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Characterisation and immunolocation of an 87 kDa polypeptide associated with UDP-glucuronic acid decarboxylase activity from differentiating tobacco cells (*Nicotiana tabacum* L.)

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Abstract

UDP-glucuronic acid decarboxylase catalyses the reaction responsible for the formation of UDP-xylose and commits assimilate for the biosynthesis of cell wall polysaccharides and glycosylation of proteins. Xylose-rich polymers such as xylans are a feature of dicot secondary walls. Thus a cell culture system of tobacco transformed with the *ipt* gene from *Agrobacterium tumefaciens* for cytokinin production and which when manipulated with auxin and sucrose leads to induction of xylogenesis, has been used as a source for purification of the enzyme. UDP-glucuronic acid decarboxylase was purified by ion-exchange, gel filtration and affinity chromatography on Reactive Brown–Agarose. The native enzyme had an apparent M_r of 220,000 which yielded a single subunit of 87,000 when analysed on SDS–PAGE using silver staining. This appears to be a novel form of the enzyme since a gene family encoding polypeptides around M_r 40,000 with homology to the fungal enzyme also exists in plants. Using an antibody raised to the native 87 kDa form of the enzyme, this decarboxylase was localised mainly to to cambium and differentiating vascular tissue in tobacco stem, consistent with a role in the provision of UDP-xylose for the synthesis of secondary wall xylan. Further analysis using immunogold electron microscopy localised the 87 kDa UDP-glucuronic acid decarboxylase to the cytosol of developing vascular tissue.

Keywords: *Nicotiana tabacum*; Solanaceae; Xylogenesis; Xylose; UDP-glucuronic acid decarboxylase; Immunolocation

1. Introduction

During the growth and differentiation of plant cells a composite of structural polysaccharides, glycoproteins and phenolic compounds are laid down to form the cell wall. A substantial number of the monosaccharides found in homo- and heteropolysaccharide components of the wall are derived from the central biosynthetic intermediate UDP-glucose by a pathway involving UDP-glucose dehydrogenase, UDP-glucuronate decarboxylase and a series of epimerase reactions (Gibeaut, 2000). UDP-glucuronate decarboxylase (EC 4.1.1.35) catalyses the conversion of UDP-glucuronic acid to form UDP-xylose and CO_2. The reaction, which is irreversible, is believed to proceed via a UDP-4-keto-hexose intermediate (Schutzbach and Feingold, 1970) and requires the cofactor NAD^+. Forms of UDP-glucuronic acid decarboxylase responsible for the formation of UDP-xylose has been demonstrated in both prokaryotes and eukaryotes. In bacteria (Ankel et al., 1967; Fan and Feingold, 1972), and fungi (Ankel and Feingold, 1966; Jacobson and Payne, 1982, Bar-Peled et al., 2001) the enzyme is involved in the formation of the extracellular polysaccharides whereas in animal tissue it is required for the synthesis of connective tissue glycoaminoglycans (John et al., 1977b; Silbert and Deluca, 1967; Kearns et al., 1993; Vertel et al., 1993). In plants, UDP-xylose is the substrate for the synthesis of UDP-arabinose by epimerisation and the donor of xylose units for the synthesis of several types of hemicelluloses. In particular, xylan is a major component of the cell walls of hardwoods, softwoods and grasses (Gregory et al., 1998), and xyloglucan is the major hemicellulose and an important structural polymer in the primary cell wall of dicotyledons (McNeil et al., 1984; Hayashi, 1989).

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The enzyme has been reported in a number of plant species (Neufeld et al., 1958; John et al., 1977a; Dalessandro and Northcote, 1977a,b,c; Amino et al., 1985; Hayashi et al., 1988; Robertson et al., 1995a) and detected in a number of plant tissues including the vascular cambium, differentiating xylem (Dalessandro and Northcote, 1977b,c) and internodes of pea seedling (Dalessandro and Northcote, 1977a). Correlation of the changes in enzyme activity and developmental processes have indicated that UDP-glucuronic acid decarboxylase could be important for the biosynthesis of UDP-xylose during cell division (Amino et al., 1985) and secondary wall formation during cell differentiation (Dalessandro and Northcote, 1977a,b,c; Robertson et al., 1995a; Blee et al., 2001), although it is not induced in response to elicitor treatment (Robertson et al., 1995b). The location of the enzyme in the plant cell, whether cytoplasmic (free or membrane associated) or within the lumen of the Golgi, is not established. Most activity is solubilised during protein extraction, but there are a number of reports of activity associated with membrane preparations (Feingold et al., 1960) and particularly the Golgi apparatus (Hayashi et al., 1988). Characterisation of UDP-glucuronic acid decarboxylase has been achieved from a small number of a diverse range of species, including the fungi *Crypotococcus laurentii* (Ankel and Feingold, 1966; Bar-Peled et al., 2001), and partially purified from the plant species *Petroselinum hortense* (Wellmann et al., 1971), *Lemna minor* (Gustine et al., 1975) and wheat germ (John et al., 1977a). As a result a number of different isoforms have been identified and their biochemical properties analysed. These earlier studies provide evidence for the existence of two generically different forms of the enzyme. Recently, examples of one form of the enzyme with homologies to the fungal enzyme have appeared in the genetic databases. These have been identified in pea and Arabidopsis and appear to encode polypeptides in the range 40–50 kDa.

Plant cell cultures have been successfully used as model systems to study enzymes involved in UDP-sugar metabolism during cell division (Amino et al., 1985), vascular differentiation (Robertson et al., 1995a; Blee et al., 2001) and elicitation (Robertson et al., 1995b). They also have advantages as a source for the purification of some proteins (Bolwell, 2001). Blee et al. (2001) characterised a tobacco cell culture system derived from a cultivar transformed with the *ipt* gene from *Agrobacterium tumefaciens,* which leads to high levels of endogenous cytokinin. The culture showed morphological, biochemical and molecular evidence of secondary wall formation. In the present study, we describe the characterisation of an 87 kDa form of UDP-glucuronic acid decarboxylase from transformed *Nicotiana tabacum* cell cultures and its immunolocation in planta.

2. Results

2.1. Purification of 87 kD UDP-glucuronic acid decarboxylase-associated polypeptide to homogeneity

UDP-D-glucuronic acid decarboxylase was assayed routinely with a radiochemical method modified from Robertson et al. (1995a), and optimised using extracts from xylogenic *Nicotiana tabacum* cell cultures. This assay was found to be more sensitive than a colormetric assay formally used by Ankel and Feingold (1966). Routine purification of the decarboxylase was carried out on three day old subcultured cells when enzyme activity was maximum (Blee et al., 2001).

Using differential centrifugation, UDP-glucuronic acid decarboxylase activity was present mainly in the 100,000 g supernatant (Table 1). However, 10% of the activity was associated with the membranes sedimenting at 100,000 g once solubilised with phosphate buffer containing 1% Digitonin, similar to findings by Feingold et al. (1960) and later by Hayashi et al. (1988). Nevertheless, the addition of 1% digitonin to the standard reaction mixture was shown to have no effect on UDP-glucuronic acid decarboxylase activity.

The enzyme was successfully purified from soluble extracts of 3 day old transformed tobacco cell cultures using ammonium sulphate precipitation, DEAE-cellulose, Gel-filtration and Reactive Brown 10 affinity chromatography (Fig. 1A–C). UDP-glucuronic acid decarboxylase was purified 254 fold, with a total recovery of 15% activity (Table 2) and eluted in a single peak from each chromatographic matrix. DEAE-cellulose had been used in previous studies to separate two isoforms of decarboxylase from wheat germ (John et al., 1977a), *Lemna minor* (Gustine et al., 1975) and *Petroeslinum hortense* (Wellmann et al., 1971). However separation of a sample from transformed *Nicotiana tabacum* cell cultures using a linear gradient up to 500 mM NaCl on DEAE-cellulose suggested only one isoform was present or isoforms had similar net negative charges (data not shown). The native molecular mass of the protein was estimated by chromatography of the enzyme on calibrated Sephacryl S-300. The size of the holoenzyme was estimated at a molecular weight of approximately 222,700 ± 18,500 (mean ± standard error of three experiments). With careful selection of active fractions for further purification, it was possible to acquire a homogeneous product with respect to subunit. SDS-PAGE analysis of this purified enzyme following silver staining showed a single polypeptide with a molecular mass of 87 kDa (Fig. 2). This polypeptide was always found in purifications but in a number of overlapping fractions a polypeptide of 40 kDa co-purified. However the 40 kDa polypeptide could not be purified free of the 87 kDa with any of the chromatographic methods used (Wheatley, 2001).

Table 1
Distribution of UDP-glucuronic acid decarboxylase activity after cell fractionation and ammonium sulphate precipitation of transformed tobacco cell cultures

Fraction	Protein (mg)	Total UDP-glucuronic acid decarboxylase (nmol/min)
Homogenate	91.0	674.2
10,000 g pellet	5.1	25.4
100,000 g pellet	9.3	56.0
100,000 g supernatant		
$(NH_4)_2SO_4$ 0–40%	16.5	13.4
40–70%	24.4	511.0
70–100%	2.7	4.0

The homogenate was centrifuged at 10,000 *g* and then 100,000 *g*, in each instance the pellet was resuspended in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.4 M sucrose, 1 mM EDTA, 5 mM mercaptoethanol and 1% Digitonin. The remaining supernatant was further fractionated by ammonium sulphate precipitation. The 0–40%, 40–70% and 70–100% fractions were desalted and assayed for UDP-glucuronic acid decarboxylase.

2.2. Properties of the purified enzyme

Reports have shown that the enzyme from fungi (Ankel et al., 1964; Ankel and Feingold, 1966) and certain isoforms from plant tissue (Gustine et al., 1975) require endogenous NAD^+ for activity. UDP-glucuronic acid decarboxylase from *Nicotiana tabacum* cell cultures was neither stimulated nor inhibited by the addition of 0.5–2.0 mM NAD^+. The pH optimum for UDP-glucuronic acid decarboxylase measured over a range of pH 5.5–8.0 was 7.0. The decarboxylase showed hyperbolic kinetics with respect to UDP-glucuronic acid. The apparent K_M of UDP-glucuronic acid decarboxylase for UDP-glucuronic acid was estimated to be 767 μM. Purified UDP-glucuronic acid decarboxylase was relatively stable at 4 °C. Activity was not effected by storage at −70 °C for several months, although repeated freeze and thawing of samples would result in significant loss of activity.

2.3. Characterisation of anti-(tobacco UDP-glucuronic acid decarboxylase) serum

UDP-glucuronic acid decarboxylase purified from *Nicotiana tabacum* cell cultures was used to raise polyclonal antibodies. The specificity of the rabbit polyclonal antiserum for UDP-glucuronic acid decarboxylase was tested by its ability to decrease decarboxylase activity in 40–70% ammonium sulphate extracts using immunoprecipitation techniques and analysed by SDS–PAGE (Fig. 3). Almost complete inhibition was obtained with 1:10 dilution of antibodies while no effect was found with the pre-immune serum controls. SDS–PAGE analysis of the Protein A IgG complexes revealed that a 87 kDa polypeptide was immunoprecipitated in increasing quantity when antiserum concentrations were increased from 1:1000 to 1:10 correlating with removal of activity. The diffuse band at 46–50 kDa represents IgG large subunit which is simultaneously removed from the protein-A Sepharose during preparation for SDS–PAGE.

2.4. Immunolocalisation using antibodies raised against native 87 kD polypeptide

Tissue prints of stem internodes from 4-week-old tobacco plants were used to localise the 87 kDa UDP-glucuronic acid decarboxylase polypeptide. Tissue prints show that during all stages of stem development the decarboxylase accumulates mainly in the cambial region of a developing *Nicotiana tabacum* stem (Fig. 4). This probably includes cambial initial and phloem and xylem mother cells. Controls showed no reaction when tissue prints were incubated with pre-immune serum.

The antibodies raised against UDP-D-glucuronic acid decarboxylase were used to locate the enzyme to differentiating tissues in the cambial region of French bean hypocotyl using electron microscopy in order to directly compare localisation with the previous enzyme in the pathway, UDP-glucose dehydrogenase (Robertson et al., 1996) and the Golgi-localised xylan synthase (Gregory et al., 2002) in exactly the *same* tissue samples. These samples were also prepared by freeze substitution which optimises preservation of subcellular structures (Gregory et al., 2002). Cells of the vascular cambium have characteristically large vacuoles and a thin cytoplasm (Fig. 5a–d). UDP-glucuronic acid decarboxylase was located to the cytoplasm in cells within the cambial region and developing xylem. Gold labelling is clearly specific to the cytoplasm and no signal is seen within the vacuole or over the cell wall. Controls to test for non-specific binding probed the same tissue with pre-immune serum and secondary antibody gold complexes or secondary antibody gold complexes alone. Gold labelling was not visible in such tissue sections.

3. Discussion

UDP-glucuronic acid decarboxylase is involved in the biosynthesis of UDP-sugar precursors for hemicellulose and glycoprotein production in both primary and secondary plant cell walls. It catalyses the conversion of UDP-glucuronic acid to UDP-xylose and hence provides xylosyl units for the synthesis of xyloglucans and xylans, both major non-cellulosic structural polymers of the cell wall. Recent interest in the metabolic pathways to hemicellulose synthesis has seen the purification and cloning of UDP-glucose dehydrogenase (Robertson et al., 1996; Tenhaken and Thulke, 1996; Seitz et al.,

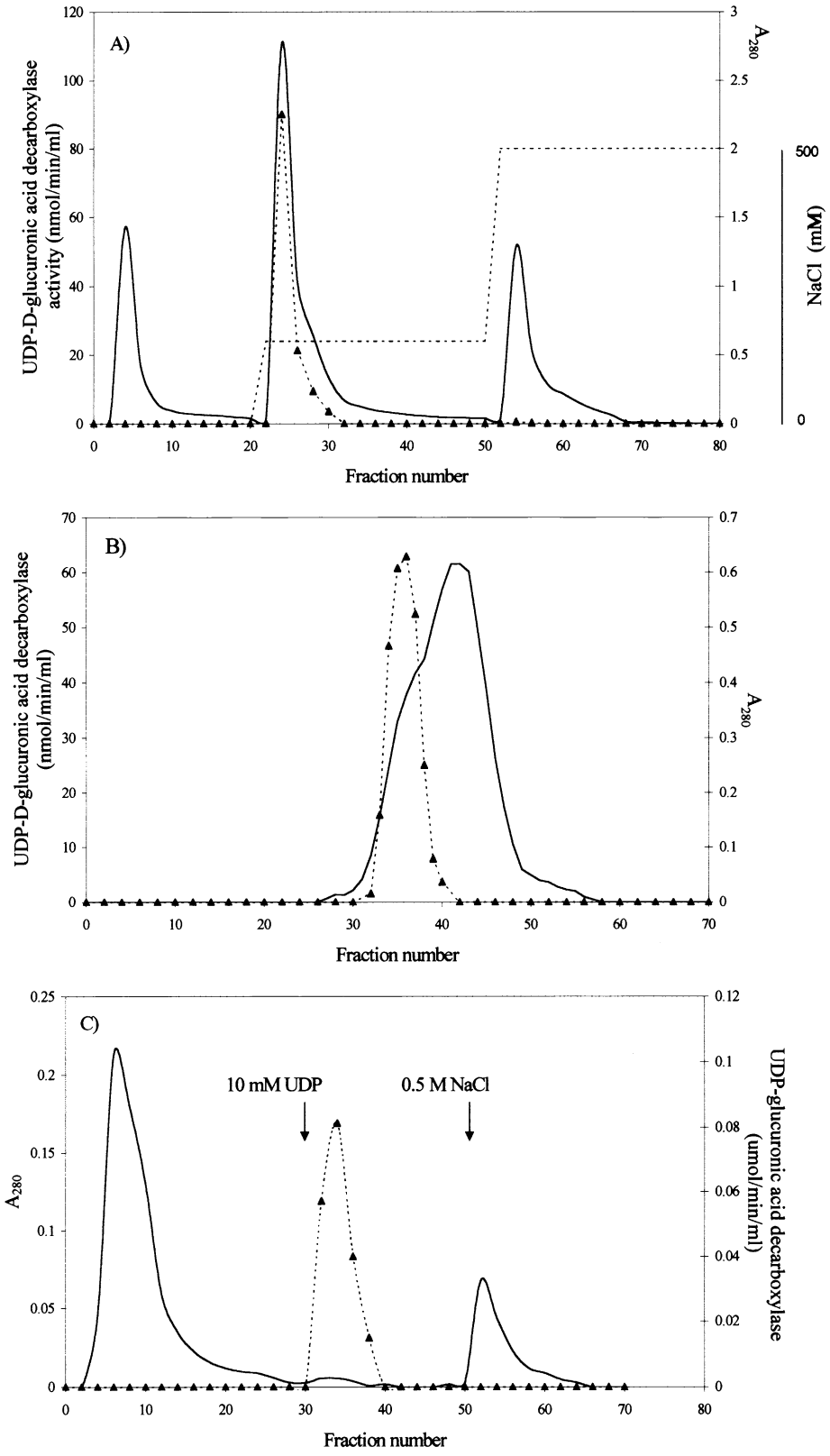

Fig. 1. Elution profiles of UDP-glucuronic acid decarboxylase purified from transformed *Nicotiana tabacum*. Soluble protein extract, precipitated between 40 and 70% $(NH_4)_2SO_4$ and desalted on PD-10 columns were subjected to a three step chromatographic procedure (A) DEAE-cellulose, (B) Sephacryl S-300 and (C) Reactive Brown 10. UDP-glucuronic acid decarboxylase activity (-▲-), protein measured as A_{280} (—).

2000), and xyloglucan fucosyltransferase (Perrin et al., 1999) and xylosyl transferase. In comparison, UDP-glucuronic acid decarboxylase remains poorly characterised. In this report we describe the purification and immunocytochemical analysis of UDP-glucuronic acid decarboxylase from transformed *Nicotiana tabacum* cell cultures.

Purified UDP-glucuronate decarboxylase from tobacco cells exhibits a number of similar biochemical properties to those previously identified from other plant species. The enzyme from tobacco did not require an exogenous supply of NAD^+ for activity, had an optimum pH of approximately 7 and eluted from DEAE-chromatography under comparable conditions (Ankel and Feingold, 1965; John et al., 1977a; Gustine et al., 1975; Hayashi et al., 1988; Wellmann et al., 1971). The apparent K_M of the soluble tobacco enzyme was 764 μM, in agreement with that of a soluble isoform from soybean calculated at 700 μM (Hayashi et al., 1988). UDP-glucuronic acid decarboxylase isolated from tobacco had a native molecular mass of 220 kDa and this was consistent with the only other report of a molecular mass of the enzyme, estimated from partially purified wheat germ extracts to be 210 kDa (John et al., 1977a). This current work suggests that tobacco contain soluble and membrane-associated forms of the enzyme, similar to reports from soybean (Hayashi et al., 1988) and mung bean (Feingold et al., 1960). Previous studies had suggested that the occurrence of multiple soluble forms of the enzyme could be a general phenomenon because two isoforms had been identified in extracts from wheat germ (John et al., 1977a), *Petroselinum hortense* (Wellmann et al., 1971) and *Lemna minor* (Gustine et al., 1975). The biochemical evidence from these studies strongly suggests the existence of two generically different forms of the enzyme with respect to physical properties, kinetics and substrate specificity. However, no data with respect to molecular size was available at that time.

The one form of UDP-D-glucuronic acid decarboxylase that could be purified to homogeneity from soluble cell fractions from suspension cultured cells of tobacco showed an 87 kDa subunit. Detailed analysis by SDS–PAGE showed the 87 kDa polypeptide correlates with activity during chromatography of the enzyme on all types of matrices investigated. Antibodies raised against the native UDP-glucuronic acid decarboxylase were found to immunoprecipitate only a 87 kDa polypeptide from crude protein extracts and inhibition of enzyme activity was found to closely correlate with antibody concentration. However, a second 40 kDa polypeptide is also expressed in these cultures and was identified as its closest match a 39 kDa UDP-glucuronic acid decarboxylase from *Pisum sativum* and a dTDP-glucose 4–6-dehydratase from chickpea following peptide fingerprinting by MALDI-TOF (unpublished data). Although this could not be purified to homogeneity and separated from the 87 kDa form, nevertheless, heterologous expression of the cognate tobacco cDNA in *E. coli* shows it has UDP-glucuronic acid decarboxylase activity (L. Bindschedler and G.P. Bolwell, unpublished data). This on-going work supports the biochemical indication that there exist two generically different forms of the decarboxylase in plants. Interestingly the previous enzyme in the pathway, UDP-glucose dehyrogenase exists in two generically different forms (Robertson et al., 1996; Tenhaken and Thulke, 1996).

Despite all the investigations to date on the enzymes of nucleotide metabolism in plant cells, the location of

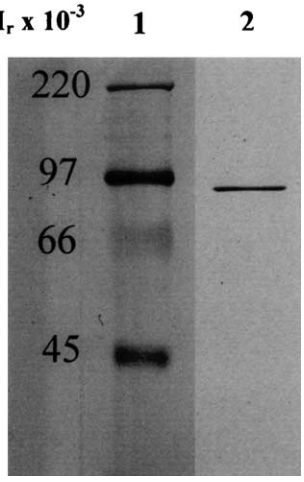

Fig. 2. SDS–PAGE analysis of purified 87 kDa UDP-glucuronic acid decarboxylase. Proteins were separated by SDS–PAGE on 10% acrylamide and subjected to silver staining. Track 1 shows marker proteins. Track 2 shows the 87 kDa subunit obtained after the reactive brown step.

Table 2
Purification of UDP-glucuronic acid decarboxylase from transformed tobacco cell suspension cultures

Stage	Total activity (μmol/min)	Protein (mg)	Specific activity (μmol/min per mg)	Recovery (%)	Purification (fold)
$(NH_4)_2SO_4$	6.05	87.09	0.07	100	1
DEAE-cellulose	5.67	35.06	0.16	93	2.3
Sephacryl S-300	1.91	6.07	0.31	31	4.5
R.Brown 10	0.93	0.05	17.57	15	254

the main enzymes involved with UDP-sugar interconversion for synthesis of matrix polysaccharides is currently still unclear. Possibilities exist that they could be either cytoplasmic (free or membrane-bound) or within the lumen of the Golgi apparatus. UDP-glucose dehydrogenase is the one exception in that it is a wholly soluble protein (Hayashi et al., 1988) and antibodies raised against a UDP-glucose dehydrogenase has shown a cytoplasmic location (Robertson et al., 1996). The current work is the first direct evidence of localisation of a UDP-glucuronic acid decarboxylase isoform to the cytoplasm. Antibodies have been raised against a native form of a homogenous preparation of soluble 87 kDa UDP-D-glucuronic acid decarboxylase polypeptide from transformed *Nicotiana tabacum* cell suspension cultures. The antibody locates the decarboxylase to the cytoplasm of cambial and developing vascular tissue cells by immunogold electron microscopy. However, it is premature to speculate on the developmental function of this form of the enzyme without further comparative study of the two forms of the enzymes at the molecular level.

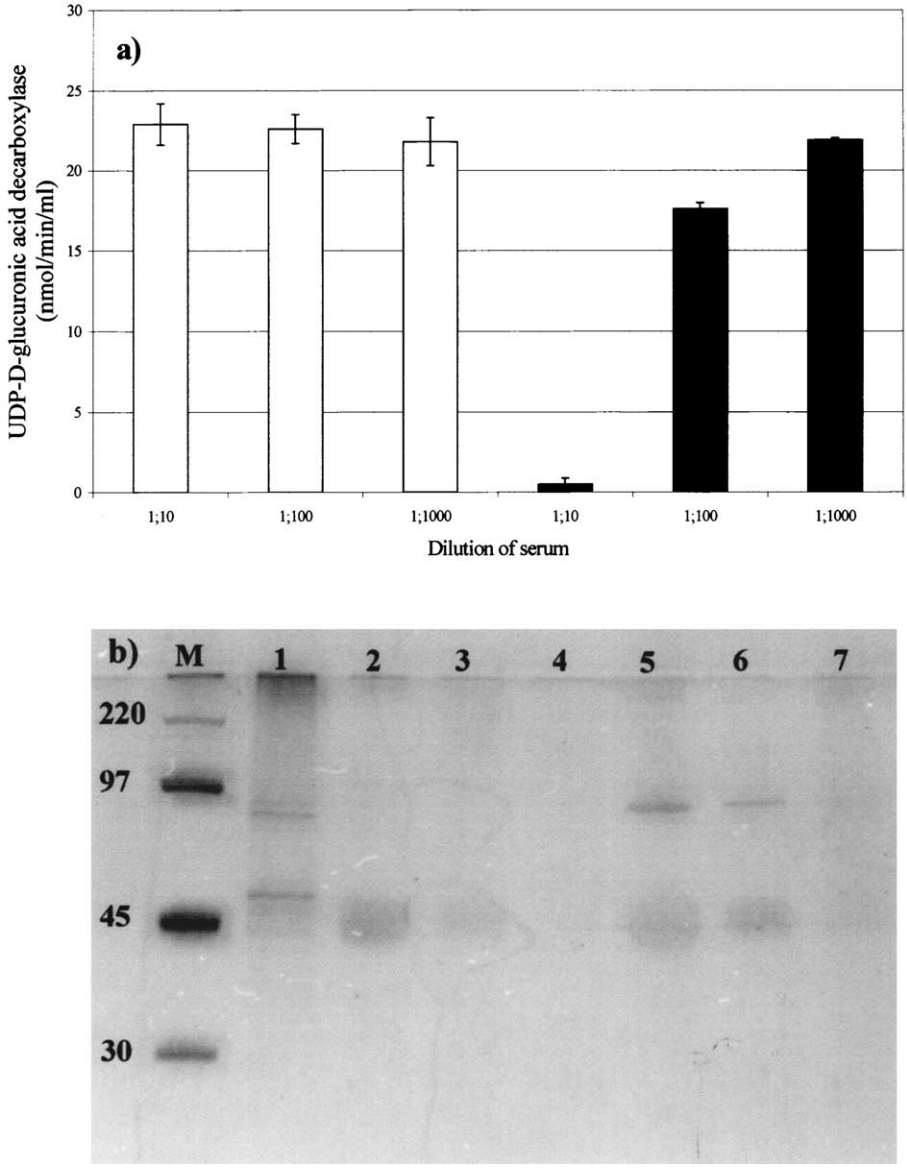

Fig. 3. Immunoprecipitation of UDP-glucuronic acid decarboxylase in a crude protein preparation extracted from transformed *Nicotiana tabacum* cell suspension culture using polyclonal antiserum raised against the 87 kD subunit. (a) UDP-glucuronate decarboxylase activity of the supernatant after incubation of protein extract with different dilutions of anti-(UDP-glucuronic acid decarboxylase) serum (■) and pre-immune serum (□). (b) SDS-PAGE analysis of immunoprecipitated protein complex. Partially purified UDP-glucuronic acid decarboxylase (**track 1**); Diluted pre-immune antiserum 1:10 (**2**), 1:100 (**3**), 1:1000 (**4**) and anti-UDP-glucuronate decarboxylase 1:10 (**5**), 1:100 (**6**), 1:1000 (**7**). M, Molecular weight markers.

4. Experimental

4.1. Materials

UDP-D-[U-^{14}C] glucuronic acid was purchased from NEN (Stevenage, Herts., UK).

4.2. Tobacco cell suspension cultures

The transformed tobacco line which contains an active *ipt* gene from *Agrobacterium tumefaciens* was obtained from Dr. Johan Memelink, Leiden University, the Netherlands (Memelink et al., 1987). The cell suspension cultures were derived from green plantlets and maintained as described previously by Blee et al. (2001).

4.3. Enzyme assays

UDP-glucuronate decarboxylase activity was assayed by measuring the amount of UDP-[U-^{14}C] xylose formed from UDP-[U-^{14}C]glucuronic acid using a method modified from Robertson et al. (1995a). The reaction was carried out for 5 min at 37 °C in a volume of 40 μl consisting of 8 μl of enzyme sample and 32 μl of the reaction mixture. The reaction mixture contained 0.4 nmol UDP-D-[U-^{14}C]glucuronic acid, 50 nmol UDP-glucuronic acid, 0.1 M sodium phosphate buffer, pH 7.0 1 mM EDTA, 5 mM 2-mercaptoethanol. A control reaction was incubated simultaneously using a boiled aliquot of the enzyme. The reaction was terminated by boiling each sample for 3 min. After centrifugation for 2 min at 20,000 *g*, 20 μl of the supernatant was added to 20 μl of 0.1 M HCl. The solution was hydrolysed for a further 15 min, at 100 °C and left to cool. Pyridine (20 μl) was added to each tube and the samples were separated by paper chromatography with authentic standards, for approximately 16

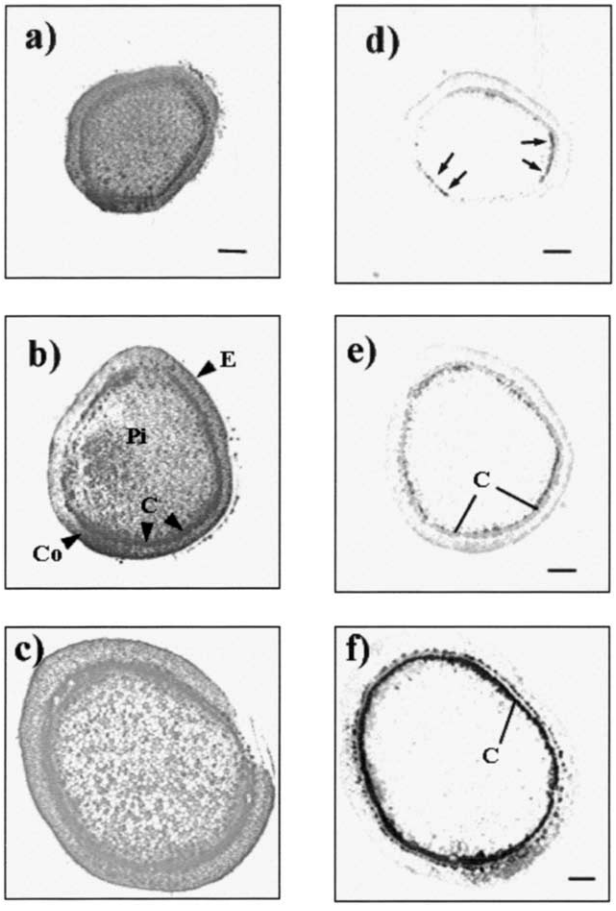

Fig. 4. Localisation of UDP-glucuronic acid decarboxylase in the internodes of 6 week old *Nicotiana tabacum* plants using tissue printing. Left (a–c) Anatomy of internodes stained with Aurodye. Right (d–f) corresponding UDP-glucuronic acid decarboxylase localisation using polyclonal antiserum. Internode 4 (a,d), Internode 6 (b,e) and internode 8 (c,f). C, cambial region; Co, cortex; E, epidermis; Pi, pith; p, phloem (Bar = 1 mm). Similar tissue prints probed with pre-immune serum resulted in no signal (not shown).

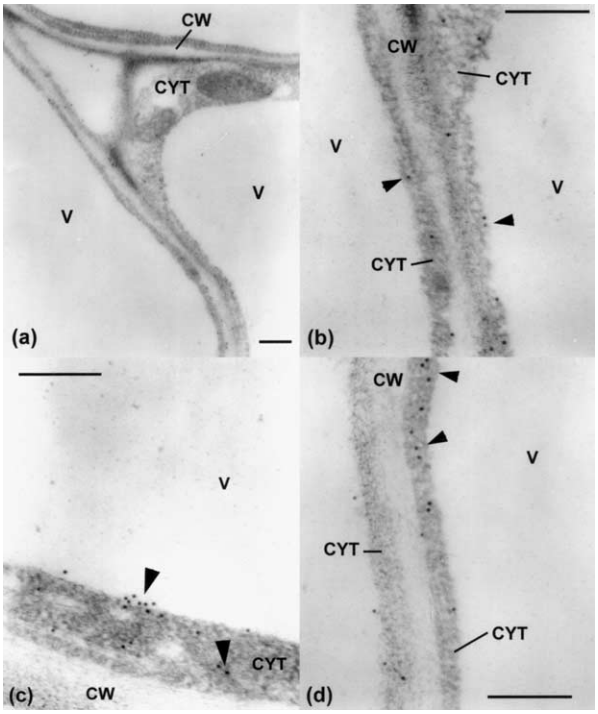

Fig. 5. Subcellular localisation by immunogold electron microscopy of UDP-glucuronic acid decarboxylase. The antigen is localised to the cytoplasm of cells in the cambial region of French bean hypocotyl, in the same tissue samples also used to locate the previous enzyme UDPglucose dehydrogenase (Robertson et al., 1996) and the subsequent enzyme in the pathway, xylan synthase (Gregory et al., 2002). Arrows show examples of gold labelling. Magnifications of each micrograph (a) ×40,000; (b) ×100,000; (c) ×100,000; (d) ×100,000. Scale bar = 0.2 μm. Abbreviations: CW, cell wall, CYT, cytoplasm; V, vacuole.

h in pyridine–ethyl acetate–water (2:8:1-v/v/v). Radioactivity that co-chromatographed with D-glucuronic acid and D-xylose standards was determined by liquid scintillation counting.

4.4. Purification of UDP-glucuronate decarboxylase

The purification procedure was carried out at 4 °C. Three-day-old transformed tobacco cells (200–250 g) were homogenised in 0.1 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 5 mM 2-mercaptoethanol (1:1 w/v) with a pestle and mortar followed by further homogenisation using a Polytron. Following filtration of the crude homogenate through 4 layers of cheesecloth, the filtrate was centrifuged at 10,000 *g*. Solid $(NH_4)_2SO_4$ was then added to the protein sample with constant stirring, to a concentration of 40% (w/v) saturation. The resulting precipitate was removed by centrifugation at 10,000 *g* for 20 min, and the supernatant retained on ice. Additional $(NH_4)_2SO_4$ was added to the supernatant to a concentration of 70% (w/v) saturation and the precipitated protein harvested by centrifugation at 10,000 *g* for 20 min The precipitate was resuspended in 10 ml of 0.1 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 5 mM 2-mercaptoethanol and the insoluble components removed by centrifugation for 5 min at 5000 *g*. The samples were desalted using PD-10 columns pre-equilibrated and eluted with 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA and 5 mM 2-mercaptoethanol.

The protein extract was applied to a column of DEAE-cellulose (16×2 cm), previously equilibrated with 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA and 5 mM 2-mercaptoethanol. Protein was eluted with a single step of 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA and 5 mM 2-mercaptoethanol at a flow rate of 1 ml/min. Fractions were assayed for UDP-glucuronate decarboxylase activity. Eluted fractions containing UDP-glucuronate decarboxylase activity were concentrated by a 70% $(NH_4)_2SO_4$ precipitation. The pellet was re-dissolved in 0.5 ml of 0.1 M sodium phosphate buffer, pH 7.0 containing 1 mM EDTA and 5 mM 2-mercaptoethanol and loaded onto a column of Sephacryl S-300 (1.5×92 cm) pre-equilibrated and eluted in 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA and 5 mM 2-mercaptoethanol at a flow rate of 0.2 ml/min. Fractions were assayed for UDP-glucuronate decarboxylase activity. The active fractions from the Sephacryl S-300 column were applied to a Reactive Brown 10 dye affinity column (1×5 cm), pre-equilibrated and washed in 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA, 5 mM 2-mercaptoethanol, and 50 mM NaCl, at a flow rate of 1ml/min. The enzyme bound to the column was eluted with 10 mM UDP in the same phosphate buffer. Fractions eluting from the column were desalted on Sephadex G-25 and assayed for UDP-glucuronate decarboxylase activity. Active fractions were analysed by SDS–PAGE on 10% acrylamide gels and visualised using either Coomassie R-250 or silver stain. In circumstances where the purified protein was required for kinetic studies the UDP was removed using a PD-10 column.

The native molecular weight of UDP-glucuronate decarboxylase was determined by gel filtration through a column of Sephacryl S-300. The column (1.5–92 cm) was calibrated with apoferritin, alcohol dehydrogenase, bovine serum albumin and cytochrome *c*. Each standard (500 μg) was applied to the column pre-equilibrated with 0.01 M sodium phosphate buffer, pH 7.5 containing 1 mM EDTA and 5 mM 2-mercaptoethanol. The column was eluted at a rate of 0.2 ml/min and 3 ml fractions were collected. The void volume was determined with Blue Dextran 2000.

4.5. Production of anti-(tobacco UDP-glucuronate decarboxylase) serum

Purified UDP-glucuronic acid decarboxylase was chromatographed on PD-10 columns pre-equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.0. Enzyme preparations were emulsified in Freud's incomplete adjuvent and administered subcutaneously to a rabbit. Initial immunisation used 100 μg protein. Two subsequent boost immunisations (100 μg) were administered at monthly intervals before collection of antisera from the ear veins 2 weeks after the final immunisation. After the clot was removed sera was clarified by centrifugation at 1000 *g* for 10 min and stored in 20 μl aliquots at−20 °C.

4.6. Immunoprecipitation

A protein sample extracted from transformed tobacco cell suspension cultures was subjected a 40–70% $(NH_4)_2SO_4$ fractionation, desalted and used to investigate the immunoprecipitation of UDP-glucuronate decarboxylase using polyclonal antiserum. Pre-immune antiserum and antiserum against UDP-glucuronate decarboxylase was diluted 1:10, 1:100 and 1:1000 into the protein extract and 0.1 M sodium phosphate buffer, pH 7.0 respectively. The samples were left on ice for 30 min with agitation at 5-min intervals. A 20-μl sample containing 2 mg protein A-Sepharose in 0.15 M Tris–HCl, pH 7.5 was added to each tube and again the samples were left on ice for 30 min with agitation at 5 min intervals. Protein A-Sepharose was collected by centrifugation at 2000 *g* for 5 min at 4 °C. Each supernatant was assayed for UDP-glucuronate decarboxylase activity. The supernatant was then removed, discarded and the pellet extensively washed with 0.15 M Tris–HCl

(pH 7.5). Finally the supernatant was removed and 10 μl of loading buffer was added to the sample and the polypeptides bound to the Protein A separated by SDS–PAGE. The gel was stained by the silver method to visualise the proteins.

4.7. Immunolocalisation of UDP-glucuronate decarboxylase in tobacco stem by tissue printing

Nitrocellulose was pre-treated with 0.2 M $CaCl_2$ for 30 min and air dried. The nitrocellulose was laid down on top of a number of sheets of Whatman paper. A new razor blade was used to cut the required section of tobacco stem. The surface was initially blotted onto spare nitrocellulose and then held down gently and evenly for 15 s on a new piece of nitrocellulose. A second print was made for anatomical comparison and control blots. The UDP-glucuronate decarboxylase was detected using similar methods to identification of proteins on western blots, with the additional step of a wash in 10 mM HCl for 15 min after membrane blocking, to inhibit endogenous alkaline phosphatase activity. The primary antibody was used at 1:2000 and a secondary antibody (anti rabbit IgG conjugated to alkaline phosphatase) at 1:10,000. The same dilution of rabbit pre-immune serum was used for the control. Aurogold staining was used for the observation of total protein content of the prints.

4.8. Preparation of tissue for immunogold localisation studies

Immunolgold localization was carried out in French bean hypocotyls as this was used to localize the previous enzyme (Robertson et al., 1996) and the next enzyme (Gregory et al., 2002) in the pathway to xylan. Tissue was prepared using freeze substitution and immunostaining. French bean hypocotyl sections were excised from 13-day-old plants and fixed in freshly prepared 1% (w/v) paraformaldehyde and 0.05% (v/v) glutaraldehyde in 0.05 M sodium phosphate buffer (pH 6.8) at 4 °C for 2 h. Following an overnight incubation in 0.05 M sodium phosphate buffer (pH 6.8), fixed stem and root material was subjected to a series of incubations in 10, 20 (1 h each) and 30% (overnight) glycerol in 50 mM sodium phosphate buffer pH 6.8 at 4 °C. The tissue was then plunged into liquid N_2 under vacuum and transferred in liquid N_2 to an Ambient Freeze Substitution apparatus (Leica, UK). The tissue was incubated sequentially in: dry methanol, methanol containing 1% uranyl acetate and methanol at −90 °C for 12 h each which was followed by incubation in methanol at −90 °C for 72 h with changes of methanol every 24 h. After increasing the temperature to −50 °C, methanol was replaced with Lowacryl HM20 resin (Polysciences, Warrington, PA) and infiltrated for 72 h with several changes of resin. Infiltrated tissue was transferred to gelatin capsules, the fresh resin was added and allowed to polymerise at −50 °C. Following gradual increase of temperature to reach ambient over the period of 24–48 h, the tissue was left at this temperature for the next 24 h to complete polymerisation. Ultrathin sections were transferred to nickel grids.

Immunogold labelling was carried out by first incubating sections at room temperature in 20 μl of 3% bovine serum albumin (BSA) /1% normal goat serum (v/v) in 20 mM Tris–HCl buffer (pH 7.6) containing 0.23 M NaCl (TBS) for 20 min. Excess solution was then drained from the section and replaced with 50 μl of anti-UDP-glucuronate decarboxylase (1:100), or pre-immune serum (1:100) in TBS–BSA-normal goat serum containing 0.05% Tween. Samples were incubated for 18 h. Sections were then washed thoroughly and repeatedly for 5 min in TBS (pH 7.6) and the area around the sections carefully dried. Sections were immersed in 20 μl goat anti-rabbit IgG conjugated to colloidal gold (British Biocell International, Cardiff, UK; 5 nm particle size; 1:200 working solution in TBS-ovalbumin-normal goat serum) for 60 min at room temperature. The grids were then washed thoroughly with TBS followed by distilled water and dried. Silver enhancement of the colloidal gold was carried out using the Biocell SE kit (British Biocell International) for 2 min at room temperature. Following a thorough washing with distilled water, the grids were counterstained with 2% aqueous uranyl acetate and lead citrate.

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