J. Plant Biotechnology(2001) *Vol. 3(1). pp. 33~38*

Journal of Plant Biotechnology

# Production and Characterizations of Somatic Hybrids between *Brassica campestris* L. ssp *pekinensis* and *Brassica oleracea* L. var *capitata*

**Yu-Ji Lian, Hak-Tae Lim***

*Division of Applied Plant Sciences & Center for the Korea Potato Genetic Resources (KPGR), Kangwon National University, Chuncheon, 200-701, Korea.*

**Key words:** GISH, chromosome, morphology, Chinease cabbage, cabbage, protoplast, somatic hybridization, cell fusion

## Abstract

**Protoplasts isolated from inbred lines of *Brassica oleracea* L. var *capitata* (cabbage) and *Brassica campestris* L. ssp. *pekinensis* (Chinese cabbage) were fused by PEG-mediated method, and somatic hybrid cells were differentiated into plants. For the identification of somatic hybrid plants, ploidy level, plant morphology, and cytological analysis were performed. All of the regenerated plants derived from fused protoplasts were shown to be 2X-4X, or higher ploidy level, presumably due to somatic hybridization or chromosome doubling. The morphology of leaves, petioles, and flowers showed an intermediate phenotype between Chinese cabbage and cabbage. Chromosome numbers in these somatic hybrids ranged mostly from 33 to 38. According to Genomic *in situ* hybridization (GISH) pattern, signals from both fusion parents of *B. campestris* or *B. oleracea* were detected in different colors when chromosomes of putative somatic hybrids were observed.**

## Introduction

Importance of the *Brassica* vegetables such as Chinese cabbage, brocolli, cabbage, cauliflower, kale, and et al. in terms of their nutrient quality for human diet and processed food has, recently, been increased. Among these *Brassica* vegetables, Chinese cabbage, the major ingredient in Kimchi along with hot pepper and garlic, is the most important vegetable in Korea, and the wide-spread of the popularity of the Kimchi as a fermented food into other countries stimulated the increase in the production of the Chinese cabbage.

It has long been the plant breeder's efforts to improve specific traits such as disease resistance, cold tolerance, male sterility, and product quality in these *Brassica* vegetables by transferring valuable genes from one species to other (Hansen and Earle, 1995; Heath and Earle, 1996; Sigareva and Earle, 1997; Brewer et al., 1999; Sigareva and Earle, 1999; Arumugam et al., 2000). There are many successful cases in crop breeding for the improvement of crop quality, with regard to sexual crosses between distantly related plant species. However, sexual crossing is often limited by physical and biological barriers of the reproductive process (Sundberg, 1991). Somatic hybridization has various advantages over sexual hybridization: i.e., elimination of laborious procedures such as synchronization of flowering times of parental species, artificial embryo rescue, and chromosome duplication of hybrids to obtain fertile amphidiploidy plants.

In addition to the recombinations of nuclear genome between parents, cytoplasmic organelles such as mitochondria and plastids can be hybridized by protoplast fusion which can provide new genetic diversity and variations in these organelles genome (Yamagishi et al., 1992). In *Brassica* species, protoplast technology has been

* Corresponding author, E-mail; limhakta@cc.kangwon.ac.kr
Received Oct. 20, 2000; accepted Dec. 12, 2000.

applied extensively, through fusion of protoplasts from *B. campestris* and *B. oleracea* (Rorbertson et al., 1987; Sundberg et al., 1987; Terada et al., 1987; Rośen et al., 1988; Richard et al., 1993). This inter-specific somatic hybridization between two species has been achieved to transfer economic traits such as cytoplasmic male sterility (Cardi and Earle, 1997; Sigareva and Earle, 1997), disease resistance (Hansen and Earle, 1995; Ren et al., 2000).

In this paper, as the first step for the production of somatic hybrids having valuable traits derived from both parents of Chinese cabbage and cabbage, conditions for the protoplast culture and fusion were established, and resulting somatic hybrids have been characterized by flow cytometric analysis, genomic *in situ* hybridization (GISH), and plant morphology.

## Materials and Methods

### Plant materials

Inbred lines of *Brassica oleracea* L. var *capitata* (kw28) and *Brassica campestris* L. ssp *pekinensis* (kw23) were used as plant materials for somatic hybridization. Seeds, supplied by Choongang seeds company, were surface-sterilized by 70% ethyl alcohol for 1 min followed by 15 min in 50% commercial chlorox bleach solution to which two drops of Tween 20 were added. The sterilized seeds were germinated and propagated *in vitro* on MS (Murashige and Skoog, 1962) medium supplemented with 1% sucrose and solidified with 0.8% agar. Right before protoplast isolation, plantlets were placed in dark for 1-2 days to reduce the starch contents.

### Protoplast isolation, fusion, and culture

Healthy germinated seedlings or young leaves of *Brassica campestris* and *B. oleracea* were used as protoplast isolation materials. Prior to protoplast isolation, leaves were cut into approximately 0.5-1 mm wide strips in a pre-plasmolysis solution of TVL (54.6 g/L sorbitol, 7.4 g/L $CaCl_2 \cdot 2H_2O$, and pH 5.6-5.8). After one to three hours of incubation in TVL solution, pre-plasmolysis solution was removed, and enzyme solution (0.4 M Mannitol, 0.5% Macerozyme, 1% Cellulysin, and pH 5.6-5.8) was added to the petridish.

The petridish was placed in an incubator of dark condition overnight (10-16 h) at 28℃. To reduce the time of isolation step, the petridish was agitated at 30 rpm in a rotary shaker. Released protoplasts were separated from undigested leaf materials by filtering the mixture through a 60 μm nylon sieve. The protoplast suspension was diluted with CPW21S (27.2 mg/L $KH_2PO_4$, 101 mg/L $KNO_3$, 1,480 mg/L $CaCl_2 \cdot 2H_2O$, 246 mg/L $MgSO_4 \cdot 7H_2O$, 0.14 mg/L KI, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 21% sucrose, and pH 5.5-5.8) and centrifuged at 1,000 rpm for 10 min. Floated protoplast suspension was transferred to a new centrifuge tube, and then centrifuged again at 800 rpm for 8 min in W5 (18.4 g/L $CaCl_2 \cdot 2H_2O$, 9.0 g/L NaCl, 1.0 g/L glucose, 0.8 mg/L KCl, and pH 5.5-5.8) solution to separate from the debris.

The protoplasts fusion was performed as described by Glimelius et al. (1986) with some modifications. The protoplasts of fusion partners were suspended in W5 solution to adjust the final concentration of $1 \times 10^6$ protoplasts/mL and mixed gently in a ratio of 1:1. Seven droplets of the mixed protoplast suspension were spaced at regular distances in a 6 cm plastic petridish. The protoplast suspension was left to be settled for 10 min, and the same amount of droplet of 40% polyethylene glycol (40% PEG w/v, mol. wt 1,500, 0.3 M glucose, 50 mM $CaCl_2 \cdot 2H_2O$, and pH 7.0) was added to each droplet. After 10 min, the PEG solution was removed with a pasteur pipette. The protoplasts attached to the bottom of the petridish were washed twice with culture medium.

Fused protoplasts were cultured in a modified K8p medium according to Glimelius et al. (1986). The protoplasts were cultured in 6 mm plastic petridish with 1-1.5 ml culture medium, and then petridishes were sealed with parafilms and incubated at 25℃ in the dark. After 5-7 days of culture, the cell begun to divide, and these dividing cells were transferred to Kao's basal medium (Kao and Michcharyluk, 1975) supplemented with 0.3 M sucrose, 0.1% agarose, and plant growth regulators. These cultures were kept under dim light with a day length of 16 h at 25℃. Small calli reached to 2-3 mm in diameter were transferred on MS medium containing plant growth regulators to obtain regenerated shoots. The calli were transferred to new media at every two week.

### Flow cytometry analysis

Flow cytometry permits measurements of the fluorescence of large number of stained nuclei within seconds (Arumugsnsthan and Earle, 1991), allowing easy determination of the ploidy levels for the somatic hybrids. For this analysis, five putative somatic hybrids and two fusion partners were used for confirmation of somatic hybrids based on their ploidy analyser (Partec PA-I, Made in Germany). Estimation of nuclear DNA content of somatic hybrids and fusion parents was done using flow cytometry

as described previously by Arumuganathan and Earle (1991).

### Chromosome observation

Root tips from actively growing plants were pretreated in 0.002 M 8-hydroxyquinolines for 1.5 h at room temperature, and then transferred to 3:1 absolute alcohol : glacial acetic acid, followed by an incubation for at least 24 h at 4℃. For determination of the chromosoume numbers, the samples were hydrolysed in 1N HCl for 10 min at 60℃, stained in Feulgen for 30 min, squashed in aceto-carmin, and observed under a microscope.

### Probe labelling and genomic *in situ* hybridization

One microgram of total DNA from either *B. campestris* or *B.oleracea* was used as a probe. Probe was labeled by random primer DNA-labelling kit (Boehringer mannheim, Germany), and *in situ* hybridization was done according to Yasuhiki (1996). Slides were incubated for 1 h at 37℃ in 3 mg/mL RNase A, washed at 2xSSC for 5 min at room temperature, dehydrated in 70%/95%/100% ethanol series for 5 min, respectively, and air-dried at room temperature for 30 min. The slides were denatured at 70℃ in 70% formamid in 2xSSC for exactly 2 min, immediately dehydrated in an cold ethanol series (75, 95, and 99%), and air-dried. The denatured probe mixture (70 μL/slide) were added to the slide, and the slide were incubated in a humid chamber overnight at 37℃.

The slides were washed in 2XSSC, 50% formamid in 2XSSC, 2XSSC, and 4XSSC, respectively for 10 min at 40℃. For the detection of hybridized sites, 600 uL/slide of 5% BSA/BT buffer (0.1 M sodium hydrogen carbonate, 0.05% tween 20, and pH 8.3) were added to slides and incubated in humid chamber for 5 min at 37℃. And then 70 μL/slide of FITC-avidin/1%BSA/4xSSC mixture were added to the slide, covered with parafilm, and incubated for 1 h at 37℃. The slides were washed twice (10 min each) in 1X BT buffer and a quick rinse in 4XSSC containing 0.2% Tween 20 and mounted with 20 μL Anti-fading agent containing 4',6-diamidino-2-phenylindole (DAPI).

### Morphology

Morphological characteristics such as leaf shape, size and color in flowers of plants derived from protoplast fusion were investigated and compared with those of the fusion parents.

## Results and Discussion

### Protopalst isolation, fusion and culture

After PEG treatment, protoplasts were washed with culture medium two times. The quality of these protoplasts was not obviously different from that of protoplasts washed with 3/1 PEG solution and then 6/1 PEG once. Some protoplasts were damaged during protoplast fusion experiments.

PEG-treated protoplasts were cultured in 1-2 mL modified K8p (Glimelius et al., 1986) medium containing 1 mg/L of 2,4-D, 0,5 mg/L of BAP, 0.1 mg/L of NAA, and 1 mg/L of kinetin. After two days of culture, the first cell division begun, and 5 to 7 day old protoplasts were transferred to Kao's medium containing 0.25 mg/L of 2,4-D 0.025 mg/L of BAP, 0.025 mg/L of NAA, and 0.25 mg/L of kinetin. The medium was supplemented with 6% mannitol and semi-solidified with 0.1% agrose. When microcalli were reached to 2-3 mm in diameter, they were transferred to the regeneration medium added with a few plant growth regulators, such as 0.2 mg/L of zeatine, 1 mg/L of BAP, 0.5 mg/L of kinetin, and 0.4 mg/L of NAA as well as 1% sucrose and 0.8% plant agar. After 90 days of culture, shoots formed on callus (Figure 1).

To obtaining regenerants from fused protoplast of, it was necessary to establish very effective culture system. In most cases in *Brassica* species, protopalsts have been cultured in liquid medium (Glimelius, 1984; Robertson and Earle, 1986; Pua, 1987), but there were also reports of successful protoplast culture using agarose beads (Yamashita et al., 1989) embedded in thin layer of agarose-solidified medium. In our experiment, fused protoplasts were cultured in liquid medium to promote cell division, but when the cells divided at 8-10 cell stages, semisolid agarose medium was added to the petridish. It was reported that the semi-solidified agarose culture could protect the cell from released polyphenolic compound to improve plate efficiency (Shillito et al., 1983).

### Flow cytometry analysis

The typical position of histograms of the fluorescence peak was obtained using the flow cytometer. *B. campestris* (A) showed two peaks ; the diploid was approximately at channel 70, and the tetraploid was at channel 100 according to the Partec User Manual. Peak of the diploid *B. oleracea* (B) was located around at channel 80. The peak of regenerated plant after protoplast fusions appeared at channel 150, indicating that somatic hybrid (C) was

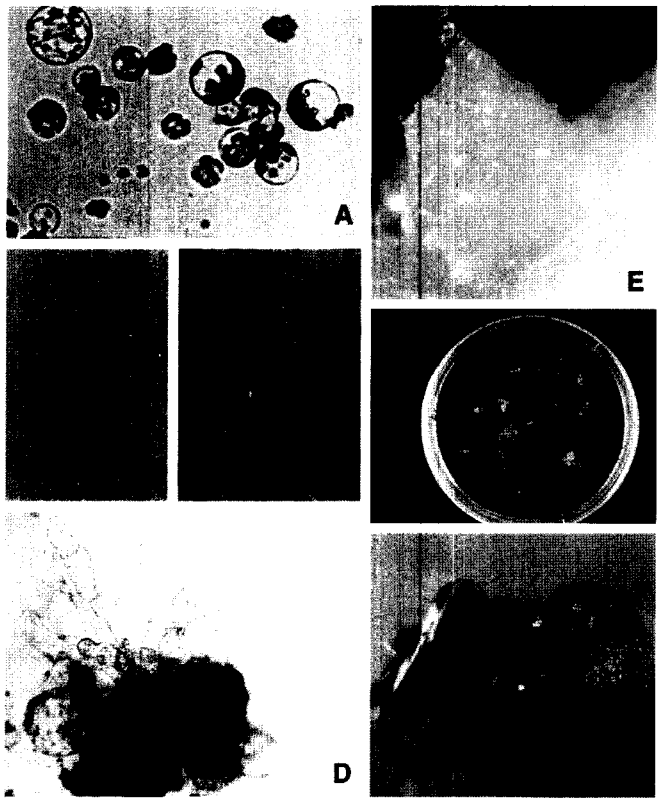


**Figure 1.** Plant regeneration from fused protoplasts (A) Isolated mesophyll protoplasts (B) Fused protoplasts (C) Cells at division (D) Microcalli from protoplasts-derived cell after 20days (E) Protoplasts-derived callus in semi-solid medium (F & G) Shoot regeneration from protoplasts-derived callus after 90days of initial protoplast culture.

derived from the combination between *B. campestris* and *B. oleracea* genomes (Figure 2). DNA contents of Chinese cabbage (A), cabbage (B), and somatic hybrids were 1.13± 4.42 pg, 1.63±2.93 pg, and 3.34±3.64 pg, respectively. DNA content of somatic hybrid was similar to the sum of the two fusion parents. Similar result was also reported by Hansen and Earle (1995).

## Chromosome counting and Genomic *in situ* hybridization (GISH)

Based on cytological analysis, several somatic hybrids seemed to have chromosome number of 2n=38, indicating the sum of chromosome of cabbage (2n=18) and Chinese cabbage (2n=20). It can be suggested that these somatic hybrids be amphidiploid like *Brasica napus*. However, various chromosome numbers ranged from 2n=33 to 2n=38 were found in somatic hybrids having various morphological differences in vein types, colors, shape, and size of leaves.

GISH was carried out on two somatic hybrids to characterize their chromosome composition. According to GISH analysis, *B.campestris* chromosome segments were showed in somatic hybrid of KBS3 (Kangwon *Brassica* Somatic hybrid 3, 2n=38. The signals were the strongest in the centrometric regions (Figure 3A), and only four chromosomes showed strong signals in the telometric region. The hybrid of KBS12 (Figure 3B) contained 36 chromosomes, and it also presented the strongest signals in the centrometric region.

By GISH, it was possible to identify and distinguish the chromosomes of Chinese cabbage and cabbage in the somatic hybrids. Some recombinant chromosomes were

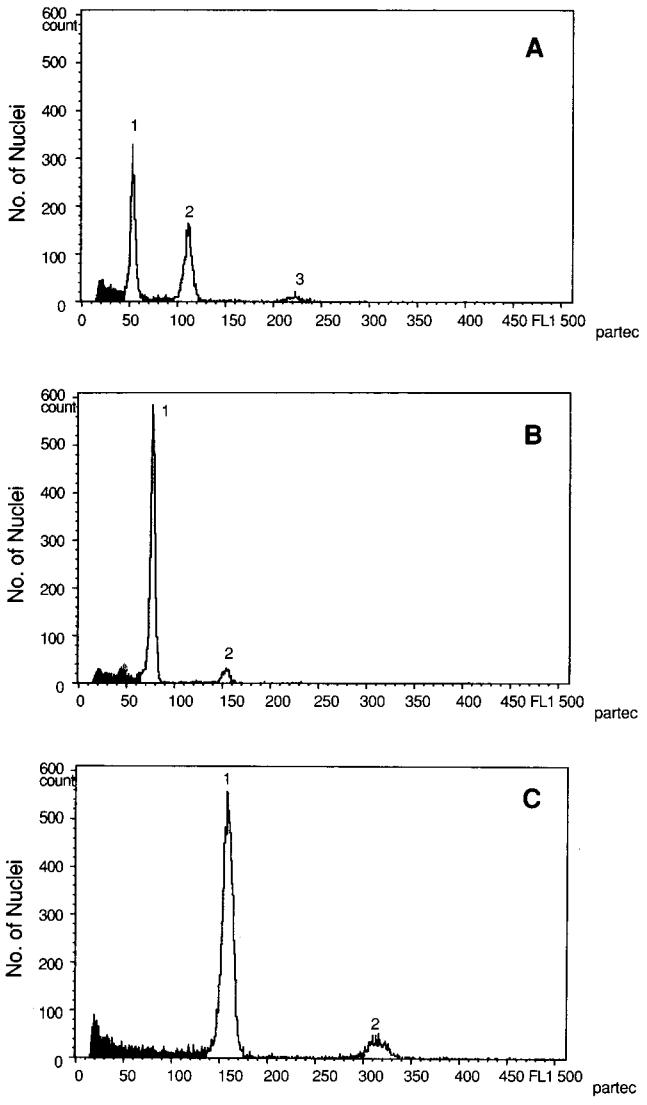


**Figure 2.** DNA-histogram of the nuclei mixture extracted from leaves tissue of *Brassica campestris* (A), *Brassica oleracea* (B), and their somatic hybrids (C).

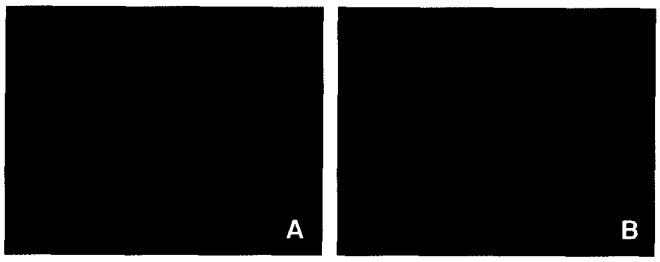


**Figure 3.** Genomic *in situ* hybridization pattern of mitotic metaphage chromosomes of somatic hybrid. Red color marks the cabbage chromosome segments and yellow color presents chromosome segments of the Chinese cabbage A: KBS3 (somatic hybrid 3. 2n=38), B: KBS12 (somatic hybrid 12, 2n=36).

observed. In this study, the strongest signals were present in the centrometric region, which could be supported by other similar result in that *Brassica* species have been found to contain a large amount of tandemly organized centrometric repeats (Lagercrnatz and Lydiate, 1996).

### Morphological characterization

All of the plants derived from protoplast fusion were determined to be somatic hybrids based on their morphologies. It was almost impossible to obtain plants from one fusion parent, since culture medium was more favorable to the growth of the fused cells. Hybrid vigour was also found in the somatic hybrids as it can be detected in the traditional crossing experiment. Somatic hybrids showed either *Brassica campestris* or *Brassica oleracea* morphology. The most striking difference between fusion parents and somatic hybrids was the leaf morphology. The morphology of somatic hybrid was intermediate to the two parents. The petiole of a somatic hybrid was also shown to be intermediate, although kw23 (*B. campestris*), one of parents, had enlarged petioles, while the other parent, kw28 (*B. oleracea*), showed narrow ones. The plant was similar to that of kw28 (*B. oleracea*), but the petiole and medivein enlargement looked like Chinese cabbage as shown in Figure 4. The color of flowers was yellow, like Chinese cabbage, and the size of flowers was larger than both species.

Based on morphological observation in somatic hybrids regenerated from callus culture, somatic hybrids regenerated after three months of callus culture were mostly turned out to be very similar to characteristics of *B. olereacea*, while those regenerated after four months of initial callus culture were very similar to characteristics of *B. campestris*. This results indicate that protoplast fusion between two different *Brassica* species can give rise to

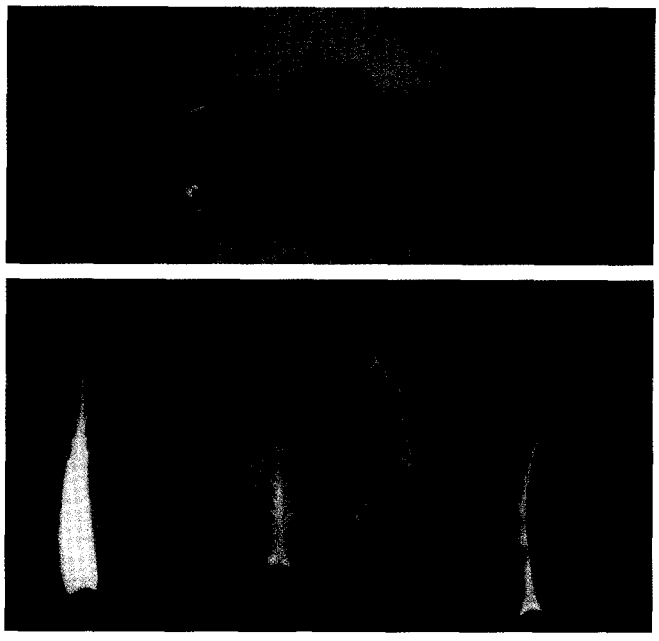

**Figure 4.** Comparison of whole plants and leaves of selected somatic hybrids (B) and its fusion parents (A; *B. campestris* and C; *B. oleracea*).

various combinations of nuclear and cytoplasmic genomes, which can be classified as symmetric and asymmetric fusions (Yamagishi et al., 1992).

These somatic hybrids will be crossed with other *Brassica* species to transfer useful traits such as disease or insect resistance. As it has been well-known, it is almost impossible to make sexual cross between *B. campestris* and *B. oreleace.* In this experiment, we successfully established somatic hybrid production system which can be a very important breeding tool to obtain new types of hybrid plants possessing diverse nuclear and cytoplasm genomes, which, otherwise, is not possible via conventional breeding methods.

## Acknowledgments

We wish to express many thanks to Mr. Nam-Kweon Baek of the Choongang seed company for kindly providing seeds, and also thanks to Prof. Nam-Soo Kim for kind help in genomic in situ hybridization (GISH) analysis. This research was funded in part by the R&D Promotion Center for Agricultural and Forestry in Korea.

## References

**Arumuganathan K, Earle ED** (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol

Rep 9: 229-233.

**Arumugam N, Mokhopadhyay A, Gupta V, Sodhi YS, Verma JK, Pental D, Pradhan AK** (2000) Somatic cell hybridizatiion of '*oxy*' CMS *Brassica juncea* (AABB) with *B. oleracea* (CC) for correction of chlorosis and transfer of novel organelle combinations to allotetraploid *Brassicas*. Thero Appl Genet 100: 1043-1049.

**Brewer EP, Saunders JA, Angle J S, Chaney RL, Mcitosh MS** (1999) Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*. Thero Appl Genet 99: 761-771.

**Cardi T, Earle ED** (1997) Production of new CMS *Brassica oleracea* by transfer of 'Anand' cytoplasm from *B. rapa* through protoplast fusion. Thero Appl Genet 94: 204-212.

**Glimelius K** (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some *Brassicae*. Plant Physiol 61: 38-44.

**Glimelius K, Mats D, Hugo FF** (1986) Selection and enrichment of plant protoplast h erokaryons of *Brassicaceae* by flow sorting. Plant Sci 45: 133-141.

**Hanen LN, Earle ED** (1994b) Novel flowering and fatty acid characters of rapid cycling *Brassica napus* L. resynthesized by protoplast fusion. Plant Cell Rep 14:151-156.

**Hansen LN, Earle ED** (1995) Transfer of resistance of *Xanthominas campestris* pv *campestris* into *Brassica oleracea* L. by protoplast fusion. Thero Appl Genet 91: 1293-1300.

**Heath DW, Earle ED** (1996) Synthesis of Ogura male sterile rapeseed(*Brassica napus* L.) with cold tolerance by protoplast fusion and effects of atrazine resistance on seed yild. Plant Cell Rep 15: 939-944.

**Kao KN, Michcharyluk MR** (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplast at very low population density in liquid media. Planta 126: 105-110.

**Lagercrantz U, Lydiate DJ** (1996) Comparative genome mapping in *Brassica*. Genetics 144: 1903-1910.

**Murashige T, Skoog F** (1962) A revised medium for rapid grown and bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

**Pua EC** (1987) Plant regeneration from stem-derived protoplasts of *Brassica alboglabra* barly. Plant Sci 50: 153-160.

**Ren JP, Dickson MH, Earle ED** (2000) Improved resistance to bacterial soft rot by protoplast fusion between *Brassica rapa* and *B. oleracea*. Thero Appl Genet 100: 810-819.

**Richard H, Ozminkowski JR, Jourdan PS** (1993) Expression of self-incompatibility and fertility of *Brassica napus* L. Resynthesized by interspecific somatic hybridization. Euphytica 65: 153-160.

**Robertson D, Earle ED** (1986) Plant regeneration from leaf protoplasts of *Brassica oleracea* var. Italica CV green comet broccoli. Plant Cell Rep 5: 61-64.

**Robertson D, Palmer JD, Earle ED, Mutschler MA** (1987) Analysis of organelle genomes in a somatic hybrid derived from cytoplasmic male-sterile *Brassica oleracea* and atrazine-resistant *B. campestris*. Theor Appl Genet 74: 303-309.

**Rosén B, Halldén C, Heneen WK** (1988) Diploid *Brassica napus* somatic hybrids: Characterization of nuclear and organellar DNA. Thero Appl Genet 76: 197-203.

**Sigareva MA, Earle ED** (1997) Direct transfer of a cold-tolerant Ogura male-sterile cytoplasm into cabbage (*Brassica oleracea* ssp. *capitata*) via protoplast fusion. Thero Appl Genet 94: 213-220.

**Sigareva MA, Earle ED** (1999) Regeneration of plants from protoplasts of *Capsella bursa-pastoris* and somatic hybridization with rapid cycling *Brassica oleracea*. Plant Cell Rep 18: 412-417.

**Sillito RD, Paszkowski J, Potrykus I** (1983) Agarose plating and a bead type culture technique enable and stimulate development of protoplast-derived colonies in a number of plant species. Plant Cell Rep 2: 244-247.

**Sunberg E** (1991) Somatic hybrids and cybrids within *Brassicacea*: Studies focused on refining production methods and identifying factors influencing the genetic composition of somatic hybrids. pp 7-44.

**Sunberg E, Langgren M, Glimelius K** (1987) Fertility and chromosome stability in *Brassica napus* resynthesised by protoplast fusion. Thero Appl Genet 75: 96-104.

**Terada R, Yamashita Y, Nishiyashi S, Shimamoto K** (1987) Somatic hybrids between *Brassica oleracea* and *B. camapestris*: Selection by the use of iodoacetamide inactivation and regeneration ability. Thero Appl Genet 73: 379-384.

**Yamagishi H, Mohammad MH, Katsuei Y** (1992) Production of somatic hybrids between southern type Chinese cabbage 'Kenshin' and cabbage 'Yoshin' and their flowering characteristics. J Japan Soc Hort Sci 16: 311-316.

**Yamashita Y, Terada R, Nishibayashi S, Shimamoto K** (1989) Asmmetric somatic hybrids of *Brassica* : Partial transfer of *B. campestris* genome into *B. oleracea* by cell fusion. Theor Appl Genet 189-194.

**Yasuhiko M** (1996) *In situ* hybridization plant chromosomes: Laboratory Methods. Chapter 8: 155-170.