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Adaptor proteins GIR1 and GIR2. II. Interaction with the co-repressor TOPLESS and promotion of histone deacetylation of target chromatin

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Abstract

Understanding how root hair development is controlled is important for understanding of many fundamental aspects of plant biology. Previously, we identified two plant-specific adaptor proteins GIR1 and GIR2 that interact with the major regulator of root hair development GL2 and suppress formation of root hair. Here, we show that GIR1 and GIR2 also interact with the co-repressor TOPLESS (TPL). This interaction required the GIR1 protein EAR motif, and was essential for the transcriptional repressor activity of GIR1. Both GIR1 and GIR2 promoted histone hypoacetylation of their target chromatin. Potentially, GIR1 and GIR2 might may link TPL to and participate in epigenetic regulation of root hair development.

Keywords

Root hair development; Epigenetic regulation; GL2-Interacting proteins; TPL; Arabidopsis

1. Introduction

Root hairs are long tubular-shaped fine structures that differentiate from root epidermal cells. They enhance plant's capacity to acquire nutrients, such as phosphorus, and water [1,2], help secure anchorage, and facilitate interactions with soil microbiome [3,4]. Understanding genetic and epigenetic control of root hair development is therefore important for understanding of many fundamental aspects of plant biology. In Arabidopsis, one of the major regulators of root hair formation is the transcriptional repressor GL2, which acts to suppress root hair differentiation [5,6]. In an accompanying paper [7], we reported identification of two novel plant-specific proteins GIR1 and GIR2 that interact with GL2 and repress root hair formation.

Negative regulation of root hair by GL2 involves numerous downstream genes [8,9], but only a few of them have been identified as direct targets of GL2 in Arabidopsis, among them

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PLDζ1 [10-13]. This suppression may involve histone modification as histone deacetylase activity [14-16], yet it is still unknown whether the direct chromatin targets of GL2 exhibit histone hypoacetylation. Here, we report that both GIR1 and GIR2 interact with the Groucho/TUP1-like transcriptional co-repressor TOPLESS (TPL), and repress the GL2 target gene, *PLDζ1*, inducing histone hypoacetylation in the promoter region of the target chromatin. Collectively, our observations suggest that GIR1 and GIR2 function as adaptor proteins that may link GL2 and TPL and participate in epigenetic control of root hair development.

2. Materials and methods

2.1. Plant material

The wild-type *Arabidopsis thaliana* (ecotype Col-0) plants, and the mutants *gl2-1*, *gir1-1* (SALK_089888), and *gir2-1* (SALK_048394) [7] were received from the *Arabidopsis* Biological Resource Center (abrc.osu.edu). The double mutant *gir1-1 gir2-1* line was described [7]. Gain-of-function transgenic GIR1-OE1 plants and genetically complemented *gir1-1 gir2-1 GIR1* plants were described [7]. Arabidopsis plants were grown as described [7]. For BiFC and GIR1 transcriptional activity assays, we used *Nicotiana benthamiana* plants grown as described [7].

2.2. RT-qPCR and gene expression assays

cDNA samples were prepared as described [7]. qPCR reactions performed as described [7,17] using primer pairs specific for *ACT8* (5′TCCAGGCATTGTCCACAGAA3’/5′ACCTGCTCCTCCTTAGACAT3′), *PLDζ1*(5′ATAGTTGATGACCGTGCTGC3’/5′TGTTCGGACCACAAAGAGAG3′), or *MYB23* (5′CAACGTCCTCCTCTCATCAA3’/5′AACTAAGCTCGAAGTCGTCT3′). All gene expression values were standardized to the expression levels of the constitutively-expressed gene *ACT8*. For each tested gene, the relative expression levels were calculated using the cycle threshold (CT) $2^{-\Delta\Delta C_T}$ method [18].

2.3. Yeast two-hybrid assay

Yeast two-hybrid constructs expressing *GIR1* or *GIR2* from the pGAD424 vector (Clontech) were described [7]. The *TPL* cDNA was subcloned into the SalI-PstI sites of pGAD424. Protein interactions were determined by the histidine prototrophy as well as by β-galactosidase assay on a nitrocellulose filter as described [19,20].

2.4. BiFC

BiFC constructs expressing *GIR1* or *GIR2* from the pSAT1-nEYFP-C1 vector [21] were described [7]. The *TPL* cDNA was cloned into the BglII-SalI sites of pSAT6-cEYFP-C1 [21]. The resulting expression cassettes were excised from pSAT1- or pSAT6-based vectors and inserted into the AscI or PI-PspI sites, respectively, of pPZP-RCS2. pRCS2-nEYFP and pRCS2-cEYFP plasmids [7] were used for negative controls. The indicated combinations of the constructs were transiently co-expressed in *N. benthamiana* leaves by agroinfiltration, followed by analysis using a Zeiss LSM 5 Pa confocal microscope. All experiments were

performed in at least three independent biological replicates, i.e., independently grown plants.

2.5. Transcriptional repression assay

Coding sequence of mGAL4-VP16 was amplified from pRTL2-mGAL4-VP16 [22] using the primer pair 5′GGATGAATTCATGAAGCTCCTGTCCTCCATCG3’/5′ACTGGTCGACGCCCCACCGTACTCGTCAATTCC3′, and cloned into the EcoRI-SalI sites of pSAT1A-MCS [23], producing pSAT1A-GV. Coding sequences of GIR1, GIR1-delEAR and GIR1-mutEAR were then cloned into the SalI-BamHI sites of pSAT1A-GV. The expression cassettes were excised from these constructs with AscI and inserted into pPZP-RCS2, producing the effector constructs. The expression cassette of pSAT6-3UAS-GUS, which contains three copies of the Gal4 UAS and the minimal CaMV 35S promoter, was excised with PI-PspI and inserted into pPZP-RCS2 to generate the p35Smin-$(UAS)_3$-GUS reporter construct (see Fig. 4D). The tested constructs together with p35Smin-$(UAS)_3$-GUS were agroinfiltrated into the leaves of *N. benthamiana*. GUS activity was assayed histochemically as described [22,24], followed by extraction of chlorophyll in 75% ethanol for 12–18 h [22,24], and the leaves were observed under a Leica MZ FLIII stereoscope. In each plant, multiple leaves were agroinfiltrated, and each experiment was performed in at least three independent biological replicates.

2.6. Agroinfiltration

Agrobacterium EHA105 strain carrying the tested expression constructs was used for agroinfiltration into the leaves of 3-4-week-old intact *N. benthamiana* plants as described [7]. Plants were grown for 24 h in the dark and 72 h in the light and then sampled for analyses.

2.7. qChIP

qChIP was performed as described [25,26]. Briefly, 14-day-old seedlings were cross-linked with formaldehyde, chromatin was isolated, sheared by sonication, and immunoprecipitated with 5–6 μg of anti-acetyl-histone H3 antibody (Millipore, 06–599). The cross-linking was heat-reversed at 65 °C, DNA purified on spin columns (Zymogen), and quantitative PCR was performed with the following primer pairs specific for regions #1–5 within the 5′ UTR of *PLDζ1*: region #1, 5′AGTTTACCTGTAGAAGAAGC3’/5′ACATGGTTGAACAAGTAACT3’; region #2, 5′GAGTAGTATCACACTTCCCA3’/5′CCCACGTATTCATAGAATCA3’; region #3, 5′GAATACGTGGGGTAAGATTG3’/5′CGGATCCGGCTATATATTAT3’; region #4.5′ATAATATATAGCCGGATCCG3’/5′ACAAAGGAAGACAAATCTGA3’; and region #5, 5′GGGAGTATAAGCATAACGAA3’/5′GATGCCATTTCTCTGATCTC3’. Relative abundance of the *PLDζ1*-specific PCR products was normalized to the amount of the *Act8*-specific product.

3. Results

3.1. GIR1 interacts with TPL via its EAR domain and promotes transcriptional repression

Our amino acid analysis identified a putative EAR motif in the N-terminal region of GIR1(Fig. 1A). One intriguing feature of this motif is that it is found in proteins that interact with and recruit the TPL co-repressor to regulate diverse developmental processes, hormonal responses, and defense [27]. We, therefore, examined whether GIR1 interacts with TPL. First, we employed the yeast two-hybrid assay, which, indeed, detected the interaction both by histidine prototrophy and β-galactosidase assay. In negative control experiments, TPL did not interact with an unrelated protein, p53 (Fig. 1B). The GIR1-TPL interaction depended on the presence of the EAR motif of GIR1 as a GIR1 mutant lacking this motif, GIR1-delEAR, showed no interaction with TPL. Furthermore, the GIR1-mutEAR mutant, in which three conserved leucine residues of the EAR sequence (Fig. 1A) were substituted with serines, also abolished the interaction (Fig. 1B), indicating the specificity of this interaction and its dependence on the EAR motif of GIR1. The interaction was confirmed by the β-galactosidase assay. As expected, in the presence of histidine, all combinations of the tested proteins resulted in the efficient cell growth (Fig. 1B).

Next, we demonstrated the interaction between GIR1 and TPL directly *in planta*, using BiFC. GIR1 and TPL were tagged with N-terminal and C-terminal fragments of YFP (nYFP and cYFP), respectively, and transiently co-expressed in *N. benthamiana* leaves. Fig. 1C shows that nYFP-GIR1 and cYFP-TPL interacted with each other, producing the BiFC signal. The interacting proteins were located in the cell nucleus, as expected from transcriptional co-repressor complexes. No BiFC signal was observed following co-expression of cYFP-TPL and free nYFP as negative control. Consistent with the functional redundancy between GIR1 and GIR2, GIR2 also interacted with TPL both in the yeast two-hybrid (Fig. 2A) and BiFC assays (Fig. 2B).

If GIR1 can bind and recruit co-repressor, it should have an inhibitory effect on gene expression. To test this idea, we utilized a variation of our *in planta* an assay for inhibition of transcriptional activation [22]. We designed a construct, p35Smin-$(UAS)_3$-GUS, that expresses the GUS reporter protein from a synthetic promoter containing three copies of the GAL4 binding site (UAS) downstream of the minimal CaMV 35S promoter (Fig. 3A). Expression of GUS is induced by co-transformation with a chimeric transcriptional activator comprising GAL4 DBD fused to the VP16 transcriptional activator from *Herpes simplex virus* (DBD-VP16). Indeed, when DBD-VP16 was co-transformed into *N. benthamiana* leaves together with the reporter construct, GUS activity was easily detected as indigoblue histochemical staining of the transformed tissues. This transcriptional activation of the reporter construct and expression of the GUS activity was completely blocked when DBD-VP16 was fused translationally to GIR1. Importantly, DBD-VP16 fused to GIR1 mutants, GIR1-delEAR and GIR1-mutEAR, still activated the GUS reporter expression (Fig. 3B). Collectively, these data suggest that GIR1 indeed can promote gene repression, and that this repressor ability depends on the EAR motif of GIR1. Because the EAR motif is also necessary for interaction between GIR1 and TPL, a known transcriptional co-repressor, GIR1 and TPL may function in a co-repressor complex.

3.2. GIR1 and GIR2 promote repression of their target gene and histone hypoacetylation of its chromatin

Do GIR1 or GIR2 repress their target genes by chromatin modification? To address this question, it was first necessary to identify an example of a GIR1/2 target gene. Presumably, such a target gene would be shared between GIR1, GIR2, GL2 and TPL if these proteins function in complex with each other. TPL affects a large number of genes [27], but GL2 has just nine known targets, one of which, *PLDζ1*, encodes an Arabidopsis phospholipase D (PLD) and represents the first-identified direct target of GL2 [10,12,13,28]. Thus, we used quantitative RT-PCR (RT-qPCR) to compare expression profiles of *PLDζ1* between the wild-type plants, the *gir1-1 gir2-1* and *gl2-1* mutants, and the *GIR1*-OE1 plants. Fig. 4A shows that the expression levels of *PLDζ1* were upregulated in the roots of both *gl2-1* and *gir1-1 gir2-1* plants whereas the *PLDζ1* levels in the roots of the *GIR1*-OE1 plants were further suppressed relative to the wild-type plants. Upregulation of the *PLDζ1* expression in the *gir1-1 gir2-1* mutant was reversed to the wild-type level by genetic complementation with the wild-type *GIR1* in the *gir1-1 gir2-1/GIR1* plants (Fig. 4A). Thus, *PLDζ1* likely represents one of the target genes shared between GL2, GIR1 and GIR2. In contrast, we detected no changes in expression of *MYB23*—the target gene of GL2 involved in formation of trichomes, but not root hair [12]—in the loss-of-function *gir1-1 gir2-1* mutant and in the genetically-complemented *gir1-1 gir2-1/GIR1* line as compared to the wild-type plants while the *gl2-1* mutant exhibited downregulated *MYB23* expression (Fig. 4B).

We then used quantitative chromatin immunoprecipitation (qChIP) to examine whether GIR1 and GIR2 repress *PLDζ1* via histone hypoacetylation. Because the regulatory elements of *PLDζ1* associated with histone modifications have not been defined, we examined five regions that span the sequence of the *PLDζ1* promoter (Fig. 4C). Using anti-acetyl-histone H3 (H3Ac) antibody, we showed that, in the *PLDζ1* chromatin, histone acetylation occurred in regions 3 and 5 (Fig. 4D). Specifically, in region 3, H3 was hyperacetylated by ca. 6-5 fold in the *gl2-1* and *gir1-1 gir2-1* mutants, respectively, as compared to the wild-type plants whereas region 5 showed a 3–5-fold increase in H3ac (Fig. 4D). The *GIR1*-OE1 plants, on the other hand, exhibited H3 hypoacetylation by 2–3 fold in the chromatin of regions 3 and 5 as compared to the wild-type plants (Fig. 4D). Furthermore, genetic complementation of the *gir1-1 gir2-1* to the wild-type root hair phenotype by *GIR1* (see Fig. 4A and B) also restored the wild-type levels of H3ac in regions 3 and 5 of the *PLDζ1* chromatin. Interestingly, region 3 of the *PLDζ1* promoter includes the L1-box, which has been shown to bind GL2 and is also recognized by ATML1 and other members of the class IV HD-Zip protein family [10,12,13], and region 5 includes the 5′ untranslated region (UTR) of *PLDζ1* (Fig. 4C). Other *PLDζ1* promoter regions that we tested, i.e., regions 1, 2, and 4 (Fig. 4C), showed no changes in H3 acetylation levels between all tested plant lines (Fig. 4D). Collectively, these data suggest that *GIR1, GIR2* and *GL2* likely repress *PLDζ1*, and potentially their other target genes, by histone deacetylation.

4. Discussion

In the accompanying paper [7], we reported identification of GIR1 and GIR2 that belong to a new plant-specific two-member family of GL2-interacting proteins that likely function

as adaptors that connect transcriptional repressors and co-repressors and suppress root hair development. GIR1 contains a conserved EAR motif found in proteins that interact with TPL, a key co-repressor involved in many aspects of the plant life cycle [27]. Indeed, GIR1 also interacts with TPL, and this interaction is dependent on the presence of the EAR motif in GIR1. Furthermore, the ability of GIR1 to promote gene repression also requires the EAR motif, suggesting that this repression involves the GIR1-TPL interaction/recruitment. We were unable to obtain meaningful and statistically significant data for root hair liner density in the temperature-sensitive *tpl-1* mutant [29,30]. For the *gir1-1 gir2-1* and *gl2-1* mutants, the elevated root hair frequency phenotypes phenocopy each other [7], and both GIR1 and GL2 share at least one common target gene, *PLDζ1*, known to be directly involved in root hair formation [10]. Taken together with the GIR1/2-GL2 interactions [7] and the GIR1/2-TPL interactions, this suggests that GIR1 may function in complex with GL2 and TPL. In this complex, GIR1/2 and GL2 presumably repress their target genes by epigenetic modifications as, in both *gir1-1 gir2-1* and *gl2-1* mutants, the promoter chromatin of *PLDζ1* exhibits hyperacetylation of the H3 histone. This is consistent with the increasing evidence of involvement of histone deacetylases (HDACs) in root hair development. For example, HDAC inhibitor trichostatin A alters epidermal cell patterning, including root hair, in the Arabidopsis root [14], and specific Arabidopsis HDACs HDA18 and HDA6 have been reported to affect root hair formation [14-16]. Furthermore, HDA6 and HDA19 have been shown to associate indirectly with TPL [29,31-33], suggesting that TPL may recruit HDACs for target gene repression. Thus, it is tempting to speculate that GIR1/2, GL2 and TPL act in a repressor complex, in which the GL2 repressor is linked to the TPL co-repressor through a novel EAR motif-containing adapter proteins, GIR1 and/or GIR2. This scenario is consistent with the emerging common mechanism of recruiting different transcription factors to TPL via specific adaptor proteins; for example, during leaf growth, PEAPOD transcription factors that negatively regulate meristemoid division are linked to TPL by KIX8 and KIX9 adaptors [34], and, during jasmonate signaling, JAZ repressors recruit TPL via the NINJA adaptor protein [35].

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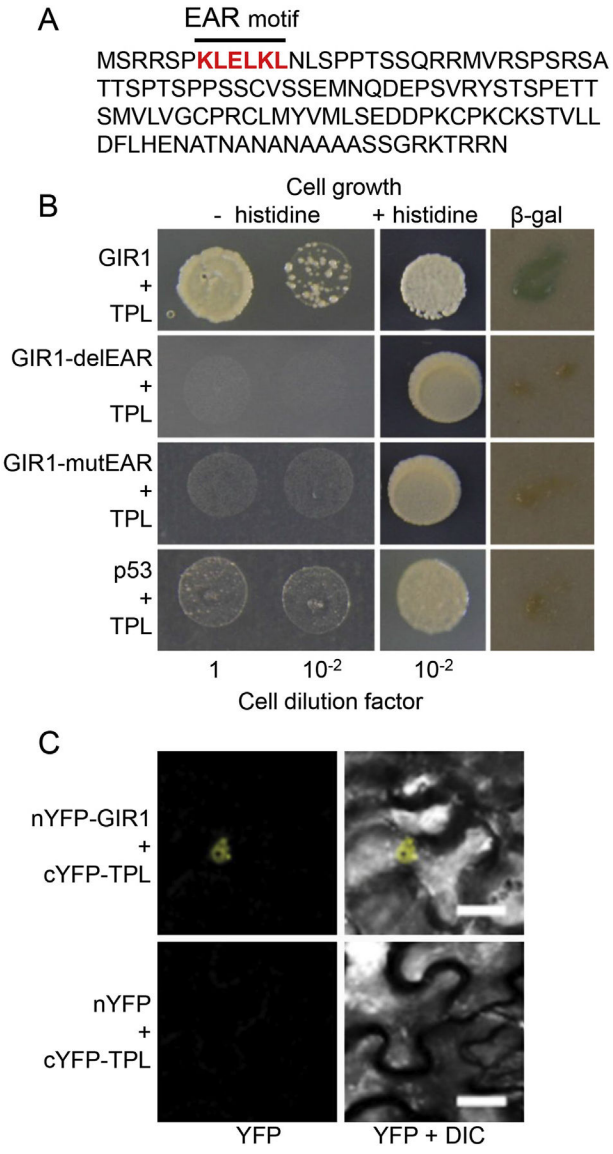

Fig. 1.

GIR1 interacts with TPL. (A) The amino acid sequence of GIR1. The conserved EAR motif is indicated and highlighted in red. (B) GIR1-TPL interaction in the yeast two-hybrid system and its dependence on the GIR1 EAR motif. The indicated dilutions of yeast cells were grown in the absence or presence of histidine as shown. Protein interaction was detected as cell growth on histidine-deficient medium and as β-galactosidase (β-gal) activity on a nitrocellulose filter. (C) GIR1-TPL interaction *in planta*, and accumulation of the interacting proteins in the cell nucleus. Protein interaction was detected by BiFC. YFP signal is in yellow. Fluorescence images are single confocal sections. Scale bars = 20 μm.

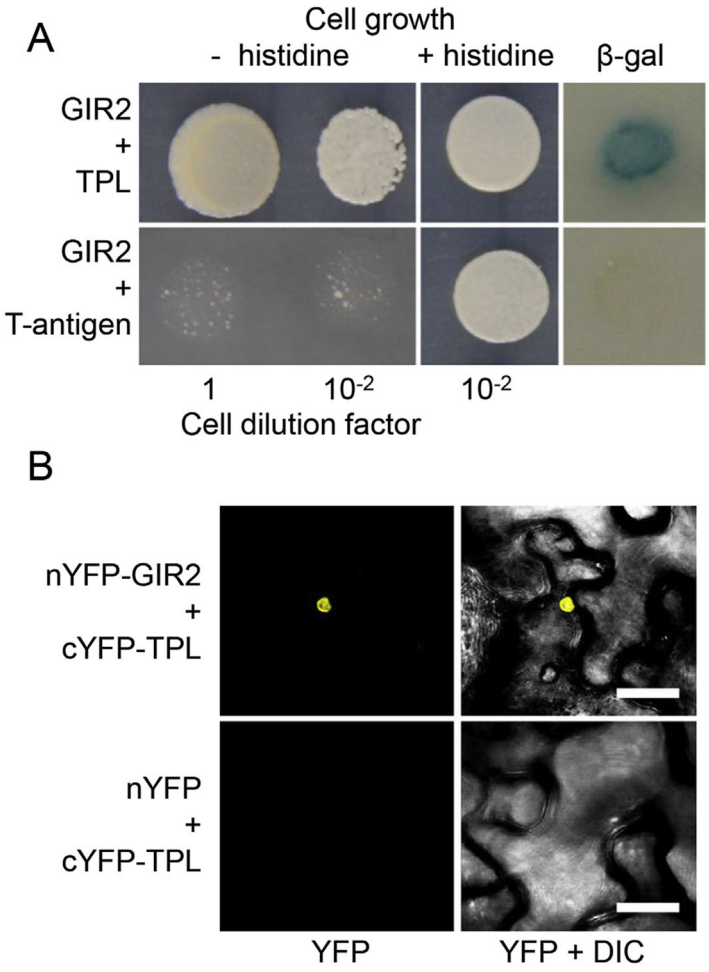

Fig. 2.

GIR2 interacts with TPL. (A) GIR2-TPL interaction in the yeast two-hybrid system. The indicated dilutions of yeast cells were grown in the absence or presence of histidine as shown. Protein interaction was detected as cell growth on histidine-deficient medium and as β-galactosidase (β-gal) activity on a nitrocellulose filter. (B) GIR2-TPL interaction *in planta*, and accumulation of the interacting proteins in the cell nucleus. Protein interaction was detected by BiFC. YFP signal is in yellow. Fluorescence images are single confocal sections. Scale bars = 20 μm.

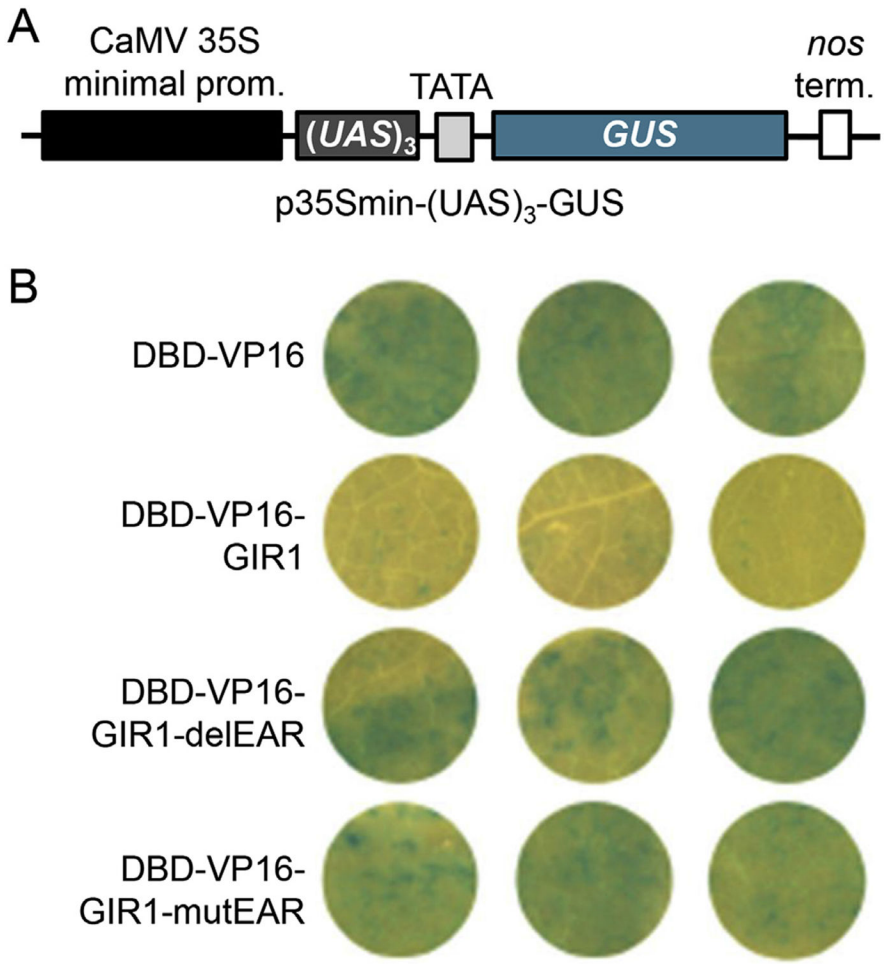

Fig. 3.

GIR1 promotes gene repression. (A) Schematic structure of the transcriptional repression reporter construct. The transcriptional activity of GIR1 was tested using the p35Smin-$(UAS)_3$-GUS reporter construct composed of the minimal CaMV 35S promoter (CaMV 35S minimal prom.), three copies of the GAL4 binding site [$(UAS)_3$], TATA box, β-glucuronidase coding sequence (*GUS*), and terminator of the nopaline synthase gene (*nos* term.). (B) GIR1-mediated transcriptional repression of *GUS*. In the presence of the DBD-VP16 transcriptional activator, p35Smin-$(UAS)_3$-GUS expressed the GUS enzymatic activity, which was detected as blue histochemical staining of leaf disks from the indicated plant lines transiently transformed with the reporter construct. Inhibition of *GUS* expression by tested GIR1 sequences fused to DBD-VP16 indicated transcriptional repression activity of these sequences.

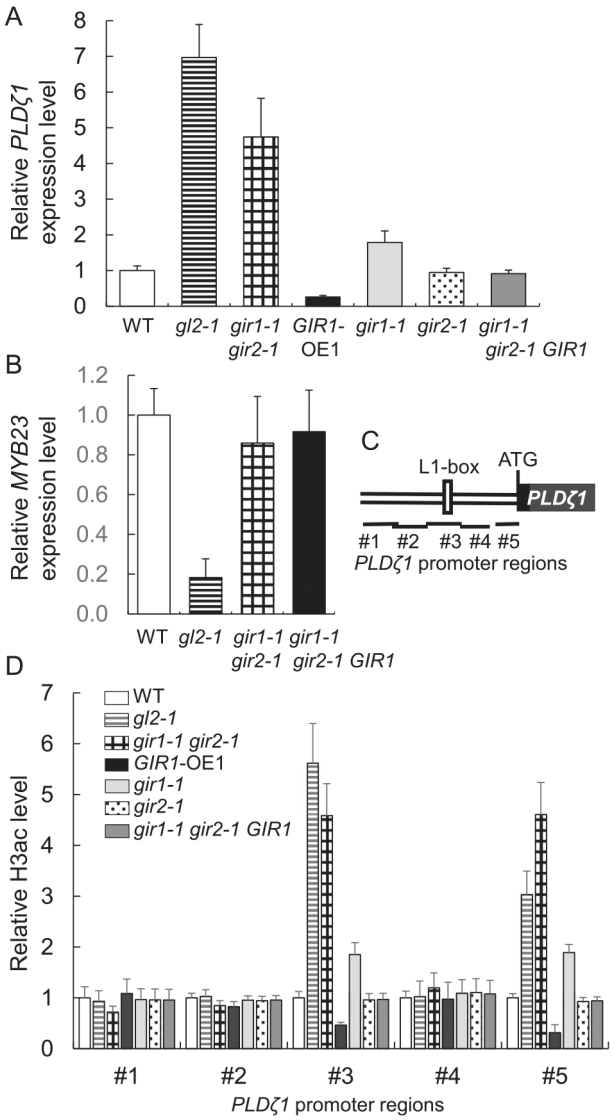

Fig. 4.

GIR1, GIR2, and GL2 repress *PLDζ1* expression via histone hypoacetylation. (A) RT-qPCR analysis of *PLDζ1* gene expression in the indicated plant lines. (B) RT-qPCR analysis of *MYB23* gene expression in the indicated plant lines. The expression level in the wild-type (WT) plants is set to 1.0, and error bars represent SEM of N = 3 independent biological replicates. (C) Schematic representation of locations of regions #1–5 within the *PLDζ1* promoter sequence used for qChIP analyses. Region #1, −1045 to −813; region #2, −859 to −594; region #3, −604 to −385; region #4, −404 to −208; region #5, −130 to +8, relative to the translation initiation codon (ATG). The conserved L1-box within region #3 and the ATG codon of *PLDζ1* are indicated. (D) qChIP analysis of relative acetylation levels of histone H3 (H3ac) in the promoter regions #1–5 of *PLDζ1* in the indicated plant lines. Error bars represent SEM of N = 3 independent biological replicates.