Photosynth Res (2015) 126:323–330
DOI 10.1007/s11120-015-0159-0

REGULAR PAPER

Effects of inefficient transcription termination of *rbcL* on the expression of *accD* in plastids of *Arabidopsis thaliana*

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Received: 19 January 2015 / Accepted: 16 May 2015 / Published online: 24 May 2015

Abstract The plastid *accD* gene encodes one subunit of a multimeric acetyl-CoA carboxylase that is required for fatty acid biosynthesis. In *Arabidopsis thaliana,* the *accD* gene is transcribed by the nuclear-encoded phage-type RNA polymerase, and the accumulation of *accD* transcripts is subjected to a dynamic pattern during chloroplast development. However, the mechanisms underlying the regulation of accD expression remain unknown. Here, we showed that the inefficient transcription termination of *rbcL* due to the absence of RHON1 impaired the developmental profile of *accD*, resulting in the constitutive expression of *accD* during chloroplast development. Moreover, the accumulation of *accD* transcripts accordingly resulted in an increase in accD protein levels, suggesting that transcript abundance is critical for *accD* gene production. Our study demonstrates that the interplay between *accD* and upstream *rbcL* regulates the expression of *accD* and highlights the significance of transcriptional regulation in plastid gene expression in higher plants.

Keywords Plastid · accD · Transcriptional regulation · Plastid transcription termination

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Introduction

Plastids are derived from a cyanobacterial ancestor and combine prokaryotic and eukaryotic features of gene expression (Barkan 2011). In vascular plant plastids, transcription is performed by a nuclear-encoded phage-type RNA polymerase (NEP) in addition to the cyanobacterium-derived plastid-encoded RNA polymerase (PEP) (Barkan 2011; Liere et al. 2011). Expression of most plastid genes involves post-transcriptional processing events, such as splicing, editing, and intercistronic processing (Stern et al. 2010). Translation has also been highlighted as an important step in plastid gene expression (Beligni et al. 2004). Several studies have suggested that plastid translation may involve mechanisms that are distinct from those in bacteria, and a number of nucleus-encoded proteins required for the translation of specific plastid mRNAs have been identified (Stampacchia et al. 1997; Schult et al. 2007; Prikryl et al. 2011).

Currently, the relative contributions of transcriptional and translational regulation to the control of plastid gene expression remain unclear. Translation initiation has been shown to be a rate limiting step in the expression of many chloroplast genes of *Chlamydomonas* (*Chlamydomonas reinhardtii*), and translational regulation largely overrides fluctuations in mRNA levels in *Chlamydomonas* (Eberhard et al. 2002). Additionally, some studies have suggested a significant role for transcriptional regulation in the chloroplasts of higher plants (Pfannschmidt et al. 1999; Tullberg et al. 2000). However, most of these studies focused on the plastid genes involved in photosynthesis. In contrast, most of the housekeeping genes are transcribed by NEP, and the relevance of transcription rates and transcript abundances to protein levels remains to be determined.

The plastid gene *accD* is a typical NEP-dependent gene. The *accD* gene encodes the β-carboxyl transferase subunit

of acetyl-CoA carboxylase (ACCase, EC 6.4.1.2), which catalyzes the formation of malonyl-CoA from acetyl-CoA. This housekeeping gene exists in the plastid genome of most eudicotyledons. Nevertheless, species belonging to the *Acoraceae* (Goremykin et al. 2005), *Campanulaceae* (Haberle et al. 2008), *Fabaceae* (Magee et al. 2010), *Geraniaceae* (Guisinger et al. 2008), and *Poaceae* (Konishi and Sasaki 1994; Martin et al. 1998) were shown to have lost plastid *accD* from their plastomes. Among them, the *Fabaceae* and *Campanulaceae* families have functionally transferred the chloroplastic *accD* gene to the nucleus (Magee et al. 2010; Rousseau-Gueutin et al. 2013). Targeted gene disruption in tobacco demonstrated the essential function of accD in plastid biogenesis and leaf development (Kode et al. 2005).

In the plastid genome of *Arabidopsis*, *accD* forms an operon with *psaI*, *ycf4*, *cemA*, and *petA* (Walter et al. 2010; Stoppel et al. 2012). These genes are polycistronically transcribed under the control of the *accD* NEP-type promoter. Two NEP promoters for *accD* have been reported in *Arabidopsis* (Swiatecka-Hagenbruch et al. 2007). The accumulation of *accD* transcripts results in a development-dependent change during chloroplast development. However, the contribution of *accD* transcription to the developmental regulation of *accD* remains to be determined. Furthermore, whether the change in *accD* transcript levels can result in a corresponding change in the protein level is unknown.

In our previous study, we found that the expression of *accD* mRNA was under the control of interplay with its upstream gene *rbcL* (encoding the large subunit of the ribulose bisphosphate carboxylase) (Chi et al. 2014). Inactivation of *RHON1* leads to enhanced *rbcL* read-through transcription and aberrant *accD* transcriptional initiation, which may result from inefficient transcriptional termination of *rbcL* (Chi et al. 2014). In this study, we found that the interplay between *rbcL* and *accD* also contributed to the developmental profile of *accD* and that the absence of RHON1 led to the constitutive expression of *accD* during chloroplast development. Additionally, the accumulation of *accD* transcripts accordingly resulted in an increase in accD protein levels, which has only rarely been observed except the expression of foreign genes in transplastomic plants. Our study may provide better understanding of the contribution of plastid transcription to plastid gene expression in higher plants.

Materials and methods

Plant materials and growth conditions

The *rhon1-2* mutant was obtained from a collection of pSKI015 T-DNA-mutagenized *Arabidopsis thaliana* (ecotype Columbia) lines. The seeds of the *rhon1-2* mutant and wild-type were incubated in darkness for 48 h at 4 °C to ensure synchronized germination. After surface sterilization, the seeds were sown on Murashige and Skoog medium containing 2 % sucrose and grown under a photoperiod of 12 h of light and 12 h of darkness at 22 °C with a photon flux density of 50 μmol m^{-2} s^{-1}.

Quantitative RT-PCR

After illumination for 2, 3, 4, 5, and 6 days, the *rhon1-2* mutant and wild-type seedlings were harvested and the total plant RNA was extracted using the RNeasy Plant Mini kit (Qiagen). The RNA was used to generate cDNA using the Superscript III cDNA synthesis system (Invitrogen) following manufacturer's instructions. Quantitative RT-PCR was performed using specific primers for *accD* and *petB*. The sequences of primers were as follows: *accD* sense, 5′-AGGATTGACTGACGCTGTTC-3′; *accD* antisense, 5′-TACTACGGATCCCATACTACCC-3′; *petB* sense, 5′-CGGCAAGTATGATGGTCCTAAT-3′; and *petB* antisense, 5′-AACCACACCAGTAACCCAAG-3′. The amplification of *ELONGATION FACTOR1-α* was used as an internal control for normalization. PCRs were performed with an ABI 7900 sequence detection system (Applied Biosystems) according to the manufacturer's protocol.

Protein isolation and immunoblot analysis

Total protein was isolated from *Arabidopsis* leaves as previously described (Chi et al. 2010). Briefly, 0.05 g of *Arabidopsis* leaf tissue from 2-week-old plants was homogenized in 200 μl E buffer (125 mM Tris-HCl, pH 8.8, 1 % [w/v] SDS, 10 % glycerol and 50 mM $Na_2S_2O_5$), then centrifuged at 12,000×*g* for 10 min; the supernatant was used for the immunoblot analysis. The protein levels were quantified using the Dc protein assay kit (Bio-Rad). The proteins resolved by SDS-PAGE were blotted onto nitrocellulose membranes and incubated with specific antibodies. The signals were detected using the enhanced chemiluminescence method.

Antiserum production

An antibody to accD produced based on the full-length recombinant accD protein was ordered from Uniplastomic. All of the other antibodies were prepared by our lab. For the production of polyclonal antibodies against D1, CP47, LHCII, LHCI, CF1β, RbcL, and PetB, the corresponding nucleotide sequences encoding the soluble part of each protein were amplified from the cDNA. The resulting DNA fragments were fused in-frame with the N-terminal His

affinity tag of pET28a. BL21 cells were harvested after the addition of 0.6 mM Isopropyl β-D-Thiogalactoside for 5 h. The fusion proteins were purified on a nickel-nitrilotriacetic acid agarose resin matrix, and antibodies were raised in a rabbit against the purified antigen. The dilution ratios for all antibodies used in the immunoblot analyses were 1:2000.

Detection of biotinylated proteins

Biotinylated polypeptides were detected according to the method of Konishi et al. (1996) with minor modification. Total proteins (approximately 10 μg) from wild-type and *rhon1-2* mutant were separated by SDS-PAGE (5–15 % polyacrylamide gradient gel) and blotted onto nitrocellulose membranes. The nitrocellulose paper was then soaked for 1 h in a solution of 0.3 % (w/v) BSA in 20 mm Tris-HCl (pH 7.5), 150 mM NaCl and incubated for another hour with horseradish peroxidase-labeled streptavidin in solution in the same buffer (0.125 μg/mL). After several washes with the Tris-NaCl buffer containing 0.05 % (v/v) Nonidet P-40, biotinylated polypeptides were detected photochemically with enhanced chemiluminescence reagents.

Polysome analysis

Polysomes were isolated according to the method of Barkan (1993). Briefly, 1 mL of polysome extraction buffer (0.2 M Tris-HCl, pH 9, 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M sucrose, 1 % Triton X-100, 2 % polyoxyethylene-10-tridecyl ether, 0.5 mg mL^{-1} heparin, 100 mM β-mercaptoethanol, 100 mg mL^{-1} chloramphenicol, and 25 mg mL^{-1} cycloheximide) was used to homogenize 1 g of leaf tissues from 2-week-old plants. After incubation for 10 min on ice, the nuclei and insoluble material were removed by centrifugation at 12,000 rpm for 5 min at 4 °C. After the centrifugation, the supernatant was collected in a new tube. Sodium deoxycholate was added to the supernatant to a concentration of 0.5 %, and the mixture was placed on ice for 5 min. The remaining insoluble materials were removed by centrifuging at 12,000 rpm for 15 min at 4 °C. The supernatant was placed on a sucrose gradient ranging from 15 to 55 % and centrifuged using a SW55Ti rotor (Beckman) at 45,000 rpm for 65 min at 4 °C. After centrifugation, the samples were separated into 12 fractions and the RNA was isolated from each fraction. Then, the RNA samples were transferred to nylon membranes for northern blot analysis.

Fatty acid analysis

The tissues of wild-type and *rhon1-2* were separately ground to a fine powder under liquid nitrogen, and then every homogenized sample was divided into two parts. One aliquot was used to determine the dry matter content, while the second sample was use to analyze the fatty acid content. Lipids were extracted from the samples by the method of Bligh and Dyer (1959), and TAG was separated from the total lipids by thin-layer chromatography (TLC). The fatty acids were analyzed by following the method of Xu et al. (2003). Briefly, the samples were transesterified with 5 % H_2SO_4 in MeOH at 90 °C for 1 h, and the fatty acid methyl esters (FAMEs) were extracted with hexane. Then, the samples were loaded on a Hewlett-Packard 6890 gas chromatography apparatus supplied with a hydrogen flame ionization detector and a capillary column (HP INNOWAX; 30 m; 0.25 mm i.d.) with an N_2 carrier at a flow rate of 20 mL min^{-1}. The temperature of the oven was maintained at 170 °C for 3 min, and then increased to 210 °C by raising the temperature 5 °C every min. The FAMEs from the TAG were identified by comparing their retention times with known standards (37-component FAME mix, Supelco 47885-U). Heptadecanoic acid (17:0; Sigma) was used as the internal standard to quantify the amount of TAG.

Results

accD constitutively accumulated in *rhon1-2* during the different stages of chloroplast development

The expression of plastid genes generally varies in response to developmental signals depending on the RNA polymerase and promoter usage (Mullet 1993; Zoschke et al. 2007). We investigated the accumulation of plastid transcripts of *accD* during different stages of chloroplast development in wild-type and *rhon1-2* mutant plants. The *accD* transcripts reached the highest level in the wild-type plants after 4 days of illumination and then dropped back down (Fig. 1). In the *rhon1-2* mutant plants, the *accD* transcripts reached their peak at the same time, but remained markedly increased after 6 days of illumination. In contrast, the expression profiles of the control gene *petB* were unaffected in the *rhon1-2* mutant (Fig. 1). Our results clearly demonstrate that *accD* is constitutively accumulated in the *rhon1-2* mutant, suggesting that RHON1 is necessary for the developmental regulation of *accD* transcript accumulation during chloroplast development. The *accD* levels of *rhon1-2* relative to the wild-type plant are somewhat less than our previous result (Chi et al. 2014). This inconsistency might result from the fact that the *Arabidopsis* seedlings used for this study differed from those in our previous study on developmental stages.

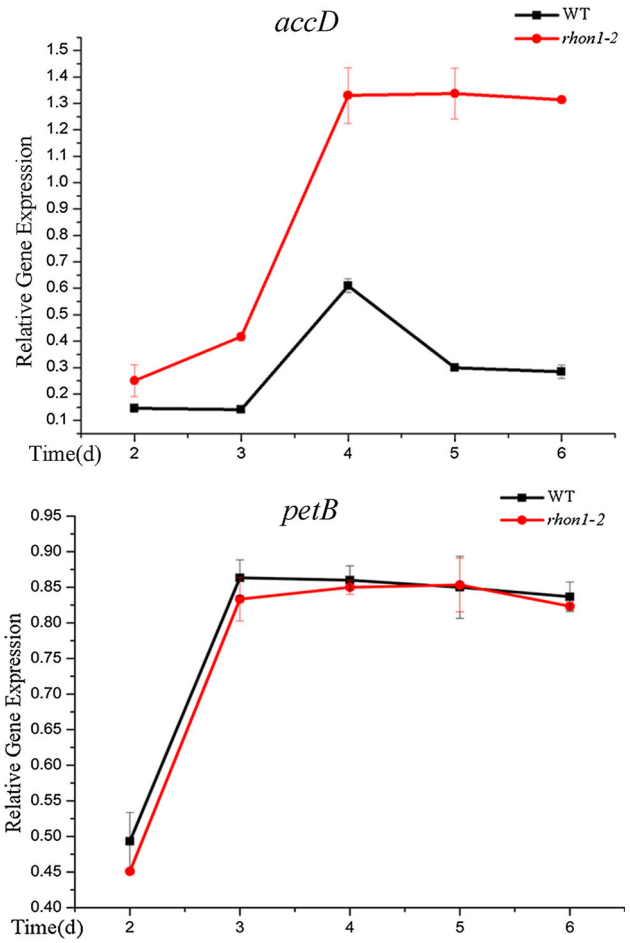

Fig. 1 Accumulation of plastid transcripts during chloroplast development in wild-type and *rhon1-2* plants. Transcript levels of plastid genes in *Arabidopsis* seedlings illuminated for 2, 3, 4, 5, and 6 days after being kept in darkness for 48 h at 4 °C were assessed by quantitative RT-PCR

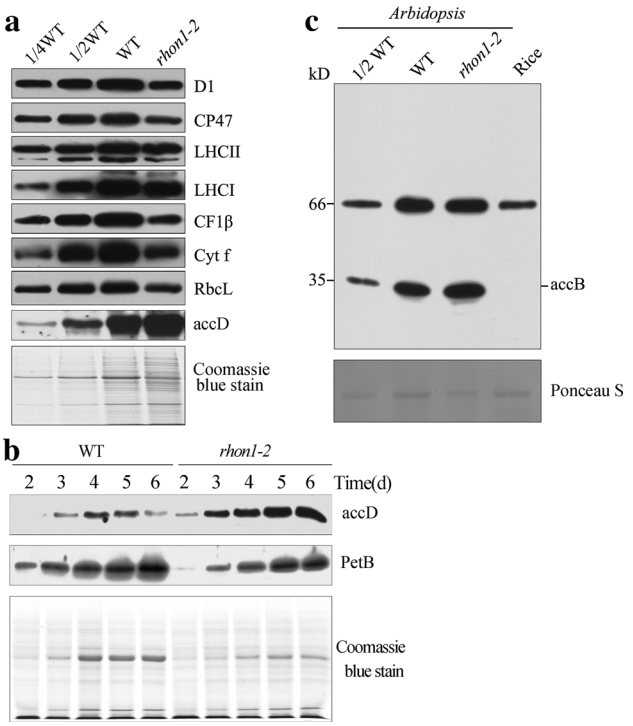

Fig. 2 The protein accumulation in *rhon1-2* plants. **a** Immunoblot analysis of chloroplast proteins in wild-type and *rhon1-2* mutants. Leaf extracts of 2-week-old leaves were separated by SDS-PAGE and immunodetected with specific antibodies directed against D1, CP47, LHC II, LHCI, CF1β, Cytf, rbcL, and accD. A replicate gel stained with Coomassie blue is shown below to provide an estimate of gel loading. **b** Accumulation of the accD and petB proteins during chloroplast development in wild-type and *rhon1-2* mutant plants. The treatment of *Arabidopsis* seedlings is described in Fig. 1. **c** The accumulation accB in *rhon1-2* and wild-type plants. The expression of accB was detected with a streptavidin probe according to the method of Konishi et al. (1996). The rice (*Oryza Sativa*) extract which lacks the biotinylated polypeptides of 35 kDa (Konishi et al. 1996) was used as a control

Overaccumulation of the accD protein in *rhon1-2*

To test whether the constitutive accumulation of *accD* transcripts in *rhon1-2* can result in accumulation of the accD protein, we extracted total proteins from wild-type and *rhon1-2* leaves and performed an immunoblot analysis. Our results showed that the accD protein level in *rhon1-2* was increased approximately 50 % compared with the wild-type plants (Fig. 2a). We also investigated the accumulation of several other chloroplast proteins. The levels of the photosystem II core subunits D1 and CP47 were reduced to approximately 30 and 25 % of the wild-type levels, respectively. The contents of the light harvesting complex I (LHCI) and II (LHCII) were reduced slightly in *rhon1-2*. The amounts of the core subunits of the ATP synthase and cytochrome *b6f* complex and RbcL were also decreased to approximately 25, 30, and 25 % of their wild-type levels, respectively.

We also examined the accumulation of the accD protein during chloroplast development (Fig. 2b). The accD protein of wild-type plants increased gradually and reached its highest expression level after 5 days of illumination before dropping back down on day 6. However, expression of the accD protein in the *rhon1-2* plants continued to increase during the illumination period and remained markedly increased after 6 days of illumination. The difference in accD developmental profiles between the wild-type and *rhon1-2* plants is in accordance with the differences in their *accD* mRNA levels.

It was found that the overexpression of accD could increase the three nuclear-encoded subunits of ACCase in tobacco (Madoka et al. 2002). In agreement with this finding in tobacco, we also found that the level of accB (one of three nuclear-encoded ACCase subunits) in *rhon1-2* was increased compared with that in wild-type plants (Fig. 2c).

Polysomal loading of *accD* mRNA in *rhon1-2*

Several studies have implicated translation as an important control point in chloroplast gene expression. Therefore, we investigated the *accD* translational capacity via a polysomal loading assay. This assay determined the coverage of mRNAs by translating ribosomes, and thus represented a measure of the translational activity (Barkan 1998; Kahlau and Bock 2008). The extracts from wild-type or *rhon1* seedlings were fractionated on 15 to 55 % sucrose density gradients and analyzed by RNA gel blotting using *accD* probes. The maximum distributions of the mature *accD* mRNA (1.6 kb) and one precursor *accD* mRNA (4.3 kb) were found in gradient fractions 4–7 in both the wild-type and *rhon1* plants. However, two precursor *accD* mRNAs were widely distributed in gradient fractions 5–11 in *rhon1-2* plants but not observed in the wild-type, which is agreement with the overaccumulation of these two precursor *accD* mRNAs in *rhon1-2* (Chi et al. 2014).

In addition to *rbcL* transcription termination, RHON1 participates in rRNA processing via interactions with the endonuclease RNase E (Stoppel et al. 2012). A plastid rRNA defect was clearly observed in the *rhon1-2* plants (Stoppel et al. 2012). Abnormal ribosome assembly or rRNA processing may affect the polysomal loading of plastid mRNAs (Barkan 1993; Beligni and Mayfield 2008); defects in polysomal loading are usually observed in mutants with impaired chloroplast ribosome functions (Fleischmann et al. 2011). Indeed, the polysomal loading of several plastid mRNAs, including *psbA* and *psbD,* was impaired in *rhon1-2*. For both transcripts investigated, the maximum mRNA distribution shifted toward the lighter fractions in the sucrose density gradient in the mutant (Fig. 3). These results suggested that distinct plastid mRNAs of *rhon1-2* behaved differently in association with ribosomes.

Fatty acid contents were increased in *rhon1-2* leaves

Based on the critical role of ACCase in fatty acid production in plants, we determined the fatty acid content in *rhon1* and wild-type plants by gas chromatography. As shown in Table 1, the contents of the six main fatty acids, including palmitic (16:0), sapienic (16:1), hexadecatrienoic (16:3), oleic (18:1), linoleic (18:2), and linolenic (18:3), in *rhon1-2* were higher than the wild-type. The total concentration of fatty acids was increased by approximately 40 % in the *rhon1-2* plants (Fig. 4). This result indicated that the fatty acid contents were significantly increased in the *rhon1-2* plants.

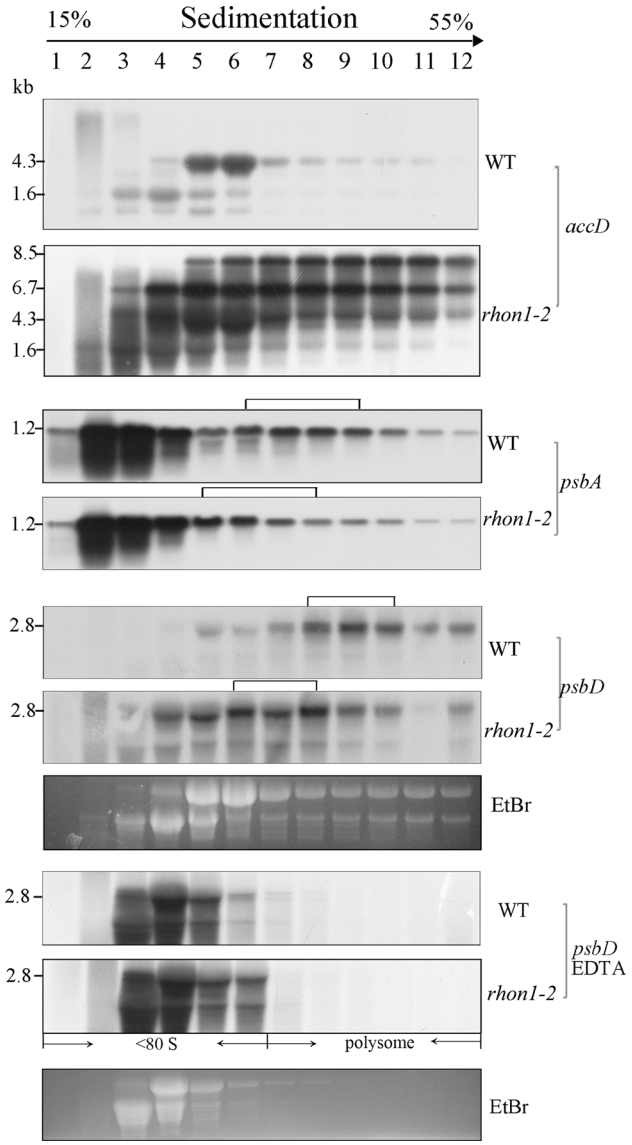

Fig. 3 Polysomal loading in the *rhon1-2* and wild-type plants. Twelve fractions of equal volume were collected from the *top to bottom* of 15–55 % sucrose gradients. Equal proportions of the RNA purified from each fraction were analyzed by gel-blot hybridization with the probes indicated at the right of each *panel*. The positions of the ribosome (<80S) and the polysomal fractions were determined by running EDTA-treated samples in parallel with the experimental samples, as indicated at the bottom. The maximums of the mRNA distributions were indicated with the *black lines* above each *panel*

Discussion

The dynamic properties of the transcriptional machinery play important roles in the regulation of chloroplast development. Changes in NEP-dependent transcription during chloroplast development have been reported (Demarsy et al. 2006; Mullet 1993; Zoschke et al. 2007), and distinct models related to the regulation of NEP activities have

Table 1 The fatty acids contents in leaves

Fatty acids composition	Wild-type (μg/mg)	*Rhon1-2* (μg/mg)
C16:0	2.32 ± 0.53	3.64 ± 0.63
C16:1	0.22 ± 0.05	0.43 ± 0.03
C16:3	1.10 ± 0.16	1.99 ± 0.34
C18:1	0.46 ± 0.14	0.71 ± 0.27
C18:2	2.47 ± 0.55	3.72 ± 0.47
C18:3	5.14 ± 1.08	8.38 ± 1.75

The data are mean ± SD of three independent experiments

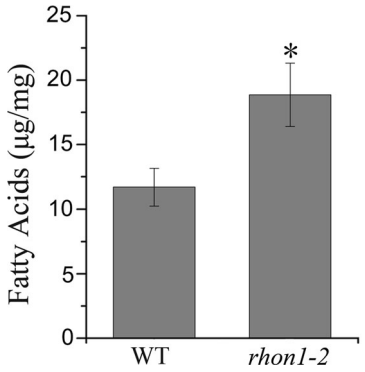

Fig. 4 Fatty acid contents in leaves of *rhon1-2* and wild-type plants. Values are based on dry weight and are provided as the mean ± SE ($n = 6$). An *asterisk* indicates that the value for *rhon1-2* is significantly different ($P < 0.05$) from the wild-type plants based on a *t* test

been proposed to explain the process (Hanaoka et al. 2005; Azevedo et al. 2008). In this study, we showed that the developmental profiles of *accD* mRNAs were affected in the *rhon1-2* mutant in which the transcription termination of the *accD* upstream gene was impaired. This finding suggested that the interplay between adjacent genes within plastid genomes also regulated the dynamic properties of plastid gene expression, which might provide alternative clues to understand the dynamic properties of the plastid transcriptional machinery. The absence of RHON1 resulted in the constitutive accumulation of accD, suggesting that RHON1 might act as a regulator of accD activity in vivo. We also noted that the expression of *RHON1* was dependent on the developmental conditions and was responsive to several perturbations, as indicated by the Genevisible Expression Data online (http://genevisible.com/perturbations/AT/AGI/AT1G06190). Therefore, it is likely that higher plants may recruit RHON1 to adjust accD levels in response to developmental cues and environmental signals.

The transcript numbers of chloroplast-encoded genes dropped significantly when transcription in *Chlamydomonas* was selectively inhibited by rifampicin, but the synthesis rates of the corresponding proteins did not decrease (Eberhard et al. 2002). This result indicated that transcript abundance did not limit the expression of chloroplast-encoded proteins in *Chlamydomonas.* Additionally, many plastid genes encode subunits of photosynthetic multiple-protein complexes that are condemned to rapid degradation if they are not properly incorporated into the complex (Goldschmidt-Clermont 1998; Wostrikoff et al. 2004). Taken together, there does not seem to be a close correlation between transcript abundance and protein accumulation levels in the chloroplast of *Chlamydomonas.* The inefficient transcription termination of *rbcL* in the *rhon1-2* mutant led to increased levels of *accD* mRNA that were accompanied by increased levels of the accD protein. However, the translation efficiency of *accD* did not appear to be increased based on the results of the polysomal loading assay (Fig. 3). These results indicated a close correlation between *accD* transcript abundance and protein levels in *Arabidopsis*. Therefore, it is likely that the point at which plastid gene expression is controlled varies somewhat between higher plants and *Chlamydomonas.* This difference may reflect the distinction between plastid transcription machineries in *Chlamydomonas* and higher plants because NEP exists in plastids of higher plants but not *Chlamydomonas* (Liere et al. 2011). In the chloroplast-to-chromoplast conversion of tomatoes, *accD* is the only plastid gene that displays a strong change in transcript abundance; this change is correlated with the high demand for lipid biosynthesis during fruit ripening to provide the storage matrix for the accumulating carotenoids (Kahlau and Bock 2008). The results of this study might also indicate the importance and significance of transcript accumulation for *accD* gene expression. Nevertheless, whether such a mechanism exists in other NEP-depended plastid genes remains unclear.

In agreement with our results obtained from *rhon1-2*, the replacement of the promoter of the *accD* operon in the tobacco plastid genome with a plastid rRNA-operon promoter that directly enhanced *accD* expression resulted in increased total ACCase levels in the plastids and increased fatty acid production in the leaves (Madoka et al. 2002). However, the mechanism underlying the increase in fatty acid production due to the overexpression of the accD protein remains an open question. It has been suggested that the level of the accD subunit is a determinant of ACCase levels and/or activity (Madoka et al. 2002; Sasaki and Nagano 2004). Indeed, we found that the level of one nuclear-encoded subunit of ACCase, accB was increased in *ronh1-2* (Fig. 2c). However, this perspective might require reevaluation. In *Arabidopsis,* a homomeric ACCase complex formed by an ACC2 protein has been found in addition to the heteromeric ACCase complex composed of accD and three nuclear-encoded subunits (Babiychuk et al. 2011; Parker et al. 2014). This homomeric complex has

been shown to possess the same function as the heteromeric complex in *Arabidopsis* mutants defective in plastid translation (Parker et al. 2014). Thus, accD expression may not be absolutely indispensable during embryo development in *Arabidopsis* as previously thought. Additionally, the mechanism by which a molar excess of the accD subunit promoted the formation of functional heteromeric ACCase complexes remains elusive. The regulation of fatty acid synthesis and accumulation in plant tissues is very complex (Guschina et al. 2014), and the relative contributions of homomeric and heteromeric ACCase complexes to fatty acid production might vary during the different developmental stages of *Arabidopsis*. The precise function of accD in fatty acid production of *Arabidopsis* awaits further investigation.

Acknowledgments We thank the Key Research Program of the Chinese Academy of Sciences and the National Natural Science Foundation of China (Grant 31370273) for financial support.

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