ULTRASTRUCTURAL AND BIOCHEMICAL ASPECTS OF CELL WALL RECONSTITUTION IN RECALCITRANT (GRAPEVINE) AND REGENERATING (TOBACCO) LEAF PROTOPLASTS

K. C. KATSIRDakis AND K. A. ROUBELAKIS-ANGELAKIS

Department of Biology, University of Crete, and Institute of Molecular Biology and Biotechnology, P.O. Box 1470, 711 10 Heraklio, Crete, Greece

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SUMMARY

To identify possible reasons that may contribute to recalcitrance in plant protoplasts, the time course of new cell wall deposition was studied by scanning electron microscopy in protoplasts of a recalcitrant species, the grapevine. Results showed that microfibrils were developed after 2 days of culture, that complete cell wall formation occurred on Day 6 to 7 of protoplast culture, and its ultrastructural appearance was identical to that of grapevine leaf-derived callus cells. In addition, a comparative study was undertaken on [U-14C]glucose uptake and incorporation in ethanol-soluble, cellulosic, and noncellulosic polysaccharide fractions in protoplasts of grapevine and of a readily regenerating species, tobacco, during culture. There was a significantly higher [U-14C]glucose uptake by tobacco than by grapevine protoplasts. The label distribution in the ethanol-soluble, cellulosic, and noncellulosic fractions of newly synthesized cell walls differed quantitatively between the two species. In particular, the labeled glucose incorporated in the noncellulosic cell wall fraction was threefold greater in tobacco than in grapevine protoplasts. Differences were also revealed in the monosaccharide composition of this fraction between the two species. Addition of dimethyl sulfoxide to the culture medium resulted in a dramatic increase in [U-14C]glucose uptake by grapevine protoplasts, whereas it exhibited a limited effect in tobacco protoplasts. It showed no effect on the ultrastructural characteristics of new cell wall nor on the incorporation rate of labeled glucose in the cellulosic and noncellulosic cell wall fractions.

Key words: grapevine protoplasts; Vitis; scanning electron microscopy; cellulosic; noncellulosic; cell wall; dimethyl sulfoxide.

INTRODUCTION

Protoplast technology can offer solutions to improving plants if generation of plants from modified protoplasts can be achieved. However, in most of the important agricultural plant species, such as most of perennials and cereals, plant regeneration has not yet been achieved. The reasons and mechanism(s) that contribute to the regeneration process of protoplasts are largely unknown.

Grapevine (Vitis spp) is one of the widely cultivated perennial plant species; plant regeneration from grapevine protoplasts has not yet been achieved. Previous work from this laboratory has developed a protocol for isolation of protoplasts from axenic leaves of grapevine plantlets grown in vitro (Theodoropoulos and Roubelakis-Angelakis, 1990); those protoplasts showed higher viability rates during culture and a greater readiness to divide than protoplasts from greenhouse or field-grown Vitis plants. In addition, by examining culture conditions, such as pH of the culture medium, osmoticum, and auxin:cytokinin ratio, a modified culture medium has been formulated that promotes cell wall regeneration (Katsirdakis and Roubelakis-Angelakis, 1992). Furthermore, protoplasts from in vitro cultured plants exhibited high uptake rates of amino acids and sugars from the culture medium and therefore misfunctioning of the plasma membrane did not seem to contribute to their recalcitrance (Theodoropoulos and Roubelakis-Angelakis, 1989; 1991).

Formation of a new cell wall in protoplasts is one of the many events that take place during the dedifferentiation phase of the regeneration process and before the induction of cell division. The deposition of fibrils on the surface of protoplasts from a variety of plant species has been visualized in many cytological studies (e.g., Willison and Groot, 1978). Criteria such as positive Calcofluor white staining have frequently been used as evidence of the cellulose nature of these fibrils; however, those criteria are not entirely rigorous; Calcofluor white is not specific for β-1,4 glucan, and polysaccharides, other than cellulose, may appear fibrillar (Darken, 1961). Also, electron microscopy studies of sectioned material can only yield limited information about the first stages of wall formation (Burgess and Fleming, 1974). Scanning electron microscopy (SEM) is one of several adequate methods for studying the effects of physical or chemical treatments on fiber formation because it is a sensitive method for detecting such fibers (Brown et al., 1983; Burgess et al., 1978; Seitz et al., 1985).

Protoplasts from a suspension culture of grapevine cells Vitis vinifera L. cv Muller Thurgau produced short fibrils after 48 h but these fibrils failed to elongate. The walls after 5 days in culture were...
atypical (Burgess and Linstead, 1976). In cell walls regenerated by protoplasts other than from Vitis, the cellulose nature of the fibrils has been substantiated by incorporation of radioactivity from labeled precursors, and total noncellulosic cell wall material has been subjected to an analysis of monosaccharide composition (Asamizu and Nishi, 1980; Blaschek et al., 1981; Mock et al., 1990; Takeuchi and Komamine, 1978). Hahne and Hoffman (1984) suggested that irregularities in the cytoskeleton of isolated protoplasts from Nicotiana plumbaginifolia, N. tabacum L., Petunia hybrida Hort., and Brassica napus L. reduce the capacity for wall synthesis and subsequent cell division. They demonstrated that cultivation of protoplasts in 2% dimethyl sulfoxide (DMSO) restored competence for protoplast division. However, Ishii (1988) added DMSO to the culture medium of rice protoplasts at the range of 0.1 to 5% without increasing the plating efficiency of the protoplasts. DMSO is a solvent that easily penetrates the membranes and it is widely employed as a carrier for many water-insoluble chemicals (Osborn and Weber, 1980).

In an attempt to further elucidate potential causes of recalcitrance, the time-course of new cell wall synthesis was followed by SEM in the presence and absence of DMSO in recalcitrant protoplasts (grapevine). In addition, tobacco protoplasts were used as a model system of regenerating protoplasts, and a comparative study was performed on the [U-14C]glucose uptake and incorporation in the ethanol-soluble, cellululosic, and noncellulosic cell wall fractions in grapevine and tobacco protoplasts. Finally, the monosaccharide composition of the noncellulosic cell wall material from the two species was identified and quantitated.

**Materials and Methods**

**Protoplast isolation and culture.** Protoplasts were isolated from leaves of in vitro cultured plants as previously described (Theodoropoulos and Roubelakis-Angelakis, 1990). Freshly isolated protoplasts were counted and plated at a density of 3 x 10^5 protoplasts/ml, in 30 x 10-mm petri dishes in 2 ml of grapevine cell wall regeneration (GCWR) culture medium (Katsiradakis and Roubelakis-Angelakis, 1992). The culture medium was sterilized by autoclaving for 20 min at 121°C. Protoplasts were cultured at 25°C in the dark. In addition, protoplasts were isolated from leaves of tobacco seedlings (N. tabacum cv Xanthi), grown in a controlled-temperature greenhouse by applying the same isolation and culture procedures. For tracer experiments, protoplasts were resuspended after the isolation at a density of 3 x 10^5 protoplasts/ml in GCWR medium supplemented with 1 µCi/ml [U-14C]glucose, with specific activity of 270 µCi/mmol. For DMSO experiments, anhydrous sterile filtered DMSO (Sigma Chemical Co., St. Louis, MO), at the range of 0.2 to 1% (vol/vol) was added to the culture medium.

**Scanning electron microscopy.** Aliquots of cultured protoplasts in defined medium were centrifuged and washed well in 0.7 M mannitol. The pellets were fixed with a double volume of 2.5% (vol/vol) glutaraldehyde in 0.7 M mannitol for 2 h at room temperature. The fixed protoplasts in suspension were filtered through a silver Nuclepore membrane using a Nuclepore filter holder (Watson et al., 1980). The suspension was pipetted onto the filter through the top of the holder with a Pasteur pipette. An air-filled syringe was then attached to gently force the protoplasts onto the filter. Protoplasts on membranes were washed thoroughly in 0.7 M mannitol and postfixed for 1 h in 1% osmium tetroxide at room temperature and washed well. For dehydration and drying, a Nuclepore membrane with fixed protoplasts was sandwiched together with a clean membrane and placed in a porous processing basket. Then a graduated ethanol concentration of 30, 50, 70, 80, 90, and 100% (vol/vol) was used. Time of immersion in each concentration was 10 to 15 min. Liquid CO2 was used for critical point drying. Segments of membrane were mounted on stubs with conductive paint, coated with gold, and examined in a Joel 840 scanning electron microscope at 10 kV.

**Cell wall isolation.** Protoplasts were collected by centrifugation for 3 min at 120 g, were washed 4 times with cold culture medium, disrupted in 80% (vol/vol) ethanol, and stored at 4°C overnight. The precipitate was collected by centrifugation for 10 min at 2000 g. A 1-ml aliquot from the supernatant was saved for determination of radioactivity. This constituted the 80% ethanol-soluble cytoplasmic content. The residual material was washed twice with 2 ml 80% (vol/vol) cold ethanol, and the last precipitate was considered as the crude cell wall fraction (Zhong and Lauchli, 1988). The cell wall preparation obtained gave a negative iodine test. These results indicated that the contamination by starch in the cell wall preparation was negligible. Noncellulosic polysaccharides were separated from cellululosic polysaccharides using an acetic-nitric reagent according to the method of Updegraff (1969). A 1-ml aliquot from the supernatant was saved for determination of radioactivity in the noncellulosic polysaccharides fraction. The acetic-nitric insoluble material was considered as cellululosic polysaccharides fraction and was collected after two subsequent washes with 2 ml of deionized water.

Radioactivity in each sample was determined by liquid scintillation spectrometry using a scintillation cocktail of 633 ml Toluene, 5.5 g 2,5 diphenyloxazole, 0.1 g [bis (2,5, diphenyl oxazolyl) benzene] and 330 ml Triton-X 100. Total uptake of glucose was calculated by adding the radioactivity of the three fractions. The data presented are from one of three similar experiments, the results of which showed a very similar pattern of response to the treatments, both in uptake of [U-14C]glucose and in the distribution of 14C in the fractions. For statistical analysis one-way analysis of variance (ANOVA) and F test were performed by using a computer package (Statgraph).

**Analysis of sugars.** The acidic and neutral sugar content of the regenerated cell wall of protoplasts and the distribution of radioactivity in sugar residues of the ethanol precipitable materials was determined after hydrolysis in 2 M trifluoroacetic acid at 120°C for 60 min. The hydrolysates were analyzed by thin layer chromatography (TLC) on cellulose plates using solvent butan-1-ol:acetic acid:water 3:1:1 (vol/vol) followed by solvent ethyl acetate:pyridine:water 10:4:3 (vol/vol) in the same dimension, and stained with orcinol ferric chloride. Spots were scraped from the plate and suspended directly in scintillation fluid for counting. All experiments were repeated 3 to 4 times and the data of one characteristic experiment in triplicate are presented in the relevant tables or figures. The results of a series of experiments differed no more than 10%.

**Results**

The distinct cytologic stages during protoplast culture in grapevine and tobacco appear in Table 1.

**Table 1**

<table>
<thead>
<tr>
<th>Cytological Phase at Which Majority of Cells are Found</th>
<th>Approximate Number of Culture Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protoplast expansion</td>
<td>Nicotiana Vitis</td>
</tr>
<tr>
<td>Loss of spherical shape</td>
<td>1-2</td>
</tr>
<tr>
<td>Chloroplastic migration to surround nucleus</td>
<td>2-6</td>
</tr>
<tr>
<td>Nuclear division</td>
<td>4-6</td>
</tr>
<tr>
<td>Plating efficiency, %</td>
<td>7.5 ± 2.5</td>
</tr>
</tbody>
</table>