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.
2007). The \textit{NYC1} gene, which is located on chromosome 1, encodes a chloroplast-localized short-chain dehydrogenase/reductase and chlorophyll \textit{b} reductases with divergent functions (Kusaba 2007). Two highly homologous genes encoding chlorophyll \textit{a} oxygenase, \textit{OsCAO1} and \textit{OsCAO2}, have been identified (Lee et al. 2005). A thermo-sensitive chlorophyll-deficient mutant gene, \textit{OsGluRS}, from T-DNA transgenic rice has been located on chromosome 2, encoding glutamyl-tRNA synthetase (Liu et al. 2007). \textit{NYC3}, which is a non-functional stay-green mutant, is located near the centromere region of chromosome 6 and encodes a plastid-localizing \alpha/\beta hydrolase-fold family protein with an esterase/lipase motif (Morita et al. 2009). \textit{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, \textit{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 \textit{tscd1} mutant, and (2) fine map the \textit{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 \textit{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.

\section*{Materials and Methods}

\subsection*{Plant materials}

The rice \textit{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 \textit{F1} and \textit{F2} populations derived from crosses between \textit{tscd1} and the \textit{japonica} cultivar ‘Sasanisiki’ and \textit{indica} cultivar ‘Habataki’ were used for the genetic analysis and molecular mapping of \textit{tscd1}.

\subsection*{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 \textit{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 \textit{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.

\subsection*{Pigment measurement}

To confirm the effect of temperature on the \textit{tscd1} phenotype, \textit{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 (chl, 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:

\begin{align*}
\text{chl} &= (12.7A_{663} - 2.69A_{645}) \times V \times (1000 \times W) \\
\text{chlb} &= (22.9A_{645} - 4.68A_{665}) \times V \times (1000 \times W) \\
\text{chl} &= \text{chl} + \text{chlb} \\
&= (20.2A_{645} - 8.02A_{665}) \times V \times (1000 \times W) \\
\text{car} &= (1000A_{470} - 2.05Ca + 14.8Cb) \times V/(245000 \times W)
\end{align*}

where chla, chlb, chl and car are the chlorophyll \textit{a}, chlorophyll \textit{b}, total chlorophyll and carotenoid contents in units of mg/g. \textit{A663}, \textit{A645} and \textit{A470} are measured absorbance values at 663, 645 and 470 nm, respectively. \textit{V} is the volume of the extraction solution (mL), and \textit{W} is the weight of leaves (mg).

\subsection*{Measurement of chlorophyll synthesis precursors}

ALA (\delta-aminolevulinic acid) content was measured as described by Xu \textit{et al.} (2006). PBG (porphobilinogen) content was assayed as described by Peng \textit{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:

\[ \text{PBG} = (226 \times A_{553}/\text{MEC}) \times \frac{V}{(1000 \times W)} \] (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:

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

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

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

Transmission electron microscopy (TEM) analysis

Chloroplast ultrastructure was observed using leaves from \textit{tscd1} and the wild type during the seedling stage, following the methods in Zhang et al. (2006). The yellow and green parts of the \textit{tscd1} mutant and the same parts in \textit{Sasanisiki} and \textit{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 \textit{tscd1} mutant was crossed with \textit{Habataki} and \textit{Sasanisiki} for genetic analysis, and then crossed with \textit{Habataki} for gene mapping. The segregation ratio of the \textit{tscd1} mutant phenotype in the F1 and F2 plants from the crosses \textit{tscd1} × \textit{Habataki}, \textit{tscd1} × \textit{Sasanisiki} and \textit{Sasanisiki} × \textit{tscd1} was analyzed.

DNA isolation and PCR analysis

Genomic DNA of the parents and F2 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, 0.2 μL 5 U/μL Taq DNA polymerase (Tiangen Biotech, Beijing, China) and 1.5 μL ddH2O. 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 \textit{tscd1} according to 236 RFLP markers distributed on the 12 chromosomes. Solid black bars and open bars represent the \textit{Habataki} segments and \textit{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 F2 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:

\[ RF = \frac{2N_1 + N_2}{2N} \]  

(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...
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

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

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

### 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   |
| tscd1          | 0.056 ± 0.004 | 0.307 ± 0.055      | 0.169 ± 0.013**        | 0.185 ± 0.0147**|

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

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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.

Fig. 5. Photosynthetic pigments content of tscd1 mutant and wild-type plants at different temperatures. ** and * mean difference significant at 1% and 5% level, respectively.
biosynthesis was defective in tscl 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 tscl mutant

Under a transmission electron microscope, the wild type and tscl 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 tscl mutant contained little of the Habataki background), the chloroplast number of mesophyll cells in the tscl mutant was obviously decreased, while the starch grain number of chloroplasts in tscl was obviously increased. (Fig. 6A, 6B, 6D–6F). However, chloroplast development in virescent leaves of tscl was almost normal and was similar to the wild type and Habataki (Fig. 6B–6F). The growth environment had a significant effect on the tscl mutant phenotype.

Genetic analysis and fine mapping

The tscl 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 tscl genotype distributed over the 12 chromosomes, the main genetic background of tscl 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 tscl phenotype was controlled by a single recessive locus based on the results that all F1 individuals from these crosses had a normal phenotype and that the approximate segregation ratio was 3 (normal) : 1 (yellow) in the F2 populations generated from the crosses tscl × Sasanisiki, Sasanisiki × tscl and tscl × Habataki. Initial mapping of the tscl 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 tscl, 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 tscl phenotype (Fig. 7, 8A), and 3, 1 and 6 recombinants were detected in 94 F2 mutant phenotype plants in a cross between tscl and Habataki.

Furthermore, a larger F2 population of 1128 mutant individuals derived from the tscl × Habataki cross was used for physical mapping. Then, four of 16 additional SSR markers between RM3421 and RM425 were used (Table 3). Finally, the tscl 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. 8B, 8C). 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. 8C, Table 4). Further analysis of the sequence and physiological mechanisms of tscl is currently underway.
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 3. New SSR markers showing polymorphisms between Habataki and the tscd1 mutant

| Marker | Forward primer | Reverse primer |
|--------|----------------|----------------|
| MM5409 | AGCTGGACGAGATGCTTCAT | AGTTGGATATACTAGCAGGGG |
| MM5436 | TTCAGCTTTGCCATTTCCT | TGCACACATATTCTCATCCT |
| MM5442 | TAATCATCTTGAGGAGCGG | CCTCTCATTTGGCAAGTTACAGC |
| MM5444 | CGACGCTAGGATGATTTCGA | CAAACGTTGGACACGGAAAC |
| MM5538 | AATTTGGCATTTACAAGGACTT | CTGATGATATATCTCAACATCAGCT |
| MM5645 | TTCTGGCATGCATGATCATC | CTTCCTTCTGATCTCATCAGC |
| TS1    | TCTTTGGAACATTCCATATT | CGGAAATGTTATATCATCAGG |
| TS2    | ACCTTTCATTCATCATCAGT | TTCTTCAATATCCACTACA |

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                 | 2361.4         | 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|
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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