The Short-Rooted Phenotype of the *brevis radix* Mutant Partly Reflects Root Abscisic Acid Hypersensitivity[1][C][W][OA]

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To gain further insight into abscisic acid (ABA) signaling and its role in growth regulation, we have screened for Arabidopsis (*Arabidopsis thaliana*) mutants hypersensitive to ABA-mediated root growth inhibition. As a result, we have identified a loss-of-function allele of *BREVIS RADIX* (*BRX*) in the Columbia background, named *brx-2*, which shows enhanced response to ABA-mediated inhibition of root growth. *BRX* encodes a key regulator of cell proliferation and elongation in the root, which has been implicated in the brassinosteroid (BR) pathway as well as in the regulation of auxin-responsive gene expression. Mutants affected in BR signaling that are not impaired in root growth, such as *bes1-D*, *bzr1-D*, and *bsu1-D*, also showed enhanced sensitivity to ABA-mediated inhibition of root growth. Triple loss-of-function mutants affected in PP2Cs, which act as negative regulators of ABA signaling, showed impaired root growth in the absence of exogenous ABA, indicating that disturbed regulation of ABA sensitivity impairs root growth. In agreement with this result, diminishing ABA sensitivity of *brx-2* by crossing it with a *35S:HAB1* ABA-insensitive line allowed significantly higher recovery of root growth after brassinolide treatment. Finally, transcriptomic analysis revealed that ABA treatment negatively affects auxin signaling in wild-type and *brx-2* roots and that ABA response is globally altered in *brx-2*. Taken together, our results reveal an interaction between BRs, auxin, and ABA in the control of root growth and indicate that altered sensitivity to ABA is partly responsible for the *brx* short-root phenotype.

Abscisic acid (ABA) is a universal stress hormone of higher plants that also plays a key role as a regulator of growth and meristem function and in different plant developmental processes, such as embryo development, germination, vegetative development, flowering, and organogenesis (Xu et al., 1998; Finkelstein et al., 2002; Barrero et al., 2005; De Smet et al., 2006; Razem et al., 2006; Liang et al., 2007). Both positive and negative effects of ABA on growth and development have been reported, depending on tissue, concentration, and interaction with the environment (Zeevaart and Creelman, 1988; Thompson et al., 2007). For instance, in tomato (*Solanum lycopersicum*) and Arabidopsis (*Arabidopsis thaliana*), normal levels of ABA are required to maintain shoot growth independently of effects of hormone status on plant water balance (Sharp et al., 2000; LeNoble et al., 2004). ABA inhibits germination and root growth at micromolar concentrations, whereas low concentrations of ABA (<1 μM) stimulate root growth (Zeevaart and Creelman, 1988; Ephritikhine et al., 1999). This fact likely explains the variable effects on root growth obtained after exogenous application of ABA in well-watered plants, ranging from growth inhibition or little effect to growth promotion. Finally, a crucial ABA-dependent adaptive feature that promotes survival of plants under water stress is the maintenance of root elongation (Sharp et al., 2004). The manipulation of endogenous ABA levels by either chemical or genetic means has shown that ABA is crucial to maintain primary root growth at low water potentials (Saab et al., 1990). In contrast, the formation of a lateral root from a lateral root primordium is repressed as water availability is reduced, and ABA is a critical component of this repression mechanism (Deak and Malamy, 2005; De Smet et al., 2006). Finally, the importance of ABA on root growth control and root system architecture is reflected by the recent identification of a major quantitative trait locus in maize (*Zea mays*) that simultaneously affects ABA biosynthesis and root agronomical traits both under

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well-watered and water-stress conditions (Landi et al., 2007).

Plant growth and development are controlled by the concerted action of many signaling pathways, which integrate information from the environment with that from developmental and metabolic cues. In the particular case of root development, genetic analysis indicates that hormone signaling pathways functionally intersect with each other for the control of root growth. For instance, auxin controls the growth of roots by modulating cellular responses to the phytohormone gibberellin (Fu and Harberd, 2003). In this case, shoot apex-derived auxin controls root growth through the modulation of GA-mediated DELLA protein destabilization (Fu and Harberd, 2003). Auxin signaling itself shows interdependency with BR signaling (Nakamura et al., 2006, Hardtke et al., 2007). Other phytohormones (ethylene, cytokinin, ABA) interact in the regulation of plant growth and development; however, the molecular mechanisms of these interactions remain poorly understood. Hormone response mutants have been of crucial importance to dissecting the signal transduction pathways that control diverse physiological processes as well as genetic interactions among different signaling pathways (Gazzarrini and McCourt, 2001). Thus, interactions of ABA with signaling pathways of drought, salinity, cold, sugars, gibberellins, jasmonic acid, pathogenic elicitors, auxins, ethylene, and brassinosteroids (BRs) have been described (Thomashow, 1999; Beaudoin et al., 2000; Ghassemian et al., 2000; Steber and McCourt, 2001; Cheng et al., 2002; Finkelstein et al., 2002; Zhu, 2002; Rock and Sun, 2005; Adie et al., 2007; Torres-Zabala et al., 2007). In particular, ABA and BRs have been reported to act antagonistically in some plant responses. For instance, BRs promote whereas ABA inhibits germination, and both the BR biosynthetic mutant *det2-1* and the BR-insensitive mutant *bri1-1* are more sensitive than the wild type to ABA-mediated inhibition of germination (Steber and McCourt, 2001). Additionally, both BR biosynthetic and perception mutants are hypersensitive to ABA-mediated inhibition of root growth (Clouse et al., 1996; Ephritikhine et al., 1999). Finally, the expression of the BR ENHANCED EXPRESSION1 (BEE1), BEE2, and BEE3 transcription factors was repressed by ABA, and *BEE1*-overexpressing roots were hypersensitive to BRs and partially insensitive to ABA (Friedrichsen et al., 2002). However, good candidates that could explain the ABA-BR cross talk at the molecular level have not been identified.

To further extend our knowledge of the ABA signaling pathway and its effect on growth regulation, we have performed a screen for mutants hypersensitive to ABA in growth assays. As a result, we have identified a mutant in the Columbia background, named *seedling hypersensitive to ABA1* (*sha1*), which showed enhanced sensitivity to ABA-mediated inhibition of root growth and was found to be allelic to the previously identified *brevis radix* (*brx*) mutant. *BRX* is a key regulator of cell proliferation and elongation in the root, which is expressed in the phloem vasculature throughout the plant (Mouchel et al., 2006). BRX has been implicated in the interaction between the auxin and BR pathways based on the observation that in the *brx* mutant globally impaired auxin-responsive gene expression can be rescued by BR application (Mouchel et al., 2006). Exogenous application of brassinolide (BL) also partially rescued the root growth defect of *brx-1* (from approximately 30% root length of the wild-type control to >50%; Mouchel et al., 2006). Constitutive expression of a rate-limiting enzyme in BR biosynthesis, CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARF (CPD), in *brx-1* driven by the cauliflower mosaic virus *35S* promoter was slightly more efficient (approximately 60% of the wild-type control) in rescuing this phenotype (Mouchel et al., 2006). Our finding of *brx-2* as hypersensitive to ABA-mediated inhibition of root growth reveals a novel phenotype for this mutant and suggests that *BRX* and BRs play important roles in modulating root response to ABA. This is corroborated by analysis of other mutants in the BR signaling pathway. Taken together, our results reveal cross talk between ABA and BRs for root growth control and suggest that a normal response to BRs is required to prevent enhanced sensitivity to ABA-mediated inhibition of root growth.

RESULTS

brx-2 Loss-of-Function Mutant Shows Enhanced Sensitivity to ABA-Mediated Inhibition of Root Growth

A screen for mutants hypersensitive to ABA-mediated growth inhibition was performed using T-DNA lines generated with the activation-tagging vector pSKI15 in the Columbia background. Seeds were germinated vertically in the absence of ABA, and then 5-d-old seedlings were transferred to plates supplemented with 30 μM ABA. Potential ABA-hypersensitive mutants were initially identified on the basis of impaired growth compared with the wild type. After screening of approximately 20,000 lines, several candidates were selected and initially named *sha* mutants. In the absence of ABA, the *sha1* mutant showed a slight decrease in root growth at 5 d compared with the wild type, which was notably increased at further stages of development (Fig. 1A). As discussed below, *sha1* was found to be allelic to *brx*; accordingly, we renamed it *brx-2*. The presence of 10 μM ABA in the medium exacerbated the root growth defect in *brx-2* compared with the wild type (73% and 45% inhibition, respectively; Fig. 1, A and B). Interestingly, the double *hy5 hyh* mutant, which displays reduced root growth because of reduced cell proliferation in the meristem (Sibout et al., 2006), did not show enhanced ABA-mediated inhibition of root growth (Fig. 1B), suggesting that the observed ABA hypersensitivity of *brx-2* does not simply reflect dispropor-

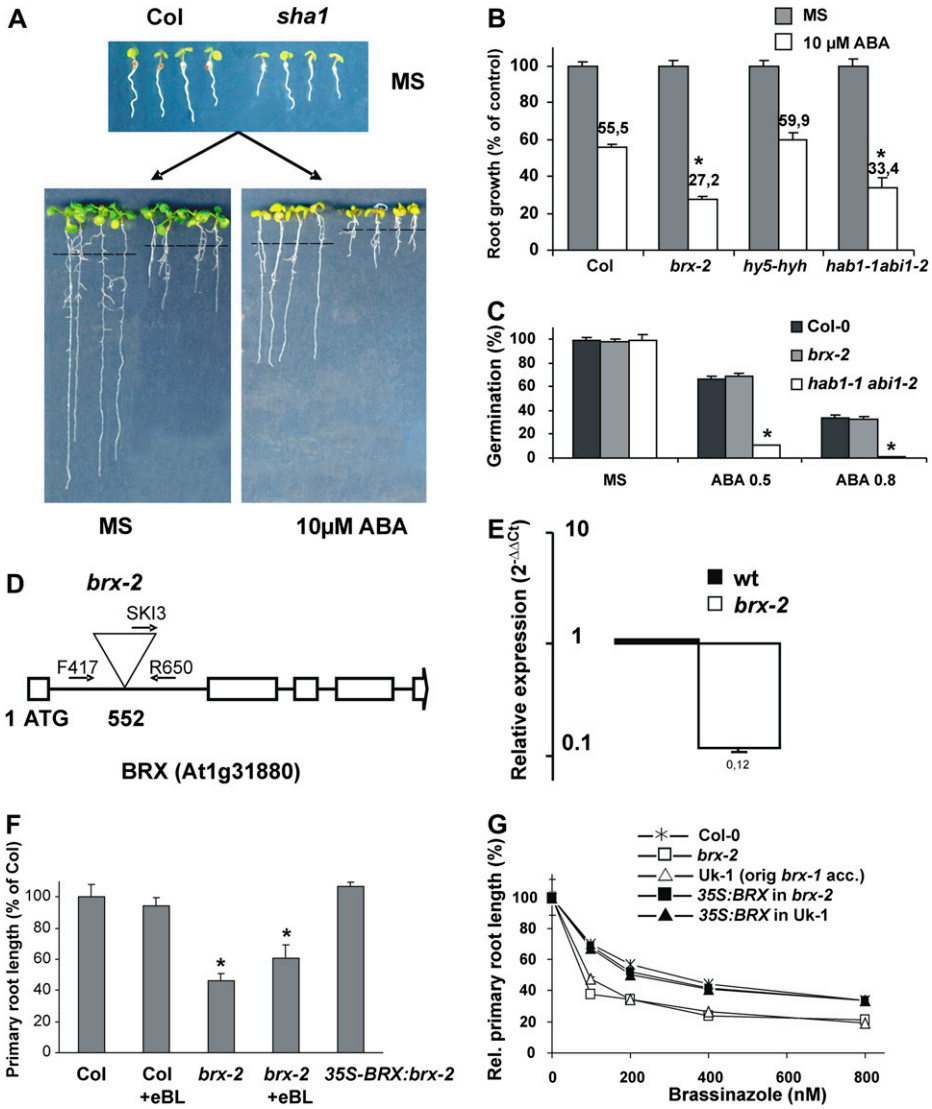

Figure 1. *brx-2* shows higher sensitivity than the wild type to ABA-mediated root growth inhibition. A, Five-day-old seedlings (top) were transferred to medium lacking or supplemented with 10 μM ABA (bottom) and grown vertically for 6 d. The dotted lines indicate the positions of root tips just after transferring to new medium. B, Quantification of root growth inhibition by ABA in the wild type, *brx-2*, *hy5 hyh*, and *hab1-1 abi1-2* mutants. Data are averages ± SE from three independent experiments ($n = 20$). C, *brx-2* shows similar sensitivity to the wild type to ABA-mediated inhibition of germination. Data are averages ± SE from three independent experiments ($n = 200$). D, Scheme of the *BRX* gene and localization of T-DNA insertion in *brx-2*. The numbering begins at the ATG translation start codon. Primers used for cosegregation analysis are indicated. E, RT-qPCR analysis of *BRX* expression in wild type and *brx-2* mRNAs prepared from 2-week-old roots. Values are relative expression levels with respect to the wild type (wt; value 1). Data are averages ± SE from three independent experiments. F, Relative root length of seedlings from the wild type (Columbia [Col] ecotype), *brx-2*, and a complemented line (*35S-BRX*::*brx-2*) grown vertically in medium lacking or supplemented with BL. Five-day-old seedlings were transferred to medium lacking or supplemented with 2 nM BL and grown vertically for 5 d. Root length of the wild type in the absence of exogenous BL was taken as 100%. Data are averages ± SE from three independent experiments ($n = 20$). G, Brassinazole hypersensitivity of *brx-1* (a natural allele in the Uk-1 accession), *brx-2*, and complemented lines (*35S-BRX*::*brx-2* and *35S-BRX* in Uk-1) in root growth assays. Data are averages from three independent experiments ($n = 20$; SE < 3%; error bars are not visible due to overlapping with legend marks). * $P < 0.05$ (Student's *t* test) when comparing data from the indicated genotype and the wild type in the same growth conditions. [See online article for color version of this figure.]

tional growth reduction due to already initially impaired root growth. Both ABA-mediated inhibition of germination and water loss kinetics were similar in *brx-2* and the wild type, in contrast to the global ABA-hypersensitive phenotype of the double *hab1-1 abi1-2* mutant (Saez et al., 2006; Fig. 1C; Supplemental Fig. S1).

The mutation was recessive because F1 seedlings showed similar root growth to the wild type both in

the absence and in the presence of ABA (data not shown). The segregation ratio in the F2 progeny was consistent with a single, recessive mutation (132 wild type to 46 short-root phenotype; $\chi^2 = 0.067$, $P = 0.79$). Homozygous *sha1* individuals were selected from the F2 generation and scored for phosphinothricin resistance. All F2 *sha1* seedlings showed cosegregation of the *sha1* phenotype and phosphinothricin resistance. Plant T-DNA-flanking sequences were isolated from the mutant by thermal asymmetric interlaced PCR, and sequence analysis revealed that the pSKI15 T-DNA was inserted at nucleotide 553 of the *BRX* (*At1g31880*) gene (Fig. 1D). A natural loss-of-function allele of *BRX* in the Arabidopsis accession Umkirch-1 (Uk-1) had been reported previously (Mouchel et al., 2004), which we named *brx-1*; therefore, we renamed *sha1* as *brx-2*. Finally, the analysis of 52 F2 *brx-2* chromosomes showed cosegregation of the short-root phenotype and the presence of the T-DNA (data not shown). Real-time quantitative PCR (RT-qPCR) analysis using primers that amplify the 3′ end of *BRX* cDNA showed that the T-DNA insertion found in *brx-2* strongly impaired the expression of *BRX* (Fig. 1E).

In the *brx-1* mutant, exogenous application of BL partially rescued the root growth defect, whereas introduction of a 35S:*BRX* transgene fully restored root growth (Mouchel et al., 2006). Similar chemical and genetic complementation assays were performed with *brx-2*, and analogous results were obtained (Fig. 1F). Finally, *brx-2* also resembles the original allele in its hypersensitivity to root growth inhibition by brassinazole, a BL biosynthesis inhibitor (Fig. 1G).

Roots of *bes1-D*, *bzr1-D*, and *bsu1-D* Mutants Are ABA Hypersensitive

Although the biochemical function of BRX has not been elucidated yet, the fact that it can localize to the nucleus and its ability to activate transcription in a heterologous yeast system have led to the suggestion that BRX might represent a novel class of transcriptional regulator (Mouchel et al., 2004). We wondered whether other transcription factors that directly affect BR action might show an ABA-hypersensitive root phenotype as well. BES1 and BZR1 are a novel class of plant-specific transcription factors that play a key role in BR signaling (Wang et al., 2002; Yin et al., 2002, 2005; He et al., 2005). Therefore, we examined ABA sensitivity of *bes1-D* and *bzr1-D* mutants in root growth assays and compared these results with *brx-2* as well as the ABA-hypersensitive double *hab1-1 abi1-2* mutant and ABA-insensitive *35S:HAB1* plants (Saez et al., 2004, 2006; Fig. 2). Interestingly, the *bes1-D* mutant showed a strong ABA-hypersensitive phenotype and, in contrast to *brx*, was not impaired in root growth in the absence of ABA. The *bzr1-D* mutant showed a weak ABA-hypersensitive phenotype at 1 and 3 μM ABA and a stronger phenotype at 10 μM ABA (Fig. 2B). The nuclear protein phosphatase BSU1 is able to modulate the phosphorylation state of BES1 (Mora-García et al., 2004). The gain-of-function *bsu1-D* mutation leads to increased steady-state levels of dephosphorylated BES1 and, thereby, modulates the magnitude of the response to BR (Mora-García et al., 2004). Interestingly, the *bsu1-D* mutant was as hyper-

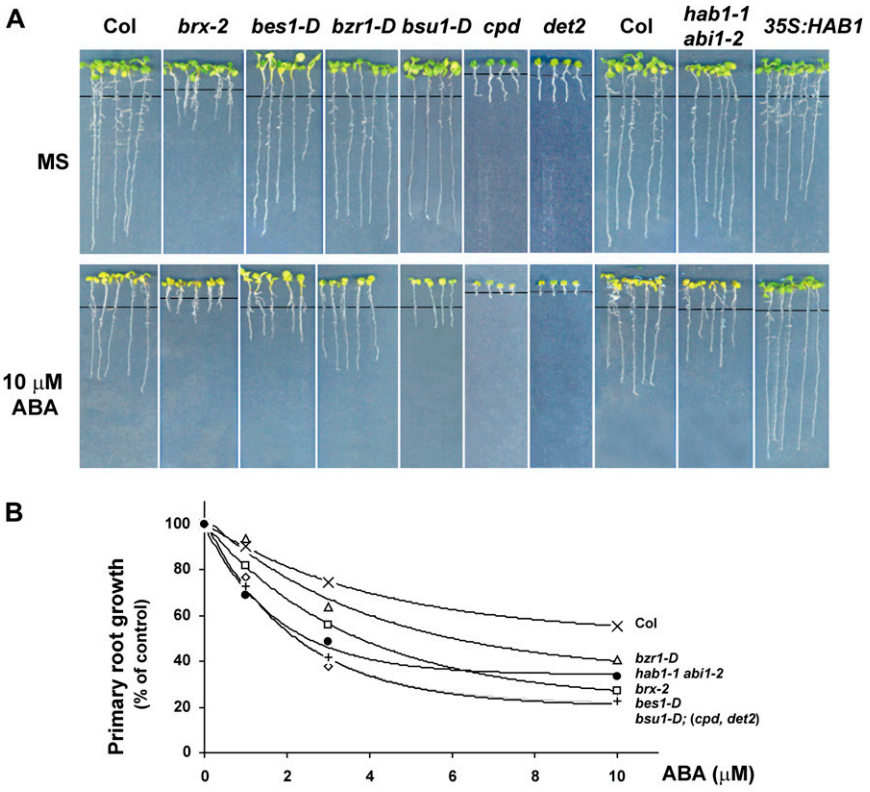

Figure 2. *bes1-D, bzr1-D, bsu1-D, cpd,* and *det2* mutants show higher sensitivity than the Columbia (Col) wild type to ABA-mediated root growth inhibition. A, Four-day-old seedlings were transferred to medium lacking or supplemented with 10 μM ABA and grown vertically for 6 d. B, Quantification of root growth inhibition by 1, 3, and 10 μM ABA in the indicated genotypes. Data are averages from three independent experiments ($n = 20$) and are expressed as percentage of root growth with respect to each genotype in the absence of ABA (SE < 4%; error bars are not visible due to overlapping with legend marks). *cpd* and *det2* graphics (data not shown) overlap those of *bes1-D* and *bsu1-D*. [See online article for color version of this figure.]

sensitive to ABA-mediated root growth inhibition as *bes1-D* (Fig. 2). It is noteworthy that all *bes1-D*, *bzr1-D*, and *bsu1-D* mutants show enhanced ABA-mediated inhibition of root growth even though they are not short-rooted mutants. Taken together, these results show that modulation of plant response to BR strongly affects ABA sensitivity of roots.

Triple *pp2c* Loss-of-Function Mutants Show Extreme ABA Hypersensitivity and Impaired Root Growth

Root sensitivity to ABA must be finely tuned to properly respond to changing environmental conditions and to prevent negative effects of ABA on root growth under well-watered conditions. As *brx-2* shows both a defect in root growth and enhanced sensitivity to ABA, we wondered whether mutants showing a hypersensitive response to ABA might be impaired in root growth. To answer this question, we have generated mutants that present different degrees of ABA hypersensitivity through combination (single, double, and triple) of loss-of-function mutations in the PP2Cs that act as negative regulators of ABA signaling (Saez et al., 2004, 2006; Fig. 3A). Thus, *hab1-1 abi1-2 pp2ca-1* and *hab1-1 abi1-2 abi2-2* triple mutants were generated and RT-qPCR analyses confirmed that expression of *HAB1*, *ABI1*, and either *PP2CA* or *ABI2*, respectively, was severely impaired (Supplemental Fig. S2). Whereas single and double *pp2c* mutants did not show a defect in root growth in the absence of exogenous ABA (Saez et al., 2004, 2006), both triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants were impaired in root growth, although less severely than *brx-2* (Fig. 3). Both triple mutants showed a constitutive transcriptional response to endogenous ABA levels (data not shown) and were extremely hypersensitive to exogenous addition of ABA in assays of root growth inhibition (Fig. 3). These results suggest that negative regulation of ABA signaling by PP2Cs is required to prevent inhibition of root growth by endogenous ABA levels.

Introduction of ABA Insensitivity into *brx-2* Improves Root Growth Rescue by BL Treatment

Exogenous application of BL partially rescued the root growth defect of both *brx-1* and *brx-2* (Mouchel et al., 2006; Fig. 1F). Taking into account the root phenotype found in triple *pp2c* mutants, we reasoned that the enhanced ABA sensitivity of *brx-2* might prevent a better rescue of its root growth defect by BL. To challenge this hypothesis, we conferred ABA insensitivity to *brx-2* by crossing it with a *35S-HAB1* transgenic line (Saez et al., 2004). Overexpression of

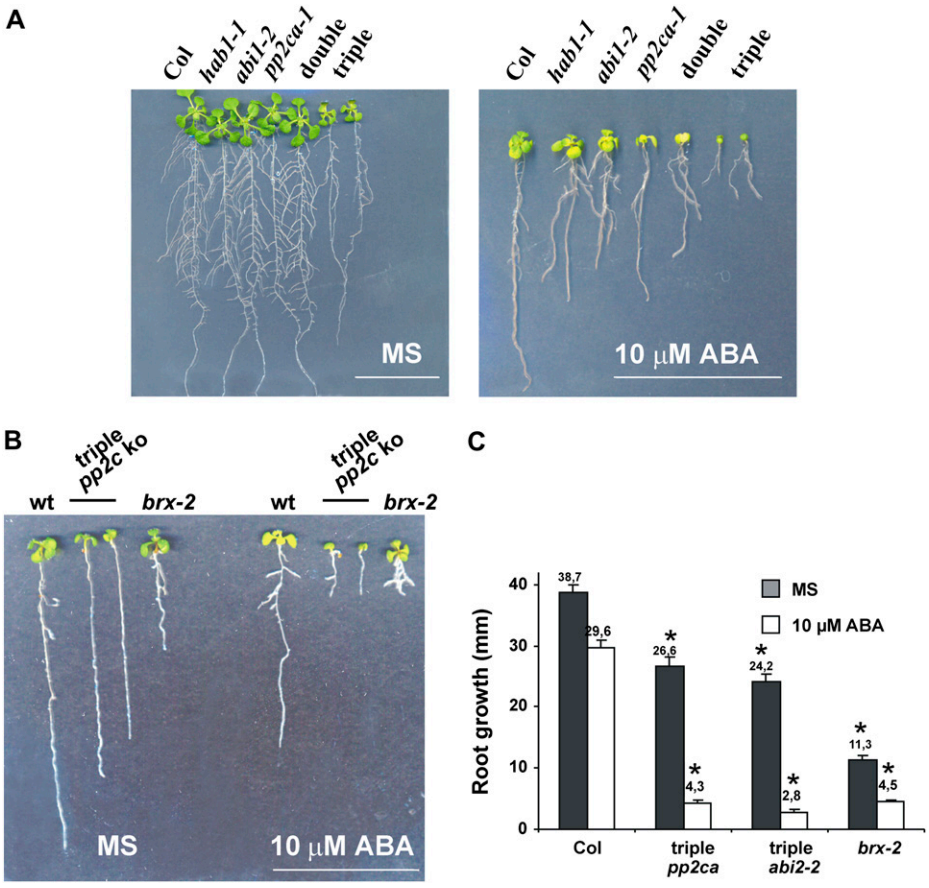

Figure 3. Impaired root growth of triple *pp2c* knockout (ko) mutants. A and B, Growth of the wild type (wt) and the ABA-hypersensitive mutants *hab1-1*, *abi1-2*, double *hab1-1 abi1-2*, and triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* in medium lacking or supplemented with 10 μM ABA. Bars = 3 cm. The photographs were taken at 12 d (A) or 6 d (B) after the transfer of 5-d-old seedlings from MS medium to plates containing 10 μM ABA. C, Quantification of root growth for the Columbia (Col) wild type, triple *pp2c* knockout mutants, and *brx-2* after 6 d in MS medium lacking or supplemented with 10 μM ABA. Data are averages $\pm$ SE from three independent experiments ($n = 20$). * $P < 0.05$ (Student's t test) when comparing data from each genotype with the wild type in the same growth conditions. [See online article for color version of this figure.]

the PP2C HAB1, which is a negative regulator of ABA signaling, leads to reduced sensitivity to ABA as compared with the wild type (Saez et al., 2004). F2 *brx-2* individuals that showed reduced sensitivity to ABA because of the presence of the *35S-HAB1* construct, and consequently enhanced expression levels of HAB1 (Fig. 4C), were selected, and their root growth in response to BL was analyzed. Figure 4 shows that 5 d after transfer onto BL medium, root growth in *brx-2* was enhanced to more than 120% of control length. By contrast, recovery of root growth to more than 150% of control length was achieved in *35S-HAB1*::*brx-2* lines. Importantly, BL treatment did not enhance root growth of *35S-HAB1* lines. These results suggest that disturbed regulation of ABA sensitivity in *brx-2* prevents full complementation of the root growth defect by exogenous addition of BL.

Transcriptomic Analysis of ABA Response in Roots of *brx-2* Compared with the Wild Type and the *cpd* Mutant

To further investigate the role of *BRX* in the modulation of root sensitivity to ABA, transcriptomic profiles of the wild type and *brx-2* were obtained from mock- or ABA-treated roots. Whole-genome long-oligonucleotide microarrays were used to compare ABA-mediated up-regulation/down-regulation of gene expression in the wild type and *brx-2* (Fig. 5A). The overlap of ABA up-regulated genes (ratio of expression >2-fold, false discovery rate $P < 0.05$) in the wild type and *brx-2* was approximately 75%. Among these genes, 507 of 1,727 were identified as differentially expressed in the wild type and *brx-2* (Fig. 5B; Supplemental Table S1): 250 genes were up-regulated by ABA to a greater extent in the *brx-2* mutant than in the wild type, whereas 257 genes showed higher induction by ABA in the wild type than in *brx-2* (Fig. 5B; Supplemental Table S1). With respect to ABA down-regulated genes (ratio of expression <0.5-fold, false discovery rate $P < 0.05$), *brx-2* showed a higher number of affected genes, 1,836, than the wild type, 1,396, and the overlap between those gene sets was 996 (Fig. 5A; Supplemental Table S1). Among them, 309 of 996 genes showed differential expression between the wild type and *brx-2*: 165 genes were down-regulated by ABA to a greater extent in *brx-2* than in the wild type, whereas 144 genes were more down-regulated in the wild type than in *brx-2* (Fig. 5B; Supplemental Table S1). Therefore, approximately 30% of genes that were ABA responsive (up-regulated or down-regulated) in both the wild type and *brx-2* showed differential expression between the two genotypes. Taken together, these data reveal a globally altered transcriptional response to ABA in *brx-2* compared with the wild type. Specifically, 250 and 166 genes showed enhanced ABA-mediated up-regulation and down-regulation, respectively, in *brx-2* compared with the wild type. However, some of these changes might be

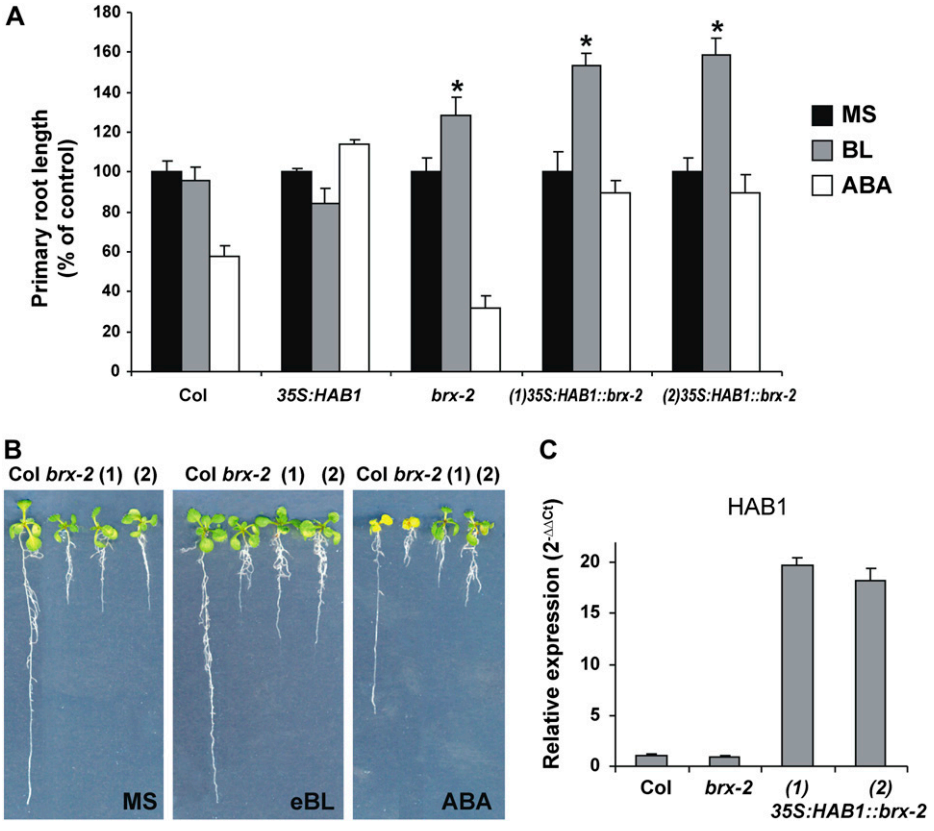

Figure 4. Improved root growth rescue by BL in *35S-HAB1*::*brx-2*. A, Relative root length of seedlings from the Columbia (Col) wild type, the *35S-HAB1* line, *brx-2*, and two representative transgenic *35S-HAB1*::*brx-2* lines. Seeds were germinated on MS medium, and 4-d-old seedlings were transferred to new MS plates lacking or containing either 2 nM BL or 10 μM ABA and grown in a vertical position for 6 d. Data are averages ± SE from three independent experiments ($n = 20$). * $P < 0.05$ (Student's t test) with respect to the same genotype in MS medium. B, Photographs of representative seedlings after 5 d of vertical growth in MS medium lacking or supplemented with either 2 nM BL or 10 μM ABA. C, RT-qPCR analysis of *HAB1* expression in the wild type, *brx-2*, and two transgenic *35S-HAB1*::*brx-2* lines. Values are relative expression levels with respect to the wild type (value 1). Data are averages ± SE from three independent experiments. [See online article for color version of this figure.]

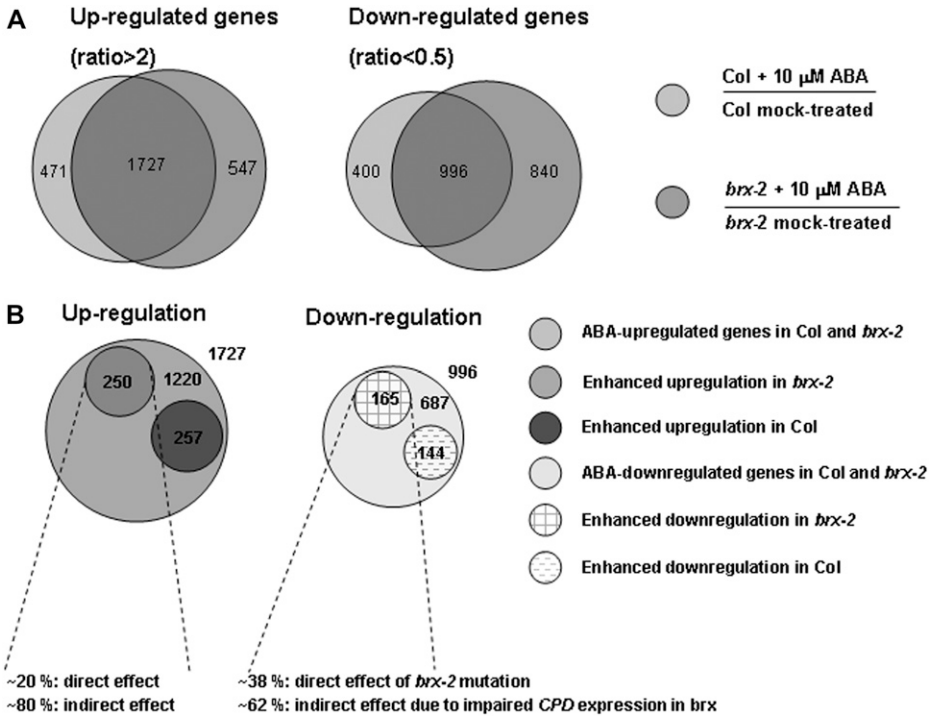

Figure 5. Transcriptomic analysis of ABA response in roots of *brx-2* compared with the Columbia (Col) wild type and the *cpd* mutant. A, Total number of ABA-responsive genes in the wild type and *brx-2* (threshold of 2-fold, ratio of >2 or <0.5 for up- and down-regulated genes, respectively; false discovery rate $P < 0.05$). B, Number of genes up- or down-regulated by ABA in both the wild type and *brx-2* that show a differential expression in *brx-2* compared with the wild type (threshold of 1.4-fold according to SAM; Tusher et al., 2001). Genes that showed enhanced response to ABA in *brx-2* were divided into two groups (direct effect of *brx-2* mutation or indirect effect due to impaired *CPD* expression in *brx*) according to the ratio of expression of *brx-2* + ABA to *cpd* + ABA (see text for a detailed explanation). A complete list of these genes is provided in Supplemental Table S1.

due to impaired expression of *CPD* in the *brx* background, and hence reduced BR biosynthesis (Mouchel et al., 2006). Indeed, the *cpd* mutant is very hypersensitive to ABA-mediated inhibition of root growth (Fig. 2A). To further explore this possibility, we have obtained the root transcriptomic profiles of *brx* + ABA compared with *cpd* + ABA. Genes that showed enhanced response to ABA in *brx-2* compared with the wild type were divided into two groups according to the ratio of expression of *brx-2* + ABA to *cpd* + ABA (Fig. 5B). Thus, when this ratio was between 0.5 and 2, we considered that the observed changes in ABA response represented an indirect effect of impaired *CPD* expression in the *brx* background. This first group comprised approximately 60% to 80% of the selected genes. However, a significant proportion of genes that showed enhanced response to ABA in *brx-2* compared with the wild type represented a direct consequence of the *brx-2* mutation, because the ratio of expression of *brx-2* + ABA to *cpd* + ABA was either greater than 2 or, to lesser extent, less than 0.5. Using this criterion, we found that 20% and 38% of genes showing enhanced ABA-mediated up-regulation or down-regulation (in *brx-2* compared with the wild type), respectively, are a direct consequence of the *brx-2* mutation. Interestingly, among the former genes, we identified several encoding RING finger E3 ligases related to the SDIR1 protein (Supplemental Table S1), which is a positive regulator of ABA signaling, since SDIR1 overexpression leads to ABA hypersensitivity (Zhang et al., 2007).

The ABA treatment of roots led to the up-regulation of genes involved in stress response: oxidative, osmotic, salt, heat shock, and cold stress as well as LEA proteins (Supplemental Table S1). ABA-mediated up-regulation of genes involved in the hyperosmotic stress response might be beneficial under low-water-potential conditions, as ABA promotes growth under those conditions (Sharp et al., 2004). On the other hand, the antagonism suggested for ABA and auxin in the development of root system architecture (Deak and Malamy, 2005; De Smet et al., 2006) was reflected in our transcriptomic analysis, as ABA treatment induced the expression of several Aux/IAA repressor proteins (IAA3, IAA6, IAA11, IAA18, IAA28, and IAA30), whereas it repressed several genes involved in auxin biosynthesis (Table I). This effect was observed in both the wild type and *brx-2*. However, taking into account that auxin-responsive gene expression is globally impaired in *brx* (Mouchel et al., 2006), it is reasonable to postulate that further impairment of auxin response by ABA will affect *brx* more negatively than the wild type.

DISCUSSION

In this work, starting from the isolation of *brx-2* as an ABA-hypersensitive mutant in root growth assays, we provide evidence for a role of BRX as a modulator of ABA sensitivity in roots. Importantly, the enhanced ABA response of *brx-2* was specific for the root, as ABA-mediated inhibition of seed germination and water loss kinetics were similar to those of the wild type. Thus, it appears likely that *BRX* mediates a root-specific branch of the ABA signaling pathway. Alternatively, it is conceivable that other *BRX-like* genes

Table I. *List of genes involved in auxin action that are up-regulated or down-regulated in brx-2 (threshold >2, ratio of >2 or <0.5 for up-regulated and down-regulated genes, respectively; false discovery rate P < 0.05)*

Category	Arabidopsis Genome Initiative Code	Gene Nomenclature	Col + ABA / Col Mock	*brx-2* + ABA / *brx-2* Mock
Aux/IAA genes	AT4G28640	IAA11	3.8	3.1
	AT5G25890	IAA28	2.1	3.1
	AT1G51950	IAA18	2.8	2.6
	AT1G52830	IAA6	4.8	2.8
	AT1G04240	IAA3	3.5	4.1
	AT3G62100	IAA30	6.1	7.2
GH3 family				
	AT5G13360		3.0	3.5
	AT5G13370		15.6	16.1
Auxin transport				
	AT5G57090	PIN2	0.6	0.4
	AT2G47000	PGP4	1.4	0.4
	AT3G28860	PGP19	0.7	0.4
F-box protein TIR1 family				
	AT4G03190	AFB1	0.6	0.4
Auxin biosynthesis				
	AT5G05730	ASA1, AMT1	0.2	0.4
	AT1G25220	TRP4	0.4	0.4
	AT3G54640	TRP3	0.2	0.4
	AT2G20610	ALF, HLS3, RTY, SUR1	0.2	0.3

(*BRXL*s) might have a role in ABA response that is masked by functional redundancy in the *brx-2* mutant. *BRX* is expressed in the columella and the phloem vasculature throughout root and shoot (Mouchel et al., 2006). Vascular expression of *BRX* was also detected in a torpedo-stage embryo (Mouchel et al., 2006). However, global expression of *BRX* as well as *BRXL1*, *BRXL2*, and *BRXL3* is low, as demonstrated by their relative expression compared with a housekeeping gene, *eIF4* (Mouchel et al., 2006), or their embryo expression compared with *HAB1*, which plays an important role in the control ABA sensitivity in seeds (Saez et al., 2004; Supplemental Fig. S3). It is likely that *BRX* expression at stomata is low, and the expression of other *BRXL*s might lead to genetic redundancy and therefore a lack of phenotype in transpiration assays (Supplemental Fig. S3).

As BRX has been implicated previously in connecting the auxin and BR pathways, our results suggest an interaction between the auxin and/or BR and ABA pathways in root development. For the BR pathway, we tested this notion directly by investigating whether other mutants affected in BR action show altered sensitivity to ABA in root growth assays. Indeed, *sax1* (Ephritikhine et al., 1999) and *det2* (Fig. 2), which are defective in BR biosynthesis, as well as *bri1-1* (Clouse et al., 1996), which is defective in BR signaling, are impaired in root growth and extremely hypersensitive to ABA-mediated inhibition. Paradoxically, an enhanced response to BR also increased the inhibitory effect of ABA on root growth, as *bzr1-D*, *bes1-D*, and *bsu1-D* showed constitutive BR response and were hypersensitive to ABA-mediated root growth inhibition. Therefore, a similar morphological response to ABA is generated when BR action is disturbed because of a BR biosynthetic/signaling defect or through mutations that lead to constitutive BR response phenotypes. These results suggest that homeostatic control of BR signaling is required for a normal response to ABA. Alternatively, it has been demonstrated that BZR1, in addition of being a positive regulator of the BR signaling pathway, also mediates negative feedback regulation of BR biosynthesis (Wang et al., 2002). Likewise, in-depth analysis of *bes1-D* microarray data reveals that several biosynthetic genes are down-regulated in this mutant (Vert et al., 2005). Therefore, it is possible that the lower BR levels found in *bzr1-D* and, presumably, *bes1-D* might be responsible of their ABA-hypersensitive phenotype. Nevertheless, the fact that *bes1-D*, *bzr1-D*, and *bsu1-D* are all ABA hypersensitive but, unlike BR biosynthesis mutants or *bri1*, do not display a defect in root growth supports the idea that a branch of the BR signaling pathway directly impinges on ABA response. Our striking observation that introduction of ABA insensitivity into the *brx-2* background significantly enhances the rescue of the short-root phenotype by exogenous BL treatment supports this idea. It will be interesting to see whether the phenotype of other genuine BR signaling mutants like *bri1* can be partially healed in a similar fashion.

Conversely, one might expect that impairment of the ABA signaling pathway would yield a root growth phenotype. Indeed, the fact that enhanced sensitivity to ABA impairs root growth appeared to be masked by genetic redundancy, as demonstrated by the phenotype of triple knockouts impaired in some of the PP2Cs that act as negative regulators of ABA signaling (Saez

et al., 2006). Both triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants were extremely sensitive to the inhibitory effect of ABA on root growth, and interestingly, root growth was notably reduced in the triple mutants in control conditions as compared with the wild type. Taken together, both our physiological and phenotypic analyses reveal that disturbed regulation of root ABA sensitivity leads to inhibition of root growth.

With respect to regulation of root growth, it has been suggested that BR biosynthesis and auxin signaling are connected through a feedback mechanism that involves BRX. Transcriptomic analysis showed that impaired auxin-responsive gene expression in the *brx-1* mutant could be restored by BL application, indicating feedback between BR levels and auxin signaling in root growth (Mouchel et al., 2006). ABA treatment in roots of the wild type and *brx-2* led to up-regulation of Aux/IAA repressor genes as well as down-regulation of genes involved in auxin biosynthesis (Table I). Aux/IAA repressor proteins are known to be negative regulators of auxin signaling through dimerization with auxin response factors, as Aux/IAA proteins prevent auxin response factors from promoting transcription of auxin-responsive genes (Tiwari et al., 2001, 2003). Therefore, ABA treatment had a negative effect on auxin signaling in roots. This effect was observed in both the wild type and *brx-2*. However, taking into account that auxin-responsive gene expression is globally impaired in *brx* (Mouchel et al., 2006), it is reasonable to suggest that further impairment of auxin response by ABA will have a stronger effect on root growth in *brx* than in the wild type. This effect might partially explain why *brx-2* is more sensitive to the inhibitory effect of ABA on root growth. Additionally, the differential ABA-mediated up-regulation of RING finger E3 ligases related to the SDIR1 protein in roots of *brx-2* might also contribute to the enhancement of ABA response. Finally, a direct comparison of transcriptomic response to ABA in the wild type and *brx-2* revealed that approximately 30% of ABA up-regulated and ABA down-regulated genes were differentially expressed in the wild type and *brx-2*. These results suggest that BRX has an important role in regulating transcription in response to ABA. Thus, it appears likely that enhanced stress perception due to impaired ABA response is partly responsible not only for the short-root phenotype but also for the pronounced perturbation of the transcriptome in *brx* mutants.

Finally, our analyses of BR biosynthesis and signaling mutants suggest that enhanced ABA and thus stress perception might significantly contribute to the root phenotypes of various hormone pathway mutants. Such perturbation of ABA sensitivity might be variable depending on the context and the level at which a given pathway is interrupted, but it could explain the seemingly disparate root growth phenotypes of signaling mutants that have been shown to reside in the same pathway. Future analyses using the transgenic approach described here might help to clarify whether this is indeed the case.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite:soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol containing 0.1% Triton X-100 for 20 min, followed by four washes with sterile distilled water. After stratification in the dark at 4°C for 2 d, seeds were sowed on plates containing Murashige and Skoog (MS) medium with 0.1% MES, 1% agar, and 1% Suc. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber at 22°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 μE m^{-2} s^{-1}.

Screening Conditions

T-DNA lines were constructed in D. Weigel's and C. Somerville's laboratories using the pSKI15 vector, which encodes a phosphinothricin resistance gene (BAR driven by 5′pMAS). Approximately 86,000 independent lines (stock nos. N21995, N21991, N23153, and N31100) were provided by the Arabidopsis Biological Resource Center. After surface sterilization, seeds were sown and grown on vertically oriented plates containing MS medium. After 5 d, seedlings were transferred to plates containing MS medium supplemented with 30 μM ABA. Potential ABA-hypersensitive mutants were selected after 5 d and left for 2 d in MS medium for recovery; finally they were transferred to soil.

Genetic Analysis

The backcross of the *sha1* mutant to the Columbia wild type was performed by transferring pollen to the stigmas of emasculated flowers. F1 and F2 seedlings were scored for root growth in the absence or presence of exogenous ABA. From the segregating F2 generation, homozygous *brx-2* individuals were selected and DNA was individually extracted to perform a cosegregation analysis between the *BRX* T-DNA insertion and the short-root phenotype. To this end, the following primers were used: F417 (5′-GTCAGTGTTTGCTTCCTCTCTATG-3′), R650 (5′-TATTTCCTTGTCTAGGTAAGAATCC-3′), and SKI3 (5′-TGATCCATGTAGATTTCCCGGACATGAA-3′). Additionally, the analysis of F2 *sha1* seedlings revealed cosegregation between the *sha1* phenotype and phosphinothricin resistance.

Generation of Triple *pp2c* Loss-of-Function Mutants

The double *hab1-1 abi1-2* mutant has been described previously (Saez et al., 2006). Lines carrying T-DNA insertions either in ABI2 (SALK_015166, *abi2-2* allele) or PP2CA (SALK_028132, *pp2ca-1* allele) were identified in the SALK T-DNA collection (Alonso et al., 2003), and homozygous mutants were kindly provided by Dr. Julian Schroeder. To generate the triple *hab1-1 abi1-2 abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants, we transferred pollen of either *abi2-2* or *pp2ca-1* to the stigmas of emasculated flowers of the double *hab1-1 abi1-2* mutant. The resulting F2 individuals were genotyped by PCR in order to identify the triple mutants.

Thermal Asymmetric Interlaced PCR

DNA was obtained either through a cetyl-trimethyl-ammonium bromide-based isolation procedure or using the DNAeasy Plant Mini Kit (Qiagen). DNA samples were treated with RNase, extracted with phenol-chloroform-isoamyl alcohol, and ethanol-sodium acetate precipitated. Plant T-DNA-flanking sequences were amplified by PCR according to the protocols of Liu et al. (2005). To this end, the following primers were used: SKI1 (5′-AATTGGTAATTACTCTTTCTTTTCCTCCATATTGA-3′), SKI2 (5′-ATATTGACCATCATACTCATTGCTGATCCAT-3′), SKI3 (5′-TGATCCATGTAGATTTCCCGGACATGAA-3′), AD1 [5′-TG(AT)G(ACGT)AG(GC)A(ACGT)CA(GC)AGA-3′], AD2 [5′-(ACGT)TCGA(GC)T(AT)T(GC)G(AT)GTT-3′], AD3 [5′-(ACGT)GTCGA(GC)(AT)GA(ACGT)A(AT)GAA-3′], AD4 [5′-AG(AT)-G(ACGT)AG(AT)A(ACGT)CA(AT)AGG-3′]; AD5 [5′-(AT)GTG(ACGT)AG-(AT)A(ACGT)CA(ACGT)AGA-3′], and AD6 [5′-(GC)TTG(ACGT)TA(GC)T-(ACGT)CT(ACGT)TGC-3′].

Complementation of *brx-2*

BRX cDNA was ordered from RIKEN (RAFL15-04-H19) and amplified using the following primers: FATG (5′-ATGTTTTCTTGCATAGCTTGTAC-3′) and Rstop (5′-TTAGAGGTACTGTGTTTGTATTC-3′). The PCR product was cloned into the pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the pMDC32 destination vector (Curtis and Grossniklaus, 2003). The *pMDC32-35S:BRX* construct was transferred to *Agrobacterium tumefaciens* C58C1 (pGV2260; Deblaere et al., 1985) by electroporation and used to transform the *brx-2* mutant (phosphinothricin resistant) by the floral-dipping method. Seeds of transformed plants were harvested and plated on hygromycin (20 μg mL^{-1}) selection medium to identify T1 transgenic plants. T3 progeny that were homozygous for the selection marker were used for further studies.

Germination and Root Growth Assays

To determine sensitivity to inhibition of germination by ABA, the medium was supplemented with 0.5 or 0.8 μM ABA. To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. Approximately 200 seeds of each genotype were sown in each medium and scored for germination and early growth 10 d later. For root growth assays, seedlings were grown on vertically oriented MS medium plates for 4 to 5 d. Afterward, 20 plants were transferred to new plates containing MS medium lacking or supplemented with the indicated concentrations of ABA or BL. After the indicated period of time, the plates were scanned on a flat-bed scanner to produce image files suitable for quantitative analysis using the NIH Image software (ImageJ version 1.37).

RNA Analysis

Root tissue was collected from 2-week-old plants that were either mock treated or treated for 3 h with 10 μM ABA and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit, and 1 μg of the RNA solution obtained was reverse transcribed using 0.1 μg of oligo(dT)$_{15}$ primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40-μL cDNA solution. RT-qPCR amplifications and measurements were performed using an ABI PRISM 7000 Sequence Detection System (Perkin-Elmer Applied Biosystems). The sequences of the primers used for PCR amplifications were as follows: for *BRX*, forward, 5′-AGTCAGATTCAGCCGGGAACG-3′, and Rstop; for *HAB1* (At1g72770), forward, 5′-AACTGCTGTTGTTGCCTTG-3′, and reverse, 5′-GGTTCTGGTCTTGAACTTTCT-3′; for *ABI1* (At4g26080), forward, 5′-ATGATCAGCAGAACAGAGAGT-3′, and reverse, 5′-TCAGTTCAAGGGTTTGCT-3′; for *ABI2* (At5g57050), forward, 5′-AGTGACTTCAGTGCGGCGAGT-3′, and reverse, 5′-CCTTCTTTTTCAATTCAAGGAT-3′; for *PP2CA* (At3g11410), forward, 5′-CTTTGTCGTAACGGTGTAGC-3′, and reverse, 5′-TTGCTCTAGACATGGCAAGA-3′; and for *β-actin-8* (At1g49420), forward, 5′-AGTGGTCGTACAACCGGTATTGT-3′, and reverse, 5′-GAGGATAGCATGTGGAAGTGAGAA-3′.

RT-qPCR amplifications were monitored using the Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the *β-actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from three independent biological replicates.

RNA Amplification and Labeling for Microarray Analysis

Total RNA (1.25 μg) from three independent biological replicates was amplified and amino allyl labeled using the MessageAmp II aRNA kit (Ambion; http://www.ambion.com) and 5-(3-aminoallyl)-2′-deoxyuridine-5′-triphosphate (Ambion), according to the manufacturer's instructions. Approximately 80 to 90 μg of amplified amino allyl RNA (aRNA) was obtained. For each sample, 7.5 μg of aRNA was resuspended in the coupling buffer and labeled with either Cy3 or Cy5 Mono NHS Ester (Cy Dye Post-labeling Reactive Dye Pack; Amersham). The samples were purified with Megaclear (Ambion) according to the manufacturer's instructions. Incorporation of Cy3 and Cy5 was measured using 1 μL of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies; http://www.nanodrop.com/). For each hybridization, 200 pmol of Cy3 and Cy5 probes was mixed and volume reduced to 5 μL in a Speed-Vac. A total of 20 μg of poly(A) and 20 μg of yeast tRNA (Sigma-Aldrich) were added. Each mixed probe was fragmented by adding 1 μL of 10× fragmentation buffer (Ambion) and incubating at 70°C for 15 min. The reaction was stopped with 1 μL of stop solution (Ambion). The 11-μL final volume of each mixed probe was diluted in 90 μL of hybridization solution.

Microarray Hybridization

Three biological replicates were independently hybridized for each transcriptomic comparison. Microarray slides were composed of synthetic 70-mer oligonucleotides from the Operon Arabidopsis Genome Oligo Set version 3.0 (Qiagen; http://www.qiagen.com/) spotted on aminosilane-coated slides (TeleChem; http://www.arrayit.com) by the University of Arizona. Slides were rehydrated and UV cross-linked according to the details on the supplier's Web site (http://ag.arizona.edu/microarray/ methods.html). The slides were then washed twice for 2 min in 0.1% SDS, in sterile water for 30 s, and dipped in ethanol for 3 min with shaking. Arrays were drained with a 2,000*g* spin for 10 min. Slides were prehybridized in 6× SSC (Sigma), 0.5% (w/v) SDS (Sigma), and 1% (w/v) BSA at 42°C for 1 h, followed by two washes with milliQ water for 1 min and one rinse with isopropanol. Excess water was drained with a 2,000*g* spin for 10 min. For the hybridization, equal amounts of dye of each aRNA labeled with either Cy3 or Cy5, ranging from 200 to 300 pmol, were mixed with 20 μg of poly(A) and 20 μg of yeast tRNA (Sigma-Aldrich) in a volume of 9 μL. To this volume, 1 μL of RNA fragmentation buffer was added (RNA Fragmentation Reagents; Ambion), and after 15 min at 70°C, 1 μL of stop solution was added. Fragmented labeled RNA was directly mixed with hybridization solution containing 50 μL of deionized formamide (Sigma), 30 μL of 20× SSC, 5 μL of 100× Denhardt's solution (Sigma), and 5 μL of 10% SDS in a final volume of 100 μL. The hybridization mixture was denatured at 95°C for 5 min, spun briefly, and applied by capillary between a pretreated slide (see above) and a 60- × 42-mm coverslip LifterSlip (Erie Scientific). Slides were incubated overnight at 42°C in a microarray hybridization chamber (ArrayIt Hybridization Cassette; TeleChem). The next morning, the slides were washed sequentially once in 1× SSC, 0.1% SDS for 5 min at 30°C; once in 0.2× SSC, 0.1% SDS for 5 min at 30°C; twice in 0.1× SSC for 2 min each at 30°C; and finally six times at 0.01× SSC for 2 min at 25°C. Slides were dried by centrifugation at 2,000*g* for 10 min at room temperature. Hybridized microarray slides were scanned right after at 532 nm for Cy3 and 635 nm for Cy5 with a GenePix 4000B scanner (Axon Molecular Devices; http://www.moleculardevices.com) at 10-nm resolution and 100% laser power. Photomultiplier tube voltages were adjusted manually to equal the overall signal intensity for each channel, to increase the signal-to-noise ratio, and to reduce the number of spots with saturated pixels. Spot intensities were quantified using GenePix Pro 6.0 microarray analysis software (Axon Molecular Devices). Data were normalized by mean global intensity and with Lowess (locally weighted scatterplot) correction (Yang et al., 2001) using GenePix Pro 6.0 and Acuity 4.0 software (Axon Molecular Devices), respectively. After image analysis, spots with a net intensity in both channels lower than twice the median signal background were removed as low-signal spots, and only probes for which we obtained valid data on at least two of the three slides were considered for further analysis.

Identification of Differentially Expressed Genes and Gene Ontology Analysis

Significance analysis of microarrays (SAM; Tusher et al., 2001) was performed on the three normalized data sets to identify differentially expressed genes. The parameters for SAM were adjusted so that the false discovery rate for every experiment was 0.05. A 2-fold expression cutoff was considered to determine up-regulated and down-regulated genes. In order to establish differences in expression between the two genotypes, a 1.4-fold threshold and a false discovery rate of 0.05 were considered. A functional category analysis of the genes simultaneously up-regulated or down-regulated in the two genotypes was carried out by the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Only overrepresented categories with *P* values smaller than 0.05 were further considered. Venn diagrams were generated to illustrate differences in expression.

Supplemental Data

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

Supplemental Figure S1. Detached leaf water-loss assays show similar water loss in *brx-2* and the wild type, whereas reduced water loss was found in the double *hab1-1 abi1-2* mutant.

Supplemental Figure S2. RT-qPCR analysis of *HAB1*, *ABI1*, *ABI2*, and *PP2CA* expression in the wild type, double *hab1-1 abi1-2*, and triple *pp2c* knockout mRNAs prepared from 2-week-old roots.

Supplemental Figure S3. Expression levels of *BRX*, *BRXL*, and *HAB1* genes at different stages of embryo development and guard/mesophyll cells.

Supplemental Table S1. Complete list of ABA-responsive genes that show enhanced up-regulation or down-regulation in *brx-2* compared with Columbia and vice versa (false discovery rate $P < 0.05$).

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