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Loss of Function in GIGANTEA Gene is Involved in Brassinosteroid Signaling

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Abstract

Brassinosteroids (BRs) are plant steroid hormones that play essential roles in growth and development. Mutations in BR-signaling pathways cause defective in growth and development like dwarfism, male sterility, abnormal vascular development and photomorphogenesis. Transition from vegetative to reproductive growth is a critical phase change in the development of a flowering plant. In a screen of activation-tagged *Arabidopsis*, we identified a mutant named *abz126* that displayed longer hypocotyls when grown in the dark on MS media containing brassinazole (Brz), an inhibitor of BRs biosynthesis. We have cloned the mutant locus using adapter ligation PCR walking and identified that a single T-DNA had been integrated into the ninth exon of the GIGANTEA (GI) gene, involved in controling flowering time. This insertion resulted in loss-of-function of the GI gene and caused the following phenotypes: long petioles, tall plant height, many rosette leaves and late flowering. RT-PCR assays on *abz126* mutant showed that the T-DNA insertion in GIGANTEA led to the loss of mRNA expression of the GI gene. In the hormone dose response assay, *abz126* mutant showed: 1) an insensitivity to paclobutrazole (PAC), 2) an altered response with 6-benzylaminopurine (BAP) and 3) insensitive to Brassinolide (BL). Based on these results, we propose that the late flowering and tall phenotypes displayed by the *abz126* mutant are caused by a loss-of-function of the GI gene associated with brassinosteroid hormone signaling.

Key words : GIGANTEA Gene, Brassinosteroids , Loss od function, *Det2*, brassinazole

1. Introduction

Steroid hormones play key roles in growth and development of eukaryotes. Brassinosteroids (BRs) are plant hormones that are ubiquitously distributed throughout the plant kingdom and regulate cellular expansion, differentiation and proliferation[14]. BR mutants in *Arabidopsis* show a characteristic phenotype that includes dwarfism, round dark green leaves, delayed development, reduced fertility and altered vascular structure[6, 24,2]. In BR-deficient mutants, all of these phenotypic defects can be rescued by exogenous application of BRs[1], demonstrating that the plant steroid hormone has an essential role for normal growth and development.

Molecular genetic studies demonstrated that mutation in a single locus, *brassinosteroid insensitive 1* (*bri1*), caused the phenotypes of steroid-deficient mutants that can not be rescued by treatment with BRs[6,17]. BRI1 is a leucine rich repeat (LRR) receptor kinase, with an extracellular domain containing 25 LRRs, a transmembrane domain and cytoplasmic kinase domain[17]. Brassinosteroids are perceived by a plasma membrane-localized receptor kinase Serine/Threonine Kinase and is encoded by BRI1 gene[6].

BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1) is another component of the BR receptor complex and a downstream target of BRI1[27,15]. The cell surface receptor kinase BRI1 and BAK1, perceive the BR signal and initiate the signal transduction cascade. The two nuclear proteins BZR1 and BES1 activate the growth response and are dephosphorylated and stabilized by BR signaling[28,31,23]. The BIN2 kinase negatively regulates BR responses by phosphorylating BZR1 and BES1 and targets them for degradation by the proteasome[31,18]. Another BSU1 phosphatase was identified as a positive regulator of BR signaling by dephosphorylating and stabilizing the BES1 protein[19]. This suggests that BES1 and BZR1 are found in the nucleus and that they modulate the transcription of BR-regulated genes.

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These proteins also appear to be kept in check by BIN2, which fine-tunes the signal strength.

In this study we isolated a late flowering mutant from the activation tagged Col-7, showing the characteristics of a BR-signaling mutant and named it *abz126*. Furthermore, molecular genetic analysis of the *abz126* mutant shows that T-DNA was inserted in GIGANTEA (GI) resulting in a knockout. GI is a nuclear-localized protein that is involved in several processes including the induction of flowering by long days, inhibition of hypocotyl elongation by red light[13], and the circadian clock[21,22]. Here, we identified that GI could either transcribe or modify BR signaling.

2. Experimental Section

2.1. Plant material and growth conditions

Arabidopsis thaliana (ecotype Col-7) plants were mutagenized by *Agrobacterium tumefaciens* (strain GV3101) mediated T-DNA transformation using the activation tagging vector pSKI015[30]. T2 Seeds of basta (20 mg/L) resistance were used for subsequent experiments. Seeds were surface sterilized in a solution of 70% ethanol containing 0.05% Triton X-100 by gently shaking for 15 to 20 min and rinsed three times with 95% ethanol and then five times with sterile water. The seeds were rinsed with an appropriate volume of sterile water (until seeds were free floating) before being sown in rows on agar plates. The agar medium contained half strength MS salts (DUCHEFA BIOCHEMIE, The Netherlands) with 1% (w/v) sucrose and phytoagar (Sigma), pH 5.8. The plates were then wrapped into three layers with aluminum foil and stored at 4°C for 3 to 4 days to ensure uniform germination before being placed in a growth room. Two weeks after germination, seedlings were hand-transferred into autoclaved soil (Sunshine mix 5, Sun Grow Horticulture, Canada) and grown to maturity in a growth room. Plants were watered twice per week with mineral nutrient solution (0.1% Hyponex). The growth room temperature was maintained at 21±1°C. Light provided by cool-white florescent tubes was 50 to 70 μmol photons m^2 sec^{-1} (constant) for seedlings on agar plates and ~10070 μmol photons m^2 sec^{-1} (16 h of light and 8 h of dark) for potted plants.

2.2. Isolation of an *abz126* mutant

Approximately 6,000 T2 activation tagging lines were screened on Brassinazole (Brz) using a hypocotyl-measurement assay. Seeds were surface sterilized and sown by spreading on plates containing one-half-strength MS medium and 2 uM Brz (Brz220). The plates were placed at 4°C for four days to break the dormancy and kept at 21±1°C for germination in the dark. Seven days later, tall hypocotyls with closed cotyledon were observed on the Brz plate and this mutant was named *abz126*.

2.3. Determination of T-DNA insertion site

Genomic DNA was isolated from dark grown T2 seedlings using a magnetic beads-based method (BioSprint 96, QIAGEN, USA) according to the manufacturer's manual. The prepared DNA samples were digested with ApoI at 50°C for 4 hours, purified once with phenol/chloroform (v/v), precipitated by adding 0.1 vol. 0.3 M Na acetate and 2.5 vol. of 100% ethanol incubated on ice for 10 min and centrifuged at 13,000 rpm for 10 min. The obtained pellet was washed once with 70% cold ethanol and air dried. The dried pellet was dissolved in 10 μl D.W. Three μl of DNA was ligated with mixed adaptors (5'-GTAATACGACTCACTATAGGG CACGCGTGGTCGACGGCCCGGGCTGC-3', 5'-AATT GCAGCCCG-(NH2)-3', 5'-AGCTGCAGCCCG-(NH2)-3') and subsequently subjected to PCR and nested PCR. The first PCR was performed with a primer set of AP1 (5'-AGAATACGACTCACTATAGGGC-3') and skLB1 (5'-TCGATCGTGAAGTTTCTCATC TAAGCCC-3'), and the PCR cycle conditions were (94°C for 25 sec, 72°C for 3 min) x 7 cycles and (94°C for 25 sec, 67°C for 3 min) x 32 cycles. The resultant PCR products were diluted approximately 30 fold in the second PCR mixture. The nested PCR was performed using primers AP2 (5'-TCGACGGCCCGGGCTGCAATTC-3') and skLB2 (5'-CCATTTGGACGTGAATG TAGACACGTCT-3') and the cycle conditions were (94°C for 30 sec, 67°C 30 sec with touch-down of 0.5°C per cycle, 72°C for 3 min) x 8 cycles, (94°C for 30 sec, 63°C for 10 sec, 72°C for 3 min) x 25 cycles, and 72°C for 10 min. The product of the nested PCR was examined on 1% agarose/EtBr gels the band was excised, purified using a gel extract kit (QIAGEN) and subjected to sequencing analysis with the SKLB2 primer using automated DNA sequencers (ABI Prism 377XL, Applied Biosystems Tokyo; Megabase 1000, Amersham Biotech, Tokyo). The sequenced data was mapped to the Arabidopsis genome at the

TAIR blast (http://www.arabidopsis.org/Blast/).

2.4. RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol (Life Technologies) from 100 mg of two weeks old leaves of *Arabidopsis* grown on soil according to the manufacturer's protocol. The concentration of the total RNA was quantified using a spectrophotometer (Smart Spec 3000, BIO-RAD, USA) at an absorbance of 260 nm. All the extracted RNA was diluted to 1 μg/μl. cDNA was synthesized using Superscript II reverse transcriptase (Life Technologies). Two μg of total RNA and 500 ng of an oligodT primer were mixed in a reaction tube, heated at 70°C for 10 min to inactivate the sample, and then chilled on ice quickly. First strand buffer (5X) and 0.1 M DTT were added, and the mixed contents of the tubes were gently incubated at 42°C for 2 min. One μl (200 units) of Superscript II was added to the tube, incubated at 42°C for 50 min and the reaction stopped by heating at 70°C for 15 min. The cDNA was stored at -20°C for further use.

2.5. RT-PCR expression analysis

Semi-quantitative RT-PCR was employed in order to determine the expression of a gene adjacent to T-DNA insertion sites. RT-PCR amplifications were performed in 20 μL reaction volumes, containing 200 ng of cDNA, 1x Taq buffer, 0.25 mM of dNTPs, 0.5 μM of each forward 5'-GAACTCGGCTGT ACTATATCCTTGTCCCA-3' and reverse 5'-ATCCACTGAAGACTAAACACCA-GACGC ACA-3' primers and 1 unit of Taq DNA polymerase. The GI gene was amplified with a denaturation of 5 min at 94°C; 30 cycles at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1min; and a final extension of 5 min at 72°C. Actin was used as a positive internal control. PCR products were electrophoresed in a 1% agarose gel containing 0.5 mg/L ethidium bromide (EtBr) and observed under ultraviolet light.

2.5. Hormone assay

For the hormone dose response, seeds of the wild-type and mutants were germinated and grown on half-strength MS plates containing different concentrations of the plant hormones Paclobutrazol [Pac (0, 0.4 and 4 uM)], benzyl aminopurine [BAP (0, 0.1, 1 and 10 uM)] and brassinolide (BL) [BL (0 M, 1 nM, 10 nM, 100 nM, 1 uM and 5 uM)] (all plant hormones except BL were purchased from Sigma, St. Louis). Hypocotyl lengths of individual seedlings were measured after 7 days grown in dark. Three replicate plates were used for each treatment and each plate contained about 40 to 50 seedlings of *Arabidopsis*. Hypocotyls were scanned and measured using SCION Image software and values were plotted as average ±SE.

3. Result

3.1. Screening of *abz126* mutant in T-DNA tagging lines

The activation tagging lines were generated by random T-DNA insertion into the *Arabidopsis* genome [30]. *Arabidopsis* activation tagged seed ecotype Columbia (Col-7) was purchased from Arabidopsis Biological Research Centre (ABRC) and examined for co-segregation of the mutant phenotype and the basta resistance marker. To obtain homozygous mutants the resulting F1 progeny were analyzed on one-half strength MS-1% sucrose medium containing basta (20 mg/L). Analysis of the basta-resistant phenotype showed that it segregated with a 3:1 ratio, indicating that there was only one T-DNA insertion at a single locus in the mutant.

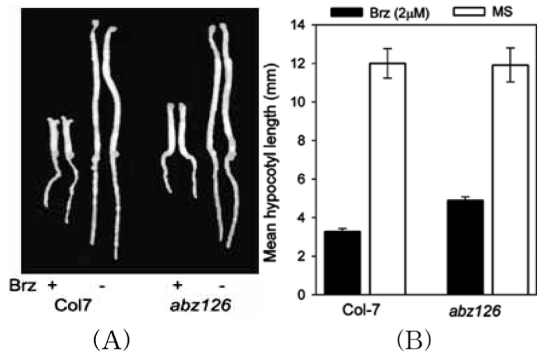

Fig. 1. Effect of Brz on Arabidopsis hypocotyol elongation in the dark. Wild-type (Col7) and *abz126* mutants were germinated and grown on one-half-strength Murashige and Skoog medium containing 2 μM Brz for 7 days in the dark. (A) Hypocotyl elongation of *abz126* mutant seedling is partially insensitive to Brz. Data are presented as the means ±SE obtained from 40 seedlings. All the hypocotyls were measured as described in "Materials and Methods". (B) Comparison of hypocotyl phenotype of the wild-type and *abz126* mutant grown on half-strength MS media supplemented with 2 μM Brz.

Brz is a triazole derivative that inhibits BRs biosynthesis and has been shown to be useful for investigating BRs function in plants. Brz specifically blocks BL biosynthesis by inhibiting the cytochrome P450 steroid C-22 hydroxylase encoded by the DWF4 gene, which causes de-etiolation and dwarf phenotypes similar to those of BRs-deficient mutants[3]. In both light and dark, Brz-induced morphological changes can be nullified by the addition of brassinosteroid hormone brassinolide[25]. In dark Brz-treated *Arabiodpsis* plants develop as if grown in light and express light regulated genes. We screened activation-tagged Col-7 seeds and isolated a dominant mutant that showed long hypocotyl when grown on Brz in dark and named this mutant as *abz126*. The phenotypes of *abz126* mutant plants grown on Brz are shown in Figure 1. Compared with wild-type seedlings, dark grown *abz126* mutant seedlings had normal hypocotyl lengths when grown on Brz-unsupplemented half-strength MS medium (Figs. 1A and 1B).

3.2. Identification of T-DNA Insertion Site

Next, we isolated the sequence flanking the T-DNA insertion by using the adapter ligation-based PCR-mediated walking method. An approximately 1.7 kb fragment was isolated by using a T-DNA specific primer located near the left border. BLAST searches using the sequence of this fragment showed identity to a region on NCBI gene locus AF076686 that is located on chromosome I. Sequence analysis indicated that the T-DNA was inserted in the GI gene, 3045 bp from its putative translation start codon (Fig. 2A).

3.3. Expression of GI and Neighboring Genes

We compared the expression pattern of the GI gene and neighbor genes on either side of the AF076686 locus between wild-type (Col-7) and *abz126* mutants. As expected, both neighbor genes on either side of the wild-type and *abz126* mutant were expressed equally at the left border, adjacent to the enhancers, At1g22760 encoding a putative poly-A binding protein, and at the right border, At1g22780, coding for the putative 40S Ribosomal protein S18 respectively. But a T-DNA insertion on At1g22770, knocked out the gene encoding for GI in *abz126* mutants (Fig. 2B). Thus, insertional disruption of the At1g22770 locus appears to be the cause of the *abz126* mutant phenotype without impact from the neighboring genes.

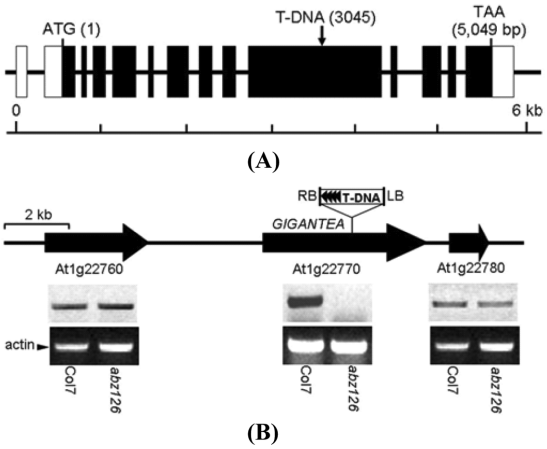

Fig. 2. Identification of *abz126*. (A) Structure of the GIGANTEA (GI) gene. The GI coding sequence (3,455 bp) consists of thirteen exons (black rectangles). White rectangles are untranslated regions. The exons and introns ranged in length from 54 to 1532 and 79 to 302 bp, respectively. Black arrow indicates T-DNA insertion position of GI. (B) Three genes surrounding the T-DNA insertion were knocked out in the region of GI. Arrows indicate the direction of transcription. (Below) RT-PCR analysis of GI and each of its neighboring genes in wild-type Col7 and *abz126*. Actin was used as (a) control.

3.4. Knock-out of the GI Gene: Effects on Plant Size and Flowering Time

To determine whether the expression of the GI gene is related to plant size and flowering time, we isolated total RNA from the leaves of wild-type (Col-7) and *abz126* mutants, and analyzed mRNA expression levels by RT-PCR. The GI gene was expressed in wild-type but completely absent in the *abz126* mutant (Fig. 3A). Knockout of the GI gene led to overall changes in phenotypes of the *abz126* mutant including changes in plant height and length of the petiole (Fig. 3, B-E). In *abz126* mutant, the numbers of the rosette leaves were increased four-fold and the days of flowering time is increased two-fold as compared to wild-type (Fig. 3F).

3.5. *abz126* responds differently to exogenous BL treatment.

To test whether the *abz126* mutant is involve in BR signaling or biosynthesis, response of BL in the mutant plant was determined using a hypocotyl elongation assay in dark-grown seedlings. We used a BL biosynthesis mutant *det2* as a control. *det2* has shorter hypocotyls

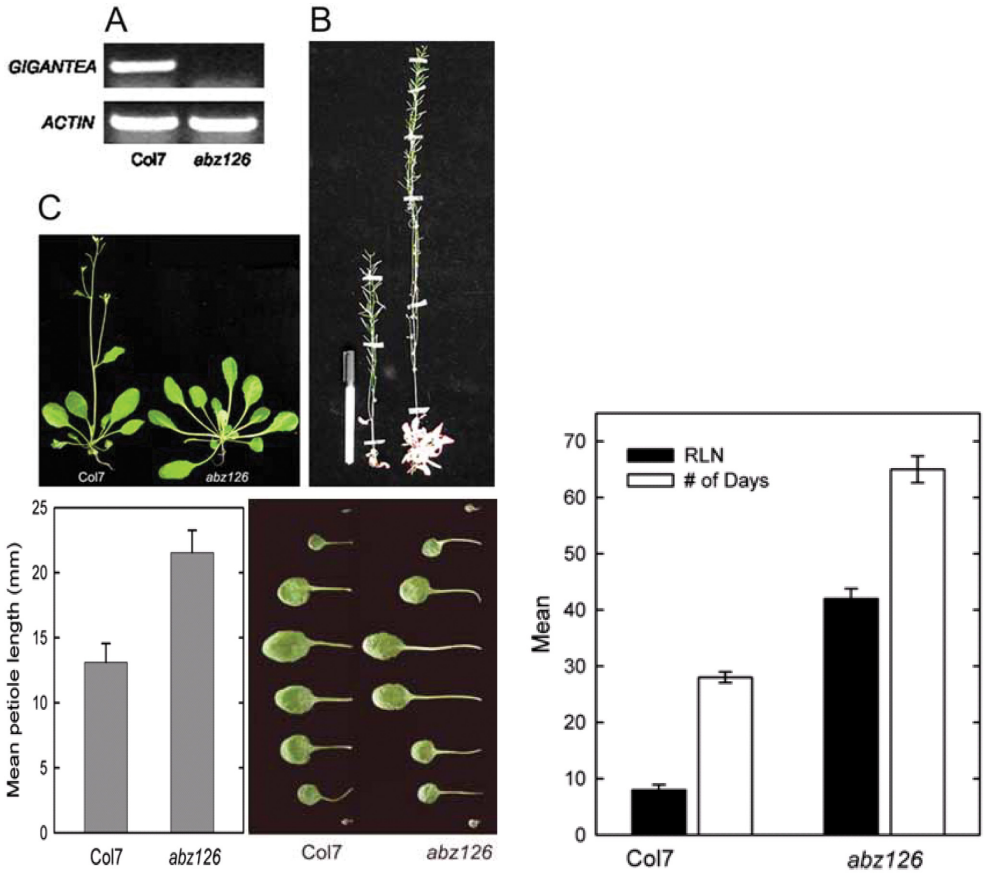

Fig. 3. RT-PCR and plant morphology. All the plants depicted are of the same age. RT-PCR analysis of wild-type (Col7) and *abz126* mutants for GIGANTEA. Reducing the expression of GIGANTEA gene increased the late flowering phenotype. Actin was used as a control. Flowering time for the wild-type (Col7), *abz126* mutant:. Rosette leaf number (RLN) and days were counted when plants (16 h light/8 h dark) at 21°C. Data are presented as mean ±SE calculated from three independent experiments with at least 15 plants each.

than WT in both dark and light. Hypocotyl elongation in BR-deficient mutant *det2* plants was rescued by increasing the concentration of BL in both light and dark conditions[26]. The hypocotyl length of *det2* increased till the concentration of 100 nM BL, whereas the hypocotyl lengths of *abz126* mutant and wild-type were altered by 10 nM BL (Fig. 4A). The *abz126* plants are not sensitive to BL-induced hypocotyl growth, suggesting that BL-induced cell elongation is impaired in the mutant. We also observed that the *abz126* mutant was less-sensitive than the wild-type at all the concentrations of BL (Fig. 4A). We examined the effect of exogenous 6-benzylaminopurine (BAP) on the elongation of *abz126*, *bzr1* and wild-type seedlings. When the concentrations were increased, the elongation of *abz126* hypocotyls were markedly less inhibited when compared to wild-type (Fig. 4B). Next, we tested *abz126* mutants under GA deficiency [(induced by treatment with the GA biosynthesis inhibitor paclobutrazol (PAC)], and they exhibited a GA-independent phenotype distinct from the wild-type, including resistance to the PAC-induced effects of hypocotyl elongation (Fig. 4C). These results suggest that act negatively in signal transduction of hypocotyl elongation through GI.

4. Discussion

Increasingly, many genes that are involved in BRs

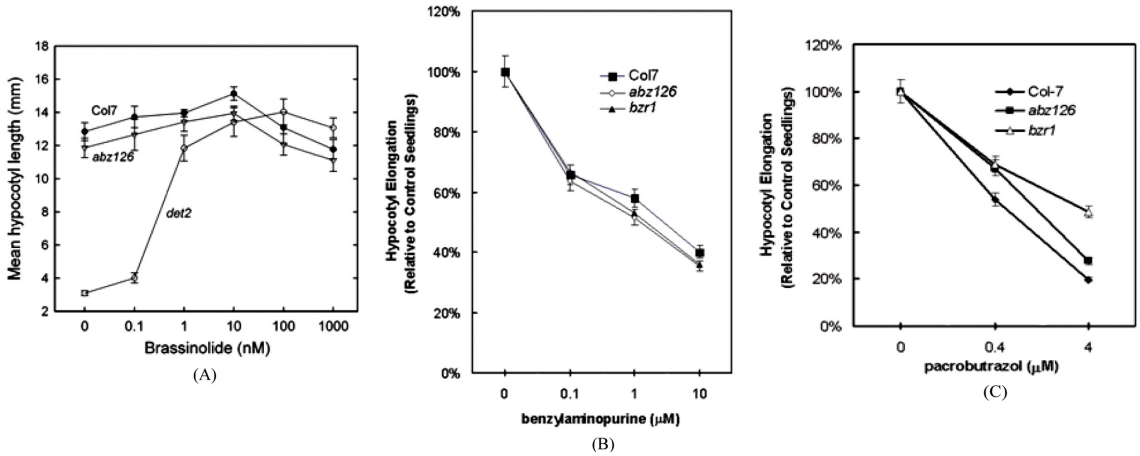

Fig. 4. Quantitative analysis of hormone sensitivity of *abz126* mutants. Seedlings of wild-type (Col7), *det2*, bzr1 and *abz126* mutants were germinated and grown on one-half-strength MS medium containing increasing concentrations of hormones. (A) Hypocotyl length of wild-type (Col7), *det2*, and *abz126* mutants in the presence of different concentrations of BL in the dark. (B) Effects of BAP (0, 0.1, 1 and 10 μM) on hypocotyl elongation. of Col7, *abz126*, and bzr1 plants. (C) Hypocotyl length of wild-type (Col7), bzr1 and *abz126* mutants in the presence of 0, 0.4, 1 and 4 μM of PAC. Hypocotyl elongation was measured 7 days after germination in the dark. Each data point represents the average hypocotyl elongation of 40 to 50 seedlings of three duplicate experiments. Wild-type and mutants were grown on medium containing the same volume of 80% (v/v) ethanol used to dilute from a stock solution. Inhibition of hypocotyl elongation by BAP and PAC are expressed relative to the hypocotyl elongation of the control. Error bar represents the SE. All the hypocotyls were measured as described in "Materials and Methods".

hormone signaling are being linked to physiological and developmental processes such as stem elongation, vascular differentiation, seed size, fertility, flowering time, senescence and resistance to biotic and abiotic stresses. Despite the rapid progress in recent years in identifying several BR-signaling components and BR-regulated genes, there is little information on brassinosteroid related genes that regulate normal flowering. In this report we described the potential role of GI, a gene that has an unknown role in BRs signaling.

Previously it was shown that GI transcript is detected throughout development in all parts of a mature plant. The analysis of GI expression suggests that it may be involved in controling photoperiodic responses[9,21,7]. In addition, the GI gene has been implicated in abiotic stress, because it is upregulated in response to low temperature[10,5]. Interestingly, *gi* mutants show enhanced tolerance to oxidative stress suggesting that the GI gene mediates the circadian clock, cold stress and oxidative stress tolerances through different signaling pathways[16]. Our results demonstrate that GI mutations increase insensitivity to Brz. Brz is a BR-biosynthesis inhibitor that induced dwarfism in *Arabidopsis* mutants that resembled BR-biosynthesis mutants that can be rescued by BR[25]. In the dark, treating *Arabidopsis* seedlings with Brz induced retardation of hypocotyl growth. Our feeding experiment on *abz126* mutants demonstrated that hypocotyl elongation was partially insensitive to Brz as compared to wild-type (Fig. 4A).

The phenotypic analysis of the *abz126* mutant demonstrated that the GI gene is negatively regulated in a divergent set of developmental processes in plants such as: 1) petiole elongation, 2) increasing the number of rosette leaves, 3) increasing plant height and 4) delaying flowering time (Fig. 3).

The plant steroid hormones brassinosteroids (BRs) are perceived by the cell surface receptor kinase BRI1[4]. BRI1 is a leucine-rich-repeat receptor-like kinase (LRR-RLK) located on the cell surface (17; 20). BRI1 has an extracellular domain containing 25 LRRs, a transmembrane domain and a cytoplasmic serine/threonine kinase domain[11]. When, BRI1 perceives the BR signal through its extracellular domain it initiates a signal transduction cascade mediated by its cytoplasmic kinase activity[12,29]. BIN2 encodes a cytoplasmic protein kinase and it negatively regulates the BR signaling pathway[18,8]. Two

nuclear proteins, BZR1 and BES1, were identified as positive regulators of the BR signaling pathway downstream of BIN2. BZR1 and BES1 are mostly found in phosphorylated forms, and BR treatment leads to dephosphorylation and accumulation of these proteins[28,31,23]. These studies demonstrate a BR signal transduction pathway leading from cell surface receptors to the nucleus. There could be many genes involved in BR signal transduction to the nucleus. The region between residues 543 and 783 contains four separate cluster of basic amino acid that function as nuclear localization signals (NLSs) leading to the localization of GI to the nuclear membrane[13]. Studies of the *abz126* mutant suggested that GI is negatively involved in growth promotion but it has positive role for flowering time. The mutation in GI causes insensitivity to the BRs biosynthetic inhibitor Brz, indicating a negative role of GI in BRs signaling. The *abz126* mutant, when grown in light, displayed constitutive BRs response phenotypes including long and bending leaf petioles and an increase in plant height. GI is a gene localized to the nuclear membrane and is likely to negatively regulate the BR signaling pathway. The role(s) of GI gene in growth and development may also involve the identification of the new additional set of BRs-responsive gene. Finally, the identification and analysis of the GI gene mutation in the *abz126* mutant showed that GI gene: 1) is involved negatively in BRs signaling, 2) results in late flowering, 3) leads to an increase in the number of rosette leaves and 4) leads to an increase in plant height. These results elucidated that knockout of GI gene enhanced the overall plant growth and development. Manupulation on GI gene could be great applicable in the field of agricultural applications for prolonging the plant life, increase the plant height and plant biomass, increase the number of leaf biomass on vegetables and tobacco without application of any plant growth substances.

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