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Research Article

Myo-inositol increases the plating efficiency of protoplast derived from cotyledon of cabbage (*Brassica oleracea* var. *capitata*)

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Abstract This study describes the effect of myo-inositol on sustained cell division and plant regeneration from cotyledon-derived protoplast of cabbage (*Brassica oleracea* var. *capitata*). Freshly isolated protoplasts were cultured in modified Murashige and Skoog (MS) medium removed ammonia ions and containing 0.4 mg l^{-1} thiamine·HCl, 100 mg l^{-1} myo-inositol, 2 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BA, 30 gl^{-1} sucrose and several concentrations of myo-inositol (2, 4, 6, 8, 10% (w/v)) as an osmotic stabilizer. After 3 weeks of culture in the dark at 25℃, the plating efficiency of cabbage protoplasts reached to 22.5 ± 2.9% when cultured in modified MS medium supplemented with 2 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BA, 30 gl^{-1} sucrose and 8% (w/v) of myo-inositol at a density of 2 x 10^5 protoplasts/ml. Rapidly growing cell colonies after 3 weeks of culture were transferred to the same culture medium removed osmoticum. To induce shoot regeneration from calluses, calluses with about 2 mm in diameter were transferred to the MS medium containing 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA. After further three weeks of incubation onto the medium in the light, green shoots were formed on the surface of calluses at a frequency of 30%. Upon transfer to half-strength MS basal medium, roots were formed onto the bottom of regenerated shoots without auxin treatments. These regenerated plantlets were successfully acclimatized to soil transfer, grown to normal mature plants. The cabbage protoplast culture system established in this study could be applied for production of somatic hybrids or cybrids by asymmetric protoplast fusion and mass proliferation of elite somatic clones of cabbage.

Keywords Cabbage (*Brassica oleracea* var. *capitata*) - Protoplast culture - Plant regeneration

Abbreviations 2,4-D: 2,4-dichlorophenoxyacetic acid; BA: 6-benzyladenine; CPW: Cell Protoplasts Washing; MS: Murashige and Skoog; NAA: α-naphthaleneacetic acid

Introduction

Brassica including broccoli, cabbage, rapeseed, and mustard is valuable vegetables and oil crops belonging to the Cruciferae. In Europe, the predominance goes to *B. oleracea*, a species that includes important crops such as cabbage, broccoli, cauliflower etc. Whereas, *B. campestris* is the most cultivated species owing to the great importance of Chinese cabbage in Asia. The vegetables have been very important in Korea as a major farmers' income source being 29% of their total farming income, and as indispensable component of their diet for many years. Korean seed market for Chinese cabbage, radish and cabbage was $12.02, $25.54 and $1.12 million respectively in 2003 (Park 2006).

Breeding strategy and targets are perfectly dependent on market trends. With an increasing number of consumers aware of the importance of diet on human health, vegetable brassicas have new appeal as a diet food with green vegetables during recent years. However, without having new genetic resources that contain horticulturally important characters, it is very difficult to develop a new vegetable variety by crossing or conventional breeding. Asymmetric cell fusion technology is to combine two cells by protoplast culture with electrical shock or PEG or radiation.

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This technique is very powerful because it generates a new crop or mutant by mixing the different genomes and ends up with a new genetic source. This technology would expand the breeding program of Cruciferae with the new CMS character (Monteiro and Lunn 1999)

Since the first successful plant regeneration from mesophyll protoplasts of rape plants (Kartha et al. 1974), lots of studies has been reported in protoplast culture and plant regeneration of the genus, *Brassica*. Li and Kohlenbach (1982) reported that plant regeneration was possible directly from mesophyll protoplast culture of *Brassica napus* via direct somatic embryogenesis. Successful plant regeneration was also established in cauliflower from various protoplast cultures including cotyledon (Vatsya and Bhaskaran 1982), root (Xu et al. 1982) and leaf explants (Bidney et al. 1983; Fu et al. 1985) of Brassica. Effects of media components and environmental factors on growth and organogenesis of protoplast-derived calli were also investigated (Lillo and Olsen 1989). Recently, protocol for rapid and efficient plant regeneration from protoplasts of cabbage was developed by a novel nurse culture method (Chen et al. 2004) and shoot cultures in large glass vessels with vented lids (Chikkala et al. 2009). A lot of successful studies are also reported from protoplast culture of Brassica for somatic cell hybridization. Gleba and Hoffmann (1978, 1979) produced Arabido-brassica, intergeneric hybrids, by protoplast fusion between *Arabidopsis thaliana* and *B. campestris*. Until now, several *Brassica oleracea* hybrids have been created through somatic cell hybridization between *B. oleracea* and other plants (Schenck and Hoffmann 1979; Hansen and Earle 1997; Ishikiwa et al. 2003; Motegi et al. 2003; Yamagishi and Nakagawa 2004; Chen et al. 2005). Thus, the techniques of protoplast culture and asymmetric protoplast fusion have provided a new way for quality improvement in Brassica species. Although techniques for protoplast culture of cabbage have advanced in some ways in recent years, there is still demand for further improvement in such areas as the choice of donor plant materials with agricultural characteristics, the optimization of culture medium and culture conditions. The present paper reports on the callus formation and plant regeneration from cotyledon- derived protoplasts of cabbage using myo-inositol as an osmotic stabilizer.

Materials and methods

Plant materials

The cabbage (*Brassica oleracea* var. *capitata* cv. 'Dongbok') seeds used in this study were purchased from Nong Woo Bio (Co.). This cultivar has been widely used in cabbage production in Korea. Seeds were surface sterilized in 70% alcohol for 3 min, soaked in a sodium hypochloride solution containing 1% active chloride for 15 min then washed 3 times with sterilized distilled water. Seeds were transferred onto the Murasige and Skoog (Murashige and Skoog 1962) MS medium containing 0.4 mg l^{-1} thiamine·HCl, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and plant agar 8 g l^{-1}, pH 5.8 (adjusted before autoclaving). Seed germination and seedling growth was maintained a tissue culture room at 25℃ under 16-h photoperiod. The cotyledon 3 ~ 7 days after sowing were used for protoplast isolation.

Protoplast isolation

Cotyledon of one week old seedlings (approximately 1 g in fresh weight) were harvested and incubated overnight with 10 ml of an enzyme solution in an 100 x 20 mm plastic Petri dish on a gyratory shaker (30 rpm) at 25℃ in the dark. For improving cell wall digestion, each cotyledon were cut into 1 ~ 2mm thick and soaked in a plasmolytic solution i.e. 13% mannitol CPW solution (Frearson et al. 1973) for 30 min which was replaced by the enzyme mixture. The enzyme solution consisted of cellulase and pectinase (Novozymes) mixture, 3 mM MES (2-[N-Morpholino] ethanesulfonic acid), and 9% mannitol in CPW (Frearson et al. 1973) salts. The enzyme solution was filter-sterilized through a 0.22 ㎛ nylon membrane and pH adjusted to 5.8 with 1N NaOH. After incubation the protoplasts were purified by passing the digested cell aggregates in the enzyme solution through 250 and 50 ㎛ stainless steel mesh screens in a plastic Petri dish. The enzyme solution containing protoplasts was transferred to a 15 ml Falcon centrifuge tube and spun at 100 Xg for 5 min. After removal of the enzyme solution with a Pasteur pipette, protoplasts packed at the bottom of the tube were resuspended in CPW solution containing 9% mannitol. The protoplast washing (centrifugation/resuspension) process was repeated three times. The final protoplast purification step using CPW containing 21% sucrose to float protoplasts was omitted. The protoplasts were collected with a Pasteur pipette and finally resuspended in 10 ml of modified MS medium fully removed ammonia ions and containing 0.4 mg l^{-1} thiamine·HCl, 60 gl^{-1} myo-inositol, 30 gl^{-1} sucrose, 2 mgl^{-1}, 2,4-D and 0.5 mgl^{-1} BA at pH 5.8 (initial protoplast culture medium). The density and the total yield of protoplasts were estimated using a hemocytometer and then adjusted to adequate protoplast densities of 2 x 10^5, 1 x 10^5, and 5 x 10^4 protoplasts/ml for

culture. After density adjustment, the protoplasts cultures were kept in 60 x 15 mm Petri dish (Falcon, 3002 tissue culture dish) at 25℃ in the dark.

Protoplast culture

One ml of protoplast suspension was plated on a plastic Petri dish (Falcon, 60 x 15 mm) as a thin layer liquid culture. Also the protoplasts were incubated by agarose embedding culture. One ml of protoplast suspension and same volume of protoplast culture medium with 1.2% agarose typeVII (Sigma, USA) at 40℃ was mixed to a plastic Petri dish (Falcon, 60 x 15 mm) (agarose embedding culture). Protoplasts were incubated at 25℃ in the dark and cell division was periodically observed with an inverted microscope. To induce sustained cell division, the initial protoplast culture medium was replaced with modified protoplast culture medium containing 20.6 mM ammonia (NH_4^+), 39.4 mM nitrate ions (NO_3^-) of the original MS salts after two weeks of culture. The plating efficiency (% of plated protoplasts which divided at least once) was determined after 3 weeks culture. Cell colonies seen with the naked eye in liquid or agarose embedding culture were then transferred onto a agar-solidified (0.4% Gelrite) protoplast culture medium supplemented with varying combinations of growth regulators in order to proliferate callus growth and induction of shoot organogenesis.

Effect of plant growth regulators and myo-inositol concentration on plating efficiency of cabbage protoplast

The effect of plant growth regulators on plating efficiency was examined. Freshly isolated protoplasts were diluted using initial protoplast culture medium at density of 1 x 10^5 protoplasts/ml. Each 200 ul of protoplast suspension without no plant growth regulators was dispensed into 24 well plate and then filter-sterilized 2,4-D and BA were add to each well. The final concentration of 2,4-D and BA was adjusted to 0, 0.1, 0.3, 1, 3 and 10 mg l^{-1} respectively. Several combinational treatment of 2,4-D and BA was also examined (Table 1). Furthermore, we also investigated the effect of myo-inositol on plating efficiency, the final concentration of myo-inositol in initial protoplast culture medium was adjusted to 20, 40, 60, 80 and 100 gl^{-1} respectively. In order to examine the effect of myo-inositol on plating efficiency, protoplasts were also resuspended in an initial protoplast culture medium in which myo-inositol was replaced with 90 g l^{-1} mannitol. All protoplasts cultures were kept in 60 x 15 mm Petri dish at 25℃ in the dark. The plating efficiency (% of plated protoplasts which divided at least once) from all treatments was determined after three weeks culture. All treatments consisted with three replicates for statistical analysis.

Plant regeneration from protoplast-derived callus of cabbage

To induce sustained callus growth, cell colonies seen with the naked eye in liquid or agarose embedding culture were then transferred onto MS medium containing 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA. After two weeks of cultures, microcalluses were transferred to MS medium supplemented with 2 mg l^{-1} BA and 0.5 mg l^{-1} NAA in order to proliferate callus growth and induction of shoot organogenesis. Also we examined the effect of gelling agent (0.4% Gelrite and 0.8% Plant agar) on shoot formation from callus during the culture. These cultures were maintained at 25℃ in the light (approximately 30 μmol $m^{-2}s^{-1}$ from cool-white fluorescent lamps with a 16-h photoperiod). The efficiency of shoot formation from protoplast-derived calluses was determined after 3 weeks culture. All treatments consisted with three replicates for statistical analysis. To regenerate whole plants, green shoots formed on calluses derived from protoplasts were transferred to MS basal medium and cultured in the light (approximately 30 μmol $m^{-2}s^{-1}$ from cool-white fluorescent lamps with a 16-h photoperiod). After 4 weeks of incubating in the light, regenerated plantlets with rooting were transplanted into potting soil (vermiculite:perlite, 3:1 mixture) and maintained in plastic container for two weeks. After emergence of new leaves from transplanted plants, acclimatized plants were transferred and maintained in a growth chamber (80 μ mol $m^{-2}s^{-1}$ from cool-white fluorescent lamps with a 16-h photoperiod and 50 ~ 70% RH).

Statistical analysis

The experiment was conducted in a completely randomized design with three replicates. Data were subjected to an analysis of variance (ANOVA). To determine the significant difference in mean ranges, one way ANOVA was performed using Origin software (Ver. 8; OriginLab Co, USA).

Results and discussion

Isolation and culture of cotyledon-derived protoplasts of cabbage

Large quantities of viable protoplasts could be obtained from cotyledon of cabbage seedlings after enzymatic digestion (Fig. 1A). The diameter of freshly isolated protoplasts was 20 ~ 50 ㎛ (Fig. 1A). After 3 to 5 days of culture, protoplasts underwent first cell division (Fig. 1B). After 1 week of culture, second cell division occurred at a frequency of approximately 30% (Fig. 1C). Cell colonies reached a diameter of 100 ~ 200 ㎛ after 3 weeks of culture (Fig. 1D), and after 4 weeks of culture, numerous micro-calluses were formed (Fig. 1E). We examined the adequate protoplast densities for prototoplast division and plant regeneration. The highest plating efficiency (about 17%) was obtained when they cultured into the initial protoplast medium at densities of 2 x 10^5 (Fig. 2). However when protoplasts cultured at a density 1 x 10^5 and 5x 10^4, the cell division efficiency was decreased to 13 to 10%. Over than 2 x 10^5 treatment, exact examination of cell division efficiency was difficult too numerous cells to count. Therefore, an optimal prototoplast density for protoplast culture was found to be the treatment of 1 to 2 x 10^5 from cotyledon-derived protoplast of cabbage.

In the preliminary studies, initial protoplast culture medium without free ammonia ions was suitable for sustained cell division. However, cell colonies showed browning and eventually dead after three weeks of culture. To induce sustained cell division, the initial protoplast culture medium was replaced with modified protoplast culture medium containing 20.6 mM ammonia (NH_4^+), 39.4 mM nitrate

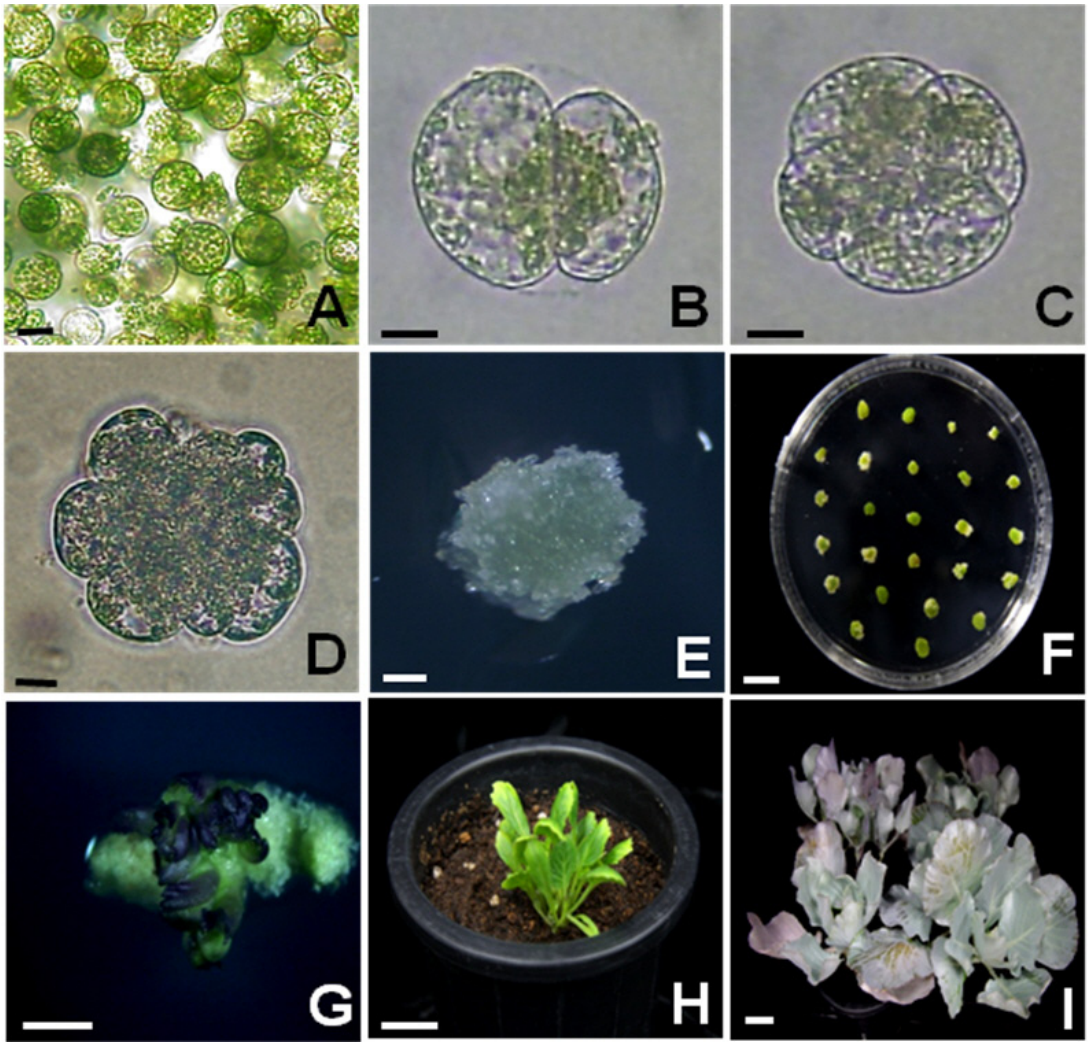

Fig. 1 Plant regeneration from cotyledon-derived protoplast culture of cabbage. A: Freshly isolated protoplasts; B: First cell division in cultured protoplasts after 3 to 5 days of culture; C: Second cell division in cultured protoplasts after 1 week of culture; D: Cell colony formation in cultured protoplasts after 4 to 6 weeks of culture; E: Microcallus formation in cultured protoplasts after 8 weeks of culture; F: Shoot induction from microcallus; G: Shoot formation from microcallus; H: Soil transfer of regenerated plantlet; I: Regenerated plantlets from protoplast-derived calluses via shoot organogenesis. Scale bars represent 20 ㎛ (A ~ D), 1 mm (E), 1 cm (F ~ G) and 2 cm (H ~ I)

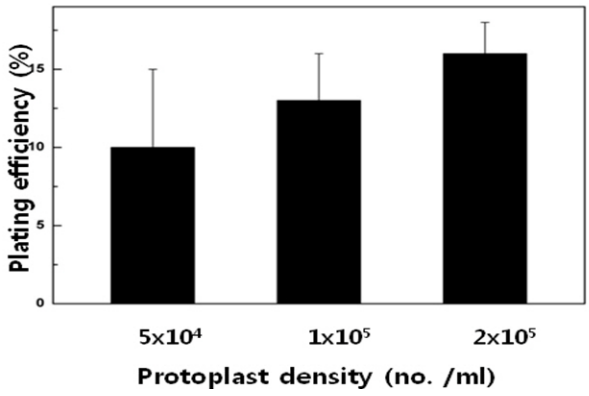

Fig. 2 Plating efficiency from cotyledon-derived protoplast culture of cabbage. Bars represent standard deviation. At the 0.01 level, the population means are significantly different (Prob = 0.009459)

ions (NO_3^-) of the original MS salts after two weeks of culture. Cell colonies from protoplast could be maintained and proliferated on a modified protoplast culture medium.

Effect of growth regulators on cell division of cabbage protoplast

We examined the optimal plant growth regulators for sustained cell division from cotyledon-derived protoplast of cabbage (Table 1). The highest plating efficiency was obtained when protoplasts were cultured onto initial protoplast culture medium containing 3 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BA. No cell division and cell colony formation occurred in 2,4-D or BA alone treatments. Cell divisions were observed in combinational treatment of 2,4-D and BA. Cell division frequency was increased in high molar ratio of 2,4-D to BA (2,4-D/BA), whereas division frequency was decreased in low molar ratio of 2,4-D to BA. These results indicated that high concentration of 2,4-D and low concentration of BA stimulate cell division from cotyledon-derived protoplast of cabbage simultaneously. Similar results were also shown in agarose embedding cultures (data not shown). Therefore we concluded that optimal plant growth regulators for cabbage protoplast culture was 1-to 3 mgl^{-1} 2,4-D and 0.5 mgl^{-1} BA. These culture conditions were used following experiments.

Effect of myo-inositol on plating efficiency of cabbage protoplast

In an initial experiment, several concentrations of osmoticum in the initial protoplast culture medium containing myo-inositol 20, 40, 60, 80 and 100 gl^{-1} respectively were examined (Fig. 3). The plating efficiency was 15, 21.5 and 22.5 % when cultured the medium containing 40, 60, and 80 gl^{-1} myo-inositol, respectively. The highest plating efficiency was obtained when protoplasts were cultured into the medium containing 80 gl^{-1} myo-inositol. The plating efficiency was slightly decreased to 20.1% when protoplasts were cultured into the medium containing 100 gl^{-1} myo-inositol. However, no cell divisions and cell colony formation occurred in case of 20 gl^{-1} treatment. These results showed that optimal concentration of myo-inositol for osmotic stabilizer was 60 to 100 gl^{-1}. These results were unchanged regardless of modification of protoplast densities (Fig. 3). We also compared the effect of myo-inositol and mannitiol on plating efficiency (Fig. 4). The plating efficiency was decreased when protoplasts were cultured in the medium containing 9% mannitol instead of 6% myo-inositol. These results showed that myo-inositol might be more suitable osmotic stabilizer for sustained cell division from cotyledon-derived protoplast

Table 1 Effect of plant growth regulators on cell division from cotyledon-derived protoplast cultures of cabbage

Plant growth regulators	Concentration of Plant growth regulators (mg l^{-1})					
	Plating efficiency *					
2,4-D	0	0.1	0.3	1	3	10
	-	-	-	-	-	-
BA	0	0.1	0.3	1	3	10
	-	-	-	-	-	-
2,4-D + BA	1 + 0.1	1 + 0.3	1 + 0.5	1 + 1	1 + 3	1 + 10
	-	+	++	+	+	-
2,4-D + BA	3 + 0.1	3 + 0.3	3 + 0.5	3 + 1	3 + 3	3 + 5
	+	++	+++	+	+	+

*Plating efficiency was determined after two weeks of culture.
-/+ symbols represent the frequency of cell division

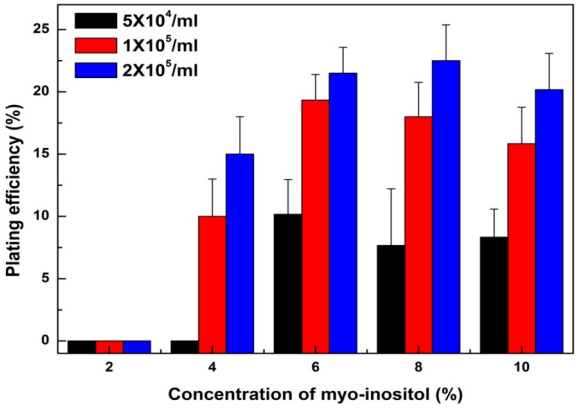

Fig. 3 Effect of myo-inositol concentration on plating efficiency from cotyledon-derived protoplast from cabbage. Vertical bars represent standard deviation. At the 0.001 level, the population means are significantly different (Prob=2.229E-7)

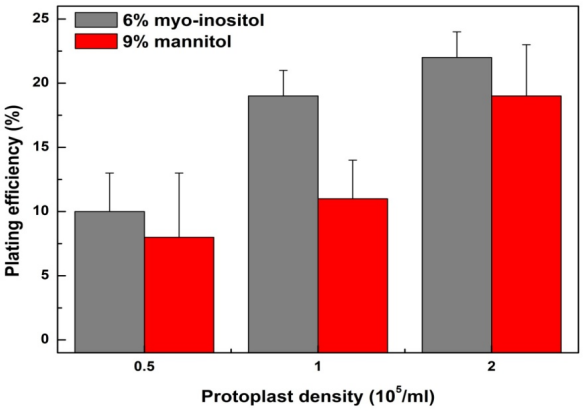

Fig. 4 Effect of osmoticums (6% myo-inositol and 9% mannitol) on plating efficiency from cotyledon-derived protoplast from cabbage. Vertical bars represent SD. At the 0.001 level, the population means are significantly different (Prob = 9.488E-5)

of cabbage. This result is in agreement with Arya et al. (1991) who reported that myo-inositol worked as an osmotic stabilizer for sustained division of ginseng protoplasts. Myo-inositol play a role as precursors to many compounds whose function in plants is linked to phosphorus storage, signal transduction, stress protection, hormonal homeostasis and cell wall biosynthesis (Hegeman et al. 2001). Myo-inositol is also considered as a growth enhancer in vitro and may be a carbohydrate source, which are good osmoticum for protoplast isolation (Azad et al. 2006). Myo-inositol oxidation pathway has a predominant role in the biosynthesis of new cell walls of the hypocotyl and roots in germinating beans (Sasaki et al. 1989). Recently, Abid et al. (2009) reported that myo-inositol phosphate synthase (MIPS), which catalyses the first step in inositol biosynthesis are involved in various physiological processes including embryogenesis, seedling growth and resistance to biotic and abiotic stresses. Previously we also reported that myo-inositol was a more efficient osmotic stabilizer for sustained cell division from protoplast cultures of *R. hybrida* (Kim et al. 2003). Furthermore, myo-inositol was used for protoplast cultures as an osmoticum including *Lycopersicon esculentum* (Bellini et al. 1990), *Picea Glauca* (Attree et al. 1989). Cell wall regeneration and resistance to abiotic stresses are necessarily required processes for successful cell division and proliferation from protoplast culture. Therefore, we inferred that application of myo-inositiol as an osmotic stabilizer in protoplast culture could have advantages not only carbohydrate metabolisms in cell walls but also inositol metabolisms in cell membrane. Considering these results we suggested that myo-inositol might be more suitable osmotic stabilizer for sustained cell division from protoplast culture.

Plant regeneration from protoplast cultures of cabbage

Callus proliferated on modified protoplast culture medium were transferred to shoot differentiation medium supplemented with several concentrations 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA (Fig. 1F). After four weeks of culture, green and reddish shoot primordia were formed on the surface of callus when cultured in the light (Fig. 1G). Upon transfer to half-strength MS basal medium, roots were formed onto the bottom of shoots without auxin treatments (Fig. 1H). These regenerated plantlets were successfully acclimatized to soil transfer, grown to normal mature plants (Fig. 1I).

In the preliminary study, we determined the optimal growth regulators conditions for shoot differentiation from cotyledon explants of cabbage. The highest frequency of shoot differentiation from cotyledon explants of cabbage was 80% when they cultured onto MS medium 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA. Also we examined the effect of gelling agent (0.4% Gelrite and 0.8% Plant agar) on shoot formation from callus during the culture. The efficiency of shoot formation from 0.4% Gelrite treatment was 2 ~ 3 times higher than that of 0.8% Plant agar (Fig. 5). Shoot differentiation could be achieved from protoplast-derived callus of cabbage when cultured on same medium used for shoot differentiation from cotyledon explants. About 30% of protoplast-derived callus formed shoots when they cultured onto MS medium containing 2 mgl^{-1} BA and 0.5 mgl^{-1} NAA.

Culture conditions for high frequency plant regeneration from cotyledon-derived protoplasts in cabbage were determined in this study. Also we found that myo-inositol was

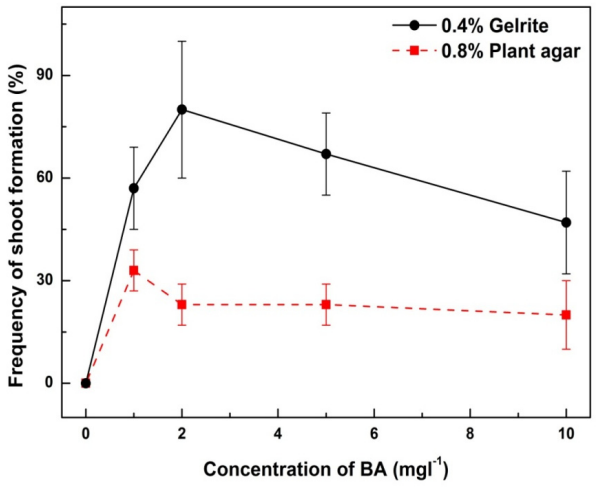

Fig. 5 Effect of BA on shoot formation from cotyledon explants cultures of cabbage. vertical bars represent SD

more efficient osmotic stabilizer for sustained cell division of cabbage protoplast than mannitol. Therefore, this plant regeneration system from protoplasts of cabbage could be applied for somatic hybridization and direct gene transfer in quality improvement of cabbage cultivars.

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