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Characterization of a Rice Chlorophyll-Deficient Mutant Using the T-DNA Gene-Trap System

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We have previously generated a large pool of T-DNA insertional lines in rice. In this study, we screened those T-DNA pools for rice mutants that had defective chlorophylls. Among the 1,995 lines examined in the T2 generation, 189 showed a chlorophyll-deficient phenotype that segregated as a single recessive locus. Among the mutants, 10 lines were β-glucuronidase (GUS)-positive in the leaves. Line 9-07117 has a T-DNA insertion into the gene that is highly homologous to *XANTHA-F* in barley and *CHLH* in *Arabidopsis*. This *OsCHLH* gene encodes the largest subunit of the rice Mg-chelatase, a key enzyme in the chlorophyll branch of the tetrapyrrole biosynthetic pathway. In the T2 and T3 generations, the chlorina mutant phenotypes are co-segregated with the T-DNA. We have identified two additional chlorina mutants that have a *Tos17* insertion in the *OsCHLH* gene. Those phenotypes were co-segregated with *Tos17* in the progeny. GUS assays and RNA blot analysis showed that expression of the *OsCHLH* gene is light inducible, while TEM analysis revealed that the thylakoid membrane of the mutant chloroplasts is underdeveloped. The chlorophyll content was very low in the *OschlH* mutants. This is the first report that T-DNA insertional mutagenesis can be used for functional analysis of rice genes.

Keywords: Chlorina — GUS — Light inducible — Magnesium chelatase — *Oryza sativa* — T-DNA.

Abbreviations: GUS, β-glucuronidase; RT-PCR, reverse transcription-PCR.

Introduction

T-DNA insertional mutagenesis has been successfully used to isolate a number of genes from *Arabidopsis* (Azpiroz-Leehan and Feldmann 1997, Takechi et al. 2000, Huang et al. 2001, Ishiguro et al. 2001, Nesi et al. 2001). T-DNA generates low copy insertions that are stable through the next generations (Azpiroz-Leehan and Feldmann 1997, Jeon et al. 2000a). Establishing T-DNA insertional mutant pools in *Arabidopsis* is simple and rapid because the in plants procedures result in high transformation frequencies (Clough and Bent 1998).

T-DNA is believed to be randomly inserted into plant chromosomes, which allows for a greater chance of finding knockouts in a given gene (Azpiroz-Leehan and Feldmann 1997, Jeon et al. 2000a). However, application of the T-DNA insertional mutagenesis to other plant species has been difficult because it requires development of an efficient transformation procedure. We recently reported the establishment of a large pool of T-DNA insertional lines in rice (*Oryza sativa* cv. Japonica), using the scutellum-driven embryonic calli (Lee et al. 1999, Jeon et al. 2000a, Jeon and An 2001). The vector used for mutagenesis carries the promoter-less β-glucuronidase (GUS) reporter gene immediately next to the right border of T-DNA. Therefore, when T-DNA is inserted into a gene in the proper orientation, gene fusion can be generated between the endogenous gene and GUS (Springer 2000). T-DNA-tagged lines that carry such a gene fusion can be verified by GUS assay. We have screened those insertional lines and have observed that 2% are GUS positive in the leaves (Jeon et al. 2000a).

Here, we report our study on isolating the T-DNA flanking sequence from a yellow-green (chlorina) mutant in order to identify the knockout gene that encodes for an enzyme involved in chlorophyll biosynthesis. Chlorophyll is the principal pigment that traps light energy; its defect results in this chlorina phenotype in various plants including *Arabidopsis* (Meinke and Koornneef 1997). The *ch42* mutant of *Arabidopsis* is deficient in chelating the Mg^{2+} ion into protoporphyrin IX to form Mg-protoporphyrin IX, which is then converted to the chlorophylls in a series of enzymatic steps (Koncz et al. 1990, Walker and Willows 1997, Rissler et al. 2002). The magnesium chelatase comprises three subunits – CHLI, CHLD, and CHLH (Zsebo and Hearst 1984, Gibson et al. 1995, Kruse et al. 1997, Papenbrock et al. 1997). The first plant gene homologous to a Mg-chelatase subunit was the T-DNA-tagged *chlI* (Koncz et al. 1990). The *CHLH* genes were found by transposon-tagged gene inactivation in snapdragon (Hudson et al. 1993) and by ethyl methanesulfonate mutagenesis in *Arabidopsis* and

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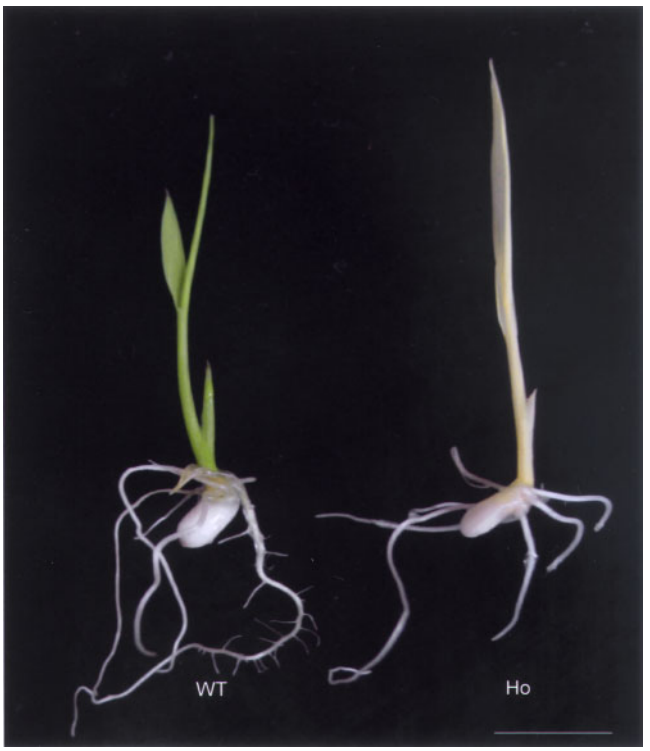

Fig. 1 Mutant phenotypes from Line 9-07117. Wild-type progeny (left, WT) and homozygous progeny (right, Ho) of a heterozygous line grown for 7 d under continuous light. Scale bar = 1 cm.

barley (Jensen et al. 1996, Mochizuki et al. 2001). The third subunit, CHLD, has proven to be essential for the formation of an active enzyme complex in tobacco, soybean, and yeast (Gibson et al. 1995, Papenbrock et al. 1997, Grafe et al. 1999, Luo et al. 1999). Genetic studies on mutant barley lines and yeast two-hybrid experiment on the three subunits of tobacco Mg-chelatase have also shown that when any one of the three subunits is mutated, Mg-chelatase activity is destroyed (Kannangara et al. 1997, Papenbrock et al. 1997). Although all the enzymes involved are nuclear encoded in higher plants, tetrapyrrole metabolism occurs in the chloroplasts.

Results

Screening chlorophyll-deficient mutants from the T-DNA insertional lines

We examined 1,995 T-DNA insertional lines for mutants in T2-generation rice seedlings. Several leaf defects were found, including 85 albino, 64 pale-green, 25 chlorina (yellow), 11 stripe, and 4 zebra. A total of 189 lines appeared to be chlorophyll deficient. If T-DNA insertion generates a gene fusion that results in a mutant phenotype, it can be identified by GUS assay. Examination of GUS activity in our seedling leaves showed that 10 lines were positive, with two of them being mesophyll specific.

Identification of a mutation in OsCHLH

We used the inverse PCR method to determine the T-DNA flanking sequences of the 10 *GUS*-expressing mutant lines (Triglia et al. 1988, Thomas et al. 1994, Akiyama et al. 2000). Sequence analysis using the Monsanto database identified one of the lines, 9-07117, as having a T-DNA insertion in a gene that showed high homology with *CHLH* genes from several plant species. Those particular genes encode for the largest subunit of magnesium chelatase. We designated this rice gene as *OsCHLH*. The phenotype of the 9-07117 mutant seedlings

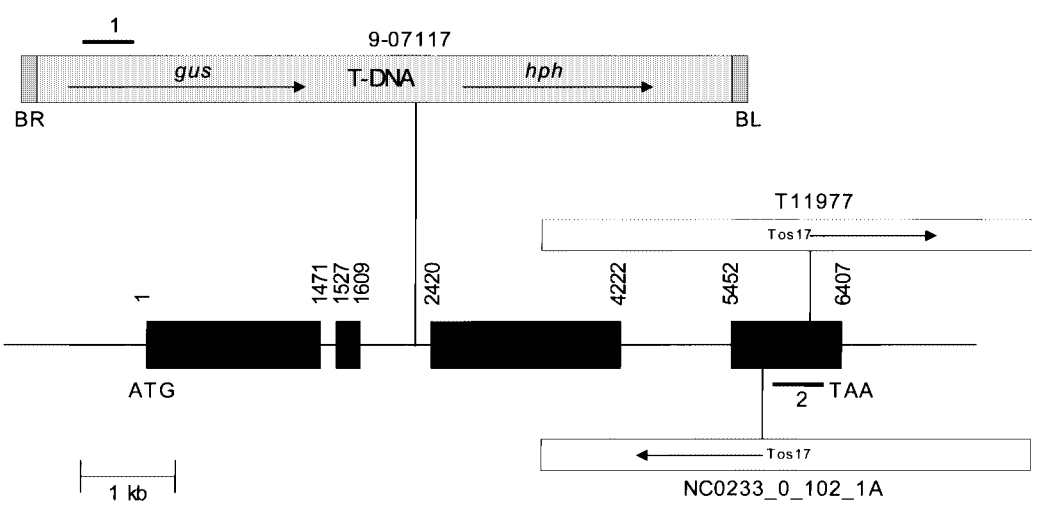

Fig. 2 Schematic representation of the *OsCHLH* gene and relative insertion positions of T-DNA and Tos17. Dark-filled boxes are exons; inter lines are introns. The ATG start codon and TAA stop codons are indicated. Insertion positions of the T-DNA Line 9-07117 and the Tos17 Lines T11977T and NC0233_0_102_1A are indicated either above or below the *OsCHLH* gene. Numbers 1 and 2 indicate the probes used for RNA blot analysis in Fig. 7. BR, T-DNA right border; BL, T-DNA left border; GUS, β-glucuronidase; hph, hygromycin resistance marker used for selection of T-DNA insertion. Scale bar = 1 kb.

Fig. 3 Predicted amino acid sequence of *OsCHLH*. The sequence was multiple-aligned with *Arabidopsis CHLH* (Z68495), soybean *CHLH* (AJ001091), tobacco *CHLH* (AF014052), and barley *XANTHA-F* (U26916). Star (*) indicate perfectly matched amino acids, colons (:) indicate highly conserved amino acids, and periods (.) indicate low-matched amino acids among the five plant *CHLHs.* The T-DNA insertion site is indicated with an open box; Tos17 insertion sites are indicated with gray-filled boxes. Underline indicates the predicted chloroplast transit peptide cleavage site.

was chlorina (Fig. 1). These plants eventually died, 4 weeks after germination (at the four-leaf stage) in greenhouse conditions, because of insufficient photosynthesis. In the T2 generation, eight plants were wild type and two were mutant. In the T3 generation, a heterozygote T2 plant generated 25 wild-type plants and 9 mutants, which fits the expectations for a single recessive locus. All the chlorina mutants were GUS-positive, whereas only some of the wild-type plants were. These *OschlH* mutant phenotypes are similar to those of barley *Xantha-f* mutants, *Arabidopsis chlH* mutants, and *CHLH* antisense tobacco plants (Jensen et al. 1996, Mochizuki et al. 2001, Papenbrock et al. 2000a).

Sequence analysis of the OsCHLH gene

A full-length genomic sequence for *OsCHLH* was obtained using the Monsanto rice blast database (OSM135260 and OSM137314). The coding sequence was deduced by using the Rice Genome Automated Annotation System, NCBI BlastX, NCBI rice EST (S13879, S10033, S13879_1A and S13667), and RT-PCR. Its primary structure consists of four exons and three introns (Fig. 2). T-DNA was inserted in the 2nd intron of *OsCHLH*. The length of the 5′ untranslated region was not identified. The promoter region (–1 to –1,360 bp from ATG) contains a putative TATA box sequence at –76 bp, nine light-responsive motifs (ATC-motif, ATCC-motif, G-box, GA-motif, GAG-motif), and 15 CAAT-boxes (Borello et al. 1993, Marcella et al. 2000).

The *OsCHLH* gene could encode a protein of 1,388 amino acids with a putative N-terminal chloroplast transit peptide (Fig. 3). A calculated molecular mass of 154 kDa corresponds to the size of the homologous precursor CHLH protein from higher plants (Hudson et al. 1993, Gibson et al. 1996, Jensen et al. 1996). Compared with other CHLH sequences, the OsCHLH protein has 88% identity with the barley XANTHA-F subunit, 84% with the soybean CHLH, 82% with the garden snapdragon CHLH, 81% with the tobacco CHLH, and 81% with the *Arabidopsis thaliana* CHLH. Although the N-terminal regions (1st to ~49th residues) of CHLHs are variable, the remaining regions are highly conserved (Fig. 3). A web server (ChloroP, http://pfam.wustl.edu; Emanuelsson et al. 1999) for the prediction of chloroplast transit peptides indicated that the proteolytic cleavage site is located between the 49th (A) and 50th (I) amino acid. Likewise, a functional domain-analysis program (Pfam 7.0, http://www.sanger.ac.uk/Software/Pfam) indicated that the region between the 330th and 1325th amino acid forms the cobN/magnesium chelatase domain, which is commonly present in both the cobN proteins (Crouzet et al. 1991, Nakayama et al. 1998) and the magnesium protoporphyrin chelatases (Hudson et al. 1993).

T-DNA co-segregated with the chlorina phenotype

We genotyped the T2 and T3 seedlings to study whether the chlorina phenotype co-segregated with the T-DNA insertion (Fig. 4). Here, Plants 1 and 2 were homozygous because only the 1.2-kb bands were amplified by the p1 and p2 primers. Plants 5 and 6 were designated as wild types because only the 0.9-kb bands were amplified by the p1 and p3 primers. In contrast, both the 1.2- and the 0.9-kb bands were amplified from Plants 3 and 4, indicating that they are heterozygotic. Plants 3, 4, 5, and 6 showed the wild-type phenotypes, and the T3 seedlings of Lines 5 and 6 were wild type. However, the progeny of Plants 3 and 4 segregated the wild-type and mutant phenotypes at a ratio of 3 : 1. In contrast, Plants 1 and 2 displayed the chlo-

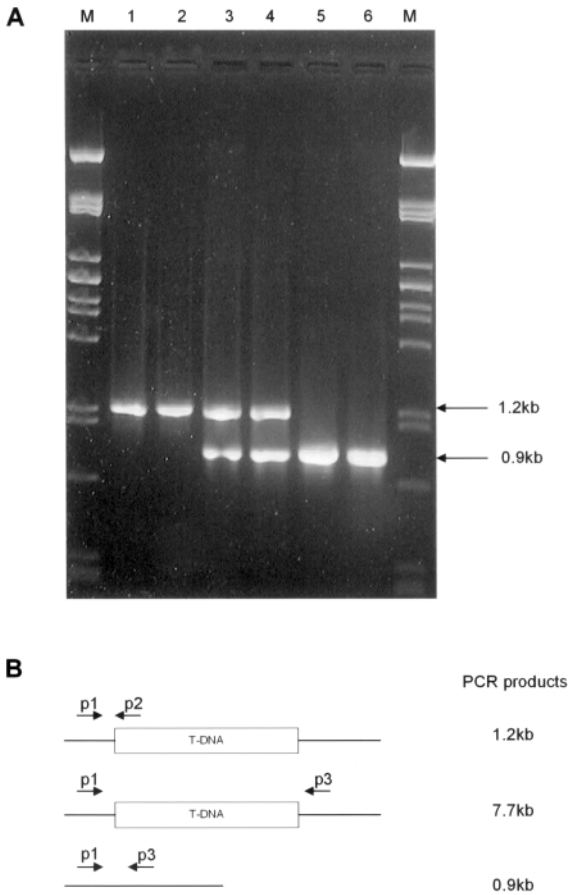

Fig. 4 Genotyping of *OsCHLH* heterozygous progeny. (A) PCR for six T2 plants: Samples 1 and 2 amplified the 1.2-kb fragment only and are *OschlH* homozygous; Samples 5 and 6 showed the 0.9-kb band only and are wild type; Samples 3 and 4 amplified both bands and are *OsCHLH* heterozygous. M, the lambda DNA size marker cut with *Pst*I. (B) Schematic diagrams of the genotyping: p1, forward primer in *OsCHLH*; p2, reverse primer in T-DNA; p3, reverse primer in *OsCHLH*. The p1 and p2 primers produced the 1.2-kb PCR fragment from the T-DNA inserted DNA template; p1 and p3 primers produced 0.9-kb PCR bands from the wild-type DNA, while no PCR band was obtained from the T-DNA inserted template because the expected fragment (7.7 kb) was too large to amplify.

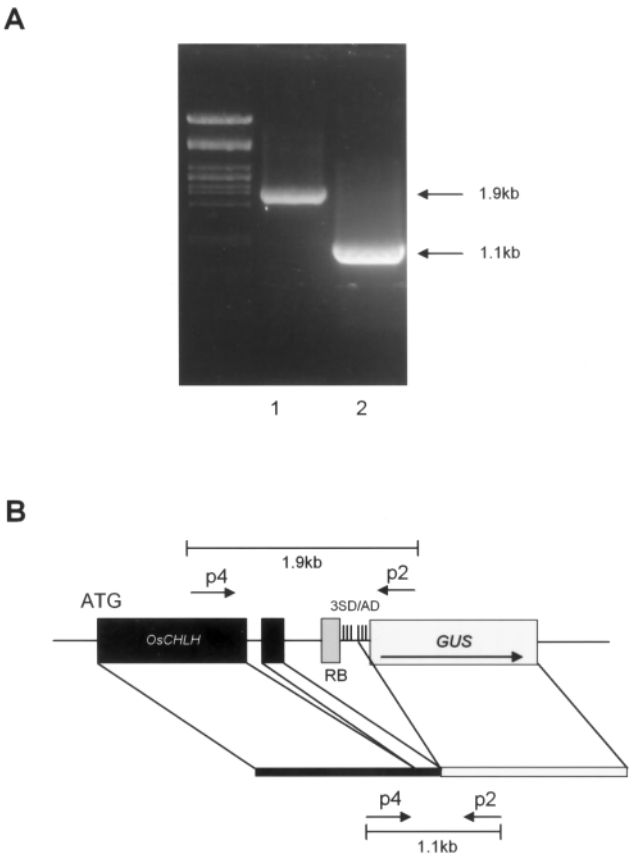

Fig. 5 RT-PCR analysis of the *OsCHLH-GUS* fusion transcript. (A) PCR products performed with the gene-specific forward primer (p4) in the 1st exon of *OsCHLH* and the *GUS* reverse primer (p2) using genomic DNA (Sample 1) or cDNA of the *OsCHLH* heterozygous plants (Sample 2) as templates. (B) Schematic diagram for the *OsCHLH-GUS* fusion gene and the fusion transcript. The upper diagram is the *OsCHLH-GUS* gene deduced from the DNA sequencing of the T-DNA insertion junction. T-DNA was inserted in the second intron of *OsCHLH*. Immediately next to the right border (RB) of T-DNA, an intron with triple-splice donor and acceptor sequences (3SD/AD) is located in front of the *GUS* gene (Jeon et al. 2000a). The lower diagram is the *OsCHLH-GUS* fusion transcript deduced from sequencing the RT-PCR products. The fusion transcript was generated by splicing between the donor of the 2nd intron and the 1st acceptor of the 3SD/AD. p4, forward primer located in the 1st exon of *OsCHLH*; p2, reverse primer located in the *GUS* gene.

rina phenotypes, and were lethal. These results demonstrate that the chlorina mutant phenotype co-segregated with the T-DNA insertion.

OsCHLH-GUS gene fusion

We conducted RT-PCR to determine whether the *OsCHLH-GUS* fusion transcript is present in the T-DNA insertional lines. The first-strand cDNAs prepared from the GUS-positive leaves were PCR-amplified using the *GUS* reverse primer (p2 of Fig. 4 and Fig. 5) and the *OsCHLH* forward primer (p4 of Fig. 5). In this experiment, the 1.1-kb fragment was amplified to be equivalent to the expected size. Sequencing this fragment showed that the end of the 2nd exon was fused to the *GUS* transcript. This indicates that the fusion transcript is formed by splicing between the donor of the *OsCHLH* 2nd intron and the 1st acceptor of the intron present in front of *GUS*. Therefore, the fusion transcript is under the control of the *OsCHLH* promoter. Because the fusion transcript produces a functional GUS enzyme, the expression pattern of the gene could be deduced through simple colorimetric assay (Dai et al. 1996).

Gus assays were conducted in seedlings of the T3 heterozygotic plants that were grown under either continuous light or in the dark. GUS-activity was light inducible in the coleoptiles, leaves, and roots of the light-grown seedlings (Fig. 6A,

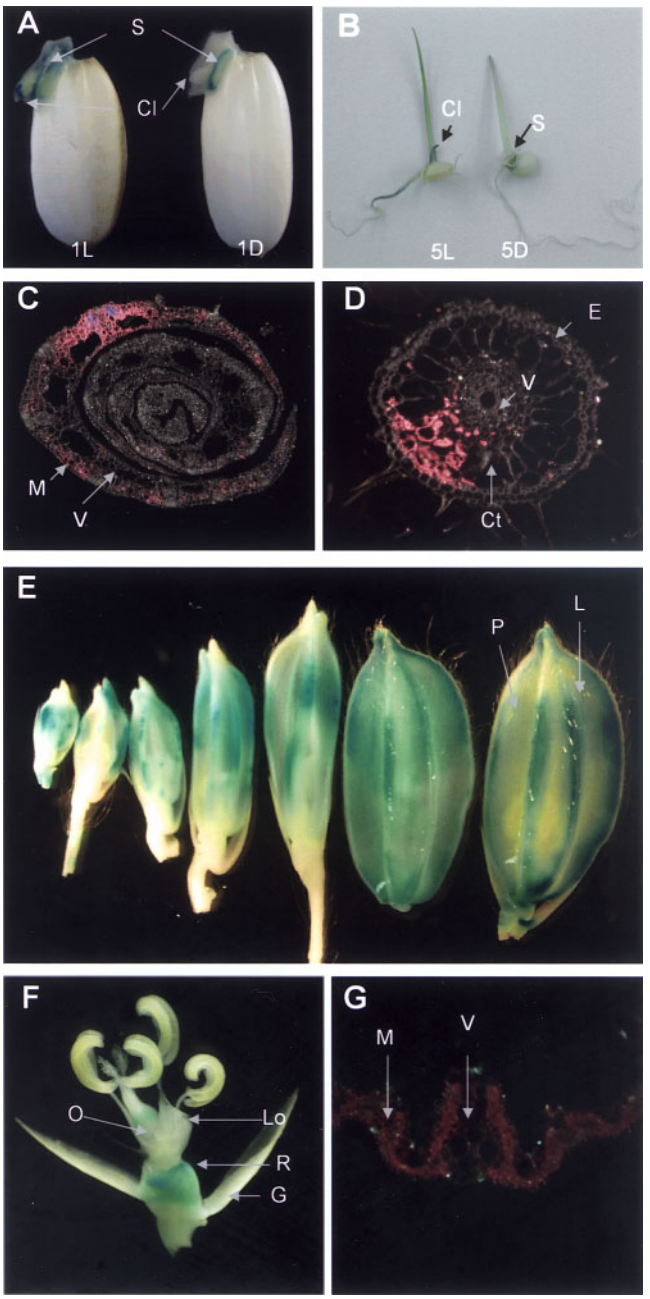

Fig. 6 Analysis of the tagged-gene expression patterns using GUS assay. (A) Germinating seeds of the T3 heterozygotic plants grown either under continuous light (1L) or in the dark (1D) for 1 d after germination. (B) Seedlings grown under continuous light (5L) or in the dark (5D) for 5 d after germination. (C) Cross-section of a shoot from a seedling grown under continuous light for 5 d after germination. (D) Cross-section of a root from a seedling grown under continuous light for 5 d after germination. (E) Flowers at various developmental stages. (F) Mature flower with palea and lemma removed to show internal floral organs. (G) Thin section of a leaf blade from a 15-day-old seedling grown under continuous light. Items featured in (C), (D), and (G) were photographed under dark-field conditions; red and orange coloration indicates GUS activity. Cl, coleoptile; Ct, cortex; E, epidermis; G, glume; L, lemma; Lo, lodicule; M, mesophyll cells; O, ovary; P, palea; R, rachilla; S, scutellum; V, vascular bundles.

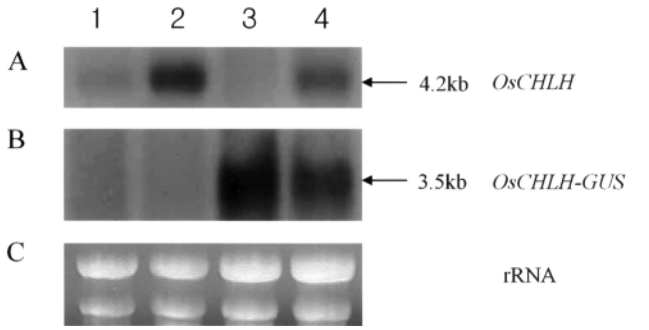

Fig. 7 RNA gel-blot analysis of *OsCHLH* homozygous, heterozygous and wild-type plants grown under either continuous light or in darkness. (A) Blot probed with ^{32}P-labeled PCR fragment of *OsCHLH* (i.e. Probe 2 in Fig. 2). (B) Blot probed with ^{32}P-labeled PCR fragment of *GUS* (Probe 1 in Fig. 2). (C) rRNA stained with ethidium bromide: Sample 1, 6-day-old wild-type plant grown in continuous darkness; Sample 2, 6-day-old wild-type plant grown in continuous light; Sample 3, 6-day-old homozygous plant grown under continuous light; Sample 4, 6-day-old heterozygous plant grown under continuous light. The size of *OsCHLH* mRNA was 4.2 kb; that of the *OsCHLH-GUS* fusion transcript, 3.5 kb.

B). Dark-grown seedlings showed a low level of GUS activity, primarily in the scutellum tissue, indicating that a basal level of the *OsCHLH* transcript still exists under darkness. The cross-section of the light-grown seedlings showed that GUS activity was prominent in the cortical tissues of the roots (Fig. 6C) and in the mesophyll cells of the coleoptiles (Fig. 6D). In the immature flower, GUS activity was observed primarily in the palea/lemma, while in the mature flower, the fusion gene was expressed strongly in the palea/lemma and weakly in the ovary (Fig. 6E, F). In the leaves of 15-day-old seedlings, expression was confined to the mesophyll cells (Fig. 6G).

To examine whether the GUS expression pattern correlates with that of the *OsCHLH* transcript in the wild-type plants, levels of *OsCHLH* mRNA were measured from 6-day-old seedlings by RNA gel-blot analysis (Fig. 7). We found that the 4.2-kb *OsCHLH* transcript was light inducible in the wild-type plants of Lines 1 and 2 of Fig. 7, a result that coincides with the GUS activity shown in Fig. 6. Likewise, the *CHLH* orthologs in barley and soybean have been reported as light inducible (Jensen et al. 1996, Nakayama et al. 1998). In the homozygotic plants (Line 3 of Fig. 7), the 4.2-kb *OsCHLH* transcript was not present because of the T-DNA insertion within the gene. Instead, the 3.5-kb *OsCHLH-GUS* fusion transcript was detected. However, in the heterozygotic progeny, both the wild-type *OsCHLH* transcript and the fusion transcript were detected (Line 4 of Fig. 7). In the heterozygotic plants, the level of the *OsCHLH* transcript was lower than that in the wild-type plants, while the level of the *OsCHLH-GUS* fusion transcript was lower than that of the homozygotic plants. This probably was due to the difference in the number of genes each type possesses.

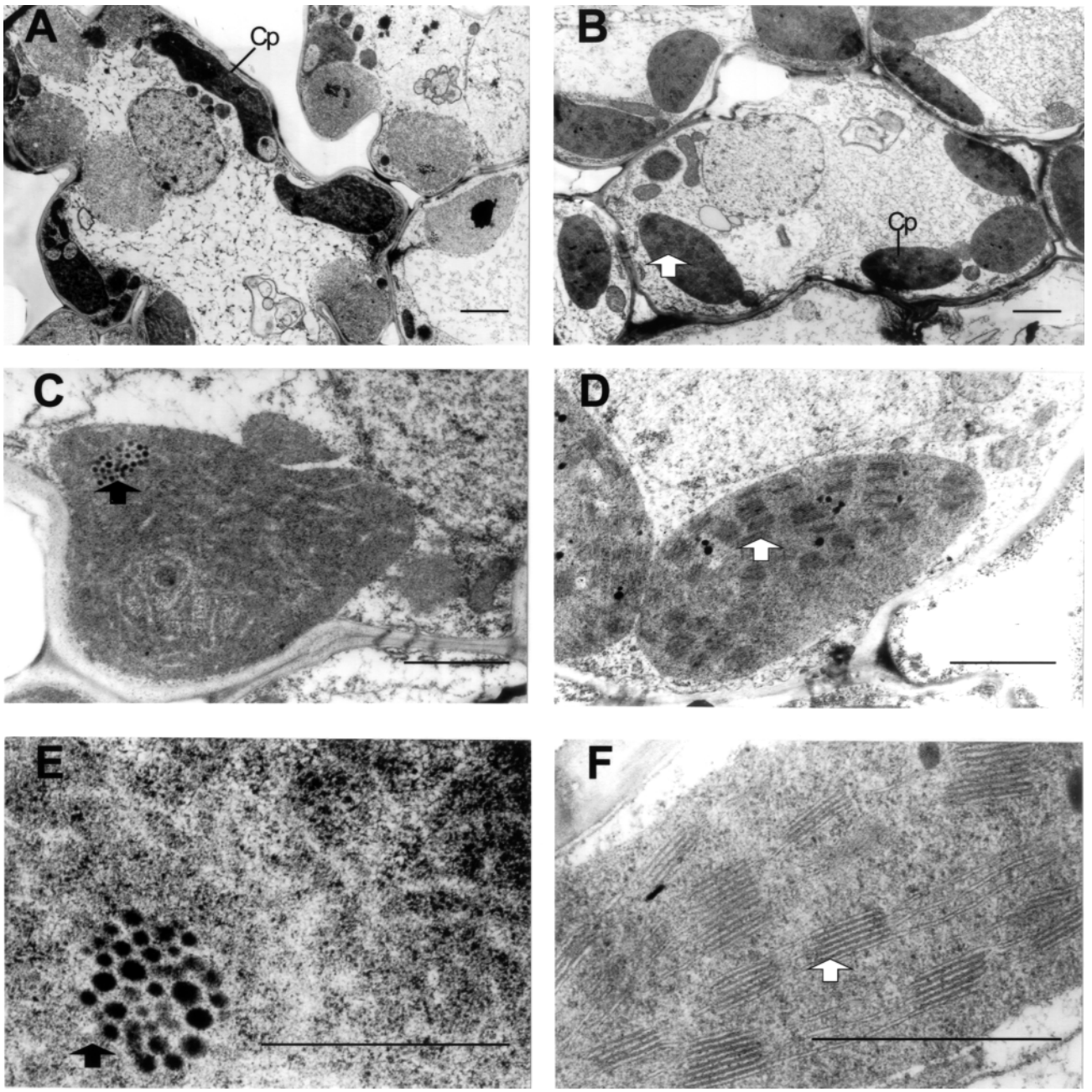

Fig. 8 TEM analysis of the *OschlH* mutant and wild-type chloroplasts. (A), (C), and (E) are sections of *OschlH* mutant chloroplasts; (B), (D), and (F) are sections of wild-type chloroplasts. Scale bars = 1 μm; open arrow, thylakoid membrane; filled arrow, prolamellar body; Cp, chloroplast.

Characterization of the OschlH insertional mutant

Light microscopic observation of the cross-section of the chlorina mutant leaf blade did not show any significant change in the size or number of mesophyll cells (data not known). However, TEM analysis revealed that, although there was no change in the number of chloroplasts, their shape in the *OschlH* mutant was irregular compared with the wild-type chloroplasts (Fig. 8). The knockout mutant did not show dispersal of prolamella bodies and retained the appearance of an etioplast in the continuous light condition. Thylakoid membranes in the mutant chloroplasts also were severely disrupted. The reduction of light harvesting complexes in the thylakoid membrane due to the lack of chlorophyll synthesis may disrupt the thylakoid ultrastructure in the mutant.

We measured chlorophyll contents in 10-day-old seedlings (Table 1). In wild-type and the heterozygotic plants, the

Table 1 Chlorophyll contents of wild-type, heterozygous, and homozygous plants

Genotypes	Growth conditions	Chlorophyll a ($\mu g\ ml^{-1}$)	Chlorophyll b ($\mu g\ ml^{-1}$)
Wild type	light	51.08±7.95 (n [a] = 8)	17.05±2.82 (n = 8)
Wild type	dark	5.36±1.94 (n = 8)	2.76±0.95 (n = 8)
Heterozygous	light	46.39±5.72 (n = 8)	14.26±1.57 (n = 8)
Homozygous	light	0.14±0.02 (n = 8)	0.29±0.10 (n = 8)

[a] The number of plants examined.

ratio between chlorophyll a and chlorophyll b was about three (Yamazaki et al. 1999). However, in homozygotic plants, the levels of chlorophylls were very low, indicating lack of chlorophyll biosynthesis.

Identification of two additional alleles generated by Tos17 insertion

To confirm that the chlorina phenotype was caused by the T-DNA insertion, we searched for additional *OschlH* mutants from the *Tos17* insertional mutant lines. *Tos17* is a useful tool for finding insertional mutants of various rice genes (Takano et al. 2001, Hirochika 2001, Agrawal et al. 2001). Out of the 3,000 *Tos17* lines whose flanking sequences have been analyzed, we found two (T11977T and NC0233_0_102_1A) that carry the *Tos17* insertion in the *OsCHLH* gene. Line T11977T has the *Tos17* insertion at 5,936 bp in the 4th exon, with the same direction as for the *OsCHLH* transcript. In contrast, the insertion for Line NC0233_0_102_1A is at 5,563 bp in the 4th exon, but in the opposite direction (Fig. 2). Both lines, however, show a 3 : 1 segregation of the wild-type and chlorina phenotypes, and the latter co-segregates with the *Tos17* insertion (data not shown).

Discussion

A number of genes have been isolated from T-DNA-tagged lines in *Arabidopsis*. In fact, the knockout facility at the University of Wisconsin (U.S.A.) has established a population of 60,480 tagged lines (Krysan et al. 1999), which is a significant step toward the production of genome-wide mutations. T-DNA insertion in *Arabidopsis* appears to be a random event, and is stable through multiple generations (Azpiroz-Leehan and Feldmann 1997). Jeon et al. (2000a) have employed the T-DNA tagging strategy in rice, a model monocot species, and have produced over 60,000 transgenic rice lines that carry that insertion. It is estimated that T-DNA is inserted into the rice chromosome at an average of 1.4 genetic loci per plant, and that approximately 84,000 taggings have now been generated.

The binary T-DNA vector used in the insertion, pGA2144 (Jeon et al. 2000a), contains the promoter-less *GUS* reporter gene with an intron, as well as multiple splicing donors and acceptors immediately next to the right border. This gene-trap vector is designed to detect fusion between *GUS* and the endogenous gene that is tagged by the T-DNA. Insertion of the promoter-less reporter not only destroys normal gene function but also activates expression of the reporter gene. This activation in a promoter-trap vector can be as high as 30% in *Arabidopsis* (Campisi et al. 1999). In rice, at least 5% of the inserted *GUS* becomes activated in various tissues (e.g. roots, leaves, flowers, and seeds) (Jeon et al. 2000a).

In this report, we described finding the first insertional mutant from the rice T-DNA tagging pool. The mutated gene is *OsCHLH*, which encodes the largest subunit of magnesium chelatase. This rice CHLH protein is highly homologous to CHLH from various other species. T-DNA is integrated to the 2nd intron of *OsCHLH*, generating a fusion transcript between *OsCHLH* and *GUS*. We found that this transcript is present in both *OsCHLH*-heterozygous and -homozygous plants, but not in the wild-type segregants. Moreover, because of the insertion, the wild-type *OsCHLH* transcript is not detectable in the T-DNA homozygotic plants.

Magnesium chelatase has an important role in chlorophyll production. Inhibition in the first step of the chlorophyll branch of the tetrapyrrole pathway can reduce its level (Papenbrock et al. 2000b), resulting in yellow to pale-green leaf phenotypes (Jensen et al. 1996, Mochizuki et al. 2001). Ultrastructural analysis has shown that the rice T-DNA insertion mutant displays a more severely disrupted chloroplast structure than that found with the *Xantha-f*[10] mutant in barley. This is probably because of a lack of enzymatic activity in the T-DNA or Tos17 insertion mutants that cause deletions of the C-terminal portion of the CHLH subunit.

Transcript levels of the *OsCHLH* gene are increased by light. We also found this induction by assaying the fusion product between *OsCHLH* and *GUS*. These observations are consistent with previous reports that expression of the barley *XANTHA-F* gene and soybean *CHLH* was light inducible (Jensen et al. 1996, Nakayama et al. 1998). GUS assay of our light-grown seedlings demonstrated that gene expression was induced in the newly green roots, 5 d after germination (Fig. 6B). Thin sections of these roots revealed that *GUS* was being expressed in the cortical tissues of the main roots (Fig. 6D). *OsCHLH* was expressed in all the green tissues, including the ovaries and the light-induced roots. The fact that the *OsCHLH-GUS* fusion transcript is light inducible and that the fusion gene is expressed in green tissues indicates that the regulatory ele-

ments controlling light inducibility and tissue specificity are present in front of the fusion point. It was previously reported that an intron is important for proper regulation of gene expression in rice (McElroy et al. 1990, Jeon et al. 2000b).

The *OsCHLH* gene is essential for rice plants. Knockout mutations of this gene result in a seedling-lethal phenotype. DNA gel-blot analysis of rice genomic DNA indicates only a single copy of the gene (data not shown). The T-DNA insertion occurs at the 2nd intron, whereas the Tos17 insertions are located in the C-terminal region of OsCHLH. Because all of these insertional mutants show similar phenotypes, we suggest that the C-terminal region is essential for CHLH enzyme activity. Based on the RGP mapping data, *OsCHLH* is positioned near the *chl2* locus on Chromosome 3. A *chl2* mutant showed a pale green phenotype. Whether *chl2* results from a mutation in the *OsCHLH* gene is yet to be determined.

Materials and Methods

Seedling growth and mutant screening

Seedlings of *Oryza sativa* cv. Japonica were grown in MSO media containing 0.44% Murashige and Skoog basal salt, 3% sucrose, 0.2% phytagel, and 0.55 mM myo-inositol from Sigma (St. Louis, MO, U.S.A.) for 1–2 weeks, at 27°C under continuous light or in the dark. The light intensity of the growth room was 30 μmol m^{-2} s^{-1}. Mutant phenotypes were screened for in the seedlings for 15 d after germination.

GUS assay and microscopic analysis

Histochemical GUS staining was performed as described by Dai et al. (1996), except for the addition of 20% methanol in the staining solution. For light-microscopic analysis, we fixed the tissues in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde, then embedded them in Paraplast (Sigma). The samples were sectioned to 16 μm thickness, and observed under a microscope using dark-field illumination.

TEM analysis

The *OsCHLH*-tagged T3 hetero plants were grown on wet filter paper in a Petri dish for 4 d in a growth chamber (32°C/26°C, 14-h day, 50% humidity). Leaves of the wild-type and the *OschlH*-homozygous plants were harvested and fixed overnight at 4°C in a solution containing 1.4% glutaraldehyde, 2% paraformaldehyde, and 50 mM PIPES (pH 7.2). They were then rinsed in 0.05 M phosphate buffer (pH 7.0) and further fixed in 1% osmium tetroxide at 4°C overnight. After rinsing again in the PIPES buffer, the samples were dehydrated with an ethanol series, from 10 to 100%, and embedded in LR white resin (London Resin Co., London. U.K.). The resin polymerization reaction was processed overnight at 60°C in a dry oven. Afterward, the embedded leaf samples were sliced into 1-μm sections with an ultra-microtome (LKB, Bromma, 2088), and stained with 0.5% toluidine blue containing 0.1% sodium carbonate. The sections were observed under a light microscope (Axiovert 100M, Karl Zeiss, Germany). Thin (40- to 50-nm-thick) sections were prepared with the ultra-microtome. They were then collected on nickel grids (1-GN, 150 mesh) and stained with 2.5% uranyl acetate for 20 min. After being washed indirectly with pure water, the specimens were stained with lead citrate for 7 min at room temperature. After the double staining, observations were made under a transmission electron microscope (TEM 100 CX-I, 80 kV).

RNA gel-blot analysis

Total RNAs were isolated from 6-day-old seedlings using Tri Reagent (MRCI Inc., Cincinnati, OH, USA). The RNAs were fractionated on a 1.3% agarose gel, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled probes by random priming using the Rediprime kit from Amersham (Buckinghamshire, U.K.). The 0.5-kb *GUS* probe (1 of Fig. 2) and the 0.6-kb *OsCHLH* probe (2 of Fig. 2) were amplified by PCR (Kang et al. 1998).

Inverse PCR

Inverse PCR was carried out as described previously (Triglia et al. 1988, Thomas et al. 1994, Akiyama et al. 2000). One μg of genomic DNA was digested with 40 units of *Eco*RI for 8 h. The reaction was stopped by ethanol precipitation, and the DNA was resuspended in 44 μl of water. Forty-four μl of the DNA sample was then ligated in 50 μl final volume using T4 DNA ligase. The PCR reaction was carried out in the 20 μl of mixture that contained 20 ng of plant DNA, 10× ExTaq buffer, 0.2 mM dNTP, 0.5 unit ExTaq polymerase from Takara (Ostu, Japan), and 1 μM of the primers. The reverse primer for the first cycle was 5′-CATCACTTCCTGATTATTGACC-3′, which is located at 311 bp from ATG of the *GUS* gene. The first forward primer was 5′-GAATTGCTACCGAGCTCGAA-3′, located at 150-bp from the *Eco*RI site in the GUS gene. The second reverse primer was 5′-ATCCAGACTGAATGCCCACAGG-3′, which is 50-bp from the first reverse primer. The second forward primer was 5′-TTTATGATTAGAGTCCCGCA-3′, 240 bp apart from the first forward primer. Samples were amplified by 30 cycles of 94°C for 60 s, 56°C for 60 s, and 72°C for 120 s. The PCR product was directly subjected to sequencing reactions using the primer 5′-GCAAGGATACAAGTCTGTACCT-3′.

Genotyping and confirming the OsCHLH-GUS fusion transcript

The PCR reaction was carried out in the 20 μl of mixture that contained 20 ng of plant DNA, 10× ExTaq buffer, 0.2 mM dNTP, 0.5 unit ExTaq polymerase (Takara), and 1 μM of the primers by 35 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 120 s. The primers for genotyping were 5′-GCAATTCCACACAGTTCCTGTA-3′ (p1, 1,601 bp downstream from the ATG start codon of the *OsCHLH* gene), 5′-GAAGACCCTCAACATTGTAGCC-3′ (p3, 2,542 bp downstream from the ATG start codon of the *OsCHLH* gene) and 5′-CATCACTTCCTGATTATTGACC-3′ (p2, 316 bp downstream from the ATG start codon of the *GUS* gene). The primers for detecting the *OsCHLH-GUS* fusion transcript were 5′-CAACCTCCAGAATTTCCTCA-3′ (p4, 773 bp downstream from the start codon of the *OsCHLH* gene) and 5′-CATCACTTCCTGATTATTGACC-3′ (p2, 316 bp downstream from the ATG start codon of the *GUS* gene).

Measurement of chlorophyll content

Chlorophyll was extracted with 80% acetone from 0.1-g samples of fresh leaves gathered from 10-day-old, greenhouse-grown seedlings. The extract was measured spectrophotometrically at 645 and 663 nm. Specific chlorophyll contents were determined according to the method of Arnon (1949).

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