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Ploidy Effects in Isogenic Populations of Alfalfa[1]

III. CHLOROPLAST THYLAKOID-BOUND COUPLING FACTOR 1 IN PROTOPLASTS AND LEAVES

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ABSTRACT

The influence of polyploidization on chloroplast coupling factor 1 (CF1) was examined in leaves and leaf protoplasts from isogenic diploid-tetraploid (DDC 2X-4X) and tetraploid-octoploid (IC 4X-8X) sets of alfalfa (*Medicago sativa* L.). Alfalfa CF1 was purified to homogeneity and was found to contain five subunits with molecular weights of 57,900, 54,300, 38,700, 23,100, and 15,200. Rocket immunoelectrophoresis with anti-spinach CF1 gamma immunoglobulins was used to quantify CF1 from protoplasts and leaves. In the DDC 2X-4X set, fresh weight per cell and cellular content of CF1, chlorophyll (Chl), and DNA doubled with ploidy. Ratios of CF1 to Chl of leaf protoplasts and leaves were similar between diploids and tetraploids. In the IC 4X-8X set, octoploid protoplasts were 90% higher in Chl than were comparable tetraploids, whereas octoploids were 50 to 60% higher than tetraploids in fresh weight per cell and cellular content of CF1 and DNA. Concentrations of CF1 and Chl in leaves and ratios of CF1 to DNA in protoplasts were similar across ploidy levels of both isogenic sets. Therefore, cellular content of CF1 increases proportionately with the amount of DNA per cell or gene dosage.

Nuclear polyploidization has been suggested to be an important component in the evolution of many species of flowering plants (10, 19). Increases in nuclear ploidy have been reported both to enhance and to decrease photosynthetic activities in higher plants (4, 14, 25). Such contradictory observations can result from genetic limitations of the plant material under investigation. Genetic differences between allopolyploid genotypes (4, 15) and inbreeding depression associated with colchicine-induced polyploids (2, 25) may confound the consequences of ploidy effects. To minimize these limitations in the study of ploidy effects, isogenic diploid-tetraploid and tetraploid-octoploid alfalfa (*Medicago sativa* L.) populations, to which heterozygosity was restored, were developed.

In these isogenic alfalfa populations, no significant differences between ploidy levels were observed in net CO_2 exchange per mg Chl of leaves (24) and O_2-evolution rates per mg Chl of leaf protoplasts and chloroplasts (23). When compared on a per leaf basis, tetraploids were 80% higher in BSP[3] and double comparable diploids in fresh weight, Chl, RuBPCase, and DNA, whereas octoploids were 33 to 80% higher than corresponding tetraploids in these parameters (22). Molin *et al.* (23) reported that the number of chloroplasts per cell doubled with a doubling in ploidy in these isogenic alfalfa populations. Ratios of RuBPCase to DNA of leaves (22) and leaf protoplasts (23) were similar across ploidy levels of both isogenic sets suggesting that cellular content of RuBPCase increases proportionately with the amount of DNA per cell or gene dosage.

Chloroplast thylakoid-bound CF1, which constitutes about 10% of the thylakoid-membrane protein (20), is the extrinsic-membrane-protein component of the ATP-synthase complex which couples proton flux across thylakoid membranes to the synthesis of ATP. The purpose of this investigation was to examine the effects of nuclear polyploidization on the quantitative expression of CF1.

MATERIALS AND METHODS

Plant Material and Culture. The derivations of isogenic diploid-tetraploid (DDC 2X-4X) and tetraploid-octoploid (IC 4X-8X) alfalfa (*Medicago sativa* L.) used in this study have been described (24). Plants were grown in an environmental chamber as in (22).

Purification of CF1. Chloroplast CF1 was prepared from alfalfa leaves as in (13) except that chloroplast membranes were washed five instead of three times with 10 mM NaPPi. Further purification was achieved by elution through two DEAE-Sephadex A-50 columns (2.3 × 22 cm) using first a 50 mM, 300 mM $(NH_4)_2SO_4$ two-step gradient and then a linear 100 to 300 mM $(NH_4)_2SO_4$ gradient. Both gradients contained 20 mM Tris (pH 7.1), 2 mM EDTA, and 1 mM ATP. After elution through the first column, the CF1-containing fraction had a heat-induced Ca^{2+}-dependent ATPase activity, as assayed by Frasch and Selman (9), of 13.1 μmol ATP hydrolyzed/mg protein·min. Elution of the CF1-containing fraction through a second ion exchange column was necessary to remove several minor contaminating polypeptides (Fig. 1). The protein was stored at 5°C as a suspension in 50% saturated $(NH_4)_2SO_4$ containing 50 mM Tricine (pH 8.0), 4 mM ATP, and 1 mM EDTA. The protein appeared to be homogeneous as determined by polyacrylamide gel electrophoresis.

Estimation of Subunit Mol Wt. Subunits of CF1 were separated on two linear polyacrylamide gradients, 7.5 to 15% and 10 to 15% with 4% acrylamide stacking gels in 0.1% (w/v) SDS, using the buffer system described by Laemmli (16). Electrophoresis was performed with a constant current of 20 mamp/gel. Gels were stained for 12 h in 0.05% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid and destained in 20% (v/v) methanol, 10% (v/v) acetic acid. Gels were then silver stained as described by Merril *et al.* (21). Mol wt of CF1 subunits were estimated from R_F values using the following reference proteins as mol wt standards: phosphorylase B, 94,000; BSA, 68,000; RuBPCase large subunit, 52,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lyso-

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[3] Abbreviations: BSP, buffer-soluble protein; RuBPCase, ribulose-1,5-bisphosphate carboxylase; CF1, coupling factor 1; IgG, gamma immunoglobulin.

zyme, 14,400; and RuBPCase small subunit, 14,000.

Extraction of CF1 and Chl from Leaves. Fully expanded trifoliolate leaves, from the 3rd to 6th nodes from the apex on plants 4 weeks after cutting, were used for the extraction of CF1 and Chl. Leaves (1 g) were washed and then homogenized in 6 ml of buffer (25 mM Tricine, pH 8.0 at 5°C) in a VirTis model 60K homogenizer (1 min at 30,000 rpm). Homogenates were filtered through a 500 μm mesh nylon screen. Triplicate 200-μl aliquots of the homogenate were added to 4.8 ml of 100% ethanol for Chl determination. The remaining homogenate was vigorously mixed with an equal volume of chloroform. The two phases were separated by centrifugation (15 min at 1,000*g*), and the aqueous phase was withdrawn. The aqueous phase was centrifuged at 6,000*g* for 15 min, and the clear supernatant was removed for the quantitative determination of CF1. Each experiment consisted of two extractions from each ploidy level of the DDC and IC sets and was repeated four times.

Protoplast Isolation and Preparation. Protoplasts were isolated from fully expanded leaves (3rd to 6th nodes from the apex) according to Molin *et al.* (23). Protoplasts were suspended in 5 ml of 0.6 M sorbitol, 50 mM Hepes (pH 7.6), 2 mM $CaCl_2$, and 0.05% (w/v) BSA. The concentrations of cells in the protoplast suspension, determined with a hemacytometer as in (23), ranged from 10^6 to 10^7 cells/ml. Duplicate 200-μl aliquots of protoplast suspension were used for the quantitative determination of DNA and Chl. The volume of the remaining suspension was determined and protoplasts were pelleted by centrifugation at 100*g* for 10 min. Pellets were suspended into a total volume of 200 μl with 25 mM Tris (pH 8.0 at 5°C) for the quantitative determination of CF1. These protoplast suspensions were vigorously mixed with an equal volume of chloroform, centrifuged, and chromatographed on Sephadex G-50 as in (8). A minimum of eight protoplast isolations were performed for each ploidy level of both isogenic sets.

Assays for Chl, DNA, and Soluble-Protein. Chl was estimated using the constants of Wintermans and Demots (27). DNA was determined according to Baer *et al.* (1). Concentration of soluble protein was determined as described by Bradford (3) using BSA as the standard.

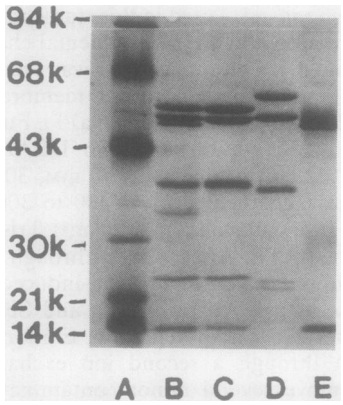

FIG. 1. SDS-electrophoresis in a linear 10 to 15% polyacrylamide gradient of: (A), mol wt standards; (B), alfalfa CF1-containing fraction after elution through the first DEAE Sephadex A-50 column; (C), alfalfa CF1 after elution through the second DEAE Sephadex column; (D), spinach CF1; and (E), alfalfa RuBPCase.

Quantitation of CF1. CF1 from alfalfa protoplasts and leaves was quantitated by rocket immunoelectrophoresis using anti-spinach CF1 IgG prepared as in (26) and purified according to (12). A 17-ml solution containing 0.9% (w/v) agarose, 40 mM barbital (pH 8.6), 5 mM NaN_3, and 170 μg anti-CF1 IgG was poured onto a glass plate (100 × 110 × 1.5 mm) heated to 40°C. The plate was cooled to 25°C for 10 min. Circular wells, 4 mm in diameter, were punched into the gel. The anode-cathode buffer contained 30 mM barbital (pH 8.6) and 4 mM NaN_3. With a potential of 70 v across the gel, 10-μl samples were applied to each well. Alfalfa CF1 standards ranged from 0.14 to 5 μg in 10 μl. Electrophoresis was performed at 90 v for 8 to 10 h at 12°C. The gel was then pressed, dried, and stained with Coomassie brilliant blue G-250. Regression equations were derived for each standard curve of peak heights.

RESULTS AND DISCUSSION

Alfalfa CF1 was found to contain five polypeptide components with apparent mol wt of 57,900, 54,300, 38,700, 23,100, and 15,200 (Fig. 1). The five polypeptides of spinach CF1 had apparent mol wt of 62,000, 55,200, 37,400, 21,700, and 16,200.

Concentrations of CF1 and Chl in leaves and ratios of CF1 to Chl and Chl *a* to *b* were similar across ploidy levels of both isogenic sets (Table I). In a previous study (22), leaf tissue concentrations of RuBPCase and DNA were similar across ploidy levels of the DDC and IC sets.

Protoplasts were isolated from leaves to permit the expression of data on a per cell basis. Tetraploids were nearly double comparable diploids in fresh weight per cell and cellular content of CF1, DNA, and Chl (Table II). The number of cells per leaf and ratios of Chl *a* to *b*, CF1 to DNA, and CF1 to Chl were similar between ploidy levels of the DDC 2X-4X set. Molin *et al.* (23) reported that DDC tetraploids were double comparable diploids in CO_2-dependent O_2 evolution per cell and cellular content of chloroplasts, Chl, DNA, and RuBPCase. CF1 and RuBPCase increase in a similar quantitative manner with polyploidization even though total soluble protein does not double with ploidy in the DDC 2X-4X set (22). CF1 and RuBPCase are similar in that both are chloroplast multisubunit complexes which require the assembly of cytoplasmically-synthesized and chloroplast-synthesized polypeptides (7). Isolated spinach chloroplasts have been reported to synthesize three of the five subunits of CF1 (6), suggesting that these gene products are encoded in the chloroplast genome. Chloroplasts of both ploidy levels of the DDC set were similar in their content of CF1, Chl (Table II), and RuBPCase (23).

Octoploids were almost double their comparable tetraploids in Chl per cell but were only 63%, 57%, and 53% greater than tetraploids in fresh weight per cell, and cellular content of CF1 and DNA, respectively. The number of cells per leaf and ratios of Chl *a* to *b*, and CF1 to DNA were similar between ploidy levels

Table I. *Effect of Ploidy Level on CF1 and Chl from Fully Expanded Trifoliolate Leaves (3rd to 6th Nodes from the Apex)*

Parameter	Ploidy			
	DDC 2X	DDC 4X	IC 4X	IC 8X
CF1 (mg/g fresh wt)	1.3 ± 0.1[a]	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.2
Chl (mg/g fresh wt)	3.6 ± 0.2	3.7 ± 0.1	3.5 ± 0.2	3.5 ± 0.1
Chl *a*:*b* ratio	3.3 ± 0.1	3.3 ± 0.1	3.4 ± 0.2	3.4 ± 0.1
CF1/Chl (mg/mg)	0.36 ± 0.03	0.41 ± 0.03	0.34 ± 0.02	0.34 ± 0.05

[a] Mean ± SE.

Table II. *Effect of Ploidy Level on Fresh Weight, CF1, DNA, and Chl in Protoplasts Isolated from Fully Expanded Trifoliolate Leaves (3rd to 6th Nodes from the Apex)*

Parameter	Ploidy			
	DDC 2X	DDC 4X	IC 4X	IC 8X
Fresh wt (ng/cell)[a]	5.8	10.9	11.2	18.2
CF1				
(pg/cell)	7.9 ± 1.1[b]	14.6 ± 1.7	15.2 ± 1.1	23.8 ± 3.0
(pg/chloroplast)[c]	0.4	0.4	0.5	0.4
DNA (pg/cell)	4.8 ± 0.8	8.2 ± 0.8	8.7 ± 1.4	13.3 ± 1.9
Chl (pg/cell)	20.2 ± 2.2	36.5 ± 2.1	39.4 ± 6.6	73.6 ± 7.6
No. cells × 10^{-6}/leaf[d]	4.04	4.35	4.66	4.73
Chl *a*:*b* ratio	3.6 ± 0.2	3.5 ± 0.3	3.7 ± 0.1	3.7 ± 0.1
CF1/DNA (pg/pg)	1.65	1.87	1.75	1.79
CF1/Chl (pg/pg)	0.39	0.40	0.39	0.32

[a] Calculated by dividing fresh weight per leaf (23) by the no. of cells per leaf.
[b] Mean ± SE.
[c] Calculated by dividing the weight of CF1 per cell by the no. of chloroplasts per cell (24).
[d] Calculated by dividing the weight of DNA per leaf (23) by the weight of DNA per cell.

of the IC 4X-8X set. The ratio of CF1 to Chl, however, was lower in octoploids compared to tetraploids. Molin *et al.* (23) reported that octoploids were 83% and 89% higher than tetraploids in cellular content of Chl and chloroplasts, respectively. They found that octoploids were only about 40% higher than comparable tetraploids in cellular content of RuBPCase and DNA. Unlike chloroplasts from the DDC isogenic set (which were similar in their content of Chl, RuBPCase [23], and CF1), IC 4X chloroplasts were 25% and 39% higher than comparable octoploids in their content of CF1 and RuBPCase, respectively.

The failure of octoploid cells to double the DNA content of tetraploid cells may be attributed to aneuploidy in the octoploid population (22). Guern and Herve (11) reported that the amount of DNA per nucleus increased only 60% with a doubling of nuclear ploidy in *Hippocrepis comosa*. Comparisons of total DNA per cell between ploidy levels may be complicated by endoreduplication of nuclear DNA observed among somatic tissues (5) and the effects of nuclear ploidy on extranuclear DNA. Chloroplast DNA was found to constitute 12% of total cellular DNA in fully-greened leaves of pea (*Pisum sativum*) (17). During pea leaf development, the number of chloroplasts per cell increased from 24 to 64 without net increases in nuclear and chloroplast DNA (18). Determining the relationship between nuclear ploidy and the amount of chloroplast DNA per cell should prove useful in understanding gene dosage effects in leaf cells.

In conclusion, ratios of CF1 to DNA were similar across ploidy levels of both isogenic sets suggesting that cellular content of CF1 increases proportionately with the amount of DNA per cell or gene dosage.

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