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Research Paper

Characterization and fine mapping of thermo-sensitive chlorophyll deficit mutant1 in rice (*Oryza sativa* L.)

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Chlorophyll content is one of the most important traits controlling crop biomass and economic yield in rice. Here, we isolated a spontaneous rice mutant named thermo-sensitive chlorophyll deficit 1 (*tscd1*) derived from a backcross recombinant inbred line population. *tscd1* plants grown normally from the seedling to tiller stages showed yellow leaves with reduced chlorophyll content, but showed no significant differences after the booting stage. At temperatures below 22°C, the *tscd1* mutant showed the most obvious yellowish phenotype. With increasing temperature, the yellowish leaves gradually turned green and approached a normal wild type color. Wild type and *tscd1* mutant plants had obviously different chloroplast structures and photosynthetic pigment precursor contents, which resulted in underdevelopment of chloroplasts and a yellowish phenotype in *tscd1*. Genetic analysis indicated that the mutant character was controlled by a recessive nuclear gene. Through map-based cloning, we located the *tscd1* gene in a 34.95 kb region on the long arm of chromosome 2, containing two BAC clones and eight predicted candidate genes. Further characterization of the *tscd1* gene is underway. Because it has a chlorophyll deficit phenotype before the tiller stage and little influence on growth vigor, it may play a role in ensuring the purity of hybrids.

Key Words: rice (*Oryza sativa* L.), thermo-sensitive chlorophyll deficit, ultrastructure, fine mapping.

Introduction

Chlorophyll content is a crucial trait and has a strong influence on crop biomass and economic yield. The chloroplast of photosynthetic eukaryotes plays an important role in photosynthesis as the most important supporter of carbon fixation and energy transformation (Douzery *et al.* 2004, Jiang *et al.* 2012, Tanaka and Tanaka 2006). Chlorophyll deficient mutants have an influence on the leaf color of rice (*Oryza sativa* L.), with direct or indirect effects on the chlorophyll biosynthesis and biodegradation pathways that reduce chlorophyll content and affect chloroplast development and photosynthesis, leading to yield reduction or even death. Many such rice mutants have been discovered and are referred to as yellow, albino, chlorine, stripe, virescent, dark green and zebra according to their phenotypes (Jung *et al.* 2003). These mutants are useful resources for studying chlorophyll biosynthesis, chloroplast structure and chlorophyll genetic mechanisms, and mapping of genes involved in these processes in plants (Eckhardt 2004, Mullet 1988). Chlorophyll biosynthesis occurs in the chloroplast and involves a large number of proteins encoded by genes from both the chloroplast and nuclear genomes. The whole pathway from glutamate to chlorophyll *a* and chlorophyll *b* is composed of 15 steps and 15 kinds of enzymes in higher plants. To date, 27 genes encoding 15 enzymes have been successfully cloned in *Arabidopsis thaliana*, as the model for dicotyledonous plants (Kumar and Soll 2000). Recently, the chlorophyll metabolic pathway and chloroplast development mechanism were defined by the major genes of these processes in Arabidopsis (Comparot-Moss and Denyer 2009, Kumar and Soll 2000).

Leaf-color mutant genes have received increasing attention throughout the world in recent decades. More than 160 leaf-color genes have been identified in rice. For example, the *OsCHLH* gene, encoding the *ChlH* subunit of Mg-chelatase, is located on chromosome 3 (Jung *et al.* 2003). In addition, through map-based cloning *chl1* and *chl9*, encoding *ChlD* and *ChlI* subunits of Mg-chelatase, were also located on chromosome 3 (Zhang *et al.* 2006). *ygl1*, which is located on chromosome 5, is a gene for a chlorophyll synthesis enzyme that affects chlorophyll *a* content (Wu *et al.*

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2007). The *NYC1* gene, which is located on chromosome 1, encodes a chloroplast-localized short-chain dehydrogenase/reductase and chlorophyll *b* reductases with divergent functions (Kusaba 2007). Two highly homologous genes encoding chlorophyll *a* oxygenase, *OsCAO1* and *OsCAO2*, have been identified (Lee *et al.* 2005). A thermo-sensitive chlorophyll-deficient mutant gene, *OsGluRS*, from T-DNA transgenic rice has been located on chromosome 2, encoding glutamyl-tRNA synthetase (Liu *et al.* 2007). *NYC3*, which is a non-functional stay-green mutant, is located near the centromere region of chromosome 6 and encodes a plastid-localizing α/β hydrolase-fold family protein with an esterase/lipase motif (Morita *et al.* 2009). *ZEBRA2* is a single-copy gene in the rice genome, encoding carotenoid isomerase, the key enzyme catalyzing the conversion of cis-lycopene to all-trans-lycopene (Chai *et al.* 2011). Though great progress has been made in the rice research field, only nine genes have been cloned and the genetic mechanisms underlying chlorophyll content remain poorly understood (Lichtenthaler *et al.* 1987, Pogson *et al.* 2011). Therefore, leaf-color mutants are still important for further research on the regulatory pathway of chlorophyll metabolism in rice. Among them, thermo-sensitive chlorophyll deficit mutants are a special type of mutant sensitive to higher or lower temperatures that could be useful tools for studying the biogenesis and biochemical processes of the chloroplast in rice (Dong *et al.* 2001, Peng *et al.* 2012). Since the rice genome was published anyone can find the location of a chlorophyll biosynthetic gene in rice and clone it, but the subject of the present study—revealing a chlorophyll deficiency gene and understanding its molecular mechanism in a rice mutant—is more challenging.

In this study, we isolated a novel thermo-sensitive chlorophyll deficit mutant, *tscd1*, with serious chlorophyll deficit at low temperature. The objectives of this study were to (1) preliminarily clarify the physiological and biochemical mechanisms of chlorophyll deficit in the *tscd1* mutant, and (2) fine map the *tscd1* gene and characterize its function. Our results provide information to help understand the molecular mechanisms governing the biogenesis and biochemical processes of the chloroplast in rice. In addition, the *tscd1* gene has a chlorophyll deficit phenotype before the tiller stage and little influence on growth vigor, so it may play a role in ensuring the purity of hybrids.

Materials and Methods

Plant materials

The rice *tscd1* mutant is a novel thermo-sensitive chlorophyll deficit mutant 1, derived from a backcross recombinant inbred line population as a spontaneous mutant, which has inherited stability after three generations of self-crossing. Three F_1 and F_2 populations derived from crosses between *tscd1* and the *japonica* cultivar 'Sasanisiki' and *indica* cultivar 'Habataki' were used for the genetic analysis and molecular mapping of *tscd1*.

Field trials and trait measurement

Experiments were conducted in 2012 and 2013 in the field at the Rice Research Institute, Shenyang Agricultural University in Shenyang, Liaoning Province, China. The *tscd1* mutant, Sasanisiki and Habataki were grown in the field. The sowing date was April 18 and young plants were transplanted to their final locations on May 20. Field management and fertilizer application essentially followed the normal standard agricultural practice in Shenyang. The agronomic traits including plant height, number of tillers per plant, panicle length, number of spikelets per panicle, number of filled spikelets per panicle, seed setting rate, 1000-grain weight, grain length, grain width, and grain thickness were investigated for *tscd1* and the wild type at the mature stage. In addition, chlorophyll content (SPAD values) was measured during the whole growth period. The seedling, tiller, jointing, booting, heading, and mature stages corresponded to 45, 65, 80, 95, 110 and 135 days after imbibition.

Pigment measurement

To confirm the effect of temperature on the *tscd1* phenotype, *tscd1* and the wild type were placed in growth chambers at four different temperatures (22°C, 24°C, 26°C and 28°C). Photosynthetic pigment contents (chla, chlb, chl and car) were measured, using the method of Arnon (1949). Fresh leaf samples were cut into pieces of about 1–2 mm and immersed into 10 mL chlorophyll extraction buffer (95% ethanol, v/v) for 48 h with slight shaking, and then the extraction solution was used to measure the absorbance values at 470, 645 and 663 nm with a spectrophotometer, with the extraction buffer as a control. Each sample was assayed with three biological replications. After that, the chla, chlb, chl and car contents were calculated by the following equations:

$$\text{chla} = (12.7A_{663} - 2.69A_{645}) \times V \times (1000 \times W) \quad (1)$$

$$\text{chlb} = (22.9A_{645} - 4.68A_{665}) \times V \times (1000 \times W) \quad (2)$$

$$\text{chl} = \text{chla} + \text{chlb} = (20.2A_{645} - 8.02A_{665}) \times V \times (1000 \times \text{W}) \quad (3)$$

$$\text{car} = (1000A_{470} - 2.05\text{Ca} + 14.8\text{Cb}) \times V/(245000 \times W) \quad (4)$$

where chla, chlb, chl and car are the chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid contents in units of mg/g. A_{663}, A_{645} and A_{470} are measured absorbance values at 663, 645 and 470 nm, respectively. *V* is the volume of the extraction solution (mL), and *W* is the weight of leaves (mg).

Measurement of chlorophyll synthesis precursors

ALA (δ-aminolevulinic acid) content was measured as described by Xu *et al.* (2006). PBG (porphobilinogen) content was assayed as described by Peng *et al.* (2012). Fresh

leaves (0.5 g) were collected and mixed with 5 mL extraction buffer (0.6 mol/L Tris-HCl, 0.1 mol/L EDTA, pH 8.2), and then centrifuged at 13,000 *g* for 10 min. An equal volume of Ehrlich reagent was added to the supernatant and incubated in the dark for 15 min. Then, the absorbance at 553 nm was measured. The PBG content was calculated as follows:

$$PBG = (226 \times A_{553}/MEC) \times V/(1000 \times W) \quad (5)$$

Proto-IX (protoporphyrin IX), Mg-protoIX (Mg-protoporphyrin IX) and Pchl (chlorophyllide *a*) contents were measured as described by Hodgins and Huystee (1986). Fresh leaves (0.5 g) were collected and ground in 5 mL extraction buffer (80% acetone), diluted to 10 mL, and then centrifuged at 13,000 *g* for 10 min. The absorbance values of the supernatants were measured at 575, 590 and 628 nm, respectively. The Proto-IX, Mg-Proto-IX and Pchl contents were calculated by the following equations:

$$\text{Proto-IX} = 0.18016 \times A_{575} - 0.04036 \times A_{628} - 0.04515 \times A_{590} \quad (6)$$

$$\text{Mg-Proto-IX} = 0.06077 \times A_{590} - 0.01937 \times A_{575} - 0.003423 \times A_{628} \quad (7)$$

$$\text{Pchl} = 0.03563 \times A_{628} + 0.007225 \times A_{590} - 0.02955 \times A_{575} \quad (8)$$

Transmission electron microscopy (TEM) analysis

Chloroplast ultrastructure was observed using leaves from *tscd1* and the wild type during the seedling stage, following the methods in Zhang *et al.* (2006). The yellow and green parts of the *tscd1* mutant and the same parts in Sasanisiki and Habataki were sampled from seedlings, and fixed in a solution of 2.5% glutaraldehyde and 1% osmic acid [prepared with 0.2 mol/L phosphoric acid buffer solution (pH 7.2)] at 4°C. After 5 h, they were dehydrated by 50%, 70%, 80%, 95% and 100% ethyl alcohol and acetone, and then embedded into epoxy resin. After being sliced, the samples were examined using a transmission electron microscope (H7650, Hitachi, Japan).

Genetic analysis

The *tscd1* mutant was crossed with Habataki and Sasanisiki for genetic analysis, and then crossed with Habataki for gene mapping. The segregation ratio of the *tscd1* mutant phenotype in the F_1 and F_2 plants from the crosses *tscd1* × Habataki, *tscd1* × Sasanisiki' and Sasanisiki × *tscd1* was analyzed.

DNA isolation and PCR analysis

Genomic DNA of the parents and F_2 individuals was extracted from fresh leaves using the CTAB method. The parents, a mutant DNA pool and a wild type DNA pool were used as templates for the polymerase chain reaction (PCR). DNA amplification was performed using a Gene Amp PCR system 9700 thermo cycler. PCR was performed in a 15 μL reaction mix including 25 ng genomic DNA, 2 μL of each primer, 1.0 μL 10× PCR buffer, 0.1 mmol/L dNTP, 0.2 μL 5 U/μL Taq DNA polymerase (Tiangen Biotech, Beijing, China) and 1.5 μL ddH_2O. Amplification conditions consisted of an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 40 sec, 55–60°C for 40 sec, and 72°C for 40 sec, followed by a final extension at 72°C for 10 min, and saving at 12°C forever. The PCR products were separated on 4–5% agarose gels, stained with EB and visualized under ultraviolet light.

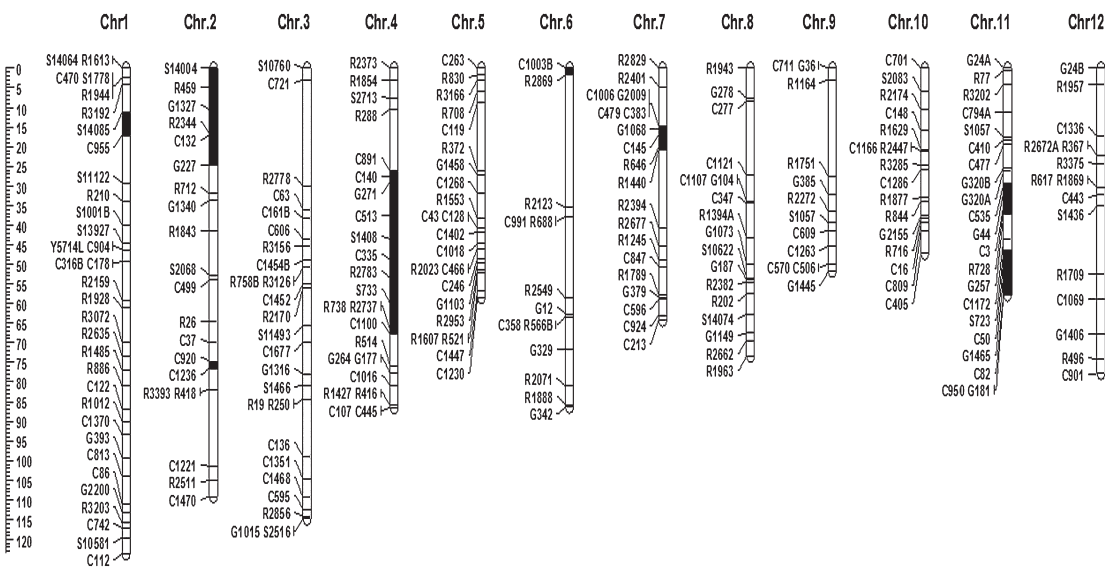

Fig. 1. Genotype of *tscd1* according 236 RFLP markers distributed on the 12 chromosomes. Solid black bars and open bars represent the *Habataki* segments and *Sasanisiki* segments, respectively.

Molecular mapping

For genetic mapping, a total of 500 simple sequence repeat (SSR) markers (Chen *et al.* 1997, Zhang *et al.* 2007) distributed evenly on the 12 rice chromosomes were used. New markers were developed using the program primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) to construct a high-density linkage map and for fine mapping. The new markers were developed according to sequences differences between *japonica* var. 'Nipponbare' and *indica* var. '93-11' (http://www.gramene.org/). Bulked segregant analysis (BSA) was used to determine the approximate map position of *tscd1* (Zhang *et al.* 1994). DNA pools were constructed by combining 15 samples selected from normal green and mutant type plants in the F_2 population derived from a cross between *tscd1* and Habataki. Linkage analysis was performed using the MAPMAKER/EXP 3.0 software (Lander *et al.* 1987). Recombination frequencies (RF) between the gene and molecular markers were calculated using the following equation:

$$RF = (2N_1 + N_2)/2N \quad (9)$$

Where N is the total number of recessive plants, N_1 is the number of recessive plants with the marker genotype of the dominant parent, and N_2 is the number of recessive plants with the heterozygous marker genotype.

Physical map construction and candidate gene analysis

A physical map of the target region was constructed by bioinformatics analysis using bacterial artificial chromosome (BAC) clones of 'Nipponbare' (*japonica*) from the International Rice Genome Sequencing project (IRGSP). Candidate genes were predicted using available sequence annotation databases (http://rice.plantbiology.msu.edu/).

RNA isolation and RT-PCR

Total RNA was prepared from leaves of *tscd1* and wild type plants using an RNA Prep Pure Plant kit (Tiangen). The mRNAs (2.0 μg each sample) were used to synthesize first-strand cDNA, which was reverse transcribed from the total RNA with a RT primer mix. PCR was performed in a total volume of 20 μL with 1 μL of the RT products, 1.0 μL gene-specific primers, and 1 unit of ExTaq (TaKaRa, Otsu, Japan). The qRT-PCR was conducted with gene-specific primers (Bio-Rad, Hercules, CA, USA). The PCRs were carried out with 40 cycles of 95°C for 30 s, 55–58°C for 60 s, and 72°C for 30 s. The primers for the related genes *GTR*, *CHLH*, *CHLD*, *CHLI*, *POM*, *CAOI*, *cabIR*, *Cab2R*, *PsbA*, *PsbA*, *RbcL* and *Rbcs* were according to Liu *et al.* (2012).

Results

Phenotypic analysis of the tscd1 mutant

The novel thermo-sensitive chlorophyll deficit mutant in rice, *tscd1*, showed yellow leaves. **Figs. 2** and **3** show that the wild type chlorophyll content was significantly greater than that of *tscd1* from the seedling stage to the booting stage. However, at the jointing stage, all leaves recovered to a normal green. The chlorophyll contents of *tscd1* and the wild type showed a non-significant difference. Compared with the wild type, key yield-related agronomic traits such as plant height, heading date, tiller number, and seed setting rate were significantly reduced in *tscd1* plants (**Table 1**).

Chlorophyll and carotenoid contents of the tscd1 mutant

To confirm the effect of temperature on the *tscd1* phenotype, the wild type and *tscd1* were grown in a growth chamber at temperatures of 22°C, 24°C, 26°C and 28°C. As shown in **Figs. 4** and **5**, the *tscd1* plant leaves changed from

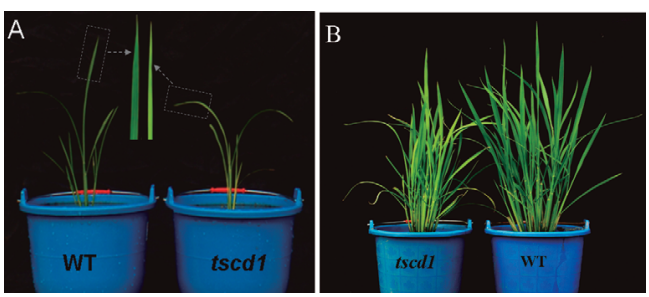

Fig. 2. Comparisons of phenotype between the *tscd1* mutant and wild-type plants. A, seedling of the tscd1 and its wild type; B, Phenotype of the *tscd1* and wild type at the tiller stage.

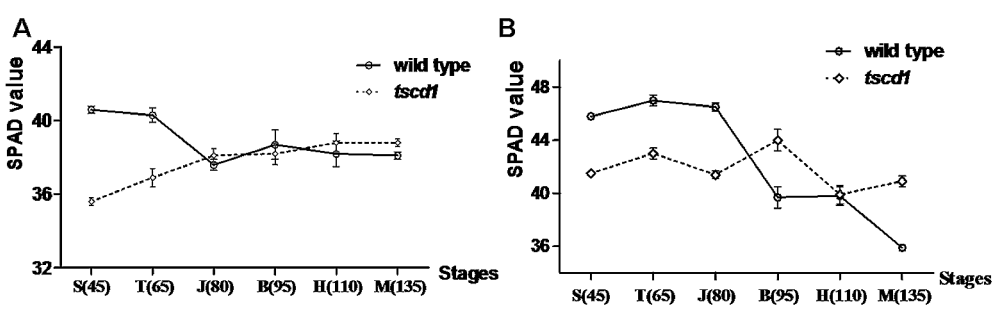

Fig. 3. Comparison of phenotype between the *tscd1* mutant and wild-type plants. A and B represent for chlorophyll content top leaf and second leaf at the different development stages, respectively. S, Seeding; T, Tiller; J, Jointing; B, Booting; H, Heading; M, Maturing, respectively. Brackets(n) representative days after imbibition.

Table 1. Comparison of the keys agronomic traits between the *tscd1* and wild-type plants

Traits	Wild type	*tscd1*	Traits	Wild type	*tscd1*
Plant height (cm)	107.08 ± 2.07	95.58 ± 2.11*	No. of filled spikelets per panicle	107.90 ± 27.20	98.60 ± 11.67
Heading date (d)	108.00 ± 0.0	116.00 ± 1.41*	Seed-setting rate (%)	90.75 ± 3.72	77.06 ± 6.13*
No. of tillers per plant	18.80 ± 6.16	15.70 ± 3.13*	Grain length (mm)	6.70 ± 0.10	6.90 ± 0.08
Panicle length (cm)	18.90 ± 1.84	18.70 ± 1.12	Grain weight (mm)	3.38 ± 0.03	3.20 ± 0.03
Panicle weight (g)	3.05 ± 0.51	2.89 ± 0.33	Grain thickness (mm)	2.20 ± 0.06	2.15 ± 0.08
No. of spikelets per panicle	118.9 ± 30.34	131.10 ± 14.93*	1000-grain weight (g)	24.65 ± 0.25	23.42 ± 0.06

* mean difference significant at 5% level. The values are mean ± S.D. of three biological replicates.

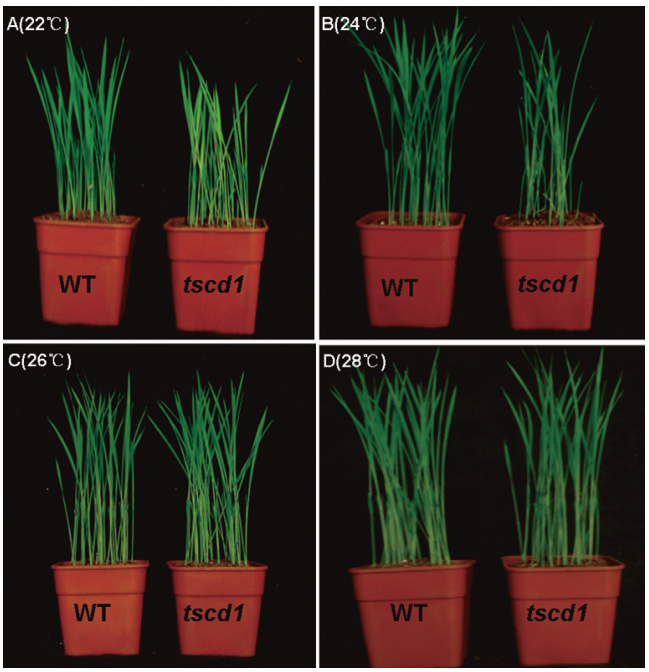

Fig. 4. Photosynthetic pigments content of *tscd1* and wild-type plants at different temperatures. A, B, C and D represent the tscd1 and wild type of plant in growth chamber at 22°C, 24°C, 26°C and 28°C, respectively.

yellow to green and the chlorophyll content gradually increased with increasing temperature. The *tscd1* plants showed an obvious yellow leaf phenotype under temperatures lower than 24°C, but developed normal green leaves under higher temperatures. In contrast, the wild type showed normal green leaves at all four temperatures. To determine whether the yellow leaf phenotype of *tscd1* was due to chlorophyll content deficiency, we measured chla, chlb, chl and car at the different temperatures. As shown in **Fig. 5**, the chl, chla, chlb and car contents of *tscd1* were only about 70%, 68%, 82% and 84% of those of the wild type at 22°C, respectively. The chlorophyll content gradually increased as the temperature increased, and 24°C was the cut-off point between the normal or abnormal phenotypes.

Changes in the main chlorophyll precursor contents in the tscd1 mutant

To determine whether the impaired chloroplasts in the *tscd1* mutant were affected by chlorophyll precursor contents, the levels of chlorophyll precursors were examined at the seedling stage. The results indicated that the Proto-IX, Mg-ProtoIX and Pchl contents were significantly deceased (**Table 2**). However, no significant difference between *tscd1* and the wild type in ALA and PBG contents was observed (**Table 2**). Thus, our results indicated that chlorophyll

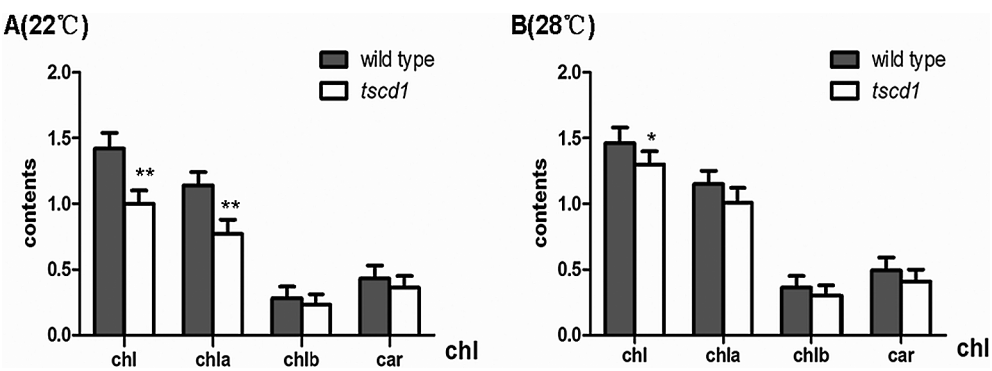

Fig. 5. Photosynthetic pigments content of tscd1mutant and wild-type plants at different temperatures. ** and * mean difference significant at 1% and 5% level, respectively.

Table 2. Comparison of the main photosynthetic pigments precursors content between the tscd1 and wild-type at the seedling stage

	ALA (nmol/gFW)	PBG (ug/g)	Proto-IX (nmol/gFW)	Mg-Proto IX (nmol/gFW)	Pchl (nmol/gFW)
Wild type	0.064 ± 0.001	0.369 ± 0.041	0.269 ± 0.048	0.298 ± 0.055	0.422 ± 0.082
tscd1	0.056 ± 0.004	0.307 ± 0.055	0.169 ± 0.013**	0.185 ± 0.0147**	0.263 ± 0.022**

** mean difference significant at 1% level. The values are mean ± S.D. of three biological replicates.

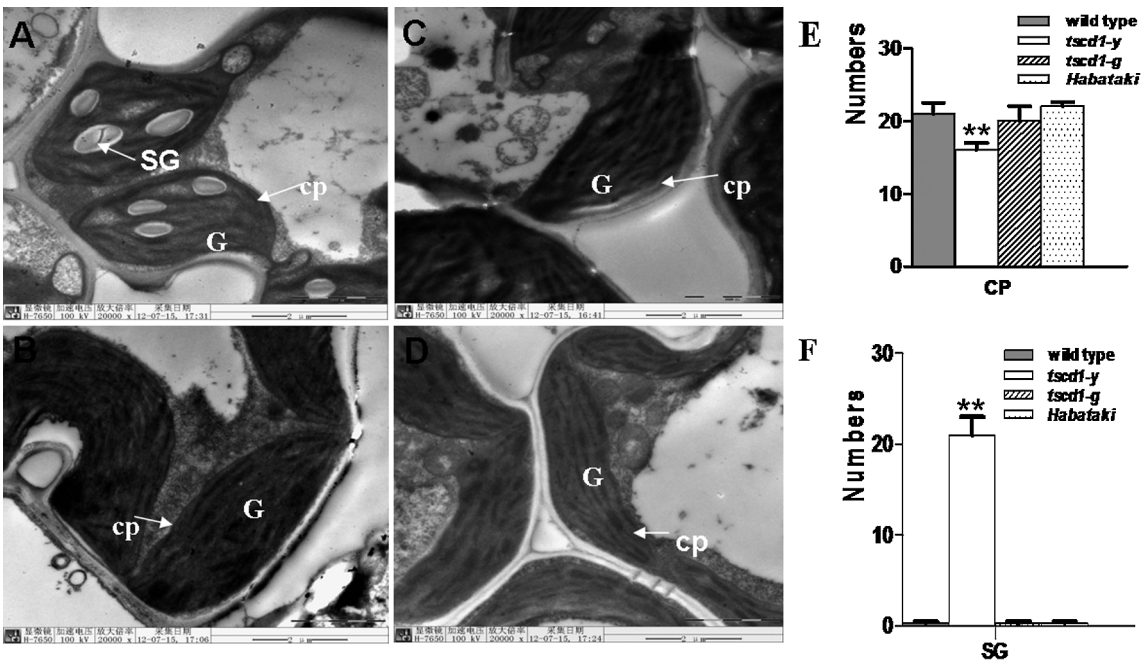

Fig. 6. Transmission electron microscopic observation of the *tscd1*, wild type and *Habataki*. (A, C) were extracted to chloroplast ultra-structure of the tscd1 of yellow and normal leaf; (B, D) were extracted to chloroplast ultra-structure for wild types and Habataki, respectively. (cp, chloroplast; G, grana; SG, starch grain). E, F were measure to the number of the chloroplast and starch grain, respectively. The number of chloroplast and starch grain was counted on the areas 9.0×10^{-6} cm^2. ** mean difference significant at 1% level.

biosynthesis was defective in *tscd1* at the seedling stage under cold stress, which might be related to gene expression in the PBG to Proto-IX steps of the photosynthetic pathway.

Transmission electron microscopic (TEM) observation of chloroplasts in the tscd1 mutant

Under a transmission electron microscope, the wild type and *tscd1* mutant showed obviously different chloroplasts. Compared with the wild type and Habataki (which acted as contrast because it is a higher photosynthetic efficiency variety and the *tscd1* mutant contained little of the Habataki background), the chloroplast number of mesophyll cells in the *tscd1* mutant was obviously decreased, while the starch grain number of chloroplasts in *tscd1* was obviously increased. (**Fig. 6**A, **6**B, **6**D–**6**F). However, chloroplast development in virescent leaves of *tscd1* was almost normal and was similar to the wild type and Habataki (**Fig. 6**B–**6**F). The growth environment had a significant effect on the *tscd1* mutant phenotype.

Genetic analysis and fine mapping

The *tscd1* mutant was identified from a backcross recombinant inbred lines population produced from Sasanisiki (ssp. *japonica*) and Habataki (ssp. *indica*). According to 236 RFLP markers in the *tscd1* genotype distributed over the 12 chromosomes, the main genetic background of *tscd1* was derived from the *japonica* parent Sasanisiki (**Fig. 1**). Thus, Sasanisiki was used as the wild type strain for comparing phenotypes.

Genetic analysis showed that the *tscd1* phenotype was controlled by a single recessive locus based on the results that all F_1 individuals from these crosses had a normal phenotype and that the approximate segregation ratio was 3 (normal) : 1 (yellow) in the F_2 populations generated from the crosses *tscd1* × *Sasanisiki*, *Sasanisiki* × *tscd1* and *tscd1* × *Habataki*. Initial mapping of the *tscd1* target gene with approximately 500 SSR and Indel markers evenly distributed on the 12 chromosomes was performed using the BSA method to analyze the polymorphic loci among *tscd1*, *Habataki* and two DNA pools. Three markers (RM3421, R2M50 and RM425) on the long arm of chromosome 2 were found to be linked with the *tscd1* phenotype (**Fig. 7**, **8**A), and 3, 1 and 6 recombinants were detected in 94 F_2 mutant phenotype plants in a cross between *tscd1* and *Habataki*.

Furthermore, a larger F_2 population of 1128 mutant individuals derived from the *tscd1* × *Habataki* cross was used for physical mapping. Then, four of 16 additional SSR markers between RM3421 and RM425 were used (**Table 3**). Finally, the *tscd1* gene was narrowed down to a 34.95 kb physical distance between markers MM5444 and RM13942; no recombinants were found near the marker RM13941 and the interval contained two BAC/PAC clones, P0487D09 and P0684F11 (**Fig. 8**B, **8**C). According to the Rice Genome Annotation Project information, eight candidate genes were predicted within the target region, including five expressed proteins, one retrotransposon protein, one hypothetical protein and one immediate-early fungal elicitor protein CMPG1 (**Fig. 8**C, **Table 4**). Further analysis of the sequence and physiological mechanisms of *tscd1* is currently underway.

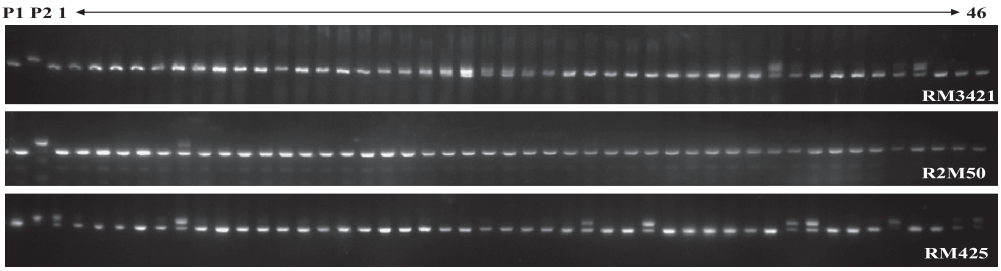

Fig. 7. Segregation of SSR markers amplification fragments in recessive F_2 plants from the cross between the *tscd1* and *Habataki*. P_1, *tscd1*; P_2, *Habataki* ; 1–46, Selected F_2 plants with the thermo-sensitive chlorophyll deficit mutant leaf.

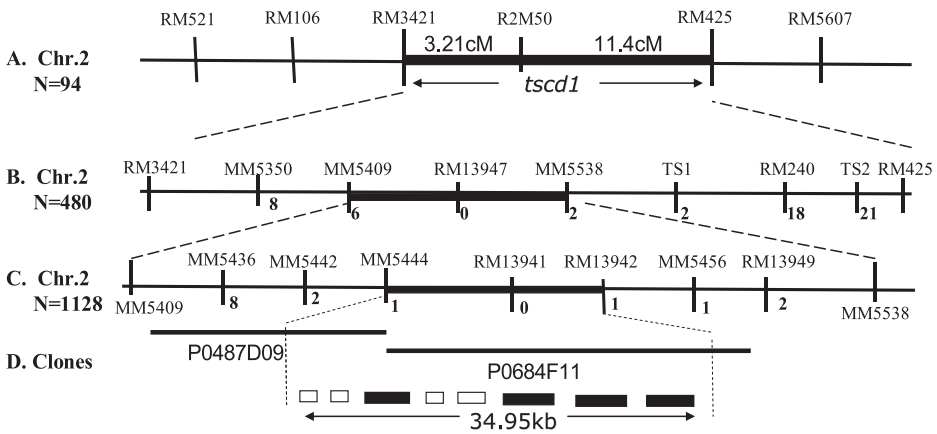

Fig. 8. Molecular mapping of the *tscd1* gene on rice chromosome 2. A, Primary molecular mapping, B and C, Fine molecular mapping, D, BAC clones and candidate genes were predicated, respectively.

Table 3. New SSR markers showing polymorphisms between Habataki and the tscd1mutant

Marker	Forward primer	Reverse primer
MM5409	AGCTGGACGAGATGCTTCAT	AGTTGGATATACATGCCGGG
MM5436	TTCAGCTTTGCCCATTTTCT	TGCCACCATTAATTCACCCT
MM5442	TAATCATCTTGGATGAGGCG	CCTTCAGTTGCAAGGTAGCAC
MM5444	CGACGCTATGAGCATTTTGA	CAAACGTTGGACACGGAAAC
MM5538	ATTTTGGCATTTCAACCCCT	GATGCAATGGCAAGAAGTGA
MM5456	TTCTGCACTTGCAGTCCATC	CTCTCTTTCTCGATCGTGATCA
TS1	TCTTTTGACAATTTCCCATT	CGCAATGACCTTATCTGATT
TS2	ACGTGTCCATCTCCATGAT	TTTTCTCAATCTCCATCACC

Transcript expression of related genes in the tscd1 mutant

To examine whether the impaired chloroplasts and photosynthetic system in the *tscd1* mutant was reflected at the gene expression level, we examined the transcript levels of genes associated with photosynthesis and chloroplast development in the *tscd1* mutant and wild type plants by quantitative PCR analysis. Our results indicated that there were no significant differences between *tscd1* and wild type plants in *GTR*, *CHLH* and *PSBA* gene expression. However, in the *tscd1* mutant, the transcript levels of the *CHLD*, *CHLI*, *POM*, *CAOI*, *Cab2R*, and *PsaA* genes were significantly increased, whereas photosynthesis-associated transcripts such as *RbcL* and *Rbcs* were significantly reduced. Furthermore, the expression of PEP- or NEP-dependent genes (*RbcL*, *Rbcs* and *Cab1R*) was decreased, but some chlorophyll content gene transcripts, such as *CabIR*, *GTR*, *CHLH* and *PsbA*, were not significantly altered (**Fig. 9**). These results showed that the transcript levels of components of the plastid translation and photosynthetic mechanism were significantly affected in the *tscd1* mutant.

Table 4. Gene function annotation in the mapped region

OFR	Genes ID	Gene function annotation	Protein length (aa)	Protein weight	Location
OFR1	LOC_Os02g50400	expressed protein	76	8086.6	30,781,924-30,782,154
OFR2	LOC_Os02g50404	expressed protein	55	5726.8	30,784,651-30,785,350
OFR3	LOC_Os02g50410	expressed protein	213	23961.4	30,785,650-30,788,012
OFR4	LOC_Os02g50420	expressed protein	92	10014.8	30,788,831-30,789,109
OFR5	LOC_Os02g50430	expressed protein	145	15976.8	30,790,494-30,791,315
OFR6	LOC_Os02g50440	retrotransposon protein, putative, unclassified	160	18903.4	30,793,385-30,796,528
OFR7	LOC_Os02g50450	hypothetical protein	232	24902.2	30,799,275-30,802,535
OFR8	LOC_Os02g50460	immediate-early fungal elicitor protein CMPG1, putative, expressed	456	49132.8	30,805,987-30,807,610

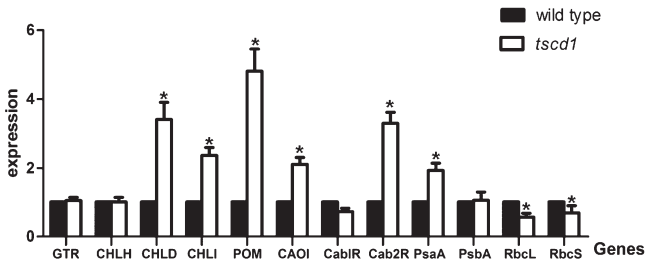

Fig. 9. Transcript analysis of related genes by quantitative polymerase chain reaction at different temperatures. The expression level of genes in wild type plant was set as 1.0 and *tscd1* were calculated accordingly. * mean difference significant at 5% level.

Discussion

Rice is not only an important staple food crop but also a monocotyledon model plant. Chlorophyll deficiency and abnormal chloroplast structure have a direct influence on plant photosynthesis and other agronomic traits. Chlorophyll deficient mutants are excellent materials for researching photosynthesis, chlorophyll biosynthesis, and chloroplast structure, heredity and molecular mechanisms. Additionally, rice leaf-color mutants have been the focus of studies for a long time, but only a few have been characterized at the molecular level. The virescent phenotype is defined as young leaves with reduced chlorophyll levels but with the chlorophyll content gradually returning to normal at a later development stage (Archer and Bonnett 1987). Virescent mutants are useful tools for studying the biogenesis and biochemical processes of chloroplasts in rice. Temperature sensitive mutants are a particular type of virescent, which exhibit normal or near normal phenotypes at permissive temperatures, but mutational phenotypes at lower or higher temperatures; thus, they have been used to study genetic and biochemical processes. In our study, we isolated a new thermo-sensitive chlorophyll deficit mutant 1, *tscd1*, whose chlorophyll content was decreased before the tiller stage at temperatures below 24°C, and then gradually recovered as the temperature rose or when grown in the field. In nature, leaf color is the result of evolution in plants, and chlorophyll deficiency would lead to decreased rice biomass and economic yield, and weaker competitiveness (Jiang *et al.* 2012, Kusaba 2007). However, *tscd1* can mature normally without significantly affecting key related-yield traits. Chloroplast structure analysis revealed that the number of mesophyll cells, thylakoids and mitochondria was obviously decreased and thylakoid structure was irregular. However, when *tscd1* plants were grown at a temperature higher than 24°C or grown until the tiller stage, they had almost normal leaves and similar chloroplast structure and chlorophyll content to the wild type. These results indicated that the control of the *tscd1* phenotype may be temperature-dependent and that it may be a novel mutation.

Chlorophyll biosynthesis occurs in the chloroplast and involves a large number of proteins encoded by genes from both the chloroplast and nuclear genomes. The whole pathway from glutamate to chlorophyll *a* and chlorophyll *b* is composed of 15 steps and 15 kinds of enzymes. In *A. thaliana*, 27 genes encoding 15 enzymes have already been successfully cloned (Kumar 2000). More than 160 chlorophyll deficient mutants or genes have been mapped onto the chromosomes, but only nine related genes have been cloned in rice. These genes are related to enzymes and subunits of chlorophyll biosynthesis (http://www.ncbi.nlm.nih.gov/). It has been found that cold stress interferes with protein biosynthesis in plastids by delaying translational elongation. Some reports have shown that deficiency in plastid translation often causes a cold-sensitive phenotype in leaves, but few genes responsible for these mutational phenotypes have been reported (Dong *et al.* 1994, Liu *et al.* 2007, Peng *et al.* 2012, Wu *et al.* 2012). In this study, the chlorophyll deficit mutant *tscd1* gene was fine mapped to a physical distance of 34.95 kb, which included eight candidate genes. Because of a lack of polymorphic markers and a low recombination rate in this fine mapping region, it was difficult to further narrow down the *tscd1* gene region. Among these candidate genes, none were directly or indirectly related with the chlorophyll biosynthesis pathway. In addition, *tscd1* is a virescent mutant that has a chlorophyll deficit phenotype before the tiller stage and has a little influence on growth vigor, so it might have many practical applications in rice hybrid breeding. For example, it could be used as a recessive phenotype marker for monitoring seed purity in hybrid rice seed production, especially in two-line hybrid rice affected by temperature (Chen *et al.* 2007, Liu *et al.* 2007, Sugimoto *et al.* 2004). During the seedling stage, plants are usually exposed to very low temperatures; therefore, it would be easy to identify hybrids with or without the *tscd1* mutant gene using leaf color as a selection index. In a summary, our results indicate that *tscd1* may be involved in a protective mechanism under low temperature and responsible for chloroplast deficiency. Further fine mapping and physiological investigation of *tscd1* is currently underway.

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