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ARTICLE

Controlling plant architecture by manipulation of gibberellic acid signalling in petunia

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Since stem elongation is a gibberellic acid (GA) response, GA inhibitors are commonly used to control plant height in the production of potted ornamentals and bedding plants. In this study, we investigated interfering with GA signaling by using molecular techniques as an alternative approach. We isolated three putative *GID1* genes (*PhGID1A*, *PhGID1B* and *PhGID1C*) encoding GA receptors from petunia. Virus-induced gene silencing (VIGS) of these genes results in stunted growth, dark-green leaves and late-flowering. We also isolated the *gai* mutant gene (*gai-1*) from *Arabidopsis*. We have generated transgenic petunia plants in which the *gai* mutant protein is over-expressed under the control of a dexamethasone-inducible promoter. This system permits induction of the dominant *Arabidopsis gai* mutant gene at a desired stage of plant development in petunia plants by the application of dexamethasone (Dex). The induction of *gai* in Dex-treated T1 petunia seedlings caused dramatic growth retardation with short internodes.

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INTRODUCTION

Gibberellic acid (GA), a plant hormone, regulates many crucial growth and developmental processes, including seed germination, leaf expansion, induction of flowering and stem elongation.[1] A common problem in the production of ornamental potted plants is undesirably tall growth, so inhibitors of GA biosynthesis including A-rest (ancymidol), B-nine (daminozide), Bonzi (paclobutrazol), Cycocel (chloromequat chloride) and Sumagic (uniconazole), are commonly used to control plant height.[2,3] To provide an alternative strategy for managing plant architecture and preventing postharvest 'stretching', we propose to investigate genetic manipulation of the GA response pathway.

In the current model of GA signaling, GA binds to a soluble GID1 receptor, which in turn binds to the DELLA repressor protein. The bound DELLA protein is then targeted for degradation by the 26S proteasome, thus relieving DELLA-mediated repression of GA-dependent growth processes.[4,5] The genes encoding the GA response cascade have been identified using dwarf mutants of *Arabidopsis*, wheat and rice.[6,7] A soluble GA receptor was identified as the basis of the rice *GA-insensitive dwarf1* (*GID1*) mutant.[8] In *Arabidopsis*, there are three *GID1* orthologs (*AtGID1a*, *AtGID1b* and *AtGID1c*); the *gid1a/gid1b/gid1c* triple mutant was severely dwarfed [9] and showed high levels of RGA (REPRESSOR OF GA1-3) and GAI (GA-INSENSITIVE) proteins.[10] These proteins, characterized by the conserved DELLA domain at their N termini, function as repressors in GA signalling.[11,12] Loss-of-function mutants such as rice *slr1*,[13] and *Arabidopsis gai-t6* and *rga-24*,[14] are DELLA deficient, and are taller and earlier flowering than wild-type plants. Conversely, DELLA gain-of-function mutants or transgenic plants are dwarfed, and flower late. Such a mutant, the DELLA protein mutant *gai-1* from *Arabidopsis* has a 17-amino acid deletion in the conserved DELLA domain.[11]

Previous researchers showed that heterologous expression of the *Arabidopsis gai* mutant gene reduced plant height and altered GA response in transgenic rice,[15] tobacco,[16] chrysanthemum[17] and apple.[18] However, the native or constitutive promoters used in these studies resulted in permanent inhibition of GA responses, which resulted in severe dwarfing and other undesirable phenotypes. To use this approach in practice would require that expression of the mutant gene be coupled to an inducible system,[19] such as the dexamethazone-inducible promoter[20] or the alcohol-inducible promoter,[21] which permits the expression of transgenes to be turned on or off at desired stages of development of an organism or tissue.

This study tested the hypothesis that interfering with GA signalling by silencing *GID1*-like receptor genes using the virus-induced gene silencing (VIGS), with GA signal transduction by over-expression of the *Arabidopsis gai* mutant gene under the control of the dexamethasone (Dex)-inducible promoter, would modulate plant growth and architecture in petunia.

MATERIALS AND METHODS

Plant material and growth conditions

Petunia (*Petunia x hybrida* cv. Primetime Blue) seeds were obtained from Goldsmith Seeds (Gilroy, CA, USA). Plants were grown from seed in growth chambers under a 16-h photoperiod (*ca* 350 μmol m^{-2} s^{-1} PPFD) with a day/night temperature regime of 22℃/18℃. VIGS experiments used the purple-flowered 'Primetime Blue' cultivar, but studies on stable transformants used white-flowered cultivar 'Mitchell Diploid'.

Isolation of *GID1*-like genes from petunia

Total RNA was extracted from petunia tissues using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and was treated with RNase-free DNase (Promega, Madison, WI, USA) to remove any contaminating genomic DNA. First strand cDNA was synthesized using 2 μg total RNA, oligo d(T) primer, random hexamer, and then replicated by superscript reverse transcriptase (Invitrogen), as previously described.[3,22] *GID1*-like gene fragments were amplified from the cDNA by RT-PCR using degenerate primers designed from the three *GID1* receptor gene sequences of *Arabidopsis* or partial EST sequences of petunia. The full-length sequences of *GID1*-like genes were isolated by standard techniques using 3′ or 5′ rapid amplification of cDNA end (RACE) with the Clontech kit (Clontech, Mountain View, CA, USA), and following the manufacturer's instructions. The sequences were analysed by the sequencing service of the College of Biological Science at UC Davis, and are deposited in Genbank as accession numbers JX501238 (*PhGID1A*),

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JX501239 (*PhGID1B*), JX501240 (*PhGID1C*). BLAST and CLUSTALW analysis tools were used to compare the nucleotide and deduced amino acid sequences of *PhGID1*-like genes with those of *GID1* genes from other organisms.

Expression analysis of PhGID1-like genes from petunia

Total RNA was extracted from different plant tissues including young leaves, mature leaves, stem, root, pollen, petal and stigma using TRIzol Reagent (Invitrogen). The isolated RNA was treated with RNase-free DNase (Promega) to remove any contaminating genomic DNA. First-strand cDNA was then synthesized from 2 μg total RNA, oligo d(T) primer and random hexamers using Superscript III reverse transcription kit (Invitrogen) according to the manufacturer's protocol. This cDNA was used as template for semi-quantitative PCR using primers (Supplementary Table S1) for *PhGID1A* (1526 bp, 5′-TCT ATG GCA AGA AAT AAT GAA GCT G-3′ and 5′-GAA GCA AAC ATA GTT CTA TAT AA-3′), *PhGID1B* (1432 bp, 5′-ACC AGT CAA ACT TGG TCA AAC TC-3′ and 5′-CAA GTG CCA ATT CCA CAA ATT AC-3′) and *PhGID1C* (1079 bp, 5′-TTG TGT AAT AGT CAT GGC TGG TG-3′ and 5′-GCT GCT TGT ATA TGA TGT TAA AG-3′). The abundance of 26S ribosomal RNA was used as an internal control and the amplification primers were 5′-AGC TCG TTT GAT TCT GAT TTC CAG-3′ and 5′-GAT AGG AAG AGC CGA CAT CGA AGG-3′ (185 bp).

VIGS

The TRV1 and TRV2 VIGS vectors were kindly provided by Dinesh-Kumar, Yale University, and have been described in detail previously.[3,22,23]

To silence all three *GID1-like* genes in petunia, a 199 bp fragment of the *PhGID1* gene was amplified from total petunia leaf cDNA using the primers listed in Supplementary Table S1. The resulting product was cloned into the pGEM-T Easy vector (Promega) for amplification, sequencing and subcloning. The fragment was excised from this plasmid by *Sac* I and *Xho* I digestion, then sub-cloned in the antisense orientation into a modified TRV2 vector with the *CHS* fragment (TRV2/*CHS*) constructed by Chen *et al*.[23] as a visual reporter to generate TRV2/*CHS/GID1* in a tandem manner. The constructs, TRV1, TRV2, TRV2/*CHS* and TRV2/*CHS/GID1* were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Agroinfection of petunia plants was then performed as described by Chen *et al*.[23] Cultures of *Agrobacterium* transformed with pTRV1 or the relevant pTRV2 construct were grown separately to an optical density of 2.0 at 600 nm, then mixed. Primary leaves of petunia seedlings (infected when the plants had two true leaves) were infected with the mixed culture using a 1 mL disposable syringe without a needle. Infected plants were grown under controlled conditions (22℃ day/18℃ night with a 16 h photoperiod). For the evaluation of plant growth, the phenotypic appearance was observed every day and the length of three branches from each infected plant was recorded.

To evaluate silencing efficacy, RT-PCR analysis was performed to measure the relative transcript levels of all three *GID1*-like genes. Total RNA was extracted from petunia leaves. Total cDNA was synthesized as previously described.[24] The cDNA was then used as a template for semiquantitative PCR using primers for a different region of each target gene than the region used for gene silencing.

The primers for amplifying *PhGID1* transcripts were designed outside the region targeted for gene silencing to avoid amplification of RNA from the TRV2/*CHS/GID1*construct. The VIGS primers (Supplementary Table S1) used for *PhGID1A* were 5′-CTT AGC TGG AGA TAG CTC TGG TG-3′ and 5′-GAA GCA AAC ATA GTT CTA TAT AA-3′ (957 bp); for *PhGID1B* were 5′-ATA GAG TCT ATC GAC ATG CTC CC-3′ and 5′-ACC AAA CAT TGG ATG AAG ATG G-3′ (434 bp); for *PhGID1C* were 5′-TTG TGT AAT AGT CAT GGC TGG TG-3′ and 5′-GCT GCT TGT ATA TGA TGT TAA AG-3′ (1079 bp).

Generation of plants overexpressing *gai*

The *gai* mutant gene (*gai-1*) was isolated from *Arabidopsis*. We confirmed a 17 amino acid deletion in the conserved DELLA domain of the putative GAI protein, and used the full-length sequence together with the GVG inducible system,[20] to generate a GVG::*gai* construct. This construct was then used to transform into 'Mitchell diploid' petunia plants by UC Davis plant transformation facility. Seeds were collected from self-pollinated transgenic plants and T_1 seedlings containing the transgene were selected on MS medium containing 20 mg L^{-1} hygromycin. Three T1 transgenic lines were selected and sprayed with 30 μM dexamethazone (the GVG inducer) for 10 days and the resulting plant architecture was compared with sprayed wild-type and H_2O-sprayed transgenic plants; three mature leaves of each seedling were selected for the measurement of leaf size. Total RNA was extracted from leaf tissues and semiquantitative RT-PCR was used to compare the abundance of each *gai* transcript using primers (Supplementary Table S1) forward 5′-GTG TAA GCT GGC TCA TTT AGC TG-3′ and reverse 5′-GTT GAA CAG AGC CAA AAG CAT AC-3′ (535 bp).

RESULTS

Identification of GID1-like genes in petunia

The sequences of the three *GID1* receptor genes from *Arabidopsis* and the *GID1*-like sequences from other species were used to identify consensus region and to design degenerate primers based on the conserved regions of the *GID1* gene. Multiple fragments were amplified by PCR from petunia, and their identity was confirmed by sequencing. The 3′ and 5′ RACE PCR was used to extend both 5′ and 3′ ends of the genes to obtain full-length sequences of all petunia *GID1*-like genes.

Three putative *GID1* genes were identified in petunia and named *PhGID1A*, *PhGID1B* and *PhGID1C* (Supplementary Figure S1). The deduced amino acid sequences were aligned with three *Arabidopsis* GID1 genes; one of the GID1-like sequences shares 72% identity with both *At*GID1A (accession number NP_187163) and *At*GID1C (accession number NP_198084) and the other two petunia GID1-like proteins share 74% and 77% identities with *At*GID1B (accession number NP_191860) (Supplementary Figure S2). The nucleotide sequences similarities are 81% between *PhGID1B* and *PhGID1C*; 64% between *PhGID1A* and *PhGID1C*; and 48% between *PhGID1A* and *PhGID1B*.

To determine the tissue distribution of the three *PhGID1* transcripts, semiquantitative RT-PCR was performed using gene-specific primers. With the exception of pollen, the three *PhGID1* transcripts were detected at moderate abundance in all tested petunia tissues, including stems, leaves, roots and flowers. Transcripts of *PhGID1A* and *PhGID1B* were barely detected in pollen (Figure 1).

VIGS of *GID1*-like genes in petunia

To examine the function of *PhGID1* in petunia, we used VIGS to silence expression of these genes. We cloned different *PhGID1*

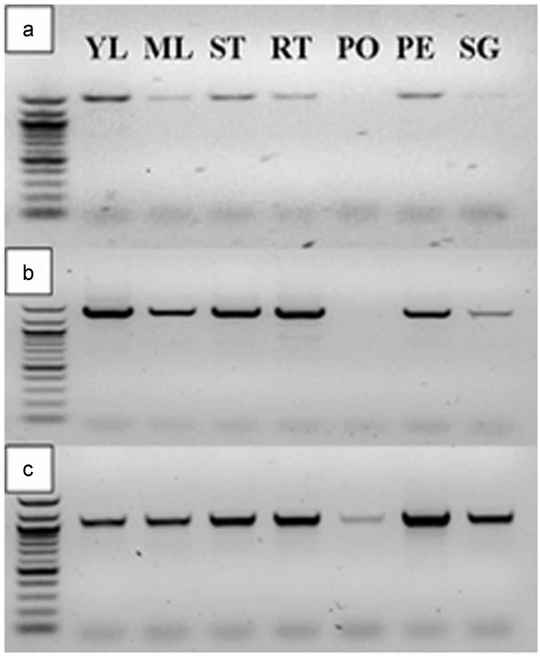

Figure 1. Abundance of transcripts of three putative *PhGID1* genes in different petunia tissues. (**a**) *PhGID1A*; (**b**) *PhGID1B*; (**b**) *PhGID1C*. Total RNA was extracted from tissues of young leaves (YL), mature leaves (ML), stem (ST), root (RT), pollen (PO), petal (PE) and stigma (SG) and cDNA was synthesized for evaluation of transcript abundance. 26S rRNA abundance determined by real-time PCR was used to normalize loading for each cDNA sample.

fragments into the TRV *CHS* vector (silencing of *CHS* was used as a visual reporter for cosilencing the *GID1* target), and infected young petunia seedlings with *Agrobacterium* transformed with silencing constructs containing single or multiple *GID1*-like genes.

Silencing of *PhGID1* had a strong impact on plant architecture, resulting in plants with short branches and internodes, small, dark leaves and late flowering (Figures 2 and 3). A range of dwarf phenotypes was observed from single or double silencing of *GID1*-like genes (Figure 2) and the frequency of silenced phenotype was relatively low (data not shown). We therefore tested the effects of simultaneously silencing all three *PhGID1* genes. A severely dwarfed phenotype was observed (Figure 3b–3d) in these plants and the silenced branches were significantly shorter than the controls (Table 1).

A semiquantitative RT-PCR analysis confirmed that the abundance of *PhGID1A, PhGID1B* and *PhGID1C* transcripts in infected plants were all strongly reduced (Figure 4). The severely dwarfed phenotypes observed in plants where all three *PhGID1* genes were silenced (Figure 3b and 3d) were correlated with reduction in abundance of transcripts of the three *PhGID1* genes (samples 4 and 5 of Figure 4), particularly *PhGID1A* (Figure 4a).

Overexpression of the *gai* mutant gene from *Arabidopsis* in petunia A GA-insensitive mutant gene (*gai*) isolated from *Arabidopsis* was cloned into a GVG inducible system, using Dex as an inducer, to generate 10 independent lines of GVG::*gai* transgenic plants (T_0). The presence of the *gai* transgene was confirmed by PCR in all T_0 lines. Since a phenotype of growth retardation was observed in some T_0 lines under normal growth conditions, *gai* expression was examined in all lines, and three lines for induction experiments, including two normal lines (#1 and #4—showing no *gai* expression) and one leaky line (#6—showing *gai* expression in the absence of the Dex inducer).

GVG::*gai* T_1 petunia seedlings were sprayed daily with 30 μM dexamethasone for 10 days. The resulting over-expression of *gai* caused a dramatic retardation of plant growth in the treated seedlings (Figure 5c) and result in smaller leaf size in line 4 and 6, but there was no significant difference in line 1 (Table 2). In the control plants (no Dex-induction), plant growth rate and leaf size were similar to non-transformed controls except for the leaky line 6 (Table 2). After Dex spraying was discontinued, line 4 plants recovered gradually from the dwarf phenotype but the leaky line 6 plants continued to grow slower than wild-type control plants (Figure 6b).

To confirm that the dwarf phenotype was caused by *gai* overexpression, the abundance of *gai* transcripts was measured from GVG::*gai* plants with or without Dex induction. The expression of *gai* was strongly induced in treated plants of line 4 and 6 (Figure 5, D4a, D4b, D6a and D6b), compared to the treated wild-type plants (WT) and the non-treated transgenic plants (C1, C4, C6a and C6c) (Figure 5a). However, *gai* transcripts were not induced in line 1 plants (D1a and D1c) by Dex treatment (Figure 5a). In correlation with recovery from the dwarf phenotype, *gai* transcripts were

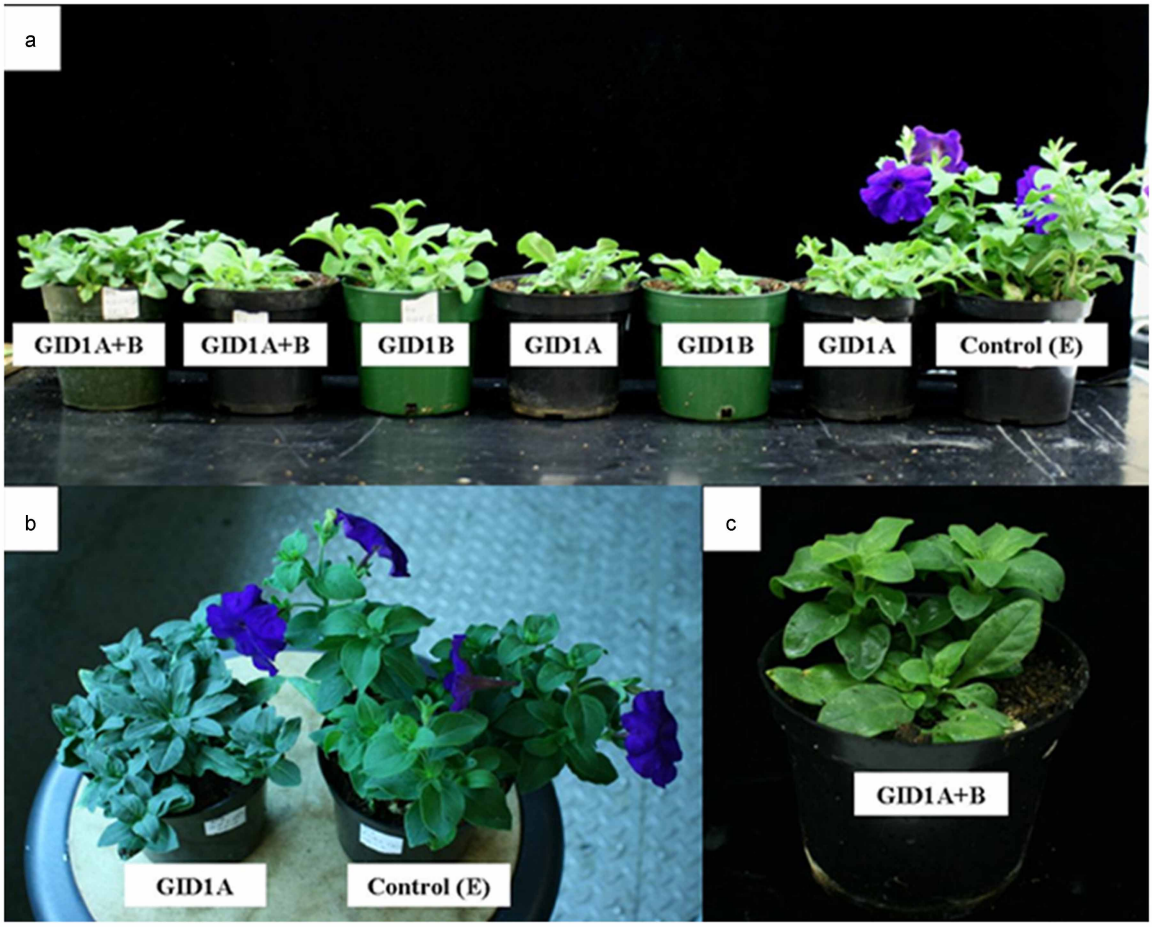

Figure 2. Effect of silencing *GID1*-like genes in petunia. The phenotypes resulting from silencing one (*PhGID1A* or *PhGID1B*) or two (*PhGID1A+B*) *GID1* genes is shown in comparison with control (E) plants transformed with the empty vector.

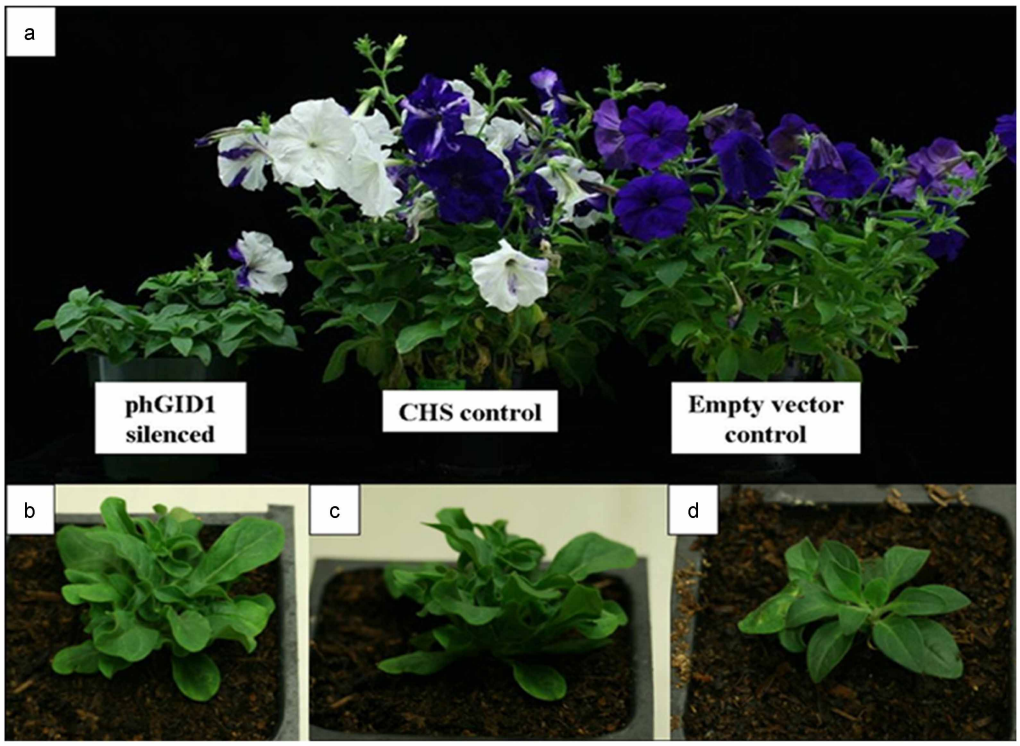

Figure 3. Effect of silencing three *GID1*-like genes in petunia. (**a**) The phenotype resulting from silencing with a TRV vector containing fragments of all three *PhGID1* genes and the *CHS* reporter gene (left) is compared with that of the *CHS* and empty vector controls. (**b**–**d**) The severely dwarfed phenotype of plants silenced with all *PhGID1* gene fragments.

barely detected in line 4 after spraying ceased, but were still abundant in line 6 (Figure 6a).

DISCUSSION

Interfering with GA signal transduction through downregulation of *GID1*-like genes in petunia

Three *GID1*-like genes, named *PhGID1A*, *PhGID1B* and *PhGID1C*, were identified in petunia (Supplementary Figure S1). In *Arabidopsis*, the three GID1 proteins have redundant function as GA receptors; *gid1* double mutants are partially impaired in GA responses but *gid1* triple mutants are totally insensitive to GA.[10] The sequences of *AtGID1A* and *AtGID1C* (A/C type) are highly similar with 73% identity but *AtGID1B* (B type) is quite different (about 40% identity with the A/C type); *gid1a/gid1c* double mutants show a strong dwarf phenotype than the other two double mutants.[10] In petunia, the sequences of *PhGID1B* and *PhGID1C* are much closer to *AtGID1B* (above 74% identity) and have 81% similarity between them (Supplementary Figure S2). It seems that there are two B-types of GID1 receptors existing in petunia. VIGS-induced silencing of single or double *PhGID1* genes resulted in only modest dwarfing and a low silencing frequency (data not shown). Silencing of all three *PhGID1* genes showed a severely dwarfed phenotype (Figures 3 and 4).

Table 1. Effect of silencing *PhGID1* on the branch length in petunia. Data collected at 60 days after infection

	Branch length (cm)		
	PhGID1	*PhCHS*	H_2O
Experiment 1	18.7±8.2***	27.0±2.6	28.7±2.4
	(*n*=15)	(*n*=10)	(*n*=10)
Experiment 2	13.1±8.3***	21.3±3.3	22.4±3.7
	(*n*=30)	(*n*=10)	(*n*=10)

Data are means±s.d.; *n* represents 10–30 plant replicates of each treatment.
*** indicates significant differences ($P<0.05$) analyzed by Tukey test.

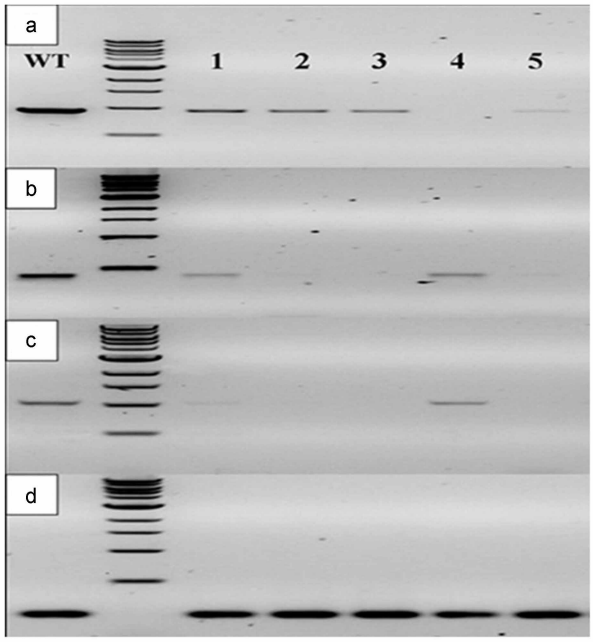

Figure 4. Virus-induced gene silencing of *GID1-like* genes in petunia. (**a**) *PhGID1A*; (**b**) *PhGID1B*; (**c**) *PhGID1C*; (**d**) 26S ribosomal RNA; cDNA samples 1–5 were synthesized from total RNA extracted from leaf tissues of five independently silenced plants. WT, wild-type.

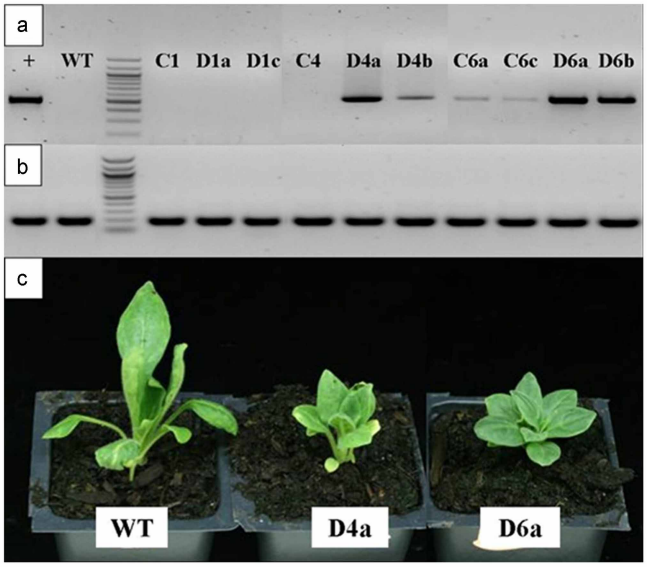

Figure 5. Induction of *gai* expression and the phenotype of GVG::*gai* T1 petunia after 10 days of spray treatment with 30 μM dexamethasone. (**a**) Abundance of *gai* transcripts (WT: wild-type; D1, D4 and D6: Dex-treated plants; C1, C4 and C6: no Dex-treated plants); (**b**) 26S rRNA transcripts; (**c**) Phenotype of GVG::*gai* T_1 petunia lines 4 and 6 after 10-days treatment with Dex compared with that of a wild-type control plant.

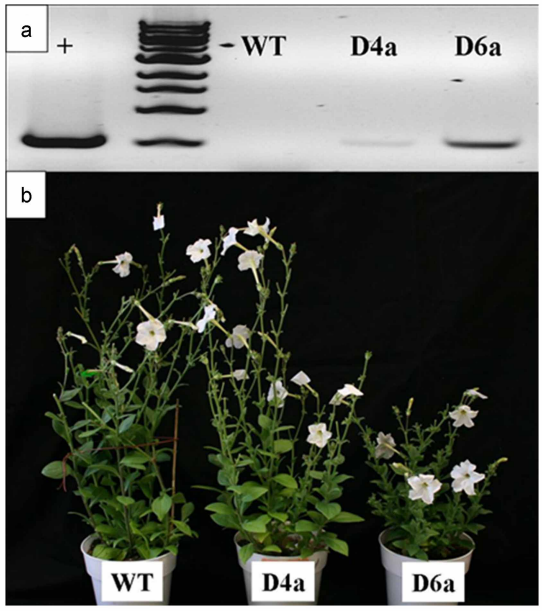

Figure 6. Recovery of the *gai* overexpressed phenotype following Dex-induction in GVG::*gai* petunia. The photograph was taken 70 days after termination of Dex treatments (**b**). Abundance of *gai* transcripts was determined using RT-PCR on cDNA prepared from total RNA extracted from tissues at the same time (**a**).

All three *PhGID1* genes are expressed throughout the plants except for in pollen (Figure 1). Although the genes are expressed in flower organs, downregulation had no visible effect on floral development (data not shown). Future experiments will examine the phenotype in transgenic petunia plants transformed with antisense *GID1* under the control of an inducible promoter. Such plants could be treated at the desired stage so that inhibition of *GID1* expression would reduce stem elongation without negative effects on other aspects and stages of plant growth and development.

Interfering with GA signal transduction by the over-expression of the *Arabidopsis* gai in petunia

This study demonstrated that heterologous expression of the mutant *Arabidopsis gai* inhibited plant growth in petunia. One line of transgenic GVG::*gai* petunia seedlings (line 4) showed the anticipated phenotype of stunted growth only when *gai* expression was induced by dexamethasone. No *gai* expression was detected in line 4 seedlings not treated with Dex, and they had the same growth rate as non-transgenic wild-type petunia. Line 1, although it contained the transgene, showed no phenotypic response or *gai* expression in response to Dex. This may be the result of loss of GVG control of the transgene during transformation, or could reflect epigenetic interference. Line 6 GVG::*gai* seedlings appeared to be 'leaky', expressing *gai* even before the Dex-induction, and showing a slower growth rate. After Dex-induction, line 6 seedlings showed strong *gai* expression and a dramatic retardation of seedling growth. Therefore, it suggested that the phenotype of growth retardation was caused by induced *gai* expression (Figure 5).

It took about 2 months after the end of the Dex treatment for petunia plants to return to their normal growth rate (Figure 6). This could be the effect of residual inducing Dex on the leaves or in the soil. The question of how the induced mutant *gai* proteins are degraded remains to be answered. The dwarfed plants of line 6 had shorter internodes but no difference in number of nodes (Table 3) compared to the wild-type plants. The data confirmed that GA plays an important role on stem elongation. It is worth mentioning that we detected the *gai* expression in the absence of the dexamethasone inducer in some transgenic lines. This is most likely caused by integration sites of the transgene in the genome of the petunia, leading to the leaky expression of the *gai*.

This study showed the potential for commercial application of biotechnology to prevent plant stretching. An inducible promoter system could provide flexible control of plant height that would allow grower to inhibit GA signalling during finishing but avoid adverse effects on growth and flowering during production. An ethanol-inducible system was used to control *gai* expression in *Arabidopsis*,[21] and this could be the basis for a practical system to

Table 2. Effect of overexpression of the *Arabidopsis gai* on leaf size in GVG-*gai* T_1 petunia plants. Data were collected after 10 days of Dex treatment

	WT	Line 1	Line 4	Line 6
Leaf length (cm)				
Dex	7.6±0.7 a	7.1±0.7 a	5.4±0.9 b	4.8±1.2 b
Non-Dex	7.4±0.8 a	7.2±1.0 a	7.5±0.5 a	5.5±0.7 b
Leaf width (cm)				
Dex	2.6±0.2 a	2.5±0.2 ab	2.1±0.3 b	2.2±0.2 b
Non-Dex	2.5±0.1 a	2.4±0.2 a	2.5±0.1 a	2.3±0.2 a

Data are means±s.d. (n=9). Different letters indicate significant differences (P<0.05) analyzed by Tukey test.

Table 3. Effects of GVG::*gai* induction on branch length and internode number in the leaky line (line 6). Data were collected 80 days after Dex induction

	WT	Line 6
Branch length (cm)	56.9±6.0	26.0±3.3***
Node numbers	14.7±2.1	14.7±1.8

Data are means±s.d..

*** indicates significant differences (P<0.05) analyzed by Tukey test.

make use of the effects of *GID1* silencing or *gai* over-expression in petunia and other ornamentals. Control of plant architecture using these molecular strategies could be of considerable benefit to commercial producers by reducing costs and environmental contamination, and permitting height control only when desired.

COMPETING INTERESTS

The authors declare no conflict of interest.

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