PROGRESS TOWARDS SUGAR BEET IMPROVEMENT THROUGH SOMATIC HYBRIDIZATION

1. INACTIVATION OF NUCLEI AND CYTOPLASM IN DONOR AND RECIPIENT PROTOPLASTS

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ABSTRACT

The isolation and culture of suspension-derived protoplasts from two sugar beet (*Beta vulgaris* L.) genotypes are described. Immobilization of protoplasts in agarose resulted in high frequency divisions and microcallus regeneration, with plating efficiency (PE) being clearly genotype-dependent. In further studies towards asymmetric fusion experiments, the effect of different doses of ultraviolet radiation (UV) and iodoacetic acid (IA) on protoplast physiology was assessed. Viability of both treated (UV, IA) and untreated protoplasts (control) was determined by FDA staining, and the biological effect was evaluated by testing the ability of protoplasts to divide and to form calli. The results are discussed in terms of the applicability of the methods for the production of asymmetric protoplasts suitable for somatic hybridization within the genus *Beta*.

KEY WORDS: *Beta vulgaris* L., suspension cultures, protoplasts, asymmetric protoplasts.

INTRODUCTION

In sugar beet breeding, cytoplasmic male sterile lines (CMS) are used for hybrid seed production. The reproduction of CMS plants requires maintainer lines – O type (OT) – which, according to a simplified model of inheritance, carry the same sterility nuclear genes as the male steriles, but have a different cytoplasmic genetic background (Owen, 1945). The identification of O type lines within populations of monogerm pollinators is troublesome and time-consuming, because of the low frequency (from 3 to 5%) with which these lines occur. Traditionally, OT plants have to be selected by testing progeny from crosses between pollinators and CMS lines. This takes 6-8 years, and is the most expensive part of sugar beet breeding.

Recent progress in protoplast techniques has created new possibilities to obtain plants with specific nucleus-cytoplasm combinations via asymmetric somatic hybridization. The aim of our studies is to determine whether the combining of nuclei from CMS genotypes with the cytoplasm of a fertile monogerm pollinator would result in desirable hybrid material showing O type characteristics. To achieve this goal, several steps in the protoplast technique had to be optimized: (1) isolation of high quality protoplasts from two genotypes of interest, (2) successful culture of protoplasts and their subsequent regeneration into calli and plants, (3) production of asymmetric protoplasts by physical and/or chemical factors, (4) fusion

of asymmetric protoplasts and regeneration of fusion products. In this paper we focus on the three steps of the procedure which precede fusion experiments. Special emphasis is given to the procedure to obtain metabolically inactivated, but highly viable asymmetric protoplasts from two selected genotypes of agricultural interest.

Nuclei from protoplasts of fertile plants were damaged by ultraviolet radiation (UV), and the cytoplasm of CMS protoplasts was inactivated by iodoacetic acid treatment (IA).

MATERIALS AND METHODS

Plant material

Cytoplasmic male sterile, diploid line 491D (Maribo Breeding Company, Denmark) and male fertile diploid pollinator JP3 (Plant Breeding and Acclimatization Institute) were the materials studied. Explants from mature plants, i.e. ovaries of 491D and shoot tips of JP3, were used for callus induction. Explants were placed on modified Saunders and Dole medium (mSD) supplemented with 4.4 μ M BAP. White friable callus was used to initiate suspension cultures, by mixing portions of callus in liquid mSD medium containing the same amount of BAP. The established cultures were kept on a rotary shaker (100 rpm) in the light, and transferred once a week to fresh liquid medium.

Isolation and culture of protoplasts

Protoplasts of both genotypes were isolated from two or three-day-old suspensions. The enzyme solution used to obtain protoplasts from 491D contained 0.6% Macerozyme R-10 (Yakult Honsha Co., Tokyo) and 0.8% Cellulase R-10 (Yakult Honsha Co., Tokyo). Protoplasts of JP3 were isolated in the solution of 0.4% Macerozyme R-10, 2% Cellulase Onozuka R-10 and 0.2% Cellulase Onozuka RS (Yakult Honsha Co., Tokyo). Protoplasts were isolated and purified as described by Majewska-Sawka et al. (1994). Protoplasts were plated at a density of $1-1.5 \times 10^5 \text{ ml}^{-1}$ in SD medium supplemented with 5 μM naphthalenacetic acid (NAA) and 2 μM 6-benzylaminopurine (BAP) solidified by 1.25% agarose (Sea Plaque, FMC Bioproducts, Rockland, USA). Agarose disks were then cut into segments and floated on liquid mSD medium, which was replaced with fresh medium after 14 days. All solutions used for protoplasts isolation, washing and culture contained 0.1 mM n-propylgallate (nPG) as an antioxidant. The culture dishes were incubated in the dark at 25°C. Plating efficiency (PE) was determined after two weeks of culture and expressed as the percentage of protoplasts which developed microcolonies. After 3-4 weeks, agarose segments with visible microcalli were transferred onto 0.5% agarose medium (Sigma Chemical Co., St. Luis, USA) and cultured for about two weeks in the light. Individual calli were picked up and placed onto 1% agarose medium and then transferred to regeneration media containing no hormones or supplemented with auxins, cytokinins and gibberellic acid in several combinations. In two cases, 50 mg or 100 mg of AgNO_3 was added to 1 litre of medium.

Metabolic inactivation of protoplasts

Nuclei of protoplasts from the JP3 genotype were damaged by ultraviolet radiation (UV). The source of UV emission was a GmbH Hanau quartz lamp with filter transmitting light at 254 nm. Freshly isolated and purified protoplasts were suspended at the density $3 \times 10^5 \text{ ml}^{-1}$ in 2 ml of culture medium and placed in a 60 mm Petri dish on ice. Subsequently, protoplasts were exposed to UV light by keeping the open Petri dish at a distance of 7.5 cm from the source of radiation.

Times of radiation tested ranged from 5 to 60 min. After that, protoplasts were cultured both in liquid medium and in agarose segments. The viability of treated and untreated (control) protoplasts was determined using FDA staining both immediately after exposure to UV light and on four successive days. The percentage index of viable protoplasts in relation to total number of protoplasts observed in the microscope was calculated for each day of the experiment. These values were subjected to linear regression analysis to evaluate the significance of changes observed in protoplasts staining. The physiological effect of UV was also assessed by observations of agarose cultures, and by determining the ability of irradiated protoplasts to divide and form callus microcolonies.

Protoplasts from the 491D genotype were metabolically inactivated by iodoacetic acid treatment (IA). Purified protoplasts were suspended in a small volume of CPW solution and placed in 10 ml Pyrex tubes on ice. Then a fixed volume of stock IA solution was added to the final desired concentration. The concentrations used in our experiments ranged from 2.5 to 20 mM. Inactivation was ensured by keeping protoplasts in CPW-IA solution during 10 min on ice. Subsequently, the protoplasts were centrifuged for 5 min, washed three times in CPW, and placed in liquid or agarose solidified media. The physiological effect of IA treatment on protoplasts was assessed by viability testing with FDA and by observing protoplast response to culture conditions. The statistical analysis of protoplast viability in control and IA experiments was performed as described above.

RESULTS AND DISCUSSION

Isolation and culture of protoplasts

The experimental conditions used to isolate protoplasts from suspensions of two sugar beet genotypes resulted in the release of numerous protoplasts of good quality, characterized by suitable morphological features (Fig. 1) and displaying high viability both immediately after isolation and in the following days of culture (Tables 2, 3).

TABLE 1. Hormonal composition of media used for regeneration of protoplast-derived calli.

CYTOKININS (+ inorganic additives)	GIBBERELLINS	CYTOKININS AND AUXINS	CYTOKININS, AUXINS AND GIBBERELLINS
4.4 μM BAP	0.44 μM GA_3	8.8 μM BAP 4.4 μM NAA	17.6 μM BAP 8.8 μM IBA 4.4 μM GA_3
4.4 μM BAP (+50 mg/l AgNO_3)	4.4 μM GA_3	8.8 μM BAP 8.8 μM NAA	
4.4 μM BAP (+100 mg/l AgNO_3)		17.6 μM BAP 8.8 μM IBA	
4.4 μM Kin		8.8 μM BAP 4.4 μM Zea 4.4 μM Kin 4.4 μM NAA	
4.4 μM Zea			

BAP – 6-benzylaminopurine, GA_3 – gibberellic acid, Kin – kinetin, NAA – naphthalenacetic acid, Zea – zeatin

Fig. 1. Freshly purified protoplasts of 491D, 120x

Fig. 2. Microcalli of 491D observed after two weeks of culture, 120x

Fig. 3. Protoplast-derived microcalli within agarose segments.

Fig. 4. UV irradiated JP3 protoplasts after six weeks of culture in agarose. No callus colonies are visible, 120x

Fig. 5. IA inactivated protoplasts of 491D after six days of culture. No protoplast divisions are visible, 120x

TABLE 2. Physiological effect of UV radiation on protoplast viability (FDA staining)*.

Control protoplasts			UV treated protoplasts	
Day of experiment	Number of protoplasts	Protoplasts viability (%)	Number of protoplasts	Protoplasts viability (%)
0	209	96.17	195	96.41
1	215	93.02	264	91.35
2	187	91.44	266	90.15
3	313	74.76	331	67.98
4	277	67.89	330	64.24

*Results are the means of two replicates

The method of protoplast culture we used proved to be very efficient in terms of protoplast division (Fig. 2). Plating efficiency, expressed as the percentage of protoplasts which developed microcalli after two weeks of culture, was high and reached values from 6.2% to 9.3% for JP3, and from 17.3% to 26.6% for 491D. It is difficult, however, to compare the PE obtained by us with values reported for other *Beta* genotypes. This is due not only to the well-known genotype-related ability of protoplasts to divide (Krens et al., 1990), but also to differences between the systems used to determine PE. Whereas some authors count real microcolonies which developed after a given period of culture, e.g. 14 or 21 days (Bhat et al., 1985; Majewska-Sawka, 1994), others include protoplasts which underwent only one mitotic division (Szabados and Gaggero, 1985). Other factors, such as composition of the culture media, hormone content and use of the nurse system, also influence the PE (Szabados and Gaggero, 1985; Bhat et al., 1985; Bhat et al., 1986). However, the most conspicuous enhancement of PE was obtained by immobilizing protoplasts within agarose or alginate instead of culturing them in liquid medium (Lindsey and Jones, 1989; Schlangstead et al., 1992; Hall et al., 1993a; Majewska-Sawka et al., 1994). The addition of 0.1 mM n-propylgallate to all solutions and media proved not to be a prerequisite for sustained division and successful culture of protoplasts – as suggested by Krens et al. (1990) – but did result in a slight increase in PE in comparison with cultures without this compound (data not shown). This effect is thought to be due to lipoxygenase inhibition and a reduction in the rate of membrane lysis (Saleem and Cutler, 1987).

Agarose segments with developing microcalli were transferred to soft agarose medium (0.5%) containing the same hormones, and kept in light for two more weeks (Fig. 3). The individual callus colonies were then transferred to 1% agarose

medium, and subsequently to regeneration media without hormones, or with different combinations of hormones (Table 1). The possible influence of two doses of AgNO₃ on morphogenic capacity was also tested (Chi and Pua, 1989). Regeneration of protoplast-derived calli into plants was not achieved with any of these media. Similar results have been described by other authors who were able to regenerate calli from suspension-derived protoplasts of several *Beta* genotypes, but organogenesis has not been reported so far (Szabados and Gaggero, 1985; Bhat et al., 1985; Majewska-Sawka et al., 1994).

The application of mesophyll as a source for protoplast isolation seemed to overcome the difficulties in regenerating whole sugar beet plants (Krens et al., 1990). It turned out, however, that organogenesis could be achieved only for selected line – SVP31-188NF – that showed an exceptionally high capacity to regenerate (Krens et al., 1990; Lenzner et al., 1994; Zoglauer, pers. comm.). The difficulty of regenerating sugar beet protoplasts led many researchers to consider this species as very recalcitrant with regard to its response to in vitro conditions (Krens et al., 1990; Hall et al., 1993b). It cannot be excluded, however, that the fusion of protoplasts from two genotypes that lack regenerative ability will result in a fusant possessing such a capacity. This situation has been found in several species, including in rice (Mori, pers. comm.).

Metabolic inactivation of protoplasts

Asymmetric fusion of protoplasts has been widely used in the production of both intra- and interspecific somatic hybrids displaying valuable agronomic traits. This method has been successfully used to transfer desirable genes such as those encoding antibiotic resistance (Medgyesy et al., 1980), clubroot disease resistance (Hagimori et al., 1992) or tolerance for cold

TABLE 3. Physiological effect of IA on protoplast viability (FDA staining)*.

Control protoplasts			UV treated protoplasts	
Day of experiment	Number of protoplasts	Protoplasts viability (%)	Number of protoplasts	Protoplasts viability (%)
0	656	96.65	560	91.07
1	693	85.28	688	72.82
2	763	81.00	837	61.77
3	820	75.61	891	54.55
4	746	65.68	668	45.96

*Results are the means of two replicates

(Walters et al., 1992) from mutants or wild species into cultivated varieties. In addition, CMS has been introduced as a result of asymmetric hybridization in rice (Yang et al., 1988) and a species of brassica (Sakai and Imamura, 1990).

The production of asymmetric hybrids within the genus *Beta* still presents many difficulties (Hall et al., 1993b; Lenzner et al., 1994) and thus requires successive steps of the protoplast technique to be perfected. We report our experiments designed to optimize conditions for obtaining asymmetric protoplasts with damaged nuclei and inactivated cytoplasm. The elimination or inactivation has been achieved in several species by selecting the technique appropriate for genotypes of interests:

1. High speed centrifugation on continuous or discontinuous gradients (Hall, 1989; van Ark et al., 1992),
2. Isolation of protoplasts in solutions of high osmotic value, which causes plasmolysis, intracellular budding and the formation of several protoplasts from one cell (Sundberg and Glimelius, 1991),
3. Exposing cells/protoplasts to ionizing radiation, i.e. X-rays and γ -rays (Melchers et al., 1992),
4. Exposing cells/protoplasts to nonionizing radiation, i.e. UV-rays.

The effect of UV radiation on protoplast physiology has been studied in detail in *Hordeum vulgare* (Bornman et al., 1982), *Petunia hybrida* (Staxen et al., 1993) and *Beta vulgaris* (Hall et al., 1992a; Hall et al., 1992b). Depending on the dose applied, UV rays may damage of several cell constituents such as the thylacoid system of chloroplasts (Bornman et al., 1982) and microtubular arrangement – due to the loss of the ability of the dimers to polymerize (Staxen et al., 1993). Ultraviolet radiation can also profoundly influence DNA structure (Hall et al., 1992c). The effect of UV on DNA is known to be not only dose-dependent, but also affected by the amount of DNA in the nucleus and its three-dimensional arrangement (Kapiszewska, 1990; Hall et al., 1992c).

Recently, UV radiation has been used as an alternative to ionizing radiation, and is potentially applicable in asymmetric hybridization experiments in the genus *Beta* (Hall et al., 1992a; 1992b; 1992c). Appropriate doses of UV induce desired physiological effects in sugar beet protoplasts, preventing resynthesis of the cell wall and consequently obviating cell division, without having lethal effects on the protoplasts themselves. The effect of UV on DNA involves changes in the structure of nucleic acid bases such that they form intrachain pyrimidine dimers, mainly between adjacent thymidilate residues. This makes the RNA polymerases terminate transcription at these dimers, which results in the formation of short incomplete transcripts (Alberts et al., 1989). Additionally, as shown by electrophoretic analyses, UV causes substantial numbers of randomly situated single and double strand breaks, and consequently extensive chromosome and chromatid breakage. Moreover, minimal doses of UV radiation which inhibit protoplast divisions have a much stronger effect on DNA structure than biologically equivalent doses of gamma rays (Hall et al., 1992c), and are therefore advantageous in obtaining asymmetric protoplasts.

For the reasons mentioned above, we undertook experiments to confirm the potential suitability of UV treatment in asymmetric fusion experiments, and to determine the optimal dose of UV to inactivate the nucleus in protoplasts of selected genotypes.

Under our experimental conditions, the optimal time of UV radiation for JP3 protoplasts was established as 15 min. This

treatment completely prevented protoplast divisions (Fig. 4), but had no detrimental effect on membrane integrity, as shown by FDA staining. Inactivated protoplasts displayed high viability, which immediately after irradiation was approximately the same as that observed in control samples, although viability gradually decreased thereafter, most conspicuously on the third day (Table 2). This interdependence was described by the equation $Y = 99.620 - 7.512X$ ($r = -0.944$) for control protoplasts and $Y = 99.568 - 8.771X$ ($r = -0.938$) for irradiated ones. We conclude that the number of viable protoplasts decreased in the similar fashion for control and UV-treated protoplasts. Shorter radiation times, i.e. 5 and 10 min. resulted in several escapes.

Metabolic inactivation of protoplasts from the second parental genotype – 491D – was accomplished by iodoacetic acid treatment. Although iodoacetic acid (IA), iodoacetate (IAA) and iodoacetamide (IOA) have been widely used as inhibitors to obtain metabolically deficient protoplasts, very little is actually known about the molecular mechanisms involved in this deficiency. According to Hall (1989) and Melchers et al. (1992), mitochondria are the organelles most strongly affected by these chemicals. As a consequence, protoplast divisions are inhibited and no colony formation takes place. The kind of inhibitor, as well as the appropriate dose, have to be determined experimentally for each species and genotype (Cella et al., 1983; Hall et al., 1993b). There is little data concerning the inactivation of sugar beet genotypes. According to a protocol developed by Hall in 1989, 0.2 mM iodoacetate effectively inhibited sugar beet mesophyll protoplasts, however, 10 mM iodoacetamide was recommended in a more recent study (Hall et al. 1993b).

We tested the influence of various doses of IA on the physiology of 491D suspension-derived protoplasts. Among the concentrations tested, 5 mM was the most suitable. Treatment with IA as described in "Material and methods" resulted in the complete inhibition of protoplast divisions (Fig. 5). However they displayed high viability immediately after exposure to IA, although viability was slightly lower thereafter (Table 3). Protoplast viability in relation to the day of the experiment was described by equations $Y = 95.166 - 7.161X$ ($r = -0.986$) for control protoplasts and $Y = 86.932 - 10.849X$ ($r = -0.981$) for IA-treated ones. We conclude that the number of viable protoplasts decreased faster in IA samples than in control ones. Lower dose of IA resulted in a low frequency of division and in the formation of several callus colonies. After the optimal dose of IA had been determined, we repeated the inactivation protocol several times, and obtained similar results. This observation contrasts with previous reports, according to which the results of treatment of sugar beet protoplasts with IOA or IA were difficult to reproduce (Hall et al., 1993b; Krens, pers. comm.). Our experiments with IA indicate that this inhibitor is suitable for producing metabolically deficient asymmetric protoplasts from the selected genotype.

In light of our data, both UV radiation and IA treatment seem to be relatively simple methods applicable for obtaining asymmetric protoplasts in sugar beets. Moreover, these treatments yield reproducible results both in terms of the inhibition of cell division and the high degree of viability observed after treatment. Further assessment of the usefulness of these methods will be based on additional studies at the physiological, biochemical and molecular levels. Physiological experiments will attempt to obtain asymmetric fusants able to grow and develop due to metabolic complementation. Biochemical and molecular studies will focus on determining the precise nature of the damage in both nuclear and organellar

DNA as a consequence of UV and IA treatment, and will also involve the measurement of enzymatic activities in inactivated and control protoplasts.

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DOSKONALENIE BURAKA CUKROWEGO
Z WYKORZYSTANIEM SOMATYCZNEJ HYBRYDYZACJI
1. INAKTYWACJA JĄDER I CYTOPLAZMY PROTOPLASTÓW RODZICIELSKICH

STRESZCZENIE

Przedstawiono warunki izolacji i kultury protoplastów zawiesinowych dwóch genotypów buraka cukrowego (*Beta vulgaris* L.). W wyniku zatopienia protoplastów w agarozie uzyskano wysoką zdolność do podziałów i tworzenia kolonii. Wartość PE była wyraźnie zależna od genotypu.

W toku badań nad uzyskaniem protoplastów asymetrycznych oceniano wpływ różnych dawek promieni ultrafioletowych (UV) oraz kwasu jodooctowego (IA) na fizjologię protoplastów. Metodą barwienia FDA określano żywotność protoplastów bezpośrednio po izolacji oraz w kolejnych dniach kultury. Biologiczny skutek działania UV i IA oceniano na podstawie zdolności protoplastów do dzielenia się i formowania kalusów. Uzyskane wyniki przedyskutowano pod kątem zastosowania opisanych metod w pracach nad somatyczną hybrydyzacją w obrębie rodzaju *Beta*.

SŁOWA KLUCZOWE: *Beta vulgaris* L., kultury zawiesinowe, protoplasty, protoplasty asymetryczne.