

The ABA Receptor PYL8 Promotes Lateral Root Growth by Enhancing MYB77-Dependent Transcription of Auxin-Responsive Genes

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The phytohormone abscisic acid (ABA) regulates plant growth, development, and abiotic stress responses. ABA signaling is mediated by a group of receptors known as the PYR1/PYL/RCAR family, which includes the pyrabactin resistance 1–like protein PYL8. Under stress conditions, ABA signaling activates SnRK2 protein kinases to inhibit lateral root growth after emergence from the primary root. However, even in the case of persistent stress, lateral root growth eventually recovers from inhibition. We showed that PYL8 is required for the recovery of lateral root growth, following inhibition by ABA. PYL8 directly interacted with the transcription factors MYB77, MYB44, and MYB73. The interaction of PYL8 and MYB77 increased the binding of MYB77 to its target MBSI motif in the promoters of multiple auxin-responsive genes. Compared to wild-type seedlings, the lateral root growth of *pyl8* mutant seedlings and *myb77* mutant seedlings was more sensitive to inhibition by ABA. The recovery of lateral root growth was delayed in *pyl8* mutant seedlings in the presence of ABA, and the defect was rescued by exposing *pyl8* mutant seedlings to the auxin IAA (3-indoleacetic acid). Thus, PYL8 promotes lateral root growth independently of the core ABA-SnRK2 signaling pathway by enhancing the activities of MYB77 and its paralogs, MYB44 and MYB73, to augment auxin signaling.

INTRODUCTION

Abscisic acid (ABA) is an important phytohormone that regulates many growth and developmental processes, including seed longevity, dormancy and germination, vegetative growth, root architecture, and abiotic stress responses (1–4). ABA, together with auxin and other hormones, regulates root growth and architecture to acclimate to the variable growth environment, such as drought and salt stress, nutrient availability, gravity, and light (5–12). The root system of higher plants consists of primary, lateral, and adventitious roots. High concentrations of ABA inhibit both primary and lateral root growth (5, 9, 13, 14). Osmotic stress and salt stress inhibit primary and lateral root growth in a partially ABA-dependent manner (1, 6, 9, 15). During salt stress, both primary roots and lateral roots that have emerged from the primary root undergo a quiescent phase followed by a recovery phase (9, 15). ABA signaling functions in growth suppression during the quiescent phase of primary roots and lateral roots that have emerged from the primary root (post-emergence), although the quiescent phases for primary and lateral roots last for different periods and show different sensitivities to salt (9, 15). A low concentration of ABA is required for primary root elongation in response to water deficit and salt stress (15, 16). ABA is required for the growth recovery of primary root from inhibition and for long-term growth of primary roots during salt stress (15). In addition, the phytohormone gibberellic acid promotes growth recovery of both primary and lateral roots (9, 15).

In contrast to the inhibitory role of ABA on lateral root initiation and growth, auxin stimulates lateral root initiation and promotes lateral root growth (8, 12). The repression of lateral root formation under osmotic

stress can be overcome by exogenous application of the synthetic auxin 1-naphthaleneacetic acid (6), implying that the balance between the repression by ABA and promotion by auxin may determine the fate of the lateral root primordium. As reported previously, ABA antagonizes auxin by promoting production of reactive oxygen species (ROS), leading to the inhibition of primary root growth (17–20). ABA also reduces auxin transport in roots by suppressing the expression of *PIN-FORMED 1* through *ABA-insensitive (ABI) 4*, resulting in suppression of lateral root formation and elongation (21). Furthermore, ABA greatly reduces the auxin reporter *ProDR5:GUS* expression in roots, indicating a reduction in auxin concentration or response (20, 22). In contrast, ABA can enhance auxin signaling without increasing the amount of auxin by activating auxin-responsive promoters to repress embryonic axis elongation (23). Similarly, auxin has both antagonistic and synergistic effects on responses to ABA. Auxin response factor (ARF) 2 directly suppresses the expression of *HOMEODOMAIN PROTEIN 33*, which is a positive regulator in ABA repression of primary root growth (22). However, auxin enhances ABA signaling through ARF10- and ARF16-mediated activation of *ABI3* expression in the regulation of lateral root formation and seed dormancy (24, 25). Plants with mutations in the *auxin resistant (AXR)* genes, *axr2-1* and *axr3-1*, and auxin transport mutants *aux1* and *pin2* are insensitive to the effects of ABA and auxin on embryonic axis elongation and root growth (23, 25, 26).

The core ABA signaling pathways consist of ABA-specific receptors and their downstream protein phosphatases and kinases. The pyrabactin resistance 1 (PYR1) and PYR1-like proteins (PYLs), also known as the regulatory component of ABA receptor (RCAR) family proteins, are ABA receptors (27, 28). PYR1/PYL/RCARs interact with and inhibit clade-A protein phosphatase type 2Cs (PP2Cs) in the presence of ABA and release the SnRK2 protein kinases from inhibition by the PP2Cs (27, 28). Activated SnRK2s phosphorylate transcription factors such as ABA-responsive element binding factors (29), *ABI5* (30), enhanced late embryogenesis abundant level (30), and flowering basic helix-loop-helix-type transcription factor 3 (30) to alter gene expression. Activated SnRK2s also phosphorylate ion channels such as slow anion channel 1 (31–36), K⁺ channel in

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Arabidopsis 1 (37), and mechanosensitive channel of small conductance-like 9 (38) to stimulate stomatal closure. SnRK2s also phosphorylate the nicotinamide adenine dinucleotide phosphate oxidase RBOHF, leading to the production of ROS (17–19). In addition, several other substrates of SnRK2s include proteins related to RNA binding, microRNA and epigenetic regulation, chloroplast function, and many other processes based on phosphoproteomics analyses (38, 39). Thus, PYR1/PYL/RCARs are thought to primarily regulate cellular functions using the PP2C-SnRK2 pathway.

Here, we found a previously uncharacterized function of PYL8 (also known as RCAR3) in the regulation of lateral root growth of *Arabidopsis thaliana* seedlings exposed to ABA treatment. PYL8 has a high affinity for ABA (40) and was recently shown to mediate the ability of ABA to inhibit primary root growth through the PP2C-SnRK2 pathway (7). We found that PYL8 mediates a synergistic action of ABA and auxin in promoting the growth recovery of post-emergence lateral roots. In addition, we found evidence for the function of the core ABA signaling components, PYR1/PYL/RCARs, PP2Cs, and SnRK2s, in the inhibition of lateral root

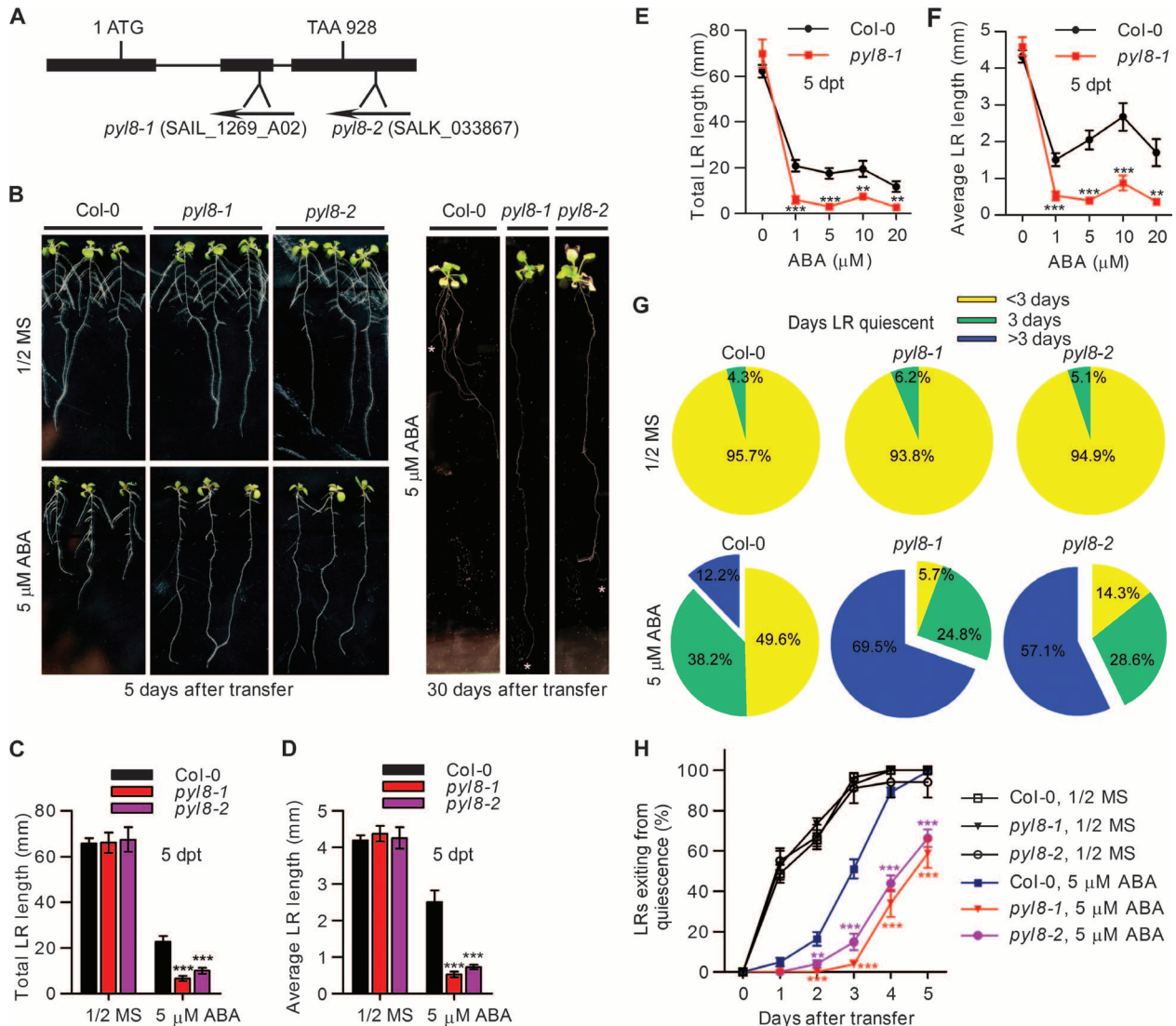


Fig. 1. *pyl8* mutant seedlings have enhanced sensitivity to ABA-induced inhibition of lateral root growth. (A) Diagram of T-DNA insertions in the *PYL8* gene. The T-DNA insertions in the two SALK lines are inserted in exons, which are presented as closed boxes. (B) The root architecture of wild-type (Col-0) or *pyl8* mutant seedlings was documented at 5 dpt (days post-transfer) (left panel) or 30 dpt (right panel) to media (1/2 MS, 1% sucrose) with or without ABA. The primary root tips are marked by white asterisks. (C and D) Lateral root (LR) length of Col-0 and *pyl8* mutant seedlings at 5 dpt to media with or without ABA. $n \geq 12$ seedlings. Data are means \pm SEM. *** $P < 0.001$, Student's *t* test. (E and F) Lateral root length of Col-0 and *pyl8-1* mutant

seedlings at 5 dpt to media with varying concentrations of ABA. $n = 9$ seedlings. Data are means \pm SEM. ** $P < 0.01$, *** $P < 0.001$, Student's *t* test. (G) Pie charts showing the percentage of quiescent lateral roots for the indicated number of days for seedlings grown in media with or without ABA media. $n = 10$ to 24 seedlings per condition. (H) Percentage of quiescent lateral roots (<0.5 mm at 0 dpt) that exited quiescence within 5 dpt to the media with or without ABA. Percentage was calculated as total lateral roots exiting quiescence (>0.5 mm) at the indicated days divided by total number of quiescent lateral roots at 0 dpt. $n = 10$ to 24 seedlings per condition. Data are means \pm SEM. ** $P < 0.01$, *** $P < 0.001$, analysis of variance (ANOVA).

growth. Our results suggest a signaling pathway in which the ABA receptor PYL8 directly interacts with a group MYB transcription factors to mediate crosstalk between ABA and auxin signaling to promote the growth recovery of lateral root from inhibition.

RESULTS

PYL8 promotes recovery from lateral root growth inhibition by ABA

ABA regulates the architecture of plant root systems during abiotic stress. To investigate how ABA controls root architecture, we analyzed the lateral

root growth of *A. thaliana* plants with mutations in components of the ABA signaling pathway. To assess the role of PYL8 in ABA signaling, we analyzed two plants with transferred DNA (T-DNA) insertion mutations in *PYL8* (*pyl8-1* and *pyl8-2*) (Fig. 1A) that result in loss of function of PYL8 (7). We found that *pyl8* mutant seedlings showed more suppression of lateral root growth than did wild-type seedlings when exposed to ABA for 5 days (Fig. 1B). Seedlings of *pyl8* mutant seedlings grown on ABA-containing media for 30 days exhibited altered root architecture compared to wild-type seedlings (Fig. 1B, right panel). Lateral root growth was severely suppressed, whereas primary root growth was less inhibited, in *pyl8* mutant seedlings exposed to ABA (Fig. 1B, right panel). Both the total and average lateral root lengths of *pyl8* mutant seedlings were

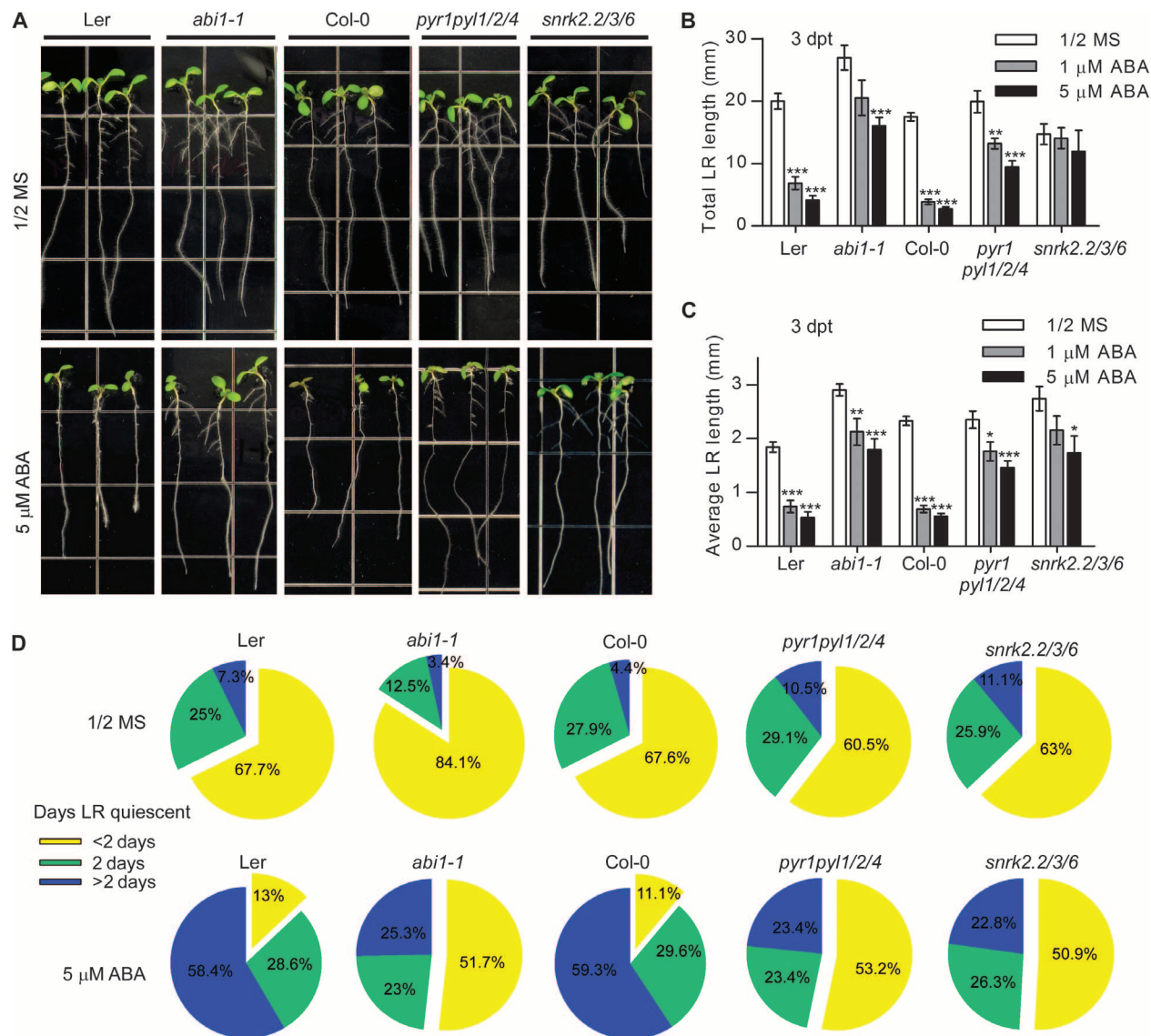


Fig. 2. Lateral root growth is not suppressed by exposure to ABA in ABA-resistant mutant seedlings. (A) The root architecture of wild-type (Col-0 or Ler) or *abi1-1*, *pyr1pyl1/2/4*, or *snrk2.2/3/6* mutant seedlings was documented at 3 dpt to media (1/2 MS, 1% sucrose) with or without ABA. (B and C) Lateral root (LR) length of seedlings at 3 dpt to media

with varying concentrations of ABA. $n \geq 11$ seedlings. Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test. (D) Pie charts for the percentage of quiescent lateral roots for the indicated number of days on seedlings grown in media with or without ABA. $n = 10$ to 12 seedlings per condition.

reduced compared to the wild-type seedlings when grown on ABA-containing media for 5 days (Fig. 1, C and D), suggesting that the lateral root growth of *pyl8* mutant seedlings is more sensitive to ABA. To confirm this observation, we examined lateral root growth with different concentrations of ABA. The lateral root growth of *pyl8* mutant seedlings was more sensitive to ABA inhibition at all concentrations tested (Fig. 1, E and F). Thus, PYL8 promotes lateral root growth in response to ABA.

A previous study suggests that after being shifted to salt stress, post-emergence lateral roots undergo a quiescent phase followed by a recovery phase, and their growth is spatially and temporally regulated (9). To test whether the retarded lateral root growth of *pyl8* mutants exposed to ABA resulted from an extended quiescent phase, we monitored lateral roots for 5 days after emergence. Lateral roots that were less than 0.5 mm in length were considered quiescent, which is defined as stage C in the development of lateral root (5, 9). We found that the quiescent phase was extended in *pyl8* mutant compared to wild-type seedlings when grown on ABA-containing media, whereas the quiescent phase of *pyl8* mutant seedlings was comparable to that of wild-type seedlings when grown on control media (Fig. 1G). As a result, growth recovery was delayed in *pyl8* mutant compared to wild-type seedlings when exposed to ABA (Fig. 1H). Thus, inhibition of lateral root growth by ABA in *pyl8* mutant seedlings may be caused by prolonged quiescence, implying that PYL8 may function in promoting lateral root growth recovery in the presence of ABA.

Because salt or osmotic stress is known to induce ABA accumulation (41, 42), we analyzed the lateral and primary root growth of *pyl8* seedlings in media supplemented with various concentrations of NaCl or mannitol. However, we did not observe significant differences in lateral root growth between *pyl8* mutant and wild-type seedlings (fig. S1). Plant responses to these stressors are more complicated than responses to ABA because NaCl causes both osmotic stress and ion toxicity (43) and mannitol causes osmotic stress and other effects due to mannitol entry into cells (44). These differences may explain the differences in the response of lateral root growth in *pyl8* mutant plants to ABA and these other stressors.

PYLs, PP2Cs, and SnRK2s are required for lateral root growth suppression by ABA

To test whether the lateral root phenotype of *pyl8* mutant seedlings is due to reduced response to ABA, we analyzed lateral root growth of seedlings with mutations in the core ABA signaling pathway, including *pyr1*, *pyl1*, *pyl2*, and *pyl4* (*pyr1pyl1/2/4*); *snrk2.2*, *snrk3*, and *snrk6* (*snrk2.2/3/6*); or *abi1-1*, each of which is resistant to ABA-induced inhibition of seed germination and seedling growth (13, 27, 45). Unlike *pyl8* mutant seedlings, the lateral root growth of *abi1-1*, *pyr1pyl1/2/4*, or *snrk2.2/3/6* mutant seedlings was less sensitive than that of wild-type seedlings to inhibition by ABA (Fig. 2, A to C). The lateral roots of wild-type and *abi1-1*, *pyr1pyl1/2/4*, or *snrk2.2/3/6* mutant seedlings had similar length of quiescent phases when grown in control media. However, the quiescent phase was shorter in *abi1-1*, *pyr1pyl1/2/4*, or *snrk2.2/3/6* mutant compared to wild-type seedlings when grown on ABA-containing media (Fig. 2D). Thus, the core ABA signaling pathway promotes lateral root quiescence in plants exposed to ABA.

Suppression of primary root growth and lateral root formation by ABA is reduced in *pyl8* and ABA-resistant mutants

Primary and lateral roots use common and distinct molecular mechanisms to initiate and grow (5, 6, 9, 46, 47). We compared primary root elongation and the number of lateral roots in *pyl8* or ABA-resistant mutant seedlings. As reported previously (7, 13, 14, 48), the primary root growth of *pyl8-1*, *abi1-1*, or *snrk2.2/3/6* mutant seedlings was less sensitive to ABA

exposure compared to that of wild-type seedlings (Fig. 3, A and B). In wild-type seedlings, exposure to ABA reduced the number of lateral roots. In contrast, in *pyl8-1*, *abi1-1*, or *snrk2.2/3/6* mutant seedlings, the number of lateral roots was less affected by exposure to ABA (Fig. 3, C and D). We found that the expression of ABA-responsive genes was similar in the roots of *pyl8* mutant and wild-type plants exposed to ABA (fig. S2), suggesting that PYL8 has functional redundancy with other PYLs in mediating ABA-responsive gene expression. Thus, PYL8 likely plays a similar role to other PYLs in primary root growth and lateral root initiation.

We also assessed whether exposing wild-type seedlings to ABA could affect the expression of PYL8. We found that growing seedlings for 12 hours on ABA-containing media reduced the abundance of PYL8 transcripts to about 33% of that in seedlings grown on control media (fig. S3). However, because ABA stabilizes PYL8 protein by reducing ubiquitination (49), the abundance of PYL8 protein may not substantially decrease in response to ABA.

Application of exogenous auxin promotes lateral root growth in *pyl8* mutant seedlings exposed to ABA

Suppression of lateral root development by osmotic stress can be overcome by exogenous application of auxin (6). To test whether the inhibition of lateral root growth recovery in *pyl8* mutants could be rescued by auxin, we analyzed the lateral root growth of *pyl8* seedlings grown on ABA-containing media supplemented with different concentrations of the auxin IAA (3-indoleacetic acid). We found that 10 nM IAA was sufficient to

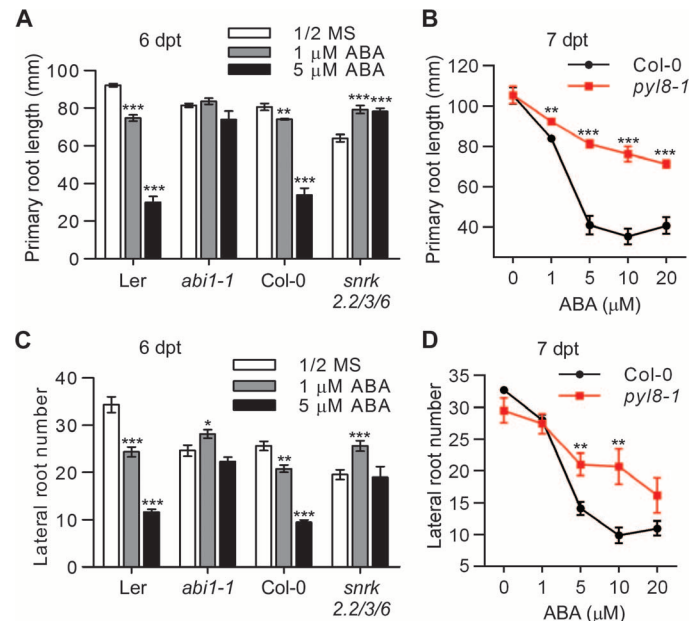


Fig. 3. Suppression of primary root growth and lateral root formation by exposure to ABA is reduced in *pyl8* and ABA-resistant mutant seedlings. (A and C) The primary root length and lateral root number of wild-type (Col-0 or Ler) or *abi1-1* or *snrk2.2/3/6* mutant seedlings were documented at 6 dpt to media (1/2 MS, 1% sucrose) with varying concentrations of ABA. $n \geq 10$ seedlings. Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test. (B and D) The primary root length and lateral root number of Col-0 and *pyl8-1* mutant were documented at 7 dpt to media with varying concentrations of ABA. $n = 9$ seedlings. Data are means \pm SEM. ** $P < 0.01$, *** $P < 0.001$, Student's t test.

rescue the inhibition of lateral root growth in *pyl8* mutant seedlings exposed to ABA for 5 days (Fig. 4, A to C). We also analyzed lateral root growth over time and found that wild-type and *pyl8* mutant seedlings showed similar lateral root growth on control media, and that lateral root elongation of *pyl8* mutant seedlings was suppressed by exposure to ABA (Fig. 4, D and E). However, in *pyl8* mutant seedlings exposed to both IAA and ABA, lateral root growth was enhanced compared to that of wild-type seedlings (Fig. 4, D and E).

To test whether IAA treatment alters the quiescent or recovery phases of lateral roots, we monitored the lateral roots of wild-type and *pyl8* mutant seedlings for 5 days. The *pyl8* mutant seedlings had a similar length of quiescent phase as wild-type seedlings in the presence of both IAA and ABA (Fig. 4F). The recovery of post-emergence lateral root growth in *pyl8* mutant seedlings exposed to ABA treatment was accelerated by exposure to IAA compared to exposure to ABA alone, resulting in comparable lengths of lateral roots to wild-type seedlings (Figs. 1H and 4G). Thus, the prolonged quiescent phase in lateral roots of *pyl8* mutant seedlings exposed to ABA was shortened by exposure to IAA, suggesting that the delayed recovery of lateral root growth may be due to auxin deficiency or reduced response to auxin.

PYL8 enhances MYB77 activity to promote lateral root growth

Auxin signaling can activate the transcription factor MYB77 to promote lateral root growth (50). Recently, systematic yeast two-hybrid screens found MYB77 as an interacting protein of PYL8 (51). To test whether the interaction between PYL8 and MYB77 was specific, we fused several PYLs to the GAL4 DNA binding domain (BD) and MYB77 or MYB61 to the GAL4-activating domain (AD) and performed yeast two-hybrid assays. We found that PYL8, but not other PYLs, interacted with MYB77, but not MYB61, in an ABA-independent manner (Fig. 5A). To further characterize the interaction between PYL8 and MYB77 in plant cells, we fused PYL8 to the N-terminal domain of firefly luciferase (LUC) (PYL8-nLUC) and MYB77 to the C-terminal domain of LUC (MYB77-cLUC) and co-expressed the two constructs in *A. thaliana* protoplasts. Coexpression of PYL8-nLUC, but not nLUC, with MYB77-cLUC produced measurable luciferase activity, comparable to coexpression of MYB77-cLUC with

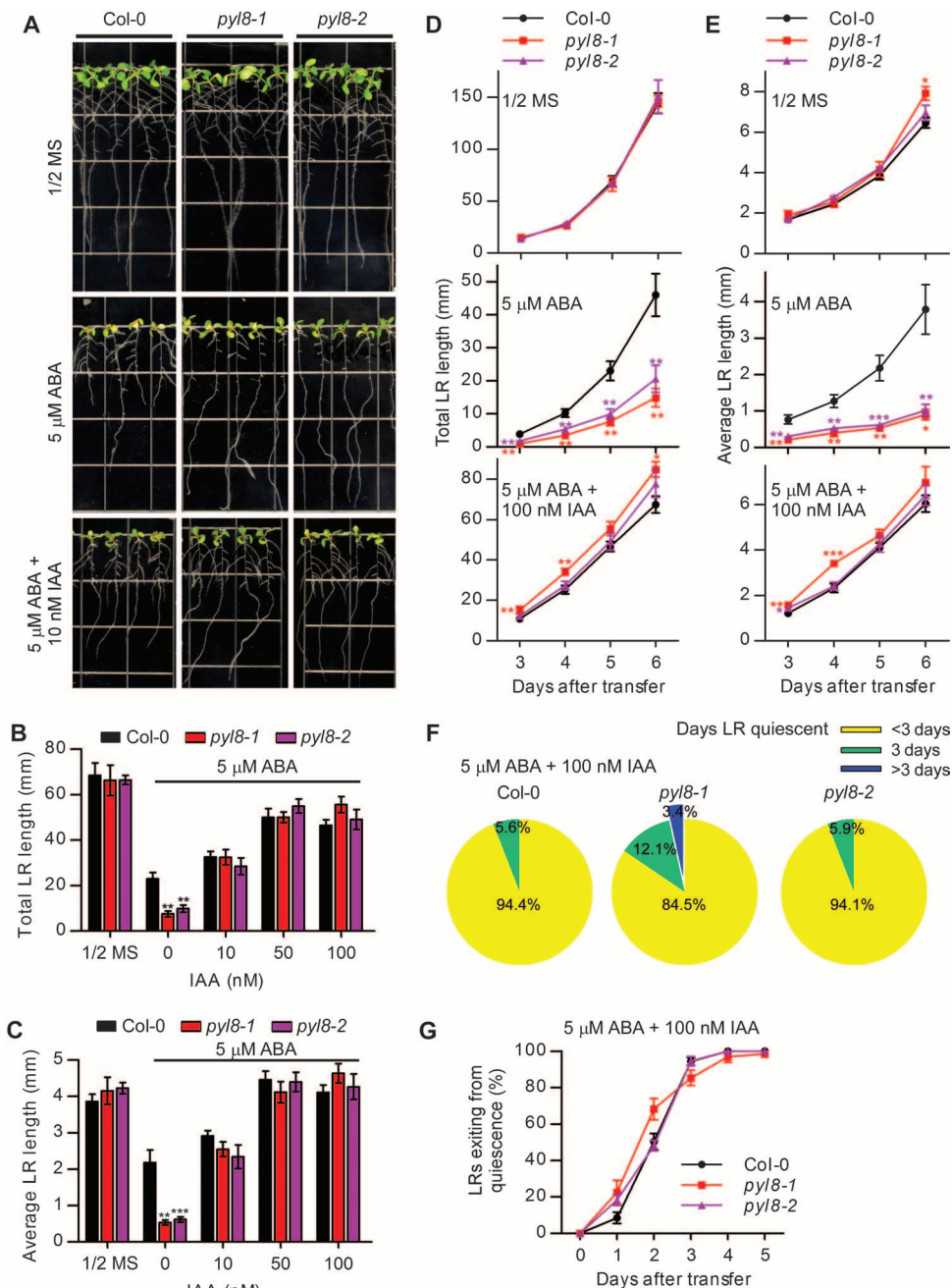
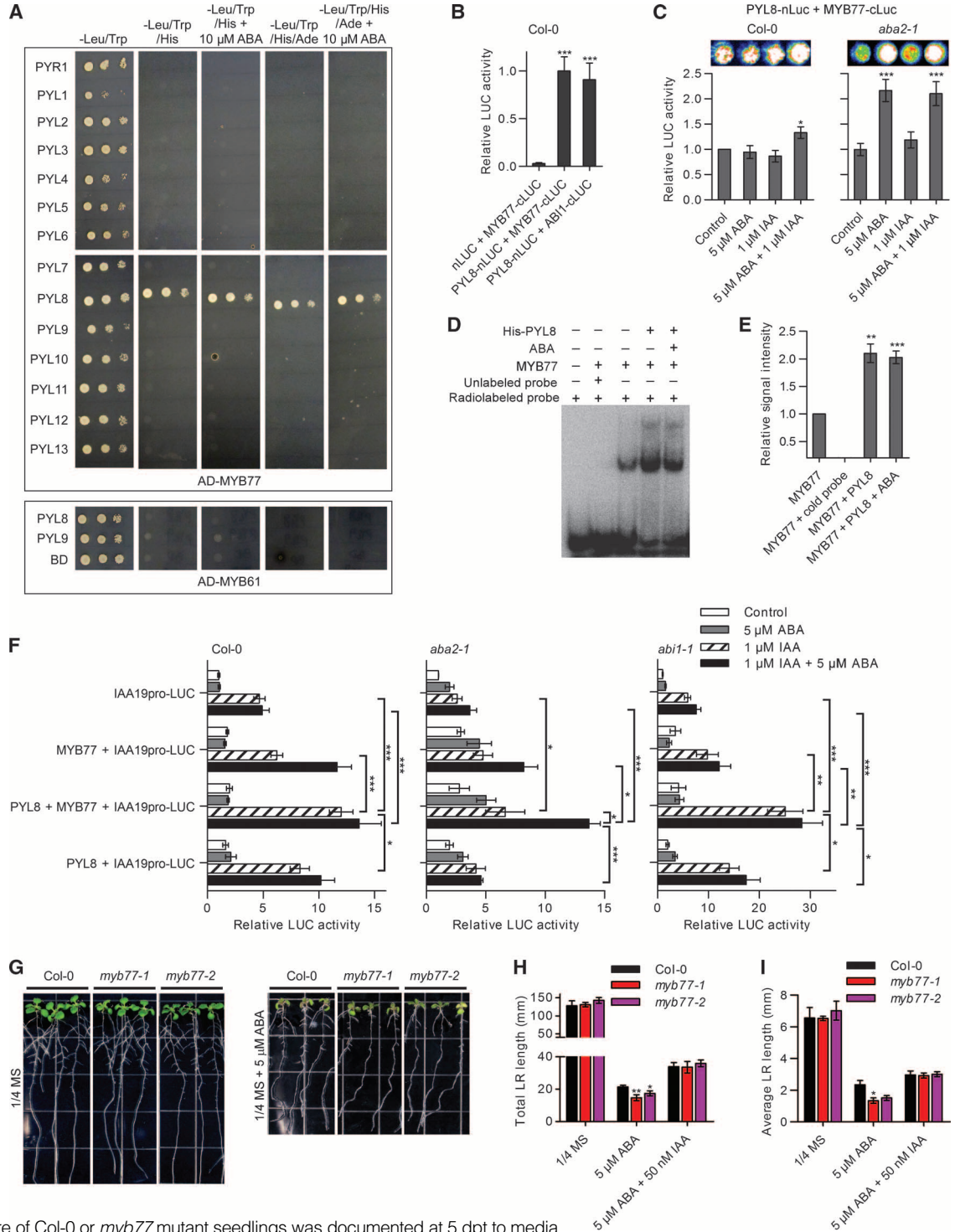


Fig. 4. Exogenous IAA rescues the lateral root growth defects of *pyl8* mutant seedlings. (A) The root architecture of wild-type (Col-0) or *pyl8* mutant seedlings was documented at 5 dpt to media (1/2 MS, 1% sucrose) with or without ABA or ABA plus IAA. (B and C) Lateral root (LR) length of seedlings at 5 dpt to media with or without ABA or ABA plus IAA. $n \geq 11$ seedlings for Col-0 and *pyl8-2*, and $n = 6$ seedlings for *pyl8-1*. Data are means \pm SEM. $**P < 0.01$, $***P < 0.001$, Student's *t* test. (D and E) The lateral root length of seedlings was measured at the indicated days after transfer to media with or without ABA or ABA plus IAA. $n \geq 11$ seedlings for Col-0 and *pyl8-2*, and $n = 6$ seedlings for *pyl8-1*. Data are means \pm SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's *t* test. (F) Pie charts for the percentage of quiescent lateral roots for the indicated number of days for seedling grown in media with or without ABA or ABA plus IAA. $n = 6$ to 18 seedlings per treatment. (G) Percentage of quiescent lateral roots (<0.5 mm at 0 dpt) that exited within 5 dpt to the media with ABA plus IAA. $n = 6$ to 18 seedlings per treatment. Data are means \pm SEM. $*P < 0.05$, ANOVA.

Fig. 5. PYL8 enhances auxin signaling through MYB77. (A) PYL-MYB77 interactions in the yeast two-hybrid assay. Interaction was determined by yeast growth in media lacking His or His and Ade in the presence and absence of ABA. Dilutions (10^{-1} , 10^{-2} , and 10^{-3}) of saturated cultures were spotted onto the plates, which were photographed after 5 days. (B) PYL8-MYB77 interaction in LUC complementation assays in wild-type Col-0 protoplasts. $n = 4$ experiments. Data are means \pm SEM. $***P < 0.001$, Student's t test. (C) PYL8-MYB77 interaction in Col-0 wild-type (left panel) and *aba2-1* mutant protoplasts (right panel) with or without ABA in LUC complementation assays. $n \geq 7$ experiments. Data are means \pm SEM. $*P < 0.05$, $***P < 0.001$, Student's t test. (D and E) Binding between MYB77 and the MBSI motif-containing DNA oligonucleotides with or without PYL8 in EMSA assay. $n = 4$ experiments. Data are means \pm SEM. $**P < 0.01$, $***P < 0.001$, Student's t test. (F) *IAA19pro-LUC* expression in Col-0 wild-type (left panel), *aba2-1* mutant (middle panel), and *abi1-1* mutant (right panel) protoplasts cotransformed with the indicated combinations of *PYL8*, *MYB77*, and *IAA19pro-LUC* with varying concentrations of ABA and IAA. $n \geq 3$ experiments. Data are means \pm SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Student's t test. (G) The root architecture of Col-0 or *myb77* mutant seedlings was documented at 5 dpt to media (1/4 MS, 1% sucrose) with or without ABA. (H and I) Lateral root (LR) length of Col-0 and *myb77* mutant seedlings at 5 dpt to media with or without ABA. $n \geq 11$ seedlings. Data are means \pm SEM. $*P < 0.05$, $**P < 0.01$, Student's t test.



the positive control ABI1-nLUC (7) (Fig. 5B). To test whether the interaction between PYL8 and MYB77 was altered in the presence of ABA and auxin in vivo, we treated wild-type protoplasts coexpressing PYL8-nLUC and MYB77-cLUC with different combinations of ABA and IAA.

We found that LUC activity was enhanced in cells exposed to both ABA and IAA, but not in cells exposed to either ABA or IAA alone (Fig. 5C, left panel). To investigate the effect of endogenous ABA, we performed LUC complementation assays in protoplasts derived from the

ABA-deficient mutant *aba2-1* (52) and found that ABA exposure promoted the interaction between PYL8 and MYB77 in the absence or presence of IAA (Fig. 5C, right panel). The interaction between PYL8 and MYB77 was not reduced by overexpression of ABI1 or ABI2 in *aba2-1* mutant protoplasts (fig. S4), suggesting that MYB77 and the PP2Cs may interact with different regions of PYL8. Thus, PYL8 specifically interacts with MYB77, and this interaction may be enhanced by ABA and IAA.

We asked whether the interaction between PYL8 and MYB77 affected the ability of MYB77 to bind to DNA and activate transcription. A previous study found that MYB77 Δ C1 (amino acids 1 to 200) recognizes several DNA motifs, including MBSI (CNGTTR) and MBSII (GTTAGTTA), and preferentially binds to MBSI motif-containing promoters (53). Using an electrophoretic mobility shift assay (EMSA), we found that full-length MYB77 bound to MBSI motif-containing DNA oligonucleotides (CRGTTA), but not to oligonucleotides containing M1 (CTGTTC), M2 (CAGTGA), or M3 (CCATTA) motifs (fig. S5, A and B). To test whether PYL8 affects the ability of MYB77 to bind to MBSI motifs, we preincubated recombinant MYB77 and PYL8 with or without ABA before performing the EMSA and found that PYL8 enhanced the binding of MYB77 to MBSI motifs in an ABA-independent manner (Fig. 5, D and E). PYL8 alone did not bind to MBSI motifs (fig. S5C). We identified putative MBSI and MBSII motifs in the 500–base pair (bp) promoter regions of multiple auxin-responsive genes, including *IAA1*, *IAA7*, *IAA17*, *IAA19*, *GH3.2*, *PIN1*, and *HAT2* (fig. S5, D and E). To test whether PYL8 can enhance MYB77 transcriptional activity, we fused a 439-bp fragment of *IAA19* promoter to the gene encoding LUC (*IAA19pro-LUC*, fig. S5D) and used it as an auxin-responsive reporter. We expressed *PYL8*, *MYB77*, *IAA19pro-LUC*, or combinations thereof in wild-type protoplasts. As expected, IAA, but not ABA, enhanced LUC activity in control protoplasts expressing only the reporter (Fig. 5F, left panel). However, co-overexpression of *PYL8* and *MYB77* increased LUC activity in the presence of IAA (Fig. 5F, left panel). To investigate the effects of endogenous ABA, we repeated the experiments in protoplasts from *aba2-1* mutant plants and found that ABA increased the ability of *PYL8* and *MYB77* overexpression to enhance LUC activity in the presence of IAA (Fig. 5F, middle panel). Because ABA can inhibit auxin-responsive gene expression (20, 22), we performed reporter assays in protoplasts of *abi1-1* mutant plants (54), which show inhibition of the core ABA signaling pathway (45), and found that co-overexpression of *PYL8* and *MYB77* increased LUC activity with or without ABA in the presence of IAA in these cells (Fig. 5F, right panel). Thus, *PYL8* enhances the binding of *MYB77* to its target motif MBSI, leading to increased response to auxin in an ABA-enhanced manner.

To further understand whether *MYB77* can activate auxin responses to control lateral

root growth in response to ABA, we analyzed the lateral root growth of two plants with T-DNA insertion mutations in *MYB77* (50). We found that lateral root growth in *myb77* mutant seedlings was more sensitive to inhibition by ABA than that in wild-type seedlings and that exposure to IAA could reverse ABA-induced inhibition of lateral root growth in *myb77* mutant seedlings (Fig. 5, H and I). Thus, *MYB77*, like *PYL8*, promotes lateral root growth in seedlings exposed to ABA.

PYL8 interacts with paralogs of MYB77 and enhances their transcriptional activity

We asked whether *PYL8* could bind paralogs of *MYB77* and modify their transcriptional activity. *MYB77*, *MYB73*, *MYB70*, and *MYB44* belong to the R2R3 MYB family subgroup 22, which is defined by two conserved motifs, TGLYMSPxSP and GxFMxVvQEMixxEVRSYM (fig. S6) (55). *PYL8* is a putative interacting protein with *MYB44* (56). To test whether *MYB73* and *MYB44* could specifically bind *PYL8*, we performed yeast two-hybrid assays and found that *PYL8*, but not other *PYLs*, bound to both *MYB44* and *MYB73* in an ABA-independent manner (Fig. 6, A and B). *PYL8* showed a much stronger interaction with *MYB77* and *MYB44* than with *MYB73* (Figs. 5A and 6B). To test whether *MYB44* and *MYB73* could enhance the transcriptional response to auxin, we performed LUC assays in *IAA19pro-LUC*-expressing protoplasts from *aba2-1* and *abi1-1* mutant plants. We found that co-overexpression with *PYL8* enhanced

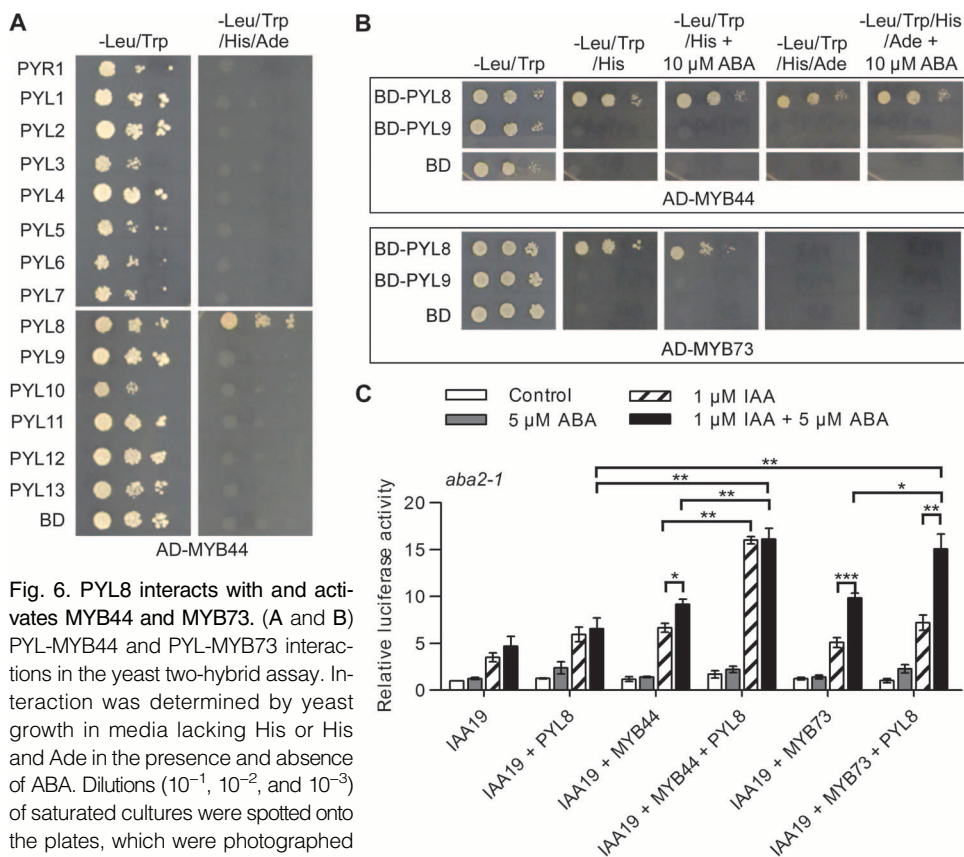


Fig. 6. PYL8 interacts with and activates MYB44 and MYB73. (A and B) PYL-MYB44 and PYL-MYB73 interactions in the yeast two-hybrid assay. Interaction was determined by yeast growth in media lacking His or His and Ade in the presence and absence of ABA. Dilutions (10^{-1} , 10^{-2} , and 10^{-3}) of saturated cultures were spotted onto the plates, which were photographed after 5 days. (C) *IAA19pro-LUC* expression in *aba2-1* mutant protoplasts cotransformed with the indicated combinations of *PYL8*, *MYB44*, *MYB73*, and *IAA19pro-LUC* with varying concentrations of ABA and IAA. $n = 3$ experiments. Data are means \pm SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test.

the ability of MYB44 or MYB73 to increase LUC activity in the presence of IAA (Fig. 6C and fig. S7) and that exposure to ABA enhanced the ability of PYL8 to activate MYB73 (Fig. 6C). Thus, PYL8 likely binds to and enhances the activities of paralogs of MYB77, which may function redundantly with MYB77 to enhance auxin signaling.

DISCUSSION

The root system of plants is one of the most sensitive organs in sensing the availability of water and nutrients or other adverse soil conditions. As a critical part of plant responses to environmental cues, phytohormone signaling finely controls the growth and development of roots for acclimating to environmental changes. Here, we found a synergistic action of ABA and auxin in controlling the growth of plant lateral roots. Our study reveals that ABA promotes lateral root growth recovery through a pathway mediated by a previously uncharacterized action of ABA receptor PYL8. PYL8 directly interacts with a group of transcription factors, MYB77, MYB44, and MYB73, leading to the enhancement of auxin-dependent transcription. Together with evidence from previous studies (5, 6, 9), our results suggest that ABA has dual roles in controlling lateral root growth via two different pathways (Fig. 7). In this model, ABA signaling inhibits lateral root growth during the quiescent phase and promotes lateral root growth during the recovery phase of post-emergence lateral root growth during abiotic stress.

The growth of post-emergence lateral roots under salt stress undergoes a quiescent phase followed by a recovery phase. Endogenous ABA stimulated by salt stress promotes lateral root quiescence, and application of exogenous ABA treatment mimics this effect (9). Therefore, together with our results, it is likely that lateral root growth undergoes quiescent and recovery phases when exposed to exogenous ABA. We found that lateral root growth was less sensitive to ABA in ABA-resistant mutant seedlings including *pyr1pyl1/2/4*, *abi1-1*, and *snrk2.2/3/6* (Fig. 2, A to C). Consistent with this result, lateral root growth is less sensitive to salt stress in transgenic seedlings expressing *ABI1-1* in the root endodermis (9). The *ABI4* gene encodes an ABA-regulated AP2 domain transcription factor, and lateral root growth of *abi4* mutant seedlings is insensitive to ABA (21). Similarly, the double mutant seedlings *abi4-1* and *fus3-3* are less sensitive to salt stress (9, 21), suggesting that *ABI4* may be the downstream regulator in controlling lateral root quiescence during exposure to ABA or salt stress. Thus, lateral root quiescence is likely controlled by core ABA signaling through the PYL receptors, PP2C co-receptors, SnRK2 protein kinases, *ABI4* transcription factor, and downstream ABA-responsive genes, and our results suggest that PYL8-mediated lateral root growth recovery is independent of the ABA core signaling pathway. Instead, PYL8 interacts directly with the MYB77 paralogs to enhance auxin-dependent gene expression.

In support of the proposed model, our data showed that the recovery of post-emergence lateral root growth was delayed in *pyl8* mutant seedlings exposed to ABA and that the application of exogenous IAA accelerated the lateral root growth of *pyl8* mutant seedlings exposed to ABA (Figs. 1 and 4). The direct interaction of PYL8 with the MYB transcription factors bridges ABA and auxin signaling in controlling lateral root growth. We showed that ABA enhances the effects of PYL8 on the activities of MYB73 and MYB77 in the presence of IAA (Figs. 5F and 6C), suggesting a synergistic action of ABA and IAA on MYB44, MYB73, and MYB77 activities through their interactions with PYL8. It is known that MYB77 positively regulates auxin signaling to promote lateral root formation (50). MYB77 forms a heterodimer with ARF7 and increases the transcription of ARF7 target genes (50), as indicated in our model (Fig. 7). In addition, both ARF7 and ARF19 increase the expression of early

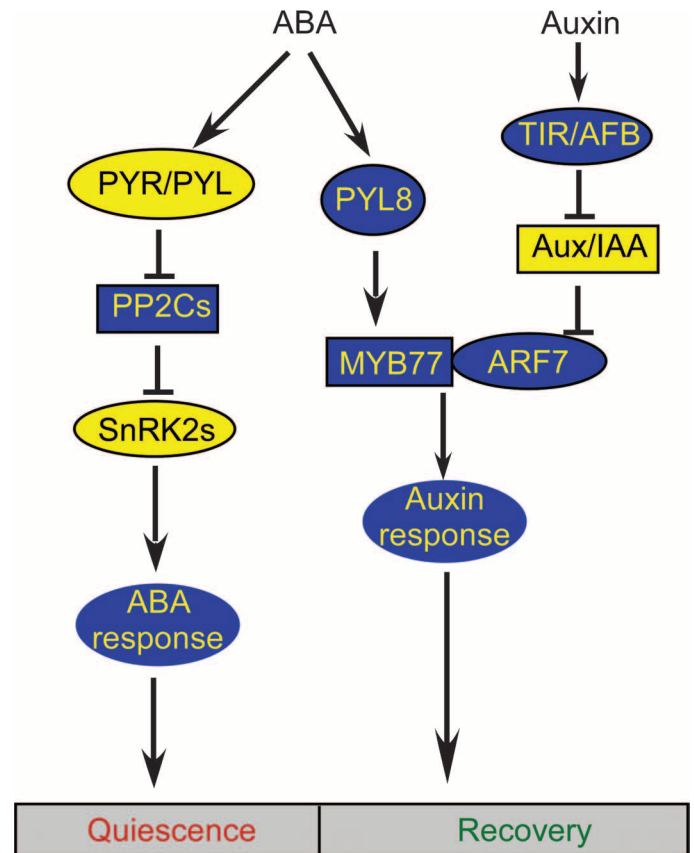


Fig. 7. Proposed model of ABA response pathways in regulating lateral root growth. ABA has dual roles in controlling lateral root growth in *Arabidopsis*. ABA exposure promotes periods of quiescence and growth recovery in post-emergence lateral roots. The inhibitory effect of ABA on lateral root growth is mediated by the core ABA signaling pathway through the PYL receptors, PP2C co-receptors, and SnRK2 protein kinases. PYL8 has a previously undescribed function in accelerating lateral root growth during the recovery phase through activating MYB77. PYL8 directly interacts with MYB77 and enhances the activity of MYB77 to up-regulate the expression of multiple auxin-responsive genes at the time when lateral roots enter the recovery phase. MYB77 forms heterodimers with ARF7 and promotes the transcription of ARF7 target genes. At high concentrations of auxin, Aux/IAA is degraded by transport inhibitor response 1 (TIR1)- and auxin signaling F-box (AFB)-mediated ubiquitination to release the inhibition on the ARFs and induce auxin-responsive gene expression, bypassing PYL8.

auxin response genes such as *LBD16* (also known as *ASL18*) and *LBD29* (also known as *ASL16*) in lateral roots, promoting lateral root formation and elongation (57–59).

ABA signaling can enhance auxin-dependent responses by activating an auxin-responsive promoter (23). Truncated MYB77 and MYB44 proteins bind to the motifs of MBSI and MBSII (53, 60) that were identified in the 500-bp fragments of the promoters of multiple auxin-responsive genes (fig. S5, D and E). Auxin-responsive genes were highly induced by salt stress in plants overexpressing *MYB44* (61), suggesting that salt stress or ABA can enhance auxin signaling when MYB44 abundance is high. *MYB73* and *MYB77* are expressed in lateral roots (50, 62), whereas *PYL8* is expressed extensively in roots (7). The spatial expression patterns of

MYBs and PYL8 indicate that the interaction of PYL8 and MYBs may occur specifically in roots. In addition, we observed that the inhibition of lateral root growth in *myb77* mutant seedlings was not as severe as that in *pyl8* mutant seedlings when exposed to ABA, suggesting that MYB44 and MYB73 may be functionally redundant with MYB77. In protoplasts, ABA enhanced the ability of IAA and PYL8 overexpression to increase the ability of MYB73 and MYB77 to activate an auxin reporter gene (Figs. 5F and 6C). This result implies that the synergistic action of ABA and IAA can be mediated by PYL8-MYB73 and PYL8-MYB77. Furthermore, the expression of *MYB44*, *MYB73*, and *MYB77* is strongly induced by dehydration, salt, cold, ABA, or auxin (50, 61, 63).

PYL8 has been shown to mediate ABA inhibition of primary root growth (7). This role is likely mediated through the PP2C-SnRK2 pathway because primary root elongation in *abi1-1* mutant seedlings and *snrk2.2/3/6* mutant seedlings was not sensitive to ABA exposure (Fig. 3, A and B). Our results show that PYL8 also has a role in promoting lateral root elongation exposed to ABA, and suggest that this function is mediated through the previously uncharacterized PYL8-MYB77 pathway. Therefore, PYL8 has different roles mediated through distinct pathways in the ABA-dependent regulation of different aspects of root growth and development.

MATERIALS AND METHODS

Plant materials

The *pyl8-1* (SAIL_1269_A02) (7, 14), *pyl8-2* (SALK_033867) (7), *pyr1pyl1/2/4* quadruple mutant (27), *snrk2.2/3/6* triple mutant (13), *myb77-1* (50), and *myb77-2* (50) were in Col-0 background, except *pyl2* (27) and *abi1-1* (45), which were in the Landsberg erecta (Ler) background. The *pyl8* and *myb77* mutants were obtained from the Arabidopsis Biological Resource Center (ABRC).

Plant growth conditions

Seeds were surface-sterilized for 10 min in 20% bleach and then rinsed in sterile deionized water four times. Sterilized seeds were grown on 0.6% Phytigel (Sigma) media containing 1/2 MS nutrients or 1/4 MS nutrients as indicated (catalog no. M524, PhytoTechnology Laboratories), 1% sucrose adjusted to pH 5.7 (control media), and kept at 4° to 8°C for 3 days. Seedlings were grown vertically for 3 days before transfer to control media or media with the indicated concentrations of ABA (Sigma, A1049) or IAA (Sigma, I2886). Seedlings were grown in a Percival CU36L5 incubator at 23°C under a 16-hour light, 8-hour dark photoperiod. Plates were sealed with micropore tape (3M).

For protoplast analysis, seedlings were grown on Jiffy 7 peat soil (42mm Pellets) in a Percival chamber with a relatively short photoperiod (12 hours of light at 23°C, 12 hours of dark at 20°C) under low light (about 100 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 50 to 70% relative humidity under well-watered conditions (no drought and no flooding). Protoplasts were isolated as described (64, 65).

Phenotype analysis

The length of all lateral roots was quantified using a Leica EZ4HD stereo microscope. The total and average lateral root lengths of individual plants were quantified by summing or averaging the lengths of all the lateral roots on each plant. Lateral roots shorter than 0.5 mm were categorized as quiescent (9).

Plasmid constructs

All primer sequences are shown in table S1. To generate *IAA19pro-LUC* construct, the 439-bp *IAA19* promoter fragment amplified from Col-0

genomic DNA with primers IAA19proF and IAA19proR was cloned into the Bam HI and Nco I of the *RD29B-LUC* vector (65). To generate pET28a-PYL8 construct, the coding sequence of *PYL8* was amplified from *His-PYL8* (65) using PYL8 primers and cloned into pET28a vectors. To generate pENTR-MYB77 construct, *MYB77* CDS was amplified using pENTR-MYB77 primers and cloned into pENTR vector using the pENTR/D-TOPO Cloning Kit (Invitrogen). *MYB77* was recombined into the pEarley-nLUC and pEarley-cLUC binary vectors using an LR reaction (Invitrogen). *MYB44*, *MYB73*, and *MYB77* were cloned into pHBT95 using transfer polymerase chain reaction with pHBT-MYBs primers, cloned into pGADT7 with AD-MYBs primers, and cloned into pGEX-6P1 with 6P1-MYB77 primers. *ABI1*, *ABI2*, BD-PYLs, and PYL8-nLUC were the same as reported (64). *ABI1* was cloned into pHBT95-cLUC using cLUC-ABI1 primers. *His-PYL8* was the same as reported (65). *ZmUBQ::GUS* was provided by J. Sheen.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described (66). pGBKT7-PYLs were the same as reported (64). PYLs fused to the GAL4 DNA binding domain were used as baits. MYB44, MYB73, MYB77, and MYB61 fused to the GAL4-activating domain were used as preys. Interaction was determined by growth assay on media lacking His or His and Ade in the presence and absence of 10 μM ABA. Dilutions (10^{-1} , 10^{-2} , and 10^{-3}) of saturated cultures were spotted onto the plates and photographed after 5 days.

Transient expression assay in *Arabidopsis*

Assays for transient expression in protoplasts were performed as described (64). All steps were kept at room temperature. *IAA19pro-LUC* was used as the auxin-responsive reporter. *ZmUBQ-GUS* was used as the internal control. After transfection, protoplasts were incubated in washing and incubation solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7) with or without ABA and IAA at the indicated concentrations with 12 hours of light. LUC complementation assays were performed as described (64).

Electrophoretic mobility shift assay

GST-MYB77 and His-PYL8 were purified as described (67). EMSA assay was performed as described (64, 68). The radiolabeled DNA probes were labeled with [γ - ^{32}P]adenosine 5'-triphosphate using T₄ polynucleotide kinase and purified using Micro Bio-Spin 6 columns (Bio-Rad, 732-6221). EMSAs were performed with radiolabeled oligonucleotides (0.6 nM) and recombinant GST-MYB77 protein (17 nM) with or without the indicated unlabeled oligonucleotides (40 nM). Recombinant His-PYL8 protein (17 nM) was preincubated with GST-MYB77 (17 nM) for 10 min with or without ABA (0.2 mM) on ice before the radiolabeled oligonucleotide was added. The DNA binding reactions were performed in binding buffer containing 10 mM Hepes-KOH (pH 7.5), 0.1 mM EDTA, 75 mM KCl, 1.25 mM MgCl₂, 0.2 mM dithiothreitol, bovine serum albumin (2 $\mu\text{g/ml}$), and 5% glycerol. The reaction was incubated with the radiolabeled oligonucleotide for 30 min on ice and then loaded onto 5% polyacrylamide native gels, which had been pre-run at 180 V in 0.5 \times tris-borate EDTA buffer for 30 min at 4°C, and run at 180 V for 35 min at 4°C. After electrophoresis, the gel was dried under vacuum at 80°C for 15 min on a filter paper and exposed to a phosphorimager for 2 to 10 hours.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/7/328/ra53/DC1

Fig. S1. *pyl8* mutants show no difference in root growth compared to wild type when exposed to NaCl or mannitol stress.

Fig. S2. Expression of stress-responsive genes in roots of *pyl8* mutant seedlings.

Fig. S3. Expression of *PYL8* in wild-type seedlings exposed to ABA.

Fig. S4. The interaction between PYL8 and MYB77 is not reduced by overexpression of ABI1 or ABI2.

Fig. S5. The promoter region of auxin-regulated genes contains MBSI elements recognized by MYB77 and MYB44.

Fig. S6. Sequence alignment of MYB77 and its paralogs.

Fig. S7. PYL8 enhances the abilities of MYB44 and MYB73 to activate *IAA19pro-LUC* expression in *abi1-1* mutant protoplasts.

Table S1. Primers used for plasmid constructions.

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Supplementary Materials for

The ABA Receptor *PYL8* Promotes Lateral Root Growth by Enhancing *MYB77*-Dependent Transcription of Auxin-Responsive Genes

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The PDF file includes:

Fig. S1. *pyl8* mutants show no difference in root growth compared to wild type when exposed to NaCl or mannitol stress.

Fig. S2. Expression of stress-responsive genes in roots of *pyl8* mutant seedlings.

Fig. S3. Expression of *PYL8* in wild-type seedlings exposed to ABA.

Fig. S4. The interaction between *PYL8* and *MYB77* is not reduced by overexpression of *ABI1* or *ABI2*.

Fig. S5. The promoter region of auxin-regulated genes contains MBSI elements recognized by *MYB77* and *MYB44*.

Fig. S6. Sequence alignment of *MYB77* and its paralogs.

Fig. S7. *PYL8* enhances the abilities of *MYB44* and *MYB73* to activate *IAA19pro-LUC* expression in *abil-1* mutant protoplasts.

Table S1. Primers used for plasmid constructions.

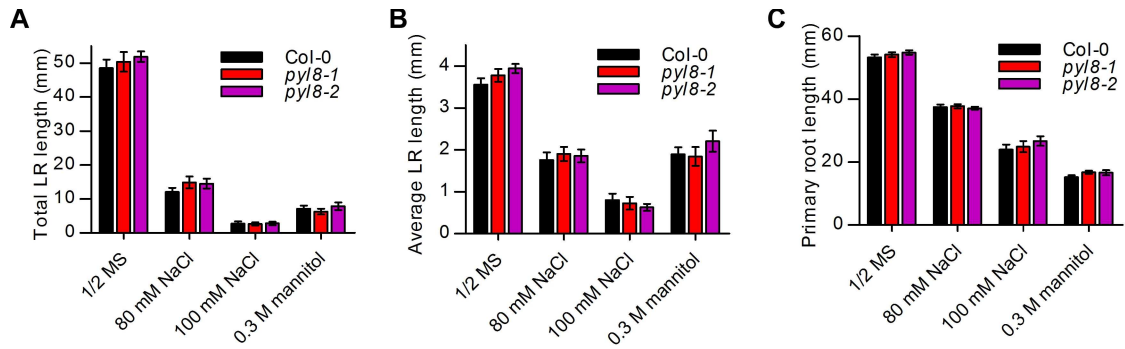


Fig. S1. *pyl8* mutants show no difference in root growth compared to the wild type when exposed to NaCl or mannitol stress. Lateral root length (A-B) and primary root length (C) of Col-0 and *pyl8* mutant seedlings at five dpt to media with or without NaCl or mannitol. $N \geq 8$ seedlings. Data are mean \pm S.E.M., $*p < 0.05$, Student's *t*-test.

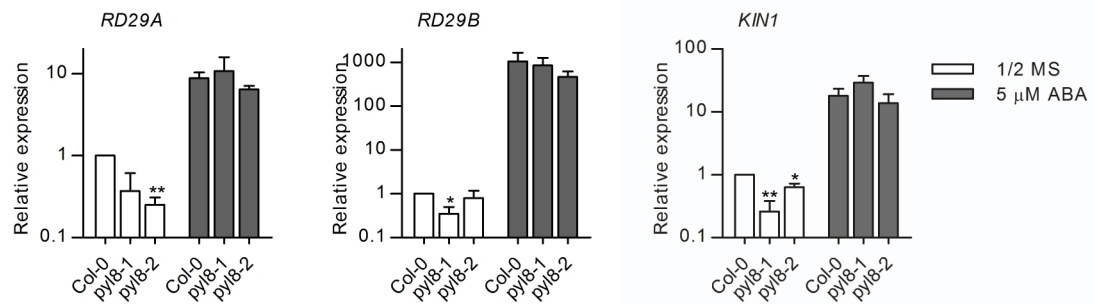


Fig. S2. Expression of stress-responsive genes in roots of *pyl8* mutant seedlings.

Quantitative RT-PCR was conducted on 3-day-old seedlings at 12 hour post transfer to media (1/2 MS, 1% sucrose) with or without 5 μ M ABA. The expression levels of *RD29A*, *RD29B* and *KIN1* in Col-0 without ABA treatment were set as 1. N = 3 experiments. Data are mean \pm S.E.M., * p <0.05, ** p <0.01, Student's *t*-test.

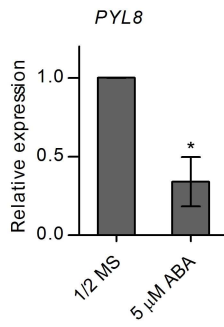


Fig. S3. Expression of *PYL8* in wild-type seedlings exposed to ABA. Quantitative RT-PCR was conducted on 3-day-old seedlings at 12 hour post transfer to media (1/2 MS, 1% sucrose) with or without 5 μM ABA. The expression level of *PYL8* in Col-0 without ABA treatment was set as 1. N = 3 experiments. Data are mean ± S.E.M., * $p < 0.05$, Student's *t*-test.

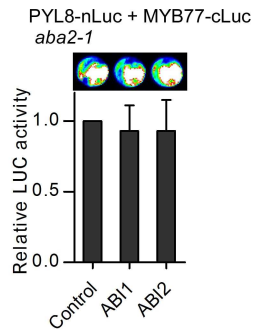


Fig. S4. The interaction between PYL8 and MYB77 is not reduced by overexpression of ABI1 or ABI2. PYL8-MYB77 interaction in LUC complementation assays in *aba2-1* mutant protoplasts with 5 μ M ABA. N = 6 experiments. Data are mean \pm S.E.M., * p <0.05, Student's *t*-test.

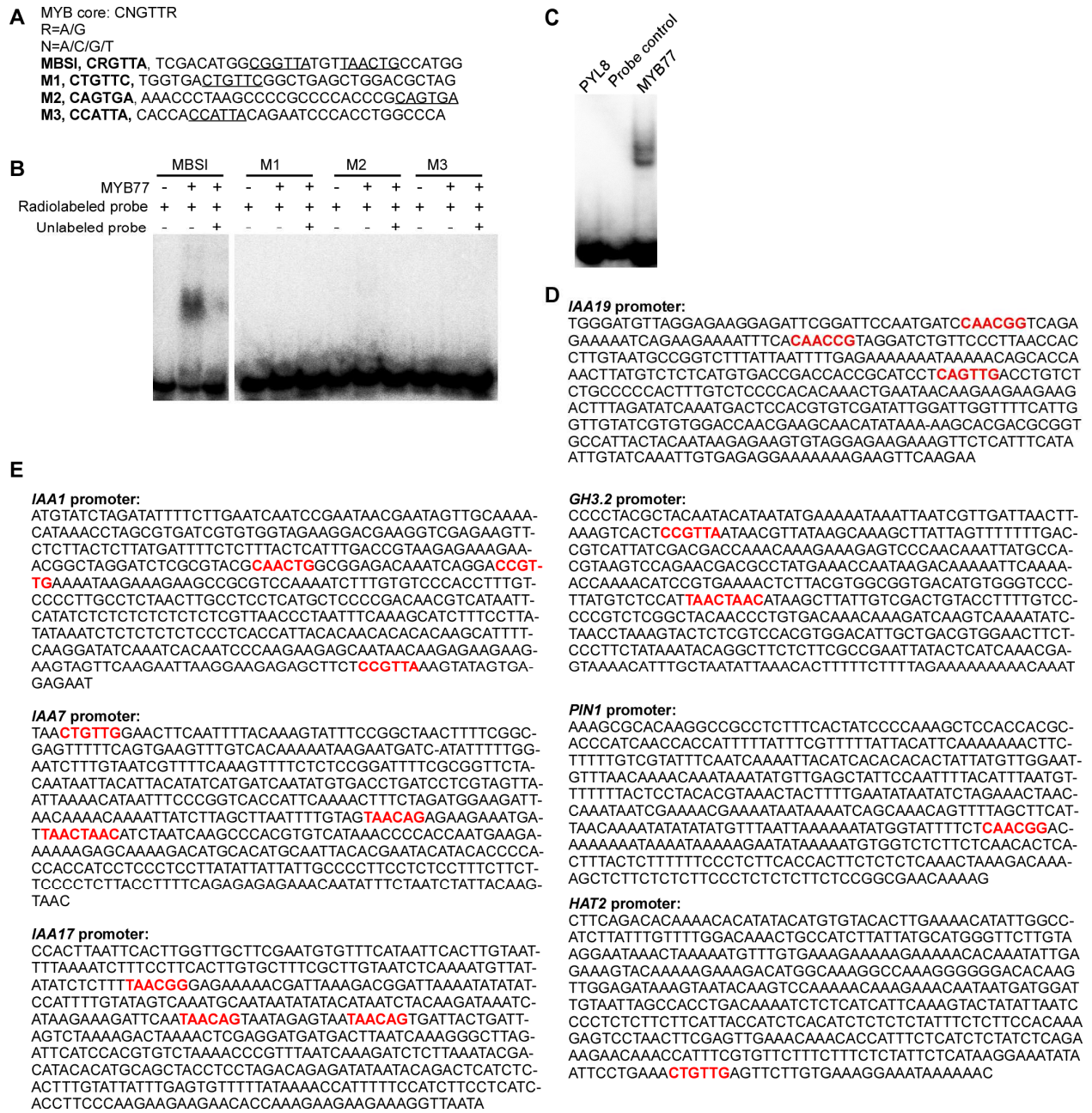


Fig. S5. The promoter region of auxin-regulated genes contains MBSI elements recognized by MYB77 and MYB44. (A) Oligonucleotides used in EMSA assay. Sequences of interest are underlined. (B) Binding between MYB77 and DNA oligonucleotides containing MBSI motif and mutated MBSI motif (M1-3) in EMSA assay. (C) Binding between MYB77 and MBSI motif-containing DNA oligonucleotides. (D) The 439-bp region of the translational start of *IAA19* is shown. MBSI elements are marked with red. (E) The 500-bp regions of the translational start of the indicated auxin-regulated genes are shown. MBSI and MBSII (GTTAGTTA) elements are marked with red.

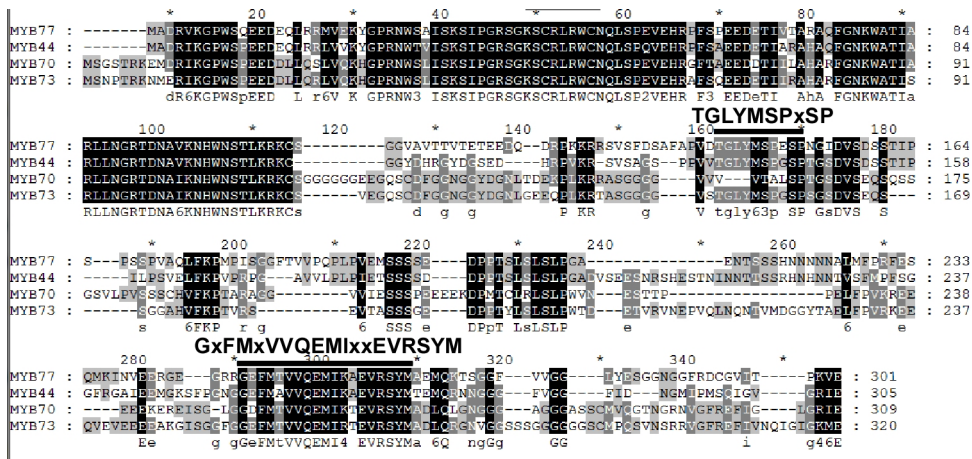


Fig. S6. Sequence alignment of MYB77 and its paralogs. MYB77, MYB44, MYB70 and MYB73 from *Arabidopsis* were aligned using ClustalX 2.0.5 with the default settings and were viewed using the GeneDoc software (<http://www.nrbc.org/gfx/genedoc/>). R2R3 MYB subgroup 22 contains two conserved motifs: TGLYMSPxSP and GxFMxVVQEMixxEVRSYM.

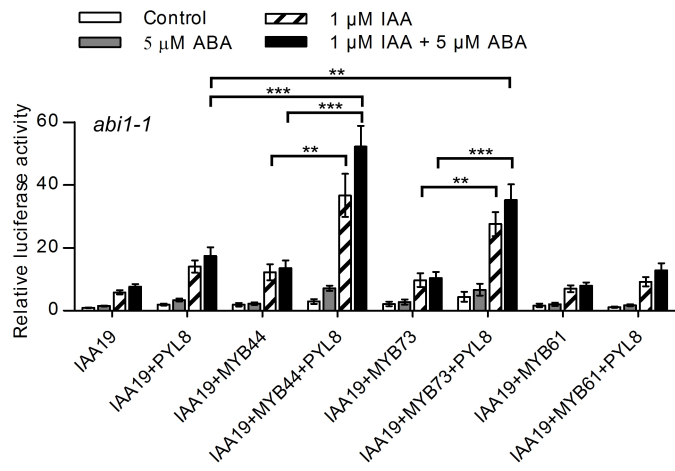


Fig. S7. PYL8 enhances the abilities of MYB44 and MYB73 to activate *IAA19pro-LUC* expression in *abi1-1* mutant protoplasts. *IAA19pro-LUC* expression in *abi1-1* mutant protoplasts co-transformed with the indicated combinations of *PYL8*, *MYB44*, *MYB73* and *IAA19pro-LUC* with varying concentration of ABA and IAA. N = 7 experiments. Data are mean \pm S.E.M., ** $p < 0.01$, *** $p < 0.001$, Student's *t*-test.

Table S1. Primers used for plasmid constructions.

Plasmids	Forward primers	Reverse primers
<i>IAA19pro-LUC</i>	<i>IAA19proF</i> : AGAGGATCCTGGGATGTTAGGAGAAGGAGATTCG	<i>IAA19proR</i> : CTTCCATGGTTCTTGAACCTCTTTTTTTCCTCTCAC
pHBT95-MYB44	CGGCTCCCTCTCCCCTTGCTCCGTGGATCC ATGGCTGATAGGATCAAAGGTCCAT	GTAGTCTGGAACGTCGTATGGGTAAGGCCT CTACTCGATTCTCCCAACTCCAATTTGAC
pHBT95-MYB73-HA	CGGCTCCCTCTCCCCTTGCTCCGTGGATCC ATGTCAAACCCGACCCGTAAG	GTAGTCTGGAACGTCGTATGGGTAAGGCCT CTCCATCTTCCCAATTCCG
pHBT95-MYB77	CGGCTCCCTCTCCCCTTGCTCCGTGGATCC ATGGCGGATCGTGTTAAAGG	GTAGTCTGGAACGTCGTATGGGTAAGGCCT CTACTCAACCTTAGGTGTTATTACTCC
pHBT95-MYB61-HA	CGGCTCCCTCTCCCCTTGCTCCGTGGATCC ATGGGGAGACATTCTTGCTGTTAC	GTAGTCTGGAACGTCGTATGGGTAAGGCCT AAGGGACTGACCAAAAAGAGACG
AD-MYB44	GTCATATGGCCATGGAGGCCAGTGAATTC ATGGCTGATAGGATCAAAGGTCCAT	ATTCATCTGCAGCTCGAGCTCGATGGATCC CTACTCGATTCTCCCAACTCCAATTTGAC
AD-MYB73	GTCATATGGCCATGGAGGCCAGTGAATTC ATGTCAAACCCGACCCGTAAG	ATTCATCTGCAGCTCGAGCTCGATGGATCC CTCCATCTTCCCAATTCCG
AD-MYB77	GTCATATGGCCATGGAGGCCAGTGAATTC ATGGCGGATCGTGTTAAAGG	ATTCATCTGCAGCTCGAGCTCGATGGATCC CTACTCAACCTTAGGTGTTATTACTCC
AD-MYB61	GTCATATGGCCATGGAGGCCAGTGAATTC ATGGGGAGACATTCTTGCTGTTAC	ATTCATCTGCAGCTCGAGCTCGATGGATCC CTAAAGGGACTGACCAAAAAGAGACG
6P1-MYB77	GAAGTTCTGTTCCAGGGGCCCTGGGATCC ATGGCGGATCGTGTTAAAGG	ATGCGGCCGCTCGAGTCGACCCGGGAATTC CTACTCAACCTTAGGTGTTATTACTCC
pET28a-PYL8	ATCGGATCC ATGGAAGCTAACGGGATTG	ATCGAATTC CTATAGTCTCGGGGTGAAG
pENTR-MYB77	CACCATGGCGGATCGTGTTAAAGG	CTCAACCTTAGGTGTTATTACTCC
cLUC-ABI1	CAGATCTCGTACGCGTCCCGGGCGGATCC ATGGAGGAAGTATCTCCGGC	TGTTTGAACGATCTGCAGTCTAGAGTCGACTTA TCAGTTCAAGGGTTTGCTCTTG