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Identification and Fine Mapping of a Gene Related to Pale Green Leaf Phenotype near the Centromere Region in Rice (*Oryza sativa*)

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Abstract: A thermo-insensitive pale green leaf mutant (*pgl2*) was isolated from T-DNA inserted transgenic lines of rice (*Oryza sativa* L. subsp. *japonica* cv. Nipponbare). Genetic analysis indicated that the phenotype was caused by a recessive mutation in a single nuclear-encoded gene. To map the *PGL2* gene, an F_2 population was constructed by crossing the mutant with Longtefu (*Oryza sativa* L. subsp. *indica*). The *PGL2* locus was roughly linked to SSR marker RM331 on chromosome 8. To finely map the gene, 14 new InDel markers were developed around the marker, and *PGL*2 was further mapped to a 2.37 Mb centromeric region. Analysis on chlorophyll contents of leaves showed that there was no obvious difference between the mutant and the wild type in total chlorophyll (Chl) content, while the ratio of Chl a / Chl b in the mutant was only about 1, which was distinctly lower than that in the wild type, suggesting that the *PGL2* gene was related to the conversion between Chl a and Chl b. Moreover, the method of primer design around the centromeric region was discussed, which would provide insight into fine mapping of the functional genes in plant centromeres.
Key words: centromere; gene; fine mapping; pale green leaf mutant; chlorophyll a; chlorophyll b; rice

Despite of lacking conserved DNA sequence, the centromeres from most multicellular eukaryotes, such as *Arabidopsis* [1-3], rice [4-7], maize [8-9], *Drosophila* [10-11], human [12-13] and so on, share very similar structural features. In most multicellular eukaryotes, centromere domains contain long arrays of repetitive DNA elements, for instance, the pAL1 satellite repeat in *Arabidopsis* [14], the CentO satellite repeats in rice [4], the CentC satellite repeat in maize [8] and the satellite repeat in human [15], which are recalcitrant to DNA sequencing. Because of the highly heterochromatin structure of centromeres, completely cloning, sequencing, and assembling their genomic components have remained a significant challenge [6]. However, the copy numbers of satellite monomers vary dramatically across species, and within an organism, or in a same chromosome from different subspecies or varieties [1, 4, 8, 11-12, 14-21]. The limited amount of satellite DNA sequence allowed us to obtain a chance to investigate the structure and sequence of centromeres and to find active genes. Indeed, not a single centromere that has been mapped in a multicellular eukaryote has been completely sequenced [5]. Among the 12 rice chromosomes, several chromosomes contain less satellite DNA sequences. To date, three rice centromeres, which contain less than 150 kb of CentO repeat among the 12 rice centromeres, have been sequenced to high quality [21]. Most importantly, active genes have been discovered in the functional domains of several rice centromeres. These resources provide an unprecedented opportunity to study the function and evolution of centromeres and centromere-associated genes [22].

Leaf plays an important role in photosynthesis and development of plant. All kinds of leaf color mutants should be important sources for functional genetic studies to connect chlorophyll biosynthesis and chloroplast development related gene structure with gene functions and to further analyze their molecular mechanisms, regulation and how they cooperate in complex biological processes in a systematic manner. In the recent years, several genes were cloned using leaf color mutants, such as *PIF1* [23], *PCB2* [24], *DVR* [25], *PORB*, *PORC* [26] and *ATHCOR1* [27] in *Arabidopsis*, and *Xantha-1* [28] in *Hordeum vulgare*

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L. In rice, however, few reports on the leaf color gene were found. Jung et al [29] first reported that the gene in the transgenic rice line, 9-07117, a chlorina seedling mutant with a T-DNA insertion in a gene, showed high homology with *CHLH* genes from several plant species. The particular gene encoded the largest subunit of magnesium chelatase, being designated as *OsCHLH*. Zhang et al [30] mapped two rice *Chl1* and *Chl9* genes on chromosome 3 and isolated these two genes by map-based cloning, which encode ChlD and ChlI subunits of Mg-chelatase, a key enzyme for chlorophyll synthesis and chloroplast development. They found that a missense mutation occurred in a highly conserved amino acid of ChlD in the *chl1* mutant and ChlI in the *chl9* mutant [30]. Lee et al [31] had identified two genes (*OsCAO1* and *OsCAO2*) from the rice genome that are highly homologous to previously studied chlorophyll a oxygenase (*CAO*) genes.

Temperature sensitive mutants, which exhibit normal or near normal phenotypes at permissive temperatures, but mutational phenotypes at higher or lower temperatures, have been used to study genetic and biochemical processes. Temperature sensitive chlorophyll mutants are useful tools for studying biogenesis and biochemical processes of the chloroplasts in higher plants. Numerous temperature sensitive chlorophyll mutants have been identified in higher plants [33-36]. In our laboratory, a thermo-insensitive pale green leaf mutant (*pgl2*), not co-segregated with the T-DNA insertion, was isolated from T-DNA inserted transgenic lines (*Oryza sativa* L. subsp. *japonica* cv. Nipponbare). Genetic analysis indicated that the phenotype was caused by a recessive mutation in a single nuclear-encoded gene. To map the *PGL2* gene, an F_2 population was constructed by crossing the mutant with Longtefu (*Oryza sativa* L. subsp. *indica*). The *PGL2* locus was linked to SSR marker RM331 on chromosome 8. In order to fine map the gene, 14 new InDel markers were developed around the marker in this study, and *PGL2* was further mapped to a 2.37 Mb centromeric region. Not only did these results prove that there exist active genes in centromeric region, but also provided insight into the evolutionary and functional analysis of plant centromeres.

MATERIALS AND METHODS

Plant materials

The *pgl2* mutant was found in one of T_1 lines [32]. T_1 progeny of the *pgl2* mutant line and F_2 population from a cross between the *pgl2* mutant and Longtefu (*Oryza sativa* L. subsp. *indica*) were used to determine whether it was controlled by a single gene or by multiple genes. Plants used for genetic analysis and F_2 population for physical mapping were planted in a paddy field at the experimental station of the China National Rice Research Institute (CNRRI), Fuyang, Zhejiang Province. F_3 population for physical mapping was grown in the greenhouse of CNRRI, Fuyang, Zhejiang Province.

The temperature sensitivity characterization of the mutant

To determine whether variability of the phenotype was indeed affected by ambient temperature, the *PGL2* mutant and wild type were grown in a temperature gradient chamber (TG-100-A, Nippon Medical & Chemical Instruments, Japan) at equivalent light intensity and photoperiod but different temperatures (24℃, 27℃, 30℃, 33℃, 36℃).

Measurement of pigment content

The mutant plants of the transgenic rice line and non-transgenic wild type plants of Nipponbare were used. The fresh leaves (0.1-0.5 g) sampled from field-growing plants at the booting stage and from plants at the seedling stage grown in a temperature gradient chamber (TG-100-A, Nippon Medical & Chemical Instruments, Japan) were extracted with 80% acetone for 48 h at about 26℃. The extract was measured spectrophotometrically at 645 and 663 nm. Total chlorophyll (Chl), Chl a and Chl b contents were determined according to the method of Arnon [37].

Determination of photosynthetic rate

The net photosynthetic rates of flag leaves for field-growing *pgl2* mutant and the wild type plants were measured at the booting stage with a portable photosynthesis system (LiCor-6400, LiCor Inc.

Lincoln, Nebraska, USA) under a LED (light-emitting diode) light source, 6400-02.

Rice total genomic DNA extraction

DNA was extracted from fresh leaf tissues following the procedure described by Lu and Zheng [38] and then used as templates for polymerase chain reaction (PCR) amplification.

Analysis of co-segregation of mutant phenotype and T-DNA

To determine whether mutant phenotype was co-segregated with T-DNA, hygromycin phosphotransferase (*hpt*) gene was amplified by using primers HPT1 (5′-GTTTATCGGCACTTTGCATCG-3′) and HPT2 (5′-GGAGCATATACGCCCGGAGT-3′). The reaction mixture (15 μL) for PCR consisted of 20 mmol/L Tris-HCl, 10 mmol/L $(NH_4)_2SO_4$, 10 mmol/L KCl, 2 mmol/L $MgCl_2$，1% Triton X-100，pH 8.8，0.6 U of *Taq* DNA polymerase, 0.17 mmol/L dNTPs, 0.33 μmol/L of primers, 100 ng of template DNA. The amplification was carried out in an ABI 9600 PCR thermocycler under the conditions of pre-denaturation at 94℃ for 4 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing for 30 s (annealing temperature determined by primer pair sequence), and extension at 72℃ for 1 min, with a final extension at 72℃ for 10 min. Reaction products were separated by electrophoresis on 1% agarose gels.

Linkage analysis

A total of 213 SSR markers scattered on the whole rice chromosomes according to Temnykh et al [39] and McCouch et al [40] were used to determine the approximate map position of the *pgl2* gene on a rice chromosome. The 98 SSR markers showing polymorphisms between Nipponbare and Longtefu were selected for linkage analysis. Linkage analysis was done with 20 *pgl2* mutant individuals of F_2 generation derived from the cross of the *pgl2* mutant with Longtefu. PCR reaction system for mapping was made as follows: pre-denaturation at 94℃ for 4 min, followed by 35 cycles of denaturation at 94℃ for 30 s, annealing for 30 s (annealing temperature determined by primer pair sequence), and extension at 72℃ for 1 min, with a final extension at 72℃ for 10 min. The PCR products were separated on 6% or 8% polyacrylamide gels. After electrophoresis, the amplified DNA bands were detected using the silver staining method.

Design of SSR and InDel markers

Primers were newly developed based on the rice genome sequences according to the Rice Genome Program (RGP) and Beijing Genomics Institute (BGI). To identify more markers for fine mapping, the BAC sequences were blasted against *Oryza sativa* L. ssp. *indica* WGS contigs (http://www.ncbi.nlm.nih.gov/blast/oldblast/Genome/PlantBlast.shtml?10), and 49 new InDel markers were developed. The primers of these newly developed markers were designed using the software Primer Premier 5.0. The reaction mixture and conditions for PCR were the same as described above in *Analysis of co-segregation of mutant phenotype and T-DNA*.

RESULTS

Characterization of the *pgl2* mutant and genetic analysis

A pale green leaf mutant (*pgl2*) was initially observed and isolated from T_1 generation of the transgenic line Fg10688. The mutant was characterized by pale green leaves, reduced plant height and delayed heading (Fig. 1, Table 1) and all these characteristics were co-segregated in all progenies tested. This

Fig. 1. Phenotypes of the mutant *pgl2* and the wild type at the seedling stage.

Table 1. Characterization of the mutant *pgl2*.

Material	Plant height (cm)	Days to heading (d)	No. of effective tillers per plant	Chl a content (mg/g)	Chl b content (mg/g)	Chl a/Chl b	Chl a+ Chl b (mg/g)	Net photosynthetic rate (μmol/m^2 • s)
pgl2	65.8±1.8	85±0	5.7±0.2	1.66±0.001	1.66±0.001	1.00	3.32	23.16±0.034
Nipponbare (CK)	93.3±4.4	68±0	12±0.6	2.39±0.003	0.95±0.001	2.52	3.34	19.18±0.549

chlorophyll deficient mutant was tentatively designed as *pgl2* mutant.

To determine whether *pgl2* was controlled by a single gene or by multiple genes, 50 T_1 plants of the line Fg10688 were used for genetic analysis. Of the tested plants, 38 plants displayed wild type and 12 plants showed mutant phenotype, fitting to a 3:1 segregation ratio (χ^2= 0<$\chi^2_{0.05}$=3.84). Thus, *pgl2* was considered to be a single recessive gene. Similarly, among 300 F_2 individuals from the cross between the *pgl2* mutant and wild type Longtefu, 231 wild type individuals and 69 mutants were found respectively. The segregation of wild type individuals and mutants in the F_2 showed a good fit to a 3:1 segregation ratio (χ^2= 0.537<$\chi^2_{0.05}$=3.84), which further confirmed that the mutation was controlled by a single recessive gene.

In order to determine whether the phenotype mutant resulted from the T-DNA insertion, co-segregation analysis was done in T_2 generation based on the leaf response to hygromycin. Leaf sections from all non-transgenic control plants and 7 individuals from 30 *pgl2* plants became necrotic and those from transgenic control plants and the remaining 23 *pgl2* mutant individuals were green. The results suggested that the mutation was not caused by T-DNA insertion. Considering that *hpt* gene might be silenced in transgenic plants, PCR amplification was further carried out for determining whether mutant plants sensitive to hygromycin contained the *hpt* gene. The PCR result showed that the *hpt* gene was absent in the above 7 hygromycin sensitive plants. Both hygromycin resistance assay and PCR analysis indicated that the mutation was not caused by T-DNA insertion. Thus, we used a map-based cloning strategy to isolate the *pgl2* gene.

Chlorophyll content and photosynthetic rate of the *pgl2* mutant

To further characterize the *pgl2* mutant, T_3 generation mutant and the wild type plants were grown in the field during the rice-growing season of summer for determining chlorophyll contents and photosynthetic rates in the flag leaves of the mutant and the wild type plants at the booting stage. There was no obvious difference between the mutant and wild type in total chlorophyll content (Chl a+Chl b), whereas the leaves of the mutant contained about 30% less chlorophyll a and 75% more chlorophyll b than those of the wild type plants, resulting in the ratio of Chl a / Chl b in the mutant being only about 1, which was distinctly lower than that in the wild type. On net photosynthetic rate, the mutant was 23.16 μmol/ (m^2 • s), increasing by 21% compared with the wild type (Table 1).

The leaf color of the mutants and wild type control plants did not change under the five different temperature conditions (with equivalent light intensity and photoperiod), suggesting that the *pgl2* mutant was a thermo-insensitive mutant (Data not shown).

Linkage analysis of the *pgl2* gene

By using 98 SSR markers showing polymorphisms between Nipponbare and Longtefu, the chromosomal location of *pgl2* was determined by observing the genotypes of 20 *pgl2* mutants. The results showed that the *PGL2* locus was linked to SSR marker RM331 on chromosome 8 (Fig. 2).

Fine mapping of the *pgl2* gene

To further confirm the map location of the *pgl2* gene, 49 new InDel markers were developed around the marker. Of these markers, 14 markers showed polymorphisms between Nipponbare and Longtefu and were used to screen 384 homozygous *pgl2* mutants of F_2 and 4590 F_3 individuals (Table 2). *PGL2* was further mapped to a 2.37 Mb centromeric region. PH72 was mapped on the one side of the *pgl2* gene with two recombinants, while PH78 was localized on the other side of the *pgl2* gene with one

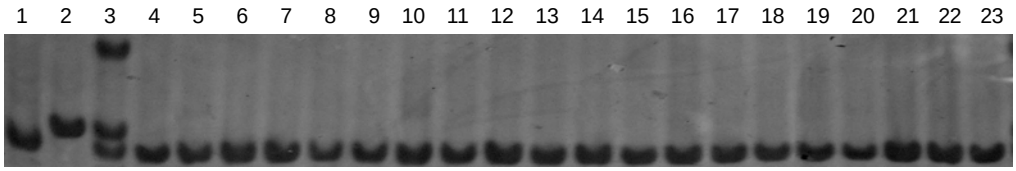

Fig. 2. SSR marker RM331 linked with the *pgl2* locus in primary mapping.

Lane 1, Nipponbare; Lane 2, Longtefu; Lane 3, F_1 (*pgl2*×Longtefu); Lanes 4 to 23, Mutant individuals in F_2 population.

recombinant. Other marker recombinants were showed in Fig. 3.

DISCUSSION

In preparation for sequencing the entire rice genome, many studies have focused on constructing a physical map of each rice chromosome. Among the 12 rice chromosomes, the centromere of rice chromosome 8 contains the least amount of satellite DNA sequence estimated to be only 64 kb based on fluorescence *in-situ* hybridization [4]. During construction and chromosomal mapping of yeast artificial chromosome contigs using rice EST and centromere-specific satellite sequences, the size of the recombination-suppressed domains of this chromosome, which locate at 54.3 cM on the linkage map, was found to be confined to 2 Mb [6, 41]. Nagaki et al [5] constructed a ~1.65 Mb virtual contig, in which 72% was repetitive sequences. Single-copy sequences include 47 gene models, of which 19 are similar in sequence to known genes and 28 are predicted solely by *ab initio* gene finders. Analysis of 1.97 Mb of contiguous nucleotide sequence revealed 48 genes within the region, showing high BLAST homology to known proteins or to rice full-length cDNAs, and some were close to the CentO clusters. Two putative genes, TGF-beta receptor-interacting protein and defective chloroplasts and leaves protein chloroplast precursor, were located very near the centromere domain (only 8 kb and 4 kb, respectively, away from the CentO clusters) [6]. Yan et al [42] showed that rice *Cen8* contains a ~750-kb core domain associated with CENH3. There are at least 16 active genes within this domain. Although, many studies had focused on constructing a physical map and completely sequencing of chromosome 8, the Cen8 accurate position had not been confirmed yet.

With accomplishment of the rice genome sequence project, cloning gene with map-based cloning strategy becomes more and more rapidly and efficiently. Nevertheless, the presence of abundant long tracts of satellite repeats has made centromeres the last frontiers of higher eukaryotic genomes and the design of molecular markers were confined within the centromere region. Here, we reported that the *PGL2* locus was finely mapped to a 2.37 Mb centromeric region on chromosome 8 by using 14 new InDel markers which were developed around the centromeric region. These results provide the essential information for the final isolation of this important gene in the rice Cen8 region. Moreover, the ratio of primer polymorphisms between Nipponbare and Longtefu were investigated.

Table 2. Markers used for fine mapping of the *PGL2* gene.

Marker	Primer sequence	Distance (Mb)
PH87	TCCCTGGTGTTACAATCAT	9.064
	TGGCATCTCCGAATCAAAA	
PH7-1	GCAGGGAAAAAATACAGCA	9.524
	ACTAGCAAAATTGAAAGCC	
PH7-2	TTGGATCGTCTCCCTCAA	10.950
	CTCCCATGCCTTCTTCTC	
PH72	CAATAGTCAACCAAAGATAA	11.782
	GAAAGAAAAGAAGGGAGATA	
PH56	TAAGATAGCCACTGATAAAGT	12.009
	GAATATGTGAGAGTAAGCATG	
PH74	GCACTGATTCTGTGGTGGTA	12.258
	CTTCGTTAGCCTCATGTCTG	
RM331	GAACCAGAGGACAAAAATGC	12.288
	CATCATACATTTGCAGCCAG	
PH75	AAGAAGCGGCAATTATAGACCC	12.711
	CTGCAACCCTCAACCAAACG	
PH77	AATGTTGCGGAAATATCTGG	13.809
	GGCTTACTCGGTGATGATTG	
PH78	CAACAGTGAATCTGCATATTG	14.153
	TTATGGATTTATGACCTGCTC	
PH79	GAGCCCTATCTAGCGTCACTG	14.412
	ATAGAAGCACCCGCCAATCGT	
PH80	AGGAAATAATTGTCCATAAC	14.732
	CAACTCAAAAGGTAAAACTG	
PH7-5	CAAACCGCACTGCTCACT	15.098
	AGGATGCACAGCCTACCG	
PH7-6	GGGAGCTGCTGCATCGTCA	15.652
	CGCTGTTGGTCGCTGTCTGTC	
PH7-8	ACCGAAGTAGGCCAAGAT	17.118
	TTGAGAAAACCAGGGTGC	

Note: Information comes from NCBI (http://www.ncbi.nlm.nih.gov/).

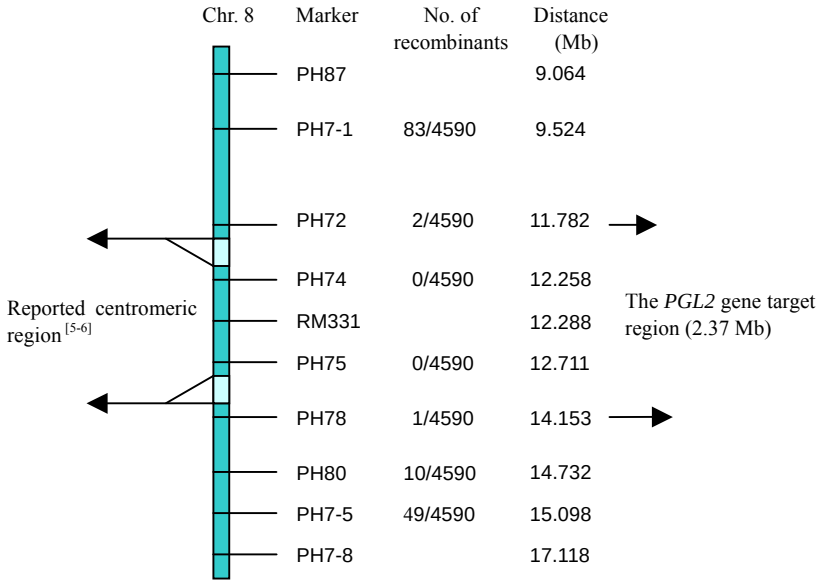

Fig. 3. Physical map around the*PGL2* locus on chromosome 8.

We found that the sequence of primers located in ORF often showed higher proportion polymorphisms based on the results of Nagaki et al [5] and Wu et al [6], while the sequence of primers located in CENO, TE, LTR often displayed lower proportion. This result would provide insight into the fine mapping of the functional genes in plant centromeres. Furthermore, when primers were designed based on small insertion/deletion polymorphisms (InDel) between *Oryza sativa* L. ssp. *indica* and *Oryza sativa* L. ssp. *japonica*, the 10-20 bp distinction of products often showed the higher proportion polymorphisms.

The *PGL2* gene target region (2.37 Mb) compared with sequenced Cen8 region by Wu et al [6] was displayed in Fig. 4. These regions were all located at 54.3 cM on the genetic map. Based on gene function predicted in NCBI, there were 17 genes with full-length cDNA besides 48 putative genes in 1.97 Mb (Table 3). We did not observe any genes related with chlorophyll biosyntheses and degradation in all predicted genes of target region. Therefore, the function of *PGL2* gene has not been confirmed.

Chlorophyll is ubiquitous among photosynthetic organisms, and enzymes required for chlorophyll biosynthesis have been extensively studied. The chlide a undergoes a phytylation reaction catalyzed by chlorophyll synthase (CHS), resulting in the formation of Chl a, and in vascular plants and green algae a portion of the Chl b is synthesized from Chl a by chlorophyll a oxygenase. Moreover, Chl b also can be converted to Chl a by Chl b reductase. Many reports showed that the ratio of Chl a / Chl b was corporately adjusted by Chl a oxygenase, Chl b reductase and hydroxychlorophyll a reductase [43-44]. In our experiment, it was found that there was no obvious difference between the mutant and the wild type in total chlorophyll content, whereas the ratio of Chl a / Chl b in the mutant was only about 1, distinctly lower than that in the control. These data suggested that the *PGL2* gene might be related to the transformation

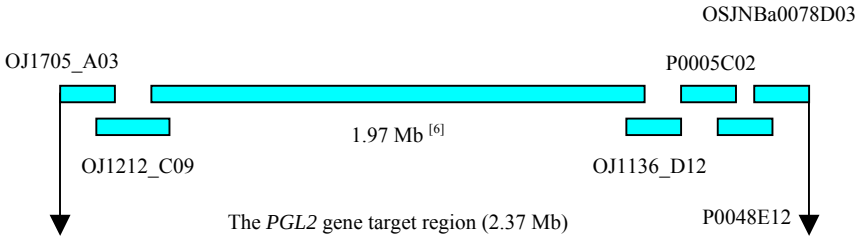

Fig. 4. The *PGL2* gene target region and reported centromeric region.

Table 3. Predicted genes in other six BAC/PAC clones in the *PGL2* gene target region.

BAC/PAC	Predicted function	cDNA
OJ1705_A03	putative ATP binding	AK073881
	unknown protein	AK069295
	unknown protein	AK062297
	putative Threonyl-tRNA synthetase (Threonine-tRNA ligase) (ThrRS)	AK070378
		AK059095
OJ1212_C09	putative NBS-LRR disease resistance protein homologue	AK066438
OJ1136_D12	unknown protein similar to cyclase-like protein	AK108030
	putative Tyrosyl-tRNA synthetase	AK101379
		AK101530
	putative RNA binding / nucleic acid binding	AK121802
		AK104999
	putative ATOPT1; oligopeptide transporter	AK121257
	unknown protein	AK069354
P0005C02	unknown protein similar to endoribonuclease E-like protein	AK099426
		AK059447
	unknown protein	AK107815
	putative FLA1 (fasciclin-like arabinogalactan-protein 1)	AK068096
		AK104404
P0048E12	unknown protein similar to porin-like protein	AK120116
	putative ATPPT1; prenyltransferase	AK069839
	unknown protein similar to signal recognition particle 68K protein	AK064573
OSJNBa0078D03	putative electron transporter/ metal ion binding	AK068722
		AK104226

Note: Information comes from http://rgp.dna.affrc.go.jp/E/index.html.

between Chl a and Chl b. However, further research should be done for determining the function of *pgl2* in leaf color change. The characterization of pale green mutant *pgl2* and fine mapping of *PGL2* gene provide the essential information for the final isolation of this important gene and confirmation of gene function in rice. Moreover, these results would provide insight into the evolutionary and functional analysis of plant centromeres.

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