Controlling plant architecture by manipulation of gibberellic acid signalling in petunia

Yin-Chih Liang\textsuperscript{1}, Michael S Reid\textsuperscript{1} and Cai-Zhong Jiang\textsuperscript{2}

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 (\textit{PhGID1A}, \textit{PhGID1B} and \textit{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 \textit{gai} mutant gene (\textit{gai-1}) from \textit{Arabidopsis}. We have generated transgenic petunia plants in which the \textit{gai} mutant protein is over-expressed under the control of a dexamethasone-inducible promoter. This system permits induction of the dominant \textit{Arabidopsis gai} mutant gene at a desired stage of plant development in petunia plants by the application of dexamethasone (Dex). The induction of \textit{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.\textsuperscript{1} A common problem in the production of ornamental potted plants is undesirably tall growth, so inhibitors of GA biosynthesis including A-rest (anymidol), B-nine (daminozide), Bonzi (paclbutrazol), Cycocel (chloromequat chloride) and Sumagic (uniconazole), are commonly used to control plant height.\textsuperscript{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 signalling, 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.\textsuperscript{4,5} The genes encoding the GA response cascade have been identified using dwarf mutants of \textit{Arabidopsis}, wheat and rice.\textsuperscript{6,7} A soluble GA receptor was identified as the basis of the rice GA-insensitive dwarf1 (\textit{GID1}) mutant.\textsuperscript{8} In \textit{Arabidopsis}, there are three \textit{GID1} orthologs (\textit{AtGID1a}, \textit{AtGID1b} and \textit{AtGID1c}); the \textit{gid1a/gid1b/gid1c} triple mutant was severely dwarfed\textsuperscript{9} and showed high levels of RGA (REPRESSOR OF GA1-3) and GAI (GA-SENSITIVE) proteins.\textsuperscript{10} These proteins, characterized by the conserved DELLA domain at their N termini, function as repressors in GA signalling.\textsuperscript{11,12} Loss-of-function mutants such as rice \textit{slr1},\textsuperscript{13} and \textit{Arabidopsis gai-1} and \textit{rga-24},\textsuperscript{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 \textit{gai-1} from \textit{Arabidopsis} has a 17-amino acid deletion in the conserved DELLA domain\.\textsuperscript{11}

Previous researchers showed that heterologous expression of the \textit{Arabidopsis gai} mutant gene reduced plant height and altered GA response in transgenic rice,\textsuperscript{15} tobacco,\textsuperscript{16} chrysanthemum\textsuperscript{17} and apple.\textsuperscript{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,\textsuperscript{19} such as the dexamethasone-inducible promoter\textsuperscript{20} or the alcohol-inducible promoter,\textsuperscript{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 \textit{GID1}-like receptor genes using the virus-induced gene silencing (VIGS), with GA signal transduction by over-expression of the \textit{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 (\textit{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 \textmu m$^2$ s$^{-1}$ PPFD) with a day/night temperature regime of 22\textdegree C/18\textdegree C. VIGS experiments used the purple-flowered ‘Primetime Blue’ cultivar, but studies on stable transformants used white-flowered cultivar ‘Mitchell Diploid’.

Isolation of \textit{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 (Invitrogen), as previously described.\textsuperscript{3,22} First strand cDNA was synthesized using 2 \textmu g total RNA, oligo d(T) primer, random hexamer, and then replicated by superscript reverse transcriptase (Invitrogen), as previously described.\textsuperscript{2,23} \textit{GID1}-like gene fragments were amplified from the cDNA by RT-PCR using degenerate primers designed from the three \textit{GID1} receptor gene sequences of \textit{Arabidopsis} or partial EST sequences of petunia. The full-length sequences of \textit{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 (\textit{PhGID1A}), JX501239 (\textit{PhGID1B}) and JX501240 (\textit{PhGID1C}).
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’-ACG AGT CCT AGG TCA AAC TG-3’ and 5’-CAT GCA ATT CCA ATT CAA ATC-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 265 ribosomal RNA was used as an internal control and the amplification primers were 5’-AGC TGG TTT GAT TCT GAT TTT 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.\(^{2,23}\)

To silence all three PhGID1-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 SacI and XhoI 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 transform petunia plants. (Supplementary Figure S1). The 26S rRNA abundance determined by real-time PCR was used to normalize loading for each cDNA sample.

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 AtGID1A (accession number NP_187163) and AtGID1C (accession number NP_198084) and the other two petunia GID1-like proteins share 74% and 77% identities with AtGID1B (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 forward 5’-GTG TAA GCT GGC TCA TTT AGC TG-3’ and reverse 5’-GTT GAA CAG CAC CAA AGG CAT AC-3’ (535 bp).

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 (T0).

The presence of the gai transgene was confirmed by PCR in all T0 lines. Since a phenotype of growth retardation was observed in some T0 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 T1 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.
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).

| Branch length (cm) | PhGID1 | PhCHS | H2O |
|-------------------|--------|-------|-----|
| **Experiment 1**  |        |       |     |
| (n=15)            |        |       |     |
| 18.7±8.2***       | 27.0±2.6 | 28.7±2.4 |     |
| **Experiment 2**  |        |       |     |
| (n=30)            |        |       |     |
| 13.1±8.3***       | 21.3±3.3 | 22.4±3.7 |     |

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

**Table 1. Effect of silencing PhGID1 on the branch length in petunia.**

Data collected at 60 days after infection

**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.

**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.
Table 2. Effect of overexpression of the Arabidopsis gai on leaf size in GVG:gai T1 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.

Control of plant architecture via GA signalling

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, and this could be the basis for a practical system to...
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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