

Molecular evaluation of chemically induced male sterility in *Brassica juncea* (L.) Czern & Coss.

Vandana Singh, S.V.S. Chauhan

Department of Botany, School of Life Sciences, Dr. B. R. Ambedkar University, Khandari Campus, AGRA-282002, India
Email: svcs250@rediffmail.com; svsc@sancharnet.in

Abstract

Plants of *Brassica juncea* var. Pusa bold were sprayed with some chemical hybridizing agents (CHAs), namely, surf excel – a detergent; benzotriazole; ethrel and gibberellic acid. All the treatments induced significantly high extent of pollen sterility (94.6-100). Surf excel treated plants exhibited changes in floral morphology which were used to promote out-crossing. Yield components were insignificantly different from control once in plants sprayed with lower concentrations of surf excel and benzotriazole. However, lower doses of ethrel and gibberellic acid brought about significant increase in yield as compare to control.

LM and TEM studies revealed that pollen abortion in CHA treated plants was associated with abnormal behaviour of tapetum, endothecium and pollen grains. Development of endothecium was inhibited in sterile anthers. This was associated with malformed tapetum supporting programmed control of tapetum on endothelial development. The cells in tapetum failed to degenerate and remained intact, however, sometimes they elongated radially or form pseudoperiplasmodium. Ultrastructural studies revealed the presence of increased number of mitochondria and enlarged degenerated plastids in the tapetal cells in the anthers of surf excel treated plants. On the other hand, tapetum in benzotriazole and ethrel treated plants exhibited pre-mature degeneration of mitochondria and plastids. Secretion of higher quantity of sporopollenin was recorded in the tapetal cells of anthers of benzotriazole treated plants as marked by their accumulation at several places in anther locule. Pollen grains of the treated plants consisted of degenerated protoplast. The intine in sterile pollen grains was lacking while the exine was abnormally thick but poorly differentiated into tectum and baculae.

The molecular studies based on RAPD, Dot-blot and RT-PCR analysed two fertility related anther specific genes i.e. *Bcp1* and *callase*. These experiments indicated that there was a transitional change in the intensity of bands. Both the genes were present in anthers of CHAs treated plants. However, their activity was either suppressed or over expressed. Since tapetal mitochondria exhibited abnormal behaviour, analysis of mt-DNA has also been initiated.

Key words: *Brassica juncea*, chemical hybridizing agents, pollen sterility, anther development, gene expression.

Introduction

Male sterility is a reproductive deficiency of some plants where male organs in hermaphrodite flowers are rendered defunct (Kaul, 1988). It relates particularly to non-viable pollen grains, which are formed through a chain of vital processes during microsporogenesis. These processes are so delicately balanced under genetic control of many loci that mutation at any one locus may throw the entire process of microsporogenesis astray resulting in the formation of non-functional microspores and thus causing male sterility.

A large number of anther specific genes have been identified in various plants, which are associated with the fertility of the anthers (Tschuiya et al., 1992). There are several molecular markers and techniques, which used to tag anther specific fertility genes in CMS and GMS lines, however, their application in chemically induced male sterile plants is not known. Keeping this in view, during present investigation the expression of two anther specific genes i.e. *Bcp 1* and *Callase* were analyzed in the anthers of male sterile plants of *Brassica juncea* treated with different chemical hybridizing agents by using RAPD, dot-blot analysis and RT-PCR.

Materials and Methods

The seeds of *Brassica juncea* (L.) Czern & Coss. Pusa Bold were grown at Botanical garden, School of Life Sciences, Dr. B. R. Ambedkar University, Agra. The seeds were sown in different plots with spacing of 20 cm in a randomized row design, maintaining 35 cm distance between rows and 20 cm distance between plants.

Aqueous solutions of a detergent- surf excel (2.0, 4.0 and 6.0% w/v); benzotriazole (0.5, 1.0 and 1.5% w/v); ethrel (0.1, 0.2 and 0.3%v/v) and gibberellic acid (0.01,0.02 and 0.03 %w/v) were prepared in distilled water. A few drops of Tween 20 as a wetting agent were added in each solution. A group of 90 plants were sprayed, 21 days after sowing i.e. a week before the appearance of floral bud primordia with each concentration. Leaving a group 45 plants after first spray (T₁), the other 45 plants were sprayed with same concentration of the CHAs again after 33 days (T₂). Individual plants were sprayed with 25 ml of the concerned solution to run off. Simultaneously, a group of 45 plants were treated with distilled water to run off to serve as control. Each treatment was replicated thrice. A row of another variety (T-59) of *B. juncea* was left untreated between the rows of treated plants to facilitate cross-pollination. A beehive was also placed between the rows to help in this process.

Morphological Studies

Pollen viability was checked at regular intervals throughout flowering by 1% tetrazolium chloride (TTC) in 0.15 M Tris-HCl buffer at pH 7.5 and fluorochromatic reaction (FCR) test as described by Shivanna and Rangaswamy (1992). Pollen fertility was checked by Alexander's stain (1980).

Anther Development and Microsporogenesis

Anther development was studied in floral buds fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 6.8. The samples were rinsed twice in the same phosphate buffer for 5 minutes. Post fixation was done with 1% osmium tetroxide in the same buffer for 2 hours. Samples were dehydrated in an ethyl-propylene oxide series, embedded in Spurr's low viscosity embedding media and were polymerized at 60° C overnight.

Semi-ultra thin sections were cut at 1 µm and stained with a solution of 0.5%w/v toluidine blue in 1 % w/v sodium borate. For TEM studies sections were cut at 60-80 nm and picked up on gold-coated copper grids. These were stained with uranyl acetate and alkaline lead citrate. Observations were made on Philips Cryo CM10 electron microscope at All India Institute of Medical Science, New Delhi.

Molecular studies

1. DNA and RNA Isolation from anthers:

DNA and RNA from the anthers of different chemically induced male sterile and fertile plants were fractionating by following the strategy of stepwise isolation proposed by Prabhu et al. (1998) and Robert et al. (1989).

2. RAPD Analysis:

Three arbitrary 10 bp long oligonucleotides namely OPH07, OPK15 and OPZ06 were used as primers (Operon Technologies, Inc., Alameda, CA, USA) for amplification of extracted DNA. The procedure for RAPD analysis was as described in Foisset et al. (1996). RAPD bands were then scored as present or absent.

3. Dot-Blot Analysis:

Known primer sequences for anther specific genes were labeled through 3-end tailing. Isolated DNA/RNA loaded on nylon membrane. In a poly pack labeled probe was hybridized with loaded samples of DNA/RNA in hybridization buffer (2.5% formamide). It was washed after 2 hrs with different concentrations of SSC and SDS. Following a series of incubations in different buffer solutions, it was detected by after final incubation of 15 min in NBT/BCIP in dark. After 15 minutes of incubation DNA/RNA dots develop a colour and the intensity of colour showed the expression (suppression or over expression) of particular gene.

4. RT – PCR Analysis:

For RT-PCR, RNA from anthers of male fertile and male sterile plants were reverse transcribed and amplified by PCR with a pair of sequence specific primers by using the Access RT-PCR system (Promega). A pair of 21 mer and 24 mer forward and reverse primers were used for Bcp1 gene.

A 25 l reaction, reaction buffer for RT-PCR containing 7.55 µl RNase free dH₂O, 5 µl TflR Buffer, 0.5 µl dNTPs, 0.2 µl RT-AMV enzyme, 0.25 µl Tf.Taq, 0.25 µl each forward and reverse primers, 1.0 µl MgSO₄ and 10 µl of sample RNA. Based on the signal intensity of the amplified products, the relative amount of Bcp1 mRNA in fertile and sterile anthers was estimated.

Observations

The effect of different chemical hybridizing agents (CHAs) on pollen fertility, yield and anther development has been evaluated with special reference to their effect on gene activity in *Brassica juncea*.

Pollen Fertility:

Foliar applications of all the chemical hybridizing agents induced significantly high pollen sterility in *B. juncea* (Table 1). All the treatments of surf excel (2, 4 and 6%) and benzotriazole (0.5, 1.0 and 1.5%) and one or two sprays with 0.2 and 0.3 % ethrel induced 100 % pollen sterility (Table 1). Higher concentrations of these CHAs induced permanent sterility. However, sterility induced by lower concentrations declined with the age.

Hybrid Seed Production:

The lower concentrations of all the CHAs either caused insignificant reduction or slight increase in total yield along with almost complete pollen sterility (Table 1).

Plants sprayed once with 2 % surf excel, 0.5 % benzotriazole and 0.3 % ethrel exhibited insignificant reduction in total yield with 100 % pollen sterility. On the other hand, single spray of 0.2 and 0.3 % ethrel enhanced yield. It is also important to note that the seeds obtained from CHAs treated male sterile plants produced better offspring in next generation.

Anther Development and Microsporogenesis:

There was three types of tapetal abnormalities associated with anther development in CHAs treated male sterile plants, these were persistent tapetum (surf excel and benzotriazole treated plants), formation of pseudotapetal periplasmodium (benzotriazole treated plants) and hypertrophied tapetum (ethrel treated plants).

The intact tapetal cells of anthers of plants treated with surf excel showed an increase in the number and size of mitochondria and these mitochondria exhibited tubular cristae (Fig. 1). The size of plastids enhanced, however, these were in

degenerated form as they failed to possess specific lamellae and filled with lipids and starch. The cytoplasm of pollen grains of surf excel treated plants was devoid of cell organelles and nucleus (Fig.2). However, they only possessed lipid inclusions and large number of small vacuoles. Both exine and intine were thick and exine was poorly differentiated (Fig. 2).

Anthers of benzotriazole treated plants exhibited degenerated tapetal cytoplasm with non-functional and degenerated cell organelles including mitochondria and plastids. Plastids were of small sized and filled with lipid like material. Mitochondria possessed disintegrated cristae (Fig. 3). Another important feature is the secretion of large amount of sporopollenin by tapetum of benzotriazole treated plants and this extra sporopollenin get accumulated in anther cavity at several places. Intine was absent and exine was well developed (Fig. 4).

In the anthers of ethrel treated plants, hypertrophied tapetum was highly vacuolated and possessed disintegrated cell organelles (Fig. 5). Microspore tetrads were devoid of primexine, but the development of exine was not much influenced. Pollen grains were highly deformed, vacuolated and the nucleus was degenerated. Cell organelles were poorly developed. The exine was thick, but intine was absent (Fig. 6).

Molecular Studies:

RAPD Analysis:

For RAPD analysis, three oligonucleotide primers viz. OPK15, OPH07 and OPZ¹ have been used. OPH07₁₁₀ and OPH07₃₅₀ act as marker for male sterility in DNA band of ethrel treated plants. However, DNA of other CHAs treated sterile plants showed similar activity for this marker as in male fertile control plants (Fig.7). OPK15₇₄₀ was found to be polymorphic in nature was absent in DNA of surf excel and gibberellic acid treated male sterile plants, however, DNA of other two male sterile plants (benzotriazole and ethrel treated) at the same band were quite suppressed. On the other hand OPK15₂₀₀ was faint only in DNA of gibberellic acid treated plants (Fig 8). DNA bands in male sterile plants with OPZ06₉₀₀ were less intense in comparison to bands of male fertile plants (Fig.9). DNA bands were faint in plants sprayed with ethrel and gibberellic acid at OPZ05₁₀₀₀ and in benzotriazole with OPZ06₅₀₀. However, OPZ06₁₀₀ was absent in DNA of benzotriazole treated plants.

Analysis of anther specific fertility related genes:

Dot Blot Analysis:

The expression pattern of *Bcp1* and *Callase* was investigated by using DNA dot blot and RNA dot blot analysis. A faint and intense signal was visible in anthers of CHAs treated male sterile plants in comparison to male fertile control plants.

DNA Dot Blot Analysis:

DNA of male fertile and sterile anthers showed that gene *Bcp1* remain suppress in anthers of surf excel and ethrel treated plants. Whereas, it found to be over expressed in the anthers of benzotriazole and gibberellic acid treated plants (Fig. 10).

On the other hand, gene *Callase* remain suppress in the DNA of anthers of surf excel and gibberellic acid treated plants. However, anthers of benzotriazole and ethrel treated plants showed its over expression.

RNA Dot Blot Analysis:

Expression of *Bcp1* gene found to be suppress in RNA of anthers of all the CHAs treated plants except in the anthers of ethrel treated plants where it found to be over expressed (Fig. 11). On the other hand, *Callase* gene remain suppressed in the anthers of surf excel and benzotriazole treated male sterile anthers and over expressed in the anthers of ethrel and gibberellic acid treated male sterile plants in comparison to the anthers of male fertile control plants.

RT-PCR Analysis:

Utilization of more sensitive RT-PCR analysis was carried out for further examination of expression of *Bcp1* gene in male fertile and male sterile anthers (Fig. 12). RT-PCR amplifications were performed with controlled amounts of RNA from anthers of male fertile and sterile plants and with two gene-specific primers. Anthers of CHAs treated male sterile plants showed a faint signal for male fertility specific gene *Bcp1*.

Discussion

Foliar applications of different concentrations of all the CHAs were found to be highly effective in inducing complete pollen sterility in *Brassica juncea*. Ethrel, benzotriazole and surf excel have been successfully used to induce male sterility in several crops (Singh and Chauhan, 2000; Chauhan and Chauhan, 2003; Chauhan *et al.*, 2003; Agnihotri and Chauhan, 2004).

Anther Development and Microsporogenesis:

Alterations in mitochondrial ultrastructure are associated with the changes in the energy requirement of the cell (Smith and Ord, 1983). Degeneration in mitochondria seems to be responsible for the decrease in oxygen uptake in the sterile anthers and associated with lower metabolic activity of tapetal cells. On the other hand, increase in the number of mitochondria in the tapetal cells of surf excel treated plants results in the increase in respiratory mechanism leading to tapetal malfunctioning. This is supported by findings of Lee and Warmke (1979). They proposed that fertile and sterile anthers differ in their capacity to cope with the demands associated with rapid mitochondrial multiplication.

Molecular Analysis:

RAPD markers appear to be suitable for mapping and tagging of genes and have been utilized to identify fertility restorer and sterility maintainer genes in CMS lines (Williams *et al.* 1990).

In present study all the primers showed polymorphic bands, however, the frequency of polymorphism is very low. Only a few bands were randomly absent in CHAs treated male sterile plants. Each CHA showed different activity with different markers. Thus, these RAPD markers linked with genes responsible for sterility in CHAs treated plants. Delourme *et al.* (1994)

identified six additional dominant RAPD markers, two of which were linked to the fertility restorer allele and the other four to the sterility maintainer allele. Activity of two anther specific fertility genes namely *Bcp1* and *Callase* has been analyzed in CHAs treated male sterile plants. These genes either suppressed or over expressed in male sterile plants in comparison to male fertile plants.

Bcp1, isolated from *Brassica campestris* show a unique pattern of expression in the diploid tapetum and haploid microspores and its expression in both cell types is essential for production in functional pollen (Xu et al. 1995). Xu et al. (1999) identified male gametic cell-specific gene expression in flowering plants.

Thus, these anther specific male fertility genes provide a tool for gaining an understanding of the molecular control of pollen development especially in chemically induced male sterile plants.

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Table 1. Effect of different CHAs on pollen sterility and total grain yield.

S.No.	Chemical Hybridizing Agents	Concentrations (%)	Pollen Sterility (%)		Total Grain Yield (g)	
			T ₁	T ₂	T ₁	T ₂
1.	Surf excel	2	100	100	24.47	11.99
		4	100	100	19.26	9.88
		6	100	100	6.75	4.65
		0.5	100	100	23.31	16.99
2.	Benzotriazole	1.0	100	100	12.72	8.76
		1.5	100	100	9.85	6.35
		0.1	94.6	96.8	36.66	12.73
		0.2	100	100	31.88	9.86
3.	Ethrel	0.3	100	100	24.43	8.13
		0.01	96.1	98.5	46.66	22.43
		0.02	100	100	27.30	15.90
		0.03	100	100	21.03	10.50
5.	Control		8.2		27.48	

Legend to Plate 1

Figs. 1-6. TEM photographs of anther development in CHAs treated plants.

Fig. 1. Tapetal cells of anthers of surf excel treated plants showing increased number of mitochondria (M) and enlarged degenerated plastids (P). 4200X.

Fig. 2. Pollen grain of surf excel treated plants showing thick exine (Ex) and intine (In). 1450X.

Fig. 3. Tapetum of anthers of benzotriazole treated plants showing degenerated mitochondria (M), plastids (P) filled with lipids and endoplasmic reticulum (Er). 4600X.

Fig. 4. Pollen grain of benzotriazole treated plants showing thick exine (Ex) and absnce of intine (In) with deformed nucleus (n) and degenerated cytoplasm. 1950X.

Fig. 5. Tapetum of anthers of ethrel treated plants showing degenerated mitochondria (m) and vacuoles (v). 3400X.

Fig. 6. Mature highly vacuolated (v) pollen grain of ethrel treated plants showing absnce of intine. 2650X.

Legend to Plate 2

Figs. 7-9. Electrophoretic pattern of RAPD analysis with different oligonucleotide primers in the anthers of control and CHAs treated male sterile plants.

- Fig.7. RAPD pattern with primer OPH – 07.
- Fig.8. RAPD pattern with primer OPK – 15.
- Fig.9. RAPD pattern with primer OPZ – 06.

Where M: Marker

- Lane 1: Electrophoretic pattern of male fertile control plants.
- Lane 2: Electrophoretic pattern of surf excel treated plants.
- Lane 3: Electrophoretic pattern of benzotriazole treated plants.
- Lane 4: Electrophoretic pattern of ethrel treated plants.
- Lane 5: Electrophoretic pattern of gibberellic acid treated plants.

Legend to Plate 3

Figs. 10-11. DNA and RNA dot blot analysis in the anthers of male fertile and CHAs treated male sterile plants for anther specific genes *Bcp1* (A) and *Callase* (B).

Fig. 10. DNA dot blot analysis for anther specific genes *Bcp1* (A) and *Callase* (B).

Fig. 11. RNA dot blot analysis for anther specific genes *Bcp1* (A) and *Callase* (B).

Where,

- 1: DNA and RNA of male fertile control plants.
- 2: DNA and RNA of male sterile surf excel treated plants.
- 3: DNA and RNA of male sterile benzotriazole treated plants.
- 4: DNA and RNA of male sterile ethrel treated plants.
- 5: DNA and RNA of male sterile gibberellic acid treated plants.

Legend to Plate 4

Fig. 12. RT- PCR analysis for anther specific gene *Bcp1*.

Where,

M: Marker

- 1: RNA of male fertile control plants.
- 2: RNA of male sterile surf excel treated plants.
- 3: RNA of male sterile benzotriazole treated plants.
- 4: RNA of male sterile ethrel treated plants.
- 5: RNA of male sterile gibberellic acid treated plants.



