Theor Appl Genet (1985) 71:495–499

© Springer-Verlag 1985

Plant regeneration from mesophyll protoplast culture of cabbage (*Brassica oleracea* var 'capitata')

You-ying Fu, Shi-rong Jia and Yun Lin

Vegetable Research Institute, Chinese Academy of Agricultural Sciences, Beijing, China

Received June 7, 1985; Accepted July 26, 1985
Communicated by H. F. Linskens

Summary. Protoplasts were enzymatically isolated from the first leaves of cabbage (*Brassica oleracea* var 'capitata', F1 hybrid 'Baochun'). Sustained cell division and somatic embryogenesis were obtained after culturing the protoplasts in modified liquid DPD medium supplemented with $CaCl_2 \cdot 2H_2O$ 800 mg/l, 2,4-D 0.5 mg/l, kinetin 1 mg/l, 0.3 M mannitol and sucrose 20 g/l. Upon transferring cell colonies onto a modified Murashige and Skoog (MS) agar medium, small calli were gradually formed. Callus proliferated on MS medium supplemented with hormone combinations of 2,4-D 0.1–0.5 mg/l and kinetin 3–4 mg/l. Multiple shoots were induced on differentiation medium supplemented with 3 mg/l of kinetin and 0.1 mg/l of gibberellic acid GA_3. After transferring differentiated shoots onto MS medium supplemented with indoleacetic acid (IAA), kinetin, GA_3 at 0.1 mg/l each and 500 mg/l of N.Z. amine, intact plants were eventually produced.

Key words: Cabbage (*Brassica oleracea* L.) – Protoplast culture – Plant regeneration

Introduction

Brassica is an important plant genus in which many valuable vegetable and oil crops, such as cabbage, rapeseed, and mustard, are included. Up to the late 1970s, it was difficult to induce plant regeneration from *Brassica* protoplast culture. In most cases only callus formation or root differentiation was obtained (Gatenby and Cocking 1977; Schenck and Hoffmann 1979; Ulrich et al. 1980). Even in *B. napus,* in which plant regeneration had been reportedly achieved at that time (Kartha et al. 1974; Thomas et al. 1976), it was a rare event.

Recently, progress has been made in *Brassica* protoplast culture and fusion. Plant regeneration from protoplast culture of *B. napus* has succeeded in various laboratories. Li and Kohlenbach (1982) obtained somatic embryogenesis directly from protoplast culture of this species, while Yu et al. (1982), Lu et al. (1982) and Bidney et al. (1983) reported on plants regenerated from protoplast culture of *B. oleracea* using root, cotyledon or leaf as material for protoplast isolation. Vatsya and Bhaskaran (1982) also obtained plant regeneration from cotyledon protoplasts of cauliflower (*B. oleracea* var 'botrytis'). In the field of protoplast fusion, a synthetic species, *B. napus* (AACC, 2n=38), has been created through cell hybridization between *B. oleracea* (CC, 2n=18) and *B. campestris* (AA, 2n=20) (Schenck and Röbbelen 1982). Symmetric and asymmetric intergeneric hybrids, *Arabido-brassica,* have also been produced by protoplast fusion between *Arabidopsis thaliana* and *B. campestris* (Gleba and Hoffmann 1978, 1979). Thus, the techniques of protoplast culture and fusion have provided a new way for crop improvement in *Brassica* species.

Although techniques for protoplast culture of cabbage have advanced in some ways in recent years, there is still room for further improvement in such areas as the choice of donor plant material, the optimization of culture medium and culture conditions when compared with tobacco, petunia and carrot, for example, in which protoplast culture techniques are well developed. The present paper reports on the embryogenesis, callus formation and plant regeneration from mesophyll protoplasts of cabbage using the first leaves as a source for protoplast isolation.

Materials and methods

An early maturing F1 hybrid 'Baochun', which has been widely used in cabbage production in China, was used in this study. Seeds were kindly provided by the cabbage breeding group of our institute. First true leaves, taken from greenhouse grown seedlings 2–4 weeks after sowing, were used for protoplast isolation. Leaves were surface sterilized in 70% alcohol

for several sec, soaked in a sodium hypochloride solution containing 0.7% active chloride for 8–10 min, then washed four times with sterilized distilled water. The lower epidermis of the leaves was peeled off under aseptic conditions.

The pre-prepared enzyme solution consisted of 4% cellulase Onozuka R10, 1% Pectinase or Macerozyme R10 dissolved in a solution containing 0.7 mM KH_2PO_4, 7 mM $CaCl_2 \cdot 2H_2O$, 0.5 M mannitol, and 3 mM MES (2-(N-morpholino)ethanesulfonic acid), pH = 5.8 and an osmotic pressure of 796 ± 3.2 mmol/kg. Before enzymatic digestion, the above enzyme solution was mixed in a ratio of 1:1 with modified DPD medium in which the concentration of $CaCl_2 \cdot 2H_2O$ was increased to 800 mg/l and where 0.5 mg/l 2,4-D, 1 mg/l of kinetin, 0.3 M of mannitol, and 20 g/l of sucrose were added. The pH value of the modified DPD medium was adjusted to 5.8; the osmotic pressure 466 ± 2.6 mmol/kg. Leaves peeled of their lower epidermis were placed in the above enzyme mixture and then shaken on a gyrotory shaker at a speed of 100 rpm for 5 h under room temperature to obtain cell wall digestion. After digestion, the enzyme mixture containing the protoplasts was passed through a stainless steel mesh with a pore size of 62 μ to remove large undigested debris. The filtrate was centrifuged and the protoplast pellet was resuspended in modified DPD medium. This washing procedure was repeated four times before the protoplast suspension was transferred into a ∅ 60 mm Petri dish (Falcon, 3002 tissue culture dish) for drop or thin layer culture at 25–26 °C in the dark.

To induce sustained cell division, after one week the cultures were diluted with a medium in which the osmotic pressure and concentration of 2,4-D were reduced and the amount of kinetin was increased (DPD medium with 800 mg/l of $CaCl_2 \cdot 2H_2O$, 0.2 mg/l of 2,4-D, 3 mg/l of kinetin, 0.25 M mannitol, and 20 g/l of sucrose, pH 5.8). After dilution, the Petri dish was placed under a light condition (light intensity = 1,500 lux). Cell colonies seen with the naked eye in liquid culture were then transferred onto a modified Murashige and Skoog (MS) agar medium (MS salts, B5 vitamins, sucrose 30 g/l, pH 5.8) supplemented with varying combinations of growth regulators in order to test for callus formation and the proliferation and induction of organogenesis. The cultures were kept under a 25°/26 °C, 16/8 h day/night photoperiod in a LH-200-RDCT growth chamber.

Results

Isolation and culture of protoplasts

Large quantities of viable protoplasts having a survival percentage over 95% could be obtained after enzymatic digestion by using final concentrations of 2% cellulase and 0.5% pectinase (Fig. 1 a). After 2–3 days in culture, cells started to swell and first division was found at day 4–5 (Fig. 1 b). Second (Fig. 1 c) and third division were seen at 7–8 days after initiation of culture. From then onwards, cell division abruptly accelerated, resulting in the formation of numerous dividing cell colonies, 80% of which were characterized with smooth surfaces and globular shapes which could be considered as a pro-embryo structure. The size of this pro-embryo structure could further increase and develop into a globular or heart-shaped embryoid (Figs. 1 f, g, h). Simultaneously, a part of the cell population formed unorganized callus (Fig. 1 d).

It was found that diluting the culture at days 5–8 using a medium with lowered osmotic pressure and auxin content and increased concentration of cytokinin could facilitate cell division and growth of globular embryoids which might succeedingly develop to heart stage embryoids. Compared to those cultures continuously maintained in the dark, where the cells of the surface layers of the globular embryoids elongated and protruded and never reached the heart stage, and in some cases, even browned and eventually died, cell growth and division were more vigorous when, after dilution, cultures were transferred from dark conditions to illuminated conditions.

In an initial experiment, three different concentrations of osmoticum in the culture medium containing mannitol 0.25, 0.35 and 0.5 M with osmotic pressure of 360, 406 and 670 mmol/kg, respectively, were tested. The best result was obtained when 0.35 M mannitol was used. Both higher and lower osmotic pressure resulted in the death of large number of protoplasts or no division.

Callus proliferation

Calli could be induced from cell colonies when they were transferred from liquid culture onto an agar medium containing 2,4-D 0.01 + BA 1 (or kinetin 3), or IAA 0.2 + BA 2 (or zeatin 1–2) mg/l. However, callus could not be subcultured and proliferated on the above-mentioned media. Callus proliferation requires a certain level of auxin which has been proven to be a limiting factor for callus growth. For example, callus would show browning and eventually die after a certain period of culture when it was transferred onto a modified MS medium supplemented with naphthaleneacetic acid (NAA) 0.001–0.005 plus BA 1–3 mg/l, or BA 0.01–15.0 mg/l without auxin, or onto a medium in which no growth hormones were added. In the later case, the callus died very rapidly. Callus could be maintained and proliferated on a medium containing 2,4-D 0.1–0.5 plus kinetin 3–4 mg/l (Fig. 1 e). Callus proliferated on this medium would give rise to shoot regeneration 20 days after transfer onto a differentiation medium.

Plant regeneration

Shoot differentiation (Fig. 1 i) could be generated from cabbage mesophyll protoplast-derived callus when callus was cultured on a differentiation medium containing kinetin 3–4 mg/l and GA_3 0.1–0.5 mg/l com-

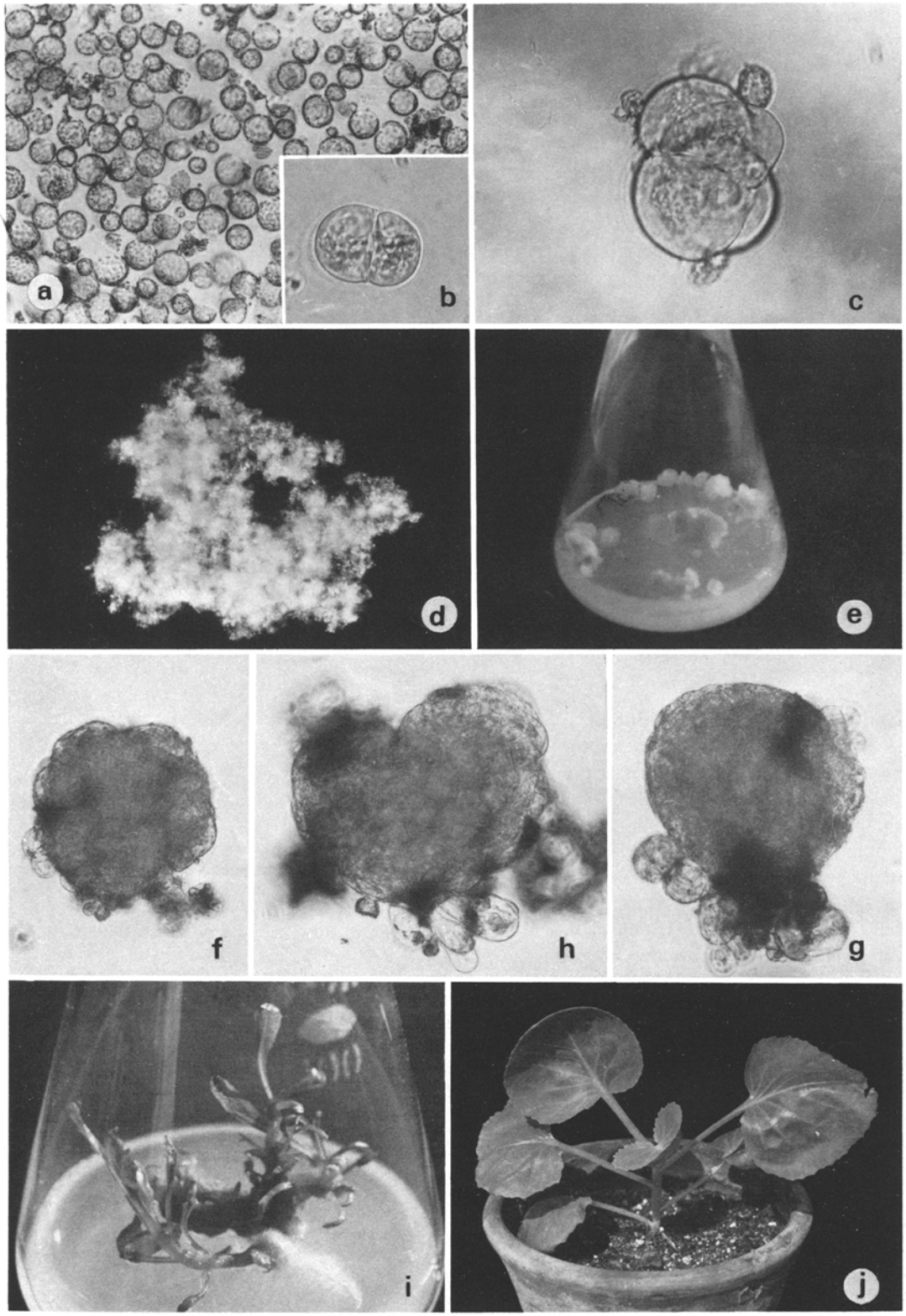

Fig. 1 a–j. Plant regeneration from mesophyll protoplast culture of cabbage (*Brassica oleracea* var 'capitata'). **a** Freshley isolated protoplasts from first true leaves; **b** first division; **c** second division; **d** small calli; **e** callus proliferation on agar medium; **f, g, h** embryoid at globular and heart stage; **i** shoot differentiation; **j** regenerated plant

bined with a reduced concentration of 2,4-D 0.01 mg/l, or without auxin. An optimal hormone combination was found to be the addition of kinetin (3 mg/l) and GA_3 (0.1 mg/l) and the absence of 2,4-D. The frequency of callus differentiation on this medium was relatively high: 20% on average and in some cases up to 100% of callus produced shoots. Multiple shoots were produced on this medium with a mean of 11.5 shoots per callus. Although shoots were able to regenerate when 0.01 mg/l 2.4-D was present in the differentiation medium, callus differentiation frequency and shoots produced per callus were both dramatically reduced with a mean of 4.0–7.1% and 2 shoots/callus, respectively. Plant regeneration was also observed in the experiment from protoplast-derived somatic embryoids, however, the frequency was relatively low indicating that an optimization of culture conditions for further development of heart-stage embryoids was obviously needed.

When differentiated, shoots were transferred onto modified MS medium supplemented with 0.1 mg/l each of IAA, kinetin, GA_3 and 500 mg/l of N.Z. amine, the intact plants were eventually produced (Fig. 1j).

Discussion

One characteristic of cabbage mesophyll protoplast culture found in this experiment was that viable protoplasts mostly aggregate and float on the surface of the culture medium after 1–2 days in culture, while protoplasts which lose their viability generally sink to the bottom. This phenomenon contrasts sharply with the characteristics found in pea mesophyll protoplast culture where sedimentation of protoplasts is a prerequisite for sustained cell division (Jia 1982). Ulrich et al. (1980) and Vatsya et al. (1982) also reported aggregation in protoplast culture of *B. rapa* and *B. oleracea* var 'botrytis', respectively. Chuong et al. (1985), while studying hypocotyl protoplast culture of *Brassica* spp., found that the intact protoplasts floated to the surface of the medium but cell wall debris and lysed cells sank to the bottom of the culture dish. They obtained good results by using a culture medium containing 13% sucrose and 5 g/l Ficoll: floating protoplasts subsequently formed microcalli on the surface of the medium. This result suggests that gas exchange might be an important factor controlling the division and development of *Brassica* protoplasts.

Another characteristic of cabbage mesophyll protoplast culture is that cell division suddenly speed up 8–10 days after the initiation of culture resulting in the formation of globular embryoids characterized by smooth surfaces. These might further develop into heart-stage embryoids.

Embryogenesis directly induced from protoplast culture is of great importance: the embryoids are able to germinate and form plantlets under appropriate conditions thus avoiding the difficulties in differentiation and ploidy and chromosome changes in callus culture. To the best of our knowledge, plant regeneration from protoplast culture has been achieved in more than eighty plant species, of these, only a few species, such as *Asparagus officinalis* (Bui-Dang-Ha et al. 1975), *Brassica napus* (Li and Kohlenbach 1982), *Daucus carota* (Kameya and Uchimiya 1972), *Medicago sativa* (Kao and Michayluk 1980), and *M. glutinosa* (Arcioni et al. 1982) are through embryogenesis. Therefore, it is worthwhile to further optimize culture conditions which may allow globular and heart-stage embryoids produced in the cabbage mesophyll protoplast culture to develop directly towards plants.

Acknowledgement. The authors wish to thank Ms. Ann Jenks-Coupland for correcting the English manuscript.

References

Arcioni S, Davey MR, dos Santos AVP, Cocking EC (1982) Somatic embryogenesis in tissues from mesophyll cell suspension protoplasts of *Medicago coerulea* and *M. glutinosa*. Z Pflanzenphysiol 106:105–110

Bidney DL, Shepard JF, Kaleikau E (1983) Regeneration of plants from mesophyll protoplasts of *Brassica oleracea*. Protoplasma 117:89–92

Bui-Dang-Ha D, Norreel B, Masset A (1975) Regeneration of *Asparagus officinalis* L. through callus cultures derived from protoplasts. J Exp Bot 26:263–270

Chuong PV, Pauls KP, Beversdorf WD (1985) A simple culture method for *Brassica* hypocotyl protoplasts. Plant Cell Rep 4:4–6

Durand J, Potrykus I, Donn G (1973) Plantes issues de protoplastes de *Petunia*. Z Pflanzenphysiol 69:26

Gatenby AA, Cocking EC (1977) Callus formation from protoplasts of marrow stem kale. Plant Sci Lett 8:275–280

Gleba YY, Hoffmann F (1978) Hybrid cell lines *Arabidopsis thaliana* + *Brassica campestris* asexual hybrid no evidence for specific chromosome elimination. Mol Gen Genet 165: 257–264

Gleba YY, Hoffmann F (1979) *Arabidobrassica:* Plantgenome engineering by protoplast fusion. Naturwissenschaften 66: 547–554

Jia SR (1982) Factors affecting the division frequency of pea mesophyll protoplasts. Can J Bot 60:2192–2196

Kameya T, Uchimiya H (1972) Embryoids derived from isolated protoplast of carrot. Planta 103:356–360

Kao KN, Michayluk MR (1980) Plant regeneration from mesophyll protoplasts of alfalfa. Z Pflanzenphysiol 96: 135–141

Kartha KK, Michayluk MR, Kao KN, Gamborg OL, Constabel F (1974) Callus formation and plant regeneration from mesophyll protoplasts of rape plants *Brassica napus* cultivar Zephyr. Plant Sci Lett 3:265–271

Li L-C, Kohlenbach HW (1982) Somatic embryogenesis in quite a direct way in cultures of mesophyll protoplasts of *Brassica napus* L. Plant Cell Rep 1:209–211

Lu DY, Pental D, Cocking EC (1982) Plant regeneration from seedling cotyledon protoplasts. Z Pflanzenphysiol 107: 59–63

Schenck HR, Hoffmann F (1979) Callus and root regeneration from mesophyll protoplasts of basic *Brassica* species: *B. campestris, B. oleracea* and *B. nigra.* Z Pflanzenphysiol 82:354–360

Schenck HR, Röbbelen G (1982) Somatic hybrids by fusion of protoplasts from *Brassica oleracea* and *B. campestris.* Z Pflanzenzücht 89:278–288

Thomas E, Hoffmann F, Potrykus I, Wenzel G (1976) Protoplast regeneration and stem embryogenesis of haploid androgenetic rape. Mol Gen Genet 145:245–247

Ulrich TH, Chowdhury JB, Widholm JM (1980) Callus and root formation from mesophyll protoplasts of *Brassica rapa.* Plant Sci Lett 19:347–354

Vatsya B, Bhaskaran S (1982) Plant regeneration from cotyledonary protoplasts of cauliflower (*Brassica oleracea* L. var *botrytis* L.). Protoplasma 113:161–163

Xu ZH, Davey MR, Cocking EC (1982) Plant regeneration from root protoplasts of *Brassica.* Plant Sci Lett 24: 117–121