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

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Received: 20 June, 1997; revised: 07 October, 1997; accepted: 20 November, 1997

Key words: cell wall proteins, extracellular matrix, *Lupinus albus*, stress-related proteins, suspension culture, wall protein secretion

A suspension culture of white lupin cells has been established, and proteins of the extracellular 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 extracellular 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 pre-existing ECM components are used, generally most of the observable changes resides in gene activation de novo, and post-translational protein processing [2].

*\(^\ast\)This work was funded by the State Committee for Scientific Research Grant 6 P04C 107 08 to P.W.*
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**Abbreviations:** ECM, extracellular 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 extracellular 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 extracellular proteins.** Cell cultures were filtered on a funnel through Miracloth. Ionically-bound cell wall proteins were isolated by the CaCl₂ elution method, which does not affect integ-
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 × 10^{-14}; [15]), *Vigna angularis* (P = 5.2 × 10^{-12}; Ishige, Mori, Yamazaki, Imaseki, unpublished; PIR, accession: S38932), *Vigna unguiculata* (P = 3.2 × 10^{-11}; Vo, Broughton, Krause, unpublished; PIR, accession: S57468), and *Psophocarpus tetragonolobus* (P = 4.8 × 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 result 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.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Initial yield (pmol)</th>
<th>Sequence</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCA51</td>
<td>38</td>
<td>D L C N P Q D K R V L L Q I K K D L N N</td>
<td>Polygalacturonase-inhibiting protein (PGIP)</td>
</tr>
<tr>
<td>LCA33</td>
<td>61</td>
<td>S D P D P L Q D F N V A D L T S V V K</td>
<td>Germin/oxalate oxidase</td>
</tr>
<tr>
<td>LCF41</td>
<td>–</td>
<td>N-terminus blocked</td>
<td></td>
</tr>
<tr>
<td>LCF27</td>
<td>020</td>
<td>A G I V I Y W G Q N G N E C S L A D A X Y N N Y Q Y V N I</td>
<td>Acidic extracellular chitinase</td>
</tr>
</tbody>
</table>
The N-terminal sequence of LCA51 protein (Table 1) indicated its high similarity to polygalacturonase-inhibiting proteins (PGIPs) identified in various dicotyledonous plants. Again the highest sequence similarity was observed with two PGIPs isolated from leguminous plants: *Phaseolus vulgaris* (P = 1.2 x 10^{-5}; [17]) and *Glycine max* (P = 4.2 x 10^{-5}; [18]), and also with PGIPs from *Citrus sinensis* (P = 1.7 x 10^{-5}; Mayer, unpublished; EMBL, accession: Y08618) and *Lycopersicon esculentum* (P = 2.3 x 10^{-5}; [19]). Interestingly, a high degree of similarity (P = 2.0 x 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 CaCl2 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 x 10^{-3} for *Triticum aestivum* pseudogermin [22], P = 1.5 x 10^{-2} for germin/oxalate oxidase from *Hordeum vulgare* [23], P = 3.0 x 10^{-2} for *H. vulgare* germin-like protein (Zhang, Wie, Collinge, Smedegard-Petersen, Thordal-Christensen, unpublished; EMBL, accession: X93171), and P = 4.0 x 10^{-2} for germin precursor GF.2.8 from *T. aestivum* [24, 25].

Although both chitinase and PGIPs 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.

We thank Chris Gerrish at Division of Biochemistry, Royal Holloway, University of London for protein sequencing.

**REFERENCES**


