Proteomic analysis reveals a novel set of cell wall proteins in a transformed tobacco cell culture that synthesises secondary walls as determined by biochemical and morphological parameters

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Abstract. A cell suspension culture of a tobacco (Nicotiana tabacum L. cv. Petit Havana) cell line derived from a cultivar transformed with the Tcyl gene from Agrobacterium, which leads to high endogenous levels of cytokinin, has been established. This cell line shows increased cell aggregation, elongated cells and a 5-fold increase in wall thickness. If allowed to carry on growing it can form a single mass without shedding cells into the medium. When analysed at an earlier growth stage, these cultures were found to produce improved levels of vascular nodule formation than in other systems that employ exogenous cytokinin. This differentiation was optimised with respect to sucrose and auxin signals in order to induce maximum production of cells with thickened walls and a morphology characteristic of fibre cells and tracheids, in addition to cells that remain meristematic. In order to establish the validity of this system for studying secondary wall formation, the walls and associated biosynthetic changes were analysed in these cells by chemical analysis of the walls, changes in activities of enzymes of xylan and monolignol synthesis, and expression of mRNAs coding for enzymes of lignin biosynthesis. The wall composition of the transformed cells was compared with that determined for primary walls from a typical untransformed tobacco cell line. Recovery of wall material was 50% greater in the transformed culture. In this material a major difference was found in the pectin fraction where there was a distinct difference in size distribution together with a lower level of methylation for the transformed line, which may be related to increased adhesiveness. There were increased amounts of xylan, although the ratio of xyloglucan to xylan content was not substantially different due to the mixture of cell types. There was also an increase in cellulose and phenolic components. Increased activity of enzymes involved in the synthesis of xylan as a marker for the secondary wall occurred around the time of tracheid differentiation and coincided with a broad peak of cinnamyl alcohol dehydrogenase activity. The expression of mRNAs coding for enzymes of the general phenylpropanoid pathway, phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, catechol O-methyltransferase was relatively constitutive in the cultures while transcripts of ferulate 5-hydroxylase, cinnamoyl CoA-reductase, cinnamyl alcohol dehydrogenase and lignin peroxidase were induced. The walls of the transformed cells also showed considerable differences in the subset of extractable proteins from that found in primary walls of tobacco when these were subjected to proteomic analysis. Many of these proteins appear to be novel and not present in primary walls. However an M₉⁻32,000 chitinase, an M₉⁻34,000 peroxidase, an M₉⁻65,000 polyphenoloxidase/laccase and possibly an M₉⁻68,000 xylanase could be identified as well as structural proteins.

Key words: Nicotiana (differentiation) – Cytokin – Secondary cell wall – Polysaccharide – Cell wall proteins – Lignin

Introduction

The plant cell wall is now recognised as a dynamic structure having roles not restricted to the maintenance of shape and rigidity (Bolwell 1993). The cell wall changes in composition during expansion growth, differentiation into specific cell types and during responses to environmental stress and pathogen attack (Fry 1988; Bolwell 1993; Roberts 1994; Sakurai 1998). It also constitutes a source of information on the form of
intercellular signalling molecules involved in self and non-self recognition. Biochemical analysis of localised changes in the wall components in the plant is, however, complicated by the existence of different tissue types. Similarly, understanding the regulation of these processes is complex due to the multiplicity of endogenous and exogenous signals. In the case of vascular differentiation, and in particular xylemogenesis, it is possible to achieve the sequential extraction of differentiating layers from poplar to allow mRNA extraction and construction of an expressed sequence tag (EST) data base (Sterky et al. 1998). These types of studies are not easily duplicated in other important faster-growing model species due to the accessibility and amount of available differentiating tissue. On the other hand, analysis of tissue layers in tree species is essentially descriptive and limited experimentally. Hence tissue culture systems that model developmental changes in a dynamic way make an important contribution to molecular studies of vascular differentiation.

As suspension-cultured cells have primary cell walls that are similar to those found in the meristematic cells of the plant, they can be used to study some of the changes dependent upon individual signals. Extensive morphological changes, such as xylemogenesis, can be modelled in a number of cell culture systems (Bolwell and Robertson 1999), the most spectacular of which is the cytokinin-induced differentiation of _Zinnia_ mesophyll cells (Fukuda 1996, 1997). Other changes can be modelled. Thus the walls of elongated and spherical cell lines have been compared (Suzuki et al. 1990). Similarly, changes in wall components due to the action of stress-inducing compounds such as elicitors (Bolwell et al. 1985; Wojtaszek et al. 1995) and salt (Iriki et al. 1989) have also been analysed. Thus phenotypic changes due to cell wall modification that would be difficult to explore in the intact plant can become accessible in such systems.

Tobacco has been adopted as a model for study of the effects of modification of lignification as much of the preliminary work was assessed in this species before transfer of the technology to tree species such as poplar and Eucalyptus (Boudet 1998). Understanding and manipulation of the process of secondary wall formation as a whole is desirable, however, and would have major implications for forestry and pulp and paper making processes in the foreseeable future (O’Connell et al. 1998). Thus a tobacco cell culture system would have use for identifying genes and proteins specific for vascular differentiation and secondary wall formation. The constitutive expression of the _Tcry_ gene derived from _Agrobacterium_ has profound morphological effects on tobacco (Memelink et al. 1987). When induced into suspension culture, the cells show different morphology in comparison to normal tobacco cell lines and the transformed cell line grows as gigantic masses. By manipulating the sucrose content of the medium, cells can be produced with very thick walls and a substantial number of cells differentiate into tracheids. The endogenous expression of high cytokinin levels avoids the metabolism associated with exogenous cytokinin that reduces the efficiency of other model systems (Bolwell and Robertson 1999) and enables the study of the effects of cytokinin on wall biosynthesis, which would be experimentally difficult in the intact plant. Large amounts of cells can also be obtained, which facilitates protein work. The present study characterises some of the changes in biosynthesis in the tobacco culture and shows that the walls contain a set of proteins very different from that seen in primary walls of tobacco cells in a previous study (Robertson et al. 1997).

Materials and methods

Plant material

Seeds of tobacco _Nicotiana tabacum_ L. cv. Petit Havana (SR1) were germinated aseptically and the plant line maintained by cuttings which were transferred to solid LS-medium (Linsmaier and Skoog 1965) without growth factors. The transformed line was derived as described previously (Memelink et al. 1987) and was a kind gift from Dr. J. Memelink and Professor J.H.C. Hoge, Leiden University, The Netherlands. The line was also maintained as green plantlets on solid LS-medium.

Derivation and maintenance of suspension cultures

Callus cultures were derived from stems of untransformed plants and maintained aseptically. These callus cultures and the resultant suspension cultures originating from this callus were both maintained on MS-medium (Murashige and Skoog 1962) supplemented with 10 mg/l cysteine, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 3% (w/v) sucrose. The transformed suspension cultures were derived from the green callus material surrounding the minute plantlets and maintained on the same medium as untransformed cells.

Phenotypic observations and electron microscopy

Clump sizes were determined using a binocular microscope, which was also used to count lignified vascular nodules following staining with phloroglucinol (saturated phloroglucinol in 6 M HCl). Numbers of tracheids were determined following disruption of the clumps. Samples of 100 mg FW of cells were incubated overnight in 0.1 M EDTA at 60 °C. The clumps were gently macerated and sonicated for 1 s. Cells were then harvested by centrifugation at 2,500 g for 5 min. The supernatant was removed and cells stained with 500 μl 0.04% Safranin O at 50 °C for 30 min. Cells were de-stained for 1 h using 1 M HCl at 50 °C. The numbers of tracheary elements were then counted. For embedding in epoxy resin for transmission electron microscopy, suspension-cultured cells were harvested by gentle aspiration and rapidly fixed by vacuum infiltration with 4% (w/v) paraformaldehyde and 3% (v/v) glutaraldehyde in 50 mM Pipes-KOH buffer, pH 7.2 (1 h, room temperature), and post-fixed in 1% (w/v) OsO4 in the same buffer for 1 h. Following dehydration in a graded series of ethanol, the tissue was rinsed in a propylene oxide, infiltrated for 16 h with propylene oxide:TAAB resin (TAAB Laboratories, Berks., UK) mixture (1:1, v/v), and with pure TAAB resin for 8 h. Finally, cells were embedded in BEEM capsules (TAAB Laboratories), and polymerised for 24 h at 60 °C. Sections were counterstained with uranyl acetate and lead citrate and observed on a Hitachi H 600 transmission electron microscope operating at 75 kV.