High frequency production of microspore derived doubled haploids (DH) and its application for developing low glucosinolate lines in Indian *Brassica juncea*

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Abstract

The Indian varieties of *Brassica juncea* are high in erucic acid (EA) and glucosinolate (GSL) contents and are, therefore, nutritionally inferior. Breeding of 'canola' quality *B. juncea* (low EA and low GSL) requires the manipulation of more than seven recessive genes and hence, can be conveniently handled only through the production of doubled haploids. We developed an efficient protocol for the production of microspore derived doubled haploids in *B. juncea*, with reasonably high embryogenic and very high embryo conversion frequencies and applied it in a backcross breeding programme for the introgression of low glucosinolate trait from an un-adapted canola quality *B. juncea* line Heera to a popular Indian variety Varuna. The parents differed significantly in their average embryogenic response and that of the F_1DH was close to Varuna. Microspores treated with colchicine at the initiation of culture for 24h showed 65-70% doubled haploid production. Maintenance of high humidity in the immediate environment of the cultured microspores was crucial for the induction of embryogenesis. Addition of activated charcoal after colchicine treatment improved the embryogenic frequency. Subjecting the fully differentiated embryos to ABA at low temperature (15°C) for physiological maturation eliminated embryo malformations such as secondary embryogenesis, hypertrophy or unipolar embryo 'germination'. The entire process, from microspore to plantlet development spanned 3-4 months. The doubled haploids were transferred to the field without any prior hardening with a mortality rate of about 10%. Till date, following this protocol through five backcross generations coupled with stringent selections, we have developed several low GSL (⩽10 µmol/g of seed) lines in *B. juncea*.

Key words: *Brassica juncea*, microspore culture, high frequency doubled haploids, backcross breeding, low glucosinilate lines

Introduction

The instant homozygosity obtained using a doubled haploid (DH) system greatly facilitates the fixation of desirable traits controlled by recessive alleles. It also reduces the size of the population required to identify individuals carrying the desired recessive traits. Hence, the DH technique can be conveniently employed in a recurrent backcross breeding programme for important recessive traits, especially for plants amenable to in vitro manipulations. Indian varieties of *Brassica* juncea are high in glucosinolates (GSL), a trait governed by at least six recessive genes (Sodhi et al., 2002). Since *B. juncea* is highly amenable to in vitro manipulations, we developed a breeding strategy utilizing the doubled haploid technique to incorporate the low GSL trait from an un-adapted canola quality *B. juncea* line Heera to a popular Indian variety Varuna in order to improve the seed meal quality. Few reports have been published dealing with application of DH technique to *B. juncea* (Thiagarajah and Stringam, 1990; Purnima and Rawat, 1997; Hiramatsu et al., 1995; Lionneton et al., 2001). However, these publications either do not mention the embryogenic and the embryo conversion frequencies or describe methods that are not very efficient in fulfilling both these criteria.

In this paper we report the development of an efficient, high throughput protocol, based on the protocol described by Iqbal (1993), for the production of DH plants in *B. juncea* that ensures high embryogenic and embryo conversion frequencies. We applied this technique for five successive backcross generations using Indian B.juncea var. Varuna as the recurrent parent and have been able to derive several low glucosinolate lines with high percentage of Varuna genome reconstituted.

Materials and Methods

Plant materials: Plants of an Indian type high glucosinolate containing *Brassica* juncea var. Varuna and a canola quality east European *B. juncea* line Heera, their F_1 and five subsequent backcross generations using Varuna as the recurrent parent, were used as the microspore donors. Plants were grown in growth chambers (Conviron, Canada) under controlled environmental conditions. During the vegetative phase of growth plants were maintained at 20°- 15°/ 14h-10h day and night cycle, and at 85% relative humidity. With the emergence of the inflorescence axis the plants were shifted to a regime of 10°-5° / 10h-14h day and night cycle and were maintained for at least 15 days prior to harvesting buds for microspore culture. Buds measuring 3.5- 4.0mm containing mid- to late uninucleate pollen grains were collected from primary as well as secondary flowering axes and were used to initiate the cultures.

Culture protocol: To sterilize, the buds were first rinsed thoroughly in a dilute solution of cetavelon (150 µl in 50ml of distilled water) for 15 min followed by washing in running water. Next, the buds were treated briefly (3min) with 70% alcohol,

drained and rinsed with sterile distilled water. Finally, the buds were treated with 4% Na- hypochlorite solution for 10min and thoroughly washed with sterile distilled water. Following sterilization, the buds were placed in a sieve (~60µ) and crushed gently in NLN medium (Gland et al., 1988) without iron and vitamins containing 13% sucrose (wash medium) to release the microspores. This suspension was centrifuged twice (1000 rpm) using wash medium to remove the debris and was finally suspended in NLN medium containing 13% sucrose (NLN-13) and 0.01% colchicine and incubated for 24h at 31.5°C in dark. After 24h colchicine was removed by repeated washing in NLN-13 and microspores were plated in sterile 60mm petridishes at a final density of 25-40,000/5ml medium containing 100µl of sterile 1% activated charcoal. These petridishes were in turn placed in larger glass petridishes (120mm) along with a petridish containing water and sealed. Such assemblies were incubated at 31.5°C in dark for seven days after which the cultures were suitably diluted with fresh NLN-13 medium and cultured at 24°C and 80 rpm under 14h/10h day-night cycle for next 15 days. The number of embryos was kept around 25 in each dish at this stage.

After 21 days the fully differentiated embryos were transferred to NLN-13 containing ABA (15µl of 1mM ABA/ml of medium) and cultured at 15°C and 80 rpm under 14h/10h day-night cycle for next 15 days. Subsequently, the embryos were transferred to Schenck medium (Schenck and Robbelen, 1982) supplemented with GA_3 (1.0 mg/l) for 'germination'. After 3-4 weeks the germinated embryos were subcultured to MS (Murashige and Skoog, 1962) medium containing IBA (2mg/l) and maintained till the time of transplantation.

Ploidy analysis: Second or third leaf from 3-4 week old individual putative doubled haploid plants was taken for analyzing its ploidy level following Arumuganathan and Earle (1991) and the diploidized plants were transplanted in the field.

Result

The parents differed greatly in their ability to produce microspore derive embryos, the east European line Heera being highly recalcitrant compared to the Indian variety Varuna. The F_1 plants were closer to Varuna in their embryogenic ability. The embryogenic frequency improved gradually during subsequent generations (Table 1). It was observed that maintenance of high humidity was absolutely essential for initiating the microspores in the sporophytic developmental pathway. No embryo differentiation was observed if the microspores were cultured in the absence of water containing petridishes as described above. Addition of activated charcoal also improved the embryogenic frequency. Colchicine treatment for 24h induced diploidization in about 65% of the microspores. Increasing the duration of colchicine treatment did not improve the frequency of diploidization but enhanced the production of embryos of higher ploidy (data not shown). Addition of activated charcoal in the culture medium was essential for embryo differentiation.

Timely dilution of cultures was crucial for subsequent development of the embryos. It was observed that about 20-25 embyos cultured in 8ml of medium in a 60mm petridish produced optimum result. It was also observed that segregating the embryos according their size class was beneficial and caused less stress. In a mixed population of embryos the cultures often turned brown.

The fully developed embryos were transferred to ABA containing medium for the physiological maturation of the embryos. At this stage it was no longer necessary to maintain the high humidity condition around the culture plates. It was observed that the embryos treated with ABA 'germinated' and grew normally whereas those without ABA treatment generally became hypertrophied following germination and also developed secondary embryos. Presence of GA_3 in the germination medium improved the embryo conversion frequency. On an average 88% embryos germinated on Schenck medium containing GA_3 (1.0 mg/l) and when subcultured on MS+ IBA (2.0mg/l) developed into healthy plantlets. Diploid plants were transferred to soil after analyzing their ploidy status.

At each generation DH plants with glucosinolate contents lower than the low GSL parent and with maximum in put of Varuna genome were stringently selected and used as the donor for microspores for the next cycle of DH production. At the end of fifth back cross we could obtain several low GSL lines (1.2µ mol /g seed – 8.0µ mol /g seed) having around 85% of Varuna genome reconstituted.

Table1: Production of doubled haploid embryos from *B. juncea* var. Heera and Varuna, their F_1 and five subsequent backcross generations*

Source material	No. of microspores cultured ($x10^5$)	No. of embryos obtained	Embryogenic frequency (%)	Plant conversion frequency (%)	Diploidization frequency (%)
Heera	187	45	0.00024	100	60
Varuna	105	2383	0.022	98	64
F_1DH	325	5179	0.016	79	65
BC1DH	258	6114	0.027	81	65
BC2DH	94	1686	0.026	82	67
BC3DH	53	9856	0.18	90	66
BC4DH	297	13720	0.046	85	67
BC5DH	346	9608	0.027	87	68

*Data average of at least seven experiments

Discussion

The main highlight of the present study is the highly reproducible rate of high frequency of embryo conversion on

Schenck medium containing GA_3 (1.0mg/l). Another crucial observation is the absolute necessity for the presence of high humidity in the immediate vicinity of the microspore culture dishes, without which no embryo would differentiate under our lab conditions. The frequency of doubled haploids obtained in the present study could perhaps be further improved by increasing the colchicine concentration for shorter duration of treatment (Mollers et al., 1994).

Using the protocol described above we were able to generate several thousand DH plants at each generation which enabled us to stringently select the most desirable plant type from a wide amplitude of low glucosinolate plants. BC1DH onward, at each generation, we selected 2-4 DH plants with highest Varuna genome and lowest GSL contents, as the microspore donors for the next cycle of breeding. The DH lines from advanced backcross generation are presently being used for mapping and tagging of genes involved in glucosinolate biosynthesis.

Acknowledgement

This work was supported by the Dhara Vegetable Oil and Food Company Ltd (DOFCO), a fully owned company of the National Dairy Development Board (NDDB), India.

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