Theor Appl Genet (1999) 99:1281–1286 

**Z. Yan · Z. Tian · B. Huang · R. Huang · J. Meng**

# Production of somatic hybrids between *Brassica oleracea* and the $C_3$–$C_4$ intermediate species *Moricandia nitens*

Received: 20 January 1999 / Accepted: 16 June 1999

**Abstract** Protoplast fusion between *Brassica oleracea* and *Moricandia nitens*, a $C_3$–$C_4$ intermediate wild species, was carried out. Four hundred and twenty five plants were regenerated from 1995 calli. More than 90% of the regenerated plants were verified as true intergeneric hybrids on the basis of morphological observation and molecular-marker analysis. The hybrids were morphologically intermediate between both fusion parents. Variations in flower color and petal number were also observed. The chromosome number and pollen fertility varied across the individual hybrids. Although after self-pollination pollen germinated on the stigma and pollen tubes were visible in the style, the pods did not develop properly without in vitro culture. Measurements of the $CO_2$ compensation point revealed that six out of eight hybrid plants expressed a gas-exchange character that was intermediate between the $C_3$–$C_4$ *M. nitens* and $C_3$ *B. oleracea* parents.

**Key words** *Brassica oleracea* · $C_3$–$C_4$ intermediate character · $CO_2$ compensation point · *Moricandia nitens* · Somatic hybridization

Communicated by K. Glimelius

Zhun Yan · Zhihong Tian · Ronggui Huang · Jinling Meng (✉)
National Key Laboratory of Crop Genetic Improvement,
Huazhong Agricultural University, Wuhan 430070,
People's Republic of China
e-mail: jmeng@public.wh.hb.cn

Bangquan Huang
School of Life Sciences, Hubei University,
Wuhan 430070, P.R. China

*Present address:*
Zhun Yan, Institute of Vegetables and Flowers,
Chinese Academy of Agricultural Sciences,
Beijing 100081, P.R. China

## Introduction

*Brassica oleracea* is one of the most important vegetable crops in the world and includes several subspecies such as cabbage, kohlrabi, broccoli and cauliflower. *B. oleracea*, together with *Brassica campestris* and *Brassica nigra*, is also a basic species constituting the amphidiploid oilseed crops *Brassica napus* and *Brassica carinata* (U 1935; Olsson 1960). It is attractive to *Brassica* crop breeders to increase the production of *B. oleracea* by modifying its photosynthetic characteristics.

The genus *Moricandia*, classified in the subtribe Moricandiiae of the tribe Brassiceae, was reported to be unique within the Cruciferae since it contains $C_3$–$C_4$ intermediate species (Hylton et al. 1988). In total includes five $C_3$–$C_4$ intermediate species, viz. *Moricandia arvensis*, *Moricandia nitens*, *Moricandia sinaica*, *Moricandia spinosa* and *Moricandia suffruticosa* (Hylton et al. 1988; McVetty et al. 1989; Rawsthorne 1992). The differential expression of active glycine decarboxylase (GDC) in the cells of mature leaves and the combination of Kranz-like leaf anatomy with a differential distribution of organelles (mitochondria, chloroplasts and peroxisome) in the bundle-sheath cells result in the efficient recapture of photorespiratory $CO_2$ and account for the low $CO_2$ compensation point of these species (Rawsthorne et al. 1988). It has also been suggested that the greater efficiency of $CO_2$ recapture observed in $C_3$–$C_4$ intermediate species could, if introduced into crop species, improve their water-use efficiency compared to $C_3$ forms of the same crops, especially under conditions of water stress (McVetty et al. 1989; O'Neill et al. 1996).

In order to transfer $C_3$–$C_4$ genes into crops, many hybridizations between *M. arvensis* and *Brassica/Raphanus* species have been carried out (Apel et al. 1984; Toriyama et al. 1987; Takahata 1990; Takahata and Takeda 1990; Kirti et al. 1992; Takahata et al. 1993; Bang et al. 1996; O'Neill et al. 1996; Meng et al. 1998). However, up to now, $C_3$–$C_4$ genes have not been transferred into crops due to the low fertility of the hybrids or to the difficulty in expressing the genes from the $C_3$–$C_4$ parent in

the hybrids (Meng et al. 1998). There are only two previous papers concerning the expression of the $C_3$–$C_4$ character in such hybrids (Apel et al. 1984; O'Neill et al. 1996) and, total, only 4 out of 14 hybrids showed a limited expression of the $C_3$–$C_4$ character.

*M. nitens* has the lowest $CO_2$ compensation point ($\Gamma$=5 $\mu l CO_2 \cdot l^{-1}$) in the genus *Moricandia* and has a close relationship with *Brassica* crops (Rawsthorne 1992; Meng and Gan 1998). There is only one recent report about hybridization involving *M. nitens* and *Brassicas*. After hundreds of pollinations followed by embryo rescue, a single plant was obtained from a sexual cross between *M. nitens* and *B. napus*, a tetraploid *Brassica* crop (Rawsthorne et al. 1998). In the present paper, we report on the mass-production of somatic hybrids between *M. nitens* and a diploid *Brassica* crop, *B. oleracea*.

## Materials and methods

### Plant material

The seeds of *M. nitens* were supplied by Dr. S. Rawsthorne, John Innes Centre, Norwich, UK. Seeds of *B. oleracea* var. Italica cultivar 'Lude dihaploid' were supplied by Dr. Gengyi Li, Institute of Horticulture, Henan Academy of Agricultural Sciences, Zhengzhou, China. The other seeds were bought from a seed market in Wuhan. The seeds of *M. nitens* were sown in a greenhouse. Shoots from one plant were then transferred to hormone-free B5 medium (Gamborg et al. 1968) after surface sterilizing for 12 min and washing three times. The seeds of *B. oleracea* were germinated directly on MS medium (Murashige and Skoog 1962) after surface sterilizing. Both seedlings and shoot tips were maintained under a photoperiod cycle of 16-h light/8-h dark, with a light intensity of 80 $\mu Em^{-2}s^{-1}$, at 25°C.

### Protoplast isolation, fusion, culture and plant regeneration

*M. nitens* protoplasts were isolated from fully expanded leaves of 40-day-old shoot cultures as described by Tian and Meng (1999). *B. oleracea* protoplasts were isolated from 4–7-day-old hypocotyls. About 100 hypocotyls were transversely cut into 2–3-mm segments, and pre-plasmolysed in CPW-13 M for 0.5–1 h. The pre-plasmolysis solution was then replaced with 10 ml of enzyme mixture solution. The enzyme mixture consisted of CPW-9 M solution (Power and Davey 1979) with 1% Celluase R-10 (Onozuka, Yakult Honsha Co. Ltd, Tokyo, Japan), 0.2% Macerozyme R-10 (Onozuka, Yakult Honsha Co. Ltd, Tokyo, Japan), 0.5% Hemicellulase (Sigma), 5 mM of MES, and 0.5 $gl^{-1}$ of BSA, pH 5.6. Digestion was carried out on a gyratory shaker (40 rpm) at 25°C in the dark for 16 h. The crude products of digestion were purified according to the method described for *M. nitens* (Tian and Meng 1999), with the exception that the pellets were washed first in CPW-9 M and then in W5 salt solution (Menczel and Wolfe 1984).

Protoplast fusion was done as described by Kirti et al. (1995) with the following modifications. Protoplasts of *B. oleracea* and *M. nitens* were mixed together in equal quantities ($2\times10^6$ $ml^{-1}$). Three 150-µl droplets of PEG solution [20% (w/v) PEG, MWt 8000 (sigma); 10% DMSO (dimethylsulphoxide); 3.6% (w/v) Glucose; 0.17% (w/v) $CaCl_2 \cdot 2\,H_2O$; 0.0095% (w/v) $KH_2PO_4$, pH 5.8] were then placed on the base of a 6-cm Pertri dish to form the corners of a triangle, with 1 cm separating each drop. The same volume of mixed protoplast suspension was placed on each droplet of PEG solution. After 5 min, the mixture was diluted with 3 ml of W5 solution and left undisturbed for 25 min. The fusion mixture was then collected by centrifugation (500 rpm, 5 min) and washed once with modified KM8p medium before culture.

The culture method employed was that of Tian and Meng (1999). When microcalli were visible to the naked eye, the microcalli were transferred to K3 amplification medium (Tian and Meng 1999) for enlarging. When they had grown to about 4–5 mm in diameter, calli were large enough to be transferred to K3 regeneration medium (Tian and Meng 1999) to promote shoot morphogenesis. Calli with shoots were then transferred to B5 medium for shoot elongation and rooting.

### Characterization of somatic hybrids

Total DNA was isolated from the parents and regenerated plants following the procedure of Rogers and Bendich (1988). The hybrid nature of the regenerates was established through RAPD analysis following Tu et al. (1997) using 40 10-mer primers supplied by the Shanghai Sangon Biotech. Co., China.

Cytological observations were made on young leaves or shoots cultured in MS medium supplemented with sucrose (3% w/v), agar (0.8% w/v), BA (6-benzylaminopurine, 3 $mgl^{-1}$) and NAA (0.2 $mgl^{-1}$). Young leaves and shoots were treated with 2 mM of 8-hydroxyquinoline for 3–4 h and fixed in Carnoy's solution. Before squashing, they were hydrolysed in 1 M HCl at 60°C for about 10 min and then stained with acetocarmine.

The $CO_2$ compensation point ($\Gamma$) measurement was done according to Moss (1962) and Jiao and Ku (1991) at 28.5°C and with a light intensity of 180 $\mu Em^{-2}s^{-1}$ using a infra-red gas analyzer.

To measure the pollen-germination ability, fresh pollen grains were placed in pollen-germinating medium. The medium, modified from Roberts et al. (1983) and Zhao et al. (1986), consisted of 25% (w/v) sucrose, 100 ppm $H_3BO_4$, 200 ppm $CaCl_2$, 100 ppm $KNO_3$, 50 ppm BA and 30 ppm $GA_3$ (gibberellin), pH 7.9. After culture for 1 h, the pollen grains were observed with a microscope and the pollen-germination percentage counted. Pollen germination on the stigma and pollen-tube penetration into the style were observed with the ABF method according to Dumas and Knox (1983). After pollination, pistils were fixed in ethanol-acetic acid solution (3:1, v/v), treated with 8 M NaOH for 8 h, stained with 0.1% aniline blue solution, and examined with a fluorescence microscope.

## Results

### Protoplast fusion, in vitro culture and plant regeneration

In five independent fusion experiments the first cell divisions were observed at 4–7 days after protoplast fusion. After 4 weeks in culture, many microcalli were present (Fig. 1A). Microcalli were then transferred to K3 amplification medium. After 3-weeks culture on the medium, the calli were transferred to K3 regeneration medium. One-month later, many shoots regenerated from these calli with an average of one shoot per five calli (Fig. 1B; Table 1).

### Verification of the somatic hybrids

Thirty four out of forty random primers showed a distinctive polymorphism of the PCR products between the parent lines. RAPD markers amplified by the informative primers were used to identify the somatic hybrids from the regenerated plantlets. From 94 analyzed plantlets, 89 plants with the specific bands of both parents were identified amongst the putative somatic hybrids

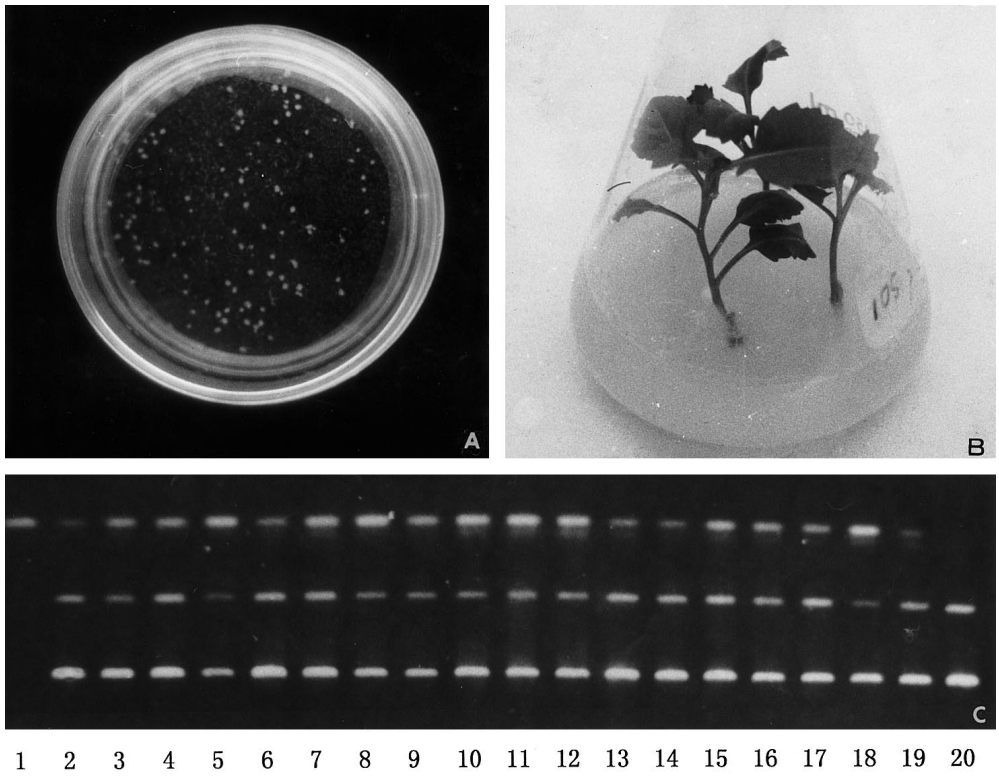


**Fig. 1** **A** Calli which appeared in the dish after protoplast fusion and culture for 4 weeks. **B** Some shoots regenerated from the callus which were ready for rooting. **C** RAPD banding pattern of intergeneric hybrids and their fusion parents using the 10-mer oligonucleotide primer $S_{1195}$. *B. oleracea* var. *italica* cv Lude dihaploid (*lane 1*), *M. nitens* (*lane 20*) and their hybrids (*lanes 2–19*); the RAPD pattern of the hybrids includes one band from *B. oleracea* var. *italica* cv Lude diphaploid and two bands from *M. nitens*

**Table 1** Numbers of calli and regenerated plants produced from the protoplast fusion between *M. nitens* and *B. oleracea*

| Combination | Code number | No. of calli produced | No. of shoots regenerated | Regeneration frequency |
|---|---|---|---|---|
| *M. nitens*+*B. oleracea* var. *italica* cv Lude dihaploid | MO30 | 468 | 111 | 23.7% |
| *M. nitens*+*B. oleracea* var. *italica* cv Yazhi | MO36 | 366 | 105 | 28.7% |
| *M. nitens*+*B. oleracea* var. *italica* cv Meiluxilanhua | MO37 | 227 | 84 | 37.0% |
| *M. nitens*+*B. oleracea* var. *gongylodes* cv Yuanyi | MO43 | 99 | 17 | 17.2% |
| *M. nitens* + *B. oleracea* var *capitata* cv Jingfeng 1 | MO44 | 835 | 108 | 12.9% |
| Totals | | 1995 | 425 | 21.3% |

(Fig. 1C, Table 2). The hybrid nature of these plants, selected by RAPD analysis, was further demonstrated by morphological observations of the plantlets grown in the test-tube. The leaves of the hybrid plantlets were fleshy with only small midribs, like *M. nitens*. At the same time, the hybrid plants also had a distinguishable petiole, like *B. oleracea*.

## Characterization of the somatic hybrids

While most of the hybrid plantlets were maintained in the test tube prior to planting out in the greenhouse, some of the first somatic-fusion plantlets that were regenerated were rooted and transplanted into soil. The morphologically intermediate feature of the hybrids became increasingly visible as the plants grew. *M. nitens* has small and fleshy leaves whose petioles cannot be discriminated from the blade, while *B. oleracea* has large leaves with a symmetric cleft and a distinguishable petiole. The leaves of hybrids were intermediate in size, half-fleshy with a symmetric cleft and a remarkable petiole (Fig. 2A, B). To-date 11 hybrids have flowered. Most of the petals in the hybrids were white with violet veins, while *M. nitens* has purple flowers and cauliflower has light-yellow flowers. Variations in flower color and petal number were also observed in the hybrids, e.g. light-yellow flower (MO36–8) and five-petalled flower (MO30–8).

Five hybrids were cytologically checked. The chromosome number in somatic cells varied between individual plants and even within an individual plant. While cells of MO30–41 had 46 chromosomes, as expected, most cells of MO36–4 and MO37–1 had 92 chromo-

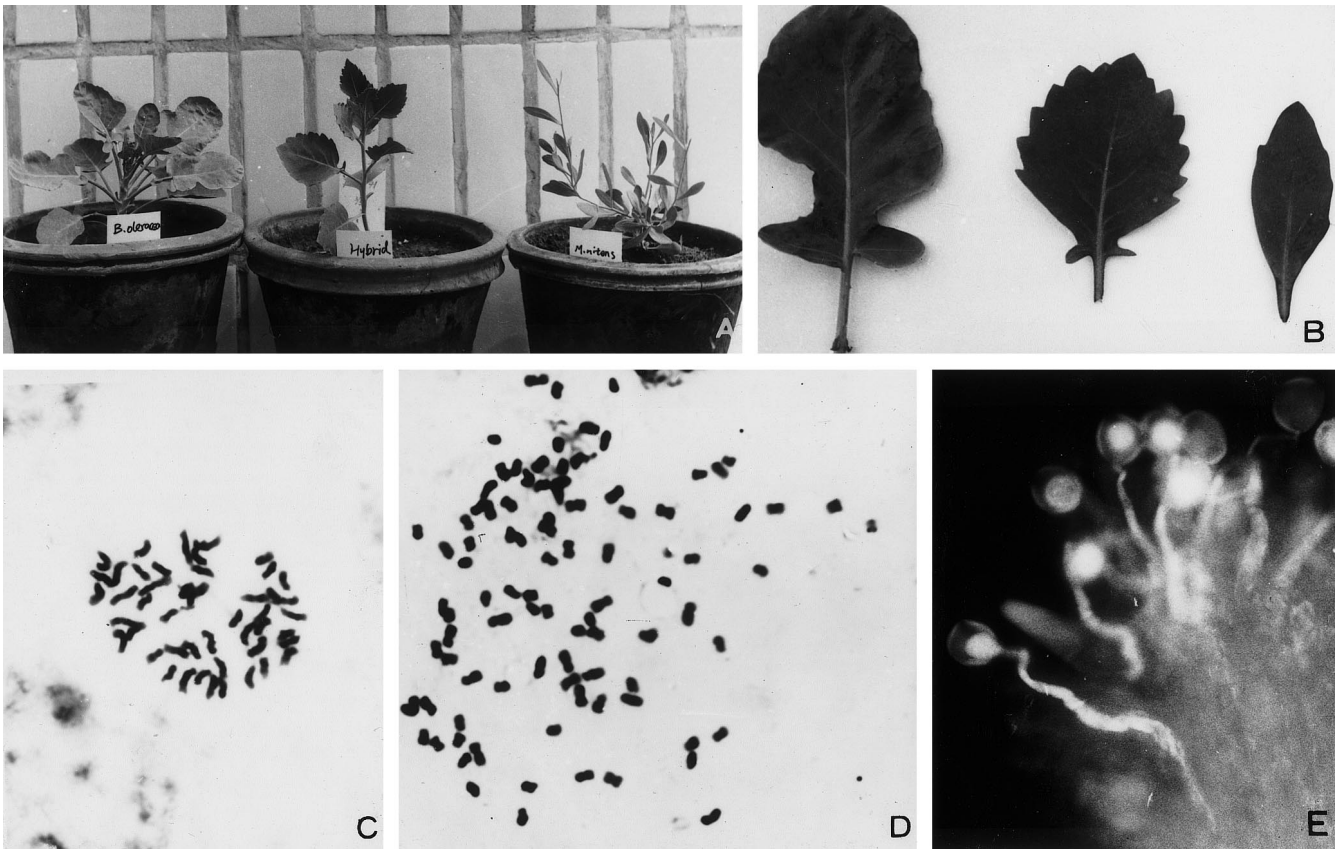


**Fig. 2** **A** A hybrid plant and its fusion parents grown in soil. Left: *B. oleracea* var. *italica* cv Lude di-haploid; middle: the hybrid plant; right: *M. nitens*. **B** Leaf morphology of the hybrid plant and its fusion parents. Left: *B. oleracea* var. *italica* cv Lude di-haploid; middle: hybrid plant; right: *M. nitens*. **C** Metaphase cell with 46 chromosomes from the hybrid plant MO30–41. **D** Metaphase cell with 92 chromosomes from the hybrid plant MO30–65. Cells with 46 chromosomes were also observed in this plant (not shown in the fig.). **E** Pollen grains of *B. napus* which germinated normally on the stigma of somatic hybrid plant MO37–17

**Table 2** The results of the fusion products by morphology and RAPD analysis

| Code number | Number of plantlets been examined | Number of hybrid plantlets identified | Number of plantlets of *M. nitens* | Number of plantlets of *B. oleracea* | Frequency of hybrid plantlets |
|---|---|---|---|---|---|
| MO30 | 60 | 57 | 0 | 3 | 95% |
| MO36 | 13 | 11 | 0 | 2 | 84.6% |
| MO37 | 13 | 13 | 0 | 0 | 100% |
| MO44 | 4 | 4 | 0 | 0 | 100% |
| MO48 | 4 | 4 | 0 | 0 | 100% |
| Totals | 94 | 89 | 0 | 5 | 94.6% |

somes and no cell with 46 chromosomes was observed, probably due to fusion between two cells of *M. nitens* and two cells of *B. oleracea*. However, both 92 ( in most cases) and 46 chromosomes were observed in the cells of MO30–65; chromosome doubling during subculture might account for this variation (Fig. 2C, D). There were 74 chromosomes in cells of MO30–38 indicating a fusion event between two cells of *M. nitens* and one cell of *B. oleracea*. Interestingly, this plant had the lowest $CO_2$ compensation point ($\Gamma$=24±0.81 $\mu l CO_2 \cdot l^{-1}$) among all of the hybrid plants measured. The chromosome ploidy coincided with the pollen fertility of the plants, i.e. the amphidiploid hybrid plant MO30–41 produced fully fertile pollen, the hexaploid plant MO30–38 was half sterile, and the octoploid plants MO36–4 and MO37–1 yield no pollen grains (Table 3).

In most cases the anthers were stunted and contained few fertile pollen grains. However, in hybrids MO30–1, MO30–40 and MO36–8 the anthers were normal and produced plenty of pollen. More than 70% of these pollen grains germinated in pollen-germinating medium. When the hybrid plants (MO36–8) were self-pollinated, pollen grains germinated on the stigma and pollen tubes penetrated down to the style. However, several abnormal phenomena were also observed such as pollen tubes winding on the surface of the stigma, short pollen tubes

**Table 3** Summary of chromosome numbers, $CO_2$ compensation point and fertility data from particular somatic hybrids and their fusion parents

| Species or fusion combination number | Chromosome number in somatic cells | $CO_2$ compensation point | Pollen fertility |
|---|---|---|---|
| *M. nitens* | 28 | 6±0.05 | Fertile |
| *B. oleracea* | 18 | 78±1.84 | Fertile |
| MO30–1 | | | Sterile |
| MO30–38 | 74 | 24±0.81 | Half-sterile |
| MO30–40 | | | Sterile |
| MO30–41 | 46 | | Fertile |
| MO30–65 | 46 and 92 | 73±0.75 | |
| MO36–4 | 92 | 38±2.37 | Sterile |
| MO36–8 | | | Fertile |
| MO37–1 | 92 | 51±1.92 | Fertily |
| MO37–2 | | 52±0.27 | |
| MO37–15 | | | Fertile |
| MO37–17 | | | Fertile |
| MO43–3 | | 62±1.78 | |
| MO44–4 | | 51±0.36 | |
| MO44–5 | | 82±1.34 | |

rejected by papilla cells, heavy callose deposited within the pollen tube, and with tubes stopping growth in the style. The same phenomena were observed when the hybrid plants MO37–15 and MO37–17 were pollinated with pollen of *B. napus* (Fig. 2E). The pods that developed on the plants continued growth for 3–4 days after pollination but then turned yellow and died.

The $CO_2$ compensation point (Γ) of eight hybrid plants was measured. The Γ values of six hybrids were significantly intermediate between their parents. It is apparent that the genes determining $C_3$–$C_4$ character in *M. nitens* were not completely suppressed by the genes of *B. oleracea* in the somatic hybrids.

## Discussion

In this series of experiments, protoplast fusion between *M. nitens* and *B. oleracea* led to the regeneration of 425 plantlets from 1995 calli in five interspecific fusion combinations. Overall, more than 90% of the putative hybrid plants were confirmed to be true hybrids according to their morphological characters and DNA fingerprinting analysis even though no selection for hybrids was made. That hybrid plants regenerated at high frequency had also been observed in a previous fusion combination between *M. arvensis* and *B. oleracea* (Toriyama et al. 1987) and a series of interspecific and intergeneric protoplast fusion combinations involving *Citrus* (Deng et al. 1992; Ling and Iwamasa 1994; Guo et al. 1998). When the mixed protoplasts of *M. nitens* and *B. oleracea* were cultured, many more calli were derived from *M. nitens*. It suggested that the modified KM8p medium was more adaptable to *M. nitens* at the callus growing stage. We also found that the plantlets of *B. oleracea* growing in K3 and B5 medium, which were used in shoot regeneration, produced more leaves and roots than that of *M. nitens* (data not shown). It is therefore possible that only somatic hybrids containing the genomes of both *M. nitens* and *B. oleracea* would have superiority during both the development and differentiation of calli. As such this could lead to a degree of selection for the hybrid plants at the stage of establishing plantlets.

To-date four papers have presented data about $CO_2$ compensation-point measurement on hybrids between *M. arvensis* or *M. nitens* and *Brassica* crops. Only five of these hybrid plants had a photosynthetic character that was intermediate between those of their parents (Apel et al. 1984; Meng et al. 1998; O'Neill et al. 1996; Rawsthorne et al. 1998). Apel et al. (1984) obtained a hybrid plant between *M. arvensis* and *B. oleracea* var. *alboglabra*, and this hybrid had a Γ value between both of the parents. O'Neill et al. (1996) and Meng et al. (1998) obtained a range of hybrid plants from somatic and sexual hybridization involving *M. arvensis* and *B. napus*. Of these only three hybrids had a lower $CO_2$ compensation point than *B. napus* (O'Neill et al. 1996). Rawsthorne et al. (1998) have recently reported that the $CO_2$ compensation point of a hybrid between *M. nitens* and *B. napus* was only slightly less than that of the $C_3$ parent, *B. napus*. However, partial confinement of glycine decarboxylase activity to the bundle sheath cells was observed revealing that the $C_3$–$C_4$ character was being expressed, at least partially, in the hybrid. Furthermore, the $CO_2$ compensation point of six somatic hybrids between *M. nitens* and *B. napus* produced in our laboratory also had a Γ value like that of *B. napus* (paper in preparation).

In contrast to the above reports, six out of the eight hybrids between *M. nitens* and *B. oleracea* produced in this study have a much lower $CO_2$ compensation point than that of the $C_3$ parent. It is possible that the $C_3$–$C_4$ character could be expressed more easily in the presence of the CC diploid genome than that of AACC amphidiploid genome. There may be respectively one and two sets of genes in the *B. oleracea* (CC) and *B. napus* (AACC) genomes that interact with the genes controlling the expression of the $C_3$–$C_4$ character. The presence of both sets in the amphidiploid could, therefore, lead to a stronger suppression in the gene-dosage response. Interestingly, the hybrid plant with the lowest Γ value in our

experiment was the one with 74 chromosomes, involving the sum of two sets of choromosomes of *M. nitens* and one set of *B. oleracea*. On the basis of the data presented here on the successful hybridization produced between *M. nitens* and *B. oleracea*, it is suggested that the most practical way for transferring the $C_3$–$C_4$ character into amphidiploid *Brassica* crops would be to use a diploid species as an initial acceptor. Since most of the hybrid plants showed normal flowers in the hybrids of *M. nitens+B. oleracea*, some recombinants would be expected and could be screened out in the offspring after cross-pollination followed by embryo rescue.

**Acknowledgements** This research has been financially supported by the National Nature Science Foundation of China. Our thanks to Dr. S. Rawsthorne, Dr. Gengyi Li and Mr. Muqiang Gao who provided gift of seed sample. Thanks also to Prof. Demao Jiao and Miss Xia Li, Jiangsu Academy of Agricultural Sciences, Nanjing, China, for their help and technical advice on $CO_2$ compensation-point measurements. Finally, we are grateful to Dr. S. Rawsthorne for critical comments and his reading of the manuscript.

## References

Apel P, Bauwe H, Ohle H (1984) Hybrids between *Brassica* alboglabra and *Moricandia arvensis* and their photosynthetic properties. Biochem Physiol Pflanz 179:793–797

Bang SW, Kaneko Y, Matsuzawa Y (1996) Production of intergeneric hybrids between *Raphanus* and *Moricandia*. Plant Breed 115:385–390

Deng XX, Grosser JW, Gmitter FG Jr (1992) Intergeneric somatic hybrid plants from protoplast fusion of *Fortunella crassifolia* cultivar "Meiwa" with *Citus sinensis* cultivar "Valencia". Sci Hort 49:55–62

Dumas C, Knox RB (1983) Callose and determination of pistil viability and incompatibility. Theor Appl Genet 67:1–10

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Guo WW, Deng XX, Shi YZ (1998) Optimization of electrofusion parameters and interspecific somatic hybrid regeneration in *Citrus*. Acta Bot Sinica 40:417–424

Hylton CM, Rawsthorne S, Smith AM, Jones DA, Woolhouse HW (1988) Glycine decarboxylase is confined to the bundle-sheath cells of $C_3$–$C_4$ intermediate species. Planta 175:452–459

Jiao DM, Ku MS (1991) The characteristics of photosynthetic $CO_2$ exchange of the $F_1$ Hybrids between $C_3$–$C_4$ intermediate and $C_4$ Species in *Flaveria*. Acta Phytophysiol Sinica 17:225–231

Kirti PB, Narashimhrlu SB,Parakash S, Chorpra VL (1992) Somatic hybridization between *Brassica juncea* and *Moricandia arvensis* by protoplast fusion. Plant Cell Rep 11:318–321

Kirti PB, Mohapatra T, Khanna H, Parakash S, Chorpra VL (1995) *Diplotaxis catholica+Brassica juncea* somatic hybrids: molecular and cytogenetic characterization. Plant Cell Rep 14:593–597

Ling JT, Iwamasa M (1994) Somatic hybridization between *Citrus reticulata* and *Citropsis gabunensis* through electrofusion. Plant Cell Rep 13:493–497

McVetty PBE, Austin RB, Morgan CL (1989) A comparison of the growth, photosynthesis, stomatal conductance and water-use efficiency of *Moricandia* and *Brassica* species. Ann Bot 64:87–94

Menczel L, Wolfe K (1984) High frequency of fusion induced in freely suspended protoplast mixtures by polyethylene glycol and dimethylsulphoxide at high pH. Plant Cell Rep 3:196–198

Meng J, Gan L (1998) Studies on the relationships between *Moricandia* and *Brassica* species. Acta Bot Sinica 40:508–514

Meng J, Yan Z, Gan L (1998) Studies on obtaining intergeneric hybrids of *Moricandia arvensis×Brassica napus* and their biological characteristics. Acta Agron Sinica 24:396–401

Moss ON (1962) The limiting carbon dixide concentration for photosynthesis. Nature 193:587

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

Olsson G (1960) Species crosses within the genus *Brassica*. II. Artfical *Brassica napus* L. Hereditas 46:351–386

O'Neill CM, Murata T, Morgan CL, Mathias RJ (1996) Expression of the $C_3$–$C_4$ intermediate character in somatic hybrids between *Brassica napus* and the $C_3$–$C_4$ species *Moricandia arvensis*. Theor Appl Genet 93:1234–1241

Rawsthorne S (1992) $C_3$–$C_4$ intermediate photosythesis: linking physiology to gene expression. Plant J 2:267–274

Rawsthorne S, Hylton CM, Smith AM, Woolhouse HW (1988) Photorespiratory metabolism and immunogold localisation of photorespiratory enzymes in leaves of $C_3$ and $C_3$–$C_4$ intermediate species of *Moricandia*. Planta 173:298–308

Rawsthorne S, Morgan CL, O'Neill CM, Hylton CM, Jones DA, Frean ML (1998) Cellular expression pattern of the glycine decarboxylase P protein in leaves of an intergeneric hybrid between the $C_3$–$C_4$ intermediate species *Moricandia nitens* and the $C_3$ species *Brassica napus*. Theor Appl Genet 96:922–927

Roberts IN, Gaude TC, Harrod D, Dickinson HG (1983) Pollen-stigma interactions in *Brassica oleracea*: a new pollen germination medium and its use in elucidating the mechanism of self-incompatibility. Theor Appl Genet 65:231–238.

Rogers S, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant Mol Biol A6:1–10

Takahata Y (1990) Production of intergeneric hybrids between a $C_3$–$C_4$ intermediate species *Moricandia arvensis* and a $C_3$ species *Brassica oleracea* through ovary culture. Euphytica 46:259–264

Takahata Y, Takeda T (1990) intergeneric (intersubstribe) hybridization between *Moricandia arvensis* and *Brassica* A and B genome species by ovary culture. Theor Appl Genet 80:38–42

Takahata Y, Takeda T, Kaizuma N (1993) Wide hybridization between *Moricandia arvensis* and *Brassica* amphidiploid species (*B. napus* and *B. juncea*). Euphytica 69:155–160

Tian Z, Meng J (1999) Plant regeneration from cultured protoplasts of *Moricandia nitens*. Plant Cell Tissue Org Cult 55(3): 217–221

Toriyama K, Hinata K, Kameya T (1987) Production of somatic hybrid plants, "Brassicamoricandia" through protoplast fusion between *Moricandia arvensis* and *Brassica oleracea*. Plant Sci 48:123–128

Tu J, Zheng Y, Fu T (1997) RAPD markers linked to the genetic male-sterile gene of rapeseed. J Huazhong Agric Univ 16:112–117

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7:389–452

Zhao QH, Huang JH, Yan CJ (1986) Studies on pollen germination of rapeseed. Acta Agron Sinica 12:15–20