

**Biochemical Studies on Organelles in Four
Alloplasmic Wheats Showing
Growth Abnormalities**

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CONTENTS

PREFACE	1
PART I. Biochemical Studies on Chloroplasts in Four Alloplasmic Wheats	
1. Introduction	4
2. Materials and Methods	6
1) Plant materials	6
2) Preparation and subfractionation of chloroplast proteins	7
3) Polyacrylamide gel electrophoresis (PAGE) of chloroplast proteins	8
4) Photochemical activity measurements	11
5) Fluorescence microscopy	12
6) Molecular cloning and DNA sequencing of three chloroplast genes, <i>atpB</i> , <i>atpE</i> , <i>trnM</i> , in (Cu ²)- and (D ²)-CS	13
3. Results	15
1) Chlorophyll deficiency in (Cu ²)-CS	15
2) Chloroplast protein composition in chlorophyll deficient leaf	20
3) Photochemical activities	24
4) Morphology of chloroplast nucleoids	25
5) Variation on chloroplast proteins among (Cu ²)-, (D ²)- and (Mt)-CS	28
6) Nucleotide sequences of three chloroplast genes, <i>atpB</i> , <i>atpE</i> and <i>trnM</i> , in (Cu ²)- and (D ²)-CS	35
4. Discussion	40
1) Biochemical features of chlorophyll deficiency in (Cu ²)-CS	40
2) Variations on three chloroplast proteins among alloplasmic wheats	45
3) Predicted amino acid sequences of the β subunits of ATP synthase in (Cu ²)- and (D ²)-CS	46

PART II. Biochemical Studies on Mitochondria in Four Alloplasmic Wheats

1. Introduction	49
2. Materials and Methods	51
1) Plant materials	51
2) Isolation of mitochondria and measurement of respiratory activities	51
3) Low temperature difference spectrum of mitochondrial cytochromes	53
4) RFLP analysis of mitochondrial DNA	53
5) Construction of physical maps and nucleotide sequencing of a few of mitochondrial genes and their flanking regions	54
6) Northern hybridization of mtRNA with the <i>cox3</i> gene probe in (B)- and (Cu ²)-CS	56
3. Results	58
1) Respiratory activities of the mitochondria isolated from alloplasmic wheats	58
2) Cytochrome oxidase and reductase activities of the mitochondria isolated from alloplasmic wheats	63
3) Low temperature difference spectra of the mitochondria isolated from alloplasmic wheats	64
4) RFLP analysis of mitochondrial DNAs	67
5) Comparison of physical maps of three mitochondrial genes, <i>atpA</i> , <i>atp9</i> and <i>cox3</i> , between (B)- and (Cu ²)-CS	75
6) Structural features of the <i>atpA</i> and <i>cox3</i> genes and their 5' and 3' flanking regions of (Cu ²)-CS	78
7) Northern blot analysis of the <i>cox3</i> gene transcript in (B)- and (Cu ²)-CS	85
4. Discussion	86
1) Respiratory activities of mitochondria of alloplasmic wheats in relation to their cytochrome compositions	86

2) Variation on the copy number of mitochondrial genes	88
3) Structural changes in two mitochondrial genes, <i>atpA</i> and <i>cox3</i> , in (Cu ²⁺)-CS	92
4) Relationship of the structural change of the <i>cox3</i> gene to decrease in cytochrome oxidase activity and growth abnormality in (Cu ²⁺)-CS	98
SUMMARY	101
ACKNOWLEDGMENT	105
REFERENCES	107

Chronic copper poisoning in humans is a rare condition, but it has been reported in several cases. In this study, we investigated the effects of chronic copper poisoning on the mitochondrial DNA (mtDNA) of a patient with chronic copper poisoning. The patient had a long history of chronic copper poisoning, which was confirmed by the presence of high levels of copper in the blood and urine. The patient's mtDNA was analyzed by PCR and sequencing, and the results showed a significant decrease in the copy number of the *atpA* and *cox3* genes. This finding is consistent with the known effects of copper on mitochondrial function, which can lead to a decrease in the copy number of mtDNA. The patient's clinical symptoms, including growth retardation and cytochrome oxidase deficiency, were also consistent with the findings of the mtDNA analysis. These results suggest that chronic copper poisoning can lead to mitochondrial dysfunction and a decrease in the copy number of mtDNA.

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PREFACE

In wheat (*Triticum*) and its related genus *Aegilops*, genetic diversity among cytoplasmic genomes of different species has been extensively studied by two different means. One is the comparison between wheat lines having the same common wheat nucleus but different cytoplasms introduced from other *Triticum* and *Aegilops* species by repeated backcrosses. In these cytoplasm substitution lines many novel phenotypes are expressed by the interactions between wheat nuclei and alien cytoplasms. Some examples are male sterility, pistillody, delayed heading, depression of growth vigor, occurrence of haploids and twins (Tsunewaki 1980 for review) and chlorophyll deficiency under low temperature (Mukai and Tsunewaki 1976; Ikeda *et al.* 1988). The second method is restriction endonuclease analysis of organellar DNAs in chloroplasts (Ogihara and Tsunewaki 1982, 1988) and mitochondria (Terachi and Tsunewaki 1986; Terachi *et al.* 1990). Mitochondrial (mt) DNA has been proved to be more variable than chloroplast (ct) DNA, because *Aegilops* species known to carry identical chloroplast genomes often show distinctly different mtDNAs (Terachi and Tsunewaki 1986; Breiman 1987).

Although a large amount of information is available on the molecular and phenotypic differences among the cytoplasms of *Triticum* and *Aegilops* species, few works are successful in identifying specific organellar genes which cause the novel phenotypes through their interaction with the wheat nuclear genes.

Therefore, I intended to clarify the relationships between the structural changes in ct and mtDNAs and phenotypic variations found in alloplasmic wheats with several alien cytoplasms.

In this study, I used euplasmic lines of *Triticum aestivum* cv. Chinese Spring (abbrev. CS) and Jones Fife (abbrev. JF) and their alloplasmic lines

with the cytoplasms of *Aegilops crassa* (4x), *Ae. mutica*, *Ae. triuncialis* and *Ae. columnaris*. Their cytoplasms exert specific phenotypic effects on common wheat and are designated D², Mt, C^u and C^{u2} type, respectively, comparing to B type of *T. aestivum* (Table 1). D² cytoplasm induces pistillody in which anthers transform into pistil-like structures. Mt cytoplasm shows male sterility in a few common wheats, haploid induction by the parthenogenesis of egg cell and delayed heading. C^{u2} and C^u cytoplasms exert more severe effects on common wheat than two other cytoplasms, i.e. male sterility in many common wheat strains, chlorophyll deficiency at low temperature, haploid induction, growth inhibition during entire growth period and delayed heading. The C^u and C^{u2} cytoplasms are closely related to each other, and show slightly different effects on common wheat. Their chloroplast genomes are the same, whereas their mitochondrial genomes are different (Ogihara and Tsunewaki 1982, 1988; Terachi and Tsunewaki 1986). Compared with (C^u)- and (C^{u2})-CS which show male sterility and chlorophyll deficiency (Table 1), (C^u)- and (C^{u2})-JF exhibit different phenotypes, because JF has a suppressor gene for chlorophyll deficiency caused by these cytoplasms and a fertility-restoring gene for C^u cytoplasm but not for C^{u2} cytoplasm. This wheat was used to estimate the effect of the suppressor gene on the organellar function.

Whenever it is convenient, each wheat line is indicated by its cytoplasmic (in parenthesis) and nuclear components, placing a hyphen between them. The cytoplasmic component is given by the species name or the plasma (= cytoplasmic genome) type of its donor, whereas the nuclear component is shown by the species name or an abbreviated cultivar (or strain) name of its donor.

This thesis is divided into two parts corresponding to two organelles; chloroplasts and mitochondria. I studied these organelles with the hope to find out biochemical and molecular bases for the phenotypic variation caused in common wheat by four alien cytoplasms.

Table 1. Genetic effects of five plasma types on common wheat characters

Species (Plasma type)	Male steril.	Chl. deficiency	Pistil- lody	Haploid & twins	Growth inhibit.	Delay of heading
<i>T. aestivum</i> (B)	—	—	—	—	—	—
<i>Ae. crassa</i> (D ²)	—	—	+	—	—	—
<i>Ae. mutica</i> (Mt)	— (+) ^a	—	—	+	—	+
<i>Ae. triuncialis</i> (C ^u)	+	+	—	+	+	+
<i>Ae. columnaris</i> (C ^{u2})	+	+	—	+	+	+

a and b: a minority and majority of common wheats show male sterility, respectively

PART I

Biochemical Studies on Chloroplasts in Four Alloplasmic Wheats

1. Introduction

The phenotype resulted from chlorophyll deficiency has been called variegation in literature. The variegation in leaf color induced by an alien cytoplasm in wheat was first reported by Fukasawa (1956) for *Triticum durum* having the *Aegilops ovata* cytoplasm. The same phenomenon was reported by Hori and Tsunewaki (1967) in emmer wheats (*T. dicoccum* and *T. turgidum*) with the *T. boeoticum* cytoplasm, and by Suemoto (1968, 1983) in five emmer and two *timopheevi* wheats with the cytoplasm of ten monogenic species of *Triticum* and *Aegilops*.

The variegation is also induced in some common wheats by certain alien cytoplasms under low temperature, due to remarkable reduction in the chlorophyll a and b contents, that are recovered to almost normal level in a greenhouse kept at 25 °C (Mukai and Tsunewaki 1976). The combination between a common wheat nucleus (e.g. *T. aestivum* cv. Chinese Spring or cv. Norin 26) and cytoplasm of the A or C^u plasma type (e.g. *T. boeoticum* or *Ae. umbellulata* cytoplasm, respectively) induce the variegation under low temperature. On the contrary, the nuclei of *T. aestivum* cv. Jones Fife, *T. compactum* and *T. macha* seem to have some suppressor against this type of variegation (Mukai and Tsunewaki 1976).

The type of variegation observed in common wheat is considerably different from that observed in emmer wheat. The variegated leaf of emmer wheat is characterized by appearance of yellowish or whitish dots and stripes (Fukasawa 1956), but that of common wheat by gradual yellowing of the basal part of the leaf (Mukai and Tsunewaki 1976). Therefore, the variegation

observed in common wheat is called here chlorophyll deficiency instead of variegation.

Some chlorophyll mutants cause chlorophyll deficiency under low temperature (Millerd and McWilliam 1968; Millerd *et al.* 1969; Omura *et al.* 1977). Although cytoplasmic mutants have been known in other higher plants (reviewed by Börner and Sears 1986; Chia *et al.* 1986), none of them was reported to be temperature sensitive. The basic mechanism of temperature-dependent, cytoplasmically induced chlorophyll deficiency observed in the above alloplasmic common wheats is not clear yet.

First, I studied this phenomenon by several biochemical means, using common wheat cultivar Chinese Spring with the C^{u2} cytoplasm of *Ae. columnaris* (abbrev. (C^{u2})-CS).

Next, I investigated some chloroplast proteins and the chloroplast genes encoding them. In previous works (Chen *et al.* 1975; Hirai and Tsunewaki 1981) two electrophoretic variants, H- and L-type, of the large subunit of ribulose 1, 5-biphosphate carboxylase/oxygenase (EC 4.1.1.39), hereafter Rubisco, were reported among *Triticum* and *Aegilops* species. The gene, *rbcL*, coding for this subunit has been sequenced using four *Triticum* and *Aegilops* species having divergent chloroplast genomes (Terachi *et al.* 1987, 1988). From the results, they assumed two amino acid substitutions and one amino acid addition that occurred in this subunit in three *Aegilops* species. The comparison of chloroplast proteins among alloplasmic wheats by two-dimensional gel electrophoresis showed that the β subunit of chloroplast ATP synthase also is differentiated into two types, and their distribution among *Triticum* and *Aegilops* species is the same as that of the Rubisco large subunit (Bahrman *et al.* 1988). However, so far, no specific chloroplast protein is known to be responsible for the chlorophyll deficiency. Therefore, I analyzed thylakoid membrane proteins, different subunits of which are known to be encoded by chloroplast genes, by two-dimensional gel electrophoresis using (C^{u2})-, (D^2)- and (Mt)-CS as materials. Thirdly, complete nucleotide

sequences of the genes coding for the β and ϵ subunits of chloroplast ATP synthase and the gene for chloroplast methionine tRNA were determined using (C^{u2}) - and (D^2) -CS which are different in chlorophyll deficiency expression, in order to clarify the relationship between the structural differences in the chloroplast genes and their corresponding products.

2. Materials and Methods

1) Plant Materials

In the study on chlorophyll deficiency, a normal line of Chinese Spring (abbrev. (B)-CS) and its alloplasmic lines with *Ae. columnaris* cytoplasm ((C^{u2}) -CS) and *Ae. triuncialis* cytoplasm ((D^u) -CS) were used. Seeds soaked in water at 25 °C overnight were sown on wet paper towels in a plastic case, and kept in an incubator in dark at 0 °C for given periods. After the treatment, seedlings were transferred to a plastic container filled with vermiculite and placed under fluorescent illumination in a cold storage room kept at 5 °C unless otherwise indicated. The seedling leaves were collected when the first leaves fully developed.

When the seedlings grew to full development of the first leaf at 5 °C after 30 days treatment at 0 °C, they were transplanted into flats in a greenhouse adjusted at 21 °C for the study of recovery of chlorophyll deficiency. At the time of transplantation, the base of the first leaf was marked with magic ink to distinguish the already developed part from the newly developed part at 21 °C. In this experiment, chlorophyll content was determined after Moran (1982) by direct immersion of the intact leaf into the solvent, N,N-dimethylformamide (DMF).

In the study of thylakoid proteins, (B)-, (C^{u2}) -, (D^2) - and (Mt)-CS were used as the source of thylakoid membrane. Seeds soaked in water at 25 °C overnight

were sown in a container filled with vermiculite and placed in a greenhouse at 21 °C.

Molecular cloning and nucleotide sequencing of the DNA fragments that contain the chloroplast genes, *atpB*, *atpE* and *trnM*, were carried out using (C¹⁸)- and (D²)-CS. In this case, seedlings were grown in dark in a greenhouse for two weeks at 21 °C.

2) Preparation and subfractionation of chloroplast proteins

i) Preparation of thylakoid membrane

Chloroplast thylakoid membrane was prepared as follows: The leaves were cut to about 5 mm long pieces and homogenized by a Polytron for 10 sec at high speed in an isolation buffer containing 0.4 M sucrose, 50 mM KH₂PO₄, 15 mM NaCl, 5 mM MgCl₂, 1 mM EDTA and freshly prepared 12 mM L-ascorbic acid, and finally adjusted to pH 7.8 by NaOH. The ratio of the leaves to buffer was about 1:5 (W/V). The homogenate was filtrated through four layers of cheesecloth, and then centrifuged at 4,000 x g for 10 min. The pellet was resuspended and washed once or twice in the same buffer. The resulted pellet was resuspended in the isolation buffer containing 30 % glycerol and stored at -70 °C. Chlorophyll concentration of the membrane suspension was determined after Arnon (1949).

ii) Subfractionations of thylakoid membrane

Photosystem II membrane (grana fraction) was prepared from isolated thylakoid membrane after Berthold *et al.* (1981) with slight modifications as follows: Twenty percent Triton X-100 was added dropwise, on ice, to thylakoid membrane suspended in the isolation buffer (see above) to the weight ratio of 25:1 for the detergent to chlorophyll. The condition was chosen in such a way that the final chlorophyll concentration became 2-2.5 mg/ml. After 1 hr of stirring

on ice, the suspension was centrifuged at 12,000 x g for 10 min. The supernatant was centrifuged at 140,000 x g for 1 hr. The pellet was resuspended in the isolation buffer and chlorophyll concentration was determined after Arnon (1949).

Sodium bromide (NaBr) soluble proteins were prepared from isolated thylakoid membrane after Kamienietzky and Nelson (1975) with the following modifications: Solution of 2 M NaBr was added to the membrane pellet to give a final chlorophyll concentration of 9 mg/ml. After 30 min of stirring on ice, the mixture was centrifuged at 20,000 x g for 10 min. The supernatant was centrifuged at 15,000 x g for 20 min to remove insoluble fraction. The supernatant was precipitated with 80 % acetone followed by centrifugation at 1,500 x g for 4 min. The pellet was solubilized by a sample buffer containing 5 % SDS, 5.5 M urea and 5 % 2-mercaptoethanol.

iii) Isolation of prolamellar bodies from etiolated and greened leaves

Isolation of prolamellar bodies was carried out after Redlinger and Apel (1980) and Apel *et al.* (1980) with the following modifications: Etiolated or greened leaves (illumination for 7, 12, 20 or 24 hr) were homogenized in 0.5 M sucrose, 1 mM MgCl₂, 0.1 % BSA, 100 mM potassium phosphate (pH 7.8) by a blender. The solution was filtrated through four layers of cheesecloth, and then centrifuged at 200 x g for 5 min. The supernatant was centrifuged at 4,000 x g for 10 min. The etioplast pellet was washed once in the same buffer and centrifuged again at 4,000 x g for 10 min. The etioplasts were suspended in a mixture of 50 mM potassium phosphate (pH 7.8) and 20 % glycerol (V/V). The suspension was underlayered with a mixture of 0.5 M sucrose, 1 mM MgCl₂, 1 mM EDTA, 30 mM potassium phosphate (pH 7.2), and centrifuged at 39,100 x g for 20 min. The pellet was resuspended in a mixture of 10 % glycerol, 1 % 2-mercaptoethanol and 65 mM Tris-HCl (pH 6.8).

Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Protein samples were stored at -20 °C until use.

3) Polyacrylamide gel electrophoresis (PAGE) of chloroplast proteins

i) SDS-urea-PAGE of chloroplast proteins

Analysis of thylakoid membrane polypeptides using SDS-urea-PAGE was carried out using the discontinuous buffer system of Laemmli (1970). Four molar urea was added to the stacking and separation gels that contained 6 % and 14 % acrylamide, respectively. Thylakoid membrane (15-20 mg chlorophyll) were pelleted by centrifugation at 7,000 x g for 1 min. The pellet was solubilized in the sample buffer containing 5.5 M urea, 5 % SDS and 5 % 2-mercaptoethanol with strong mixing, and then centrifuged at 7,000 x g for 5 min. The supernatant was electrophoresed with 1 mm thick gel at room temperature with a constant current of 10 mA. The gel was stained with Coomassie brilliant blue R250.

ii) Two-dimensional gel electrophoresis of chloroplast proteins

(a) *Solubilization of membrane proteins*

Thylakoid proteins containing 100 mg chlorophyll (about 400 mg proteins) were precipitated by an ice-cold 80 % acetone (v/v) containing 35 mM 2-mercaptoethanol following centrifugation at 1300 x g for 2 min. This treatment was repeated three times in order to remove pigments and lipids which could interact with proteins and change their characteristics, resulting in some electrofocusing artifacts (Remy and Ambard-Bretteville 1983).

The protein pellet was solubilized in 25 ml of the solubilization buffer containing 2 % SDS and 50 mM Tris-HCl (pH 6.8). The protein was incubated at 70 °C for 20 min and placed in water for a moment for cooling. Then 20 mg of solid urea were added to each sample. The solubilized sample was diluted with two volumes of the sample dilution buffer which contained 9.5 M urea, 2 % ampholines (LKB, pH 5-8), 5 % 2-mercaptoethanol (v/v), and 8 % Triton X-100. Triton X-100 was added to give a final Triton/SDS ratio of 8:1 (Ames and Nikaido 1976). The sample mixture was then centrifuged at 10,000 x g for 2 min

to remove the insoluble fractions and the supernatant was subsequently used for isoelectric focusing.

(b) Isoelectrofocusing

Isoelectric focusing (the first dimension) was carried out as described by O'Farrel (1975) with the following modifications: For five gel tubes (130 mm length, 2.5 mm inner diameter) sealed their bottom with cellophane, 3 ml of the gel solution containing 9.2 M urea, 0.59 ml distilled water, 0.6 ml 10% Triton X-100, 0.4 ml 30% acrylamide (28.38 % acrylamide and 1.62 % bis acrylamide), 150 μ l ampholine (LKB, pH 5-8), 5 ml 10 % ammonium persulfate (freshly prepared) and 3.5 μ l N,N,N',N'-tetramethylene diamine (TEMED) were prepared. The tubes were filled with this solution and overlaid with 10 μ l 8 M urea. After polymerization (about 1 hr), 8 M urea was removed and 50 μ l solubilized sample was loaded. The samples were overlaid with 10 μ l of the sample overlay solution containing 8 M urea and 1 % ampholine (LKB, pH 5-8) and filled with 0.02 M NaOH. The tubes were placed in a standard tube gel electrophoresis chamber. The upper cathode reservoir was filled with 0.02 M NaOH, and the lower reservoir with 0.01 M H₃PO₄. The electrophoresis was carried out at 400 V for 16-17 hr, followed by 800 V for 0.5-1 hr. After the electrophoresis, the gel was removed from the tube applying water pressure through the needle of a 5 ml syringe.

The gel was pushed out with the cap of a Pasteur pipette into the 5 ml equilibration buffer containing 5 % 2-mercaptoethanol, 2.3 % SDS, 62.5 mM Tris-HCl (pH 6.8) and 5 M urea, and kept at room temperature for 1 hr, and stored at -70 °C.

(c) SDS-urea-PAGE

The electrophoresis applied for the second dimension (SDS-urea-PAGE) was essentially the same as described in i) of this section. An equilibrated

isoelectric focusing gel was loaded onto the stacking gel with the equilibration buffer which prevented bubble formation between the gels. The electrophoresis with 1 mm thick gel was carried out at 20 mA as usual.

iii) Peptide mapping by SDS PAGE

The 56 kD protein spots separated by two-dimensional electrophoresis from (B)- and (D²)-CS were compared by the digestion with *Staphylococcus aureus* V8 protease according to Gooderham (1984). The spots were cut out from the gel and equilibrated in 5 ml of equilibration buffer containing 0.1 % SDS, 1 mM EDTA and 12.5 mM Tris-HCl (pH 6.8). After 1 hr the buffer was replaced with a further 5 ml of fresh buffer and the equilibration continued for further one hour. And then, the gel slices were placed in the sample wells of 2 mm thick SDS polyacrylamide gel with 5 cm stacking gel. The gel slices were covered with the equilibration buffer containing 20 % glycerol followed by 10 ml of the equilibration buffer containing 10 % glycerol and 0.05 mg of the V8 protease. The samples were then electrophoresed at a constant current of 20 mA until the marker dye reached the top of the separating gel when the current was increased to 40 mA for the remainder of the run. Peptides were fixed and subsequently visualized by silver staining using the kit, Silver Stain Wako (Wako Pure Chemical Industry Co. Ltd.)

4) Photochemical activity measurements

Whole chain, photosystem II (PSII) dependent, and photosystem-I (PSI) dependent electron transport activities were measured. Freshly prepared thylakoid membrane (15 mg chlorophyll/run) was used for this study. For measurement of the whole chain electron transport, a reaction mixture containing 25 mM methyl viologen as an autooxidation reactant of the terminal electron acceptor, 1 mM NaN₃ as a catalase inhibitor and 5 mM NH₄Cl as an uncoupler was used. For measurement of the PSI electron transport, the

reaction mixture containing 25 μ M DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) as PSII inhibitor, 0.1 mM DCIP (2,6-dichlorophenol indophenol) with 5 mM ascorbate as an electron donor and methyl viologen as an electron acceptor and 5 mM NH_4Cl was used. The reaction mixture for measuring the PSII electron transport contained 0.3 mM phenyl-*p*-benzoquinone as an electron acceptor and 5 mM NH_4Cl . All the reactions were carried out in the isolation buffer containing 30 % glycerol (see above). The whole-chain and PSI electron transport were measured as oxygen uptake mediated by the Mehler reaction, and the PSII activity as oxygen evolution at 25 °C, using a Clark type oxygen electrode.

5) Fluorescence microscopy

The observation of chloroplast nucleoids stained with 4'6-diamidino-2-phenylindole (DAPI) was made with a Nikon epifluorescent microscope equipped with a 100 W Hg lamp. Observation of DAPI fluorescence was carried out with a UV excitation filter (Nikon, 330-380 nm) in combination with a 420 nm suppression filter. Autofluorescence of chlorophyll was observed by a Blue excitation filter (Nikon, 420-490 nm) and a 520 nm suppression filter. Photographs were taken with Kodak Ektachrome (400 ASA) film.

These observations were carried out using protoplasts isolated from intact leaves. Protoplasts were isolated according to the method of Okuno and Furusawa (1977) as follows: The abaxial epidermis of the first leaves was incised with a razor blade and peeled off by hand. These leaves were soaked in the isolation buffer containing 1 % Cellulase Onozuka R-10 and 0.6 M mannitol at 25 °C for 2-3 hr. Subsequently, the suspension was filtrated through one layer of Miracloth and centrifuged at 150 x g for 2 min. The pellet was washed once with the washing medium containing 0.6 M mannitol and 10 mM CaCl_2 , and resuspended in the same medium. The protoplast suspension was placed on a slide glass, then mixed with the buffer-NS (Kuroiwa *et al.* 1981) containing

0.25 M sucrose, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.1 mM ZnCl₂, 0.8 mM PMSF, 0.05 % 2-mercaptoethanol, 0.5 % glutaraldehyde and 50 mg/ml DAPI.

6) Molecular cloning and DNA sequencing of three chloroplast genes, *atpB*, *atpE* and *trnM*, of (C^{U2})- and (D²)-CS

Isolation of chloroplast and purification of chloroplast DNAs were made after Terachi *et al.* (1987). All the three genes are located in the 2.3 kb *Bam*HI-*Pst*II fragment and 2.0 kb *Pst*II-*Pst*II fragment (ref. Figure 13). Using plasmid pUC119, each of these fragments of *Ae. crassa* ctDNA was subcloned from its B2I fragment (Terachi *et al.* 1987).

In case of (C^{U2})-CS, these fragments were cloned directly into the corresponding restriction sites of the plasmid pUC119, and the inserts were confirmed by the colony hybridization (Maniatis *et al.* 1982).

Preparation of deletion derivatives from these two subfragments and that of single stranded DNAs were made after Terachi *et al.* (1987), using the competent *E. coli* MV1184.

Dideoxy chain termination reaction using single strand DNAs or denatured double strand DNAs was carried out with the sequencing kit (Takara Shuzo, Japan) and Sequenase ver. 2 (U.S.B., U.S.A.), under the conditions indicated by the manufacturers, using universal primer and synthetic oligonucleotides (17 mer). The synthetic oligonucleotides were prepared using a Model 391 PCR Mate DNA synthesizer (Applied Biosystems). The oligonucleotide was eluted from the reaction column with 25% ammonia solution and was incubated at 55 °C overnight. After evaporating the solution, the sample was dissolved in water, then purified by ethanol-precipitation. The concentration of the oligonucleotide was adjusted to 1 pmol/μl.

The data analysis of the nucleotide sequences and estimation of isoelectric

3. Results

1) Chlorophyll deficiency in (Cu²)-CS

i) Induction of chlorophyll deficiency under different conditions

Seedlings of the alloplasmic lines grown at 5 °C for about 30 days after the 30-day treatment at 0 °C exhibited chlorophyll deficiency in the middle part of the first leaves, and eventually became almost completely albinic. The tip and basal parts of the leaves contained some chlorophyll (Figure 1). Under a microscope, chlorophyll was usually found along the veins even in the albinic part of the leaves.

The chlorophyll deficiency was induced in (Cu)- and (Cu²)-CS, when grown at 5 °C after the 17-, 23- or 30-day treatment at 0 °C. The degree of chlorophyll deficiency induced by these treatments varied somewhat among seedlings of the same alloplasmic lines, from normally green to almost white when grown at 5 °C after the treatment (Figure 2). But the seedlings of (Cu²)-CS derived from the same ear did not show such wide variation. Chlorophyll deficiency was induced more clearly in (Cu²)-CS than in (Cu)-CS, although the growth inhibition was less in the former than in the latter under these conditions (Figure 1). Therefore, (Cu²)-CS was used in further studies.

The chlorophyll content of (Cu²)-CS grown at 21 °C without the 0 °C treatment was not significantly different from (B)-CS. Compared to (B)-CS, the chlorophyll content of (Cu²)-CS decreased by 20 % in the seedlings grown at 5 °C without the 0 °C treatment, and by 51 % and 56 % in the seedlings grown at 5 °C after the 0 °C treatment for 30 and 40 days, respectively (Table 2).

Chlorophyll deficiency was not induced in the seedlings kept at 18 °C after the 0 °C treatment, but the leaves of seedlings developed at 5 °C for 33 days after the 0 °C treatment for 30 days did not recover from chlorophyll

deficiency even after transfer to 21 °C (Table 3). The chlorophyll a/b ratio was slightly higher in the alloplasmic line than in (B)-CS under these conditions.

ii) Effect of a suppressor gene on the chlorophyll deficiency

In the seedlings of (C^{u2})-JF, chlorophyll deficiency was not conspicuous even with the severe cold treatment, and its chlorophyll content was 77 % of (B)-JF when grown at 5 °C after 0 °C treatment for 40 days (Table 2). When (C^{u2})-CS, as female, was crossed with JF, the F₁ hybrids also exhibited the chlorophyll deficiency at low temperature. The progenies of a cross between the F₁ hybrids and JF as male segregated into 1:1 ratio for the normally green vs. chlorophyll deficient. From these results, it is assumed that JF carries a single recessive gene for chlorophyll deficiency suppression in good agreement with the result of Tsujimoto (1983).



Figure 1. Chlorophyll deficiency induced in the first leaves of alloplasmic CS by growing at 5 °C after the 30-day treatment at 0 °C
 B: (B)-CS, C^u: (C^u)-CS, C^{u2}: (C^{u2})-CS.

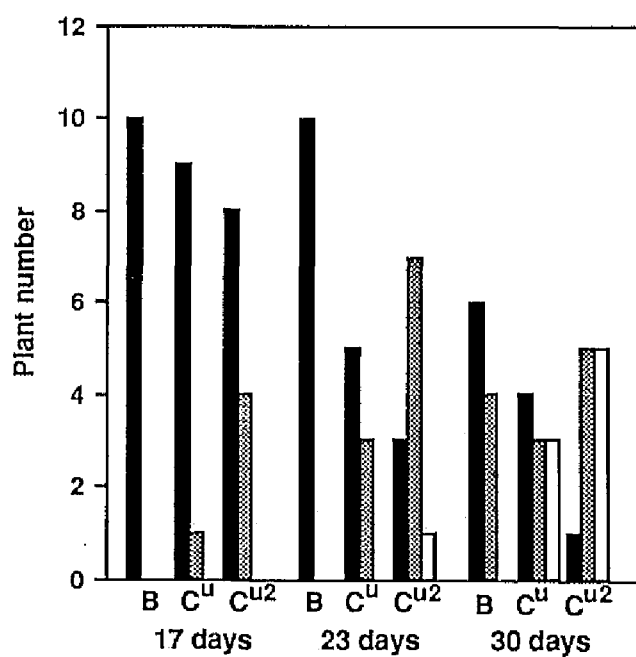


Figure 2. Segregation of chlorophyll deficiency in (B)-, (C^u)- and (C^{u2})-CS treated for indicated periods at 0°C. ■: Normally green, □: white, ▨: intermediate between normally green and white

Table 2. Effect of temperature on chlorophyll content of (B)-CS and (C^{u2})-CS

Treatment	Total chlorophyll content (mg/g fr.wt.)	a/b ratio
Under 21°C		
(B)-CS	1.18	3.48
(C ^{u2})-CS	1.23 (104%)	3.51
Under 5°C		
(B)-CS	1.88	3.31
(C ^{u2})-CS	1.51 (80%)**	3.60**
0°C, 30 days → 5°C		
(B)-CS	1.88	3.19
(C ^{u2})-CS	0.95 (51%)**	3.26**
0°C, 40 days → 5°C		
(B)-CS	1.19	3.25
(C ^{u2})-CS	0.66 (56%)**	3.38
(B)-JF	2.21	3.53
(C ^{u2})-JF	1.70 (77%)	3.70
0°C, 30 days → 18°C		
(B)-CS	1.31	3.53
(C ^{u2})-CS	1.29 (98%)	3.78**

(): Percent of (B)-CS.

** : Significantly different from (B)-CS at 1 % level.

Table 3. Recovery of chlorophyll content at 21°C in the initially chlorophyll deficient leaves

Treatment	Total chlorophyll content (mg/g fr.wt.)	a/b ratio
0°C, 30 days → 5°C, 43 days		
(B)-CS	1.79	2.69
(C ^{u2})-CS	0.29 (16%)**	3.38**
0°C, 30 days → 5°C, 33 days → 21°C, 10days		
(B)-CS	2.37	2.40
(C ^{u2})-CS	0.73 (31%)**	2.87**

(): Percent of (B)-CS.

** : Significantly different from (B)-CS at 1 % level.

2) Chloroplast protein composition in chlorophyll deficient leaf

In order to analyze the thylakoid membrane protein composition, thylakoid membrane and its subfractions were prepared from the seedlings of (B)-CS and (Cu²)-CS grown at 21 °C or under low temperature. The membrane proteins were resolved into more than 20 bands by SDS-urea-PAGE using 4 M urea (Figure 3). The predominant proteins were PSI reaction center (CPI), CF1 α and β subunits, chlorophyll protein II (CPII, 47, 42 kDa), PSII reaction center, oxygen evolving complex (33, 23, 17kDa) and light harvesting chlorophyll a/b complex (LHCII).

The thylakoid membrane isolated from the seedlings of (Cu²)-CS grown at 21 °C without the 0 °C treatment had the same protein composition as that of (B)-CS (Figure 3). When grown at 5 °C without the 0 °C treatment, several new proteins appeared in the both seedlings, their molecular masses were 44, 42, 37, 20.5, 18.5, 17.5 and 14.0 kDa (Figure 3). The amounts of these proteins were larger in the seedlings grown at 5 °C after the 0 °C treatment for 30 days than those in the seedlings grown at 5 °C without the 0 °C treatment, especially in the alloplasmic line. On the contrary, the relative contents of PSII proteins (grana in Figure 3) decreased by the 0 °C treatment.

In order to characterize these new proteins, subfractionation of thylakoid membrane and isolation of prolamellar bodies during greening process were carried out, which were followed by SDS-urea-PAGE of their proteins. Surprisingly, the proteins appeared in the seedlings of (B)- and (Cu²)-CS grown under low temperature were also found in the 2 M NaBr soluble fraction of thylakoid membrane of (B)-CS grown at 21 °C, although the ratios between three proteins (44, 42 and 37 kDa) in the 2 M NaBr soluble fraction were different than those observed in the cold treated seedlings (Figure 3, lane a).

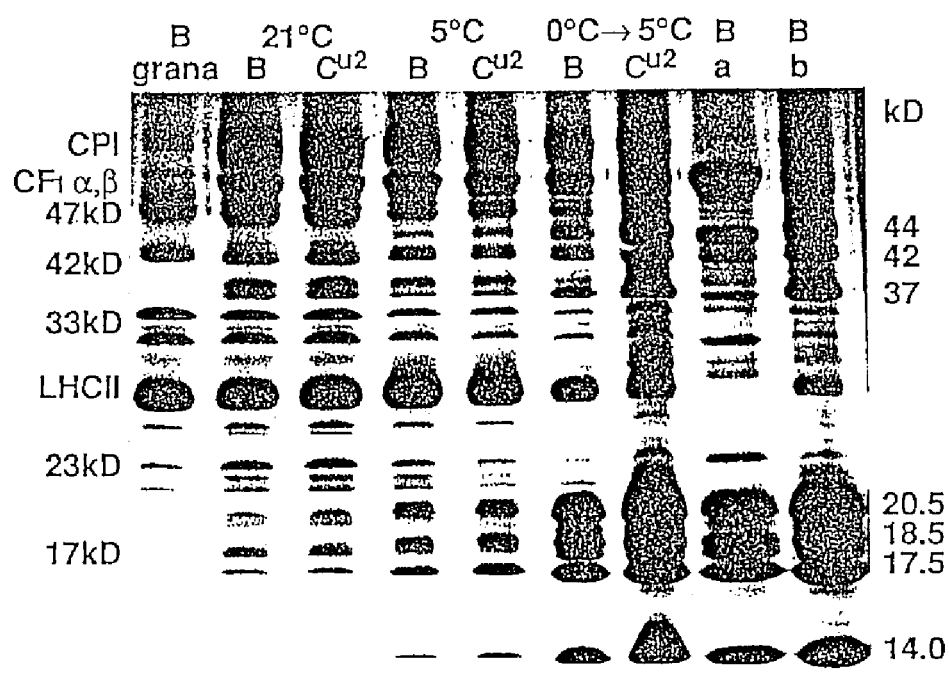


Figure 3. Protein profile of the thylakoid membrane of (B)- and (Cu²)-CS. B: (B)-CS, Cu²: (Cu²)-CS, grana: prepared from seedlings of (B)-CS grown at 21 °C, a: 2 M NaBr soluble fraction of thylakoid membrane prepared from seedlings of (B)-CS grown at 21 °C, b: thylakoid membrane prepared from seedlings illuminated for 20 hr at 25 °C.

Prolamellar bodies were isolated from the seedlings of (B)-CS grown at 25 °C in the dark or under illumination for various periods. Proteins of prolamellar bodies isolated from the etiolated leaves contained NADPH:protochlorophyllide oxidoreductase as well as the proteins appeared at low temperature (Figure 4). With continuous illumination, both proteins were replaced by photosystem-constituting proteins (Figure 4). Protein components of thylakoid membrane obtained from the seedlings greened with 20-hr illumination at 25 °C without the 0 °C treatment were very similar to those of the leaves grown at 5 °C after the 0 °C treatment, especially in (Cu²⁺)-CS (Figure 3, lane b).

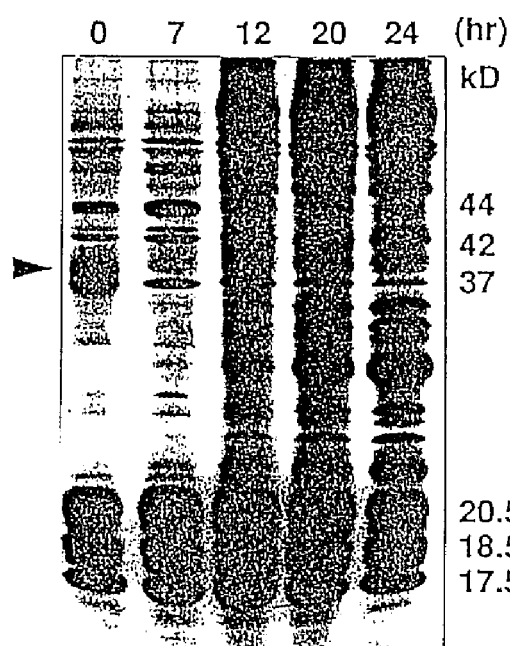


Figure 4. Protein profile of the prolamellar bodies isolated from leaves, not illuminated (0 hr) or illuminated for 7, 12, 20 and 24 hr, of (B)-CS grown at 25 °C, observed by SDS- urea-PAGE. Arrowhead indicates NADPH:protochlorophyllide oxidoreductase

Membrane and soluble protein contents per fresh leaf weight were low in the low-temperature-treated (Cu^{2+})-CS (Figure 5). The soluble fraction which includes Rubisco showed no difference between the two lines grown at a low temperature (Figure 5).

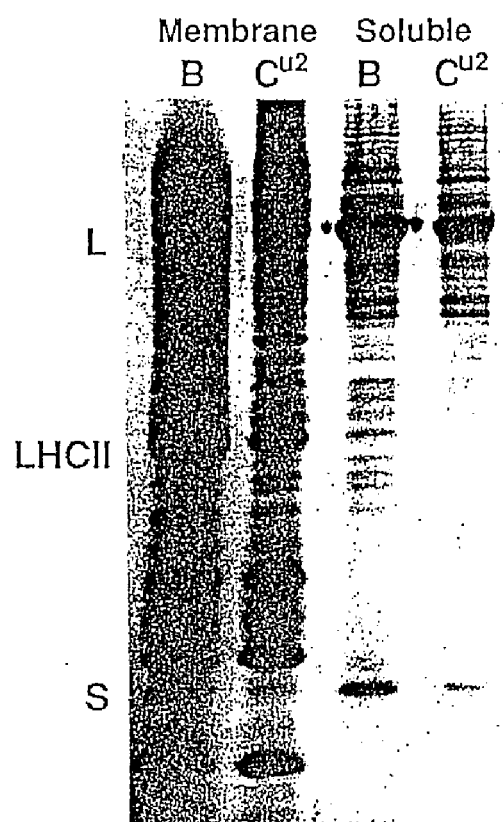


Figure 5. Protein profile of the thylakoid membrane and soluble protein fraction prepared from the same weight of fresh leaves of (B)-CS and (Cu^{2+})-CS grown at 5 °C after 0 °C treatment. L and S: Rubisco large and small subunits, respectively

3) Photochemical activities

Photochemical activities, namely, whole chain, photosystem I (PSI) and photosystem II (PSII) dependent electron transport, were measured in an attempt to identify functional lesions of the chloroplasts. Isolated thylakoid membranes were used for this assay. In the seedlings grown at 21 °C without the 0 °C treatment, no significant difference in the whole chain, PSI and PSII activities on the basis of chlorophyll was detected between (B)-CS and (C^{u2})-CS (Table 4). However, in the seedlings grown at 5 °C after the 0 °C treatment, the whole chain and PSII activities in the alloplasmic line were significantly lower (ca. 50%) and the PSI activity was slightly lower than those in (B)-CS.

Table 4. Photochemical activities of (B)-CS and (C^{u2})-CS grown under two different conditions

Treatment	Whole chain ^a	PSII ^b	PSI ^c
Under 21°C			
(B)-CS	271.8	285.6	562.1
(C ^{u2})-CS	262.6 (97%)	262.6 (92%)	571.3 (102%)
0°C, 30 days → 5°C			
(B)-CS	152.1	152.1	938.0
(C ^{u2})-CS	82.2 (54%)	71.8 (47%)	801.1 (85%)

(): Percent of (B)-CS.

a: H₂O → Methyl viologen: O₂ uptake (μmol O₂/mg Chl/hr).

b: H₂O → Phenyl-p-benzoquinone: O₂ evolution (μmol O₂/mg Chl/hr).

c: DCIP → Methyl viologen: O₂ uptake (μmol O₂/mg Chl/hr).

4) Morphology of chloroplast nucleoids

The analyses on proteins and photochemical activities on the basis of chlorophyll give informations only on the part of leaves where chlorophyll is associated. In order to know directly the characteristics of the chlorophyll deficient tissue, the morphological change in chloroplast nucleoids was studied by DAPI staining, using the first leaf developing at 5 °C after the 0 °C treatment for 30 days. In an intact leaf, DAPI stained both cell walls and nucleoids, and, therefore, observation of the chloroplast nucleoids was difficult. For this reason, protoplasts isolated from the leaves were used in most part of this study. The protoplast population was more or less heterogeneous in both lines, as to the shape of the nucleoid and its number per chloroplast. Chloroplast of most protoplasts isolated from developing and developed leaves of (B)-CS contained small spherical nucleoids which were dispersed in it, whereas chloroplast of some protoplasts contained a single large nucleoid in the developed leaf (Figure 6a-c). On the other hand, the chloroplasts isolated from the developing and developed leaves of the (Cu²⁺)-CS contained ring shaped nucleoid, and irregular shaped or degraded nucleoids, respectively (Figure 6d-f). These observations were made using the protoplasts devoid of chlorophyll by eye under visible light. However, the fluorescence of chlorophyll could be observed even in these protoplasts (Figure 7d-f). This fluorescence also decreased in the chloroplasts that contained degraded nucleoids (Figure 7f).

Many chloroplasts of the chlorophyll deficient leaves that were kept at 5 °C after the treatment at 21 °C for 1 or 2 days contained a big nucleoid. However, these nucleoids had low fluorescent intensity and obscure outline, and easily degraded under UV light.

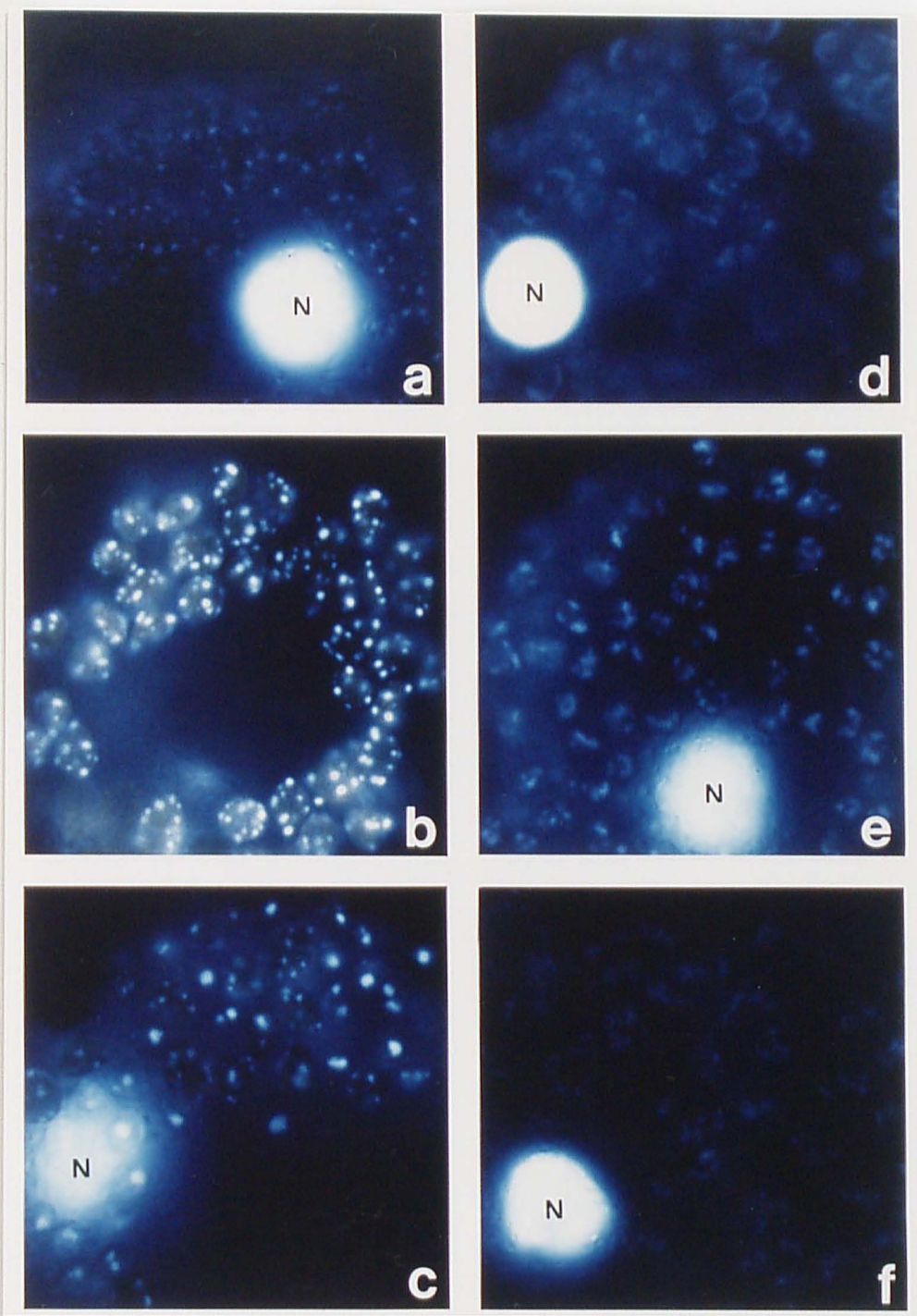


Figure 6. Epifluorescence microphotographs of chloroplast nucleoids in protoplasts of the first leaves grown at 5 °C after the 30 days treatment at 0 °C. Samples were prepared from (B)-CS (a, b and c) and (C^{u2})-CS (d, e and f) and developing (a and d) and developed leaves (b, c, e and f). N: nucleus

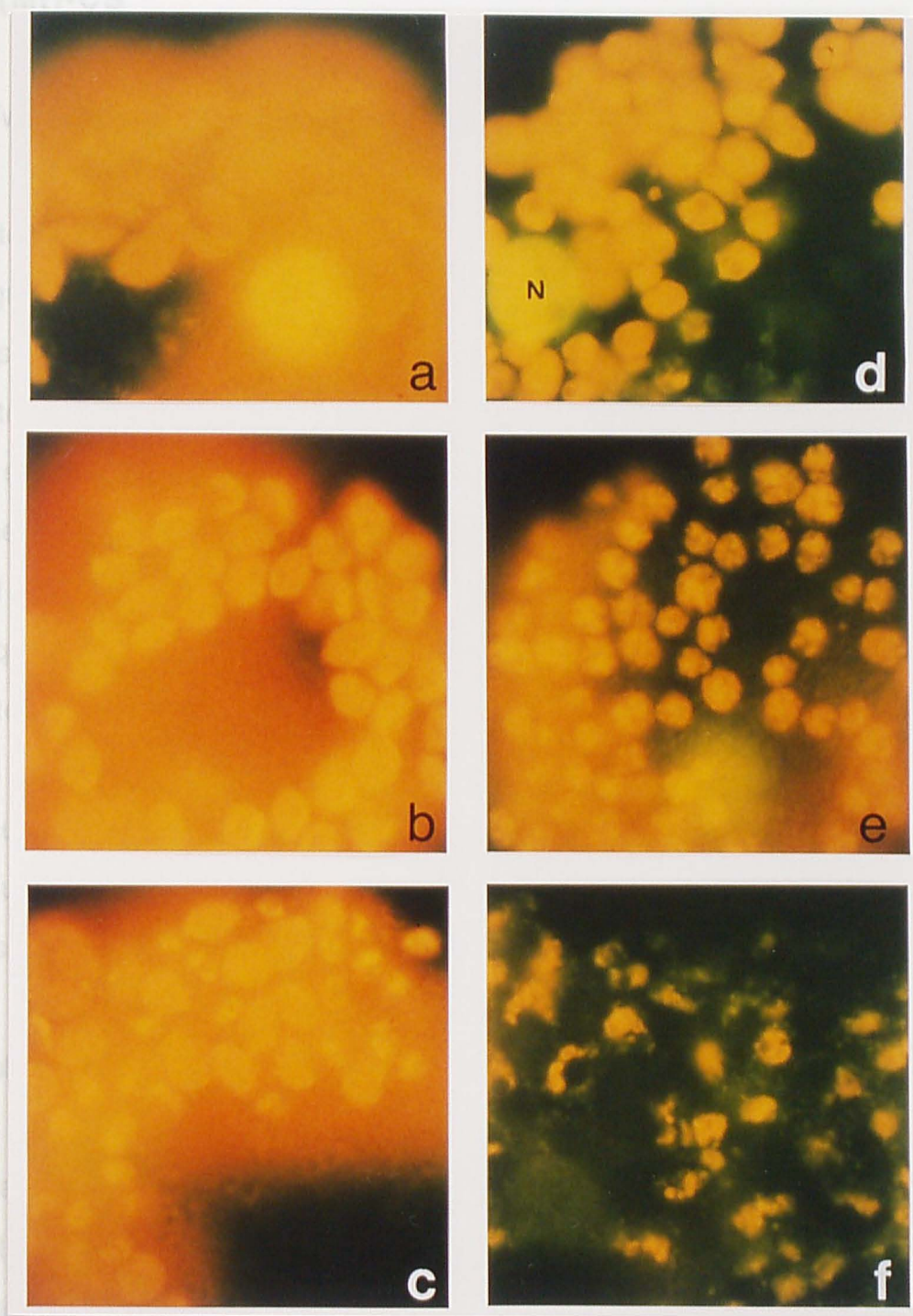


Figure 7. Fluorescence of chlorophyll in protoplasts from the chlorophyll deficient tissue of (B)-CS and (C^{u2})-CS. a to f correspond to those in Figure 6. N: nucleus

5) Variation on chloroplast proteins among (Cu²⁺)-, (D²)- and (Mt)-CS

i) Two-dimensional gel electrophoresis of chloroplast proteins

I could not find any difference in the molecular weights of the thylakoid proteins among (B)-, (Cu²⁺)-, (D²)- and (Mt)-CS using SDS-urea-PAGE (Figure 8). Therefore, in order to find out the difference in the proteins among these alloplasmic wheats, two-dimensional electrophoresis of thylakoid proteins was carried out. In this case, thylakoid membranes obtained from the seedlings grown at 21 °C were used. Although many spots were observed at the same positions among these lines, three spots (spots 1, 2 and 3 in Figure 9) showed different isoelectric point and, in addition, a single unique spot (spot 4) appeared in all alloplasmic lines, as compared to the protein profile of (B)-CS. The spot 1 had a molecular mass of about 56 kDa and shifted to alkaline side (Figure 9). The spot 2 had a molecular mass of about 55 kDa and shifted to acidic side. The spot 3 had a molecular mass of about 31 kDa and shifted to alkaline side. The unique spot 4 had a molecular mass of about 56 kDa.

To identify these proteins, subfractionated thylakoid membrane and soluble protein fraction of the leaves in (B)- and (Cu²⁺)-CS were separated. The two 56 kDa spots (spots 1 and 4) of (Cu²⁺)-CS were found in the proteins extracted from thylakoid membrane with 2 M NaBr (Figure 10). The 55 kDa spot was observed in the soluble protein fraction of both lines (Figure 11). Origin of the 31 kDa spot could not be clarified. Photosystem II-constituting proteins showed poor resolution, except 33 and 24 kDa extrinsic proteins, and the 31 kDa proteins could not be identified clearly in this fraction.

From these results, the specific proteins discovered in alloplasmic wheats seemed to correspond to the following proteins; the two 56 kDa proteins to the β subunit of CF₁, and the 55 kDa protein to the large subunit of

Rubisco.

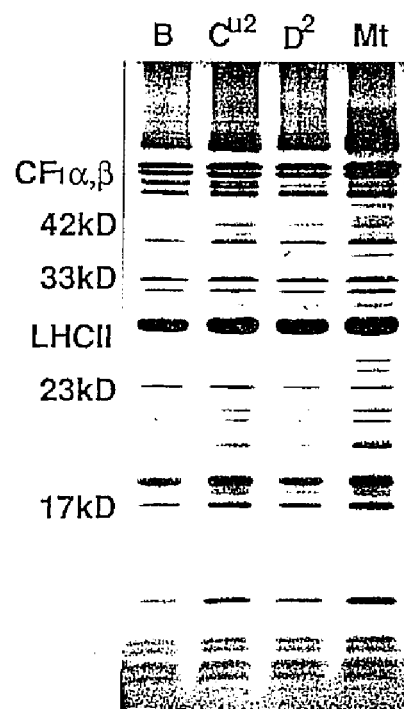


Figure 8. Protein profile of the thylakoid membranes of CS with four types of cytoplasms

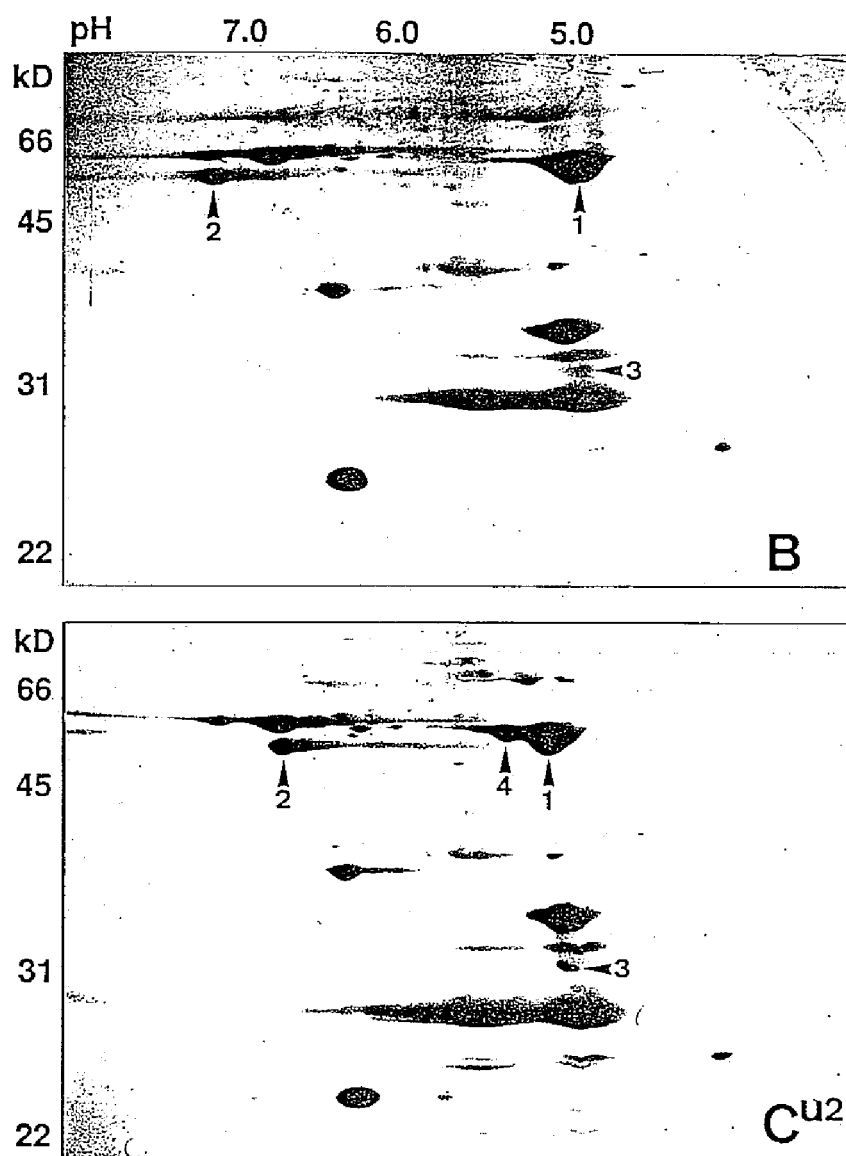


Figure 9. Two-dimensional electrophoresis of the thylakoid proteins of (B)-CS, (Cu^{2+})-CS, (D^2)-CS and (Mt)-CS grown at 21 °C. B: (B)-CS, Cu^{2+} ; (C): (Cu^{2+})-CS, Mt: (Mt)-CS, D^2 : (D^2)-CS

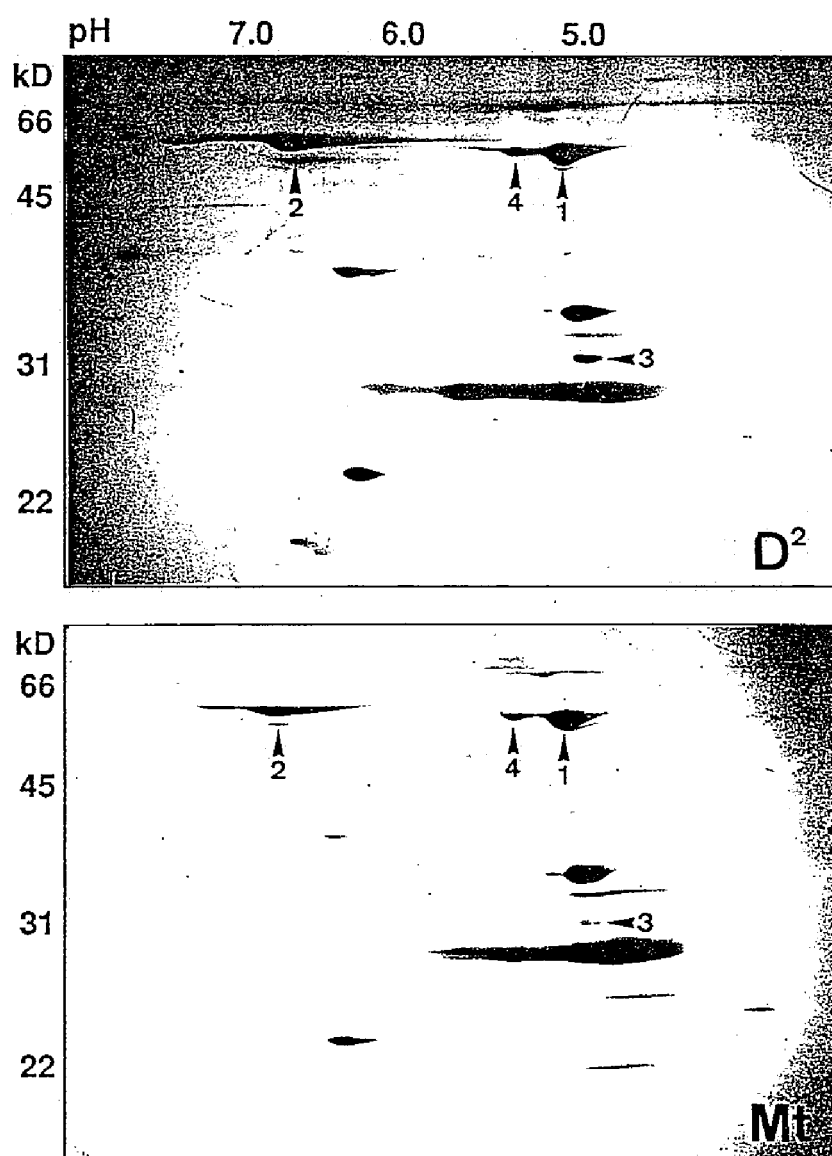


Figure 9. (continued)

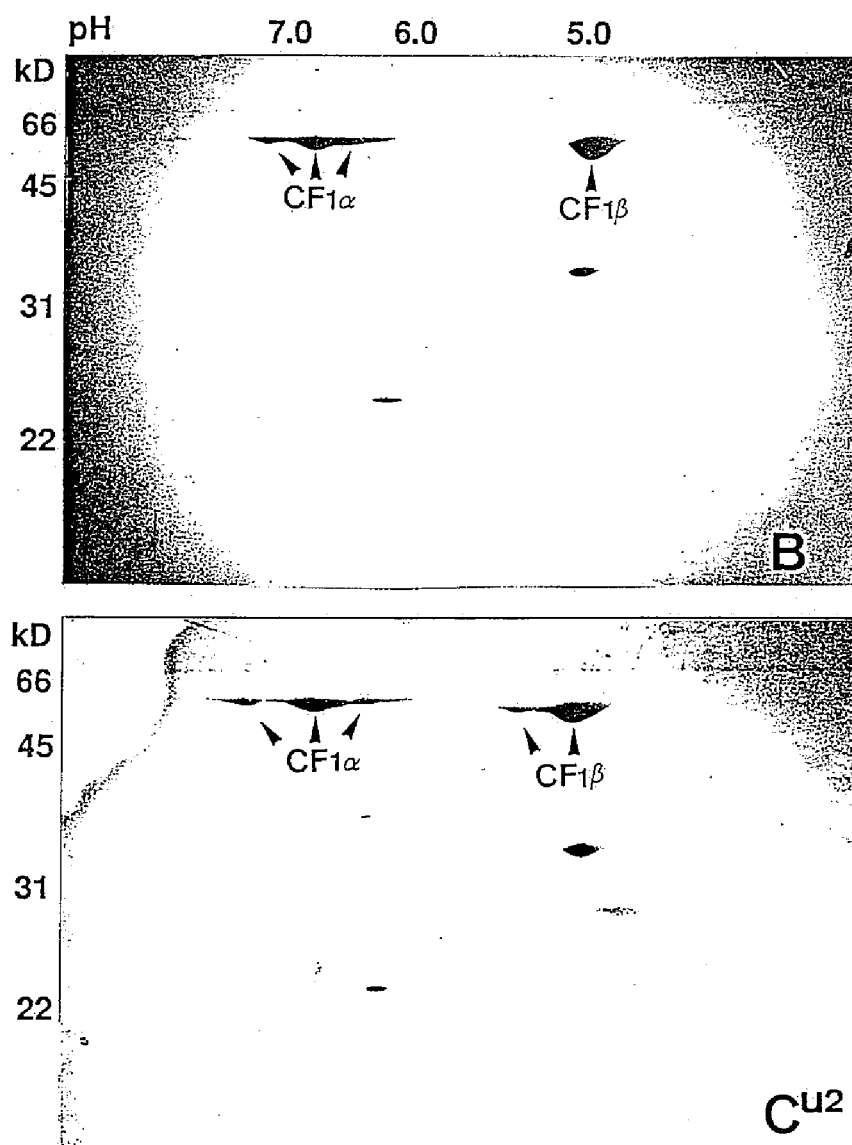


Figure 10. Two-dimensional electrophoresis of the 2 M NaBr soluble fraction of the thylakoid membrane of (B)- and (C^{u2})-CS grown at 21 °C. B: (B)-CS, C^{u2}: (C^{u2})-CS

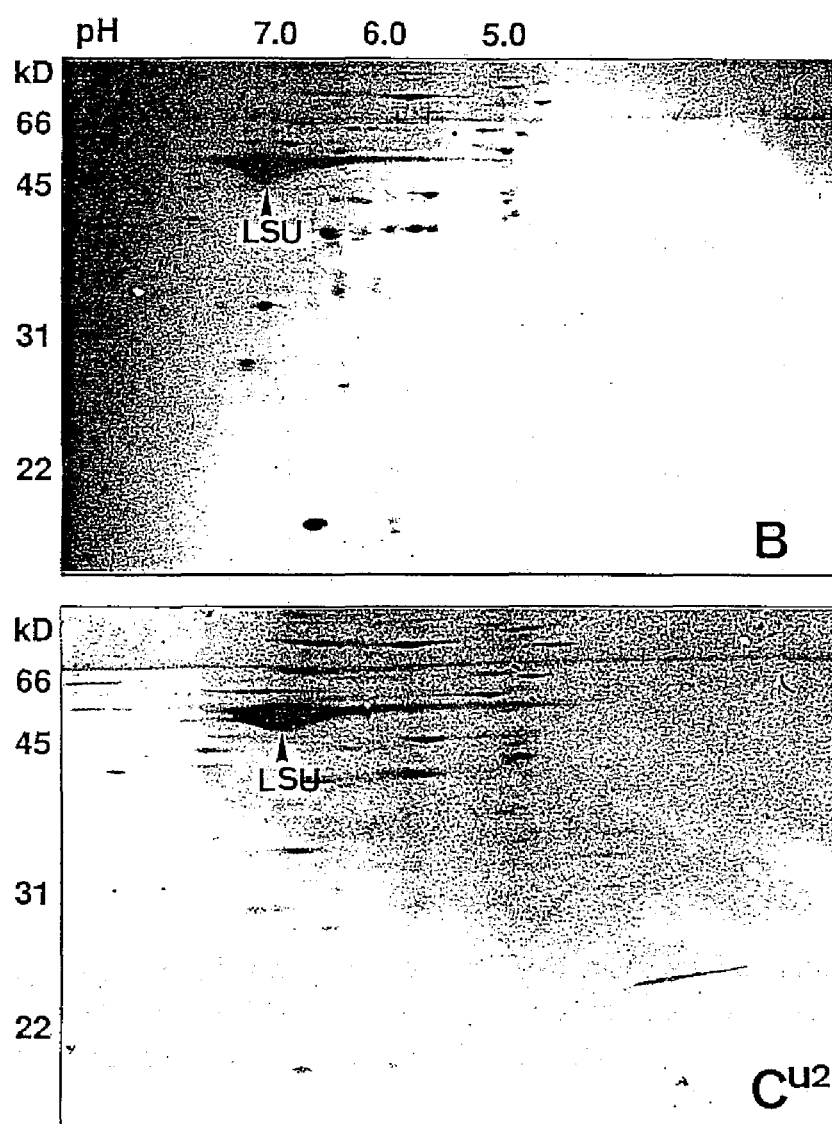


Figure 11. Two-dimensional electrophoresis of the water soluble fraction from the leaves of (B)-CS and (Cu²)-CS grown at 21 °C.
 B: (B)-CS. Cu²: (Cu²)-CS

ii) Peptide mapping by SDS-PAGE

To clarify the nature of the unique spot 4 (Figure 9) found in (D²)-CS , the 56kD spots (spots 1 and 4) were excised from the gels of (B)-CS and (D²)-CS, and digested with *Staphylococcus aureus* V8 protease. The resulted peptides were identical among the spot 1 of (B)-CS and the spots 1 and 4 of (D²)-CS (Figure 12).

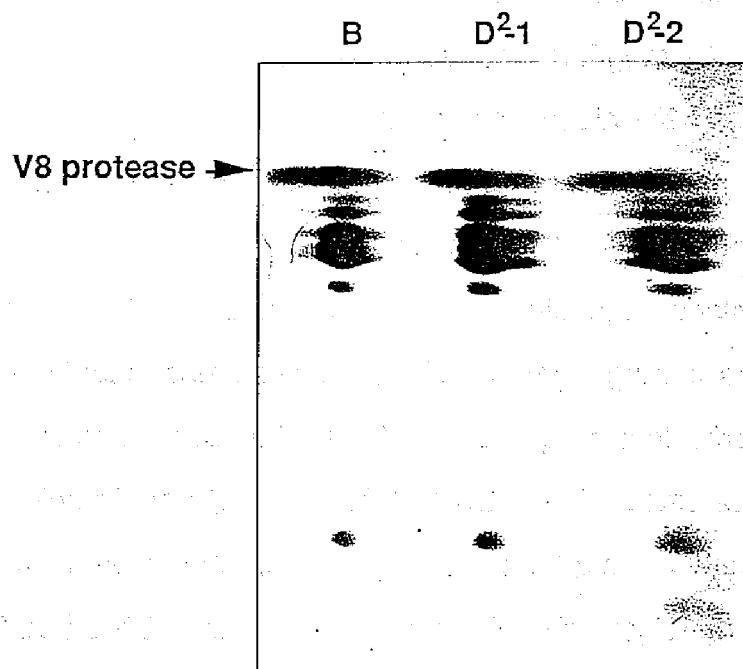


Figure 12. Peptide mapping of the β subunit of ATP synthase in (B)-CS and (D²)-CS. B: (B)-CS, D²-1 and -2; major and minor spot of (D²)-CS, corresponding to the spots 1 and 4 in Figure 9.

**6) Nucleotide sequences of three chloroplast genes,
atpB, *atpE* and *trnM*, in (Cu²)- and (D²)-CS**

The nucleotide sequences of the genes, *atpB* and *atpE*, for β and ϵ subunits of ATP synthase, respectively, and the gene, *trnM* for tRNA^{met} of (Cu²)- and (D²)-CS were determined by the dideoxy chain termination method, and compared to those of *T. aestivum* (B cytoplasm, Howe *et al.* 1985). The strategy of their sequencing is shown in Figure 13.

The nucleotide sequences of these genes and their 5' and 3' flanking regions in all three wheats are shown in Figure 14. The open reading frames for the *atpB* and *atpE* genes contain 1494 bp and 411 bp, respectively. They overlap by 4 bp, as is known in common wheat (*T. aestivum* in Figure 14). The coding regions of the *atpB* gene of D² and Cu² cytoplasms have five and seven base substitutions, respectively, when compared to that of common wheat. The coding region of *atpE* gene have the same two base substitutions in both *Aegilops* cytoplasms. The 5' flanking region of the *atpB* gene of (D²)-CS has the same sequence as that of common wheat, whereas this region has a 5 bp duplication (AAATT) in (Cu²)-CS at the -25 position. As for the 3' flanking region of the *atpE* gene, one base substitution was found in (D²)-CS, and one base substitution and one base insertion were detected in (Cu²)-CS. The gene for methionine tRNA (*trnM*) located downstream of the *atpE* gene shares the same nucleotide sequence among the ctDNAs from three different cytoplasms.

Compared to that of common wheat, predicted amino acid sequence of the β subunit in both the D² and Cu² type ctDNAs have the same three amino acid substitutions (Figure 15, Table 5). Two base substitutions found in the *atpE* gene do not alter the amino acid sequence in the ϵ subunit (Figure 15, Table 5).

Compared with those of other plants, i.e., barley (Zurawski and

Clegg 1984), maize (Krebbers *et al.* 1982) and spinach (Zurawski *et al.* 1982), the β subunit of both cytoplasms has three, 16 and 44 amino acid substitutions, respectively (Figure 15).

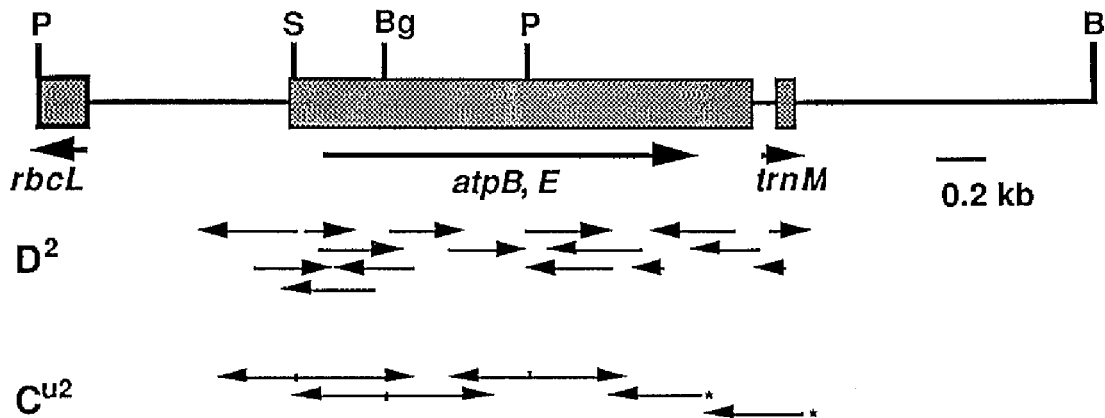


Figure 13. Restriction map of *atpB* and *atpE*, and their flanking regions in (B)-, (C^{u2})- and (D^2)-CS, all three showing the same map. Sequence strategies are also shown below the map. *: Sequencing using specific synthetic primers. B: *Bam*HI, Bg: *Bgl*II, P: *Pst*I, S: *Sma*I.

<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TCCACAAAAGGC AAAATGTATATGAAAAAAGATTGATTGAACTTTCCAACGGACTCATTCCATGAGTAAACGATTGAAT	-176
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	GGGATTCGCTTGGGCAACGAAATCAAGTCTGGTCCCTTTTCTCTCTTATTGAATTAACATAATTCATTTCCTTTTACT	-96
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TTTGGATTTTTTTTGGATTGATTGGCATTATTCAACAATAAAAAAGAAAAATTCGACAAATT*****CCTTTTTTTT	-25 -16
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	AATTATGTGATAATTATGAGAACCAATCCTACTACTTCTCCTCCCGGGGCTTCCACAATTGAAGAAAAAGTACAGGTCG	35 65
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TATCGATCAAATTATTGGACCCGTGCTGGATGTCACCTTTTCCCCCAGGCAAGTTACCTTATATTTATAACGCTTTGGTAG	145
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TCCAGAGTAGAGACACTGACGATAAGCAAATTAATGTGACTTGTGAGGTACAACAATTATTAGGAAATAATCGAGTTAGA	164 225
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	GCTGTAGCTATGAGTGTACGGACGGGTGATGAGAGGAATGGAAGTGATTGACACGGGAGCTCCTCTCAGTGTTCCGGT	305
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	CGGTGGAGCTACTCTTTGGACGAATTTTCAACGTTCTTGGGGAGCCTGTTGACAATTTGGGTCTCTAGATAGTAGTGAA	321 385
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	CGTTCCCTATTTCATAGATCTGCGCTGCCTTTATCGAGTTAGATACGAAATTATCCATCTTTGAAACAGGTATTAAGGTC	465
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	GTCGATCTTTTAGCTCCTTATCGACGTGGAGGAAAAATAGGACTATTTGGGGGGCTGGAGTAGGTAAAACAGTACTGAT	545
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	CATGGAATTAATCAATAACATTGCTAAAGCTCATGGGGCGTATCCGTATTCCGTGGAGTAGGGGAACGGACTCGTGAAG	615 625
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	GAAATGATCTTTATATGGAATGAAGGAATCCGGAGTAATTAATGAAAAAATATTGAGGAATCAAAGGTAGCTCTAGTC	651 705
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TATGGCCAAATGAATGAACCACCGGAGCTCGTATGAGAGTTGGTTTAACTGCCCTAACTATGGCGGAATATTTCCGAGA	785
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TGTTAATAAGCAAGACGTGCTTTTATTTATCGATAATATCTTTCGTTTTGTTCAAGCAGGATCAGAGGTATCCGCTTTAT	865
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	TAGGGAGAATGCCCTCCGCACTGGGTATCAACCTACTCTTAGTACAGAAATGGGTCTTTTGAAGAAAGAATTGCTTCT	945
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	ACTAAAAGGGATCTATAACTTCGATTCAAGCAGTTTATGTACCTGCAGACGATTTGACCGACCTGCCCTGCCACAAC	1025
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	ATTTGCACATTTGGATGCTACTACCGTACTTCCAGAGGATTAGCTTCCAAGGGTATTTATCCAGCAGTAGATCCTTTAG	1105
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	ATTCAACCTCGACTATGTTACAGCTCGGATCGTTGGCAACGAACATTATGAAACTGCGCAAAGAGTTAAGGAAACTTTA	1185
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	CAACGTTACAAAGAACTTCAGGACATTATTGCAATTCTTGGCTGGATGAATTATCGGAAGAGGATCGTTTAACTGTAGC	1215 1265
<i>aestivum</i> (D ²)-CS (Cu ²)-CS	AAGAGCAAGAAAAATTGAGCGTTTCTTATCACAACCGTTCTTTGTGGCAGAAGTTTTTACTGGTTCTCCAGGAAAGTATG	1345

Figure 14. Comparison of the nucleotide sequences of the genes for the β and ϵ subunits of chloroplast ATP synthase and the methionine tRNA among *T. aestivum* (Howe *et al.* 1985), (D²)-CS and (Cu²)-CS. Bold letter and lower case letter show the initiator codon and stop codon, respectively. The gene for the methionine tRNA is underlined

1425
aestivum TTGCTCTTGC GGAACTATTAGGGGATTCAACTAATCCTTTCCGGAGAATTAGACGGCTACCTGAACAGGCTTTTAT
 (D²)-CS -----
 (C^{u2})-CS -----

1489 *atpE*→ 1505
aestivum TTGGTGGGTAACATCGATGAAGCTAGCACGAAAGCTATAACCTTAGAAGAGGAGAACAATCGCAGAAAtgaATTAAAT
 (D²)-CS ----- A-----
 (C^{u2})-CS ----- A-----

1547 1585
aestivum CTTTATGTACTGACTCCTAAGCGAATTATTGGGATTGTGAGGTGAAAGAAATCATTTTATCCACTAATAGTGGCCAAAT
 (D²)-CS ----- A-----
 (C^{u2})-CS ----- A-----

1665
aestivum TGGCGTATTACCAAACCACGCCCCATTAAACACAGCAGTAGATATGGGTCCTTTGAGAATACGCCTCCTCAACGACCAAT
 (D²)-CS -----
 (C^{u2})-CS -----

1745
aestivum GGTTAACAGCGGTTCTGTGGAGCGGTTTTGCGAGAATAGTTAATAATGAGATCATCATTTTAGGAAATGATGCGGAACCTT
 (D²)-CS ----- G-----
 (C^{u2})-CS ----- G-----

1825
aestivum GGTAGTGATATTGATCCGGAAGAAGCTCAAAAGGCCTTGAAATAGCCGAAGCTAACTTGAGTAAAGCTGAGGGTACGAA
 (D²)-CS -----
 (C^{u2})-CS -----

1905
aestivum AGATTGTGTTGAAGCGAAGCTCGCTCTCAGACGAGCTAGGATACGAATCGAGGCTGTCAATTGGATTCCCCCATCCAAT
 (D²)-CS -----
 (C^{u2})-CS -----

1913 1985
aestivum gaAGACAATCCAGTGGTTTATAGTTGATACAAAGAAAAGGGAAGAGGGGTAGAAAAAGTTATTAGATAGCGAAGCGAAG
 (D²)-CS ----- G-----
 (C^{u2})-CS -----

2004 2007 *trnM*→ 2165
aestivum TAAGTCCAATGCTATCTAGTAA*TTTTTCTACCTACTTACCTACTATTGGATTGGAACCAATGACTCCCGCGGTATGAAA
 (D²)-CS ----- A-----C-----
 (C^{u2})-CS -----

2131
aestivum GCAATACTCTAACCCTGAGTTAAGTAGGCAATTTATCACCACAAAGGAAGACCCATTACTTCGATC
 (D²)-CS -----
 (C^{u2})-CS -----

Figure 14. Continued

Table 5. The positions of different nucleotides in *atpB* and *atpE* genes for chloroplast ATP synthase and possible alterations in their products among (B)-, (D²)- and (C^{u2})-CS

Gene	Codon position			
		(B)-CS	(D ²)-CS	(C ^{u2})-CS
<i>atpB</i>	12	GCT (Ala)	GTT (Val)	GTT (Val)
	55	GAC (Asp ⁻)	GCC (Ala)	GCC (Ala)
	107	CTT (Leu)	CTC (Leu)	CTC (Leu)
	205	CGG (Pro)	CGG (Pro)	CGA (Pro)
	217	AAG (Lys ⁺)	AAG (Lys ⁺)	AAA (Lys ⁺)
	405	ATT (Ile)	ATC (Ile)	ATC (Ile)
<i>atpE</i>	497	CAG (Gln)	AAG (Lys ⁺)	AAG (Lys ⁺)
	18	GAG (Glu ⁻)	GAA (Glu ⁻)	GAA (Glu ⁻)
	84	CTT (Leu)	CTG (Leu)	CTG (Leu)

+ and -: Plus and minus charged amino acids, respectively.

	10	20	30	40	50	60
<i>aestivum</i>	MRTNPTTSPPGASTIEEKSTGRIDQIIGPVLDTFPPGKLPYIYNALVVQSRDITDDKQIN					
(D ²)-CS	-----V-----					A-----
(C ^{u2})-CS	-----V-----					A-----
barley	-----R-V-S-----					
maize	-----R-I-----V-----I-----I-K-----					
spinach	--I-----D-V--L-K-NL--IA-----N-A--M-N--I-KG--AGQPN-					
	70	80	90	100	110	120
<i>aestivum</i>	VTCEVQQLLGNNRVRVAMSATDGLMRGMEVIDTGAPLSVPVGGATLGRIFNVLGEPVDN					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-I-----					
maize	-I-----E-----T-----I--					
spinach	-----T-----P-----					
	130	140	150	160	170	180
<i>aestivum</i>	LGPVDSSATFPIHRSAPAFIELDTKLSIFETGIKVVDLLAPYRRGGKIGLFGGAGVGKTV					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----T-----					
spinach	-R--TRT-S--TQ-----					
	190	200	210	220	230	240
<i>aestivum</i>	LIMELINNIKAHGGVSVFGGVGERTREGNDLYMEMKESGVINEKNIEESKVALVYGQMN					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----					
spinach	-----Q-----					
	250	260	270	280	290	300
<i>aestivum</i>	EPPGARMRVGLTALTMAEYFRDVNKQDVLFLFIDNIFRFVQAGSEVSALLGRMPSAVGYQP					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----					
spinach	-----E-----					
	310	320	330	340	350	360
<i>aestivum</i>	TLSTEMGSLQERIASTKKGSITSIQAVYVPADDLTDPAPATTFARLDATTVLSRGLASKG					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----T-----					
spinach	-----T--E-----A--					
	370	380	390	400	410	420
<i>aestivum</i>	IYPAVDPLDSTSTMLQPRIVGNEHYETAQRVKETLQRYKELQDIIAILGLDELSEEDRLT					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----					
spinach	-----E--I-----					
	430	440	450	460	470	480
<i>aestivum</i>	VARARKIERFLSQPFFVAEVFTGSPGKYVALAETIRGFQLILSGELDGLPEQAFYLVGNI					
(D ²)-CS	-----					
(C ^{u2})-CS	-----					
barley	-----					
maize	-----G-----					
spinach	-----G-----S-----					
	490	498				
<i>aestivum</i>	DEASTKAITLEENKSQK					
(D ²)-CS	-----K-----					
(C ^{u2})-CS	-----K-----					
barley	-----					
maize	-----N--S-LK--					
spinach	---TA--MN--M-S-LK--					

Figure 15. Comparison between the amino acid sequences of the β subunits of chloroplast ATP synthase in different organisms. Amino acid sequences of *T. aestivum*, barley, maize, and spinach were cited from Howe *et al.* (1985), Zurawski and Clegg (1984), Krebbers *et al.* (1982) and Zurawski *et al.* (1982), respectively

4. Discussion

1) Biochemical features of chlorophyll deficiency in (C^{u2})-CS

In CS having *Ae. umbellulata* cytoplasm which is closely related to *Ae. columnaris* and *Ae. triuncialis* cytoplasms, chlorophyll content reduced to 87 % when grown at 5.5 °C and to 47 % in winter of that of normal CS, whereas chlorophyll a/b ratio remained the same between them under both conditions (Mukai and Tsunewaki 1976). For critical study of this phenomenon, full induction of chlorophyll deficiency with a short treatment is desired. For this purpose, the soaked seeds were treated at 0 °C for various periods (like in winter), then the germinated seeds were grown at 5 °C for about one month during which the first leaf became fully developed. Chlorophyll deficiency was induced more severely in (C^{u2})-CS than in (C^u)-CS under such condition (Table 1). This result does not agree with the previous one reported by Mukai and Tsunewaki (1976), because (C^{u2})-CS showed weak variegation in winter, compared to the alloplasmic lines having the other C^u type cytoplasms including that of *Ae. triuncialis*.

Thirty-day treatment at 0 °C followed by the growth at 5 °C induced chlorophyll deficiency in the middle part of the first leaf. This suggests that the deficiency is induced in the tissue that develops at 0 °C, since the apical part of the leaf is thought to have already developed during seed maturation, and the basal part after transfer to 5 °C. The degree of the deficiency appears to depend upon the temperature and duration of cold treatment (Table 1). It should be mentioned that the chlorophyll deficiency is induced only in the first and second leaves under the present condition. In seedlings that already have developed the first leaf before the 30-day treatment at 0 °C, chlorophyll deficiency does not appear in the second and third leaves which develop at 5 °C. This observation is also inconsistent with the field observation in winter,

namely, the leaves developed in fall to early winter are normally green, whereas those developed later in mid-winter show chlorophyll deficiency (Mukai and Tsunewaki 1976).

As for the degree of the chlorophyll deficiency, all the lines studied were not uniform, showing varying degrees of the deficiency even among seedlings of the same lines. When the seeds from a single ear were used, however, the degree of deficiency did not vary so greatly within a line. At present, no reasonable explanation can be given for these differences.

The results of an experiment on chlorophyll recovery (Table 3) suggest that the present chlorophyll deficiency is reversible, at least, within a limited period of time before full development of the leaf. The high chlorophyll a/b ratio observed in (Cu²)-CS subjected to various low temperature treatments can be explained by a change in protein composition of the thylakoid membrane in chlorophyll deficient leaf, i.e., its membrane contains less photosystem II protein, including LHCII that harbors the majority of chlorophyll b (Figure 2). Even in the albinic portion of the leaf, certain amount of chlorophyll is usually found along with the vein. Some substances translocated through the vein seem to suppress the chlorophyll deficiency. Similar phenomenon is reported in a nuclear chlorophyll mutant of maize (Millerd *et al.* 1969).

The similarity in the thylakoid protein composition between the chlorophyll deficient and greening leaves suggests that the low temperature treatment inhibits the development of the thylakoid membrane at a certain stage, in much higher extent in (Cu²)-CS (Figure 3). And then, many thylakoid proteins which appeared in the leaves grown at low temperature and those illuminated for greening at 25 °C were also found in 2 M NaBr soluble fraction in the normally developed membrane of (B)-CS (Figure 3). These proteins seem to bind to thylakoid membrane by hydrophobic interaction, because 2 M NaBr solubilizes periferal proteins on the thylakoid membrane. And they may have some function in both the developed and

developing chloroplasts, because of their constant occurrence in the thylakoid membrane, although their functions remain still unknown.

In the alloplasmic line, (Cu²⁺)-CS, the PSII and whole chain activities are remarkably reduced in the thylakoid membrane of the seedlings subjected to low temperature (Table 4). Low whole chain activity appears to be caused by low PSII activity, because PSI activity is not greatly affected by low temperature (Table 4). Low PSII activity is consistent with a fact that PSII-constituting proteins decrease in the alloplasmic line subjected to severe cold treatment (Figure 3). The PSI complex is formed prior to the PSII complex during greening (Bradbeer 1981 for review). Therefore, the cold treatment seems to allow chloroplast to form the PSI complex, but stops chloroplast development before completion of the PSII complex formation in the thylakoid membrane.

The ring and irregular shaped chloroplast nucleoids found in chlorophyll deficient tissue are similar to those found in the leaf developing in the dark (Miyamura *et al.* 1986). Chloroplast development is suppressed in chlorophyll deficient tissue, but the number of the chloroplasts seems not to be affected by low temperature. Chlorophyll is detected by its autofluorescence even in chlorophyll deficient protoplasts. The intensity of autofluorescence seems to be related in some extent to the shape of chloroplast nucleoids, because the chloroplasts with degraded nucleoids show only a trace of chlorophyll autofluorescence. The inhibition of chloroplast development in the albinic cell appears to be more severe than that of thylakoid membrane development in a whole chlorophyll deficient leaf. Less inhibition of the latter is probably due to chlorophyll still remaining in the chlorophyll deficient leaf. Degradated chloroplast nucleoids in (Cu²⁺)-CS, as compared to single, large chloroplast nucleoids in (B)-CS, might not be a cause of cold sensitivity, but, instead, its result, because this difference is prominent in the developed leaf but not in the developing one. Chloroplasts of the leaves which could not recover chlorophyll at 21 °C also contained a

large nucleoid. This suggests that the loss of chlorophyll recovery is not caused by the degradation of chloroplast DNA. However, the nucleoids in chloroplasts of chlorophyll deficient leaves became very sensitive to UV light illumination given at 21 °C. Therefore, these chloroplasts seem to have structural lesions in their DNA itself or DNA-protein complex in the nucleoids.

Several possible causes are assumed for the present chlorophyll deficiency. First one is the photooxidation of chlorophyll that is known to be caused by low temperature and high light intensity (Powles 1984; Wise and Naylor 1987 a, b), which induce the inactivation of PSII activity (Powles 1984). The observed low PSII activity, chloroplast nucleoid degradation and chlorophyll reduction are in favor of this possibility. However, the degradation of both chloroplast and chlorophyll is observed only in the developed leaves. Therefore, photooxidation does not seem to be the primary cause of chlorophyll deficiency. Second one is the inhibition of chloroplast development, preventing the synthesis of proteins constituting thylakoid membrane to which chlorophyll binds. Third one is a change in D2 protein that is known in *Chlamydomonas reinhardtii* to prevent accumulation of other PSII core proteins (Erickson *et al.* 1986). On the basis of low relative content of PSII proteins in chlorophyll deficient leaves, some mutation might be assumed for chloroplast genes encoding PSII proteins in the Cu² cytoplasm. But this is not the case, because the inhibition of chloroplast development occurs before the synthesis of thylakoid proteins. In conclusion, the present chlorophyll deficiency caused by the Cu² cytoplasm is assumed to be due to severe inhibition of chloroplast development at a low temperature.

From the shape of chloroplast nucleoids in the chlorophyll deficient leaves, this inhibition is suggested to occur at the level of chloroplast gene expression that concerns with the change in chromoplast to chloroplast (Hansmann *et al.* 1985). The factor(s) responsible for this inhibition should be some mutation(s) occurred in mitochondrial or chloroplast DNA of the Cu²

cytoplasm. If the mutation has occurred in a chloroplast gene that encodes a subunit of RNA polymerase or ribosome protein (Ohyama *et al.* 1986; Shinozaki *et al.* 1986), it may affect the development of chloroplast.

However, a more plausible explanation is that the mutation of a mitochondrial gene(s) induces the inhibition of chloroplast development. During the early stage of greening, chloroplast itself can not produce the energy that is needed for the expression of chloroplast genes which code for proteins involved in photosynthesis. In this period, an intensive mitochondrial activity for ATP transport to chloroplast is observed (Hamp 1979). The present explanation is substantiated by an observation that inhibition of oxidative phosphorylation in intact leaves severely damages chlorophyll formation and chloroplast development (DeGreef and Verbelen 1977). Chloroplasts of a ribosome-deficient chloroplast mutant 'albostrians' of barley are usually in close proximity or appressed to mitochondria (Wellburn and Wellburn 1979). This positioning should be advantageous in the energy transport from normal mitochondria to chloroplast devoid of ATP synthesis by photophosphorylation (Wellburn and Wellburn 1979).

The alloplasmic line of CS with C^{u2} cytoplasm exhibits severer chlorophyll deficiency than that with a C^u cytoplasm (e.g. *Ae. triuncialis* cytoplasm), whereas the former shows stronger growth vigor than the latter, although their chloroplast genomes are identical with each other so far as the restriction fragment patterns of chloroplast DNA treated with 13 different endonucleases are concerned (Terachi *et al.* 1984; Ogihara and Tsunewaki 1988). The mitochondrial genome of C^{u2} is most differentiated among those of the species having the C^u and C^{u2} type cytoplasms (Terachi and Tsunewaki 1986). The observed difference in phenotypic effects between C^{u2} and other C^u type cytoplasms can be ascribed for their mitochondrial genome differences. Non-chromosomal stripe mutants in maize characterized by variable leaf stripe and poor growth are correlated with novel mitochondrial DNA rearrangements resulting in differential

mitochondrial gene expression (Feiler and Newton 1987). To prove the present hypothesis that the chlorophyll deficiency observed in (Cu²)-CS is caused by a mitochondrial gene mutation(s), the physiological activities of the mitochondria during greening process, and the differences in mitochondrial proteins should be studied.

2) Variation on three chloroplast proteins among alloplasmic wheats

The analysis of thylakoid proteins by two-dimensional electrophoresis showed that all of the three proteins, the large subunit of Rubisco, the β subunit of chloroplast ATP synthase and the 31 kD unknown protein are classified into two types, i.e., one type is found in the B cytoplasm which is characterized by the absence of spot 4 and the other type in all the three Cu², D² and Mt cytoplasms. This fact suggests that their differences can not be the cause of the phenotypic differences observed among eu- and alloplasmic wheats having these cytoplasms (Table 1). As for the Rubisco large subunit and the CF₁ β subunit, their distribution patterns are consistent with the results of extensive studies carried out by Bahrman *et al.*(1988). A disagreement is found on the β subunit of chloroplast ATP synthase. In my study, it appeared in two spots in all alien cytoplasms but in one spot in common wheat cytoplasm. On the contrary, Bahrman *et al.* (1988) found also two forms in common wheat. In the present experiments, thylakoid membrane from well developed green leaves was studied, whereas they studied that from etiolated tissue. It might be suspected that modification of the β subunit is different among different tissues. The protein of the molecular mass of 31 kDa also appeared in two forms which differ in isoelectric point. Further study is needed to clarify the nature and function of this protein.

3) Predicted amino acid sequences of the β subunit of ATP synthase in (Cu²)- and (D²)-CS

Amino acid sequences of the β subunits of ATP synthase in (Cu²)- and (D²)-CS were deduced from the nucleotide sequences of their *atpB* genes (Figure 15). Comparing to that of common wheat (Howe *et al.* 1985), the β subunit of both cytoplasms has three amino acid substitutions. Two of them (Asp⁻ → Ala and Gln → Lys⁺) seem to cause net charge change in this subunit, resulting in the shift of its spot to high isoelectric point in two-dimensional electrophoregram.

The present sequence data could not explain why the β subunit of both alien cytoplasms appeared in two spots. It is most probable that these two spots are the products of post-translational modification. In the large subunit of Rubisco, removal of N-terminal peptides by limited tryptic proteolysis is known in some plants (Houtz *et al.* 1989). The β subunit of ATP synthase might undergo the similar change as follows; the carboxyl terminal region of the β subunit of Cu² and D² cytoplasms has -Lys-Lys, whereas that of B cytoplasm has -Gln-Lys (Figure 15). If a trypsin-like protease cleaves the peptide bond at the carboxyl end of arginine or lysine residue, the terminal lysine residue in both *Aegilops* cytoplasms can be removed but not in *T. aestivum* cytoplasm, and the modified subunits in the former will become more acidic. The two spots of the β subunit may represent the modified and non-modified forms (Figure 16). Isoelectric points (pI) of these forms of the subunit are estimated from their deduced amino acid sequences, which are pI = 4.92 for (B)-CS, and pI = 5.03 and 4.97 for non-modified and modified forms, respectively, of (Cu²)- and (D²)-CS (Figure 16). These values are in good agreement to the pI values observed in the electrophoretic patterns (Figure 9). The modified form is also assumed to be present as the major component of the β subunit.

The effect of amino acid substitutions in both alien cytoplasms on the secondary structure of the β subunit was predicted by the method of Chou and Fasman (1978). The predicted secondary structures of the β subunit in C^{u2} and D^2 cytoplasms are identical to that in B cytoplasm (Figure 17).

These results suggest that amino acid substitutions occurred in the β subunits of chloroplast ATP synthase of C^{u2} and D^2 cytoplasms should not cause any phenotypic changes in CS wheat.

		C'	pI
B	-Ile-Thr-Leu-Glu ⁻ -Glu ⁻ -Glu ⁻ -Asn-Lys ⁺ -Ser-Gln-Lys ⁺		4.92
C^{u2} , D^2	-Ile-Thr-Leu-Glu ⁻ -Glu ⁻ -Glu ⁻ -Asn-Lys ⁺ -Ser-Lys ⁺ -Lys ⁺		5.03
		↓	↑
			trypsin
Modified	-Ile-Thr-Leu-Glu ⁻ -Glu ⁻ -Glu ⁻ -Asn-Lys ⁺ -Ser-Lys ⁺		4.97

Figure 16. A model explaining two type of the b subunit of (C^{u2})-CS and (D^2)-CS in comparison with (B)-CS. A lysine residue in the carboxyl terminal of the subunit in C^{u2} and D^2 cytoplasms is removed by trypsin. Estimated pI values are also shown.

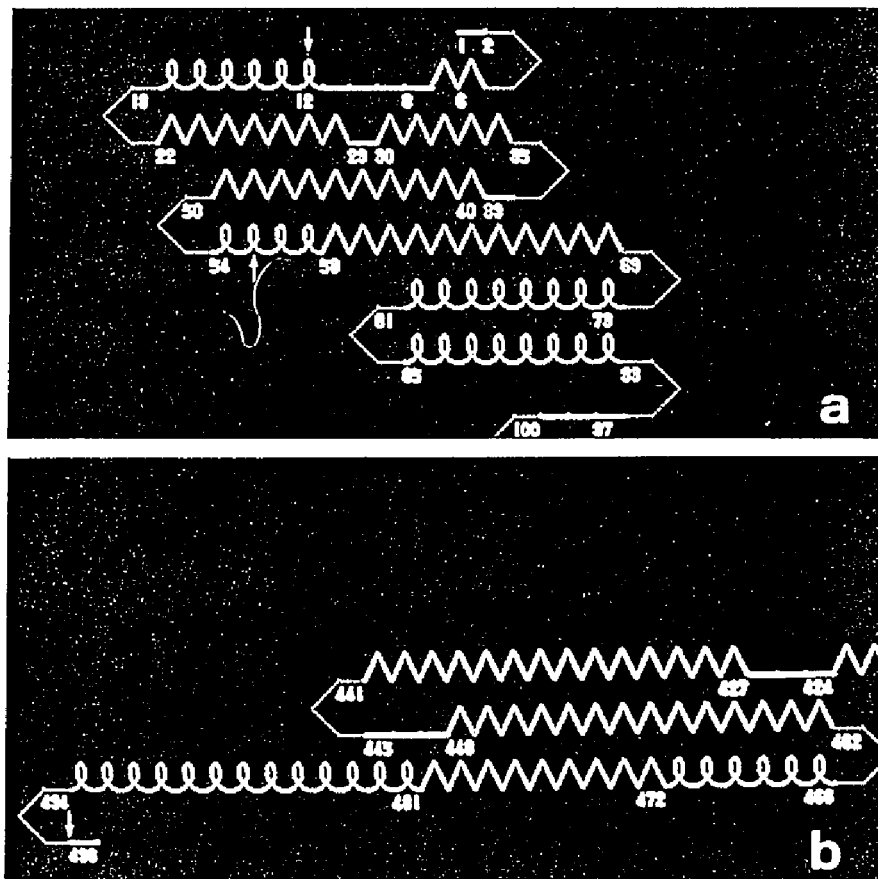


Figure 17. Predicted secondary structure of N- and C-terminal regions of β subunit of ATP synthase in (B)- and (C^{u2})-CS. a: N-terminal region, b: C-terminal region. Arrows indicate the positions of the amino acid substitutions

PART II

Biochemical Studies on Mitochondria in Four Alloplasmic Wheats

1. Introduction

The mitochondrial genome in higher plants is known to show great variation on its size that varies from 200 kb in *Brassica* (Palmer *et al.* 1983) to approximately 2500 kb in *Cucurbitaceae* (Ward *et al.* 1981), and its structure is highly complex due to intramolecular recombinations which occur between repeated sequences (Falconet *et al.* 1984; Falconet *et al.* 1985; Lonsdale 1984; Fauron and Havlik 1989) as well as the presence of chloroplast DNA sequences (Stern and Palmer 1984; Jubier *et al.* 1990). Most of the genes in plant mtDNA are involved in the electron transport and oxidative phosphorylation (Newton 1988 for review), namely, *nad1*, *nad3*, *nad5*, (subunits of NADH dehydrogenase), *cob* (subunit of cytochrome reductase), *cox1*, *cox2*, *cox3*, (subunits of cytochrome oxidase), *atpA*, *atp6*, and *atp9* (subunits of ATP synthase). However, very little is known about the mechanisms of mitochondrial gene expression in higher plant, including consensus regulatory sequences (Schuster and Brennicke 1989) and RNA editing (Mulligan *et al.* 1988; Gualberto *et al.* 1989; Covello and Gray 1989; Finnegan and Brown 1990).

Phenotypes altered by the structural changes in mtDNA have been studied extensively in relation to cytoplasmic male sterility (CMS) in maize, *Sorghum* and *Petunia* (Glab *et al.* 1990; Young and Hanson 1987; Newton 1988; Levings and Brown 1989 for review). These studies suggested that a novel chimeric gene produced by the rearrangements of mitochondrial genes causes CMS. The clear evidence for the relationship between the male sterility and the chimeric gene was recently obtained for the T-*urf13* gene of

T cytoplasm in maize, that is also responsible for the sensitivities to T-toxin and methomyl, because when this gene flanked with a sequence for a mitochondrion-targeting N-terminal peptide was introduced into yeast cells, they became sensitive to T-toxin/methomyl in a manner that mimicked the phenotype of T-type male sterile maize plants (Glab *et al.* 1990). Nuclear fertility-restoring genes (*Rf*) which suppress the male sterility exert their effect only in specific cytoplasms at the transcriptional or post-transcriptional level (Dewey *et al.* 1986; Forde and Leaver 1980; Young and Hanson 1987). A maternally inherited mutant, nonchromosomal stripe (NCS), characterized by leaf stripe, poor growth and decreased yield (Coe 1983) also carries mtDNA alterations that lead to greatly reduced synthesis of a 24 kD polypeptide of unknown function (Newton and Coe 1986; Feiler and Newton 1987).

Although a large amount of informations are accumulated, including those on molecular alterations in mtDNAs of CMS plants, only a few works have revealed how such mtDNA alterations could result in the changes in mitochondrial functions leading to abnormal phenotypes, especially in *Triticum* and *Aegilops*. Therefore, I intended to reveal the relationships between mtDNA alterations and growth abnormalities expressed in common wheat by different *Aegilops* cytoplasms.

In the present study, intact mitochondria and mtDNA were isolated from common wheat having five different cytoplasms which exert unique effects on wheat phenotypes one another (Table 1). In the beginning, the respiratory activity, and low-temperature spectrum of cytochromes in intact mitochondria were investigated. Next, Restriction Fragment Length Polymorphisms (RFLPs) of mtDNAs among the five cytoplasms were analyzed using seven mtDNA clones as probes, which carry different genes encoding some subunits of cytochrome oxidase, cytochrome reductase and ATP synthase. Thirdly, the physical maps of two mitochondrial genes, *atpA* and *cox3*, and their flanking regions, which were isolated from (B)- and (C^{u2})-CS, were constructed and their complete nucleotide sequences were determined.

Finally, the expression of the *cox3* gene in (Cu²⁺)-CS was investigated.

Based on the results of these studies, functional relationships among the phenotypes, mitochondrial activities and structural changes in the mitochondrial genes are discussed.

2. Materials and Methods

1) Plant materials

As for the measurement of mitochondrial activities, (B)-, (Cu)-, (Cu²⁺)-, (D²⁺)- and (Mt)-CS, and (B)- and (Cu)-JF were used. Seeds soaked in water at 20 °C overnight were placed in vermiculite in a plastic container, and kept in an incubator in dark at 23 °C for five days. In the study of mtDNAs, (B)-, (Cu)-, (Cu²⁺)-, (D²⁺)- and (Mt)-CS were used. Seedlings were grown in dark in a greenhouse for two weeks at 21 °C. At certain ages, the seedling foliage was collected and used.

2) Isolation of mitochondria and measurement of their respiratory activities

i) Isolation of mitochondria

Excised shoots were cut into 5 mm long pieces and ground in chilled grinding medium with a mortar and pestle. The grinding medium contained 0.5 M mannitol, 0.1 M Hepes-KOH (pH 7.5), 5 mM EDTA, 0.05 % cysteine, and 0.1 % BSA. Homogenate was filtered through a single layer of nylon sheet (25 µm mesh) and centrifuged at 1,000 x g for 10 min. The supernatant was centrifuged at 10,000 x g for 15 min. The resulted mitochondrial pellet was washed by resuspending it in the washing medium and centrifuging at 10,000 x g for 10 min. The washing medium contained 0.5 M mannitol, 50

mM Tes-KOH (pH 7.2), and 0.3 % BSA. The precipitate was resuspended in the washing medium. All operations were carried out at 4 °C.

ii) Measurement of respiratory activities

The respiratory activity of mitochondria was measured polarographically after the procedures of Shibasaka and Tsuji (1988b) using a Clark type oxygen electrode (Yellow Springs Instrument Co. 4004 Oxygen Probe). The reaction mixture (3 ml) contained 0.3 M mannitol, 4 mM MgCl₂, 5 mM potassium phosphate, 50 mM Tes-KOH (pH 7.2), 0.75 mg/ml BSA and 85 µM ATP, beside mitochondria. Succinate and malate were used as the substrate at the concentrations of 8 and 32 mM, respectively. For malate oxidation, 0.3 mM TPP (thiamine pyrophosphate) and 1 mM pyruvate were added to the reaction mixture to trap oxaloacetate.

iii) Enzyme assay

Cytochrome oxidase (EC 1.9.3.1) and cytochrome reductase (EC 1.6.99.3) activities were measured according to Shibasaka and Tsuji (1988a). The activity of cytochrome oxidase was assayed with the medium (2.5 ml) containing 30 mM phosphate buffer (pH 7.4), 0.1 % Triton X-100, 48 µM ferro cytochrome *c*, and the mitochondrial suspension, and that of cytochrome reductase was surveyed with the medium (2.5 ml) containing 5 mM phosphate buffer (pH 7.2), 1 mM KCN, 8 mM succinate, 48 µM ferri cytochrome *c*, and the mitochondrial suspension. These activities were measured as the change in absorbance at 550 minus 540 nm using a Hitachi 556 spectrophotometer with the dual wavelength mode and an extinction coefficient of 19.6 mM⁻¹ cm⁻¹.

Protein content in the mitochondrial suspension dissolved in 1 N NaOH was measured according to Lowry *et al.* (1951).

3) Low temperature difference spectrum of mitochondrial cytochromes

Reduced minus oxidized difference spectrum was measured at 77 K with a Hitachi 556 spectrophotometer after Shibasaka and Tsuji (1988 b). Mitochondrial suspension was placed in acrylic resin cuvettes with 2 mm light path. Reduction of the suspension was achieved by the addition of dithionate standing for 15 min. Oxidation of the suspension carried out using ferricyanide, and the suspension was frozen instantly in liquid nitrogen. Spectrum was scanned from 400 to 650 nm with 1.0 nm bandpass

4) RFLP analysis of mtDNAs

The isolation of intact mitochondria and the purification of mtDNA were made after Terachi and Tsunewaki (1986).

The mtDNA samples were treated with three restriction endonucleases, *Bam*HI, *Eco*RI and *Hind*III, all being the six-base cutter. The digestion was carried out according to the directions given by the suppliers (Takara Shuzo Co. Ltd. and Nippon Gene Co. Ltd.). Electrophoresis of the digests was performed at 2 V/cm for overnight, using 0.8 % agarose slab gel made with TAE buffer (Maniatis *et al.* 1982). DNA fragments stained with ethidium bromide (0.5 µg/ml) were made visible by long wave UV light illumination, and then photographed. Restriction fragments were transferred to two sheets of Nylon membrane (Amersham, Hybond-N) as described by Maniatis *et al.* (1982). Cloned mtDNA fragments that contain either one of the following seven mitochondrial genes were used as hybridization probes; pea *atpA* (Morikami and Nakamura 1987), *Oenothera atp6* (Schuster and Brennicke 1987), *Oenothera atp9* (Schuster and Brennicke 1989), common wheat *cob* (Boer *et al.* 1985), *Oenothera cox1* (3' portion, Hiesel *et al.* 1987), pea *cox2* (Morikami and

Nakamura unpublished), and *Oenothera cox3* (Hiesel *et al.* 1987). DNA labelling, Southern hybridization and detection of the hybrid fragments were carried out by the standard procedure using the Non-radioactive DNA Labelling and Detection Kit (Boehringer Mannheim Co. Ltd.). Because of low homology of the heterologous probes with the corresponding wheat sequences, hybridization of two probes containing the *Oenothera atp6* and *atp9* genes was carried out at 60 °C for 72 h. After rinsing twice in 2 x SSC and 0.1 % SDS solution at room temperature for 5 min, the membranes were washed twice in 5 x SSC and 0.1 % SDS at 60 °C for 15 min.

5) Construction of restriction maps and nucleotide sequencing of a few mitochondrial genes and their flanking regions

- i) Construction of restriction maps of three mitochondrial genes, *atpA*, *atp9* and *cox3* genes and their flanking regions from (B)- and (Cu²⁺)-CS

The isolation of intact mitochondria and the purification of mtDNA were made after Terachi and Tsunewaki (1986).

The mtDNA libraries of (B)- and (Cu²⁺)-CS were constructed by Terachi (1990) using a lambda phage vector, Lambda FIX. The plaques were transferred to Nylon membranes (Amersham, Hybond-N⁺) followed by alkali denaturation and fixation. The phages containing *atpA* and *cox3* genes were isolated by hybridization with the cloned DNAs of pea *atpA* (Morikami and Nakamura 1987) and *Oenothera cox3* (Hiesel *et al.* 1987), respectively, that were labelled using Dig-ELISA kit (Boehringer Mannheim). The isolated phages were amplified by the plate lysate method (Maniatis *et al.* 1982). The phage particles were collected by centrifugation at 40,000 rpm (140,000 x g) for 10 min at 20 °C using a Hitachi RP65T rotor. Glassy pellet of the phage particles was dissolved in the SM solution (Maniatis *et al.* 1982). The solution was mixed vigorously with buffered phenol and stood for 10 min, followed by mixing it with 100 µl of a chloroform and

isoamylalcohol mixture (24:1). The upper aqueous phase was collected and mixed with phenol, chloroform and isoamylalcohol (25:24:1) followed by ethanol precipitation.

Restriction sites of the cloned fragments were mapped by standard techniques (Maniatis *et al.* 1982) based on the electrophoretic patterns of single, double and partial digests with three six-base cutters (*Bam*HI, *Eco*RI and *Hind*III). For more detailed mapping, certain fragments were isolated by preparative electrophoresis using GENE CLEAN (BIO 101, U.S.A.) and used as probes to detect overlapping fragments in other restriction patterns by Southern hybridization. The mtDNA fragments containing the *atpA* and *atp9* genes were mapped with five additional restriction enzymes, *Kpn*I, *Pst*I, *Sac*I, *Sal*I and *Xho*I, by their single and double digestion .

ii) Nucleotide sequencing of the genes, *atpA* and *cox3*, and their flanking regions from (B)- and (Cu²)-CS

The *atpA* gene of the mitochondrial genome in (B)-CS is located in two *Bam*HI fragments of 4.8 kb and 2.1 kb, whereas that of (Cu²)-CS is in two *Bam*HI fragments of 2.9 kb and 2.5 kb (ref. Figure 25). These *Bam*HI fragments were subcloned by inserting their subfragments into the corresponding restriction sites of plasmid pUC119 for sequencing.

Single stranded DNAs were prepared as shown in Part I. Double stranded DNA templates were purified by equilibrium centrifugation in CsCl-EtBr gradients (Maniatis *et al.* 1982). Dideoxy chain termination reaction using single stranded DNAs or double stranded DNAs was carried out with Sequenase ver. 2, 7-deaza-dGTP edition (U.S.B., U.S.A.), under the conditions indicated by the manufacturer, using universal and synthetic oligonucleotides (17 mer) as mentioned in Part I.

The data analysis and homology search for the nucleotide sequences determined were carried out using the DNASIS (Hitachi software) and the IDEAS (Integrated database and extended analysis system for nucleic acids

and proteins), respectively. The prediction of RNA secondary structure was made by the Macintosh programs; MulFold (Zuker 1989; Jaeger *et al.* 1989a,b) and LoopViewer (Gilbert 1990).

6) Northern hybridization of mtRNAs of (B)- and (Cu²⁺)-CS with the *cox3* gene probe

i) Preparation of total mtRNA

A crude mitochondrial pellet that was prepared as described previously was washed in a buffer containing 0.44 M sucrose, 3 mM EDTA and 50 mM Tris-HCl (pH 8.0) and suspended in 0.5 ml of the same buffer. Total mtRNA was prepared by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987; Goodall *et al.* 1990) with the following modifications:

The mitochondrial suspension was lysed with twice volume (1 ml) of the denaturing solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5 % sarcosyl, 0.1 M 2-mercaptoethanol in a 2 ml Eppendorf tube with vigorous vortexing. To this added was 40 µl 2 M sodium acetate (pH 4.0), 400 µl water-saturated phenol, and 80 µl chloroform-isoamyl alcohol (49:1 v/v). After vigorous mixing, the sample was kept on ice for 15 min, then centrifuged at 14,000 x g for 20 min. The supernatant aqueous phase, excluding the portion near the interphase, was transferred to two 1.5 ml Eppendorf tubes and mixed with the same volume of 2-propanol. After standing at -20 °C for 15 min, RNA was pelleted by centrifugation at 12,500 x g for 5 min. The pellet was washed with 70 % ethanol and dissolved with 50 µl of the denaturing solution, then mixed with the same volume of 2-propanol and left at -20 °C for 15 min. After centrifugation at 12,500 x g for 5 min, the pellet was washed with 70 % ethanol, briefly air dried, and dissolved in 50 µl DEPC-treated water. An aliquot of 2 µl was used to check RNA concentration by measuring the O.D. 260.

ii) Electrophoresis and hybridization analysis of mtRNA

Electrophoresis of total mtRNA (15 µg per lane) was carried out using 1.2 % agarose gel in the presence of 0.66 M formaldehyde. After the electrophoresis, mtRNA was transferred to nylon membrane (Hybond-N, Amersham). The *Oenothera cox3* containing clone (Hiesel *et al.* 1987, ref. Figure 23) was labelled with ³²P-dCTP using the Random Primed DNA Labelling Kit (Boehringer Mannheim), followed by removing the unincorporated deoxynucleotide triphosphate through Sephadex G50 (Maniatis *et al.* 1982). The membrane was prehybridized in a sealed plastic bag containing 5 x SSPE, 0.1 % sodium N-lauroyl sarcosinate, 0.02 % SDS, 50 % formamide and 5 % Blocking Reagent (Boehringer Mannheim) at 42 °C overnight. The prehybridization solution was replaced with the same solution of 5 ml per 50 cm² filter size. The ³²P-labelled probe, denatured by heating in boiling water for 10 min, was added to the hybridization solution. After hybridization overnight at 42 °C, the membrane was washed at 65 °C, once with a mixture of 2 x SSC and 0.1 % SDS for 30 min followed by rinse with a change of the washing solution. Signal intensity of the membrane was monitored with a GM-survey-meter. Fuji X-ray film was then exposed to the membrane, using one intensifying screen at -70 °C for one day.

3. Results

1) Respiratory activities of the mitochondria isolated from alloplasmic wheats

Oxidation of succinate and malate by mitochondria isolated from the common wheats having five different cytoplasms was compared (Table 6, Figure 18). Mitochondria isolated from the lines having C^U and C^{U2} cytoplasms oxidized the substrates in the presence of ADP (state 3) at about a half of the rate of the euplasmic lines. The rate of oxidation without ADP supply (state 4), the ratio of state 3 to state 4 (respiratory control ratio, RCR) and the ratio of synthesized ATP to consumed oxygen atom (P/O) also showed the suppression of respiratory activity in mitochondria of alloplasmic lines, especially in those of C^U and C^{U2} cytoplasms, but the degrees of their suppressions by both cytoplasms were smaller than that of state 3. The differences between two nuclear backgrounds of CS and JF, i.e., between (B)-CS and (B)-JF, and between (C^U)-CS and (C^U)-JF, were not clear. (B)-CS had slightly higher respiratory rate than (B)-JF, whereas (C^U)-CS showed slightly lower value in RCR and P/O ratio than (C^U)-JF (Table 6, Figure 18).

The cyanide-resistant respiration (Douce 1985 for review) can be estimated by the reduction of respiration in the presence of 1 mM SHAM (salicylhydroxamic acid) from that of control, because SHAM inhibits cyanide-resistant respiration. The relative cyanide-sensitive respiratory activities of the alloplasmic lines were very similar to those obtained without the inhibitor (Table 6, Figures 18 and 19).

Table 6. Respiratory activities of the mitochondria isolated from seedlings of eu- and alloplasmic wheats

Line	State 3†	State 4†	RCR	P/O
Succinate				
(B)-CS	533.2(100) a	292.5 (100) a	1.85 (100) a	1.07 (100) a
(D ²)-CS	500.7 (94) a	291.2 (100) a	1.73 (94) a	0.98 (92) a
(Mt)-CS	467.9 (88) a	264.5 (90) a	1.77 (96) a	0.98 (92) a
(C ^u)-CS	329.3 (62) b	238.2 (81) b	1.39 (75) c	0.88 (82) a
(C ^{u2})-CS	296.6 (56) b	198.0 (68) b	1.53 (83) b	0.86 (80) a
(B)-JF	466.1 (87) a	262.7 (90) ab	1.77 (96) a	1.18 (110) a
(C ^u)-JF	329.0 (62) b	214.3 (73) b	1.54 (83) b	0.98 (92) a
Malate				
(B)-CS	519.5 (100) a	168.1 (100) a	3.16 (100) a	1.75 (100) a
(D ²)-CS	509.1 (98) a	188.0 (112) a	2.71 (86) a	1.58 (90) a
(Mt)-CS	456.4 (88) a	157.8 (94) a	2.90 (92) a	1.60 (91) a
(C ^u)-CS	341.6 (66) b	170.8 (102) a	2.01 (64) b	1.45 (83) ab
(C ^{u2})-CS	289.5 (56) b	134.3 (80) b	2.16 (68) b	1.35 (77) b
(B)-JF	497.8 (96) a	185.0 (110) a	2.70 (85) a	1.71 (98) a
(C ^u)-JF	356.6 (69) b	167.5 (100) a	2.14 (68) b	1.60 (91) a
Succinate +1mM SHAM				
(B)-CS	412.1 (100) a	248.2 (100) a	1.68 (100) a	1.15 (100) a
(D ²)-CS	371.3 (90) a	230.9 (93) a	1.61 (96) ab	0.91 (79) a
(Mt)-CS	322.3 (78) ab	221.4 (89) ab	1.46 (87) ab	0.84 (73) a
(C ^u)-CS	252.7 (61) c	174.0 (70) b	1.47 (88) ab	0.85 (74) ab
(C ^{u2})-CS	214.0 (52) c	152.8 (62) b	1.40 (83) ab	0.72 (63) ab
(B)-JF	380.7 (92) a	242.3 (98) a	1.57 (93) ab	1.05 (91) ab
(C ^u)-JF	269.2 (65) bc	181.1 (73) ab	1.50 (89) ab	0.88 (77) ab
Malate +1 mM SHAM				
(B)-CS	422.0 (100) a	181.5 (100) a	2.39 (100) a	1.51 (100) a
(D ²)-CS	384.6 (91) a	183.1 (101) a	2.10 (88) ab	1.50 (99) a
(Mt)-CS	349.2 (83) a	173.1 (95) ab	2.01 (84) ab	1.47 (97) a
(C ^u)-CS	230.1 (55) bc	134.2 (74) ab	1.72 (72) ab	1.38 (91) ab
(C ^{u2})-CS	202.9 (48) bc	112.5 (62) ab	1.85 (77) ab	1.32 (87) ab
(B)-JF	369.8 (88) a	168.8 (93) ab	2.19 (92) ab	1.68 (111) a
(C ^u)-JF	278.8 (66) b	145.4 (80) ab	1.96 (82) ab	1.50 (99) ab

†: Nanoatomes oxygen mg protein⁻¹ min⁻¹.

RCR: Respiratory control ratio.

(): Percent of (B)-CS.

a, b and c: Significantly different from each other at 5 % level.

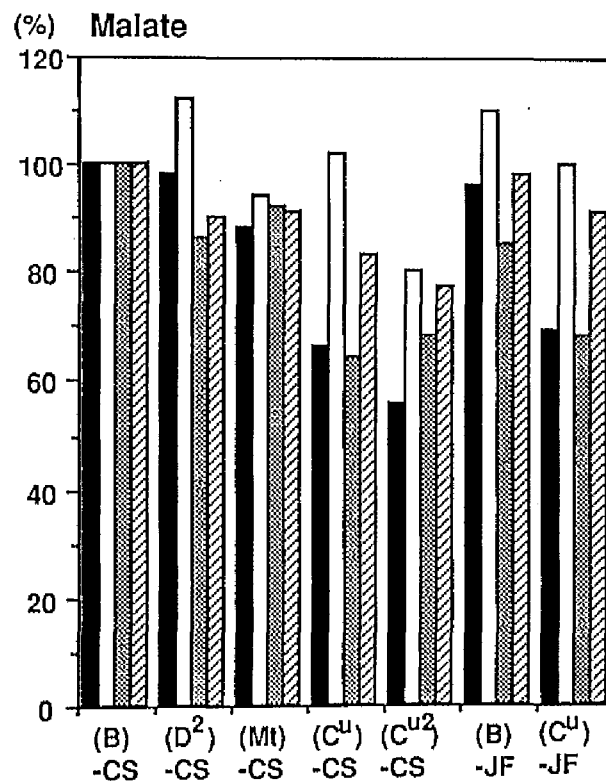
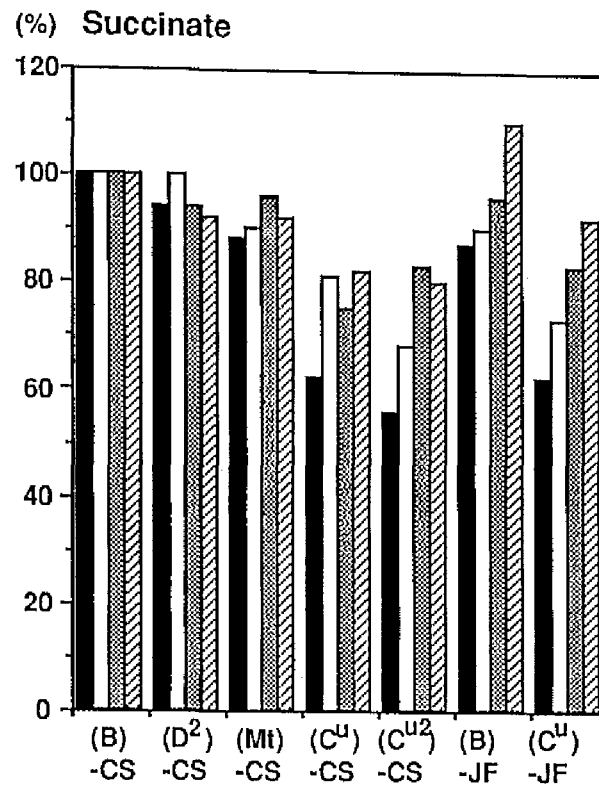


Figure 18. Relative respiration activities of eu- and alloplasmic lines of two common wheats when succinate and malate were used as the substrate with or without 1 mM SHAM

■: State 3, □: State 4, ▨: RCR, ▩: P/O

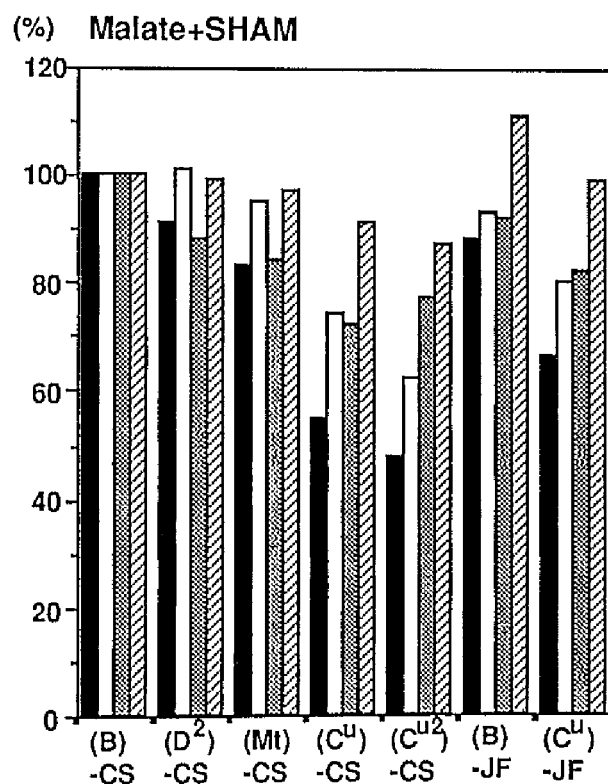
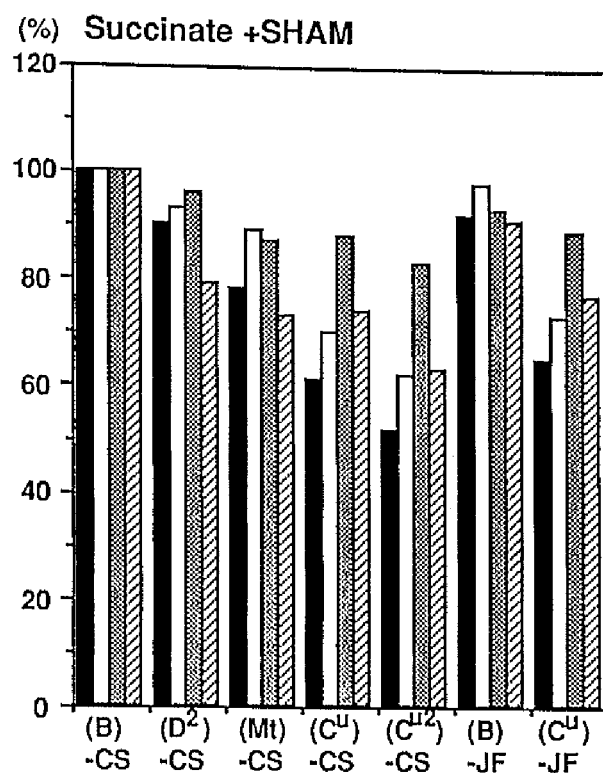


Figure 18. Continued

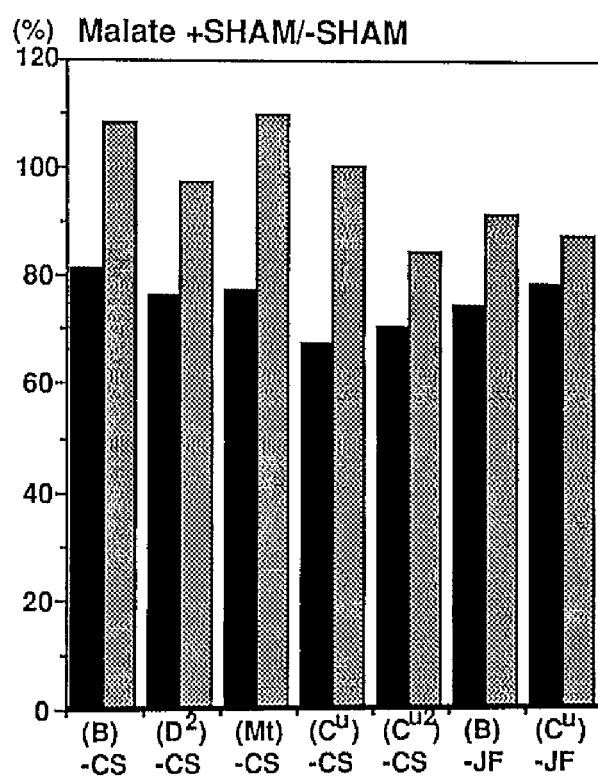
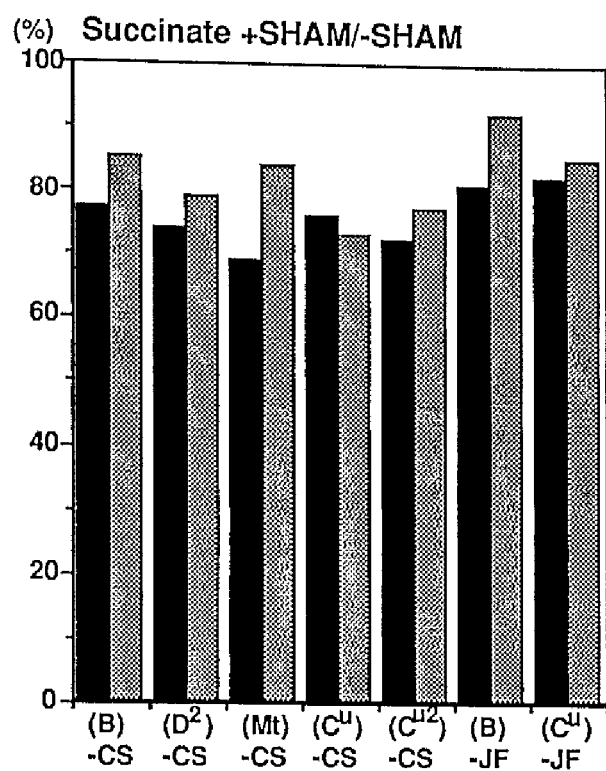


Figure 19. Relative ratio between the respiratory activities observed in the presence and absence of 1 mM SHAM in two common wheats having different cytoplasms. Succinate and malate were used as substrate

■: State 3, ▨: State 4

2) Cytochrome oxidase and reductase activities of the mitochondria isolated from alloplasmic wheats

The activities of cytochrome oxidase and cytochrome reductase, both of which constitute the terminal complexes of the electron transport chain, were measured. The cytochrome oxidase activity decreased considerably in (C^U)- and (C^{U2})-CS (Table 7, Figure 20). Although the cytochrome reductase activity was not different significantly among (B)-, (D²)-, (Mt)- and (C^U)-CS, it decreased in (C^{U2})-CS to 73 % of (B)-CS (Table 7, Figure 20).

Table 7. Cytochrome reductase and cytochrome oxidase activities in mitochondria isolated from CS having five cytoplasms

Line	Cyt c reduced (nmol /mg protein/min)	Cyt c oxidized (nmol /mg protein/min)
(B)-CS	326.5 (100%) a	1406.5 (100%) a
(D ²)-CS	349.2 (107%) a	1499.4 (107%) a
(Mt)-CS	320.4 (98%) a	1522.5 (108%) a
(C ^U)-CS	355.6 (109%) a	361.6 (26%) b
(C ^{U2})-CS	239.9 (73%) b	333.3 (24%) b

(): Percent of (B)-CS.

a and b: Significantly different at 5 % level.

3) Low temperature difference spectra of the mitochondria isolated from alloplasmic wheats

The difference spectra at 77 K of dithionate-reduced mitochondria against the ferricyanide-oxidized mitochondria are shown in Figure 21. In (B)-CS, distinct peaks of cytochrome aa_3 at 598 nm (cytochromes in the cytochrome oxidase), cytochrome b at 557 nm (b -557) and 554 nm (b -554) (cytochromes in the cytochrome reductase) and a soluble cytochrome c at 550 nm were observed. Mitochondria from (D²)-CS showed almost the same spectrum as those of (B)-CS, except the peak of cytochrome b at 554 nm was not apparent. In (Mt)-CS, the peak height of cytochrome aa_3 was slightly low in relation to those of cytochrome b and c . In (C^U)-CS, peaks of cytochrome b and c were obvious, but the peak height of cytochrome aa_3 was extremely low. The spectrum of (C^{U2})-CS was almost the same as that of (C^U)-CS. The peak heights of aa_3 per unit mitochondrial protein also indicated reduced aa_3 values in (C^U)- and (C^{U2})-CS (Table 8).

Table 8. Peak heights of cytochrome b -557 (corresponding to cytochrome reductase) and cytochrome aa_3 (corresponding to cytochrome oxidase) in the mitochondria isolated from CS having five cytoplasms

Material	A557x10 ³ /mg protein ml ⁻¹	A598x10 ³ /mg protein ml ⁻¹
(B)-CS	5.90 (100%) a	2.72 (100%) a
(D ²)-CS	5.41 (92%) a	2.51 (92%) a
(Mt)-CS	6.31 (107%) a	1.71 (63%) b
(C ^U)-CS	6.96 (118%) a	0.82 (30%) c
(C ^{U2})-CS	6.47 (110%) a	0.72 (26%) c

(): Percent of (B)-CS.

a, b and c: Significantly different from each other at 5 % level.

Note) A557 and A598 correspond to the cytochrome b -557 and cytochrome aa_3 , respectively

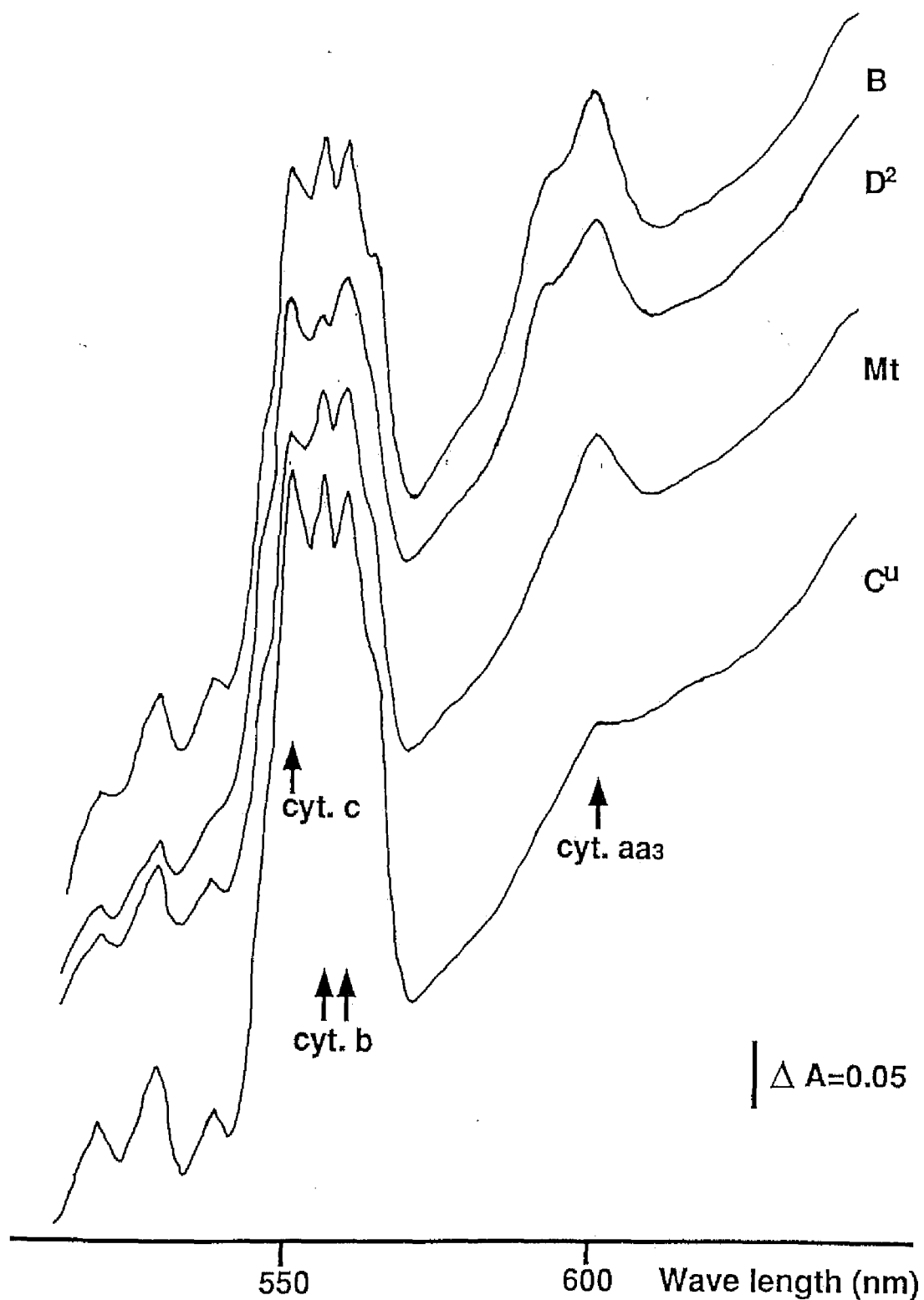


Figure 21. Comparison between the the dithionate-reduced minus ferricyanide-oxidized difference spectra at 77K of the mitochondria of (B)-, (D²)-, (Mt)- and (C^u)-CS

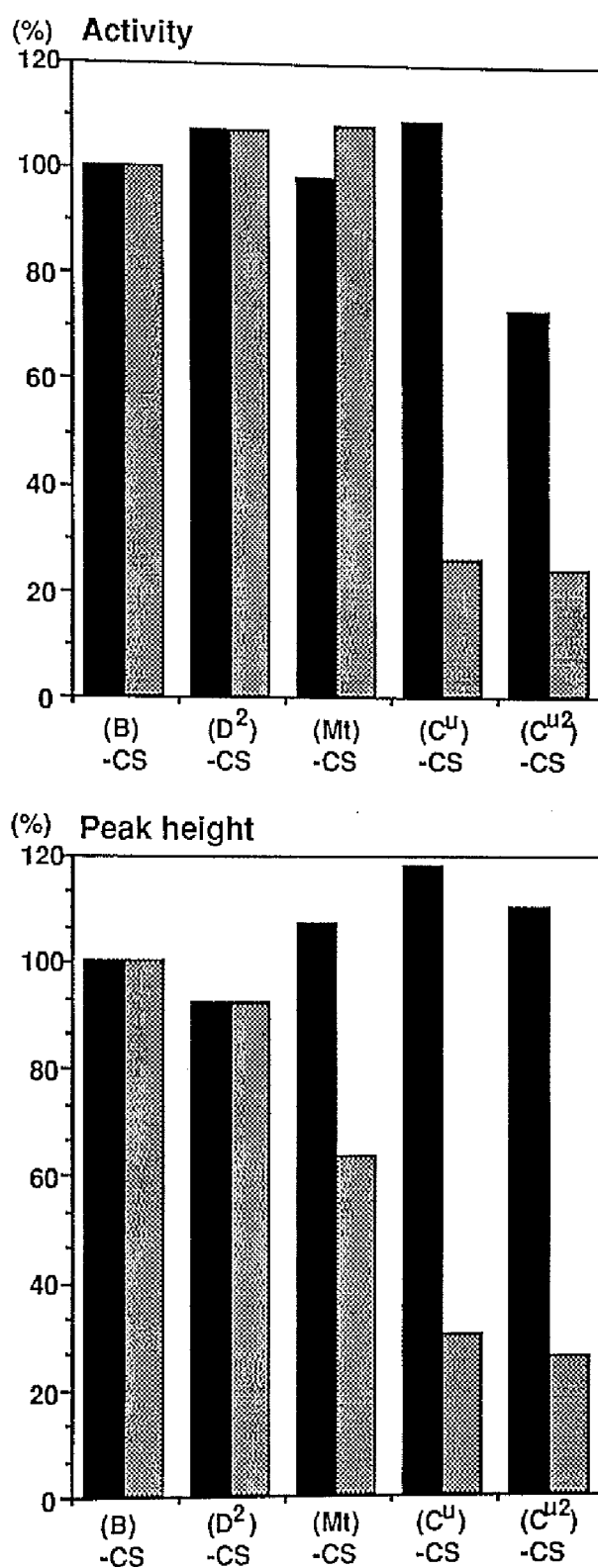


Figure 20. Relative activities and peak heights of cytochrome reductase (*b*-557) and cytochrome oxidase (*aa*₃)

■: Reductase, ▨: Oxidase.

4) RFLP analysis of mitochondrial DNAs

MtDNAs isolated from the eu- and alloplasmic lines of CS were electrophoresed after *Bam*HI, *Eco*RI and *Hind*III digestions (Figure 22). Using seven mtDNA clones as probes (Figure 23), Southern hybridization of the *Bam*HI, *Eco*RI and *Hind*III digested mtDNAs was carried out to reveal the structural differences in the seven mitochondrial genes, *atpA*, *atp6*, *atp9*, *cob*, *cox1* (3' portion), *cox2* and *cox3* among the five cytoplasms (Figure 24). All but a single probe carrying the *cox2* gene revealed clear variation on the number or molecular size of the hybrid bands or the intensity of hybrid signals. Cytoplasms which showed extra hybrid bands were; D² cytoplasm probed with *atpA*, B cytoplasm probed with *atp6*, Mt cytoplasm probed with *cox1* and C^U and C^{U2} cytoplasms probed with *cox3* (Figure 24). Extra weak hybrid bands were also found in C^U cytoplasm probed with *atpA*, B, C^U and C^{U2} cytoplasms probed with *cob* and all cytoplasms probed with *atp9* and *cox2*. The C^U and C^{U2} cytoplasms which have identical chloroplast genome (Ogihara and Tsunewaki 1982, 1988) showed different RFLP patterns of their mtDNAs when probed with *atpA* (one extra minor band in C^U), *cob* (difference in fragment size) and *cox3* (difference in the fragment size and number).

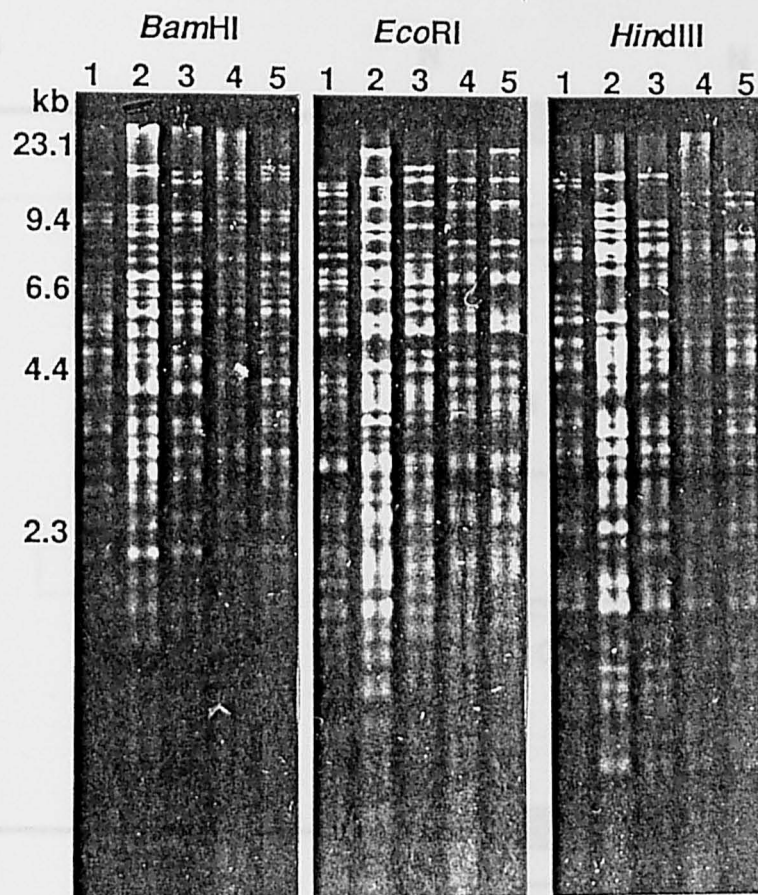


Figure 22. *Bam*HI, *Eco*RI and *Hind*III restriction fragment patterns of mtDNAs isolated from five cytoplasms
1: B type, 2: D² type, 3: Mt type, 4: C^u type, 5: C^{u2} type

Figure 23. Physical maps of seven mtDNA fragments which carry the *atpA*, *atp6*, *atp9*, *cox1*, *cox2* and *cox3* genes, and their subfragments used as the hybridization probes. The symbols of the restriction sites are as follows: B: *Bam*HI, E: *Eco*RI, H: *Hind*III, M: *Mlu*I, N: *Nhe*I, P: *Pst*I, S: *Sac*I and X: *Xba*I.

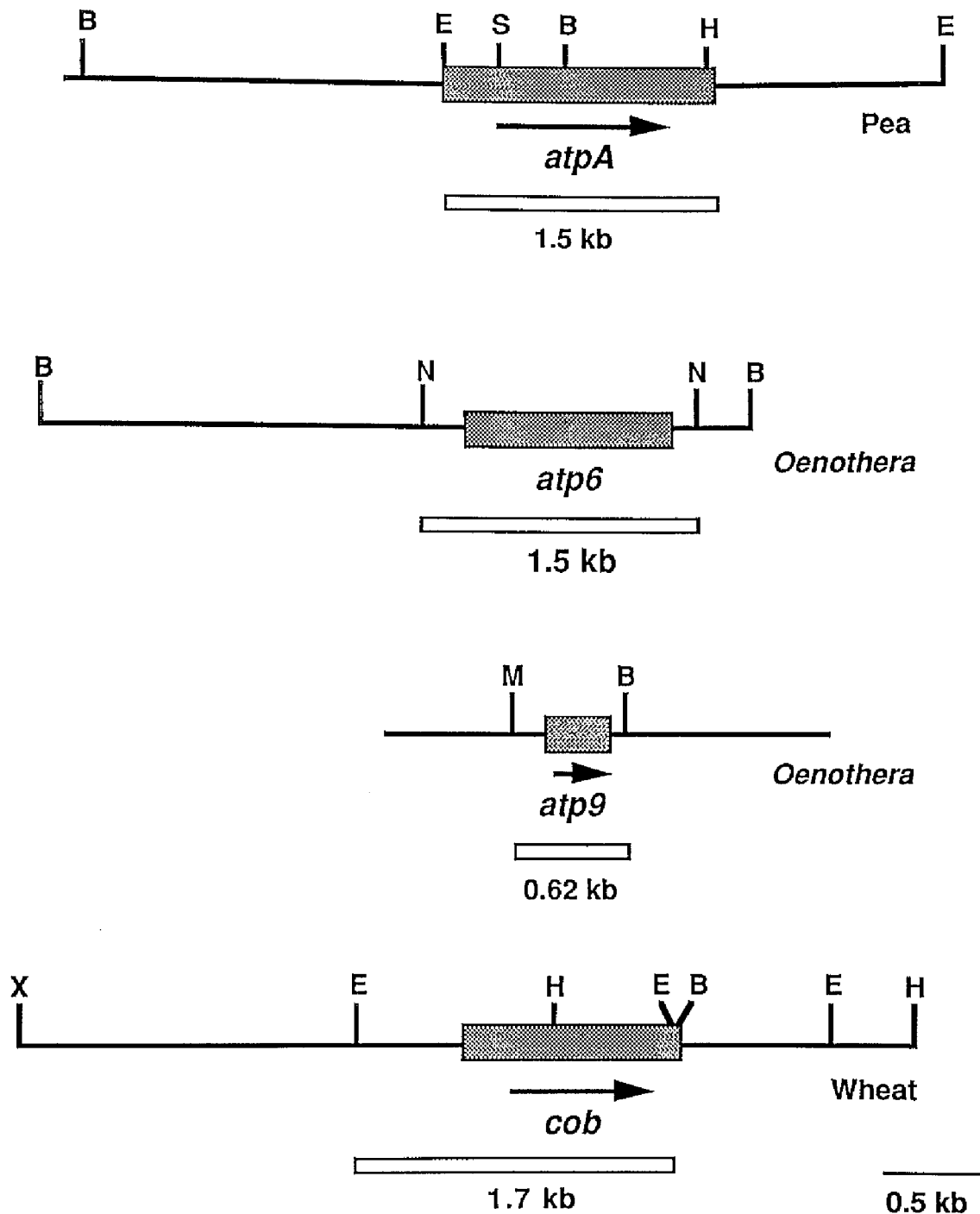
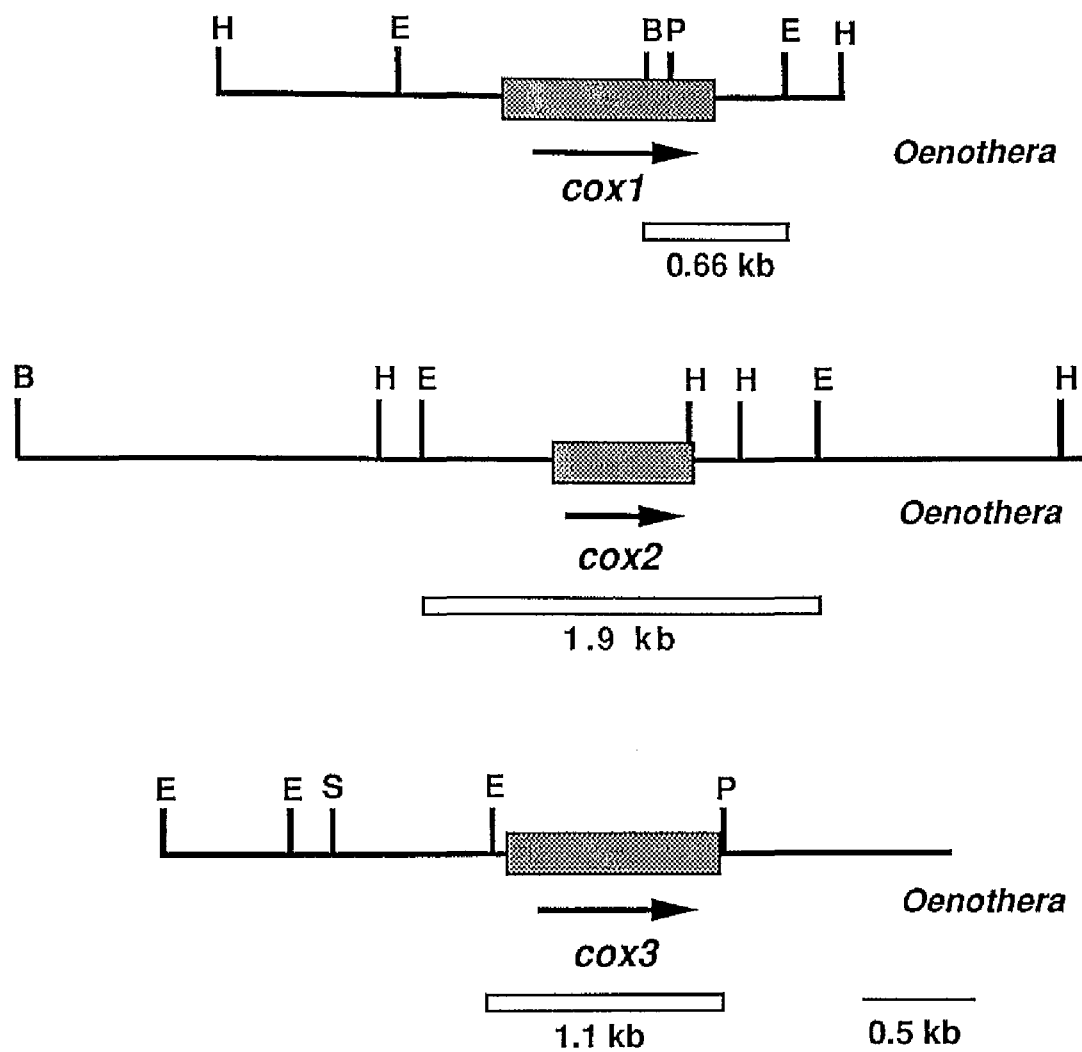


Figure 23. Physical maps of seven mtDNA fragments which carry the *atpA*, *atp6*, *atp9*, *cob*, *cox1*, *cox2* and *cox3* genes, and their subfragments used as the hybridization probes. The symbols of the restriction sites are as follows; B: *Bam*HI, E: *Eco*RI, H: *Hind*III, M: *Mlu*I, N: *Nhe*I, P: *Pst*I, S: *Sal*I and X: *Xho*I



(Figure 23. Continued)

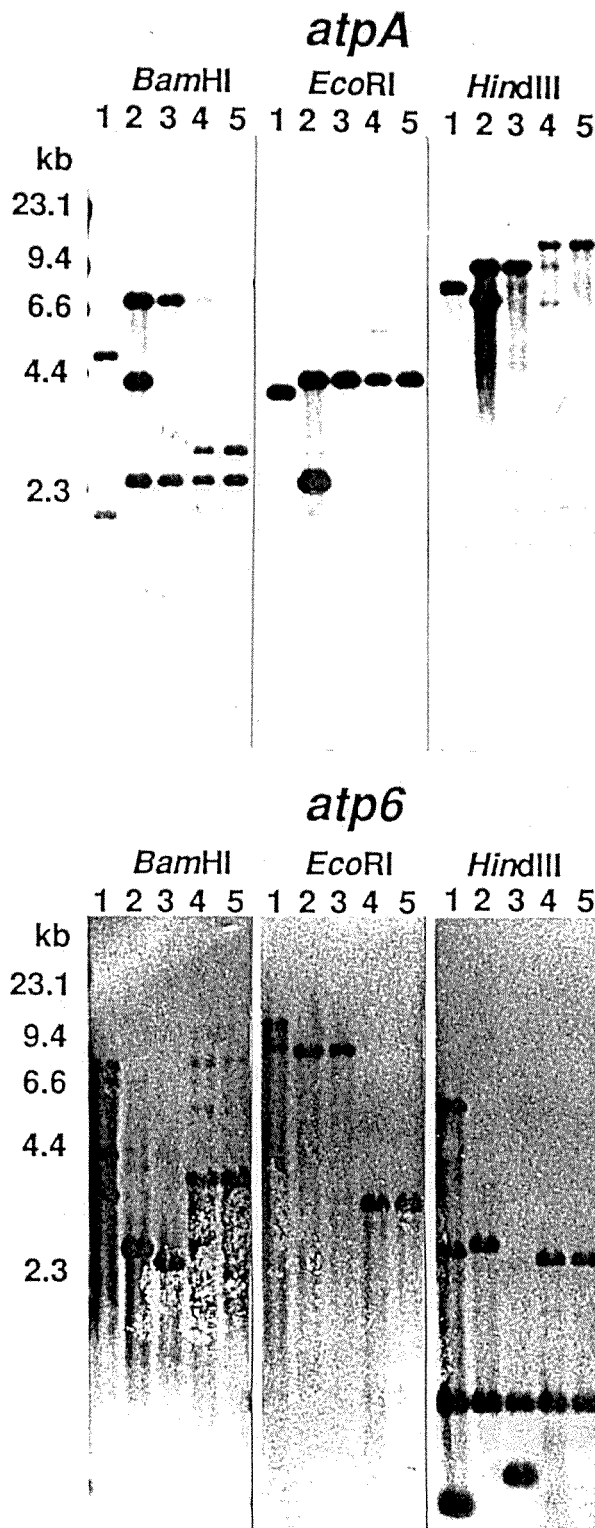


Figure 24. Southern blots of total mtDNA isolated from CS with five cytoplasms, digested with the three enzymes and probed with seven mtDNA clones shown in Figure 23
 1: B, 2: D², 3: Mt, 4: C^u, 5: C^{u2}

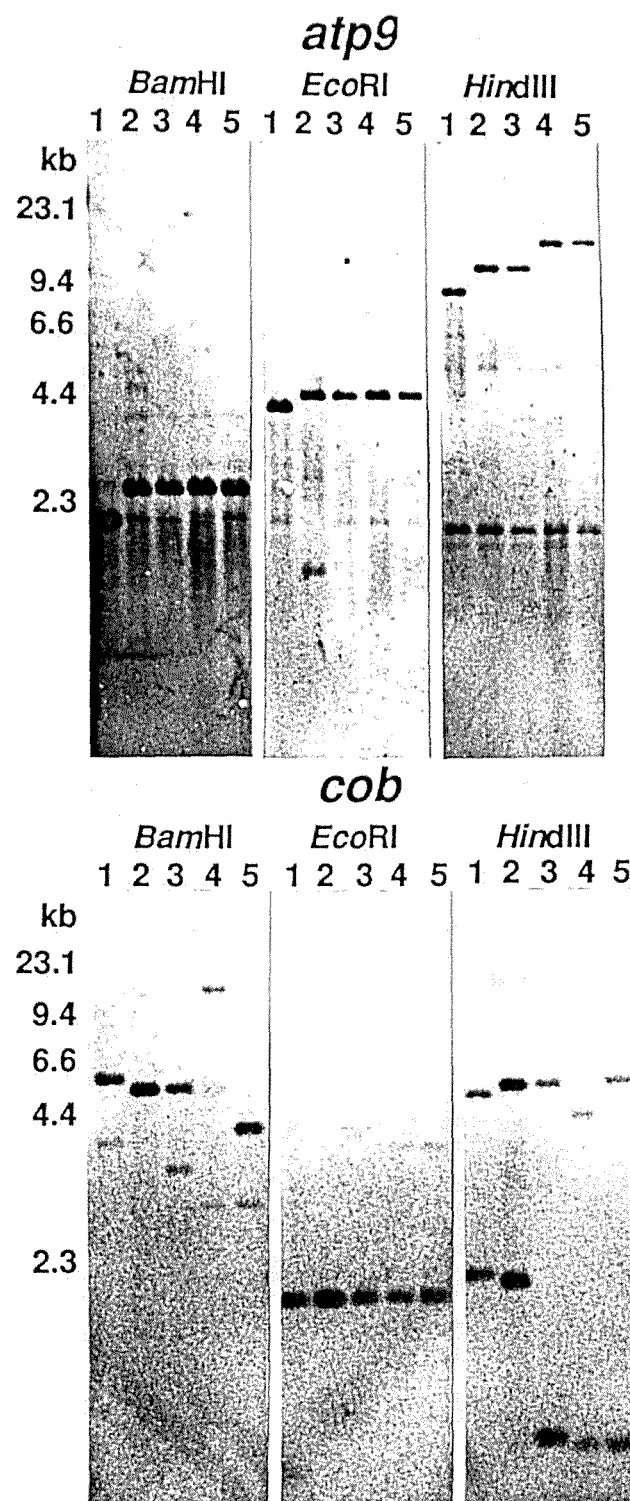


Figure 24. Continued

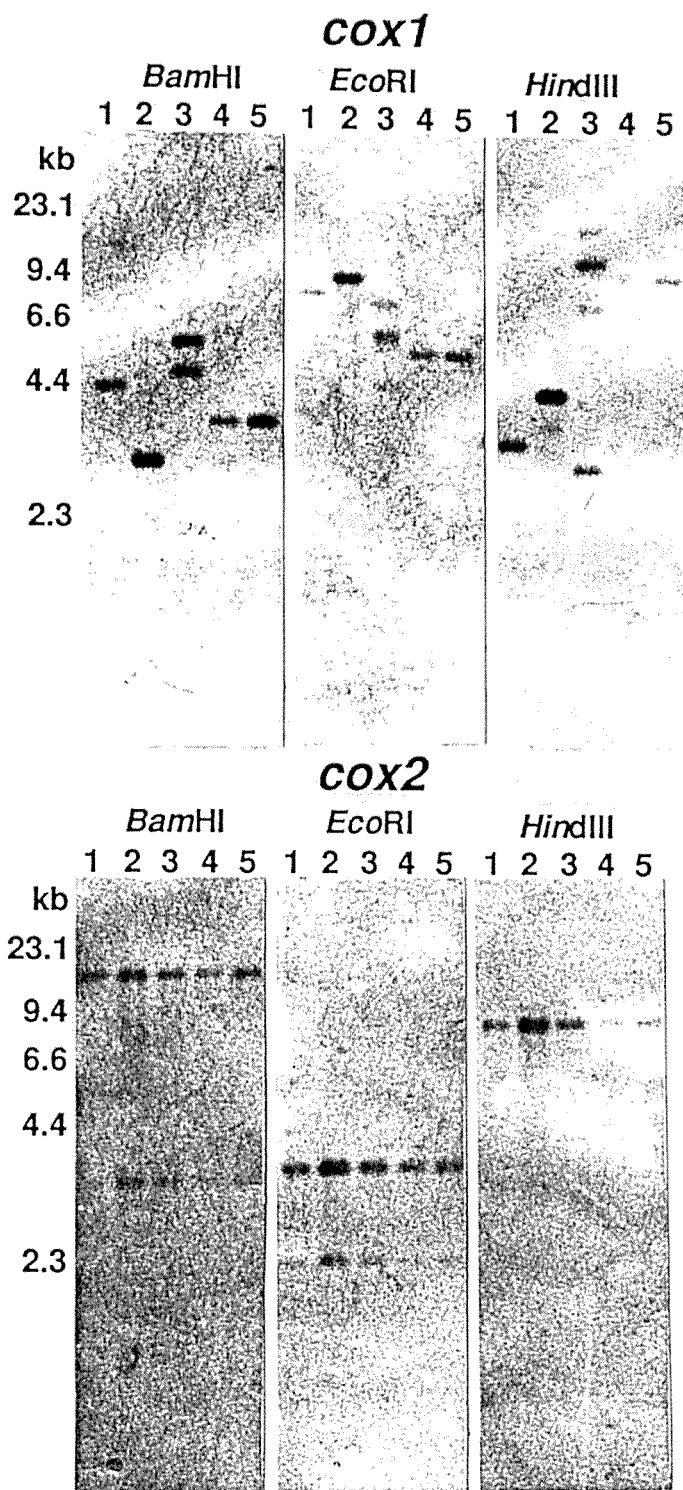


Figure 24. Continued

Figure 24

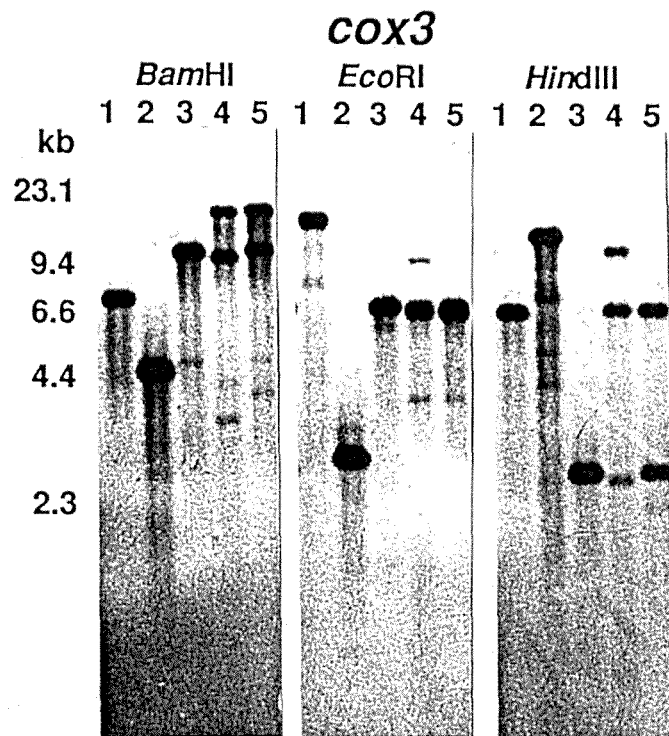


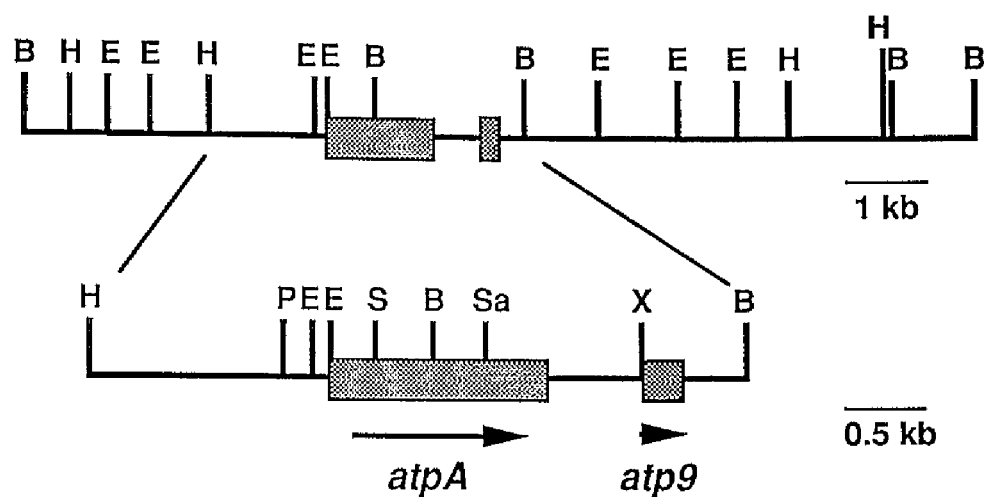
Figure 24. Continued

5) Comparison of physical maps of the three genes, *atpA*, *atp9* and *cox3*, between (B)- and (C^{u2})-CS

The linkage between the *atpA* and *atp9* genes in (B)-CS (Ishii unpublished) is also conserved in (C^{u2})-CS (Figure 25). Although the maps of the 5' flanking region of the *atpA* gene and the 3' flanking region of the *atp9* gene were different between (B)- and (C^{u2})-CS, all the four restriction sites in the coding region of *atpA* gene (*Bam*HI, *Eco*RI, *Sac*I and *Sal*I sites) were identical between them.

In the case of the *cox3* gene, two different maps were constructed using the cloned mtDNA of (C^{u2})-CS (Figure 26). The sizes of the isolated DNA fragments carrying the *cox3* gene digested with *Bam*HI, *Eco*RI and *Hind*III were equal to that of the fragments found in the Southern hybridization pattern of these three endonuclease-treated total mtDNAs probed with the *Oenothera cox3* gene (Figure 24). The two copies of the *cox3* gene in (C^{u2})-CS shared the same restriction sites in their 5' flanking regions, whereas their 3' flanking regions showed different maps. The maps of both copies of the *cox3* gene in (C^{u2})-CS differed from the corresponding map of (B)-CS in both 5' and 3' flanking regions (Figure 26).

B



C^{u2}

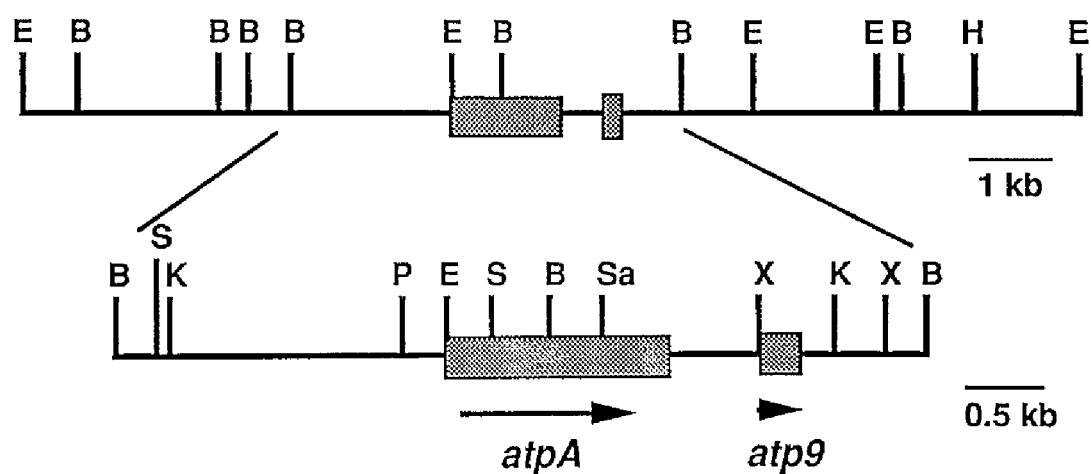
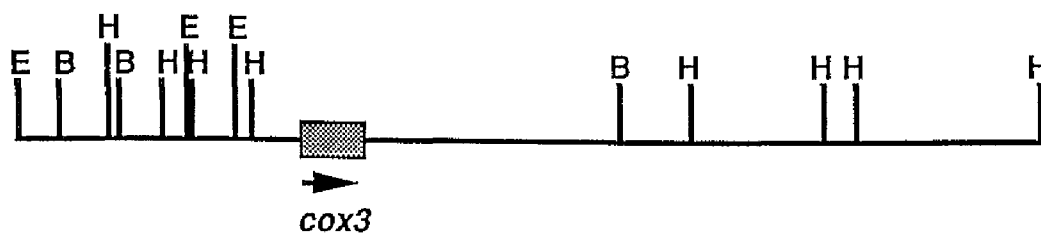


Figure 25. Physical maps of mtDNA segments carrying the *atpA* and *atp9* genes of (B)-CS and (C^{u2})-CS

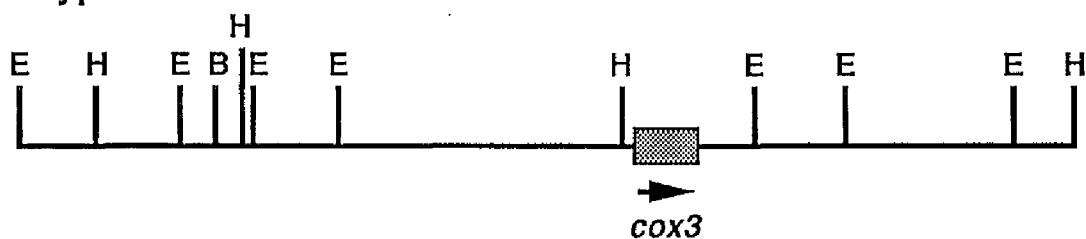
B: *Bam*HI, E: *Eco*RI, H: *Hind*III, K: *Kpn*I, P: *Pst*I, S: *Sal*I, Sa: *Sad*, X: *Xho*I

B

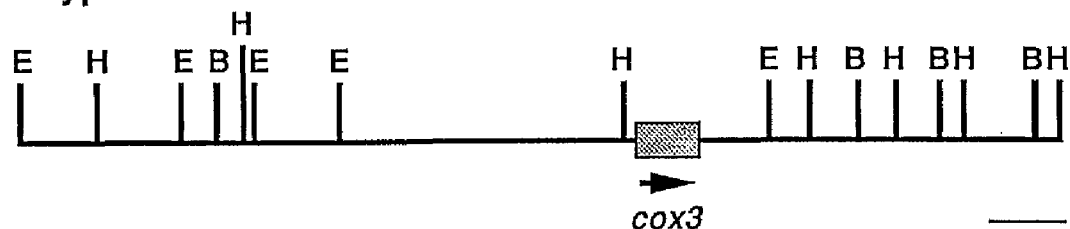


C^{u2}

Type I



Type II



1 kb

Figure 26. Physical maps of the mtDNA segments carrying the *cox3* gene of (B)-CS and (C^{u2})-CS. Two different segments, Type I and II, were obtained in (C^{u2})-CS

B: *Bam*HI, E: *Eco*RI, H: *Hind*III

6) Structural features of the *atpA* and *cox3* genes and their 5' and 3' flanking regions of (C^{u2})-CS

The complete nucleotide sequences of the *atpA* genes of (B)- and (C^{u2})-CS and the two copies of the *cox3* gene of (C^{u2})-CS were determined by the dideoxy chain termination method (Figure 27).

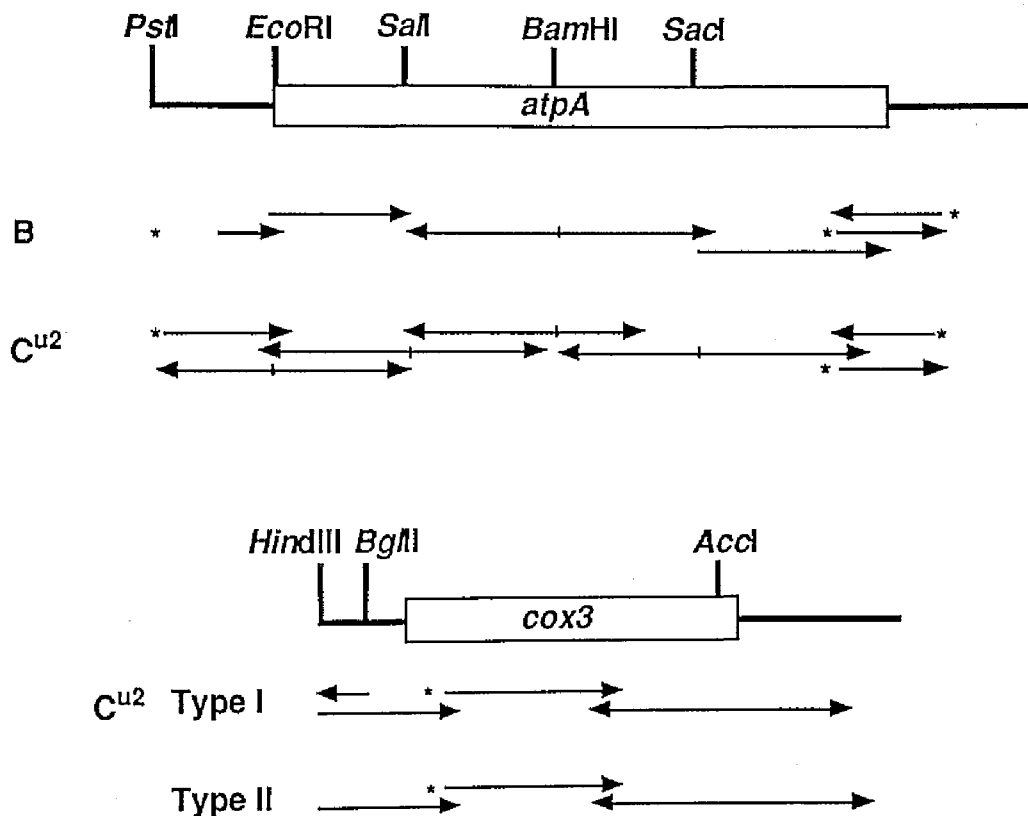


Figure 27. Physical maps of the DNA fragments carrying the *atpA* gene of (C^{u2})-CS, and the *cox3* gene of (C^{u2})-CS. Sequencing strategies are also shown below the maps

*: Sequenced using specific synthetic primers

The *atpA* genes of (B)- and (C^{u2})-CS are almost identical to each other in both the coding and noncoding regions (Figure 28). Four base substitutions were found between them, i.e., at -89 corresponding to the *EcoRI* site in (B)-CS (Figure 25), -5, 1433 and 1537 positions.

(B)-CS	TTTCCCATCTCTTCTTGTATTGTCTTTGTCCGAGATGCCCGCTTCACCAAATGAGAATTCAAATCGAGATTGTGTGAAG	-89	-65
(C ^{u2})-CS	-----T-----		
(B)-CS	TAAAGATCTTTTCTTGAAACGACGATTCTCCCTTCAAAATATCATCCATCTAATTTGTTAAAGTATGGAATTCTCACCC	-5	atpA 15
(C ^{u2})-CS	-----C-----		
(B)-CS	AGAGCTGCGGAACCTCAGACTCTATTAGAAAGTAGAATGACCAACTTTTACACGAATTTTCAAGAGGATGAGATCGGTCG		95
(C ^{u2})-CS	-----		
(B)-CS	AGTGGTCTCAGTTGGAGATGGGATTGCACGTGTTTATGGATTGAACGAGATTCAAGCAGGAGAAATGGTGAATTTGCCA		175
(C ^{u2})-CS	-----		
(B)-CS	GCGGTGTGAAAGGAATCGCCTTAAATCTTGAGAATGAGAATGTAGGTATTGTTGTCTTTGGTAGTGATACCGCTATTAAA		255
(C ^{u2})-CS	-----		
(B)-CS	GAAGGAGATCTTGTCAAGCGCACTGGATCTATTGTGGATGTTCCCTGCGGGAAAGGCCATGTTAGGCCGTGTGGTCGACGC		335
(C ^{u2})-CS	-----		
(B)-CS	CTTGGGAGTACCTATTGATGGAAAAGGGGCTCTAAGCGATCACGAACGAAGACGTGTCGAAGTGAAAGCCCCAGGGATTA		415
(C ^{u2})-CS	-----		
(B)-CS	TTGAACGTAATCTGTGCACGAACCAATGCAAACAGGCTTGGGGGAGTGGATAGCCTGGTTCCTATAGGCCGTGGTCAA		495
(C ^{u2})-CS	-----		
(B)-CS	CGAGAACTTATAATCGGGGACAGACAAACTGGAAAACTGCAATAGCTATCGATACTATATTAAACCAAAGCAAATGAA		575
(C ^{u2})-CS	-----		
(B)-CS	CTCAAGGGGCACAAATGAGAGTGAGACATTGTATTGTGTCTATGTTGCGATTGGACAAAAACGCTCGACTGTGGCACAAAT		655
(C ^{u2})-CS	-----		
(B)-CS	TAGTTCAAATCTTTTCAAGCGAATGCTTTTGAATATTCCATTCTTGTAGCAGCCACCGCTTCGGATCCTGCTCCTCTG		735
(C ^{u2})-CS	-----		
(B)-CS	CAATTTCTGGCCCCATATTTCAGGGTGTGCCATGGGGGAATATTTCCGCGATAATGGAATGCACGCATTAATTATATATGA		815
(C ^{u2})-CS	-----		
(B)-CS	TGATCTAAGTAAACAGGCGGTGGCATATCGACAAATGTCATTATTGTTACGCCGACCACCAGGCCGTGAGGCTTTCCAG		895
(C ^{u2})-CS	-----		
(B)-CS	GGGATGTTTTCTATTTTACATTCCCGTCTCTTGAAGAGCCGCTAAACGATCGGACCAGACAGGTCGAGGTAGCTCGACT		975
(C ^{u2})-CS	-----		
(B)-CS	GCGTTACCCGTGATTGAAACACAAGCTGGAGACGTATCGGCCTATATCCCCACCAATGTGATCTCCATTACAGATGGACA		1055
(C ^{u2})-CS	-----		
(B)-CS	AATCTGTTTGGAAACAGAGCTCTTTTATCGCGGAATTAGACCAGCTATTAACGTTGGCTTATCCGTCAGTCGCGTGGGT		1135
(C ^{u2})-CS	-----		
(B)-CS	CTGCCGCTCAGTTGAAAGCTATGAAACAAGTCTGCGGTAGTTCAAACTGGAATTGGCACAATATCGCGAAGTGGCCGCC		1215
(C ^{u2})-CS	-----		
(B)-CS	TTGCTCAATTTGGGTCAGACCTTGATGCTGCGACTCAGGCATTACTCAATAGAGGTGCAAGGCTTACAGAAGTGCCCAA		1295
(C ^{u2})-CS	-----		
(B)-CS	ACAACCACAATATGAACCACTTCCAATTGAAAAACAAATTGTTGTGATTTATGCTGCTGTCAACGGCTTCTGTGATCGAA		1375
(C ^{u2})-CS	-----		
(B)-CS	TGCCACTAGACAGAATTCTCAATATGAAAAAGCCATTCTAAGTACTATTAATCCAGAAATTACAAAAATCCTTCTTAGAA	1433	1455
(C ^{u2})-CS	-----T-----		
(B)-CS	AAAGGTGGCTTAACTAACGAAAGAAAGATGGAACCAGATGCTTCTTTAAAGAAAGCACTTTGCCTTACCTGTGAATTAA		Stop 1535
(C ^{u2})-CS	-----		
(B)-CS	TCAAAAAGGTTGCCCTTACTCGCAGATGTAGGTTCAAGGCTTTTCTCGCCAACTGAACCCCAATAGGCTATTTTGGACT	1538	2015
(C ^{u2})-CS	-A-----		

Figure 28. Nucleotide sequence comparison between the *atpA* genes of (B)- and (C^{u2})-CS. Bold letters show the initiation and stop codons

The amino acid sequences of the α subunit of ATP synthase, which were deduced from those nucleotide sequences, are identical between (B)- and (C^{u2})-CS (Figure 29).

	10	20	30	40	50	60
B and C^{u2}	MEFSPRAAELTTLLLESRTNFYTNFQVDEIGRVVSVGDGIARVYGLNEIQAGEMVEFASG					
Rice	-----					
Maize	-----I-----LK-----					
	70	80	90	100	110	120
B and C^{u2}	VKGIALNLENENVGIVVFGSDTAIKEGDLVKRTGSIVDVPAGKAMLGRVVDALGVPIIDGK					
Rice	-----					
Maize	-----					
	130	140	150	160	170	180
B and C^{u2}	GALSDHERRRVEVKAPGIIERKSVHEPMQTGLKAVDLSVPIGRGQRELIIGDRQTGKTAI					
Rice	-----					
Maize	-----					
	190	200	210	220	230	240
B and C^{u2}	AIDTILNQKQMNSRGTNESETLYCVYVAIGQKRSTVAQLVQILSEANALEYSILVAATAS					
Rice	-----					
Maize	-----M-----					
	250	260	270	280	290	300
B and C^{u2}	DPAPLQFLAPYSGCAMGEYFRDNGMHALIIYDDLKQAVAYRQMSLLLRPPGREAFPGD					
Rice	-----					
Maize	-----					
	310	320	330	340	350	360
B and C^{u2}	VFYLHSRLLERAARKRSDQTGAGSSSTALPVIETQAGDVSAYIPTNVISITDGQICLETELF					
Rice	-----L-----					
Maize	-----L-----					
	370	380	390	400	410	420
B and C^{u2}	YRGIRPAINVGLSVSRVGSAAQLKAMKQVCGSSKLELAQYREVAFAAQFGSDLDAAATQAL					
Rice	-----					
Maize	-----					
	430	440	450	460	470	480
B and C^{u2}	LNRGARLTEVPKQPQYEPLPIEKQIVVIYAAVNGFCDRMPLDRISQYEKAILSTINPELQ					
Rice	-----L-----					
Maize	-----L-----					
	490	500	509			
B and C^{u2}	KSFLEKGGLTNERKMEPDASLKESTLPYL*					
Rice	-----I-----QTAKEIN*					
Maize	-----A-NL*					

Figure 29. Comparison between the predicted amino acid sequences of the α subunit of ATP synthase among (B)-CS, (C^{u2})-CS, rice (Kadowaki *et al.* 1990) and maize (Braun and Levings 1985)

The two copies of the *cox3* gene in (C^{u2})-CS shared the same nucleotide sequences in both the 5' flanking and coding regions, whereas their 3' flanking regions differed at many positions downstream the position 1059 in their nucleotide sequences (Figure 30).

The *cox3* gene of common wheat (Gualberto *et al.* 1990a) and its two copies in the C^{u2} cytoplasm showed three base substitutions in their coding regions, i.e., at the positions 157, 686 and 687 (Figure 30). In their 5' flanking regions, only a single base substitution (at -212 position) was found up to the -218 position, although the upstream regions from this position differed greatly between the sequences of common wheat and C^{u2} mtDNAs. In the 3' flanking region, (B)- and (C^{u2})-CS had non-homologous sequences downstream the position, +866.

<i>aestivum</i> (C ^{u2})-CS I, II	TTGAAGTCAGAGTCTGGGTGTGTTTTTCTTACTAATACGGAGAGGGTCCGAATGATAAAGACCAATCAAAAACTTCTTC AA-CTT-AGCGCAAGTTTC-----G-G-C-TA--GA-GCATCTATCA-TAT-CGCTGTTTAT-----C-----	-201
<i>aestivum</i> (C ^{u2})-CS I, II	<u>GTTTCGTTGGTAGAAGCCACGCCAACTCTTTCTCTAAATAATAGAGAGATCTTTTGATAGTCTCACTTCTATCAATGCA</u> -----	-121
<i>aestivum</i> (C ^{u2})-CS I, II	ATGAAAGAACTATCCCTTCCTATTGTGTTGTCTTAATGAGACAAAAAGAGCCTCCTTCTTTACCACTTTAGGGGATGGG -----	-41
<i>aestivum</i> (C ^{u2})-CS I, II	cox3 → GGTGAAGGGGGGTTTACATACAACCGGGGCAAAGTGGTTTATGATTGAATCTCAGAGGCATTCTTATCATTTGGTAGATC -----	40
<i>aestivum</i> (C ^{u2})-CS I, II	CAAGTCCATGGCCTATTTCGGGTTCACTCGGAGCTTTGGCAACCACCGTAGGAGGTGTGATGTACATGCACTCATTTCAA -----	120
<i>aestivum</i> (C ^{u2})-CS I, II	GGGGGTGCAACACTTCTCAGTTTGGGCCTAATATTTATCCCTTTATACCATGTTCTGTATGGTGGCGGGATGTTCTACGTGA -----C-----	157 200
<i>aestivum</i> (C ^{u2})-CS I, II	ATCCACGTTGGAAGGGCATCATACAAAAGCTGTACAATTAGGACCTCGATATGGTTCTATTCTCTTCATAGTCTCGGAGG -----	280
<i>aestivum</i> (C ^{u2})-CS I, II	TTATGTTTCCTTTTGTCTTTTTTTGGGCTTCTTCTCATCTTCTTTGGCACCTACGGTAGAGATCGGAGGTATTGGCCC -----	360
<i>aestivum</i> (C ^{u2})-CS I, II	CCAAAAGGGATTGGGGTTTGTAGTCCTTGGGAAATCCCTCTTCTTAATACCCCTATTCTCCCTTCATCCGGAGCTGCCGT -----	440
<i>aestivum</i> (C ^{u2})-CS I, II	AACTTGGGCTCATCATGCTATACTCGCGGGGAAGGAAAAACGAGCAGTTTACGCTTTAGTAGCAACCGTTTCACTGGCTC -----	520
<i>aestivum</i> (C ^{u2})-CS II	TAGTATCCACTGGCTTTCAAGGAATGGAATATTACCAAGCACCCCTCCACTATTTCGGATAGTATTTATGGTTCTACCTTT -----	600
<i>aestivum</i> (C ^{u2})-CS I, II	TTCTTAGCAACTGGCTTTCATGGTTTTCATGTGATTATAGGTACTCTTTTCTTGATCGTATGTGGTATTCGCCAATATCT -----	680
<i>aestivum</i> (C ^{u2})-CS I, II	686 TGGTCAGATGACCAAGAAGCATCACGTTGGCTTTGAAGCAGCTGCATGGTACTGGCATTTTGTAGACGTGGTTTCGGTTAT -----TC-----	760
<i>aestivum</i> (C ^{u2})-CS I, II	stop (798) TCCCATTGTCTCTATCTATTGGTGGGGAGGTATATGAAGAAGGGGAACGAATAAGTGGATTGAGGAATAAAAGCTCGAA -----	840
<i>aestivum</i> (C ^{u2})-CS I, II	866 GACAAAGAGAACTTCTCCGGGTACTCAAGATATTGTGTTTGGAGAGGTGTAGATTGTAGATTCCAGCCGAGCCAGACCCA -----A-TGGATCGCCCC-GCACTGTG-T-TA-TGAGCGGA-AA--CTAA-AG--C-GG-G	920
<i>aestivum</i> (C ^{u2})-CS I, II	GGAAGAATACGAAGTCCAATTTCTCAACCGCGCACACGGATAAGACGCGAGACAGTTTCTGAGGGATAGAAGTCTTGAT ATGT-C-GGAA-TAG-GGTCC-AAAATGAGT-T-C-C-ATGATCATTTTACAT--CGA-A-CTTTTTCTTCT-GT-ATGC	1000
<i>aestivum</i> (C ^{u2})-CS I	CGGGCCAAGCCGGCATAACAGTTAGTAGTTTCGATTACTTTCTCTTCCGAAGCGGCACATTCGAGTTCAGTATGGATGC GTTTT-CTCTTCA-T-TT-TT--TA-TTC-TTT--GGAA-CG--CGGGC-GAAA--GGG--AGTGA--G-TGGCAT-GAG	1059 1080
<i>aestivum</i> (C ^{u2})-CS II	GTTTT-CTCTTCA-T-TT-TT--TA-TTC-TTT--GGAA-CG--CGGGC-GAAA--GGC-AGAATC-CTTC-G-C-TA-A -----	1125
<i>aestivum</i> (C ^{u2})-CS I	ACTGAGCGCTTAAGACCACTCGCTGGATGATATGGTCAATCATAC TT-TGATAGAA--CT-GTA--AAAATCAT-CCAAAAG--AGTCT-	
<i>aestivum</i> (C ^{u2})-CS II	GGCCTCGT-GAGT-CTTCGCTA-GCC-G--G---A-G-TCA-CGA	

Figure 30. Comparison between the nucleotide sequences of the *cox3* genes among *T. aestivum* (Gualberto *et al.* 1990a) and (C^{u2})-CS (Type I and II). Bold letters show the initiation and stop codons. Common sequences found upstream and downstream the coding regions of other mitochondrial genes are underlined (ref. Figure 31)

The *cox3* gene of common wheat is flanked by two short homologous sequences, one to a sequence upstream the maize *cox3* gene and common wheat *cob* gene, and the other to downstream the wheat *atp6* gene (Gualberto *et al.* 1990a, Figure 31). It is interesting to note that the upstream sequence of the *cox3* gene in (C^{u2})-CS showed homology in a further extended region to the common wheat *cob* gene than that of the common wheat *cox3* gene, whereas its 3' flanking region retained only 8 bp homology to the wheat *atp6* gene due to a possible recombination at 864 position, whereas the corresponding region of the wheat *cox3* gene revealed homology much more extended. Further downstream the *cox3* gene, (C^{u2})-CS showed homologies with several other genes, namely, a part of the sequence including a portions of the 5' exon of the wheat *cox2* gene (Bonen *et al.* 1984, position 955 to 991), 5' flanking region of the *Oenothera cox3* gene (Hiesel *et al.* 1987, position 986 to 1017) and the maize *atp6* gene (McCarty *et al.* 1988, position 1055 to 1100).

Upstream

		-247	-228	-218	-212	-176
Wheat <i>cox3</i>	-252	TTACTAATACGGAGAGGGTTCCGAATGATAAAGACCAATCAAAAACCTTCTTCGTTTCGTTGGTAGAACCCACGCC				.AACTCTTTTCTCTAAA
Maize <i>cox3</i>	-250	CTTTCGGGGGGGTATCCCATGACATGATAAAGACCAATCCAAAACCTTCTTCGTTTCGTTGGTAGAA				.CAACGCC.GACTCTTCTCT.AAAA
<i>C^{u2}</i> <i>cox3</i> I,II	-252	CTTATAGAAGCATCTATCATTATGCGCTGTTTATCCAATCCAAAACCTTCTTCGTTTCGTTGGTAGAACCCACGCC				.AACTCTTTTCTCTAAA
Wheat <i>cob</i>	-576	GCCGAGAGAAGCGTCTATCATTATACGCTGTTTATCCAATCAAAAACCTTCTTCGTTTAGTTGGTAGAACCCACGCCCAATATCATATTTGGACT				

Downstream

Downstream		856	864		930	
Wheat <i>cox3</i>	+848	AGAACTTCTCCGGGTACTCAAGATATT.....GTGTTTGGAGAGGTGTAGATTGTAGATTCCAGCCGAGCCAGACCAGGAAGAATACGAA				
Wheat <i>atp6 a</i>	+1218	AACATGGTTCGGGTACTCAAGATATTGCGTTTGTGTTTGGAGAGGTGTAGATTGTAGATTCCAGCCGAGA.AGACC.AGGAAAGATAAGG				
Wheat <i>atp6 b</i>	+1218	AACATGGTTCGGGTACTCAAGATATT.....GTGTTTGGAGAGGTGTAGATTAGATAGGACGTAGTCTTGCTCG				
<i>Cu² cox3</i> I,II	+848	AGAACTTCTCCGGGTAAATTGGATCGCCCCGGCACTGTGGTTTAATGAGCGGAAACCCTAAAAGCACAGGCGATGTGCAGGAAATAGCGGT				
		955	986	991	1017	
<i>Cu² cox3</i> I,II	+940	CCTAAATGAGTCTCCCCATGATCATTTTACATCAGATATCTTTTCTTCTGGTTATGCGTTTTCCTCTTCACTTTCTTTTTATTTCCTT				
Wheat <i>cox2</i> 5'		AAACAATGTGTCCCCATGATCATTTTACATCAGATATCTTTTCTTCTCTCATTCTTATTTTGGTTTTCGTATCACGGATGT				
<i>Oenothera cox3</i>		-998	AAATTTACTTATTTTACACAAATCTTCTCTGGTTCATGCCTTTTCCTCTTACTTTCTATATTTCCCATAT			
		1045	1055	1059	1064	1100
<i>Cu² cox3</i> I	+1032	TTATGGAATCGTCCGGGCAGAAACGGGGATAGTGATTGATGGCATAGAGTTTGTATAGAAAACCTCGTATCAAATCATACCAAAGAAAGTC				
Maize <i>atp6</i>		-762	GGATTCTATCCCGAGGATAGAGATTGATGGGTAATAGTTTGTATAGAAAACCTCGTATGTTAAGTTCTCTAGTTG			
<i>Cu² cox3</i> II	+1032	TTATGGAATCGTCCGGGCAGAAACGGGCAAGAATCTCTTCTGTCGTAGAGGCTCGTCGAGTGCTTCGCTACGCCAGGAGATGATCAACGAA				

Figure 31. Homologies between the nucleotide sequences of the flanking regions of the *cox3* genes of common wheat (Gualberto *et al.* 1990a), (*C^{u2}*)-CS, maize (MaCarty *et al.* 1988) and *Oenothera* (Hiesel *et al.* 1987), the *cob* (Boer *et al.* 1985) and partial copy of wheat *cox2* genes of common wheat (Bonen *et al.* 1984), and *atp6* genes of common wheat and maize (Dewey *et al.* 1986). Vertical thick arrows indicate the possible recombination sites. Underlines indicate putative stem-loop structure. Short homologous sequences are indicated by horizontal arrows

7) Northern blot analysis of the *cox3* gene transcript in (B)- and (C^{u2})-CS

In order to clarify the effect of the rearrangements occurred in the both flanking regions of the *cox3* genes in (C^{u2})-CS, northern blot analysis of their transcripts which were isolated from the etiolated seedlings were carried out, using the cloned mtDNA fragment containing the *Oenothera cox3* gene (Hiesel *et al.* 1987, Figure 23) as the probe. Two major bands were found in (B)-CS, their sizes being about 1.2 kb (Figure 32). On the contrary, (C^{u2})-CS showed a single major band of about the same size as in (B)-CS, with much reduced intensity.

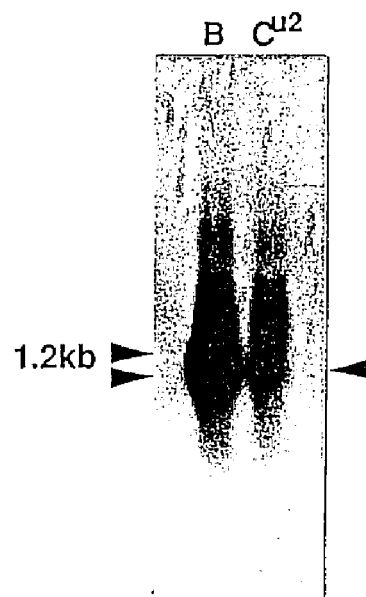


Figure 32. Northern blot of total mtRNAs of (B)- and (C^{u2})-CS hybridized with the *Oenothera cox3* gene

4. Discussion

1) Respiratory activities of mitochondria of alloplasmic wheats in relation to their cytochrome compositions

Cytoplasm-specific variation on the mitochondrial activities has been revealed among the cytoplasms of five *Triticum* and *Aegilops* species. The decrease in the state 3 and cytochrome oxidase activities in the C^U and C^{U2} cytoplasms were most evident. The low cytochrome oxidase activity in both cytoplasms is consistent with the low peak height of cytochrome aa_3 in the low temperature difference spectra. Because the state 3 activity represents the electron chain activity, the impaired cytochrome oxidase activity may suppress the electron transport, causing the low state 3 activity. The decrease in cytochrome reductase activity observed in (C^{U2}) -CS may result in the lower state 3 activity than in (C^U) -CS. As for the other enzymes, NADH dehydrogenase and succinate dehydrogenase, both of which constitute the initial complex of the electron chain, may not have appreciable effects on the variation on the respiratory activity, because the relative respiratory activity of, for example, state 3 did not differ when succinate and malate (NADH produced by its dehydrogenation) used as the substrates (Table 6, Figure 18).

These results suggest that the growth inhibition in (C^U) - and (C^{U2}) -CS is caused by the suppression of respiratory activity of their mitochondria. However, higher respiratory activity of (C^U) -CS than that of (C^{U2}) -CS does not agree with a fact that growth inhibition is more severe in (C^U) -CS than in (C^{U2}) -CS. Other factors may be involved in growth inhibition.

In maize, a close relationship between male sterility and cytochrome oxidase activity during pollen development is reported (Bino *et al.* 1986; Ohmasa *et al.* 1976). In (C^U) - and (C^{U2}) -CS, both of which show weak male

sterility (40 to 60 % decrease of selfed seed fertility, Tsunewaki 1980), the decrease in oxidase activity (more than 70 % reduction, ref. Figure 20) is also correlated with the male sterility, but even such severe decrease in the oxidase activity does not induce complete male sterility. In general, male sterility and growth inhibition are not strictly correlated to each other. To clarify the effect of cytochrome oxidase activity on these characters, it is necessary to measure its activity in anther during microsporogenesis on one hand and that in the intact plant at different stages of growth on the other.

The pathway resistant against cyanide is also related to cytoplasmic male sterility in several plants (Musgrave *et al.* 1986; Connet and Hanson 1990; Kishitani and Konno 1990). From the present results (Figures 18 and 19), however, this pathway does not seem to contribute for the variation on the respiratory activity. CS with *T. timopheevii* cytoplasm which causes severe male sterility in common wheat should be used to clarify a relationship between this pathway and male sterility.

Relatively low peak heights of cytochrome *b* (*b*-557) in (D²)-CS and of cytochrome *aa*₃ in (Mt)-CS are not reflected on the activities of cytochrome reductase and cytochrome oxidase, respectively (Figure 21, Tables 7 and 8). Low respiratory activity and growth abnormality are found in (Mt)-CS in less extent than those in (C^U)- and (C^{U2})-CS (Tables 1 and 6) which show remarkable reduction of cytochrome *aa*₃ peak height. It suggests that the reduction of cytochrome *aa*₃ peak height in Mt cytoplasm should be related to the low respiratory activity and growth abnormality. Further study is needed to clarify the relationship between variation on cytochrome composition and growth abnormality, for example, using other cytoplasms which show the same growth abnormalities as D² and Mt cytoplasms to identify common variation on cytochrome composition.

As for the effect of the JF nucleus which carries a gene for suppressing chlorophyll deficiency and growth inhibition (Mukai and Tsunewaki 1976), the respiratory control ratio (RCR) and P/O ratio of (C^U)-JF were slightly higher

than those of (C^U)-CS (Table 6). It seems to suggest that this suppressor improves the coupling between electron transport and ATP synthesis in mitochondria.

2) Variation on the copy number of mitochondrial genes

All three restriction enzymes used for mtDNA digestion revealed extensive RFLPs among the five different cytoplasms when seven mitochondrial genes encoding the functional proteins related to respiratory chain were used as probes (Figure 24). Based on the numbers of hybrid bands, the copy numbers of these genes in each cytoplasm can be estimated (Table 9). Duplications of the following genes have been disclosed in some cytoplasms shown in parentheses; *atpA* (D²), *atp6* (B), *cox1* (Mt), and *cox3* (C^U and C^{U2}). At least three and two copies of *cox3* are present in (C^U)- and (C^{U2})-CS, respectively. Weak hybrid bands detected with *atpA*, *atp9* and *cox2* probes suggest the presence of their intact copy in substoichiometrical state as sublimons (Small *et al.* 1987) or the presence of their small portion in stoichiometrical number. The *cox2* gene of, at least, common wheat (B cytoplasm) seems to be the latter case, because its mitochondrial genome contains a portion of the 5' exon of the *cox2* gene in addition to its intact copy (Bonnen *et al.* 1984). Further study is needed to clarify the origin of the weak hybrid bands, such as construction of physical maps including these fragments.

From the Southern hybridization patterns given in Figure 24, identical hybrid fragments found in common between all pairs of the five cytoplasms have been scored, in addition to the total hybrid fragments (Table 10). From these data, Nei's genetic distances have been calculated (Table 11). A dendrogram showing the phylogenetic relationships between these mitochondrial genomes is drawn by the UPGMA method (Sokal and Michener 1958) based on their genetic distances (Figure 25). MtDNA of B

cytoplasm is the most differentiated among all others, whereas C^U and C^{U2} cytoplasms are most closely related to each other. The percentage of common fragments (93.5 %) observed here between C^U and C^{U2} cytoplasms is very close to that (92 %) reported by Terachi and Tsunewaki (1986) from restriction fragment pattern analysis.

Because of very complex nature of the mitochondrial genome due to frequent intramolecular recombinations between repeated sequences (Lonsdale 1984; Fauron and Havlik 1989), its RFLP data obtained by either Southern hybridization or restriction pattern will give the overestimated evolutionary rate as compared with that of their chloroplast genome (Ogihara and Tsunewaki 1988).

Among the three genes, *cox1*, *cox2* and *cox3*, for the subunits of cytochrome oxidase, *cox3* is only the one in which more than one copy of a gene are found in C^U and C^{U2} cytoplasms (Table 9). It is known that the duplicated genes generated from a rearrangement of mtDNA often have novel gene structure leading to alteration of their function (Dewey *et al.* 1986; Levings and Brown 1989 for review). The *cox3* genes in these cytoplasms may be the case, and their altered structures seem to have caused change in the cytochrome oxidase activity. The peak height of cytochrome *aa₃* of Mt cytoplasm is low (63 % of control, Table 8). The mitochondrial genome of this cytoplasm has at least two copies of the *cox1* gene (Table 9). In this case, too, gene duplication and functional alteration seem to have occurred.

Table 9. Estimated copy numbers of seven mitochondrial genes in the euplasmic and four alloplasmic lines of CS

Material	Estimated copy number*						
	<i>atpA</i>	<i>atp6</i>	<i>atp9</i>	<i>cob</i>	<i>cox1</i>	<i>cox2</i>	<i>cox3</i>
(B)-CS	1	2	1(1)	1(1)	1	1(1)	1(1)
(D ²)-CS	2	1	1(1)	1	1	1(1)	1(1)
(Mt)-CS	1	1	1(1)	1	2	1(1)	1(1)
(C ^u)-CS	1(1)	1	1(1)	1(1)	1	1(1)	3(2)
(C ^{u2})-CS	1	1	1(1)	1(1)	1	1(1)	2(2)

*: Number of the copies showing weak hybrid signal are given in parentheses

Table 10. The total (above the diagonal) and the common (below the diagonal) hybrid fragments revealed among the mitochondrial genomes in five cytoplasms by Southern blotting using 21 probe-enzyme combinations

	Mitochondrial genome (cytoplasm)				
	B	D ²	Mt	C ^u	C ^{u2}
B		89	89	93	86
D ²	18		105	102	95
Mt	18	36		101	100
C ^u	18	28	26		124
C ^{u2}	18	28	32	58	

Table 11. Genetic distances estimated among the mitochondrial genomes in five cytoplasms from the data given in Table 10

	Mitochondrial genome (cytoplasm)			
	B	D ²	Mt	C ^u
D ²	0.25			
Mt	0.25	0.52		
C ^u	0.25	0.38	0.35	
C ^{u2}	0.26	0.42	0.47	0.88

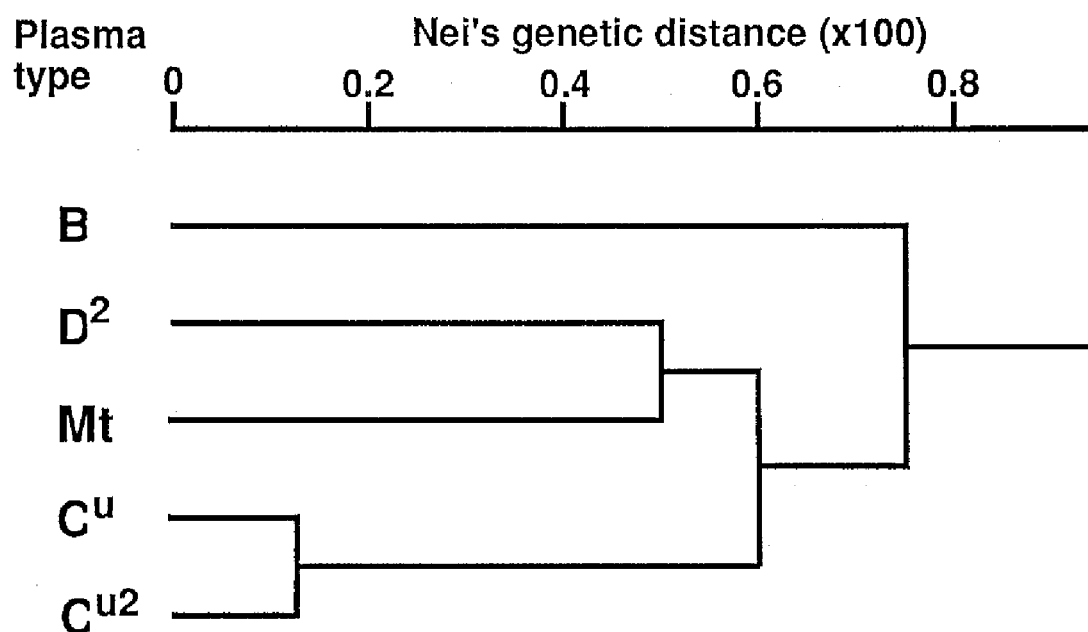


Figure 33. A dendrogram showing the phylogenetic relationships among the mitochondrial genomes in five cytoplasms (indicated by their plasma types) using genetic distances (Nei 1987)

3) Structural changes in two mitochondrial genes, *atpA* and *cox3*, in (C^{u2})-CS

i) The *atpA* gene

Close linkage between the two genes, *atpA* and *atp9*, found in (C^{u2})-CS is conserved in other *Aegilops* species (Ishii unpublished). Selection pressure may have been in favor of its maintenance, or no repeated sequences necessary for recombinations exist in this region.

Compared with that of (B)-CS, the *atpA* gene of (C^{u2})-CS has one and three base substitutions in its coding and flanking regions, respectively (Figure 28). The observed base substitution rate is somewhat smaller than that (seven substitutions) observed in the chloroplast *atpB* gene of the same cytoplasm, that has similar coding region in size (Figure 14). These results are in agreement with a known fact that the base substitution rate is lower in mtDNA than in ctDNA in higher plants (Palmer and Herbon 1987; Wolfe *et al.* 1987; Fauron and Havlik 1989). Therefore, it is concluded that the extensive RFLPs found in mtDNAs should be mainly resulted from frequent recombinations as the ones found in upstream the *atpA* gene and downstream the *atp9* gene (Figure 25).

Because the predicted amino acid sequence is identical between (B)- and (C^{u2})-CS (Figure 29), the α subunits of ATP synthase in their mitochondria should have the same function. The α subunits of both (B)- and (C^{u2})-CS have four and six amino acid substitutions, when compared with those of rice (Kadowaki *et al.* 1990) and maize (Braun and Levings 1985), respectively, which are resulted from base substitutions. Besides these an extensive variation is found in the carboxyl terminal region of this subunit due to DNA rearrangements occurred in these species (Figure 34).

B, Cu ²	ATGGAACCAGATGCTTCTTTAAAAGAAAGCACTTGCCTTACCTGTGAATTAAT
	M E P D A S L K E S T L P Y L STOP
Rice	<u>ATTGAACCTGATGCTTCTTTAAAACAAACTGCGAAGGAGATTAATTAGATAGAC</u>
	I E P D A S L K Q T A K E I N STOP
Maize	<u>ATGGAACCTGATGCTTCTTTAAAAGAAAGCGCTTTAAATTTATGAGAAGCAAAA</u>
	M E P D A S L K E S A L N L STOP

Figure 34. Comparison between the nucleotide sequences of the 3' terminal of the *atpA* gene and amino acid sequences deduced from them among (B)-CS, (Cu²)-CS, rice (Kadowaki *et al.* 1990) and maize (Braun and Levings 1985)

ii) The *cox3* genes

Remarkable structural differences in both the flanking regions of the *cox3* gene are found between (B)- and (Cu²)-CS. The 5' and 3' flanking sequences of the common wheat *cox3* gene is also present in the 5' flanking sequences of the *cob* gene (position -542 to -500), and 3' flanking sequence of the *atp6* gene (position 856 to 930) of common wheat, respectively. Therefore, active recombination sites seem to locate in these regions resulting in the structural divergence of these genes (Gualberto *et al.* 1990 a). Both 5' and 3' flanking sequences of the *cox3* gene of (Cu²)-CS also seem to be involved in recombination (Figure 31). A 3' flanking sequence which distinguishes the two types of the *cox3* gene of (Cu²)-CS shows homology to a 5' flanking sequence of the maize *atp6* gene that is known to be duplicated in maize cms-T cytoplasm creating a novel sequence TURF 2H3 (Dewey *et al.* 1986). In addition, a 3' flanking sequence from the position +955 to +991 of the *cox3* gene of (Cu²)-CS is homologous to the partial copy of the common wheat *cox2* which carries a portion of its 5' exon sequence (Bonen *et al.* 1984). It means that this DNA fragment is repeated at least three times in Cu² cytoplasm. These repeats may have contributed to the mtDNA diversity and complexity in this cytoplasm. It is interesting to note that all these homologous sequences found have been originated from the flanking or coding regions of mitochondrial genes and are located in the

same direction as in the *cox3* gene of (Cu²)-CS. A similar case is also found in the 3' flanking region of the *atp9* gene of other *Aegilops* species (Ishii unpublished). Schuster and Brennicke (1987) reported that *Oenothera* mtDNA has homologous sequence with the gene for reverse transcriptase. If the mtDNA of the present materials carries the gene for this enzyme, it is possible to assume that reverse-transcribed DNAs might have been inserted into mitochondrial genome, resulting in the repeated sequences as observed here.

Short homologous sequences reported at the recombination sites in the vicinity of the wheat *trnP* (Joyce *et al.* 1988) and *atp6* genes (Bonen and Bird 1988, Figure 31) are also found in the putative recombination site of the *cox3* genes of (Cu²)-CS at positions 992 and 1059. A short sequence TTCTTC found in the downstream of this gene (position 986 to 991, Figure 31) is also present in the partial copy of the common wheat *cox2* gene (Bonen *et al.* 1984) and 5' flanking region of the *Oenothera cox3* gene (Hiesel *et al.* 1987). The presence of these short homologous sequences suggests that the recombinations had occurred by homologous recombination between these sequences (Figure 35). In the region where the two types of the *cox3* gene of Cu² cytoplasm differ from each other, the 9 bp incomplete tandem repeats are found in the type II *cox3* gene, and 4 bp incompletely homologous sequences are present among the two types of *cox3* gene and the 5' flanking region of the maize *atp6* gene (at the positions from 1045 to 1059 in Figure 31). Among these genes, two different rearrangements can be postulated as follows: The first one is a homologous recombination occurred in the 4 bp sequence at the position 1059 (small vertical arrow in Figure 31 and 35) between the type II *cox3* gene and the putative Cu² homologous sequence of the maize *atp6* gene, resulting in the type I *cox3* gene of Cu² cytoplasm. The second one is a recombination occurred at the 9 bp tandem repeats which might have played as a target sequence for a recombination or excision of a transposable element. In this

case, the break point should be at the position 1055 (thick vertical arrow in Figure 31 and 35). However, other regions in which rearrangements of the nucleotide sequences are found do not show any specific homologous sequences, and, therefore, these rearrangements may have occurred by different mechanisms.

The predicted amino acid sequence of the product of *cox3* in (Cu²)-CS is shown in Figure 36, together with those of the common wheat (Gualberto *et al.* 1990 a, b), *Oenothera* (Hiesel *et al.* 1987) and maize (MaCarty *et al.* 1988). Compared with the amino acid sequence deduced from the nucleotide sequence of wheat *cox3* genomic DNA, three amino acid substitutions due to the corresponding base substitutions are assumed for that of Cu² cytoplasm. Recently, Gualberto *et al.* (1990 b) reported that the wheat *cox3* transcript has 13 nucleotide modifications due to the RNA editing. In the case of Cu² cytoplasm, RNA editing may not correct the substituted bases, because these substitutions do not correspond to a C to T or T to C conversion, for which RNA editing is known to occur in the wheat *cox3* gene (Figure 30). These substitutions seem not to have critical effect on the function of cytochrome oxidase, because the same amino acid substitutions are found at the same positions in *Oenothera* and maize as in Cu² cytoplasm (Figure 36).

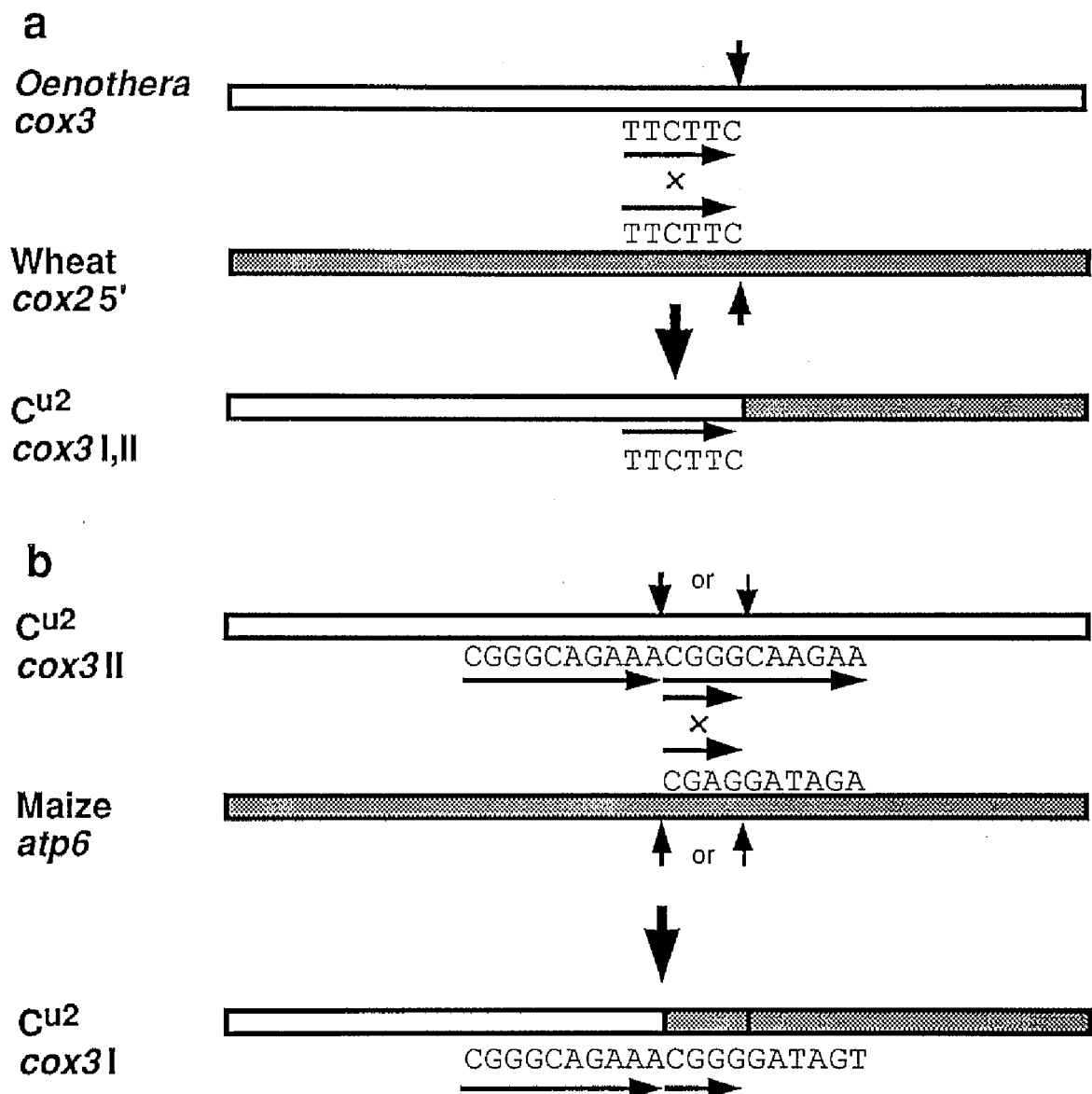


Figure 35. Models of recombinations at the short direct repeats, between homologous sequence of the *Oenothera cox3* gene and the partial copy of the common wheat *cox2* gene (a), and the 3' flanking region of type II *Cu*² *cox3* gene and homologous sequence of the 5' flanking region of maize *atp6* gene (b), generating 3' flanking region of the two types of the *Cu*² *cox3* gene, and 3' flanking region of type I *Cu*² *cox3* gene, respectively. Horizontal arrows indicate the short direct repeats. Vertical arrows indicate the recombination sites

	10	20	30	40	50	60
<i>aestivum</i>	MIESQRHSYHLVDESPWPISGSLGALATTVGGVMYMHSEFQGGATLLSLGLIFILYTMFVW					
<i>aestivum</i> cDNA	-----					
(C ^{u2})-CS I, II	-----L-----					
<i>Oenothera</i>	-----P-----L-----					
Maize	-----L-----					
	70	80	90	100	110	120
<i>aestivum</i>	WRDVLRESTLEGHHTKAVQLGPRYGSILFIVSEVMFLFAFFWASSHSSLAPTVEIGGIWP					
<i>aestivum</i> cDNA	-----L-----F-----FF-----					
(C ^{u2})-CS I, II	-----					
<i>Oenothera</i>	-----V-----L-----R-----					
Maize	-----SF-L-----					
	130	140	150	160	170	180
<i>aestivum</i>	PKGIGVLDPWEIPLLNTPILPSSGAAVTWAHHAILAGKEKRAVYALVATVSLALVSTGFQ					
<i>aestivum</i> cDNA	-----L-----L-----F-----					
(C ^{u2})-CS I, II	-----					
<i>Oenothera</i>	-----F-----L-----I-F-----					
Maize	-----L-----					
	190	200	210	220	230	240
<i>aestivum</i>	GMEYYQAPSTISDSIYGSTFFLATGFHGHFVVIIGTLFLIVCGIRQYLGQMTKKHHVGFEA					
<i>aestivum</i> cDNA	-----F-----					
(C ^{u2})-CS I, II	-----HL-----					
<i>Oenothera</i>	-----P-----Y-----S-I-----HL-----E-----					
Maize	-----L-----HL-----					
	250	260	265			
<i>aestivum</i>	AAWYWHFVDVVRLEPFVSIYWGGI					
<i>aestivum</i> cDNA	-----W-----L-----					
(C ^{u2})-CS I, II	-----					
<i>Oenothera</i>	-----L-----					
Maize	-----T-----					

Figure 36. Predicted amino acid sequence of the product of *cox3* in (C^{u2})-CS, as compared with those of *T. aestivum* (from both genomic and complementary DNA, Gualberto *et al.* 1990 a, b), *Oenothera* (Hiesel *et al.* 1987) and maize (MaCarty *et al.* 1988)

4) Relationships among the structural changes in the *cox3* gene, the decrease in cytochrome oxidase activity and the growth abnormality in (Cu²)-CS

The 5' end of the wheat *cox3* mRNA is mapped 323 bp upstream the initiation codon (Gualberto *et al.* 1990 a). In the 3' flanking regions of the *cox3* and *atp6* genes of common wheat, partially homologous sequence which can be folded to form a stem-loop structure and probably involved in the regulation of transcript processing (Gualberto *et al.* 1990 a) are found (Figure 37). A sequence similar to these is not found in the 3' flanking region of the *cox3* gene in (Cu²)-CS. This fact suggests that the *cox3* gene (both copies) of (Cu²)-CS does not have the sequence important for the expression of this gene in common wheat mitochondria. A recent study showed that the maize nuclear genome influenced the size and quantity of the *cox2* mRNA produced by *Zea perennis* or *Z. diploperennis* mitochondria (Cooper *et al.* 1990). It is presumed that the efficiency of the *cox3* transcription in Cu² cytoplasm that normally occurs under the presence of *Ae. columnaris* nucleus is altered by the common wheat nucleus present in the (Cu²)-CS. This hypothesis is supported by the result of the northern hybridization that revealed a small amount of *cox3* mRNA in (Cu²)-CS than in (B)-CS (Figure 32). It means that the genetic incompatibility between common wheat nucleus and Cu² cytoplasm is due in part to ill transcription of the *cox3* gene. Further study is need to test this hypothesis using *in vitro* assay system (Hanic-Joyce and Gray 1991).

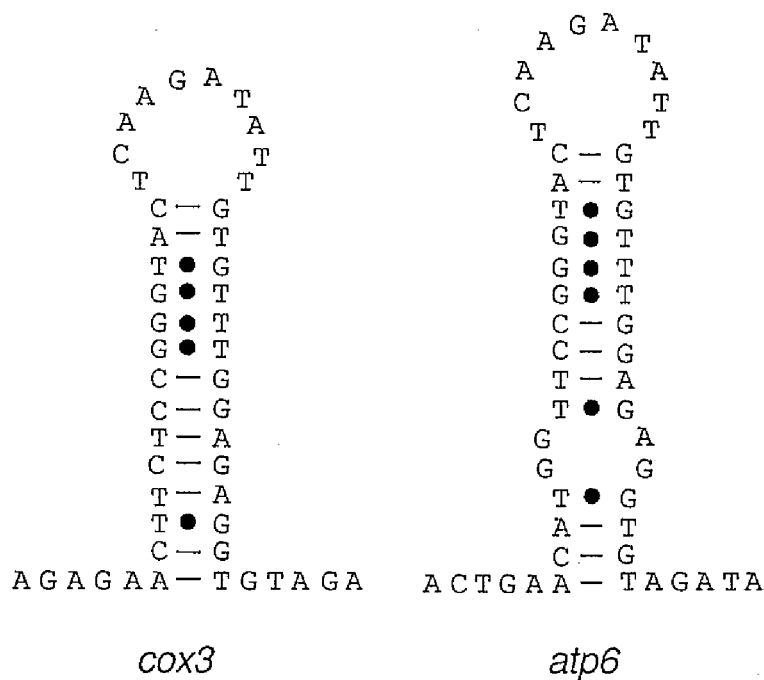


Figure 37. Potential secondary structures of the 3'-terminal regions of the *cox3* and *atp6* genes of common wheat (ref. Figure 31)

Although other factors can not be ruled out, the observed decrease in the amount of *cox3* mRNA most probably causes the reduction in synthesis of the subunit 3 of cytochrome oxidase in (Cu²)-CS, that leads to the impaired cytochrome oxidase activity. In turn, it reduces the mitochondrial activity, and finally results in growth inhibition of a common wheat (Figure 38).

Photosynthesis may not be directly related to growth inhibition of this alloplasmic wheat, because the activities of photosystems in chloroplast of Cu^u cytoplasm are not influenced at 21 °C and in addition the growth inhibition also occurs when the seedlings are grown in dark, as compared to those of the euplasmic line. Chlorophyll deficiency expressed in (Cu^u)- and (Cu²)-CS at low temperature is also explained by the low mitochondrial activity, as

follows: respiration is more seriously affected by low temperature than normal temperature, and as a result, ATP synthesis needed for chloroplast development is also decreased severely. Consequently, chloroplast stops its development at early stage, resulting in chlorophyll deficiency.

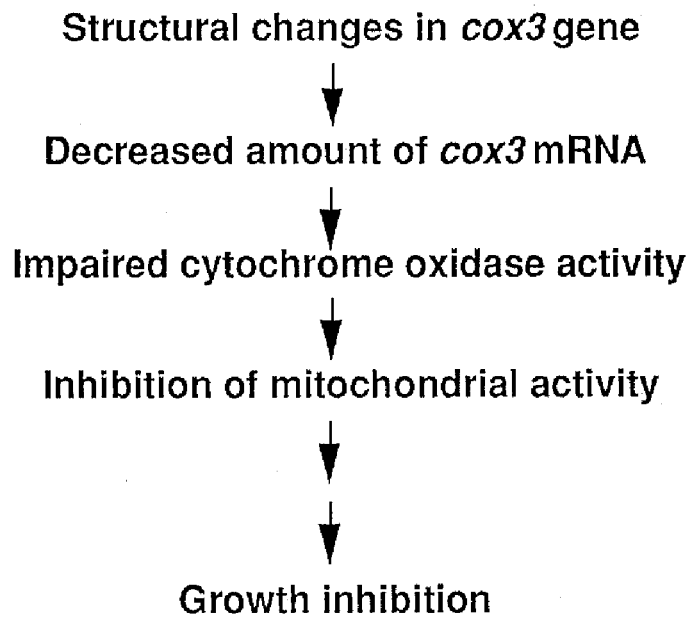


Figure 38. Possible sequence of the events leading to growth inhibition in (Cu²⁺)-CS

SUMMARY

With the final aim to clarify the relationships between the structural changes in chloroplast (ct) and mitochondrial (mt) DNAs and phenotypic variations found in common wheats with several alien cytoplasms, functional differences of these organelles and structural changes in their genomes have been studied using the following materials: euplasmic lines of two common wheats, *Triticum aestivum* cv. Chinese Spring (abbrev. CS) and Jones Fife (abbrev. JF) and their alloplasmic lines with the cytoplasms of *Aegilops crassa* (4x), *Ae. mutica*, *Ae. triuncialis* and *Ae. columnaris*. Their cytoplasms are known to have specific phenotypic effects on common wheat and are designated D², Mt, C^u and C^{u2} type, respectively, comparing to B type of *T. aestivum*.

In the first part of the present thesis, chloroplasts of these cytoplasms have been investigated. First of all, chlorophyll deficiency induced by low temperature in (C^{u2})-CS has been studied by several biochemical means. The first leaves of seedlings of this line, which are grown at 5 °C after leaving the soaked seeds at 0 °C for 30 days, show a chlorophyll content ca. 50 % lower than that of normal CS. The composition of thylakoid membrane proteins of the chlorophyll deficient leaves is very similar to that of the leaves greened under 20 hr illumination at 25 °C. The photosystem II activity in the chlorophyll deficient leaves of (C^{u2})-CS is remarkably low, comparing with that of the normal line. Plastid nucleoids in the chlorophyll deficient tissue have the same shape as those found in developing leaves. These results suggest that the present type of chlorophyll deficiency is resulted from severe inhibition of chloroplast development in its early stage.

Next, chloroplast proteins have been studied by two-dimensional electrophoresis. Three proteins, i.e., the large subunit of Rubisco, the β subunit of chloroplast ATP synthase and a 31 kDa unknown protein, are electrophoretically classified into two types, one type is found in the B

cytoplasm and the other type in all the three cytoplasms (Cu^2 , D^2 and Mt).

Other proteins appear to be the same among all four cytoplasms

Thirdly, complete nucleotide sequences of the genes coding for β and ϵ subunits of ATP synthase and methionine tRNA have been determined using cDNAs isolated from Cu^2 and D^2 cytoplasms which differ in chlorophyll deficiency expression from each other. Amino acid sequences of the β subunits of both *Aegilops* cytoplasms which have been deduced from the nucleotide sequences, have the same three amino acid substitutions when compared to that of common wheat (Howe *et al.* 1985). Two of them ($Asp^- \rightarrow Ala$ and $Gln \rightarrow Lys^+$) cause net charge change in this subunit, resulting in shift of the β subunit spot toward alkaline side in two-dimensional electrophoregram. From these results, no key chloroplast factor responsible for the phenotypic changes in the alloplasmic lines has been identified.

In the second part of the present thesis, mitochondria of common wheat and four *Aegilops* cytoplasms have been studied. First of all, respiratory activity and cytochrome composition of mitochondria isolated from CS having five different cytoplasms have been investigated. Cytoplasm-specific variation on the mitochondrial activities has been revealed among the eu- and alloplasmic lines. The decreases in respiratory and cytochrome oxidase activities are most evident in Cu^1 and Cu^2 cytoplasms both of which are known to cause growth inhibition. The low cytochrome oxidase activity is consistent with low cytochrome aa_3 content in these cytoplasms. However, relatively low peak height of cytochrome *b* (*b*-557) in (D^2)-CS and that of cytochrome aa_3 in (Mt)-CS are not reflected on the activities of cytochrome reductase and cytochrome oxidase, respectively. Cyanide resistant pathway does not seem to contribute for the variation on the respiratory activity.

Next, restriction fragment length polymorphisms (RFLPs) among mtDNAs isolated from the five cytoplasms have been studied using seven mtDNA clones as probes, which carry different genes encoding some subunits of cytochrome oxidase (*cox1*, *cox2* and *cox3*), cytochrome

reductase (*cob*) and ATP synthase (*atpA*, *atp6* and *atp9*). All but a single probe carrying the *cox2* gene have revealed clear variation on the number or molecular size of the hybrid bands or the intensity of their signals. The C^u and C^{u2} cytoplasms are the only ones in which more than one copy of the *cox3* gene are found.

Thirdly, the physical maps of two mitochondrial genes, i.e., *atpA* and *cox3*, of (B)- and (C^{u2})-CS and their flanking regions have been constructed. Followingly, the complete nucleotide sequences of the *atpA* genes of (B)- and (C^{u2})-CS and the two copies of the *cox3* gene of (C^{u2})-CS have been determined. Linkage between the *atpA* and *atp9* genes is found to have been conserved in C^{u2} cytoplasm as in B cytoplasm (Ishii unpublished). Compared with (B)-CS, (C^{u2})-CS has one and three base substitutions in its coding and flanking regions of the *atpA* gene, respectively. The amino acid sequence of the α subunit of ATP synthase, which has been deduced from the above nucleotide sequences, is identical between (B)- and (C^{u2})-CS. As for *cox3*, remarkable structural differences in both flanking regions of the *cox3* gene are found between (B)- and (C^{u2})-CS, which are assumed to have been caused by recombinations between them and some sequences in other mitochondrial genes. Three amino acid substitutions are assumed from the above base substitutions for the *cox3* gene of C^{u2} cytoplasm, when compared to that of common wheat. RNA editing may not correct the substituted bases, because those substitutions do not correspond to a C to T or T to C conversion, for which RNA editing is known to occur.

Lastly, expression of the *cox3* gene in (C^{u2})-CS has been studied to know the effect on it of the rearrangements occurred in the both flanking regions of the *cox3* gene. Northern hybridization has revealed smaller amount of the *cox3* mRNA produced in (C^{u2})-CS than in (B)-CS.

Although other factors can not be ruled out completely, the observed

decrease in the amount of *cox3* mRNA most probably causes the reduction in synthesis of the subunit 3 of cytochrome oxidase in (Cu²)-CS, that leads to the impaired cytochrome oxidase activity. In turn, it reduces the mitochondrial activity, and finally results in growth inhibition of a common wheat.

In conclusion, cytoplasm-specific growth abnormalities induced by the four alien cytoplasms seem to be caused by their mitochondrial but not by chloroplast genome.

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