RESEARCH PAPER

Albino Leaf 2 is involved in the splicing of chloroplast group I and II introns in rice

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Abstract

Chloroplasts play an essential role in plant growth and development through manipulating photosynthesis and the production of hormones and metabolites. Although many genes or regulators involved in chloroplast biogenesis and development have been isolated and characterized, identification of novel components is still lacking. We isolated a rice (Oryza sativa) mutant, termed albino leaf 2 (al2), using genetic screening. Phenotypic analysis revealed that the al2 mutation caused obvious albino leaves at the early developmental stage, eventually leading to al2 seedling death. Electron microscopy investigations indicated that the chloroplast structure was disrupted in the al2 mutants at an early developmental stage and subsequently resulted in the breakdown of the entire chloroplast. Molecular cloning illustrated that AL2 encodes a chloroplast group IIA intron splicing facilitator (CRS1) in rice, which was confirmed by a genetic complementation experiment. Moreover, our results demonstrated that AL2 was constitutively expressed in various tissues, including green and non-green tissues. Interestingly, we found that the expression levels of a subset of chloroplast genes that contain group IIA and IIB introns were significantly reduced in the al2 mutant compared to that in the wild type, suggesting that AL2 is a functional CRS1 in rice. Differing from the orthologous CRS1 in maize and Arabidopsis that only regulates splicing of the chloroplast group II intron, our results demonstrated that the AL2 gene is also likely to be involved in the splicing of the chloroplast group I intron. They also showed that disruption of AL2 results in the altered expression of chloroplast-associated genes, including chlorophyll biosynthetic genes, plastid-encoded polymerases and nuclear-encoded chloroplast genes. Taken together, these findings shed new light on the function of nuclear-encoded chloroplast group I and II intron splicing factors in rice.

Key words: Albino leaf, chloroplast development, CRS1, group I intron, group II intron, rice, RNA splicing.

Introduction

Chloroplasts are essential for plant development and growth, through manipulating the fixation of CO₂ and biosynthesis of carbon skeletons and other physiological processes (Sakamoto et al., 2008; Jarvis and Lopez-Juez, 2013). Accumulating evidence has shown the importance of chloroplast biogenesis and development during germination for plant vitality, seed set and growth (Lopez-Juez and Pyke, 2005; Pogson and Albrecht, 2011). Chloroplast biogenesis
is initiated from proplastids through endosymbiosis from an ancestor of extant cyanobacteria and is dependent on the coordinated expression of genes encoded in both nuclear and plastid genomes (Lopez-Juez and Pyke, 2005; Sakamoto et al., 2008; Kessler and Schnell, 2009). The development of chloroplasts differs between organs and species depending on the specialization of tissues and stage of development. For example, distinct phenotypes between cotyledons and true leaves were observed in variegated (var) and snowy cotyledon (sco) mutants in Arabidopsis, respectively (Albrecht et al., 2006, 2008, 2010; Liu et al., 2010), i.e. chlorotic true leaves but green cotyledons in the var and chlorotic or bleached cotyledons but green true leaves in the sco mutants. It is evident that nucleus-encoded polymerases (NEPs) and plastid-encoded polymerases (PEPs) involved in gene transcription, RNA maturation, protein translation and modification have great effect on the biogenesis of chloroplasts (Hedtke et al., 1997; Pogson and Albrecht, 2011; Yu et al., 2014). Recent studies indicated that a large group of nuclear-encoded pentatricopeptide repeats proteins (PPRs) involved in RNA processing, splicing, editing, stability, maturation and translation are critical for chloroplast development (Pogson and Albrecht, 2011). Besides PPRs, a group of chloroplast RNA splicing and ribosome maturation (CRM) domain proteins were also shown to be required for chloroplast development, which regulate the splicing of certain introns in chloroplasts and/or mitochondria (de Longevialle et al., 2010). In general, primary RNA transcripts in chloroplasts are spliced by a group of ribozymes via the same chemical steps as spliceosome-mediated splicing in the nucleus (de Longevialle et al., 2010; Borner et al., 2015). Based on the conserved structures and different splicing mechanisms, the introns of certain chloroplast and mitochondrial genes are classified into two main families in plants, group I and group II. Twenty group II introns and only one group I intron have been identified in the chloroplast genome of Arabidopsis, whereas 17 group II introns and one group I intron have been described in maize and rice (de Longevialle et al., 2010; Bonen and Vogel, 2001).

Splicing of group I introns is mediated through a two-step phosphoryl transfer reaction (Kruger et al., 1982). So far, only the intron of trnL has been characterized as a group I intron in chloroplasts and its splicing requires the function of matK, ZmRNC1, ZmWTF1 and AtCFM2 (Asakura and Barkan, 2007; Watkins et al., 2007; Asakura et al., 2008; Kroeger et al., 2009; Zoschke et al., 2010). Group II introns are divided into four subgroups: IIA, IIB, IIC and IID (Michel et al., 1989; Toor et al., 2001). Many group II intron splicing factors have been identified and characterized, including AtmMat2 (Keren et al., 2009), ORGANELLAR TRANSCRIPT processing 43 (OTP43) (de Longevialle et al., 2007; Keren et al., 2012), mCSF1 (Zmudjak et al., 2013), RUG3 (Kuhn et al., 2011) and ABA overly-sensitive 5 (ABOS) (Liu et al., 2010) in mitochondria, and chloroplast RNA splicing 1 (CRS1) (Ostersetzer et al., 2005; Till et al., 2001), ZmRNC1 (Watkins et al., 2007), AtCFM2 (Asakura and Barkan, 2007) and Zm-mTERF4 (Hammani and Barkan, 2014) in chloroplasts. Among these proteins, CRS1 is the first defined CRM protein from maize, which contains three CRM domains (Jenkins et al., 1997; Till et al., 2001). Sixteen and fourteen proteins containing one or more CRM domains are identified in Arabidopsis and rice, respectively. Besides CRS1, four more CRM proteins have been characterized as chloroplast splicing factors: CRM Family Member 2 (CFM2), CFM3, CRS2-associated factors I (CAF1) and CAF2 (Barkan et al., 2007; de Longevialle et al., 2010). CRS1 only functions in the splicing of the atpF intron (group IIA), whereas the function of maize CFM2 is associated with one group I intron (trnL intron) and two group II introns (ndhA intron, ycf3 intron 1), and Arabidopsis CFM2 also promotes the splicing of the additional group II intron (clpP intron) (Asakura and Brakan, 2007; Asakura et al., 2008). Differing from other splicing factors in the CRM family, CFM3 is dual-targeted to chloroplasts and mitochondria. In chloroplasts, the function of CFM3 is associated with a subset of group II introns (ndhB, rpl16, rps16, trnG, petB and petD introns) and regulates their splicing in rice (de Longevialle et al., 2010). CSR2 binds to CAF1 and CAF2 to form the complexes CRS2-CAF1 and CRS2-CAF2, respectively, which are required for the splicing of a subset of group IIB introns, including the introns of ndhA, ndt1B, petB, petD, rpl16, rps16, trnG and ycf3 in maize and rpoCl1 and ClpP in Arabidopsis (Ostheimer et al., 2003; Asakura and Barkan, 2006; de Longevialle et al., 2010). To the best of our knowledge, most of the group II intron splicing factors were identified from Arabidopsis or maize, whereas these factors, particularly the CRM proteins in rice, are either not identified or their functions are not fully characterized.

We describe the function of a nuclear-encoded splicing factor termed Albino Leaf 2 (AL2) that encodes a chloroplast group IIA intron splicing factor CRS1, which is required for chloroplast development in rice. Our results suggest that in contrast to the function of the orthologous CRS1 in maize and Arabidopsis, AL2 probably participates in the splicing of both chloroplast group I and II introns in rice. In addition, AL2 appears to coordinate the expression of a subset of chloroplast-associated genes to regulate chloroplast development in rice.

Materials and methods

Plant materials and growth conditions

The rice japonica cultivar Zhonghua 11 was used as the wild type. The T-DNA insertion library with Zhonghua 11 background was obtained from the Shanghai Institute of Plant Physiology and Ecology, Chinese Academy of Sciences. All rice seeds in this study were propagated in a paddy field in Guangzhou, China. For laboratory work, rice plants were grown in the greenhouse under a 16-h light/8-h dark cycle at 30 °C with the given light intensity (1000 mmol m⁻² s⁻¹). No significant differences were observed when albino leaf (al2) mutants were grown in the greenhouse versus the paddy field.

Electron microscopy

The samples prepared for electron microscopy were fixed overnight at 4 °C with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and then the samples were dehydrated in a graded ethanol series followed by substitution with a graded isoamyl acetate series. The samples were critical point dried, sputter coated with gold and observed using a HITACHI S-3 000N scanning electron microscope.
at 10 kV. For transmission electron microscopy analysis, samples were fixed overnight at 4 °C with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After washing with phosphate buffer three times, samples were fixed overnight in 2% (v/v) OsO4 in phosphate buffer. After staining and dehydration in a grade ethanol series, the samples were submerged with LR White resin and polymerized for 2 d. Ultrathin cross-sections were prepared with a Leica EM UC6 ultra-microtome and observed under a Tecnai Spirit (120 kV) transmission electron microscope.

Analysis of the T-DNA insertion locus in al2 mutant
Inverse polymerase chain reaction (IPCR) was used to isolate the flanking sequence of T-DNA (Ochman et al., 1988). Nested primers of the T-DNA right border primers were C1 and C2, and those of the left border primers were H1 and H2. The genomic DNA was digested by HindIII. Primers for testing of the T-DNA inserting locus were AL2-11310 and 5TF1 for the left site and AL2-11655 and 5TR1 for the right. Primers sequences are listed in Supplementary Table S1 at JXB online.

Plasmid construct and transformation
To produce the AL2 complementation transgenic plants, a 6.2 kb DNA fragment of AL2 was amplified from wild type using their corresponding primer pairs listed in Supplementary Table S1. The amplified DNA fragment was confirmed by DNA sequencing and cloned into the binary vector pCAMBIA1301 (Promega, Madison, WI, USA) and then transformed into al2 heterozygotes. To generate the interfering AL2 construct, a 549-bp fragment from the specific coding region of AL2 was amplified from wild-type complementary DNA (cDNA) templates using their corresponding primer pairs listed in Supplementary Table S1. The DNA fragment was confirmed by sequencing and cloned into the binary vector pCAMBIA1301-35S. The final constructs were electroporated into Agrobacterium tumefaciens strain EHA105 for rice transformations that were conducted as previously described (Toki et al., 2006).

Histological β-glucoronidase (GUS) assay
GUS activity analysis was performed following a standard protocol (Jefferson et al., 1987). Transgenic plant tissues were incubated in X-gluc buffer [0.1 mol L−1 K3HPO4 (pH 7.0), 0.1 mol L−1 KH2PO4 (pH 7.0), 5 mmol L−1 K3Fe(CN)6, 5 mmol L−1 K4Fe(CN)6•3H2O, 0.1% Triton X-100, 20% methanol, 1 mg ml−1 X-Gluc] at 37 °C for 2 h and then cleared by ethanol. Stained samples were photographed using a Cannon digital camera and stereoscope (OLYMPUS SZX12) and further sliced using resin sections (Leica HistoResin) and analysed under a light microscope (OLYMPUS BX51).

RNA extraction and quantitative real-time (qRT)-PCR assay
Total RNA was extracted using the RNeasy Plant Mini kit (Qiagen) according to the manufacturer’s instructions. The first strand of cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) and qRT-PCR was performed as previously described (Chen et al., 2015). The relative expression level of the target gene was normalized to that of rice gene actin1. All primers used in qRT-PCR are listed in Supplementary Table S1.

Chlorophyll detection
Total chlorophyll was determined in triplicate according to the method described previously (Hartmut, 1983). Extracts were obtained from the sixth leaves or seedlings at different growth stages. Approximately 0.2 g of fresh tissue was homogenized in 5 ml of 80% acetone for 12 h in darkness. Spectrophotometric quantification was carried out in a Gene Quant spectrophotometer (GE Healthcare) and followed with calculations: Chl a=12.21×A663−2.81×A646, and Chl b=20.13×A646−5.03×A663 (μg ml−1).

Results
Phenotype of al2 mutants
To identify novel genes or regulators involved in chloroplast development, a rice T-DNA insertion population (Zhonghua 11 background) was screened for mutants that exhibited defects in leaf colour, termed albino leaf mutants (als). Dozens of als were obtained through phenotypic investigation. Among these als, the al2 mutant has an interesting and distinct phenotype compared to the others. The al2 mutant showed no significant difference compared to the wild type at the germination stage (Fig. 1A, B), but it had an apparent albino phenotype in the young buds (Fig. 1C). During seedling growth, the albino phenotype became more obvious and spread around the entire leaf at the third-leaf stage in the al2 mutant (Fig. 1D), which eventually led to the seedling lethal al2 mutant. To quantify the changes in this albino phenotype, chlorophyll contents were measured in al2 mutant and wild type. Consistent with their phenotypes, the chlorophyll contents were significantly reduced in al2 compared with that of

Fig. 1. Characterization of al2. Phenotype of al1 during germination at (A) 1 d after germination (DAG), (B) 2 DAG, (C) 4 DAG and (D) 10 DAG. (E) Chlorophyll content of al2 at 4 and 10 DAG. In each panel, from left to right is wild type and al2, respectively. Bar: 1 cm (A–C); 2 cm (D).
the wild type (Fig. 1E). These data suggested that the albino phenotype of al2 mutants might be caused by defects in chlorophyll metabolism or the breakdown of entire chloroplasts.

**Chloroplasts are disrupted in al2 mutants**

To further investigate the albino leaf phenotype of al2, we observed the chloroplast structure of al2 mutants and wild type in leaves by electron microscopy. Because the albino phenotype of the al2 mutant was developed from the germination to the third-leaf stage, leaves of al2 and wild type from 3 d after germination (DAG), 5 DAG and 10 DAG were selected for microscopy analysis. Our results showed that the development of chloroplasts was significantly affected in al2 leaves at 3 DAG (Fig. 2A, D), and chloroplasts were severely disrupted in al2 at 5 DAG, particularly in the formation of thylakoids (Fig. 2B, E). The most apparent defective phenotype of chloroplasts in al2 was observed at 10 DAG compared to that in wild type (Fig. 2C, F). Taken together, our results indicated that the albino leaf phenotype of the al2 mutant resulted from the abnormal development of chloroplasts, suggesting that the AL2 gene is required for chloroplast development in rice.

**Molecular cloning of the AL2 gene**

We backcrossed the al2 mutant with wild type and did a segregation analysis in the F2 population based on the al2 mutant phenotype. The segregation ratio between al2 mutant and wild type was ~1:3 (χ²=0.120), indicating that al2 was controlled by a single recessive gene. We then performed inverse PCR and isolated the genomic DNA fragment flanking the T-DNA insertion region in the al2 mutant. Analysis of the resulting sequences via the BLAST function in the NCBI database revealed that T-DNA was inserted into the ninth exon of the Os09g19850 gene, which is comprised of nine exons and eight introns (Fig. 3A). Semi-quantitative PCR analysis indicated that the transcript level of Os09g19850 was completely eliminated in the al2 mutants (Fig. 3B). These data all suggested that Os09g19850 is a strong candidate for the AL2 gene. Gene annotation showed that Os09g19850 encodes a chloroplast group IIA intron splicing facilitator CRS1 in rice (http://rice.plantbiology.msu.edu) containing three CRS1-YhbY domains (also called the CRM domain) (Fig. 3C). Protein alignment analysis indicated that AL2/CRS1 proteins were highly conserved among various organisms. Moreover, phylogenetic analysis demonstrated that the AL2-like proteins in the plant kingdom were grouped into two clusters: monocot plants and dicot plants (Supplementary Fig. S1).

To verify the identity of AL2, a genetic complementation assay was performed. A 6225-bp wild-type genomic DNA fragment of Os09g19850, containing the putative promoter (2318 bp), the coding sequences (including the intron sequences) and a part of the 3′-untranslated region (246 bp), was cloned into a binary vector and transformed into al2 heterozygous plants due to the lethality of the al2

![Fig. 2.](image-url) Electron microscopy observation of al2 leaf. Chloroplasts in wild type mesophyll cells at the (A) 3 DAG, (C) 5 DAG and (E) 10 DAG, respectively. Chloroplast in al2 mutant mesophyll cells at (B) 3 DAG, (D) 5 DAG and (F) 10 DAG, respectively. Chloroplasts of the wild type have abundant and well-ordered thylakoid, whereas those of the al2 mutant have no similar structures. cp, chloroplast; DAG, day after germination; pg, plastoglobuli; st, stroma; th, thylakoid. Bar, 1 µm.
AL2 regulates introns splicing in rice

homozygous plants. Five independent transgenic lines within the al2 homozygote background were obtained, and two of them were used as representatives in the following studies. In these two transgenic lines, the albino leaf phenotype of al2 was fully rescued by the AL2 transgene (Fig. 3D, E), and the chlorophyll changes (Supplementary Fig. S2). Therefore, we concluded that Os09g19850 is the AL2 gene, and the disruption of its function results in the albino leaf phenotype.

Disruption of AL2 leads to an al2-like phenotype

The al2 mutants were seedling lethal, which caused difficulty in the further study of the AL2 functions. To address this issue, we produced weak al2 alleles by specific knockdown of AL2 gene expression. Finally, six independent knockdown lines (KD) were obtained with different expression levels of the AL2 gene (Supplementary Fig. S3). In accordance with their relevant expression levels of AL2, the KD plants exhibited different degrees of the colourless phenotype. Of these 11 KD lines, KD1, KD7 and KD10, with the most significant down-regulation of AL2, showed a highly similar phenotype to that of the al2 mutants (Fig. 4A–D). These results further confirmed that AL2 is required for chloroplast development.

Expression pattern of AL2

To explore the expression pattern of the AL2 gene, its promoter was fused with the GUS reporter and transformed into wild-type plants. Consistent with the appearance of the al2 phenotype, GUS staining revealed that the AL2 gene was expressed during germination (Fig. 5A–D). Because plastids are also present in the root and root hairs, GUS staining was also observed in the root and root hairs in addition to the chloroplasts (Fig. 5E). Subsequently, the AL2 gene was highly expressed in the mature leaf (Fig. 5F) and also detected in the panicle, spikelet, culm and leaf sheath (Fig. 5G–J). Histological analysis indicated that AL2 was specifically expressed in the endodermis of the culm (Fig. 5K). Additionally, a qRT-PCR analysis of the AL2 gene expression in various tissues revealed the similar expression pattern observed in the promoter analyses (Supplementary Fig. S4). Taken together, our results demonstrate that AL2 is constitutively expressed in various tissues and mainly functions in the green tissues.

AL2 is likely to be involved in the splicing of chloroplast group I and II introns

According to the gene annotation (http://rice.plantbiology.msu.edu), AL2 encodes a putative chloroplast group IIA intron splicing facilitator CRS1 in rice. It has been
implicated that CRS1 is required for the splicing of the group IIA atpF intron via direct interaction (Till et al., 2001). To validate the function of AL2, we detected the expression level of chloroplast group IIA atpF and rpl2. Our results indicated that the expression level of atpF and rpl2 was significantly reduced in al2 compared with that in wild type, suggesting that AL2 is a functional CRS1. We also analysed the expression levels of chloroplast-associated genes containing group IIB introns, including ndhA, ndhB, petD and ycf3, and found that the expression of these genes was also significantly reduced in al2 (Fig. 6), suggesting that AL2 may also be involved in the splicing of chloroplast group IIB introns. We further tested the expression level of trnL containing the chloroplast group I introns. Interestingly, trnL expression was also predominately eliminated in the al2 mutant (Fig. 6). Taking these results together, we propose that AL2 is likely to be involved in the splicing of both chloroplast group I and II introns.

Discussion

RNA splicing is a modification process of the nascent precursor messenger RNA transcript in which the introns are removed and exons are joined. In plants, certain chloroplast and mitochondrial genes, encoding either tRNAs or proteins, are interrupted by introns. Based on their conservation of structure and different splicing mechanisms, these introns are divided into group I and group II introns (de Longevialle et al., 2010). We characterized the function of the AL2 gene encoding CRS1 in rice. Our results suggested that AL2 is likely to be involved in the splicing of both chloroplast group I and II introns, and it coordinates a subset of chloroplast-associated genes to regulate the development of chloroplast in rice.

The maize CRS1 contains three CRM domains and is required for the splicing of group IIA atpF intron via direct interaction (Ochman et al., 1988; Till et al., 2001;
AL2 regulates introns splicing in rice (Ostheimer et al., 2003). AL2 also contains three typical CRM domains. Moreover, multiple alignments of AL2-like proteins among various species indicated that AL2 protein is highly conserved in evolution. The albino leaf phenotype is found not only in the rice al2 mutant but also in the Arabidopsis crs1 mutant (Asakura and Barkan, 2006), suggesting that AL2 may be a functional CRS1 in rice. Validation of the expression of atpF suggested that AL2 is involved in regulating the splicing of chloroplast group IIA introns. We thus propose that AL2 is a functional CRS1 in rice. Surprisingly, our results also showed that the expression of ndhA, ndhB, petD, ycf3 and trnL, was significantly reduced in al2, suggesting that AL2 probably participates in the splicing of group IIB and I introns. Therefore, we also propose that AL2 not only has an overlapping role as the Arabidopsis CRS1 but also functions distinctly. However, further studies are still required to understand the function of AL2 in the splicing of group I and IIB introns. In addition, two additional orthologous CRS1 are present in rice, including Os08g27150 and Os05g47850 (de Longevialle et al., 2010). Characterization of these two genes would extend our knowledge about the function of CRS1 in chloroplast development in rice.

Phylogenetic analysis revealed that AL2-like proteins in the plant kingdom were grouped into two clusters – monocots and dicots – implying that AL2 may also have distinct roles across these plant groups. However, studies of CRS1 in the two representative monocot and dicot plants, maize and Arabidopsis, have implicated both maize and Arabidopsis CRS1 in the specific function of splicing of the chloroplast group II intron (Ostheimer et al., 2003; Ostersetzer et al., 2005; Asakura and Barkan, 2006). Therefore, we propose that the role of AL2 in intron splicing in rice may be different from the above plants. This assumption would be an interesting issue to explore in the future. So far, numerous genes have been shown to be crucial for chloroplast biogenesis and development, and mutations of these genes cause chlorotic or bleached leaf phenotypes. Similar to al2, snow-white leaf1 (swl1) and albino lethal 1 (al1) also exhibit albino leaf and seedling lethality phenotype in rice (Hayashi-Tsugane et al., 2014; Zhao et al., 2016), whereas other mutants, such as sco and var, generate a leaf phenotype restricted to one leaf organ in Arabidopsis. Therefore, characterization of many more monocot mutants will facilitate the discovery of genes functioning in chloroplast biogenesis and/or development that have not yet been clarified in dicot research.

The most abundant transcript of AL2 is found in green tissues, such as the leaf and culm. However, AL2 is also expressed in non-green tissues, such as the root. This suggests a possible involvement of AL2 in the regulation of RNA processing in different kinds of plastids in addition to the chloroplast. However, this hypothesis requires further determination as it remains unclear whether chloroplast intron splicing factors participate in the regulation of other chloroplast-associated genes. In this study, we showed that AL2 is
also involved in the regulation of PEPs and nuclear-encoded chloroplast genes. By contrast, our results indicate that AL2 is not involved in the regulation of the NEPs, suggesting that AL2-mediated chloroplast development is independent of NEPs. Additionally, we found that a group of CBGs was significantly down-regulated in the al2 mutants, which may be due to the absence of apoproteins for chlorophyll rather than to the direct involvement of AL2 in regulating these CBGs.

Overall, our results showed that AL2 is likely to be involved in the splicing of both group I and II introns in rice, which is distinct from the function of orthologous CRS1 in maize and Arabidopsis. Moreover, our results also indicated that AL2 coordinates a subset of chloroplast-associated genes to regulate the development of chloroplast in rice.

**Supplementary data**

Supplementary data are available at *JXB* online.

- Figure S1. Phylogenetic tree of AL2-like proteins among multiple organisms.
- Figure S2. Chlorophyll contents of complementation lines.
- Figure S3. Expression pattern of the *AL2* gene in the *AL2* knock-down lines.
- Figure S4. Expression pattern of the *AL2* gene in various tissues.
- Table S1. Primers used in this study.

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