

WHITE PANICLE1, a Val-tRNA Synthetase Regulating Chloroplast Ribosome Biogenesis in Rice, Is Essential for Early Chloroplast Development¹[OPEN]

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Chloroplasts and mitochondria contain their own genomes and transcriptional and translational systems. Establishing these genetic systems is essential for plant growth and development. Here we characterized a mutant form of a Val-tRNA synthetase (OsValRS2) from *Oryza sativa* that is targeted to both chloroplasts and mitochondria. A single base change in OsValRS2 caused virescent to albino phenotypes in seedlings and white panicles at heading. We therefore named this mutant *white panicle 1* (*wp1*). Chlorophyll autofluorescence observations and transmission electron microscopy analyses indicated that *wp1* mutants are defective in early chloroplast development. RNA-seq analysis revealed that expression of nuclear-encoded photosynthetic genes is significantly repressed, while expression of many chloroplast-encoded genes also changed significantly in *wp1* mutants. Western-blot analyses of chloroplast-encoded proteins showed that chloroplast protein levels were reduced in *wp1* mutants, although mRNA levels of some genes were higher in *wp1* than in wild type. We found that *wp1* was impaired in chloroplast ribosome biogenesis. Taken together, our results show that OsValRS2 plays an essential role in chloroplast development and regulating chloroplast ribosome biogenesis.

Plastids possess their own genomes and the transcriptional and translational systems to synthesize the encoded proteins. Plastidic genes are transcribed by two types of RNA polymerases (Shiina et al., 2005; Zubo et al., 2011). One is the bacterial-type plastid-encoded RNA polymerase (PEP). The PEP catalytic core is composed of four subunits (α , β , β' , β'') encoded

by the plastidic genes *rpoA*, *rpoB*, *rpoC1*, and *rpoC2*, respectively. Functional PEP requires one of the sigma factors encoded by the nuclear genome that facilitate promoter recognition (Shiina et al., 2005). The other is the bacteriophage-type nuclear-encoded polymerase (NEP; Hedtke et al., 1997). NEP is a single protein that is responsible for the transcription of genes encoding plastidic PEP subunits, ribosomal proteins, and other plastidic “housekeeping” proteins. During de-etiolation, PEP transcribes genes involved in the formation of thylakoid membranes and the photosynthetic machinery (Swiatecka-Hagenbruch et al., 2007; Liere et al., 2011). Proteins involved in the PEP complex, such as plastid transcriptionally active chromosome 3 (pTAC3), pTAC10, pTAC12, polymerase-associated protein 6 (PAP6), and PAP10, are essential for PEP activity. Mutants of these genes displayed albino phenotypes, suggesting an essential role of the PEP complex in early chloroplast development (Arsova et al., 2010; Steiner et al., 2011; Yagi et al., 2012; Pfalz and Pfannschmidt, 2013). Proteins encoded by the plastid genome are synthesized by plastidic prokaryotic-type 70S ribosomes that are composed of 30S and 50S subunits. Defects in components of the 70S ribosome result in severe developmental phenotypes or even lethality. In *Arabidopsis thaliana*, knocking out RNA HELICASES 22 (RH22) resulted in embryo lethality, while a knock-down mutant of RH22 accumulated precursors of 23S

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J.W., YiHua Wang, and YunLong Wang designed the research; YiHua Wang mapped the *WP1*; M.Z., J.L., and M.N. performed western blot; W.L. and D.W. performed real-time PCR; Y.X. and X.L. performed the rRNA analysis; YunLong Wang performed all other experiments; H.W., W.T., YiHua Wang, J.W., and YunLong Wang analyzed the data and wrote the paper.

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and 4.5S rRNA and displayed a virescent seedling phenotype. RH22 indirectly affected ribosome assembly due to its role in rRNA metabolism (Chi et al., 2012). The null mutant of AtObgC (a protein that could coprecipitate with 23S rRNA) resulted in embryo lethality, and knocking down AtObgC with RNAi resulted in a chlorotic phenotype. In rice (*Oryza sativa*), the OsObgC mutant exhibited a severe chlorotic phenotype in early leaf development but no embryo lethality, and ObgC directly participated in 70S ribosome assembly (Bang et al., 2012). Thus, 70S ribosome biogenesis is essential for early chloroplast development in higher plants.

Chloroplast differentiation is a process whereby chloroplasts develop from proplastids. During chloroplast differentiation, the proplastids first establish the plastidic genetic system. At this stage, NEP transcribes genes encoding PEP and ribosomal proteins and other nuclear genes that are necessary for the development of the plastidic genetic systems. The transcriptional and translational activity of the plastid is dramatically increased. The second stage is the establishment of the photosynthetic apparatus. At this stage, PEP transcribes plastid-encoded photosynthetic genes, and nuclear genes encoding photosynthetic proteins are expressed at high levels (Mullet, 1993; Majeran et al., 2010).

Aminoacyl-tRNA synthetases (AARSs) are essential components of protein synthesis that catalyze the attachment of amino acids to their cognate tRNAs (O'Donoghue and Luthey-Schulten, 2003) and in some cases perform additional roles in translational regulation, prevention of mistranslation, RNA splicing, immune responses, cytokine activity, and tRNA proofreading (Szymański et al., 2000; Guo et al., 2010). All cells must contain a complete set of 20 AARSs. Eukaryotic cells possess more than the basal set of 20 AARS genes, because organelles such as plastids and mitochondria possess their own translational systems (Duchêne et al., 2009). In Arabidopsis, 45 expressed AARSs genes have been identified in the nuclear genome and none in the mitochondrial and chloroplast genomes. These 45 proteins are expected to catalyze the formation of the 20 sets of amino-acylated tRNAs in the three cellular compartments. Accordingly, each gene product cannot be uniquely targeted to a single compartment, and dual-targeting is the rule for AARSs in Arabidopsis (Duchêne and Giritch et al., 2005). Several mutant Arabidopsis AARS genes have been characterized. *twm2* had altered expression of Val-tRNA synthetase and showed suspensor-derived polyembryony (Zhang and Somerville, 1997). *edd1* inactivated a Gly-tRNA synthetase and was arrested in embryonic development (Uwer et al., 1998). Other AARS mutants were characterized by aborted ovules, defective embryos, or viable homozygotes. Mutants in genes encoding plastidic AARSs resulted in seed abortion at the transition stage of embryogenesis with minimal effects on gametophytes, and those in genes encoding mitochondrial AARSs resulted in ovule abortion before and immediately after fertilization. Mutations in genes encoding cytoplasmic AARSs were not identified due to

their important roles in the development of male and female gametophytes (Berg et al., 2005). However, the molecular details of how AARSs affect plant development have not been reported.

In this study, we report a *white panicle 1* (*wp1*) mutant that displays white panicles at the heading stage and virescent to albino phenotypes during early leaf development. *WP1* encodes a Val-tRNA synthetase that is targeted to both mitochondria and chloroplasts and is indirectly essential for chloroplast 70S ribosome biogenesis in rice because of its role in plastidic protein synthesis. Our results suggest that *WP1* plays an essential role in the establishment of the genetic system during the differentiation of proplastids into chloroplasts.

RESULTS

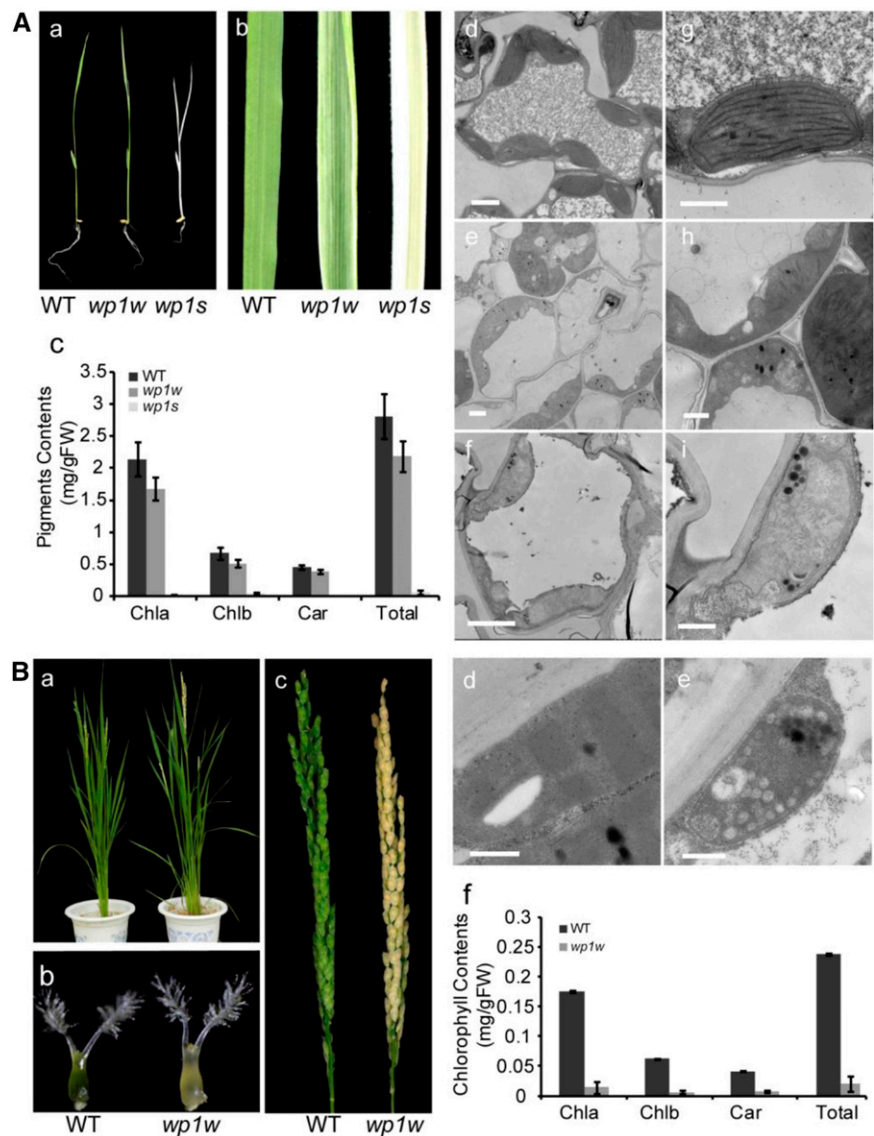
Phenotypic Characterization of the *wp1* Mutant

The *wp1* mutant was a somaclonal variant derived from the *japonica* variety Nipponbare by tissue culture. After backcrossing three times with Nipponbare, it still exhibited a virescent phenotype or a more severe albino phenotype during early leaf development. Seedlings showing the severe albino phenotype (*wp1s*) died when they reached the four-leaf stage (Fig. 1A, a–b; Supplemental Figure S1A), while *wp1* seedlings showing the weak virescent phenotype (*wp1w*) could reach the flowering stage when they displayed white panicle (Fig. 1B, a and c). The ovaries of *wp1w* mutants were chlorotic (Fig. 1B, b). Leaves of *wp1s* mutants could not develop the normal yellow color when grown in the dark (Supplemental Figure S1B), and the chlorophyll and carotenoid levels were remarkably lower in *wp1* seedlings and panicles (Fig. 1A, c, and B, f). Transmission electron microscopy (TEM) revealed that wild-type chloroplasts were orbicular-ovate shaped and contained well-developed thylakoid membrane systems, whereas the plastids in *wp1s* leaves and *wp1w* panicles were small and abnormally shaped with no thylakoid membranes. Instead, large amounts of small vesicle-like structures were present. The plastid morphology and ultrastructure in *wp1w* leaves was intermediate between wild-type and *wp1s* chloroplasts (Fig. 1A, d–i, and B, d–e). Other agronomic characters of *wp1w* were similar to wild type (Fig. 1B, a; Supplemental Table S1). Therefore, *WP1* specifically regulates chloroplast development in early leaf and panicle development.

WP1 Is Involved in Early Chloroplast Development

In rice, leaf morphology changes drastically and early chloroplast differentiation occurs during the P4 stage (Itoh et al., 2005; Kusumi et al., 2010). We therefore observed chlorophyll autofluorescence, chloroplast morphology, and ultrastructure in wild type and *wp1s* during the germination and P4 stages. Chlorophyll autofluorescence could not be observed in the seed embryos of wild type and *wp1*; however, during germination

Figure 1. Phenotypic characterization of wild type and *wp1*. A, Phenotypes of 10-d-old plants. a, b, Wild type and *wp1* were photographed 10 d after sowing. c, Pigment contents of wild-type, *wp1w*, and *wp1s* leaves. Ultrastructure of chloroplasts in wild-type (d, g), *wp1w* (e, h), and *wp1s* (f, i) leaves. Scale bars = 2 μm (d, E, f), 1 μm (g, H, i). B, Phenotypes of plants after heading. a, Wild type and *wp1w* were photographed after heading. b, Pistils of wild type and *wp1w* viewed through a stereomicroscope. c, Panicles of wild type and *wp1w*. Ultrastructure of chloroplasts in wild type (d) and *wp1w* (e) glumes. f, Pigment contents of wild-type and *wp1w* panicles. Scale bars = 0.5 μm (d, e).



autofluorescence could be observed in wild-type but not in *wp1s* seedlings (Fig. 2A, a and b). Autofluorescence could be observed in wild type throughout the P4 stage, whereas it was very weak in *wp1s* leaves until the late P4 stage. Only the leaf tip region showed autofluorescence comparable to wild type, indicating that chloroplast development was much slower in *wp1s* leaves than in wild type (Fig. 2A, c–g). No visible difference between the seed embryos of wild type and *wp1* was detected by TEM during the P4 stage (Fig. 2B, a and e). At the P4-2 stage, wild-type chloroplasts contained several internal membranes, while *wp1s* chloroplasts were much smaller without any internal membranes (Fig. 2B, b and f). At the P4-4, P4-8, and P4-10 stages of *wp1s*, small vesicle-like structures were present instead of the well-developed thylakoid membranes seen in wild type. The shape of *wp1s* chloroplasts was abnormal and smaller (Fig. 2B, c, d, g, h, i, and j). During the P4 stage, the proplastids developed into early chloroplasts (Kusumi et al., 2010). In our

study, the chloroplasts in the *wp1s* were quite different from the wild type at the P4-2 stage, suggesting an earlier defect in chloroplast development. Therefore, WP1 plays an important role in the transition of proplastids into early chloroplasts.

Map-Based Cloning of WP1

To identify the WP1 locus, 89 F2 individuals showing the mutant phenotype derived from the cross between *wp1* (*japonica*) and 93-11 (*indica*) were used for gene mapping. The WP1 locus was restricted to a 2.0-cM region between two simple sequence repeat markers, Bs1 and Bs8, on the short arm of chromosome 7. The WP1 locus was further delimited to a 43-kb region between Bs12 and Bs17 using 958 F2 recessive plants. Five expressed and hypothetical genes were predicted in this region by the Rice GAAS Program (<http://ricegaas.dna.affrc.go.jp>; Fig. 3A). Sequence analysis showed that

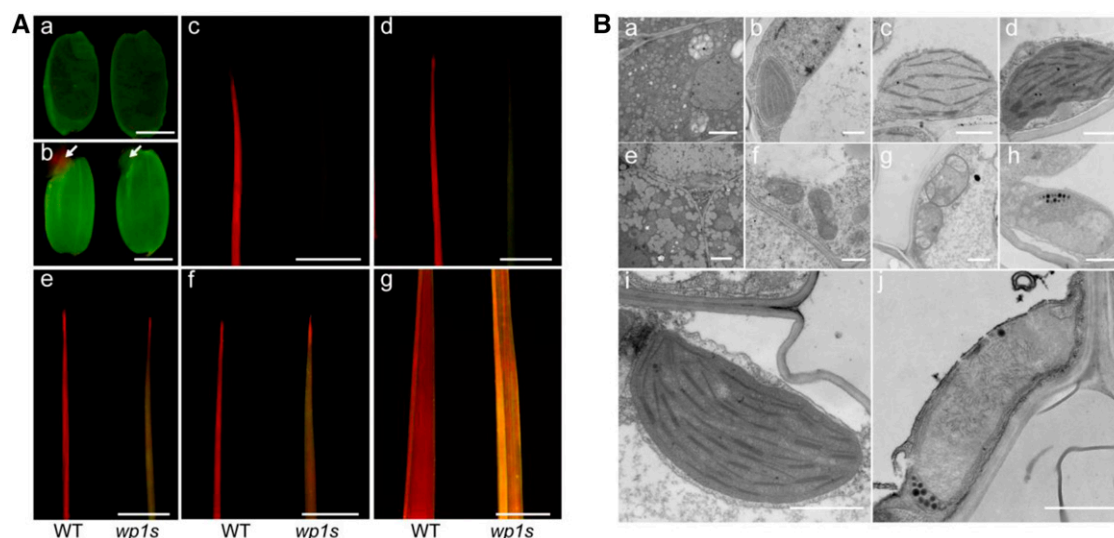


Figure 2. Chlorophyll autofluorescence and TEM observations of wild type and *wp1s*. A, Observation of autofluorescence in wild-type and *wp1s* seedlings. Seeds of wild type and *wp1* before (a) and 2 d after imbibition (b). c-g, Leaf tips of wild type and *wp1s* at P4-2 stage (c), P4-4 stage (d), P4-8 stage (e), P4-10 stage (f), and mature fourth leaf stage (g). Scale bars = 3 mm. B, Transmission electron micrographs of plastids in wild-type and *wp1s* seedlings. a, B, c, D, I, Wild type. e, F, g, H, j, *wp1s*. Tissues were collected from the seedlings at the following developmental stages: embryo (a, e), P4-2 (b, f), P4-4 (c, g), P4-8 (d, h), and P4-10 (i, j). Scale bars = 2 μm (a, e), 0.5 μm (b, f), and 1 μm (c, D, g, F, i, j).

only one open reading frame (ORF) encoding a putative Val-tRNA synthetase was different between wild type and *wp1* in this region. We validated the mutation by digesting a derived cleaved amplified polymorphic sequence marker with *NcoI* (Fig. 3C). A single base transition (G to T) changed a Trp into a Cys. We designated the locus *OsValRS2* (Fig. 3B).

To confirm that the mutation of *OsValRS2* was responsible for the *wp1* phenotype, an expression plasmid containing the full-length cDNA of *OsValRS2* driven by the *ubiquitin* promoter was constructed and transformed into *wp1*. Eight transgenic lines were obtained and all of them were restored to the wild-type phenotypes (Fig. 3, D and E). Therefore, *OsValRS2* is the gene responsible for the *wp1* phenotypes.

Expression Analysis and Subcellular Localization of WP1

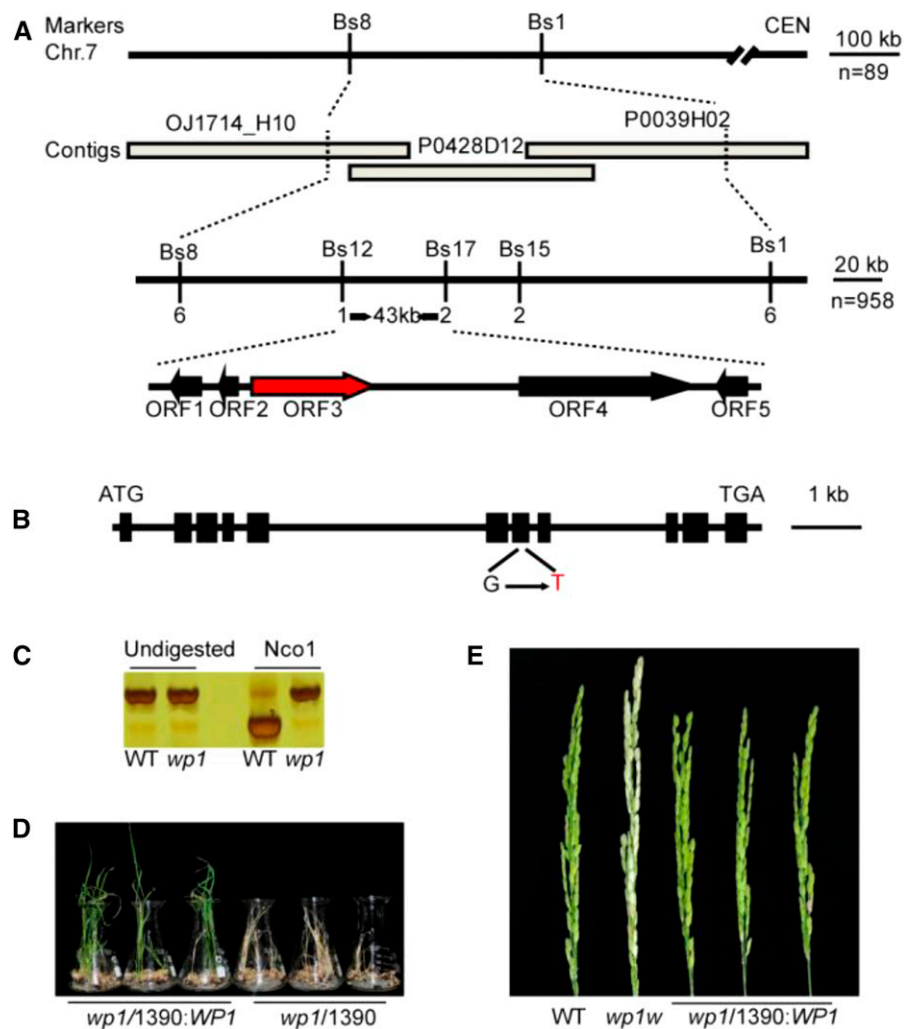
The mRNA level of *WP1* was detected by quantitative RT-PCR (qPCR) in various organs, including seedlings, roots, stems, leaves, panicles, and leaf sheaths. The expression of *WP1* was higher in the organs with more chloroplasts (Fig. 4Aa). The expression of *WP1* increased during the P4 stage up to P4-8 then decreased in mature P4 leaves (Fig. 4Ab).

To determine the subcellular localization of WP1, transient expression assays were performed in protoplasts prepared from rice seedlings. We first compared localization of GFP with that of full-length WP1 fused to the N terminus of GFP (WP1-GFP). Free GFP signal was dispersed through the cytoplasm and nuclei in the protoplasts, whereas GFP fluorescence of the protoplasts transformed with WP1-GFP partially merged

with chlorophyll autofluorescence, indicating chloroplast localization. GFP signal was also detected in some punctate structures similar in shape and size to mitochondria. To test the hypothesis that WP1-GFP also localized to mitochondria, we used Mito-Tracker Orange as a mitochondrial marker and found that the GFP signal from the punctate structures merged with that from Mito-Tracker Orange. We therefore concluded that WP1 was targeted to both chloroplasts and mitochondria. The transit PEptide of AARS was reported to be in the N-terminal region (Duchêne and Giritch et al., 2005), so we fused the N-terminal 1-100 amino acids of WP1 to the N terminus of GFP. The fluorescent signal of WP1 1-100 was the same as that of the full-length fusion protein (Fig. 4B). In *Nicotiana*, the Arabidopsis homolog of WP1 *AtValRS2* was targeted to mitochondria with weak signal in chloroplasts (Duchêne and Giritch et al., 2005). We found that WP1 had the same subcellular localization as *AtValRS2* in *Nicotiana* and that *AtValRS2* was targeted to chloroplasts only in rice protoplasts (Supplemental Fig. S2).

The amino acid sequences of ValRSs are highly conserved in rice and Arabidopsis (Supplemental Fig. S3). There are three *ValRS* genes in the rice genome, including *LOC_Os03g48850* (*OsValRS1*), *LOC_Os10g36210* (*OsValRS3*), and *LOC_Os07g06940* (*WP1*). *OsValRS3* was mostly expressed in anthers, and its expression level was much lower than the other *ValRSs*. *OsValRS1* was highly expressed in all organs (Supplemental Fig. S4), and *OsValRS1* protein was targeted to the cytosol (Fig. 4B). The differential localization of *OsValRS1* and WP1 suggested that *OsValRS1* and WP1 functioned independently without redundancy.

Figure 3. Positional cloning of the *WP1* gene. **A**, Map-based cloning of the *WP1* gene. The *WP1* locus was initially mapped to the short arm of chromosome 7 between two simple sequence repeat markers, Bs8 and Bs1. The *WP1* locus was narrowed to a 43-kb region between Bs12 and Bs17 on BAC clone P0428D12 using 958 homozygous mutant plants. **B**, Structure of *WP1*. ATG and TGA represent the start and stop codons, respectively. Black boxes indicate the exons and the lines between them indicate introns. A single nucleotide replacement led to an amino acid change. **C**, Verification of the change between wild-type and *wp1* genomic DNAs with the derived cleaved amplified polymorphic sequence marker *jc1*. **D**, Functional complementation of *WP1*: Seedlings of transformation experiments. **E**, Panicle phenotypes of the wild-type, *wp1w*, and three independent transgenic complementation lines.



Expression of Nuclear Genes Involved in Photosynthesis Is Repressed in *wp1s*

Chloroplast development is dependent on the coordinated expression of both chloroplast and nuclear genes and is largely under the control of nuclear genes (Pogson and Albrecht, 2011). To analyze the effect of the *wp1* mutation on gene expression, we compared the transcriptional profiles of nuclear and plastidic genes in *wp1s* and wild-type seedlings using RNA-seq. 17.5 million reliable clean reads were obtained from both *wp1s* and wild type (Fig. 5A-C). The results showed that, in seedlings, 2038 nuclear genes ($q < 0.005$; $|\log_2(\text{FoldChange})| > 1$) exhibited an over 2-fold difference in their expression (repressed 972; induced 1066) between *wp1s* and wild type (Supplemental Data S1).

Many genes involved in photosynthesis were significantly repressed, including genes encoding proteins of the light-harvesting complex, PSI and PSII, electron carriers, ATP synthase, ferredoxin-NADP⁺ reductase, and proteins involved in carbon fixation (Supplemental Data S2a), which are necessary for photosynthesis. The reduction in expression of photosynthetic genes could

be caused by the stagnancy of chloroplast development. Meanwhile, the expression levels of many genes involved in both synthesis and degradation of Val, Leu, and Ile were remarkably increased in *wp1s*, which is consistent with the result that *WP1* encodes a Val-tRNA synthetase. It was interesting that the chlorophyll synthesis genes were not changed, which was verified using real-time PCR (Supplemental Fig. S5), whereas the chlorophyll content of *wp1s* mutants was much lower (Fig. 1Ac).

WP1 Affects the mRNA levels of Plastid-Encoded Genes

The *wp1* mutant had an abnormal chloroplast structure similar to *pap* (polymerase-associated protein) mutants (Allison et al., 1996). We therefore compared the expression profiles of plastidic genes in *wp1s* and wild type using RNA-seq. The expression levels of many plastidic genes differed between *wp1s* and wild type (Supplemental Data S3). Plastidic genes are transcribed by NEP and PEP. Accordingly, plastidic genes can be divided into three types. Class I genes are

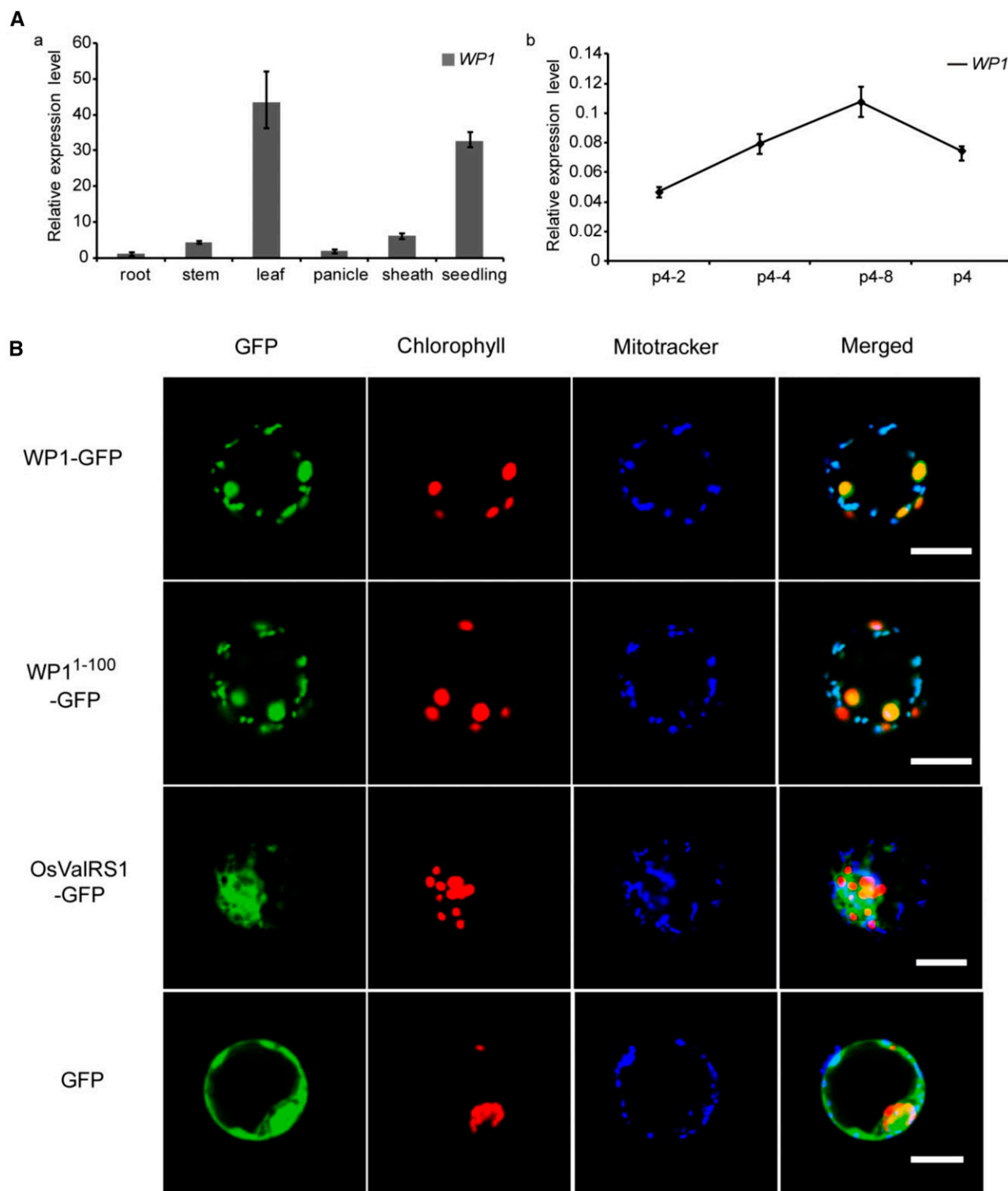


Figure 4. Expression levels of *WP1* in different tissues and localization of *WP1* in rice protoplasts. **A**, Expression analysis of *WP1*. **a**, qPCR analysis of *WP1* expression in roots, seedlings, stems, leaves, panicles, and sheaths of wild type. **b**, Relative expression of *WP1* in P4 stage leaves. Values are means \pm SD of three replicates relative to rice *ubiquitin*. **B**, Localization of *WP1*-GFP, *WP1*¹⁻¹⁰⁰-GFP, *OsVaIRS1*, and GFP in rice protoplasts. Constructs encoding the indicated fusion proteins were transformed into rice protoplasts prepared from 10-d-old seedlings and incubated in the dark at 28°C for 16 h before examination with a confocal microscope, Green fluorescence signals, chlorophyll red autofluorescence signals, Mito-tracker Orange CM-H2Xros fluorescence, and merged images. Bar = 10 μ m.

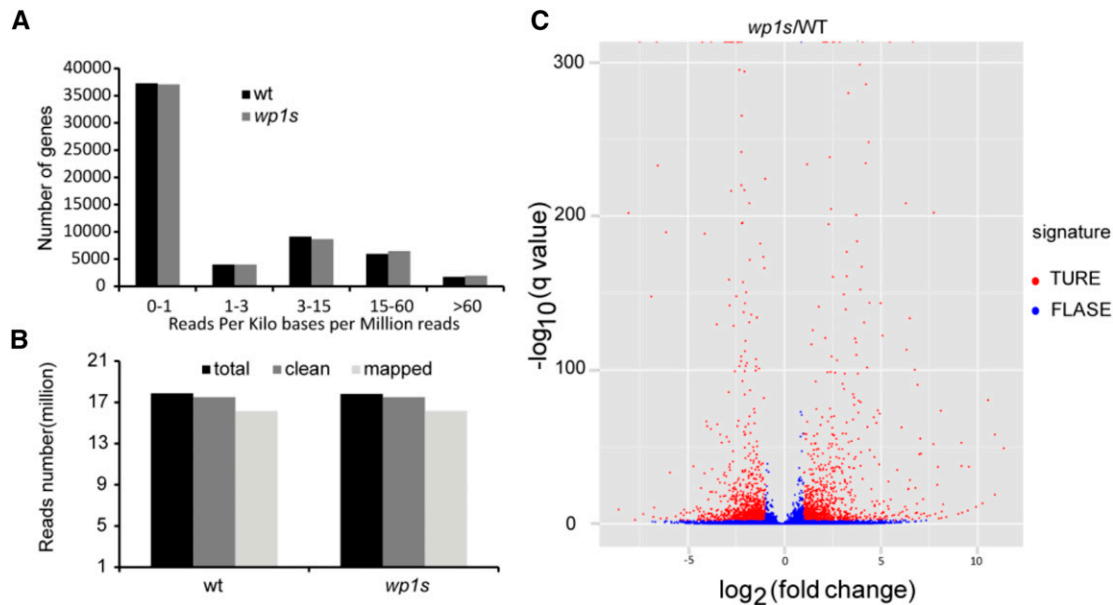


Figure 5. RNA-seq analysis of wild-type and *wp1s* seedlings. mRNA enriched from total RNA isolated from 10-d-old seedlings of wild type and *wp1s* using oligo-(dT) was fragmented and reverse-transcribed using random hexamer primers. The library was then constructed and sequenced using an Illumina HiSeq2000. A, Frequencies of detected genes sorted according to their expression levels. B, Read numbers of wild-type and *wp1s* sequences. C, Volcano plot showing the overall alterations in gene expression in wild type and *wp1s*.

predominantly transcribed by PEP, class II genes are transcribed by both NEP and PEP, and class III genes are exclusively transcribed by NEP. In *wp1s*, the expression levels of class III genes including RNA polymerase and genes encoding ribosomal proteins were strongly increased, while class I genes including PSI, PSII, and the large subunit of Rubisco (RbcL) decreased (Fig. 6). These results suggest that *wp1* was defective in PEP activity.

The *wp1* Mutant Was Defective in Plastidic Protein Synthesis

Since *WP1* encodes a ValRS located in chloroplasts and mitochondria, we tested the accumulation of chloroplast proteins in *wp1* mutants and wild type using western-blot analysis. As shown in Figure 7, the levels of RbcL, Rubisco small subunit (RbcS), and Rubisco activase (RCA) were reduced in *wp1* (Fig. 7, A and B), although RbcL was a plastid-encoded protein while RbcS and RCA were encoded by the nucleus. The RNA-seq results showed that the expression levels of *RbcS* and *RCA* were significantly repressed. We therefore inferred that the lower levels of RbcS and RCA proteins were due to the decreased expression levels of both genes.

We next tested the levels of other plastid-encoded proteins, including A1 of PSI, D1 of PSII, alpha and beta' subunits of RNA polymerase, NADH dehydrogenase subunit 4, and ATP synthase CF1 beta (AtpB). We found that the levels of all plastid-encoded proteins

were strikingly reduced in *wp1s* seedlings (Fig. 7C) as well as in young panicles of *wp1w* (Supplemental Fig. S6). To determine whether the reduced protein levels in *wp1* mutants resulted from reduced mRNA levels, we examined the expression levels of these genes in *wp1s* and wild type using qPCR. We found that the expression levels of the class I genes *rbcL*, *psaA*, *psaB*, and *psbA* were remarkably reduced, while the expression of class III genes *rpoA*, *rpoC2* and class II genes *ndhD*, *atpB* increased or was unchanged (Fig. 7E). The expression level of *psaA* was greatly increased in wild type but increased only slowly in *wp1s* during the P4 stage (Fig. 7F). Conversely, the expression of *rpoB* decreased in wild type but increased in *wp1s* during the P4 stage (Fig. 7G). These results suggested that the lower accumulation of chloroplast proteins in *wp1* was most likely due to the defects in chloroplast protein biosynthesis.

The *wp1* Mutant Was Defective in Biogenesis of Chloroplast Ribosomes

The expression levels of class III genes increased, yet the accumulation of these proteins was strikingly decreased. This feature was very similar to the *obgC* mutant, which is defective in biogenesis of plastidic ribosomes, probably because ObgC acts as a 70S ribosome assembly factor (Bang et al., 2012). Chloroplast ribosomes consist of 50S large subunit and 30S small subunit. The 50S and 30S subunits are comprised of rRNAs (23S, 16S, 5S, and 4.5S) and ribosomal proteins.

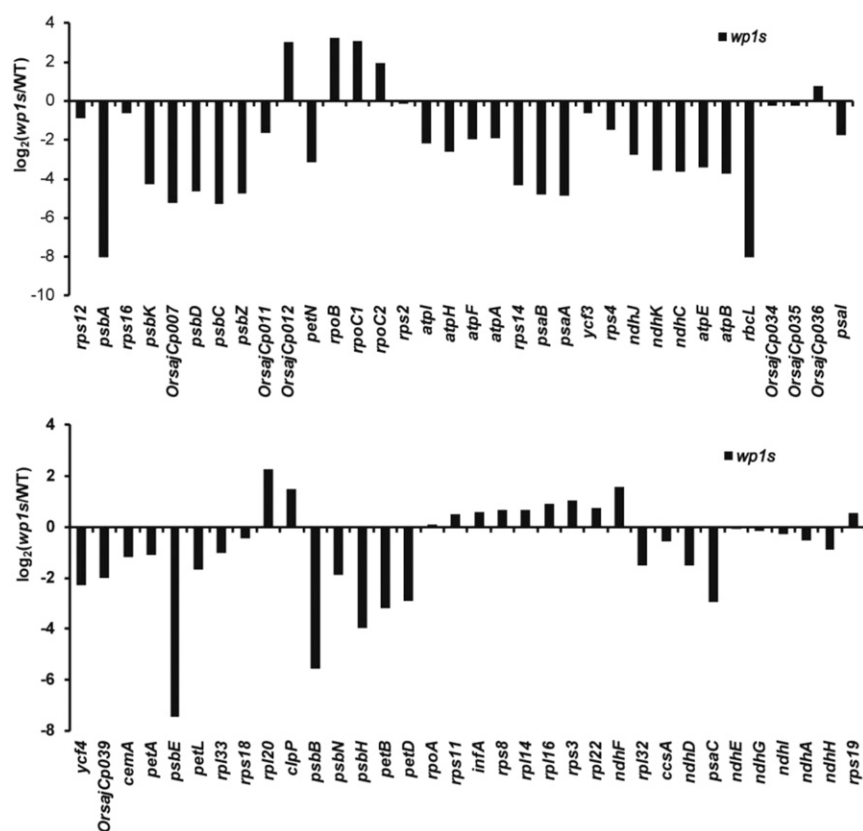


Figure 6. Differential expression of plastid-encoded genes in wild type and *wp1s*. mRNA enriched from total RNA isolated from 10-d-old seedlings of wild type and *wp1s* using Ribo-Zero rRNA Removal Kits (Plant Leaf) was fragmented and reverse-transcribed using random hexamer primers. The library was then constructed and sequenced using an Illumina HiSeq2000. The graph shows the \log_2 ratio of transcript levels in *wp1s* mutants compared with wild type. Raw data are shown in Supplemental Data S3.

We analyzed the composition and content of rRNAs using an Agilent 2100. The rRNAs, including 23S and 16S rRNAs, were decreased in *wp1s* seedlings displaying the albino phenotype and slightly decreased in *wp1w* leaves showing the virescent phenotype (Fig. 8A, a–c). Consistently, the rRNAs were decreased in *wp1w* glumes (Fig. 8A, d and e). The chloroplast rRNAs could not be detected in roots as a negative control (Fig. 8A, f). We tested the protein levels of some plastid-encoded ribosomal proteins, including RPL2 (ribosomal protein large subunit 2), RPS3 (ribosomal protein small subunit 3), and RPS18 (ribosomal protein small subunit 18) by western-blot analysis. The levels of these ribosomal proteins were significantly decreased in *wp1s* seedlings and *wp1w* glumes and slightly decreased in *wp1w* seedlings (Fig. 8B, a and b). The mRNA levels of chloroplast ribosomal proteins were increased in *wp1s* seedlings compared to wild type (Fig. 6; Supplemental Fig. S7). These results clearly indicated that severe defects in plastidic ribosome biogenesis in *wp1* lead to lower levels of protein biosynthesis in chloroplasts. Because WP1 is also targeted to mitochondria, we examined biogenesis of mitochondrial ribosomes. The results showed little difference between 26S and 18S rRNA levels in *wp1* and wild type (Supplemental Fig. S9A), while the ribosomal protein levels were the same in *wp1* and wild type (Supplemental Fig. S9B). These results indicated that

mitochondrial ribosome biogenesis was almost normal in *wp1*.

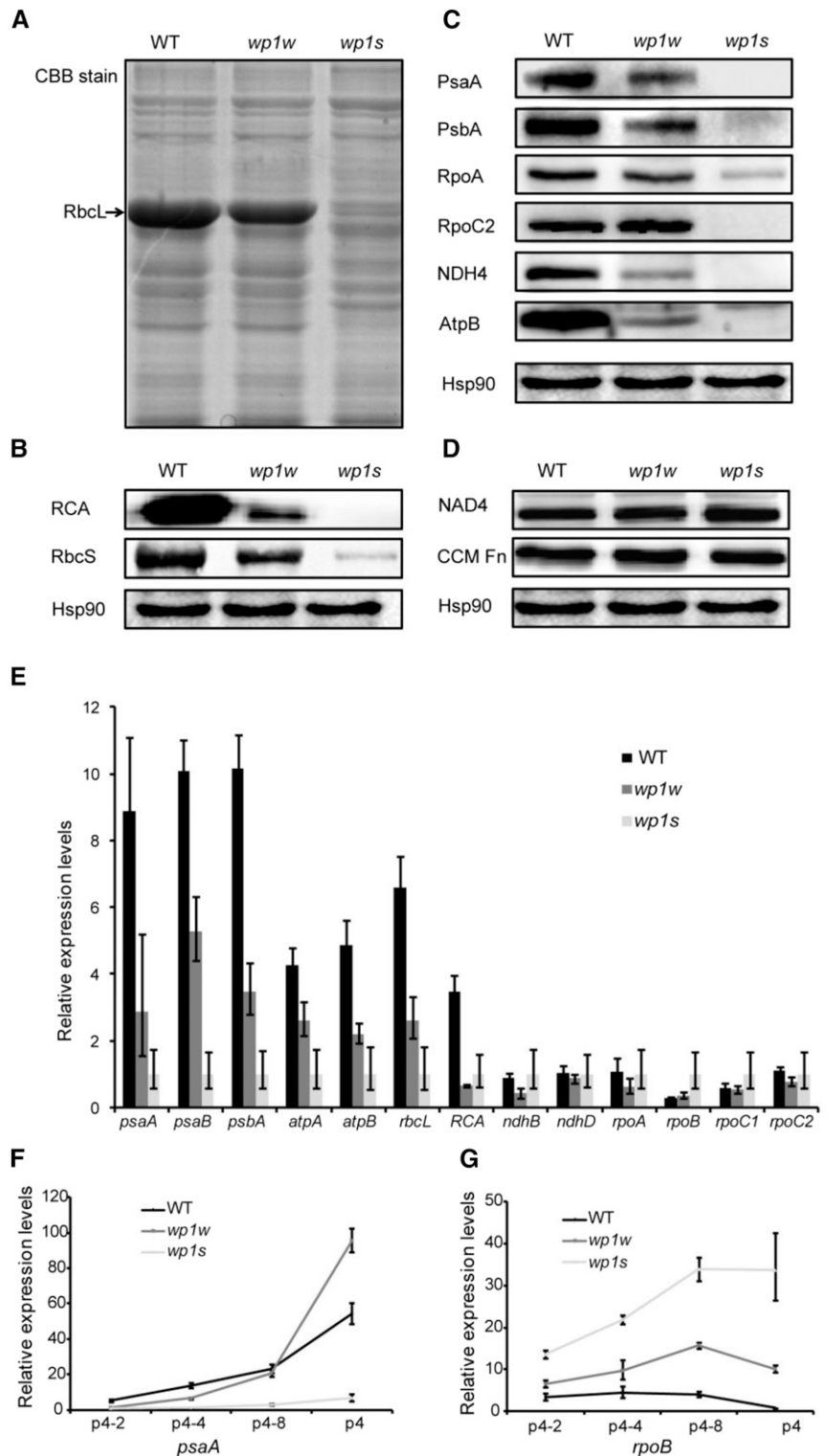
DISCUSSION

WP1 Plays an Important Role during Early Chloroplast Development

In this study, we isolated the *wp1* mutant, which displays virescent to albino phenotypes in seedlings and white panicles at heading. We found that *wp1* chloroplasts were defective in the P4-2, P4-4, and P4-8 stages (Fig. 2). Further, chlorophyll fluorescence could be observed in wild type 2 d after germination but not in *wp1* mutant seedlings (Fig. 2A, a and b). Thus, the defect in chloroplasts could occur earlier than the P4-2 stage. Strikingly, the morphologies and ultrastructures of *wp1s* mutant chloroplasts were very similar to those of mutants in PEP-associated proteins such as *pap5/ptac12* and *pap8/ptac6* (Pfalz et al., 2006), *pap7/ptac14* (Gao et al., 2011; Steiner et al., 2011), *pap4/fad3* and *pap9/fsd2* (Myouga et al., 2008), *pap6/fln1* (Arsova et al., 2010; Steiner et al., 2011), *pap10/trxz* (Arsova et al., 2010), and *pap11/atmurE* (Garcia et al., 2008). These results indicated that WP1 plays an important role during early chloroplast development.

The severity of the phenotype varied between plants, resulting in the fact that some seedlings were albino

Figure 7. Transcript and protein levels of some representative genes. A, Rubisco in wild-type and *wp1* seedlings. CBB, Coomassie Brilliant Blue-stained gel. B, Western-blot analysis of RCA and RbcS. C, Western-blot analysis of chloroplast proteins. D, Western-blot analysis of mitochondrial proteins. Hsp90 was used as an internal control. The seedlings were grown for 10 d at 30°C. E, Relative expression of plastidic genes in wild-type and *wp1* seedlings grown for 10 d at 30°C. Relative expression levels of *psaA* (F) and *rpoB* (G) in P4 stage leaves. Error bars indicate SD ($n = 3$).



(*wp1s*) and others were virescent (*wp1w*), perhaps due to conditions in the parent as the embryos developed. However, back-crossing sequencing, transformation, and

several other analyses confirmed that they were caused by a single mutation to the same gene. In addition to the virescent or albino leaf phenotype, the *wp1w* mutant also

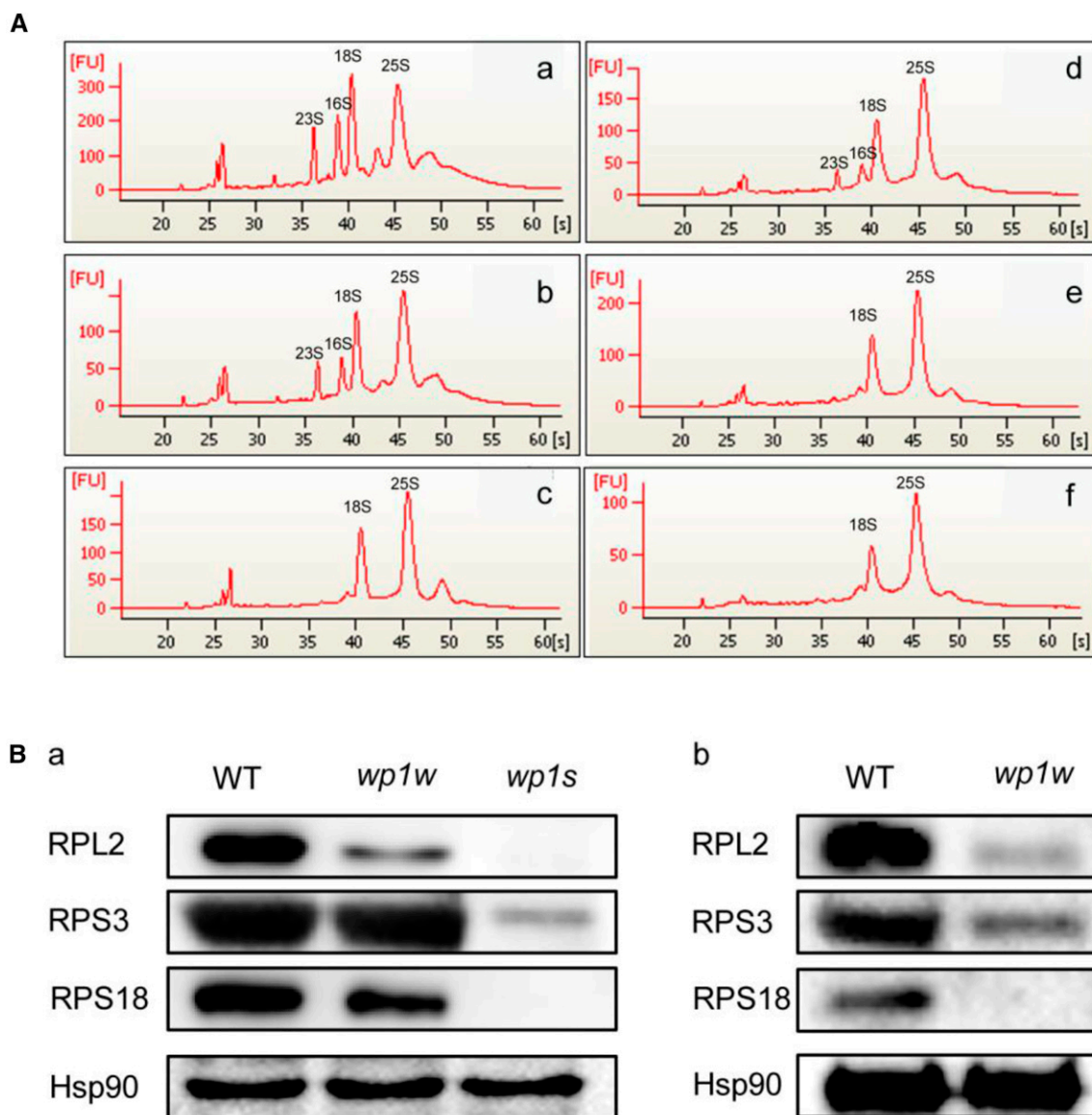


Figure 8. Analyses of rRNA and ribosomal proteins from wild type and *wp1*. A, rRNA analysis using Agilent 2100. RNA was isolated from 10-d-old wild-type seedlings (a), *wp1w* seedlings (b), *wp1s* seedlings (c), wild-type panicles (d), *wp1w* panicles (e), and wild-type roots as control (f). B, Western-blot analysis of chloroplast ribosomal proteins. Proteins were extracted from 10-d-old seedlings (a) and panicles (b). The proteins were hybridized with antibodies against RPL2, RPS3, and RPS18, respectively. Hsp90 was used as an internal control.

had a white panicle phenotype (Fig. 1B, b). In rice, white panicle mutants are rarely reported and no gene was isolated. We have obtained another virescent mutant that has a white panicle phenotype as well (Wang YL, Wang YH, Wan JM, unpublished data). This mutant has a defect in *trxz*, which is part of the PEP/PAP complex (Arsova et al., 2010; Pfalz and Pfannschmidt, 2013). In Arabidopsis, the *trxz* mutant showed pale yellowish leaves and also played an important role in chloroplast development (Arsova et al., 2010). Therefore, we deduced that chloroplast development is frail in seedlings and young panicles in rice, and mutations in genes

participating in chloroplast development might lead to the white panicle phenotype.

WP1 Encodes OsValRS2 and Is Required for Plastidic Protein Biosynthesis and Gene Expression

Plants synthesize proteins in three cellular compartments: the cytosol, the mitochondria, and the chloroplasts. Thus, full sets of AARSs, which catalyze the addition of amino acids to their cognate tRNAs, need to be localized in each of these compartments. In plants, all

AARs are nuclear-encoded and posttranslationally targeted to their respective compartments. In this study, we show that *WP1* encodes a Val-tRNA synthetase (*OsValRS2*) that is targeted to both chloroplasts and mitochondria and that the first N-terminal 100 amino acids are sufficient to ensure its proper targeting (Fig. 4B).

We found that the *wp1* mutant harbors a single base transition (G to T) that causes the change of a Trp into a Cys in the coding sequence of *OsValRS2*. This amino acid substitution might alter the enzymatic activity of *OsValRS2* protein and lead to a defect in plastidic Val-tRNA and in protein biosynthesis in the chloroplasts. The RNA-seq results showed that the expression levels of both the genes for synthesis and degradation of Val, Leu, and Ile were increased (Fig. 5, D and E). We infer that the mutation in *WP1* may lead to lack of Val-tRNA. The increased biosynthesis and degradation of Val, Leu, and Ile could maintain the Val, Leu, and Ile flow at a high level.

Consistent with this, we found that the levels of chloroplast genome-encoded proteins transcribed by NEP or PEP were severely reduced (Fig. 7C). In addition, ribosomal proteins encoded by the chloroplast genome and chloroplast rRNAs were also reduced in the *wp1* mutant (Fig. 8). In early chloroplast development, a number of housekeeping genes are transcribed and translated and in this stage chloroplast development is rapid and sensitive. The decrease in chloroplast Val-tRNA restrained the synthesis of these plastid-encoded housekeeping proteins, including chloroplast ribosomal proteins. This could lead to a deficiency in functional ribosomes, which would inhibit protein synthesis in chloroplasts. It could establish a vicious circle leading to the breakdown of protein synthesis during early chloroplast development. Thus, *WP1* could indirectly regulate the biogenesis of chloroplast ribosomes. Moreover, we found that in *wp1* mutants the expression levels of PEP-dependent genes were decreased, whereas those of NEP-dependent genes were increased or unchanged (Fig. 7). These results suggested that the *wp1* mutant is severely impaired in PEP activity and *WP1* is important for plastidic gene expression.

Reduced expression of class I genes and increased expression of class III genes has also been described in other mutants, such as PEP-related mutants (Pfalz et al., 2006; Steiner et al., 2011), *obgc* (Bang et al., 2012), *rh3* (Asakura et al., 2012), and some PPR protein-related mutants (Chateigner-Boutin et al., 2008; Zhou et al., 2009; Chateigner-Boutin et al., 2011; Pyo et al., 2013). These mutants were all involved in the “housekeeping” functions of chloroplast development. The switch of RNA polymerase usage in chloroplasts is mediated by the plastid-encoded Glu-tRNA whose expression depends on PEP and directly binds to and inhibits the transcriptional activity of NEP. Compromised PEP activity in plants would lead to decreased Glu-tRNA expression, thus preventing inhibition of NEP and eventually leading to higher expression levels of class

III genes compared to control plants with normal PEP activity (Hanaoka et al., 2005; Arsova et al., 2010). Therefore, the *wp1* mutation leads to impaired PEP activity resulting in reduced expression of class I genes and increased expression of class III genes, which is a general phenomenon in most mutants with important roles in establishment of basic chloroplast functions including transcription and translation. Chloroplast development depends on the coordination of nuclear and plastidic gene expression. Most chloroplast proteins are encoded by the nuclear genome, while the plastid genome only encodes a few proteins (Sugiura, 2003). The status of the chloroplast influences the transcription of nuclear genes through retrograde signaling. Some retrograde signaling factors such as tetrapyrrole signaling and redox signaling have been reported (Strand et al., 2003; Laloi et al., 2007). In *wp1s* expression of many nuclear genes changed (Supplemental Data S1). The RNA-seq results showed that the expression of photosynthetic genes was reduced (Fig. 5F). This was also observed in the rice *obgc* mutants (Bang et al., 2012). Conversely, expression of nuclear-encoded chloroplast ribosomal protein genes increased (Supplemental Fig. S7). These changes in expression levels could be due to retrograde signaling. The *wp1* chloroplasts were defective in mRNA and protein levels, and chloroplast development was stagnant. Some form of retrograde signaling could conduct the signal that the chloroplast’s genetic system was not established and restrained the expression of nuclear-encoded photosynthetic genes. Expression levels of the nuclear-encoded chloroplast ribosomal protein genes were increased. In *wp1s* the expression levels of these nuclear genes maintain the chloroplasts’ status during the early stages of development.

It is interesting to note that earlier studies reported that the knock-out mutant of Arabidopsis ValRS2 (*AtValRS2*) showed an embryo lethality phenotype (Berg et al., 2005), whereas the rice *wp1* we reported here showed a virescent phenotype or a more severe albino phenotype in seedlings. This indicates that the mutation in *wp1* may represent a partial loss-of-function mutation caused by a single amino acid substitution. Alternatively, other *OsValRS* homologs (*OsValRS1* and *OsValRS3*, which share high homology) may play a partially redundant role with *OsValRS2*. *OsValRS3* was predominantly expressed in anthers, and its expression level was much lower than the other *OsValRS1* and *OsValRS2*. *OsValRS1* was highly expressed in all tissues, but its product was only targeted to the cytosol (Fig. 4B). Therefore, *OsValRS1* and *WP1/OsValRS2* may have evolved nonredundant function to support protein synthesis in the cytosol and the plastids and mitochondria, respectively.

Since *WP1* is also targeted to mitochondria, we measured mitochondrial 26S and 18S rRNA levels using real-time PCR and found that these rRNAs were slightly decreased in *wp1s* but were not changed in *wp1w* (Supplemental Fig. S9A). We tested levels of mitochondrial ribosomal proteins using western blot.

The results showed that the levels of ribosomal proteins were unchanged or decreased slightly in *wp1* (Supplemental Fig. S9B). We also examined the expression of some genes encoded by the mitochondrial genome. We found that the expression levels of these genes increased in *wp1w* and increased even more in *wp1s* (Supplemental Fig. S9C). The levels of the proteins encoded by these genes were almost the same in wild type and *wp1* (Fig. 7D; Supplemental Fig. S9B). Thus, mitochondrial protein synthesis might be impaired in *wp1* and mitochondrial ribosomal biogenesis might be slightly impaired. Hence, we deduced that WP1 functioned in both chloroplasts and mitochondria, and WP1 and OsValRS1 functioned without redundancy. However, we found no differences between wild-type and *wp1s* mitochondrial ultrastructures. Similar results were observed in the rice VIRESCENT2 (V2) mutant (Sugimoto et al., 2007). V2 encodes a guanylate kinase (GK) that is dual-targeted to chloroplasts and mitochondria, while there is another GK that is targeted to the cytosol. V2 played an important role in translation of plastidic transcripts at an early stage of chloroplast differentiation (Sugimoto et al., 2004). The phenotypes of *v2* mutants were most apparent during early plastidic rather than mitochondrial development. AEF1/MPR25 is implicated in editing of plastidic *atpF* and mitochondrial *nad5* transcripts and promotes *atpF* splicing (Toda et al., 2012; Yap et al., 2015). In *mpr25* the NADH dehydrogenase activity of complex I was not affected, but photosynthesis was defective. The NADH dehydrogenase genes were up-regulated in *mpr25*. The reason why we could observe only slight differences between wild-type and *wp1s* mitochondria could be that mitochondrial differentiation is different from chloroplast differentiation during early development. In chloroplast differentiation, the genetic system is rebuilt. We therefore speculate that the single base mutation in WP1 is important for establishment of the plastidic genetic system but has little effect on mitochondrial differentiation. Hence, we could not observe any differences between the ultrastructures of wild-type and *wp1s* mitochondria.

In conclusion, we report herein that WP1, which plays an important role in expression of plastidic genes and biogenesis of plastidic ribosomes, is essential for differentiation from proplastids to chloroplasts.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *wp1* mutant was isolated from a Nipponbare T-DNA insertion mutant library (Yang et al., 2004), but no T-DNA insertion was detected (Supplemental Fig. S1C). The seedlings were grown in a paddy field or growth chambers. The chamber conditions were as follows: 12-h light ($300 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 30°C and 12-h dark at 28°C (SD).

Fluorescent Stereoscopic and TEM Analyses

Wild type and *wp1* were grown in growth chambers. Leaves of wild type and mutants were harvested to observe fluorescence and fixed overnight at 4°C in a

phosphate buffer containing 2.5% glutaraldehyde (pH 7.4). The sampling time was performed as described previously (Kusumi et al., 2010). The fluorescence was observed using a NIKON AZ100. The samples fixed in 2.5% glutaraldehyde were further fixed in 1% OsO_4 , stained with uranyl acetate, dehydrated in an ethanol series, and finally embedded in Spurr's medium prior to ultrathin sectioning. The samples were stained again and observed using a Hitachi H-7650 transmission electron microscope.

Measurement of Photosynthetic Pigments

For pigment extraction, equal fresh weights of plant tissues were extracted with equal volumes of 95% ethanol. Specific chlorophyll contents were determined with 800 UV/Vis Spectrophotometers (Beckman Coulter) according to the method of Lichtenthaler (1987).

Map-Based Cloning and Complementation Analysis

A total of 1047 plants showing the mutant phenotype derived from the F2 progeny of the cross *wp1* × 93-11 (*indica*) were used for genetic mapping. Ten F2 recessive plants were used for preliminary mapping based on 200 genome-wide microsatellite markers. New genetic markers were designed by comparing the sequences of 93-11 and Nipponbare posted at NCBI (<http://www.ncbi.nlm.nih.gov>) and listed in Supplemental Table S2.

For complementation of the *wp1* mutation, the WP1 cDNA was cloned into the pUbi-1390 binary vector. The pUbi:WP1 plasmid was introduced into *Agrobacterium tumefaciens* (strain EHA105) and used to transform calli prepared from *wp1*. Hygromycin-resistant calli were regenerated and seedlings were grown in a greenhouse to obtain transgenic plants.

qPCR Analysis

Total RNA was isolated using the RNA prep pure plant kit (TIANGEN). The first-strand cDNA was synthesized using random hexamer primers (TaKaRa) for chloroplast-encoded genes and oligo(dT) (TaKaRa) for nuclear-encoded genes and reverse-transcribed using Prime scriptase (TaKaRa). Rice (*Oryza sativa*) ubiquitin (UBQ) was used as an internal control. The real-time PCR analysis was performed using an ABI 7500 real-time PCR system with SYBR Green MIX (BIO-RAD) and three biological repeats. Primers used for real-time PCR analysis are listed in Supplemental Table S3.

Subcellular Localization

The coding sequences of OsValRS2, OsValRS2¹⁻¹⁰⁰, OsValRS1, and AtValRS2 were amplified and fused to the N terminus of GFP under control of the double CaMV35S promoter in the transient expression vector pAN580-GFP, referred to as WP1-GFP, WP1¹⁻¹⁰⁰, OsValRS1-GFP, and AtValRS2-GFP. WP1 was also fused to the N terminus of GFP in the binary vector pCAMBIA1305-GFP for agro-infecting tobacco (*Nicotiana tabacum*) mesophyll cells. The cDNA fragments were PCR-amplified using the corresponding primer pairs shown in Supplemental Table S4. The rice protoplasts were isolated from 10-d-old seedlings. The transient expression constructs were separately transformed into rice protoplasts and incubated in the dark at 28°C for 16 h before examination (Chiu et al., 1996; Chen et al., 2006). Tobacco mesophyll cells were agro-infected by injection and incubated in the chamber (12 h light/ 12 h dark, 25°C) for 2 d, and protoplasts were isolated from injected leaves. The fluorescence of GFP was observed using a confocal laser scanning microscope (Zeiss LSM 780). To stain mitochondria, protoplasts were incubated with 0.5 μM MitoTracker Orange CM-H₂TMros (Invitrogen) for 30 min and washed before observation.

Western-Blot Analysis

Total proteins were isolated from 10-d-old wild-type and *wp1* seedlings and panicles before flowering. The tissues were ground in liquid nitrogen and thawed in equal volumes of extraction buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 0.5 (v/v) β -mercaptoethanol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 μg /mL antipain, 2 μg /mL leupeptin, 2 μg /mL aprotinin] for 30 min on ice. Cell debris was removed by centrifugation at 12,000g for 15 min at 4°C. Total proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, immunoblotted with various antibodies, and detected using the Clarity western ECL Substrate (BIO-Rad). Antibodies were obtained from BGI (<http://www.proteomics.org.cn/>).

RNA-seq Analysis

Total RNA was isolated from 10-d-old wild-type and *wpl1s* seedlings. mRNA was enriched using oligo-(dT) primer for nuclear-encoded genes and Ribo-Zero rRNA Removal Kits (Plant Leaf) for chloroplast-encoded genes, then fragmented into segments approximately 200 nucleotides in length and reverse-transcribed using random hexamer primers. The library was then constructed and sequenced using an Illumina HiSeq 2000 (Novogene-Beijing). The raw sequence data were collected and filtered. A total of 17.5 million reads each from wild type and *wpl1s* were obtained for nuclear-encoded genes and 33 million reads each from wild type and *wpl1s* were obtained for chloroplast-encoded genes. Levels of gene expression were calculated using the RPKM method. The significance of differentially expressed genes (DEGs) was determined using q values < 0.005 and $|\log_2(\text{FoldChange})| > 1$. Gene Ontology (<http://www.geneontology.org/>) analyses were performed referring GOseq (Wang et al., 2009). Pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes database (Kanehisa et al., 2008). The rice chloroplast genes were identified by referring to the chloroplast genome database (<http://chloroplast.ocean.washington.edu/>). DEGs with more than 2-fold differences are listed in Supplemental Data S1 and chloroplast genome DEGs are listed in Supplemental Data S3.

RNA Analysis

Total RNA was isolated from 10-d-old seedlings of wild type and *wpl1* panicles of wild type and *wpl1* and roots of wild type using the RNA prep pure plant kit (TIANGEN). The RNA samples were diluted to 10 ng/ μ L and analyzed by Agilent 2100. The RNA 6000 Nano Total RNA analysis Kit (Agilent) was used for analysis.

Supplemental Data

The additional supporting information is available in the online version of this article.

Supplemental Data S1. List of genes that are differentially expressed in wild type and *wpl1s*.

Supplemental Data S2a. List of photosynthesis genes that are differentially expressed in wild type and *wpl1s*.

Supplemental Data S2b. List of genes involved in synthesis and degradation of Val, Leu, and Ile that are differentially expressed in wild type and *wpl1s*.

Supplemental Data S3. List of plastid-encoded genes that are differentially expressed in wild type and *wpl1s*.

Supplemental Figure S1. Phenotypes of wild type and *wpl1*.

Supplemental Figure S2. Subcellular localization of ValRS2.

Supplemental Figure S3. Alignment of OsValRS1, WP1, AtValRS1, and AtValRS2.

Supplemental Figure S4. Expression profiles of *OsValRS1*, *WP1*, and *OsValRS3*.

Supplemental Figure S5. Expression levels of chlorophyll synthesis genes in wild type and *wpl1*.

Supplemental Figure S6. Western-blot analysis of chloroplast proteins in wild type and *wpl1w* glumes.

Supplemental Figure S7. Expression of nuclear-encoded chloroplast ribosomal protein genes in seedlings

Supplemental Figure S8. TEM observation of mitochondria in wild type and *wpl1s*.

Supplemental Figure S9. Analysis of mitochondrial ribosome and mRNA levels of some mitochondrial genes.

Supplemental Table S1. Agronomic characteristics of WT and *wpl1w*.

Supplemental Table S2. Primers used in mapping.

Supplemental Table S3. Primers used in real-time PCR.

Supplemental Table S4. Primers used in vector construction.

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LITERATURE CITED

- Allison LA, Simon LD, Maliga P (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. *EMBO J* **15**: 2802–2809
- Arsova B, Hoja U, Wimmelbacher M, Greiner E, Ustün S, Melzer M, Petersen K, Lein W, Börnke F (2010) Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in *Arabidopsis* and *Nicotiana benthamiana*. *Plant Cell* **22**: 1498–1515
- Asakura Y, Galarnau E, Watkins KP, Barkan A, van Wijk KJ (2012) Chloroplast RH3 DEAD box RNA helicases in maize and *Arabidopsis* function in splicing of specific group II introns and affect chloroplast ribosome biogenesis. *Plant Physiol* **159**: 961–974
- Bang WY, Chen J, Jeong IS, Kim SW, Kim CW, Jung HS, Lee KH, Kweon HS, Yoko I, Shiina T, et al (2012) Functional characterization of ObgC in ribosome biogenesis during chloroplast development. *Plant J* **71**: 122–134
- Berg M, Rogers R, Muralla R, Meinke D (2005) Requirement of aminoacyl-tRNA synthetases for gametogenesis and embryo development in *Arabidopsis*. *Plant J* **44**: 866–878
- Chateigner-Boutin AL, des Francs-Small CC, Delannoy E, Kahlau S, Tanz SK, de Longevialle AF, Fujii S, Small I (2011) OTP70 is a pentatricopeptide repeat protein of the E subgroup involved in splicing of the plastid transcript *rpoC1*. *Plant J* **65**: 532–542
- Chateigner-Boutin AL, Ramos-Vega M, Guevara-García A, Andrés C, de la Luz Gutiérrez-Nava M, Cantero A, Delannoy E, Jiménez LF, Lurin C, Small I, et al (2008) CLB19, a pentatricopeptide repeat protein required for editing of *rpoA* and *clpP* chloroplast transcripts. *Plant J* **56**: 590–602
- Chen S, Tao L, Zeng L, Vega-Sanchez ME, Umemura K, Wang GL (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. *Mol Plant Pathol* **7**: 417–427
- Chi W, He B, Mao J, Li Q, Ma J, Ji D, Zou M, Zhang L (2012) The function of RH22, a DEAD RNA helicase, in the biogenesis of the 50S ribosomal subunits of *Arabidopsis* chloroplasts. *Plant Physiol* **158**: 693–707
- Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. *Curr Biol* **6**: 325–330
- Duchêne AM, Giritch A, Hoffmann B, Cognat V, Lancelin D, Peeters NM, Zaepfel M, Maréchal-Drouard L, Small ID (2005) Dual targeting is the rule for organellar aminoacyl-tRNA synthetases in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **102**: 16484–16489
- Duchêne AM, Pujol C, Maréchal-Drouard L (2009) Import of tRNAs and aminoacyl-tRNA synthetases into mitochondria. *Curr Genet* **55**: 1–18
- Gao ZP, Yu QB, Zhao TT, Ma Q, Chen GX, Yang ZN (2011) A functional component of the transcriptionally active chromosome complex, *Arabidopsis* pTAC14, interacts with pTAC12/HEMERA and regulates plastid gene expression. *Plant Physiol* **157**: 1733–1745
- García M, Myouga F, Takechi K, Sato H, Nabeshima K, Nagata N, Takio S, Shinozaki K, Takano H (2008) An *Arabidopsis* homolog of the bacterial peptidoglycan synthesis enzyme MurE has an essential role in chloroplast development. *Plant J* **53**: 924–934
- Guo M, Yang XL, Schimmel P (2010) New functions of aminoacyl-tRNA synthetases beyond translation. *Nat Rev Mol Cell Biol* **11**: 668–674
- Hanaoka M, Kanamaru K, Fujiwara M, Takahashi H, Tanaka K (2005) Glutamyl-tRNA mediates a switch in RNA polymerase use during chloroplast biogenesis. *EMBO Rep* **6**: 545–550
- Hedtko B, Börner T, Weihe A (1997) Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. *Science* **277**: 809–811
- Itoh J, Nonomura K, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. *Plant Cell Physiol* **46**: 23–47

- Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T, et al (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res* **36**: D480–D484
- Kusumi K, Hirotsuka S, Shimada H, Chono Y, Matsuda O, Iba K (2010) Contribution of chloroplast biogenesis to carbon-nitrogen balance during early leaf development in rice. *J Plant Res* **123**: 617–622
- Laloi C, Stachowiak M, Pers-Kamczyc E, Warzych E, Murgia I, Apel K (2007) Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* **104**: 672–677
- Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol* **148**: 350–382
- Liere K, Weihe A, Börner T (2011) The transcription machineries of plant mitochondria and chloroplasts: composition, function, and regulation. *J Plant Physiol* **168**: 1345–1360
- Majeran W, Friso G, Ponnala L, Connolly B, Huang M, Reidel E, Zhang C, Asakura Y, Bhuiyan NH, Sun Q, et al (2010) Structural and metabolic transitions of C4 leaf development and differentiation defined by microscopy and quantitative proteomics in maize. *Plant Cell* **22**: 3509–3542
- Mullet JE (1993) Dynamic regulation of chloroplast transcription. *Plant Physiol* **103**: 309–313
- Myouga F, Hosoda C, Umezawa T, Iizumi H, Kuromori T, Motohashi R, Shono Y, Nagata N, Ikeuchi M, Shinozaki K (2008) A heterocomplex of iron superoxide dismutases defends chloroplast nucleoids against oxidative stress and is essential for chloroplast development in *Arabidopsis*. *Plant Cell* **20**: 3148–3162
- O'Donoghue P, Luthy-Schulten Z (2003) On the evolution of structure in aminoacyl-tRNA synthetases. *Microbiol Mol Biol Rev* **67**: 550–573
- Pfalz J, Liere K, Kandlbinder A, Dietz KJ, Oelmüller R (2006) pTAC2, -6, and -12 are components of the transcriptionally active plastid chromosome that are required for plastid gene expression. *Plant Cell* **18**: 176–197
- Pfalz J, Pfannschmidt T (2013) Essential nucleoid proteins in early chloroplast development. *Trends Plant Sci* **18**: 186–194
- Pogson BJ, Albrecht V (2011) Genetic dissection of chloroplast biogenesis and development: an overview. *Plant Physiol* **155**: 1545–1551
- Pyo YJ, Kwon KC, Kim A, Cho MH (2013) Seedling Lethal1, a pentatricopeptide repeat protein lacking an E/E+ or DYW domain in *Arabidopsis*, is involved in plastid gene expression and early chloroplast development. *Plant Physiol* **163**: 1844–1858
- Shiina T, Tsunoyama Y, Nakahira Y, Khan MS (2005) Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *Int Rev Cytol* **244**: 1–68
- Steiner S, Schröter Y, Pfalz J, Pfannschmidt T (2011) Identification of essential subunits in the plastid-encoded RNA polymerase complex reveals building blocks for proper plastid development. *Plant Physiol* **157**: 1043–1055
- Strand A, Asami T, Alonso J, Ecker JR, Chory J (2003) Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. *Nature* **421**: 79–83
- Sugimoto H, Kusumi K, Tozawa Y, Yazaki J, Kishimoto N, Kikuchi S, Iba K (2004) The virescent-2 mutation inhibits translation of plastid transcripts for the plastid genetic system at an early stage of chloroplast differentiation. *Plant Cell Physiol* **45**: 985–996
- Sugimoto H, Kusumi K, Noguchi K, Yano M, Yoshimura A, Iba K (2007) The rice nuclear gene, VIRESCENT 2, is essential for chloroplast development and encodes a novel type of guanylate kinase targeted to plastids and mitochondria. *Plant J* **52**: 512–527
- Sugiura M (2003) History of chloroplast genomics. *Photosynth Res* **76**: 371–377
- Swiatecka-Hagenbruch M, Liere K, Börner T (2007) High diversity of plastid promoters in *Arabidopsis thaliana*. *Mol Genet Genomics* **277**: 725–734
- Szymański M, Deniziak M, Barciszewski J (2000) The new aspects of aminoacyl-tRNA synthetases. *Acta Biochim Pol* **47**: 821–834
- Toda T, Fujii S, Noguchi K, Kazama T, Toriyama K (2012) Rice MPR25 encodes a pentatricopeptide repeat protein and is essential for RNA editing of nad5 transcripts in mitochondria. *Plant J* **72**: 450–460
- Uwer U, Willmitzer L, Altmann T (1998) Inactivation of a glycyl-tRNA synthetase leads to an arrest in plant embryo development. *Plant Cell* **10**: 1277–1294
- Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **10**: 57–63
- Yagi Y, Ishizaki Y, Nakahira Y, Tozawa Y, Shiina T (2012) Eukaryotic-type plastid nucleoid protein pTAC3 is essential for transcription by the bacterial-type plastid RNA polymerase. *Proc Natl Acad Sci USA* **109**: 7541–7546
- Yang YZ, Peng H, Huang HM, Wu JX, Jia SR, Huang DF, Lu TG (2004) Large-scale production of enhancer tapping lines for functional analysis of rice genome. *Plant Sci* **167**: 281–288
- Yap A, Kindgren P, Colas des Francs-Small C, Kazama T, Tanz SK, Toriyama K, Small I (2015) AEF1/MPR25 is implicated in RNA editing of plastid atpF and mitochondrial nad5, and also promotes atpF splicing in *Arabidopsis* and rice. *Plant J* **81**: 661–669
- Zhang JZ, Somerville CR (1997) Suspensor-derived polyembryony caused by altered expression of valyl-tRNA synthetase in the twm2 mutant of *Arabidopsis*. *Proc Natl Acad Sci USA* **94**: 7349–7355
- Zhou W, Cheng Y, Yap A, Chateigner-Boutin AL, Delannoy E, Hammani K, Small I, Huang J (2009) The *Arabidopsis* gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of chloroplasts during early growth. *Plant J* **58**: 82–96
- Zubo YO, Kusnetsov VV, Börner T, Liere K (2011) Reverse protection assay: a tool to analyze transcriptional rates from individual promoters. *Plant Methods* **7**: 47