Pergamon

0031-9422(94)00874-4

Phytochemistry, Vol. 39, No. 1, pp. 21–28, 1995

0031-9422/95 $9.50 + 0.00

REGULATION OF THE ENZYMES OF UDP-SUGAR METABOLISM DURING DIFFERENTIATION OF FRENCH BEAN

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(*Received in revised form* 6 *October* 1994)

Key Word Index—*Phaseolus vulgaris*; Leguminosae; French bean; cell walls; differentiation; cytokinin; UDP-sugars.

Abstract—A study has been made of changes in the enzymes of UDP-sugar metabolism in suspension cultured cells of French bean during their normal growth following subculture. These are compared to cells of the same cell suspension subcultured into a cytokinin enriched media which induces changes in their cell walls that resemble xylogenesis. The same enzymes have also been measured in seedling hypocotyls undergoing elongation growth and differentiation. Evidence was obtained for sucrose synthase and possibly amylase for having roles in the mobilization of sugar reserves during growth changes in suspension cultures. Furthermore, UDP-glucose dehydrogenase showed characteristic increased activity that indicated a key role in the provision of sugar nucleotides for cell wall biosynthesis. Less significant changes were observed for other enzymes involved in sugar nucleotide interconversion.

INTRODUCTION

Mechanisms of the regulation of the biosynthesis of plant cell wall components are relatively obscure especially with respect to the polysaccharides. There is currently a lack of information on the enzymes involved in quantitative and differential carbon flux into wall polysaccharides. However, the plant cell wall undergoes profound changes under diverse conditions such as during growth and differentiation and in response to stress and pathogenic attack [1–3]. These characteristic changes are likely to be partly regulated by changes in the underlying synthetic systems. Besides these important developmentally and environmentally regulated qualitative changes, the accumulation of cell wall polymers represents a significant sink for the quantitative flow of nonretrievable carbon. With perhaps the exception of the products of the phenylpropanoid pathway, little is known at present of the quantitative and qualitative control of the accumulation of wall polymers when compared with the progress being made on the understanding of the regulation of the flow of hexose into storage compounds such as sucrose and starch [4, 5].

Possible control points of quantitative carbon flux into polysaccharides may occur in the pathway which supplies the polysaccharide synthases with their relevant UDP-sugar substrates [6, 7]. UDP-glucose is the probable substrate for cell wall glucan synthases. Further elaboration of UDP-glucose provides the precursors for the polysaccharides that contain arabinose, galactose and galacturonic acid, broadly the pectin group, and those that contain glucose, xylose and glucuronic acid, the hemicellulose group. These nucleotide sugars are also precursors for ER and Golgi-mediated protein glycosylations. UDP-glucuronate biosynthesis may arise directly from the oxidation of UDP-glucose by UDP-glucose dehydrogenase (EC 1.1.1.22) or from the indirect *myo*-inositol route [8]. *In vivo* the direct oxidation of UDP-glucose is believed to be the favoured pathway [9, 10]. UDP-glucose is also converted to UDP-galactose by the action of a specific 4-epimerase (EC 5.1.3.2). UDP-glucuronic acid can be decarboxylated to UDP-xylose. All three intermediates for hemicellulose biosynthesis can be converted to their 4-epimers by specific epimerases that provide all the precursors of the pectin group.

UDP-glucose is thus a central intermediate. It could be derived from several sources such as the action of sucrose synthase (EC 2.4.1.13) on sucrose, or from the activation of glucose, either derived from sucrose by the action of invertase (EC 3.2.1.26) or from starch by amylase (EC 3.2.1.1)/phosphorylase (EC 2.4.1.1) activities. Possible key control points in the supply of UDP-sugars in relation to cell wall biosynthesis could be the reactions catalysed by UDP-glucose dehydrogenase and UDP-glucuronate decarboxylase (EC 4.1.1.35), both of which are irreversible [6, 7]. UDP-glucose dehydrogenase is also subject to feedback inhibition by UDP-xylose implicating that the pathway is highly regulated [6].

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The present work investigates the involvement of the enzymes involved in UDP-sugar interconversion in seedlings undergoing secondary cell wall differentiation and suspension cultures undergoing xylogenesis. Our experimental approach was to take cells freshly subcultured into normal and induction media and determine the effects on growth and cell wall composition. In turn this was compared to the underlying metabolism of UDP-sugar formation and interconversion through the period when changes in growth were occurring. Similar analysis was also conducted in hypocotyls from the time of emergence through to complete cessation of elongation and the subsequent period of continuing differentiation. In both the suspension cultured cells and the hypocotyls the results are interpreted in the light of previous work which has determined the induction of arabinosyl and xylosyl transferases as markers for wall polysaccharide biosynthesis [11, 12]. Changes in the activities of amylase, acid invertase, starch phosphorylase and sucrose synthase were also determined in each experimental system and these are discussed in relation to the supply of UDP-glucose.

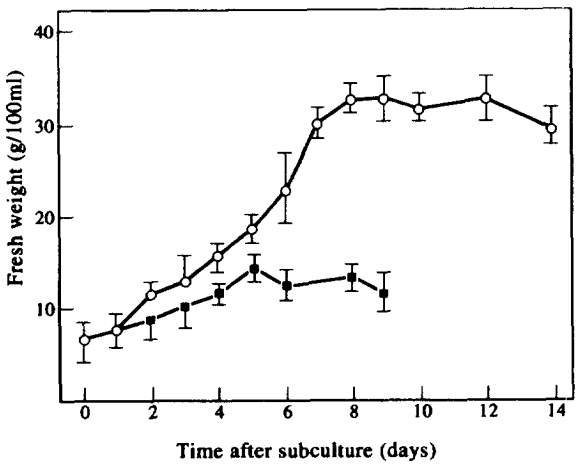

Fig. 1. The growth of suspension cultured French bean cells. Growth was determined for cells grown in maintenance media (○) and cytokinin induction media (■). Each value is the mean of 3 ± s.e.

RESULTS

Growth of suspension cultures and hypocotyls

Suspension cultures were routinely maintained and grown as previously described [12]. On subculturing the suspension growth continued for up to nine to 10 days before entering the stationary phase. When subcultured into cytokinin-enriched induction-medium [12] to bring about differentiation, growth was considerably slowed (Fig. 1).

Under the growth conditions employed the hypocotyls emerged at about five days after imbibition and underwent rapid elongation growth (Fig. 2). At about 10 days, extension growth had totally ceased and the hypocotyl had become hollow. During the period immediately before cessation of hypocotyl elongation, vascular bundles form from procambium tissue and at about 11 days secondary cambium forms.

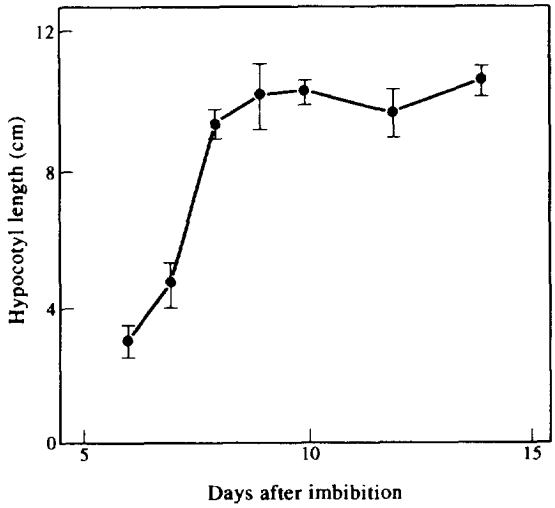

Fig. 2. The growth of French bean seedling hypocotyls. Each value is the mean of 15 ± s.e.

Analysis of cell wall changes induced in suspension cultured cells treated with cytokinins

Comparison has been made in the cell wall composition of cells grown in maintenance media and cells subcultured into cytokinin induction media (Table 1). The fractionation of the cell walls was carried out on several lots of pooled cell wall material derived from batches of cells remaining after removal of a portion of cells for enzyme analysis. This approach of pooling cell wall material from separate experiments was adopted in order to optimize recoveries since previous experience indicated that larger samples were easier to process without incurring excessive losses and that variation between replicates was found to be low. [13].

The most striking observation was the 32-fold increase in phenolic material associated with the cell walls from cells grown in induction media compared to the control cells. Other changes included increase in the hydroxyproline content, cellulose and hemicellulose contents of the cell walls from cells induced into undergoing xylogenesis. The nature of the hemicellulosic material also showed changes characteristic of secondary cell walls, there being about six times more xylan and half as much xyloglucan content in the cell walls from cytokinin treated cells than in comparative control cell walls. This is also reflected in the five-fold increase in the ratio of xylose:glucose present in the xylogenic cell walls. However, there was less pectinaceous material produced in these cell walls. Also, the cell walls from the control cells contained four to five times more acidic than neutral pectin, whereas the walls from the cytokinin treated cells had three to four fold more neutral than acidic pectin.

Table 1. Cell wall composition of suspension cultured French bean cells grown in maintenance media and cytokinin induction media.

	Treatment			
	Maintenance medium		Induction medium	
Fraction	mg dry wt (g fr. wt)$^{-1}$		mg dry wt (g fr. wt)$^{-1}$	
Neutral pectin	4.0		12.0	
Acidic pectin	18.0		4.3	
Hemicellulose	52.0		62.0	
Xyl:Glc		0.4		1.9
Xyloglucan	46.3		24.2	
Xylan	5.7		37.8	
Cellulose	40		58	
Hydroxyproline	0.10		0.13	
Total phenolics (rel. level)		1.0		32

The cell wall analysis was carried out on cells harvested seven days after subculture. The cells remaining from three separate experiments for which the enzyme levels were determined were pooled and the resulting cell wall material fractioned. Each fraction represents the total material recovered expressed on a g fr. wt basis.

Optimization of enzyme determinations

Throughout this study activities of the enzymes measured were optimized with respect to pH and buffer components in batches of untreated cells harvested four days after subculture. Similar experiments were also conducted on seedling hypocotyl tissue eight days after imbibition. Where crude extracts were subject to ammonium sulphate fractionation, analysis of the distribution of enzyme activities in the various fractions showed that the recovery of activity in those used for the determination of enzyme levels was greater than 90%. With either hypocotyl or cultured cells the optimum conditions for each individual enzyme did not vary. In all cases it was also ensured that each activity was directly proportional to time and amount of extract.

Changes in level of enzymes involved in UDP-sugar interconversion

UDP-glucose dehydrogenase, UDP-glucuronate decarboxylase, UDP-glucose epimerase and UDP-xylose epimerase enzyme activities were measured in cells grown in maintenance media and cells subcultured into cytokinin induction media (Fig. 3). UDP-glucose dehydrogenase, showed a two-fold increase and subsequent decrease in activity initiated between three and six days after subculturing, almost paralleling the period where the logarithmic growth phase enters the stationary phase. Compared to cells subcultured into cytokinin induction media, UDP-glucose dehydrogenase activity also increased to the same extent as the control cells. However, in the induction media the maximal increase in activity appeared at least three days sooner than found in the control cultures (Fig. 3a). The other enzyme activities measured in the induced cells showed no significant variation from the relevant control cells, perhaps with the exception of UDP-glucuronate decarboxylase. This enzyme showed slightly higher rates of activity than those in the control cells at two and three days post subculture (Fig. 3b). In cells grown in both the induction and maintenance media the activities of UDP-glucuronate decarboxylase (Fig. 3b) and UDP-xylose epimerase (Fig. 3d) showed peaks of activity coinciding at three days after subculture. The activities were approximately double the initial basal level and they had returned to normal by about the sixth day after subculture.

UDP-glucose dehydrogenase, UDP-glucuronate decarboxylase, UDP-glucose epimerase and UDP-xylose epimerase enzyme activities were also measured in hypocotyls undergoing elongation and differentiation (Fig. 4). UDP-glucose dehydrogenase showed a transient increase in activity of about three-fold coinciding with the stage where elongation growth was terminating and vascularization and secondary cell wall thickening was underway (Fig. 4a). UDP-glucuronate decarboxylase gradually increased in activity from the early stages of elongation growth having almost doubled in activity by the time the hypocotyls were differentiating (Fig. 4b). In contrast UDP-glucose epimerase (Fig. 4c) and UDP-xylose (Fig. 4d) activities appeared to remain unchanged throughout the development of the hypocotyls.

Changes in enzymes involved in sucrose and starch degradation

Acid invertase activity (pH optimum 4.5) was measured both in the soluble extracts and in further sequential extracts of the particulate material using 1 M salt. Typically, the salt extract contained 80% of the total acid invertase and this fraction is represented in Figs 5c and

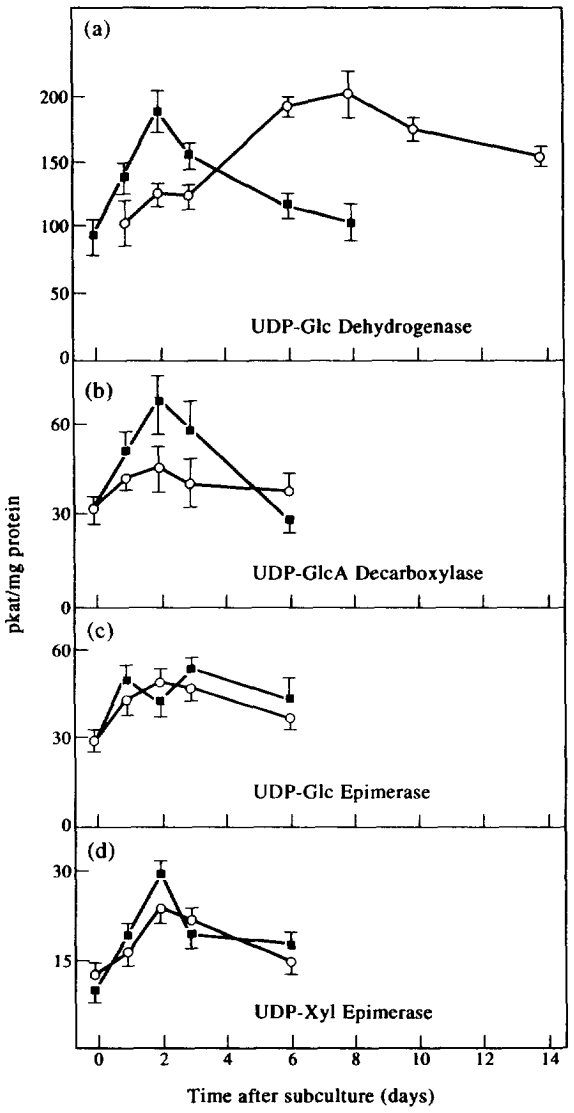

Fig. 3. Enzyme activities of UDP-sugar metabolism in suspension cultured French bean cells. Activities were determined from cells grown in maintenance media (○) and cytokinin induction media (■). Each value is the mean of 3 ± s.e.

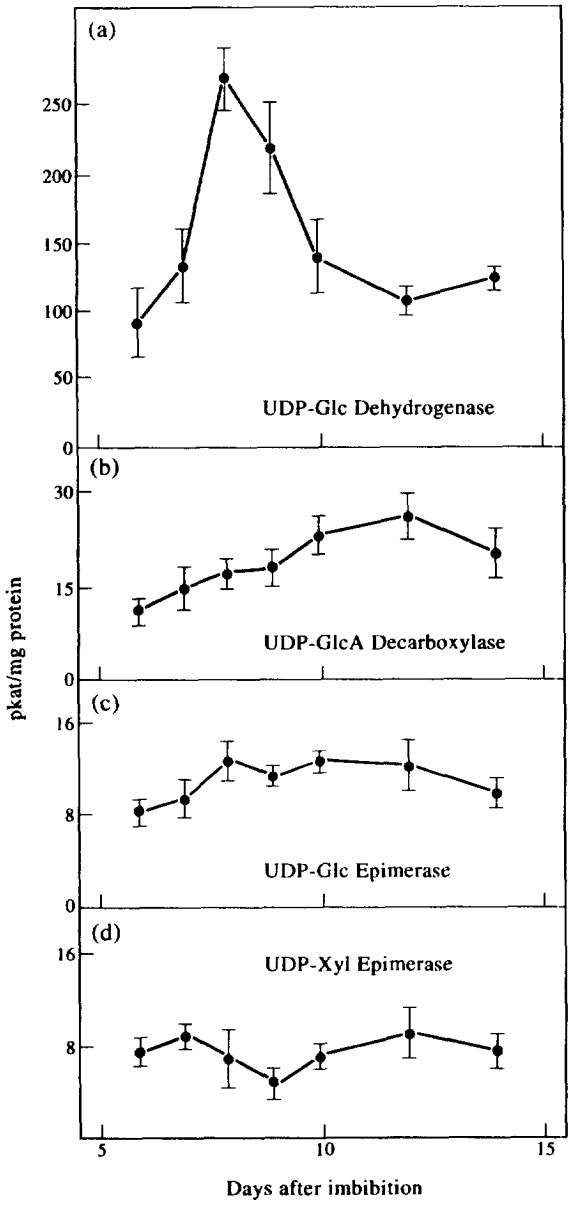

Fig. 4. Enzyme activities of UDP-sugar metabolism in hypocotyls of French bean. Each value is the mean of 3 ± s.e.

6c. The acid invertase from the soluble extract (pH optimum 4.5) when measured in either cell cultures or hypocotyl tissues showed identical patterns to the salt extractable invertase over the time courses measured (data not shown).

Starch phosphorylase, sucrose synthase and amylase enzyme activities were also measured in cells grown in maintenance media and cells subcultured into cytokinin induction media (Fig. 5). For either cells grown in maintenance media or induction media no changes in specific activities or the levels of activity were detected for invertase (Fig. 5a) or starch phosphorylase (Fig. 5c). However, in the maintenance media, sucrose synthase activity increased dramatically from about three days after subculturing (Fig. 5b). The increase in activity more than doubled by day 6 from whence the activity started to decline. The period from three to six days post subculture coincided with the logarithmic phase in growth. In sharp contrast, the cell subcultured into induction media showed sucrose synthase activity to gradually decline up until four days after subculture where the activity remained at basal levels (Fig. 5b). Amylase activity measured in cells grown in maintenance media was fairly constant compared to the activity found in cells grown in induction media over the respective time period. In the cells grown in the induction media amylase activity showed a three-fold transient rise in activity starting from the time of subculturing and returning to the initial levels

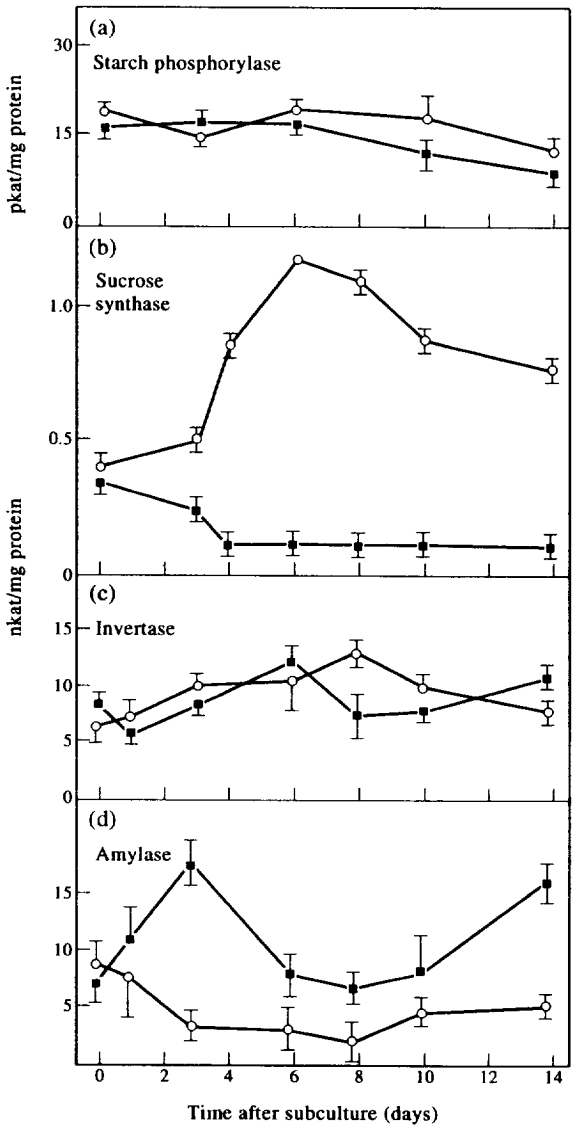

Fig. 5. Enzyme activities associated with starch and sucrose degradation in suspension cultured French bean cells. Activities were determined from cells grown in maintenance media (○) and cytokinin induction media (■). Each value is the mean of 3 ± s.e.

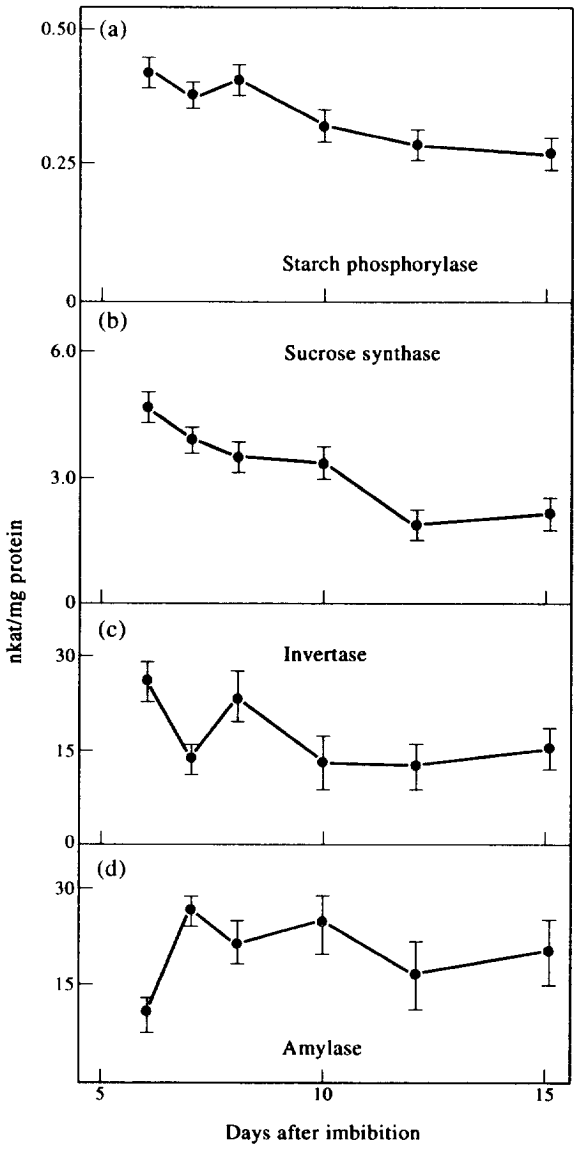

Fig. 6. Enzyme activities associated with starch and sucrose degradation in hypocotyls of French bean. Each value is the mean of 3 ± s.e.

by day 6. By day 10 and towards the end of the experiment at day 14 the levels of amylase activity had risen again (Fig. 5d).

Starch phosphorylase, sucrose synthase, invertase and amylase activities were also measured in hypocotyls undergoing rapid extension growth and differentiation (Fig. 6). Starch phosphorylase (Fig. 6a) and sucrose synthase (Fig. 6b) activities showed a gradual and relatively small decline in activity from when the hypocotyls had emerged at about day 6, to the end of the growth period at day 15. Invertase (Fig. 6c) and amylase (Fig. 6d) activities remained relatively constant throughout the growth and development phase of the hypocotyls, although amylase activity showed a modest increase at the onset of extension growth. However, all these enzyme activities were found to be higher than when measured in suspension cultured cells. This was especially so of starch phosphorylase where the activity was about 20 times greater in the green hypocotyl tissue compared to the non-photosynthetic suspension cultured cells. With sucrose synthase, invertase and amylase hypocotyl tissue generally showed a two to three-fold increase in activity over suspension cultured cells.

DISCUSSION

Present evidence suggests that the major controlling factor in the qualitative production of plant cell wall polysaccharide synthesis resides in the complement of

synthases present at any one time in the various membrane systems [2]. However, the availability of nucleotide diphosphate sugars appears to be under some fine control. For example, the biosynthesis of UDP-glucose *in vivo* can be dependent on several factors. The presence of UDP-glucose pyrophosphorylase throughout the growth cycle in French bean suspension cultures, at levels which are much higher than the estimates of maximum sucrose breakdown, has the potential to supply all the cellular UDP-glucose requirement [14]. Yet sucrose synthase may also be involved in the supply of UDP-glucose, since the sucrose synthase reaction when considered in conjunction with glycolysis, requires less ATP relative to the catabolism of sucrose than by invertase [15]. Furthermore, in suspension cultures the increase in activity of sucrose synthase, which coincided with the logarithmic growth phase, has been shown not to be dependent on the presence of sucrose [14]. It may be that sucrose synthase has a key role in supplying the glycolytic pathways but an alternative, or additional role may be in supplying the glucan synthases with UDP-glucose at the period of maximum growth [7]. It is worth noting that in the cells subcultured into induction media, where growth was slowed, there was a severe decline in sucrose synthase activity compared to the control cultures. This could be interpreted as a sign of differential losses of the enzyme activity during extraction, especially when considering that the induced cells have an increased phenolic content. However, other enzymes measured between the control and induced cells appeared not to be subjected to such inactivation. Significantly, amylase activity in the cytokinin treated cells increased relative to the control cells, whereas starch phosphorylase and invertase activities remained at similar levels between the two cell lines. Similarly, the amylase activity level increased in the hypocotyls at the onset of extension growth and was sustained at this level throughout development while invertase activity remained fairly constant and sucrose synthase and phosphorylase activities only declined slightly over the same period. In these cases the role of amylase in the supply of sugars appeared to increase in importance. Of course without carrying out the necessary recovery experiments using purified enzymes as internal standards, if they were available for each enzyme measured, differential losses during extraction cannot totally be ruled out. In view of other studies on soluble enzymes involved with carbohydrate metabolism in stressed suspension cultured cells [16], where recoveries were greater than 88%, it would seem likely that our determinations were made under near optimal conditions.

The oxidation of UDP-glucose by UDP-glucose dehydrogenase, a reaction potentially subject to feedback inhibition by the level of UDP-xylose [6], may act as a key step for the provision of UDP-sugars from membrane-bound glycosylations [2, 6, 7]. This enzyme activity has been seen to be induced during differentiation of cambium to xylem in sycamore and popular [6] and in synchronized cell suspension cultures of *Catharanthus roseus* [7]. An extensive mapping of the changes in enzyme activities in relation to the cell cycle in *C. roseus* has revealed that accumulation of glycan polymers occurs during G1, preceded by the necessary metabolic activity involving UDP-sugar levels [7, 17–19].

In suspension cultured French bean cells [12] and in developing French bean hypocotyls [11] changes in the levels of arabinosyl and xylosyl transferase activities have been observed to vary during the normal growth cycle. In the suspension cultures arabinosyl transferase activity rapidly increased several fold by the second day after subculture and had returned to the initial rate by the fourth day. Compared to cells grown in induction media, the arabinosyl transferase activity was only marginally induced over the same time period. On the other hand, the xylosyl transferase activity rapidly increased several-fold in cells grown in the induction media reaching a maximum about three days after subculturing. The xylosyl transferase activity was also much higher than in comparative cells grown in maintenance media and was also present at higher rates for a much longer period after subculture. These differential changes in the expression of two key transferases have been linked to cell wall biosynthesis and consequently, their action was observed here by the distinct changes in cell wall composition found between the normal and induced suspension cultures (Table 1).

In this present study we have measured the enzymes associated with the production of the relevant UDP-sugars in the same type of French bean suspension cultured cells (Fig. 3). The most striking observation was found to be induction of UDP-glucose dehydrogenase activity in cells grown in the cytokinin enriched media. This may also be relevant to the supply of UDP-xylose since there was also a small increase in UDP-glucuronate decarboxylase activity. Similarly, the increases in UDP-glucose dehydrogenase activity were also observed in developing hypocotyls at about eight days after imbibition (Fig. 4). It is at this stage of development when there is increased activity associated with arabinosyl and xylosyl transferases involved with xylogenesis [11]. In conclusion, UDP-glucose dehydrogenase may occupy a key control point in regulating the flux of UDP-sugars destined for use by the relevant transferases. Since the reaction it catalyses is irreversible and therefore rate limiting, it may be strategic in exerting coarse control over polymer synthesis.

EXPERIMENTAL

Materials. The substrates, co-factors and enzymes were obtained from Sigma. Uniformly labelled UDP-[^{14}C]Glc (295 mCi mmol^{-1}), UDP-[^{14}C]GlcA (285 mCi mmol^{-1}) and UDP-[^{14}C]Xyl (250 mCi mmol^{-1}) were supplied by NEN Stevenage, U.K.

Cell cultures and plant material. Suspension cultures were derived and maintained as previously described [12]. Biochemical changes associated with secondary wall formation were induced by transfer to a xylogenesis induction media which was B5 [20] supplemented with

3% sucrose, 1 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin. Seedlings of French bean (*Phaseolus vulgaris*. L.) var. The Prince were grown in vermiculite under a 16 hr light, 8 hr dark regime at 15°. Seeds were not presoaked but planted directly into wet vermiculite in standard seed trays and watered every 3 days.

Analysis of cell walls. The cell wall analyses were carried out as described previously [13]. Crude cell wall pellets of cells were washed ×3 in 85% EtOH, sequentially extracted with cold H_2O followed by warm (60°) 0.5% ammonium oxalate and then with 0.75 M NaOH for 16 hr at 4° followed by 4.25 M NaOH for 16 hr at 4°. Neutral pectin was recovered from the cold H_2O extract by adding EtOH to a final concn of 85%. Acidic pectin was recovered from the ammonium oxalate extract by addition of EtOH to 50% and the hemicellulose from the pooled NaOH extracts by addition of EtOH to 70% followed by washing in ice-cold H_2O to remove any pptd alkali. Polysaccharide fractions were lyophilized and weighed. The residual cellulose fraction, which also contains the fraction of the hydroxyproline rich material which is not extracted in the NaOH extracts, was washed with 50% EtOH before lyophilization and weighing.

The hemicellulose fractions were separated into xylan and xyloglucan fractions by chromatography on powdered cellulose [21]. The xylan did not bind and the xyloglucan was released with urea–NaOH elution. The Xyl and Glc content of the hemicellulose fractions was determined by TLC [13] after partial hydrolysis in 0.1 M HCl at 100° for 30 min (xylan), as described previously [11]. Hydroxyproline was estimated colorimetrically by the method of ref. [22] following acid hydrolysis of whole cell wall material in 6 M HCl at 105° for 24 hr.

Phenolic material bound to the hemicellulosic and cellulosic fractions was extracted by saponification in 4.25 M NaOH at 4° for 7 days and at 37° for 16 hr. This procedure extracted all the brown material from the polysaccharide fraction. Contaminants were precipitated in 70% EtOH and absorption spectra of the coloured supernatants determined over the range 240–340 nm. The level of phenolic material was estimated from the absorbance value at 310 nm.

Measurement of growth. Samples (20 ml) of suspension cultures were vacuum filtered on mira-cloth, the fr. wt recorded and the cells immediately frozen in liquid N_2. Hypocotyls from 15 seedlings per determination were excised, measured with a ruler and immediately frozen in liquid N_2. On harvest all the hypocotyls for each time point were ground to a powder in a mortar and pestle under liquid N_2. All tissue samples were stored at −70° until required.

Preparation of extracts. Portions of the hypocotyl powders or cells from the suspension cultures were taken and in the case of invertase or amylase homogenized in 2 vols 50 mM NaOAc (pH 6.0). After centrifugation, 10000 g for 10 min the pellet was resuspended in 2 volumes of the same buffer containing 1 M NaCl, stirred for 1 hr and centrifuged as before. The supernatants were dialysed against 3 changes of NaOAc (pH 6.0) overnight before assay.

Alternatively the tissue was homogenized in 2 vols 0.1 M Tris–HCl, 5 mM 2-mercaptoethanol, 2 mM EDTA (pH 7.5) and centrifuged for 10 min at 10000 g. The supernatant was immediately assayed in the case of starch phosphorylase, sucrose synthase and UDP-Glc dehydrogenase. A portion of the same supernatant was 30–70% $(NH_4)_2SO_4$ fractionated, dialysed overnight against 3 changes of Tris–HCl homogenization buffer and assayed for UDP-Glc, UDP-Xyl 4-epimerases and UDP-GlcA decarboxylase. Protein concentrations in supernatants were monitored by the Bio-Rad assay using BSA as the standard [23].

Enzyme assays. Except those with radiolabelled substrates all reaction mixtures were of 1 ml volume. Invertase [24], 50 mM NaOAc, 3% sucrose (pH 4.5) with extract and amylase [24], 50 mM NaOAc, 25 mM KCl, 0.5% amylopectin (pH 6.0) with extract were incubated at 37° and the amount of reducing groups quantified as Glc equivalents [25]. The assays dependent on measuring the conversion of NAD to NADH were monitored at 340 nm in a double beam spectrophotometer with the appropriate blank and the reaction mixtures were: starch phosphorylase [26], 50 mM HEPES (pH 6.8), 20 mM Na-PO_4 (pH 7.4), 0.1% amylopectin, 55 μg P-glucomutase, 0.46 units Glc-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), 1 mM NAD and extract: sucrose synthase [16], 0.1 M Tris–HCl (pH 7.5), 0.4 M sucrose, 1 mM NAD, 0.5 mM UDP, 0.03 units UDP-Glc dehydrogenase and extract: UDP-Glc dehydrogenase [17], 0.1 M glycine–NaOH (pH 8.5), 5 mM UDP-Glc, 2 mM NAD and extract.

The assays dependent upon radiolabelled substrates were carried out for 5 min at 37° in reaction vols of 20 μl. The reaction mixtures were: UDP-GlcA decarboxylase [6], 0.2 M Na–Pi (pH 7.0), 0.4 nmol UDP-[^{14}C]GlcA, 50 nmol UDP-GlcA and extract: UDP-Glc 4-epimerase [6], 0.1 M glycine–NaOH (pH 9.0), 0.4 nmol UDP-[^{14}C]Glc, 50 nmol UDP-Glc and extract: UDP-Xyl 4-epimerase [6], 0.1 M Tris–HCl (pH 8.0), 0.31 nmol UDP-[^{14}C]Xyl, 10 nmol UDP-Xyl and extract. The reactions were stopped by immersion in a boiling water bath for 3 min. After centrifuging for 10 min at 20000 g the supernatants were hydrolysed with 0.1 M HCl at 100° for 15 min. The solution was then dried under vacuum, extracted with 20 μl pyridine and subjected to paper chromatography. The radioactivity which co-chromatographed with authentic standards was determined by liquid scintillation counting.

REFERENCES

1. Carpita, N. and Gibeaut, D. M. (1993) *Plant J.* **3**, 1.
2. Bolwell, G. P. (1993) *Int. Rev. Cyt.* **146**, 261.
3. Iiyama, K., Lam, T. B. and Stone, B. A. (1994) *Plant Physiol.* **104**, 315.
4. Woodrow, E and Berry, J. A. (1988) *Annu. Rev. Plant Physiol. Mol. Biol.* **39**, 533.
5. Stitt, M. and Quick, W. P. (1989) *Physiol. Plant.* **77**, 633.

6. Dalessandro, G. and Northcote, D. H. (1977) *Biochem. J.* **162**, 267.
7. Amino, S., Takeuchi, Y. and Komamine, A. (1985) *Physiol. Plant.* **64**, 111.
8. Feingold, D. S. and Avigad, G. (1980) in *The Biochemistry of Plants*, Vol. 3 (Preiss, J., ed.), pp. 101–170. Academic Press, New York.
9. Biffen, M. and Hanke, D. E. (1991) *Plant Sci.* **75**, 203.
10. Wakabayashi, K., Sakurai, N. and Kuraishi, S. (1989) *Plant Cell Physiol.* **30**, 99.
11. Bolwell, G. P. and Northcote, D. H. (1981) *Planta* **152**, 225.
12. Bolwell, G. P. and Northcote, D. H. (1983) *Biochem. J.* **210**, 509.
13. Bolwell, G. P., Robbins, M. P. and Dixon, R. A. (1985) *Eur. J. Biochem.* **148**, 571.
14. Botha, F. C., O'Kennedy, M. M. and du Plessis, S. (1992) *Plant Cell Physiol.* **33**, 477.
15. Huber, S.C. and Akazawa, T. (1986) *Plant Physiol.* **81**, 1008.
16. Mohanty, B., Wilson, P. M. and ap Rees, T. (1993) *Phytochemistry* **34**, 75.
17. Amino, S., Fujimara, T. and Komamine, A. (1984) *Physiol Plant.* **60**, 326.
18. Amino, S., Takeuchi, Y. and Komamine, A. (1985) *Physiol. Plant.* **64**, 197.
19. Amino, S., Takeuchi, Y. and Komamine, A. (1985) *Physiol. Plant.* **64**, 202.
20. Gambourg, O. L., Miller, R. A. and Ojima, K. (1968) *Exp. Cell. Res.* **50**, 151.
21. Aspinall, G. O., Molloy, J. A. and Craig, J. W. T. (1969) *Can. J. Biochem.* **47**, 1063.
22. Kivirikko, K. I. (1963) *Acta Physiol. Scand. Suppl.* **219**, 1.
23. Bradford, M. (1976) *Anal. Biochem.* **72**, 248.
24. Robinson, N. L., Hewitt, H. J. D. and Bennet, A. B. (1988) *Plant Physiol.* **87**, 727.
25. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
26. Levi, C. and Preiss, J. (1978) *Plant Physiol.* **61**, 218.