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BRX modulates root growth sensitivity to ABA

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The short-rooted phenotype of the *brevis radix* mutant partly reflects root ABA hypersensitivity

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Abstract

To gain further insight into ABA signaling and its role in growth regulation, we have screened for *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 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 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 that 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 BL treatment. Finally, transcriptomic analysis revealed that ABA treatment negatively affects auxin signaling in wt 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.

Introduction

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; Razem et al., 2006; de Smet 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 and *Arabidopsis*, 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 concentration, whereas low concentrations of ABA (<1 M) stimulate root growth (Zeevaart and Creelman, 1998; 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 QTL in maize that simultaneously affects

ABA biosynthesis and root agronomical traits both under 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 modulation of GA-mediated DELLA protein destabilization (Fu and Harberd, 2003). Auxin signaling itself shows interdependency with BR signaling (Nemhauser et al., 2004; Nakamura et al., 2006, Hardtke et al., 2007). Other phyothormones (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 dissect 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; Ghassemian et al., 2000; Beaudoin et al., 2000; Steber and McCourt, 2001; Cheng et al., 2002; Zhu, 2002; Finkelstein et al., 2002; Rock and Sun, 2005; Torres-Zabala et al., 2007; Adie 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 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 EXPRESSION BEE1, BEE2 and BEE3 transcription factors was repressed by ABA, and *BEE1* over-expressing roots were hypersensitive to BRs and partially insensitive to ABA (Friedrichsen et al., 2002). However, good candidates that could explain the ABA-BR crosstalk at the molecular level have not been identified.

To further extend our knowledge on 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 Columbia background, named *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 *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 ~30% root length of wt 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 CaMV *35S* promoter was slightly more efficient (~60% of wt control) to rescue 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 a 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 a Columbia background. Seeds were germinated vertically in the absence of ABA and then 5-day-old seedlings were transferred to plates supplemented with 30 M ABA. Potential ABA-hypersensitive mutants were initially identified on the basis of impaired growth compared to wild type. After screening of approximately 20000 lines, several candidates were selected and initially named *seedling hypersensitive to ABA* (*sha*) mutants. In the absence of ABA, the *sha1* mutant showed a slight decrease in root growth at 5 days compared to wt, which was notably increased at further stages of development (Fig. 1A). As it is discussed below, *sha1* was found to be allelic to *brx* and accordingly, we renamed it as *brx-2*. The presence of 10 M ABA in the medium exacerbated the root growth defect in *brx-2* compared to wt (73% and 45% inhibition, respectively) (Figure 1A and 1B). 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 (Figure 1B), suggesting that the observed ABA hypersensitivity of *brx-2* does not simply reflect disproportional 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 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 than wild type both in the absence and presence of ABA (data not shown). The segregation ratio in the F2 progeny was consistent with a single, recessive mutation (132 wild type: 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 co-segregation of the *sha1* phenotype and phosphinothricin resistance. Plant T-DNA flanking sequences were isolated from the mutant by TAIL-PCR and sequence analysis revealed that the pSKI15 T-DNA was inserted at nucleotide 553 of the *BREVIS RADIX* (*BRX*, *At1g31880*) gene (Fig. 1D). A natural loss-of-function allele of *BRX* in the *Arabidopsis* accession Umkirch-1 (Uk-1) had been previously reported (Mouchel et al., 2004), which we name as *brx-1*, and therefore we have renamed *sha1* as *brx-2*. Finally, the analysis of 52 F2 *brx-2* chromosomes showed co-segregation 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 *brx-1* mutant, exogenous application of brassinolide (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 (Yin et al., 2002 and 2005; Wang et al., 2002; He et al., 2005). Therefore, we examined ABA sensitivity of *bes1-D* and *bzr1-D* mutants in root growth assays and we 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 and 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 M ABA, and a stronger phenotype at 10 M 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 hypersensitive 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; Saez et al., 2006) (Figure 3A). Thus, *hab1-1 abi1-2 pp2ca-1* and *hab1-1 abi1-2 abi2-2* triple mutants were generated and real-time quantitative (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; Saez et al., 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) (Figure 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). Over-expression of the PP2C HAB1, which is a negative regulator of ABA signaling, leads to reduced sensitivity to ABA as compared to 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 (Figure 4C), were selected and their root growth in response to BL was analysed. Figure 4 shows that 5 days after transfer onto BL media, root growth in *brx-2* was enhanced to >120% of control length. By contrast, recovery of root growth to >150% of control length was achieved in *35S-HAB*1::*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 to wild type and *cpd* mutant

To further investigate the role of *BRX* in the modulation of root sensitivity to ABA, transcriptomic profiles of wild type and *brx-2* were obtained from mock- or ABA-treated roots. Whole-genome long-oligonucleotide microarrays were used to compare ABA-mediated upregulation/downregulation of gene expression in wild type and *brx-2* (Figure 5A). The overlap of ABA-upregulated genes (ratio of expression

>2-fold, false discovery rate p<0.05) in wild type and *brx-2* was approximately 75%. Among these genes, 507 out of 1727 were identified as differentially expressed in wild type and *brx-2* (Figure 5B; Supplemental Table S1): 250 genes were upregulated by ABA to a greater extent in the *brx-2* mutant than in wild type, whereas 257 genes showed higher induction by ABA in wild type than *brx-2* (Figure 5B; Supplemental Table S1). With respect to ABA-downregulated genes (ratio of expression <0.5-fold, false discovery rate p<0.05), *brx-2* showed a higher number of affected genes, 1836, than wild type, 1396, and the overlap between those gene sets was 996 (Figure 5A; Supplemental Table S1). Among them, 309 out of 996 showed differential expression between wild type and *brx-2*: 165 genes were downregulated by ABA to a greater extent in *brx-2* than in wild type, whereas 144 genes were more downregulated in wild type than in *brx-2* (Figure 5B; Supplemental Table S1). Therefore, approximately 30% of genes that were ABA-responsive (upregulated or downregulated) in both 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 to wild type. Specifically, 250 and 166 genes showed enhanced ABA-mediated upregulation or downregulation, respectively, in *brx-2* compared to wild type. However, some of these changes might be due to impaired expression of *CPD* in *brx* background, and hence reduced BR biosynthesis (Mouchel et al., 2006). Indeed, *cpd* mutant is very hypersensitive to ABA-mediated inhibition of root growth (Figure 2A). To further explore this possibility we have obtained the root transcriptomic profile of *brx*+ABA compared to *cpd*+ABA. Genes that showed enhanced response to ABA in *brx-2* compared to wt were divided in two groups according to the ratio of expression *brx-2* + ABA/ cpd + ABA (Figure 5B). Thus, when this ratio was comprised between 0.5 and 2, we considered that the observed changes

in ABA response represented an indirect effect of impaired *CPD* expression in *brx* background. This first group comprised ~60-80% of the selected genes. However, a significant proportion of genes that showed enhanced response to ABA in *brx-2* compared to wt represented a direct consequence of the *brx-2* mutation because the ratio of expression *brx-2* + ABA/ cpd + ABA was either >2 or, to lesser extent, <0.5. Using this criterion, we found that 20% and 38% of genes showing enhanced ABA-mediated upregulation or downregulation (in *brx-2* compared to wt), respectively, are a direct consequence of the *brx-2* mutation. Interestingly, among the former genes we identified several one 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 upregulation of genes involved in stress response: oxidative stress, osmotic, salt, heat shock and cold as well as LEA proteins (Supplemental Table S1). ABA-mediated upregulation of genes involved in 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 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, IAA30), whereas it repressed several genes involved in auxin biosynthesis (Table I). This effect was observed in both wt 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 wt.

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 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 (*BRXLs*) 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 *BRX-like* genes (*BRXL1*, *BRXL2*, *BRXL3*) is low, as demonstrated by their relative expression compared to a housekeeping gene, *eIF4* (Mouchel et al., 2006), or embryo expression compared to *HAB1,* which plays an important role to control ABA-sensitivity in seed (Saez et al., 2004; Supplemental Fig. S3). Likely, *BRX* expression at stomata is low and expression of other *BRXLs* might lead to genetic redundancy and therefore lack of phenotype in transpiration assays (Supplemental Fig. S3).

As BRX has been previously implicated in connecting the auxin and brassinosteroid 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* (Figure 2), which are defective in BR biosynthesis, and *bri1-1* (Clouse et al., 1996), 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* or *bsu1-D* show 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 so far, 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 to 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 wild type and *brx-2* led to upregulation of Aux/IAA repressor genes as well as downregulation 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 (ARFs), as Aux/IAA proteins prevent ARFs from promoting transcription of auxin-responsive genes (Tiwari et al., 2001 and 2003). Therefore, ABA-treatment had a negative effect on auxin signaling in roots. This effect was observed in both wt 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 wt. 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 wild

type and *brx-2*, revealed that approximately 30% of ABA-upregulated and ABA-downregulated genes were differentially expressed in 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 not only partly responsible 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 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 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 4ºC for 2 d, seeds were sowed on plates containing Murashige-Skoog (MS) medium with 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar, and 1% sucrose. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16-h light, 8-h dark photoperiod at 80 to 100 E m^{-2} sec^{-1}.

Screening conditions

T-DNA lines were constructed in D. Weigel and C. Somerville laboratories using the pSKI15 vector, which encodes a phosphinothricin resistance gene (BAR driven by 5´pMAS). Approximately 86000 independent lines, stock numbers N21995, N21991, N23153 and N31100, were provided by the *Arabidopsis* Biological Resource Center (ABRC, Ohio, USA). After surface sterilization, seeds were sowed and grown on vertically oriented plates containing MS medium. After 5 days, seedlings were transferred to plates containing MS medium supplemented with 30 μM ABA. Potential ABA-hypersensitive mutants were selected after 5 days and left 2 days in MS medium for recovery, and finally they were transferred to soil.

Genetic analysis

The backcross of *sha1* mutant to 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 co-segregation analysis between the *BRX* T-DNA insertion and the short root phenotype. To this end, the following primers were used: F417, 5´-GTCAGTGTTTGCTTCCTCTCTATG, R650, 5´- TATTTCCTTGTCTA GGTAAGAATCC and SKI3, 5´-TGATCCATGTAGATTTCCCGGACA TGAA. Additionally, the analysis of F2 *sha1* seedlings revealed co-segregation between the *sha1* phenotype and phosphinothricin resistance.

Generation of triple *pp2c* loss-of-function mutants

The double *hab1-1abi1-2* mutant has been previously described (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 *hab1-1abi1-2* mutant. The resulting F2 individuals were genotyped by PCR in order to identify the triple mutants.

Thermal asymmetric interlaced PCR (TAIL-PCR)

DNA was obtained either through a CTAB-based isolation procedure or using the DNAeasy Plant Minikit (Qiagen). DNA samples were treated with RNase, extracted with phenol:chloroform:isoamylalcohol 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; SKI2, 5´-

ATATTGACCATCATACTCATTGCTGATCCAT; SKI3, 5´-TGATCCA TGTAGATTTCCCGGACATGAA; AD1, 5´-TG(AT)G(ACGT)AG(GC)A (ACGT)CA(GC)AGA; AD2, 5´-(ACGT)TCGA(GC)T(AT)T(GC)G(AT)G TT; AD3, 5´-(ACGT)GTCGA(GC)(AT)GA(ACGT)A(AT)GAA; AD4, 5´-AG(AT)G(ACGT)AG(AT)A(ACGT)CA(AT)AGG; AD5, 5´-(AT)GTG (ACGT)AG(AT)A(ACGT)CA(ACGT)AGA and AD6, 5´-(GC)TTG (ACGT)TA(GC)T(ACGT)CT(ACGT)TGC

Complementation of *brx-2*

BRX cDNA was ordered from RIKEN (RAFL15-04-H19) and amplified using the following primers: FATG, 5´-ATGTTTTCTTGCATAGCTTGTAC and Rstop, 5´-TTAGAGGTACTGTGTTTGTATTC. 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 g/ml) selection medium to identify T1 transgenic plants. T3 progenies 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 sowed in each medium and scored for germination and early

growth 10 days later. For root growth assays, seedlings were grown on vertically oriented MS medium plates for 4 to 5 days. Afterwards, 20 plants were transferred to new plates containing MS medium lacking or supplemented with the indicated concentrations of ABA or brassinolide. After the indicated period of time, the plates were scanned on a flatbed scanner to produce image files suitable for quantitative analysis using the NIH Image software (ImageJ v1.37).

RNA analysis

Root tissue was collected from two-week old plants that were either mock- or 10 M ABA-treated for 3 h and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy Plant Mini Kit and 1 g of the RNA solution obtained was reverse transcribed using 0.1 g oligo$(dT)_{15}$ primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40 l 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 the following ones: for *BRX*, forward 5´-AGTCAGATTCAGCCGGGAACG and Rstop, for *HAB1* (At1g72770), forward 5´-AACTGCTGTTGTTGCCTTG and reverse 5´- GGTTCTGGT CTTGAACTTTCT; for *ABI1* (At4g26080), forward 5´-ATGATCAGCAG AACAGAGAGT and reverse 5´-TCAGTTCAAGGGTTTGCT; for *ABI2* (At5g57050) forward 5´-AGTGAC TTCAGTGCGGCGAGT and reverse 5´- CCTTCTTTTTC AATTCAAGGAT; for *PP2CA* (At3g11410), forward 5´-CTTTGTCGTAACGGTGTAGC and reverse 5´-TTGCTCTAGACA TGGCAAGA, and for *β-actin-8* (At1g49420), forward 5´-AGTGGTCGT ACAACCGGTATTGT and reverse 5´-GAGGATAGCATGTGGAAG TGAGAA.

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 g) from three independent biological replicates was amplified and amino allyl-labeled using MessageAmp® II aRNA kit (Ambion, http://www.ambion.com) and 5-(3-aminoallyl)-2'-deoxyuridine-5'-triphosphate (aa-dUTP, Ambion), according to manufacturer's instructions. Approximately 80-90 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-labelling Reactive Dye Pack, Amersham). The samples were purified with Megaclear™ (Ambion) according to manufacturer's instructions. Incorporation of Cy3 and Cy5 was measured using 1 µl of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.; http://www.nanodrop.com/). For each hybridization 200 pmol of Cy3 and Cy5 probes was mixed and volume reduced to 5 l in a speed-vac. 20 g Poly(A) and 20 g of yeast tRNA (Sigma Aldrich) were added. Each mixed probe was fragmented by adding 1 µl of 10X fragmentation buffer (Ambion) and incubating at 70°C for 15 min. The reaction was stopped with 1 µl of stop solution (Ambion). The 11 l final volume of each mixed probe was diluted in 90 l 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-crosslinked according to the details on the supplier's web page 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 sec and dipped in ethanol for 3 minutes with shaking. Arrays were drained with a 2000 g spin for 10 min. Slides were pre-hybridized in 6X SSC (Sigma), 0.5% SDS (w/v) (Sigma), and 1% BSA (w/v) at 42°C for 1 h., followed by 2 washes with milliQ water for 1 minute and one rinse with isopropanol. Excess water was drained with a 2000 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 polyA 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. Fragmented labeled RNA was directly mixed with hybridization solution containing 50 µl deionized formamide (Sigma), 30 µl 20 × SSC, 5 µl 100 × Denhardt's solution (Sigma) and 5 µl 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 pre-treated 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,

slides were washed sequentially once in 1 × SSC 0.1% SDS 5 min at 30°C; once in 0.2 × SSC 0.1% SDS 5 min at 30°C; twice in 0.1 × SSC 2 min each at 30°C; and finally 6 times at 0.01 × SSC for 2 min at 25°C. Slides were dried by centrifugation at 2000g 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 scatter plot) 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 at least in the 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 (FDR) for every experiment was 0.05. A 2 fold expression cut-off 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 FDR of 0.05 was considered. A functional category analysis (FunCat) analysis of the genes simultaneously

up-regulated or down-regulated in the two genotypes was carried out by MIPS (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). Only over represented categories with a p-value smaller than 0.05 were further considered. Venn diagrams were generated to illustrate differences in expression.

Supplemental material

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

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

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

Supplemental Figure S3. Expression levels of *BRX*, *BRX-like* (*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-or down-regulation in *brx-2* compared to Col and viceversa (false discovery rate $p<0.05$). Genes that showed enhanced response to ABA in *brx-2* were divided in two groups according to the ratio of expression *brx-2* + ABA/ *cpd* + ABA. Those genes whose ratio was >2-fold are labeled in red.

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Figure 1. *brx-2* shows higher sensitivity than wild type to ABA-mediated root growth inhibition. A, Five-day-old seedlings (top panel) were transferred to medium lacking or supplemented with 10 M ABA (lower panels) and grown vertically for 6 days. The dotted line indicates the position of root tip just after transferring to new medium. B, Quantification of root growth inhibition by ABA in wild type, *brx-2*, double *hy5hyh* and *hab1-1abi1-2* mutants. Data are averages ±SE from three independent experiments (n=20). C, *brx-2* shows similar sensitivity than wild type to ABA-mediated inhibition of germination. Data are averages ±SE from three independent experiments (n=200). D, Scheme of *BRX* gene and localization of T-DNA insertion in *brx-2*. The numbering begins at the ATG translation start codon. Primers used for co-segregation 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 Col (value 1). Data are averages ±SE from three independent experiments. F, Relative root length of seedlings from wt (Col ecotype), *brx-2* and 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 brassinolide (BL) and grown vertically for 5 days. Root length of 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* (natural allele in Uk-1 accession), *brx-2* and complemented lines (*35S-BRX*::*brx-2; 35S-BRX* in Uk-1) in root growth assays. Data are averages from three independent experiments (n=20) (SE <3%, SE 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 WT in the same growth conditions.

Figure 2. *bes1-D*, *bzr1-D*, *bsu1-D*, *cpd* and *det2* mutants show higher sensitivity than wild type to ABA-mediated root growth inhibition. A, Four-day-old seedlings were transferred to medium lacking or supplemented with 10 M ABA and grown vertically for 6 days. B, Quantification of root growth inhibition by 1, 3 and 10 M ABA in the indicated genotype. 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%, bars are not visible due to overlapping with legend marks). *cpd* and *det2* graphics (not shown) overlap to those of *bes1-D* and *bsu1-D*.

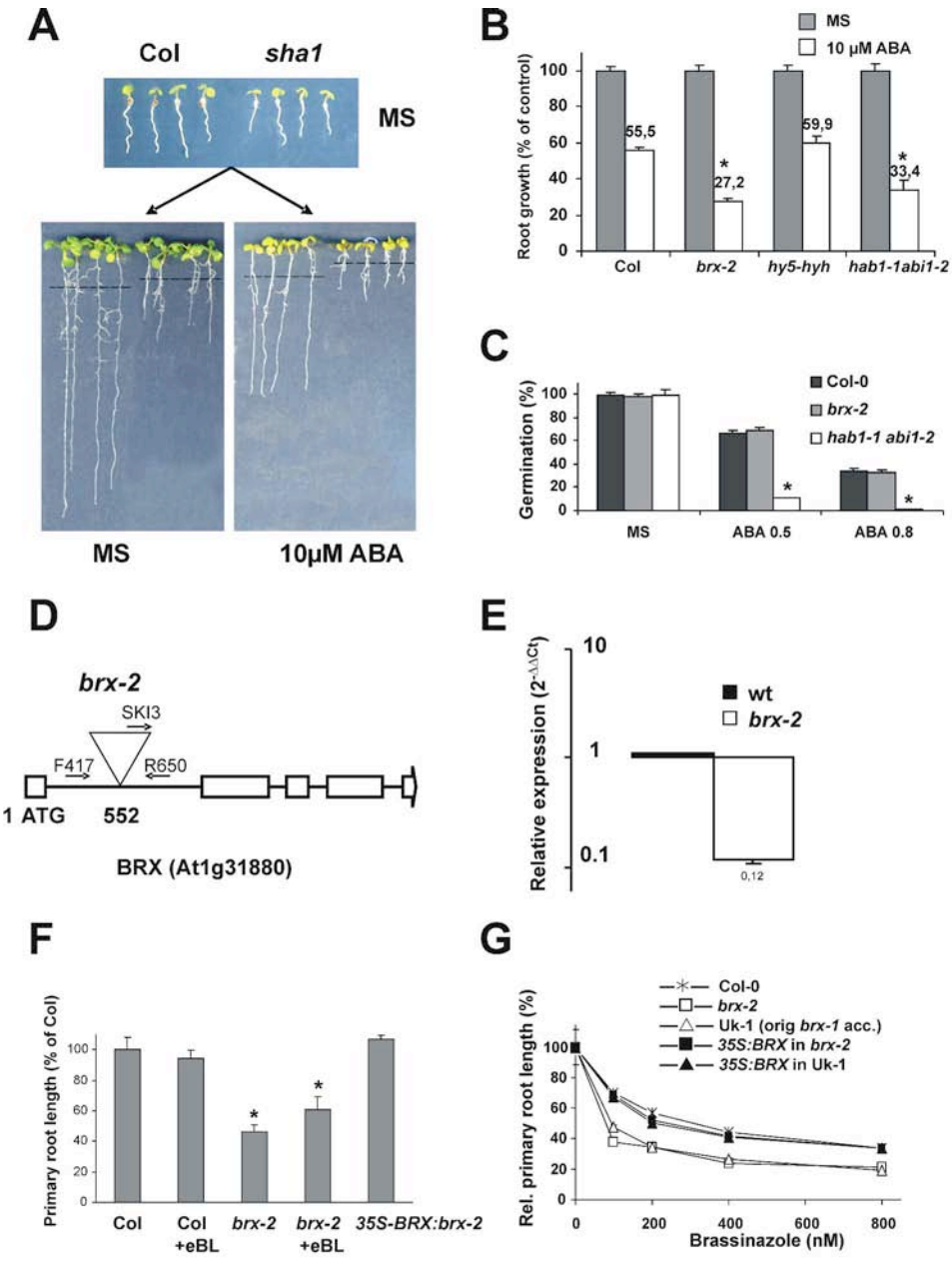
Figure 3. Impaired root growth of triple *pp2c* knockout (ko) mutants. A and B, Growth of wild type and ABA-hypersensitive mutants *hab1-1*, *abi1-2*, double *hab1-1abi1-2* and triple *hab1-1abi1-2abi2-2* and *hab1-1 abi1-2 pp2ca-1* mutants (triple *pp2c* ko) in medium lacking or supplemented with 10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

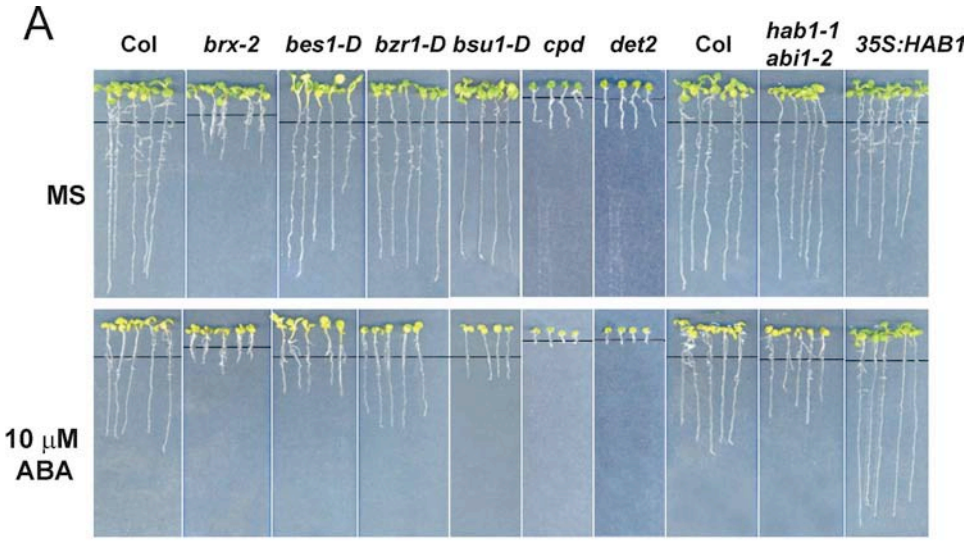
Figure 4. Improved root growth rescue by brassinolide (BL) in *35S-HAB1*::*brx*-2. A, Relative root length of seedlings from wt, *35S-HAB1* line, *brx-2* and two representative transgenic lines *35S-HAB1*::*brx-2*. Seeds were germinated on MS medium and four-day-old seedlings were transferred to new MS plates lacking or containing either 2 nM BL or 10 M ABA and grown in vertical position for 6 days. Data are averages ±SE from three independent experiments (n=20). * indicates P<0.05 (Student´s *t* test) with respect to the same genotype in MS medium. B, Photographs of representative seedlings after five days of vertical growth in MS medium lacking or supplemented with either 2 nM brassinolide (BL) or 10 M ABA. C, RT-qPCR analysis of *HAB1* expression in wt, *brx-2* and two transgenic lines *35S-HAB1::brx-2*. Values are relative expression levels with respect to Col (value 1). Data are averages ±SE from three independent experiments.

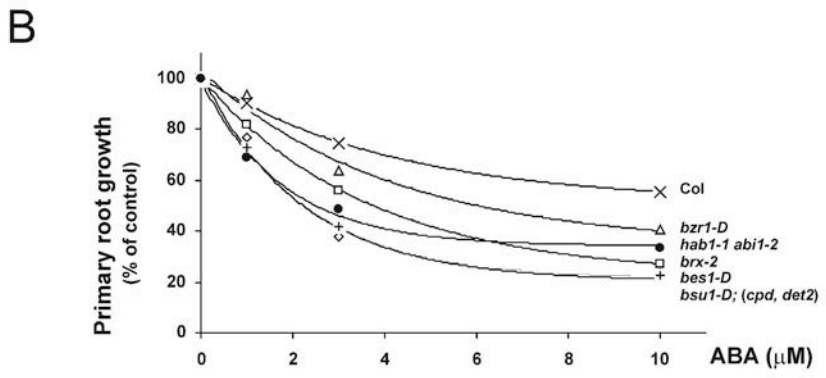
Figure 5. Transcriptomic analysis of ABA-response in roots of *brx-2* compared to wild type (Col) and *cpd* mutant. A, Total number of ABA-responsive genes in wt and *brx-2*. Threshold of 2-fold, ratio>2 or <0.5 for up- and down-regulated genes, respectively (false discovery rate $p<0.05$). B, Number of genes up- or down-regulated by ABA in both wt (Col) and *brx-2* that show a differential expression in *brx-2* compared to 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 in two groups (direct effect of *brx-2* mutation or indirect effect due to impaired *CPD* expression in *brx*) according to the ratio of expression *brx-2* + ABA/ *cpd* + ABA (see text for a detailed explanation). A complete list of these genes is provided in Table S1.

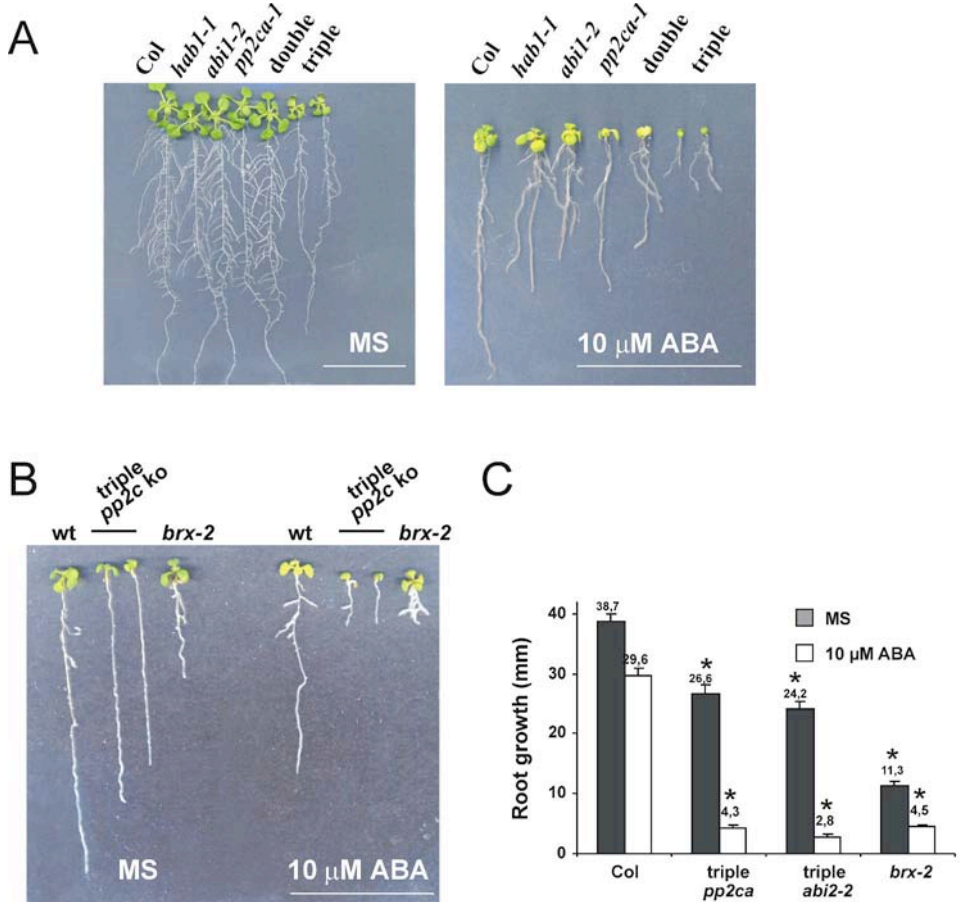
Table I. List of genes involved in auxin action that are up- or down-regulated in *brx-2* (threshold >2, ratio>2 or <0.5 for up- and down-regulated genes, respectively, false discovery rate p<0.05).

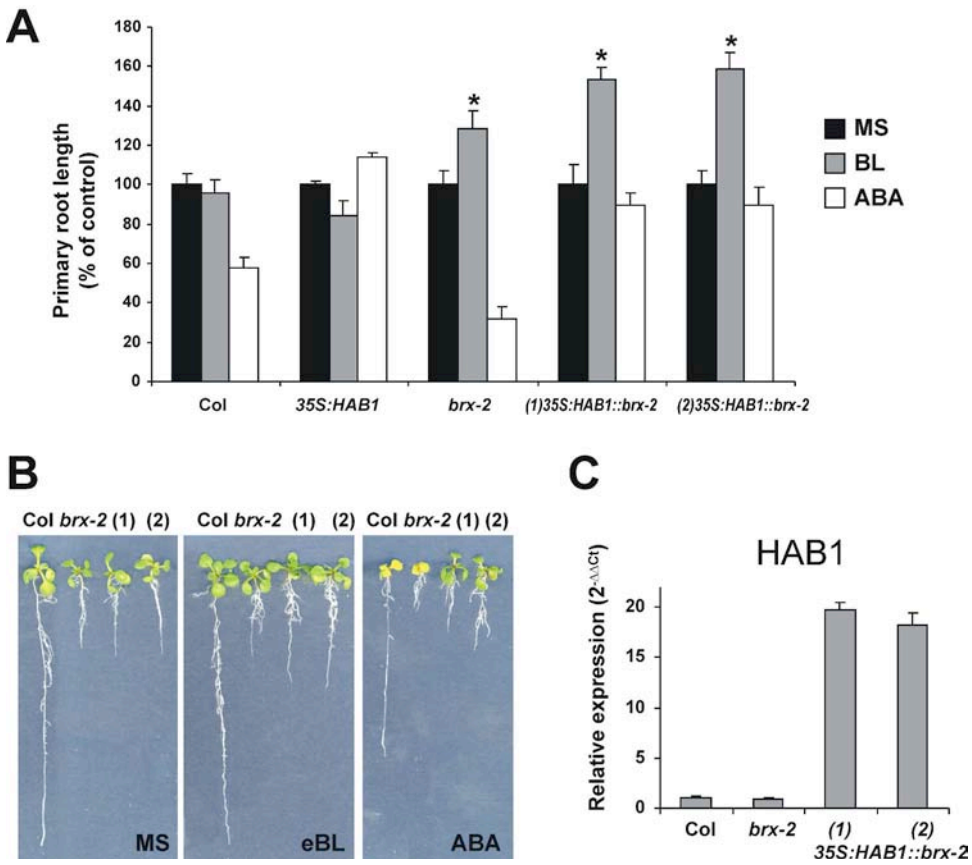
Category	AGI Code	Gene Nomenclature	Col + ABA / Col mock	*brx-2* + ABA / *brx-2* mock
Aux/IAA genes	AT4G28640	IAA11	3.8	3.1
	AT5G25890	IAA28	2.1	3.1
	AT1G51950	IAA18	2.8	2.6
	AT1G52830	IAA6	4.8	2.8
	AT1G04240	IAA3	3.5	4.1
	AT3G62100	IAA30	6.1	7.2
GH3 family				
	AT5G13360		3.0	3.5
	AT5G13370		15.6	16.1
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

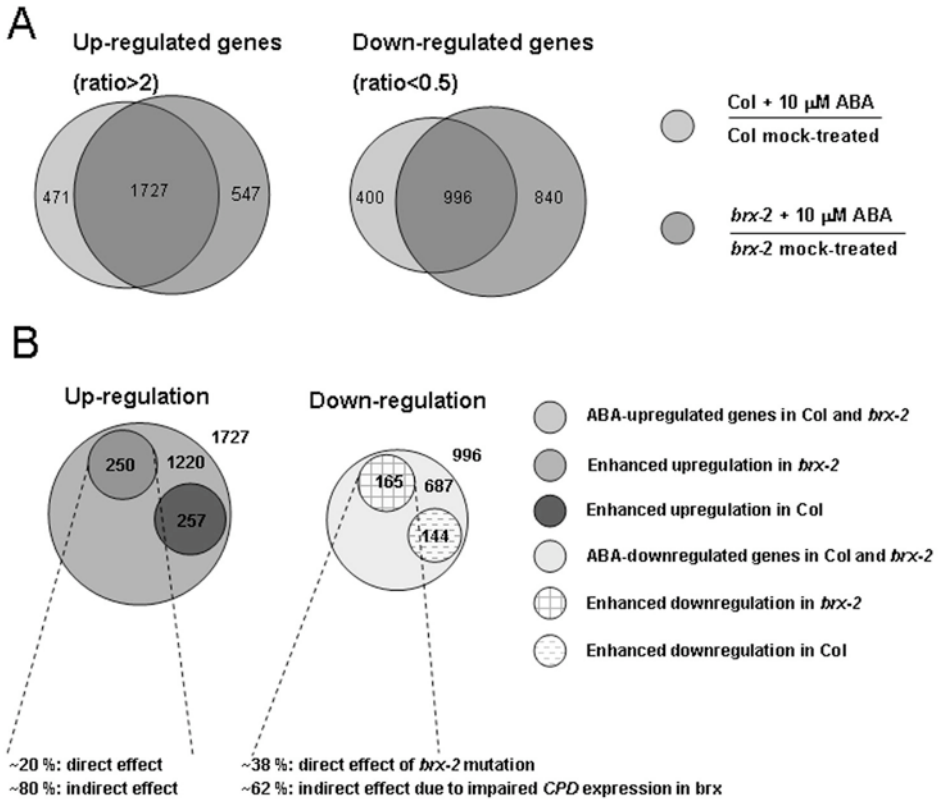

A
Col
sha1
MS
MS
10µM ABA
B
MS
10 µM ABA
Root growth (% of control)
55,5
27,2
59,9
33,4
Col
brx-2
hy5-hyh
hab1-1abi1-2
C
Germination (%)
Col-0
brx-2
hab1-1 abi1-2
MS
ABA 0.5
ABA 0.8
D
brx-2
SKI3
F417
R650
1 ATG
552
BRX (At1g31880)
E
Relative expression (2-ΔΔCt)
wt
brx-2
0,12
F
Primary root length (% of Col)
Col
Col +eBL
brx-2
brx-2 +eBL
35S-BRX:brx-2
G
Col-0
brx-2
Uk-1 (orig brx-1 acc.)
35S:BRX in brx-2
35S:BRX in Uk-1
Rel. primary root length (%)
Brassinazole (nM)


A
Col
brx-2
bes1-D
bzr1-D
bsu1-D
cpd
det2
Col
hab1-1
abi1-2
35S:HAB1
MS
10 μM
ABA


B
Primary root growth
(% of control)
100
80
60
40
20
0
0
2
4
6
8
10
ABA (μM)
Col
bzr1-D
hab1-1 abi1-2
brx-2
bes1-D
bsu1-D; (cpd, det2)


A
Col
hab1-1
abi1-2
pp2ca-1
double
triple
MS
10 μM ABA
B
triple pp2c ko
wt
brx-2
C
Root growth (mm)
MS
10 μM ABA
38,7
29,6
26,6
4,3
24,2
2,8
11,3
4,5
Col
triple pp2ca
triple abi2-2
brx-2


A
Primary root length (% of control)
180
160
140
120
100
80
60
40
20
0
*
*
*
MS
BL
ABA
Col
35S:HAB1
brx-2
(1)35S:HAB1::brx-2
(2)35S:HAB1::brx-2
B
Col brx-2 (1) (2)
Col brx-2 (1) (2)
Col brx-2 (1) (2)
MS
eBL
ABA
C
HAB1
Relative expression (2-ΔΔCt)
20
15
10
5
0
Col
brx-2
(1)
(2)
35S:HAB1::brx-2


A
Up-regulated genes
(ratio>2)
471
1727
547
Down-regulated genes
(ratio<0.5)
400
996
840
Col + 10 µM ABA
Col mock-treated
brx-2 + 10 µM ABA
brx-2 mock-treated
B
Up-regulation
1727
250
1220
257
Down-regulation
996
165
687
144
ABA-upregulated genes in Col and brx-2
Enhanced upregulation in brx-2
Enhanced upregulation in Col
ABA-downregulated genes in Col and brx-2
Enhanced downregulation in brx-2
Enhanced downregulation in Col
~20 %: direct effect
~80 %: indirect effect
~38 %: direct effect of brx-2 mutation
~62 %: indirect effect due to impaired CPD expression in brx