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Aux/IAA Proteins Contain a Potent Transcriptional Repression Domain

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Aux/IAA proteins are short-lived nuclear proteins that repress expression of primary/early auxin response genes in protoplast transfection assays. Repression is thought to result from Aux/IAA proteins dimerizing with auxin response factor (ARF) transcriptional activators that reside on auxin-responsive promoter elements, referred to as AuxREs. Most Aux/IAA proteins contain four conserved domains, designated domains I, II, III, and IV. Domain II and domains III and IV play roles in protein stability and dimerization, respectively. A clear function for domain I had not been established. Results reported here indicate that domain I in Aux/IAA proteins is an active repression domain that is transferable and dominant over activation domains. An LxLxL motif within domain I is important for conferring repression. The dominance of Aux/IAA repression domains over activation domains in ARF transcriptional activators provides a plausible explanation for the repression of auxin response genes via ARF-Aux/IAA dimerization on auxin-responsive promoters.

INTRODUCTION

Aux/IAA genes are, in general, classified as early auxin response genes that are upregulated within 5 to 30 min after auxin application to plants or excised plant organs (reviewed in Hagen and Guilfoyle, 2002). *Arabidopsis thaliana* contains 29 *Aux/IAA* genes that are referred to as *IAA1* to *IAA20* and *IAA26* to *IAA34* (Liscum and Reed, 2002). These genes are predicted to encode 18- to 35-kD short-lived nuclear proteins (Reed, 2001; reviewed in Hagen and Guilfoyle, 2002; Kepinski and Leyser, 2002), which appear to function as transcriptional repressors by interacting with auxin response factors (ARFs) on promoters containing TGTCTC auxin-responsive promoter elements (AuxREs) (Ulmasov et al., 1997b; Tiwari et al., 2001, 2003).

Most Aux/IAA proteins have four conserved motifs or domains, referred to as I, II, III, and IV (reviewed in Hagen and Guilfoyle, 2002; Liscum and Reed, 2002). Domain II interacts with an F-box protein, TIR1, which is a component of the SCF^{TIR1} ubiquitin ligase complex (Gray et al., 2001). Auxin increases this interaction in a dose-dependent manner, promoting the rapid degradation of Aux/IAA proteins through the ubiquitin-proteasome pathway (Gray et al., 2001; Zenser et al., 2001, 2003). Mutations in domain II result in increased stability of Aux/IAA proteins (Zenser et al., 2001, 2003), leading to increased repression on TGTCTC AuxREs (Tiwari et al., 2001). Domains III and IV have a similar amino acid sequence to motifs III and IV found in the C-terminal domains of ARF proteins, and these motifs mediate dimerization between Aux/IAA and ARF proteins (Kim et al., 1997; Ulmasov et al., 1997a, 1997b; Morgan et al., 1999; Ouellet et al., 2001). The role played by domain I in Aux/IAA proteins is less clear, but mutations in this domain have been shown to partially suppress phenotypes resulting from mutations in domain II (Rouse et al., 1998; Nagpal et al., 2000), to decrease homodimerization of a domain II mutant Aux/IAA protein in *Saccharomyces cerevisiae* (yeast) two-hybrid assays (Ouellet et al., 2001), and to decrease the capacity of Aux/IAA proteins to repress transcription in protoplast transfection assays (Tiwari et al., 2001).

We have shown previously that all 20 different Aux/IAA proteins tested repressed transcription of auxin-responsive reporter genes in protoplast transfection assays (Ulmasov et al., 1997b; Tiwari et al., 2001). Repression is thought to occur by Aux/IAA repressors interacting with ARF transcriptional activators, which are bound to AuxREs in promoters of early auxin response genes. When Aux/IAA proteins were directly targeted to a promoter by fusion with a Gal4 DNA binding domain (DBD), they repressed transcription of constitutive reporter genes containing Gal4 DNA binding sites. These latter results are consistent with Aux/IAA proteins being active repressors. Mutations in domain II increased repression, whereas mutations in domains I and III decreased repression mediated by Aux/IAA proteins on auxin-responsive reporter genes. These same mutations in chimeric Gal4 DBD-Aux/IAA proteins had similar effects on expression of constitutive reporter genes containing Gal4 DNA binding sites. Although it is likely that mutations in domain III interfere with dimerization, these latter results do not distinguish whether mutations in domain I interfere with dimerization, repression, or something else.

Here, we have used protoplast transfection assays to better elucidate the function of domain I in Aux/IAA proteins. Our results show that domain I functions as a repression domain and that this repression domain is dominant over activation domains, whether the activation domain is present as an intramolecular

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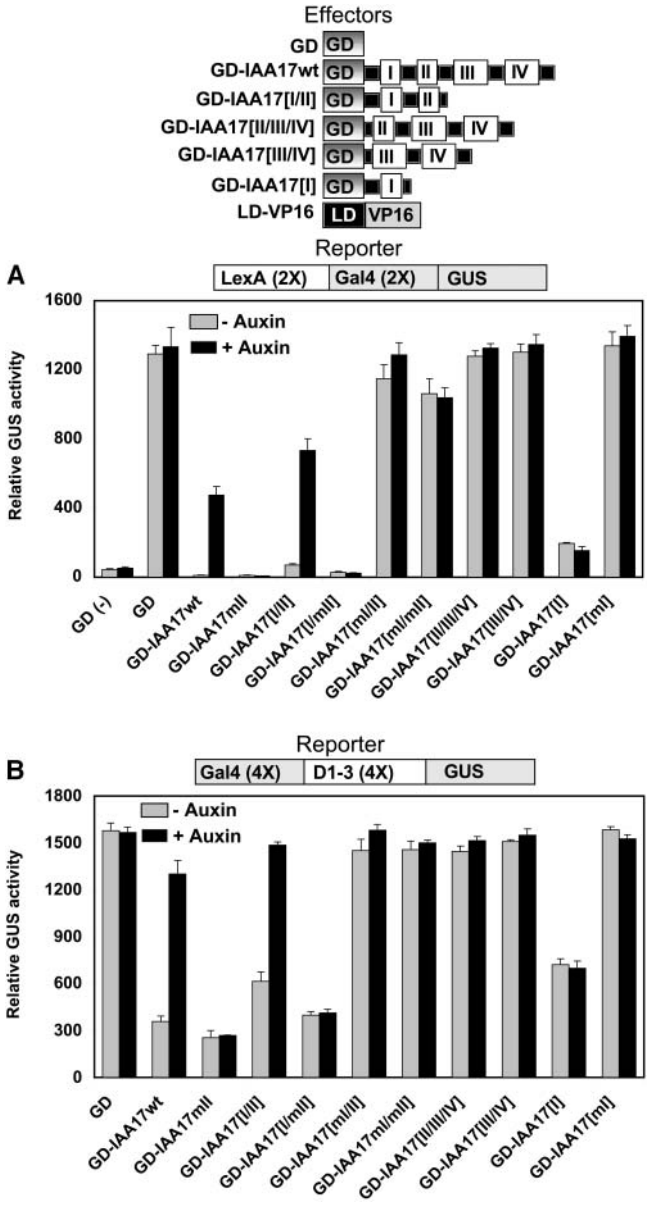

Figure 1. Domain I in IAA17 Is an Active Repression Domain.

Effector genes (diagrammed at top) and reporter genes (diagrammed above bar graphs) were cotransfected into *D. carota* suspension cell protoplasts and assayed in the presence (+auxin) and absence (−auxin) of 10 μM 1-naphthaleneacetic acid. GUS activities were measured 24 h after transfections. Standard errors are indicated. The GD-IAA17wt and GD-IAA17mII effector genes encoded a full-length IAA17 and a full-length domain II mutant IAA17mII, respectively, fused in frame with the Gal4 DBD (Tiwari et al., 2001). The GD effector gene contained only the Gal4 DBD. The GD-IAA17[I], GD-IAA17[I/II], GD-IAA17[II/III/IV], and GD-IAA17[III/IV] effector genes encoded the Gal4 DBD fused to truncated versions of IAA17 containing domain I [I], domains I and II [I/II], domains II, III, and IV [II/III/IV], and domains III and IV [III/IV], respectively (see Methods). The GD-IAA17[mI], GD-IAA17[I/mII], GD-IAA17[mI/II], and GD-IAA17[mI/mII] effector genes encoded the Gal4 DBD fused to truncated versions of IAA17 with site-specific mutations in either (or both) domain I or domain II (see Tiwari et al., 2001).

(A) Cotransfections of GD-IAA17 effector genes with an effector gene encoding the LexA DBD fused to VP16 (LD-VP16) and a LexA(2X)-Gal4(2X):GUS reporter gene. All assays contained the LD-VP16 effector gene with the exception of GD(−).

(B) Transfections with the Gal4(4X)-D1-3(4X):GUS reporter gene.

domain or as an intermolecular domain. Our results also indicate that conserved Leu residues in domain I are important for repression. Our results further reveal that the repression domain in Aux/IAA proteins bears a resemblance to the ethylene response factor (ERF)–associated amphiphilic repression (EAR) motif repression domains found in some ERFs and SUPERMAN (Ohta et al., 2001; Hiratsu et al., 2003). Dominant repression domains in Aux/IAA proteins provide a plausible explanation for repression of early auxin response genes under conditions that promote ARF–Aux/IAA interactions.

RESULTS

Domain I in IAA17 Is Sufficient to Repress a VP16 Transactivator

To test what is required for repression by *A. thaliana* Aux/IAA proteins, we chose to analyze IAA17 as a representative example. When referring to Aux/IAA proteins, the IAA nomenclature used here is as described by Liscum and Reed (2002). We first designed experiments to test whether domain I in IAA17, if targeted to one DNA binding site in a promoter, could repress the activity of a transactivator targeted to another site in the promoter. We used a heterologous *Daucus carota* (carrot) suspension cell protoplast transfection assay system with a β-glucuronidase (GUS) reporter gene that was driven by the −46 35S promoter of *Cauliflower mosaic virus* (CaMV) with both LexA and Gal4 DNA binding sites (LexA[2X]-Gal4[2X]:GUS reporter gene). The GUS reporter gene was cotransfected with one effector gene that encoded a chimeric protein consisting of the LexA DBD fused to the *Herpes simplex virus* VP16 activation domain (LD-VP16) and a second effector gene encoding a chimeric protein consisting of the *S. cerevisiae* Gal4 DBD fused to IAA17 (GD-IAA17wt) or a truncated and/or mutated version of IAA17 (GD-IAA17[I], GD-IAA17[I/II], GD-IAA17[II/III/IV], GD-IAA17[III/IV], GD-IAA17mII, GD-IAA17[mI], GD-IAA17[I/mII], and GD-IAA17[mImII]; see Figure 1 and Methods for details on reporter and effector genes and Tiwari et al. [2001] for details on site-specific mutations in IAA17).

Transfection of the LD-VP16 effector gene along with the reporter gene (i.e., no Gal4 effector gene) resulted in strong, auxin-independent activation of the reporter gene (data not shown), which was similar to the result obtained if a second effector gene encoded only the Gal4 DBD (cf. GD with GD[−], Figure 1A). Cotransfection of an effector gene encoding Gal4 DBD fused to the IAA17 wild-type protein (GD-IAA17wt) resulted in total repression of the reporter gene in the absence of auxin and reduced repression in the presence of auxin. Reduced repression in the presence of auxin presumably occurs because auxin increases the instability of the IAA17 wild-type protein

(Tiwari et al., 2001; Zenser et al., 2001). This is supported by results obtained with an effector gene encoding Gal4 DBD fused to IAA17 containing a mutation in domain II (GD-IAA17mII), which is known to stabilize the Aux/IAA protein (Ouellet et al., 2001; Tiwari et al., 2001; Zenser et al., 2001). With GD-IAA17mII, total repression of the reporter gene occurred in both the presence and absence of auxin.

To determine which domains in IAA17 are required for repression, truncated versions of IAA17 or IAA17 mutants were fused to the Gal4 DBD and tested in cotransfection assays. For truncated IAA17 proteins, domains present in the effector genes are indicated by brackets. Effector genes with a truncation of domain I (GD-IAA17[II/III/IV]) and truncations of domains I and II (GD-IAA17[III/IV]) failed to repress expression of the reporter gene, suggesting that domain I is required for repression. An effector gene encoding Gal4 DBD fused to IAA17 domains I and II (GD-IAA17[I/II]) repressed expression of the reporter gene in an auxin-dependent manner like the GD-IAA17wt effector gene. A mutation in domain II of the effector gene containing only domain I and II (GD-IAA17[I/mII]) functioned like the GD-IAA17mII effector gene, causing nearly complete repression in an auxin-independent manner. Mutations in domain I of either construct containing only domains I and II (GD-IAA17[mI/II] and GD-IAA17[mI/mII]) resulted in complete loss of repression in the presence and absence of auxin. Finally, domain I in isolation from other IAA17 domains (GD-IAA17[I]) functioned as an auxin-independent repressor, and a mutation in domain I relieved repression (GD-IAA17[mI]).

A second reporter gene was used to support the results presented in Figure 1A. A constitutive reporter gene containing Gal4 DNA binding sites (Gal4[4X]-D1-3[4X]:GUS) was cotransfected with the GD-IAA17 effector genes (Tiwari et al., 2001, 2003). In this case, the activity of the reporter gene did not depend on cotransfection of a second effector gene encoding a transactivator, like LD-VP16, but was presumably activated by natural transcriptional activators present in the *D. carota* cells. Results shown in Figure 1B are consistent with results shown in Figure 1A, although the levels of repression achieved with the various effector constructs containing domain I are lower.

These results indicate that an N-terminal portion of IAA17, containing only conserved domain I, functions as a repression domain. Furthermore, the results show that the repression domain in IAA17 is dominant over an acidic activation domain (i.e., VP16) as well as an uncharacterized activation domain(s) (i.e., associated with natural transactivators that are targeted to the D1-3 constitutive element) and that the repressor can function downstream (Figure 1A) and presumably upstream (Figure 1B) of the activation domain. In these assays, the activation domains would be present on transcription factors that are targeted to DNA binding sites, which are independent of the sites targeted by the Gal4 DBD-IAA17 chimeric repressors and are examples of intermolecular dominant repression.

Domain I Is, in General, a Repression Domain in Aux/IAA Proteins

To determine if domain I of IAA proteins other than IAA17 also functioned as repression domains, the N-terminal portions of IAA2, IAA8, IAA13, IAA18, and IAA28 were tested using the reporter gene systems shown in Figure 1. These IAA proteins were chosen to be representative of Aux/IAA proteins, in general, and included those with some different versions of conserved domain I (Table 1). Domain I of the IAA proteins tested contains the following variations, with Leu presented in bold: IAA2, TE**L**C**L**G**L**PG; IAA8, TE**L**R**L**G**L**PE; IAA13, SE**L**E**L**G**L**GL; IAA17, TE**L**C**L**G**L**PG; IAA18, K**L**E**L**K**L**GPP; and IAA28, R**L**E**L**R**L**APP. Each of the domain I effector genes (e.g., GD-IAA2[I]) functioned as a repressor with the Gal4(2X)-LexA(2X):GUS reporter gene system (Figure 2A) and the Gal4(4X)-D1-3(4X):GUS reporter gene (Figure 2B). The reason for the slightly lower activities observed in the presence of auxin with the Gal4(4X)-D1-3(4X):GUS reporter gene is not known, but the results, nevertheless, show that domain I confers repression to the reporter gene.

Table 1. LxLxL Motifs in Aux/IAA and ERF Repression Domains

IAA	Domain I
1.	TE**L**R**L**G**L**PG
3.	TE**L**R**L**G**L**PG
4.	TE**L**R**L**G**L**PG
5.	TE**L**R**L**G**L**PG
6.	TE**L**R**L**G**L**PG
19.	TE**L**R**L**G**L**PG
16.	TE**L**R**L**G**L**PG
27.	TE**L**R**L**G**L**PG
8.	TE**L**R**L**G**L**Pe
2.	TE**L**c**L**G**L**PG
14.	TE**L**c**L**G**L**PG
17.	TE**L**c**L**G**L**PG
7.	TE**L**c**L**G**L**PG
9.	TE**L**t**L**G**L**PG
15.	TE**L**t**L**a**L**PG
20.	Td**L**R**L**G**L**sf
30.	Td**L**R**L**G**L**sf
10.	TE**L**d**L**a**L**gl
12.	sE**L**e**L**G**L**gl
13.	sE**L**e**L**G**L**gl
29.	mE**L**d**L**G**L**sl
11.	lE**L**g**L**t**L**sl
34.	id**L**g**L**s**L**rt
31.	vn**L**s**L**s**L**tf
18.	kk**L**e**L**k**L**gp
26.	kk**L**e**L**r**L**hr
28.	kr**L**e**L**r**L**ap
NtERF3.	id**L**d**L**n**L**ap
AtERF4.	ld**L**e**L**n**L**pp
AtSUPR.	qd**L**d**L**e**L**rl

Conserved Leu in the LxLxL motif are in bold. The most conserved amino acids in domain I of *A. thaliana* IAA proteins are in uppercase. IAA32 and IAA33, which lack a LxLxL motif, are not shown. For nomenclature of *A. thaliana* IAA proteins, see Liscum and Reed (2002). NtERF3, AtERF4, and AtSUPR correspond to the EAR motifs within the repression domains of *N. tabacum* ERF3, *A. thaliana* ERF4, and *A. thaliana* SUPERMAN, respectively.

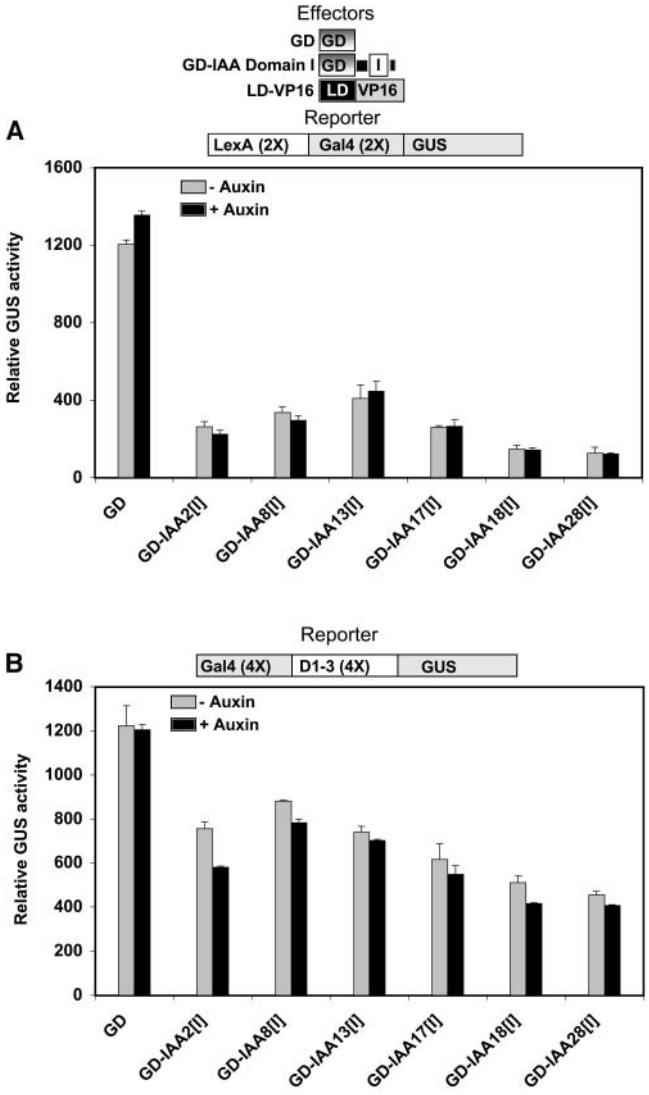

Figure 2. Domain I Is an Active Repression Domain in Other Aux/IAA Proteins.

Effector genes are diagrammed at the top, and reporter genes are diagrammed above the bar graphs. Protoplast transfection assays with the two GUS reporter genes were as described in Figure 1 and Methods, using effector genes that encoded the Gal4 DBD fused in frame with domain I of *A. thaliana* IAA2 (GD-IAA2[I]), IAA8 (GD-IAA8[I]), IAA13 (GD-IAA13[I]), IAA17 (GD-IAA17[I]), IAA18 (GD-IAA18[I]), and IAA28 (GD-IAA28[I]). Standard errors are indicated.

(A) Transfections of GD-IAA[I] effector genes with an effector gene encoding the LexA DBD fused to VP16 (LD-VP16) and a LexA(2X)-Gal4(2X):GUS reporter gene.

(B) Transfections of GD-IAA[I] effector genes with a Gal4(4X)-D1-3(4X):GUS reporter gene.

Leu in Domain I of IAA17 Are Important for Repression

To assess the importance of each amino acid in domain I of IAA17, individual residues within the sequence ETE**L**C**L**G**L**PG were mutated to Ala. N-terminal portions of IAA17 containing the domain I mutations were fused to the Gal4 DBD and tested as effector genes with the Gal4(4X)-D1-3(4X):GUS reporter gene. Figure 3A shows that mutation of any of the three Leu in the LxLxL motif resulted in total loss of repression or strongly reduced repression in the case of the third Leu in the motif. Mutation of Thr also resulted in a strong reduction in repression, whereas mutation of other residues had either no effect or a more modest effect on repression.

The LxLxL motif in the repression domain of IAA28, R**L**E**L**R-**L**APP, differs considerably in amino acid sequence from that in IAA17 and several other IAA proteins (Table 1). To test whether the first Leu in the LxLxL motif of IAA28 was critical for repression like that in IAA17, this amino acid was mutated. Figure 3B shows that an Ala substitution for the first Leu in domain I of IAA28 resulted in complete loss of repression similar to a mutation in the first Leu in domain I of IAA17.

The Repression Domains in ERFs and IAA Proteins Contain a Similar LxLxL Motif

As documented above, conserved domain I in IAA proteins contains an LxLxL motif, which plays an important role in repression. Similar LxLxL motifs, D**L**D**L**N**L** and D**L**D**L**E**L**, are found in the AtERF4 and SUPERMAN repression domains (referred to as the EAR motif), respectively (Hiratsu et al., 2003; Table 1). Because of the LxLxL similarity within the EAR motif repression domains and in domain I of IAA proteins, we tested whether a 29–amino acid fragment containing the EAR motif from AtERF4 could repress Gal4(4X)-D1-3(4X):GUS reporter gene expression like domain I from IAA proteins. Figure 3B shows that an effector gene encoding Gal4 DBD fused to the repression domain of AtERF4 (GD-ERF4RD) repressed reporter gene expression like GD-IAA17[I] (Figure 3A) and GD-IAA28[I]. To determine if the LxLxL motif played a role in conferring repression within the EAR motif of AtERF4, Ala was substituted for each Leu. Each mutation resulted in loss of repression, but like results observed with IAA17, an Ala substitution for the third Leu was less effective in relieving repression than an Ala substitution for the first or second Leu. These results suggest that the repression domains in IAA and ERF proteins may bring about repression in a similar fashion and that one (or more) Leu in the LxLxL motif is an important component of the repression domain of both types of repressors.

The Repression Domain in IAA17 Functions as a Short-Range Repressor and Is Active Both Upstream and Downstream of a Transcriptional Activator

To determine if the IAA17 repression domain was functional when targeted upstream as well as downstream of a VP16 activator in a promoter, we tested both binding site arrangements using a LexA(2X)-Gal4(2X):GUS reporter gene. In one case, the Gal4 DBD was used to target the IAA17 repression domain (GD-IAA17[I]) to Gal4 DNA binding sites located downstream of a VP16 activation domain, which in turn was targeted to LexA DNA binding sites by a LexA DBD (LD-VP16). In the second case, the LexA DBD was used to target the IAA17 repression domain (LD-IAA17[I]) to LexA DNA binding sites located upstream of the VP16 activator (GD-VP16). Figures 4A

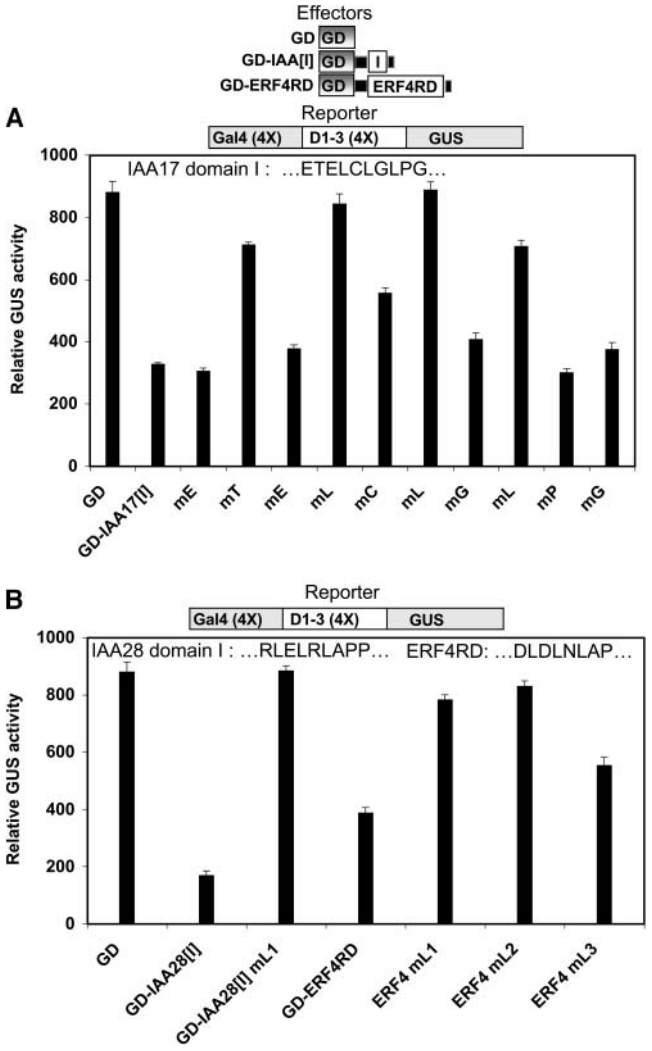

Figure 3. The LxLxL Motif Is Important for Repression in Both Aux/IAA and AtERF4 Repressors.

Effector genes are diagrammed at the top, and reporter genes are diagrammed above the bar graphs. Effector genes encoded the Gal4 DBD fused in frame to domain I of IAA17 (amino acids 1 to 29), IAA28 (amino acids 1 to 24), or the repression domain of AtERF4 (amino acids 199 to 222). The reporter gene was the constitutive Gal4(4X)-D-1-3(4X):GUS. Transfections were performed as described in Figure 1 and Methods. Standard errors are indicated.

(A) Effect of Ala substitutions in domain I of IAA17. Ala substitutions for individual amino acids are indicated by the letter code. GD-IAA17[I] is the unmutated control. GD contains only the Gal4 DBD. m, mutation.

(B) Effect of site-specific Ala substitutions that were introduced into the first, second, or third Leu in the LxLxL motifs found in domain I of IAA28 and the EAR motif of AtERF4. GD-IAA28[I] and GD-ERF4RD are unmutated controls. An mL indicates the position of the Ala substitution in GD-IAA28[I] (Table 1) or GD-ERF4RD (D**L**D**L**N**L**AP).

and 4B show that the VP16 activated transcription when targeted to either position in the promoter (cf. GUS activities with no GD-VP16 effector gene, GD[−], or no LD-VP16 effector gene, LD[−], with GUS activities with the VP16 effector genes [GD and LD]). In cotransfection experiments with the IAA17 effector genes, domain I of IAA17 repressed the VP16 activator whether the repressor was located upstream or downstream of the activator binding sites, although the repression domain may be more potent if located downstream of the activator.

We next examined whether the repression domain in IAA17 could function if located at more distal upstream positions from GD-VP16. In the LexA(2X)-Gal4(2X):GUS reporter gene (Figures 4A and 4B), the LexA DNA binding sites (−146 to −104 from the transcription start) were separated from the Gal4 DNA binding sites (−93 to −57) by 10 bp. Figures 4C and 4D show results with reporter genes in which the LexA DNA binding sites were separated from the Gal4 DNA binding sites by 63 bp (LexA DNA binding sites at −199 to −157 from the transcription start site) and 370 bp (LexA DNA binding sites at −506 to −464 from the transcription start site), respectively. Repression by LD-IAA17[I] was reduced when the distance between the LD and GD DNA binding sites was increased from 10 to 63 bp (cf. Figures 4B and 4C) and was eliminated when the distance increased to 370 bp (Figure 4D). Mutation of the repression domain (GD-IAA17[mI] or LD-IAA17[mI]) resulted in loss of repression in all cases.

The Repression Domain in IAA17 Is Dominant over an Activation Domain within a Chimeric Protein

We have shown in Figure 1 that the IAA17 repressor is dominant over a transactivator when both proteins are targeted separately to a promoter (i.e., intermolecular repression). To assess if this same dominance is observed when the repression and activation domains are part of the same protein (i.e., intramolecular repression), we constructed chimeric proteins containing both repression and activation domains.

To determine if the IAA17 repression domain could repress the activity of an ARF with a Gln-rich activation domain, an N-terminal fragment of IAA17 containing domain I was fused to ARF5 and tested in transfection assays with a natural auxin-responsive promoter, *Glycine max* (soybean) GH3 (Hagen and Guilfoyle, 2002). An effector gene encoding the full-length wild-type IAA17 (IAA17wt) repressed GH3:GUS reporter gene expression (cf. the no-effector control with IAA17wt in Figure 5A), but a mutation in domain I of IAA17 (IAA17mI) relieved the repression. An effector gene encoding full-length ARF5 activated the reporter gene as described previously (Tiwari et al., 2003). When the IAA17 repression domain was present at the C terminus of ARF5 (ARF5-IAA17[I]), the reporter gene was repressed in the presence of auxin to below that seen without an effector gene. Mutation of the repression domain (ARF5-IAA17[mI]) restored activation by ARF5. The reason for increased activity observed with the effector gene encoding an activator with a mutated repression domain (cf. ARF5 with ARF5-IAA17[mI]) is not known.

As another example of repressor domain dominance, we tested effector genes encoding VP16-IAA17 fusion proteins with a GAL4(4X):GUS reporter gene in the absence and presence of

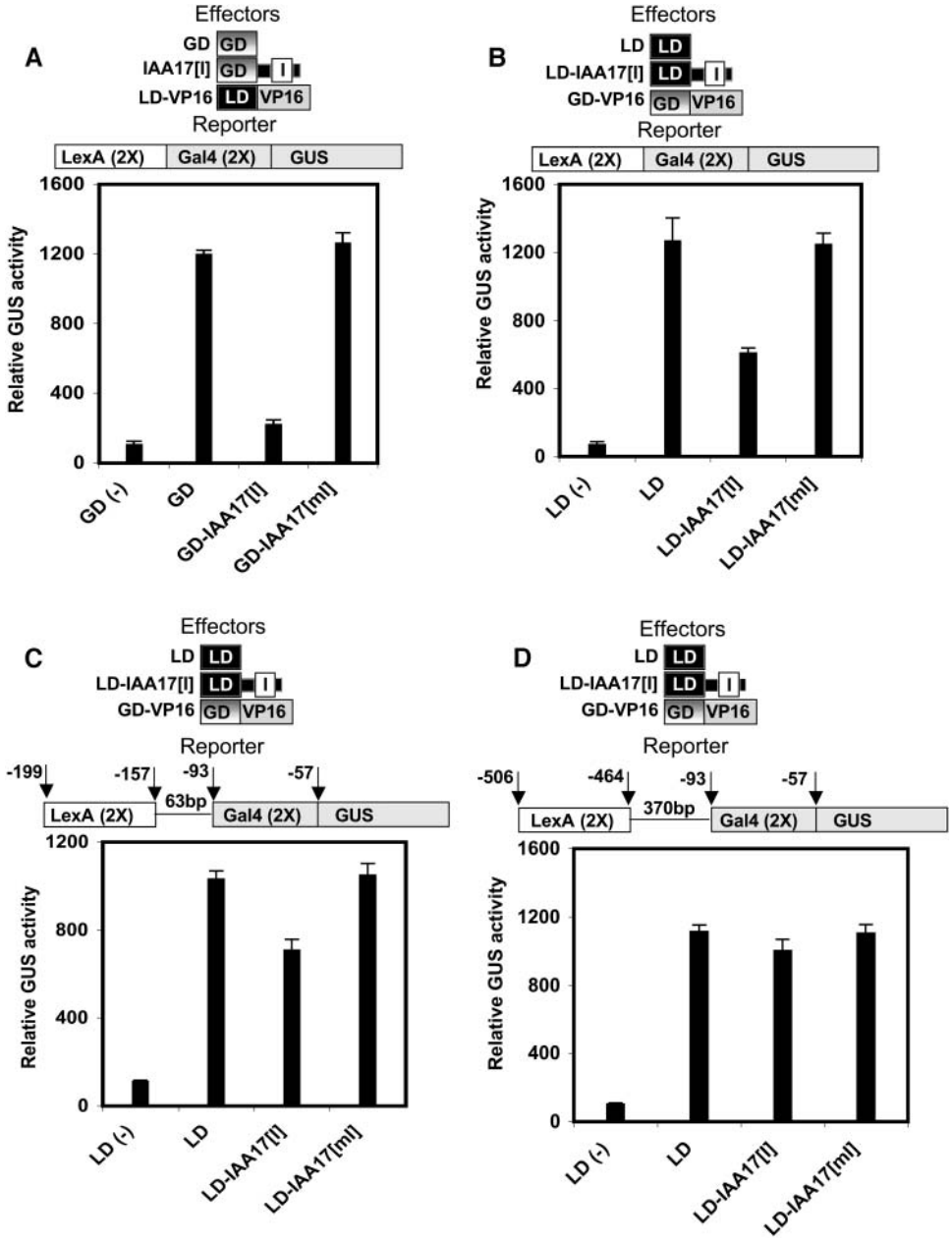

Figure 4. The Repression Domain from IAA17 Functions as a Short-Range Repressor and Is Active Both Downstream and Upstream of a VP16 Activator.

Effector genes and reporter genes are diagrammed above the bar graphs. Transfections were performed as described in Figure 1 and Methods. Standard errors are indicated. GD and LD indicate effector genes encoding only the DBDs of Gal4 and LexA, respectively. GD(−) and LD(−) indicate transfection assays performed with a Gal4 DBD or LexA DBD effector gene in the absence of an effector gene encoding a VP16 activator. All other transfection assays contained an effector gene encoding either the LD-VP16 **(A)** or GD-VP16 (see **[B]**, **[C]**, and **[D]**). Effector genes GD-IAA17[I] and LD-IAA17[I] contained an unmutated domain I from IAA17 fused in frame to the Gal4 DBD or LexA DBD. GD-IAA17[mI] and LD-IAA17[mI] contained a mutation in IAA17 domain I that compromises its repressor activity (see Tiwari et al., 2001).

(A) Transfection assays with the IAA17 repression domain (GD-IAA17[I]) targeted to DNA binding sites downstream of the VP16 activation domain (LD-VP16) DNA binding sites using a LexA(2X)-Gal4(2X):GUS reporter gene. A 10-bp spacer separates the LexA DNA binding sites from the Gal4 DNA binding sites.

(B) Transfection assays with the IAA17 repression domain (LD-IAA17[I]) targeted to DNA binding sites upstream of the VP16 activation domain (GD-VP16) DNA binding sites using a LexA(2X)-Gal4(2X):GUS reporter gene identical to that described in **(A)**.

(C) Transfection assays as in **(B)** but with a LG63 reporter gene. The LG63 reporter gene is identical to the Lex(2X)-Gal(2X):GUS reporter gene except the spacer between the LexA and Gal4 DNA binding sites is 63 bp. Numbers above the reporter gene indicate the distance from the transcription start site.

(D) Transfection assays as in **(B)** but with a LG370 reporter gene. The LG370 reporter gene is identical to the Lex(2X)-Gal(2X):GUS reporter gene except the spacer between the LexA and Gal4 DNA binding sites is 370 bp. Numbers above the reporter gene indicate the distance from the transcription start site.

a second effector gene encoding a chimeric protein, which consisted of a GAL4 DBD fused to an ARF5 transactivator lacking its normal DBD (GD-5MC; Ulmasov et al., 1999; Tiwari et al., 2003). These experiments were designed to determine if a VP16 fusion would convert an IAA repressor into an activator and, if so, what was required for this conversion. In the absence of a GD-5MC effector gene, transfection of VP16-IAA17 effector genes resulted in activity equivalent to the no-effector control (data not shown). Figure 5B shows that transfection of the GD-5MC effector gene resulted in a 10-fold increase in reporter gene activity (cf. GD with GD-5MC). Cotransfection with VP16-IAA17wt and VP16-IAA17mII effector genes caused repression of the reporter gene in the absence of auxin compared with transfections with the GD-5MC effector gene alone. Repression was not observed in the presence of auxin with the VP16-IAAwt effector gene, most likely because of its increased instability in the presence of auxin. Repression was observed, however, with the VP16-IAA17mII protein in the presence of auxin, and this probably resulted from the increased stability of the IAA17 protein with the domain II mutation compared with the wild-type protein in protoplasts treated with auxin (Tiwari et al., 2001). When domain I was mutated along with domain II (VP16-IAA17mImII), repression was relieved and activation achieved, indicating that the full activation potential of VP16 is dependent on the inactivation of the IAA17 repression domain. The greater activation achieved by cotransfection with both GD-5MC and VP16-IAA17mImII may be explained by the presence of two independent activation domains (i.e., the Gln-rich middle region of ARF5 and the VP16 acid-rich activation domain on IAA17mI-mII). We have repeated these experiments with IAA7 and obtained results that show the same patterns presented in Figure 5B (e.g., a mutation in domain I was required to achieve activation with a VP16-IAA7 chimeric effector gene), indicating that these results are not an exclusive property of IAA17 (data not shown).

Although the results described in Figures 5A and 5B are consistent with domain I of IAA17 being able to function as an intramolecular repression domain, the experiments do not rule out that repression might be the result of intermolecular interactions because of the multiple ARF5 DNA binding sites in the natural GH3:GUS reporter gene (Hagen and Guilfoyle, 2002) and multiple Gal4 DNA binding sites in the Gal4(4X):GUS reporter gene. The multiple binding sites in the two promoters leave open the possibility that repression was brought about by intermolecular interactions between proteins bound at different sites in the promoters. To provide further support that domain I of IAA17 can function by intramolecular repression, chimeric activators that did or did not contain the IAA17 repression domain were targeted via the Gal4 DBD to a minimal CaMV −46 35S promoter:GUS reporter gene with a single Gal4 DNA binding site (Gal4[1X]:GUS).

As shown in Figure 5C, transfection of an effector gene encoding Gal4 DBD fused to a truncated ARF5 that consisted of a Gln-rich activation domain and a C-terminal dimerization domain but lacked the ARF5 DBD (GD-5MC) brought about activation in an auxin responsive manner with the Gal4(1X):GUS reporter gene. Fusion of domain I of IAA17 to the truncated ARF protein (GD-5MC-IAA17[I]) reversed the activation, but activation was again observed if domain I was mutated (GD-5MC-IAA17[mI]). In a second experiment, we tested whether fusion of IAA17 domain I to VP16 could override an acidic activation domain. Figure 5D shows that an effector gene encoding Gal4 DBD fused to VP16 resulted in activation of the Gal4(1X):GUS reporter gene; however, if the effector gene contained both a VP16 activation domain and the IAA17 repression domain (GD-VP16-IAA17[I]), activation of the reporter gene was reduced. A mutation in the repression domain (GD-VP16-IAA17[mI]) restored reporter gene expression equal to or greater than that observed with GD-VP16. GUS activities are lower with the Gal4(1X):GUS reporter gene because of the single Gal4 DNA binding site.

Together, these results suggest that the IAA17 repression domain is dominant over an activation domain in the same protein (i.e., intramolecular repression), whether that activation domain is acid rich (VP16) or Gln rich (ARF5). The results in Figure 5B also suggest that the dominance of the repression domain over an activation domain can be transmitted intermolecularly through protein–protein interactions (i.e., through ARF5-IAA17 dimerizaton).

DISCUSSION

We originally suggested that domain I in Aux/IAA proteins might function as a repression domain because effector genes encoding *A. thaliana* IAA proteins with mutations in this domain exhibited a decreased capacity to repress reporter genes to which they were targeted in transfected protoplasts (Tiwari et al., 2001). However, direct evidence that domain I was a repression domain could not be concluded from the types of experiments conducted. It was clear from other experiments that domain I mutations did not interfere with the nuclear localization of Aux/IAA proteins or with their ability to dimerize with ARF transcriptional activators (Ouellet et al., 2001; our unpublished data). Results presented here reveal that domain I in IAA proteins is an active repression domain that can function independently when transferred to a heterologous DNA binding protein or to a protein that can dimerize with a DNA-bound transcriptional activator. Our results suggest that domain I can confer repression by both intermolecular and intramolecular mechanisms (i.e., repression of a transactivator protein that is a separate moiety and repression of a transactivator that is the same moiety). Although our results are consistent for both types of repression, our experiments do not definitively show that domain I is dominant over an activation domain within the same transcription factor. Because our transfected protoplasts may contain more than one copy of the reporter gene, even with a single DNA binding site, intermolecular interactions might still be possible between two reporter gene complexes that contain the chimeric proteins with both an activation domain and repression domain I. In any case, our results show that the IAA17 repression domain is dominant over both a VP16 acidic activation domain and an ARF5 Gln-rich activation domain.

The molecular mechanisms involved in dominant repression by domain I of IAA17 remain unknown; however, our current results make some mechanisms seem more plausible than others. Our results suggest that Aux/IAA proteins may function only as short-range repressors because they appear to lose their

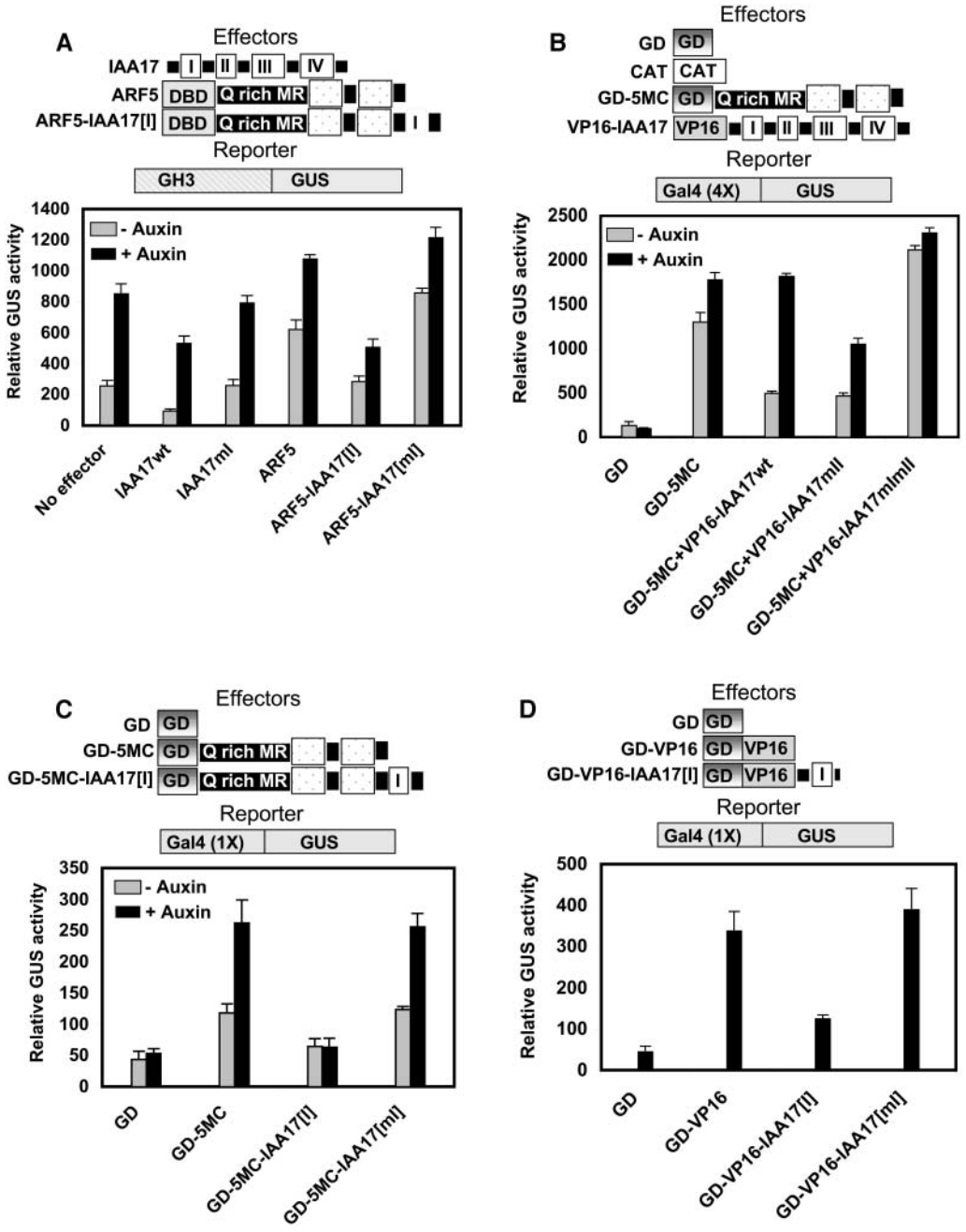

Figure 5. The Repression Domain in IAA17 Is Dominant over Activation Domains in the Same Protein or Dimerized Protein.

Effector genes and reporter genes are diagrammed above the bar graphs. Transfection assays were performed as described in Figure 1 and Methods. Standard errors are indicated.

(A) Transfection assays with a natural auxin-responsive GH3 promoter:GUS reporter gene. The GH3 promoter is from *G. max* (Liu et al., 1994). The GH3:GUS reporter gene was transfected alone (no effector) or cotransfected with an effector gene encoding the full-length wild-type IAA17 (IAA17wt), a domain I mutant version of IAA17 (IAA17mI; Tiwari et al., 2001), full-length auxin response factor ARF5 (Tiwari et al., 2003), domain I (amino acids 1 to 29) from IAA17 fused to the C terminus of ARF5 (ARF5-IAA17[I]), and a mutated version of domain I from IAA17 fused to the C terminus of ARF5 (ARF5-IA17[ml]).

(B) Transfection assays with a minimal promoter Gal4(4X):GUS reporter gene and two effector genes. One effector gene encoding the Gal4 DBD fused in frame to the ARF5 middle region and C-terminal domain (GD-5MC) was present in all assays except the GD(−) (Gal4 DBD only) control. The second effector gene encoded VP16 fused in frame to the full-length wild-type IAA17 (VP16-IAA17wt), a domain II mutation in IAA17 (VP16-IAA17mII), or domain I and domain II mutations in IAA17 (VP16-IAA17mImII).

ability to repress when artificially targeted to distal sites from the activator and/or basal promoter (e.g., TATA element and transcriptional start site). One possibility is that the repression domain of Aux/IAA proteins interacts directly or indirectly with ARF activation domains and prevents the ARF activator from contacting its coactivator or general transcription factor target(s) in a promoter-specific fashion. Alternatively, Aux/IAA repressors may bring about repression by interacting with a general transcription factor or RNA polymerase II holoenzyme when targeted to promoters that contain ARF binding sites. These possibilities seem feasible based on results presented here and results presented previously (Ulmasov et al., 1997b; Tiwari et al., 2003); however, we cannot not rule out other mechanisms, like chromatin modification or remodeling. It seems unlikely that Aux/IAA repressors can bring about repression by competing with ARF activators for DNA binding sites because there is no evidence that Aux/IAA repressors are DNA binding proteins, and there is evidence that Aux/IAA proteins do not bind to ARF DNA binding sites (Ulmasov et al., 1997b). It also seems unlikely that the dominance of the IAA17 repression domain over activation domains in our experiments could be explained by some type of steric hindrance resulting from multiple artificial binding sites within proximity of one another because one might expect steric interference would occur whether domain I was a wild type or mutated form.

There are many examples of repression domains in eukaryotic transcription factors that are both dominant and portable or transferable to heterologous DBDs (John et al., 1995; Ghosh et al. 1999; reviewed in Gaston and Jayaraman, 2003). Repression domains that are dominant over activation domains have been used to create dominant negative variations of transcriptional activators (reviewed in Chandler and Werr, 2003). As one example, the engrailed (En) homeodomain protein is an active transcriptional repressor in *Drosophila melanogaster* (Tolkunova et al., 1998), and the repression domain in En has been transferred to a *D. melanogaster* transactivator, converting it to a repressor (John et al., 1995). The En repression domain has also been used to convert plant transcriptional activators into dominant repressors that can confer loss-of-function phenotypes in transgenic plants (Markel et al., 2002; Wunderlich et al., 2003). The EAR repression motif from *Nicotiana tabacum* (tobacco) ERF3 has been shown to be dominant over ERF activators and to confer repression on a VP16 activation domain in a chimeric ERF3-VP16 protein (Ohta et al., 2001). The EAR motif or a modified, stronger version of the EAR motif, consisting of only 12 amino acids, has been used to convert a number of plant transcriptional activators into dominant negative repressors, resulting in loss-of-function phenotypes (Hiratsu et al., 2003). Experiments are currently in progress to determine if domain I of IAA proteins functions as a dominant negative domain when fused to transactivators in transformed *A. thaliana* plants.

Conserved domain I in IAA proteins is enriched in Leu residues that are spaced with the general pattern LxLxL (Table 1). Interestingly, the LxLxL amino acid sequence pattern is also found in the EAR repression domain of several class II ERF and SUPERMAN repressor proteins in *A. thaliana* and/or *N. tabacum* (Ohta et al., 2001; Hiratsu et al., 2002, 2003). Results from Ala substitutions for an aspartic acid that immediately preceded or followed the first Leu in the EAR motif of *N. tabacum* ERF3 (Table 1) indicated that both acidic amino acids were important for repression but that the aspartate following the first Leu was more critical (Ohta et al., 2001). Ala substitutions for other amino acids, including the Leu in *N. tabacum* ERF3, were not reported by Ohta et al. (2001). Our results indicate that the Leu in the EAR motif are important for repression in AtERF4. Although charged amino acids are commonly found in repression domains, hydrophobic amino acids have also been found to be important for the function of some repression domains (Hanna-Rose and Hansen, 1996). For example, Leu present in an LxVxL motif within two repression domains of the human c-*myc* promoter binding protein 1 (MBP-1) have been found to be required for the MBP-1 protein to function as a repressor (Ghosh et al., 1999).

In domain I of 20 different Aux/IAA proteins that have been shown to function as repressors in protoplast transfection assays (Tiwari et al., 2001), the amino acid preceding the first Leu in the LxLxL motif is glutamate as opposed to the aspartate found at that position in the EAR motif. Ala substitution for this glutamate suggests that an acidic amino acid is not critical for repressor activity in the IAA repressor domain. The lack of an aspartate or a conserved amino acid that follows the first Leu in the LxLxL motif in domain I of IAA proteins along with our Ala substitution results suggest that the type of amino acid present at this position is also not critical, unlike the critical aspartate found in the corresponding position of the EAR motif. Our Ala substitution results do indicate, however, that the Leu in both domain I of IAA proteins and the EAR motif of AtERF4 are important for repression. More detailed studies will be required to determine what combinations of amino acids are required for repression in both domain I of IAA proteins and in the EAR motif repression domain. Whether the LxLxL motif in both types of repressors is a hallmark of a conserved repression domain in plants or is simply coincidence remains to be clarified. It will be important in future studies to identify proteins (e.g., basal

Figure 5. (continued).

(C) Transfection assays with a minimal promoter Gal4(1X):GUS reporter gene and effector genes encoding Gal4 DBD (GD), Gal4 DBD fused in frame to the ARF5 middle region and C-terminal domain (GD-5MC), GD-5MC fused in frame with domain I (amino acids 1 to 29) of IAA17 (GD-5MC-IAA17[I]), or a mutated version of IAA17 domain I (GD-5MC-IAA17[mI]).

(D) Transfection assays with a minimal promoter Gal4(1X):GUS reporter gene and effector genes encoding Gal4 DBD (GD), Gal4 DBD fused in frame to the VP16 activation domain (GD-VP16), Gal4 DBD fused in frame to the VP16 activation domain followed by domain I (amino acids 1 to 29) of IAA17 (GD-VP16-IAA17[I]), and Gal4 DBD fused in frame to VP16 activation domain followed by a mutated version of IAA17 domain I (GD-VP16-IAA17[mI]).

Q-rich MR, middle region.

transcription factors, coactivator proteins, or corepressor proteins) that interact with these repression domains to bring about repression.

There are numerous examples of eukaryotic transcription factors that contain both activation and repression domains (e.g., see Ghosh et al., 1999). Because the repression domain in IAA proteins is dominant over at least two types of activation domains (i.e., VP16 and ARF5 middle regions), it is possible that IAA proteins themselves contain activation domains that could function as such under selected conditions not present in protoplast transfection assays. That this might be the case is suggested from some enhanced auxin responses observed with IAA domain II mutants, like *axr3/iaa17* (Leyser et al., 1996). Removal of the repression domain from IAA17 (Figure 1, GD-IAA17[II/III/IV]) or mutation of the repression domain in IAA17 and other IAA proteins (Tiwari et al., 2001, 2003) failed to reveal any activation of reporter gene expression by the truncated proteins. These results suggest that Aux/IAA proteins function solely as repressors, at least in protoplast transfection assays. Our experiments do not rule out the possibility that activation domains exist in Aux/IAA proteins but only function under conditions that are restricted to specific types of cells in plant tissues or to specific developmental programs.

A part of our current model for auxin-regulated gene transcription implies that Aux/IAA repressors interact with ARF activators bound to AuxREs at low auxin concentrations, preventing transcription of early auxin response genes (see Tiwari et al., 2001, 2003). Support for such a model would require that repression conferred by Aux/IAA proteins be dominant over activation by ARF transactivators. Identification of a dominant repression domain in conserved domain I of IAA proteins adds support for this model. Demonstration that domain I of IAA17 is dominant over the activation domain in ARF5, whether it is translationally fused to the ARF transactivator or targeted to an ARF5 chimeric protein via a dimerization domain (i.e., domains III and IV in the C termini of ARF5 and IAA17), is consistent with repression of early auxin response genes by Aux/IAA proteins. Accumulation of Aux/IAA proteins to relatively high levels at low auxin concentrations would promote their dimerization with ARF transactivators bound to AuxRE target sites and prevent the activator from functioning.

METHODS

Reporter Genes

The GH3:GUS, Gal4(4X):GUS, and Gal4(4X)-D1-3(4X):GUS reporter genes have been described previously (Liu et al., 1994; Ulmasov et al., 1995, 1997a; Tiwari et al., 2001, 2003). The LexA(2X)-Gal4(2X):GUS reporter gene, which contains two LexA and two Gal4 DNA binding sites upstream of a CaMV 35S minimal promoter, was provided by William Gurley (University of Florida). The LexA and Gal4 DNA binding sites are separated by 10 bp of spacer DNA in this promoter. Reporter genes LG63 and LG370 are the same as LexA(2X)-Gal4(2X):GUS, except they contain 63 and 370 bp of spacer DNA from the GUS open reading frame cloned between the LexA and Gal4 binding sites, respectively. The Gal4(1X):GUS reporter gene was prepared by cloning a single Gal4 DNA binding site upstream of the CaMV −46 35S minimal promoter.

Effector Genes

All effector genes were placed under the control of the CaMV 35S double-enhancer promoter and translational enhancer as described (Skuzeski et al., 1990; Tiwari et al., 2001, 2003). Effector genes encoding full-length ARF5, GD-5MC, GD-IAA17wt, GD-IAA17mII, and VP16-IAA17mImII have been described previously (Ulmasov et al.,1999; Tiwari et al., 2001, 2003). Full-length IAA17 protein and IAA17 proteins containing mutations in different domains have been described by Tiwari et al. (2001). Effector genes encoding VP16-IAA17wt and VP16-IAA17mII contained an N-terminal fusion of the VP16 activation domain (amino acids 413 to 490) from *Herpes simplex virus* to the full-length wild-type IAA17 and a domain II mutant version of IAA17 (Tiwari et al., 2001, 2003). Truncated IAA17 effector genes encoding GD-IAA17[I], GD-IAA17[I/II], GD-IAA17[II/III/IV], and GD-IAA17[III/IV] consisted of domain I (amino acids 1 to 29), domains I and II (amino acids 1 to 95), domains II, III, and IV (amino acids 30 to 229), and domains III and IV (amino acids 97 to 229) of IAA17, respectively. Mutations in GD-IAA17[mI], GD-IAA17[mI/II], GD-IAA17[I/mII], and GD-IAA17[mI/mII] effector genes are the same as those described by Tiwari et al. (2001). Additional site-specific mutations in GD-IAA17[I] were made using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The IAA17 deletion fragments were fused in frame at the C terminus of the Gal4 DBD. Truncated IAA effector genes encoding GD-IAA2[I], GD-IAA8[I], GD-IAA13[I], GD-IAA18[I], and GD-IAA28[I] consisted of *A. thaliana* IAA2 domain I (amino acids 1 to 31), IAA8 domain I (amino acids 1 to 69), IAA13 domain I (amino acids 1 to 31), IAA18 domain I (amino acids 1 to 57), and IAA28 domain I (amino acids 1 to 24), respectively. These were fused in frame at the C terminus of the Gal4 DBD.

The EAR repression domain (Ohta et al., 2001) from the C terminus (amino acids 199 to 222) of *A. thaliana* ERF AtERF4 (At3g15210) was amplified by reverse transcriptase–PCR and cloned in frame at the C terminus of the Gal4 DBD. The GD and LD effector genes encoded only the Gal4 DBD and LexA DBD, respectively. The GD-VP16 effector gene encoded the Gal4 DBD fused in frame with the N terminus of the VP16 activation domain (amino acids 413 to 490). The effector genes GD-VP16-IAA17[I] and GD-VP16-IAA17[mI] were identical to GD-VP16 but contained a C-terminal in-frame fusion of IAA17[I] or IAA17[mI], respectively. The LD-VP16 effector gene encoded the LexA DBD (amino acids 1 to 202) from the *Escherichia coli* LexA repressor fused in frame with VP16 activation domain (provided by William Gurley, University of Florida). Effector genes encoding LD-IAA17[I] and LD-IAA17[mI] contained the wild-type domain I (amino acids 1 to 29) or a mutated version of domain I from IAA17 (Tiwari et al., 2001) fused in frame at the C terminus of the LexA DBD. Effector genes encoding ARF5-IAA17[I], ARF5-IAA17[mI], GD-5MC-IAA17[I], and GD-5MC-IAA17[mI] consisted of the wild-type IAA17 domain I (amino acids 1 to 29) or a mutant version (Tiwari et al., 2001) fused in frame to the C terminus of full-length ARF5 or a truncated version of ARF5 lacking its DBD (GD-5MC).

Protoplast Transfection Assays

Isolation of protoplasts from *D. carota* suspension culture cells, transfections, and GUS assays has been described previously (Liu et al., 1994; Ulmasov et al., 1995). Reporter and effector plasmid DNA used for protoplast transfections were prepared using Endofree Plasmid Maxi kit (Qiagen, Valencia, CA). Ten micrograms each of effector and reporter plasmids were used in transfection assays. Effector plasmids expressing chloramphenicol acetyltransferase or the Gal4 DBD were used to equalize the amount of DNA in transfection assays in which no effector gene or only a single effector gene were tested. Each transfection assay was performed in triplicate, and at least two independent transfection assays were performed for each experiment.

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