Plant Science 259 (2017) 1–11

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

The rice *TCD11* encoding plastid ribosomal protein S6 is essential for chloroplast development at low temperature

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ARTICLE INFO

Article history:
Received 21 September 2016
Received in revised form 18 February 2017
Accepted 20 February 2017
Available online 27 February 2017

Keywords:
Rice (*Oryza sativa* L.)
Plastid ribosomal protein (PRP)
Thermo-sensitive
Chloroplast development
Map-based cloning
Subcellular localization

ABSTRACT

Plastid ribosome proteins (PRPs) are important components for chloroplast biogenesis and early chloroplast development. Although it has been known that chloroplast ribosomes are similar to bacterial ones, the precise molecular function of ribosomal proteins remains to be elucidated in rice. Here, we identified a novel rice mutant, designated *tcd11* (*thermo-sensitive chlorophyll-deficient mutant 11*), characterized by the albino phenotype until it died at 20 °C, while displaying normal phenotype at 32 °C. The alteration of leaf color in *tcd11* mutants was aligned with chlorophyll (Chl) content and chloroplast development. The map-based cloning and molecular complementation showed that *TCD11* encodes the ribosomal small subunit protein S6 in chloroplasts (RPS6). *TCD11* was abundantly expressed in leaves, suggesting its different expressions in tissues. In addition, the disruption of *TCD11* greatly reduced the transcript levels of certain chloroplasts-associated genes and prevented the assembly of ribosome in chloroplasts at low temperature (20 °C), whereas they recovered to nearly normal levels at high temperature (32 °C). Thus, our data indicate that *TCD11* plays an important role in chloroplast development at low temperature. Upon our knowledge, the observations from this study provide a first glimpse into the importance of RPS6 function in rice chloroplast development.

1. Introduction

The chloroplast is a semi-autonomous organelle that possesses its own genome (plastome) and protein synthesis machinery. Since the plastome remains only about 120 genes, which mainly function in photosynthesis and gene expression, the majority of chloroplast proteins are imported from the cytosol through the TOC (translocon at the outer envelope membrane of chloroplast) and TIC (translocon at the inner envelope membrane of chloroplasts) complexes [1]. Thus, chloroplast development is regulated by coordinated expression of nuclear and plastid genes. It is well documented that plastid genes are transcribed by two types of RNA polymerase, namely the plastid-encoded RNA polymerase (PEP) and the nucleus-encoded RNA polymerase (NEP) [2–4]. At the beginning of chloroplast development, NEP is mainly responsible for transcription of house-keeping genes, such as genes encoding subunits of PEP, rRNA, and tRNA. Then, PEP becomes active to transcribe photosynthetic genes. NEP activity is directly feedback inhibited by the PEP product tRNA for glutamate that is used for both protein and chlorophyll biosynthesis during chloroplast development [5]. mRNAs in chloroplasts are translated by 70S ribosomes, which are similar to bacterial-type ribosomes composing of the 50S large and 30S small subunits [6]. The large subunit consists of three rRNAs (23S, 4.5S and 5S rRNAs) and 33 proteins, of which 8 are plastid-encoded while the rest are nuclear-encoded and imported from the cytosol [7]; the small subunit is made up of 16S rRNA and 24 proteins, of which 12 are plastid-encoded while 12 are imported from the cytosol [8–10]. Besides orthologs in *Escherichia coli*, plastid specific ribosomal proteins have been identified, three for the small ribosomal subunit while two for the large ribosomal subunit [11]. The essentiality and non-essentiality of ribosomal proteins are partially conserved in bacteria and chloroplasts [12]. It is worth to note that, some orthologues of non-essential bacterial ribosomal proteins are essential in plant chloroplasts, such as RPL1, RPL21,

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http://dx.doi.org/10.1016/j.plantsci.2017.02.007
0168-9452/

RPS13 and RPS20 [12]. However, any defect in essential or non-essential plastid ribosomal proteins (PRPs) leads to lethality or chlorophyll deficiency in plants [12–14] and translation in grasses is only essential for autotrophic (photoautotrophic) growth, but not for heterotrophic growth [15].

To date, a number of PRP mutants that are defective in chloroplast ribosomes have been reported in plants. Schultes et al. described the first plant PRP mutant (*hcf60*) in maize, where *hcf60* mutants exhibited an unsteady pale green seedling lethality, caused by the lack of the plastid small subunit protein 17 (RPS17) [16]. Thereafter, four of the plastid small subunits (RPS5, 9, 13 and 20) and ten of the large plastid subunits (RPL1, 4, 6, 10, 13, 19, 22, 28, 32 and 36) were proved to be essential for embryogenesis in *Arabidopsis* [12,14,17–20]. Also, *Arabidopsis* RPL28 was reported to be essential at the latest phase of embryo-seedling development during the seedling greening process [14]. In addition, *Arabidopsis* RPS1, 17, 24 and 33 were apparently not essential for basal ribosome activity and could complete their entire life cycle, but plastid protein synthesis and photosynthesis were impaired in these mutants [14,21]. Notably, two plastid-encoded ribosomal proteins (RPL33 and RPS15) in tobacco were required under cold stress conditions [22,23]. In rice, we previously reported the first PRP mutant (*asl1*), which exhibited an albino and seedling lethality phenotype. The *ASL1* gene encodes the plastid 30S ribosomal protein S20 (RPS20) [24]. Later, we identified the second rice PRP mutant (*asl2*) with the same seedling lethality and the *ASL2* gene encodes the 50S ribosome protein L21 [25]. Moreover, the 50S ribosomal protein L13 (RPL13) was proved to be essential for chloroplast development in rice at low temperature [26]. Recently, the disruption of the 50S ribosomal protein L12/AL1 (RPL12) in rice were demonstrated to seedling lethality [27]. However, though the importance of PRPs in higher plants, only a few of rice PRPs have been functionally analyzed [12,25,27].

In this study, we uncovered a new rice thermo-sensitive chlorophyll-deficient mutant *tcd11* which exhibits the albino phenotype until death below 20 °C, but can grow as the wild type plants at 32 °C. Map-based cloning revealed that *TCD11* encodes the plastid 30S ribosomal protein S6 (RPS6). Our findings suggest that rice nuclear-encoded chloroplast-localized RPS6 *TCD11* plays a vital role in chloroplast development at low temperature.

2. Materials and methods

2.1. Plant materials and growth conditions

The *thermo-sensitive chlorophyll-deficient* (*tcd11*) mutant used in this study was obtained from a ^{60}Co gamma ray-irradiated mutant pool of the genetic background, Jiahua 1, which was a leading *japonica* rice variety in Shanghai, China in 2006. Rice plants were grown at the experimental station of the Shanghai Normal University (summer-autumn season, temperate climate), China, using conventional methods. The F_2 genetic mapping population was generated from a cross between Pei'ai 64S (*indica*) and the *tcd11* mutant. For analysis of phenotype and pigment, DNA and RNA extraction, wild type (WT) and *tcd11* seedlings were grown in growth chambers (GXZ, Ningbo, China) under the condition of controlled 12 h of light and 12 h of dark with light intensity of 120 μmol of photons m^{-2} s^{-1} at a constant temperature of 20 °C or 32 °C.

2.2. Chlorophyll (Chl) and carotenoid (Car) content measurement

For Chl analysis, 200 mg of fresh leaves were sampled from the 3-leaf-stage seedlings grown at 20 °C and 32 °C, respectively, and incubated with 5 mL of extraction buffer (ethanol:acetone:water = 5:4:1) at 4 °C in the dark for 18 h. The pigment content was measured with a DU 800 UV/Vis spectrophotometer (Beckman Coulter, Danvers, MA, USA) at 663, 645, and 470 nm, respectively. Total Chl, Chl *a*, Chl *b* and Car contents were determined according to the methods of Arnon and Wellburn [28,29].

2.3. Transmission electron microscopy (TEM) analysis

For TEM analysis, tissues were sampled from the 3rd leaves of the 3-leaf-stage WT and *tcd11* seedlings grown at 20 °C and 32 °C, respectively, and fixed in 2.5% glutaraldehyde in a phosphate buffer (pH 7.4) at 4 °C first then in 1% OsO_4. After staining with uranyl acetate, tissues were further dehydrated in an ethanol series and finally embedded in the Spurr's medium prior to ultrathin sectioning. Samples were stained again and examined with a Hitachi-7650 transmission electron microscope (Hitachi, Tokyo, Japan).

2.4. Positional cloning of TCD11

Rice genomic DNA was extracted from young seedlings with the modified CTAB method [30]. Briefly, an initial set of 120 albino F_2 seedlings from Pei'ai 64S/*tcd11* randomly was selected for coarse linkage analysis, subsequently the fine mapping was carried out using 3560 homozygous mutant F_2 segregants. In this study, a total of 81 SSR primers were adopted based on Gramene web (http://www.gramene.org), and six InDel (insertion–deletion) markers were designed using Primer 5.0 on the basis of the genomic sequences of the *japonica* Nipponbare variety and the *indica* variety 9311 [31,32]; the primer sequences were listed in Supplementary Table S1. The candidate genes' function and full length cDNA were acquired using TIGR (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) and KOME (http://cdna01.dna.affrc.go.jp/cDNA/index.html), respectively.

2.5. Complementation of the TCD11 mutant

For complementation test, a 4400 bp the WT genomic DNA fragment containing the entire *TCD11* coding region, plus a 1255 bp upstream region and a 771 bp downstream sequence was amplified using the gene specific primers, 5′- CGGGGTACCTTTTCCTACTTACGACC-3′ and 5′-GCTCTAGATGATGTCACCCAAGAAC −3′. The underlined sequences were represented the cleavage site of *Kpn*I and *Xba*I. PCR products were first cloned into the pMD18-T vector (TaKaRa, Dalian, China). Then, the pMD18T-TCD11 plasmids were digested with *Kpn*I and *Xba*I and ligated into the *Kpn*I and *Xba*I site of a binary vector pCAMBIA1301 (CAMBIA, http://www.cambia.org.au). The pCAMBIA1301-TCD11 plasmids were transferred into *Agrobacterium* EHA105 and introduced into the *tcd11* mutant by *Agrobacterium tumefaciens*-mediated transformation [33]. The genotype of transgenic plants was determined using PCR amplification of the *hygromycin phosphotransferase* gene (*hpt*) with primers HPTF (5′-GGAGCATATACGCCCGGAGT-3′) and HPTR (5′-GTTTATCGGCACTTTGCATCG-3′) and GUS gene with primers GUSF (5′-GGGATCCATCGCAGCGTAATG-3′) and GUSR (5′-GCCGACAGCAGCAGTTTCATC-3′) as selection.

2.6. Subcellular localization of TCD11

To investigate the subcellular localization of TCD11, a cDNA fragment encoding the N-terminal region (amino acids 1–110) of *TCD11* was amplified from total RNA in WT plants using primer pair 5′-GAAGATCTATGCCGCTCCTCCTCCAC-3′ (*Bgl*II); 5′-GGGGTACCCCACCAACGTCTGCAACCAC-3′ (*Kpn*I) and introduced into vector pMON530-GFP (the underlined sequences represent cleavage sites of *Bgl*II and *Kpn*I, respectively). The resultant