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Shigeo TANAKA and Koichiro TSUNEWAKI

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Genetic diversity of the cytoplasm in *Triticum* and *Aegilops*. XI. The SDS gel electrophoretic patterns of chloroplast proteins prepared from the alloplasmic common wheat lines[1)]

Shigeo TANAKA[2)] and Koichiro TSUNEWAKI

Laboratory of Genetics, Faculty of Agriculture,
Kyoto University, Sakyo-ku, Kyoto 606

Introduction

In two related genera, *Triticum* and *Aegilops*, interspecific as well as intergeneric crosses are successful, due to which a large number of alloplasmic lines of common wheat with cytoplasms of various *Triticum* and *Aegilops* species have been produced, since the work of KIHARA (1951). Using these alloplasmics of common wheat, TSUNEWAKI *et al.* (1976, 1978, 1980, 1984) investigated the effects of cytoplasms from 33 *Triticum* and *Aegilops* species (43 accessions in total) on various morphological and physiological characters of 12 common wheats, and classified them into 12 types based on those effects. Furthermore, OGIHARA and TSUNEWAKI (1982, 1984) and TSUNEWAKI and OGIHARA (1983) have studied restriction fragment patterns of chloroplast DNAs isolated from the alloplasmic lines of common wheat, and classified the chloroplast genomes of the same 33 species into 11 major types plus five subtypes. Combining all the results so far obtained, TSUNEWAKI and TSUJIMOTO (1984) have proposed to classify cytoplasms of all the *Triticum* and *Aegilops* species into 16 plasma types.

Comparing to such extensive investigations on the cytoplasmic variability among *Triticum* and *Aegilops* species as to the phenotypic effects and chloroplast DNA molecules, little work has been done so far on the variability of proteins encoded by the organellar DNAs among those species, except the electrophoretic study of Rubisco (ribulose 1, 5-bisphosphate carboxylase/oxygenase) large subunit (HIRAI and TSUNEWAKI 1981). To complement this, the present investigation has been planned to study the variability of chloroplast proteins which are isolated from the alloplasmic lines of common wheat with the same nuclear genotype. The results will be reported below.

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2) Present address : The Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565.

Materials and Methods

1. *Plant materials*

Alloplasmic lines of a common wheat, *Triticum aestivum* cv. Chinese Spring (CS in abbreviation, 2n=42, genome constitution AABBDD), which are shown in Table 1, were used in the present investigation. All of them were backcrossed with Chinese Spring six times or more before use. Their cytoplasms represent eight of the 16 plasma types proposed by TSUNEWAKI and TSUJIMOTO (1984). As the control, euplasmic lines of *T. aestivum* cv. Chinese Spring, *T. dicoccoides*, *Aegilops umbellulata* and *Ae. squarrosa* were also studied.

For each line, 50 seeds were sown in a plastic case filled with vermiculite, and were grown at 20°C in a growth chamber. Fresh leaves of three leaf-stage seedlings were collected and used for chloroplast isolation.

Table 1. Materials used in the present investigation

Nucleus donor	Cytoplasm donor		Abbreviation
	Species	Plasma type[1)]	
Euplasmic line			
T. aestivum cv. CS	*T. aestivum*	B	CS
T. dicoccoides	*T. dicoccoides*	B	*dicoccoides*
Ae. umbellulata	*Ae. umbellulata*	C^u	*umbellulata*
Alloplasmic line			
T. aestivum cv. CS	*Ae. umbellulata*	C^u	(*umbellulata*)-CS
do	*Ae. squarrosa*	D	(*squarrosa*)-CS
do	*Ae. speltoides*	S	(*speltoides*)-CS
do	*Ae. sharonensis*	S^l	(*sharonensis*)-CS
do	*T. dicoccum*	B	(*dicoccum*)-CS
do	*T. dicoccoides*	B	(*dicoccoides*)-CS
do	*Ae. triuncialis*	C^u	(*triuncialis*)-CS
do	*Ae. cylindrica*	D	(*cylindrica*)-CS
do	*Ae. ovata*	M^o	(*ovata*)-CS
do	*Ae. kotschyi*	S^v	(*kotschyi*)-CS
do	*Ae. variabilis*	S^v	(*variabilis*)-CS
do	*Ae. ventricosa*	D	(*ventricosa*)-CS
do	*Ae. juvenalis*	D^2	(*juvenalis*)-CS
do	*Ae. crassa* 6x	D^2	(*crassa*)-CS
do	*Ae. vavilovii*	D^2	(*vavilovii*)-CS

[1)] After Tsunewaki and Tsujimoto (1984).

2. Preparation of chloroplast and its protein fractions

The collected seedling leaves were homogenized with 0.015% M Tris-HCl buffer (pH 7.2) containing 0.001 M NaCl and 0.45 M sucrose. The homogenate was filtered through two layers of cheese cloth. After centrifugation at 2,000×g for 10 min, the pellet was resuspended in the same buffer. This suspension was layered on the discontinuous sucrose density gradient in a 30 ml tube and centrifuged at 90,000×g for 100 min. The gradient was prepared by layering five 5.5 ml layers of sucrose of the concentrations of 2.0, 1.8, 1.6, 1.4 and 1.2 M in 0.015 M Tris-HCl buffer (pH 7.2), respectively.

The pure chloroplast fraction forming a dark green band in the interphase between 1.8 and 1.6 M layers, was collected and diluted with the three times volume of the same buffer. To adjust the number of chloroplasts, the optical density at 660 nm was measured, and a certain volume of the fraction was taken as the sample in such a way that the product of (optical density)×(volume in ml) became 10. Then, the sample was centrifuged at 2,000× g for 10 min.

The pellet was treated in two ways. (1) The pellet was suspended in a test-tube containing the cracking buffer, i.e., 0.05 M Tris-HCl (pH 7.2) containing 2% sodium dodecyl sulphate (SDS), 10% glycerol and 2% 2-mercaptoethanol, and was placed in boiling water for 2 min in order to dissociate proteins completely. The resulted solution was used as the sample for total chloroplast proteins. (2) The pellet was resuspended in a hypotonic solution, by which chloroplasts were swelled and broken. Their membrane and lamella were sedimented by centrifugation at 5,000×g for 15 min. The pellet was suspended in the cracking buffer in a test-tube, and was placed in boiling water in the same way as in the first method. The resulted solution was used as the sample for chloroplast membrane and lamella proteins.

3. SDS gel electrophoresis

The electrophoresis was carried out according to the method of LAEMMLI (1970) as follows; the slab gel of 0.2 mm thick and 100 mm long was made between two glass plates. The separation gel containing 10 or 12.5% acrylamide was prepared from a stock solution of 30% acrylamide and 0.8% N-N' bisacrylamide by weight. The gel was polymerized by adding 0.055% tetramethylene-diamine (TEMED) and 0.04% ammonium persulphate in volume. The stacking gel that was 10 mm long and contained 0.125 M Tris-HCl (pH 6.8) and 0.1% SDS was polymerized by adding 0.1% TEMED and 0.03% ammonium persulphate in volume.

The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Electrophoresis was carried out with 40 volt until the bromophenol blue marker reached the bottom of the gel (for about 16 h). The proteins were fixed in the gel with 50% trichloroacetic acid (TCA) overnight, stained for 1 h at 37°C with 0.1% Coomassie brilliant blue solution freshly made with 50% TCA. The gel was destained by repeated washing with 7% acetic acid and 30% methanol.

Results

1. SDS eletrophoretic patterns of total chloroplast proteins from three alloplasmic lines and their nucleus and cytoplasm donors

The SDS electrophoretic patterns of total chloroplast proteins from three alloplasmic lines of Chinese Spring, i.e., (*umbellulata*)-, (*squarrosa*)- and (*dicoccoides*)-CS, were compared with those of euplasmic lines of Chinese Spring, *Ae. umbellulata*, *Ae. squarrosa* and *T. dicoccoides*, using 10% acrylamide gel. The patterns obtained are shown in Fig. 1. Four lines, i.e., (*umbellulata*)-CS, *Ae. umbellulata*, (*squarrosa*)-CS and *Ae. squarrosa* showed an

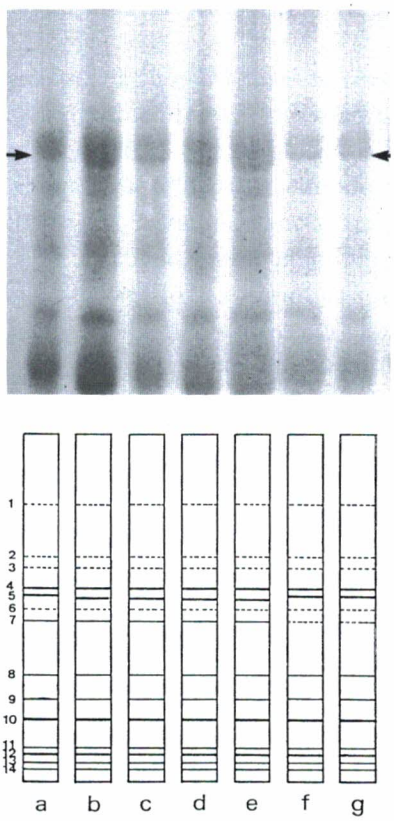

Fig. 1 The SDS electrophoretic patterns of total chloroplast proteins prepared from a euplasmic and three alloplasmic lines of *T. aestivum* cv. Chinese Spring (CS), compared with those of the three cytoplasm donors (10% acrylamide gel is used). (Band 5 arrowed)
a-g: Euplasmic CS, (*umbellulata*)-CS, *Ae. umbellulata*, (*squarrosa*)-CS, *Ae. squarrosa*, (*dicoccoides*)-CS and *T. dicoccoides*, respectively.

identical electrophoretic pattern with each other, which differed from that of normal Chinese Spring only in one band, namely, band 5. This band showed higher mobility in the first four lines than in normal Chinese Spring. Therefore, it is concluded that band 5 is encoded by an organellar DNA. For all other bands, the five lines showed the identical pattern so that it could not be determined whether they are encoded by the nuclear or organellar DNA.

Two lines, (*dicoccoides*)-CS and *T. dicoccoides* showed the identical band pattern to that of normal Chinese Spring. This fact does not contradict with the above conclusion that band 5 is encoded by organellar DNA. It is clear from the present results that two B type cytoplasms of *T. aestivum* and *T. dicoccoides* produce band 5 of a high molecular weight (estimated from its low mobility), comparing to the corresponding band of a C^u and D type cytoplasm of *Ae. umbellulata* and *Ae. squarrosa*, respectively.

2. SDS electrophoretic patterns of total chloroplast proteins from 15 alloplasmic lines

The SDS electrophoretic patterns of total chloroplast proteins of 15 alloplasmic lines of Chinese Spring were studied, using 10 and 12.5% acrylamide gel. The patterns of 12 alloplasmic lines which were obtained with 10% acrylamide gel are shown in Fig. 2 and 3. No variation was found among normal Chinese Spring and its alloplasmic lines on three major bands, 4, 10 and 12, and eight minor bands, 1, 2, 3, 8, 9, 11, 13 and 14. On the contrary, band 5 appeared in two forms, i.e., of low and high molecular weight. Normal, (*speltoides*)-, (*dicoccoides*)- and (*dicoccum*)-CS had the band 5 of high molecular weight, whereas all other lines had that of low molecular weight.

The patterns obtained with 12.5% acrylamide gel from 14 alloplasmic lines of Chinese

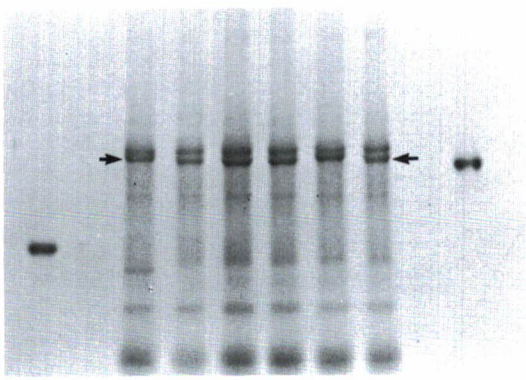

Fig. 2 The SDS electrophoretic patterns of total chloroplast proteins prepared from a euplasmic and five alloplasmic lines of *T. aestivum* cv. Chinese Spring (CS) (10% acrylamide gel is used). (Band 5 arrowed)
Left to right: Marker protein (mol. wt. 48 kd), euplasmic, (*umbellulata*)-, (*squarrosa*)-, (*ovata*)-, (*speltoides*)-, (*sharonensis*)-CS and marker protein (mol. wt. 68 kd).

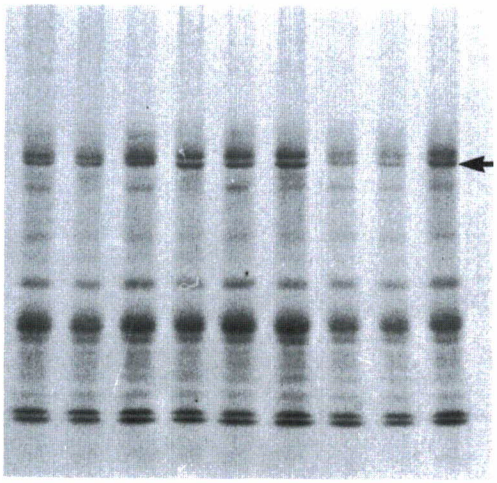

Fig. 3 The SDS electrophoretic patterns of total chloroplast proteins prepared from a euplasmic and eight alloplasmic lines of *T. aestivum* cv. Chinese Spring(CS) (10% acrylamide gel is used). (Band 5 arrowed)
Left to right: Euplasmic, (*dicoccoides*)-, (*dicoccum*)-, (*triuncialis*)-, (*ovata*)-, (*kotschyi*)-, (*variabilis*)-, (*ventricosa*)- and (*crassa* 6x)-CS.

Spring, except (*dicoccum*)-CS, were studied. The patterns of (*cylindrica*)-, (*ovata*)-, (*kotschyi*)-, (*variabilis*)-, (*ventricosa*)-, (*juvenalis*)-, (*crassa* 6x)- and (*vavilovii*)-CS which were obtained by 12 h electrophoresis are shown in Fig. 4. In this experiment, seven additional bands were identified below band 14, which were named bands 15 to 21 in their order of mobility. All the eu- and alloplasmic lines of Chinese Spring showed the identical electrophoretic patterns on both the intensity and mobility of those bands. Thus, no protein fractions of low molecular weights were confirmed to be encoded by organellar DNAs.

3. SDS electrophoresis of chloroplast-membrane proteins

A normal and six alloplasmic lines of Chinese Spring were studied as to the electrophoretic patterns of their chloroplast-membrane proteins (Fig. 5). Though the intensities of minor bands were weak, the entire band patterns appeared to be the identical to those of total chloroplast proteins (ref. Fig. 2 and 3). Band 5 of normal, (*speltoides*)- and (*dicoccoides*)-CS showed low mobility, comparing to that of (*umbellulata*)-, (*squarrosa*)-, (*sharonensis*)- and (*cylindrica*)-CS.

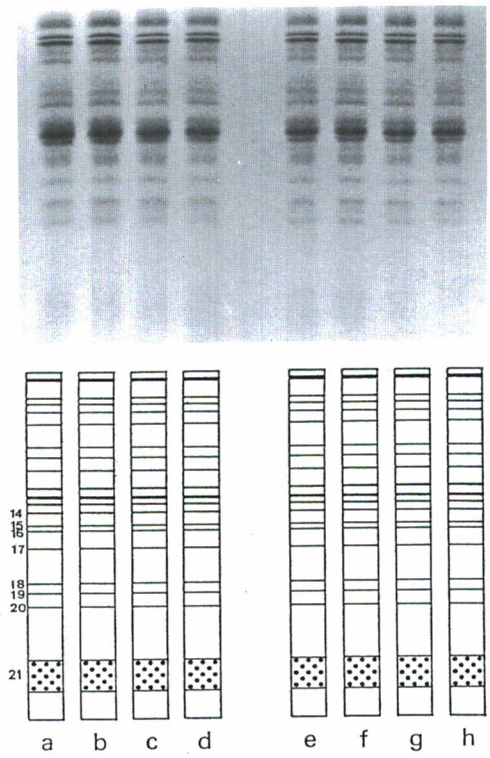

Fig. 4 The SDS electrophoretic patterns of total chloroplast proteins prepared from eight alloplasmic lines of *T. aestivum* cv. Chinese Spring (CS) (12.5% acrylamide gel is used).
a-h: (*cylindrica*)-, (*ovata*)-, (*kotschyi*)-, (*variabilis*)-, (*ventricosa*)-, (*juvenalis*)-, (*crassa* 6x)- and (*vavilovii*)-CS.

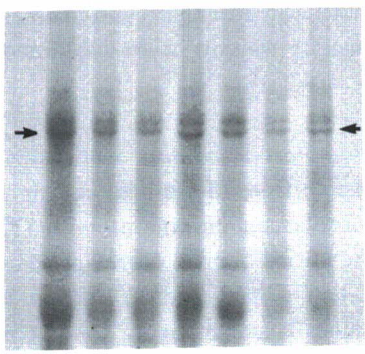

Fig. 5 The SDS electrophoretic patterns of chloroplast-membrane proteins prepared from a euplasmic and six alloplasmic lines of *T. aestivum* cv. Chinese Spring (CS) (10% acrylamide gel is used). (Band 5 arrowed)
Left to right: (*cylindrica*)-, (*dicoccum*)-, euplasmic, (*squarrosa*)-, (*umbellulata*)-, (*speltoides*)- and (*sharonensis*)-CS.

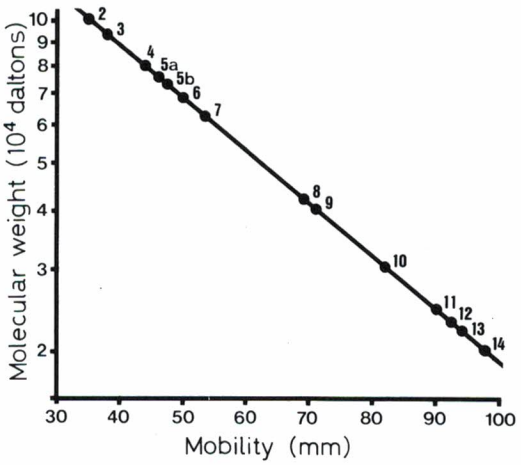

Fig. 6 Estimation of the molecular weights of individual protein bands from a linear regression of the molecular weight (in log) on the mobility (in mm), that has been obtained from the data of three marker proteins.

Discussion

1. Estimation of molecular weights of individual chloroplast proteins

From the mobilities of three marker proteins with known molecular weights, the molecular weight ($Y \times 10$ kilo daltons (kd)) of a given band can be estimated from the following formula;

$$\log Y = -0.011\,X + 1.38,$$

where X is mobility in mm of the band. As shown in Fig. 6, bands 2 to 14 are plotted on the above regression line, based on their mobilities. The molecular weights estimated for them are as follows; 99, 92, 79, 75, 73, 68, 62, 42, 40, 30, 25, 23, 22 and 20 kd for band 2, 3, 4, 5a, 5b, 6, 7, 8, 9, 10, 11, 12, 13 and 14, respectively.

As described in "Results", band 5 encoded by an organellar DNA exists in two forms, 5a and 5b, the former being about 2 kd larger than the latter. Euplasmic lines of *T. aestivum*(CS) and *T. dicoccoides*, and three alloplasmic lines of Chinese Spring, namely, (*speltoides*)-, (*dicoccoides*)- and (*dicoccum*)-CS, all have the band 5a, whereas euplasmic lines of *Ae. umbellulata* and *Ae. squarrosa* and 12 alloplasmic lines of Chinese Spring, namely, (*umbellulata*)-, (*squarrosa*)-, (*sharonensis*)-, (*triuncialis*)-, (*cylindrica*)-, (*ventricosa*)-, (*ovata*)-, (*kotschyi*)-, (*variabilis*)-, (*juvenalis*)-, (*crassa* 6x)- and (*vavilovii*)-CS, all have the band 5b.

2. Nature of the band 5 chloroplast protein

The SDS electrophoresis using 10% acrylamide gel proved that this protein is encoded

by an organellar DNA. In order to know the nature of this protein, chloroplast-membrane proteins, including lamella proteins, were studied. The electrophoretic band patterns of these membrane proteins of a euplasmic line and six alloplasmic lines of Chinese Spring are all comparable to those of their total chloroplast proteins (ref. Fig. 2, 3 and 5), though intensities of minor bands become weak in the former patterns. This fact indicates that the band 5 protein is a component of chloroplast membrane or lamella.

It is the well established fact that chloroplast (ct) DNA codes for chloroplast RNAs (rRNAs and tRNAs) and chloroplast proteins, including some enzymes, whereas mitochondrial DNA codes for mitochondrial RNAs and proteins. Thus, it is reasonable to assume that the band 5 chloroplast protein is encoded by ctDNA. So far, ctDNA is known to encode about a dozen of polypeptides, such as Rubisco large subunit, cytochrome b and f, four subunits (α, β, ε and proton-translocating) of ATP synthase, three subunits (α, β and ε) of CF_1, one subunit (III) of CF_0, one component (PSI_{70}) of photosystem I, and three subunits ($PSII_3$, $PSII_{44}$ and $PSII_{51}$) of photosystem II (Uchimiya *et al.* 1976, Howe *et al.* 1983, Nelson *et al.* 1984). Molecular weight of Rubisco LS is 56 kd, and one of photosystem II components is 32 kd. The band 5 protein is apparently different from both polypeptides. However, we do not know yet whether this protein is the same as any one of other ctDNA-encoded polypeptides. This is a point that must be studied in future.

3. Origin of the two forms of the band 5 chloroplast protein

The larger molecular form (75 kd) of band 5, i.e., band 5a is found only in chloroplasts of *T. aestivum* (CS), *T. dicoccoides*, *T. dicoccum* and *Ae. speltoides*, all having the B or S type cytoplasm. On the contrary, the lower molecular weight form (73 kd), namely, band 5b distributes widely among the C^u type cytoplasms of *Ae. umbellulata* and *Ae. triuncialis*, the D type cytoplasms of *Ae. squarrosa*, *Ae. cylindrica* and *Ae. ventricosa*, the D^2 type cytoplasms of *Ae. juvenalis*, *Ae. crassa* and *Ae. vavilovii*, the S^1 type cytoplasm of *Ae. sharonensis*, and the S^v type cytoplasms of *Ae. kotschyi* and *Ae. variabilis*.

The restriction fragment pattern analysis of ctDNA indicates that chloroplast genomes in D^2, S^1 and S^v type cytoplasms are in the center of chloroplast genome diversification, and that the chloroplast genomes in B and S type cytoplasms are highly specialized ones (Ogihara and Tsunewaki 1982, 1984). These data strongly suggest that the prototype of the band 5 chloroplast protein in the *Triticum-Aegilops* complex is of the smaller molecular form, and that the higher molecular form found in *Ae. speltoides* and polyploid wheats is of a recent origin.

Summary

In order to get an information on the genetic diversity among organellar DNA-encoded proteins of *Triticum* and *Aegilops* species, chloroplast proteins of a euplasmic line and 15 alloplasmic lines of a common wheat, *T. aestivum* cv. Chinese Spring and the euplasmic lines of a few cytoplasm donors have been analyzed by the SDS gel electrophoresis. The

results are summarized as follows:

(1) In order to identify chloroplast proteins encoded by organellar DNAs, total chloroplast proteins of the euplasmic lines of Chinese Spring, *T. dicoccoides*, *Ae. umbellulata* and *Ae. squarrosa*, and three alloplasmic lines of Chinese Spring having the cytoplasms of last three species are analyzed by the SDS electrophoresis with 10% acrylamide gel. All the electrophoretic patterns consist of 14 bands (band 1 to 14), of which only band 5 shows variation among the seven lines, i.e., euplasmic lines of Chinese Spring and *T. dicoccoides* and an alloplasmic line, (*dicoccoides*)-CS, give band 5a of high molecular weight, whereas all other lines have the band 5b of low molecular weight, indicating that the band 5 is encoded by an organellar DNA.

(2) To clarify further the variation on chloroplast proteins among cytoplasms of different sources, total chloroplast proteins of 15 alloplasmic lines of Chinese Spring have been analyzed by the same method. Band 5 again shows variation among the alloplasmic lines, while the remaining 13 bands do not. The B type cytoplasms of *T. aestivum*, *T. dicoccum* and *T. dicoccoides* and an S type cytoplasm of *Ae. speltoides* give the band 5a, whereas all other 12 cytoplasms of C^u, D, D^2, M^0, S^1 and S^v plasma types show the band 5b.

(3) Molecular weights (Y) in 10 kilo daltons (kd) of individual chloroplast proteins, corresponding to bands 2 to 14, are estimated from their mobilities (X), using the equation, $\log Y = -0.011\,X + 1.38$. Two forms of band 5, namely, 5a and 5b, are found to have the molecular weight of 75 and 73 kd, respectively.

(4) To get a clue on the nature of the band 5 protein, chloroplast membrane (including lamella) proteins prepared from cracked chloroplasts are studied by the same method. The results indicate that the band 5 protein is a component of chloroplast membrane or lamella, and is assumed to be encoded by chloroplast (ct) DNA. Referring to its molecular weight, this protein differs from both Rubisco LS and 32 kd protein of photosystem II. However, its identity with any other known ctDNA-encoded proteins is not clear at present.

(5) Because chloroplast genomes of D^2, S^1 and S^v cytoplasms are assumed to be in the center of chloroplast genome diversification, and those of B and S cytoplasms are highly specialized forms, the prototype of the band 5 protein is probably the 73 kd protein (band 5b), from which the 75 kd protein has evolved.

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