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Map-based cloning and characterization of *YGL22*, a new yellow-green leaf gene in rice (*Oryza sativa*)

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Abstract

Leaf-color mutants have been extensively studied in rice (*Oryza sativa* L.) and many corresponding genes have been identified and cloned. However, the mechanism of complex leaf-color mutations requires further study. In this study, we obtained an introgression line (TIL22) from a set of introgression lines raised using African cultivated rice (*O. glaberrima* Steud.) as the donor parent and *O. sativa* subsp. *indica* Teqing as the recipient. Compared with Teqing, TIL22 showed a yellow-green leaf phenotype at the seedling stage, but the leaves gradually changed to green after the five-leaf stage. The photosynthetic pigment contents of yellow-green leaves at the seedling stage were significantly reduced and chloroplast development was retarded compared with those of Teqing. Genetic analysis indicated that the introgression line phenotype was controlled by a single nuclear gene, temporarily designated *YGL22*. Map-based cloning and sequence analysis suggested that the candidate gene was likely to be LOC_Os01g15390, which encodes a chloroplast protein. Real-time quantitative polymerase chain reaction (qPCR) analysis revealed that the *YGL22* transcript level was significantly lower in TIL22 than that of Teqing at the seedling stage. We generated the *ygl22* knockout mutant using the CRISPR/Cas9 system. The *ygl22* mutants showed a similar phenotype to that of TIL22 at the seedling stage, suggesting that *YGL22* may be involved in the early development of chloroplasts in rice. In addition, plant height, number of panicles per plant, and grain yield per plant of *ygl22* were not significantly affected. The results indicate that *YGL22* can be used as a trait marker gene in hybrid rice production.

1 | INTRODUCTION

Abnormal leaf colors are widely known in higher plants, including rice (*Oryza sativa* L.), which is an important model plant. Given their unique physiological features, plants with an abnormal leaf-color phenotype are extensively used in

Abbreviations: Chl *a*, chlorophyll *a*; Chl *b*, chlorophyll *b*; CRISPR, clustered regularly interspaced short palindromic repeat; InDel, insertion/deletion; PBS, phosphate buffer; qPCR, real-time quantitative polymerase chain reaction; SSR, simple sequence repeat.

fundamental research, including studies of photosynthesis and the mechanism of plant stress resistance (Singh, Prithiviraj, & Sarma, 2000; Wu et al., 2018). In addition to selection of natural leaf-color mutants, introgression breeding is a widely used method to generate suitable materials (Li et al., 2005) for such studies. Leaf-color traits in rice can be applied as a visual marker in practical breeding, which is of considerable importance to maintain the genetic purity of hybrid rice and reduce the cost of hybrid seed production (Zhao, Wang, Zhang, Du, & Pan, 2000).

Given that the phenotype of rice leaf-color mutants is predominantly detectable at an early growth stage, the mutant types are generally classified based on the leaf phenotype at the seedling stage, including yellow, yellow-green, green-yellow, white, light green, striped, and white phenotypes (Awan, Konzak, Rutger, & Nilan, 1980). On the basis of the physiological mechanism of the color mutation, the mutants can be divided into four types: total chlorophyll-deficient, chlorophyll *a*-deficient, chlorophyll *b*-deficient, and increased total chlorophyll (Falbel & Staehelin, 1994).

The normal green phenotype of leaves is attributable to the large amount of chlorophyll stored in the chloroplast (Kusaba et al., 2007), which is the semi-autonomous organelle in green plants that conducts photosynthesis. Chlorophyll, distributed in and around the thylakoid membranes of chloroplasts, is the primary photosynthetic pigment that absorbs and converts light energy into stable chemical energy. Chlorophyll consists of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*). Chlorophyll *a* absorbs red light and Chl *b* absorbs red-blue light. Chlorophyll biosynthesis involves a complex of catalytic enzymes comprising more than 15 enzymes and 27 genes (Leister, 2003). In Arabidopsis, the *ch42* mutant cannot efficiently chelate Mg^{2+} ions into protoporphyrin IX to form Mg-protoporphyrin IX, and subsequently fails to synthesize chlorophylls (Rissler, Collakova, DellaPenna, Whelan, & Pogson, 2002). The *OsCHLH* gene (Jung et al., 2003) encodes the largest subunit of rice Mg-chelatase and knockout mutation of the gene is lethal. In general, the phenotype of rice leaf-color mutants is caused by mutations in genes associated with the regulation of chloroplast development, directly or indirectly affecting the metabolic pathway of chlorophyll and resulting in an alternative leaf color.

Several genes associated with chloroplast development and chlorophyll anabolism have been isolated and identified in previous studies on leaf-color mutants. The gene *YGL1* encodes a chlorophyll synthase and affects accumulation of chlorophyll (Wu et al., 2007). In the *ygl1* mutant, the yellow leaves at the seedling stage may gradually change color to green as growth progresses, and as a result the leaf color is similar to that of the wild type at grain ripening. Compared with the wild type, the *ygl1* mutant retains high photosynthetic efficiency and strong tolerance of light inhibition, which contributes to the comparable yield of the mutant and wild type. In the *ygl7* mutant, the leaves remain yellow throughout the entire growth period, but yield does not decline because the photoelectron utilization and photosynthetic rates are higher in the mutant compared with those of the wild type (Deng et al., 2014). The gene *NYC1* encodes a chloroplast-localized Chl *b* reductase that represses degradation of chlorophylls. The *nyc1* mutant maintains a green leaf color during senescence and the chloroplast contains large, dense grana even in senescent leaves (Kusaba et al., 2007). *OsCAO1* (Oster, Tanaka, Tanaka, & Rüdiger, 2000), which shows high homology to Arabidopsis *CAO*, encodes a Chl *a* oxygenase and affects Chl *b* biosynthesis. Homozygous plants in which *OsCAO1* expression is disrupted show pale green leaves and retarded growth (Lee et al., 2005). *OsHAP3* encodes the HAP3 subunits of the CCAAT-box binding complex in rice. Transformation with an antisense or RNAi construct of *OsHAP3* causes a pale green leaf phenotype in which the chlorophyll content is reduced and chloroplast development is disrupted (Miyoshi, Ito, Serizawa, & Kurata, 2003). Analysis of chlorophyll-related genes has important implications for understanding chloroplast development in rice leaves, pathways for chlorophyll anabolism, and rice yield.

In this study, we report on an introgression line (TIL22) displaying a yellow-green leaf phenotype compared with the recipient parent Teqing. TIL22 harbored six chromosomal segments on chromosomes 1, 2, 3, 7, 8, and 10 from the donor parent, a cultivar of African rice (IRGC102305, *O. glaberrima* Steud.) TIL22 developed yellow-green leaves before the five-leaf stage and showed reduced chlorophyll content. Further observation of the leaves using transmission electron microscopy revealed that chloroplast development was retarded in TIL22. We localized the candidate gene, which we designated *YGL22*, in a 3 kb region on the short arm of chromosome 1 using genetic analysis and gene mapping. The YGL22 protein is a nuclear-encoded chloroplast-targeted protein that plays possible roles in early leaf color development.

Core Ideas

- We consider that *YGL22* could be used as a leaf-color marker gene in commercial two-line hybrid rice production.
- Study of *YGL22* could contribute to an improved understanding of biological function and mechanism of chloroplast development.
- As rice is staple food worldwide, each identified gene in the rice genome helps us better understand this plant and then use genetic improvement to increasing its yield.

2 | MATERIALS AND METHODS

2.1 | Plant materials and mapping population

TIL22 was an introgression line isolated from the repeated backcross progeny derived from a cross between a cultivar of African rice (IRGC102305, *O. glaberrima*) × *O. sativa* subsp. *indica* Teqing (Supplemental Figure S1). The leaf color of TIL22 and Teqing differed conspicuously at the seedling stage (yellow-green and green, respectively). To analyze the genetic and molecular basis of the difference, F_2 and $F_{3:4}$ populations were developed from the cross between TIL22 and Teqing for gene mapping. All plant materials were grown under field conditions in experimental fields of Fudan University at Shanghai (31°11′ N, 121°29′ E) and Sanya (18°14′ N, 109°31′ E), China.

2.2 | Measurement of chlorophyll *a* and *b* and carotenoids contents

Contents of Chl *a*, Chl *b*, and carotenoids (Car) were measured in accordance with Peng's method with modifications (Peng et al., 2012). Fresh leaf tissue was cut into small sections and 50 mg of each sample was immersed in 25-ml extraction buffer (absolute ethanol/acetone/water, 5:4:1, v/v/v) and incubated for 18 h at 4 °C in the dark. To quantify Chl *a*, Chl *b*, and Car in the extracts, the absorbance of the extract solution at 663, 645, and 470 nm, respectively, was measured with a spectrophotometer. The extraction buffer was used as the control. Three biological replicates were performed for each sample. The contents of Chl *a*, Chl *b*, and Car were calculated using the following equations:

$$Chla = \left(12.21D_{663} - 2.81D_{645}\right) \times 0.025/w$$

$$Chlb = \left(20.12D_{645} - 5.03D_{663}\right) \times 0.025/w$$

$$Car = \left(1000D_{470} - 3.27Chla - 104Chlb\right)/229 \times 0.025/w$$

where D is absorbance at 470, 645, or 663 nm and w is the weight of leaf tissue (g).

2.3 | Transmission electron microscopy

We sampled leaves of TIL22 and Teqing at the seedling and mature stages. All leaf samples were cut into sections of about 2 by 2 mm and fixed in 2.5% glutaraldehyde in 0.1 mol L^{-1} phosphate buffer (PBS; pH 7.4) at 4 °C for 4 h, followed by three washes with 0.1 mol L^{-1} PBS (pH 7.4). The samples were transferred to 1% osmium tetroxide in 0.1 mol L^{-1} PBS (pH 7.4) for 2 h. After three washes with 0.1 mol L^{-1} PBS (pH 7.4), the samples were dehydrated with a gradient ethanol series (50, 70, 80, 90, 95, and 100% ethanol) and embedded in Spurr's resin for 48 h. The embedded samples were sectioned to 60–80 nm thickness using an ultramicrotome, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope.

2.4 | Positional cloning of *YGL22*

Total genomic DNA was extracted from young leaves using the cetyltrimethylammonium bromide method. We used bulked extremes to identify the chromosomal region containing target gene. Two DNA bulks were made by selecting extreme individuals from the F_2 population from a TIL22 × Teqing cross during the seedling stage. The first bulk (yellow-green DNA bulk) was made by mixing equal amounts of DNA from 35 yellow-green-leaf plants, and the other (green DNA bulk) was made by mixing equal amounts of DNA from 35 green-leaf plants. The 331 simple sequence repeat (SSR) markers scattered on rice chromosomes were used for primary mapping. The locus was mapped between SSR markers RM577 and RM582 on chromosome 1. Among this region, additional SSR markers and new insertion/deletion (InDel) markers were designed from publicly available rice genome sequences accessed on the National Center of Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST). Based on the F_2 mapping population, we further developed a set of $F_{3:4}$ families for the target locus. Then the gene was finally mapped between InDel makers TIL22-15 and TIL22-16 based on 2,100 recessive individuals from $F_{3:4}$ population. All primers used in this study were designed using Oligo 7 software.

2.5 | RNA isolation and quantitative real-time polymerase chain reaction

Total RNAs were extracted from leaf samples using the MiniBEST Universal RNA Extraction Kit (TaKaRa). Real-time quantitative polymerase chain reaction (qPCR) analysis was performed using a Bio-Rad CFX96 real-time PCR system (Bio-Rad) using the Advanced Universal SYBR Green Supermix (Bio-Rad). The rice gene *UBQ5* was used as the internal control for normalization of transcript levels. The relative quantification method (ΔΔCt) was used to evaluate quantitative variation of replicates examined. All assays were performed with three biological replicates. Primers are listed in Supplemental Table S1.

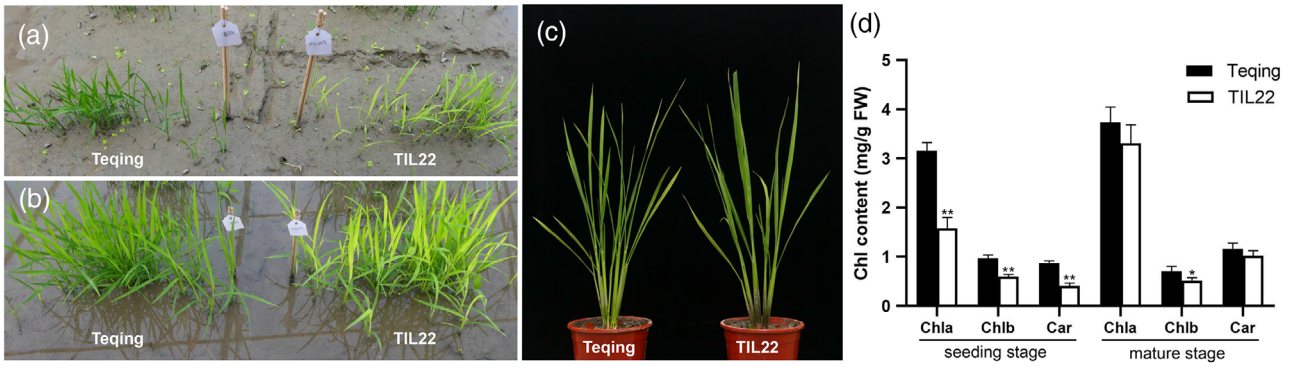

FIGURE 1 Phenotypes and pigment contents of wild type (Teqing) and TIL22. (a) Morphology of wild type (WT) Teqing and TIL22 at the three-leaf stage. (b) Morphology of WT (left) and TIL22 (right) plants at the four-leaf stage. (c) Morphology WT (left) and TIL22 (right) at the tillering stage. (d) Pigment contents of WT and TIL22 plants at the seedling stage and the mature stage. chl, chlorophyll; car, carotenoid. Values are means ± SD of three biological replicates. Significant differences compared with WT (Teqing) were determined using Student's *t* test: $*P < .05$. $**P < .01$

2.6 | Subcellular localization of *YGL22*

To investigate the subcellular localization of *YGL22*, the coding region sequence of *YGL22* without the termination codon was cloned into the PYL332-GFP binary vector. The fusion construct (35S::YGL22-GFP) and control construct (35S::GFP) were transformed separately into rice protoplasts. The transformed cells were examined using a confocal fluorescence microscope (Leica Microsystems) after culture for 14 h.

2.7 | Vector construction and transformation in rice

We used the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein 9 (Cas9) multiplex editing system (Xie, Minkenberg, & Yang, 2015) to knockout the *YGL22* gene. The 20-bp gene-specific spacer sequences (CTATAGAGGATTGAGGAGCA) were selected using the CRISPR-PLANT database (http://www.genome.arizona.edu/crispr/) (Xie, Zhang, & Yang, 2014) and cloned into the pRGEB32 vector. The vector was introduced into embryogenic calli from Teqing by means of an *Agrobacterium*-mediated transformation method. Successfully transformed calli were selected by culturing on the selection medium containing 50 mg L^{-1} hygromycin for 2 wk under dark conditions (28 ˚C). Drug-resistant calli were selected to transfer into Murashige and Skoog (MS) medium under long-day conditions (16 h: 8 h, light: dark) to grow into plants in the greenhouse. The mutation sites of positive transgenic individuals were confirmed by PCR amplification and DNA-sequencing analyses. Primers are listed in Supplemental Table S1.

3 | RESULTS

3.1 | Yellow-green leaf phenotype of TIL22

Plants of TIL22 grown in paddy fields developed yellow-green leaves before the five-leaf stage and thus were clearly distinguishable from the green leaves of the wild-type Teqing (Figure 1a and 1b). With subsequent growth, the leaves of TIL22 gradually changed color to green (Figure 1c) and ultimately showed no obvious difference in color at maturity in comparison with leaves of Teqing.

Chlorophyll deficiency is a common cause of leaf-color mutant phenotypes. To investigate whether the yellow-green leaf phenotype of TIL22 results from impaired chlorophyll biosynthesis, we measured the contents of Chl *a*, Chl *b*, and Car at different growth stages for TIL22 and Teqing. The contents of Chl *a*, Chl *b*, and Car of TIL22 were approximately half of those of Teqing at the seedling stage (Figure 1d). However, the Chl and Car contents of TIL22 increased gradually with subsequent growth to be similar to those of Teqing at maturity. These results were consistent with the observed change in leaf phenotype.

3.2 | Ultrastructural changes in chloroplasts of TIL22

Using transmission electron microscopy, we compared the chloroplast ultrastructure of TIL22 and Teqing grown under identical conditions. At the seedling stage, TIL22 mesophyll cells contained a smaller number of normal chloroplasts than corresponding cells of Teqing (Figure 2a and 2b) and showed hypogenetic thylakoids that lacked a well-stacked structure (Figure 2c and 2d). During the tillering period, the chloroplast ultrastructure of TIL22 reverted to normal with

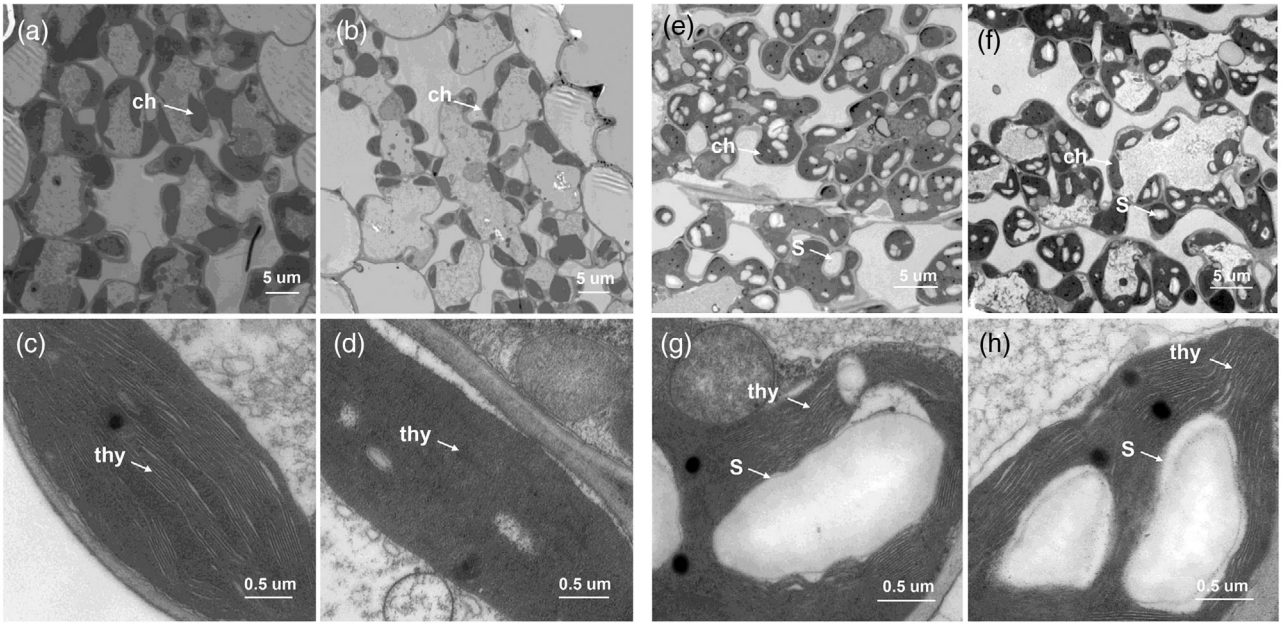

FIGURE 2 Ultrastructures of chloroplasts in the mesophyll cells. Transmission electron microscope analysis of (a and c) wild type (WT) cultivar Teqing and (b and d) TIL22 leaves at the seedling stage. Transmission electron microscope analysis of (e and g) Teqing and (f and h) TIL22 leaves at the tillering stage.ch: chloroplast, thy: thylakoid membrane, S: starch granule

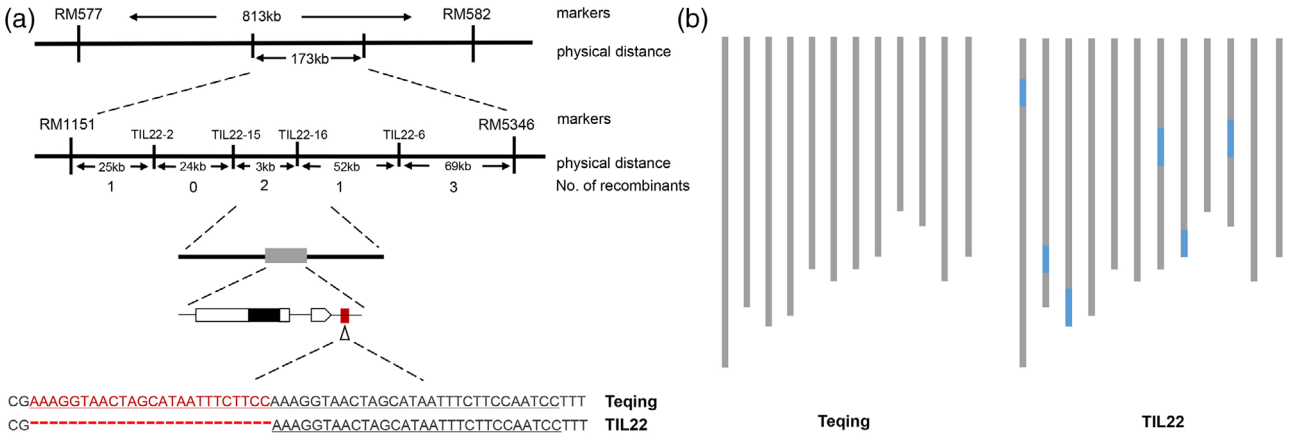

FIGURE 3 Fine mapping and mutation site analysis of the *YGL22* gene. (a) The target gene was delimited to an interval of 3 kb between TIL22-15 and TIL22-16 on chromosome 1. The gray rectangle indicates the *YGL22* gene (LOC_Os01g15390). White boxes are untranslated regions and black box is the coding region; The red dashed line indicates the 25 bp deletion in the TIL22. (b) Graphical genotypes of Teqing and TIL22. The blue boxes indicate chromosomal segments from African cultivar (*Oryza glaberrima* Steud.)

well-structured thylakoids identical to those of Teqing (Figure 2e–h). These results indicated that the abnormal chloroplasts of TIL22 seedlings was caused by delayed formation of thylakoid membranes.

3.3 | Identification of rice *YGL22* by map-based cloning

Firstly, we used about 331 SSR markers evenly distributed on the 12 chromosomes to analyze the introgression line TIL22 and its two parents. Graphical genotype from the GGT software was carried out to determine the fragment size of the total genome in TIL22 that came from each parent (Figure 3b). Then to identify the genetic basis of the leaf-color phenotype in TIL22, 295 individuals from the TIL22 × Teqing F_2 population were used for genetic analysis. Phenotypic segregation for leaf color fitted a 3:1 ratio (225 green:70 yellow-green, $\chi^2 = 0.221 < \chi^2_{0.05,1} = 3.84$), which suggested that the phenotype was controlled by a single recessive gene. A bulked segregant analysis (BSA) suggested that the locus was possibly linked to the markers RM582 and RM577 on chromosome 1 (Supplemental Figure S2), with an African cultivated

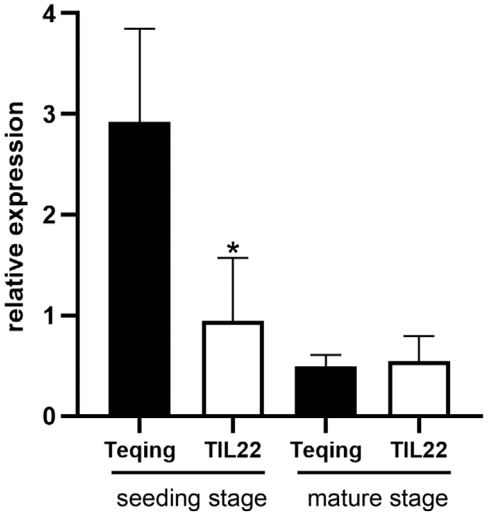

FIGURE 4 Expression analysis of *YGL22* in the leaves of wild type (Teqing) and TIL22; Values are means ± SD of three biological replicates. Significant differences compared with wild type (Teqing) were determined using Student's *t* test: *$P < .05$. **$P < .01$

rice-derived allele contributing to the phenotype (Figure 3a and 3b). We temporarily designated the locus *Yellow Green Leaf 22 (YGL22)*. To map *YGL22* precisely, based on the F_2 mapping population, we further developed a set of $F_{3:4}$ families for the target locus. For high-resolution mapping of *YGL22*, additional SSR markers were used in the previous restricted region to analyze these populations and linkage analysis with genotyping showed that the target locus was between RM1151 and RM5346 markers. Further, we developed nine new Indel markers in this region (Supplemental Table S1). Using 2,100 individuals with the yellow-green leaf phenotype from the $F_{3:4}$ populations, we further mapped *YGL22* to a 3 kb region between the markers TIL22-15 and TIL22-16, which contained a single putative gene, LOC_Os01g15390, encoding a protein of unknown function. Sequence analysis of the 3 kb region revealed duplication of a 25 bp Indel polymorphism downstream of LOC_Os01g15390 in Teqing and only one copy of the polymorphism in TIL22.

3.4 | Quantitative polymerase chain reaction analysis of the candidate gene

To investigate whether expression of LOC_Os01g15390 (*YGL22*) differed between Teqing and TIL22, we quantified the abundance of LOC_Os01g15390 mRNA by qPCR analysis. The abundance of LOC_Os01g15390 transcripts in TIL22 at the seedling stage was considerably lower compared with that of Teqing, whereas abundance at the mature stage was similar between Teging and TIL22 (Figure 4). This result was consistent with the observed leaf-color phenotypes. The gene expression pattern and molecular analysis strongly supported the hypothesis that LOC_Os01g15390, designated *YGL22*, was the likely candidate gene.

3.5 | Knockout mutant shows yellow-green leaf phenotype

To evaluate whether expression of *YGL22* was associated with the leaf-color phenotype, we used the CRISPR/Cas9 system to knockout *YGL22* in the Teqing background and generated the *ygl22* knockout mutant. We confirmed the identity of positive gene-edited individuals from the primary transformed T_0 seedlings by sequence analyses and then raised the T_1 generation. The knockout mutant showed a single-base adenine insertion in *YGL22* that resulted in a frameshift mutation and early termination of translation (Figure 5a). In contrast to the wild type, seedlings of the *ygl22* mutant showed the same yellow-green leaf phenotype as TIL22 (Figure 5b). And the leaves of *ygl22* gradually changed color the green at maturity, showing no obvious difference with leaves of wild-type Teqing. The wild-type Teqing and *ygl22* were planted in the same paddy field with the same management.

Phenotypic analysis showed that the plant height, number of panicles per plant, and grain yield per plant of the *ygl22* mutant were similar to those of Teqing (Figure 5c and 5d). These data suggested that loss-of-function of *YGL22* had no negative effect on plant yield, and thus *YGL22* could be used as a trait marker gene in commercial hybrid rice production.

3.6 | Subcellular localization of the YGL22 protein in rice protoplasts

The subcellular localization of the YGL22 protein was examined by fusing the *YGL22* coding sequence with the green fluorescent protein (GFP) gene and transiently expressing the fusion protein in rice protoplasts. The GFP fluorescence of the fusion protein was co-localized with chlorophyll autofluorescence (Figure 6), whereas signal from transient expression of GFP alone was detected in the nucleus and cytoplasm. This result indicated that the YGL22 protein was localized in the chloroplast and may be involved in chloroplast development.

4 | DISCUSSION

In this study, we isolated an introgression line, TIL22, that exhibited a yellow-green leaf phenotype at the seedling stage from a set of introgression lines of African cultivated rice (IRGC102305, *O. glaberrima*) with the genetic background of Teqing (*O. sativa* subsp. *indica*). We observed that the leaf color gradually changed to green after the five-leaf stage in TIL22 (Figure 1a–c).

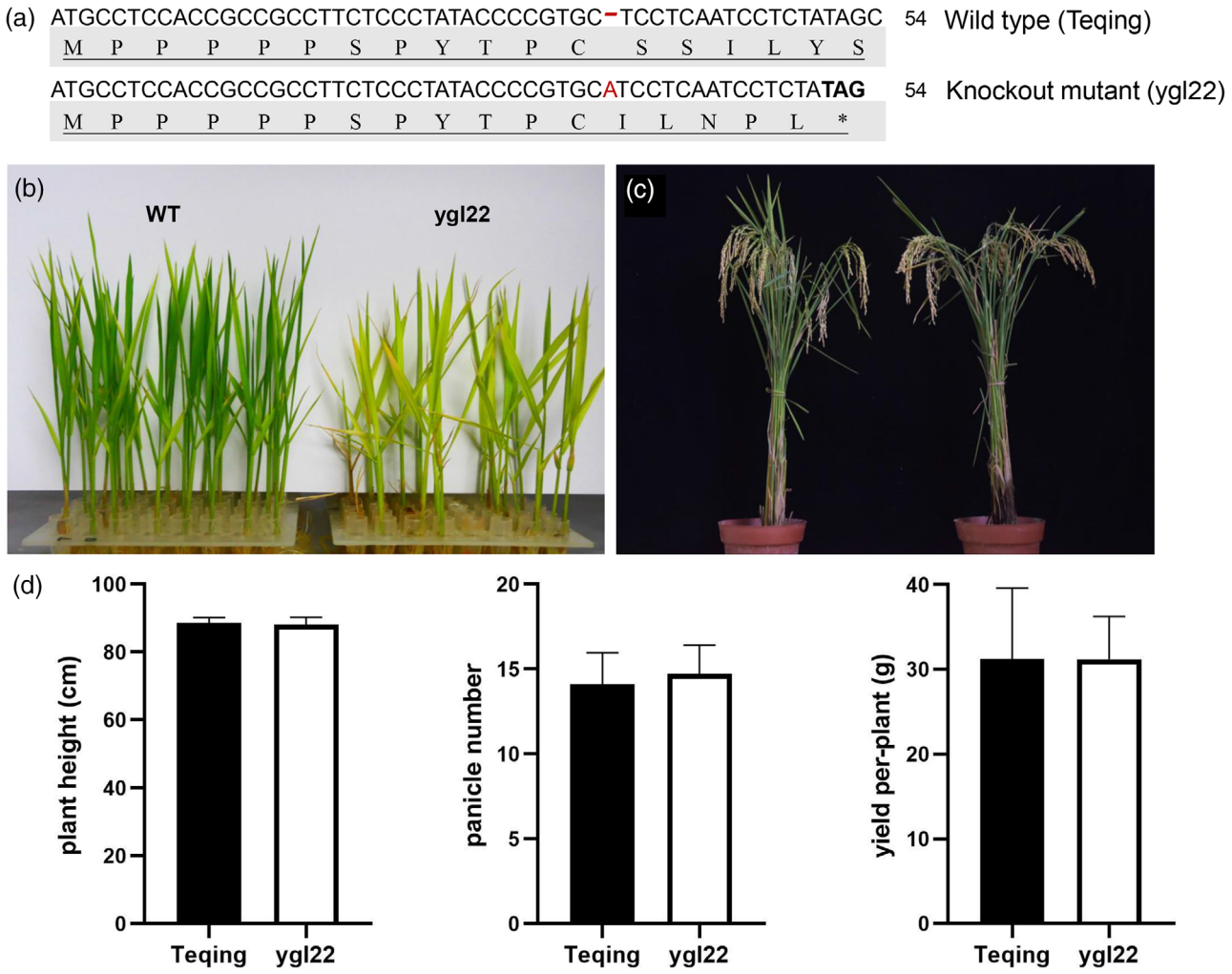

FIGURE 5 Characterization of ygl22 plants. (a) The red character indicates the 1 bp insertion in the *ygl22* mutant which lead to early termination of the translation. (b) Morphology of wild type (WT) cultivar Teqing (left) and the knockout mutant *ygl22* (right) at the three-leaf stage. (c) Morphology of wild type (WT) cultivar Teqing (left) and the knockout mutant *ygl22* (right) at the mature stage. (d) Plant height, panicle number and yield per plant. Significant differences compared with wild type (Teqing) were determined using Student's *t* test: $*P < .05$. $**P < .01$

Comparison of Chl *a*, Chl *b*, and Car contents in TIL22 and Teqing revealed that, at the seedling stage, the Chl *a* content in TIL22 was substantially lower than that of Teqing, but subsequently increased to be identical to that of Teqing at the mature stage (Figure 1d). The development of chloroplast ultrastructure showed a similar pattern. Thus, the chloroplasts developed more slowly in TIL22 compared with those of Teqing, as shown by the fewer and retarded development of thylakoids in cells at the seedling stage (Figure 2). These results indicated that the gradual change in leaf color to green in TIL22 was a result of continued accumulation of chloroplasts, although the chloroplasts developed more slowly than in Teqing.

The present mapping results showed that the most likely candidate gene was LOC_Os01g15390, designated *YGL22*, which encodes a domain of unknown function (Figure 3a). We noted that the coding sequence of *YGL22* in TIL22 and Teqing was identical. The two parents differed in number of copies of a 25 bp Indel polymorphism downstream of LOC_Os01g15390 (Figure 3a). Teqing harbored two copies, whereas only one copy was present in TIL22. A qPCR analysis revealed that the *YGL22* transcript level in Teqing was significantly higher than that in TIL22 at the seedling stage, whereas the transcript levels were similar at the mature stage (Figure 4). This result suggested that *YGL22* may predominantly participate in the early development of chloroplasts.

Knockout of *YGL22* in the wild type (Teqing) and observation of the phenotype of the T_1 generation at the seedling stage revealed that loss-of-function of *YGL22* caused a yellow-green leaf phenotype identical to that of TIL22 (Figure 5b). Subcellular localization analysis confirmed that *YGL22* encodes a chloroplast protein (Figure 6). These results indicated that *YGL22* might be the primary cause of the leaf-color phenotype. However, the reason for the difference in

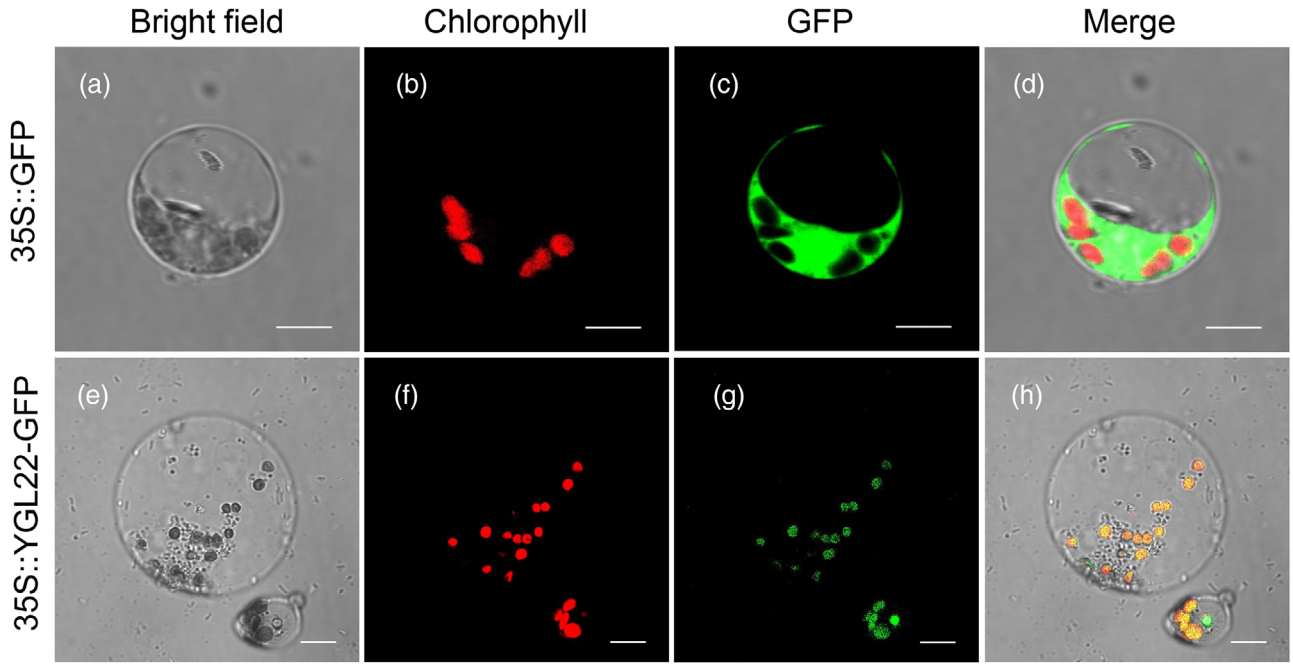

FIGURE 6 Subcellular location of 35S::GFP and 35S::YGL22-GFP in rice protoplasts. (a and e) Bright field, (b and f) chlorophyll fluorescence, (c and g) GFP fluorescence in rice protoplasts were observed separately by confocal laser scanning microscopy, and (d and h) the images were merged. Scale bars are 5 μm

expression levels of YGL22 between the two parents, despite the identical sequence of the coding region, remains unclear. We speculate that the 25 bp fragment acted as a regulatory element and affected the expression of *YGL22*. In a previous study, a rice quantitative trait locus, *SGDP7* (Bai et al., 2017), contained an 18 bp fragment designated CNV-18 bp, which acted as an upstream silencer of *FZP*. The CNV-18 bp fragment inserted upstream of *FZP* resulted in a tandem duplication, which repressed *FZP* expression, prolonged the rice panicle-branching period, and boosted grain yield. Previous studies have shown that sequence changes in the regions upstream and downstream of a gene may affect its transcription level. Wang et al. (2015) inserted the first intron of rice *Act1* downstream of $P_{Osrbcs\text{-}62}$ and observed sharply increased activity of $P_{Osrbcs\text{-}62}$ in the stem.

There are many potential genomic locations for regulatory elements (Ogbourne & Antalis, 1998). Such elements may act to increase or decrease transcription of neighboring genes (Biłas, Szafran, Hnatuszko-Konka, & Kononowicz, 2016). Whether the 25 bp repetitive sequence regulates the transcript level of *YGL22* requires further study, which could contribute to an improved understanding of gene regulation. In addition, further efforts to conduct functional characterization of *YGL22* will help to elucidate its biological function and the mechanism of chloroplast development in rice.

Notably, the donor parent *O. glaberrima* showed the normal green leaf phenotype, whereas the introgression line TIL22 displayed a yellow-green leaf phenotype. Initially, we considered that a natural mutation may have occurred during the construction of the introgression population. However, BLAST analysis (http://ensembl.gramene.org/Oryza_glaberrima/Tools/Blast) showed that two identical copies of *YGL22* (ORGLA01G0090100 and ORGLA07G0227300) are located on chromosomes 1 and 7, respectively, in *O. glaberrima*. Then, we compared the sequences of *YGL22* coding sequence and its 1-kb upstream and 1-kb downstream in *O. glaberrima* chromosome 1 and 7, which showed no differences. Thus, we speculated that the both copies function in *O. glaberrima*, making the leaves appear green at the seedling stage, while there is only one copy in TIL22 which cannot meet the requirement of maintaining normal green leaves. However, the exact reason for the different color phenotypes between TIL22 and the donor (*O. glaberrima*) is still unknown. Whether this is because of the different copy number needs further study and more evidence.

In addition, the present results indicated that YGL22 performed distinct functions at the seedling stage, but had little effect on important agronomic traits, such as plant height and number of panicles per plant, at the mature stage (Figure 5d). This phenomenon is similar to that reported for a number of rice leaf-color mutants described previously, such as *ys83* and *ygl7* (Deng et al., 2014; Ma et al., 2017). Hence, we consider that *YGL22* could be used as a leaf-color marker gene in commercial two-line hybrid rice production.

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AUTHOR CONTRIBUTIONS

Y.Z. performed the research and wrote the article; P.Y. participated in the DNA and total RNA extraction and subcellular localization; S.D. and Z.H. contributed to helping with the field work and analyzed the data; Y.W. assisted the rice transgenic experiments; J.Y. contributed to modifying the manuscript. X.L. and X.X. designed the experiments and revised the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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