EVACUOLATION OF MESOPHYLL PROTOPLASTS*

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SUMMARY

The differential sedimentation of mesophyll protoplasts through a continuous Percoll gradient can lead to 100% evacuolation of protoplasts which subsequently could be regenerated to whole plants.

Key words: Petunia — Protoplasts — Evacuolation

INTRODUCTION

Many of the present somatic cell genetic manipulations and techniques are hindered by the presence of the vacuole within each plant cell. For example, it is generally believed that a cell may have difficulty dividing if it has a large vacuole [1]. In addition, large vacuoles may hinder somatic hybridization by inhibiting post-fusion cytoplasmic mixing [1]. Vacuoles are also known to be a rich reservoir of many secondary metabolites. This large pool of metabolites may make it very difficult to obtain auxotrophic mutants [2]. Vacuoles accumulate many toxic chemicals, thereby reducing their toxic effects on the cytosol. This may explain the problems involved in obtaining mutants through mutagen treatment. Finally, vacuoles produce a large number of hydrolytic enzymes which may explain the difficulty in producing genetically transformed plant cells [2].

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Abbreviations: BAP, benzylaminopurine; CPW salts mg/l, 27.2 KH2PO4, 101 KNO3, 246 MgSO4·7H2O, 1480.2 CaCl2·2H2O; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; NAA, naphthaleneacetic acid.

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Somatic cell genetics would benefit from a rapid method for producing viable cells lacking a vacuole. There are many techniques for isolating vacuoles [3,4]; such techniques also rupture the cell. Likewise, there are several methods for isolating minicells without a vacuole. However, most of the cytoplasm is also lost in the process.

This paper describes a simple procedure for removing the vacuole from protoplasts without a loss in cytoplasm or a reduction in viability.

MATERIALS AND METHODS

Protoplasts were enzymatically isolated from Petunia parodii leaves as previously described [6]. One half of a milliliter of $2 \times 10^6$ protoplasts/ml were mixed with 4.5 ml of Percoll containing 100 mM CaCl$_2$, 5 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.0) and 9% manitol. The Percoll solution was filter sterilized (45-μm millipore filter). The Percoll/protoplast suspension was pipetted into a 5-ml polycarbonate centrifuge tube and centrifuged for 60 min at 23°C at 40 000 rev./min (SW 56 rotor - Beckman). In order to do an ultracentrifuge run under sterile conditions, the following steps were taken. The centrifuge tube, centrifuge adapter and adapter top were sterilized by submersion in 70% ethanol for 10 min and air dried in a sterile laminar air flow hood. The sterilized tube was then loaded and balanced aseptically in a flow hood. After the centrifuge run, the centrifuge adaptor was opened in a flow hood and 70% ethanol pipetted on top of the Percoll gradient (the pressure changes during centrifugation can force bacteria and fungi into the adapter and tube). The band of evacuolated protoplasts was aseptically removed with a pasteur pipette, diluted 10-fold in CPW salts with 9% mannitol and washed via centrifugation at $70 \times g$ for 5 min.

The washed evacuolated protoplasts were recovered, counted, diluted and plated at $5 \times 10^5$/ml for regeneration in Murashige and Skoog medium supplemented with 2 mg/l naphthaleneacetic acid (NAA), 0.5 mg/l benzylaminopurine (BAP) and 9% mannitol [6]. After 5 days viability was determined by staining with methyl blue. When 2 mm in diameter, green colonies were transferred to Murashige and Skoog medium supplemented with 2 mg/l zeatin for shoot regeneration. Once a shoot reached 1 cm in length it was excised and placed on hormone free medium for rooting. Established plantlets were transferred to soil and placed on a mist bench for 1 week before being placed in the greenhouse.

RESULTS

There are several stages in the evacuolation procedure as revealed by recovering protoplast samples at various time periods during centrifugation. Firstly, the mesophyll protoplasts banded at their corresponding density. This stage takes about 5 min. Secondly, the protoplasts remain banded,
but the chloroplasts aggregate within each protoplast (Fig. 1a). After about 30 min more of centrifugation, the vacuole begins to extrude (Fig. 1a). Thirdly, the vacuoles pinch off from the plasmalemma and float to the surface of the Percoll gradient. The evacuolated protoplasts, now denser, move toward the bottom of the gradient and band at the interphase with the silica pellet (Percoll gradients typically produce a silica pellet after centrifugation). Figure 1b shows both a vacuolated and evacuolated mesophyll protoplast. This procedure produces 100% evacuolation. The

Fig. 1. Evacuolation of mesophyll protoplasts of Petunia parodii. (a) After 15 min of centrifugation at 150,000 x g, the chloroplasts pellet within the protoplast and the vacuole begins to pinch off. (b) A normal vacuolated protoplast (40 μm diameter) and an evacuolated protoplast (20 μm diameter).
viability of these evacuolated protoplasts is 40%, while the viability of normal vacuolated protoplast is 60%. The evacuolated mesophyll protoplasts of Petunia parodii have been regenerated without difficulty into mature plants. A slightly more mild procedure can also produce about 35% evacuolated suspension-cultured tobacco cells. The viability of these cells was 33% (M. Aly and L. Owens, unpublished data).

DISCUSSION

Evacuolated protoplasts can be useful in many situations. For example, in somatic hybrid selection systems, one could use evacuolated mesophyll protoplasts as one parent and suspension-cultured vacuolated protoplasts as the other parent. Experiments with Petunia parodii and P. inflata indicate that vacuolated suspension-cultured protoplasts will not pellet through 30% sucrose and will float in 20% sucrose. Evacuolated protoplasts, on the other hand, will pellet through 30% sucrose. If after fusion, the protoplasts are resuspended in 20% sucrose, layered on top of 30% sucrose and centrifuged, the evacuolated parental protoplasts will pellet, the vacuolated parental protoplasts will float and the hybrid protoplasts will band at the 30% sucrose interphase. Over 50% of the hybrid cells can be recovered by this procedure. The use of these procedures following fusion may eliminate the need for highly complex selection schemes in some species. This system for the bulk selection of hybrid fusion products will also offer advantages over manual heterokaryon selection. A larger number of cells can be processed in a given period of time using this procedure.

Evacuolated protoplasts may also be helpful in mutagenesis. Plant vacuoles are known to sequester toxic materials and be a reservoir for metabolites [2]. This may be one of the reasons why it is so difficult to isolate true genetic mutants from cultured plant cells. Mutagens, being toxic, could be quickly transported into the vacuole. Several studies [7,8] indicate that toxic substances once inside the cell are indeed quickly relocated to the vacuole. The use of evacuolated protoplasts may make it possible to keep a mutagen within the cytoplasm or nucleus for a longer period of time. This should allow a much higher mutation frequency to be obtained.

Similarly, vacuoles are a rich reservoir of many metabolites [2]. For example, in Hevea brasiliensis the basic amino acids are predominantly located with the vacuole [9]. In addition, the transport of these amino acids is mediated by a permease [10]. The release of amino acids from the vacuole would make it extremely difficult to select amino acid auxotrophs. The slow release of amino acids from the vacuole would require a long period of time before the cellular pool of amino acids were depleted. Most of the so called 'auxotrophs' would turn out to be the result of physiological changes in the permease and not true genetic auxotrophs. When selecting for methionine auxotrophs, only one plant in ten proved to be a true mutant when crossed sexually [11]. The use of evacuolated protoplasts could eliminate the flow
of metabolites from the vacuole into the metabolic pool. In this way, the production of true auxotrophs should be enhanced.

Finally, evacuolated protoplasts will be very useful in microinjection studies. The microinjection of normal vacuolated protoplasts is not very successful for two reasons. Firstly, it is very difficult to inject material directly into the cytoplasm. Most protoplasts have a very large central vacuole with a thin periphery of cytoplasm. It is almost impossible to place a microneedle within this thin layer of cytoplasm. In most instances, the microneedles are placed within the vacuole and most of the foreign material injected there. The second problem with the microinjection of vacuolated protoplasts also concerns the vacuole. The vacuole contains a large number of toxic materials. The acidic pH of vacuoles is also deleterious to many cytoplasmic enzymes. Many times during microinjection, the vacuolar membrane is damaged. Many of these toxic compounds then leak out into the cytoplasm. When this happens, the cell usually dies. The use of evacuolated protoplasts solves many of these problems. Preliminary experiments indicate a higher viability when evacuolated instead of normal vacuolated protoplasts are used in microinjection. In addition, microinjections are much quicker when evacuolated protoplasts are used for one does not need to restrict the microneedle to the periphery of the protoplast.

The use of evacuolated protoplasts in both biochemical studies and in somatic manipulations should be quite advantageous. Many of the restrictions associated with protoplast research is the result of the vacuole. By removing the vacuole, these restrictions are removed.

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