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Secretion of stress-related proteins by suspension-cultured *Lupinus albus* cells[o]

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A suspension culture of white lupin cells has been established, and proteins of the exocellular matrix analysed. Based on homologies of N-terminal amino-acid sequences, three stress- or defence-related proteins: acidic class III chitinase, polygalacturonase-inhibiting protein, and germin/oxalate oxidase, secreted by lupin cell culture, were identified.

The plant exocellular matrix (ECM) [1] is a dynamic structure and its composition is altered in response to various internal and/or external stimuli. It changes during expansion growth, differentiation into different cell types, and in response to environmental stress and pathogen attack. In plant-pathogen interactions, this often results in the formation of the barrier "papilla" structure limiting the spread of pathogen to the site of infection [2]. On the other hand, successful establishment of plant-microbe symbiotic interaction requires formation of a new apoplastic compartment [3]. Although in some cases preexisting ECM components are used, generally most of the observable changes resides in gene activation *de novo*, and post-translational protein processing [2].

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Abbreviations: ECM, exocellular matrix; LCA, ionically-bound lupin cell wall proteins; LCF, lupin wall proteins secreted into culture media; PGIP, polygalacturonase-inhibiting protein; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis.

Various aspects of the biology of plant species from genus *Lupinus*, especially those related to secondary metabolism [4] or to symbiotic interactions [5, 6], are considerably different from those commonly observed within *Leguminosae*. However, no data are as yet available on the interactions of lupins with pathogenic microbes. To study these issues in more detail, a model system of suspension-cultured white lupin (*Lupinus albus* L.) cells has been established, and the ECM composition analysed. Major ECM proteins were isolated and subjected to N-terminal amino acid sequencing. In this paper we report on identification of constitutively secreted exocellular stress- or defence-related proteins in the absence of a pathogen or pathogen-derived signalling molecule by suspension-cultured lupin cells.

MATERIALS AND METHODS

Derivation and maintenance of lupin suspension culture. White lupin (*Lupinus albus* L. cv Bac) callus was initiated from pieces of roots of 3-day-old seedlings on the Murashige & Skoog [7] medium supplemented with 2% (w/v) sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin, and 0.6% (w/v) agar, and incubated at 24°C in the dark. After 2 passages, a growing callus tissue was transferred to the same medium without agar and cultures were maintained at 22–24°C on an orbital shaker at 120 r.p.m. in the dark. An optimal growth of the culture was obtained on the Murashige and Skoog medium supplemented with 3% (w/v) sucrose, 2 mg/l 2,4-D, and 0.2 mg/l kinetin. Cells were subcultured biweekly, and since initiation above 50 passages were performed. The cells of 4–5 day-old cell culture were analysed.

Isolation and characterisation of exocellular proteins. Cell cultures were filtered on a funnel through Miracloth. Ionically-bound cell wall proteins were isolated by the $CaCl_2$ elution method, which does not affect integrity of the cells [8], and proteins secreted into culture media were recovered by precipitation with trichloroacetic acid as described by Wojtaszek *et al.* [9]. Protein content was determined using dye-binding assay [10] with bovine serum albumin as a standard. Proteins (30- or 10-μg samples) were separated using discontinuous SDS/polyacrylamide gels [11], and detected with Coomassie Brilliant Blue or silver staining, respectively. For sequencing, SDS/PAGE-separated proteins were electroblotted onto polyvinylidene difluoride membranes using semi-dry transferring system, and 50 mM borate buffer, pH 9.0, containing 20% (v/v) methanol. The excised protein bands were subjected to N-terminal sequencing using a gas-phase sequencer (model 477A; Applied Biosystems, U.S.A.). Sequence homology was searched using all the major sequence databases through BLITZ [12], and BLAST+BEAUTY [13,14] network servers.

RESULTS AND DISCUSSION

Two subsets of cell wall proteins: soluble proteins secreted into culture media, and ionically-bound wall proteins washed off with $CaCl_2$ from intact cells, were isolated from suspension-cultured lupin cells. Both extracts were separated by SDS/PAGE (Fig. 1). The pattern of ionically-bound proteins revealed about 30 protein bands with the major ones (designated LCA) of 51, 33, and 27 kDa (Fig. 1A). Only a few protein bands were observed in culture filtrates with the dominating proteins (designated LCF) of 27, 33, and 41 kDa (Fig. 1B). Major proteins were subjected to N-terminal amino acid sequencing following their separation by SDS/PAGE and electroblotting. These included: LCA51 and LCF27 – the dominant protein bands in the cell wall extract and culture filtrate, respectively, LCF41 – another major protein secreted into culture medium, and LCA33 – the protein band found in both exocellular compartments (Fig. 1). No sequence was obtained

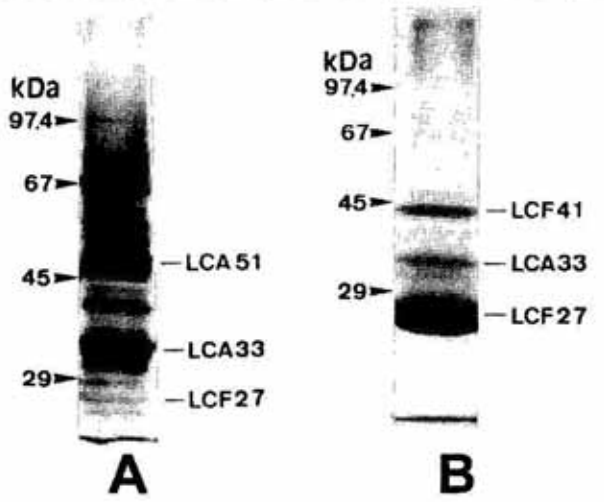

Figure 1. Patterns of exocellular proteins isolated from cell walls of intact lupin cells or culture filtrates.

A. Extracted cell wall proteins (10 μg) were separated on 10% (w/v) SDS/polyacrylamide gel and visualised by silver staining. B. 30 μg of culture filtrate proteins were separated on 12% (w/v) SDS/polyacrylamide gel and stained with Coomassie Brilliant Blue. The distribution in both exocellular compartments and positions of protein bands subjected to N-terminal amino acid sequencing are indicated and designated as described in the text. Note that LCF27 is poorly stained with silver and the intensity of this band in (A) does not reflect its true abundance in cell wall protein extract.

for LCF41 protein. The remaining three proteins were sequenced, and based on similarity searches were found to correspond to: chitinase, polygalacturonase-inhibiting protein and germin/oxalate oxidase, known as the plant stress- or defence-related proteins (Table 1).

Thirty N-terminal amino acids were sequenced for LCF27 protein. When compared with sequence databases, this protein revealed high sequence similarity to class III acidic exocellular chitinases and dual function chitinase/lysozymes. As expected the highest similarity was found with the respective enzymes from other legumes: *Cicer arietinum* ($P = 3 \times 10^{-14}$; [15]), *Vigna angularis* ($P = 5.2 \times 10^{-12}$; Ishige, Mori, Yamazaki, Imaseki, unpublished; PIR, accession: S36932), *Vigna unguiculata* ($P = 3.2 \times 10^{-11}$; Vo, Broughton, Krause, unpublished; PIR, accession: S57-468), and *Psophocarpus tetragonolobus* ($P = 4.8 \times 10^{-11}$; Esaka, Teramoto, unpublished; DDBJ: accession: D49953). The same protein (designated IF3) was identified recently in the intercellular spaces of stems and roots of healthy white lupin plants, and in culture media of lupin cell culture [16]. Although the sequence of the first 16 amino acids was demonstrated in this culture, our results revealed that, apart from longer amino acid sequence, two ambiguous residues have now been identified (Asn-12 and Ser-15).

Table 1. Summary of the N-terminal amino acid sequencing of exocellular proteins isolated from cell walls and culture media of suspension-cultured white lupin cells.

Respective protein fractions were separated by SDS/PAGE, blotted onto PVDF membranes and stained with Coomassie Brilliant Blue. Major lupin proteins, indicated on Fig. 1, were excised and subjected to amino acid sequencing on an Abi 477A Protein Sequencer. The possible identity of the sequenced proteins was determined on the basis of similarity searches done using BLITZ and/or BLAST + BEAUTY network servers.

Protein	Initial yield (pmol)	Sequence	Identification
LCA51	38	D L C N P Q D K K V L L Q I K K D L N N	Polygalacturonase-inhibiting protein (PGIP)
LCA33	61	S D P D P L Q D F N V A D L T S V V K	Germin/oxalate oxidase
LCF41	–	N-terminus blocked	–
LCF27	620	A G I V I Y W G Q N G N E G S L A D A X N T N N Y Q Y V N I	Acidic extracellular chitinase

The N-terminal sequence of LCA51 protein (Table 1) indicated its high similarity to polygalacturonase-inhibiting proteins (PGIPs) identified in various dicotyledoneous plants. Again the highest sequence similarity was observed with two PGIPs isolated from leguminous plants: *Phaseolus vulgaris* ($P = 1.2 \times 10^{-5}$; [17]) and *Glycine max* ($P = 4.2 \times 10^{-5}$; [18]), and also with PGIPs from *Citrus sinensis* ($P = 1.7 \times 10^{-5}$; Mayer, unpublished; EMBL, accession: Y08618) and *Lycopersicon esculentum* ($P = 2.3 \times 10^{-5}$; [19]). Interestingly, a high degree of similarity ($P = 2.0 \times 10^{-3}$) was noted also with the product of FIL2 gene from *Antirrhinum majus* shown to be a leucine-rich repeat protein [20] corroborating recent data that PGIPs belong to the class of plant proteins specialised in recognition of pathogen signals [21].

A similarity search for the N-terminal sequence of LCA33 protein (Table 1) – obtained from the band excised from the blots of $CaCl_2$ protein extracts, gave rather unexpected results since LCA33 appeared to be a putative analogue of germin-like oxalate oxidases thought to be specific for monocotyledonous species. The levels of sequence similarities were reasonably high giving: $P = 1.4 \times 10^{-3}$ for *Triticum aestivum* pseudogermin [22], $P = 1.5 \times 10^{-2}$ for germin/oxalate oxidase from *Hordeum vulgare* [23], $P = 3.0 \times 10^{-2}$ for *H. vulgare* germin-like protein (Zhang, Wie, Collinge, Smedegard-Petersen, Thordal-Christensen, unpublished; EMBL, accession: X93171), and $P = 4.0 \times 10^{-2}$ for germin precursor GF-2.8 from *T. aestivum* [24, 25].

Although both chitinase and PGIP were individually found in the suspension-cultured cells, to our knowledge this set of proteins, including germin-like oxalate oxidase, has not been demonstrated previously as being constitutively secreted together by a single suspension cell culture in the absence of externally applied stress factor, either biotic or abiotic. Moreover, acidic chitinase and thaumatin-like antifungal protein were also found in the intercellular fluids of healthy white lupin plants [16], while PGIP was identified in roots of white lupin [26]. The occurrence of those proteins in healthy plants or suspension cultures cannot be easily explained, particularly if their participation in plant response to stress in general, and to microbial infection in particular, would be considered as the only one aspect of their activity. The evidence originating from the studies on plant-microbe symbiotic interactions [3, 27] or somatic embryogenesis [28] suggest that these proteins might act in regulation of plant growth and development. Moreover, this role for germins as wall-associated protein markers of wheat embryo development has been already established [29]. Studies aiming at elucidation of these issues are now in progress. It is intended however that this early presentation of N-terminal amino acid sequences of lupin exocellular proteins, especially germin, will hasten the identification and cloning of respective genes and enable a deeper insight into the putative dual function of defence-related proteins.

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