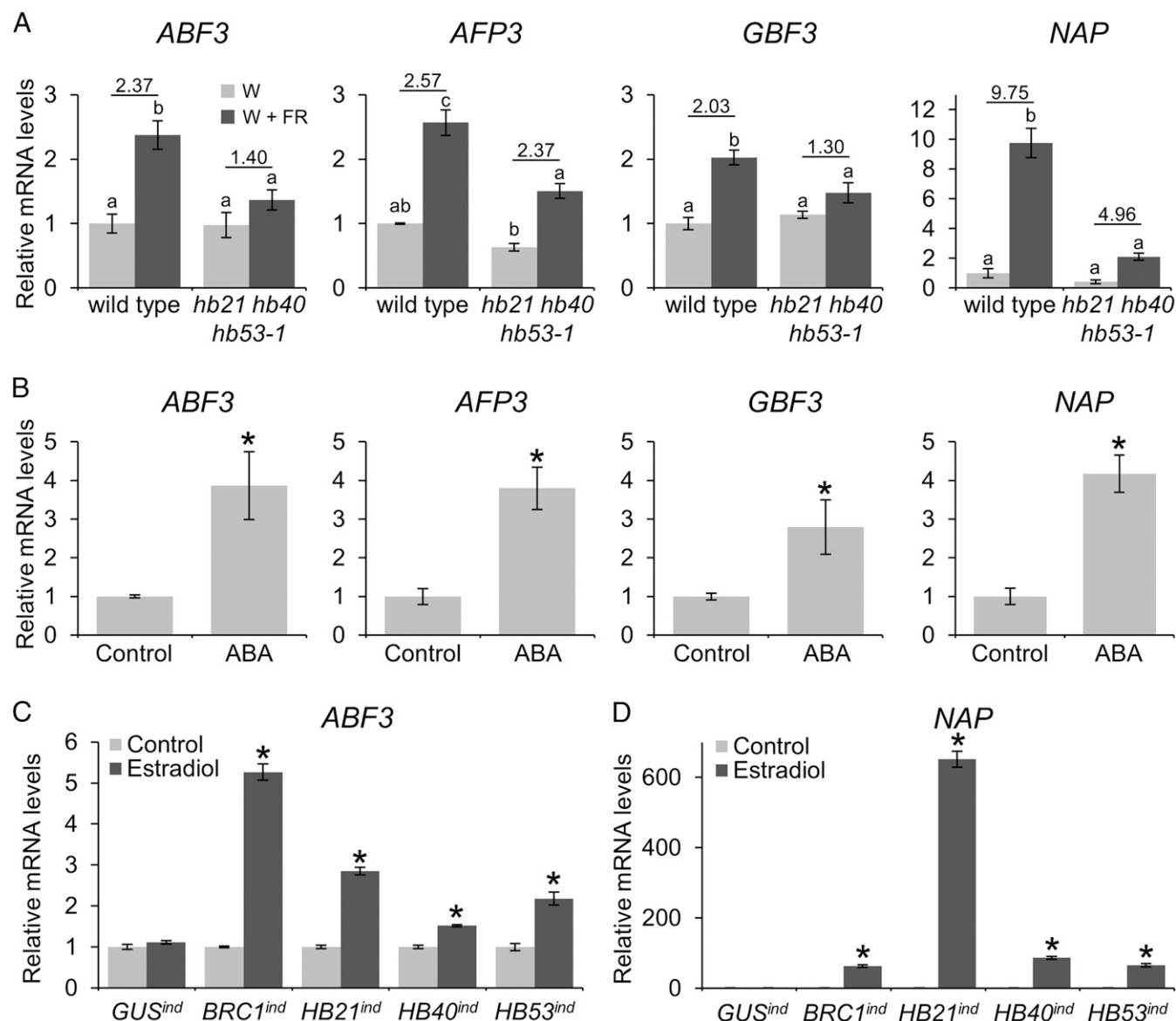


Fig. 5. *BRC1*, *HB21*, *HB40*, and *HB53* regulate the expression of ABA-related genes encoding TFs. (A and B) mRNA levels of *ABF3*, *AFP3*, *GBF3*, and *NAP* analyzed by quantitative PCR in axillary buds in wild-type and *hb21 hb40 hb53-1* buds treated with W or W+FR light for 8 h (A) or in wild-type buds after an application of 50 μM ABA for 8 h (B). (C and D) Expression of *ABF3* (C) and *NAP* (D) in 7-d-old *GUS^{ind}*, *HA:BRC1^{ind}*, *HA:HB21^{ind}*, *HA:HB40^{ind}*, and *HA:HB53^{ind}* seedlings treated for 8 h with 10 μM estradiol. Error bars show the SEM of three biological replicates. Asterisks indicate significant differences ($P < 0.05$; student's *t*-test) between control and treated plants. Letters denote significant differences ($P < 0.05$; one-way ANOVA) among means.

HB53, are necessary for the normal expression of these factors in W+FR light-treated dormant buds.

We then studied their response to W+FR light in *nced3-2* mutants and found reduced up-regulation compared with the response in wild-type plants (*SI Appendix, Fig. S15B*). This reduced response suggested that *NCED3*, and presumably ABA, are required for the full induction of these genes. Therefore, we studied their mRNA levels after direct application of ABA to axillary buds. All four genes were responsive to ABA (*Fig. 5B*), confirming that *NCED3*-induced ABA accumulation could contribute to their transcriptional induction.

Using estradiol-inducible lines, we also examined whether *BRC1*, *HB21*, *HB40*, or *HB53* alone was sufficient to boost their expression in seedlings. We treated 7-d-old seedlings with estradiol for 8 h and measured *ABF3*, *AFP3*, *GBF3*, and *NAP* mRNA levels. All four genes were significantly up-regulated after *BRC1* or *HB53* induction, and *ABF3* and *NAP* also were up-regulated by *HB21* and *HB40* (*Fig. 5 C and D and SI Appendix, Fig. S15 C and D*).

***HB21*, *HB40*, and *HB53* Do Not Mediate All *BRC1*-Induced Responses.** *HB21*, *HB40*, and *HB53* do not seem to mediate all the *BRC1*-induced responses. The expression of a group of *BRC1*-dependent genes related to cell division and consistently down-regulated after *BRC1* induction (3) was not affected in buds of the *hb21 hb40 hb53-1* triple mutants (*SI Appendix, Fig. S16A*) or in estradiol-induced *HB21^{ind}*, *HB40^{ind}*, or *HB53^{ind}* lines (*SI Appendix, Fig. S16B*). These results indicate that *HB21*, *HB40*, and *HB53* mediate only a subset of the gene responses promoted by *BRC1* (*SI Appendix, Fig. S17*).

Discussion

Little is known about the genetic mechanisms acting inside axillary buds during the growth-to-dormancy transition. In *Arabidopsis*, this process is regulated by the class II TCP transcription

factor *BRC1*, which regulates the expression of several GRNs (*SI Appendix, Fig. S17*) and which, among other effects, causes a local enhancement of the ABA response (3). Although ABA has been classically associated with dormancy in seeds and buds in many different species, including *Arabidopsis* (4, 6), this hormone is not yet fully integrated into the current molecular and genetic models of the hormonal control of shoot branching. Moreover, how *BRC1* controls the response to ABA was completely unknown.

Here we provide evidence that *Arabidopsis* *BRC1* directly triggers an HD-ZIP-mediated cascade that results in a local boost of *NCED3* expression and ABA biosynthesis inside axillary buds in conditions of low R:FR or short photoperiods. ABA, along with *BRC1* and HD-ZIP activity, may promote the induction of a GRN required for bud dormancy (*Fig. 6*). *NCED3* encodes an enzyme catalyzing a key regulatory, rate-limiting step of ABA biosynthesis: *NCED3* loss of function compromises ABA accumulation, and its overexpression is sufficient to increase ABA levels (14, 17). We have shown that *HB21*, *HB40*, and *HB53* are essential for wild-type *NCED3* transcription levels in buds. Moreover, ectopic expression of *BRC1*, *HB21*, *HB40*, or *HB53* in seedlings is sufficient to cause a significant accumulation of *NCED3* transcripts and ABA within 8 h, providing compelling evidence of the causal relationship between the function of these genes and *NCED3* activity. Furthermore, these four genes may control *NCED3* transcription directly, because our ChIP data and available Dap-Seq data (15) indicate that all four proteins bind the *NCED3* promoter.

ABA measurements have confirmed an inverse correlation between bud growth potential and bud ABA levels in *Arabidopsis* (4, 6). The increased branching phenotypes of *nced3* mutants further support a critical role for ABA in this process (refs. 4 and 6 and this work). Moreover, the observation that the *hb21 hb40 hb53* mutant phenotype in low R:FR is rescuable by ABA application supports the idea that this phenotype is caused by a failure to accumulate this hormone in buds, indicating that an important role

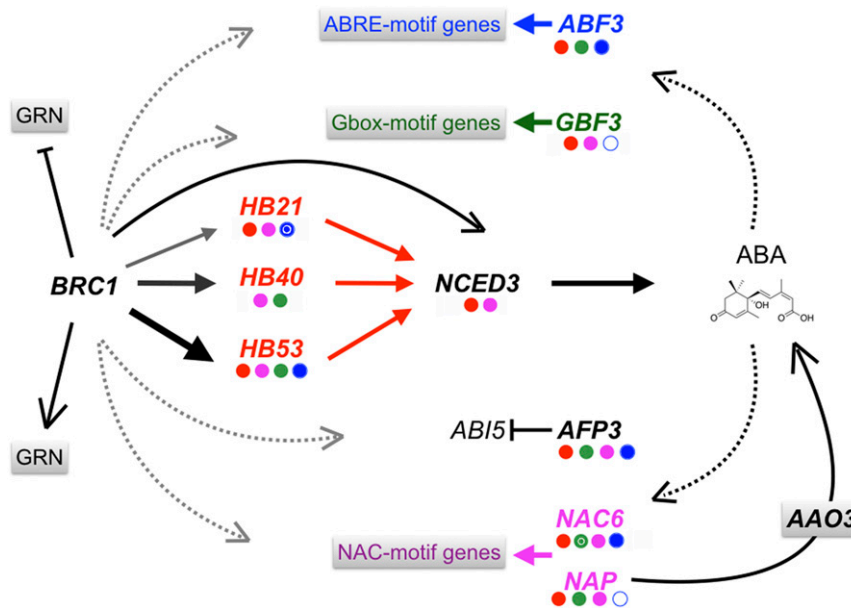


Fig. 6. *BRC1* regulation of ABA signaling in dormant buds. A working model is shown of how *BRC1* regulates ABA signaling and response in buds. *BRC1* and some core transcriptional regulators of the GRN (*SI Appendix, Fig. S18*) are represented. Solid arrows indicate (i) direct protein–DNA interactions based on ChIP (this work) or DAP-seq data (15); (ii) protein–protein interactions (24); and (iii) known metabolic pathways (14, 28). Potential cross-regulation between *HB21/40/53*, *ABF3*, *NAP*, *GBF3*, and *NAC6* is indicated by the colored dots below genes based on direct binding (15, 29) of the TF with the same color coding. Red dots, *HB21/40/53* binding; green dots, *GBF3* binding; purple dots, *NAP/NAC6* binding; open blue circles, binding only without ABA; white inner circles, binding only with ABA. Dotted arrows indicate indirect or untested regulation, e.g., *BRC1* also may promote ABA signaling via direct control of *ABF3*, *NAP*, *NAC6*, *GBF3*, and *AFP3* (also see *SI Appendix, Fig. S18*). In addition, *BRC1* controls GRNs other than the one analyzed in this work (*SI Appendix, Fig. S17*).

of *HB21/40/53* is to cause a local rise in ABA. It is noteworthy that *HB40* and *HB53* also are expressed in stomata guard cells, where cell-autonomous ABA synthesis occurs to control stomata closure (18). Local ABA synthesis contrasts with that of auxin, known to control shoot branching systemically, and with strigolactones that can be transported from the roots to suppress branching (19). ABA transport nevertheless may contribute to strengthen ABA accumulation in buds because the nitrate transporter *NRT1.2* (*At1g69850*), which also mediates ABA cellular uptake (20), is induced in dormant buds and repressed in active buds (3, 21).

A rise in ABA may activate a positive feed-back loop, because several ABA synthesis genes including *NCED3* and *HB21/40/53* (but not *BRC1*) are induced by ABA (Fig. 6) (ref. 22 and this work). Furthermore, most of the genes in the ABA-related GRN are induced by ABA, according to public microarray data (23), so it is likely that ABA accumulation causes a general up-regulation of the GRN. Indeed the response to *NCED3* and ABA of four members of the GRN encoding TFs (*ABF3*, *GBF3*, *NAP*) or TF-interacting proteins (*AFP3*) supports this model (refs. 16 and 24 and this work). *ABF3* is a bZIP master regulator of ABA signaling that controls ABRE-dependent gene expression (16), *GBF3* is a bZIP factor that binds G-box motifs (25), and *AFP3* interacts with the bZIP protein *ABI5* to fine-tune the ABA response (24). *NAP*, an NAC TF associated with stress and senescence (26, 27), controls the expression of *ABSCISIC ALDEHYDE OXIDASE3* (*AAO3*), an enzyme that catalyzes the final steps of ABA synthesis (28), and thereby may contribute further to ABA accumulation (Fig. 6). Up-regulation of these genes probably has a great impact in the GRN: *ABF3*, *GBF3*, and *NAP* bind 76, 83, and 29% of the GRN promoters, respectively (SI Appendix, Fig. S18 A and B) (15, 29). Cross-talk also may involve *HB21*, *HB40*, and *HB53*, which bind 46, 63, and 42% of the GRN gene promoters, respectively (15), including those of *GBF3*, *NAP*, *AFP3*, and *NAC6* (SI Appendix, Fig. S18 A and B). Further ChIP-seq studies and high-resolution transcriptomic analyses of mutants and ABA-treated and inducible lines will allow a better understanding of the sequential gene activation and the relationships among the genes of the GRN.

This response seems essential in low R:FR light and short photoperiods, conditions associated with reduced photosynthe-

sis, energy deprivation, and stress in which ABA is required (30). The molecular mechanisms by which this hormone controls bud growth are yet to be determined. However, the antagonistic roles of ABA and cytokinin, which locally promotes bud activity, in several developmental processes such as seed germination and seedling development are well known (31, 32). Moreover, it is worth noting that loss-of-function mutants of the ortholog gene of *HB21/40/53* in maize, *GRASSY TILLERS1* (*GT1*), have increased branching and that *GT1* has been proposed to act genetically downstream of *TEOSINTE BRANCHED1* (*TB1*) in the control of shoot branching. Moreover *GT1* orthologs in both sorghum and teosinte are induced in plants treated with FR-rich light, and this gene has been proposed to mediate the reduced branching associated with the shade-avoidance response in the grasses (33). It remains to be tested whether this pathway also controls ABA synthesis and response in monocots. Nevertheless these results indicate that the genetic module *TB1/GT1*, *BRC1/HB21/40/53*, which is enhanced under shade conditions, is probably conserved throughout flowering plants. Conservation of the *BRC1*-binding sites in the genomic regions of the corresponding *HD-ZIP* genes in several Brassicaceae supports the conservation of this regulatory module and suggests the existence of a general strategy to promote branch suppression as a plant adaptation to light-limiting conditions.

Materials and Methods

Details about plant lines, growth conditions, cloning, *LUC* assays, histochemical analyses, treatments, quantitative PCR, ABA measurements, and other techniques are in SI Appendix, Materials and Methods.

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