Disruption of a Rice Pentatricopeptide Repeat Protein Causes a Seedling-Specific Albino Phenotype and Its Utilization to Enhance Seed Purity in Hybrid Rice Production\textsuperscript{1[W][OA]}

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The pentatricopeptide repeat (PPR) gene family represents one of the largest gene families in higher plants. Accumulating data suggest that PPR proteins play a central and broad role in modulating the expression of organelar genes in plants. Here we report a rice (\textit{Oryza sativa}) mutant named \textit{young seedling albino} (\textit{ysa}) derived from the rice thermo/photoperiod-sensitive genic male-sterile line Pei’ai4S, which is a leading male-sterile line for commercial two-line hybrid rice production. The \textit{ysa} mutant develops albino leaves before the three-leaf stage, but the mutant gradually turns green and recovers to normal green at the six-leaf stage. Further investigation showed that the change in leaf color in \textit{ysa} mutant is associated with changes in chlorophyll content and chloroplast development. Map-based cloning revealed that \textit{YSA} encodes a PPR protein with 16 tandem PPR motifs. \textit{YSA} is highly expressed in young leaves and stems, and its expression level is regulated by light. We showed that the \textit{ysa} mutation has no apparent negative effects on several important agronomic traits, such as fertility, stigma extrusion rate, selfed seed-setting rate, hybrid seed-setting rate, and yield heterosis under normal growth conditions. We further demonstrated that \textit{ysa} can be used as an early marker for efficient identification and elimination of false hybrids in commercial hybrid rice production, resulting in yield increases by up to approximately 537 kg ha\textsuperscript{-1}.

The completion of the genome sequence of several higher plants species, including Arabidopsis (\textit{Arabidopsis thaliana}; Arabidopsis Genome Initiative, 2000), rice (\textit{Oryza sativa}; Goff et al., 2002; Yu et al., 2002), maize (\textit{Zea mays}; Schnable et al., 2009), sorghum (\textit{Sorghum bicolor}; Paterson et al., 2009), castor bean (\textit{Ricinus communis}; Chan et al., 2010), soybean (\textit{Glycine max}; Schmutz et al., 2010), potato (\textit{Solanum tuberosum}; Xu et al., 2011), salt cress (\textit{Thellungiella halophila}; Dassanayake et al., 2011), and most recently \textit{Medicago truncatula} (Young et al., 2011), has opened an unprecedented opportunity for functional genomics studies in higher plants. Meanwhile, it poses a significant challenge to the plant science community to elucidate the functions of all plant genes. One of the largest and perhaps the most mysterious gene families uncovered by bioinformatic analyses is the pentatricopeptide repeat (PPR) protein family, which is characterized by the tandem array of a PPR motif, a highly degenerate unit consisting of 35 canonical amino acid. The PPR motif is predicted to resemble the structure of the tetratricopeptide repeat motif that consists of a protein sequence constituting two antiparallel α-helices. This motif is found in a few animal and fungal proteins but the family has expanded greatly in higher plants, with 466 members in Arabidopsis and 480 in rice (Small and Peeters, 2000; Lurin et al., 2004). The vast majority of these proteins are predicted to localize to...
chloroplasts or mitochondria, but a small portion is predicted as untargeted and they likely localize outside the organelles of the plant cells (Lurin et al., 2004; Ding et al., 2006).

Functional studies of PPR proteins in higher plants remain very sparse. Accumulating data point to an involvement in posttranscriptional processes in organelles. Fisk et al. (1999) first reported a maize PPR gene, (for CHLOROPLAST RNA PROCESSING1), which was implicated by genetic analysis in processing and translation of plastid pet transcripts. Similar effects on plastid transcripts were subsequently observed in other mutants from Arabidopsis (Hashimoto et al., 2003; Meierhoff et al., 2003; Yamazaki et al., 2004; Chatteigner-Boutin et al., 2008, 2011; Chi et al., 2008; Okuda et al., 2009, 2010; Yu et al., 2009; Johnson et al., 2010), rice (Kazama and Toriyama, 2003; Komori et al., 2004; Gothandam et al., 2005), and maize (Williams and Barkan, 2003; Schmitz-Linneweber et al., 2006; Pfalz et al., 2009; Prikryl et al., 2011). Additional evidence for a role of PPR proteins in regulating organelle gene expression has also come from positional cloning of several cytoplasmic male sterility (CMS) restorer genes from petunia (Petunia hybrida; Bentolila et al., 2002) and radish (Raphanus sativus; Brown et al., 2003; Desloire et al., 2003; Koizuka et al., 2003). Genetic and biochemical data, and structural modeling of PPR tracts based on established tetratricopeptide repeat proteins together suggest that PPR proteins typically bind directly to specific organellar RNA sequences through a surface created by the stacked helical repeating units. However, still very little is known about the functions, substrates, and regulatory mechanisms for the vast majority of PPR proteins.

Food security is a global concern. The development and utilization of hybrid rice has made a great contribution to ensuring food sufficiency in China, and the annual planting area of hybrid rice has been maintained at about 16 million ha since the early 1990s. In 2010, hybrid rice accounted for about 58% of China’s 196 million tons of rice production. The average yield of hybrids is 7.1 to 7.2 tons per ha compared with 5.8 to 5.9 tons per ha for inbred varieties (Normile, 2008). Recently, hybrid rice has been adopted in other countries. So far, more than 40 countries have participated in the introduction, research, and promotion of hybrid rice worldwide. In 2008, the cultivation area of hybrid rice has reached 1.3 million ha in India, 0.65 million ha in Vietnam, and 0.34 million ha in Philippines. Compared with the traditional CMS-based three-line hybrid rice system, the development of two-line hybrid rice system based on thermo/photoperiod-sensitive genic male-sterile (T/PGMS) lines has raised the yield potential of rice by another 10% (Sing and Limani, 1990; Yuan, 2004). However, a major constraint in utilizing two-line hybrid rice, especially for indica rice, is the unpredictable influence of prevailing temperature and photoperiod conditions on the fertility of the T/PGMS line during the male-sterility-inductive period (Yuan, 1997). Normally, the T/PGMS lines are male sterile under high-temperature and long-day conditions, but they become fertile under lower-temperature and short-day conditions—these conditions are used for the propagation and maintenance of T/PGMS lines. This means that a significant fall in temperature during the male-sterility-inductive period could cause the male-sterile lines to become partially fertile, resulting in contamination of the hybrid seeds by T/PGMS selfed seeds and, as a consequence, a decrease in hybrid yield (Bi et al., 1990).

Great efforts have been made to minimize the environmental effect in hybrid rice production. However, previous attempts using leaf-color mutants as screening markers to monitor seed purity in two-line hybrid rice production have achieved limited success (Wu et al., 2002). To search for markers better suited for use in early removal of false hybrids, we sought to identify markers that allow early detection of false hybrids resulted from selfed T/PGMS seeds. In this study, we isolated a rice mutant named young seedling albino (ysa), which develops albino leaves before the three-leaf stage. Map-based cloning revealed that YSA encodes a PPR protein. We demonstrated that the ysa mutation could be used as an early marker for efficient identification and elimination of false hybrids in commercial hybrid rice production, resulting in a grain yield increase of up to approximately 537 kg ha$^{-1}$.

RESULTS

Phenotypic Analysis of the ysa Mutant

Leaves of ysa mutant appeared albinic albino before the three-leaf stage (Fig. 1A), but gradually turned green from the four-leaf stage onward (Fig. 1B) and appeared normal green like the leaves of parental Pei’ai64S plants after the six-leaf stage (Fig. 1C). The contents of chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid in ysa plants before the three-leaf stage were drastically lower than those in the parental Pei’ai64S plants (Fig. 1D). After regreening, the levels of these pigments in the ysa mutant increased and became similar to Pei’ai64S plants (Fig. 1E). These results suggest that the albino phenotype of the young ysa seedlings is caused by a reduction in total chlorophyll content, rather than reduction of a particular pigment.

We next examined whether the lack of photosynthetic pigments in the ysa mutant were accompanied by ultrastructural changes in the chloroplasts. We compared the ultrastructure of chloroplasts in the ysa mutant and Pei’ai64S at three developmental stages using transmission electron microscopy. Granal stacks in Pei’ai64S plants were dense and well structured at all three developmental stages (Fig. 2, A–C). The granal stacks in ysa mutant were less dense and lacked well-structured thylakoid membranes before the three-leaf stage (Fig. 2D). Subsequently, the thylakoid membranes slightly increased (Fig. 2E), and the granal stacks in ysa mutant were similar to those of wild-type plants.
after the six-leaf stage (Fig. 2F). These observations revealed that the ysa mutation primarily affects chloroplast development in the first few leaves.

Cloning of the YSA Gene

To understand the molecular basis of the phenotype, we used map-based cloning to isolate the YSA gene. Genetic analyses from reciprocal crosses between the ysa mutant and the two indica rice cultivars 93-11 and Taiziyuzhu showed that the albino phenotype in the ysa mutant is recessive and controlled by a single gene (Supplemental Table S1). Molecular analysis of an F2 population from the cross Taiziyuzhu × ysa placed the YSA locus between the markers RM411 and RM8208 on chromosome 3 (Fig. 3A). Five simple sequence repeat markers, four derived cleaved amplified polymorphic sequence (dCAPS) markers, and one insertion-deletion polymorphism (InDel) marker were developed between RM411 and RM8208 (Supplemental Table S2). The YSA locus was further narrowed down to a 45-kb region, which includes 10 putative open reading frames (ORFs; Fig. 3, B–D). We sequenced all ORFs and found a 5-bp deletion in Os03g40020, causing a premature stop codon (Fig. 3D). A newly developed InDel marker P9 can be used to distinguish ysa mutant and wild-type Pei’ai64S by the size of the amplified genomic DNA fragment (Fig. 3E). Molecular complementation experiment showed that all of the 25 independent transgenic lines transformed with vector pYSA containing the wild-type Os03g40020 gene completely rescued the ysa mutant phenotype as judged by the color of the first few leaves and their thylakoid structure, whereas 20 independent lines transformed with vector pYSAT containing the shifted ORF all failed to rescue the ysa mutant (Fig. 3, F and G). These results suggested that the 5-bp deletion in Os03g40020 is responsible for the albino phenotype of ysa mutant.

Figure 1. Phenotypic analysis of the ysa mutant plants. A to C, Phenotypes of Pei’ai64S (left) and ysa mutant (right) seedlings at 1 (A), 2 (B), and 3 (C) weeks after sowing. D, The pigment contents in leaves of 1-week-old ysa mutants are much lower than that in Pei’ai64S. E, The pigment contents in leaves of 6-week-old ysa mutants are similar to that of Pei’ai64S plants. Chla, Chlorophyll a; Chlb, chlorophyll b; Chl, total chlorophyll; Car, carotenoid. Bars represent ±s of three measurements. Student’s t test was performed on the raw data; asterisk indicates statistical significance at P < 0.01.

Figure 2. Transmission electron microscopic images of chloroplasts of wild-type Pei’ai64S and ysa mutant plants at 10 d (A and D), 18 d (B and E), and 30 d (C and F) after sowing. Chloroplasts of Pei’ai64S have abundant, well-ordered stacks membranes after sowing for 10, 18, or 30 d, but ysa have normal stacked membranes only after 30 d.
The predicted ORF of YSA encodes a 742-amino acid polypeptide with a calculated molecular mass of 82.5 kD. Database searches using Pfam (Finn et al., 2006) revealed that YSA encodes a member of the F subfamily of PPR-containing proteins. The gene product contains a tandem repeat of 15 PPR motifs with varying degrees of conservation (Fig. 4A). The second to 15th repeats are contiguous, and the sequence is not well conserved (Fig. 4B). The PPR motif is a degenerate 35-amino acid repeat often arranged in tandem arrays of two to 27 repeats per polypeptide. Thus YSA is a new member of the superfamily of PPR proteins.

BLAST searches of the available genome sequences revealed that in the rice genome, there is another gene (Os11g03850) encoding a PPR protein that shares high sequence homology to YSA (33.4% amino acid identity). Close homologs of YSA were also identified in sorghum, maize, Arabidopsis, Populus trichocarpa, M. truncatula, and soybean (Supplemental Fig. S1); however, the functions of these genes are largely unknown. Notably, YSA and the Arabidopsis protein MEE40 (encoded by At3G53700) possess a similar domain structure and they share the highest sequence homology across their entire lengths (51% amino acid identity; Fig. 4C). Interestingly, MEE40 protein was previously reported to be essential for embryogenesis (Pagnussat et al., 2005).

**Expression Analysis of YSA**

To examine the tissue-specific expression pattern of YSA, we generated transgenic plants expressing a GUS reporter gene driven by the YSA promoter (pYSA::GUS). Histochemical assay revealed that YSA is highly expressed in young leaves and stems, but not in the roots (Fig. 5, A–D). Quantitative real-time reverse transcription (RT)-PCR analysis revealed that the expression of YSA peaked in the fourth leaf (Fig. 5E). Thus, the expression pattern of YSA is consistent with the seedling-stage-specific albino phenotype of ysa mutant and further supports the notion that YSA plays an important role in chloroplast development in the first few leaves of rice seedlings, but plays more minor roles in later stages.

Light is one of the most important environmental signals that trigger the differentiation of nonphotosynthetic proplastids into fully functional photosynthetic chloroplasts. To examine the effects of light on the expression of YSA, we examined the accumulation of YSA transcripts during light-induced greening of Pei’ai64S seedlings. As shown in Figure 5E, YSA transcript level was high under light, but much lower in the dark in 10-d-old etiolated seedlings. Furthermore, the expression level of YSA was highly induced after illumination for 4 h and reached maximal levels after 16 h of illumination (Fig. 5F). These observations suggest that light plays a major role in regulating YSA expression.

We next examined the transcription levels of other genes associated with chlorophyll biosynthesis, chloroplast development, or photosynthesis in the ysa mutant. Nine genes were selected, including those encoding a glutamyl-tRNA reductase (HEMA1), CHLOROPHYLLIDE A OXYGENASE1 (CAO1), NADPH: PCHLIDE OXIDOREDUCTASE, light-harvesting Chi a/b-binding protein of PSI (CAB1R and CABI2R, Matsuoka,
1990), two reaction center polypeptides (psaA and psbA), Rubisco large subunit, and Rubisco small subunit (Kyozuka et al., 1993). Quantitative real-time PCR analysis showed that the transcripts of both nuclear-encoded genes (such as CAB1R, CAB1R, HEMA1, CAO1, and Rubisco small subunit) and plastid-encoded genes (such as psaA, psbA, and Rubisco large subunit) were all severely suppressed at the two-leaf stage in the ysA mutant (Fig. 6), but the suppression became alleviated in the ysA mutant at the four-leaf stage, and was fully restored to wild-type levels at the seven-leaf stage. These results indicated that YSA plays an important role in regulating gene expression associated with chlorophyll biosynthesis, chloroplast development, and photosynthesis in the first few leaves.

Subcellular Localization of YSA Protein

Most PPR proteins are predicted to be targeted to either mitochondria or chloroplasts (Small and Peeters, 2000). The YSA protein is predicted to localize to chloroplasts by ChloroP (Emanuelsson et al., 1999) and TargetP (Emanuelsson et al., 2000). To investigate the actual cellular localization of YSA, we constructed a chimeric gene expressing a fusion protein consisting of the 200 N-terminal amino acids of YSA and GFP under the control of the 35S promoter. The plasmid containing the chimeric gene was transformed into Arabidopsis protoplasts, and confocal microscopy was used to observe the fluorescent signals 16 h after transformation. The green fluorescent signals of YSA-GFP fusion proteins colocalized with the autofluorescent signals of chlorophylls in the chloroplasts, consistent with the results obtained for GFP fused to the transit peptide of the small subunit of Arabidopsis ribulose bisphosphate carboxylase (Fig. 7, A and B). When GFP fused to the nuclear localization signal of the fibrillarin protein, GFP signals located specifically in the nucleus of Arabidopsis protoplasts (Fig. 7C). In addition, the protoplasts transformed with the empty GFP vector without a specific targeting sequence had green fluorescent signals in both the cytoplasm and the nucleus (Fig. 7, D and E). To further confirm the subcellular localization of YSA protein, we transformed the plasmid containing the YSA-GFP fusion constructs into rice protoplasts. Confocal microscopy observations revealed that GFP-YSA was exclusively detected in the chloroplasts (Fig. 7F). These findings suggest that YSA protein is localized to the chloroplast.
Utilization of ysa as an Early Marker to Remove Off-Type Seedlings during T/PGMS Line Propagation and Hybrid Seed Production

To use the albino phenotype of ysa mutant as a leaf-color marker in hybrid rice production, we first examined the potential effect of the ysa mutation on several important agronomic traits of T/PGMS lines. There were not any discernible differences between the parental Pei’ai64S plants and the ysa plants in heading date, plant height, tiller number, panicle length, floret number, stigma extrusion rates, selfed seed-setting rate, hybrid seed-setting rate, 1,000-grain weight, and yield per plant (Supplemental Table S3). In addition, we examined the potential effect of the ysa mutation on male sterility, using the standard methods of T/PGMS authentication used by the China National Rice Research Institute. The results showed that Pei’ai64S and the ysa mutant had similar seed-setting rates when...
grown under different temperature and photoperiod conditions (Supplemental Table S4). These data indicated that the ysa mutation did not affect the agronomic traits of the parental Pei'ai64S T/PGMS line.

Yield heterosis is the key trait for the male-sterile parent in hybrid rice production and F1 hybrid yield is the main measurement for yield heterosis in the field. Using the same restorer line 93-11, we found that all F1 hybrid plants derived from Pei'ai64S or ysa displayed normal green leaves and similar grain yields and other agronomic traits, indicating that the ysa mutation did not have any negative effects on yield heterosis (Supplemental Table S3). We next tested the ysa mutation as a marker for removing off-type seedlings in T/PGMS line propagation. Results showed that only the true male-sterile plants derived from self-pollination develop albino leaves, whereas off-type seedlings from cross-pollination are green (Fig. 8A). We randomly sampled 5,000 seeds from self-pollinated ysa T/PGMS line grown under conditions necessary for full fertility, and sowed them in different fields in 2006 and 2007. Off-type seeds arising from biological mixtures (cross-pollination) or mechanical mixtures were identified at the seedling stage. This sampling showed that the seeds produced from selfed ysa were approximately 99.8% pure each year, and the seed purity reached 100% after removal of the false-positive green seedlings (Table I). It is worth noting that although the false-positive seedlings were only approximately 0.12% of the total seedlings in the trials, the improvement in the self-pollinated ysa T/PGMS seeds is significant in commercial seed production. With an average planting density of 300,000 plants ha⁻¹, and at an average productivity of 500 seeds plant⁻¹, there will be approximately 168,000 off-type T/PGMS seeds produced per ha in commercial seed production.

On the other hand, for hybrid seed production, it is the albino seedlings that are off-type seeds (false hybrid) and derived from self-pollination of the ysa mutant, and it is the green seedlings that are true F1 hybrids derived from cross-pollination with the restorer (Fig. 8B). We also randomly sampled 5,000 F1 seeds generated from crosses involving ysa with the 93-11 restorer line and sowed them in a field. We found 183 and 121 albino seedlings in the 2006 and 2007 trial, respectively (Table I). Although these off-type seedlings were only approximately 3.66% of the total samples, their removal increased the hybrid seeds purity to 100%. According to these data, we estimated that the use of the ysa mutation increased hybrid seed yield by up to 537 kg/ha and 367 kg/ha in 2006 and 2007, respectively (Table I).

To further test and expand the utility of the ysa marker, we introduced the ysa mutation into Guangzhan63S, which is one of the most popular commercial T/PGMS lines currently used for hybrid seed production in China. Analysis of agronomic traits and the heterosis confirmed that ysa worked well as an efficient marker for T/PGMS propagation and hybrid seed production (Supplemental Table S3). Recently, a new hybrid rice variety with good grain quality and high yield potential, harboring the ysa marker, was released, and tested in provincial trials. It is estimated that approximately 10,000 ha of hybrid rice developed by utilizing the ysa marker are now being grown in the middle and lower regions of Yangtze River, and that the acreage of hybrid rice based on this ysa system will reach approximately 0.2 million ha by 2012 or shortly thereafter.

Figure 7. Subcellular localization of YSA protein. Fluorescence signals were visualized using confocal laser-scanning microscopy. Green fluorescence shows GFP, red fluorescence indicates chloroplast autofluorescence, and yellow fluorescence indicates images with the two types of fluorescence merged. A, GFP signals of the YSA-GFP fusion protein. B, GFP signals from the transit peptide of ribulose bisphosphate carboxylase small subunit (control). C, GFP signals from the nuclear localization signal of fibrillarin (control). D, Empty GFP vector without a specific targeting sequence. E, Untransformed chloroplasts. F, Subcellular localization of YSA protein in rice protoplasts. Bars = 5 μm. A to E are Arabidopsis protoplasts and F is rice protoplasts.
DISCUSSION

Here, we showed that a rice PPR protein, YSA, is required for chloroplast development in early seedling leaves, and disruption of its function causes a seedling-stage-specific albino phenotype, but the plant recovers and develops normal green leaves from the four-leaf stage onward. We showed that the ysa mutation could be used as an early marker for efficient identification and elimination of false hybrids in commercial hybrid rice production.

YSA Encodes a PPR Protein Essential for Chloroplast Biogenesis in Early Leaves

The PPR protein family is one of the largest families in plants. Although PPR proteins are found in other eukaryotes, their large number (466 members in Arabidopsis and 480 in rice) is probably required for plants to meet the specific needs of organelar gene expression. Most PPR proteins are predicted to localize to plastids or mitochondria. It is generally believed that the repeats of PPR proteins form a superhelical structure to bind a specific ligand, probably a single-stranded RNA molecule, and modulate its expression (Lurin et al., 2004).

Functional analyses on this group of proteins in plants have just begun. PPR proteins have been implicated in many crucial functions including chloroplast biogenesis, embryogenesis fertility restoration in CMS plants, and plant development (Saha et al., 2007). An emerging theme of PPR protein function is regulation of various aspects of gene expression in plastids, including transcription, splicing, RNA cleavage, RNA editing, translation, and RNA stabilization (Schmitz-Linneweber and Small, 2008). For example, the Arabidopsis dg1 (for delayed greening1) and ys1 mutants (for yellow seedling1) display a seedling-stage-specific albino and yellow seedling phenotype, respectively. Both DG1 and YS1 encode chloroplast-targeted PPR proteins and YS1 has been shown to be required for editing of rpoB transcripts (Chi et al., 2008; Zhou et al., 2009). The null mutation of the maize PPR2 gene leads to albino seedlings, but does not affect seed development or gametogenesis (Williams and Barkan, 2003). Maize PPR4 (ZmPPR4) is a chloroplast-targeted protein harboring both a PPR tract and an RNA recognition motif and it facilitates transsplicing of chloroplast rps12 premRNA (Schmitz-Linneweber et al., 2006). ZmPPR10 bound to its native binding site in the chloroplast atpI-atpH intergenic region stabilizes RNA and activates translation (Pfalz et al., 2009; Prikryl et al., 2011).

So far, only one PPR protein affecting chloroplast development in rice has been reported. OsPPR1 is a chloroplast-targeted PPR protein with 11 PPR motifs, and is required for chloroplast biogenesis (Gothandam et al., 2005). This study reports the second PPR protein, YSA, to be required for chloroplast biogenesis in rice. This finding adds further support to a central role of PPR proteins in regulating chloroplast development in a staple crop plant. However, there are several important differences between OsPPR1 and YSA proteins and their respective mutant phenotypes. First, although both OsPPR1 and YSA belong to the PPR gene family and share similar secondary structure, they do not share significant primary sequence homology (amino acid identity is only 15.64%). Phylogenetic analysis revealed that the two proteins are grouped in different clades (Supplemental Fig. S1), suggesting that they might have been evolved from distinct immediate ancestors. Second, the albino phenotype of the ysa mutant is seedling stage specific and the mutant is viable, whereas the OsPPR1 antisense transgenic plants are lethal and their pigmentation is not reversible. Third, no internal membranes or clear prolamellar body were detected, with only proplastid-like structures being detected in the chloroplast of

Figure 8. Application of the ysa marker in T/PGMS propagation and two-line hybrid seed production. A, The ysa marker identifies the green off-type seedlings during propagation of T/PGMS seeds. B, ysa identifies albino off-type seedlings during two-line hybrid seed production.
OsPPR1 antisense transgenic plants (Gonthadam et al., 2005), whereas chloroplasts with reduced thylakoid membrane were detected in the ysa mutant leaves. Further, the expression levels of a number of chloroplast development-associated genes were significantly down-regulated in the two-leaf-stage ysa mutant as compared with Pei’ai64S, but the differences became less at the four-leaf stage, and disappeared at the seven-leaf stage (Fig. 6). However, it was reported that the expression level of psaA and psbA rarely decreased in the OsPPR1 antisense plants (Gonthadam et al., 2005). Taken together, these results suggest that YSA and OsPPR1 may play distinct roles in regulating chloroplast development. Future studies will be required to further dissect the functional relationship between YSA and OsPPR1 and the molecular mechanisms linking YSA to chloroplast development.

Why the ysa mutant displays a seedling-stage-specific albino phenotype is an intriguing question. A possible explanation is that other related genes may compensate for the absence of YSA during later developmental stages. It is also possible that YSA is not required for later developmental stages of chloroplast development, as the expression level of YSA decreased as their development proceeded (Fig. 5E). This phenotype, in essence, is very similar to a previously described virescent (v) phenotype, which is defined as presenting young leaves with reduced chlorophyll but can accumulate almost normal amounts of chlorophyll as they mature (Archer and Bonnett, 1987). Due to the unique features of their chloroplast biogenesis and potential value in agriculture, v mutants have been studied for more than half a century in various plant species, including maize, rice, cotton, tobacco (Nicotiana tabacum), peanut (Arachis hypogaea), and Arabidopsis (Zhou et al., 2009). To date, only a few genes responsible for v phenotype have been isolated and their molecular mechanisms remain largely unknown. For example, the rice yellow-green leaf1 (ygl1) mutant showed yellow-green leaves in young plants with decreased chlorophyll synthesis and delayed chloroplast development. The ygl1 mutant carries a missense mutation (Pro-198 to Ser) in the rice chlorophyll synthase gene (Wu et al., 2007). Such mutants became green with near-normal chlorophyll accumulation at the mature stage. Why the ygl1 mutation, like ysa, dramatically affects chlorophyll biosynthesis in the early developmental stages but appears to be dispensable in the later stages is also not clear. The rice v2 mutant develops chlorotic leaves at the restrictive temperature (20°C), but develops nearly normal green leaves at the permissive temperature (30°C; Iba et al., 1991). The v2 mutation affects activation of the chloroplast translation machinery and/or expression of the plastid-to-nucleus signaling pathway during the P4 stage of leaf development (Sugimoto et al., 2004). The V2 gene was cloned and shown to encode a novel type of guanylate kinase (Sugimoto et al., 2007). Clearly, chloroplast development and pigmentation is under complex genetic control and elucidating the molecular mechanisms regulating chloroplast and leaf development in higher plants remains a major task for future studies.

On the other hand, despite the overwhelming accumulated evidence supporting the notion that the majority of PPR proteins are involved in organellar RNA metabolism, studies of their biochemical function have remained quite limited. Attempts to identify the in vivo RNA ligands have only been made for a few PPR proteins (Schmitz-Linneweber and Small, 2008; Okuda et al., 2009, 2010; Pfalz et al., 2009; Yu et al., 2009; Johnson et al., 2010; Chateigner-Boutin et al., 2011; Prikryl et al., 2011). Many of the postulated activities of PPR proteins still lack direct biochemical proof and thus leave open the possibility that their roles are more indirect than suspected and may require additional, unidentified factors. Further studies will be aimed at identifying the RNA ligands and/or interacting proteins that YSA binds to and determining the biochemical activity of YSA.

Advantages of Utilizing ysa as a Seedling-Stage-Specific Marker for Enhancing Seed Purity during Hybrid Seed Production

The discovery of T/PGMS lines opened up a new avenue for the development of two-line hybrid rice

### Table 1. Utilization of the ysa marker to remove off-type plants in T/PGMS line propagation and F1 hybrid rice production

| Parameters Investigated | ysa T/PGMS Line | F1 (ysa × 93-11) |
|-------------------------|----------------|-----------------|
| Year                    | 2006          | 2007           |
| Seed number             | 5,000         | 5,000          |
| Green seedlings         | 6             | 3              |
| No. of off-type plants  | 6             | 3              |
| Original seed purity (%)| 99.88         | 99.94          |
| Seed purity after removal of the false-positive seedlings (%) | 100 | 100 |
| Increase in seed purity after early removal of off-type seedlings utilizing the ysa marker (%) | 0.12 | 0.06 |
| Estimated off-type seeds produced per ha | 168,000 | 84,000 |
| Estimated yield increase (kg/ha) | NA | 537 |

The number of off-type seeds were estimated according to the formula: 300,000 plants/ha × 500 seeds/plant × percentage of off-type seedlings (6/5,000 and 3/5,000 for 2006 and 2007, respectively). The estimated yield increase was calculated based on the formula: 300,000 plants/ha × percentage of false hybrids (183/5,000 or 121/5,000 for 2006 and 2007, respectively) × yield per plant (average yields of ysa × 93-11 F1 hybrid were 48.6 and 48.3 g for 2006 and 2007, respectively). NA, Data not available.
systems (Sing and Limani, 1990). However, a major constraint in utilizing two-line hybrid rice, especially for indica rice, is the unpredictable influence of prevailing temperature and photoperiod conditions on the fertility of the T/PGMS line during the male-sterility-inductive period (Liao et al., 2001). Previous efforts using leaf-color mutations as screening markers in two-line hybrid rice production achieved only limited success (Dong et al., 1995; Cao et al., 1999; Wu et al., 2002; Song and Song, 2007). These mutations affect pigmentation throughout the life cycle of the rice plant, resulting in lower photosynthetic rates and decreased grain yields in both T/PGMS lines and hybrids developed from them. Additionally, the pale-green and light-yellow leaves are not easily distinguishable when grown in the field, making the mutations inefficient as the screening tools.

Compared with other methods currently used to remove false hybrids in the two-line hybrid rice production system, the ysa marker offers two major advantages. In ysa T/PGMS line propagation and hybrid seed production, off-type contaminants arising from biological mixtures or mechanical mixtures can be easily identified visually and removed prior to transplanting (which is still a largely manual process in China and many other Asian countries), ensuring pure line in the paddy field. Assuming that 1 ha T/PGMS seedlings in a nursery will be transplanted to 20 ha paddy field, and that 30 man hours are needed to eliminate all the off-type contaminants per ha, at least 600 man hours of labor are needed for each 20 ha of paddy rice in traditional cultivation. Utilizing the ysa marker, only 15 man hours are needed to eliminate the false positives, equivalent to a 40-fold increase in efficiency. In addition, identification and removal of the green seedlings does not require any special training. Thus, the ysa marker can be the basis of a simple, rapid, and economic seed purity determination method requiring only 1 week to achieve results. In comparison, other practices require examination of the hybrid plants at the heading and flowering stages, which is costly, time consuming, and not reliable.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The rice (Oryza sativa) albino leaf mutant ysa was identified from a M2 population of the indica cultivar Per’ai64S irradiated with Cobalt-60 γ rays at a total dose of 300 Gy and a dosage rate of 5 Gy/min. M2 seeds were sown on a seedling bed under standard field conditions. Mutants displaying an albino phenotype at the two-leaf or three-leaf stage were labeled and transplanted to the paddy rice field at the five-leaf or six-leaf stages. Mature M3 seeds were then harvested from each plant developed as plant lines uniformly showing the trait. One mutant expressing young seedling albino termed ysa was observed in subsequent M4 to M7 generations. Homozygous M8 ysa mutants were selected and used for in-depth analysis. Rice plants were cultivated in experimental fields in Beijing (39°54′N, summer season, temperate climate), Zhejiang (30°15′N, summer season, temperate climate), and Hainan (18°16′N, winter season, subtropical climate) under local growing conditions.

Cloning of YSA

For genetic analysis, F2 populations were generated from crosses between the ysa mutant and three wild-type varieties, Per’ai64S, 93-11, and Taiziyuizhu. The population from the cross between ysa and Taiziyuizhu, which includes 7,760 normal green individuals and 2,240 mutant plants, was used for fine mapping of the YSA locus. Genomic DNA was extracted from F2 plants and analyzed for cosegregation using available simple sequence repeat markers (McCouvh et al., 2002).

New markers were developed based on the entire genomic sequences of the Nipponbare variety (Goff et al., 2002) and the indica variety 93-11 (Yu et al., 2002). dCAPS markers were designed using the Web server program dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/dcaps.html). Full-length cDNAs for candidate genes were amplified using the GeneRacer kit following the manufacturer’s instructions (Invitrogen, L1500-02).

Complementation of the ysa Mutant

Plasmids for transgenic plants were constructed using standard molecular techniques. To create the complementation construct pYSA, the genomic fragment encoding YSA was amplified by RT-PCR from Per’ai64S with the primers 5′-cagcaaccttcgagggagacagcgatgctggg-3′ and 5′-ctggacatctggacattcagggggtg-3′. To create the pYSA::GFP construct, the frame-shifted coding region of ysa was amplified by RT-PCR with the primers 5′-cagcaacctttcagggagacatgctg-3′ and 5′-ctggacatcctttatgtaattgtacttcgaggacattcagggggtg-3′. The resulting fragments were inserted into the binary vector pCUbi1390 in which the YSA gene was under the control of the constitutive maize (Zea mays) ubiquitin promoter. The constructs were introduced into ysa mutant by Agrobacterium tumefaciens-mediated transformation as described previously (Jeon et al., 2000).

Chlorophyll and Carotenoid Content Measurement

Chlorophyll and carotenoid contents were determined according to the method of Wu et al. (2007). Leaves (approximately 0.2 g fresh weight) were cut and homogenized in 5 ml of 9:1 acetone:0.1 M NaOH, and centrifuged at 3,000 g for 20 min. The supernatants were combined and washed successively with an equal volume of hexane for three times and then the pigment content was measured with a DU 800 UV/Vis spectrophotometer (Beckman Coulter).

Transmission Electron Microscopy

Samples of wild-type and ysa mutant leaves were prepared for transmission electron microscopy by cutting into small pieces, fixed in 2.5% glutaraldehyde in a phosphate buffer at 4°C for 4 h, rinsed and incubated overnight in 1% OsO4 at 4°C, dehydrated through an ethanol series, and infiltrated with a graded series of epoxy resin, and then embedded in Epon 812 resin. Thin sections were obtained using a diamond knife and a Reichert OM, ultramicrotome, stained in 2% uranyl acetate, and 10 ms lead citrate, pH 12, and then viewed with a JEOL 100 CX electron microscope.

Subcellular Localization of GFP Proteins

To investigate the subcellular localization of YSA, a gene fragment encoding the N-terminal region (amino acids 1–200) of the YSA protein was amplified by PCR and ligated into the pAP7-GFP vector (kindly provided by Dr. Hongquan Yang), in frame with GFP, resulting in pAP7-YSA-GFP. The primer pair used for YSA was 5′-gggagacactgagccagcgcggggctgccttcgagggaggc-3′ and 5′-catactagtgattcctcccaagacccagtgg-3′. The vectors for the nuclear localization signal of the Arabidopsis thaliana (Arabidopsis thaliana) promoter were designed using the GeneRacer kit following the manufacturer’s instructions (Invitrogen, L1500-02).

To create the pYSA::GFP reporter gene construct, a 1,855-bp genomic sequence upstream of the YSA transcription start site was amplified from Per’ai64S and inserted into the binary vector pCAMBIA1304. The primers used were: 5′-ctgctgtcgacagctttcctccccaggccacagcgtg-3′ and 5′-ggatactctgtcgacagcgcggggc-3′.
Transgenic rice plants were produced by the Agrobacterium-mediated cocultivation method as described previously (Jeon et al., 2000).

Histochemical assays in transgenic lines were performed as described (Ai et al., 2009). Seedlings or adult plants were incubated at 37°C in 100 mM sodium phosphate pH 7.5, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM EDTA, and 0.1% Triton X-100 containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid. After incubation for 10 h, the samples were vacuum infiltrated. Samples were subsequently transferred to 70% ethanol to remove the chlorophyll. The stained tissues were photographed using an OLYMPUS MVX10 stereomicroscope, with a color CCD camera.

RNA Preparation and RT-PCR Analysis

Total rice RNA was extracted with a RNA prep pure kit (Tiangen) according to the manufacturer’s instructions. The first-strand cDNA was synthesized from 2 μg of total RNA using the QuantiTect reverse transcription kit (QIagen). Primer pairs were designed using Primer Express (Applied Biosystems) and listed in Supplemental Table S5. Real-time PCR analysis was conducted using ABI7300HT fast real-time PCR system with the SYBR Premix Ex Taq (TaKaRa; RR814). A program was as follows: initial polymerase activation for 10 s at 95°C followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. For each sample, real-time PCR was performed with three technical replicates on three biological replicates. Ef was used to quantify relative differences in transcript accumulation. Representative PCR products for all sample types and gene targets were sequenced to confirm target specificity.

Sequence data of YSA can be found in the GenBank library under the accession number YN167987.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic tree of OsYSA and other PPR proteins in plants.

Supplemental Table S1. Segregation of wild-type and mutant plants in the F2 populations derived from four different crosses.

Supplemental Table S2. The PCR-based molecular markers developed for fine mapping of ysa.

Supplemental Table S3. Major agronomic traits and yield heterosis ability of ysa mutant and its F1 hybrids.

Supplemental Table S4. Comparison of the male-sterility trait between ysa mutant and Pei'ai64S grown in the phytotrons (PAR 384 μmol m⁻² s⁻¹).

Supplemental Table S5. Primers used in quantitative real-time RT-PCR.

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