Scanning Electron Microscopy of Cell Wall Formation Around Isolated Plant Protoplasts

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Summary. The process of cell wall regeneration around two species of higher plant protoplasts has been studied using reflection scanning electron microscopy. The first stage in the process is the formation of short fibres from randomly spaced centres. With protoplasts of tobacco leaf (Nicotiana tabacum L., cv White Burley) these fibres then elongate and interlace apparently at random to give rise to a matted continuous layer of wall. Protoplasts of a suspension culture of grapevine cells (Vitis vinifera L. cv Müller Thurgau) produce short fibres but these fail to elongate. Budding is observed during wall regeneration around vine protoplasts. The results are discussed in terms of the mechanical properties of the wall and its relationship to changes in plasmalemma morphology which are observed during wall formation.

Introduction

Protoplasts of several species of higher plants are capable of regenerating a cell wall which is competent to contain the expansion of the protoplast and allow cell division and transfer to osmotically unbuffered media (Nagata and Takebe, 1971; Constabel et al., 1973; Poirier-Hamon et al., 1974; Fowke et al., 1974). In other species the wall is in some way incompetent and division and development of the protoplasts does not occur, or occurs in a very small percentage of individuals only (Horine and Ruesink, 1972; Pearce and Cocking, 1973; Skene, 1974). Failure of prolonged culture is frequently associated with the observation of "bud" formation as the first sign of regeneration of a wall like structure (Horine and Ruesink, 1972; Pearce and Cocking, 1973, Hanke and Northcote, 1974). Bud formation has been explained (Hanke and Northcote, 1974) in terms of an incompetent wall failing to accommodate expansion due to growth of the protoplast; the plasmalemma is extruded through a weakened part of the wall and so forms a bud. Subsequent buds are then more likely to form on the initial bud (Bawa and Torrey, 1971).

Conventional electron microscopy of sectioned material can only yield limited information about the first stages of wall formation (Burgess and Fleming, 1974). Techniques for visualising the surface of protoplasts such as freeze-etching have suggested that the microfibrils may not arise from one end by growth but rather crystallise and "lift out of the membrane surface" (Grout, 1975). This is in contradiction to the observations of Robinson and Preston (1971) and Brown and Montezinos (1976) on cellulose biosynthesis in algae. This paper presents results obtained by examining whole fixed protoplasts by scanning electron microscopy. This enables early stages of wall formation to be detected and also confirms the mechanism of bud formation in non-competent protoplasts.

Materials and Methods

a) Protoplast Preparation

Protoplasts from tobacco leaves (Nicotiana tabacum L., cv White Burley) and a tissue culture of grapevine (Vitis vinifera L., cv Müller Thurgau) were prepared as described previously (Burgess and Linstead, 1976). Leaf material was surface sterilised and enzyme mixtures sterilised by Millipore filtration (Burgess et al., 1973). Protoplasts were cultured in the medium of Nagata and Takebe (1971), containing 6 mg/l 1-naphthyl-acetic acid, 1 mg/l 6-benzyl-aminopurine and 100 ml/l coconut milk.

b) Electron Microscopy

Protoplasts were fixed in glutaraldehyde, postfixed in osmic acid and dehydrated into amyl acetate through an alcohol series (Bur-
gess et al., 1973). Samples were critical point dried from carbon
dioxide using amyl acetate. Dried protoplasts were mounted on
stubs using double sided sellotape and then coated with carbon
and gold. The gold was evaporated from a heated tungsten wire
whilst the stub was rotated. Specimens were examined in a JEOL
JEM 100B fitted with ASID scanning attachment. The accelerating
voltage was 20 kV. Photographs were recorded on Ilford HP4 roll
film and developed in Ilford Microphen developer for 9 min at
20°C.

Results

a) Freshly Isolated Protoplasts

Newly isolated protoplasts of tobacco leaf and vine
tissue culture cells present a quite distinct appearance
in SEM. The protoplasts of tobacco leaf are easily
recognisable by the presence of chloroplasts lying be-
neth the surface of the plasmalemma (Fig. 1). The
finer structure of the surface itself is variable between
different protoplasts of the same preparation and be-
tween different preparations. It is largely featureless,
but may show “holes” (Fig. 2) or projections (Fig. 3).
These projections may consist of large or small
spheres or clumps of more amorphous material which
appears to lie on the surface.

Vine protoplasts by contrast have a much smoo-
ther outline (Fig. 4), and are more uniform in appear-
ance within and between preparations. This would
appear to reflect the comparatively greater uniformity
of growth conditions in tissue culture. At the finer
structural level the surface shows only small pro-
jections (Fig. 5). Extensive “holing” and large projec-
tions were not seen in vine protoplasts.

Examination of large number of both types of
protoplast fixed immediately following isolation and
washing failed to reveal any fibrillar residue which
might correspond to retained wall fragments.

b) 24 h of Incubation

Within 24 h of incubation within a medium known
to support cell wall regeneration, the appearance of
the tobacco protoplast surface is quite different from
that of the freshly isolated protoplast. “Holes” are
absent from the surviving protoplasts, and whilst
some large spherical projections remain which appear
to correspond to those present on fresh protoplasts,
the main characteristic of the surface is the presence
of large numbers of small projections (Fig. 6). Fibril-
lar material is seen in small amounts on the surface
at this stage (Figs. 6, 7, 8). The fibres may be very
short (Fig. 7) and rather straight, or they may be
longer and flexuous (Fig. 8). The shorter fibres in
particular are always clearly associated with one or
more of the small projections from the surface
(Figs. 7, 8), although by no means all the projections
are associated with fibres. The fibre-associated projec-
tions were not necessarily placed at one end of the
fibre. Neither the projection nor the fibres showed
any apparent ordering in their distribution or direc-
tion. Fibres up to about one micron in length could
be seen at this stage of wall regeneration around to-
bacco protoplasts.

Vine protoplasts behave quite differently to tobac-
co following incubation (Burgess and Linstead, 1976;
Skene, 1974); in particular at the 24 h stage a high
proportion of the protoplasts have formed single
buds. The appearance of such a budded protoplast
in SEM is shown in Figure 9. The protoplast has
two distinct types of surface morphology. Examina-
tion of the junction between the two regions shows
that one is covered with a mat of short fibrous mate-
rial whilst the other is devoid of this material and
has a number of small surface projections (Fig. 10).
The junction itself is indistinct at the fine structural
level and is marked by a rapid decrease in the concen-
tration of the fibres. The fibres are once again asso-
ciated with the small projections (Fig. 11). A notice-
able and consistent difference between the fibres seen
on the surface of the two species was that those on
the vine protoplasts were more uniform in length and
far shorter than the longest fibres seen on the tobacco
surface.

c) 48 h

By 48 h most tobacco protoplasts had formed a con-
tinuous mat of fibres across their entire surface
(Fig. 12). The thickness of this mat varied from indi-
vidual to individual. The structure of the mat ap-
peared to be random; areas with a relatively dense
covering of fibres occurred with blank areas in be-
tween (Fig. 13). A few small projections could be seen
on the protoplast surface in the more blank areas.

Vine protoplasts showed very little change from
their appearance at 24 h. A continuous mat of fibres
did not develop over the entire surface, and areas
persisted where surface projections and amorphous
material predominated over a small number of fibres,
which remained rather short (Fig. 14). This situation
did not change with prolonged incubation, to 5 days.

Longer times of incubation of tobacco protoplasts
lead to the formation of a wall in which the fibrous
structure was not visible by the techniques used. This
corresponds to the appearance of the normal wall
around the undigested cells (see Discussion).