Cell wall polysaccharide biosynthesis and related metabolism in elicitor-stressed cells of French bean (*Phaseolus vulgaris* L.)

Duncan ROBERTSON, Barbara A. McCORMACK and G. Paul BOLWELL*
Department of Biochemistry, Royal Holloway and Bedford New College, University of London, Egham, Surrey TW20 0EX, U.K.

Enzyme activities involved in quantitative and qualitative flux of sugars into cell wall polysaccharides were determined following elicitor treatment of suspension cultured cells of French bean (*Phaseolus vulgaris* L.). Two subsets of activities were examined: the first were involved in synthesis and metabolism of UDP-glucose and the provision of the pool of UDP-sugars, and the second a selection of membrane-bound glycosyltransferases involved in the synthesis of pectins, hemicelluloses and glucans of the primary cell wall. Of the first group, only UDP-glucose dehydrogenase (EC 1.1.1.22) showed any significant induction in response to elicitor treatment, sucrose synthase (EC 2.4.1.13), UDP-glucuronate decarboxylase (EC 4.1.1.35), UDP-glucose and UDP-xylose 4-epimerases (EC 5.1.3.2 and EC 5.1.3.5 respectively) did not change in activity significantly over the time course. In contrast, enzymes of the second group showed a more complex response. Callose synthase (glucan synthase II, EC 2.4.1.12) increased in activity, as has been shown in other systems, while arabinan synthase (EC 2.4.1.-), xylan synthase (EC 2.4.1.72), xyloglucan synthase (EC 2.4.1.72) and glucan synthase I (EC 2.4.1.12) activities were rapidly depleted from membranes within 3 h following elicitor action. This rapid turnover of activity was striking, indicating that the half-life of such enzymes can be short and that elicitor action causes substantial perturbation of some membrane activities. Glucan synthase I activity appears to increase in the later stages over the time period measured, indicating some recovery of this metabolism.

INTRODUCTION

Regulation of cell wall polysaccharide biosynthesis appears to be subject to both quantitative and qualitative modulation of flux. During the cell cycle, increases in pectin synthesis are observed during cell division, while glucan polymer accumulation occurs during G_1 phase. These events are preceded by the necessary metabolic activity determining UDP-sugar levels (Amino et al., 1984, 1985a,b,c). Some of the events have been similarly mapped during xylogenesis. In a number of systems, cessation of pectin synthesis and increased xylan synthase activity have been observed during secondary thickening (Bolwell, 1993). In such stages of development, an increase in UDP-glucose dehydrogenase was observed, but not in UDP-glucuronate decarboxylase and UDP-sugar epimerases, indicating that flux may increase and the pool of UDP-sugars is maintained to supply the demand for increased matrix polysaccharide and cellulose synthesis.

Metabolic activity in relation to the cell wall is known to be modulated in response to stress, and changes have been documented in composition where this has been due to salt or other osmotic factors. Another source of stress is that in response to pathogens, where new material deposited on to the cell wall may form barriers and are an important component of the resistance response. The effect of elicitor on suspension-cultured French bean (*Phaseolus vulgaris* L.) cells gives rise to a number of striking changes in cell wall composition (Bolwell et al., 1985). Increased phenolic deposition has been particularly well studied in such systems, but other than the accumulation and deposition of callose, the fate of biosynthesis and deposition of matrix polysaccharides is relatively unknown. The present work further examines the changes in enzyme activities associated with quantitative and qualitative flux into glycosylated products following elicitor action on suspension-cultured French bean cells.

MATERIALS AND METHODS

Materials

Cell suspension cultures of French bean were derived as described previously (Dixon and Lamb, 1979). Elicitor preparations from the fungus *Colletotrichum lindemuthianum* were prepared as described previously (Dixon and Lamb, 1979). Uniformly labelled UDP-[^{14}C]glucose (295 mCi/mmol), UDP-[^{14}C]glucuronate (285 mCi/mmol) and UDP-[^{14}C]xylose (250 mCi/mmol) were obtained from NEN, Stevenage, Herts., U.K. UDP-[^{3}H]-arabinose (8 Ci/mmol) was made as described by Bolwell and Northcote (1981). UDP-glucose dehydrogenase, 6-phosphoglucomutase, glucose-6-phosphate dehydrogenase (from the bacterium *Leuconostoc mesenteroides*) were obtained from Sigma.

Elicitation of cultures

Cell suspension cultures were subjected to biotic stress between 4 and 6 days after subculture. Elicitor was added to a concentration of 30 μg/ml glucose equivalents, and cell samples were taken at intervals. Over the period of treatment, no gross effects on cell growth were observed (Bolwell et al., 1985).

Preparation of extracts

Cells were harvested by vacuum filtration, frozen in liquid nitrogen and stored below −70 °C. All subsequent procedures were carried out at 4 °C. Cells (5 g fresh weight) were homo-

Abbreviation used: GSI and GSII, glucan synthases I and II.
* To whom correspondence should be addressed.

genized in 10 ml of 0.1 M Tris/HCl (pH 7.5) containing 250 mM sucrose, 5 mM 2-mercaptoethanol and 2 mM EDTA. The brei was centrifuged for 20 min at 20000 *g* and the supernatant re-centrifuged for 1 h at 100000 *g*. The supernatant was used to determine the activities of the soluble enzymes, and the membrane pellet was resuspended in the requisite buffer and used to measure those enzymes associated with the microsomes.

Enzyme assays

Invertase

Invertase was assayed as described by Robinson et al. (1988). The assay mixture contained extract and 3% sucrose in 50 mM sodium acetate, pH 4.5, in a final volume of 0.2 ml. Before use, the extract was dialysed overnight against two changes of 50 mM sodium acetate, pH 6.0. The reaction was carried out at 37 °C for 30 min, and the amount of products determined using the reducing-sugar assay described by Nelson (1944).

Amylase

Amylase was assayed by the method of Robinson et al. (1988). The reaction mixture contained 50 mM sodium acetate, pH 6.0, 25 mM KCl, 0.5% amylopectin and extract in a final volume of 0.2 ml. Before use the extract was dialysed overnight against two changes of 50 mM sodium acetate, pH 6.0, and the procedure was carried out as for invertase.

Starch phosphorylase

Starch phosphorylase was assayed as described by Levi and Preiss (1978) with slight modification. The reaction mixture, total volume 1 ml, contained 50 mM Hepes, pH 6.8, 20 mM sodium phosphate, pH 7.4, 0.1% debranched amylopectin, 55 μg of phosphoglucomutase, 0.46 unit of glucose-6-phosphate dehydrogenase and 1 mM NAD^+. The reaction was monitored at 340 nm.

Sucrose synthase

Sucrose synthase was determined by a procedure modified from that described by Mohanty et al. (1993) in a final volume of 1 ml containing extract, 0.1 M Tris/HCl, pH 7.5, 0.4 M sucrose, 2 mM NAD^+, 0.5 mM UDP, 0.03 unit UDP-glucose dehydrogenase and the assay was continuously monitored at 340 nm over a 5 min period.

UDP-glucose dehydrogenase

UDP-glucose dehydrogenase was determined as described by Amino et al. (1985a). The reaction mixture contained extract, 0.1 M glycine/NaOH, pH 8.5, 5 mM UDP-glucose and 2 mM NAD^+ in a volume of 1 ml. The assay was monitored continuously at 340 nm over a 5 min period. A control cell containing everything except UDP-glucose was simultaneously assayed.

UDP-glucuronate decarboxylase, UDP-glucose 4-epimerase and UDP-xylose 4-epimerase

Assays for the above three enzymes were modified from those described by Dalessandro and Northcote (1977). Soluble extract was fractionated and concentrated by $(NH_4)_2SO_4$ precipitation; the 30–70%-satd. fraction was dialysed overnight and used to determine the relevant enzyme activity. In the case of UDP-glucuronate decarboxylase the reaction mixture contained extract, 0.2 M sodium phosphate, pH 7.0, 0.4 nmol of UDP-[^{14}C]glucuronic acid and 50 nmol of UDP-glucuronic acid in a final volume of 20 μl. UDP-glucose 4-epimerase was assayed in a final volume of 20 μl containing extract, 0.1 M glycine/NaOH, pH 9.0, 0.4 nmol of UDP-[^{14}C]glucose and 50 nmol of UDP-glucose. UDP-xylose 4-epimerase was assayed in a final volume of 20 μl containing extract, 0.1 M Tris/HCl, pH 8.0, 0.31 nmol of UDP-[^{14}C]xylose and 10 nmol of UDP-xylose. The reactions were carried out for 5 min at 37 °C and stopped by immersion in a boiling-water bath for 3 min. After centrifuging for 10 min at 10000 *g*, the supernatants were hydrolysed with 0.1 M HCl at 100 °C for 15 min. The solution was then dried under vacuum, extracted with 20 μl of pyridine and subjected to paper chromatography (Dalessandro and Northcote, 1977). The radioactivity which co-chromatographed with authentic standards was determined by liquid-scintillation counting.

Glucan synthase I (GSI)

GSI was determined by a modification of the method of Ray (1980). Reaction mixtures contained membrane fractions in 40 mM Tris/HCl, pH 8.0, 3 mM $MgCl_2$ and 0.7 μM UDP-[^{14}C]glucose (final concn.) in a total volume of 50 μl. Reactions were terminated after 10 min by the addition of 0.5 M EDTA to 10 mM, and incorporation into polymeric products was determined by high-voltage paper electrophoresis (Bolwell and Northcote, 1981). Incorporation into lipid, glycoprotein and polysaccharide was determined after fractionation of the products as described by Bolwell and Northcote (1983a).

Glucan synthase II (GSII)

GSII was determined by a modification of the method of Delmer (1987). Membranes were incubated in 100 mM Hepes buffer, pH 6.8, containing 3 mM $CaCl_2$, 1 mM UDP-glucose and 0.7 μM UDP-[^{14}C]glucose (final concn.) in a total volume of 50 μl. Reactions were terminated after 10 min with the addition of 0.5 M EDTA to 10 mM. Total incorporations were determined by high-voltage paper electrophoresis. The products were also fractionated as described for GSI.

Arabinan synthase

Arabinan synthase was assayed by the method described by Bolwell (1986). Membranes were incubated in 50 mM phosphate, pH 7.2, 1 mM dithiothreitol, 10 mM $MgCl_2$, 4 μM UDP-[^{3}H]arabinose and 0.1 mM UDP-arabinose in a total volume of 50 μl. Reactions were terminated after 10 min, and the products were fractionated into lipid, glycoprotein and polysaccharide, and incorporation was determined as described by Bolwell and Northcote (1983a).

Xylan synthase

Xylan synthase was assayed under the conditions described by Bolwell and Northcote (1983a). Membranes were incubated in 50 mM sodium phosphate buffer, pH 7.2, 1 mM dithiothreitol, 10 mM $MgCl_2$, 4 μM UDP-[^{14}C]xylose and 0.5 mM UDP-xylose (final concn.) in a total volume of 50 μl. Reactions were terminated after 10 min, and the products were fractionated into lipid, glycoprotein and polysaccharide as described by Bolwell and Northcote (1983a) for determination of incorporations.

Xyloglucan synthase

Xyloglucan synthase the xylosyltransferase involved in xyloglucan synthesis was determined by a modification of the method

of Ray (1980). Membranes were incubated as for xylan synthase with the addition of 1 mM UDP-glucose. Reactions were terminated after 10 min by the addition of 0.5 M EDTA to a final concentration of 10 mM. Incorporation was determined by high-voltage paper electrophoresis. Enzyme activity was taken to be the amount of incorporation stimulated by the presence of UDP-glucose.

UDP-sugar breakdown

UDP-sugar breakdown was determined by the method of Rodgers and Bolwell (1992). Remaining UDP-sugar, monosaccharide 1-phosphate and monosaccharide were determined following incubations as described for xylan synthase and GSII. This was performed as a check on substrate losses and to give an indication of changes in the level of particulate hydrolytic enzymes.

RESULTS

Experimental design and optimization of enzyme determinations

Elicitor treatment leads to considerable changes in the composition of the cell walls of suspension-cultured cells of French bean (Bolwell et al., 1985). The present investigation studied the changes in enzyme levels involved in glycoconjugate biosynthesis in such cells. Throughout the present study, activities of the enzymes measured were optimized with respect to pH and buffer components in batches of untreated cells harvested 4 days after subculture. Where crude extracts were subject to $(NH_4)_2SO_4$ 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%. Similarly, it was established that the microsomal enzymes were exclusively membrane-associated and that no losses could be attributed to changes in distribution into other particulate or soluble cell fractions. In all cases it was also ensured that each activity was directly proportional to time and amount of extract.

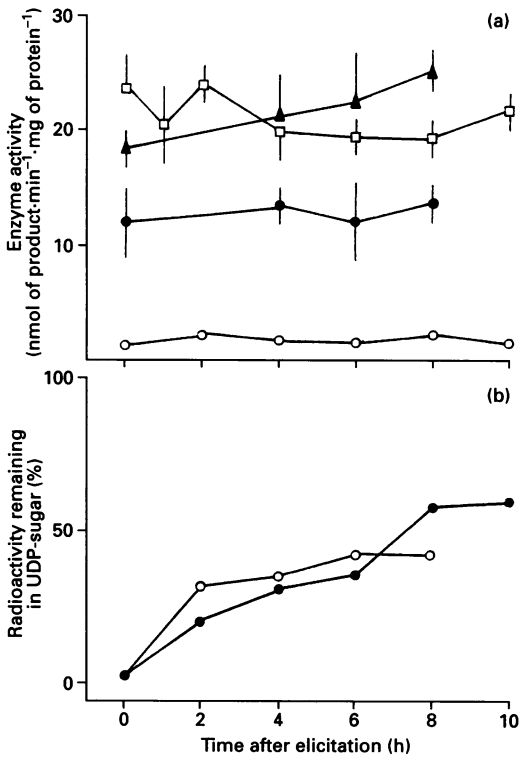

Figure 1 Changes in the enzyme activities of (a) sucrose and starch degradation and (b) the relative rates of UDP-xylose and UDP-glucose degradation following treatment with elicitor

Soluble extract preparations (**a**) were used to determine the activities of amylase (▲), starch phosphorylase (○), sucrose synthase (□) and invertase (●). Means and S.E.M. for five determinations are shown. (**b**) Typical rates of UDP-sugar hydrolysis determined under the assay conditions of the glycosyltransferases. Levels of UDP-glucose (○) and UDP-xylose (●) remaining after 10 min incubation were estimated by high-voltage paper electrophoresis.

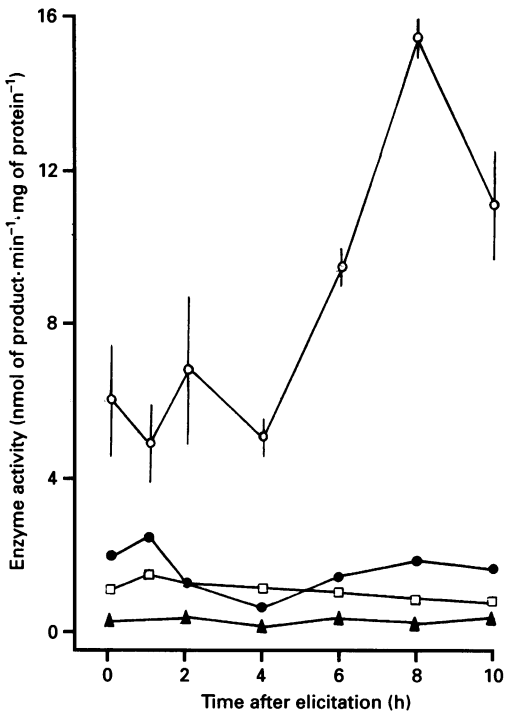

Figure 2 Changes in the activities of UDP-sugar interconversion and formation following treatment with elicitor

Soluble extract preparations, precipitated between 30 and 70%-satn. of $(NH_4)_2SO_4$ and dialysed overnight, were used to determine the activities of UDP-glucose dehydrogenase (○), UDP-glucuronate decarboxylase (●), UDP-glucose epimerase (□) and UDP-xylose epimerase (▲). Means and S.E.M. for five determinations are shown.

Changes in levels of enzymes involved in UDP-sugar provision

Activities following elicitation were monitored for enzymes involved in UDP-glucose production, UDP-sugar interconversion and UDP-sugar breakdown (Figures 1 and 2). No significant changes in level of invertase or sucrose synthase were observed over the examined time course. However, a slight increase in the levels of amylase (but not starch phosphorylase) occurred, indicating possible involvement in mobilization of reserves (Figure 1a). Similarly, UDP-glucuronate decarboxylase, UDP-glucose epimerase and UDP-xylose epimerase did not change to any great extent over the same time period (Figure 2). However, one enzyme, namely UDP-glucose dehydrogenase, showed significant induction, an approx. 2.5-fold increase over the early stages of elicitation, reaching its maximum rate 8 h after addition of the elicitor (Figure 2).

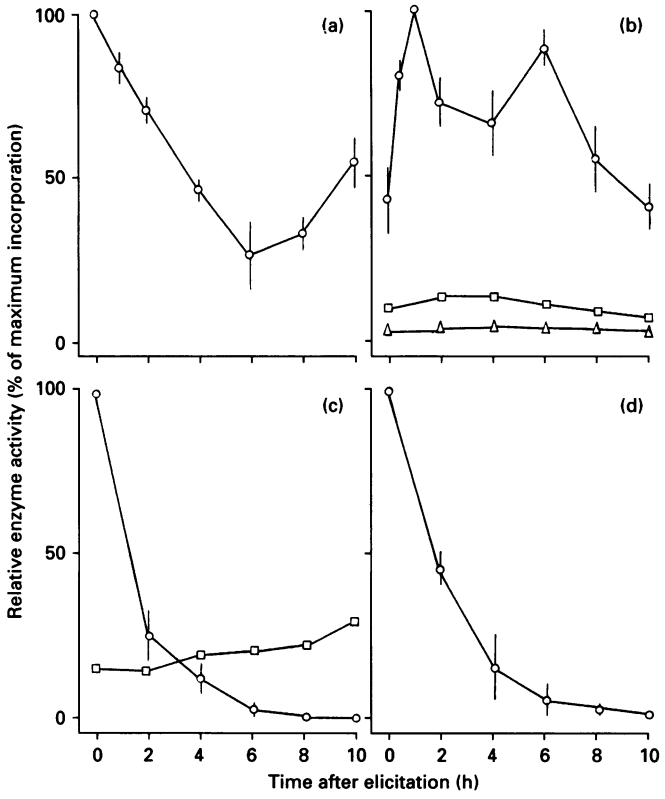

Figure 3 Changes in the activities of glycosyltransferases following treatment with elicitor

Resuspended membrane preparations were assayed for glycosyltransferases in membranes isolated from cells harvested at different times following addition of elicitor. Products of incubations were fractionated into polysaccharide (○), glycoprotein (□) and lipid (△). Where data is absent for incorporation into any these fractions, this signifies that the amount of label incorporated was negligible. (**a**) GSI (initial activity for incorporation into polysaccharide at zero time was 850 ± 51 d.p.m. $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}). Means and S.E.M. are shown for three determinations. (**b**) GSII (initial activity for incorporation into polysaccharide: 1.3 ± 0.5 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}). Means and S.E.M. for five determinations are shown. Also, under the assay conditions used to measure glucan synthase II activity, the amount of substrate incorporated into lipid and protein was determined and is shown. (**c**) Xylosyltransferase under conditions for xylan synthase (intial incorporation into polysaccharide: 0.11 ± 0.02 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}). The incorporations (means and S.E.M. for three determinations) are shown for either polysaccharide (○) or protein (□). (**d**) Xyloglucan xylosyltransferase was also measured over the same period after elicitation (initial activity 0.14 ± 0.03 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}). Means and S.E.M. for three determinations are shown.

Changes in levels of enzymes involved in cell wall polysaccharide synthesis

Unlike the enzymes of sugar-nucleotide metabolism and because of the nature of the radiometric assay used to assay them, it is not always possible to measure activities of glycosyltransferases at substrate saturation, owing to loss of sensitivity at high dilution with non-radioactive substrate. Consequently, in studies of this type, reactions are carried out at about double the K_m. The K_m for these enzyme systems have been previously determined and, with the exception of GSI, are all in the range 0.2–0.6 mM (Ray, 1980; Bolwell and Northcote, 1981; Kamat et al., 1992). The data described in Figures 3 and 4 present relative incorporations into polymeric material under the same incubation conditions for membranes isolated from cells under differing physiological conditions.

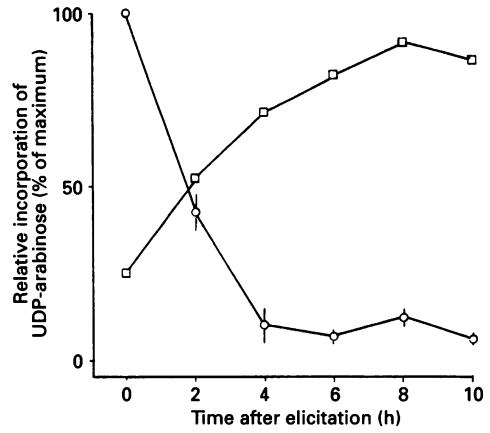

Figure 4 Changes in the activities of arabinosyltransferases following treatment with elicitor

Resuspended membrane preparations were used to determine arabinosyltransferase activities where incorporation was either into polysaccharide (○) or protein (□). The initial incorporation into polysaccharide was 0.3 ± 0.01 nmol $\cdot$ min^{-1} $\cdot$ mg of protein^{-1}. The levels show means and S.E.M. for five determinations.

Incorporation of UDP-glucose, UDP-xylose and UDP-arabinose into polymeric material was determined under differing assay conditions for microsomal fractions isolated at different time points following addition of elicitor (Figures 3 and 4). There was rapid depletion of the enzyme activities known to be wholly associated with Golgi membranes. Thus arabinan synthase, xyloglucan xylosyltransferase and xylan synthase activities rapidly turn over with a half-life of about 1.5 h. This was less true of GSI activities, which may represent a combination of the Golgi-localized β1-4-glucosyltransferase involved in xyloglucan synthesis and cellulose synthase. In this case activity fell, but the half-life was 3 h. GSII activity, which is thought to represent callose synthase, increased in membranes isolated from cells following elicitor action. This has been observed in a large number of systems where callose synthesis has been shown to increase in response to stress (Delmer, 1987; Kauss, 1987). There are thus profound changes in the synthesis and secretion of wall polysaccharides following elicitor action.

These findings of a total loss of the capacity for incorporation of arabinose and xylose into polysaccharide and for glucose under the conditions of incorporation for 'GSI' contrast with the increased incorporation of glucose under the conditions of GSII during the same period. This has important consequences for the interpretations, because each of these incorporations were carried on single batches of membranes and so are directly comparable. Thus, although the capacity for substrate hydrolysis increases in the membranes isolated from cells (Figure 1b), it clearly does not impede the detection of increased incorporation catalysed under GSII conditions. For those situations where there is loss of activity, it is likely that the loss in the amount of substrate available during the progress of the assay is insufficient enough to account for the nearly total loss in detectable activity. The concentration of substrate does not fall below the K_m for each of the enzyme conditions over the 10 min incubation period. The total loss of polysaccharide synthase activity cannot be wholly based upon UDP-sugar breakdown in the assays.

Regulation of other membrane-associated glycosylations

When incorporation of monosaccharides from UDP-sugars into

lipid was determined, the only significant incorporation and increases observed were under the assay conditions for GSII (Figure 3b). Glucosyl-conjugates of a large number of lipophilic compounds are a feature of stressed cells, although in this case they were not characterized further.

Much lower incorporations of UDP-sugars into glycoproteins were observed compared with polysaccharide by membranes from unelicited cells. After elicitation, the UDP-sugar incorporation into glycoprotein was seen to dramatically increase in the case of UDP-arabinose (Figure 4). This has been demonstrated previously, and is repeated here for comparison with the utilization of the other UDP-sugars which have not previously been demonstrated in this system. There was relatively lower level of incorporation of UDP-glucose into glycoprotein as measured under the assay conditions of GSII (Figure 3b), and only in the case of xylose incorporation into glycoprotein was there any increase after elicitation (Figure 3c). The most striking shift in synthesis was that of UDP-arabinose, where there was a 3-fold increase in incorporation into glycoprotein by isolated membranes after 6–8 h of elicitation, while incorporation by arabinan synthase fell to levels shown by the other Golgi-localized synthases (Figure 4).

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 (Bolwell, 1993). However, the availability of nucleotide diphosphate-sugars appears to be under some fine control. Some of the basis of this regulation is beginning to be understood, but this has mainly been aimed at the synthesis and metabolism of UDP-glucose (Stitt and Quick, 1989). Thus examination of the relative roles of invertase, sucrose synthase, reversed UDP-glucose pyrophosphorylase and sucrose phosphate synthase in non-photosynthetic tissues appears to favour a role for sucrose synthase as a major source of UDP-glucose (Huber and Akazawa, 1986). Oxidation of UDP-glucose by UDP-glucose dehydrogenase, a reaction potentially subject to feedback inhibition by the level of UDP-xylose (Dalessandro and Northcote, 1977), probably acts as a key step for the provision of UDP-sugars for membrane-bound glycosylations. The levels of extractable enzyme activity for some of these reactions have been studied throughout the growth cycle of suspension-cultured cells. Thus sucrose metabolism has been studied with respect to sucrose synthase and other activities in French bean, where a strong correlation between the exponential phase of growth and a several-fold induction of sucrose synthase was observed (Botha et al., 1992). Furthermore, in this system the induction of sucrose synthase was seen to be independent of the presence of sucrose in the medium. An extensive mapping of the characteristic changes in enzyme activities in relation to the cell cycle has shown, in *Catharanthus roseus* (Madagascan periwinkle), accumulation of glucan polymers occurs during phase G_1, preceded by the necessary metabolic activity involving UDP-sugar levels (Amino et al., 1985a–c). Changes in the levels of arabinan and xylan synthase activities were also observed to vary during the growth cycle in French bean (Bolwell and Northcote, 1983a).

Regulation of such activities has now been studied in elicitor-treated French-bean cells over a period of 24 h. Changes in the pectin, hemicellulose and cellulosic fractions of the cell wall of elicitor-treated cells have previously been examined (Bolwell et al., 1985). No changes were observed in the pectin components, but the treatment was carried out during the period, 4–6 days after subculture, when the bulk of the pectin synthesis as measured by arabinan synthase (Bolwell and Northcote, 1983b) or polygalacturonan synthase (G. P. Bolwell and D. H. Northcote, unpublished work) had probably ceased. There were also changes that reflected a redistribution of compounds between hemicellulosic and cellulosic fractions, namely an apparent loss in extractable hemicellulose and an increase in the cellulosic fraction. The latter increase may partly be accounted for by increased synthesis of insoluble glycoprotein and phenolics. Differences in extractability may also be a consequence of oxidative cross-linking in the walls of elicited French-bean cells (Bolwell, 1987; Bradley et al., 1992). However, the present investigation indicates that some of the changes in cell wall composition are also determined by alterations in the underlying synthetic capacity and not solely by turnover and extracellular modifications.

There were no significant changes in the levels of enzyme activities possibly involved in the provision of UDP-glucose in response to elicitor action. These are unlikely to be rate-limiting. Of those responsible for UDP-sugar interconversion, only the levels of UDP-glucose dehydrogenase activity increased significantly after extended incubation in the presence of elicitor. This would lower the level of UDP-glucose only if the provision of this intermediate were rate-limiting. However, it would increase the flux into other UDP-sugars, since the level of the enzymes involved in interconversion remained unchanged and is probably not rate-limiting (Northcote, 1985; Bolwell, 1988). The utilization of the pool of UDP-sugars, however, changed significantly. Increased levels of callose synthase activity were observed, which in other systems is probably reflected as an increase in callose synthesis *in vivo* (Kauss, 1987). Concomitant with this was a loss in GSI activity, which appeared to recover after 8 h. This may indicate a resumption of Golgi-localized β1-4-glucan and perhaps cellulose synthesis. There were striking changes in the complement of other synthase activities associated with the Golgi. This was shown by the near-total loss of arabinan, xylan and xyloglucan synthase activities, with a turnover having a half-life of about 1.5 h. The polysaccharides of these primary walls of these French-bean cells contain 3% xylan in total, and the majority of the hemicellulose is xyloglucan (G. P. Bolwell and D. H. Northcote, unpublished work). Elicitation therefore appears to have profound effects on synthesis and secretion of polysaccharides through the endomembrane system which may be temporarily halted during the initial response. However, arabinosylation and xylosylation of extracellular glycoproteins, some of which are known to be induced by elicitor action, appears to increase and thus increased UDP-glucose dehydrogenase activity could be related to the provision of the requisite sugar-nucleotides required.

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