Constitutive Repression and Activation of Auxin Signaling in Arabidopsis[1[C][W][OA]]

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Aux/IAA proteins are proposed to be transcriptional repressors that play a crucial role in auxin signaling by interacting with auxin response factors and repressing early/primary auxin response gene expression. In assays with transfected protoplasts, this repression was previously shown to occur when auxin concentrations in a cell are low, and derepression/activation was observed when auxin concentrations are elevated. Here we show that a stabilized version of the Arabidopsis (*Arabidopsis thaliana*) IAA17 repressor, when expressed constitutively or in a specific cell type in Arabidopsis plants, confers phenotypes similar to plants with decreased auxin levels. In contrast, a stabilized version of IAA17 that was converted to a transcriptional activator confers phenotypes similar to plants with increased auxin levels, when expressed under the same conditions in Arabidopsis plants. Free auxin levels were unchanged compared to control (*DR5:β-glucuronidase*), however, in the seedlings expressing the IAA17 repressor and activator. These results together with our previous results carried out in transfected protoplasts suggest that the hormone auxin can be bypassed to regulate auxin signaling in a cell-autonomous manner in plants.

The hormone auxin plays a central role in regulating a wide variety of growth and developmental responses/processes during the life span of plants. Many of the responses to auxin are mediated by what appears to be a simple, streamlined pathway that results in degradation of Aux/IAA transcriptional repressors and derepression of auxin response genes (Guilfoyle and Hagen, 2007; Kepinski, 2007). Aux/IAA repressors are proposed to function by interacting with transcriptional activators, the auxin response factors (ARFs), which are targeted to TGTCTC auxin response elements in promoters of auxin response genes (Tiwari et al., 2001, 2003, 2004; Guilfoyle and Hagen, 2007). Entry of auxin into a cell results in its binding to the Transport Inhibitor Response1 (TIR1) receptor/F-box ubiquitin ligase or related auxin signaling F-box (AFB) receptors/F-box ubiquitin ligases in a concentration-dependent manner (Gray et al., 2001; Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Binding of auxin to its receptors promotes the recruitment of the Aux/IAA repressors to TIR1 or AFB auxin receptor/ubiquitin ligase complex (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005; Tan et al., 2007). This recruitment, in turn, triggers the destruction of the repressors via the ubiquitin-proteasome pathway (Parry and Estelle, 2006; Mockaitis and Estelle, 2008), resulting in derepression/activation of auxin response genes (Guilfoyle and Hagen, 2007).

There are 29 *Aux/IAA* genes in Arabidopsis (*Arabidopsis thaliana*; Remington et al., 2004) and 31 *Aux/IAA* genes in rice (*Oryza sativa*; Jain et al., 2006), the bulk of which encode proteins with four conserved domains, referred to as domains I, II, III, and IV (Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Domain I is an active, portable repression domain containing a LxLxL motif (Tiwari et al., 2004) that interacts with the TOPLESS corepressor, at least in the case with IAA12/BDL (Szemenyei et al., 2008), to bring about repression of auxin response genes. Domain II confers instability to Aux/IAA proteins by targeting the repressors to the TIR1/AFB auxin receptors in an auxin dose-dependent manner, where they enter the ubiquitin-proteasome pathway (Parry and Estelle, 2006; Mockaitis and Estelle, 2008). A few of the Aux/IAA proteins do not have a recognizable domain II, and those that lack a domain II display increased stability (Dreher et al., 2006) and confer defective auxin signaling phenotypes when constitutively expressed in Arabidopsis (Sato and Yamamoto, 2008). Domains III and IV are protein-protein interaction domains found in ARFs as well as Aux/IAA proteins (Kim et al., 1997; Ulmasov et al., 1997a, 1997b).

Dominant or semidominant mutations in many of the 29 Arabidopsis *Aux/IAA* genes have been identified (Reed, 2001; Liscum and Reed, 2002; Woodward

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and Bartel, 2005; Uehara et al., 2008). These mutations reside in domain II and result in enhanced stability of the repressors due to their decreased affinity for the TIR1/AFB auxin receptors (Dharmasiri et al., 2005a, 2005b; Kepinski and Leyser, 2005). Domain II mutants display a range of auxin-related phenotypes with some being unique and some overlapping for each Arabidopsis *iaa* mutant (Reed, 2001; Liscum and Reed, 2002; Woodward and Bartel, 2005; Uehara et al., 2008). In some cases, phenotypes of different *iaa* mutants may be opposite to one another (e.g. root hair formation in *iaa3/shy2* versus *iaa17/axr3*; Knox et al., 2003). The range of phenotypes may be partially explained by the different expression patterns of *Aux/IAA* genes, but even when expressed from the same promoter, *iaa* mutants have nonidentical phenotypes (Knox et al., 2003; Weijers et al., 2005; Muto et al., 2007), suggesting that intrinsic properties of different Aux/IAA repressors may also contribute to the mutant phenotypes.

In previous studies with transfected protoplasts, we showed that expression of Aux/IAA proteins from *35S* promoter:effector plasmids resulted in repression of auxin-responsive reporter gene expression (Tiwari et al., 2001, 2004). Stabilized versions of Aux/IAA proteins with mutations in domain II (e.g. IAA17mII) functioned as stronger repressors than wild-type Aux/IAA proteins, while point mutations in the repression domain (e.g. IAA17mImII) compromised, but did not eliminate repression by stabilized Aux/IAA proteins (Tiwari et al., 2001). We also showed that the compromised repressor, IAA17mImII, could be converted to an activator by fusing the herpes simplex virus VP16 activation domain to its N terminus (i.e. VP16-IAA17mImII; Tiwari et al., 2003). These results suggested that a stabilized version of VP16-IAA17 can activate auxin response genes in an auxin-independent manner, and thus, bypass the hormone signal in the auxin signaling pathway. Here, we report on bypassing the auxin signal in transformed Arabidopsis plants by expressing a stabilized form of a VP16-IAA17 activator, and contrast these plants with those expressing a stabilized form of an IAA17 repressor. Our results provide support for a model in which natural Aux/IAA proteins function through an active repression domain to regulate auxin-responsive gene expression in planta and provide a strategy to control auxin signaling in an auxin-independent and cell-autonomous manner.

RESULTS

Constitutive Expression of a Stabilized IAA17 Repressor and a Stabilized IAA17 Activator Confer Low and High Auxin Phenotypes, Respectively, in Transformed Arabidopsis Plants

We used the same version of *35S:VP16-IAA17mImII* gene described by Tiwari et al. (2003) that functioned as an activator of auxin-responsive reporter gene expression, to transform Arabidopsis plants (Fig. 1A). More than 10 transformed lines were selected that

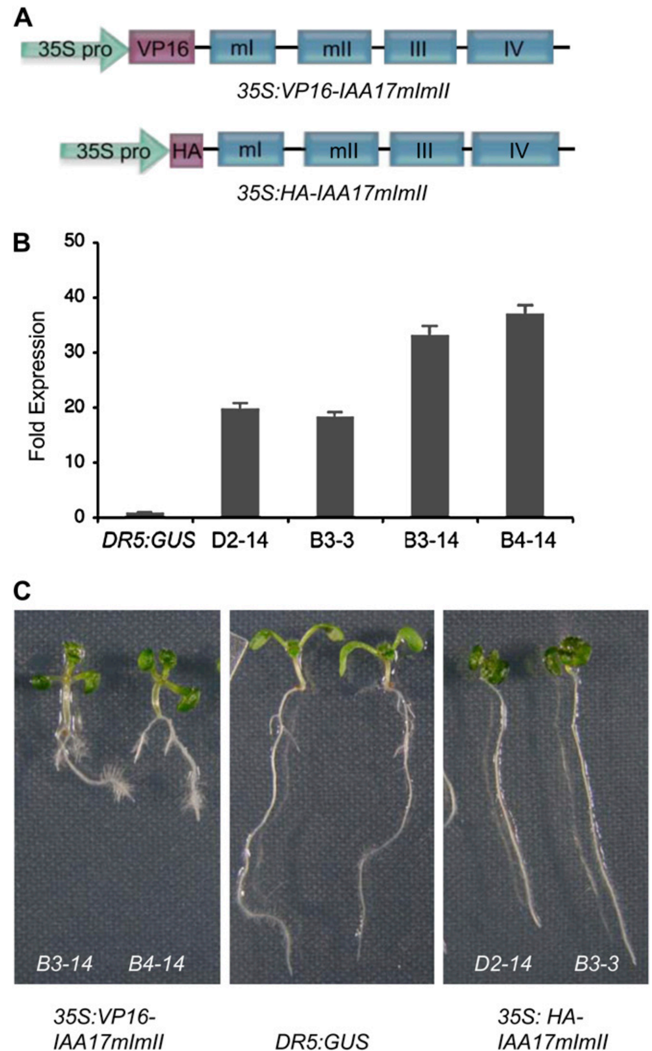

Figure 1. Constructs and phenotypes of Arabidopsis seedlings transformed with *35S:HA-IAA17mImII* and *35S:VP16-IAA17mImII*. A, Schematic diagrams of the *35S:HA-IAA17mImII* and *35S:VP16-IAA17mImII* constructs used to transform Arabidopsis plants. Expression of the modified IAA17 proteins was driven by the *35S* cauliflower mosaic virus promoter, and constructs are identical to those described by Tiwari et al. (2001, 2003). The *35S:HA-IAA17mImII* construct has a HA epitope tag at the N terminus of IAA17 in place of the VP16 activation domain in the *35S:VP16-IAA17mImII* construct. The four conserved domains, I to IV, are indicated, and m indicates a missense mutation(s) in domains I and II. In domain I, ETELCLGL was changed to ETVRCLGL, and in domain II, GWPPV was changed to GWSPV. B, Constitutive expression of *HA-IAA17mImII* (lines D2-14 and B3-3) and *VP16-IAA17mImII* (lines B3-14 and B4-14). qRT-PCR was used to determine the gene expression levels of *IAA17* in control seedlings (*DR5:GUS*) and *IAA17* plus the transgene in transformed seedlings. The expression level of *IAA17* in control seedlings was set at 1.0, and expression levels of the transgenes are presented relative to the *DR5:GUS* control. C, Seven-day-old *DR5:GUS* control seedlings and seedlings transformed with *35S:HA-IAA17mImII* and *35S:VP16-IAA17mImII*. A seedling from two *35S:HA-IAA17mImII* (lines D2-14 and B3-3) and *35S:VP16-IAA17mImII* (lines B3-14 and B4-14) independent lines is shown.

expressed the construct and had similar phenotypes. Analyses of two representative lines that overexpress the single-copy IAA17 transgene at levels 30- to 40-fold higher than wild-type IAA17 (Fig. 1B) are reported here.

Compared to the *DR5*:*GUS* line, which had a phenotype indistinguishable from wild type and was used as a control in experiments presented here, the two *35S*:*VP16-IAA17mImII* lines had long hypocotyls, long petioles, small epinastic cotyledons and leaves, short, highly branched roots, and dense root hairs (Fig. 1C). Hypocotyl length of 7-d-old light-grown seedlings transformed with the *35S*:*VP16-IAA17mImII* transgene was about 60% to 70% greater than control (Fig. 2A). The number of lateral roots/cm of primary root was approximately 1.5- to 2-fold higher in 7-d-old seedlings expressing the transgene compared to control (Fig. 2B). Adult plants transformed with the *35S*:*VP16-IAA17mImII* gene had narrow, twisted leaves, apically dominant inflorescences with a zigzag pattern of short siliques on the inflorescence stalks, and poor fertility (Fig. 3, A and C). In several ways, the seedling and adult phenotypes of the *35S*:*VP16-IAA17mImII* lines resembled *yucca1*, *yucca4*, *yucca6*, *CYP79B2ox*, *sur1*, *sur2*, *FZYox*, and *iaaM* mutants that overproduce auxin (Klee et al., 1987; Boerjan et al., 1995; King et al., 1995; Barlier et al., 2000; Zhao et al., 2001, 2002; van der Graaff et al., 2003; Kim et al., 2007; Li et al., 2008). The phenotypes observed in the *35S*:*VP16-IAA17mImII* seedlings did not result from increased free indole-3-acetic acid (IAA) levels, however, because free IAA levels in the *35S*:*VP16-IAA17mImII* lines were equal to or marginally less than IAA levels in the *DR5*:*GUS* control seedlings (Fig. 2C).

That the above phenotypes result from the addition of the VP16 activation domain to IAA17 is supported by analysis of plants transformed with a single-copy *35S*:*HA-IAA17mImII* gene (Fig. 1A), which contains an N-terminal hemagglutinin (HA) epitope tag in place of the VP16 moiety. We have previously shown that expression of the *35S*:*HA-IAA17mImII* construct represses auxin-responsive reporter gene expression in transfected protoplasts, and that the mutation in domain I (mI) weakened, but did not eliminate repression (Tiwari et al., 2001). Seedlings in two lines expressing the *35S*:*HA-IAA17mImII* transgene at levels 15- to 20-fold higher than the wild-type gene (Fig. 1B) had short hypocotyls and petioles and unbranched primary roots (Fig. 1C). Seven-day-old seedlings expressing the *35S*:*HA-IAA17mImII* transgene had hypocotyls that were about half the length of control hypocotyls (Fig. 2A) and had no lateral roots, in contrast to control seedlings (Fig. 2B). Adult plants were bushy dwarfs with short inflorescence stalks and short, wrinkled siliques (Fig. 3). The phenotypes of *35S*:*HA-IAA17mImII* plants contrast markedly with the phenotypes observed for the *35S*:*VP16-IAA17mImII* lines and resemble phenotypes of mutants with decreased auxin levels (Romano et al., 1991; Rampey et al., 2004). The low auxin-like phenotypes observed

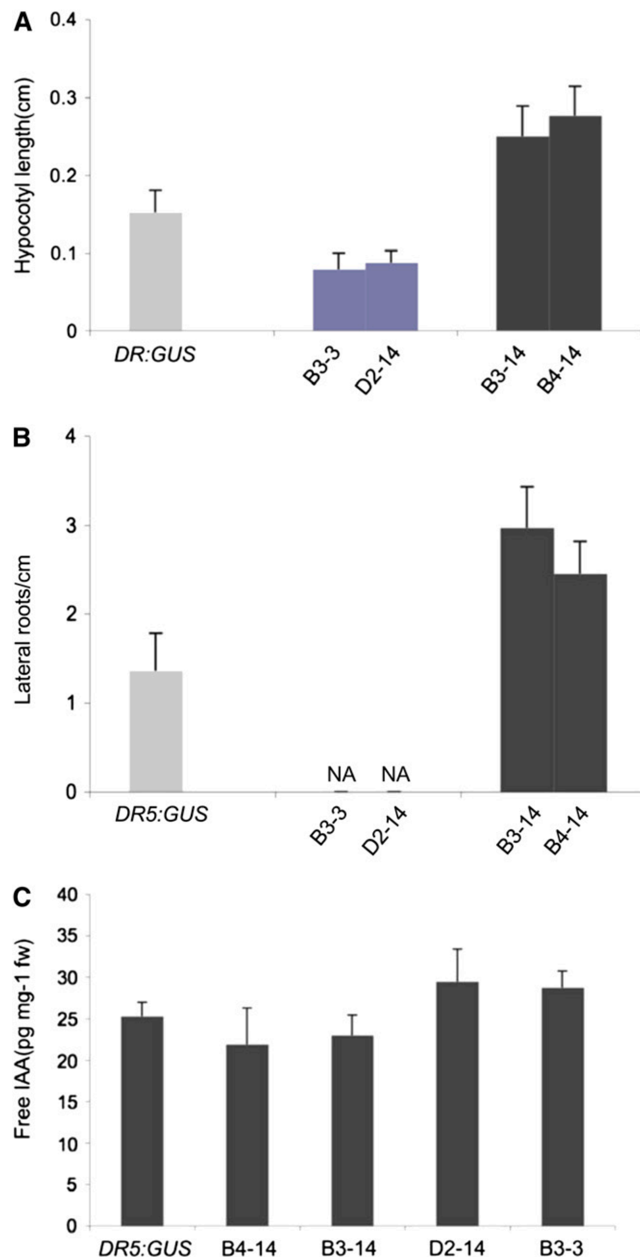

Figure 2. Quantitative characteristics of *DR5*:*GUS* and transgenic seedlings expressing *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII*. A, Measurement of hypocotyl length in control (*DR5*:*GUS*), *35S*:*HA-IAA17mImII* (lines B3-3, D2-14), and *35S*:*VP16-IAA17mImII* 7-d-old seedlings (lines B3-14, B4-14; $n \geq 15$, ANOVA test, $P < 0.01$). B, Lateral root numbers per centimeter in control, *35S*:*HA-IAA17mImII* (lines B3-3, D2-14), and *35S*:*VP16-IAA17mImII* 7-d-old seedlings (lines B3-14, B4-14). NA, 0.00 ± 0 ($n \geq 15$, ANOVA test, $P < 0.01$). C, Free IAA levels in control, *35S*:*HA-IAA17mImII* (lines B3-3, D2-14), and *35S*:*VP16-IAA17mImII* (lines B3-14, B4-14) seedlings. ANOVA analyses indicate that the values are not different from each other (F = 3.693, P = 0.43) in post hoc pairwise comparisons (Tukey and SNK) or comparison to controls (Student's, Bonferroni, P = 0.285, 0.257, 0.152, 0.078, respectively). [See online article for color version of this figure.]

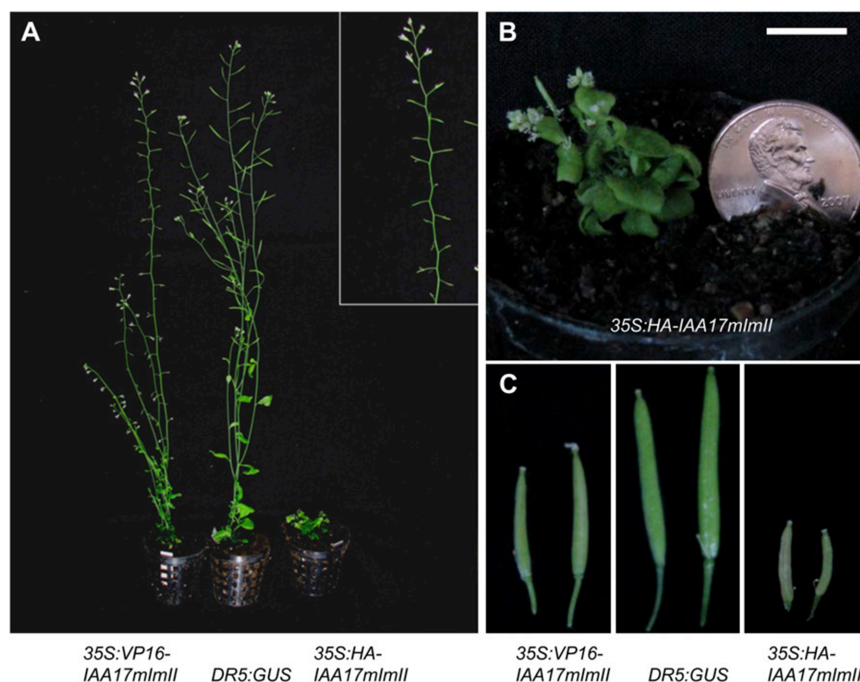

Figure 3. Phenotypes of adult Arabidopsis plants expressing *35S*:*HA-IAAA17mImII* and *35S*:*VP16-IAA17mImII*. A, An adult *DR5*:*GUS* control plant and adult plants transformed with *35S*:*HA-IAA17mImII* (line B3-3) and *35S*:*VP16-IAA17mImII* (line B3-14). Inset, Zigzag pattern of the inflorescence stalk in a *35S*:*VP16-IAA17mImII* plant. B, Small stature of a *35S*:*HA-IAA17mImII* plant. Bar = 1 cm. C, Representative siliques of *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII* compared to *DR5*:*GUS* control.

in the *35S*:*HA-IAA17mImII* seedlings did not result from reduced free IAA levels, because *35S*:*HA-IAA17mImII* lines had free IAA levels that were equal to or slightly greater than IAA levels in the *DR5*:*GUS* control seedlings (Fig. 2C).

We also examined first generation plants (T1) that were transformed with *35S*:*HA-IAA17mII* (i.e. IAA17 with only a domain II mutation, which functions as a stronger repressor than IAA17 with mutations in both domains I and II; Tiwari et al., 2001). These plants, like plants transformed with *35S*:*HA-IAA17mImII* had phenotypes similar to mutants with decreased auxin levels; however, the phenotypes of plants transformed with the *35S*:*HA-IAA17mII* gene were lost in T2 generation plants (i.e. T2 lines were indistinguishable from wild type). While we have not investigated the reason for this loss of phenotype, it may be that constitutive expression of these strong repressors compromises the auxin response to such an extent that expression of the transgenes is eliminated following the T1 generation. In contrast to unstable phenotypes observed with the *35S*:*HA-IAA17mII*, phenotypes observed with *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII* lines were maintained at least through the T4 generation.

DR5:*GUS* Reporter Gene Expression Is Constitutively Activated in Seedlings Expressing the *35S*:*VP16-IAA17mImII* Transgene and Constitutively Repressed in Seedlings Expressing the *35S*:*HA-IAA17mImII* Transgene

The phenotypes described above suggest that plants expressing the *35S*:*VP16-IAA17mImII* transgene display a constitutively high auxin response in the absence of applied auxin, while plants expressing the *35S*:*HA-IAA17mImII* transgene display a constitutively low auxin response. To examine this further, the *DR5*:*GUS* auxin-responsive reporter gene line (Ulmasov et al., 1997b) was crossed into *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII* lines. Figure 4 shows *DR5*:*GUS* expression in control seedlings compared to seedlings expressing either the *35S*:*VP16-IAA17mImII* or the *35S*:*HA-IAA17mImII* transgene. Compared to *DR5*:*GUS* control seedlings, *35S*:*VP16-IAA17mImII* lines showed strongly enhanced expression of *DR5*:*GUS* in seedlings that were not exposed to auxin. In contrast, *DR5*:*GUS* expression observed in leaf margins, primary root tips, and developing lateral roots (i.e. auxin maxima) in control seedlings was strongly reduced or eliminated in the *35S*:*HA-IAA17mImII* seedlings. These results indicate that the auxin-responsive reporter gene is constitutively activated in the *35S*:*VP16-IAA17mImII* lines and constitutively repressed in *35S*:*HA-IAA17mImII* lines.

When control seedlings containing the *DR5*:*GUS* reporter gene were treated with the synthetic auxin, 1-naphthalene acetic acid (1-NAA), over a concentration range of 10^{-7} to 10^{-4} M, the reporter gene was strongly activated at 10^{-6} to 10^{-4} M 1-NAA (Fig. 5). Unlike control seedlings, the *DR5*:*GUS* reporter gene was only weakly activated by applied auxin even at 10^{-4} M 1-NAA in lines expressing the *35S*:*HA-IAA17mImII* transgene. With seedlings expressing the *35S*:*VP16-IAA17mImII* transgene, *DR5*:*GUS* gene expression was already high without applied auxin and increased only slightly in response to auxin over the concentration range tested.

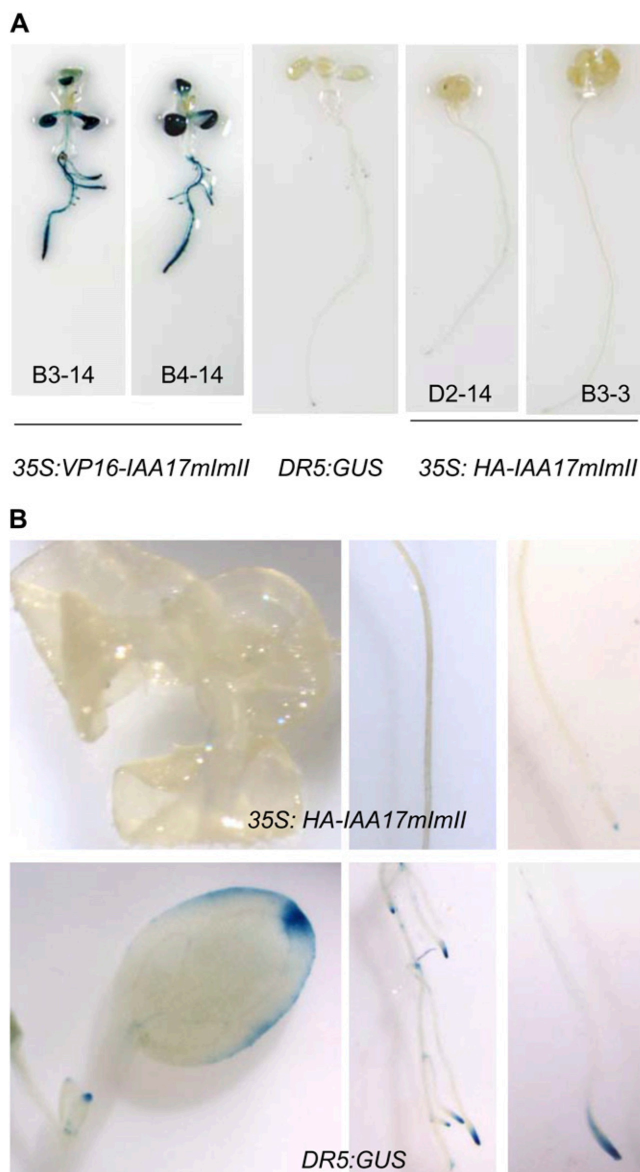

Figure 4. Expression of *DR5*:*GUS* auxin-responsive reporter gene in a wild-type seedling and seedlings transformed with *35S*:*VP16-IAA17mImII* and *35S*:*HA-IAA17mImII*. A, Seven-day-old seedlings from two *35S*:*VP16-IAA17mImII* (lines B3-14 and B4-14) and two *35S*:*HA-IAA17mImII* (lines D2-14 and B3-3) independent lines are shown. Seedlings were removed from agar plates and histochemically stained for GUS. B, Loss of *DR5*:*GUS* expression (auxin maxima) in *35S*:*HA-IAA17mImII* (B3-3) compared to expression in leaf margin, developing lateral roots, and primary root tip of *DR5*:*GUS* control seedlings.

A Variety of Natural Auxin Response Genes Are Constitutively Activated in Seedlings Expressing the *35S*:*VP16-IAA17mImII* Transgene and Constitutively Repressed in Seedlings Expressing the *35S*:*HA-IAA17mImII* Transgene

To determine if natural genes were constitutively repressed and constitutively activated like the *DR5*:*GUS* reporter gene in seedlings transformed with *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII* lines, respectively, we initially carried out microarray analyses with *DR5*:*GUS* control seedlings (7-d-old) not treated or treated for 1 h with 1 μM 1-NAA (the synthetic auxin, naphthalene acidic acid), a *35S*:*HA-IAA17mImII* line not treated or treated for 1 h with 1 μM 1-NAA, and a *35S*:*VP16-IAA17mImII* line not treated with auxin. The microarray data were used as a guide to identify genes that were up-regulated in auxin-treated *DR5*:*GUS* seedlings, down-regulated in *35S*:*HA-IAA17mImII* seedlings compared to *DR5*:*GUS* whether not treated or treated with auxin, and up-regulated in *35S*:*VP16-IAA17mImII* seedlings compared to wild-type seedlings. A set of genes was then selected to carry out quantitative reverse transcription (qRT)-PCR for analysis of gene expression (Supplemental Table S1). Figure 6 shows that selected *GH3*, *SAUR*, *Aux/IAA*, and other auxin response genes, all of which have been identified in previous microarray analyses to be strongly induced by auxin (Nemhauser et al., 2004, 2006), were down-regulated in the *35S*:*HA-IAA17mImII* line and up-regulated in the *35S*:*VP16-IAA17mImII* line. The down-regulation in the *35S*:*HA-IAA17mImII* line is not large with most genes analyzed because these genes, like the *DR5*:*GUS* reporter gene, are not highly expressed in seedlings that have not been exposed to exogenous auxin. Application of exogenous auxin to *35S*:*HA-IAA17mImII* seedlings failed to activate the natural auxin response genes, unlike what is observed with control seedlings (Fig. 7). These results indicated that expression of the *35S*:*HA-IAA17mImII* transgene in Arabidopsis seedlings results in constitutive repression of a spectrum of auxin response genes and that this repression is not relieved by treating seedlings with exogenous auxin, while expression of the *35S*:*VP16-IAA17mImII* transgene results in constitutive activation of these same auxin response genes.

To gain insight into how robustly the *35S*:*VP16-IAA17mImII* transgene functioned in conferring a constitutive auxin response, we compared expression of the auxin response genes in a *35S*:*VP16-IAA17mImII* line with that in control seedlings exposed to exogenous auxin over a 24-h period. A number of early auxin response genes have been shown to display transient activation when exposed to exogenous auxin, with expression returning to basal or near-basal levels following long exposure times to auxin (Abel et al., 1995; Nakamura et al., 2003; Nemhauser et al., 2006). Figure 8 shows a time course for auxin-induced expression of genes analyzed in Figures 6 and 7. The bulk of these genes, including all of the *GH3*, *SAUR*, and *IAA* genes examined, were most highly expressed within 1 to 3 h of auxin treatment, and expression levels declined thereafter (i.e. 3–24 h of treatment). These results along with previous results (Abel et al., 1995; Nakamura et al., 2003; Nemhauser et al., 2006) suggest that some feedback regulation must function in desensitizing the early response genes to auxin. Figure 8 also includes data showing gene expression

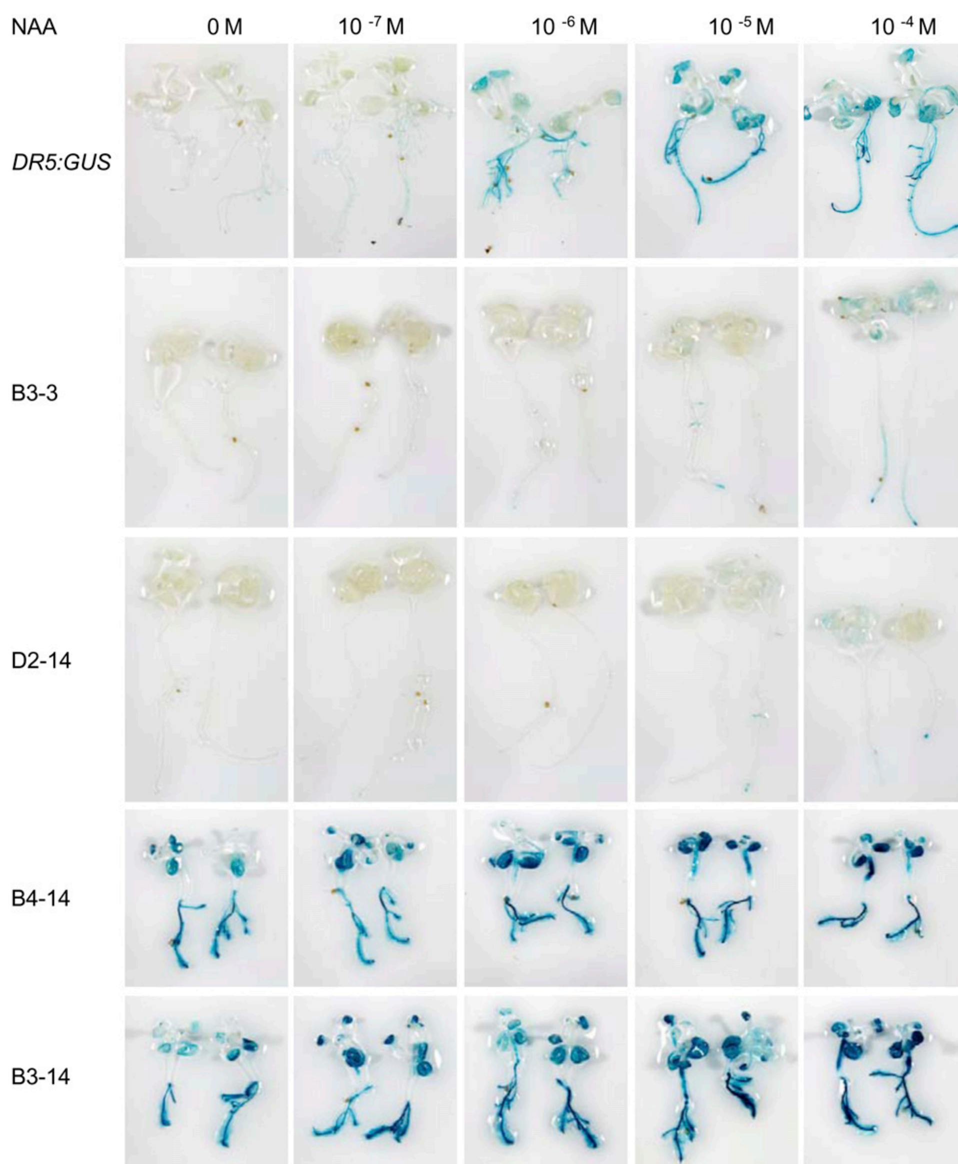

Figure 5. Comparison of auxin-responsive *DR5*:*GUS* expression in control, *35S*:*HA-IAA17mImII* (lines B3-3, D2-14), and *35S*:*VP16-IAA17mImII* (lines B3-14, B4-14) seedlings. Seven-day-old seedlings were removed from agar plates and incubated in a solution containing the concentration of 1-NAA indicated for 12 h and then histochemically stained for GUS.

for seedlings containing the *35S*:*VP16-IAA17mImII* transgene (i.e. replotted from data presented in Fig. 6). These latter results indicate that, in general (i.e. *SAUR23* being an exception), seedlings containing the *35S*:*VP16-IAA17mImII* transgene expressed the auxin response genes at levels considerably less than the maximal expression levels observed with seedlings exposed to applied auxin. With plants expressing the *35S*:*VP16-IAA17mImII* transgene, it is possible that a full auxin response is attenuated to a level that allows the transformed plants to survive.

Expression of the *HA-IAA17mImII* and *VP16-IAA17mImII* Transgenes under Control of the *EXPANSIN7* Promoter Has Dramatic Effects on Root Hair Growth and Development

The *EXPANSIN7* (*EXP7*) gene and promoter (i.e. *EXP7*:*GFP* and *EXP7*:*GUS*) have been reported to be expressed primarily in root hair and trichoblast cells (Cho and Cosgrove, 2002; Kim et al., 2006; Lee and Cho, 2006; Singh et al., 2008). Lee and Cho (2006) showed that when a PINOID protein kinase-GFP fusion protein (i.e. PID-GFP) or a stabilized version of IAA7 (i.e. AXR2) was expressed under control of the *EXP7* promoter, root hair formation and elongation was suppressed. To determine if expression of *EXP7*:*HA-IAA17mImII* and *EXP7*:*VP16-IAA17mImII* transgenes altered root hair development and growth, homozygous seedlings expressing these transgenes were analyzed. Figure 9 shows that expression of the *EXP7*:*HA-IAA17mImII* transgene suppressed root hair formation and growth, while expression of the *EXP7*:*VP16-IAA17mImII* transgene enhanced root hair elongation compared to *DR5*:*GUS*. Our results with the *EXP7*:*HA-IAA17mImII* transgene are consistent with those of Lee and Cho (2006), which indicate that auxin signaling is required for root hair growth and devel-

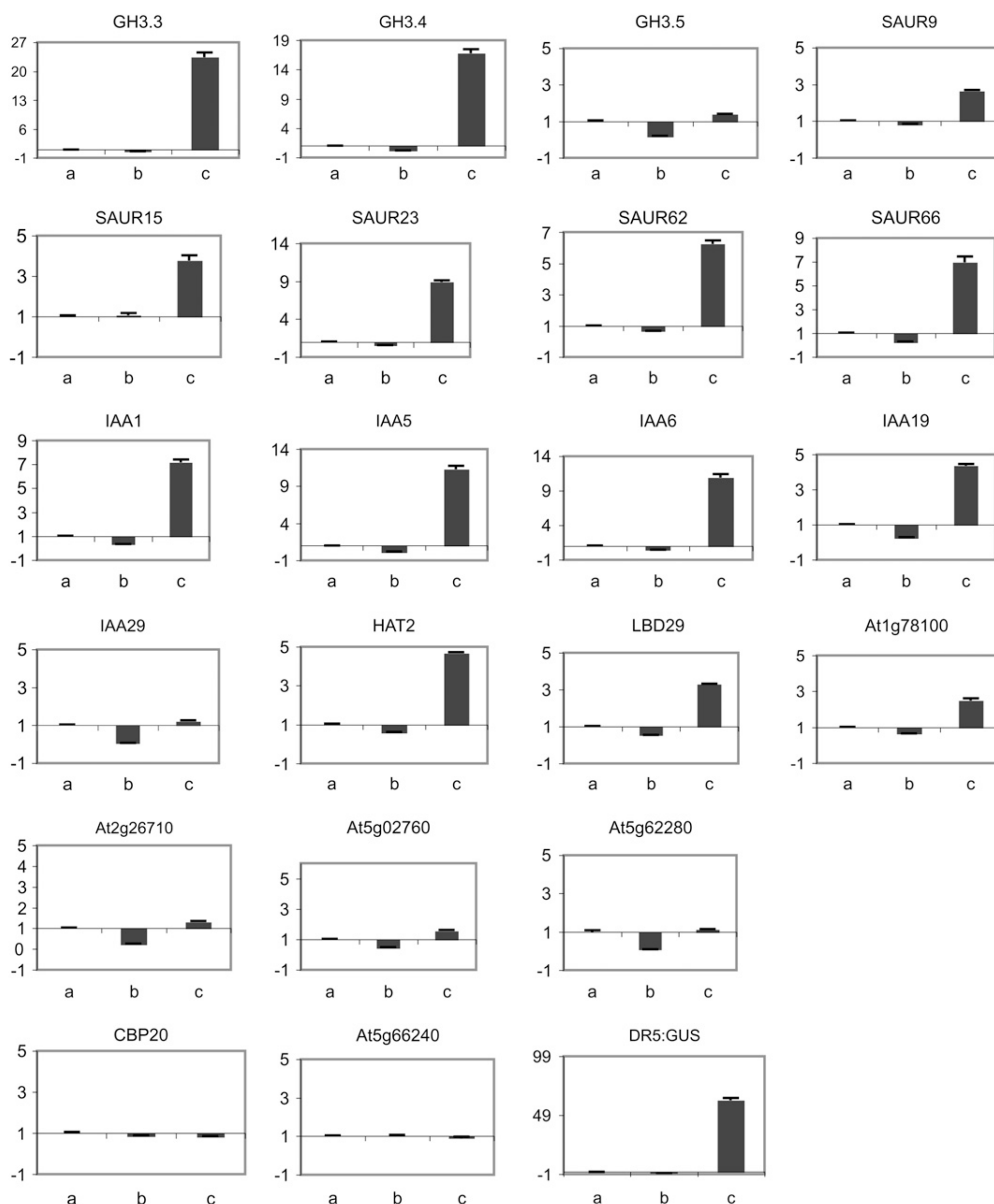

Figure 6. Expression levels for selected auxin response genes in control (*DR5*:*GUS*) seedlings and seedlings transformed with *35S*:*HA-IAA17mImII* (line D2-14) and *35S*:*VP16-IAA17mImII* (line B3-14). Seven-day-old seedlings were used for qRT-PCR. Gene expression levels in *DR5*:*GUS* seedlings were set at 1.0, and gene expression levels in seedlings containing the modified IAA17 transgenes are presented relative to the *DR5*:*GUS* control. Expression levels of *DR5*:*GUS* and two non-auxin response genes, *CBP20* (At5g44200) and At5g66240, are also shown. a, *DR5*:*GUS* control; b, *35S*:*HA-IAA17mImII*; c, *35S*:*VP16-IAA17mImII*.

opment, and further suggest that there is nothing inherently different with regard to how two different Aux/IAA repressors function in Arabidopsis root hairs. In addition, our results with the *EXP7*:*VP16-IAA17mImII* transgene indicate that root hair growth can be enhanced by constitutively activating the auxin response pathway in root hairs. Our results with the *EXP7*:*HA-IAA17mImII* and *EXP7*:*VP16-IAA17mImII* transgenes taken together suggest that auxin signaling can be effectively suppressed or enhanced in a cell-autonomous manner and provide support for an important role of auxin in root hair growth and development.

DISCUSSION

Because the auxin signal transduction pathway is streamlined, simple, and ultimately dependent on the stability of the Aux/IAA transcription factors, we

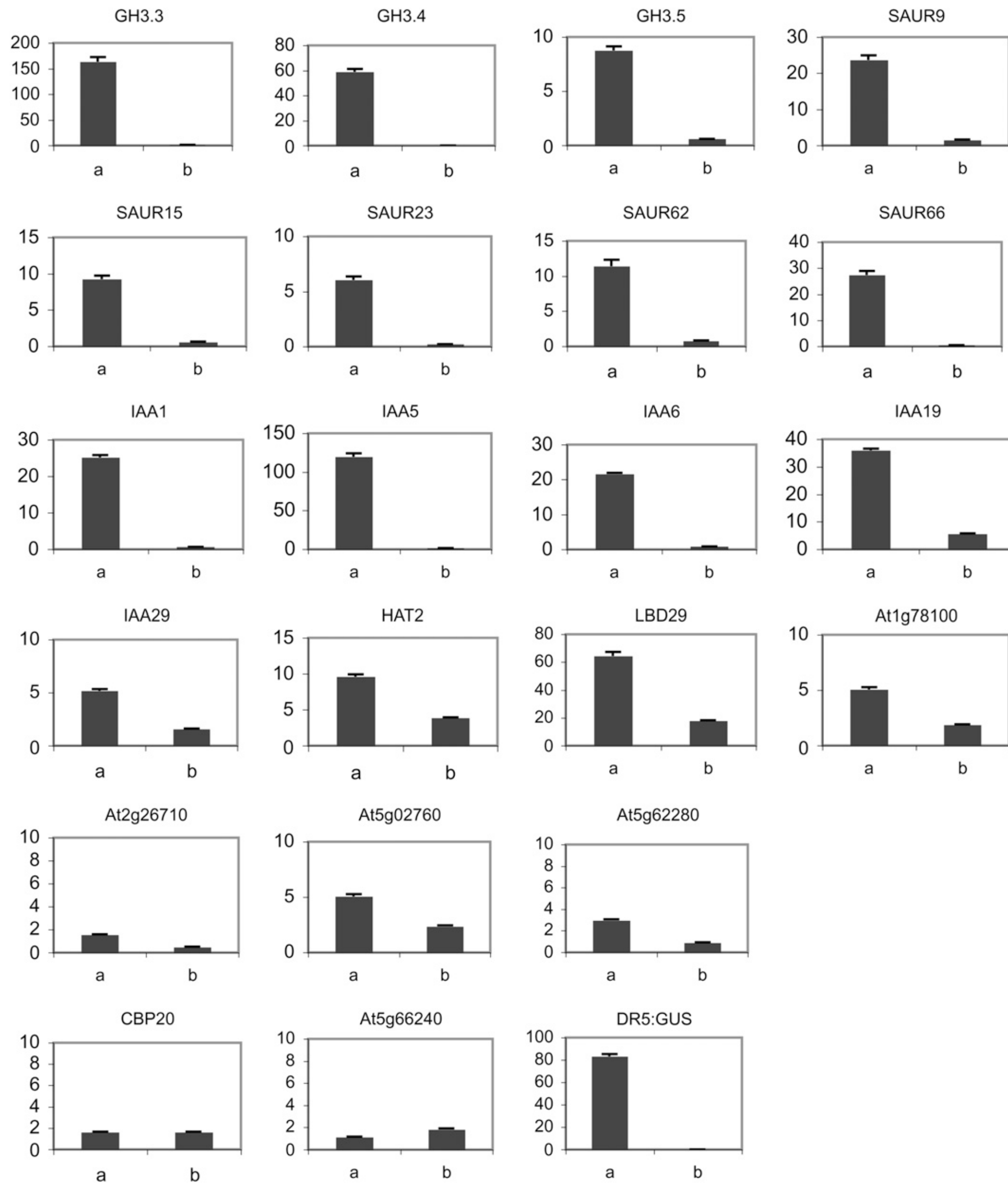

Figure 7. Auxin-induced expression levels for selected auxin response genes in control (*DR5:GUS*) seedlings and seedlings transformed with *35S:HA-IAA17mImII* (line D2-14). Seven-day-old seedlings were removed from agar plates and incubated in a solution containing 1 μM 1-NAA for 1 h. qRT-PCR was used to determine the expression levels of genes indicated. See Figure 6 for additional details on the genes analyzed and presentation of the data. a, *DR5:GUS* control; b, *35S:HA-IAA17mImII*.

reasoned that it should be possible to down-regulate the auxin response pathway in plants by constitutively expressing a stabilized version of an Aux/IAA repressor and to up-regulate the auxin response pathway by constitutively expressing a version of an Aux/IAA protein that had been converted from a repressor to a stabilized activator. Converting the repressor to an activator was made possible by mutating the repression domain of IAA17 and adding an activation domain in the form of VP16 (Tiwari et al., 2003). Previous results, using transfected protoplasts, indicated that the VP16 activation domain was ineffective when fused to an IAA17mII mutant, which contained a wild-type repression domain, suggesting that the wild-type repression domain in IAA17 was dominant over the VP16 activation domain (Tiwari et al., 2003).

Results presented here show that Arabidopsis plants constitutively expressing a stabilized version of an Aux/IAA protein that was converted into an activator have high auxin-like phenotypes, including enhanced expression of auxin response genes. This contrasts with plants that express a stabilized Aux/IAA repressor and have low auxin-like phenotypes with reduced auxin response gene expression. Free

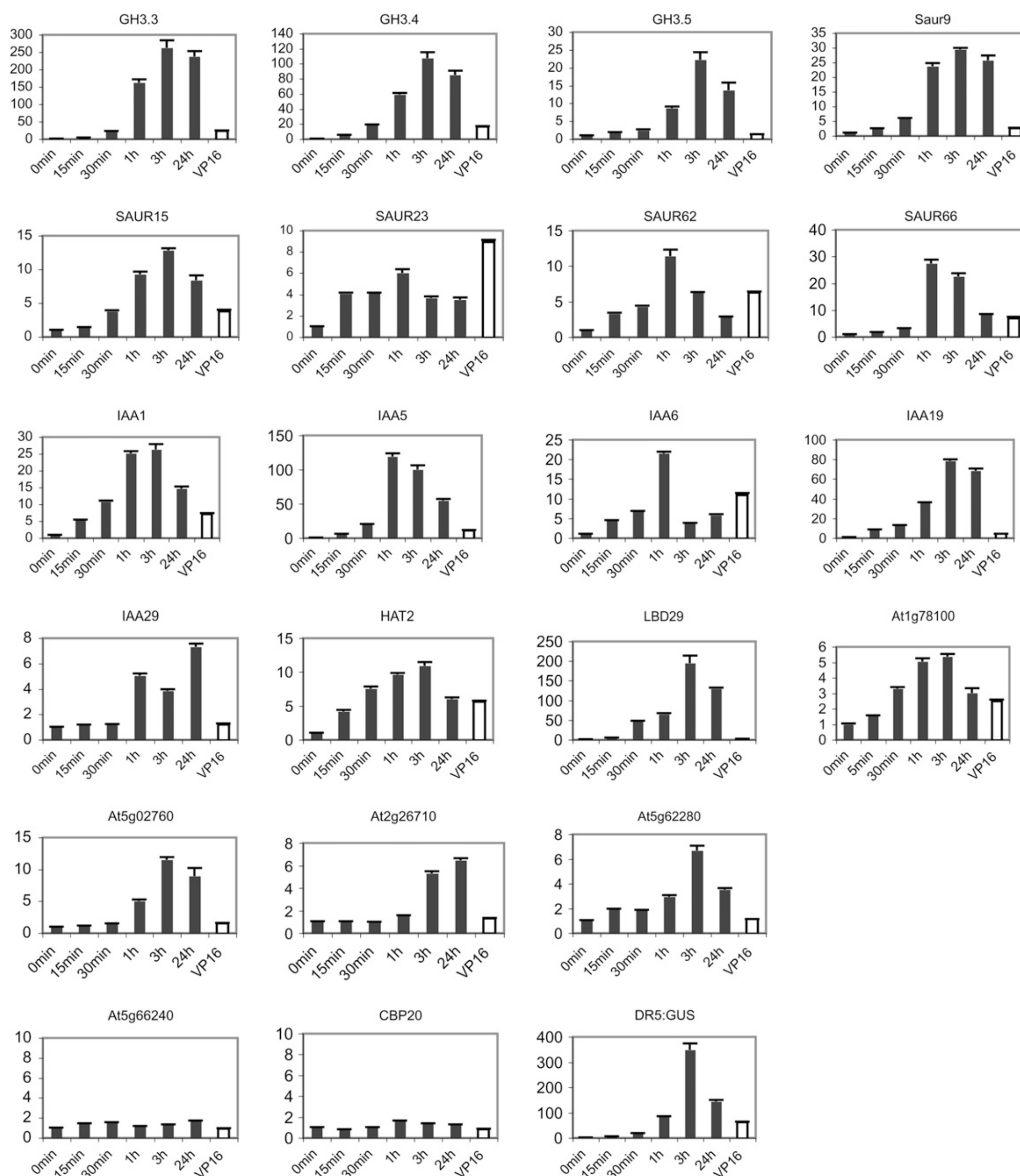

Figure 8. Time course for auxin-induced expression for selected auxin response genes in control (*DR5*:*GUS*) seedlings. Seven-day-old seedlings were removed from agar plates and incubated in a solution containing 1 μM 1-NAA for the times indicated. qRT-PCR was used to determine the expression levels of genes indicated. Gene expression levels at time 0 were set at 1.0, and gene expression levels for treatments at indicated time points are presented relative to time 0 (black bars). For comparison, the expression levels of these genes are also shown for untreated (i.e. no exogenous auxin) 7-d-old seedlings expressing the *35S*:*VP16-IAA17mImII* transgene (line B3-14; white bars, labeled as *VP16*). See Figure 6 for additional details on the genes analyzed.

auxin levels were not significantly altered in seedlings expressing the *35S*:*VP16-IAA17mImII* or *35S*:*HA-IAA17mImII* transgene, however, suggesting that the high and low auxin-like phenotypes do not result from altered free auxin levels in the IAA17 transgenic lines. Therefore, the constitutively expressed, stabilized Aux/IAA activator and repressor appear to function in an auxin-independent manner to up-regulate and down-regulate auxin response genes and confer high and low auxin-like phenotypes, respectively.

In addition, our results show that by restricting expression of the modified Aux/IAA proteins to a specific cell type (e.g. in this case, trichoblast/root hair cells), auxin signaling can be suppressed or enhanced

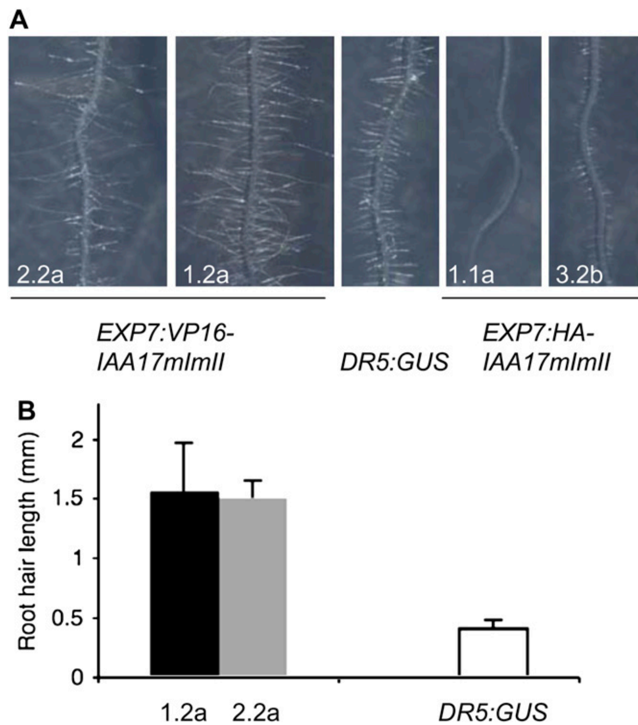

Figure 9. Root hair growth and development in *DR5*:*GUS* control seedlings and seedlings transformed with *EXP7*:*VP16-IAA17mImII* and *EXP7*:*HA-IAA17mImII*. A, Images of root hairs in control 7-d-old seedlings and seedlings transformed with *EXP7*:*VP16-IAA17mImII* (roots from two independent lines, 2.2a and 1.2a, are shown) and *EXP7*:*HA-IAA17mImII* (roots from two independent lines, 1.1a and 3.2b, are shown). B, Root hair length measurements in control 7-d-old seedlings and seedlings transformed with *EXP7*:*VP16-IAA17mImII* (two independent lines, 2.2a and 1.2a; $n \geq 20$, ANOVA test, $P < 0.001$).

in a localized manner. By selecting appropriate promoters to drive expression of modified IAA17 proteins, it should be possible to not only shut down auxin signaling in specific cell types or under specific environmental or developmental conditions (i.e. as has been demonstrated here and previously with stabilized Aux/IAA repressors; Park et al., 2002; Fukaki et al., 2005; De Smet et al., 2007; Lucas et al., 2007; Singh et al., 2008), but to enhance auxin signaling (i.e. by using stabilized Aux/IAA activators as demonstrated here). While we have shown that root hair-specific expression of an *IAA17mImII* transgene can effectively suppress root hair growth, expression of IAA17mII or other Aux/IAAmII proteins (i.e. lacking a mutation in the repression domain) would likely be more effective in suppressing auxin responses when expressed in a cell/tissue-specific manner because of their greater capacity to repress auxin response genes. It is possible that by controlling the auxin signaling pathway in a cell-autonomous, developmental-specific, or environmental-specific manner, plants might be modified to enhance lateral root or root hair formation and growth, alter plant architecture, or enhance/suppress processes that require an auxin signal.

Our results indicate that a variety of auxin response genes can be targeted for repression or activation by a single, stabilized member of Aux/IAA repressor family (i.e. namely IAA17) or converted activator, respectively. Results reported here, along with our previous results using protoplast transfection assays (Tiwari et al., 2001, 2003, 2004), support a model for auxin signaling in which Aux/IAA repressors function directly on auxin response genes to actively repress their transcription. This is also supported by recent results that show that the IAA12/BDL repressor interacts with the corepressor TOPLESS in repressing auxin-responsive gene expression (Szemenyei et al., 2008). Furthermore, if Aux/IAA proteins were passive repressors and simply sequestered ARF activators away from their target sites, then expression of *35S*:*VP16-IAA17mImII* transgenes would be expected to have the same effect as expression of *35S*:*HA-IAA17mImII* transgenes in preventing ARFs from reaching their target sites. Because expression of *35S*:*VP16-IAA17mImII* transgenes result in constitutive activation of auxin response genes, the modified IAA17 protein must function directly at the gene activation level. Whether the VP16 activation domain functions by recruiting a coactivator to auxin-responsive promoters or somehow prevents the IAA17 repression domain from interacting with a corepressor (e.g. TOPLESS) remains to be determined. It is possible that the VP16 activation domain simply interferes with the IAA17 repression domain, allowing the ARF activation domain to function even when an ARF is associated with the modified Aux/IAA protein.

MATERIALS AND METHODS

Vector Constructs and Transgenic Lines

The *35S*:*HA-IAA17mImII* and *35S*:*VP16-IAA17mImII* plasmid constructs have been previously described (Tiwari et al., 2001, 2003). For *EXP7* At1g12560 promoter:*IAA* constructs, the *EXP7* promoter was obtained by PCR amplification of Columbia genomic DNA with Fw primer 5′-TCTAGATGAT-TACAAAGGGGAAATTTAGG-3′, and Rv primer 5′-CCATGGTCGATAG-TATCCAGCGACGA-3′, and was subcloned into pPZP221 vector harboring *VP16-IAA17mImII* and *HA-IAA17mImII*, respectively. Cloning fidelity was confirmed by sequencing.

All transgenic plants used in this report are in *DR5*:*GUS* background. *EXP7* constructs were introduced into *DR5*:*GUS* background using *Agrobacterium*-mediated transformation by the floral-dip method (Clough and Bent, 1998). T3 homozygous lines were analyzed in this study unless indicated otherwise. *35S*:*HA-IAA17mImII X DR5*:*GUS* and *35S*:*VP16-IAA17mImII X DR5*:*GUS* were obtained by crossing. Plants containing the *DR5*:*GUS* transgene have been previously described (Ulmasov et al., 1997b).

Plant Growth and Treatments

Arabidopsis (*Arabidopsis thaliana*) plants were grown in growth chambers at 22°C under continuous, cool-white fluorescent light. For experiments with seedlings, Arabidopsis seeds were sterilized and germinated on horizontal or vertical plates as described previously (Wang et al., 2005). For auxin treatments, 7-d-old seedlings were removed from the agar plates and incubated with shaking in liquid media containing or lacking 1 μM 1-NAA for the times indicated. After incubation, seedlings were frozen in liquid nitrogen and stored at −70°C prior to RNA isolation. Root hair length was measured using ImageJ (http://rsb.info.nih.gov/ij/) from photographs taken with a Leica

stereomicroscope at the University of Missouri Molecular Cytology Core. For measurements of hypocotyl length and lateral root numbers, 7-d-old seedlings were scanned and analyzed using ImageJ.

Free IAA Level Determination

Free IAA determinations were performed as described in Kim et al. (2007) using 6-d-old seedlings. Three independent determinations were made for each line using 65 seedlings for each sample. The whole assay series was repeated a second time with similar results.

Analysis of variance was performed using Student's *t* test followed by post hoc analyses (Tukey, Student-Newman-Keuls pairwise, Bonferroni to control).

GUS Staining

Gus staining was carried out as previously described (Ulmasov et al., 1997b). Seedlings were placed in GUS solution for 12 h at 37°C and cleared in 70% ethanol.

RNA Isolation and Real-Time RT-PCR Analysis

RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. For the microarray analysis, hybridizations and array scanning were carried out at the University of Missouri DNA Core Facility with two biological and two technical replicates. The detection *P* value was generated, signal values for individual genes were obtained using statistical algorithms on Affymetrix GeneChip Microarray Suite version 5.0 software, and the resulting CEL files were further analyzed by GeneChip Operating Software 1.4 (Affymetrix). For qRT-PCR, during the RNA isolation, RNase-Free DNase (Qiagen) was used to eliminate any DNA contamination. First-strand cDNA was synthesized from 2 μg total RNA using an Omniscript RT kit (Qiagen). One microliter of the resulting cDNA was subjected to qRT-PCR using SYBR Green Supermix in a CFX96 Real-Time system (Bio-Rad).

Efficiency of each primer pair was determined by standard curves using serial *DR5:GUS* cDNA dilutions. PCR was performed using a three-step protocol with a melting curve. Based on primer efficiency, fold expression was generated in Bio-Rad CFX manager (version 1.0) after normalization to *TUB5* (At1g20010). Triplicates for each PCR reaction and at least two biological replicates were performed for each gene.

Supplemental Data

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

Supplemental Table S1. Primer pairs used for qRT-PCR.

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