doi: 10.17221/42/2016-CJGPB

Identification and Characterization of the *yls* Mutation in Rice (*Oryza sativa* L.) with Lower Photosynthetic Pigment Content

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Abstract

Liu S., Deng L., Fu Y., Hu G., Liu W., Zhao X. (2016): Identification and characterization of the *yls* mutation in rice (*Oryza sativa* L.) with lower photosynthetic pigment content. Czech J. Genet. Plant Breed., 52: 101–107.

Normal chloroplast development in rice is essential for photosynthesis and yield potential. To explore the physiological and molecular mechanism of chloroplast development, we isolated the rice mutant *yls*, which has yellow-green leaves at the rice seedling stage. In comparison with wild type (WT) plants, mutant plants had lower chlorophyll and carotenoid contents at the seedling stage. Transmission electron micrographs of the leaves of mutant plants showed abnormal grana stacking. We finally mapped the *YLS* gene within the BAC clone OSJNBa0032M21 of chromosome 11. Sequence analysis revealed the existence of a 33-bp deletion within the 3'-untranslated region (UTR) of the *cpSRP54* gene, which encodes the 54-kDa subunit of the chloroplast signal recognition particle (SRP). A knockdown of *cpSRP54* using RNAi technology produced the *yls* phenotypes, indicating that *cpSRP54* is responsible for the phenotypic changes found in the *yls* mutant. The study suggests the existence of a functional association between *cpSRP54* and chloroplast development in rice.

Keywords: chloroplast development; map-based cloning; SRP54 protein; yellow-green leaf mutant

In plants, the leaf is the main organ for photosynthesis and the chloroplast is the main photosynthetic organelle. In plant chloroplasts, photosynthetic pigments, such as chlorophyll a and b, play a crucial role in harvesting energy from light and in driving electron transfer within the reaction centres (Fromme *et al.* 2003; Wu *et al.* 2007).

Chlorophyll deficiency in plants usually leads to a chlorine phenotype that is characterized by yellowish-green leaves. According to the data from Gramene (Jaiswal *et al*. 2002), 62 genes associated with chlorophyll content have been identified in rice (*Oryza sativa* L. subsp. *japonica*). Among them, Wu *et al.* (2007) showed that a missense mutation in a highly conserved residue of the *Yellow-green leaf 1* (*ygl1*) mutant in rice resulted in decreased chlorophyll accumulation and delayed chloroplast development. Zhou *et al.* (2013) reported that *Young leaf chlorosis 1* (*YLC1*) was required for chlorophyll accumulation in rice. The leaves of the *ylc1* mutant exhibited a chlorosis phenotype at the seedling stage. Interestingly, from the early tillering stage, the leaves of mutants can gradually turn green; after the heading stage, these leaves can show an almost normal green colour. In comparison with the wild type (WT), *ylc1* mutants have decreased levels of chlorophyll and lutein and a clearly loose arrangement of the thylakoid lamellae (Zhou *et al.* 2013). Taken together, these findings indicate that many components function in the pathways of photosynthetic pigment biosynthesis.

In the present study, we isolated a *yls* (*Yellow-green leaves at seedling stage*) mutant in rice. The leaves

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doi: 10.17221/42/2016-CJGPB

of this mutant were yellow at the seedling stage, but gradually turned green at the reproductive stage. We measured the photosynthetic pigment contents of the mutant seedlings and examined the leaves of these seedlings microscopically. In addition, we conducted genetic and molecular analysis of the *YLS* gene. Our objective was to determine whether this gene is responsible for photosynthetic pigment biosynthesis and chloroplast development in rice.

MATERIAL AND METHODS

Plant materials. We identified the *yls* T-DNA mutant in a T_1 transgenic rice line in the background of Zhonghua 11 (*O. sativa* subsp. *japonica*). F_2 population from a cross between the *yls* mutant and Zhonghua 11 was used for the genetic analysis and for the mapping of the target gene. The above-mentioned rice plants were grown in a field at the experimental site of the China National Rice Research Institute, Fuyang, Zhejiang Province. We used Student's *t* tests to determine statistical significance.

Temperature sensitive assay. The 30 most uniformly germinated rice seeds were transferred onto a nylon net. The net was floated on water for 4 days in a growth chamber with a 13 h photoperiod and 80% relative humidity. Four days later, the seedlings were cultured with Yoshida's culture solution. The temperature in the chamber was set to 23°C or 30°C according to different treatment conditions.

Hygromycin resistance assay. 30 F_2 mutants derived from a cross between Zhonghua 11 and the *yls* mutant were used for a hygromycin resistance assay as described in detail previously (Murray & Thompson 1980; Li *et al.* 2013). For each plant, 4-cm leaf sections were excised and immersed in a solution containing 50 mg/l hygromycin and 0.5 mg/l 6-benzyl aminopurine (6-BA) (Wang & Waterhouse 1997). Two primers (5'-GTTTATCGGCACTTTGCATCG-3' and 5'-GGAGCATATACGCCCGGAGT-3') were used to amplify the *hygromycin phosphotransferase* (*HPT*) gene.

Map-based cloning of *yls*. We used 126 simple sequence repeat (SSR) markers, which were evenly distributed on whole rice chromosomes, to map the mutant gene. For fine-mapping of the target gene, we selected 5 linked SSR markers and developed 21 InDel markers based on the sequence differences between *japonica* var. Nipponbare according to our previously described procedure (Li *et al.* 2013).

Measurement of chlorophyll content. The chlorophyll contents in rice leaves were measured according to our previously described procedure (Li *et al.* 2013). Photosynthetic pigments were extracted from 0.1-g samples by using 8 ml of 80% acetone (v/v) at room temperature in the dark. We calculated the total chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoid (Car) contents based on the methods of (Arnon 1949) and (Wellburn 1994).

Transmission electron microscopy analysis. According to our previously described procedure (Li *et al.* 2013), thin sample sections (70–90 nm-thick) were stained with uranyl acetate and alkaline lead citrate for 15 min, respectively. The obtained samples were examined under a transmission electron microscope (TEM) (H-7650, Hitachi, Tokyo, Japan).

Quantitative real-time PCR analysis. In the *YLS* expression pattern analysis, total RNA of rice roots, stems, leaf blades, leaf sheaths and inflorescences at the flowering stage was isolated using a Qiagen RNeasy Plant Mini Kit (Qiagen, Duesseldorf, Germany). In the *YLS* expression level analysis, total RNA was extracted from 25-days-old WT and yls mutant leaves. First strand cDNAs were synthesized from 1.0 μg of total RNA. Real-time PCR assays were conducted using 5× TaqMan PCR Master Mix (Applied Biosystems, Warrington, UK) on the Applied Biosystems 7900HT Real-Time PCR System. The relative expression levels were determined using normalization to the *OsACT1* gene according to the comparative CT method (Livak & Schmittgen 2001). We used 2 primers (5'-CTTGGTGAACAGGTCGGTGTAC-3' and 5'-TCCACGGCATTCTTGGTTATT-3') for the quantitative real-time PCR analysis of the *yls* gene.

Construction of the RNAi vector and rice transformation. The RNAi vector p130035SI-X was modified from the pCAMBIA1300 vector. The amplified fragments were cloned into the pMD18-T simple vector, double digested with *Bam*HI-*Spe*I and *Sac*I-*Xba*I, respectively, and then successively inserted into the two sides of a linker. The plasmid was then transformed into Zhonghua 11 using the *Agrobacterium tumefaciens*-mediated genetic transformation method (Zhu *et al.* 2001).

Western blot analysis. Proteins were extracted from leaf samples (100 mg fresh weight) using PBS buffer (pH 7.4), separated by 10% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane for 2 h. A secondary anti-rabbit HRP diluted 1 : 6000 in TTBS for 1 h was used for immune detection. Super Signal West Pico Trial Kit (Thermo Fisher Pierce, Rockford, USA) enhanced chemiluminescent HRP substrate was used for immune detections.

doi: 10.17221/42/2016-CJGPB

RESULTS

Characterization of the *yls* mutant. The rice *yls* mutant exhibited yellow-green leaves (Figure 1D) at the seedling stage; these leaves gradually turned green at maturity, but to a lesser extent than did WT leaves (Figures 1A, D). In the field, mutant plants were markedly smaller, had significantly shorter first and second internodes, and produced shorter panicles compared to WT (Figure 1B). In addition, the numbers of productive panicles and grains per plant were significantly lower. In contrast, the 1000-grain weight was markedly higher (Table 1). However, the roots of mutant plants were smaller and grew more slowly than those of WT plants (Figure 1C).

A large number of rice leaf-colour mutants are sensitive to temperature. When grown at 23°C and 30°C, mutant plants showed no marked differences in leaf colour (Figure 1E). Our results indicate that the change of leaf colour in mutants is related to different growth stages, but is not influenced by temperature.

Abnormal photosynthetic pigment content and chloroplast development in the *yls* mutant. The chlorophyll a, chlorophyll b, and carotenoid contents were lower than those of WT plants (Figure 2A, B). The ratio of chlorophyll a to chlorophyll b was significantly higher in mutant plants than in WT plants, whereas the ratio of total chlorophyll to carotenoids was significantly lower (Figure 2C, D). Under TEM, the chloroplasts of *yls* plants contained markedly fewer well-stacked grana but many liposomes (Figure 2E). The defective grana phenotypes were not fully recovered at maturity (Figure 2F). Interestingly, we observed well-developed starch granules in WT plants at the seedling stage and at maturity. In contrast, *yls* mutant plants lacked starch granules at the seedling stage, but contained well-developed starch granules at maturity (Figure 2F). Our results suggest that the phenotype of the *yls* mutant is mainly derived from decreased photosynthetic pigment contents and abnormal chloroplast development.

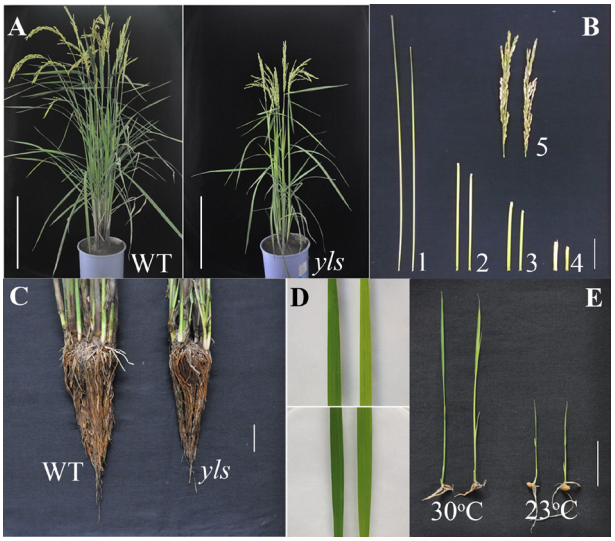

Figure 1. Phenotypes of the wild type (WT) and *yls* mutant: (A) morphology of WT and *yls* mutant plants at maturity, bar 30 cm; (B) internodes and panicles of WT (left) and *yls* mutant (right) plants (1 – first internode, 2 – second internode, 3 – third internode, 4 – fourth internode, 5 – panicle), bar 3 cm; (C) roots of WT and *yls* mutant plants, bar 3 cm; (D) leaf blades of WT (left) and *yls* mutant (right) plants at the seedling stage (top) and at maturity (bottom); (E) phenotypes of WT (left) and *yls* mutant (right) plants at 30°C and 23°C, bar 3 cm

Co-segregation analysis of the mutation and T-DNA insertion. All the F_1 progeny showed a normal phenotype like WT. In the F_2 generation, the normal and mutant phenotypes segregated at a 3:1 ratio (24 of 109 were mutant individuals; $\chi^2 = 0.517 < \chi^2_{0.05} = 3.84$), implying that the yellow-green leaf phenotype in the *yls* mutant is regulated by a recessive allele of a single nuclear gene. To determine whether the genotype was derived from the T-DNA insertion which contains the *HPT* gene in the *yls* mutant sequence, we used 30 mutant plants to conduct co-segregation analysis (Wang & Waterhouse 1997). Only 7 individual leaf sections from 30 mutants became necrotic; all other leaf sections remained green. Because the *HPT* gene may be silenced in *yls* mutant plants, we subsequently amplified the *HPT* gene by PCR. It was shown that this gene was absent in mutant and WT plants. Our results confirmed that the mutant phenotype was not caused by a T-DNA insertion.

Map-based cloning and characterization of the *yls* gene. We used 1033 F_2 mutant individuals to map the *yls* gene to an interval of 21.8 kb between

Table 1. Phenotypic analysis of the y*ls* mutant in rice

Agronomic trait	Zhonghua 11	*yls*
No. of productive panicles	10.5 ± 1.38	7.5 ± 1.38**
Grain No. per plant	484.50 ± 76.55	307.07 ± 27.00**
1000-grain weight(g)	23.61 ± 0.57	27.55 ± 1.21*

Values are the mean ± SD of five biological replicates; *$P \leq 0.05$; **$P \leq 0.01$

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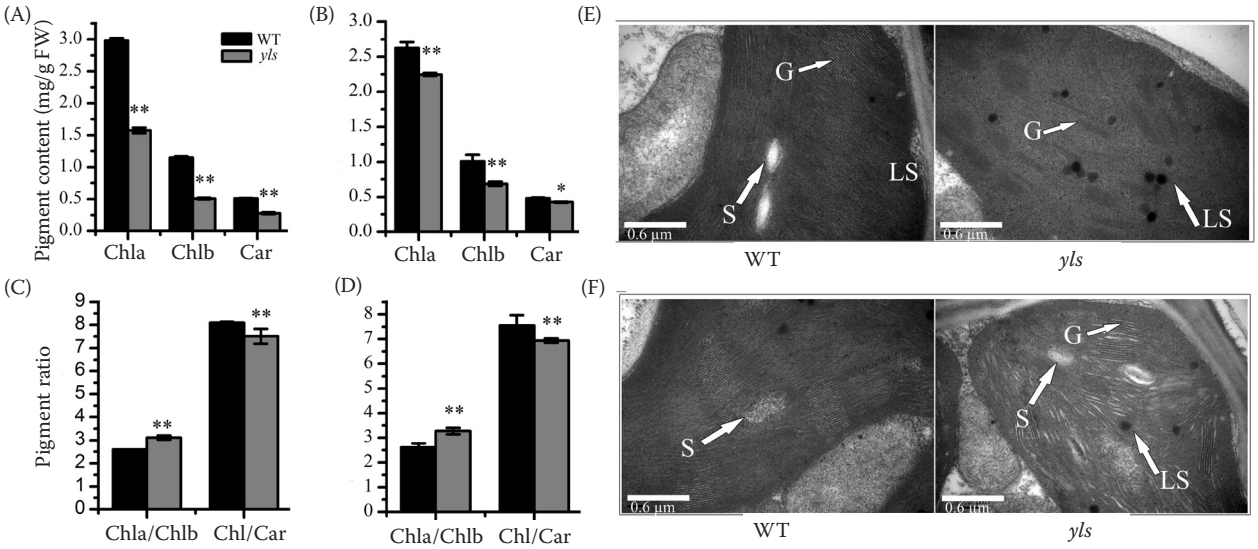

Figure 2. Characterization of photosynthetic pigment contents and chloroplast development in the wild type (WT) and *yls* mutant: (A) pigment contents of WT and *yls* mutant plants at the seedling stage and (B) at the maturity stage; (C) pigment ratios in WT and *yls* mutant plants at the seedling stage and (D) at the maturity stage; data are presented as means and SD; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; (E) representative transmission electron microscope (TEM) images of chloroplasts in WT and *yls* mutant plants at the seedling stage and (F) at the maturity stage; G – grana; LS – liposome; S – starch granule; bars 600 nm

the markers WK31 and WK44 within the BAC clone OSJNBa0032M21 (Figure 3A, B). Within this region, 4 genes, namely, 2 chloroplast precursor genes, a 40S ribosomal protein S25 gene, and a ribonuclease H2 large subunit gene, were predicted. Comparison of the nucleotide sequences of these 4 candidates in *yls*

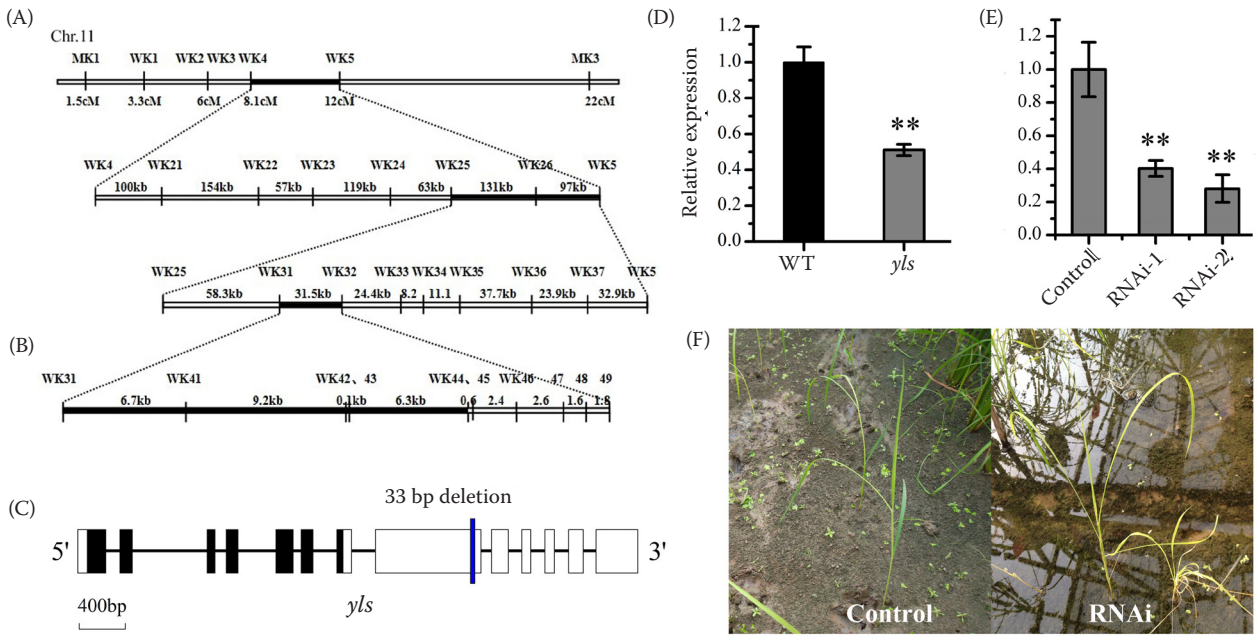

Figure 3. Map-based cloning of the *yls* gene and RNAi test: (A and B) the *YLS* locus was finally mapped to a 21.8-kb region between markers WK31 and WK44; (C) gene structure and mutation site of the candidate gene *yls*; black boxes indicate open reading frame (ORF); white boxes indicate untranslated region (UTR); (D) *YLS* expression in the leaves of wild type (WT) and *yls* mutant plants at maturity; (E) real-time PCR analysis of *YLS* expression in RNAi lines; *YLS* transcript levels were normalized to the levels of *OsACT1* gene expression; data are presented as mean ± SD (n = 3); (F) phenotypes of the RNAi plants and vector control

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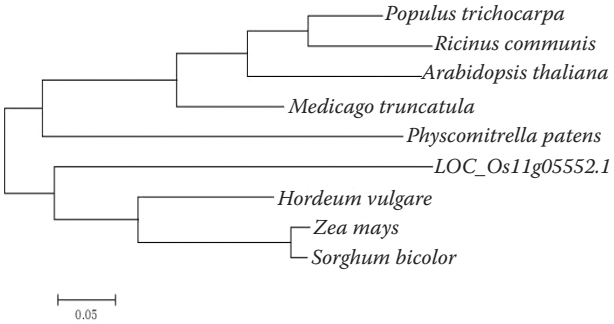

Figure 4. Phylogenetic tree of the *YLS* gene (*LOC_Os11g05552*) and its homologous proteins

mutant and WT plants revealed the existence of a 33-bp deletion within the 3'-UTR of the eighth exon of the *cpSRP54* gene (Figure 3C, Figure S1 in Electronic Supplementary Material). Quantitative real-time PCR results showed that the transcript level was significantly lower in the *yls* mutant than in WT (Figure 3D), indicating that the *cpSRP54* mRNA expression level was affected by the mutation. Then we used RNAi technology to silence *cpSRP54* in WT to study the gene function in leaf colour (Figure 3E). Most of the T_0 transgenic plants had yellow-green leaves at the seedling stage (Figure 3F). Real-time PCR results demonstrated the expression of cpSRP54 in various tissues, with relatively high expression in green tissues, particularly the leaf blades (Figure 5A). Our results confirm that the *cpSRP54* gene is responsible for the phenotype of the *yls* mutation.

Phylogenetic analysis of cpSRP54 and its homologous proteins. We used BLASTP with the Loc_Os11g05552/cpSRP54 protein as query to search for homologous proteins. We found that signal recognition particle 54-kDa proteins were widely distributed in various green plants, including algae (*Oscillatoria acuminata*), moss (*Physcomitrella patens*), and fern (*Selaginella moellendorffii*), implying a likely early origin of the spSRP54 family. We conducted phylogenetic analysis of cpSRP54 and its homologous proteins using MEGA version 6 software (Tamura *et al.* 2013). We found that rice cpSRP54 (LOC_Os11g05552) is more closely related to the homologous proteins of the monocotyledonous plants barley (*Hordeum vulgare*), maize (*Zea mays*), and sorghum (*Sorghum bicolor*) than to the homologous proteins of other species (Figure 4).

***cpSRP54* regulates the expression of photosynthesis-related proteins**. To investigate whether the steady-state levels of chloroplast proteins are affected by the mutation of *cpSRP54*, we performed a western blot assay to detect the expression of photosynthesis-related proteins. We used antibodies for 1 member of the photosystem I complex (PsaC) and 2 members of the photosystem II complex, rubisco large subunit (RbcL) and Lhcb1, which is the most abundant light-harvesting chlorophyll a/b-binding protein (LHCP). The levels of Lhcb1 were markedly lower in mutant plants than in WT plants; in addition, the levels of PsaC and RbcL were decreased (Figure 5B). Our results indicate the existence of a functional association between cpSRP54, LHCPs, PsaC, and RbcL; this functional association may be important in regulating chloroplast development.

DISCUSSION

The 3'-UTR is known to play a crucial role in mRNA stability and gene expression (He *et al.* 2012; Hou *et al.* 2013; Huh & Paek 2014). On the basis of our

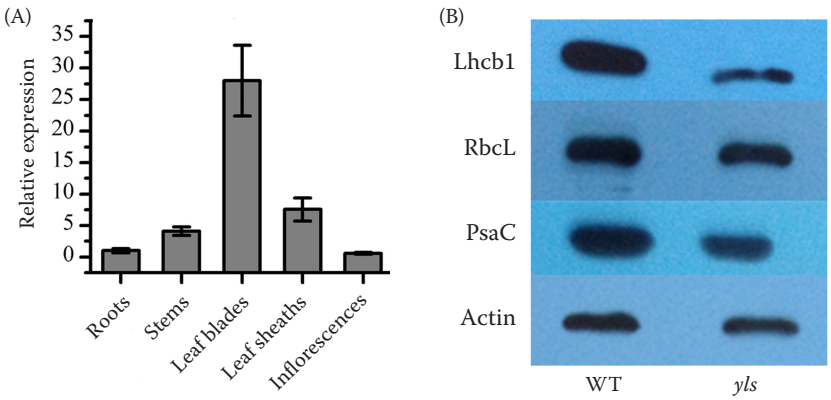

Figure 5. *YLS* gene expression patterns and related protein expression: (A) expression patterns of *YLS* in different organs; *YLS* transcript levels were normalized to the levels of *OsACT1* gene expression in respective samples; the levels of *YLS* transcript in nodes were arbitrarily set to 1; data are presented as mean ± SD of 3 replicates; (B) Western blot analysis of the expression of photosynthesis-related proteins

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results, we propose that the 3'UTR mutation at the eighth exon of *LOC_Os11g05552/cpSRP54* results in down-regulation of the *cpSRP54* transcript, thereby leading to a yellow-green leaf phenotype. Interestingly, Zhang *et al.* (2013) identified and characterized the *ygl138(t)/cpSRP54* gene in rice. An 18-bp nucleotide deletion in the coding region led to a frame shift, which resulted in the mutant phenotype. Similar to *ygl138(t),* the *yls* mutant derived from Zhonghua 11, which is also a *japonica* variety, exhibited yellow-green leaves at the seedling stage. However, the leaves of *yls* mutant plants became gradually greener as they matured. In addition, we observed a marked reduction in the carotenoid content of the *yls* mutant. It remains unclear whether the phenotypic variation between the two rice mutants is derived from the different mutation position.

The LHCP contents of *yls* mutant plants were lower than those of WT plants, implying that the thylakoid membrane assembly of light-harvesting proteins was negatively affected. The leaves of *yls* mutant plants were yellow-green at the seedling stage but became progressively greener as they matured, indicating that another mechanism can compensate for the loss of the cpSRP54 pathway during the later developmental stage. One interpretation is that cpSRP43 can function independently of cpSRP54, to accomplish the thylakoid membrane assembly process (Yu *et al.* 2012; Kirst *et al.* 2014).

When we downregulated *YLS/cpSRP54* in rice using an RNAi approach, the transgenic plants showed yellow-green leaves at the seedling stage. Interestingly, the rice *yls* mutant transformed with the cpSRP54 coding sequence (CDS) failed to exhibit normal green leaves (data not shown). In contrast, in the *ygl138* mutant transformed with genomic DNA, WT growth and colour phenotype were restored (Zhang *et al.* 2013). Similarly, in *Arabidopsis* lines transformed with the cpSRP54 CDS, the carotenoid content was not markedly altered; in contrast, overexpression of the genomic DNA of cpSRP54 led to a marked increase in the carotenoid content (Yu *et al.* 2012). Taken together, these findings suggest the existence of some key elements at the UTR or promoter region, or some alternative complex mechanisms such as RNA splicing and post-translational modification, to regulate *cpSRP54* expression. Hence, a more complex regulatory mechanism of the SRP pathway may exist in rice. We believe that further studies of these mutant or transgenic lines will contribute to a deeper understanding of the molecular mechanisms whereby SRP54 regulates chloroplast development and the SRP pathway.

Acknowledgements. This work was supported by the Central Public-interest Scientific Institution Basal Research Fund (2014RG002-5), and the National Nature Science Foundation of China (30900771).

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Received for publication March 26, 2016
Accepted after corrections September 7, 2016

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