The ABA receptor PYL9 together with PYL8 plays an important role in regulating lateral root growth

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Abscisic acid is a phytohormone regulating plant growth, development and stress responses. PYR1/PYL/RCAR proteins are ABA receptors that function by inhibiting PP2Cs to activate SnRK2s, resulting in phosphorylation of ABFs and other effectors of ABA response pathways. Exogenous ABA induces growth quiescence of lateral roots, which is prolonged by knockout of the ABA receptor PYL8. Among the 14 members of PYR1/PYL/RCAR protein family, PYL9 is a close relative of PYL8. Here we show that knockout of both PYL9 and PYL8 resulted in a longer ABA-induced quiescence on lateral root growth and a reduced sensitivity to ABA on primary root growth and lateral root formation compared to knockout of PYL8 alone. Induced overexpression of PYL9 promoted the lateral root elongation in the presence of ABA. The prolonged quiescent phase of the pyl8-1pyl9 double mutant was reversed by exogenous IAA. PYL9 may regulate auxin-responsive genes in vivo through direct interaction with MYB77 and MYB84. Thus, PYL9 and PYL8 are both responsible for recovery of lateral root from ABA inhibition via MYB transcription factors.

As sessile organisms, plants need a sophisticated regulatory network to survive unfavorable and changing environments. When the soil environment becomes unfavorable, root system is often the first to sense it. This involves phytohormones that act quickly and accurately.

The phytohormone auxin is tightly correlated with both primary and lateral root growth and development. Auxin is perceived by a small family of F-box proteins including TRANSPORT INHIBITOR RESPONSE 1 (TIR1). Auxin gradient, which forms a sink at the root apex and just below quiescent center (QC), provides essential information for cell division, polarity and cell fate. Lateral root initiation requires PIN-FORMED 1 (PIN1)-dependent auxin transport. During lateral root initiation, auxin determines both its position and frequency. After initiation, auxin gradient is also required for the correct patterning of lateral root primordium. To facilitate lateral root primordium emergence, auxin modulates cell turgor in the outer tissue layers and in the primordium, and induces the expression of cell wall remodeling enzymes. Besides auxin, other phytohormones, such as cytokinin, gibberellin, brassinosteroids, abscisic acid and strigolactones, also function during root growth. However, auxin acts as an integrator to them and these phytohormones either regulate polar auxin transport (PAT) or regulate auxin responsive genes.

Abscisic acid (ABA) is an isoprenoid plant hormone and a main regulator of responses to biotic and abiotic stress. ABA biosynthesis is one of the quickest responses of plants facing stresses and ABA, in turn, will trigger downstream ABA responsive gene expression. Besides its function on stress responses, ABA also has a role in plant development and physiological processes, including seed development and dormancy, embryo morphogenesis and stomatal movement. The core signaling pathway of ABA includes receptors, phosphatases and kinases. The 14-member family of START domain proteins known as PYR1/PYLs/RCARs, has been identified as intracellular ABA receptors. PYR1/PYLs/RCARs bind to and inhibit type 2C protein phosphatases (PP2Cs) in an ABA-dependent manner, which in turn release the inhibition of PP2Cs on SNF1-related kinase 2 (SnRK2).
Single mutant was reported to have a longer quiescent phase on pyl8 length were both decreased (Fig. 2a). The growth than the double mutant showed a slower lateral root roots (Fig. 1b). Consistent with its visible phenotypes, the mary root growth phenotypes, we have also observed that the interact with an R2R3 MYB transcription factor, MYB44. We found that PYL9 acts together with PYL8 in has a very high sequence identity with PYL8 and both of them have a relative high expression level in roots, μferent concentrations of ABA, pyl8-1pyl9 had more lateral roots than pyl8-1 double mutant showed a longer primary root than single mutant after treatment with ABA. On the 9th day post transfer (dpt) to plates with growth than the pyl8-1pyl9 double mutant showed a faster primary root pyl8-1pyl9 double mutant shows a reduced inhibition of primary root growth and lateral root formation from inhibition after ABA treatment. Our results suggest that both pyl8 and pyl9 are nodes of crosstalk between ABA and auxin, and their signal is transduced by a group of MYB transcription factors to regulate the lateral roots.

Results

The pyl8-1pyl9 double mutant shows a reduced inhibition of primary root growth and lateral root formation to exogenous ABA compared to pyl8. ABA is a key factor in regulating root architecture under stress. To dissect its role, we analyzed Arabidopsis mutants impaired in ABA signaling pathway, especially null mutants in ABA receptors. Previous studies demonstrated that PYL8 plays an important role in ABA signaling in roots and the work was focused on primary roots. We found that in addition to regulating the growth of primary roots, pyl8 could also promote lateral root recovery from ABA inhibition. Since PYL9 has a very high sequence identity with PYL8 and both of them have a relative high expression level in roots, we obtained a T-DNA knock-out mutant of pyl9 (SALK_083621) and tested the root growth of pyl8-1pyl9 double mutant in primary root growth and lateral root formation. pyl9 mutant did not have an obvious lateral root growth phenotype on ABA medium (see Supplementary Fig. S1). We also quantified the primary root length and the lateral root number as well as average lateral root length both on the control medium and on ABA medium, still found no significant difference between pyl9 and wild type (WT) (see Supplementary Fig. S1). PYL8 and PYL9 are in the same clade of the PYL phylogenetic tree. We therefore analyzed the root growth of the pyl8-1pyl9 double mutant (see Supplementary Fig. S2). Consistent with its visible phenotypes (Fig. 1b), the pyl8-1pyl9 double mutant showed a faster primary root growth than the pyl8-1 single mutant after treatment with ABA. On the 9th day post transfer (dpt) to plates with ABA, the pyl8-1pyl9 double mutant showed a longer primary root than pyl8-1 single mutant at all concentrations of ABA tested (Fig. 1c). We also quantified the lateral root number of both mutants (Fig. 1d). On the 7 dpt to different concentrations of ABA, pyl8-1pyl9 had more lateral roots than pyl8-1 on 1, 5 and 10 μM ABA medium, and both of them differ significantly from WT at all concentrations except 1 μM ABA medium. Thus pyl8-1pyl9 double mutant was less sensitive to ABA than pyl8-1 single mutant in primary root growth and lateral root formation.

Lateral root growth stays in a longer quiescence in pyl8-1pyl9 double mutant. Besides primary root growth phenotypes, we have also observed that the pyl8-1pyl9 double mutant had shorter lateral roots (Fig. 1b). Consistent with its visible phenotypes, the pyl8-1pyl9 double mutant showed a slower lateral root growth than the pyl8-1 single mutant after treatment with ABA. Lateral root growth was severely suppressed in both mutants exposed to ABA especially in the double mutant. Average lateral root length and total lateral root length were both decreased (Fig. 2a). The pyl8 single mutant was reported to have a longer quiescent phase on ABA plates before recovery. Our results here suggest that PYL9 may promote growth recovery of lateral root under ABA treatment together with PYL8. Lateral root with a length shorter than 0.5 mm is considered to be quiescent. We found that the two mutants and the wild type had similar quiescent days on control medium.
However both mutants have a prolonged quiescent phase than Col-0 under ABA treatment. Moreover, the pyl8-1 pyl9 double mutant had an even longer quiescent phase than the pyl8-1 single mutant (Fig. 2b). This suggests that PYL9 functions together with PYL8 in promoting growth recovery of lateral roots under ABA treatment.

IAA suppresses ABA-induced growth inhibition of lateral roots in pyl8-1 pyl9. Exogenous IAA can release growth inhibition of lateral roots under ABA treatment. To determine whether this happens in the pyl8-1 pyl9 double mutant, we analyzed the lateral root growth of pyl8-1 pyl9 seedlings grown on ABA-containing medium supplemented with IAA (Fig. 3a). We found that the ABA-dependent lateral root growth suppression of pyl8-1 pyl9 was partially overcome by 10 nM IAA, and was fully rescued by 100 nM IAA (Fig. 3b). We also determined lateral root growth over the course of time. We found that both pyl8-1 and pyl8-1 pyl9 mutants had a slower

Figure 1. The primary root and lateral root formation of pyl8-1 pyl9 double mutant are more insensitive upon ABA treatment. (a) A schematic diagram of T-DNA insertions in the PYL9 gene. The T-DNA insertion in the pyl9 mutant is inserted in exon, which is presented as closed box. (b) Root architecture of Col-0, pyl8-1 and pyl8-1 pyl9 mutants under ABA treatment. Root architecture of seedlings was documented at 9 dpt (days post transfer). Seedlings were transferred at 4 dpg (days post germination) to the control medium (1/2 MS, 1% sucrose) or medium with 10 μM ABA. Bar, 1 cm. (c,d) The primary root length and lateral root number of Col-0, pyl8-1 and pyl8-1 pyl9 mutants were documented with different concentrations of ABA. The concentrations of ABA in the medium are as indicated. Error bars indicate s.e.m. (n = 25 seedlings, 5 independent experiments). Asterisks indicate comparison between pyl8-1 and pyl8-1 pyl9, *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test. P-values were adjusted for multiple comparisons by "Benjamini & Hochberg" method. Primary root length and lateral root number of pyl8-1 (at 5 μM, 10 μM, 15 μM, 20 μM) and pyl8-1 pyl9 (at all ABA concentrations) were significantly larger than Col-0, P < 0.05.
lateral root elongation than wild type under ABA treatment but not under control conditions. Exogenous application of 10 nM IAA rescued the ABA-dependent growth defects of lateral root in pyl8-1 but not in pyl8-1pyl9. In contrast, in the presence of 100 nM IAA, the lateral root growth defects of both mutants were fully rescued (Fig. 3c). This means that IAA could also rescue the ABA-dependent inhibition of lateral root elongation caused by loss of PYL8 and PYL9. However, the pyl8-1pyl9 double mutant requires a higher concentration of IAA than pyl8-1 single mutant. These results suggest that the lateral root growth defect of pyl8-1pyl9 mutant may be caused by auxin deficiency.

ABA-induced overexpression of PYL9 alters both primary and lateral root growth. Since the function of PYL9 on root growth was relatively weaker than that of PYL8, we analyzed the root architecture of PYL9 overexpression lines. The PYL9 gene, under the control of RD29A promoter, was transformed into Arabidopsis. This RD29A gene was first discovered as a cold-induced gene. Later it was found to be responsive to not only cold but also dehydration and ABA. Two homozygous pRD29A:PYL9 transgenic lines with high PYL9 expression were used for further analysis. Both transgenic lines had a dramatic increase in PYL9 expression compared to Col-0 wild type under ABA treatment (see Supplementary Fig. S3).
Figure 3. High exogenous IAA complements the lateral root growth defect of pyl8-1pyl9 on ABA-containing medium. (a) Root architecture of Col-0, pyl8-1 and pyl8-1pyl9 mutants under ABA and ABA with IAA treatment. Root architecture of seedlings was documented at 7 dpt. Seedlings were transferred at 4 dpg to the control medium (1/2 MS, 1% sucrose) (upper panel) or medium supplemented with 5 μM ABA (middle panel) or 5 μM ABA and 100 nM IAA (bottom panel). Bar, 1 cm. (b) Average lateral root length of Col-0, pyl8-1 and pyl8-1pyl9 mutants grown with or without ABA treatment or ABA plus IAA treatment. Lateral root length of seedlings grown on ABA-containing medium was measured at 7 dpt. The concentrations of ABA and IAA in the medium are as indicated. Error bars indicate s.e.m. (n = 25 seedlings, 5 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001, Student's t test. (c) The lateral root length of seedlings was measured at the indicated days after transfer to media with or without ABA or ABA plus IAA. Error bars indicate s.e.m. (n = 25 seedlings, 5 independent experiments). *P < 0.05, **P < 0.01, ***P < 0.001, Student's t test. Asterisks indicate comparison between pyl8-1 and pyl8-1pyl9.
We transferred 4-day-old seedlings to both control medium and medium with 1 μM ABA (Fig. 4a). We monitored the lateral root length over time (Fig. 4b). The two transgenic lines did not have any obvious difference from wild type on control medium since the PYL9 gene was not induced. When 1 μM ABA was applied, the transgenic lines produced longer lateral root than the wild type after 3 days and the difference was more significant over time (Fig. 4b). As loss of PYL9 caused a prolonged quiescent phase, we wondered whether this increase in average lateral root length was due to a shortened quiescent phase. Under 1 μM ABA treatment, more than half of the lateral roots had a quiescent phase shorter than 3 days in both transgenic lines (Fig. 4c). Thus, overexpression of PYL9 promotes lateral roots to escape from ABA-dependent inhibition.

To test whether overexpression of ABA receptor PYL9 conferred hypersensitivity to ABA in primary root growth, we analyzed root growth of pRD29A:PYL9 transgenic lines with treatment of different concentrations of ABA. After transferring to medium supplemented with ABA and growing for a week, the primary root of transgenic lines showed an obvious reduction (Fig. 5a). After ABA treatment, both lines showed a significant reduction of lateral root number compared to the wild type (Fig. 5b). This suggests that PYL9 functions in the promotion of lateral root escaping from quiescence as well as the inhibition of primary root growth and lateral root formation under ABA treatment.

**PYL9 interacts with MYB transcription factors.** Recently, interactions between PYLs and MYBs have been reported. In our study, several different clones in each combination were tested on yeast growth medium deprived of Trp, His and Leu (Fig. 6a). All of these clones with MYB44/PYL8 had visible colonies, suggesting that PYL8 strongly interacts with MYB44. However, only one of these clones with MYB44/PYL9 had visible colonies (Fig. 6a), suggesting that PYL9 may weakly interact with MYB44. Other MYB proteins were also tested using multiple independent colonies. PYL6 was used as a negative control and had no interactions with any of the three tested MYBs (Fig. 6b, bottom panel). PYL8, as a positive control, had interactions with MYB77 and MYB73 (Fig. 6b, upper panel). Similar to clones with MYB44/PYL9, the minority of these independent clones with MYB77/PYL9 had visible colonies. However, we did not detect any interaction between MYB73 and PYL9 in the yeast two-hybrid assay (Fig. 6b, middle panel). These differences between our results and previous published results might be caused by the expression level of different plasmids. Li et al. used pGADT7-MYBs and the higher expressing pGBKTT7-PYLs, whereas Jaradat et al. used pGADT7-PYLS and the lower expressing pGBT9-MYBs. We used pGADT7-MYBs and pBD-GAL4-PYLS. Our results and the previous findings suggest that PYL8 strongly interacts with MYBs, while PYL9 has a relatively weak interaction with MYBs.

To further analyze the interactions between PYL9 and MYBs in plant cells, we used the firefly luciferase (LUC) complementation assay in Arabidopsis protoplasts. PYL9 was fused to the N-terminal domain of firefly luciferase (LUC) and MYB proteins were fused to the C-terminal domain of LUC. We co-transformed PYL9-nLUC with the MYB-cLUC into Arabidopsis protoplasts (Fig. 6c). Coexpression of PYL9-nLUC with MYB44/73/77-cLUC, but not MYB61-cLUC produced measurable luciferase activity. These results suggest that PYL9 also interacts with some MYBs in vivo.

**PYL9 enhances the activity of MYBs in the presence of ABA and IAA.** We asked whether the interaction with PYL9 might affect the regulation of downstream genes by MYBs. Previous studies showed that MYB77 could recognize cis-elements MBSI (CNGTTR) and MBSII (GTAGTTA) and preferentially binds to the MBSI motif. IAA19 is one of these downstream genes regulated by MYB77. To further understand whether PYL9 and MYB77 regulate their target genes as a protein complex, we used a transgenic line with tagged PYL9 driven by PYL9 native promoter. PYL9-PYL9-YFP-HA was introduced into the quadruple mutant ppyr1pyl1pyl2pyl4 and this tagged line was used in chromatin immunoprecipitation assay (see Supplementary Fig. S4). We pre-incubated the seedlings with 5 μM ABA and 1 μM IAA for 5 hours, and the assay was performed as described. We added extra EGS or ethylene glycol-bis (succinic acid N-hydroxysuccinimide ester) to strengthen the PYL9-MYBs interaction during cross-linking. Because MYB77 preferentially binds to MBSI, we filtered candidate genes using this criterion. We checked several DNA fragments that harbored this cis-element, including the promoter region of IAA1, IAA7, IAA17, IAA19 and HAT2. Different primer pairs were designed flanking this motif. The ChIP-PCR result showed that the promoter region of IAA7 was enriched with PYL9-YFP-HA after treatment of IAA and ABA (Fig. 7b). The ChIP-quantitative-PCR result showed nearly 10-fold enrichment in pPYL9:PYL9-YFP-HA transgenic lines than in the wild type negative control after the treatment of ABA and IAA. While in the control, there was no such enrichment (Fig. 7c). The motif in this promoter fragment was CTGTTG, belonging to MBSI. We also found another CTGTTG in the promoter of HAT2 but this one did not show enrichment in ChIP-quantitative-PCR. This might be due to the position of the motif. The one in IAA7 is located around 500 bp upstream from the start codon, however, the one in HAT2 is located within 100 bp from its start codon. This suggests that the transcription complex of PYL9 and MYB proteins may bind to promoters around 500 bp from where transcription starts. This in vivo evidence strongly supports the hypothesis that PYL9 regulates the transcriptional activity of MYB77 or other MYB proteins directly in vivo and participates in the auxin signal pathway.
Figure 4. Induced expression of PYL9 increases the average lateral root length and shortens the quiescent phase. (a) Root architecture of pRD29A:PYL9#13, pRD29A:PYL9#27 and Col-0 under ABA treatment. Seedlings were transferred at 4 dpg to the control medium (1/2 MS, 1% sucrose) (upper panel) or medium supplemented with 1 μM ABA (bottom panel). Root architecture of seedlings was documented at 7 dpt. Bar, 1 cm. (b) The lateral root length of Col-0, pRD29A:PYL9#13 and pRD29A:PYL9#27 was measured at the indicated days after transfer to media supplemented with ABA. Seedlings were transferred at 4 dpg. Error bars indicate s.e.m. (n = 25 seedlings, 5 independent experiments). **P < 0.01, ***P < 0.001, Student’s t test. Asterisks indicate comparison between Col-0 and pRD29A:PYL9#13, Col-0 and pRD29A:PYL9#27. (c) Pie charts of the percentage of quiescent lateral roots for the indicated number of days on seedlings on control medium and medium with ABA (n = 25 seedlings, 5 independent experiments). The proportion of quiescence less than 3 days of Col-0 is significantly less than that of pRD29A:PYL9#13 and pRD29A:PYL9#27, respectively (p-value < 0.001, one-tailed binomial test).
ABA and auxin are two important phytohormones; one is well known for its function under stress, and the other one is well known for its growth-inducing activity. Our study here reveals a crosstalk node between them in regulating root growth. Roots provide a tight connection between plants and the soil environment, and are often the first to sense the soil environment. Root systems of ABA-deficient mutants like aba2-1 and aba3-1 are less affected than wild type seedlings upon osmotic stress. ABA at low concentrations has been reported to promote root growth. The endogenous ABA controls root architecture both in the presence and absence of osmotic stress through maintaining meristem in dormancy and inhibiting QC division and suppressing the differentiation of stem cells in the primary root. High concentrations of ABA are well known to inhibit the growth of both primary and lateral root. ABA has a much stronger effect on lateral root than on the primary root, suggesting that different signaling mechanisms in the two types of roots. In our study, the lateral root number in all the tested ABA concentrations showed a decrease compared to control medium. What's more, the lateral root elongation is more sensitive to ABA than the inhibition of seed germination and this reversible growth arrest occurs at a specific developmental stage, that is right after the lateral root emergence with a length less than 0.5 mm. As shown in Fig. 2b, longer quiescence was observed on ABA-containing medium and this might be due to a dormant state of lateral roots. Although ABA is well known for its function in seed dormancy and growth inhibition, different ABA sensitivities suggest different pathways in lateral roots compared with other tissues.

Salt stress induces a quiescence phase in post emergence lateral root growth and then recovery takes place several days later, which involves genes of ABA biosynthesis, signaling and transcription regulation. PYL8 is responsible for this quiescence upon ABA treatment. PYL9 has a 77% amino acid sequence identity with PYL8 and particularly high GUS activity can be detected in stele cells of pPYL9:GUS transgenic plants. Although pyl9 single mutant did not show obvious difference from wild type on ABA medium (see Supplementary Fig. S1),

**Figure 5.** Induced expression of PYL9 confers hypersensitivity of primary root and lateral root initiation. (a,b) Relative primary root length and lateral root number of Col-0, pRD29A:PYL9#13 and pRD29A:PYL9#27 under ABA treatment. The concentrations of ABA in the medium are as indicated. Measurements in a are expressed as a percentage of the length under 1/2 MS conditions. Error bars indicate s.e.m. (n = 25 seedlings, 5 independent experiments). *P < 0.05, ***P < 0.001, Student’s t test. Asterisks indicate comparison between Col-0 and #13, Col-0 and #27.

**Discussion**

ABA and auxin are two important phytohormones; one is well known for its function under stress, and the other one is well known for its growth-inducing activity. Our study here reveals a crosstalk node between them in regulating root growth.
Figure 6. PYL9 interacts with MYB transcription factors. (a) PYLs interact with MYB44 in the yeast two-hybrid assay. PYLs fused to the GAL4-DNA-binding domain (BD) were used as bait. MYB44 fused to the GAL4-activating domain (AD) were used as preys. Interaction was determined by yeast growth on media lacking His, Leu and Trp. BD-PYL10 and AD-ABI1 was used as a positive control. (b) PYL9 interacts with MYBs in the yeast two-hybrid assay. PYL8 and MYB combinations were used as positive controls and PYL6 and MYBs were used as negative controls. Interaction was determined by growth on media lacking His, Leu and Trp with or without 5 μM ABA. Dilutions (10⁻¹, 10⁻², and 10⁻³) of saturated cultures were spotted onto the plates, which were photographed after 5 days. (c) Co-transform of PYL9-nLUC with MYB44-cLUC, MYB73-cLUC, MYB77-cLUC and MYB61-cLUC in Col-0 wild-type protoplasts in LUC complementation assay.
pyl8-1pyl9 double mutant had an even longer primary root and more lateral roots than pyl8-1 on ABA medium (Fig. 1c,d). The induced overexpression of PYL9 upon ABA treatment led to a shorter primary root and fewer lateral roots (Fig. 5a,b). These results suggest that PYL9 functions in the repression of lateral root formation and primary root elongation by ABA. PYL9 is a functional ABA receptor11 and the ABA signal perceived by it and other PYLs is passed down eventually to ABA-RESPONSIVE ELEMENT (ABRE)-BINDING FACTOR (ABF) proteins. And overexpression of ABF2 confers hypersensitivity to ABA in primary root63. Other mutants impaired in ABA signaling pathway have also been tested including abi1-1, pyr1/pyl1/2/4 and snrk2.2/3/662. All these ABA-resistant mutants showed a reduced repression of primary root growth and lateral root formation upon ABA treatment63.

**Figure 7.** PYL9 directly regulates MYB77 transcriptional activity in vivo. (a) PYL9 enhances the ability of MYB77 to activate IAA19 expression in Col-0 protoplasts. PYL9, MYB77, IAA19-LUC and ZmUBQ-GUS were co-expressed in protoplasts. IAA19-LUC was used as the auxin-responsive reporter. ZmUBQ-GUS was used as the internal control. After transfection, protoplasts were incubated for 12 h under light in the absence of hormone (open bars) or in the different combinations of 5 μM ABA and 1 μM IAA. Error bars indicate s.e.m. (n ≥ 3 experiments). **P < 0.01, ***P < 0.001, Student’s t test. (b,c) ChIP-PCR and ChIP-quantitative-PCR for IAA7 promoter. The 500-bp region of the translational start of IAA7 contains MBSI elements recognized by MYB77 and MYB44. pPYL9:PYL9-YFP-HA was treated with ABA and IAA before conducting ChIP assay. Col-0 was used as control. Numbers are folds compared to Col-0 adding anti-HA. Error bars indicate s.e.m. (n = 3 experiments). *P < 0.05, Student’s t test.
and this correlates with our results in pyl8-1 pyl9 double mutant. However, these ABA-resistant mutants have shorter quiescence compared to WT on ABA medium, which is opposite to our pyl8-1 pyl9 double mutant. This suggests a different pathway involving PYL9. Recently, interactions between PYLs and MYBs have been reported29,30. PYL8 strongly interacts with the MYBs29,30. Further studies found that PYL9 and probably PYL7 also interact with these MYBs29, which is different from the previous published result30. Our results also showed that PYL9 interacted with MYB77 and MYB44 (Fig. 6), however in our Y2H assay there was no obvious interaction between PYL9 and MYB73 (Fig. 6b), which is different from previous reports29. This is probably due to different yeast systems and plasmids. MYB77 is a positive regulator of lateral root growth and its interaction with ARF7 suggests that MYB77 may function through auxin signal transduction pathway27. The lateral root growth of myb77 mutants is more sensitive upon ABA treatment and exogenous IAA could reverse this like it could reverse in pyl8-1 and pyl8-1 pyl929. So the interaction between PYL9 and MYB77 suggests they may function together. MYB77 preferentially recognizes cis-elements MBSI (CNGTTR) and previous EMSA data showed that PYL8 protein enhances MYB77 binding to MBSI in an ABA-dependent manner25,27. MYB44 has a clear preference for MBSII (GTAGTTA) type but still binds to MBSII44. Our chromatin immunoprecipitation assay indicated that anti-HA antibody pulls down the protein complex of PYL9-MYB proteins (Fig. 7b,c). The exogenous IAA and ABA enhanced their interaction and the effect on the downstream gene promoters29 (Fig. 7). Quantitative PCR showed that one fragment of IAA7 promoter, containing MBSI motif, has a huge enrichment (Fig. 7b,c). IAA7 is a member of the Aux/IAA protein family, which is induced by auxin45. Besides PYL8 and PYL9, PYL7 is also an interacting protein of MYB44 in Y2H assays44. This implies that more PYLs might be involved in the PYL-MYB pathway.

Auxin and ABA are widely accepted as regulators of root growth. These two phytohormones have a lot of interactions and previous studies have revealed that auxin can potentiate ABA response in roots46. Unlike in p35S:VP1 where ABA fully inhibits auxin induced lateral root initiation47, the prolonged quiescence phase of pyl8-1 on ABA medium was shortened by application of IAA25. Osmotic stress represses lateral root development which could be overcome by auxin42, however exogenous ABA represses lateral root formation, that is the initiation of lateral root primordia, which could not be rescued by auxin41. In our study, the quiescence in pyl8-1 and pyl8-1 pyl9 was overcome by application of IAA (Fig. 3). What’s more, the severe defects in the double mutant require more IAA to be rescued (Fig. 3b,c). Whether auxin rescues or not might be dependent on the state of lateral roots and these activities might be mediated by different mechanisms. Lateral root primordia initiation is highly dependent on auxin41, however in our study, we only consider the lateral roots that are already formed when we transferred seedlings to ABA medium.

The function of PYL9 and MYB77 complex is dependent on IAA treatment. In Arabidopsis protoplasts, the reporter pIAA19:LUC showed a strong signal when PYL9 was co-transformed with MYB77 in the presence of IAA (Fig. 7a). In transgenic seedlings, PYL9 can pull down auxin responsive promoter fragments in the presence of IAA and ABA (Fig. 7b,c). Besides, PYL9 itself is a functional ABA receptor. Taken together, this suggests that PYL9 connects ABA and auxin signaling pathway. MYB77 modulates auxin response by forming a heterodimer with ARF7 and their double mutant pyl7 pyl9 (myb77-2nph4-1) has even smaller lateral root density than pyl8 pyl9-1, which is already smaller than wild type and pyl8 pyl9-1 pyl1/2/4. In myb77 mutants, auxin-responsive genes are attenuated while they are increased in over-expression lines27. Similar results were obtained in p35S:MYB44 transgenic lines where auxin-responsive genes are increased, suggesting that salt respose and auxin signaling cross-talk at the transcriptional level11. This suggests that MYB77 and MYB44 might have some overlapping functions in regulating auxin-responsive genes. IAA7, which might be the target of PYL9-MYB77 complex, is also a key component regulating ABA and auxin-dependent post-embryonic growth49. It is suppressed when ABA represses embryonic axis elongation by potentiating auxin signaling in the elongation zone49.

**Methods**

**Plant materials and growth condition.** The pyl8-1 (SAIL_1269_A02)24, pyl9 (SALK_083621)24, pyr1pyl1/2/4 quadruple mutant were in Col-0 background. The pyl8-1 pyl9 double mutant was obtained by crossing pyl8-1 with pyl9. The PRD29A::PYL9 construct was transformed into Col-0, and pPYL9::PYL9-YFP-HA construct was transformed into pyr1pyl1/2/4 by Agrobacterium tumefaciens-mediated floral-dip transformation.

Seeds were surface-sterilized for 8 min in 10% bleach and then rinsed in sterile deionized water for four times. Sterilized seeds were grown on 10% Phytagel (Sigma) or 0.8% agar media containing 1/2 MS nutrients (catalog no. M524, PhytoTechnology Laboratories), 1% sucrose adjusted to pH 5.7 (control media), and kept at 4 °C for 2 days. Seedlings were grown vertically before transfer to control media or media supplemented with the indicated concentrations of ABA (Sigma, A1049) or IAA (Sigma, I2886). Seedlings were grown at 22 °C under 16-h light/8-h dark cycles.

For protoplast analysis, seedlings were grown on Jiffy 7 peat soil (42 mm 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 μE m−2 s−1) and 50 to 70% relative humidity under well-watered conditions.

**Phenotype analysis.** The primary root length was measured from the first day after transfer. The results of lateral root were observed using HIROX KH-7700 digital microscope with MX-5040RZ lens. 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 quiescent49.
Plasmid construction. The pIAA19-LUC and pGADT7-MYBs constructs were generated as described25. The pBD-GAL4-PYLS constructs were generated as described11.

Yeast two-hybrid assay. Yeast two-hybrid assays were performed as described25. PYLs fused to the GAL4 DNA binding domain were used as baits. MYB44, MYB73 and MYB77 fused to the GAL4-activating domain were used as preys. Interaction was determined by growth assay on media lacking His or His and Ade with or without 5μM 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 described25. All steps were at room temperature. pIAA19::LUC was used as the auxin-responsive reporter. ZmUBQ-GUS was used as the internal control. After transforming, 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 reporter.

Chromatin immuno precipitation assay. Ten-day-old transgenic seedlings and anti-HA antibodies (1:100 for ChiP assay, HA-Tag, 26D11, Mouse mAb, M20003, Abmart, Shanghai, China) were used for ChiP experiments25. Briefly, the transgenic seedlings were ground into a fine powder with liquid nitrogen and resuspended in nuclei isolation buffer. 1.5 mM EGS was added into the nuclei isolation buffer for protein-protein cross-linking. The nuclei were then collected by centrifugation and resuspended with nuclei lysis buffer. 1.5 mM EGS was added into the nuclei isolation buffer for protein-protein cross-linking. The nucleus were then collected by centrifugation and resuspended with nuclei lysis buffer. The resuspended chromatin was sonicated to fragments with various sizes (250 bp–1 kb) subsequently. PYL9-HA-YFP-MYBs protein complex was precipitated from input DNA with or without anti-HA antibodies. Protein A agarose beads (Millipore, USA) were added into the incubation mixture for additional 2 h at 4°C. The immune complexes were eluted from the washed protein A beads. The DNA was purified with phenol/chloroform (1:1, v/v) and precipitated. The purified DNA and input DNA were used as templates. The enrichment of DNA fragments was determined by quantitative PCR with specific primers.

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**Author Contributions**

L.X. conducted the phenotype analysis experiments and the yeast-two–hybrid experiments. Y.Z. and J.G. did the Arabidopsis transient transformation experiments. L.X., Y.Z., J.G., C.X. and J.-K.Z. designed the study. L.X. and Y.Z. analyzed the data. All authors reviewed the manuscript.

**Additional Information**

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