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Growth Retardation of Plants Transformed by Overexpression of NtFtsZ1-2 in Tobacco

Won Joong Jeong[1,2], Suk Won Jeong[1], Sung Ran Min[1], Ook Joon Yoo[2], and Jang Ryol Liu[1]*

[1]Plant Cell Biotechnology Laboratory, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon 305-333, Korea

[2]Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

We transformed tobacco plants (*Nicotiana tabacum* L., Xanthi) by introducing a sense construct of *NtFtsZ1-2*. This tobacco nuclear gene encodes a chloroplast-localized homologue of FtsZ, the bacterial cell-division protein. The overexpressing plants contained enlarged chloroplasts in their leaf mesophyll cells. In the T_1 progeny, we observed three different phenotypes: 1) plants with cells containing many small chloroplasts, which was the same as for wild-type plants; 2) plants in which the cells contained one to three enlarged chloroplasts (severe type); and 3) plants whose cells contained a combination of many small chloroplasts and one to three enlarged chloroplasts (intermediate type). The outward appearance of the severe and intermediate types of transgenic plants did not differ noticeably from the wild-types. However, the severe-type plants were most retarded in their growth under both high- and low-light conditions, followed by the intermediate-types. Under medium levels of light, the two types of transgenic plants exhibited growth rates comparable to that of the wild types. Based on the overall results, we suggest that many small chloroplasts, rather than a few large chloroplasts, are required for efficient use of light energy in the mesophyll cells.

Keywords: chloroplast division, chloroplast number and size, *FtsZ* overexpression, plant growth

Higher plants contain plastids, which are a family of intracellular organelles that include chloroplasts (for photosynthesis), amyloplasts (starch accumulation), and chromoplasts (pigment synthesis). Typical chloroplasts are discoid-shaped (3 to 10 μm in diameter), and are most often limited to the leaf mesophyll and stomatal guard cells. In *Arabidopsis*, approximately 100 chloroplasts exist per mesophyll cell, occupying 70% of its area (Pyke and Leech, 1992). Plastid division is necessary to ensure that continuity is maintained in the cells, to accumulate photosynthetic tissues, and to produce the large number of chloroplasts required for maximum photosynthetic productivity.

Because plastids are believed to originate from the prokaryotic organism *Cyanobacterium*, they resemble such an organism in their division machinery. Prokaryotic cells divide, via a ring, through binary fission (Bramhill, 1997). Recent genetic approaches for understanding plastid division and development using *Arabidopsis* clearly indicate a close similarity with prokaryotic cells in their genetic control. Accumulation and replication of chloroplast (*arc*) mutants has been reported in mesophyll cells of *Arabidopsis thaliana* that have a few large chloroplasts (Pyke and Leech, 1992; Pyke et al., 1994; Robertson et al., 1995, 1996). In *Esherichia coli*, more than nine proteins are involved in the binary fission process (Bramhill, 1997). The best known of these, FtsZ, is a structural homologue that very likely is the evolutionary precursor of the eukaryotic tubulins (Erickson, 1998; Faguy and Doolittle, 1998). Genes encoding FtsZ have been identified in nearly all prokaryotes and in diverse eukaryotes, including unicellular algae and other protists, moss, and vascular plants (Beech and Gilson, 2000). To date, most of the identified eukaryotic *FtsZ* genes have been related to cyanobacteria, and are presumed to be involved in chloroplast division. *FtsZ* homologues in higher plants have been identified in six species. The antisense and sense orientations of *FtsZ* cDNA-transformed *A. thaliana* and the knockout FtsZ mutants of *Physcomitrella patens* (via homologous recombination) have a few enlarged chloroplasts (Osteryoung et al., 1998; Strepp et al., 1998; Stokes et al., 2000). *Arabidopsis FtsZ* transgenic plants and *arc6* mutants, with a few enlarged chloroplasts per cell, are indistinguishable from wild-type plants in their outward appearance and growth (Robertson et al., 1995; Osteryoung et al., 1998). Therefore, the question arises as to why the photosynthetic cells of higher

*Corresponding author; fax +82-42-860-4608
e-mail jrliu@mail.kribb.re.kr

plants contain so many small chloroplasts.

Previously, we introduced a sense construct of *NtFtsZ1-2* to produce transgenic tobacco plants that contained only one to three enlarged chloroplasts per leaf mesophyll cell. We were able to demonstrate that those enlarged chloroplasts could not move properly as the intensity of incidental light changed, thereby retarding plant growth (Jeong et al., 2002). In addition to this phenotype of only a few enlarged chloroplasts per cell, we found another type of plant among the T_1 progeny - an intermediate type with a mixture of many small chloroplasts and one to three enlarged chloroplasts per leaf mesophyll cell. In this study, we attempted to determine why higher-plant photosynthetic cells contain so many small chloroplasts rather than a few enlarged ones. In doing so, we systematically compared the overall growth of two different types of transgenic plants versus wild-type plants, all growing under various light conditions.

MATERIALS AND METHODS

Transgenic Tobacco Plants

We generated transgenic plants of tobacco (*N. tabacum* L. cv. Xanthi) that overexpressed *NtFtsZ1-2* (*FtsZ1-2* of *N. tabacum*) under the control of the CaMV 35S promoter (see Jeong et al., 2002). These plants, produced in a growth chamber, were self-pollinated, and their progeny were used for our growth comparisons.

Isolation and Observation of Protoplasts

Fully expanded leaves were removed with a razor blade from the transgenic and wild-type plants, 30 d after germination. For the light-microscopic observations, we isolated the protoplasts by incubating leaf segments overnight in an enzyme digestion solution [2% Cellulase Onozuka R-10 (Yakult, Tokyo, Japan), 1% Macerase R-10 (Yakult, Tokyo, Japan), 0.6 M mannitol, and 5 mM MES at pH 5.8]. The protoplasts were purified by filtering, centrifugation, and washing, and were then observed under a light microscope. Images were captured by digital camera (Nikon COOLPIX-950, Japan) and Adobe Photoshop (Adobe Systems, San Jose, CA) software.

Measurement of Plant Growth

In a preliminary analysis of the T_1 progeny, we used a light microscope to select two distinct phenotypes -- one with a few enlarged chloroplasts in the mesophyll cells (severe type); the other having a mixture of a few enlarged chloroplasts and many, typically wild-type, small chloroplasts (intermediate type). Afterward, three independent transgenic lines of the severe- and intermediate-type plants, as well as the wild-type plants, were grown in a greenhouse under natural fluctuations of sunlight for 10 weeks. Temperatures typically reached 32°C, and irradiance during the day ranged from 750 to 1500 μmol $m^{-2}s^{-1}$. The maximum daytime irradiance was defined arbitrarily as: 1) 100% relative growth irradiance (high light), 2) 20% (medium light), or 3) 5% (low light). These various levels were achieved by reducing the available irradiance with layers of shade cloth. The measured parameters included plant height, stem thickness, fresh weight and size of the youngest fully expanded leaf, soluble protein content, chlorophyll contents, and the chlorophyll a/b ratio. Chlorophyll contents were determined in 100% methanol, following the method of Porra et al. (1989). Soluble protein contents were measured using Bio-Rad protein assay reagents according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Mature transgenic plants that overexpressed *NtFtsZ1-2* under the control of the CaMV35S promoter exhibited a normal phenotype. Their isolated protoplasts contained one to three enlarged chloroplasts, whereas those from the wild-type leaf tissue typically contained 80 to 200 smaller chloroplasts (Fig. 1). The enlarged chloroplasts usually were flat, filling a thin layer of cytoplasm surrounding the vacuole (Fig. 1). Despite the enlarged size, the chloroplast ultrastructure consistently appeared normal (Jeong et al., 2002). The morphology of the enlarged chloroplasts observed in this study matches that previously found with the *Arabidopsis* plastid-division mutant *arc6* (Pyke et al., 1994; Robertson et al., 1995); transgenic *Arabidopsis* with the sense/antisense *AtFtsZ* transgene (Osteryoung et al., 1998; Stokes et al., 2000); and *FtsZ* knockout mutants from the moss *P. patens* (Strepp et al., 1998). *NtFtsZ1-2* (*FtsZ1-2* of *N. tabacum*) apparently is also associated with chloroplast division in tobacco, as is *AtFtsZ* in *Arabidopsis* (Osteryoung et al., 1998; Stokes et al., 2000); that overexpression of *NtFtsZ1-2* inhibits chloroplast division.

The T_1 plants that resulted from self-pollination were analyzed by light microscopy, and were divided into

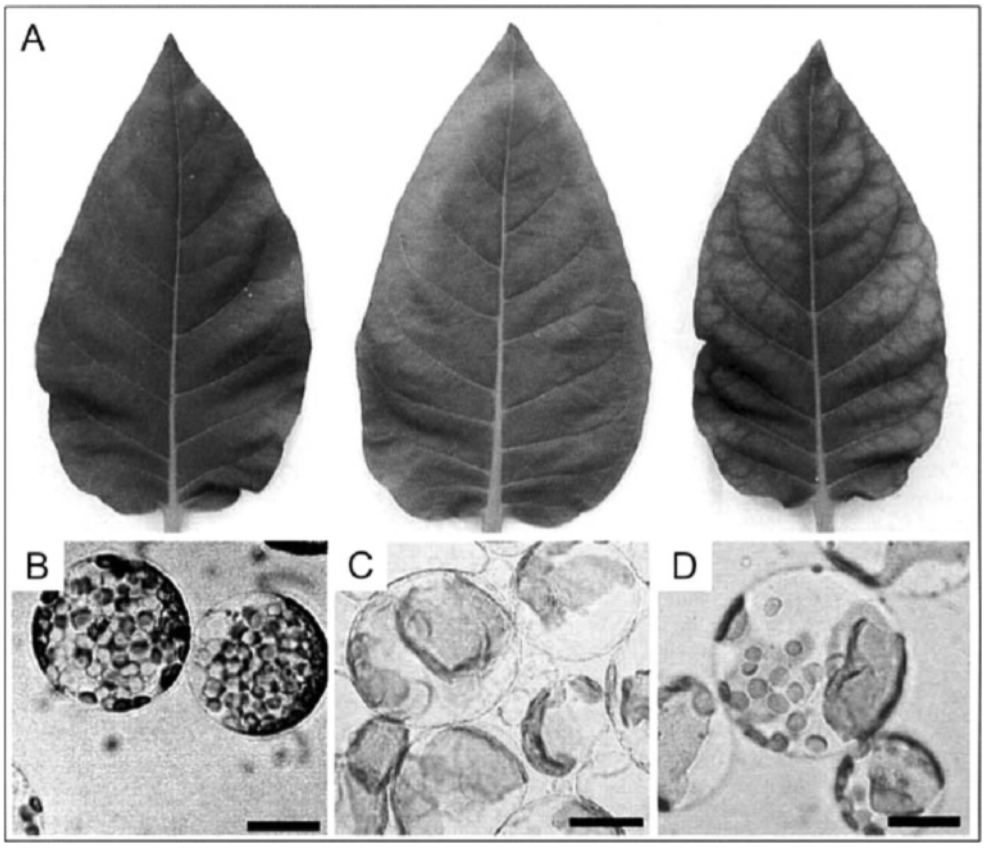

Figure 1. Phenotypes of transgenic tobacco plants. **A.** Photodamage in leaves grown under high-light conditions: wild-type (left); severe-type (middle), and intermediate-type plants (right). **B-D.** diverse chloroplasts in mesophyll protoplasts from wild-type (**B**); severe-type (**C**); and intermediate-type plants (**D**). Bars represent 20 μm.

three groups: 1) plants with leaf mesophyll cells containing many small chloroplasts (same as for the wild types, Fig. 1B); 2) those with cells containing one to three enlarged chloroplasts (severe type, Fig. 1C); and 3) plants with cells containing a mixture of many small chloroplasts and one to three enlarged chloroplasts (intermediate type, Fig. 1D). These differences probably resulted from segregation of the transgene in the T_1 generation.

Although the severe-type phenomenon in our tobacco was comparable to that found in transgenic *Arabidopsis* overexpressing FtsZ (Stokes et al., 2000), the intermediate-type tobacco (i.e., a mixture of many small and one to three enlarged chloroplasts) differed from *Arabidopsis* intermediate-type plants (in which 15 to 30 enlarged chloroplasts were seen per cell). Transgenic plants with a combination of many small chloroplasts and a few enlarged ones have not been reported with *Arabidopsis*. Likewise, the mesophyll cells of intermediate-type transgenic *Arabidopsis* contained 10 to 30 uniformly enlarged chloroplasts. However, the intermediate-type tobacco plants seemed to be similar to the *Arabidopsis arc10* mutant, with highly heterogeneous chloroplast sizes within a single cell (Rutherford, 1996). Both the intermediate-type transgenic tobacco and the *Arabidopsis arc10* mutant plants may have resulted from a subpopulation of chloroplasts that did not divide, or by abnormal, asymmetric chloroplast division (Pyke, 1999).

When growth parameters were compared, the severe-type plants showed the most retarded development (plant height, stem thickness, leaf area, and leaf fresh weight) under both high- (saturation irradiance) and low-light conditions (limiting irradiance), followed by intermediate-type plants (Table 1, Fig. 2). However, under medium-light conditions, both types of transgenic plants had growth comparable to the wild types (Table 1, Fig. 2). Under high-light conditions, the leaf blades of severe-, intermediate-, and wild-type plants were pale green, pale-green-and-deep-green variegated, and deep green, respectively (Fig. 1A). The chlorophyll content of severe-type leaves grown

Table 1. Growth measurements of wild-type and transgenic tobacco plants. Three independent lines of wild-type plants, and severe- and intermediate-type transgenic plants were grown in a greenhouse under natural fluctuations of sunlight for 10 weeks. Typical temperatures reached 32°C; diurnal irradiance ranged from 750 to 1500 μmol $m^{-2}s^{-1}$. Maximum daytime irradiance was defined arbitrarily as: 1) 100% relative growth irradiance (high light), 2) 20% (medium light), and 3) 5% (low light).

		PH	ST	LA	FW	Chl	Chl a/b	Protein
HL	WT	69.37 ± 3.81	1.56 ± 0.09	499.13 ± 71.29	13.41 ± 1.91	0.37 ± 0.030	3.70 ± 0.13	1.55 ± 0.10
	S	36.56 ± 6.65	1.22 ± 0.15	341.22 ± 89.74	9.09 ± 2.54	0.27 ± 0.006	4.22 ± 0.12	1.54 ± 0.16
	INT	54.50 ± 6.98	1.47 ± 0.12	484.12 ± 68.25	13.01 ± 1.83	0.35 ± 0.029	3.77 ± 0.07	1.52 ± 0.12
ML	WT	48.23 ± 5.01	0.85 ± 0.09	200.75 ± 47.02	4.89 ± 1.14	0.31 ± 0.010	3.30 ± 0.05	1.23 ± 0.02
	S	40.24 ± 3.23	0.80 ± 0.06	186.88 ± 33.72	4.53 ± 0.82	0.30 ± 0.009	3.47 ± 0.01	1.40 ± 0.09
	INT	46.37 ± 5.34	0.88 ± 0.08	210.85 ± 45.72	5.14 ± 1.11	0.32 ± 0.008	3.45 ± 0.03	1.46 ± 0.01
LL	WT	29.20 ± 5.05	0.47 ± 0.09	110.21 ± 17.73	2.47 ± 0.39	0.20 ± 0.005	2.91 ± 0.02	0.48 ± 0.04
	S	8.80 ± 3.14	0.28 ± 0.06	42.27 ± 14.22	0.92 ± 0.33	0.19 ± 0.005	2.98 ± 0.05	0.48 ± 0.05
	INT	19.33 ± 2.94	0.35 ± 0.03	65.48 ± 25.82	1.47 ± 0.58	0.19 ± 0.009	2.96 ± 0.17	0.45 ± 0.02

PH, plant height (cm); ST, stem thickness (cm); LS, size of the youngest, fully expanded leaf (estimated by leaf area, cm^2); FW, fresh weight (g/leaf); Chl a/b, chlorophyll a/b ratio; Chl, chlorophyll content (mmol m^{-2}); Protein, the soluble protein content (g m^{-2}); WT, wild-type plants; S, severe-type transgenic plants; INT, intermediate-type transgenic plants; HL, high light (700 to 1500 μmol photons m^{-2} s^{-1}); ML, medium light (200 to 400 μmol photons m^{-2} s^{-1}); LL, low light (30 to 150 μmol photons m^{-2} s^{-1}); Mean values (± SE) are shown for 8 to 15 plants.

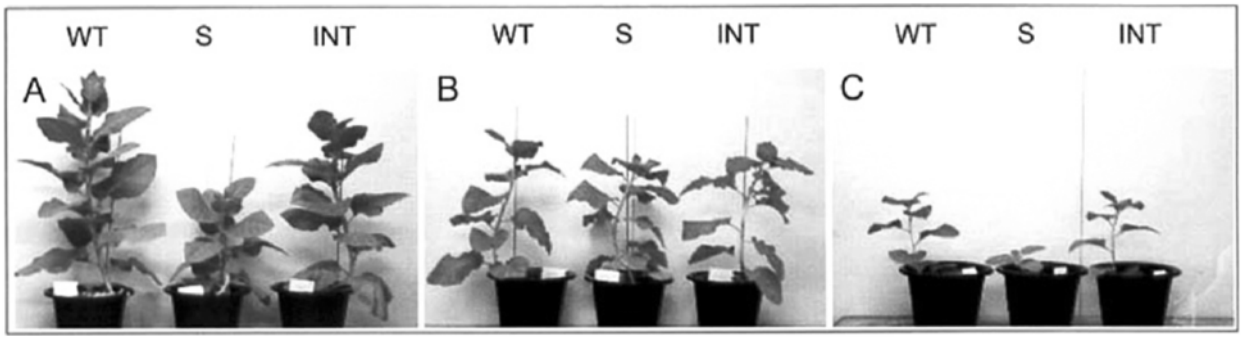

Figure 2. Typical wild-type (WT), severe- (S), and intermediate-type (INT) tobacco plants grown for 10 weeks under high- (A), medium- (B), and low-light conditions in a greenhouse.

under high-light conditions was reduced to 72.5% of the wild-type level (Table 1). Pale-green leaves, which had reduced chlorophyll contents and an increased chlorophyll a/b ratio (3.7 to 4.2) under high-light conditions, probably were a result of photodamage. Severe-type and intermediate-type plants grown under either medium- or low-light conditions had similar leaf colorations, and their chlorophyll contents were almost the same as with the wild types (Table 1). It has been reported that *Arabidopsis FtsZ* transgenic plants and *arc* mutant plants with a few enlarged chloroplasts per cell are similar to wild-type plants in their outward appearance and growth (Robertson et al., 1995; Osteryoung et al., 1998). However, those particular plants have not yet been systematically compared with wild-type plants when grown under various light conditions.

In this study we observed that growth of the severe-type transgenic tobacco plants was most retarded under both high- and low-light conditions, followed by intermediate-type plants. However, under medium-light conditions, performance was similar to that of the wild-type plants. It is obvious that the total surface area/volume ratio of all the enlarged chloroplasts within a transgenic cell is less than the ratio for all the numerous, normal chloroplasts found in a wild-type cell. Because growth of plants with a few enlarged chloroplasts per cell was lower than that of the wild-type plants under low-light conditions, the total surface area/volume ratio did seem to affect photosynthetic efficiency. However, this ratio cannot explain why, under high light, growth was less for plants with a few enlarged chloroplasts than for the wild types. This retardation under saturation irradiance seems to have resulted from photodamage (Fig. 1A).

Zurzycki (1957) has suggested that a dual function for chloroplast rearrangement exists in plants, whereby they maximize their use of limited light, while minimiz-

ing the chance of photo-damaging the photosynthetic apparatus under excess light. Such a rearrangement has been observed in many species (Haupt and Scheuerlein, 1990). We previously demonstrated that the enlarged chloroplasts in severe-type transgenic mesophyll cells of tobacco could not move properly as the incident-light irradiance changed (Jeong et al., 2002). Intermediate-type transgenic tobacco may also have a similar problem in chloroplast movement, which might explain why the development of plants with a few enlarged chloroplasts per cell was retarded under both high- and low-light conditions. Presumably, if a mesophyll cell has a few enlarged chloroplasts, the capacity of those chloroplasts to resolve their rearrangement under varying levels of irradiance would be greatly compromised at the expense of photosynthetic efficiency. Therefore, we conclude that chloroplasts in higher plants have been evolved to divide into many small ones, rather than a few enlarged ones, to support efficient light-energy utilization in their mesophyll cells.

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