Overexpression of *Constitutive Differential Growth 1* Gene, Which Encodes a RLCKVII-Subfamily Protein Kinase, Causes Abnormal Differential and Elongation Growth after Organ Differentiation in Arabidopsis[1]

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To better understand genetic regulation of differential growth of plant organs, a dominant and semidwarf mutant, *constitutive differential growth 1-Dominant* (*cdg1-D*), was isolated utilizing the technique of activation tagging. *cdg1-D* showed pleiotropic phenotype including dwarfism, exaggerated leaf epinasty, and twisted or spiral growth in hypocotyl, inflorescence stem, and petiole. Hypocotyls of *cdg1-D* were longer than those of wild type under light conditions. The phenotype was caused by activation tagging of *CDG1* gene that encodes a receptor-like cytoplasmic kinase of RLCKVII subfamily. When treated with high concentrations of brassinolide, light-grown wild-type seedlings showed long hypocotyls and strong leaf epinasty as observed in *cdg1-D* seedlings. Treatment of *cdg1-D* with brassinazole, a specific inhibitor of brassinosteroid (BR) biosynthesis, did not rescue the mutant phenotype. Gene expression of *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* involved in BR biosynthesis and *phyB ACTIVATION-TAGGED SUPPRESSOR1* that inactivates BR was repressed and induced, respectively, in *cdg1-D* plants, suggesting constitutive activation of BR signaling in the mutant. *CDG1* was expressed at a very low level in all the organs of the wild type tested. We isolated two independent intragenic suppressors of *cdg1-D*. However, they showed normal morphology and responded to BR in a similar manner to wild type. Taken together, *CDG1* gene may interfere with signal transduction of BR when overexpressed, but is not an essential factor for it in the wild type.

After differentiating in the apical meristem or embryo, plant organs continue to grow to reach their adult size, accompanying cell division and enlargement (Howell, 1998). Plant cells in an organ do not always grow uniformly, resulting in differential growth. Since plant cells are surrounded by cell walls, they do not change their position relative to neighbor cells. Thus, differential growth is one of the major means for plant organs to change their shape after cell division ceases. Plants change their form depending on their developmental stage and in response to environmental conditions. Consequently, plants often exhibit differential growth throughout their life. The process of differential growth is important for plant development and survival and represents a fundamental difference in how plants and animals react to environmental change.

Phototropism and gravitropism are the best characterized differential-growth responses induced by environmental conditions. Auxin plays a central role in these processes (Iino, 2001; Tatematsu et al., 2004). Hypocotyl cells at the apex grow differentially under dark conditions, so that a hook-like structure is formed. Hooks disappear when irradiated with light or as seedlings get old. Formation and disappearance of the hook structure is an example of differential growth caused by a developmental cue (Raz and Ecker, 1999). Ethylene has long been considered as a major factor controlling hook formation, inhibiting cell growth at the inner side of hook (Peck et al., 1998). Epinasty is another example of differential growth. Ethylene, high concentrations of auxin (Abeles et al., 1992), and brassinosteroid (BR; Schlagnhaufer and Arteca, 1985) induce leaf epinasty, which occurs as a result of differential growth of leaf blade and petiole, in many plants. Thus, several plant hormones appear to be involved in epinasty.

Activation tagging with the enhancer from the cauliflower mosaic virus (CaMV) 35S transcript promoter is a technique in plant functional genomics that

[1] This work was supported in part by a Grant-in-Aid for Scientific Research (B) (to K.T.Y. and N.Y.), and by Grants-in-Aid for Scientific Research in Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, and from the National Space Development Agency of Japan and the Japan Space Forum (Ground Research for Space Utilization; to K.T.Y.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.104.046805.

can create transgenic plants in which the T-DNA carrying 35S enhancer at its right border is spliced into the plant genome at random sites (Hayashi et al., 1992; Weigel et al., 2000). Analysis of gain-of-function mutants created through activation tagging can provide insight to a gene's function. In fact, study on activation-tagged mutants has played a pivotal role in dissecting cytokinin (Kakimoto, 1996), BR (Neff et al., 1999), and light (Nakazawa et al., 2001) signaling.

In an attempt to determine factors involved in genetic regulation of differential growth of plant organs, we have carried out screening of gain-of-function mutants by use of activation-tagging methods in this study. From 10,000 activation-tagged lines, a novel, dominant, and semidwarf mutant, *constitutive differential growth 1-Dominant* (*cdg1-D*), was isolated. Although adult *cdg1-D* mutants have a semidwarf phenotype, *cdg1-D* hypocotyls are longer than wild-type hypocotyls under white light. In *cdg1-D*, the hypocotyl, stem, petiole, and fruit grow in a spiral or twisted shape and the leaves are small and show severe epinasty. Here we show that these defects were induced by activation of a gene for a Ser/Thr protein kinase and that the kinase may interfere with BR signaling.

RESULTS

Mutant Screening of Activation-Tagging Lines

We generated approximately 10,000 activation-tagged primary lines of Arabidopsis ecotype Columbia by using pPCVICEn4HPT (Hayashi et al., 1992; Nakazawa et al., 2001). For mutant screening, 11 T_2 seeds of each transformed line were grown in the dark for 3 d on vertically oriented plates after germination. The length and shape of hypocotyls and the hook structure of the etiolated seedlings were monitored.

As a result, we isolated a dominant mutant that showed a twisted or spiral hypocotyl and an open-hook structure in the dark and excessively epinastic leaves when grown in the light condition. Three-quarters of the T_2 progeny of this line showed the mutant phenotype and hygromycin resistance. F_1 plants of the mutant allowed to backcross to Columbia also showed the mutant phenotype. These traits of the mutant are consistent with it having a single dominant allele. This dominant mutation was named *cdg1-D* after its leaf epinasty and twisted growth in the hypocotyl.

Mutant Phenotype

Defects of the *cdg1-D* mutant were pleiotropic. *cdg1-D* plants were dwarf and had small and remarkably epinastic leaves (Fig. 1, a–c). Mature leaves of the mutant were smaller than those of the wild type. Rosettes of the mutant plants showed a pineal shape because of its small, curly leaves and shorter petioles (Fig. 1c). Mutant plants bore only a small number of seeds. These phenotypes were more severe in homozygous *cdg1-D* plants (Fig. 1c) than heterozygous plants (Fig. 1b), indicating that *cdg1-D* is a semidominant mutation.

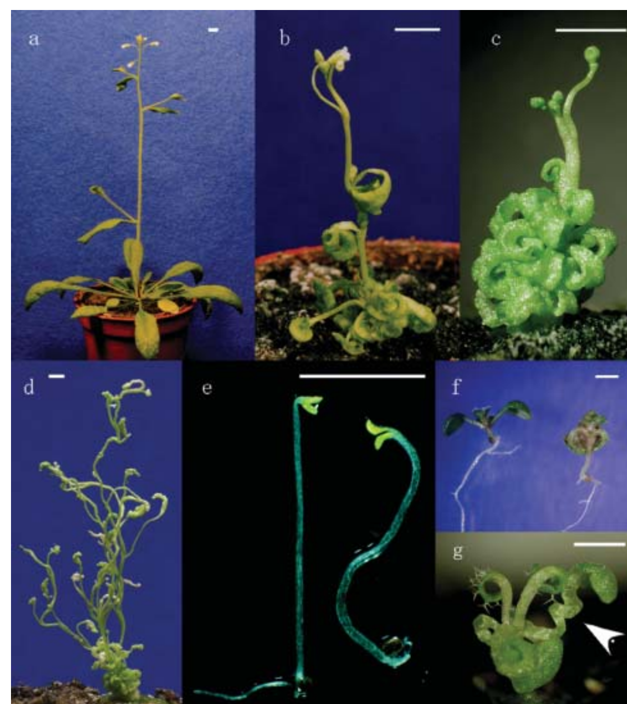

Figure 1. Morphologies of wild-type and *cdg1-D* plants. a to c, Five-week-old light-grown wild type (a), heterozygous (b), and homozygous (c) *cdg1-D*. d, Eight-week-old light-grown *cdg1-D*. e and f, Three-day-old etiolated seedlings (e) and 1-week-old light-grown seedlings (f) of wild type (left) and *cdg1-D* (right). g, Two-week-old light-grown *cdg1-D*. Plants were grown on soil (a–d and g) or on agar plates (e and f). Arrowhead in g shows spiral growth in petiole. Bars, 5 mm in all sections.

cdg1-D hypocotyls grew in a twisted manner (Fig. 1e) or sometimes spirally in the dark with a vertical screw axis showing normal gravitropic responses. The hypocotyls were as long as those of the wild type. Twisted growth was also seen in various organs of adult *cdg1-D* plants such as inflorescence stem, petiole, and fruit (Fig. 1d). Petioles of *cdg1-D* also sometimes showed spiral growth (Fig. 1g, arrowhead). All leaves of *cdg1-D*, including cotyledons, rosette, and cauline leaves, showed exaggerated epinasty (Fig. 1, c and f). *cdg1-D* leaves were bent at the junction of the blade and petiole in a longitudinal direction before the leaf blade began to expand (Fig. 1g). Then they started to expand and curl in both longitudinal and latitudinal directions.

Microscopic observation was performed to examine cellular basis of abnormal differential growth such as leaf epinasty and twisted growth of hypocotyl and inflorescence stem (Fig. 2). The overall anatomy of the mutant organs appeared unaltered, suggesting that the mutation did not affect organ differentiation. But tissue development in each organ was disordered in the mutant. Cortex and epidermal cells of *cdg1-D*

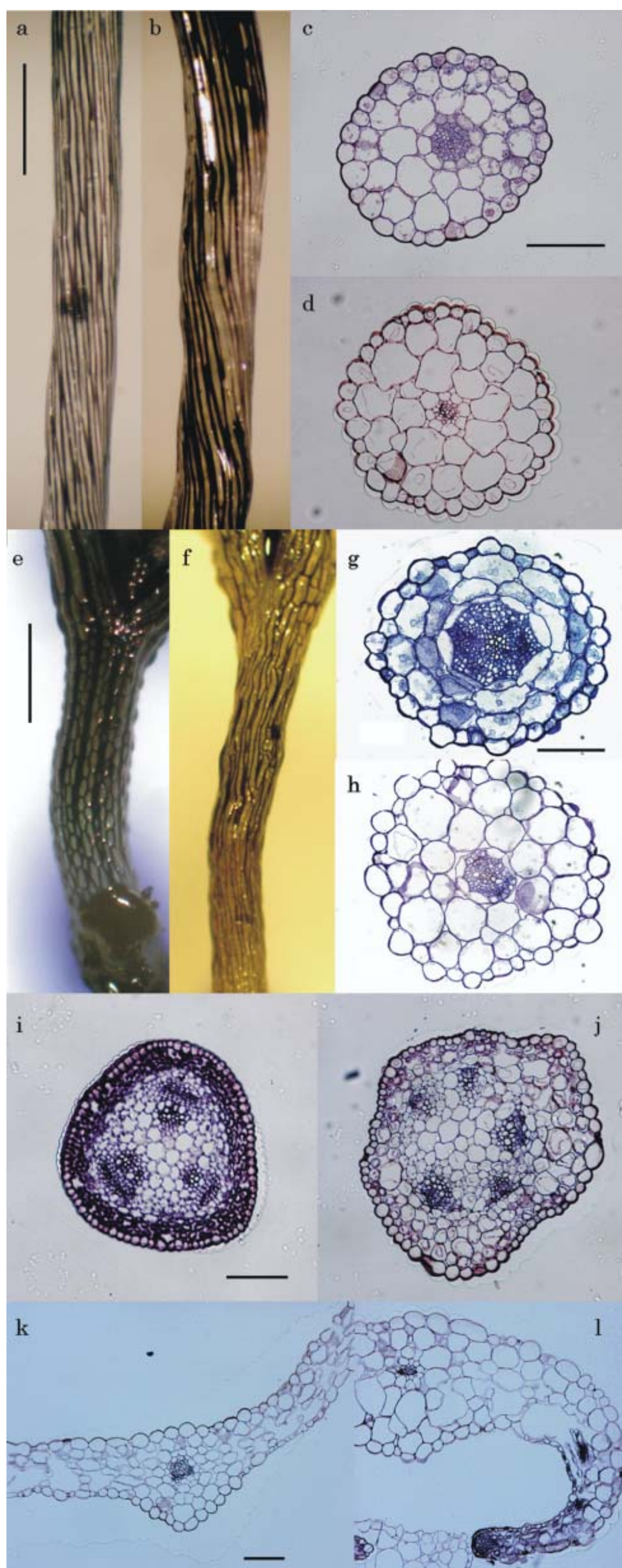

Figure 2. Structure of *cdg1-D* organs. a to h, Three-day-old etiolated (a–d) or 1-week-old light-grown (e–h) hypocotyls of wild-type (a, c, e, and g) and *cdg1-D* (b, d, f, and h) seedlings. Photographs were taken after immersion in India ink (a, b, e, and f) or cross-sectioning (c, d, g, and h). i and j, Cross-sections of inflorescence stems of 5-week-old wild-type (i) and *cdg1-D* (j) plants. k and l, Cross-sections of leaves of 3-week-old wild-type (k) and *cdg1-D* (l) plants. Bars, 500 (a and e) and 100 (c, g, i, and k) μm.

hypocotyl expanded randomly (Fig. 2, d and h). They became uneven in shape and size. *cdg1-D* tissue had extensive intercellular spaces in comparison with wild type. Consequently, the surface of *cdg1-D* hypocotyls was not as smooth as that of the wild type. Longitudinal observations of *cdg1-D* hypocotyls after immersion in India ink also showed a distortion of epidermal cell files and rough surface, which was dented and bulged from place to place (Fig. 2, b and f).

Epidermal and cortex cells of *cdg1-D* were also enlarged randomly in inflorescence stems, while those of the wild type were small and organized in order (Fig. 2, i and j). In contrast, the central stele of the mutant was relatively normal, with a smooth and round shape (Fig. 2j). These observations suggest that abnormal radial growth of the cortex and epidermal cells in *cdg1-D* disrupts epidermal cell files and causes twisted or spiral growth in both hypocotyls and inflorescence stems.

Leaf epidermal cells on the adaxial side were generally larger than those on the abaxial side in wild-type plants (Fig. 2k). In *cdg1-D*, epidermal cells on the adaxial side were larger than those of wild type, while those on the abaxial side were much smaller than those of wild type, especially in the region of the leaf margin (Fig. 2l). Namely, the difference in cell size between the adaxial and abaxial epidermal cells was expanded by the *cdg1-D* mutation. The amplified differential expansion of leaf cells appeared to be the cause of leaf epinasty in the latitudinal direction displayed in the mutant. Leaf parenchyma cells of *cdg1-D*, like the adaxial epidermal cells, also swelled. Epinasty in the longitudinal direction was also increased in a similar manner (data not shown). Underdevelopment of vascular tissues, another prominent characteristic of *cdg1-D*, was observed in all organs examined (Fig. 2, d, h, j, and l).

Responses to Growth Regulators in Light-Grown Seedlings

Hypocotyls of *cdg1-D* were about three times longer than those of the wild type under white-light condition (Fig. 1f). *cdg1-D* hypocotyls had longer epidermal cells than those of wild type (Fig. 2, e and f). Therefore, the longer *cdg1-D* hypocotyls probably resulted from an increase in length rather than an increase in the number of epidermal cells.

Since plant hormones are often involved in the long-hypocotyl phenotype, effects of growth regulators were examined on the growth of hypocotyls under white-light conditions. Seedlings were grown for 1 week on agar plates that contained various substances, including gibberellin (1 μM GA_3), BR (1 μM brassinolide [BL]), inhibitors of ethylene synthesis (1 μM aminoethoxyvinylglycine) and perception (1 μM $AgNO_3$),

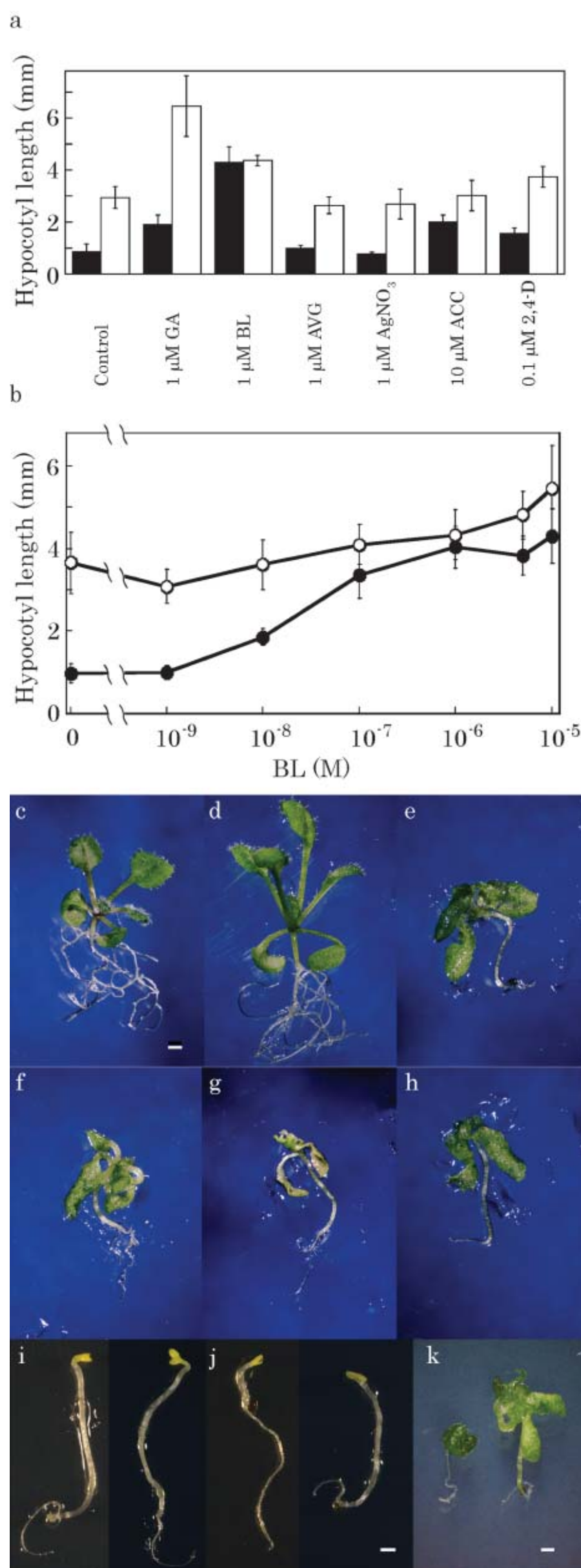

Figure 3. Effects of growth regulators on elongation of hypocotyls and epinasty of leaves. After seedlings were grown under continuous white light for 1 (a and c–h) or 2 (b) weeks on agar plates, length of hypocotyls of wild type (black bars and circles in a and b, respectively) and *cdg1-D* (white bars and circles in a and b, respectively) was measured. Wild-type (c–e) and *cdg1-D* (f–h) plants were grown in the absence (c and f) or presence (d and g) of 1 μM GA or BL (e and h). Wild-type (left) and *cdg1-D* (right) plants were grown in the absence (i) or presence (j and k) of 2 μM brassinazole for 4 d in the dark (i and j) or for 10 d under continuous white light (k). Bars, 1 mm.

ethylene precursor (10 μM 1-aminocyclopropane-1-carboxylic acid [ACC]), and a synthetic auxin (0.1 μM 2,4-dichlorophenoxyacetic acid; Fig. 3a). GA treatment increased hypocotyl length about 2-fold in both wild-type and *cdg1-D* seedlings, showing that GA exhibited an additive interaction with the *cdg1-D* mutation. GA treatment did not affect leaf morphology in either the wild type or *cdg1-D* (Fig. 3, d and g).

BR also promoted hypocotyl elongation in both wild type and *cdg1-D* (Fig. 3a). Dose response of the BR effects in Figure 3b showed that BL started to increase hypocotyl length of the wild type at 10 nM and that the promotive effects reached a plateau at 1 μM. In contrast, growth of *cdg1-D* hypocotyls was not affected as much as the wild type. Consequently, *cdg1-D* hypocotyls were as long as those of the wild type at BL concentrations higher than 1 μM. Since BL treatment also induced leaf epinasty in wild type (Fig. 3e), wild-type plants grown in the presence of 1 μM BL looked very similar to *cdg1-D* grown in the same condition (Fig. 3, e versus h) and somewhat resembled *cdg1-D* grown in the absence of the hormone (Fig. 3, e versus f). However, treatment of *cdg1-D* with 1 μM brassinazole, a specific inhibitor of BR biosynthesis (Asami and Yoshida, 1999; Min et al., 1999), did not restore the aberrant phenotype of *cdg1-D* (Fig. 3, i–k). Thus, BL treatment appeared to mimic the *cdg1-D* phenotype in wild-type seedlings under white-light conditions, and the mutant phenotype was not likely due to overproduction of BR in it.

Neither aminoethoxyvinylglycine nor $AgNO_3$ had any effects on hypocotyl elongation (Fig. 3a) or leaf epinasty (data not shown) in the wild type or *cdg1-D*. On the other hand, ACC induced hypocotyl elongation in the wild type. In the presence of ACC, hypocotyls of the wild type were more than twice as long as those of the control. They were, however, still shorter than those of *cdg1-D* (Fig. 3a). ACC also induced leaf epinasty in the wild type (data not shown), but its effects were smaller in extent than those observed by either the *cdg1-D* mutation (Fig. 3f) or BR treatment (Fig. 3e). These results suggest that ethylene is not a major factor for the expression of the *cdg1-D* phenotype, although sensitivity to ethylene was reduced in *cdg1-D*. Similarly, 2,4-dichlorophenoxyacetic acid had only a slight promotive effect on hypocotyl elongation in both the wild type and *cdg1-D* (Fig. 3a).

Cloning of *CDG1* Gene

The *CDG1* gene was cloned by plasmid rescue. Nucleotide sequences adjacent to both ends of T-DNA

perfectly matched sequences in a P1 genomic clone, MOJ10, which is mapped on chromosome 3. The tetramerized CaMV 35S enhancer sequences were perfectly conserved in the fragment. A hypothetical gene (GI, 9279618, At3g26940) with similarity to Ser/Thr protein kinases was downstream of the enhancer sequences. Since no expressed sequence tags were available for the predicted gene, cDNA was isolated by the PCR after reverse transcription of RNA (RT-PCR) using primers designed to amplify the fragment from the putative start to stop codons of this gene. Results of 3′ and 5′ RACE showed that polyadenylation occurred 146 bp downstream of the putative stop codon and that the 5′ initiation site was located 332 bp upstream of the putative start codon. These results indicate that the *CDG1* gene is transcribed into a sequence of 1,754 nucleotides and consists of 5 exons (Fig. 4a). T-DNA was inserted at 47 bp upstream of the determined 5′ end of the *CDG1* transcript. No canonical TATA box was found upstream of the initiation site. The *CDG1* transcript contained two open reading frames (ORFs) in addition to the protein kinase ORF at the 5′ end (Fig. 4a, indicated by asterisks). Start codons of these upstream ORFs (uORFs) were 242 and 79 bp upstream of the start codon of the protein kinase ORF, respectively. The uORFs can be translated into short peptides, 11 and 8 amino acid residues, respectively, that exhibit no similarity to any of the protein sequences in the GenBank database.

The longest ORF of the *CDG1* gene can be translated into a sequence of 431 amino acid residues (Fig. 4b) that contains all the 11 conserved subdomains of eukaryotic protein kinases. All the invariant amino acid residues are conserved in their proper positions (Hanks and Quinn, 1988). The putative kinase domain is flanked by short nonkinase domains on both sides. Since this protein contains no membrane-spanning regions or extra membrane domains, it is likely to be either a cytoplasmic or nuclear kinase.

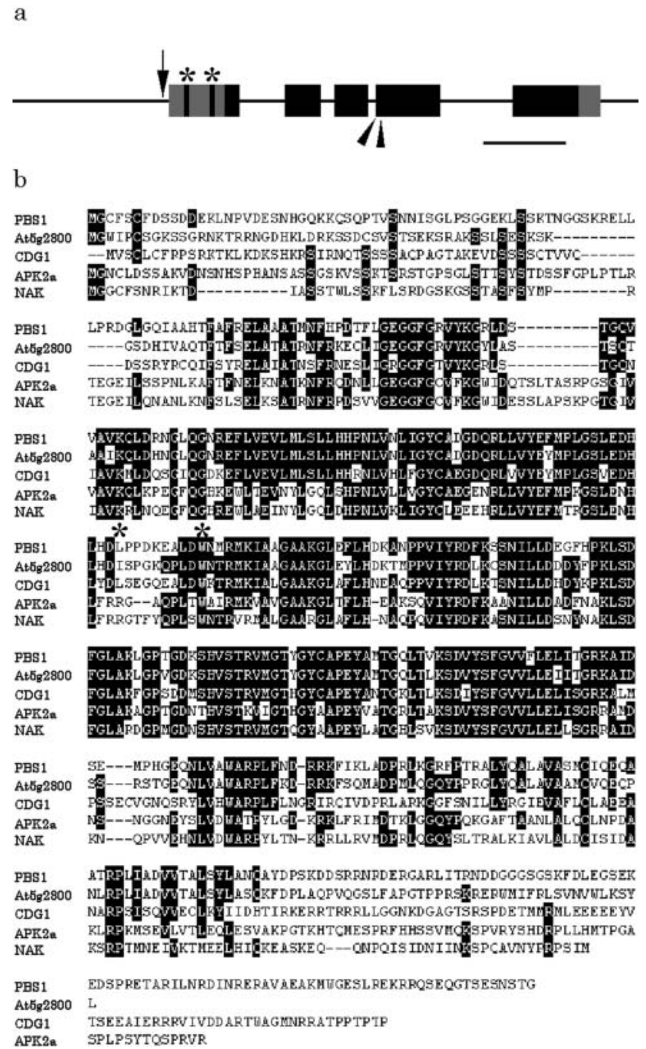

Figure 4. Structure of *CDG1* gene. a, Boxes show exons. Black and gray boxes indicate coding and noncoding regions, respectively. Arrow indicates the T-DNA insertion site. Arrowheads indicate suppressor mutation sites. Asterisks indicate upstream ORFs. Bar, 500 bp. b, Alignment of amino acid sequences of CDG1, PBS1 (Swiderski and Innes, 2001), APK2a (Ito et al., 1997), NAK (Moran and Walker, 1993), and At5g02800. Asterisks indicate suppressor mutation sites in CDG1. The alignment was made with CLUSTALW (http://www.ddbj.nig.ac.jp/E-mail/clustalw-j.html).

Expression of *CDG1* Gene

An RNA gel-blot analysis using the CDG1 kinase ORF as a probe revealed overexpression of this gene in the *cdg1-D* mutant (Fig. 5a). In the wild type, no signal of *CDG1* was detectable in 10 μg poly(A)$^+$ RNA, while a signal was observed in *cdg1-D* using 0.1 μg poly(A)$^+$ RNA. Therefore, *CDG1* mRNA appeared to accumulate more than 100-fold in *cdg1-D*. This result suggests that the abnormalities of *cdg1-D* plants result from activation tagging of the *CDG1* protein kinase gene. Expression of the *CDG1* gene was higher in homozygous *cdg1-D* than in heterozygous *cdg1-D* (data not shown), suggesting that the severity of the *cdg1-D* phenotype depends on the dose of the *CDG1* gene. Expression of the *CDG1* gene was not detected in the wild type by RNA gel-blot analysis, but it was observed by RT-PCR in various organs of the wild type, such as root, stem, leaf, flower, and fruit (Fig. 5b). The amounts of *CDG1* messages seem to be slightly higher in the flower and fruit. These results show that *CDG1* mRNA accumulates at a very low level in all organs of wild-type plants.

Under control of the CaMV 35S promoter in the sense orientation, kinase ORF of the *CDG1* mRNA was introduced into the genome of the wild type by the use of *Agrobacterium tumefaciens*. Of the nine independent T_1 lines we generated, five lines showed dwarfism and epinastic leaves as observed in *cdg1-D* mutants. Their phenotype was heritable in T_2 generation, linking with selection markers of T-DNA. Twelve-day-old plants of these transgenic lines were almost identical to *cdg1-D* plants with respect to their morphology (Fig. 6). They showed all the phenotypes seen in *cdg1-D* including

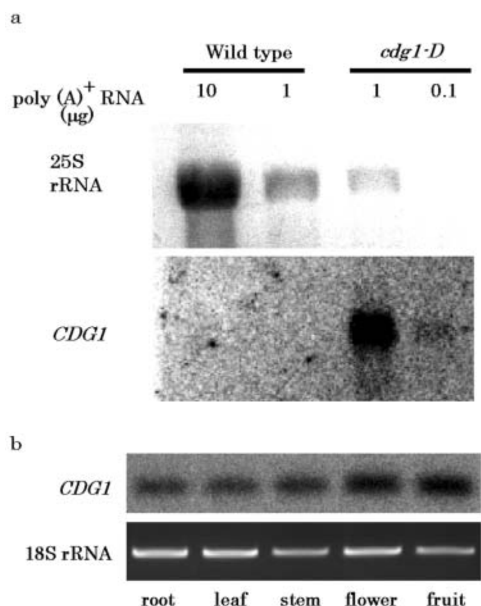

Figure 5. mRNA accumulation of *CDG1* gene. a, RNA gel-blot analysis of *CDG1* gene in the wild type and *cdg1-D*. Poly(A)$^+$ RNA was prepared from aerial tissue of wild-type and *cdg1-D* adult plants. The kinase ORF of *CDG1* was used as probe. b, *GDG1* gene expression in different organs of the wild type examined with RT-PCR. RT-PCR for amplification of *CDG1* and 18S ribosomal RNA was carried out using 20 ng of total RNA prepared from each organ of wild type as templates. The numbers of cycles were 25 for *CDG1* and 13 for 18S ribosomal RNA. RT-PCR product of *CDG1* was detected by DNA gel-blot analysis using ^{32}P-labeled probes because of its low expression level, while that of 18S ribosomal RNA was detected by staining with ethidium bromide.

dwarfism, twisted stem and fruit, and frizzy growth in dark condition and long hypocotyls under white-light condition (data not shown). Therefore, all the phenotypes seen in *cdg1-D* were reproduced in the *CDG1* overexpressors. These results indicate that the *cdg1-D* phenotype is caused by overexpression of the protein kinase encoded by the *CDG1* gene.

On the other hand, one of the nine transgenic lines showed the wild-type phenotype, and the other three lines exhibited weaker phenotype. Rosette leaves of the latter lines were as large as those of wild type; they displayed epinastic and twisted growth that was often seen in BR-treated plants, but their petioles were not as elongated as BR-treated ones (data not shown). These results clearly indicate that overexpression of *CDG1* activates signaling pathway that is overlapping, but not identical with BR signaling.

Control of Gene Expression Involved in BR Metabolism

Recently BR biosynthesis has been shown to be controlled in a feedback fashion (Fujioka and Yokota, 2003). Genes involved in BR biosynthesis are repressed and those in BR catabolism are activated in the presence of higher levels of BR. If *cdg1-D* phenotype is related to BR action, such gene regulation should be observed in *cdg1-D*. This argument prompted us to examine gene expression of *CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM* (*CPD;* Szekeres et al., 1996) and *phyB ACTIVATION-TAGGED SUPPRESSOR1 (BAS1*; Neff et al., 1999; Turk et al., 2003), which encode cytochrome P450 enzymes involved in biosynthesis and inactivation of BL, respectively. RT-PCR in Figure 7a shows that mRNA level of *CPD* and *BAS1* was decreased and increased in *cdg1-D*, respectively, compared to that of wild type. The change was very similar to that induced by treatment of wild type with 1 μM BL and was not affected by the treatment of the mutant with 1 μM brassinazole. These results suggest that *cdg1-D* phenotype is not caused by overproduction of BR, but that BR signaling is constitutively activated in *cdg1-D* seedlings. The repression of *CPD* and activation of *BAS1* were also observed in mature *cdg1-D* plants (Fig. 7b).

Isolation and Characterization of *cdg1*, a Loss-of-Function Mutation of *CDG1*

By screening 10,000 M_2 seeds, we isolated two independent suppressor mutants of *cdg1-D* that showed hygromycin resistance and suppressed all the phenotypes of *cdg1-D*. F_1 plants of both mutants that were allowed to backcross with Columbia also showed the wild-type phenotype and hygromycin resistance. These traits of the suppressors suggest two possibilities: the mutations occurred at dominant loci, or they were intragenic suppressors that had mutations within the *CDG1* gene downstream of the enhancer sequences in the T-DNA. Genomic sequencing of each line revealed a single-base substitution in *CDG1* gene that results in a premature stop codon in exon 4 or disruption of a splice site junction between intron 3 and exon 4 (Fig. 4a). These findings mean that the mutated and overexpressed *CDG1* mRNA in each allele should give rise to a nonfunctional protein that

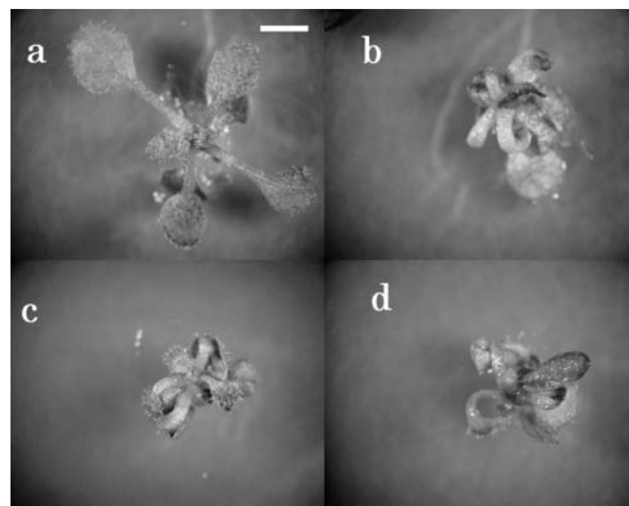

Figure 6. Overexpressors of the *CDG1* ORF for kinase. a, Wild type. b, *cdg1-D*. c and d, T_2 generation of transformant lines ovD2 (c) and ovD4 (d). Plants were grown for 12 d on agar plates. Bar, 3 mm.

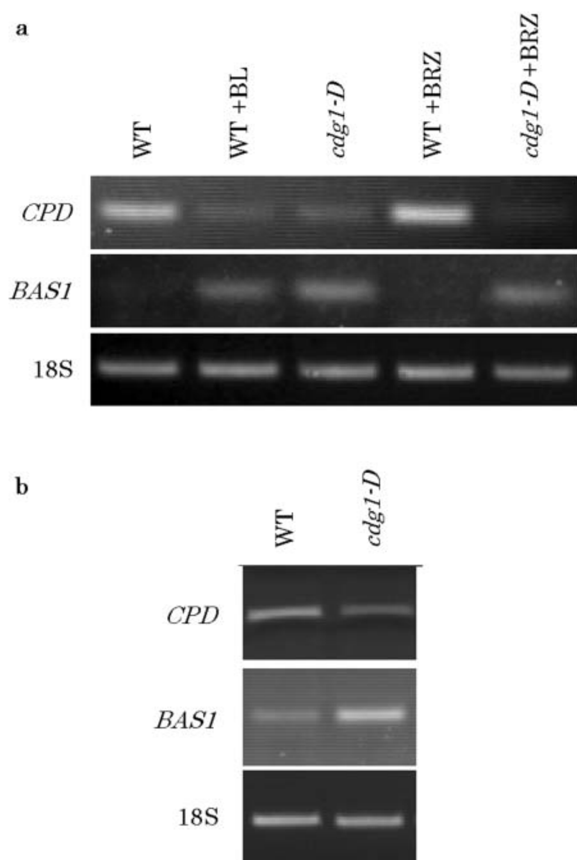

Figure 7. Expression of *CPD* and *BAS1* genes determined by RT-PCR. RT-PCR for amplification of *CPD*, *BAS1*, and 18 S ribosomal RNA was carried out using 20 ng of total RNA prepared from 10-d-old seedlings grown in the absence or presence of 1 μM BL or 2 μM brassinazole under continuous white light (a) or from aerial tissue of 5-week-old plants (b). Numbers of PCR cycles were 27 for *CPD*, 29 for *BAS1*, and 13 for 18S ribosomal RNA. RT-PCR products were detected by staining with ethidium bromide.

contains only subdomains I to V (Fig. 4b). Therefore, homozygotes of these suppressors must be loss-of-function mutants of *CDG1* and were named *cdg1-1* and *2*, respectively. In contrast to the pleiotropic effects of overexpression of the *CDG1* gene, *cdg1-1* and *2* showed normal morphology in all organs (data not shown). We also examined effects of BR and GA on growth of these mutant alleles and found that they responded to the hormones essentially in the same fashion as the wild type (data not shown). These effects suggest that *CDG1* gene possibly interferes with signal transduction of BR when overexpressed, but is not an essential factor for it in the wild type.

DISCUSSION

BR Signaling May Be Interfered within *cdg1-D* at the Seedling Stage

In this study we showed that activation of the Arabidopsis *CDG1* gene by the use of CaMV 35S enhancers produced a dominant mutant, *cdg1-D*, which displayed pleiotropic defects in organ growth regulation after organogenesis. These defects included dwarfism, exaggerated differential growth in multiple organs such as hypocotyls, leaves, and inflorescence stems, and long-hypocotyl phenotype under white-light conditions. Treatment of wild-type seedlings with only BL phenocopied the long-hypocotyl phenotype of *cdg1-D*. Application of BL also induces epinasty of cotyledons, resulting in a *cdg1-D*-like morphology. It has been well known that BR biosynthesis is controlled by an elaborate feedback regulation (Fujioka and Yokota, 2003). BL treatment of Arabidopsis seedlings decreases mRNA level of several cytochrome P450 enzymes, including CPD (Mathur et al., 1998), DWF4, and ROT3 (Bancoş et al., 2002; Goda et al., 2002; Müssig et al., 2002), which function in BR biosynthesis. On the other hand, expression of *BAS1*/*CYP72B1* gene, whose product inactivates BL through hydroxylation (Neff et al., 1999; Turk et al., 2003), is induced by exogenous BRs (Goda et al., 2002), suggesting that *BAS1* acts to maintain steady-state levels of endogenous BRs. Decrease in *CPD* mRNA level and increase in *BAS1* mRNA level were observed in *cdg1-D*, which were very similar to those observed in the wild-type seedlings treated with 1 μM BL. Furthermore, neither the change in gene expression level nor *cdg1-D* phenotype was influenced by treatment of *cdg1-D* with an inhibitor of BR biosynthesis, brassinazole (Asami and Yoshida, 1999; Min et al., 1999; Fujioka and Yokota, 2003). These findings strongly suggest that *cdg1-D* phenotype at the seedling stage results from constitutive activation of BR signaling.

Interestingly, *CDG1* encodes a receptor-like kinase (RLK), and a few kinases have been identified that constitute the signal transduction pathway of BR (Thummel and Chory, 2002). A putative receptor of BR is a plasma membrane-localized Leu-rich repeat (LRR)-RLK, BRI1 (Li and Chory, 1997; Wang et al., 2001). Another LRR-RLK, BAK1, may form a receptor complex with BRI1 on the plasma membrane (Li et al., 2002; Nam and Li, 2002). BIN2, a negative regulator of BR signaling, is a glycogen synthase kinase-3 (Li and Nam, 2002; Pérez-Pérez et al., 2002). BIN2, together with BRI1 and BAK1, may constitute a phosphorylation cascade. The presumed constitutive activation of BR signaling in *cdg1-D* may thus result from interference of the phosphorylation cascade by the overexpressed CDG1 kinase.

Although *cdg1-D* seedlings look like BL-treated wild-type seedlings, mature *cdg1-D* plants are dwarf. In contrast, transgenic plants overexpressing BRI1-green fluorescent protein fusion protein (Wang et al., 2001) and wild-type plants grown with exogenously added BR (Arteca and Arteca, 2001) appear larger than the wild type because of their longer petioles, which is probably caused by activation of BR signaling. On the other hand, the mRNA level of *CPD* and *BAS1* was decreased and increased, respectively, even in mature *cdg1-D* plants (Fig. 7b). Thus, the presumed interference

of BR signaling by the overexpressed *CDG1* may occur in mature plants of *cdg1-D*, although they are dwarf. A few overexpression lines of CDG1 kinase ORF showed weaker phenotype than *cdg1-D* and were not dwarf. In this case, however, their leaf petioles were not as elongated as those of the *BRI1*-overexpressing plants. At present, therefore, it is unknown what causes dwarfism in mature *cdg1-D*. There may exist another mechanism of dwarfism distinct from BR signaling that is also activated by CDG1.

CDG1 Is a Member of a Poorly Understood Subfamily of Receptor-Like Protein Kinases

Based on its amino acid sequence similarity, *GDG1* was judged to encode a Ser/Thr kinase of the RLK subfamily. CDG1 kinase is closely related to PBS1 of Arabidopsis (Swiderski and Innes, 2001), with 66.8% identity within the kinase domain (Fig. 4b), and is also similar to several Arabidopsis protein kinases, NAK (Moran and Walker, 1993), ARSK1 (Hwang and Goodman, 1995), APK1 (Hirayama and Oka, 1992), and APK2 (Ito et al., 1997). It is also similar to Esi47 of wild wheatgrass (*Lophophyrum elongatum*; Shen et al., 2001). All of these proteins belong to NAK subfamily of plant protein kinases (Hardie, 1999). Like the product of *CDG1*, PBS1 and all the members of the NAK subfamily have a central kinase domain flanked by short nonkinase domains on both sides.

Based on the kinase domain phylogeny, Shiu and Bleeker (2001) have recently defined 44 subfamilies of the 610 RLKs in Arabidopsis that have a monophyletic origin. They include 11 subfamilies, which are named receptor-like cytoplasmic kinases (RLCKs), with no apparent signal sequence or transmembrane domain. CDG1 and PBS1 as well as members of the NAK subfamily are classified into the RLCKVII subfamily, which consists of 47 members. The subfamily forms a monophyletic group separated from the other RLCKs. Though *CDG1* is most closely related to At5g02800 of Arabidopsis with 67.2% amino acid identity within the kinase domains, At5g02800 shows higher similarity to *PBS1* and At3g20530 with 81.5% and 73.5% identities, respectively. In addition, the highest similarity between *CDG1* and the other Arabidopsis RLCKVII genes is just as high as that observed between a monocot RLCKVII subfamily gene, *Esi47*, and Arabidopsis At3g09830 (69.3% identity). These observations indicate that *CDG1* is a rather isolated gene in the RLCKVII subfamily of Arabidopsis.

Though several genes of the subfamily have been described in the literature, physiological significance is well characterized for only one of them, *PBS1*. Limited information is available on three others: *ARSK1* may be involved in osmotic response of root (Hwang and Goodman, 1995), *APK2a* may be involved in flower development (Ito et al., 1997), and *Esi47* of wild wheatgrass may play a role in abscisic acid or GA signaling of root (Shen et al., 2001). *PBS1*, one of the most closely related genes to *CDG1* in the RLCKVII subfamily, is necessary for recognition of a bacterial pathogen, *Pseudomonas syringae* pv *phaseolicola*, by Arabidopsis (Swiderski and Innes, 2001). Pathogens secrete products of the avirulence (*avr*) genes into host plant cells. Specific recognition of the Avr protein of *P. syringae* pv *phaseolicola*, AvrPphB, requires at least two resistance genes in Arabidopsis, *PBS1* and *RPS5*. Recently AvrPphB has been shown to be a Cys protease (Shao et al., 2002). It is proposed that RPS5, which is a nucleotide binding site-LRR protein, recognizes pathogens by binding to a peptide released from proteolysis of PBS1 kinase by the AvrPphB protease (Schneider, 2002; Shao et al., 2002). According to this model the kinase activity of PBS1 may not be involved in recognition of pathogens.

CDG1 Expresses Ubiquitously at a Low Level in Wild-Type Plants

The *CDG1* transcript, in addition to a major ORF for protein kinase, contains two uORFs in the 5′ leader sequence (Fig. 4a). uORFs occur in about 7% to 10% of plant genes and may have a role in reducing the translation efficiency of the downstream major ORFs (for review, see Futterer and Hohn, 1996; Gallie, 1996; Geballe and Sachs, 2000). A uORF positioned upstream of the *Lc* gene of maize (*Zea mays*) reduces gene expression about 30-fold (Damiani and Wessler, 1993). A missense mutation that causes premature termination of uORF of Myb-like transcription factor, ATR1, results in a dominant mutant of Arabidopsis, in which *ATR1* expression is increased 2.5-fold (Bender and Fink, 1998). Interestingly, the 5′ leader of *Esi47* of wild wheatgrass also contains a 17-codon, small uORF, and the DNA sequence around the ATG start codon of this uORF has been shown to mediate the repression of basal-level expression of *Esi47* in maize callus (Shen et al., 2001). It is suggested that such repression may be relieved by abscisic acid. At3g09830, which is the gene most closely related to *Esi47* in Arabidopsis, also contains a uORF in the 5′ leader sequence (Shen et al., 2001). In contrast, *PBS1* does not include any uORFs.

The presence of two uORFs in *CDG1* gene raises the possibility that its expression is also regulated translationally. Although an RNA gel-blot analysis revealed more than a 100-fold accumulation of *CDG1* gene in *cdg1-D* plants (Fig. 5a), *CDG1* expression may be modified by translational repression in *cdg1-D* plants because transcripts of *CDG1* contain intact uORFs in *cdg1-D* (Fig. 4a). On the other hand, overexpressors of *CDG1* created in this study by the use of 35S promoter, contained only the kinase ORF of *CDG1* mRNA without uORFs but still had the *cdg1-D* phenotype (Fig. 6). Thus, it is apparent that the uORFs do not contribute to abnormal growth in transgenic plants, and this is probably also the case in *cdg1-D*. In wild-type plants, however, the *CDG1* mRNA level is quite low, and translation of the downstream ORF may be repressed by uORF. This suggests that activity of

CDG1 is tightly repressed by both transcriptional and translational control in the wild type. This is probably the reason why *CDG1* overexpression causes severe neomorphological abnormalities in *cdg1-D* mutants.

To better understand the function of *CDG1* gene in BR signaling, we have isolated loss-of-function mutants of *CDG1*. However, we could not detect any alterations of phenotype, including responses to BR, indicating that CDG1 is not an essential factor for BR in Arabidopsis. Redundancy of RLCKVII kinases in Arabidopsis might explain no aberrant phenotype of loss-of-function mutants of *CDG1*, though it is rather an isolated gene in the subfamily as described above. *cdg1-D*, at least, could be a useful tool for dissecting cellular processes involved in differential and elongation growth caused by BR.

MATERIALS AND METHODS

Plant Materials

Transformation of Arabidopsis ecotype Columbia with activation-tagging vector, pPCVICEn4HPT (Hayashi et al., 1992), was performed using vacuum infiltration methods (Bechtold and Pelletier, 1998). T_2 seeds of each hygromycin-resistant plant were used for mutant screening.

Seeds were surface sterilized with 1.5% (v/v) sodium hypochlorite and 0.02% Triton X-100 for 5 min with vigorous shaking, washed several times with sterile water, chilled in water at 4°C for 2 to 4 d, and plated onto half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with half-strength Gamborg B5 vitamins (Gamborg et al., 1968), 1% Suc, and 1% agar (Wako, Osaka). The plates were illuminated with continuous white light for 24 h at 23°C to induce germination. In some experiments, plants were grown on a 1:1 (v/v) mixture of vermiculite and Metromix 350 (Scotts-Sierra Horticultural Products, Marysville, OH) in a pot and grown at 23°C under continuous light.

DNA Preparation and Plasmid Rescue

Plants that had been stored frozen were ground with mortar and pestle with liquid N_2. Ten milliliters of cetyltrimethylammonium bromide buffer that consisted of 140 mM sorbitol, 220 mM Tris-HCl, pH 8.0, 22 mM EDTA, 800 mM NaCl, 1% sarkosyl, and 0.8% hexadecyltrimethylammonium bromide were added to the powdered tissue. The homogenate was heated at 65°C for 15 min, and the same volume of chloroform was added. The mixture was mixed gently by a rotator for 15 min and centrifuged at 3,000 rpm for 10 min. The upper aqueous phase was taken, and the same volume of chloroform was added to it again. The sample was mixed and centrifuged as described above. After centrifugation, DNA was recovered by ethanol precipitation. Then DNA was dissolved in 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA (Tris-EDTA) buffer, and centrifuged at 90,000 rpm for 16 h in 1 mg/mL CsCl (CS100GX; Hitachi, Tokyo). After centrifugation, DNA was recovered and purified and finally dissolved in Tris-EDTA buffer.

For plasmid rescue, 2 μg of DNA were digested overnight with *Eco*RI (Takara, Kusatsu, Japan). After phenol-chloroform extraction, digested DNA was ligated at 16°C overnight with T4 DNA ligase (Takara), precipitated with ethanol, and was used for transformation of *Escherichia coli* DH5α competent cells (Toyobo, Tokyo) by heat shock at 42°C. Plasmid DNA extracted from ampicillin-resistant clones was sequenced using a Thermo Sequencing Primer Cycle sequencing kit 7-deaza dGTP (Amersham, Buckinghamshire, UK) with the primer 5′-TGGACGTGAATGTAGACACGTCGA-3′, which was designed from the nucleotide sequence of the T-DNA left border. Denaturing gel electrophoresis was run by a DNA sequencer (4000 L; LI-COR, Lincoln, NE).

RT-PCR

RT-PCR was performed with the Access RT-PCR system (Promega, Madison, WI), according to the manufacturer's directions. The primers for amplification of *CDG1* cDNA were 5′-TTTACTTCACTTTCATGGAGTCG-GAGT-3′ and 5′-ATGGTTAGTTGCTTGTGTTTTCGT-3′. For amplification of *CPD*, *BAS1*, and 18S ribosomal RNA, primers were designed according to Shimada et al. (2003). The numbers of amplification cycles were optimized to be quantitative in each gene. For *CPD* and *BAS1*, PCR was carried out between 25 and 29 cycles, and PCR with 27 and 29 cycles was found optimal, respectively. We conducted PCR for *CDG1* with 20 and 25 cycles, and found the latter optimal. For 18S rRNA, we compared PCR with 13 and 15 cycles, and found the former condition optimal. RT-PCR products were detected by staining with ethidium bromide after electrophoresis or DNA gel-blot analysis using ^{32}P-labeled probes. Each experiment was repeated independently three times.

Hormone Treatment

Seeds were imbibed in water in the dark at 4°C for 4 d, surface sterilized as described above, incubated for 1 d at 23°C under continuous white light in liquid medium that contained half-strength Murashige and Skoog salt, half-strength B5 vitamins, and 1% Suc, transferred onto 1% agar medium that consisted of the above nutrients supplemented with various growth regulators, and grown at 23°C under continuous white light thereafter. Hypocotyl length was measured 1 week after the transfer.

Transgenic Plants

RT-PCR products of the *CDG1* kinase ORF were cloned into a binary vector pBI121 (CLONTECH Laboratories, Palo Alto, CA) under the control of the CaMV 35S promoter in the sense orientation. Then, the plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Arabidopsis wild-type plants, ecotype Columbia, were transformed with *Agrobacterium* by the floral dip method (Clough and Bent, 1998). T_1 plants were selected by kanamycin resistance at 20 μg/mL.

Isolation of Suppressors

About 500 mg of seeds were harvested from heterozygous *cdg1-D* plants, treated with 0.1% ethyl methanesulfonate for 12 h at room temperature, extensively washed with water, vernalized at 4°C for 2 d, and sown in the soil. Plants showing the wild-type phenotype were discarded from the M_1 population. The M_1 plants that showed heterozygous or homozygous *cdg1-D* phenotype were self-pollinated, and M_2 seeds were collected in five pools. About 10,000 M_2 seeds from each pool were plated onto agar medium containing 40 μg/mL hygromycin and grown under white light for 2 weeks. Plants that showed suppression of *cdg1-D* phenotype were selected and grown in the soil.

Microscopic Analysis

Light microscopic observations were carried out according to the method of Kurata and Yamamoto (1998). Plants were fixed overnight in formaldehyde-acetic acid and dehydrated in a graded-ethanol series at room temperature. Completely dehydrated samples were embedded in Technovit 7100 (Kulzer, Wehrheim, Germany) as described by Tsukaya et al. (1993). Sections of 5-μm thickness were cut with Histoknives (Kulzer) on a microtome (RM2135; Leica, Nubloh, Germany), affixed to glass slides, and stained with 0.1% (w/v) toluidine blue at room temperature for 1 min. Specimens were examined with a microscope (Axioplan; Zeiss, Jena, Germany), and their images were recorded by a digital camera (DXM1200; Nikon, Tokyo). Intact seedlings were examined with a dissection microscope (Stemi 2000-C; Zeiss) after immersion in India ink.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession number AB099698.

ACKNOWLEDGMENTS

We are grateful to Ms. N. Nishizaki for her technical assistance. This study was partly carried out in the Laboratory of Genetic Research, Center for Advanced Science and Technology, Hokkaido University.

Received May 26, 2004; returned for revision July 31, 2004; accepted July 31, 2004.

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