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Research article

Rice albino 1, encoding a glycyl-tRNA synthetase, is involved in chloroplast development and establishment of the plastidic ribosome system in rice

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ABSTRACT

The chloroplast is an important organelle that performs photosynthesis as well as biosynthesis and storage of many metabolites. Aminoacyl-tRNA synthetases (aaRSs) are key enzymes in protein synthesis. However, the relationship between chloroplast development and aaRSs still remains unclear. In this study, we isolated a *rice albino 1* (*ra1*) mutant through methane sulfonate (EMS) mutagenesis of rice *japonica* cultivar Ningjing 4 (*Oryza sativa* L.), which displayed albinic leaves in seedling stage due to abnormal chloroplast development. Compared with wild type (WT), *ra1* showed significantly decreased levels of chlorophylls (Chl) and carotenoids (Car) in 2-week-old seedlings, which also showed obvious plastidic structural defects including abnormal thylakoid membrane structures and more osmiophilic particles. These defects caused albino phenotypes in seedlings. Map-based cloning revealed that *RA1* gene encodes a glycyl-tRNA synthetase (GlyRS), which was confirmed by genetic complementation and knockout by Crispr/Cas9 technology. Sequence analysis showed that a single base mutation (T to A) occurred in the sixth exon of *RA1* and resulted in a change from Isoleucine (Ile) to Lysine (Lys). Real-time PCR analyses showed that *RA1* expression levels were constitutive in most tissues, but most abundant in the leaves and stems. By transient expression in *Nicotiana benthamiana*, we found that RA1 protein was localized in the chloroplast. Expression levels of chlorophyll biosynthesis and plastid development related genes were disordered in the *ra1* mutant. RNA analysis revealed biogenesis of chloroplast rRNAs was abnormal in *ra1*. Meanwhile, western blotting showed that synthesis of proteins associated with plastid development was significantly repressed. These results suggest that *RA1* is involved in early chloroplast development and establishment of the plastidic ribosome system in rice.

1. Introduction

Chloroplasts are not only the important site of photosynthesis in higher plants, but also responsible for biosynthesis and storage of metabolites (Ruuska et al., 2004; Millar et al., 2006). Chloroplasts retain their own genome and translation system, nuclear and plastid genomes collectively regulate chloroplast development (Millar et al., 2006). In addition, chloroplast development requires cooperation between the genes encoded by the nucleus and those encoded by the plastid. In plants, there are two types of RNA polymerase in the chloroplast. One type is a nuclear-encoded RNA polymerase (NEP), and the other type is a plastid-encoded RNA polymerase (PEP). PEP is a multimeric enzyme of the prokaryotic type and NEP is a monomeric enzyme of the phage type (Azevedo et al., 2006). PEP catalytic core components are composed of four subunits, i.e., α, β, β′ and β″, encoded by the plastidic genes RNA polymerase α subunit (rpoA), RNA polymerase β subunit (rpoB), RNA polymerase β′ subunit (rpoC1), and RNA polymerase β″ subunit (rpoC2), respectively. PEP are responsible for transcription of

Abbreviations: dCAPS, derived cleaved amplified polymorphic sequence; GluTR, Glutamyl t-RNA reductase; NEP, Nuclear-encoded RNA polymerase; PEP, Plastid-encoded RNA polymerase; psaA1, PS I P700 apoprotein A1; psaA2, PS I P700 apoprotein A2; RBCL, Rubisco large subunits; RBCS, Rubisco small subunit; RCA, Rubisco activase

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photosystem proteins (Shiina et al., 2005). The development of chloroplasts can be divided into three periods: plastid replication and plastid genome replication; chloroplast genesis and translation system construction and the establishment of the chloroplast light-harvesting system (Kusumi et al., 2010). Loss of function of proteins involved in the PEP and NEP could lead to the plant albinic phenotype (Liu et al., 2016; Steiner et al., 2011; Yagi et al., 2012; Pfalz et al., 2013). In Arabidopsis, plastidial *thioredoxin z* (*trx z*) had pale yellowish leaves when grown on agar plates and subsequently died. *TRX z* encodes a plastidial thioredoxin, which can interact with two fructokinase-like proteins FLN1 and FLN2 by conserved Cys residues (Arsova et al., 2010).

Plastid proteins are translated by 70S ribosomes, which consist of 30S rRNA and 50S rRNA complexes. The former is composed of a 16S rRNA molecule and 21 different proteins and the latter includes a 23S rRNA, a 5S rRNA, a 4.5S rRNA and 32 different proteins. In rice and Arabidopsis, mutations in Spo0B-associated GTP-binding protein C (ObgC) affect ribosomal biosynthesis, leading to chloroplast developmental disorders (Bang et al., 2009, 2012). Dysfunction of *Albino Leaf1* (*AL1*) resulted in altered abundances of ribosomal proteins and breakdown of chloroplast (Zhang et al., 2016).

The accuracy of mRNA translation in protein synthesis is determined by two factors, the fidelity of the amino acylation reactions binding amino acids to their cognate tRNAs, and the accuracy of the binding of the anticodons to the mRNA codons exposed at the “A” site of the ribosome. The role of aminoacyl-tRNA synthetases (aaRSs) in translation is to define the genetic code by accurately pairing cognate tRNAs with their corresponding amino acids (Ling et al., 2009). There are 20 aaRSs in eukaryotes, corresponding to each amino acid. These aaRSs not only catalyze protein synthesis, but also perform translational regulation, RNA splicing, apoptosis, and rRNA synthesis (Guo et al., 2010; Wang et al., 2016). For example, Methionine-tRNA synthetase (MetRS) is involved in protein synthesis in cytoplasm and rRNA synthesis in the nucleus (Ko et al., 2000). Glutamate-tRNA synthetase (GluRs) can regulate apoptosis (Ko et al., 2001). In Arabidopsis, *EDD1* encodes a plastid and mitochondrial localized glycyl-tRNA synthetase and *embryo defective development 1* (*edd1*) were characterized by aborted ovules and change in distal regions of the leaf. *EDD1* control the abaxial fate genes *kanadi 1* (*KAN1*) and *auxin response factor 3* (*ARF3*) (Uwer et al., 1998; Moschopoulos et al., 2012). All aaRSs are encoded by the nuclear genome and transported to the designated compartments. A complete set of 20 aaRSs are present in every cell compartment that requires protein synthesis (Duchene et al., 2005). In total, 45 expressed aaRSs genes were identified in the Arabidopsis genome. Previous reports on aaRS gene mutants were mainly focused on morphology. In rice, *white panicle 1* (*WP1*) encodes a Val-tRNA synthetase. Dysfunction of WP1 displayed virescent phenotypes in seedlings and white panicles at heading, suggesting WP1 plays an essential role in chloroplast development (Wang et al., 2016). However, little is known about aaRSs affecting of plant growth and development.

In this study, we isolated a *rice albino 1* (*ra1*) mutant that displayed albino leaves at the seedling stage and died after 18 days. *RA1* encodes a glycyl-tRNA synthetase (GlyRS), which is targeted to chloroplasts. Irregularly-shaped membranes with no clear thylakoids and vacuole-like structures were observed in *ra1* chloroplasts. In addition, establishment of the plastidic ribosome system was defective in *ra1*. We demonstrate that *RA1* is important for chloroplast development and ribosomal biogenesis.

2. Materials and methods

2.1. Plant materials and growth conditions

The *ra1* mutant was isolated from an ethyl methane sulfonate (EMS)-mutagenized *japonica* cultivar Ningjing 4 (NJ4) M_1 population. WT, *ra1* mutant and an F_2 mapping population were grown in the field or growth chambers. Growth chambers conditions were 10 h light (Light intensity was 300 μmol $m^{-2}.s^{-1}$) at 30 °C and 14 h dark at 28 °C.

2.2. Measurement of chlorophyll content and observation of chloroplast autofluorescence

Chlorophyll contents of WT and *ra1* were measured *in vitro* as described previously (Wang et al., 2016). In brief, approximately 0.5 g fresh 7-day-old WT and *ra1* seedling leaves were cut into 5 mm pieces, then transferred to a centrifuge tube, and placed in 95% ethanol at 4 °C under dark conditions for 24 h, repeatedly shaking. Absorbance of the supernatants was measured at 665, 649, and 470 nm. The equations used to calculate the chlorophyll and carotenoid concentrations were: Chlorophyll *a*: $C_a = 13.95D_{665} - 6.88D_{640}$, Chlorophyll *b*: $C_b = 24.96D_{649} - 7.32D_{665}$, Carotenoid: $C_x = (1000D_{470} - 2.05C_a - 114C_b)/245$. Fluorescence microscopy (NIKON AZ100) was used to observe chlorophyll autofluorescence of rice at different stages according to the previous method (Itoh et al., 2005). At least 2 biological and 3 technical replicates were performed.

2.3. Transmission electron microscopy (TEM)

Leaves of WT and *ra1* plants grown for 2 weeks were cut into small pieces and transferred to 2.5% glutaraldehyde (0.2 M phosphate buffer, pH 7.2). Vacuum was applied to infiltrate the solution, resulting in the samples sinking to the bottoms of the tubes. Then the samples were incubated at room temperature for 12 h, rinsed and incubated overnight in 1% OsO_4 at 4 °C. After several rounds of rinsing with 0.2 M phosphate buffer (PH 7.2), the samples were dehydrated through 30, 50, 70, 80, 90 and 100% of ethanol solutions for 20 min, and embedded in Spurr's medium before ultrathin sectioning. The samples were stained with uranyl acetate and observed using a Hitachi H-7650 transmission electron microscope (Han et al., 2006).

2.4. Map-based cloning of the RA1 gene

Since the recessive homozygous *ra1* seedlings were albino lethal, crosses were made between green heterozygous *RA1/ra1* plants and N22 (*indica*) for construction of the F_2 mapping population. Within the F_2 mapping population, 318 albino plants were used for fine mapping and genetic analysis. Based on the published simple sequence repeats (SSR) markers, the polymorphic markers between the *ra1* mutant and N22 were used for initial mapping. The DNA sequences in this region of *japonica* (http://www.ncbi.nlm.nih.gov/) and *indica* (http://www.gramene.org/) were aligned, and new genetic markers were designed using Primer Premier 5 (http://www.premierbiosoft.com/). All of the molecular markers for genetic mapping and gene cloning are listed in Supplementary Table 1.

2.5. Complementation and Crispr/Cas9 targeting of RA1

For functional complementation of the *ra1* mutant, full length WT cDNA (entire coding sequences) and a genomic fragment upstream of native promoter region were amplified and inserted into the binary vector pCUbi1390. Fusion vector was transformed into the *ra1* callus by *Agrobacterium*-mediated transformation (Hiei et al., 1994). The target sequence of *RA1* for Crispr/Cas9 deletion was “AACAGACCCACTAGA CTA”. The sequence were amplified and fused to a binary vector pCAMBIA 1305 and transformed into Nipponbare by *Agrobacterium*-mediated transformation as described above.

2.6. Subcellular localization of RA1

The full-length *RA1* cDNA (with the stop codon deleted) was amplified using the primers listed in Supplementary Table 1 and translationally-fused to the N-terminus of green fluorescent protein (GFP) in

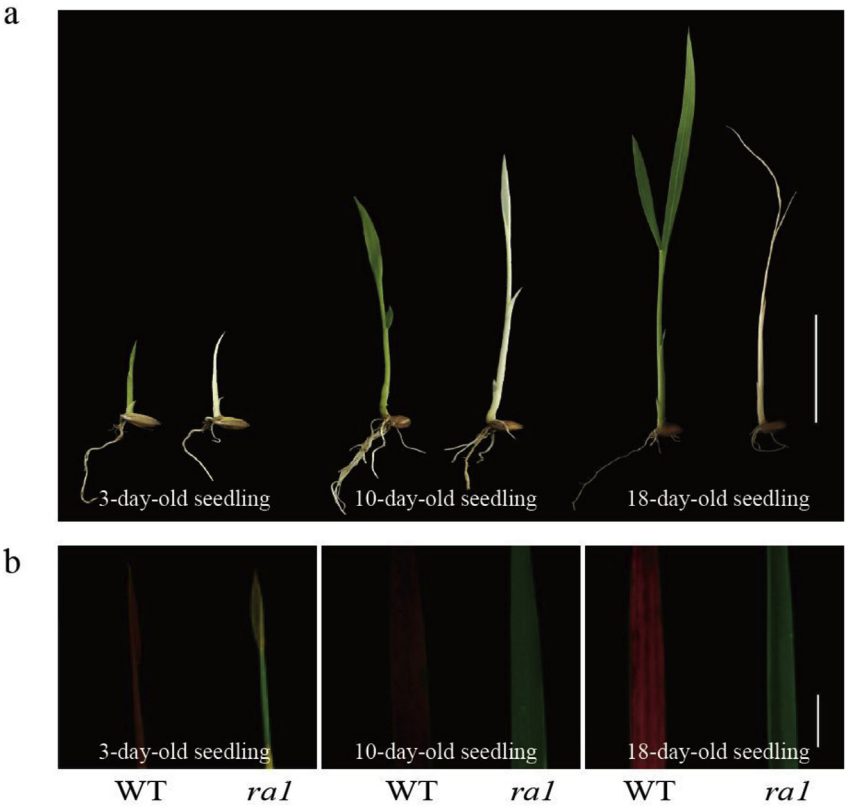

Fig. 1. Phenotypic characteristics of wild type and *ra1* mutant
a Phenotypes of 3, 10 and 18 day old seedlings. Scale bars = 3 cm **b** Chlorophyll autofluorescence of 3, 10 and 18 day old seedlings. Scale bars = 5 mm.

the binary vector pCAMBIA1305. The vectors were transiently expressed in *Nicotiana benthamiana* mesophyll cells by *Agrobacterium* infiltration. The fluorescence was observed using a confocal laser scanning microscope (Zeiss LSM 780), and red chlorophyll autofluorescence was used as a marker for reference.

2.7. Real-time PCR

According to a RNA Prep Pure Plant kit protocol (Tiangen Co, Beijing, China), total RNA was extracted from 7-day-old rice seedlings. From each rice seedling RNA sample (2 μg) was reverse-transcribed using an oligo-dT or random primer and PrimeScript I (Takara Bio, Kusatsu, Japan). Quantitative Real-time PCR (qRT-PCR) using a SYBR Premix Ex Taq™ kit (TaKaRa) was executed on an ABI prism 7900 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). At least 2 biological and 4 technical replicates were performed. The primer sequences for the genes related to chlorophyll synthesis and plastid development are listed in Supplementary Table 1, respectively. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression (Livak et al., 2001).

2.8. rRNA analysis

The concentrations and purities of 7-day-old rice seedlings RNA extracts were first assayed with a NanoDrop Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). RNAs were diluted to ~10 ng.uL^{-1} and analyzed by an Agilent 2100 Bioanalyzer. An RNA 6000 Nano Total RNA Analysis Kit (Agilgent) was used for analysis following the manufacturer's instructions. At least 2 biological replicates were performed.

2.9. Western blot analysis

Total proteins were isolated from 7-day-old WT and *ra1* seedlings. In brief, 0.2g fresh 7-day-old WT and *ra1* seedlings were ground into powder in liquid nitrogen and transferred to a centrifuge tube. Then 600 μL NBI 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^{-1} antipain, 2 μg mL^{-1} leupeptin, 2 μg mL^{-1} aprotinin) was added to centrifuge tube and incubated for 30 min on ice. The solutions were centrifuged at 12,000 g for 20 min, and then all supernatants were aspirated and centrifuged at 12,000 g for 10 min. 80 μL of supernatant were transferred to a new centrifuge tube, mixed with 20 μL 5 X protein loading buffer and incubated for 5 min at 100 °C. 10 μL of this mix were then loaded on 8–15% SDS-PAGE gradient gels and electrophoresis using Tris-Glycine Electrophoresis Buffer. Gel-membrane Sandwich model was used for transferred to polyvinylidene difluoride membrane. The proteins were immunoblotted with various specific antibodies, and detected using High-sig ECL Western Blotting Substrate (Tanon). Specific antibodies were obtained from Beijing Protein Innovation (http://www.proteomics.org.cn/). At least 2 biological replicates were performed.

2.10. Statistical analysis

Results were subjected to Student's *t*-test to compare the statistical significant differences of the mean values between WT and *ra1* (Microsoft Excel software). Statistical significance differences at $P < 0.05$ and $P < 0.01$ were indicated by asterisks * and **, respectively. The number of replicates (n) for each measured parameter refers to the number of repetitions of technical replicates. All values in figures were means ± SD (standard deviation).

3. Results

3.1. Phenotypic characterization of the ra1 mutant

The *ra1* mutant was derived from the *japonica* variety NJ4 by EMS mutagenesis. Under chamber and paddy field conditions, the *ra1* mutant exhibited obvious albinic leaf during early leaf development and died 18 days after germination (Fig. 1a). In addition, we also observed the chlorophyll auto-fluorescence of 3, 10 and 18-day-old seedlings, and found that the auto-fluorescence of chlorophyll was always observed in the WT, and showed an increasing trend as the seedlings developed. In contrast, at 3 day after sowing, only a small amount of auto-fluorescence was detected in *ra1* mutant. At 10 and 18 days after sowing, chloroplast auto-fluorescence was almost undetectable with the *ra1* mutants (Fig. 1b). Subsequently, leaves of *ra1* mutant began to wilt and died.

3.2. Early chloroplast development is affected in the ra1 mutant

We measured the pigment contents of WT and *ra1* seedlings. The various pigment content showed a very significant decrease, and Chl a, Chl b and Car did not accumulate in the *ra1* mutant seedlings as compared to WT (Fig. 2a and b). In order to determine whether the morphology and development of chloroplasts in *ra1* were affected, the chloroplast ultrastructures of both WT and *ra1* were observed by TEM. The results revealed that a large number of lamellar structures in the wild-type chloroplasts thylakoid could be observed at 2-weeks-old seedling (Fig. 2c and d). Instead, only small and irregular lamellas with no clear thylakoid structures were shown in *ra1* plastids. Besides, a large amount of vacuole-like structures and osmiophilic particles appeared in *ra1* chloroplasts (Fig. 2e and f). Clearly, these results emphasized *RA1* is essential for chloroplasts development.

3.3. Map-based cloning of the RA1 gene

Genetic analysis indicated that selfed F_1 plants corresponded to 3: 1 segregation in a segregating genetic analysis population (green plants n = 260, albino plants n = 73, $\chi^2 = 1.68 < \chi^2_{0.05,\ 1} = 3.84$). For further gene mapping of the *RA1* locus, we performed a F_2 mapping population from the cross between heterozygous green plant (*RA1/ra1*) and N22. Totally 135 polymorphic markers evenly covering the whole genome were used for initial mapping. From the F_2 mapping population, 10 green plants and 10 albino plants were randomly selected. Preliminary linkage analysis indicated that the *ra1* locus was located near to SSR markers RM1369 on the short arm of chromosome 6. To further locate the gene, 318 additional albino plants from the F_2 mapping population were analyzed. Further dissection delimited the *ra1* locus to about 266-kb region from InDel markers Bh6-12 to telomere of

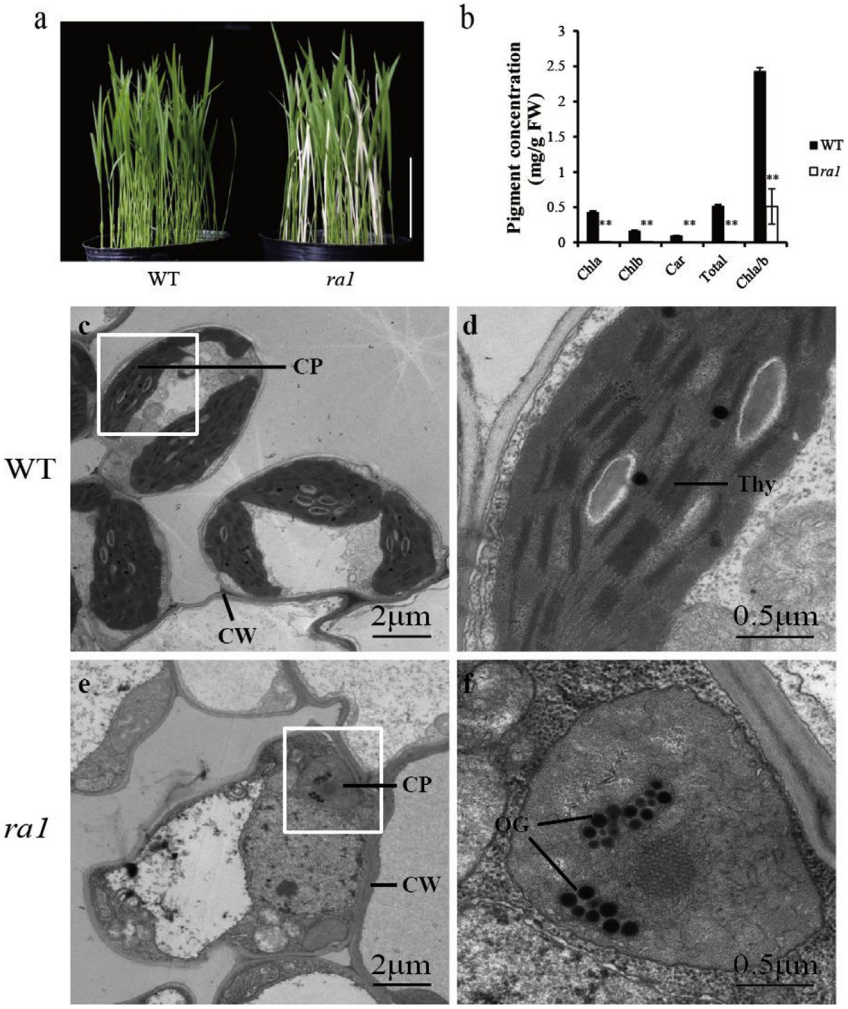

Fig. 2. Phenotypic characteristics and ultrastructure of chloroplasts of wild-type and ra1 mutant in second leaf
a Phenotypes of wild type and *ra1* mutants. Scale bars = 5 cm **b** pigment concentration in wild type and *ra1* seedlings. Error bars, ± SD (n = 3). ** indicate statistical significance at $P < 0.01$ by *t*-test. **c** Leaf cells of wild type. **d** Ultrastructures of chloroplasts of wild type. **e** Leaf cells of *ra1* mutant. **f** Ultrastructures of chloroplasts of *ra1* mutant. Cp, chloroplast; CW, cell wall; Thy, Thylakoid; OG, osmiophilic plastoglobuli.

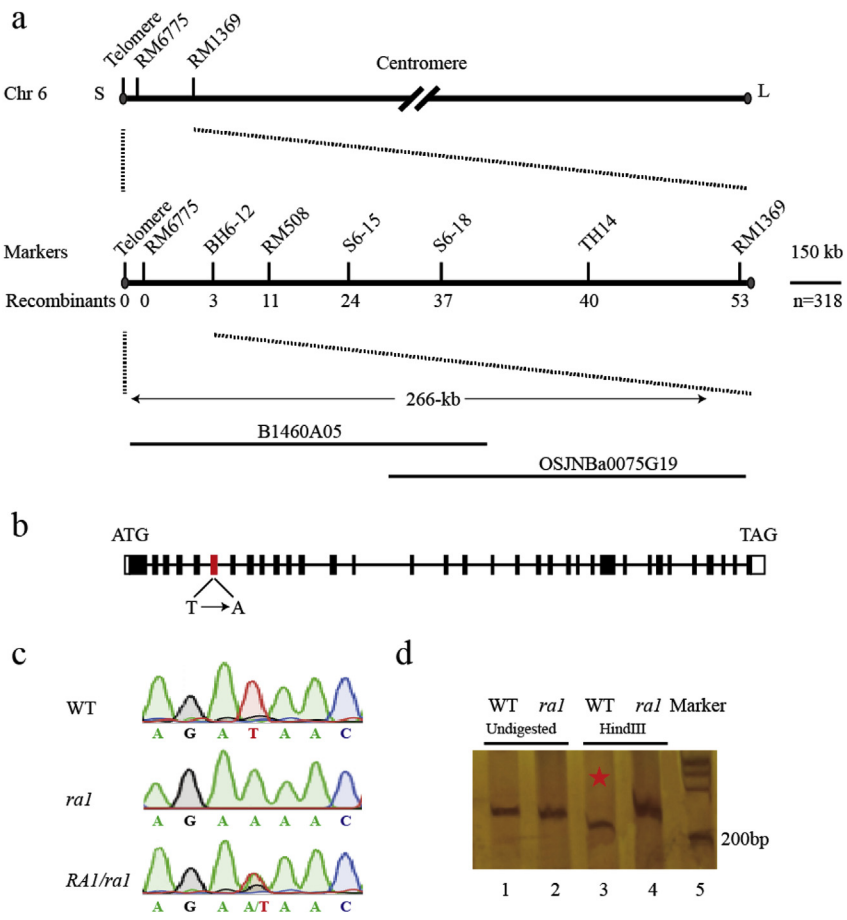

Fig. 3. Map-based cloning of the *RA1* gene
a. The *RA1* gene was located near to SSR markers RM1369 on the short arm of chromosome 6. Fine mapping, *RA1* gene was located between RM1369 and the telomere of chromosome 6. The physical distance of this interval is 266-kb. **b**. Structure of *LOC_Os06g01400*. ATG and TAG, represent the start codon and the stop codons, respectively. Black boxes represent exons and lines between black boxes represent introns. Empty boxes at the top and bottom represent the 5 'UTR and the 3′ UTR, respectively. On the sixth exon, the T to A mutation of the mutant is indicated by a red box. **c**. Sequencing peak pattern of mutation site. **d**. The dCAPs markers were used to verify the mutation sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

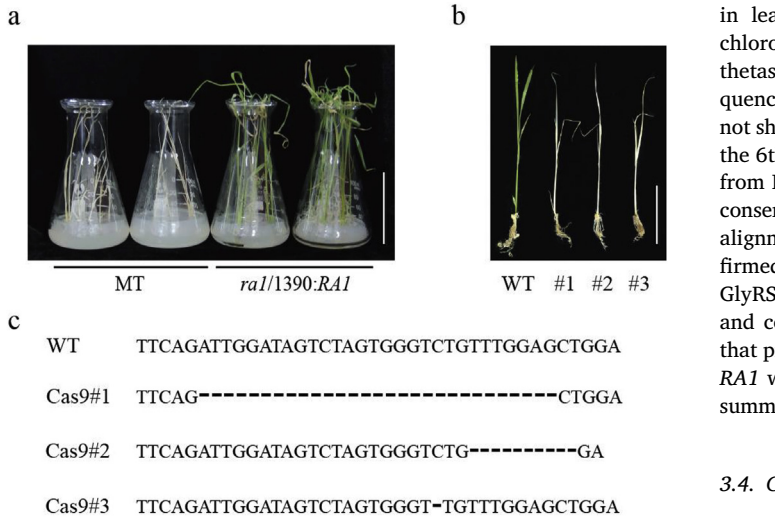

Fig. 4. Genetic complementation and CRISPR/Cas9 validate the *RA1* gene
a Complementation experiments validated the seedling phenotype **of** *RA1* gene. Scale bars = 5 cm **b** The *RA1* gene was edited using CRISPR/Cas9 technology and the phenotype of seedlings. **c** Comparisons of transgene positive and wild type sequences using CRISPR/Cas9. Scale bars = 5 cm.

chromosome 6 including two BACs B1460A05 and OSJNBa0075G19 based on the Nipponbare genome sequence (Fig. 3a). Within the target region, 17 *open reading frames* (*ORFs*) were predicted by the Rice Expression Profile Database (http://ricexpro.dna.affrc.go.jp) (Supplementary Table 2). These *ORFs* were analyzed by the online gene expression database, and only *ORF4* and *ORF15* showed high expression in leaf tissue. *ORF4* and *ORF15* were annotated for plastocyanin, chloroplast precursor (*LOC_Os06g01210*) and aminoacyl-t-RNA synthetase precursor (*LOC_Os06g01400*), respectively. No genome sequence difference was found for *ORF4* between WT and mutant (Date not shown). However, a T-to-A transition at nucleotide position 1346 in the 6th exon of *ORF15* was identified, leading to an amino acid change from Ile to Lys at 228th residue (Fig. 3b and c). The mutation was the conserved amino acid in α-core domain by amino acid sequence alignment (Supplementary Fig. 1). Moreover, a dCAPS marker confirmed the mutation site in the *ra1* mutant (Fig. 3d). *ORF15* encodes a GlyRS from known gene annotation. This gene is 10,538 bp in length and contains 34 exons and 33 introns. Phylogenetic analysis showed that proteins homologous to *RA1* exist in both monocots and dicots and *RA1* was highly conserved in higher plants (Supplementary Fig. 2). In summary, *ORF15* may be as a candidate gene.

3.4. Confirmation of RA1 function

To further demonstrate that the mutation in candidate gene *ORF15* causes the albino leaf phenotype, we conducted a complementation experiment. Because homozygous *ra1* seedlings couldn't live to maturity, we selected the phenotype of albino seedlings for transgenic complementation experiments in the callus. A 6.2-kb genomic fragment containing its native promoter and complete CDS sequence was introduced into *ra1* callus. Thirty-seven independent positive T_0 transgenic lines were obtained and they all showed the WT phenotype (Fig. 4a). In order to better confirm complementation result, CRISPR/Cas9 technology was used to editing *RA1* in the WT plants. As a result, knockout lines showed a severe albino plants phenotype and died early (Fig. 4b). Specifically, there were 29-bp, 10-bp and 1-bp deletion of mutant plants by sequencing, respectively (Fig. 4c). These sequencing

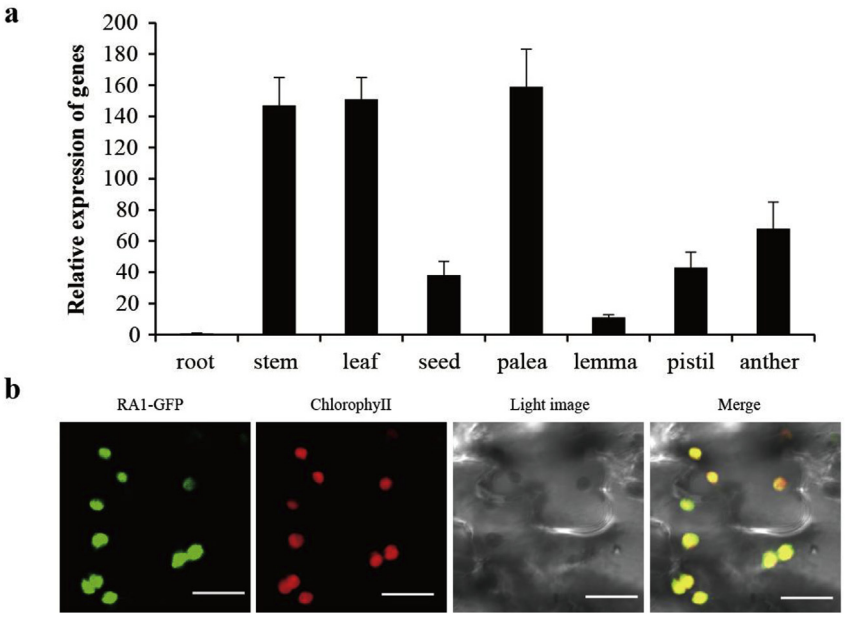

Fig. 5. Tissue analysis of *RA1* gene and subcellular localization
a Expression analysis of the *RA1* gene in various tissues. The *UBQ* gene was used as a control. Error bars, ± SD (n = 4). **b** RA1 subcellular localization signals coincide with chlorophyll autofluorescence. Scale bars = 20 μm.

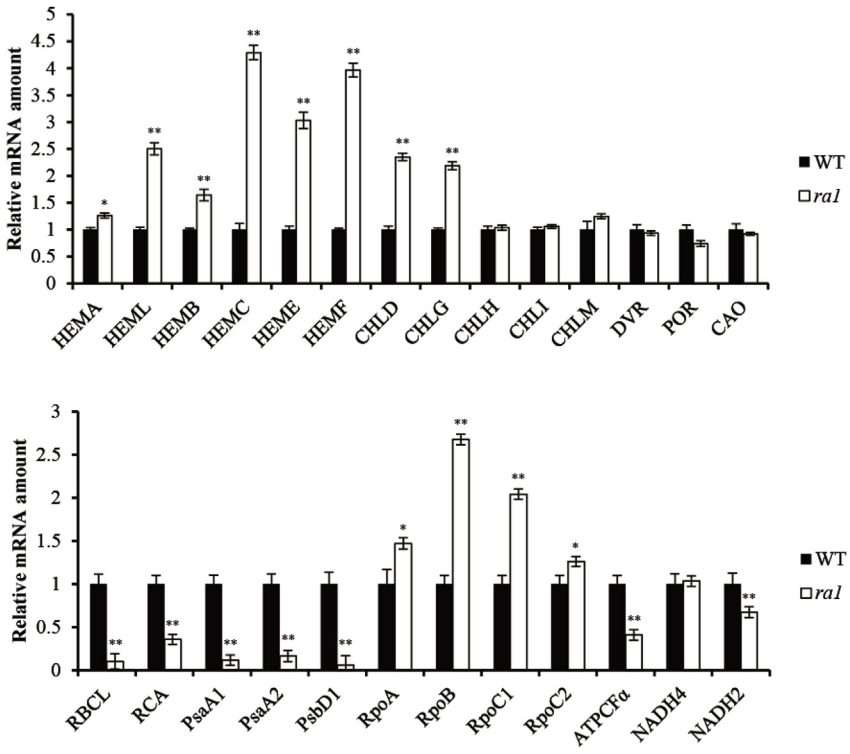

Fig. 6. Quantitative RT-PCR analysis of chlorophyll biosynthesis related gene expression levels
a Expression level of chlorophyll biosynthesis related gene, and UBQ was as a control. Error bars, ± SD (n = 4). * and ** indicate statistical significance at $P < 0.05$ and $P < 0.01$ by *t*-test. **b** Expression level of plastid development related gene, and UBQ was as a control. Error bars, ± SD (n = 4). * and ** indicate statistical significance at $P < 0.05$ and $P < 0.01$ by *t*-test.

changes led to frameshift mutations. Thus we validated the mutation in *ORF15* was the cause of albinic phenotype.

3.5. Tissue analysis of RA1 and subcellular localization

To detect the tissue expression patterns of *RA1*, we detected mRNA levels in various organs of WT by quantitative RT-PCR. *RA1* mainly expressed in stems, leaves, paleas, and other organs that contain abundant chloroplasts (Fig. 5a). Previous studies found that aaRSs had different subcellular localization results (Duchene et al., 2005). In rice, lethal albinic seedling (LAS) was chloroplast localization, while WP1 was co-localized in chloroplast and mitochondria (Wang et al., 2016; Zhang et al., 2017). In Arabidopsis, aaRSs are not only located in the chloroplast and mitochondria, but also synthesized in the cytoplasm (Duchene et al., 2005; Berg et al., 2005). To determine the subcellular localization of RA1 protein, a transient expression system was performed in *Nicotiana benthamiana*. We constructed GFP fusion proteins driven by the 35S promoter, and transiently expressed them in *Nicotiana benthamiana*. Strong green fluorescence signals were co-localized with the chlorophyll (Fig. 5b).

3.6. RA1 affects expression levels of chlorophyll biosynthesis and plastid development related genes

Normal plastids development and chlorophyll synthesis are the result of the coordination between NEP and PEP (Kusumi et al., 2014). In Arabidopsis, Glutamyl-tRNA may mediate the switch of RNA polymerase usage from NEP to PEP during chloroplast development (Hanaoka et al., 2005). We hypothesized that the expression of chlorophyll biosynthesis and plastid development related genes may be affected by defective glycyl-tRNA synthetase in the *ra1* mutant. Quantitative analysis showed that the expression of genes related to chlorophyll biosynthesis and plastid development had significantly changed in WT and *ra1*. Firstly, expression of related genes involved in the chlorophyll biosynthetic pathway, those from the synthesis of Glutamyl t-RNA (GluTR) to aminolevulinic acid (ALA) and ALA to Protoporphyrin IX in *ra1* were increased compared to WT, including *HEMA*, *HEML*, *HEMB*, *HEMC*, *HEME* and *HEMF*. However, expression of related genes for the synthesis of Protoporphyrin IX to Chlorophyll *a* and *b* in *ra1* kept the same as compared to WT, including *CHLH*, *CHLI*, *CHLM*, *DVR*, *POR* and *CAO* (Fig. 6a). Secondly, the expression levels of plastid development related genes, including class I genes *RBCL*, *RCA*, *PsaA1*, *PsaA2* and *PsbD1*, as well as class II genes *ATP synthase coupling factor1 alpha* (*ATPCFa*) and *NADH dehydrogenase subunit 2* (*NADH2*) in the *ra1* mutant, showed a great of reduction compared to wild type. Conversely, expression levels of class III genes *RpoA*, *RpoB*, *RpoC1*and *RpoC2* were increased relative to wild type (Fig. 6b). In short, expression levels of chlorophyll biosynthesis and plastid development related genes were disordered in the *ra1* mutant.

3.7. Defective plastid ribosomal RNA biogenesis system in ra1

On account of expression of plastid development related genes were disordered, we suspected the changes might affect corresponding protein levels in the *ra1* mutant. In order to verify this, total proteins from wild type and *ra1* seedlings was extracted and analyzed by western blotting. Before that, levels of RBCL protein was examined by Coomassie blue staining. The results showed that RBCLs were significantly reduced in the *ra1* mutant (Fig. 7a). Likewise, protein abundance of RBCS, RCA, subunits of photosynthetic complexes (PsbC and PsbD) and ATP synthase CF1 beta (AtpB) were dramatically decreased in *ra1* (Fig. 7b). Thus, the decrease of the protein levels may be the reason of the arrest of plastid development.

Based on the previous reports, *ra1* showed similar phenotype to *obgc1*, *wp1*, *temperature-sensitive virescent* (*tsv*), *las* mutants (Bang et al., 2012; Wang et al., 2016; Sun et al., 2017; Zhang et al., 2017). The chloroplast ribosome is composed of a 50S large subunit and another 30S small subunit, which further include four rRNA (23S, 16S, 5S and 4.5S) and ribosomal proteins. We hypothesized that the chloroplast ribosomal biosynthesis in the *ra1* might be impaired. To prove this hypothesis, we used the Agilent 2100 to analyze the composition and amount of rRNA. In the seedlings of the *ra1*mutant, 23S rRNA and 16S rRNA were marginally detected (Fig. 7c and d). Overall, the chloroplast ribosomal biosynthesis in the *ra1* mutant is severely impaired.

4. Discussion

Chloroplasts are plant organelles for photosynthesis, and are also important places for metabolism. In plants, many mutants are associated with early plastid development (Xu et al., 2013; Dong et al., 2013; Qiao et al., 2013; Motohashi et al., 2007; Pyo et al., 2013). Aminoacyl-tRNA synthetases (aaRSs) are enzymes that catalyze the binding of amino acids and their corresponding tRNAs during protein synthesis, which plays a crucial role in the protein synthesis process and is necessary for plastid development. In this study, we determined that *ra1* was a mutation of a glycyl-tRNA synthetase (GlyRS) gene. Our results suggest that *RA1* is essential for ribosomal biosynthesis and the absence of this aaRS leads to plastid developmental disorders.

It is well-known that aaRSs are conserved in function and aaRSs plays an important role in the study of biological evolution. The aaRSs are present in all cell organisms and its catalytic response is mainly divided into two steps. The first step is that the amino acids are catalyzed, under the action of enzymes and ATP, to produce a PPi and

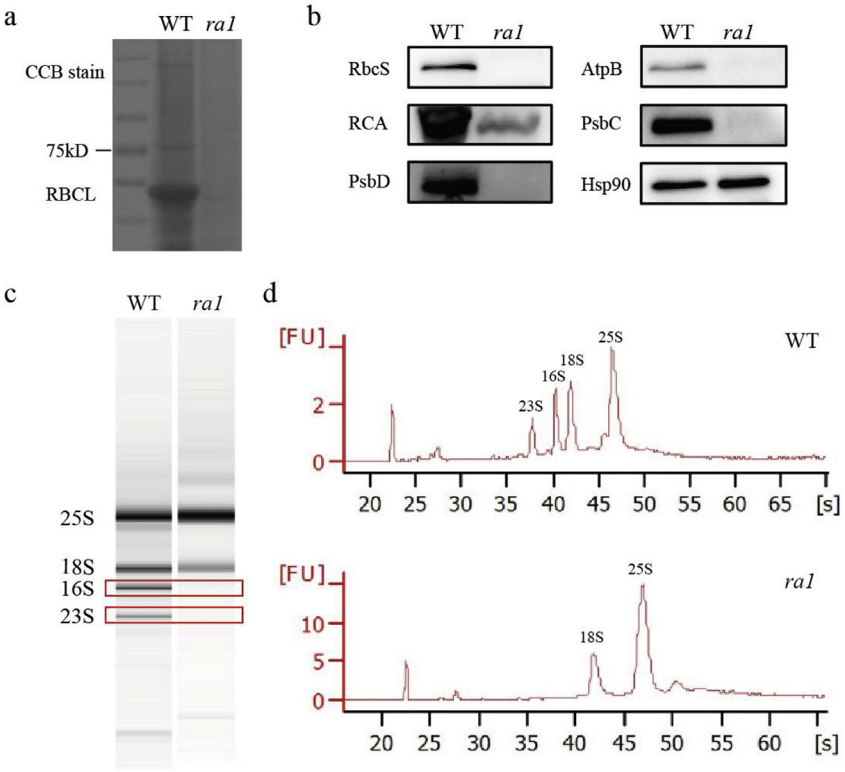

Fig. 7. Transcript and immunoblots analysis of plastid development related genes and rRNA analysis of wild type and *ra1* mutant

a RBCL was detected by Coomassie blue staining. **b** The protein level of chloroplast related genes was detected by Western blot. Hsp90 was used as an internal control. **c** Wild type and *ra1* were analyzed with Agilent 2100. The red boxes indicate the absence of 16S and 23S in *ra1*, respectively. **d** rRNA analysis results using Agilent 2100. The rRNAs isolated from 7 day old WT and *ra1* seedlings. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

aminoacyl adenylate. Generally, aminoacyl adenylate is bound firmly to the enzyme. The second step is that the aminoacyl adenylate-enzyme conjugated complex and tRNA to generate aaRSs, AMP and enzymes and both step are reversible reactions. There are 20 aaRSs in organisms, and class I and class II contain 10 aaRSs, respectively (Sugiura et al., 2000). All class I aaRSs contain a Rossman fold domain in its core site, as well as the conserved HIGH and KMSKS domains. Moreover, the class II aaRSs has an anti-parallel β-sheet structure surrounded by an alpha helix. In this study, GlyRS was classified as class II and consists of four domains (Supplementary Fig. 1a). Amino acid at 1–72 is the N-terminal signal peptide. This domain is essential for recognition of chloroplast membrane after protein synthesis in cytoplasm and transport of protein into chloroplast. The 73-351aa region is the α-core domain, which has function of glycine-tRNA ligase activity. The 386-933aa region is the β subunit, which was supposed to decompose ATP to provide energy for the normal functioning of GlyRS. The 962-1054aa region of C-terminal is anticodon-binding domain, and its role is likely to act as anticodon to recognize glycine (Hausmann et al., 2008; Ling et al., 2009). By amino acid sequence alignment, it was found that the T to A mutation resided in a highly conserved α-core domain in the *ra1* mutant (Supplementary Fig. 1b). Thus it is reasonable that the T to A mutation results in pivotal amino acid change from Ile to Lys at 228th residue and loss of enzymatic activity of the RA1, although it causes difference for *RA1* transcriptional levels between the wild type and *ra1* (Supplementary Fig. 3).

In eukaryotes, cytoplasm, mitochondria and plastids all contain their own protein synthesis systems. In Arabidopsis, some aaRSs are localized in more than one organelle. Seventeen aaRSs are localized in both mitochondria and plastids, five aaRSs are localized in both the cytosol and mitochondria, and two aaRSs are only localized to the chloroplast (Duchene et al., 2005). In rice, previous studies have found that WP1 was co-localized in both chloroplasts and mitochondria in rice, whereas OsValRS1 was only localized in the cytoplasm. Three different aaRSs localization are crucial to plants (Wang et al., 2016). In this study, strong green fluorescence signal co-localized with chlorophylls autofluorescence (Fig. 5c). In addition, we found two other glycine-tRNA synthetases in rice by database analysis (http://ricexpro.dna.affrc.go.jp/). *OsGlyRS1* (*LOC_Os04g32650*) was highly expressed in leaf and inflorescence, while *OsGlyRS3* (*LOC_Os08g42560*) mainly in ovary and anther in rice. Similar to WP1, localization of RA1, GlyRS1 and GlyRS3 may cover the cytoplasm, mitochondria and chloroplast.

Previous evidence showed that impairment of PEP activity led to a reduction in glutamate-tRNA content in the plastid (Hanaoka et al., 2005; Pfalz et al., 2006; Arsova et al., 2010; Yagi et al., 2012). The down regulation of PEP expression and the up-regulation of NEP expression in the *clb19*, *obg*, *cotp70*, *wp1*, *las* and *ys1*mutant indicated that their PEP activity was severely impaired (Bang et al., 2009, 2012; Chateigner-Boutin et al., 2008, 2011; Wang et al., 2016; Zhang et al., 2017). Similarly, expression levels of class I genes in the *ra1* were reduced compared to wild type. Moreover, expression levels of class III genes were increased (Fig. 6b). We suspect that dysfunction of RA1 may affect the activity of the PEP, which affects the expression level of genes encoded by the plastid. In addition, in the seedlings of the *ra1*, almost no 23S rRNA and 16S rRNA were detected (Fig. 7c and d). A significant decrease in the amount of its Gly-tRNA in the mutant may disturb the synthesis of early ribosomal proteins and therefore Gly-tRNA is essential for the synthesis of ribosomal rRNA.

In Arabidopsis, *EDD1* is a homolog gene of *RA1*. *EDD1* encoding a glycyl-tRNA synthetase, an essential enzymes that catalyzes the addition of amino acids to specific tRNA (Uwer et al., 1998; Moschopoulos et al., 2012). *EDD1* was targeted to plastid and mitochondrial, and dysfunction of EDD1 displayed embryo and leaves defective development, suggesting it plays an essential role in embryo and leaves development. Similar to the phenotype of the *edd1* mutant, *ra1* show abnormal chloroplast developmental. The similar localization and function of RA1 and EDD1 suggested that function of glycyl-tRNA synthetase should be conserved function in higher plants.

5. Conclusion

We identified a seedling albino mutant, whose chloroplasts showed no intact thylakoid membranes and obvious albinic leaf during early leaf development. Map-based cloning revealed that *RA1* encodes a glycyl-tRNA synthetase that is essential for plant chloroplast development. RA1 protein was located in the chloroplast. Expression levels of genes transcribed by the NEP and PEP were disrupted in the mutant and ribosome synthesis was abnormal. Hence, *RA1* is crucial for chloroplast development and ribosomal synthesis.

Author contribution statement

Hai Zheng, Zhuoran Wang and Feng Lv conducted the experiments. Hai Zheng, Peiran Wang, Xi Liu. Ling Jiang designed the research. Hai Zheng and Wenting Bai conducted Western blot experiments. Yunlu Tian and Weiyi Kong found the mutant and analyzed the data. Chaolong Wang and Xiaowen Yu guide the writing of manuscripts. Hai Zheng wrote the manuscript. Zhigang Zhao and LingLong Liu edited and modify the manuscript. Hai Zheng and Zhuoran Wang have contributed equally to this work. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.04.008.

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