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Efficient plant regeneration from hypocotyl protoplasts of broccoli (*Brassica oleracea* L. ssp. *italica* Plenck)

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Abstract. We have assessed the capacity of cultured protoplasts from two tissue sources of several commercially-grown broccoli cultivars to regenerate plants. A procedure that employs hypocotyl protoplasts and a culture medium with a high NAA:2,4-D auxin ratio was developed. The procedure permits highly efficient formation of colonies that regenerate shoots at frequencies of 8-17% with two of the four cultivars tested. The time required for the development of plants from protoplasts was 8-11 weeks. No mtDNA rearrangements were observed among any of 17 analysed regenerants. Double-stranded RNAs were detected in mitochondrial DNA (mtDNA) preparations of some, but not all, regenerants of one of the cultivars.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = 6-benzylaminopurine; GA_3 = gibberellic acid; IAA = 3-indoleacetic acid; NAA = 1-naphthaleneacetic acid; ZR = zeatin riboside; CF = colony formation; mtDNA = mitochondrial DNA

Introduction

The genus Brassica comprises a large number of important crops (Williams and Hill 1986). Considerable progress has been made in recent years in manipulating the cytoplasmic male-sterility trait in these plants through protoplast fusion and cybrid production (Kemble and Barsby 1988). Effective deployment of cell fusion technology in crop improvement requires the capacity for efficient plant regeneration from cultured protoplasts. Ideally, the regeneration procedure should be applicable to a significant number of agriculturally important genotypes and should give rise to plants that are identical in genotype and phenotype to the parent plant.

Vegetable crops of the species B. oleracea include cabbage, cauliflower, broccoli, Brussel sprouts and kale. As a first step towards the introduction of cytoplasmic genetic elements from B. napus into B. oleracea through cybrid formation, we have sought to develop an efficient method for plant regeneration from broccoli protoplasts. We have focussed our efforts on broccoli because of the ease with which traits affecting floral characteristics, such as cytoplasmic male-sterility, can be analysed. Broccoli has relatively large flowers, is an annual and requires only about 75 days to flower. By contrast, most other B. oleracea subspecies, such as cabbage, Brussel sprouts and kale either require vernalization and may take up to a year to set seed or, like cauliflower, have been bred for delayed bolting and have small flowers.

To our knowledge, only one laboratory has reported plant regeneration from protoplasts of broccoli (Robertson and Earle 1986, Robertson et al. 1988). In those studies, mesophyll or cotyledon protoplasts of a single cultivar were employed. We report here an evaluation of commercially-grown hybrid broccoli cultivars for protoplast culturability and subsequent plant regeneration using both hypocotyl and leaf sources. We have examined the plant regenerants for possible tissue-cultured induced variation in mtDNA since there have been reports of mtDNA alterations occurring in protoplast culture systems (Kemble and Shepard 1984, Morgan and Maliga 1987).

Materials and methods

Plant material. The following hybrid cultivars were employed: Green Hornet, a green broccoli lacking side shoots; Packman and Premium Crop, both standard green broccolis;

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Table 1. Percentage survival, cell division and colony formation in hypocotyl protoplast cultures of different broccoli cultivars

Cultivar[1]	Survival	Cell division	Colony formation
Premium Crop	79 ± 2	29 ± 3	5.0 ± 0.9
Green Hornet	85 ± 2	12 ± 4	---[2]
Packman	52 ± 3	22 ± 6	---[3]
Paragon	84 ± 3	37 ± 3	4.3 ± 1.1

[1]Cultured in K9 medium [salts, vitamins and organic acids of Kao's medium (1977), 85.5 g/l sucrose, 23.4 g/l glucose, 1 mg/l NAA, 0.5 mg/l BAP, 0.2 mg/l 2,4-D, pH 5.6].

[2]Slow colony growth with negligible colonies at 20 days; small colonies subsequently developed.

[3]2-4 cell clusters developed.

and Paragon, a blue green hybrid obtained by crossing green broccoli with Chinese Kale (ssp. alboglabra). Seeds were surface-sterilized for 2 min with 70% ethanol then for 20 min with 2.4% sodium hypochlorite containing one drop of Tween 20 per 100 ml. Seeds were rinsed thoroughly with sterile distilled water and grown aseptically on autoclaved germination medium [half-strength basal Murashige and Skoog's medium (1962), 10 g/l sucrose, 12 g/l agar, pH 5.8] and incubated at room temperature in darkness. When necessary, 100-200 mg/l cefotaxime and 200 mg/l ampicillin were included in the germination medium and the antibiotics were used at 5 mg/l in other media.

Protoplast isolation and culture. Procedures were performed aseptically and all solutions and media were filter-sterilized. Hypocotyl explants from 5 day-old seedlings were cut transversely into 1-2 mm pieces and incubated in 10 ml enzyme solution (salts, vitamins and organic acids of Kao's medium (1977), 0.4 g/l $CaCl_2.2H_2O$, 130 g/l sucrose, 10 g/l Onozuka R10, 1 g/l Pectolyase Y23, pH 5.6) in 100 X 15 mm petri dishes, in darkness, without agitation for 14-18 h, then with agitation on a rotary shaker (40 rpm) for 15-30 min. The mixture was filtered through a 63 um nylon screen into centrifuge tubes and an equal volume of 16% sucrose was added to each tube. Following centrifugation (ca. 200 g, 5 min), the protoplast band that formed at the top of each tube was collected. Protoplasts were washed 3 times by resuspension in 10 ml of wash solution [solution W5 of Menczel and Wolfe (1984) at a reduced strength (0.8X)] followed by centrifugation at 200 g for 3 min.

Protoplasts were resuspended in culture medium at a density of 0.5-1.0 X 10^5 per ml and dispensed in 2 ml aliquots into 60 x 15 mm petri dishes (Falcon 1007). Protoplasts were cultured in medium K9 [salts, vitamins and organic acids of Kao's medium (1977), 85.5 g/l sucrose, 23.4 g/l glucose, 1 mg/l NAA, 0.5 mg/l BAP, 0.2 mg/l 2,4-D, pH 5.6] unless otherwise specified, and incubated at 25°C, 16 h photoperiod, in dim fluorescent light (25 $uEm^{-2}s^{-1}$). After 5-8 days in culture, 1-1.5 ml of feeder medium F5 [salts and vitamins of K3 medium (Nagy and Maliga 1976), 51.3 g/l sucrose, 9 g/l glucose, 0.5 mg/l NAA, 0.25 mg/l BAP, 0.1 mg/l 2,4-D, pH 5.6] were added to each dish and the dishes were placed under brighter fluorescent light (50 $uEm^{-2}s^{-1}$). At ca. 14 d, 1-2 ml of medium were removed from each dish and 2-3 ml of feeder medium F13 [basal Murashige and Skoog's medium (1962), 30 g/l sucrose, 0.5 mg/l BAP, 0.1 mg/l NAA, pH 5.6] were added. The cultures were placed on a rotary shaker (40 rpm) under bright fluorescent light (65-70 $uEm^{-2}s^{-1}$). At ca. 21 d, the cultures were fed once more with feeder medium F13 as described above.

Plant regeneration. After 4-5 weeks in culture, colonies (1 mm or larger in diameter) were plated onto regeneration medium [basal Murashige and Skoog's medium (1962), 10 g/l sucrose, 2 mg/l ZR, 0.1 mg/l NAA, 8 g/l agarose, pH 5.6]. Plates were incubated under the conditions described for the liquid cultures. Callus colonies were transferred onto fresh regeneration medium every 2 weeks. Regenerated shoots were transferred onto autoclaved rooting medium [half-strength basal Murashige and Skoog's medium (1962), 10 g/l sucrose, 0.05 mg/l NAA, 8 g/l agar, pH 5.8) and incubated under dim fluorescent light (25 $uEm^{-2}s^{-1}$).

Plantlets were transferred to pots or flats containing PROMIX (Premier Brands Inc., Stanford, Ct.) and grown in a growthroom (20°C day/15°C night, 16 h photoperiod, 100-140 $uEm^{-2}s^{-1}$ fluorescent and incandescent light at soil level). Plantlets were covered with transparent plastic cups for one week to allow for acclimatization.

Observations. Protoplast survival (percentage of living cells/total cells) and cell division rate (percentage of dividing cells/living cells) were determined after 2 and 6 days, respectively, based on a minimum sample of 400 cells per treatment. Protoplasts were considered living when they had intact plasma membranes and organelles. Colony formation was assessed after 20 days in culture as the percentage of colonies with 8 or more cells/total initial protoplast population. Colony formation was estimated by colony counts from 60 25X magnification fields per treatment. Shoot regeneration was assessed as the percentage of shoots produced from individual calli after 4 weeks on regeneration medium. Experiments were conducted 3 or more times.

Mitochondrial DNA analysis. Mitochondrial DNA was extracted from 10-15 g flower buds by the procedure of Kemble (1987). Restriction enzyme digestions were carried out using an excess of Sal1 and EcoR1 enzymes (Bethesda Research Laboratories) for 5 h or more. The samples were treated with RNAse A (40 ug/ml) for 15 min at 65°C, unless otherwise indicated. RNAse-treated samples were purified by extracting twice with phenol:chloroform and reprecipitating with ethanol at -70°C for 30 min. Following electrophoresis in 1% agarose/TBE buffer (1 M Tris base, 1 M boric acid, 20 mM Na_2EDTA), gels were stained with 0.5 ug/ml ethidium bromide for 15-20 min and photographed. The 1 kb ladder marker DNAs were obtained from Betheseda Research Laboratories.

Table 2. Effect of NAA:2,4-D auxin ratio on the percentage survival, cell division and colony formation in hypocotyl protoplast cultures of cv. Premium Crop

Medium[1]	Auxin (mg/l)	Survival	Cell division	Colony formation
K9	1 NAA 0.2 2,4-D	80 ± 2a[2]	20 ± 4a	5.3 ± 1.5a
KM9	0.6 NAA 0.6 2,4-D	75 ± 3a	26 ± 8a	2.7 ± 0.2b
KR9	0.2 NAA 1 2,4-D	78 ± 2a	24 ± 6a	2.3 ± 0.9b

[1]Auxins added to a medium containing salts, vitamins and organic acids of Kao's medium (1977), 85.5 g/l sucrose, 23.4 g/l glucose, 0.5 mg/l BAP, pH 5.6. Fed with modified feeder medium F5 containing half the growth regulator concentrations of media K9, KM9 and KR9 respectively.

[2]Waller-Duncan's Multiple Range Test conducted on arcsine transformed data. Values with different letters within a column are significantly different at p = 0.05.

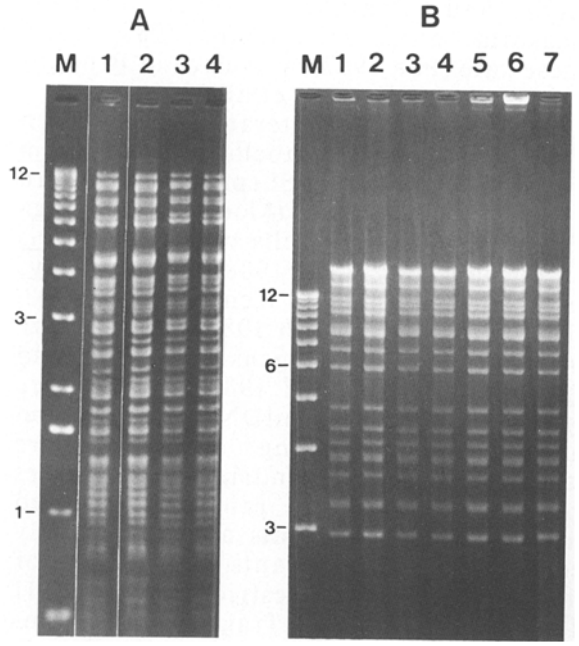

Fig. 1. EcoR1 (A) and Sal1 (B) digested mtDNAs of broccoli cv. Premium Crop. Mitochondrial DNAs were isolated from individual plants either grown from seed (lane 1) or regenerated from protoplasts (lanes 2-4 and 2-7 of A and B, respectively. M designates 1 kb ladder marker DNAs.

Results and Discussion

Viable hypocotyl protoplasts were isolated from each of the four cultivars. The average yields of protoplasts from Premium Crop and Paragon were 9 X 10^6 and 4 X 10^6 per 100 seedlings, respectively, as estimated after the washing procedure. At 2 days, survival of protoplasts from Premium Crop, Green Hornet and Paragon was high (79-85%), while survival of protoplasts from Packman was moderate (52%) (Table 1). Protoplasts of Paragon and Premium Crop started to divide after 2 days and continued to divide rapidly. At 6 days, cell division rates averaged 29% and 37% for Premium Crop and Paragon, respectively, but were lower for Packman and Green Hornet, averaging 22% and 12%, respectively. By 2-3 weeks, microcalli were visible in cultures of Premium Crop and Paragon. Consistent high frequency colony formation was observed for Premium Crop (CF=5.0%) and Paragon (CF=4.3%). Colony development with Green Hornet was slower but eventually occurred. In the case of Packman, only 2-4 cell clusters developed. Similar examples of genotypic effects on protoplast culture are well-documented in B. oleracea (Bidney et al. 1983, Jourdan and Earle 1985, Bauer 1988, Robertson et al. 1988) and other Brassica species (Pelletier et al. 1983, Glimelius 1984, Barsby et al. 1986, Chuong et al. 1987, Sproule 1987, Kao 1988).

Variations of the standard medium that differed only in the ratio of NAA:2,4-D (Table 2) were tested. No significant differences in protoplast survival or cell division rate were detected but differences in colony formation were significant. The frequency of colony formation (Table 2) and colony size were largest in the medium with the highest NAA concentration. A high NAA to 2,4-D ratio has been used in cotyledon protoplast cultures of cabbage (Lu et al. 1982) and cauliflower (Vatsya and Bhaskaran 1982), mesophyll and cotyledon protoplasts of broccoli (Robertson and Earle 1986, Robertson et al. 1988) and root protoplast cultures of cabbage (Xu et al. 1982). By contrast, high levels of 2,4-D have been found to be beneficial for colony proliferation in hypocotyl protoplast cultures of cabbage (Lillo and Shahin 1986, Yamashita and Shimamoto 1987) and leaf and callus protoplast cultures of cabbage, cauliflower and Brussel sprouts (Bauer 1988).

Protoplast-derived calli from Premium Crop and Paragon were induced to regenerate shoots. At 4 weeks, many of the calli were 1 mm or larger. These were plated on a regeneration medium consisting of Murashige and Skoog's medium (1962) supplemented with 10 g/l sucrose, 2 mg/l ZR and 0.1 mg/l NAA.

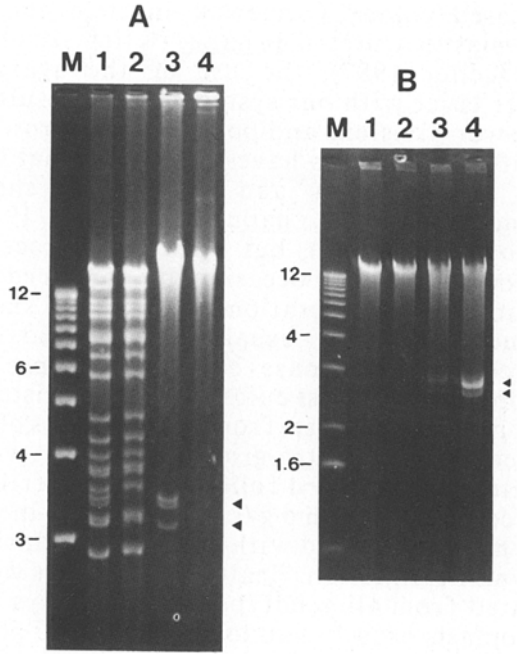

Fig. 2. (A) Sal1 digested (lanes 1,2) and undigested (lanes 3,4) mtDNA preparations of broccoli cv. Premium Crop regenerant PC11 before (lanes 1,3) and after (lanes 2,4) treatment with RNAse A. (B) Undigested mtDNA preparations of broccoli cv. Premium Crop regenerants PC5 (lanes 1,2) and PC11 (lanes 3,4) obtained at early (lanes 1,3) and late (lanes 2,4) flowering stages. Position of double-stranded RNAs are indicated by arrows. M designates 1 kb ladder marker DNAs.

Shoots were regenerated from up to 17% of the Paragon calli and up to 13% of the Premium Crop calli. Shoots formed at an average of 8-10% in both cases. Other regeneration media consisting of various combinations of Murashige and Skoog (1962) or K3 (Nagy and Maliga 1976) media components with different growth regulators (BAP, kinetin, ZR, IAA, NAA or GA_3) were tested. Shoots regenerated in these media at frequencies comparable to or lower than that obtained with the standard medium.

Most shoot primordia formed 8-14 days after plating. Eighty to 90% of the shoots were morphologically normal and rooted within 2 weeks on the rooting medium. The 10-20% abnormal shoots eventually died. A lower light intensity (25 $uEm^{-2}s^{-1}$) appeared to enhance rooting and prevent the accumulation of anthocyanins in the young shoots. One to 2 weeks after rooting, plantlets were transferred to pots containing PROMIX. Almost all plantlets survived the transplanting. The process from protoplast to plantlet took 8-11 weeks.

Various modifications to this procedure were tested. These included dispensing the protoplast suspension over an agarose feeder layer during early culture, using proliferation plates for microcalli growth and using leaf tissue as the explant source. Although layering of protoplast suspensions over agarose has increased colony formation in stem cortex protoplast cultures of B. napus (Klimaszewska and Keller 1987), the use of the agarose feeder layer with our system usually resulted in slower divisions and poorer colony growth. Proliferation plates have been beneficial for calli proliferation and subsequent shoot regeneration in B. napus (Glimelius 1984, Barsby et al. 1986), but growth of broccoli microcalli was only occasionally enhanced by culture on proliferation plates and shoot regeneration was usually unaffected or reduced. The response of the genotypes to mesophyll protoplast culture was inconsistent. Mesophyll protoplasts from young leaves of 2-4 week old in vitro-grown seedlings were isolated and cultured following the described procedures but with 5 g/l Onozuka R10 in the enzyme solution and without agitation during enzymatic digestion. Healthy protoplasts were isolated from all genotypes but by 2 days the protoplasts usually had low survival (20-50%) and colonies developed only in a few experiments. In similar experiments in B. napus however, mesophyll protoplasts have been successfully cultured and regenerated to plants (Kao and Seguin-Swartz 1987). Mesophyll protoplast cultures have been reported to be more variable in response and generally more recalcitrant in cauliflower (Vatsya and Bhaskaran 1982), cabbage (Yamashita and Shimamoto 1987), and Chinese kale (Pua 1987), as well as in B. napus (Glimelius 1984, Chuong et al. 1985).

Mitochondrial DNA alterations have been reported to occur in protoclones of Solanum tuberosum (Kemble and Shepard 1984), of an ogu CMS line of B. napus (Morgan and Maliga 1987) and in cultured cells of B. campestris (Shirzadegan et al. 1988), Zea mays (Gengenbach et al. 1981, Kemble et al. 1982, Umbeck and Gengenbach 1983, McNay et al. 1984), Glycine max (Morgens et al. 1984) and Beta vulgaris (Brears et al. 1989). We therefore sought to determine if mtDNA organization remained stable during the procedure described above. Mitochondrial DNA analyses were conducted on 11 randomly selected Premium Crop regenerants and 6 randomly selected Paragon regenerants. The mtDNA of each regenerant was restricted with SalI and/or EcoRI. The fragment patterns obtained resembled those reported for B. oleracea by others (Lebacq and Vedel 1981, Chetrit et al. 1984, Palmer 1988). In each case, the mtDNA fragments generated were identical to those of control plants grown from seed (Fig. 1). A similar absence of mtDNA alterations has been noted for Nicotiana and most B. napus protoclones (Nagy et al. 1983, Morgan and Maliga 1987, Kemble et al. 1988).

Mitochondrial DNA alterations could result from either the tissue culture system employed (Kemble et al. 1988) or from the particular plant genotype studied (Morgan and Maliga 1987). In the present study, apparently neither the tissue culture system employed nor the genotypes studied are condusive to producing mtDNA alterations.

RNAse A-sensitive bands, similar to the RNA molecules described by Kemble et al. (1986) and Monroy et al. (1990), were observed in some but not all Premium Crop regenerants (Fig. 2) and not in any of the Paragon regenerants analysed. The presence of the RNAs in certain plants appeared to increase with plant age (Fig. 2). This observation suggests that the abundance of these RNAs may be developmentally-regulated. No morphological differences were observed between regenerants that did or did not possess the RNAs.

We have improved the protoplast culture systems available in broccoli. The described procedure is relatively simple and yields efficient plant regeneration. We have regenerated over 100 plants from culture experiments. Most of these appear morphologically normal; only 2 plants were

found that were stunted in growth. In addition, we have regenerated plants from somatic cybridization experiments employing B. oleracea as the nuclear recipient. Two genetically diverse cultivars, Paragon, a blue-green broccoli obtained from a cross with Chinese kale (ssp. alboglabra) and Premium Crop, a commonly-grown green broccoli, were successfully cultured. Both cultivars are agronomically-desirable and thus, useful plant regenerants can easily be incorporated into a breeding program.

Robertson et al. (1988) reported a shoot regeneration frequency of 17-75% from mesophyll or cotyledon protoplast cultures of broccoli. However, they used plant material that had been regenerated through one or two cycles of tissue culture and thus had been "selected" for high regenerability. Plant regeneration frequency from seed-grown plants of this particular cultivar was lower (1-53%) and variable from experiment to experiment (Robertson and Earle 1986, Robertson et al. 1988). The hypocotyl protoplast culture system described in this report yields lower but relatively efficient and consistent (8-10% on average) shoot regeneration. The short growth period of seedlings avoids the variable environmental influences which may affect reproducibility of culture and regeneration. An efficient hypocotyl protoplast culture system is advantageous in that: 1) relatively high yields of protoplasts can be obtained after a short growth period; 2) hypocotyl protoplasts are more stable in culture and are more resistant to the effects of protoplast fusion (Sundberg and Glimelius 1986, Yamashita and Shimamoto 1987, Kao 1988) and 3) hypocotyl protoplasts have dense cytoplasms which make them an excellent fusion partner since they can be distinguished from mesophyll protoplasts in fusion experiments.

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