S23-015

Study on replication and morphology of chloroplasts in *Arabidopsis thaliana*

M Fujiwara, R Itoh, S Yoshida

Plant Functions Laboratory, RIKEN, Wako-shi, Saitama 351-0198, Japan. Fax: +81 (48) 4624674, E-mail: mfuji@postman.riken.go.jp

Keywords: chloroplast division, ftsZ, minE, GFP, CLSM

Introduction

Chloroplasts originated from an ancestral cyanobacterium and proliferate by binary fission. Higher plant mesophyll cells contain ten to several hundred chloroplasts, which differentiate and replicate from a few proplastids in meristem cells (Pyke et al. 1999). Ultrastructural studies have revealed that a double ring structure, called the PD ring, appears on the cytoplasmic and stromal sides of the envelope membranes of dividing chloroplasts in many plant species, indicative of a general apparatus for chloroplast division (Kuroiwa 1998). On the other hand, genetic analyses of 12 *accumulation and replication of chloroplasts* (*arc*) mutants of the higher plant *Arabidopsis thaliana* showed a hierarchy of these components in the chloroplast division process (Marrison et al. 1999; Pyke et al. 1999). However, no genes producing purified components of the PD ring or *arc* have been identified.

Our understanding of chloroplast division at the molecular level has developed with recent progress in plant genome analyses. FtsZ, which resembles eukaryotic tubulin and forms a cytokinetic ring at the midpoint of the bacterial cell, has been found in various plant species. Gene knockout or antisense expression of *FtsZ* homologs in the moss *Physcomitrella patens* and *Arabidopsis* produced decreased numbers of abnormally enlarged chloroplasts in cells, demonstrating that FtsZ proteins are required for chloroplast division and morphology (Osteryoung and Pyke 1998). FtsZ homologs in land plants are divided into two groups (FtsZ1, FtsZ2) based on their primary structure. The *Arabidopsis* nuclear genome contains at least one FtsZ1 (*AtFtsZ1-1*) and two FtsZ2 genes (*AtFtsZ2-1*, *AtFtsZ2-2*), and *in vitro* experiments showed that AtFtsZ1-1 is targeted into chloroplasts, while AtFtsZ2-1 is not. This generated an extended model for the distinct localization of FtsZ1 and FtsZ2 in chloroplast constriction (Osteryoung et al. 1998). Recently, transiently expressed FtsZ2-GFP fusions were observed to form a cytoskeleton-like network structure within chloroplasts in *Physcomitrella* (Kiessling et al. 2000). In contrast, AtFtsZ1-1 or AtFtsZ2-1 were detected immunologically forming a ring at the midpoint of chloroplasts in *Arabidopsis*, although it was uncertain whether these FtsZ proteins were inside or outside the chloroplasts (Vitha et al. 2001). Other than FtsZ, MinCDE proteins, which are required to determine the proper site of cell division, are known in eubacteria. An *Arabidopsis* homolog of MinD (AtMinD1) was found, and shown to regulate chloroplast division (Colletti et al. 2000; Kanamaru et al. 2000).

In this study, we characterized *Arabidopsis* chloroplast division factors. Green fluorescent protein (GFP), an effective marker protein, was used to monitor protein localization *in vivo*. GFP is also useful for analyzing organelle dynamics *in planta*. Notably, plastid-targeted GFP visualized tubular structures, called stromules (stroma filled tubules), extending from plastids; these transferred stroma GFP between interconnected plastids (Köhler et al. 1997). However, little is known of the molecular basis of tubular membrane extension, and there is inadequate description of the tissue- and plastid-dependent development of stromules. We also studied

GFP-labelled stromules in *Arabidopsis* tissues, and investigated the role of chloroplast division proteins in stromule formation.

Materials and methods

Plant and DNA materials –

Seeds of *A. thaliana* (Columbia background) and *Nicotiana tabacum* (Xanthi, BY4) were sown on Murashige-Skoog (MS) agar plates supplemented with 2% sucrose, as described previously (Kanamaru et al. 2000). *Arabidopsis* transformation was performed by an *Agrobacterium*-mediated floral dip method. Transformed (T_1) seeds were selected on MS plates containing kanamycin (50 mg L^{-1}) and cefotaxime (100 mg L^{-1}). cDNA sequences of *AtFtsZ1-1*, *AtFtsZ2-1*, *AtFtsZ2-2*, and *AtMinE1* were amplified by PCR from an *Arabidopsis* cDNA library (Clontech Laboratories, USA). The 5'-capping sites of *AtFtsZ2-1* mRNA were determined with Cap Site cDNA (Nippon Gene, Japan).

GFP experiments –

The cDNAs of *AtFtsZ1-1*, *AtFtsZ2-1*, *AtFtsZ2-2*, and *AtMinE1* corresponding to the N-terminal regions (90, 119, 109, and 97 amino acids (aa), respectively) or the *AtFtsZ1-1* and *AtFtsZ2-1* cDNAs corresponding to the entire coding regions (433 and 478 aa, respectively) were amplified by PCR and introduced into the *Sal*I-*Nco*I sites under the cauliflower mosaic virus 35S promoter (CaMV35S) of a CaMV35S-sGFP(S65T)-nos vector (a gift from Y Niwa). The resulting plasmids were bombarded into tobacco leaf cells using a Biolistic PDS-1000/He (Bio-Rad, USA). Leaves were incubated for 1-22 days before CLSM (TCS-NT; Leica Microsystems, Germany) (Kanamaru et al. 2000). Transgenic *Arabidopsis* plants expressing chloroplast-targeted GFP were observed by CLSM or epifluorescent microscopy (IX70; Olympus, Japan).

GUS staining –

A 1.6-kb genomic DNA upstream from the *AtMinE1*-coding region was ligated to the *uidA* gene of pBI101. The resulting plasmid was used to transform *Arabidopsis*. Intact seedlings or excised organs of the transformed plants were soaked in staining buffer containing 0.7 mM X-Gluc (Nacalai, Japan) for 24 h. These were cleared of chlorophyll with ethanol, and observed under a stereomicroscope (SZH10; Olympus).

Overexpression of AtMinE1 –

A full-length *AtMinE1* cDNA was ligated into the *Sma*I-*Sac*I site under CaMV35S of pBI121. The resulting plasmid was used to transform *Arabidopsis*. Leaf sections were processed from the T_1 plants. For fluorescent microscopy, samples were fixed in 4% paraformaldehyde, buffered with 20 mM Na-cacodylate at pH 7.2, dehydrated through an ethanol series, and embedded in Technovit 7100 resin (Kulzer and Co., Germany). Then, 0.6 μm-sections were cut on an ULTRACUT UCT ultramicrotome (Leica, Austria), stained with 100 mg L^{-1} $DiOC_6$ and 1 mg L^{-1} DAPI, and observed with IX70. For electron microscopy, samples were fixed in 4% glutaraldehyde, buffered with 20 mM Na-cacodylate at pH 7.0, post-fixed in 2% OsO_4, and embedded in Spurr's resin. Ultrathin sections were stained with 1% uranyl acetate and lead citrate solution, and observed with a JEM-2000 FX II electron microscope (JEOL, Japan).

Results

Our sequence analysis suggested that the previously predicted sequences of two *Arabidopsis* FtsZ2 both lacked N-terminal extensions. We determined the complete *AtFtsZ2-1* cDNA (no. AB052757) (Fig. 1) and the full ORF-containing *AtFtsZ2-2* cDNA sequences, and found that they encode N-terminally extended polypeptides of 478 aa (50.7 kDa) and 473 aa (50.3 kDa), respectively. The N-terminal regions of AtFtsZ2-1 and AtFtsZ2-2 deliver the fused GFP into chloroplasts. Transiently expressed GFP fusions with full-length AtFtsZ1-1 and AtFtsZ2-1 are also targeted into chloroplasts. GFP fluorescence was detected as lines or dots in mature chloroplasts; this is similar to the cytoskeleton-like FtsZ network found in *Physcomitrella* (Kiessling et al. 2000).

Moreover, in a database search, we identified a new *Arabidopsis* gene similar to eubacterial *minE*. Isolated cDNA of this gene (*AtMinE1*; no. AB046117) encoded a polypeptide of 229 aa containing a chloroplast-targeting signal revealed by a GFP experiment. We constructed transgenic *Arabidopsis* plants that express *uidA* (GUS gene) under a 5'-upstream region of the *AtMinE1* ORF or the full-length *AtMinE1* cDNA under CaMV35S. Green tissue-specific GUS activity was detected in the GUS plants. On the other hand, *AtMinE1* overexpression resulted in a reduced number of giant heteromorphic chloroplasts per cell.

We extended our study to the morphology of non-green plastids in *Arabidopsis*. A ptA5-3 transgenic plant (from Y Niwa) in which plastids were labelled with GFP was analyzed by CLSM. Stromules frequently emanated from root plastids or leucoplasts, which ranged from round to amoeboid, especially in the epidermal and cortex cells of mature roots. Leucoplasts had one or a few stromules, which connected with surrounding plastids, or extended towards the cytoplasm or cell nucleus. In contrast, non-green plastids in petals or trichomes were amorphous in shape, like root leucoplasts, and formed stromules infrequently, while mesophyll and epidermal chloroplasts in mature leaves rarely had stromules. Nevertheless, we and Vitha et al. (2001) found that transgenic *Arabidopsis* plants with elevated levels of AtFtsZ1-1-GFP extended highly developed stromule-like structures from leaf epidermal chloroplasts.

Discussion

This study showed that the prokaryotically derived FtsZ and MinE work inside chloroplasts, and that an FtsZ-GFP network is also seen in higher plants. It was recently reported that the chloroplast FtsZ network might be an artifact caused by the overproduction of FtsZ-GFP fusions (Vitha et al. 2001). However, we cannot exclude the possibility that the GFP fusions visualize pre-existing, thin, skeleton-like structures. The localization of AtMinE1 and AtMinD1 within chloroplasts is still unknown. Further analysis is required, and these data should provide a comprehensive view of the regulation of chloroplast replication and morphology in plants.

Acknowledgments

We thank Y Niwa (University of Shizuoka) for providing materials. MF and RI are supported by the Special Postdoctoral Researchers Program of RIKEN.

References

Colletti KS, Tattersall EA, Pyke KA, Froelich JE, Stokes KD, Osteryoung KW (2000) A homologue of the bacterial cell division site-determining factor MinD mediates placement of the chloroplast division apparatus. *Current Biology* **10**, 507-516.

Kanamaru K, Fujiwara M, Kim M, Nagashima A, Nakazato E, Tanaka K, Takahashi H (2000) Chloroplast targeting, distribution and transcriptional fluctuation of AtMinD1, a eubacteria-type factor critical for chloroplast division. *Plant and Cell Physiology* **41**, 1119-1128.

Kiessling J, Kruse S, Rensing SA, Harter K, Decker EL, Reski R (2000) Visualization of a cytoskeleton-like FtsZ network in chloroplasts. *Journal of Cell Biology* **151**, 945-950.

Köhler RH, Cao J, Zipfel WR, Webb WW, Hanson MR (1997) Exchange of protein molecules through connections between higher plant plastids. *Science* **276**, 2039-2042.

Kuroiwa T, Kuroiwa H, Sakai A, Takahashi H, Toda K, Itoh R (1998) The division apparatus of plastids and mitochondria. *International Review of Cytology* **181**, 1-41.

Marrison JL, Rutherford SM, Robertson EJ, Lister C, Dean C, Leech RM (1999) The distinctive roles of five different *ARC* genes in the chloroplast division process in *Arabidopsis*. *The Plant Journal* **18**, 651-662.

Osteryoung KW, Pyke KA (1998) Plastid division: evidence for a prokaryotically derived mechanism. *Current Opinion in Plant Biology* **1**, 475-479.

Osteryoung KW, Stokes KD, Rutherford SM, Percival AL, Lee WY (1998) Chloroplast division in higher plants requires members of two functionally divergent gene families with homology to bacterial *ftsZ*. *The Plant Cell* **10**, 1991-2004.

Vitha S, McAndrew RS, Osteryoung KW (2001) FtsZ ring formation at the chloroplast division site in plants. *Journal of Cell Biology* **153**, 111-120.