The Arabidopsis Transcription Factor MYB77 Modulates Auxin Signal Transduction

Ryoung Shin, Adrien Y. Burch, Kari A. Huppert, Shiv B. Tiwari, Angus S. Murphy, Tom J. Guilfoyle, and Daniel P. Schachtman

INTRODUCTION

The development of roots is altered in response to many different environmental changes that occur in soil (Casimiro et al., 2003; López-Bucio et al., 2003). Both root hair and lateral root development change in different ways according to nutrient and moisture availability in the soil. One of the main functions of roots is in the acquisition of mineral nutrients from the soil environment (Epstein, 1977). To maximize the capture of nutrients from soils, root developmental changes often result in altered overall root architecture. Lateral roots may proliferate or elongate in patches of soil that contain resources such as nitrogen or phosphorus. Lateral root density and root hairs may also increase in response to low nutrient conditions (López-Bucio et al., 2003). One conspicuous response to low nitrogen and phosphorus is a change in overall plant development that results in a shift in the resource allocation to the roots and higher root-to-shoot biomass ratios (Hodges, 2004). In contrast with nitrogen and phosphorus deficiency, the lack of potassium leads to an overall decline in root and shoot growth. In potassium-deprived conditions, lateral root growth and development decreases (Armengaud et al., 2004; Shin and Schachtman, 2004). Some of the important factors that lead to the changes in root growth under patchy nitrogen and phosphorus conditions or phosphorus-deprived conditions (Schachtman and Shin, 2007) have been identified. It is not yet known what leads to the changes in root development that result in reduced lateral root density under low potassium conditions. The control of root growth in response to nutrient supply is a vital developmental adaptation used by plants for survival under changing conditions encountered in soils (López-Bucio et al., 2003).

The hormone auxin plays a central role in lateral root development (Casimiro et al., 2003). The action of auxin depends on specific auxin receptors that function in the ubiquitin-proteasome pathway and transporters that control the uptake and polar localization of auxin (Berleth et al., 2004; Blakeslee et al., 2005; Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Woodward and Bartel, 2005; Yang et al., 2006). Transcription factors that regulate gene expression in response to auxin are also a key component in regulating the multitude of effects that auxin exerts on plant growth and development (Casimiro et al., 2003; Montiel et al., 2004). Several transcription factors, some of which are regulated by auxin, play a role in lateral root development. Studies on gene regulation in response to auxin have mainly focused on specific classes of genes regulated by auxin and auxin response factors (ARFs) that interact with Aux/IAA proteins (Guilfoyle and Hagen, 2001; Berleth et al., 2004). Both Aux/IAAs and ARFs play important roles in lateral root development (Teale et al., 2006).

MYB factors have not been implicated in lateral root development or in regulating auxin-inducible genes. These factors are...
The emergence of the lateral root, the expression of and below the emerging lateral root primordia (Figure 2B). Upon through the epidermis, the reporter gene was expressed above in the vascular tissue (Figure 2A). As the lateral roots emerged, MYB77 was strongly localized behind the lateral root tip (Figures 2C and 2D). Later in lateral root development, strong expression of MYB77 was seen in a discrete region just behind the tip (Figures 2E to 2H). MYB77 expression partially overlapped localized auxin accumulations as indicated by the DR5:GUS auxin-responsive reporter (Ulmasov et al., 1997b), especially when treated with exogenous IAA (Figures 2 and 3; see Supplemental Figure 2 online). Moreover, analysis of MYB77 expression using the Genevestigator Atlas showed that MYB77 is expressed in primary and lateral root tips. (https://www.genevestigator.ethz.ch/at/index.php?page=tairandoption=atlasandagi=&AT3G50060). The expression pattern of MYB77 in the different parts of roots was also confirmed using PCR, which showed that MYB77 was more strongly expressed in the tips of lateral and primary roots (see Supplemental Figure 1B online).

**RESULTS**

The Phenotypic Analysis of MYB77 Overexpression Plants and Spatial Analysis of MYB77 Expression in Roots

MYB77 was identified as being specifically downregulated in Arabidopsis roots by potassium deprivation (Shin et al., 2005). To gain insight into what functional processes this transcription factor may influence, MYB77 was overexpressed in Arabidopsis, and two inactivation lines, myb77-1 (Salk_67655) and myb77-2 (Salk_55373), were isolated. The roots and shoots of the lines overexpressing MYB77 (MYB77-ox) were stunted under control conditions, whereas the knockout lines showed no obvious phenotype under control conditions (Figures 1A and 1B). The stunted growth phenotype was similar to wild-type plants grown on higher concentrations of the plant hormone auxin (Figure 1B).

To determine in which cells and tissues MYB77 might function, gene expression was localized using the β-glucuronidase (GUS) reporter gene driven by the 1904-bp MYB77 promoter. Expression of MYB77 was detected in roots (Figures 2A to 2H) and leaves (see Supplemental Figure 2 online). At the initiation of lateral root emergence, MYB77 expression was visible mainly in the vascular tissue (Figure 2A). As the lateral roots emerged through the epidermis, the reporter gene was expressed above and below the emerging lateral root primordia (Figure 2B). Upon emergence of the lateral root, the expression of MYB77 became strongly localized behind the lateral root tip (Figures 2C and 2D). Later in lateral root development, strong expression of MYB77 was seen in a discrete region just behind the tip (Figures 2E to 2G), and eventually the expression of MYB77 could be seen in the vasculature (Figure 2G). In the primary root, MYB77 was expressed within the root cap and in the inner cell layers, including the stele (Figure 2H). MYB77 expression partially overlapped localized auxin accumulations as indicated by the DR5:GUS auxin-responsive reporter (Ulmasov et al., 1997b), especially when treated with exogenous IAA (Figures 2 and 3; see Supplemental Figure 2 online). Moreover, analysis of MYB77 expression using the Genevestigator Atlas showed that MYB77 is expressed in primary and lateral root tips. (https://www.genevestigator.ethz.ch/at/index.php?page=tairandoption=atlasandagi=&AT3G50060). The expression pattern of MYB77 in the different parts of roots was also confirmed using PCR, which showed that MYB77 was more strongly expressed in the tips of lateral and primary roots (see Supplemental Figure 1B online).

Altered MYB77 Expression Modulates Expression of the Auxin-Responsive Reporter

The strongly stunted root phenotype observed in MYB77-ox lines suggested that MYB77 modulates gene expression in response to auxin. MYB77-ox and myb77-1 knockout lines were crossed to plants containing the DR5:GUS auxin-responsive reporter gene (Ulmasov et al., 1997b). As MYB77-ox lines with the highest levels of expression (Figure 1B) exhibited much lower levels of fertility, MYB77-ox lines with medium levels of overexpression were used in these and subsequent experiments. MYB77-ox seedlings grown on control medium showed increased DR5:GUS expression in roots and shoots compared with the wild type (10 independent lines tested) (Figures 3A and 3B), and DR5:GUS activity was reduced in roots and shoots of

![Figure 1. Arabidopsis MYB77-ox Plants Exhibit a Phenotype Similar to Wild-Type Plants Grown with Auxin.](A) MYB77-ox lines grown in soil develop poorly and appear stunted compared with wild-type Columbia (Col-0) or the knockout myb77-1. (B) Col-0, MYB77-ox, and the myb77-1 knockout plants on medium without additional IAA and Col-0 plants on medium containing 1 μM IAA.

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reflected localized auxin concentrations, free IAA concentrations were measured in wild-type, MYB77-ox, and myb77 knockout plants grown under potassium-sufficient and -deficient conditions (Figure 3D). To eliminate differences resulting from altered lateral root number, free IAA levels were assayed in 6-d-old seedlings before lateral roots appeared. Shoot concentrations of free IAA did not vary significantly across the lines or in potassium-sufficient conditions (Figure 3D). In wild-type roots, free IAA concentrations decreased significantly under potassium-deficient conditions. In contrast with the wild type, the free IAA concentrations in the roots of MYB77-ox and myb77-1 were unchanged by potassium deficiency (Figure 3D). The free IAA levels in myb77-1 roots were reduced compared with the wild type under potassium-sufficient conditions but were the same as the wild type under potassium-deficient conditions. However, free IAA levels in MYB77-ox roots were not different from the wild type and myb77-1 under both conditions.

The observed alterations in auxin concentrations could be a result of altered auxin transport in myb77-1 and MYB77-ox lines. Basipetal auxin transport in hypocotyls and roots was assayed using [3H]-IAA (Geisler et al., 2003, 2005). Although auxin transport in hypocotyls was not significantly altered by potassium deficiency in the wild type, myb77-1, or MYB77-ox, auxin transport was reduced under potassium deficiency when myb77-1 was compared with the wild type (P < 0.02; see Supplemental Figure 4A online). In roots, basipetal auxin transport was uniformly reduced under potassium deficiency in the wild type and myb77-1 and MYB77-ox lines (P < 0.01), but no differences in transport were observed between the lines (see Supplemental Figure 4B online). Overall free IAA levels observed in MYB77-ox roots and shoots and in the myb77-1 shoots under control conditions did not differ from the wild type to the extent expected from DR5:GUS reporter activity, which was also measured under control conditions, suggesting that MYB77 regulates auxin signaling processes and auxin concentrations.

Figure 2. MYB77 Is Expressed in Lateral and Primary Roots.
ProMYB77:GUS activity in wild-type roots is localized to lateral roots throughout development ([A] to [G]) and is also expressed in the primary root tip (H). Bars = 1 mm.

**MYB77 Plays a Role in Lateral Root Development**

MYB77 is expressed in both primary and lateral roots; therefore, we determined the effects of exogenous auxin on root growth in the wild type and myb77-1 (Figure 3E) and myb77-2 lines (see Supplemental Figure 3 online). When Arabidopsis seedlings were grown with low exogenous IAA, lateral root density was significantly less in the myb77 knockout plants compared with the wild type (Figure 3E; see Supplemental Figure 3 online). At higher concentrations of auxin, the lateral root density was greater in myb77 knockout plants than the wild type. The difference in lateral root density was due to changes in the number of lateral roots and small changes in primary root length (Figure 3E). From these results, it appears that MYB77 acts as a positive regulator of lateral root responses at low auxin concentrations and has inhibitory effects at higher auxin concentrations.

MYB77 is downregulated by potassium deprivation. To determine whether the remaining expression of MYB77 plays a role in lateral root growth under nutrient deprived conditions, lateral root density was quantified. When plants were deprived of potassium, the lateral root density in myb77-1 and myb77-2 was significantly lower than the wild type (Figure 4A). Under nitrogen
or phosphorus deprivation, the lateral root density was the same for the wild type and for myb77-1 and myb77-2. To determine whether the reduced lateral root numbers under potassium-deprived conditions is an auxin-dependent process, the lateral root density of an auxin receptor mutant (tir1-1) was measured under control and deprived conditions (Figure 4B). Similar to the myb77 knockout mutant lines, tir1-1 had a significantly lower lateral root density under potassium deprivation than the wild type (Figure 4B).

**MYB77 Contributes to the Expression of Auxin Response Genes**

Since results suggested that MYB77 may be involved in lateral root growth and response to auxin, we assayed the expression of genes that are upregulated by exogenous auxin in the wild type and in myb77-1 before and after the addition of 10 μM IAA to buffered solutions containing seedlings. Our results clearly show that in the wild type, the expression of IAA1, IAA19, PIN1, GH3.2, GH3.3, SAUR-AC1, HAT2, and MYB77 (Gil et al., 1994; Abel et al., 1995; Galweiler et al., 1998; Sawa et al., 2002; Tatematsu et al., 2004; Staswick et al., 2005) was upregulated 0.5 to 24 h after the addition of IAA (Figure 5). By contrast, the induction of these genes was greatly attenuated or abolished in the myb77-1 knockout. However, the expression of non-auxin-inducible MYB transcription factors (MYB66 and MYB123; Shirley et al., 1995; Lee and Schiefelbein, 1999) (Figure 5) was not altered in myb77-1 or the Col-0 wild type with or without the addition of auxin (Figure 5). Moreover, auxin-inducible genes were expressed at higher levels in the MYB77-ox lines with different levels of MYB77 expression, even without the application of exogenous IAA (Figure 5).

Multiple putative MYB binding motifs (Williams and Grotewold, 1997) were identified in all seven of the promoters from the genes (Figure 5) that are induced by auxin treatment. MYB factor binding motifs MRE (AACC) and MYBcore (CNGTTR) were previously determined (Romero et al., 1998), and our own gel shift assay experiments confirmed those results with MYB77 (data not shown). Our data show that after IAA addition, the expression of IAA19 in myb77-1 was also greatly attenuated (Figure 5). Similarly, auxin-responsive genes are downregulated in a nph4-1 mutant (null for ARF7), including IAA19 (Okushima et al., 2005; Wilmoth et al., 2005). To determine whether ARF7 and MYB77 might interact and bind to the IAA19 promoter,

![Figure 3. MYB77 Alters Auxin Response and Lateral Root Growth in the Presence of Auxin.](image-url)
we analyzed the gene sequence 500 bp upstream of the IAA19 start codon. In that region, auxin response elements (AuxREs) (Tatematsu et al., 2004) are located in close proximity to MYB binding sites (Figure 6A). The similar attenuation of auxin-responsive genes in the myb77-1 and myb77-2 knockout than the wild type under potassium-deprived conditions but not under phosphorus and nitrogen deprivation. (B) Lateral root density decreased more significantly in myb77-1 and tir1-1 than in the wild type under potassium deprivation. Full nutrients (+NPK), with low (10 μM) K (−K), low (12 μM) phosphorus (−P), and low (100 μM) nitrogen (−N) (n = 50 plants ± se). Bars that have different letters at the top are significantly different (P < 0.05; Tukey’s honestly significant difference).

Figure 4. Lateral Root Density as Affected by Nutrient Deprivation.
(A) Mean lateral root density decreased significantly more in the myb77-1 and myb77-2 knockout than the wild type under potassium-deprived conditions but not under phosphorus and nitrogen deprivation.
(B) Lateral root density decreased more significantly in myb77-1 and tir1-1 than in the wild type under potassium deprivation. Full nutrients (+NPK), with low (10 μM) K (−K), low (12 μM) phosphorus (−P), and low (100 μM) nitrogen (−N) (n = 50 plants ± se). Bars that have different letters at the top are significantly different (P < 0.05; Tukey’s honestly significant difference).

MYB77 Interacts with ARF7 in Vitro and in Vivo

To determine if MYB77 and ARF7 interaction factors interact, in vitro pull-down assays were performed. Results showed that the MYB77 bound to ARF1 and ARF7 (Figures 6B and 6C) as well as ARF 2, 5, and 6 (data not shown), which were the five ARFs tested in this study and represent both repressors and activators of transcription. In vitro MYB77 also binds to IAA19 (Figure 6C), but we did not detect this interaction between IAA19 and MYB77 in vivo using a bimolecular fluorescence complementation (BiFC) system (tested in four independent experiments). Further studies showed that the interaction between MYB77 and ARF7 was dependent on domains III and IV, which are present in most ARF proteins and also Aux/IAA proteins (Figures 6B and 6C) (Hagen and Guilfoyle, 2002; Berleth et al., 2004). An unrelated His-tagged protein (glyoxalase; At1g11840; data not shown) and two other MYB transcription factors (Figure 6C) (MYB66 and MYB123; Shirley et al., 1995; Lee and Schiefelbein, 1999) did not bind in vitro to ARF7, indicating some specificity in the interaction between MYB77 and ARF7 proteins. The binding of MYB77 and ARFs in vitro suggest that the interaction between these proteins may contribute to the modulation of auxin-responsive genes in planta.

To determine whether MYB77 interacts with ARF7 in vivo, BiFC was used in both Arabidopsis roots and onion epidermal cells (Walter et al., 2004). Co bombardment of MYB77 and ARF7 fused to parts of yellow fluorescent protein (YFP) (pUC-SPYCE; N terminus of YFP, pUC-SPYNE; C terminus of YFP) was performed. Fluorescence was observed in the nucleus of cells in which the MYB77 in pUC-SPYNE and ARF7 in pUC-SPYCE (or MYB77 in pUC-SPYCE and ARF7 in pUC-SPYNE) were expressed, indicating complementation of YFP (Figure 7A). As positive controls, ARF7 in pUC-SPYNE and IAA19 in pUC-SPYCE (or ARF7 in pUC-SPYCE and IAA19 in pUC-SPYNE) were tested, which also resulted in fluorescence in the nucleus (Figure 7A). As a negative control, pUC-SPY constructs containing ARF or MYB77 were bombarded with the relevant empty vector (either pUC-SPYCE or pUC-SPYNE), and no fluorescence was observed (data not shown).

The Interaction between MYB77 and the ARF C Terminus Enhances Gene Expression

To further demonstrate the activity of MYB77 plant protoplasts, we used Arabidopsis leaf mesophyll protoplasts (Tiwari et al., 2006). Transfection of the effector gene encoding MYB77 enhanced expression of the auxin-responsive 2XD0 reporter gene, which contains four TGTCTC AuxREs, the binding sites for ARFs (Murfett et al., 2001) (Figures 7B and 7C). The activity was severalfold higher with the MYB77 effector gene compared with the control effector gene, CAT, in both the absence and presence of auxin, 1-naphthalene acetic acid (Figure 7C). The enhanced activity with MYB77 was dependent on the TGTCTC AuxREs in the 2XD0 reporter gene because a Gal4-D1-3 reporter gene, which contains four mutated AuxREs, showed no response to the MYB77 effector gene (GD in Figure 7D). If a truncated version of the ARF5 activator with a Gal4 DNA binding domain (DBD) substituted for its own DBD, but containing its activation and C-terminal dimerization domains was targeted to Gal4 binding sites in the D1-3 promoter, transfection of the MYB77 effector gene did enhance transcription of the D1-3 reporter gene (GD-5A in Figure 7D). By contrast, transfection of a truncated ARF5 lacking both its own DBD and C-terminal dimerization domain resulted in stronger activation of the D1-3 reporter gene, but there was little response to the transient expression of the MYB77 effector gene (GD-5A in Figure 7D), indicating that the C...
terminus was required for enhanced expression of the reporter
gene in the presence of MYB77. There was also no effect of the
MYB77 effector gene if a DBD truncated ARF1 consisting of its
repression domain and C-terminal dimerization domain was
targeted to Gal4 sites in the D1-3 promoter (Figure 7D).

Coimmunoprecipitation assays were performed to identify
which part of MYB77 interacts with ARF7 (Figure 8). His-tagged
full length of MYB77, N-terminal R2R3 domain (R2R3), and
N-terminal deletion (ΔN) of MYB77 (Figure 8A) were precipitated
with hemagglutinin (HA)-tagged ARF7 using protein A beads and
HA antibodies. The C-terminal region of MYB77 (ΔN) coimmuno-
precipitated with ARF7 (Figure 8B).

Analysis of MYB77 and ARF7 Double Mutants
To provide further evidence for potential interactions between
MYB77 and ARF7 in planta, a homozygous double mutant be-
tween myb77-1 knockout plants and arf7 (nph4-1) was created.

The lateral root density under control conditions was measured
in the wild type, myb77-1 knockout plants, arf7 (nph4-1) null, and
the double mutant of MYB77 and ARF7 (myb77-1 nph4-1) (Figure
9). Lateral root density was lower in nph4-1 compared with the
wild type or myb77-1. Knockout of MYB77 in the nph4-1 back-
ground further decreased the lateral root density. This result
implies that MYB77 and ARF7 interact synergistically in planta.

DISCUSSION
To elucidate the function of MYB77, we constructed multiple
overexpression lines and isolated several knockout alleles.
Overexpression of MYB77 using a constitutive promoter re-
vealed a very strong stunted root phenotype under control con-
ditions. The knockout lines showed no obvious phenotype under
control conditions. The stunted root phenotype was similar to
wild-type Arabidopsis plants grown on elevated levels of auxin
(Estelle and Somerville, 1987). The plant hormone auxin is well

![Figure 5. The Expression of Auxin-Regulated Genes Is Attenuated in the myb77-1 Knockout and Increased in MYB77-ox Lines.](image_url)

RNA gel blots of Arabidopsis wild type, knockout myb77-1, and MYB77-ox-1, -2, and -3 lines grown for 7 d and treated with 10 μM IAA for 0, 0.5, 1, 2, 6, and 24 h. The MYB77-ox lines were grown without IAA treatment. rRNA loading control is shown at the bottom.
known to stimulate growth at very low concentrations and to reduce growth at higher concentrations (Teale et al., 2006). Therefore, we tested the hypothesis that \textit{MYB77} was involved in the auxin signal transduction pathway.

**MYB77 Plays a Role in Lateral Root Growth**

To determine where \textit{MYB77} plays a role in whole plants, we constructed promoter:GUS lines and then studied the expression...
MYB77 was most highly expressed in the primary root tip and throughout the development of lateral roots. The localization of MYB77 expression in lateral roots, the decrease in expression under potassium-deprived conditions, and the decrease in lateral root density measured in other studies (Armengaud et al., 2004; Shin and Schachtman, 2004) were correlated. Based on these results, we focused our experiments on changes in lateral root development and tested whether MYB77 may play a role in auxin-mediated lateral root growth.

It is well known that lateral root growth is regulated by auxin (Laskowski et al., 1995; Casimiro et al., 2003). Several transcription factors related to auxin signal transduction have been linked to lateral root development (Montiel et al., 2004). Many lateral root mutants in Arabidopsis have been found to contain defects in auxin-related genes (Casimiro et al., 2003). Auxin plays a role in lateral root initiation and is required during specific stages of lateral root development. It appears that auxin is not produced in lateral roots because excised lateral root primordial do not continue to divide in the absence of added IAA (Dubrovsky et al., 2001). Transport of auxin, most likely from the root apex or developing regions of the root closer to the lateral root, supply the auxin that is needed for lateral root initiation and for cell elongation (Marchant et al., 2002; Biliou et al., 2005). Since MYB77 was expressed in lateral roots, we tested how lateral root growth responds to the application of exogenous auxin in the wild type and in myb77 knockout lines. We found that the pattern of response to auxin was complex. At low concentrations, lateral root density increased more in the wild type than in the knockout, which may be due to the lower free IAA concentrations that we measured under control conditions in the roots of the myb77-1. At higher IAA concentrations, the lateral root density was greater in the knockout, which may in part be due to the initially lower free IAA, since IAA both promotes and inhibits root growth. Higher concentrations of auxin were required to stimulate maximal lateral root density in myb77-1, which implies that MYB77 is a positive regulator of lateral root growth and may function through auxin signal transduction pathways.

MYB77 Plays a Role in Root Response to Nutrient Deprivation

Since the expression of MYB77 is decreased, but not completely abolished when plants are deprived of potassium, we reasoned that there might be a detectable difference in lateral root density between the wild type and the myb77 knockout lines. More detailed studies were conducted on the knockout lines to test whether nutrient concentrations also play a role in lateral root development. Comparison between the wild type and the myb77 knockout confirmed that the low level of MYB77 expression maintained in wild-type plants under nutrient-deprived conditions modulates lateral root development and may continue to modulate auxin responses. A similar decrease in lateral root density under potassium-deprived conditions was observed in tir1-1, which contains a mutation in an auxin receptor (Ruegger et al., 1998; Dharmasiri et al., 2005; Kepinski and Leyser, 2005), indicating that the decrease is an auxin-dependent process. In the wild type, the free IAA concentrations decreased under potassium-deprived conditions. In myb77-1 knockout and MYB77-ox plants, free IAA concentrations were lower than the wild type under control conditions and did not decrease further under potassium-deprived conditions, indicating that MYB77 plays a role in the modulation of auxin responses during the transition from nutrient-sufficient to -deficient conditions. Based on the differences in lateral root density under potassium-deprived conditions, where free IAA concentrations were the

![Figure 8. ARF7 Interacts with the C-Terminal Domain of MYB77.](image)

(A) Full-length and deletion constructs of MYB77 are shown. R2R3 domain of MYB77 (R2R3; amino acids 1 to 110) and N-terminal deletion of MYB77 (ΔN; amino acids 111 to 301). (B) In vitro coimmunoprecipitation of ARF7 and MYB77. The [35S]Met-labeled in vitro–translated proteins of HA:ARF7, MYB77, R2R3, and ΔN were incubated and then analyzed on an SDS-PAGE gel.

![Figure 9. Lateral Root Density of myb77-1, arf7/nph4-1, and the Double myb77-1 arf7-nph4-1 Mutant.](image)

Mean lateral root density was significantly lower in the double knockout plants of myb77-1 and arf7/nph4-1 than the wild type, myb77-1, and nph4-1 single knockout (n = 66 to 70 plants ± SE). Bars that have different letters at the top are significantly different (P < 0.05; Tukey’s honestly significant difference).
same in the all the lines, we suggest that MYB77 modulates sensitivity to auxin. Changes in lateral root development also occur under nitrogen- and phosphorus-deprived conditions (López-Bucio et al., 2003). Although the lateral root density decreased under the nitrogen- and phosphorus-deprived conditions used in this study, MYB77 expression does not decrease (Shin et al., 2005), and there were no differences between the wild type and the knockout lines. Therefore, the expression of this transcription factor that decreases under potassium-deprived conditions may play a specific role in potassium deprivation responses that lead to reduced lateral root development. The changes in root architecture due to low phosphorus or patchy nitrogen are well documented (Casimiro et al., 2003; López-Bucio et al., 2003). Under patchy nitrogen conditions, a MADS box transcription factor, ANR1, may be important in controlling the proliferation of roots (Zhang and Forde, 1998; Remans et al., 2008). Auxin has been previously implicated in response to phosphate deprivation (López-Bucio et al., 2002; López-Bucio et al., 2005). A very detailed study on low phosphate responses concluded that changes in lateral root growth were due to alterations in auxin transport (Nacry et al., 2005).

**MYB77 Modulates Auxin-Inducible Genes**

To determine which genes the transcription factor MYB77 might be activating in an auxin-dependent manner, we characterized the expression of a number of genes whose expression is regulated by auxin. We confirmed that MYB77 expression was induced by auxin (Kranz et al., 1998) over the time course of 24 h after incubation with exogenous IAA. MYB77 has also been shown to be induced by stress (Kamei et al., 2005) and to be expressed during the late embryogenesis (Kirik et al., 1998). In wild-type Arabidopsis, seven auxin-responsive genes tested (IAA1, IAA19, PIN1, GH3.2, GH3.3, SAUR-AC1, and HAT2) were upregulated, but in the myb77-1 knockout, the expression of all the auxin inducible genes tested was attenuated or in some cases completely abolished. In the MYB77-ox lines, these genes were upregulated in the absence of added auxin. This showed that MYB77 plays a role in auxin signal transduction through the modulation of known auxin-inducible genes. Even though the expression of these genes is attenuated in the knockout, the phenotype of these lines under control conditions was indistinguishable from the wild type. This may suggest that under control conditions there are other factors redundant to MYB77, which is a common finding in auxin metabolism and signaling (Normany and Bartel, 1999). We characterized one potential redundant factor MYB44 (At5g67300) (Stracke et al., 2001). MYB44 is also upregulated by exogenous auxin (Kranz et al., 1998). However, the double knockout plants of MYB77 and MYB44 (myb77-1 myb44-1) didn’t change the lateral root phenotypes of myb77-1 under control conditions or with the addition of exogenous IAA (data not shown). In addition, the Genevestigator Response Viewer (https://www.genevestigator.ethz.ch/at/index.php?page= responseviewer) showed that MYB77 and MYB44 are not induced or repressed by the same stimuli except auxin treatment. From these analyses, MYB44 does not appear to be a redundant with MYB77.

**MYB77 Interacts with ARFs**

Analysis of the promoter regions of the auxin-regulated genes that we used for expression analysis revealed the presence of numerous MYB transcription factor binding sites. Further analysis of the IAA19 promoter indicated that there were several AuxREs (Remington et al., 2004) and at least one was in close proximity to a MYB binding motif. This raised the possibility that MYB77 might bind to ARFs that are known to bind to the AuxREs (Ulmasov et al., 1997a). In addition to the physical proximity of the MYB and ARF transcription factor binding elements, it is also relevant to note that in the nph4-1 mutant in which ARF7 is inactivated, the expression of auxin-inducible genes is also attenuated, which is similar to what we observed with the myb77 knockout.

To test the hypothesis that MYB77 interacts with ARFs, we used several different approaches. First we showed in vitro that MYB77 interacts with ARF7. ARF7 is a transcriptional activator that belongs to a family of transcription factors involved in auxin responses (Guilfoyle and Hagen, 2001). There are 22 ARF genes with five that function as transcriptional activators, while the remaining 17 are thought to function as transcriptional repressors. ARFs contain a DNA binding domain, a middle region involved in activation or repression, and a C-terminal dimerization domain. In vitro, we determined that MYB77 interacts with both the activator and the repressor types of ARFs. These interactions were specific to MYB77 and not with two other R2R3 MYB transcription factors that are expressed in Arabidopsis roots. R2R3 MYB transcription factors contain a conserved DNA binding domain. However communoprecipitation experiments showed that the specificity of interaction between MYB77 and ARF7 is likely to arise from motifs found in the C-terminal domain of MYB77.

To demonstrate the interaction between MYB77 and ARF7 in vivo, we used a BiFC assay (Walter et al., 2004). These assays showed that a physical interaction between MYB77 and ARF7 occurs in vivo (onion epidermal and root cells). A protoplast transfection assay was used to determine whether the expression of MYB77 could alter reporter gene activation using several different constructs. Expression of **MYB77** in Arabidopsis mesophyll cells clearly stimulated the expression of the auxin-responsive promoter 2XD0:GUS (Murfett et al., 2001) in the presence and absence of added NAA. These results suggest that MYB77 interacts with an ARF activator to enhance transcription on the 2XD0 reporter gene or that MYB77 stimulates the transcription of another factor involved in the enhancement of 2XD0 expression. MYB77 activated the reporter gene when both the activation and C-terminal dimerization domains of ARF5 were present (GD-5AC) but not when only the activation domain (GD-5A) was present, further implicating the C-terminal domain in activation. This indicates that the C terminus is required for enhanced expression of the reporter gene in the presence of MYB77. Failure to observe enhanced expression of the reporter gene with Gal4-1RC (GD-1RC) plus the MYB77 effector gene might be due to dominance of the ARF1 repression domain over MYB77 activity. The activation of transcription in a protoplast system by MYB77 provides additional evidence for the role of MYB77 in auxin signal transduction and highlights a relationship
between MYB77 and ARFs. MYB77 may interact in vivo specifically with ARF7 as well as other ARF transcriptional activators. It has been shown that the arf7 arf19 double mutant has a reduced lateral root phenotype (Okushima et al., 2005; Wilmoth et al., 2005; Fukaki et al., 2006), so we speculate that MYB77 may interact with ARF7 and/or ARF19. The results from the in vitro pull-down assays, the BIFC experiments, and the protoplast transfections show that MYB77 interacts with ARFs to promote the expression of auxin-regulated genes.

The in vitro pull-down assays showed that the C termini of ARFs that contain domains III and IV are necessary and sufficient for the interaction with MYB77. Domains III and IV are also the regions of interaction between ARFs and the transcriptional repressors Aux/IAAs (Berleth et al., 2004; Teale et al., 2006). Therefore, an alternative explanation for our results could be that MYB77 titrates out the Aux/IAA and through that mechanism there is a corresponding activation of ARF binding and auxin-regulated transcription. This hypothesis is not favored because (1) auxin stimulates the expression of MYB77 and the degradation of Aux/IAAs, so it seems unlikely that these molecules would be present in the same cell concurrently; (2) we did not find evidence for this interaction in vivo using the BIFC system; and (3) it is possible that the in vitro interactions between ARFs or Aux/IAAs with MYB77 may be more promiscuous than those that occur under natural conditions in plants. This could result from the elevated amounts of the interactors used in pull-down assays compared with those that exist in cells as well as the non-physiological environment existing in pull-down assays. It remains formally possible that MYB77 exerts its effect on auxin responses through the titration of Aux/IAA proteins in vivo, and further analysis will be needed to determine whether MYB77 and IAA19 interact in vivo.

**Combinatorial Interactions**

Genome analyses have revealed the presence of numerous binding sites for specific transcription factors, including in introns and the upstream regions of genes that are not regulated by the specific factor (Martone et al., 2003; Eusukirchen and Snyder, 2004). One conclusion from the genome analyses of transcription factor motifs is that not all the sites are actively recruiting transcription factors. The specificity of these sites may in part be due to a requirement for multiple transcription factors to bind. Many transcription factors have been shown to function as hetero- and homomultimers. In some cases, multiple transcription factors interact in the form of enhancosomes to regulate the expression of genes (Thanos and Maniatis, 1995). Little is known in plants about the combinatorial effects of interactions between transcription factors that selectively regulate gene expression (Guilfoyle, 1997; Singh, 1998). Combinatorial interactions of transcription factors have been predicted to occur on auxin-responsive promoters that appear to function as composite elements (Ullmasov et al., 1995; Guilfoyle, 1999). These composite elements appear to function in combination with an AuxRE (TGTCCT) to which ARFs bind (Tatematsu et al., 2004). Our results show that MYB77 is involved in auxin signal transduction and that it participates in a combinatorial transcription factor interaction in plants. While it is known that MYB factors in plants interact with basic helix-loop-helix factors (Goff et al., 1992; Diaz et al., 2002; Ramsay and Glover, 2005), this work demonstrates that an R2R3 MYB factor interacts with ARFs, providing evidence that ARFs interact with proteins outside the Aux/IAA family. In addition to directly binding to DNA in the proximity of AuxREs on auxin-responsive genes, it is also possible that MYB77 could be recruited to auxin response genes by dimerizing with ARFs that are already bound to DNA. The recruitment of Aux/IAA to ARFs occurs by the process of dimerization between proteins, which highlights this mode of interaction (Tiwari et al., 2003) in auxin signal transduction. It is also possible that the proximity of MYB binding sites to AuxREs in some promoters might have no function or could enhance but not be essential for effective interactions between ARFs and MYB77.

In this study, we show that the transcription factor MYB77 is involved in auxin signal transduction in Arabidopsis. MYB77 modulates lateral root development, which is known to be dependent on auxin. MYB77 acts in combination with ARFs to alter auxin-responsive gene regulation, and this provides a novel mechanism for further modulating plant development in response to auxin. This newly identified function for a member of the R2R3 MYB transcription factor family and the interaction with ARFs provide new insight into how plant response to auxin is modulated, defines a new combinatorial interaction between transcription factors in plants, and provides a better understanding of how plants modify root development in response to environmental changes.

**METHODS**

**Plant Materials, Treatments, and Growth Conditions**

The Arabidopsis thaliana Columbia ecotype (Col-0) was used for this study. Transgenic plants carrying constitutively overexpressing MYB77 (MYB77-ox) and ProMYB77::GUS (MYB77 promoter with GUS) were generated. For constitutive expression, we used pCambia2300 (CAMBIA) with figwort mosaic virus promoter (Sanger et al., 1990) and nopaline synthase terminator (NOS). For the localization of gene expression, a 1904-bp region upstream from the start codon of the MYB77 gene was amplified from Arabidopsis genomic DNA. The binary vector constructs were introduced into Arabidopsis plants by the Agrobacterium tumefaciens-mediated floral dip method. Seeds of the mb77-1 knockout plant (Salk_67655) and myb77-2 (Salk_55373) were obtained from the ABRC. MYB77 has only one exon, and in both inactivation lines, the T-DNA was inserted into the exon. MYB77 transcripts were not present in either knockout, as determined by RNA gel blot analysis (Figure 5; data not shown). The isolation of arf7/rph4, which is a null mutant of ARF7, was described previously (Harper et al., 2000). DR5:GUS transgenic plants were used to test for auxin responsiveness (Ulmasov et al., 1997). Plants containing DR5:GUS were crossed to mb77-1 or MYB77-ox plants with DR5:GUS, and homozygous lines were generated. For the auxin sensitivity assay, seeds of Col-0, myb77-1, and myb77-2 were grown on plates containing nutrient solution (Shin et al., 2005), 2% sucrose, and 0.8% SeaKem agarose (Cambrex) at 22°C with a 16-h daylength at 200 µmol·m⁻²·s⁻¹. Seedlings of Col-0, myb77-1, and myb77-2 were transferred to medium of the same composition containing various concentrations of auxin 4 d after germination (DA4). The length of primary roots and lateral root number were measured 7 d after transfer (n = 50 to 70 plants). Quarter-strength Murashige and Skoog medium was also used for auxin response experiments. The results were similar, except that
higher concentrations of IAA were required to elicit responses. The 4-d-old Col-0, myb77-1, and tir1-1 were transferred to potassium-deficient and -sufficient plates, and the primary root length and the number of lateral roots were measured to determine the lateral density 7 d after transfer. For potassium deficiency growth assays, 10 μM KCl instead of 1.75 mM KCl was used, 12 μM phosphoric acid instead of 0.5 M phosphoric acid was used for phosphate-deficient growth assay, and 100 μM Ca(NO₃)₂ instead of 2 mM Ca(NO₃)₂ was used for nitrogen-deficient growth.

ProMYB77:GUS and DRS Expression
To localize GUS activity, plants were stained with 1 mM X-Gluc solution 4 DAG for primary root localization and 12 DAG for lateral root localization. The pictures were taken using a Nikon SMZ1500 dissecting microscope. For these assays, plants were transferred 3 or 11 DAG to full nutrient (1.75 mM KCl) medium and then grown for 7 d before staining. For the DRS analysis, 10 progeny for each of three overexpressor and three knockout lines were analyzed. For the ProMYB77:GUS experiments, >10 lines were tested, and expression patterns were found to be consistent among the lines. MUG fluorometric assays for the quantitative analysis of GUS activity (Shin et al., 2003) were used to quantify GUS activity. To confirm the spatial localization of MYB77 in roots based on ProMYB77:GUS activity, RT-PCRs of MYB77 (3’ untranslated region) were performed with 1 mm of primary and lateral root tips and other parts of primary roots and lateral roots. Images were quantified using ImageJ.

Free IAA Quantification
For free auxin quantifications, hypocotyl and root segments of 300 seedlings were collected and pooled for each replicate. For the K⁺ deprivation treatment, seedlings were transferred to K⁺-sufficient or K⁺-deficient media for 24 h. Auxin transport assays and free IAA determinations were as described by Geisler et al. (2005) except using an Agilent 6890N/5973 gas chromatograph/mass selective detector and a LECO Peg-asus IV GCXGC time-of-flight mass spectrometer for gas chromatography-mass spectrometry analysis.

RNA Gel Blot Analysis
Seven-day-old Arabidopsis Col-0, myb77-1, and MYB77-ox-1, 2, and 3 were transferred to 10 μM MES solution, pH 5.8, with or without 10 μM IAA (Sigma-Aldrich). The seedlings were kept in the light and put on a slow shaker and harvested after 0.5, 1, 2, 6, and 24 h. The amplified 3’ untranslated regions of the cDNAs were labeled with [α-32P]dCTP (Amersham), and RNA gel blot analyses were performed as described by Shin et al. (2005). RNA gel blots to characterize the expression of MYB77 in the myb77-1 knockout were performed with both a full-length cDNA and the 3’ untranslated region to confirm that the allele was a null.

Bacterial Expression of MYB77
The MYB77 cDNA was amplified by PCR and cloned into the NdeI and SacI sites of the pET28 vector (Novagen). The MYB77 in-frame fusion in pET28 was transferred into Escherichia coli strain BL21 (DE3). Overexpression of MYB77:HIS was induced by 0.5 mM isopropyl-β-D-thiogalactoside at 20°C for 4 h. Bacteria were pelleted after the induction period, suspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole), and subjected to sonication on ice for 1 min. Bacterial lysates were then centrifuged. The His-tagged fusion protein was recovered by affinity chromatography using nickel-nitriotriacetic acid agarose resin (Sigma-Aldrich). The MYB66 cDNA and MYB123 cDNA were amplified by PCR and cloned into the SacI and HindIII sites of pET28 vector. N-terminal His tag–fused MYB66 and MYB123 were obtained in the same way as MYB77 and used for in vitro pull-down assay controls.

In Vitro Pull-Down Assay
Bacterial extracts of MYB77:His were immobilized on a nickel-nitriotriacetic acid agarose column and then incubated for 90 min at 4°C with in vitro–translated [35S]Met-labeled ARF1, ARF7, and IAA19 (ARF2, ARF5, and ARF6; data not shown) that were obtained using the TntT-coupled reticulocyte lysate system (Promega). Beads were washed, and proteins were analyzed by SDS-PAGE, exposed to an imaging plate, and scanned using the Typhoon 9410 system (Amersham Biosciences). An unrelated His-tagged protein (glyoxalase) and two other MYB proteins (MYB66 and MYB123) were used as controls in the pull-down assays.

BiFC
To clone into BiFC vectors, MYB77, ARF7, and IAA19 were amplified and fused to part of YFP at both the N or C terminus into pUC-SPYNE (N-terminal) and pUC-SPYCE (C-terminal) via SpeI and Xmal (Waller et al., 2004). Fusion to either the N or C terminus gave the same results. All constructs were fully sequenced. For transient expression, particle bombardment was performed using onion cells and Arabidopsis roots. Gold particles (1.0-μm diameter) were washed with 100% ethanol and coated with 5 μg of each DNA. Gold-coated DNAs were bombarded using a Bio-Rad PDS1000/He particle gun. To stain the nucleus of onion cells and Arabidopsis roots, Hoescht dye (Sigma-Aldrich) was used for 30 min. The bright-field and fluorescent images were taken using a Nikon Eclipse E800 microscope with appropriate YFP and Hoescht filters.

Protoplast Assays
The 2XD0:GUS and Gal4-D1-3:GUS [previously referred to as Gal4(4X)-D1-3(4X):GUS] reporter genes have been described previously (Murfett et al., 2001; Tiwari et al., 2004). The Pro35S:HA-MYB77-NOS effector construct was made by cloning the MYB77 open reading frame in front of the cauliflower mosaic virus 3SS double enhancer promoter and terminated by NOS. The HA epitope was cloned in frame at the N terminus of the MYB77 open reading frame. The ARF effector genes and modified versions have been described (Tiwari et al., 2003). Isolation of protoplasts and transfection assays with Arabidopsis leaf mesophyll protoplasts were performed as described (Tiwari et al., 2006). Protoplasts were incubated in the presence and absence of 1 μM NAA. Pro35S:CAT-NOS was used as an inactive control effector gene used to equalize the amount of DNA transfected into cells. All transfections were performed in triplicate, and three independent transfections were performed for each of the effector genes tested.

In Vitro Commmunoprecipitation
HA:ARF7, MYB77:His, R2R3 domain of MYB77 (R2R3; amino acids 1 to 110), and N-terminal deletion of MYB77 (NΔN; amino acids 111 to 301) were translated with [35S]Met using the TntT-coupled reticulocyte lysate system. The in vitro–translated HA:ARF7 was incubated with in vitro–translated MYB77, R2R3, and NΔN for 16 h at 4°C. HA antibody was added (1:150) to each reaction and incubated for 16 h at 4°C. Protein A beads (Fluka) were equilibrated using 1 M glycine and 2 M NaCl solution, pH 9, and 5 μL of 50% slurry of Protein A beads were added to the reaction mixture with HA antibodies and incubated for 16 h at 4°C. Each reaction was washed three times with 500 μL of 1 M glycine and 2 M NaCl buffer, pH 9, and three times with 500 μL of 0.02 M NaH₂PO₄ and 0.15 M NaCl, pH 8, buffer. The beads were resuspended in 20 μL of SDS-PAGE loading buffer and then denatured at 95°C for 10 min. Reactions were run on SDS-PAGE gels (15%), dried, exposed to a phosphor imaging screen, and scanned using the Typhoon 9410 system.
Accession Numbers

The Arabidopsis Genome Initiative locus identifiers for the genes from this article are as follows: MYB77 (At3g50060), ARF7 (At5g20730), ARF1 (At1g59750), ARF5 (At1g19850), IAA1 (At4g14560), IAA19 (At3g15540), Fni1 (At1g73590), GHI3.2 (At4g57390), GHI3.3 (At2g31770), SaurAC (At4g38850), HAT2 (At5g17370), MYB123 (At5g35550), MYB66 (At5g14750), and MYB44 (At5g67300).

Supplemental Data

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

Supplemental Figure 1. MUG Assay of DR5:GUS Plants Crossed with MYB77-ox and myb77-1, and RT-PCR Analysis for MYB77 Expression in Roots.

Supplemental Figure 2. ProMYB77:GUS Activity Is Downregulated by K+. Deprivation and Upregulated by IAA Treatments.

Supplemental Figure 3. Lateral Root Density under Control Conditions and with Various Concentrations of IAA on Low Salt Medium in the Wild Type and in myb77-1 and myb77-2 Lines.

Supplemental Figure 4. Measurement of Auxin Transport in Wild-Type Col-0, myb77-1, and MYB77-ox Lines.

ACKNOWLEDGMENTS

We thank Joe Jez for assistance with protein purification and for the ARF cDNA clones, the Monsanto Company and Danforth Center for funding to D.P.S., the National Science Foundation (Grant IOB-0550417 to T.J.G.), Brad Fabbri and Gary Lee at Monsanto for their support of funding to D.P.S., the National Science Foundation (Grant IOB-0550417 to T.J.G.), and E. Liscum for nph4 seed, E. Liscum for nph4 seed, E. Epstein for nph4 seed, E. for nph4 seed, and T. Hoyt for assistance with the auxin transport assays. We also thank K. Mauch-Mani and L. Schaller for comments on the manuscript, and Josh Blakeslee for assisting with setting up the auxin transport assays.

Received February 6, 2007; revised July 5, 2007; accepted July 12, 2007; published August 3, 2007.

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The Arabidopsis Transcription Factor MYB77 Modulates Auxin Signal Transduction
Ryoung Shin, Adrien Y. Burch, Kari A. Huppert, Shiv B. Tiwari, Angus S. Murphy, Tom J. Guilfoyle and Daniel P. Schachtman
Plant Cell 2007;19;2440-2453; originally published online August 3, 2007;
DOI 10.1105/tpc.107.050963

This information is current as of December 31, 2017

Supplemental Data /content/suppl/2007/08/03/tpc.107.050963.DC1.html
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