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The Effects of Nuclear Mutation on Chloroplast Development

Part 1: Chloroplast DNA, Proteins, Ribosomes, Hormones and Tissue Cultures

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Summary. Under greenhouse conditions the dark green wild type (su/su) tobacco grows 2-3 times faster than the yellow mutant (Su/su) and contains five-fold more chlorophyll. On a fresh weight basis, however, both genotypes contain similar amounts of RuBPCase and fraction 11 protein in approximately equal proportion and have similar levels of 70s and 80s ribosomes. When seedlings are cultured on agar medium supplemented with sucrose and equal concentrations of IAA and kinetin or kinetin alone, a drastic reduction of RuBPCase and free 70s ribosomes, but not of chlorophyll content, were observed. Moreover, albino (Su/Su) seedlings developed on supplemented media still contain appreciable amounts of RuBPCase and free 70s ribosomes although chlorophyll levels are extremely low indicating no correlation between RuBPCase and chlorophyll content. RuBPCase crystallized from both wild type and yellow mutant plants seem to have identical composition and structure when examined by isoelectric focusing, amino acid analysis or peptide mapping techniques. The slow-growing yellow mutant is apparently deficient only in chlorophyll of the light harvesting chlorophyll-protein complex but with no alteration of the protein moiety or chlorophyll a/b ratio.

Key words: Chloroplast DNA – RuBPCase – Ribosomes – Photosystems – Tissue culture

Introduction

The dominant aurea mutant of tobacco, designated Su by Burk and Menser (1964) is a nuclear mutation found in *Nicotiana tabacum* var. 'John Williams Broadleaf'. Homozygous albino (Su/Su) seedlings do not survive under greenhouse conditions; however, the heterozygous yellow mutant (Su/su) will grow, although at a rate much reduced from that of the wild type (su/su). Wide use of this mutation in studies of photosynthesis (Schmid and Gaffron 1966), photorespiration (Zelitch and Day 1968), cell culture (Schaeffer 1977) and protoplast fusion (Evans et al. 1980) has generated much ultrastructural and functional information on chloroplasts from the mutant plants. These organelles have been shown to have altered membrane development with few grana and reduced chlorophyll levels accompanied by a chlorophyll a/b ratio higher than that of the wild type (Schmid and Gaffron 1966). Under high light intensity and high CO_2 concentration the yellow mutant plants photosynthesize at a more rapid rate per mg chlorophyll than the wild type and can be induced to green and appear indistinguishable from wild type plants (Schmid 1967). While these lines of evidence indicate involvement of the chlorophyll biosynthetic pathway, they do not preclude a role for his nuclear mutation in altering other cell components thought to be directly correlated with the capacity of a cell to grow.

In view of the reduced growth rate and altered photosynthetic capacity of the mutant plants, we have examined whether this nuclear mutation may have affected levels of ribulose 1,5 bisphosphate carboxylase-oxygenase (RuBPCase), other soluble proteins, and the protein moiety of the light-harvesting chlorophyll-protein complex (LHCP). In addition, we looked for the presence and relative proportion of 70s and 80s ribosomes in these genotypes. Since the homozygous dominant (albino) can be lethal, comparisons involving all three genotypes were possible only when seedlings were cultured on agar.

Materials and Methods

Plants

Nicotiana sylvestris and *N. tabacum L.* cv. 'Maryland 609', cv. 'Turkish Samsun', as well as the wild type and yellow mutant of

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cv. 'John Williams Broadleaf' (J.W.B.) were grown in a greenhouse. Seedlings having 4-6 leaves were transplanted into 5-in. soil pots. Leaves were harvested 2-4 weeks after transplanting.

Seeds from self-pollinated 'J.W.B.' Su/su plants were surface sterilized with 20% household Clorox for 15 min. and placed on 1% (w/v) agar containing a modified Murashige-Skoog medium (1962) with or without auxin, kinetin, or both. The medium was adjusted to pH 5.5 and autoclaved for 15 min. at 15 psi. Approximately 15 seeds were placed in each 125 ml Erlenmeyer flask containing 30 ml solid agar medium. Fourteen days after germination, with or without sucrose, excess seedlings were removed with forceps leaving only the desired su/su, Su/su, or Su/Su phenotype, usually 3-5 plants per flask.

Examination of Chloroplast DNA

Chloroplast DNAs were prepared by the method of Rhodes and Kung (1981) and digested with EcoRI, HindIII, BamHI, or SmaI, as directed by the supplier, New England Biolabs. The digested fragments were separated in a 1% agarose slab gel according to the procedure of Helling et al. (1974). Ethidium bromide-stained bands were then visualized with a model C-62 Chromato-vue transilluminator (Ultra-violet Products, Inc.). The EcoRI and HindIII fragments or lambda phage DNA were used as molecular weight markers.

Quantitative Analysis of Soluble Protein, Free Ribosomes and Chlorophyll

The protein and ribosome content of the leaf extract obtained by grinding in a buffer containing 80 mM Tris, 40 mM KCl and 20 mM $MgCl_2$ pH 8.5, was determined by analytical ultracentrifugation. Photographs were taken 15 min. after centrifugation at 44,770 RPM and the area under each peak was measured. The amount of protein in each sample was obtained by comparison to areas on a standard concentration curve prepared from known concentrations of RuBPCase (Kung and Tso 1978). Ribosomal RNAs (rRNAs), prepared by the method of Bourque et al. (1973), were fractionated by polyacrylamide gel electrophoresis in 30 mM NaH_2PO_4, 1 mM EDTA, 36 mM Tris-HCl, pH 7.2. Ratios were calculated from the area of each peak. Chlorophyll content was measured in 80% acetone by the method of Arnon (1949).

Crystallization and Analysis of RuBPCase

RuBPCase was prepared from leaves of the wild type and mutant plants of 'J.W.B.' by a modified direct crystallization procedure (Kung et al. 1980). The recrystallized enzyme was dissociated in 0.5% SDS and the subunits were separated on a Sephadex G-100 column according to the method of Rutner and Lane (1967). The separated large and small subunits were then lyophilized to a salt-free white powder and hydrolyzed in 1 ml of HCl (5.7 M) at 110°C for 20 hrs under vacuum. Amino acid analyses were performed on a Beckman 119C amino acid analyzer. The large and small subunits of RuBPCase were also subjected to trypsin digestion, lyophilized, and spotted on Whatman paper. Two-dimensional chromatography of the peptides was carried out as previously described (Kung et al. 1974).

Electrophoresis of Chloroplast Thylakoid Components

Chloroplast thylakoids, prepared by the method of Kung et al. (1972), were solubilized in 50 mM Tris (pH 8.0) containing 1% SDS such that the final SDS: chlorophyll ratio was 10:1 (W/W). This SDS extract was then analyzed by polyacrylamide gel electrophoresis. After the position of chlorophyll was determined by scanning at 670 nm, the tube gels were stained for protein with amido black and scanned at 600 nm using a Gilson 250 spectrophotometer equipped with linear transport. Peak areas were determined by cutting and weighing the absorbance tracings.

Results

The nuclear character of the Su mutation was affirmed by our own observations of segregation with the self-pollinated heterozygous mutant. Moreover, we examined the chloroplast DNAs from these genotypes and found them to be identical to each other as well as to chloroplast DNAs from *N. sylvestris* and *N. tabacum* cv. MD609 (Rhodes et al. 1981). Several restriction enzymes were used but in no case were any differences in the restriction pattern detected (Fig. 1).

The slow growth rate of the heterozygous yellow mutant is accompanied by a five-fold reduction in total

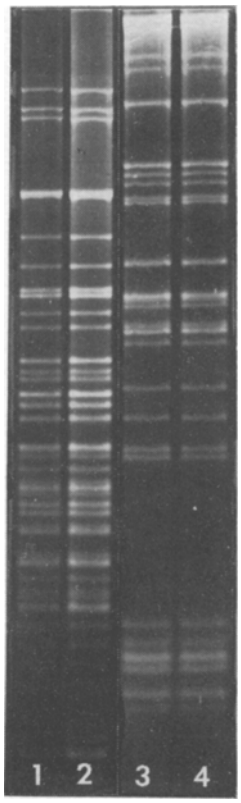

Fig. 1. Restriction fragment patterns of ct-DNA from *N. tabacum* cv. 'JWB' wild type (su/su) (*1, 3*) and yellow mutant (Su/su) (*2, 4*). They were digested with ECORI (*1, 2*) and BamHI (*3, 4*)

chlorophyll (Table 1) and a high a/b ratio which varied widely among Su/su plants tested. Although the chlorophyll content is quite different, both plants contain approximately equal proportions of RuBPCase and fraction II protein (F II protein) (Table 1) similar in amount to the total soluble protein found in other cultivars and species of tobacco on a fresh weight basis. When sedimentation profiles of extracts from unexpanded leaves 1-2 cm in length are examined, comparable amounts of total soluble protein portioned equally between RuBPCase and F II proteins are found (Fig. 2, a, c). At a later stage of development the expanded leaves (10-12 cm) contain only slightly more RuBPCase than F II protein (Fig. 2, b, d). This ratio remains constant until maturation.

Table 1. Chlorophyll and soluble leaf protein content from different cultivars of *N. tabacum*[a]

Cultivars	mg ch1/g FW	ch1 a/b	Total soluble protein mg/g FW	RuBPCase/ F11 protein
'J.W.B.' wild type (su/su)	1.37	3.25	19.65	1.01
'J.W.B.' yellow mutant (Su/su)	0.26	7.71	16.70	0.85
'Maryland 609'	0.96	2.95	17.50	0.80

[a] Results are the mean of 3-5 experiments

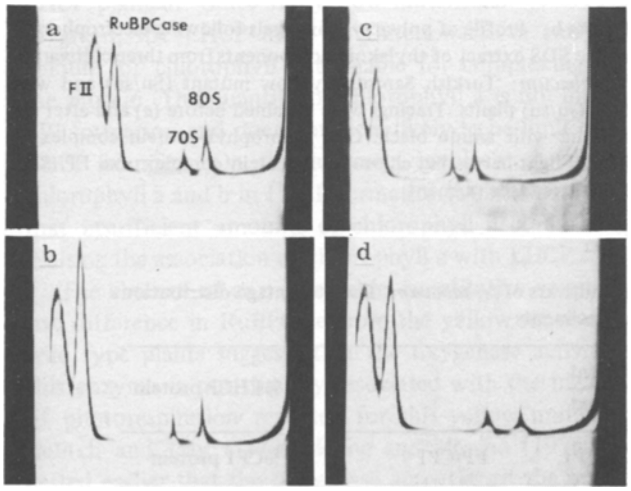

Fig. 2a-d. Analytical ultracentrifuge schlieren pattern of total soluble protein and free ribosomes extracted from leaves of the wild type (su/su) (a, b) and yellow mutant (Su/su) (c, d) plants. Leaves were harvested at different stages of development (1-2 cm in a, c; 10-12 cm in b, d). Photographs were taken at a bar angle of 55°

Since RuBPCase comprises 50% of the total soluble protein in the chloroplast and remains unaffected by this mutation, the chloroplast protein synthetic capacity of the yellow mutant plants must be equivalent to that of the wild type tobacco. Indeed, these tobacco plants have similar levels of chloroplastic (70s) and cytoplasmic (80s) ribosomes in relation to fresh weight, total soluble protein, and RuBPCase content at different stages of development (Fig. 2). When soluble non-membrane bound ribosomes or total ribosomal RNAs (rRNAs) were compared, a similar ratio of 70s to 80s ribosomes, approaching 1 to 3, was observed in both yellow mutant and wild type plants. The rRNAs from both genotypes were intact as evidenced by ratios of 25s to 18s and 23s to 16s rRNA which were close to 2. Thus, even though the yellow mutant growth rate is reduced by as much as 60%, there seems to be little or no apparent reduction in the population of either chloroplastic or cytoplasmic ribosomes.

Comparisons involving all three genotypes were made by studying seedlings cultured on agar. The seedlings of wild type and yellow mutant plants grow equally well and become indistinguishable in color when cultured on an agar medium supplemented with sucrose, with or without hormones (IAA, kinetin, or both). Without hormones the RuBPCase and F II protein are consistent with the proportions found in greenhouse-grown plants (Figs. 2, 3) but have a somewhat reduced amount of 70s and 80s ribosomes (Compare Fig. 2a with Fig. 3a). When equal concentrations (1 mg/L) of IAA and kinetin (Fig. 3b) or kinetin alone (Fig. 3c) were added to the medium, a dramatic reduction of RuBPCase and disappearance of 70s ribosomes was observed in both genotypes. Interestingly, when the homozygous albino seedlings (Su/Su) which can be grown only under these conditions, were treated with kinetin they remain deficient in chlorophyll content and yet exhibit appreciable amounts of both RuBPCase and 70s ribosomes (Fig. 3d). Even though RuBPCase is a

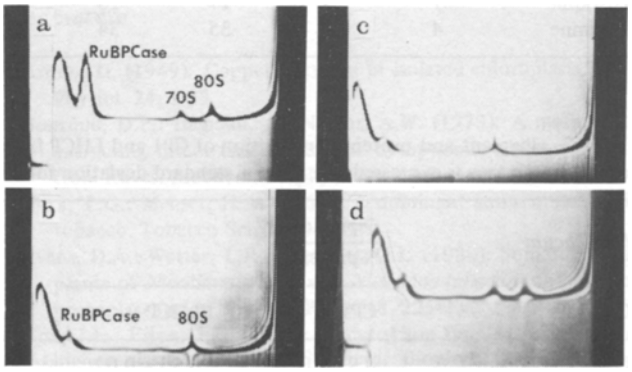

Fig. 3a-d. Comparison of RuBPCase and free ribosome levels in tobacco seedlings cultured on supplemental media. a Wild type (su/su) without added hormones (control). b Wild type (su/su) with equal amounts of IAA and kinetin. c Wild type (su/su) with kinetin alone. d Albino (Su/Su) with kinetin alone

major photosynthetic chloroplast protein, there is no correlation between RuBPCase and chlorophyll content.

Several reports (Kung and Marsho 1976; Okabe, 1977; Okabe and Schmid 1980) have associated differences in RuBPCase enzymatic activity with the Su mutation, but such differences were not detected by Koivuniemi et al. (1980). We have examined RuBPCase crystallized from both wild type and yellow mutant plants. When analyzed by isoelectric focusing techniques, the patterns obtained are identical to those of other cultivars of *N. tabacum* (Kung et al. 1974). The amino acid composition (Table 2) and peptide map (not shown here) of both subunits of the wild type and yellow mutant enzyme also appear to be similar to other cultivars of *N. tabacum* (Kung et al.

Table 2. Amino acid composition of the large and small subunits of RuBPCase from wild type (su/su) and yellow mutant (Su/su) 'J.W.B.' tobacco plants. Results are expressed as probable numbers of residue in a 1.2×10^4 dalton (small subunit) and in a 5.5×10^4 dalton (large subunit) peptide

	Small subunit		Large subunit	
Amino Acid	Wild type (su/su)	Mutant (Su/su)	Wild type (su/su)	Mutant (Su/su)
Aspartic acid	8	8	40	43
Threonine	4	5	28	28
Serine	4	4	15	15
Glutamic acid	17	17	51	52
Proline	8	8	25	24
Glycine	7	7	53	52
Alanine	6	6	44	42
Valine	6	6	44	42
Methionine	1	1	4.5	5.0
Isoleucine	5	5	25	25
Leucine	9	9	44	45
Tyrosine	8	9	20	19
Phenylalanine	5	5	26	25
Lysine	8	8	26	24
Histidine	1	1	17	15
Arginine	4	4	35	34

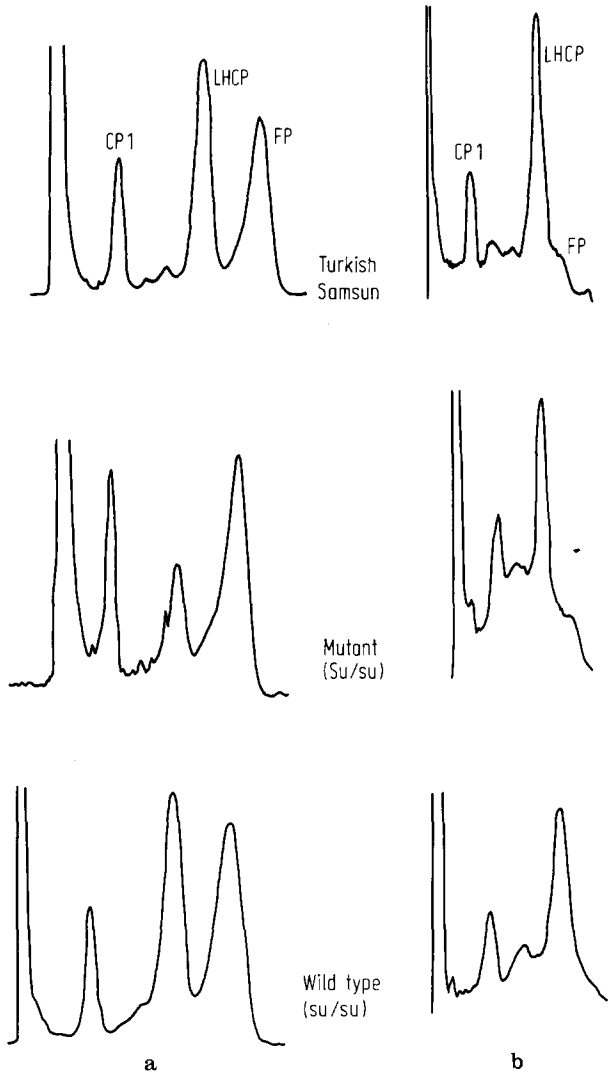

Fig. 4a-b. Profile of polyacrylamide gels following electrophoresis of the SDS extract of thylakoid components from three cultivars of *N. tabacum*; 'Turkish Samsun', yellow mutant (Su/su) and wild type (su/su) plants. Tracings were obtained before (*a*) and after (*b*) staining with amido black. CPI: chlorophyll-protein complex 1; LHCP: light-harvesting chlorophyll-protein complex; and FP: SDS complexed free pigment

Table 3. Pigment and protein composition of CP1 and LHCP from three cultivars of *N. tabacum*. The percentage distribution of total peak area is expressed as a mean ± standard deviation for 3 to 4 experiments

N. tabacum	%pigment / %protein		%pigment / %pigment		%LHCP protein
Cultivars	CP1	LHCP	LHCP/CP1	FP/CP1	%CP1 protein
'Turkish Samsun'	0.92 ± 0.34	0.55 ± 0.02	2.55 ± 0.76	2.26 ± 0.83	3.76 ± 0.76
'J.W.B.' yellow mutant (Su/su)	1.04 ± 0.41	0.28 ± 0.03	0.78 ± 0.22	2.06 ± 0.78	2.75 ± 0.60
'J.W.B.' wild type (su/su)	0.60 ± 0.30	0.51 ± 0.03	3.19 ± 0.69	3.98 ± 1.35	3.51 ± 1.22

1974). The reported differences in enzyme activity are certainly not attributable to structural changes in the enzyme.

This nuclear mutation does, however, affect pigmentation as reflected by the association between pigment and thylakoid proteins. This association was examined by separating the chlorophyll-protein complex I (CPI) from the LHCP, on polyacrylamide gels in the presence of SDS. The distribution of pigment and protein among these two fractions is illustrated in Fig. 4. The highly variable CPI pigment content in each experiment (Table 3) was traced to the generation of free chlorophyll a (FP) during electrophoresis. In fact, the ratio of the sum of CPI pigment and FP to CPI protein (3:1) was not significantly different among these plants. Evidently, however, there has been a striking loss of as much as 70% of the pigment associated with LHCP protein in the yellow mutant (Table 3). This loss was not accompanied by a change of chlorophyll a/b ratio in LHCP which remained similar to that from wild type tobacco leaves. Since the yellow mutant can green under the appropriate environmental conditions, the LHCP protein moiety is apparently present in normal amounts and must be fully functional.

Discussion

Results presented here show that the heterozygous mutant contains approximately the same proportion of RuBPCase, 70s ribosomes, 80s ribosomes, and the protein moiety of LHCP as are found in the wild type on a fresh weight basis. The 80% reduction in total chlorophyll (Table 1), however, was accounted for by the loss of LHCP pigment. Since the yellow mutant can be induced to green, the LHCP must be functional if a sufficient amount of chlorophyll is available for complexing with the protein. The quantity of chlorophyll a is not limiting CPI formation and does not seem likely to be limiting the LHCP complex. On the contrary, involvement of both chlorophyll a and b in LHCP formation leads us to propose that insufficient amounts of chlorophyll b could be inhibiting the association of chlorophyll a with LHCP.

The absence of any detectable quantitative or qualitative difference in RuBPCase from the yellow mutant and wild type plants suggests that the oxygenase activity of this enzyme is not directly associated with the high rate of photorespiration reported for this yellow mutant by Zelitch and Day (1968). Kung and Marsho (1976) suggested earlier that the oxygenase activities of the isolated enzyme and the respective photorespiratory rates are not correlated. A reportedly higher K_m (CO_2) and lower K_m (O_2) for RuBPCase from the yellow mutant was attributed to differences in amino acid composition of the small subunit (Okabe and Schmid 1980). But in our hands, no differences in either large or small subunits of the RuBPCase from both genotypes were detected by isoelectric focusing, peptide mapping, or amino acid analysis. Therefore, it is quite certain that both enzymes are identical. Moreover, recent data in our laboratory on the K_m (CO_2), K_m (O_2) and K_m (RuBP) of RuBPCase from both the wild type and yellow mutant (LeBon et al. unpublished data) tend to agree with findings of Koivuniemi et al. (1980) in which no differences in the kinetic properties were detected. If one accepts that the photorespiration rates differ but that the kinetic properties of crystallized RuBPCase are not different between su/su and Su/su plants, then two possibilities remain: either here is a source other than RuBPCase for the photorespiratory oxygen consumption or there are physiological factors which can alter the carboxylase/oxygenase ratio of RuBPCase in vivo.

The effects of kinetin in reducing the RuBPCase and free 70s ribosomes in seedlings of both genotypes are striking. The mechanism for this is unknown at this time; however, it is conceivable that the free ribosomes particularly the 70s ribosomes may be a target of the hormonal action since cytokinins will bind to higher plant ribosomes (Fox and Erion, 1975). Such modifications of the protein synthesizing machinery which result in the reduction of RuBPCase synthesis may provide the impetus for further research in the area of plant hormone and its mode of action.

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