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Genetics

Rice gibberellin-insensitive dwarf mutant gene *Dwarf 1* encodes the α-subunit of GTP-binding protein

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ABSTRACT **A rice *Dwarf 1* gene was identified by using a map-based cloning strategy. Its recessive mutant allele confers a dwarf phenotype. Linkage analysis revealed that a cDNA encoding the α-subunit of GTP-binding protein cosegregated with *d1* in 3,185 *d1* segregants. Southern hybridization analysis with this cDNA as a probe showed different band patterns in several *d1* mutant lines. In at least four independent *d1* mutants, no gene transcript was observed by Northern hybridization analysis. Sequencing analysis revealed that an 833-bp deletion had occurred in one of the mutant alleles, which resulted in an inability to express GTP-binding protein. A transgenic *d1* mutant with GTP-binding protein gene restored the normal phenotype. We conclude that the rice *Dwarf 1* gene encodes GTP-binding protein and that the protein plays an important role in plant growth and development. Because the *d1* mutant is classified as gibberellin-insensitive, we suggest that the GTP-binding protein might be associated with gibberellin signal transduction.**

Dwarf mutants in plants are crucial for elucidating regulatory mechanisms for plant growth and development. This character is also favored in breeding. Dwarf mutants have been isolated in many species and have been extensively analyzed for their mode of inheritance and their response to plant hormones. There are various reasons for their dwarf phenotypes, associated with, for example, gibberellins (1–3), brassinosteroids (4, 5), abnormal cell walls (6), and abnormal cell elongation (5, 7).

In rice, at least 54 dwarf mutants are known, but only two, *d18* and *d35*, are known to have a deficiency in their gibberellin biosynthetic pathway (8, 9). Rice dwarf mutant Daikoku, carrying the *d1* gene, was first isolated as a spontaneous mutant that was not only short but had broad, dark green leaves, compact panicles, and short, round grains (Fig. 1). These phenotypes are all induced by a recessive allele (*d1*) of the *Dwarf 1* (*D1*) gene and are thought to reflect aberrant physiological and biochemical pathways in plant growth and development. The rice *d1* mutant was classified as gibberellin-insensitive (10). Gibberellins (GAs) are a large family of tetracyclic diterpenoid plant growth regulators and are associated with a number of plant growth and development processes such as seed germination, stem elongation, flowering, and fruit development (1–3) and regulation of gene expression in the cereal aleurone layer (11). Many GA-related mutants have been isolated from many plant species and have been classified. GA-insensitive mutants that did not respond to exogenous GAs were defined as dwarf mutants: *GAI* in *Arabidopsis*, *D8* and *D9* in maize, *Rht3* in wheat (3), and *d1* in rice (10).

These mutants were thought to be associated with GA-signal transduction. The mechanisms of signal transduction triggered by GAs are still unknown. Isolation and characterization of genes defining the dwarf mutation caused by GA insensitivity can help to clarify not only the molecular mechanisms of plant growth and development but also signal transduction of GAs.

We report here the map-based cloning of the rice *D1* gene. The amino acid sequence of *D1* revealed that it encodes the α-subunit of GTP-binding protein (G protein). Although the α-subunit of G protein homologues have been isolated from several plants (12, 13), their functions are so far not clear. The most intriguing finding in this study is that the α-subunit of G protein plays an important role in plant growth and development.

MATERIALS AND METHODS

Plant Materials. Six *d1* mutant lines (HO532, HO533, HO537, HO538, HO541, and HO552) have been maintained for more than 60 years at Kyushu University (14). These mutants were all *japonica* and all spontaneous mutants. FL2 (*d1*) is a marker line derived from HO538. Two *d1* mutant lines (CM392 and CM1729) induced by *N*-methyl-*N*-nitrosourea (mutants of Kinmaze) were provided by H. Satoh (Institute of Genetic Resources, Kyushu University, Japan).

Mapping Population. Substitution lines that substitute chromosomes of Kasalath (*indica*) for that of Nipponbare (*japonica*) were used for crossing. Given that *d1* was located on chromosome 5, substitution line SL18, which substitutes chromosome 5 of Kasalath (*indica*), was crossed with line FL2 (*d1*) (*japonica*). About 13,000 F_2 seeds were sown in a nursery. At the seedling stage, 3,185 dwarf plants were selected and transplanted into a paddy field. One month after transplanting, two green leaves of each individual were collected, and leaves of five plants were combined to obtain 637 pooled samples (15).

DNA Extraction, Southern Hybridization, and Linkage Analysis. The cetyltrimethylammonium bromide (CTAB) method (16) was used with minor modifications for extracting total DNA from rice leaves. The extracted DNA was digested with restriction enzymes. Three micrograms of each digested DNA was loaded on a 0.8% agarose gel and run for 16 h at 20 V and then blotted onto a nylon membrane. Southern hybridization (17) was done with horseradish peroxidase-labeled restriction fragment-length polymorphism (RFLP) markers according to the protocol for the Enhanced Chemiluminescent (ECL) direct nucleic acid labeling and detection system (Amersham Pharmacia). Linkage analysis was done by using the pooled-sampling method (15).

Screening of Yeast Artificial Chromosome (YAC) Library and P1-Derived Artificial Chromosome (PAC) Library. A rice

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Abbreviations: *D*1, *Dwarf 1*; GA, gibberellin; G protein, GTP-binding protein; YAC, yeast artificial chromosome; EST, expressed sequence tag; PAC, P1-derived artificial chromosome; RFLP, restriction fragment-length polymorphism; cM, centiMorgan.

Data deposits: The sequences reported in this paper have been submitted to the DNA Data Bank of Japan (accession nos. AB028602 and AB028603).

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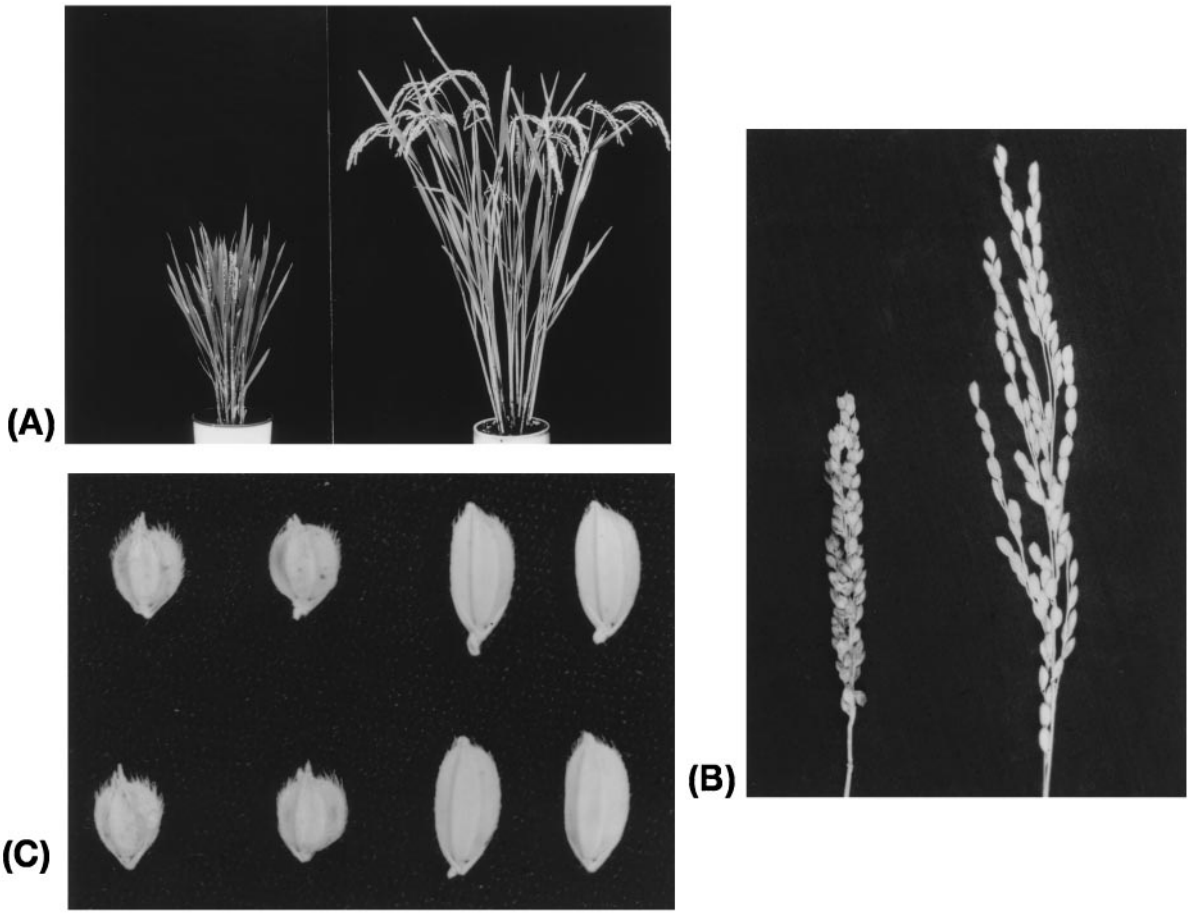

FIG. 1. Phenotypes of *d1* mutant, Daikoku. In each photograph, Daikoku is on the left and Nipponbare (wild-type variety) is on the right. (*A*) Plant height. (*B*) Panicle. (*C*) Seeds.

YAC library containing 6,934 clones with an average insert size of 350 kilobases and covering six times the haploid genome was constructed previously (*japonica* cultivar Nipponbare; ref. 18). All YAC clones were blotted on five high-density filters, multiplied on many replica filters, and screened by colony hybridization with RFLP markers or a three-step PCR with YAC end-fragment DNA.

A rice PAC library (*japonica* cultivar Nipponbare) was kindly provided by the Rice Genome Research Program (RGP). From this library, 18,432 clones were screened by PCR with Sequence Tagged Site (STS) primers.

Three-step PCR Screening of Expressed Sequence Tags (ESTs). A screening system using a three-step PCR to isolate YAC clones with STS markers (19) was modified. The 20-base primers were designed from the 3′-end sequences of rice ESTs. In the first screening, a PCR reaction was done with nine superpools of YAC DNA mixture (W pools), each coming from 9 × 96 clones, yeast host strain AB1380, and rice cultivar Nipponbare. In the second screening, 29 subpools (8 X pools, 12 Y pools, and 9 Z pools) of each W pool that was positive in the first screening were surveyed with the same primers. The YAC clones passing the second PCR were considered as candidates carrying ESTs, and the positive clones were finally confirmed by a third PCR using the individual candidate YAC DNAs as templates.

Isolation of YAC End DNA Fragment. YAC end DNA fragments were amplified by using a slightly modified cassette-PCR method (20) and were cloned to the TA vector (Invitrogen).

RNA Extraction and Northern Hybridization. Total RNA was extracted from 2-week-old seedlings by using a single-step method (21) with minor modifications. Three micrograms of poly(A)$^+$RNA was recovered from total RNA by Oligotex-dT30 (Takara Shuzo, Kyoto) and was electrophoresed on 1.0% agarose containing 18% formaldehyde for 3 h at 75 V and then blotted onto a nylon membrane. Northern hybridization was done in Rapid hybridization buffer (Amersham Pharmacia) at 65°C overnight with ^{32}P-labeled cDNA as a probe. The nylon membrane was washed twice in 1× SSPE (3M NaCl/173 mM $NaH_2PO_4{\cdot}2H_2O$/25 mM EDTA, pH 7.4) buffer containing 1% SDS at 65°C for 20 min then twice in 0.1× SSPE buffer containing 1% SDS at 65°C for 30 min. The banding patterns were detected on x-ray film after a week-long exposure at −80°C.

Sequence Analysis. The primers were designed from the genomic sequences of the α-subunit of G protein in rice (IR36, *indica*) (22). Genomic regions of the α-subunit of G protein in Nipponbare (*japonica*) and a *d1* mutant HO541 (*japonica*) were amplified by PCR with the primers. Amplified products were cloned to the TA vector (Invitrogen) and were sequenced.

Isolation of Cosmid Clone Carrying the *D1* Gene and Complementation Test. A cosmid vector (pPZP2CH-lac) for complementation testing was kindly provided by T. Fuse of the Bio-oriented Technology Research Advancement Institution, Japan. A PAC clone carrying the candidate *D1* gene was partially digested with *Sau*3AI, and fragment DNAs were ligated into the *Bam*HI cloning site of the cosmid vector. The cosmid clones were packaged *in vitro* with GigaPak III Gold (Stratagene) and infected into competent *Escherichia coli* XL1-BlueMRF′. Cosmid clones carrying the candidate *D1* gene were screened by using PCR with STS primers.

Transformation was used for complementation testing with the *Agrobacterium* system (23). The cosmid DNA carrying the *D1* gene was infected into callus of a *d1* mutant line in darkness

at 25°C for 3 days. The callus was regenerated in a growth chamber, and the regenerated plants were grown in an isolated greenhouse.

RESULTS

High-Resolution Mapping and Physical Mapping. In general, variations in genotypes of individual F_2 plants derived from a cross between *japonica* and *indica* varieties create large variations in phenotypes. This variation causes difficulties in identifying true mutants among F_2 plants, such as mutants for culm height. However, individual F_2 plants derived from crosses between *japonica* marker line FL2 (*d1*) and SL18, which substitutes chromosomes of Kasalath (*indica*) outside the target region of chromosome 5 for those of Nipponbare (*japonica*), showed clear phenotypic differences for *d1*. We could identify dwarfism in individual plants with high reliability. Of about 13,000 F_2 plants derived from the cross, 3,185 dwarf (*d1* homozygous) plants were identified. DNA of the 637 pools was extracted for pooled-sample mapping. Southern hybridization and linkage analysis were done with 13 RFLP markers between C309 and G1458, which were mapped around *d1* (24) on chromosome 5 (25). Of the 637 pools, 37 and 71 recombinant pools were identified with RFLP markers V147 and G5004, respectively, as the closest markers flanking either side of *d1*. *d1* was located between these markers within 1.65 centiMorgan (cM) (Fig. 2). To select the individual recombinant plants from each pool, DNA was extracted from the 540 individual plants (5 plants × 108 recombinant pools), and each genotype was surveyed by using V147 and G5004 as probes. As a result, 37 and 71 recombinant plants were identified individually and used for fine mapping between V147 and G5004.

A YAC library was screened for construction of YAC contigs covering the *d1* region. Four YACs (Y5483, Y3401, Y4480, and Y4336) were identified with RFLP marker V147, and two (Y2287 and Y4611) were identified with G5004 (Fig. 2). Both end sequences of these YACs were amplified by using the cassette-PCR method, and the products were also used for linkage mapping by RFLPs. Fragment Y5483R was mapped between *d1* and G5004 and linked to *d1* within 0.03 cM. By using Y5483R as a probe, three YACs (Y1988, Y3745, and Y4488) were also identified. End-fragment DNAs of Y3745R and Y4488R were both mapped between *d1* and V147 with genetic distances from *d1* of 0.12 and 0.19 cM, respectively. As a result, *d1* was located between Y3745R and Y5483R within a 0.15-cM genetic distance (Fig. 2).

EST Mapping. We used large-scale EST mapping on a YAC physical map. Three ESTs (E50508, S32, and S5933) were mapped onto the YAC clones in the *d1* region. YACs Y5483, Y3401, Y4336, and Y4480 carried the sequences of E50508 and S32. YACs Y5483, Y1988, Y4488, and Y3745 carried the sequence of S5933. The three ESTs were used for genetic mapping in the same population as above. As a result, S5933, E50508, and S32 were mapped to within 0, 0.5, and 0.5 cM, respectively, of *d1* in the 3,185 *d1* segregants (Fig. 2). The cosegregated cDNA S5933 (350-bp insert) was derived from green shoots, and its translated amino acid sequence completely coincided with the sequence from Phe-332 to Thr-380 of the α-subunit of rice G protein *RGA1* (22, 26).

Mutant Analysis. Allelic tests were used to confirm the *d1* allelism among the nine *d1* mutant lines (FL2, HO532, HO533, HO537, HO538, HO541, HO552, CM392, and CM1729). F_1 hybrids from crosses between FL2 and each of the other eight lines were all *d1* dwarf. Allelic testing confirmed that these lines were caused by the mutation at the *D1* locus. Genomic Southern hybridization analysis of these *d1* mutant lines and two wild-type varieties, Nipponbare (*japonica*) and Kasalath (*indica*), was done with S5933 as a probe. No polymorphism was observed between the wild types, but five different band patterns were observed among the nine mutants (Fig. 3*A*). These results strongly suggested that several types of structural variations occurred in the region probed by S5933 in the nine *d1* mutant lines.

Poly(A)$^+$RNA was extracted from four *d1* mutants (FL2, HO532, HO541, and HO552) that showed different types of banding patterns in genomic Southern hybridization (Fig. 3*A*) and from Nipponbare and Kasalath. Northern analysis was done with S5933 as a probe. A 1.8-kilobase translated product that coincided well with the reported size for mRNA of the α-subunit of rice G protein (22, 26) was observed in Nipponbare and Kasalath but not in the independent four *d1* mutant lines (Fig. 3*B*).

The genomic region of the α-subunit of rice G protein in both Nipponbare and HO541 was cloned and sequenced. Deletion of 833 bp was detected in HO541 between the first exon and intron.

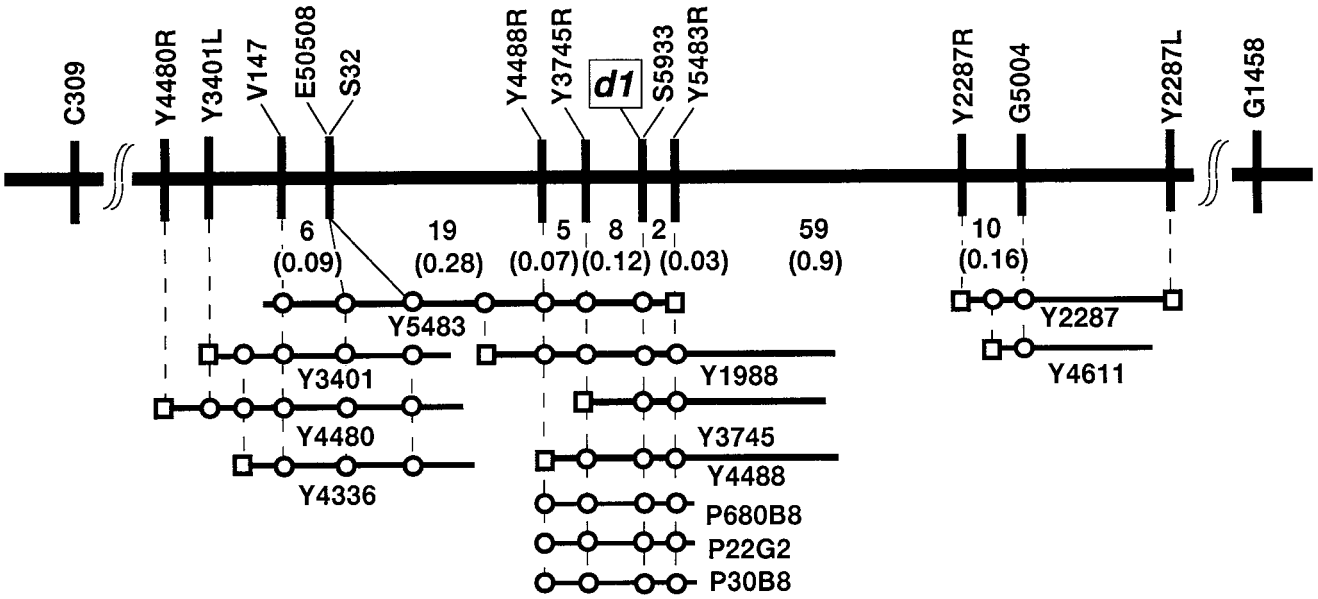

FIG. 2. High-resolution RFLP linkage map and physical map of the *d1* locus. The vertical bar represents the RFLP markers, and the numbers of recombinants are indicated under the linkage map. Genetic distances between adjacent markers are shown in parentheses. □, End-fragment DNA of YAC clone; ○, RFLP markers or YAC end-fragment DNA contained in the YAC clones (Y number) and PAC clones (P number).

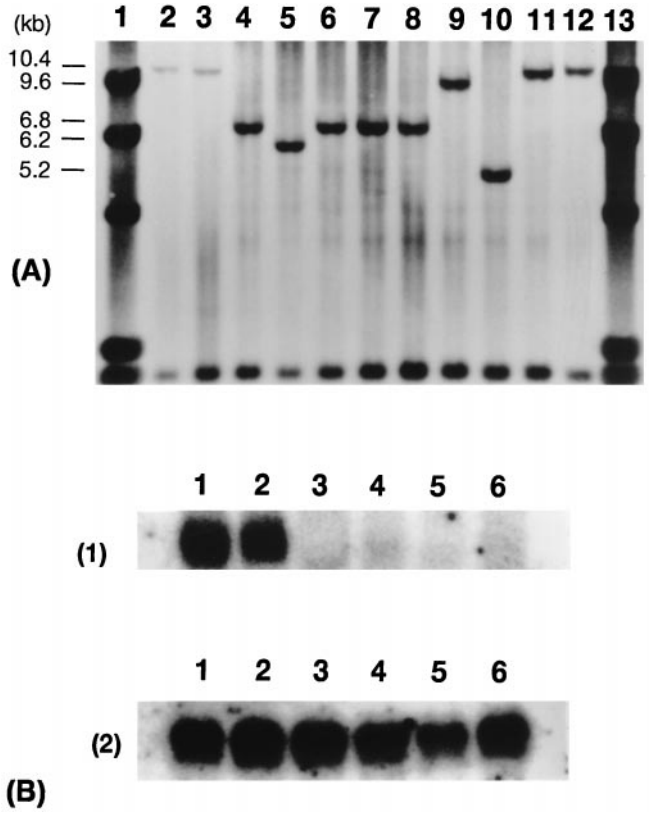

FIG. 3. (*A*) Genomic Southern hybridization analysis of nine *d1* mutants using S5933 as a probe. Lanes: 1, λ/*Hin*dIII; 2, Nipponbare; 3, Kasalath; 4, FL2; 5, HO532; 6, HO533; 7, HO537; 8, HO538; 9, HO541; 10, HO552; 11, CM382; 12, CM1792; 13, λ/*Hin*dIII. Three micorgrams of total DNA were digested with *Hin*dIII. (*B*) Northern hybridization analysis using S5933 (*1*) or S14002 (*2*) (actin) as a probe. Lanes: 1, Nipponbare; 2, Kasalath; 3, FL2; 4, HO532; 5, HO541; 6, HO552.

Complementation Test. A PAC library was screened for covering the *d1* gene. Three PACs (P680B8, P22G2, and P30B8) were identified by PCR with STS primers of EST S5933 (Fig. 2). The flanking markers on both sides of the *d1* gene, Y3745R and Y5483R, were contained in these PACs. A PAC clone (P22G2) was subcloned into cosmid vector (pPZP2CH-lac), and a cosmid clone carrying the α-subunit of rice G protein (C6) was screened by PCR with STS primers of EST S5933. Because this cosmid clone had the flanking markers on both sides of the *d1* gene Y3745R and Y5483R, the *D1* gene was included in the cosmid clone.

The cosmid clone was transformed into a *d1* mutant line (HO541). Twenty-one transgenic plants with C6 clone in HO541 were obtained. Of 21 plants, 14 plants showed a normal phenotype and 7 plants showed a dwarf phenotype. Eleven transgenic plants with only vector in HO541 were obtained as control, and all showed a dwarf phenotype (Fig. 4*A*, *1* and *2*). Those transgenic plants that showed a normal phenotype were tested if they carried the cosmid clone. Primers were designed between the first exon and the second exon of the region of α-subunit of rice G protein, because HO541 has an ~833-bp deletion here. DNA of transgenic plants was extracted, and PCR amplification was performed with the primers. Transgenic plants showing a normal phenotype had the band of cosmid clone. Transgenic plants with the vector did not have the bands of cosmid clones (Fig. 4*B*). To test inheritance of transgenic plants, the segregation of the second generations of the transgenic plants was observed. The segregations of the second generation of the three normal transgenic plants were 60 normal and 20 dwarf, 46 normal and 18 dwarf, and 40 normal and 10 dwarf; all segregations were close to a 3:1 ratio, as expected.

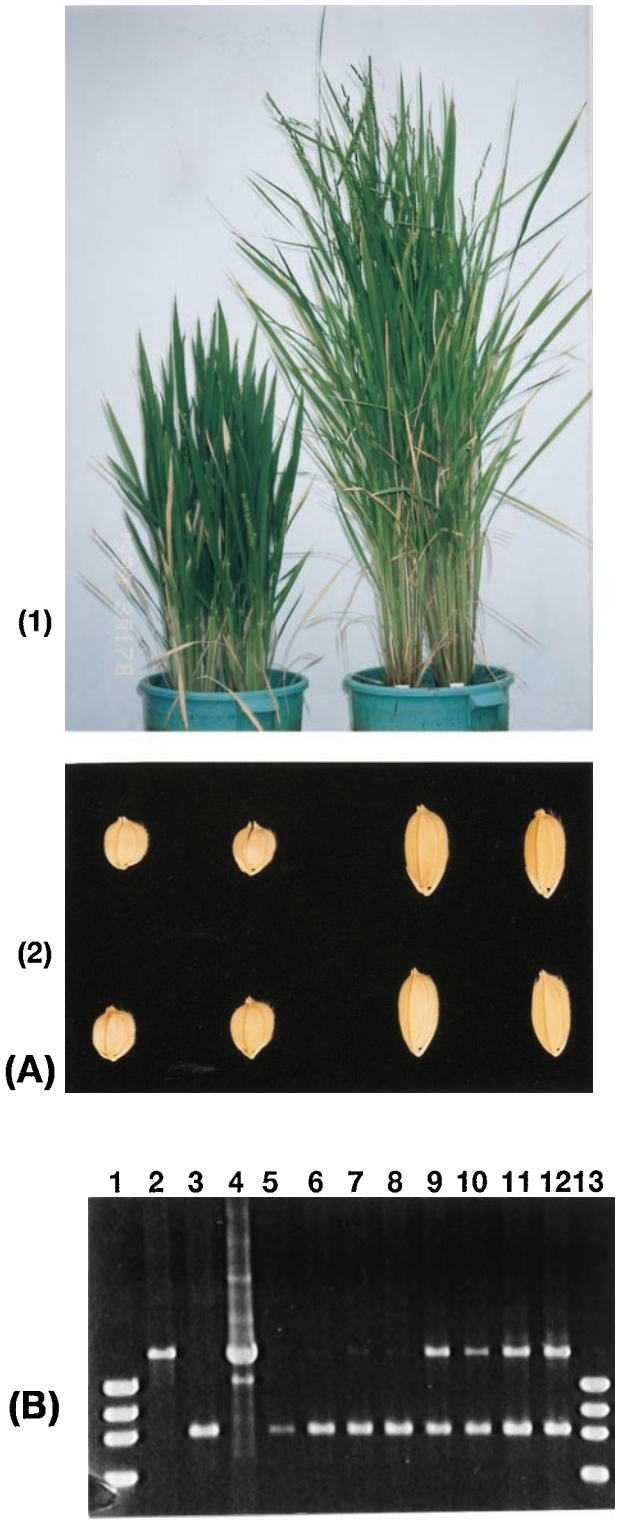

FIG. 4. Complementation test. (*A*) Transgenic plants of *d1* mutant (HO541). (*1*) Left, transgenic plant with cosmid vector; right, transgenic plant with cosmid clone (C6) containing *D1* genomic region. (*2*) Left, seeds of transgenic plant with cosmid vector; right, seeds of transgenic plant with cosmid clone (C6). (*B*) Detection of integrated *D1* gene in transgenic plants. Lanes: 1, marker (φX174/*Hae*III); 2, Nipponbare (normal); 3, HO541 (*d1* mutant); 4, cosmid clone (C6); 5–8, transgenic plants with cosmid vector in HO541 (phenotypes were all dwarf); 9–12, transgenic plants with cosmid clone C6 (phenotypes were all normal); 13, marker (φX174/*Hae*III).

DISCUSSION

Linkage analysis revealed that a cDNA encoding the α-subunit of G protein cosegregated with *d1* in 3,185 *d1* segregants. Southern hybridization analysis using this cDNA as a probe showed different band patterns in several *d1* mutant lines. In at least four independent *d1* mutants, no gene transcript was observed by Northern hybridization analysis. Sequence analysis revealed that an 833-bp deletion had occurred in one of the mutant alleles (HO541). This result coincides well with the genomic Southern hybridization pattern using S5933 as a probe, in which an ~800-bp difference in length was observed between Nipponbare (10.4 kilobase) and HO541 (9.6 kilobase) (Fig. 3*A*). Based on these results, we conclude that the rice *D1* gene encodes the α-subunit of G protein. Transgenic plants restored the normal phenotype. This result finally confirmed the responsibility of the α-subunit of G protein for the *d1* dwarf mutation in rice.

It is well known that G protein plays an important role in signal transduction in animals and microbes (27, 28). Heterotrimeric G protein has also been identified in several plant species (12, 13), and evidence of relatedness of G protein to signal transduction, such as in the K^+ channel, has been shown (29).

Rice dwarf mutant *d1* has dark green leaves, compact panicles, and short, round grains. Because these forms are all induced by *d1* as pleiotropic effects of this gene, the heterotrimeric G protein in rice that *D1* encodes is likely to play a key role in controlling rice growth and development. The abnormal phenotype was exhibited as a result of the lack of G protein as a signal associated with cellular growth, differentiation, and development in *d1* mutants.

GAs are well known as regulators of plant growth and development, including seed germination, stem elongation, flowering, and fruit development (1–3), and regulation of α-amylase expression in the cereal aleurone layer (11). Recently, it has been shown that heterotrimeric G proteins are implicated in inducing the expression of α-amylase in oat aleurone by GA (32). This result is very significant. The rice mutant *d1* was characterized as GA-insensitive, because while production of α-amylase by application of GA_3 was saturated at 10^{-8} M GA_3 in Nipponbare (normal cultivar) and Tanginbouzu dwarf (*d18*-gibberellin sensitive), no production of α-amylase in the *d1* mutant was detectable at that concentration (10). Recently, putative G protein-coupled receptors were isolated from *Arabidopsis* (30), and one of these G protein-coupled receptors that influences sensitivity to cytokinins, one of the plant hormones, has been identified in *Arabidopsis* (31).

The G protein that the *D1* gene encodes in rice might be associated with GA signal transduction. Many GA-associated mutants have been isolated and classified (3). GA biosynthetic pathways are well studied, and several genes coding enzymes catalyzing steps in GA biosynthesis have been isolated from GA-deficient mutant plants (33–37). For example, GA-sensitive dwarf mutant *le* in pea, first identified by Gregor Mendel, is reversed by GA_1 and was shown to be deficient in GA-3-β-hydroxylase, which converts GA_{20} to GA_1 in the GA biosynthetic pathway (38, 39). Even in this case, however, the molecular mechanism of signal transduction after triggering by GA is so far unknown. However, GA-insensitive and GA-constitutive mutants are useful for elucidating the mechanism of signal transduction of GAs; analysis of these mutants can help to clarify the molecular mechanisms. Several mutants have been defined as unresponsive to GA, for example, *GAI* in *Arabidopsis*, *D8* and *D9* in maize, *Rht3* in wheat, or as constitutive GA-responsive mutants, such as *spy* and *rga* in *Arabidopsis* and *sin* in barley (3). *GAI*, *spy*, and *rga* were isolated in *Arabidopsis* (40–42). *GAI* works as a repressor of GA responses. *spy* contains a tetratricopeptide repeat region, which suppresses GA signal transduction and is epistatic to *gai* (41), and *RGA* is also a negative regulator of GA signalling (42). Regulators of GA signal transduction have been accumulating, but the mechanisms of signal components in GA-signal pathway triggered by GAs are still not clear. Our identification in rice of the G protein gene responsible for plant growth and development might help elucidate the signal-transduction pathway related to GA if the dwarfism is caused by a deficiency in this pathway. It is necessary to present genetical and biochemical evidence to prove direct participation of G protein to GA signaling in rice. We have several rice elongated mutants showing phenotype similar to that of *Spy* mutants in *Arabidopsis*. These rice mutants may associate with GA-signal transduction. Genetic analysis of epistasis with *d1* and cloning of the *spy*-like gene are required. Also, further analysis of the structure of a plausible GA receptor and of the biochemical interactions between the receptor and G proteins is required to clarify the signal-transduction system mediated by the G protein in plants.

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