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Chitin-binding proteins in potato (*Solanum tuberosum* L.) tuber

Characterization, immunolocalization and effects of wounding

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Tubers of potato (*Solanum tuberosum* L.) contain a number of chitin-binding proteins which have possible functions in defence against pathogens. A major protein of the tuber is the chitin-binding lectin which has been further characterized with respect to its antigenicity and *N*-terminal amino acid sequence. By using an antiserum monospecific for tuber lectin in unwounded potato the protein was found in the cytoplasm and vacuole, unusually for a hydroxyproline-rich glycoprotein, but consistent with its soluble nature in subcellular extracts. Little increased synthesis of the lectin precursor or the post-translationally modified form could be demonstrated in excised potato tuber discs. However, after wounding there is increased synthesis of another hydroxyproline-containing glycoprotein of M_r 57000, which binds to chitin and shares common epitopes with the lectin. In comparison with the tuber lectin, this novel glycoprotein contains less hydroxyproline, but from its overall composition it is clearly not an underhydroxylated form of the tuber lectin. It differed in its *N*-terminal amino acid sequence and was much less glycosylated, although arabinose was still present. Synthesis of the M_r-57000 polypeptide began after the initial burst of protein synthesis and increased, reaching a peak at 24 h after wounding. The protein was produced with its enzymes of post-translational modification, prolyl hydroxylase and arabinosyltransferase, concomitantly with the marker enzymes for wounding, phenylalanine ammonia-lyase and membrane-bound phenol oxidase and peroxidase.

INTRODUCTION

A number of chitin-binding proteins have been identified in potato (*Solanum tuberosum*, L.) on the basis of their affinity, enzymic properties or amino acid sequence. These include the tuber lectin (Allen, 1983) chitinase (Schlumbaum *et al.*, 1986) and two wound-inducible gene products (Standford *et al.*, 1989). A number of roles for these products have been postulated, particulary in defence. A major chitin-binding component of the tuber is the lectin, which is a glycoprotein of M_r 50000 with an unusual composition, being particularly rich in hydroxyproline, cystine and glycine, and with more than 50% of its weight being carbohydrate (Allen & Neuberger, 1973). The lectin consists of at least two distinct domains: a binding-site region with a β-turn structure dependent upon disulphide bridges for its stability, and a highly glycosylated region with polyhydroxyproline II conformation (Allen *et al.*, 1978; Ashford *et al.*, 1982*a*; Van Holst *et al.*, 1986). It has thus been classified as a hydroxyproline-rich glycoprotein (HRGP; Showalter & Varner, 1988) on the basis of chemical analysis, whereas others have been identified from sequence analysis and include extensins and arabinogalactans and a new class of proline-rich proteins (Hong *et al.*, 1989; Datta *et al.*, 1989). All these HRGPS may have a role in wall structure and its modification in response to stress and pathogen attack.

The differential expression of these types of HRGPs is therefore of some interest. In this work, use has been made of polyclonal antibodies to the tuber lectin to immunolocalize and investigate changes in patterns of synthesis after wounding. The tuber lectin *per se* was not found to be induced, but a novel lectin HRGP of M_r 57000 has been identified and characterized as part of the wound response. The timing of the appearance of this lectin subunit has been compared in wounded tissues with that of its enzymes of post-translational modification and other enzymes of the wound response.

MATERIALS AND METHODS

Materials

Potato cultivars (King Edward, Ulster Sceptre and Cara) were obtained from local markets. All three cultivars show negligible differences in chemical analysis of lectin and cross-reactivity towards antisera (Ashford *et al.*, 1982*b*). Structural studies were carried out on the lectin prepared from the varieties showing highest abundance, whereas Cara was used in expression studies because of lower levels of production of phenolics whose presence in extracts prevented recovery of lectin subunits. Translabel (95% [^{35}S]methionine, 5% cystine) (39 TBq/mol) was obtained from ICN. ^{14}C-Labelled amino acid mix (1.85 GBq/mg-atom carbon) and [^{3}H]arabinose (700 GBq/mmol) were obtained from Amersham.

Isolation of lectin subunits

Native lectin was prepared from potato tubers of the Ulster Sceptre or other varieties by affinity chromatography (Desai & Allen, 1979; Desai *et al.*, 1983). The composition of the lectin

Abbreviations used: dopa, dihydroxyphenylalanine; HRGP, hydroxyproline-rich glycoprotein; PAL, phenylalanine ammonia-lyase; PVDF, poly(vinylidene difluoride); PBS, phosphate-buffered saline (20 mM-potassium phosphate/0.15 M-NaCl, pH 7.2); TBS, Tris-buffered saline (for composition, see the text).

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and its domains have been given previously (Allen *et al.*, 1978; Allen, 1983; Van Holst *et al.*, 1986).

Deglycosylated lectin was prepared from a batch of Ulster Sceptre tubers different from that used above. It was deglycosylated by the use of trifluoromethanesulphonic acid (Edge *et al.*, 1981). This procedure removed at least 99% of the carbohydrate, but did not result in any loss of amino acids or cleavage of the polypeptide chain.

Preparation of wounded potato discs and radioactivity incorporation *in vivo*

Batches of sliced potato discs (1 cm diameter; 2 mm thickness) were incubated in 2 ml of sterile distilled water in 5 cm-diameter Petri dishes. Dishes were maintained at 25 °C under illumination at 10000 lux. Discs were labelled with 1.85 MBq of Translabel/dish for 3 h before harvesting at each time point. Discs were washed with distilled water and then snap-frozen in liquid N_2.

Preparation of cell fractions and enzyme extracts

Non-radioactive discs were homogenized (1 g tissue/ml of buffer) in either 20 mM-potassium phosphate buffer, pH 7.2, containing 0.15 M-NaCl for peroxidase and phenolase, or 50 mM-Tris/HCl (pH 8.0)/2 mM-2-mercaptoethanol for phenylalanine ammonia-lyase (PAL). Extracts were centrifuged at 10000 *g* for 10 min and the supernatants assayed for enzyme activities immediately. Microsomal membranes were prepared from discs homogenized in 50 mM-Tris/HCl, pH 8.0, containing 25 mM-$MgCl_2$, 1 mM-dithiothreitol, 300 mM-KCl and 0.4 M-sucrose as described previously (Bolwell *et al.*, 1985). All extractions were performed at 4 °C. Radioactive discs were extracted for immunoprecipitation of proteins from supernatants, as described previously (Shaw *et al.*, 1990).

Enzyme assays

PAL was assayed by measuring the formation of cinnamic acid at 290 nm. Hydroxylation of poly-L-proline by proline,2-oxoglutarate dioxygenase was estimated by the stoichiometric decarboxylation of 2-[^{14}C]oxoglutarate (Bolwell *et al.*, 1985). Protein arabinosyltransferase activity in membrane fractions was measured by determining the initial rates of transfer of [^{3}H]-arabinose from UDP-β-L-[1-^{3}H]arabinose to endogenous proteinaceous acceptors (Bolwell, 1986). Phenolase [dihydroxyphenylalanine (dopa) oxidase] was measured spectrophotometrically at 470 nm as described by Bolwell & Butt (1983). Peroxidase was assayed by the method of Espelie *et al.* (1986).

Other assays

Protein was determined by the method of Read & Northcote (1981).

Immunoprecipitation

Extracts of radiolabelled discs were incubated with the respective antibody at a titre of 1:50 at 4 °C overnight. PAL subunits were precipitated using an anti-[French-bean (*Phaseolus vulgaris*) PAL] (Bolwell *et al.*, 1986) serum, which inhibits the potato enzyme (results not shown); prolyl-hydroxylase subunits were precipitated using a combination of anti-(French-bean hydroxylase) (Bolwell & Dixon 1986) and anti-(*Chlamydomonas* hydroxylase) (kindly given by Dr. Debbie Kaska, University of California, Santa Barbara, CA, U.S.A.) sera. Both cross-react with partially purified potato PAL subunits (results not shown). After overnight incubation at 4 °C, 4 mg of Protein A–Sepharose that had been preincubated in a buffer [20 mM-Tris/HCl (pH 7.8)/0.75 M-NaCl/1% Triton X-100], containing 1% (w/v) BSA, was added and the solution was further incubated for 2 h at room temperature. Immobilized complexes were harvested by centrifugation and washed three times with 1 ml of wash buffer before preparation for analysis by SDS/PAGE and fluorography.

Dot-blots

Solutions of the glycoproteins which were of interest were applied in 1 μl aliquots at dilutions of 1, 5, 25 and 125, to a grid marked on nitrocellulose paper (Schleicher and Schuell B85) and allowed to dry. The paper was blocked for 2 h in a solution of 2% defatted milk powder in phosphate-buffered saline (PBS), then immersed for 2 h in rabbit anti-(potato lectin) antiserum (Ashford *et al.*, 1982*b*), which had been diluted 1:200 in PBS, and then, after washing, immersed in ^{125}I-Protein A solution (10^6 d.p.m./ml) for a further 2 h. Finally the paper was washed for 3 h with PBS, dried, and exposed to pre-flashed Kodirex X-ray film for 1–7 days.

Western blots

Samples of potato lectin and deglycosylated lectin were subjected to SDS/PAGE. Polypeptides were then transferred to poly(vinylidene difluoride) (PVDF) paper (Immobilon; Millipore, Harrow, Middx., U.K.) using Caps buffer, pH 11.0, containing 10% (v/v) methanol for 1 h at 250 mA (Legendre & Matsudaira, 1988). The blots was treated with a blocking solution containing 1% (w/v) BSA in Tris-buffered saline I (TBS I; 20 mM-Tris/HCl, pH 7.5, containing 500 mM-NaCl) for 1 h. The blots were then incubated in TBS I/1% (w/v) BSA containing rabbit anti-(potato lectin) serum (1:200) for 2 h and then washed (4 × 15 min) in TBS I. The washed blots were incubated for 1 h with goat anti-rabbit IgG–alkaline phosphatase (Promega, Liverpool, U.K.) diluted 1:2000 in TBS I/1% (w/v) BSA. After washing (4 × 15 min in TBS I), immunodetection was carried out using 0.35 mM-Nitroblue Tetrazolium, 0.45 mM-5-bromo-4-chloroindol-3-yl phosphate in 50 mM-Tris/HCl (pH 9.0)/100 mM-$MgCl_2$. M_r values were determined by running parallel tracks containing rainbow markers (Amersham International). Blots were also stained with Aurodye (Amersham International).

Analysis of subunits

Immobilized anti-(tuber lectin) IgG was produced by the method of Bolwell *et al.* (1986). Immunopurification was also carried out as described by Bolwell *et al.* (1986), except that bound lectin was released in 50 mM-triethylamine, pH 11.2. Lectin subunits were recovered by freeze-drying, subjected to SDS/PAGE and blotted on to PVDF membrane (Legendre & Matsudaira, 1988).

Glycoprotein bands (200–1000 ng) that had been detected by Coomassie Blue staining or autoradiography were excised from PVDF paper, briefly immersed in methanol and then water and hydrolysed in 6 M-HCl for 16 h at 110 °C. Hydrolysates were analysed on an LKB Alpha-Plus Analyzer using sodium buffers and ninhydrin detection in accordance with the manufacturer's recommendations. Carbohydrates were analysed from the blotted bands by methanolysis in 1 M-methanolic HCl for 16 h at 90 °C followed by trimethylsilylation (Chambers & Clamp, 1971) and analysis, by capillary g.l.c. on a Varian 3300 gas chromatograph as described elsewhere (Allen *et al.*, 1991). *N*-Terminal amino acid sequencing was carried out on an ABI gas phase sequencer (model 470).

Preparation of tissue for immunomicroscopy

Wounded or unwounded potato-tuber material was fixed in freshly prepared 1% (v/v) paraformaldehyde/0.05% (v/v) glutaraldehyde in 0.05 M-sodium phosphate buffer, pH 6.8, at 4 °C for 2 h. After an overnight incubation in 0.05 M-sodium phosphate buffer, pH 6.8, the tissues were dehydrated through

graded alcohols and placed in several changes of hydrophilic resin [three parts of LRGold (London Resin Co, Woking, Surrey, U.K.), two parts GMA glycol methacrylate (low acid; Polysciences, Warrington, PA, U.S.A.) and 0.1% (v/v) benzoin ethyl ether]. The tissue was finally embedded in gelatin capsules or flat-bottomed BEEM (Taab Laboratories, Aldermaston, Berks., U.K.) capsules and polymerized for 24 h at room temperature by illumination with u.v. light at 360 nm.

Immunostaining/optical microscopy

Clean dry microscope slides were prepared by dipping them into a solution (3%, v/v, in pure water) of Ortho Tissue Adhesive (Ortho Diagnostics, High Wycombe, Bucks., U.K.), the excess solution allowed to drain and the slides dried overnight at 40 °C. Sections (1 μm) of tuber were cut on a glass knife and transferred to drops of water on a prepared slide. The water was evaporated slowly using a warm hotplate and sections were kept overnight at 30 °C. Immunogold labelling was carried out by first incubating sections at 37 °C in 20 μl of 3% (v/v) ovalbumin in 50 mM-Tris buffer, pH 7.4, containing 0.15 M-NaCl (TBS I), for 30 min. Excess solution was then drained from the sections and replaced with 20 μl of anti-(potato lectin) serum [1:4000 (v/v) dilution in TBS I] for 60 min at 37 °C. They were then washed thoroughly and repeatedly for 5 min in TBS I, pH 7.4, and the area around the sections carefully dried. Sections were immersed in 20 μl of goat anti-rabbit IgG conjugated to colloidal gold (Janssen; ICN Biomedical, High Wycombe, Bucks., U.K.; 5 nm particle size, 1:200 working solution in TBS, pH 7.4) for 30 min at 37 °C and then washed as described above, followed by a final rinse in distilled water.

Silver enhancement was then carried out using a modification of the method of Danscher (1981). This method employed incubation of slides with a mixture of 2 ml of 1 M-sodium citrate buffer, pH 3.5, 3 ml of 30% (w/v) gum Arabic (Sigma), 1.5 ml of 100 mM-silver lactate, freshly prepared, and 1.5 ml of 50 mM-hydroxyquinoline, freshly prepared, in total darkness. The exact incubation time was determined by periodic monitoring of the enhancement reaction under the optical microscope. Sections were revealed under confocal optics (Bio-Rad; model MRC500).

Immunostaining/electron microscopy

Ultrathin sections of tuber tissue were collected on Formvar (2% in amyl acetate)-coated nickel grids and placed in 10 μl aliquots of 1% (w/v) ovalbumin in 0.2 M-Tris/HCl (pH 7.4) 450 mM-NaCl (TBS II) for 20 min at 37 °C. The grids were then transferred without washing to 10 μl aliquots of rabbit anti-(potato lectin) serum (1:4000 in TBS II containing 0.1% Tween 20) for 60 min at 37 °C. Grids were then washed thoroughly with TBS II and then incubated with goat anti-rabbit IgG colloidal gold at 1:200 dilution in TBS II/ovalbumin for 30 min at 37 °C. The grids were then washed thoroughly with TBS II followed by distilled water and dried. Silver enhancement of the colloidal gold was carried out using IntenSE II (Janssen) for 2–4 min at room temperature. After a thorough washing with distilled water the grids were counterstained with aq. 2% uranyl acetate and lead citrate.

RESULTS

Immunological studies

The anti-serum to potato lectin was previously described by Ashford *et al.* (1982*b*) and has been shown to recognize both a glycosylated and a non-glycosylated domain. Immunodiffusion tests showed that the antibodies did not cross-react with any proteins, other than the lectin, which were present in aqueous extracts of potato tubers. In dot-blots (Fig. 1) native lectin and its *S*-carboxylmethylcysteinyl derivative gave very strong reactions, the deglycosylated lectin gave a much weaker reaction and an M_r-42000 French-bean chitin-binding protein also gave a weak, but positive, reaction. However, an extensin preparation from bean showed very poor cross-reactivity.

After SDS/PAGE on 10% gels and transfer to PVDF paper, potato lectin polypeptide that had been affinity-purified on immobilized chitin oligomers from unwounded tuber discs, were detected with the antiserum. Native lectin comprises a lower-M_r (90000) component together with higher-M_r aggregates, all of which react on Western-blot analysis (Fig. 2). The deglycosylated lectin was detected with a lower M_r of about 70000 attributable to the material being completely devoid of carbohydrate.

Immunocytochemical localization

No binding of gold particles were observed when tissue was incubated with preimmune serum and second-antibody–gold complexes or with second antibody alone. Similarly, no binding was found using antiserum which had been absorbed out with

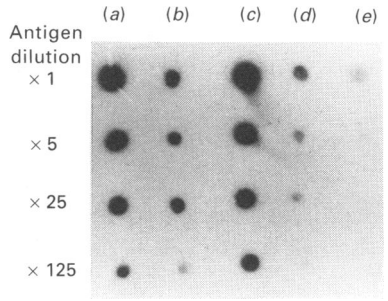

Fig. 1. Dot-blot of polypeptide antigens to anti-(potato lectin) serum

For dot-blots, antigens were as follows: (*a*) *S*-carboxymethylcysteinyl-lectin (1 μg); (*b*) deglycosylated lectin (2 μg); (*c*) native lectin (1 μg); (*d*) 42 kDa arabinosylated-HRGP lectin from bean (1 μg; Bolwell, 1987); (*e*) crude extensin preparation from bean prepared by the method of Stafstrom & Staehelin (1988) (1 μg).

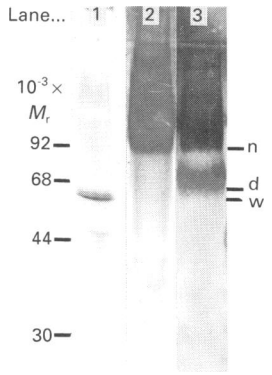

Fig. 2. Western analysis of cross-reactive antigens separated on a 10%-acrylamide gel and transferred to the same PVDF membrane from control and wounded tubers

Immunopurified subunits were prepared and treated as follows. Lane 1, autoradiograph of ^{35}S-subunits radiolabelled between 21 and 24 h after excision and immunopurified. Lane 2, native potato tuber lectin, and lane 3, deglycosylated potato tuber lectin from unwounded tissue, detected using antiserum at 1:1000 dilution and alkaline phosphatase-conjugated goat anti-rabbit IgG as second antibody (because the lectins stain poorly on blots). The immunopurified ^{35}S-subunit from wounded tuber (w) has a lower M_r than native (n) or deglycosylated (d) tuber lectin.

native lectin subunits (10 mg of lectin/ml of antiserum used at 1:2000 dilution).

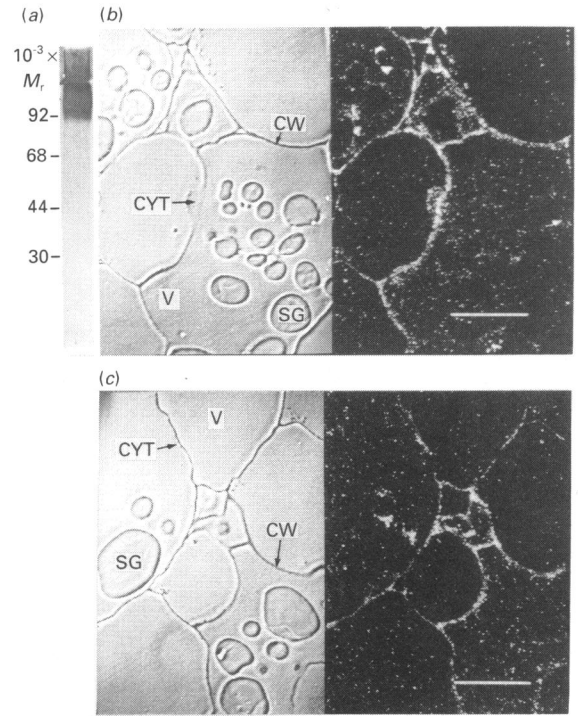

Fig. 3. Immunolocalization of tuber lectin: optical microscopy

(*a*) Western blot of total cell protein separated on a 12%-acrylamide gel. Note lack of lower-M_r cross-reacting material. (*b*, *c*) Confocal imaging (left), phase-contrast/immunogold deposition (right) in unwounded tuber parenchyma. Abbreviations: CW, cell wall; SG, starch grain; V, vacuole; CYT, cytoplasm. Note immunogold deposits, especially in the vacuole, and heavy deposition associated with parietal cytoplasm at the inner face of the wall. The scale bar indicates 50 μm.

A Western blot of total soluble cell protein is shown in Fig. 3(*a*) and confirms the monospecific nature of the anti-lectin serum. The distribution of potato lectin was determined in unwounded tissue by immunogold localization and observed by optical microscopy with silver enhancement (Figs. 3*b* and 3*c*), The level of binding to unwounded tissue was most dense in the region of parietal cytoplasm and the cell wall. However, there was also substantial binding in the large vacuole when observed under confocal optics.

When gold complexes were examined in the electron microscope (Fig. 4) the association with a layer of denser cytoplasm on the inner surface of the cell wall and vacuole in the unwounded tissue was resolved, but, unlike other hydroxyproline-rich glycoproteins such as extensin (Stafstrom & Stahelin, 1988), little binding was seen in the wall. Although this lack of association with wall found in the microscopic analysis may be a result of lectin constituting an intrinsic wall protein in which the epitopes are masked by an association with other wall polymers and are inaccessible to detection by the methods used, it is highly consistent with the ease of extraction of the lectin, which is readily solubilized from potato tuber (Desai & Allen, 1979). Close inspection of the electron micrographs shows that some of the silver particles in the cytoplasm were associated with structures that resemble membranes and vesicles. These results constitute a new location for an HRGP. Other confirmatory evidence that largely rules out non-specific binding of serum components is shown in French-bean tissue, where the anti-(potato lectin) serum was found to be cross-reactive with a known wall protein (Fig. 1) and bound to internal wall material, thus showing a different distribution (result not shown).

Effect of wounding on chitin-binding proteins

When material was purified from wounded potato-tuber discs 24 h after excision by affinity chromatography on chitin oligomers, a concentration of material of average M_r 57000 was observed in addition to the tuber lectin on immunodetection (Fig. 5). A Western blot of immunopurified lectins from wounded

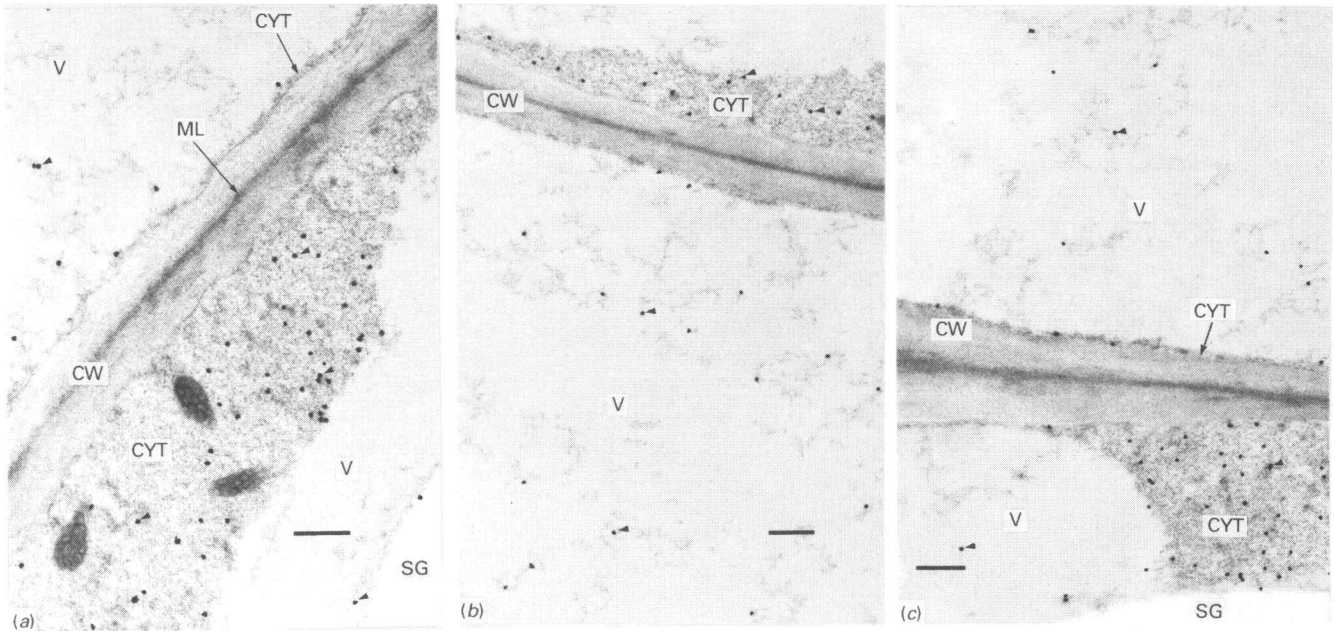

Fig. 4. Immunolocalization of tuber lectin: electron microscopy

(*a–c*) Unwounded tissue. Abbreviations: CW, cell wall; ML, middle lamella; CYT, cytoplasm; SG, starch grain; V, vacuole. Arrows indicate examples of gold-particle deposition. The scale bar indicates 1 μm. Magnifications: (*a*) 20000×; (*b*) 16000×; (*c*) 16000×.

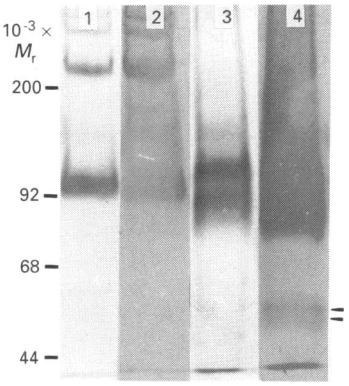

Fig. 5. Western analysis of chitin-binding proteins from control and wounded tubers

Chitin-binding proteins were prepared from extracts of unwounded and wounded tuber discs by affinity chromatography, analysed on the same 7.5%-acrylamide gel, and blot-transferred to PVDF membrane. Duplicate tracks from unwounded (1 and 2) and wounded (3 and 4) tissues were stained with Aurodye (Amersham) (1 and 3) or immunodetected with anti-(tuber lectin) serum (2 and 4). The M_r-57000 polypeptides (arrowed), which stain poorly, are readily detected with the antibody (4) or when radiolabelled (Fig. 2, lane 1).

Table 1. Comparison of the amino acid and monosaccharide compositions of potato chitin-binding lectins

The composition of the tuber lectin has been recalculated from the results of Allen (1983). The composition of tuber lectin polypeptides blotted on to PVDF was found to be identical, showing a lack of artefacts (cf. Ozals, 1990). The composition of the M_r-57000 polypeptide is the mean of five determinations from up to 5 μg of blotted protein. Abbreviation: n.d., not determined.

Amino acid or monosaccharide	Composition (mol/100 mol of amino acid)	
	Tuber lectin	M_r-57000 polypeptide
Hyp	20.4	7.9
Asx	4.5	6.3
Thr	5.7	2.2
Ser	10.2	4.3
Glx	8.6	10.0
Pro	9.0	10.1
Gly	11.8	19.2
Ala	4.1	8.8
Cys	10.6	2.8
Val	0.8	5.0
Met	0.4	n.d
Ile	1.6	1.9
Leu	1.2	3.1
Tyr	2.5	0.3
Phe	0.2	2.3
His	0.4	0.9
Lys	4.1	2.7
Trp	3.3	n.d.
Arg	0.8	5.2
GlcNAc	0	1.2
Ara	70.0	< 1.0
Gal	4.0	2.5
Man	0	2.5

tuber radiolabelled between 21 and 24 h after excision is also shown in Fig. 2. The newly synthesized isolectin is confirmed as

Table 2. *N*-Terminal amino acid sequence of chitin-binding proteins

The *N*-terminus of the tuber lectin was determined from native and deglycosylated lectin and a tryptic peptide with initial yields of 62, 75 and 43 pmol respectively. The first eight residues of 28 that have been sequenced for the *N*-terminus of deglycosylated tuber lectin are shown. The M_r-57000 lectin has only been sequenced as far as the eighth residue and had a clearly different *N*-terminal sequence which was not present in any database inspected. The initial yield was 10 pmol. X, unidentified residue; O, hydroxyproline.

Protein	Residue...	Amino acid							
		1	2	3	4	5	6	7	8
Tuber lectin		D	A	S	T	O	S	P	P
M_r-57000 polypeptide		P	X	D	M	V	Y	A	N

of lower M_r (57000) when compared with that for the tuber lectin or the chemically deglycosylated form separated on the same blot (Fig. 2). It was clearly of lower M_r than the tuber lectin, which was the only species isolated from dormant tuber. These experiments also show that the M_r-57000 polypeptide is a lectin by virtue of its chitin-binding properties and is not a degradation product of the tuber lectin, since it is newly synthesized at this M_r value.

Immunolocalization of the epitope in wounded tissue showed a large increase in binding especially in the vacuole (results not shown). However, since multiple proteins are recognized in wounded tissue, predictions about the relative distribution are not valid.

Composition and analysis of the M_r-57000 subunit

Immobilized anti-(potato lectin) IgG was used to purify the M_r-57000 polypeptide from wounded tubers, which was then subjected to blotting on to PVDF, stained with Coomassie Blue and radioactive bands detected by autoradiography. The requisite bands were excised and subjected to amino acid analysis (Table 1) and *N*-terminal-sequence analysis (Table 2). Tuber lectin was prepared similarly from unwounded tuber, and the applicability of the methodology was checked by comparing the analysis of the blotted immunopurified tuber lectin with affinity-purified lectin that had not been blotted on to PVDF membrane. Compositions of amino acid and sugars were very similar in this case. However, amino acid analysis showed that, whereas the M_r 57000 polypeptide contained only 7% hydroxyproline, its overall composition clearly showed that it is not an underhydroxylated form of the tuber lectin and differs considerably. It was also relatively very low in arabinose. In fact it showed greater similarity to the protein from bean (Bolwell, 1987), with which the antiserum is cross-reactive. The M_r-57000 component is therefore a novel wound-inducible chitin-binding protein distinct from, but sharing epitopes with, the tuber lectin. Attempts to epitope-map the M_r-57000 protein in comparison with the native lectin did not succeed, since both lectins were refractory to trypsin or *Staphylococcus aureus* V8 proteinase during analysis by Cleveland mapping. However, there was also no sequence similarity in the *N*-terminal amino acid sequence (Table 2), although the possibility remains that the M_r-57000 protein could be an *N*-terminal cleavage product that is produced so extremely rapidly that the radiolabelled precursor is not seen.

Changes in the rates of synthesis of proteins in response to wounding

Changes in the rates of the synthesis of individual proteins were measured by immunoprecipitation of labelled enzyme

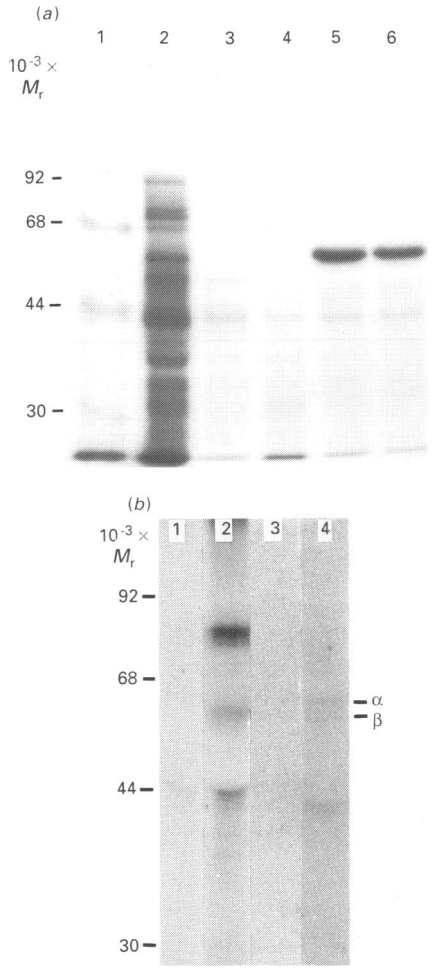

Fig. 6. Analysis of subunits immunoprecipitated by homologous and heterologous antisera from extracts of ^{35}S-labelled potato tuber tissue

(*a*) Track 1, markers; 2, total proteins synthesized 21–24 h after excision; tracks 3–6: 3, M_r-57000 lectin polypeptides immunoprecipitated from soluble extracts of discs labelled 0–3 h after excision; 4, as 3, extracts 9–12 h after excision; 5, immunoprecipitated M_r-57000 polypeptides from soluble extracts of discs labelled 21–24 h after excision; 6, as 5, soluble extracts 33–36 h after excision. (*b*) Tracks 1 and 2, PAL subunits immunoprecipitated from soluble extracts of discs labelled (1) 0–3 h after wounding, (2) 21–24 h after wounding. Tracks 3 and 4, prolyl hydroxylase subunits immunoprecipitated from soluble extracts of discs labelled (3) 0–3 h or (4) 21–24 h after wounding.

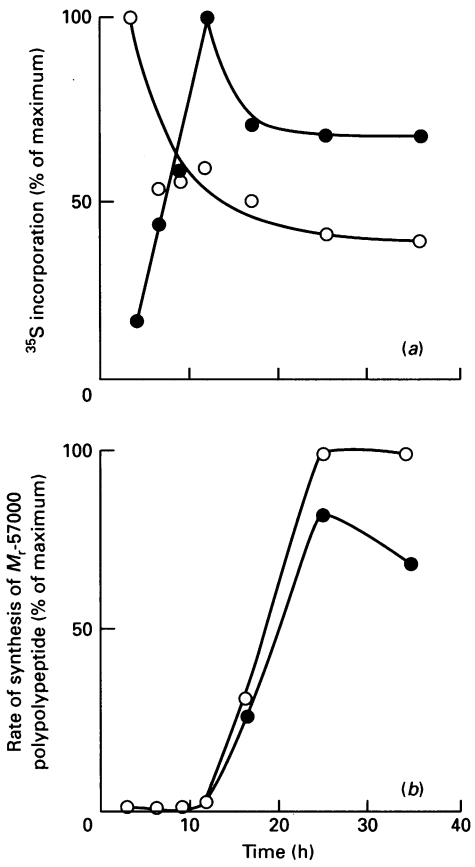

Fig. 7. (*a*) Changes in rates of protein synthesis during pulse-labelling of excised tuber discs and (*b*) time-course induction of the synthesis of protein subunits of the M_r-57000 lectin

(*a*) Discs were pulse-labelled for 3 h before extraction and determination of (○–○) relative total uptake of Translabel into tissue and (●–●) relative incorporation into material insoluble in 10% (w/v) trichloroacetic acid. (*b*) (○) Total detergent-soluble material and (●) material soluble in extraction buffer. Discs were pulse-labelled for 3 h before harvesting and immunoprecipitations were performed on cell extracts for each time point indicated. The level of incorporation was determined (by densitometry at 310 nm) for each immunoprecipitated band. Incorporations were corrected for total uptake of radiolabel into the tissue and for incorporation into total protein. Incorporations were then normalized with respect to maximum levels for each subunit.

subunits from extracts of tuber discs that had been exposed to 1.85 MBq of Translabel for 3 h immediately before harvesting. Use of Translabel was essential for the potato lectin, since it contains only one methionine residue (although it is rich in cystine). Polypeptides were immunoprecipitated by homologous lectin antiserum and heterologous lectin antiserum and heterologous cross-reacting antisera for PAL and prolyl hydroxylase (Figs. 6*a* and 6*b*). All three polypeptides were found to be precipitated. PAL (M_r 83000) and the M_r 57000 lectin showed relatively much greater abundance than the hydroxylase (M_r 65000/60000). The M_r-60000 subunit of the hydroxylase is a protein disulphide-isomerase in animal systems; such an activity is probably required for cross-linking of the cysteine-rich domain and the establishment of lectin activity.

Wounding induces general protein synthesis in excised tuber discs (Fig. 7*a*). Pulse-labelling studies show that, although the amount of uptake in each 3 h pulse fell, incorporation into total protein reached a maximum at 12 h after excision. However, no synthesis of the M_r-57000 lectin was observed at this time (Fig. 7*b*). Indeed, rates of synthesis for all three components reached similar maximum incorporation into each subunit and are shown in Figs. 7 and 8. Newly synthesized M_r-57000 polypeptides could be immunoprecipitated from total and soluble cell fractions in a way that also suggests they are not particularly associated with the wall like other HRGPs. No radiolabelled subunits of M_r-90000 were immunoprecipitated, even when ^{14}C-labelled amino acids or [^{3}H]arabinose were used to label discs to high specific radioactivity (results not shown). This was used to check whether lack of observed induction was due to lack of methionine content in the lectin and/or low incorporation of cystine. There was no induction of the tuber lectin, and the induced form is a novel species in wounded tissue.

Changes in some of the enzyme activities associated with wounding are also shown in Fig. 8. The induction kinetics for PAL in this tissue are similar to those already reported (Borchert,

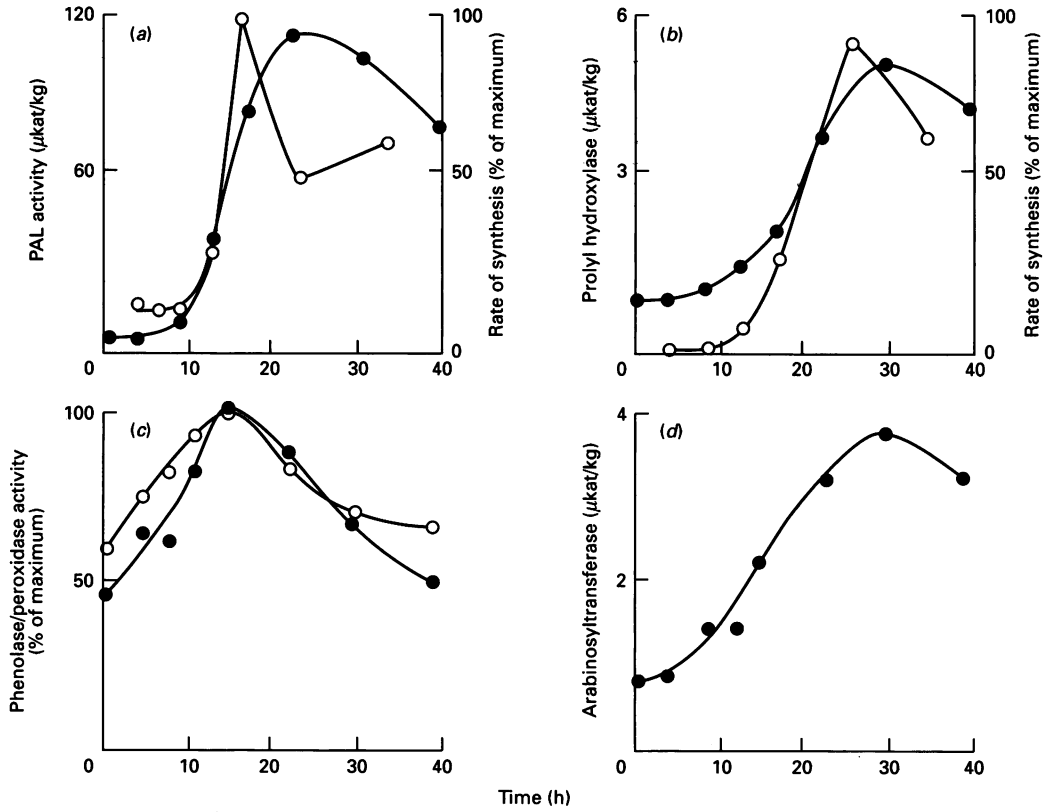

Fig. 8. Changes in the activities of wound-specific enzymes and enzymes related to the post-translational modification of potato lectin

Potato discs were excised and the enzyme activities determined subsequently on extracts corresponding to the time points shown. (*a*) ●, PAL activity; ○, rate of synthesis; (*b*) ●, prolyl hydroxylase activity; ○, rate of synthesis; (*c*) ●, microsomal peroxidase; ○, phenolase; (*d*) microsomal arabinosyltransferase. Rates of synthesis were calculated as in Fig. 7(*b*).

1978) and are included here to study the level of co-ordination of the induction. Both the activities of prolyl hydroxylase and protein arabinosyltransferase increased in microsomal fractions extracted from wounded tubers with a time course similar to the appearance of glycosylated lectin subunits of M_r 57000. These changes were co-ordinated with membrane-bound wound-induced peroxidase and phenolase activities.

DISCUSSION

Chitin-binding proteins have a probable role in plant defence. The relative roles of lectins, chitin-binding proteins and chitinases have not been fully elucidated (Boller, 1985; Schlumbaum *et al.*, 1986; Broekaert *et al.*, 1988; Roby *et al.*, 1990), but present knowledge suggests that they are differentially expressed in response to different forms of stress such as mechanical wounding and pathogenic attack. In potato, the tuber lectin differs from the other chitin-recognizing proteins, since it is an HRGP-requiring extensive post-translation modification catalysed by prolyl 4-hydroxylase and arabinosyltransferase within the endomembrane system (Allen, 1983; Bolwell, 1988; Showalter & Varner, 1988). However, the lectin does not seem to be an integral wall protein to the same extent as other HRGPs, since it can readily be isolated in soluble extracts in low-salt buffers (Desai & Allen, 1979) and it can thus be only loosely associated at the very least. Although previous evidence purports to indicate that potato lectin is associated with both soluble and wall fractions when isolated from aerated potato discs (Casalongue & Pont Lezica, 1985), a comparison of the overall cellular distribution of immunoreactive lectin using immunogold labelling with silver enhancement showed dense concentrations associated with the parietal cytoplasm in the region of the cell wall and less densely in the vacuole in unwounded tuber when observed by confocal optics. When observed at the subcellular level in the electron microscope, these localizations were clarified, subunits appearing to be largely confined to a layer of dense cytoplasm apposed to the inner wall surface and plasmalemma and in the vacuole. Although it is possible that some lectin molecules may be present in structures deeper in the wall but are inaccessible to antibody detection without experimental modification of the tissue, this indicates a novel localization for an HRGP. In common with other glycoproteins it is probable that lectin subunits are synthesized, modified and transported through the endomembrane system, but this does not preclude transport to the vacuole. The presence in the cytoplasm is less easily explained, but may reflect the paucity of observed membrane structures in these storage-tissue cells. Close inspection shows the association of cytoplasmic silver particles with discernible membrane and vesicle structures. The validity of these studies depends on the specificity of the antibody for lectin. Two obvious candidates for cross-reactivity would be arabinose residues on polysaccharides, and other HRGPs. The former is unlikely, since the linkages observed in polysaccharides are different from those found in the lectin. The latter is also unlikely. Ashford *et al.* (1982*b*) found no evidence for the presence of any extensin-like macromolecules that were cross-reactive in tuber tissue examined. It is probable that putative extensins are sufficiently divergent or in relatively low abundance in solanaceous species. Sequence data available (C. Sidebottom, A. R. Slabas, A. K. Allen & G. P. Bolwell, unpublished work) and those presented, combined with the amino

acid analysis of the domains, give little scope for the existence of the same repeated sequences, as have been found in the many extensin-like HRGPs now sequenced (Showalter & Varner, 1988; Varner & Lin, 1989; Cooper, 1988), so that the arabinose residues may also be presented as a different structural epitope although containing similar linkages. The antiserum was only weakly cross-reactive with a bean extensin fraction.

The lack of association with the wall contrasts with the localization of a number of glycoprotein components in cells of other species using immunogold technologies. The extensins (Stafstrom & Staehelin, 1988; Mauch & Staehelin, 1989) have been shown to be largely located in the wall. In these studies, components are seen to be asymmetrically deposited and associated with differing layers. In actively secreting tissues these macromolecules can also be observed within the structures of the endomembrane system. In a relatively dormant tissue such as potato tuber there is not enough membrane present to confirm such a secretory pathway, but it is probably responsible for the synthesis, modification and secretion of the lectin subunits. There must be additional controls and factors required for the final destination in the cell. Association with cytoplasm and vacuole would be in full accordance with a defensive role, since chitinases and β1-3 glucanases show this distribution (Mauch & Staehelin, 1989).

We have carried out further biochemical studies utilizing this antiserum which, in Western blots, recognizes M_r-90000 glycosylated and M_r-70000 deglycosylated forms of the lectin as well as lectins from other species of the family Solanaceae with common epitopes (Ashford *et al.*, 1982*b*). Upon wounding, cross-reacting lectins exhibit variable M_r values on gels, and polypeptides newly synthesized in excised tuber discs were found to be of M_r 57000. Previous work (Ashford, 1982; Ashford *et al.* 1982*b*) has shown that, after potato lectin had been removed from the soluble extracts of potato tubers by affinity chromatography (using a chitin-oligosaccharide column; Desai & Allen, 1979), no further precipitation with the antigen could be seen. This indicated that molecules (such as HRGPs) which might have similar epitopes to those of potato lectin were not present in the soluble extract of the tubers. The M_r-57000 protein was found to be a lectin, binding to immobilized chitin oligomers, and is not present to any great extent in unwounded tuber. There are clearly distinct differences in the amino acid and sugar compositions, even though the novel lectin shares epitopes with both native and deglycosylated tuber lectin. They also diverged in *N*-terminal sequences, although there is a possibility that the M_r-57000 isolectin may be an *N*-terminal cleavage product of the native lectin. The tuber lectin may therefore be a part of the passive defence of the tuber and the novel lectin one of the subset of stress-response gene products and therefore part of active defence. Further support for this is shown by the induction of the tuber lectin during tuber development (Pont Lezica *et al.*, 1991) and the fact that it is not expressed in cultured cells of potato (N. N. Desai & A. K. Allen, unpublished work).

Wounding of potato tuber tissues brings about an increase in a number of proteins whose accumulation depends upon a process of gene activation, as has been established for the proteinase inhibitor (Peña-Cortes *et al.*, 1988) and anionic peroxidase (Roberts *et al.*, 1988). In general, some of these proteins are involved in suberization of the tissue that occurs at the wound site as part of a battery of defence responses, of which the induction of PAL is an essential component (Butler *et al.*, 1990; Shaw *et al.*, 1990; Rumeau *et al.*, 1991). We have shown that increases in lectin subunits, together with its enzymes of post-translational modification, prolyl hydroxylase and arabinosyl transferase, accompany and are co-ordinately induced with PAL, peroxidase and phenolase activities. The soluble enzyme activities (Borchert, 1978) increase co-ordinately with the components involved in the long-term defence responses, whereas the activities of membrane-bound oxygenases may be a part of a more rapid response involving membrane changes (Bolwell, 1988; Apostol *et al.*, 1989). The inducibility of the M_r-57000 lectin together with its properties as a potential microbial agglutinin demonstrate it to be involved in defence mechanisms, although other aspects, particularly with regard to the existence and comparative role of other chitin-binding proteins, as discussed above, require elucidation. Subcellular localization is particularly relevant in regard to such roles (Mauch & Staehelin, 1989), but requires a specific antibody for the M_r-57000 isolectin.

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REFERENCES

Allen, A. K. (1983) in Chemical Taxonomy, Molecular Biology and Function of Plant Lectins (Goldstein, I. J. & Etzler, M. E., eds.), pp. 71–85, Alan R. Liss, New York
Allen, A. K. & Neuberger, A. (1973) Biochem. J. **135**, 307–314
Allen, A. K., Desai, N. N., Neuberger, A. & Creeth, J. M. (1978) Biochem. J. **171**, 665–674
Allen, A. K., Ellis, J. & Rivett, D. E. (1991) Biochim. Biophys. Acta **1074**, 331–333
Apostol, I., Heinstein, P. F. & Low, P. S. (1989) Plant Physiol. **90**, 109–116
Ashford, D. (1982) Ph.D. Thesis, University of London
Ashford, D., Desai, N. N., Allen, A. K., Neuberger, A., O'Neill, M. A. & Selvendran, R. R. (1982*a*) Biochem. J. **201**, 199–208
Ashford, D., Allen, A. K. & Neuberger, A. (1982*b*) Biochem. J. **201**, 641–645
Boller, T. (1985) Molecular Biology of Plant Stress (Key, J. L. & Kosuge, T., eds.), pp. 247–262, A. R. Liss, New York
Bolwell, G. P. (1986) Phytochemistry **25**, 1807–1813
Bolwell, G. P. (1987) Planta **172**, 184–191
Bolwell, G. P. (1988) Phytochemistry **27**, 1235–1253
Bolwell, G. P. & Butt, V. S. (1983) Phytochemistry **22**, 37–45
Bolwell, G. P. & Dixon, R. A. (1986) Eur. J. Biochem. **159**, 163–169
Bolwell, G. P. Robbins, M. P. & Dixon, R. A. (1985) Biochem. J. **229**, 693–699
Bolwell, G. P. Sap, J., Cramer, C. L., Lamb, C. J., Schuch, W. & Dixon, R. A. (1986) Biochim. Biophys. Acta **881**, 210–221
Borchert, K. (1978) Plant Physiol. **62**, 789–793
Broekaert, W. F., Van Parijs, J., Allen, A. K. & Peumans, W. J. (1988) Physiol. Mol. Plant. Pathol. **33**, 319–331
Butler, W., Cook, L. & Vayda, M. E. (1990) Plant Physiol. **93**, 264–270
Casalongue, C. & Pont Lezica, R. (1985) Plant Cell Physiol. **26**, 1533–1539
Chambers, R. E. & Clamp, J. R. (1971) Biochem. J. **125**, 1009–1018
Cooper, J. (1988) in Temporal and Spacial Regulation of Plant Genes (Verma, D. P. S. & Goldberg, R. B., eds.), pp. 235–251, Springer, Vienna
Danscher, G. (1981) Histochemistry **71**, 81–88
Datta, K., Schmidt, A. & Marcus, A. (1989) Plant Cell **1**, 945–952
Desai, N. N. & Allen, A. K. (1979) Anal. Biochem. **93**, 88–90
Desai, N. N., Allen, A. K. & Neuberger, A. (1983) Biochem. J. **211**, 273–276
Edge, A S. B., Faltynek, C. R., Hof, L., Reichert, L. E. & Weber, P. (1981) Anal. Biochem. **118**, 131–137
Espelie, K. E., Francheschi, V. R. & Kolattukudy, P. E. (1986) Plant Physiol. **81**, 487–492
Hong, J. C., Nagao, R. T. & Key, J. L. (1989) Plant Cell **1**, 937–943
Legendre, N. & Matsudaira, P. (1988) Biotechniques **6**, 154–159
Mauch, F. & Staehelin, L. A. (1989) Plant Cell **1**, 447–457
Ozals, J. (1990) in Protein Purification (Deutscher, M. P., ed.), pp. 587–601, Academic Press, San Diego, New York, Berkeley, Bonn, London, Sydney, Tokyo and Toronto
Peña-Cortes, H., Sanchez-Serrano, J., Rocha-Sosa, M. & Willmitzer, L. (1988) Planta **174**, 84–89

Pont-Lezica, R. F., Taylor, R. & Varner, J. E. (1991) J. Plant Physiol. **137**, 453–458
Read, S. M. & Northcote, D. H. (1981) Anal. Biochem. **116**, 53–64
Roberts, E., Kutchan, T. & Kolattukudy, P. E. (1988) Plant. Mol. Biol. **11**, 15–26
Roby, D., Broglie, K., Cressman, R., Biddle, P., Chet, I & Broglie, R. (1990) Plant Cell **2**, 999–1007
Rumeau, D., Maher, E. A., Kelman, A. & Showalter, A. M. (1991) Plant Physiol. **93**, 1134–1139
Shaw, N. M., Bolwell, G. P. & Smith, C. G. (1990) Biochem. J. **267**, 163–170
Schlumbaum, A., Mauch, F., Vogeli, U. & Boller, T. (1986) Nature (London) **342**, 365–367
Showalter, A. M. & Varner, J. E. (1988) in Biochemistry of Plants: A Comprehensive Treatise, vol. 15 (Molecular Biology) (Marcus, A., ed.), pp. 485–520, Academic Press, San Diego
Stafstrom, J. P. & Staehelin, L. A. (1988) Planta **174**, 321–332
Standford, A., Bevan, M. & Northcote, D. (1989) Mol. Gen. Genet. **215**, 200–208
Van Holst, G. J., Martin, S. R., Allen, A. K., Ashford, D., Desai, N. N. & Neuberger, A. (1986) Biochem. J. **234**, 481–484
Varner, J. E. & Lin, L. S. (1989) Cell (Cambridge, Mass.) **56**, 231–239

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