BIOLOGIA PLANTARUM 35 (3): 349-354, 1993

Differences in the synthesis of total and poly(A)$^+$RNA in the Kennebec potato and its dihaploid

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Abstract

RNA synthesis as measured by the incorporation of tritiated uridine into trichloroacetic acid insoluble material was studied in the leaf protoplasts of cv. Kennebec and its parthenogenically derived dihaploid. Protoplasts of cv. Kennebec incorporated tritiated uridine at a greater rate and accumulated more than twice the amount of radioactive materials than did the dihaploid over a 6-h incubation period. Poly(A)$^+$RNA, isolated from the total RNA of the tetraploid and of the dihaploid, by oligo(dT)-cellulose column chromatography, was present in amounts of 11.3 and 5.2 % respectively. The tetraploid synthesized 4.8 times the amount of poly(A)$^+$RNA that was synthesized by the dihaploid.

Introduction

Nebiolo *et al.* (1982/83) found that total RNA synthesis in a heterotic maize hybrid was intermediate to that of the inbred parents, but the quantity of poly(A)$^+$RNA synthesized was much greater in the heterotic hybrid. This investigation was initiated to determine if a similar relationship existed in a highly heterotic tetraploid like the cultivated potato, *Solanum tuberosum*, and in its less vigorous derived dihaploid.

S. tuberosum (2n = 4x = 48) (Hermsen 1984) is a segmental alloploid whose tetrasomic state contributes to a high degree of heterosis in both tuber yield and vegetative vigor (Bajaj and Sopory 1986). With four alleles at each locus, the potential for heterozygosity and consequently heterosis is greatly increased (Peloquin 1981).

Though the dihaploid has similar genetic information as its tetraploid parent it is diallelic at most loci (Dunbier and Bingham 1975), and as a consequence much of the heterotic interaction has been lost (Mendiburu *et al.* 1974).

Received 6 July 1992, *accepted* 18 February 1993.
Acknowledgement: This investigation was supported by Federal Hatch Project 288 and is published with the approval of the Director of the University of the West Virginia Agricultural Experiment Station as Scientific Paper No. 2262.
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The multiallelic system permits a variety of heterotic interactions not found in diploids. Only one allelic interaction is possible in dihaploids *versus* eleven theoretical ones in tetraploids (Galston *et al.* 1980, Mendiburu *et al.* 1974). Statistical studies indicate that heterosis in the tetraploid potato is predominantly due to epistasis and non-additive gene action (Dunbier and Bingham 1975).

Many of the metabolic activities of protoplasts in suspension are identical or closely parallel those of cells in tissues of whole plants (Kaiser and Heber 1983). DNA and RNA synthesis (Fuchs and Galston 1976, Mendiburu 1974), the properties of the plasmalemma (Kaiser and Heber 1983), and the effects of various endogenous factors on the biochemical activities are alike. However, important biochemical differences between protoplasts and whole cells in plant tissues result from cell to cell contact and the stress of the isolation procedure (Meyer 1985, Pilet 1985). Also variation in the degree of differentiation exists. The mesophyll cells in well developed potato leaves are highly differentiated and involved mainly with photosynthesis; they are arrested in the G_0 phase of the cell cycle. Isolated potato protoplasts "dedifferentiate" by stopping photosynthesis and synthesis of ribulose diphosphate carboxylase (Meyer 1985). The protoplasts enter G_0, begin DNA synthesis, cell division and new cell wall synthesis (Bajaj and Sopory 1986, Galston *et al.* 1980).

Materials and methods

The tetraploid Kennebec potato and its derived dihaploid progeny *US-W-495* (from R.E. Hanneman, Jr., Sturgeon Bay, USA) were sources of leaf protoplasts. Tubers were planted in 23 cm pots in a sterile greenhouse potting mix and grown in an incubator at 21 °C. A 12 h photoperiod was provided by fluorescent tubes giving a photon fluence rate of 655 μmol m^{-2} s^{-1} at pot level. Leaf tissue was taken 20 - 23 d after sprouts emerged. The ploidy status of the plants were confirmed by counting chloroplasts in the pairs of guard cells of 15 - 20 leaf stomata (Frandsen 1968, Wenzel and Foroughi-Wehr, 1986) from a 1 cm^2 section of a young leaf from each of four plants of each strain.

Isolation of mesophyll protoplasts followed the procedure of Shephard and Totten (1975). The composition of the isolation solution (IS) and osmoticum (OS) is modified Shephard and Totten Medium A (1977) without sucrose, agar, organic nutrients or plant hormones but with 63.77 g dm^{-3} sorbitol (0.35 M) added.

After an 18 h incubation in darkness at 10 - 15 °C, the soak solution was replaced with 3 % cellulase solution (0.3 g cellulysin + 9.7 cm^3 IS) (*Cellulysin*™ *Cellulase, Calbiochem-Behring*, San Diego, USA). Prior to use the cellulysin solution was centrifuged at 4000 *g* in order to remove insoluble salts and precipitates. Incubation resumed at 28 °C for 4 h with a *Grow-lux* fluorescent tube providing a photon fluence rate of 36.5 μmol m^{-2} s^{-1} at the level of the protoplasts. All solutions contained 10 μg cm^{-3} chloramphenicol.

Leaves were teased apart after digestion and the suspension filtered through one layer of Mirocloth and four layers of cheese cloth. The suspension was centrifuged

(100 *g*) for 8 min at ambient temperature. The supernatant was aspirated and the protoplasts washed twice in 8 cm^3 of IS each time; the final pellet was resuspended in 3 cm^3 of osmoticum. Cell viability was determined by Evans blue dye (0.1 % in OS, pH 5.6) exclusion over a 6 h period. Cell number was determined with a hemocytometer. One gram of tissue yielded 2 - 3 × 10^6 protoplasts from Kennebec and 5 - 6 × 10^6 protoplasts from the dihaploid. Cell size was determined by photographing protoplasts on a hemocytometer and measuring their diameters with a vernier caliper. Three sets of protoplasts from three different plants were measured for each strain. For the dihaploid and for Kennebec 372 and 373 cells were measured, respectively.

RNA synthesis in Kennebec and dihaploid protoplasts was measured by the incorporation of ^{3}H-uridine over an 6 h period, using a modified version of Fuchs' method (1976). For the isolation and quantification of poly(A)$^+$RNA tubes containing 2 × 10^6 protoplasts in 10 cm^3 of OS were pre-incubated for 1 h when 3.7 MBq of ^{3}H-uridine were added and incubation continued in a closed water bath for 6 h at 37 °C under a *Grow-lux* light. Incorporation of isotope was stopped by chilling in an ice bath for 5 min after which the protoplasts were pelleted at 100 *g* for 4 min at 4 °C. The supernatant was removed, 2 cm^3 of ice-cold NET buffer (0.1 M NaCl, 0.1 mM Na-EDTA, 10 mM Tris-HCl, 1 % m/v SDS, pH 7.4) were added and the cells vortexed for 30 s. This homogenate was extracted with 2 cm^3 of phenol:chloroform (1:1, v:v) saturated with ANE buffer (0.01 M Na-acetate, 0.1 M NaCl, 1 mM EDTA, pH 6.0) (Kulikowsky and Mascarenhas 1978). The mixture was vigorously agitated on a rotary shaker for 5 min at ambient temperature followed by centrifugation at 160 *g* for 10 min. The aqueous layer containing the nucleic acid was removed and saved, but the phenol layer was re-extracted with an additional 2 cm^3 of NET buffer. The two layers were combined and extracted a final time with 4 cm^3 of phenol-chloroform. The aqueous phase was made 0.3 M with Na-acetate and 2.5 volumes of ice-cold 95 % ethanol were added. After precipitating overnight at -20 °C the RNA was recovered by centrifugation at 12 000 *g* for 35 min at 10 °C. The supernatant was removed, the pellet washed once with 70 % ethanol containing 0.1 M Na-acetate, and then dried under vacuum. The sample was resuspended in 1 cm^3 of binding buffer (0.01 M Tris-HCl, 0.5 M NaCl, 1 mM EDTA, pH 7.5) by swirling in a 37 °C water bath for 5 min. An aliquot was taken and counted to determine total incorporation of ^{3}H-uridine.

Total RNA in binding buffer [0.33 M NaCl, 10 mM Tris acetate, 0.5 % (m/v) SDS, pH 7.5] was chromatographed on a 2 cm^3 column of oligo(dt)-cellulose Type 3 (*Collaborative Research, Inc.*, Lexington, USA) and equilibrated with 7 cm^3 of binding buffer for isolation of poly(A)$^+$RNA. 2 cm^3 of binding buffer were used to rinse the sample tube and added to the column. The flow through volume was collected and added back to the column once. 6 cm^3 of binding buffer were used to wash through any unbound RNA.

The bound poly(A)$^+$RNA was eluted with 5.2 cm^3 of elution buffer (0.01 M Tris-HCl, 1.0 mM EDTA, 0.5% SDS, pH 7.5). The sample was made 0.3 M Na-acetate and 2.5 volumes of ice-cold 95 % ethanol were added and mixed. After precipitation overnight at -20 °C, the sample was centrifuged at 12 000 *g* for 35 min at 10 °C,

dried, and resuspended in 0.5 cm^3 of double-distilled water. 2 cm^3 of 15 % trichloroacetic acid were added, followed by a 30 min precipitation on ice. The precipitate was filtered and counted as described for the incorporation study.

Results and discussion

Clean, viable protoplasts were obtained from plants of both strains grown under artificial light. The polyploid and the diploid status of cv. Kennebec and of the dihaploid were confirmed by counts of the chloroplast number per pair of guard cells and by protoplast diameters and volumes. Kennebec had an average number of 22.7 and the dihaploid had 12.2 chloroplasts per pair of guard cells respectively. These numbers are in agreement with those of Frandsen (1968). The mean diameters for Kennebec and for the dihaploid protoplasts were 26.0 $\pm$ 8.2 and 22.8 $\pm$ 4.6µm respectively. These differences were statistically significant ($P \leq 0.01$) as determined by the Behrends-Fisher analyses for data with unequal variances (Hartley's F-max Test). The mean protoplasts volumes of Kennebec and of the dihaploid were 9203 and 6206 μm^3 respectively. The Kennebec protoplasts therefore have a 48.0 % greater volume than those of the dihaploid. These statistics also confirm the tetraploid and dihaploid status of Kennebec, respectively.

The viability of protoplasts, as tested by Evans blue dye exclusion, declined from 100 to 90 % for both strains after 6 h of incubation. The addition of ^{3}H-uridine or of ^{3}H-leucine did not affect viability (data not shown). The incorporation of ^{3}H-leucine was used as a second test of viability. Protoplasts incorporated ^{3}H-leucine linearly for an 8 h period (data not shown) indicating that the complex energy requiring protein synthesizing mechanisms were functional and the cells were viable (Table 1).

Table 1. Total RNA and poly(A)$^+$RNA as measured by scintillation counting of ^{3}H-uridine incorporation over a 6 h period into 2 × 10^6 protoplasts of potato cv. Kennebec and a derived dihaploid.

	Count rate [s^{-1}]		
	total RNA	poly (A) $^+$RNA	% poly (A) $^+$RNA
cv. Kennebec	5928[1]	633	10.7
Dihaploid	2416[2]	132	5.5

[1] Means of two different plants and three replications each per analysis.
[2] Means of five different plants and three replications each per analysis.

The tetraploid synthesized 2.45 times more RNA than did the dihaploid. This difference is probably best attributed to the effects of four genomes and heterotic interactions in the tetraploid.

Scintillation counting of the solution eluted from the oligo (dT)-columns showed that Kennebec synthesized 4.8 times the amount of poly(A)$^+$RNA that was produced by the dihaploid (Table 1). This result supports those of Nebiolo *et al.* (1982/83), *i.e.*,

hybrid systems. Of course, the dihaploid is not a homozygous system like maize inbreds, but it is less heterozygous than its Kennebec parent and consequently it cannot produce many of the epistatic and non-additive interactions that are found in the tetraploid potato (Dunbier and Bingham 1975). The increased synthesis of poly(A)$^+$RNA is not just the result of doubling of the potato genomes which, without interaction or dosage factor, would be expected to yield twice the amount of poly(A)$^+$RNA. The increase is probably best attributed to the epistatic and non-additive gene actions as well as the more numerous interactions that are possible in a tetraploid (Peloquin 1981, Mendiburin *et al.* 1974).

The greater amount of poly(A)$^+$RNA produced by Kennebec may be a manifestation of the tetraploid state of heterosis. Supposedly, the only genomic difference between these two plants is that the dihaploid has half the number of alleles of each gene (most often diallelic) and that Kennebec is most often tetrallelic (Dunbier and Bingham 1975). While it is possible that some difference may occur in poly(A)$^+$RNA turnover rate or rate of hmRNA processing, the most likely cause of an increase in the amount of poly(A)$^+$RNA is that more is being transcribed. This may be due to an increased template availability, to a more active RNA polymerase II, or to differences in the functioning and number of promotor, regulator and structural genes. In Kennebec, any multimeric enzyme may exist with a much wider range of forms, since theoretically, 4 different alleles may be present that code for polypeptide sub-units, as opposed to 2 in the dihaploid. RNA polymerase II is a complex multimer (Levin 1990). The genomic complexities of the potato could therefore give rise to great varieties of isozymic forms. The isozymes of RNA polymerase have been compared in hybrid maize and its inbred parents (Epperly 1984). Differences in the isozymic profiles and their activities have been suggested as a possible mechanism of heterosis in that system.

In addition to the RNA normally produced by the cells, some new poly(A)$^+$RNA may be synthesized in response to the stresses of protoplast isolation. The tetraploid may be able to respond more efficiently to these stresses because it may possess twice the number of different alleles and up to eleven times the number of theoretical interactions. The greater amount of poly(A)$^+$RNA produced may be a manifestation of this.

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Communicated by T. GICHNER