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ENZYMES OF STARCH METABOLISM IN *NICOTIANA TABACUM* CALLUS

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Abstract—Activities of enzymes of starch metabolism were determined in tobacco callus grown in light or darkness and in the presence or absence of gibberellic acid There was a higher rate of starch turnover in light-grown cultures, as judged by the activities of synthetic and degradative enzymes. Gibberellic acid-treated tissues contained a lower level of starch than the corresponding control tissue This decrease could be correlated with the activity of phosphorylase Cultured tissue and seedling material were found to have comparable levels of activity for the starch metabolizing enzymes

INTRODUCTION

TISSUE cultures have been used for many years to study the requirements for growth and differentiation One such requirement is the need for an exogenous source of carbon and energy, which is usually supplied as sucrose.[1] However, very little is known about carbohydrate metabolism in cultured tissue. Our interest in this area has come from our previous studies, which indicated the importance of carbohydrate metabolism in organized development. We have shown that shoot-forming tobacco callus accumulated starch, and that there was a positive correlation between this accumulation, shoot formation and respiration of the tissue.[2–6] In association with these studies, we have been examining the role of carbohydrate metabolism in proliferating callus, i.e. in growing tissue in the absence of organized development. We have previously shown that the breakdown of sucrose in tobacco callus during growth was more related to the activity of sucrose synthetase than to the activity of the invertases.[7] More recently we have shown that differences in growth of tobacco callus under various culture conditions could not be correlated with changes in the specific activities of enzymes of the Embden–Meyerhof–Parnas glycolytic or the pentose phosphate pathways.[8] Proliferating tobacco callus maintains a low level of stored starch throughout culture,[2–5] and this starch (along with free sugars in the tissue) is used for maintenance in tissue cultured in the absence of any carbon source.[9] In this paper, we

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have examined the activities of starch metabolizing enzymes in tobacco callus cultured under various conditions, and have also compared the activities in the cultured tissue with those of the intact plant

RESULTS

Some characteristics of tobacco callus grown in light or darkness in the presence or absence of gibberellic acid (GA_3) are shown in Table 1. Fresh weight increases were greater for dark-grown than the corresponding light-grown tissue, and higher for GA_3-treated tissue than the corresponding tissue cultured in its absence. Largest fresh weight to dry weight ratios were observed in dark-grown GA_3-treated tissue, followed by dark-grown tissue, followed by light-grown GA_3-treated tissue and finally light-grown tissue No treatment effects were observed in the protein contents of the cultured tobacco callus. The protein contents per g fresh weight were lowest at 21 and 28 days of culture The cultured tissues accumulated very little starch during growth However, the starch content of GA_3-treated tissues was lower than in its absence in both light and dark-grown cultures

TABLE 1 CHANGES IN FRESH WEIGHT (FW), FRESH WEIGHT/DRY WEIGHT RATIOS (FW DW), PROTEIN AND STARCH CONTENTS OF TOBACCO CALLUS DURING GROWTH IN THE LIGHT (L), DARK (D) AND IN THE ABSENCE OR PRESENCE OF GA_3 (G, 5×10^{-7} M) Data are the averages of duplicate experiments, which gave similar results

		Culture					
	Treatment	0	7	14	21	28	35
FW	L		0 157	0 271	0 337	0 416	0 428
g/callus	LG		0 211	0 348	0 524	0 699	0 808
piece	D	0 060	0 145	0 292	0 582	1 100	1 332
	DG		0 157	0 351	0 804	1 338	1 748
FW/DW	L		12 7	10 5	10 3	11 4	9 6
	LG		14 7	12 8	12 1	12 6	11 7
	D	15 1	15 8	17 1	17 1	17 7	17 9
	DG		16 8	18 0	20 4	22 3	21 5
Protein	L		8 58	8 90	4 84	6 26	9 09
mg/g FW	LG		8 16	8 21	3 90	5 38	8 66
	D	6 90	9 33	8 55	4 14	5 25	7 22
	DG		8 55	8 27	3 89	4 77	7 68
Starch	L		0 34	0 34	0 58	0 72	1 04
mg/g FW	LG		0 50	0 58	0 40	0 37	0 44
	D	0 65	0 51	0 69	0 53	0 55	0 70
	DG		0 30	0 24	0 32	0 30	0 38

Changes in the activities of starch metabolizing enzymes in the cultured tobacco callus are shown in Table 2. The activity of soluble starch synthetase varied during culture, with light-grown tissue reaching a peak of activity earlier in culture than the dark-grown tissues In addition, the activity in light-grown tissue was higher initially, but by the end of the culture period the activity of soluble starch synthetase was higher in dark-grown tissues Furthermore, the activity of this enzyme was higher in the absence of GA_3 than in the corresponding-GA_3-treated tissue up to 28 days in culture. With respect to insoluble or granule-bound starch synthetase, the activity in light-grown tissue was higher than in dark-grown tissue, and the activity in the absence of GA_3 was higher than in the corresponding tissue grown on the GA_3-medium The activity of Q- or branching enzyme fluctuated during culture. No treatment differences in activity were observed, except that 35-day-old cultures had higher activities in the absence of GA_3 than in its presence.

TABLE 2 CHANGES IN SPECIFIC ACTIVITIES OF STARCH METABOLIZING ENZYMES IN TOBACCO CALLUS DURING GROWTH IN THE LIGHT (L), DARK (D) AND IN THE ABSENCE OR PRESENCE OF GA_3 (G, 5×10^{-7} M)
Specific activity of enzymes defined in Experimental section

Enzyme	Treatment	Days in culture 0	7	14	21	28	35
Soluble starch synthetase $\times 10^{-10}$	L		13 65	17 21	25 57	14·24	1·67
	LG		9·60	6 23	10 37	9·28	2 62
	D	6 28	7 97	7 36	9·61	15 36	4 42
	DG		7 87	2 93	5 38	8 50	9 29
Insoluble starch synthetase $\times 10^{-10}$	L		3 30	12 08	25 42	7 87	0 39
	LG		1 77	4 25	12 42	6·48	1 61
	D	4 10	1 17	2 22	6 87	3 20	2·17
	DG		0·23	0 33	5 25	2 73	2 85
Q-enzyme $\times 10^{-3}$	L		0 14	0 52	1·36	0 67	1 26
	LG		0 25	0 59	1·22	0 37	0 52
	D	0 14	0 82	0 29	1·26	0 48	1 23
	DG		0·44	0 61	1 12	0·41	0 74
R-enzyme $\times 10^{-3}$	L		*	0 98	5 99	3 48	2 74
	LG		*	1 41	6 41	4·08	3·84
	D	1·24	0·08	0 80	4·59	2 21	0 38
	DG		*	0·86	1 23	2·78	0·77
Phosphorylase $\times 10^{-10}$	L		4·65	4 09	5 76	3 53	3·53
	LG		7·58	4·81	4 81	4·77	2·86
	D	5 03	4 71	4 79	10·30	10·21	4·62
	DG		6·49	8 14	8·73	10·53	7·71
α-Amylase $\times 10^{-1}$	L		3·79	5 08	12·58	5·93	5·45
	LG		3·05	5·05	13 29	6·35	5·86
	D	2·36	1·88	1·72	4 73	3 26	3·65
	DG		1·59	2·76	7·09	3·94	3·08
Maltase (α-glucosidase) $\times 10^{-1}$	L		0 35	0 56	2 29	0·55	0·40
	LG		*	0 92	1·69	1·19	0·17
	D	0 80	1 19	0 53	0·65	*	0·19
	DG		1·47	0·28	0·50	0 11	0·16

* Trace of activity. Data are the averages of duplicate experiments, which gave similar trends

The specific acitivity of R-or debranching enzyme was low during the early culture period but increased dramatically during culture. In general, the activities were higher in light-grown tissue and in GA_3-treated tissue than in the corresponding dark-grown and non-GA_3-treated cultures. With respect to phosphorylase, the activity tended to be higher in dark-grown tissue than in light-grown tissue, particularly later in culture. In addition, the activity was higher in GA_3-treated tissue than in the corresponding tissue in the absence of the growth regulator. The activity of α-amylase was higher in light-grown tissue than in dark-grown cultures. No pronounced effects of GA_3 on α-amylase activity were apparent. β-amylase was not detected in tobacco callus. The pattern of activity of maltase or α-glucosidase was different in light- and dark-grown cultures. For light-grown tissues activity was low at 7 and 35 days, with peak activities in 21-day-old cultures. With dark-grown cultures peak activities were found in 7-day-old tissue after which there was a more or less steady decline in activity. No apparent effects of GA_3 were observed on maltase levels in light or darkness.

The levels of activity of the starch metabolizing enzymes in pith and leaf tissue of greenhouse grown tobacco seedlings, and of the 35-day-old callus and leafy vegetative shoot material of cultures, originally derived from pith was determined (Table 3). As can be seen, leaves had higher soluble and insoluble starch synthetase activities than pith tissues. This

same relationship held for shoot and callus tissues In addition, the relative levels of activity were of the same order. With respect to Q-enzyme, the specific activities in pith, leaves and shoots were of the same order, while that in the light-grown callus was higher The level of activity of R-enzyme was higher in tobacco leaves than pith. However, while the activity of this enzyme in the callus was higher than the pith; the level in the shoot tissue was very much lower than even in the pith The level of activity of phosphorylase was the same in leaves and shoot tissue, and was higher than in pith However, the activity of this enzyme in light-grown callus was much reduced. The activities of α-amylase in pith and leaf tissue were low, whereas much higher activities were observed in the cultured tissues Finally, whereas the levels of activity of maltase in callus and shoot tissue were of the same order, the activity in pith was about twice as great and in leaves about 6 times less

TABLE 3 COMPARISON OF SPECIFIC ACTIVITIES OF STARCH METABOLIZING ENZYMES IN PITH AND LEAF TISSUE OF TOBACCO PLANTS AND OF CALLUS AND SHOOTS OF TOBACCO CALLUS GROWN FOR 35 DAYS IN THE LIGHT Specific activity of enzymes defined in Experiment section Values are the averages of duplicate determinations

Enzyme	Plant material			
	Pith	Leaves	Callus	Shoots
Soluble starch synthetase $\times 10^{-10}$	1 84	6 63	1 67	6 31
Insoluble starch synthetase $\times 10^{-10}$	0 58	4 90	0 39	3 82
Q-enzyme $\times 10^{-3}$	0 24	0 36	1 26	0 25
R-enzyme $\times 10^{-3}$	1 86	3 96	2 74	0 71
Phosphorylase $\times 10^{-10}$	12 01	16 95	3 53	16 56
α-Amylase $\times 10^{-1}$	0 17	0 08	5 45	2 20
Maltase $\times 10^{-1}$	1 02	0 08	0 40	0 58

DISCUSSION

Recently,[8] growth curves of tobacco callus in light and darkness and in the presence or absence of GA_3 were published In the present studies, similar growth curves were obtained, except that the light-grown GA_3-treated tissue did not show as much fresh and dry weight increase FW/DW ratios showed that the dark-grown tissue had higher ratios than light-grown tissue, and that GA_3 increased these ratios over the corresponding tissue in the absence of this growth regulator Despite the differences in growth, there were no pronounced treatment effects on the protein content of the tissues, although light-grown tissue possibly had slightly higher protein contents later in culture The protein contents of the tissues were lowest in the 21- to 28-day period The significance of this finding is not clear, although it might be related to the fact that the tissues were in the logarithmic phase of growth The proliferating callus accumulated very little starch, thus confirming earlier reports.[2–5] However, GA_3 reduced the amount of starch in the tissue in both light- and dark-grown cultures. GA_3 has also been shown to reduce starch levels in shoot-forming tobacco callus.[3] Of interest was the apparent slight increase with time of starch in light-grown tissue in the absence of GA_3.

The enzymes of starch metabolism studied were active throughout the culture period. With respect to the synthesis of starch, the activities of soluble and insoluble or granule-bound starch synthetases and Q- or branching enzyme suggested that active synthesis was going on throughout culture. Of interest was the higher levels of activity of the starch synthetases observed in light-grown tissue, where approximately 2-fold differences in activity were observed over the corresponding dark-grown cultures GA_3 in light or darkness repressed the enzyme levels, so that again approximately 2-fold differences in activity were

obtained between the GA_3-treated tissues in the 14–28 day culture period. No treatment effects were observed in relation to the activity of Q-enzyme. This could indicate that the type of starch formed, i.e. the proportion of amylopectin in the starch, might be different under the different culture conditions. This aspect has not yet been examined.

With respect to the degradation of starch; the activities of R- or debranching enzyme, α-amylase and maltase were generally higher in light-grown cultures than in those grown in darkness. This would indicate a higher degree of degradation of starch in these light-grown cultures. There were no pronounced effects of GA_3 on the levels of activity of the above enzymes.

The activity of phosphorylase did not fit into the above picture. The activity of this enzyme tended to be higher in dark-grown cultures than in light, and higher in GA_3-treated tissues than in non-GA_3-treated tissues throughout most of the culture period. Phosphorylase is now generally considered to be important in the degradation of starch,[10] thus its role in dark-grown and GA_3-treated tissues assumes major significance. The relatively higher levels in GA_3-treated tissues could account for the decreased starch content in these tissues. In addition, the relatively lower level of activity in light-grown tissue could possibly account for the slight increase of starch in this tissue. The possible importance of phosphorylase in controlling the level of starch in GA_3-treated tissues is worthy of further investigation. However, it should be noted that in shoot-forming tobacco cultures α-amylase is apparently of greater importance than phosphorylase in starch degradation.[11]

Nevertheless, it would appear that light-grown and dark-grown tissues maintained more or less the same level of starch in culture through an increased rate of starch turnover in the light-grown tissues. Light stimulation of enzyme activity in plants is quite common, e.g. light stimulates the activity of ribulose-1,5,diphosphate carboxylase in barley, nitrate reductase in corn, and phenylalanine ammonia lyase in gherkin.[12] We have also found a light-stimulation of the levels of activity of glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase in cultured tobacco tissue.[8]

Tobacco seedlings were found to have different levels of activity for starch metabolizing enzymes in the pith and leaves; with leaf tissue, in general, having higher levels of activity. Of interest was the finding that tissue cultured under shoot-forming and non-shoot-forming conditions, reflected these differences. Tissue cultured for 5 weeks in light was selected for comparison, because at that time the non-shoot-forming or callus proliferating tissue was essentially in the stationary phase of growth and would therefore, metabolically be similar to the pith tissue. After 5 weeks in culture, leafy vegetative shoots could easily be separated from callus and being green, would also be actively photosynthetic. Cultured tissues are often noted for the absence or greatly reduced level of activity of enzymes.[13] With respect to the enzymes of starch metabolism, this was not borne out in cultured tobacco callus which, therefore, offers potential for further studies on starch metabolism.

EXPERIMENTAL

Plant material and culture conditions The method of obtaining callus from tobacco (*Nicotiana tabacum* L., cv Wisconsin *38*), the maintenance of that tissue in culture, and experimental conditions for shoot production and callus proliferation had been reported [4–8] 10 Pieces of tissue per treatment, per experimental day, were weighed and then dried for 48 hr at 80° to obtain FW and DW values

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Starch was determined by the anthrone reagent procedure [14]

Enzymes The enzymes examined were starch synthetase (ADP glucose α-1,4 glucan α-4 glucosyltransferase, E.C. 2.4.1.21), Q-enzyme (α-1,4 glucan, α-1,4-glucan 6-glycosyltransferase or branching enzyme, E.C. 2.4.1.18), R-enzyme (amylopectin 6-glucanohydrolase or debranching enzyme, E.C. 3.2.1.9), phosphorylase (α-1,4-glucan orthophosphate glucosyltransferase, E.C. 2.4.1.1), α-amylase (α-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1) and maltase (α-D glucoside glucohydrolase or α-glucosidase, E.C. 3.2.1.20)

Preparation of enzymes 3–4 g of tissue was ground with *ca* 15 ml of 0·1 M Tris–HCl buffer (pH 7); and 0·2 g polyvinylpyrrolidone (PVP) in a mortar at 0°. The homogenate was filtered through glass wool and centrifuged at 27 000 *g* for 10 min at 0°. The supernatant was collected and made up to 25 ml with the Tris–HCl buffer. The residue was suspended in 2 ml, 0·1 Tris–glycine buffer (pH 7·2), and used for insoluble starch synthetase determination. A 5 ml aliquot of the supernatant was dialyzed against 4 l. of 50 mM Tris–HCl for 3 hr, and used for the maltase assay. Protein was precipitated from the remaining 20 ml of supernatant by the addition of solid $(NH_4)_2SO_4$ to 80% saturation for 30 min at 0°. Equal portions of the suspension were placed into 4 centrifuge tubes and the protein collected by centrifuging for 10 min at 20 000 *g* at 0°. The ppts were dissolved in appropriate buffers and used for the assays of α-amylase, R-enzyme, Q-enzyme, phosphorylase and soluble starch synthetase.

Enzyme determinations All assays were carried out at 30° for the lengths of time required. All controls consisted of boiled enzyme blanks. The protein ppt used for α-amylase and R-enzyme was dissolved in 0·2 M acetate buffer (pH 6·6). The enzyme levels were assayed colorimetrically,[15] using as substrates 0·15% amylase for α-amylase and 4 mg/ml β-limit dextrin for R-enzyme. The assay for α-amylase was carried out at a pH 5·3. The R-enzyme solution was incubated for 45 min with $2·5 \times 10^{-2}$ M EDTA to inactivate α-amylase, and then assayed at pH 6·6. 10 units of purified α-amylase (Sigma, type I-B) were added to the reaction mixture prior to incubation. Activities are expressed as decrease in A at 660 nm/min for α-amylase, and at 540 nm/min for R-enzyme. Maltase activity was assayed using maltose as substrate. The reaction mixture consisted of 0·5 ml, 0·2 M acetate buffer, maltose (Sigma) 2 mg/ml and 0·5 ml enzyme solution. The glucose liberated was measured by the glucose oxidase peroxidase method using Glucostat (Worthington Biochemicals Corporation). Activity is expressed as μg glucose produced/min. Q-enzyme was prepared by dissolving a portion of the protein ppt in 0·15 M sodium citrate buffer (pH 7·0). The enzyme soln was incubated for 45 min with $2·5 \times 10^{-2}$ M EDTA to inactivate α-amylase and then assayed.[16] Activity is expressed as decrease in A at 660 nm/min. The phosphorylase assay[17] was slightly modified. The assay mixture contained 30 μl 0·1 M Tris maleate buffer, pH 8, 20 μl soluble starch solution (2%), 0·25 μmol (20 000 cpm) uniformly labelled glucose-[^{14}C]-1-phosphate (International Nuclear Corporation) and 30 μl enzyme solution in a total vol of 100 μl. The enzyme soln was prepared by dissolving a portion of the protein ppt in 0·5 ml of the Tris–maleate buffer. For the starch synthetases, precipitated protein dissolved in 0·5 ml of 0·1 M Tris–glycine buffer (pH 7·2) containing 70 mM EDTA was used as soluble enzyme and the prepared starch suspension was used as the granule-bound enzyme for assay.[18] The reaction mixtures consisted of 30 μl of 0·1 M Tris–glycine buffer, pH 8, for the soluble and pH 7·2 for the insoluble enzyme with 0·07 M EDTA, 0·25 μmol (5000 cpm) uniformly labelled ADP-glucose[^{14}C] (International Nuclear Corporation), and 40 μl starch suspension in a total vol of 80 μl for granule-bound starch synthetase, and 30 μl enzyme soln plus 20 μl soluble starch (2%), to act as primer in a total vol of 100 μl for soluble starch synthetase. After incubation the starch was precipitated and washed.[19] All results obtained from the carbohydrate assays are expressed as mmol of glucose incorporated/min. Protein was measured by the Folin phenol method.[20] The insoluble fraction was dispersed in 10% $HClO_4$ prior to protein determination.

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