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Disruption of tobacco chloroplast *clpP* gene encoding a proteolytic subunit of ATP-dependent protease

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Introduction

ATP-dependent Clp protease is highly conserved among divergent organisms and presents in chloroplasts and mitochondria in higher plants (Schirmer *et al*. 1996, Suzuki *et al*. 1997). Clp complex consists of two heptameric proteolytic subunits, which are flanked at one or both sides by hexameric rings of regulatory subunits. Regulatory subunit genes consist of small gene family, which makes it easier for the Clp complex to become involved in divergent cellular functions. In higher plants, proteolytic subunits are encoded both by the chloroplast *clpP* gene (*clpP1*) and the nuclear gene family (Adam *et al*. 2001). Proteolytic ring is a complex mixture of divergent subunits (Peltier *et al*. 2001).

In microorganisms, Clp protease has two distinct physiological functions; (1) degradation of denatured or abnormal proteins, (2) specific proteolysis of regulatory proteins with short half-lives (Schirmer *et al*. 1996, Suzuki *et al*. 1997). To analyze the physiological function of Clp protease in chloroplasts, we disrupted tobacco *clpP1*.

Materials and methods

Chloroplast transformation. A 5.5-kb *Sal*I-*Sph*I fragment containing *clpP1* was isolated from *Nicotiana tabacum* cv. Xanthi and was cloned in pUC19. Vectors for the gene disruption and the control transformation were constructed as shown in Fig. 1. The vectors were introduced into tobacco chloroplasts using chloroplast transformation (Svab and Maliga 1998, Shikanai *et al*. 1998). Transformants were maintained on the MS medium containing 3% sucrose and 500 mg L^{-1} spectinomycin.

Fluorescence microscopy Transverse sections of a leaf were examined by fluorescence microscopy (BX50, Olympus) using a blue excitation beam (460-490 nm).

Results and discussion

The second and third exons of *clpP1* were replaced by the chimeric *aadA* gene conferring spectinomycin resistance in chloroplasts and were introduced into tobacco (Fig. 1a, *clpP1* disruption). To assess the possible secondary effects of the transformation, the *aadA* gene was also introduced at the downstream of *clpP1*, which did not disrupt any gene (Fig. 1a, vector control). Resulting four lines transformed with the disruption vector (ΔclpP lines) and two control lines were analyzed.

Southern analysis showed that the chloroplast genome of ΔclpP consisted of the mixture of the wild-type and transformed copies (Fig. 1b, lane 4). The regeneration process under the spectinomycin pressure did not function to alter the heteroplasmic genome composition (Fig. 1b,

lane 5). In contrast, vector control lines were homoplasmic even after the first screening (Fig. 1b, lanes 2 and 3). We conclude that *clpP* is essential for cellular survival and complete segregation of the wild-type copy is impossible, as was in Chlamydomonas (Huang *et al*. 1994).

Although the half of the wild-type genome was still remaining, ΔclpP exhibited the severe phenotype in the leaf morphology (Shikanai *et al*. 2001). The leaf surface was rough by clamping and the lateral leaf expansion was irregularly arrested leading to the slender leaf shape. In contrast, morphology of vector control lines was unaffected. In the absence of spectinomycin pressure, the transformed genome copy rapidly disappeared, which was accompanied by the restoration of the wild-type leaf morphology (Shikanai *et al*. 2001).

Theses results confirmed that the morphological phenotype in ΔclpP was directly related with the decrease in the *clpP1* copy number.

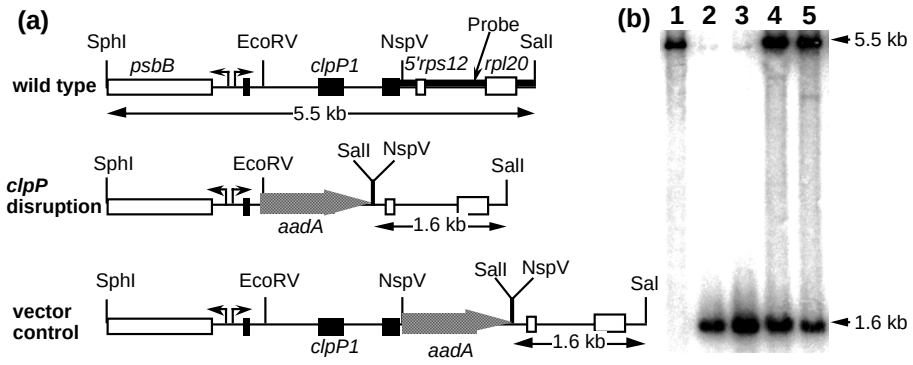
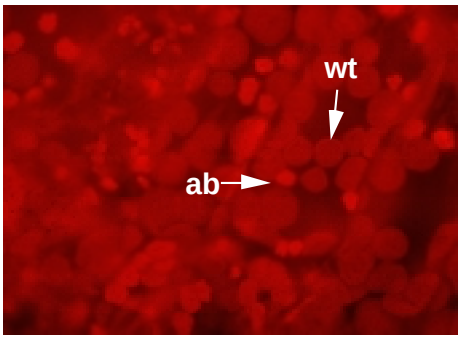


Fig. 1 Gene disruption of *clpP1* by chloroplast transformation (a) genome structures. (b) Southern analysis of the wild type (lane 1), vector control line (lanes 2 and 3), and *clpP1* disruption line (lanes 4 and 5). Total DNA was extracted from seedlings after the first (lanes 2 and 4) and second spectinomycin screenings (lanes 3 and 5).

Fig. 2 Fluorescence microscopy analysis. Transverse section of the ΔclpP leaf was examined by fluorescence microscopy. Chloroplasts consist of two populations; wild-type-like (wt) and abnormal (ab) chloroplasts.



To analyze the phenotype in the chloroplast level, chloroplasts were examined by fluorescence microscopy. In the wild type and vector control lines, size and chlorophyll fluorescence levels were rather uniform (Shikanai *et al*. 2001). In contrast, chloroplasts consisted of two populations, small chloroplasts emitting high fluorescence and wild-type-like chloroplasts (Fig. 2). These results suggest that incomplete segregation of the chloroplast genome induced the difference in the ClpP1 expression levels among chloroplasts, leading to heterogeneous chloroplast population. The high chlorophyll fluorescence emitted from small chloroplasts indicates that electron transport is severely affected in abnormal chloroplasts. It was confirmed by ultrastructure analysis of chloroplasts showing that thylakoid membranes exclusively consisted of tightly stacked membranes in the small chloroplasts (Shikanai *et al*. 2001). We consider that Clp protease is essential for thylakoid membrane biogenesis.

Chloroplast differentiation is not essential for plant cells, as far as sucrose is supplemented as a carbon source. We could not segregate clpP1 even under heterotrophic culture conditions, indicating that Clp protease functions also in housekeeping of various types of plastids, as well as in chloroplasts.

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