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Stoloniferous shoot protoplast, an efficient cell system in potato for somatic cell genetic manipulations

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ABSTRACT

The present study reports that protoplasts isolated from stoloniferous shoots (SS) of potato represent an efficient system for somatic cell genetic manipulations. SS were established from single-node cuttings on MS medium supplemented with either 0.1 or 0.2 M sucrose (Suc), and protoplasts were isolated and cultured within the alginate strip, following an improved method. SS induced by 0.1 M Suc yielded 8–22 $\times$ 10^5 protoplasts g^{-1} fresh mass, with a high morphogenic competence. However, 0.2 M Suc-induced SS yielded protoplasts that contained large amounts of starch grains, resulting in their high degree of fragility, delayed cell division and poor morphogenic competence. For symmetric somatic hybridization (electrofusion) between *Solanum tuberosum* Gp. Tuberosum androgenic (di)haploid ($2n = 2x = 24$) 'C-13' and diploid ($2n = 2x = 24$) wild species *S. pinnatisectum*, protoplasts isolated from 0.1 M Suc-induced SS were also found to be most responsive. Out of several putative somatic hybrids, there were two tetraploids and five diploids, with 48 and 24 chromosomes, respectively at all the three shoot layers (L_1–L_3). This precluded the occurrence of mixoploidy vis-à-vis chimaerism in regenerants, as common in somatic fusion involving mesophyll protoplasts of *S. pinnatisectum*. Nuclear microsatellite analyses based on the two single-locus nSSR loci (*STM0037* and *STM2030*) confirmed that one of the tetraploids was a true nuclear hybrid (heterokaryon), while the other a homokaryon of the Tuberosum parent 'C-13'. The use of 0.2 M Suc-induced SS protoplasts for fundamental studies on tissue- and/or cell type-specific transient gene expression underlying tuberization has been discussed.

1. Introduction

Protoplasts represent physiological and versatile cell systems for somatic cell genetic manipulations in potato (*Solanum tuberosum* L.; $2n = 4x = 48$) including its monoploids ($2n = 1x = 12$), (di)haploids ($2n = 2x = 24$) and related wild diploid ($2n = 2x = 24$) *Solanum* species (Wenzel, 2006). They are mainly used for symmetric somatic hybridization between (di)haploids of *S. tuberosum* and diploid wild *Solanum* species to achieve targeted whole genome complementation across various sexual and endosperm balance number (EBN) barriers (Millam et al., 1995; Pehu, 1996; Cardi, 2001; Rokka, 2009; Pandey et al., 2010). Protoplasts are also used for direct gene transfer through chemical or electroporation methods, not only to carry out genetic transformation (Jones, 1995; Ghislain and Golmirzaie, 1998) but also to develop transient expression systems for high-throughput screening and systematic characterization of gene functions (Jones et al., 1989b; Sheen, 2001). Since the first report on potato protoplast isolation, culture and regeneration (Shepard and Totten, 1977), these protoplast-based technologies have been successfully used in a large number of potato cultivars, advanced breeding lines, monoploids, (di)haploids and wild *Solanum* species (reviewed in Orczyk et al., 2003; Nouri-Ellouz et al., 2006).

Mesophyll protoplasts isolated from leaf tissues of in vitro-grown microplants are universally used for somatic cell genetic manipulation in potato. Higher protoplast yield per unit leaf tissue (Haberlach et al., 1985) combined with their higher totipotency across the species (Orczyk et al., 2003) make mesophyll protoplasts ideal for most of the fundamental studies in potato. However, despite a large volume of scientific and technical information about mesophyll protoplast isolation, culture and regeneration in potato, the knowledge is broadly descriptive, and the methodologies still continue to be empirical. Mesophyll protoplasts are, in general, rich in chloroplasts, usually about 50–100 per protoplast, and as a result, they are fragile, making their isolation, purification and further downstream applications not only cumbersome, but also highly demanding in terms of expert handling that involves a high degree of technical skills. This may be one of the reasons for

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so-called 'genotypic effect' on mesophyll protoplast isolation, culture and regeneration in potato (Rokka, 2009). Even today, after more than three decades of research, obtaining the sustained division of cultured mesophyll protoplasts leading to successful plant regeneration is one of the fundamental challenges in plant biology. Although the possible outcome of a protoplast-based experiment can be envisaged well in advance, the results of that experiment cannot be predicted *a priori*.

As a rule, excised potato leaves (Haberlach et al., 1985) or shoot cultures with expanded leaves (Oberwalder, 1996; Sarkar et al., 2007) are pre-conditioned in the dark for varying periods (usually 48 h) before mesophyll protoplast isolation. This is done to reduce the number of chloroplasts per isolated protoplast so as to make it physically more stable during subsequent manipulations while suspended in a suitable osmoticum. Dark pre-conditioning of leaf tissues may increase the cell division vis-à-vis morphogenic competence of mesophyll protoplasts in culture. However, there appear to be no supporting data in the literature, except that starch accumulation impairs division activity of isolated protoplasts (Foulger and Jones, 1986; Jones et al., 1989a; Gram et al., 1996).

In potato, attempts have also been made to isolate and regenerate plants from protoplasts of other tissues without chloroplasts. Single cell suspensions (Jones et al., 1989b), in vitro-induced microtubers (Jones et al., 1989a) and true potato seedling-derived hypocotyl tissues (Dai and Sun, 1994) have been successfully used for protoplast isolation, culture and regeneration in potato. However, hypocotyl protoplasts can be isolated only from true potato seedlings, while isolating protoplasts from single cells requires parallel culture efforts in terms of time-consuming and labor-intensive preparation and maintenance of batch suspension cultures in vitro. In contrast, the isolation and regeneration of protoplasts from storage parenchyma tissues of potato tubers is rather attractive (Jones et al., 1989b) because of relative ease with which microtubers can be induced in vitro in a broad range of potato genotypes including its wild relatives. However, tuber protoplasts typically contain many starch grains (Jones et al., 1989a). Although most of the starch grains are metabolized during the first seven days of culture (Jones et al., 1989a), they make the tuber protoplasts initially too fragile to process at various steps of isolation, purification and downstream applications. As a result, tuber protoplasts are seldom used for somatic cell genetic manipulations in potato.

In this paper, we report the development of a procedure for the isolation and regeneration of protoplasts from stoloniferous shoots of potato single-node cultures in vitro. The objectives were to assess the morphogenic competence of stoloniferous shoot protoplasts in vitro and to investigate whether they can be routinely used for symmetric somatic hybridization by electrofusion. We also report the development of an ideal cell system in potato, for undertaking fundamental studies (transient expression) on signal transduction pathways underlying tuberization and other developmental responses.

2. Materials and methods

2.1. Plant material

S. tuberosum L. Gp. Tuberosum androgenic (di)haploid ($2n = 2x = 24$) 'C-13' of tetraploid ($2n = 4x = 48$) potato cv. 'Kufri Chipsona-2' (Sharma et al., 2010), and diploid ($2n = 2x = 24$) 1EBN wild species *S. bulbocastanum* L. 'clone 10' (CGN18310) and *S. pinnatisectum* Dun. 'clone 15' (CGN17745) were used in the present study. Both the diploid species were procured from Centre for Genetic Resources (CGN), Wageningen under a material transfer agreement with the Indian Council of Agricultural Research (ICAR), New Delhi. They were established in vitro from true potato seeds followed by the selection of clones based on optimum microplant growth. The genotypes were maintained and multiplied in vitro on semisolid (2.0 g l^{-1} gelrite) MS medium (Murashige and Skoog, 1962) containing 20.0 g l^{-1} sucrose and 250 mg l^{-1} cefotaxime, through single-node cuttings (SNCs) under a 16-h photoperiod (60 μmol m^{-2} s^{-1} PPFD) at 20 °C following the method as described earlier (Sarkar et al., 2004).

2.2. Stoloniferous shoot culture

SNCs without any subtended leaves were prepared from 4-week-old microplants and cultured on semisolid (2.0 g l^{-1} gelrite) MS medium supplemented with 0.1 or 0.2 M Suc and 500 mg l^{-1} cefotaxime. The pH was adjusted to 5.8 before autoclaving at 121 °C for 20 min, and cefotaxime was added to the medium by filter-sterilization (0.22 μm PVDF; Millipore India Pvt. Ltd., Bangalaru, India). Ten SNCs were cultured per 90-mm disposable Petri dish (Greiner Bio-One GmbH, Frickenhausen, Germany) containing 40 ml of medium, sealed with Parafilm M™ (American National Can, Chicago, USA) and incubated in the dark at 20 °C. All chemicals used in the study were from Sigma–Aldrich, St. Louis, MO, USA.

2.3. Protoplast isolation and purification

Stoloniferous shoots (1.5 g) harvested from 3-week-old cultures were minced in 15 ml of protoplast digestion solution (PDS) in a 90-mm Petri dish and incubated overnight in the dark at 25 °C. The PDS was based on Cheng et al. (1995) with modifications: modified MS macronutrients (0.95 g l^{-1} KNO_3, 0.085 g l^{-1} KH_2PO4, 0.185 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 0.66 g l^{-1} $CaCl_2 \cdot 2H_2O$), 1/2-strength MS micronutrients, full-strength MS vitamins, 5.0 g l^{-1} PVP, 5.0 mM MES, 0.4 M mannitol, 0.1 M glucose, 1.0% cellulase 'Onozuka' RS (L0011; Yakult Pharmaceuticals Ind. Co. Ltd., Tokyo, Japan) and 0.5% macerozyme R-10 (L0021; Yakult). Prior to use, the PDS was heated at 55 °C for 10 min to precipitate nucleic acids and proteins. The protoplast suspension was diluted with 15.0 ml of 0.3 M KCl, filtered through a 60 μ nylon mesh (Millipore) and centrifuged at 50 × *g* for 5 min. They were resuspended in 10.0 ml of 0.6 M Suc, and 1.0 ml of 0.3 M KCl was carefully layered onto this protoplast suspension followed by centrifugation at 50 × *g* for 5 min. The protoplasts were recovered from the Suc–KCl interface using a sterile 9-in. Pasteur pipette (S6143; Sigma–Aldrich, St. Louis, MO, USA), diluted with 10.0 ml of 0.3 M KCl and centrifuged at 50 × *g* for 5 min. The purified protoplasts were resuspended in 0.5 M mannitol (pH 7.0) and adjusted to a final density of 10^6 ml^{-1} using a Bright Line counting chamber (Hausser Scientific, Horsham, USA).

2.4. Symmetric protoplast electrofusion

Symmetric protoplast fusion was carried out between 'C-13' and *S. pinnatisectum*. The fusion suspension was prepared by mixing the protoplasts in a 1:1 ratio (5×10^5 ml^{-1} protoplasts per clone), and electrofusion was performed in a 3.2-mm-gap glass microslide (BTX model 453; Harvard Apparatus, MA, USA) using the BTX Elecrto Cell Manipulator ECM 2001 (Harvard Apparatus). Protoplasts were aligned at 100 V cm^{-1}/1.0 MHz alternating current (AC) for about 30 s, and then fused by the application of a square direct current pulse of 1250 V cm^{-1} for 60 μs followed by a post-fusion AC field for 10 s. After 15 min recovery period, the fusion suspension was centrifuged at 50 × *g* for 5 min to collect the protoplasts.

2.5. Alginate strip culture

Fused or unfused protoplasts (100 μl) were layered onto a 100 μl droplet of 2.8% sodium alginate (Sigma) and gently spread in the well of a 6-well Multiwell plate (Greiner); 2.0 ml complexing solution (50 mM $CaCl_2 \cdot 2H_2O$) was slowly added by the wall to form a