

Full Length Research Paper

Development of male sterility by silencing *Bcp1* gene of *Arabidopsis* through RNA interference

Muhammad Tehseen^{1*}, Muhammad Imran¹, Mazhar Hussain¹, Shazia Irum¹, Shahid Ali²,
Shahid Mansoor¹ and Yusuf Zafar¹

¹National Institute for Biotechnology and Genetic Engineering (NIBGE), Jhang Road, Faisalabad, Pakistan.

²Department of Genetics, The University of Melbourne, Australia.

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The development of male sterility is one of the most important steps in hybrid seed production. Several methods for the abortion of pollens based on conventional as well as genetic engineering are reported for the various crop species. Here we have investigated the use of RNA interference (RNAi) technology to silence a male specific gene, *Bcp1* in the model host *Arabidopsis thaliana*. *Bcp1* is active in both diploid tapetum and haploid microspores. Three batches of explants (*A. thaliana*) were selected on herbicide glufosinate ammonium and putative transgenes were confirmed through PCR and Southern hybridization. About 49 out of 58 *Arabidopsis* lines transformed with RNAi construct containing *Bcp1* sequences were male sterile. Transgenic plants were phenotypically indistinguishable from non-transgenic plants and by crossing with non-transgenic fertile pollens successful seed set was observed. The *Bcp1* gene was also amplified from chilies, tomato and brassica. The present study resulted in developing male sterile *A. thaliana* (*Eco. Columbia*) line through genetic engineering. The diversity of *Bcp1* gene will help us to produce transgenic male sterile plants in crop plants.

Key words: RNA interference, male sterility, *Bcp1*, aborted pollen, *Arabidopsis thaliana*.

INTRODUCTION

Male sterile mutants are of agricultural importance for the production of hybrids to improve crop yield. The emasculation of anthers is very labor intensive, and makes it very difficult to produce hybrid seed on large scale. There are some natural mechanisms also that breeders can use to develop male sterile plants, (e.g., cytoplasmic male sterility, chromosomal abnormality, etc.) but they are not available for many crops and require additional steps to restore fertility in the hybrid. Male sterility can be cytoplasmic, nuclear (genetic) and genetic-cytoplasmic (Jain, 1959). In cytoplasmic male sterility (CMS), reciprocal differences are observed and male sterility is inherited maternally. This form of male sterility is usually observed among back-crosses and has been shown to be associated with mitochondrial DNA rearrangements (Vedel et al., 1994).

In the genetic-cytoplasmic male sterility system, both nucleus and cytoplasmic genes are involved. But compatibility between the cytoplasm and nucleus is important. All the species capable of donating sterile cytoplasm are also the sources of corresponding restorer genes (Edwardson, 1970). In other words, the varieties with sterile cytoplasm are self-fertile due to the presence of dominant genes for fertility restoration. While in case of genetic male sterility, Mendelian inheritance is due to nuclear (but not cytoplasmic) genes, arisen as spontaneous mutants in most cases and few reciprocal differences observed. It can be produced through genetic engineering like use of antisense technology (Van der Meer et al., 1992; Xu et al., 1995), Barnase system (Mariani et al., 1992), by the chemical treatment (Kriete et al., 1996; O'Keefe et al., 1994) and by the temperature (Francois et al., 2003; Wang et al., 1995).

Male sterility has been reported across a broad spectrum of higher plants (Kaul, 1988) and its genetic basis remains of technological interest to the plant breeding industries, as well as providing insights into the regulation of developmental biology. The molecular study of anther

*Corresponding author. E-mail: tehseenbt@yahoo.com.

Abbreviations: RNAi, RNA interference; CMS, cytoplasmic male sterility; CTAB, cetyltrimethyl ammonium bromide.

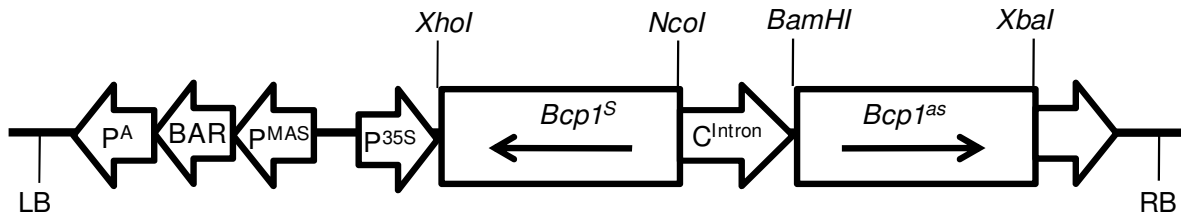


Figure 1. Production of the *Bcp1* RNAi cassette. Two pairs of primers were used to PCR-amplify a portion of the 163 nucleotide of *Bcp1* gene, yielding fragments with BamHI-XbaI and NcoI-XhoI ends. These fragments were directionally cloned into the RNAi vector pFGC5941, yielding the final pTA29RNAi cassette. Shown are the *Bcp1* sense (*Bcp1^S*) and antisense (*Bcp1^{as}*) amplification fragments, the *Agrobacterium tumefaciens* promoter (PMAS) and polyadenylation signal (PA), the Cauliflower mosaic virus 35S promoter (P35S), the petunia chalcone synthase A gene intron (C intron), the *Agrobacterium tumefaciens* polyadenylation signal, gene encoding BASTA (BAR), T-DNA left border (LB) and T-DNA right border (RB).

development currently estimates around 3500 anther-specific transcripts in *Arabidopsis* (Sanders et al., 1999). These large estimates, along with the high percentage of sterility-associated genes in *Arabidopsis* that encode transcription factors (Bowman et al., 1989; Wilson et al., 2001), reflect the complex cascade regulating the differentiation of the specialized cell types within the anther. Detailed histological analysis of anther development has shown that cell differentiation occurs in a precise chronological order, with distinct stages (Goldberg et al., 1993). Engineered male sterility is an alternate method for developing hybrids in cases where natural male sterility is not available. Numerous genes have been identified, in a diverse range of plant species, which show anther specific expression. For example, Male Sterility 1 (MS1) in *Arabidopsis* (Takuya and Kazuo, 2002), AMS gene (Anna et al., 2003), TA29 (Mariani et al., 1990; Shivana et al., 1997), Tapetum Determinant1 (TPD1) in *Arabidopsis* anther (Yang et al., 2003), *Bcp1* in *Arabidopsis* (Xu et al., 1995), TAZ1 (Tapetum Specific Zinc Finger Protein1) (Kobayashi et al., 1998) and Male Meicyte Death 1 (MMD1) (Yang et al., 2003).

Interference with the expression of anther-specific genes or, alternatively, expression of cytotoxic genes from anther-specific promoter has been used to obtain male sterility. For example, expression of *barnase* leads to degradation of the tapetum and inhibition of pollen maturation (Shivana et al., 1997). By using RNAi which is more convenient than using cytotoxic genes, Nawaz-Ul-Rehman et al., (2007) achieved male sterility by silencing tapetum specific TA-29 gene. We have investigated the potential for producing male-sterile lines by specific down-regulation of the anther-specific gene *Bcp1* of *Arabidopsis* by RNAi. *Bcp1* is an anther-specific gene expressed in both diploid tapetum and haploid microspores (Xu et al., 1995). RNA interference is a post-transcriptional gene silencing initiated by dsRNA which is cleaved by dicer in to siRNA of 21-23 nucleotides. The siRNA with the help of RISC and ATP target cognate mRNA sequence (Agrawal et al., 2003; Baulcomb, 2004; Hannon, 2002). In plant genetic engineering, dsRNA can

be formed with in plant cell by introducing a gene cloned in sense and antisense orientation in same vector (Wang and Waterhouse, 2000). siRNA are highly sequence specific and guide RISC to degrade homologous mRNA (Wang and Carmichael, 2004). The phenomenon of RNAi is being used to silence endogenous (Ogita et al., 2004) or viral genes (Vanitharani et al., 2003), to understand genome immunity (Plasterk, 2002) and in functional genomics (Holen et al., 2002). Recently, it has been demonstrated that expression of both sense and antisense fragments, separated by an intron, yields more efficient silencing than only antisense (Smith et al., 2000).

Here we show that targeting of the coding sequences of *Bcp1* using RNAi leads to male sterility, without detectable deleterious effects on other developmental processes. This strategy provides an alternative mechanism for producing the female lines required for hybrid production.

MATERIALS AND METHODS

Cloning gene constructs

Total DNA was isolated from tobacco (*Nicotiana tabacum* cv. Samsun) by the CTAB method (Doyle and Doyle, 1990). Primers used for amplification of *Bcp1* were BCP1sf (5'-CTTTCTCGA GTTCTGAGGTGTTGATTTT-3') and Bcp1sr (5'-ACTACCA TGGTATTGCTAAGGAAAGTTTAA-3') for the sense orientation and Bcp1af (5'-CTTTGGATCCTATTGCTAAGGAAA GTTTAA-3') and Bcp1ar (5'-ACTATCTAGATT TCTGAGGTGT TGTATTTT-3') for the anti-sense orientation. Restriction endonuclease recognition sequences (underlined) were included in the primers to allow cloning of the amplified fragments into appropriately digested vector. Purified amplification products were ligated into pTZ57R/ T (Fermentas) and the complete sequence of the "sense" clone was determined. Subsequently the separate sense and anti-sense fragments were transferred into the RNAi vector pFGC5941, as described in Figure 1.

Agrobacterium-mediated plant transformation

The gene construct Bcp1/pFGC was transformed in *Agrobacterium tumefaciens* strain LBA4404 by electroporation. The transformants

were confirmed through PCR using forward and reverse primers. Seeds of *Arabidopsis thaliana* Eco. Columbia were sterilized and placed in the glass jars having seed germination media (MSO having MS medium supplemented with B5 vitamins, 20 g/L sucrose, phytigel 6 g/L and pH 5.8) placing 20 seeds per jar. Jars were kept in the growth chamber at 22°C. Seedlings were grown for 3 weeks under long day and blue light conditions. After three weeks, plantlets were taken out of the jars one by one each time closing the lid to prevent desiccation of the plantlets. Largest leaves were cut off from the plantlet and directly transfer these to liquid C1 medium (MS medium supplemented with B5 vitamins, 20 g/L sucrose, pH 5.8) in a standard Petri dish (Greiner 94 x 16 mm). All leaves were collected from one jar in one dish with liquid C1. Leaves were cut longitudinally splitting the main vascular bundle along the length and subsequently transferred the leaf explants to solid C1 medium (MS medium supplemented with B5 vitamins, 30 g/L sucrose, phytigel 6 g/L, pH 5.8, NAA 0.1 mg/L and BAP 1 mg/L). Plates were sealed with parafilm and incubated for 2 days under white light conditions. The 50 ml culture of *A. tumefaciens* (strain LBA4404) containing Bcp1/pFGC construct was grown in LB supplemented with 50 ug/ml acetosyringon, 50 ug/ml kanamycin and 50 ug/ml rifampicin. After two or three days, leaf explants were transferred to this 50 ml of diluted bacterial suspension and incubated for 5 - 10 min with gentle shaking. Leaf explants were removed using forceps, briefly blot dried on filter paper and transferred leaf explants to fresh solid C1 medium plates. The leaf explants were pressed into the medium taking care that the cut edge with the vascular tissue was completely in contact with the medium. The plates were sealed with parafilm and incubated for two days in dark at 28°C. After two days, leaf explants were collected in a sterile glass beaker (250 ml size) for washing. 50 ml of liquid C1 medium supplemented with cefotaxime (250ug/ml) was added in a flask and shaken gently. Fresh liquid C1 medium supplemented with antibiotic was added and again shaken vigorously. Leaf discs were blotted in groups of 10 - 15 pieces to complete dryness. Leaf discs were transferred to regeneration media (solid C1 medium) at a density of 10 - 15 leaf discs per plate and were placed with upside up with cut edge in contact with the phytigel, nutrient and drug. The cefotaxime kills the *Agrobacterium* and glufosinate ammonium (5ug/ml) that reduced glutamine and increased ammonia levels in the plant tissues and thus only leaf discs having transgene were survived. This causes photosynthesis to stop and the plant dies within a few days. Plates were incubated at 22°C under white light conditions. Plates were sub-cultured every 3 weeks till the shoots were appeared and become large enough for Petri dishes. Shoots of regenerated *Arabidopsis* (*Eco. Columbia*) were cut from callus when at least 1 internode was formed. Then these shoots were transferred into rooting media (MS having half strength of MS salt mixture, 10 g/L sucrose, phytigel 4 g/L and pH 5.8). Regenerated plants of *A. thaliana* (*Eco. Columbia*) were transplanted into soil, after roots have been established. Plants were covered with plastic bags and were kept in growth chamber under controlled conditions of white light and day/night conditions (16 h light (3000 lux)/8 h night at 22°C.

Confirmation of transgenic plants

DNA from transgenic *Arabidopsis* plants was extracted by Doyle and Doyle CTAB method (Doyle and Doyle, 1990). Polymerase chain reaction (PCR) and Southern hybridization were employed for the confirmation of the putative transgenic plants through *bar* gene specific primers and probes. AlkPhos Direct™ non-radioactive kit of Amersham was used. In Southern hybridization, DNA was run on 1% agarose gel and transferred on to Hybond N⁺ nylon membrane under alkaline conditions. Probe was prepared from amplified PCR product of *bar* gene by denaturing the DNA (10 ng/μl, 10 μl) for 5 min, snap cool for 5 min and then reaction buffer (10 μl), labelling

reagent (2 μl), cross-linker (10 μl) was added and mix gently and incubated at 37°C for 30 min. Blot was hybridized with AlkPhos Direct™ hybridization buffer (add NaCl/blocking reagent) for 15 min at 55°C and probe was added and hybridized overnight at 55°C. Blot was washed two times with primary wash buffer (2 M Urea, 0.1% SDS, 50 mM Na phosphate, 1 mM MgCl₂ and 0.2% Blocking reagent) for 10 min at 55°C and then washed with secondary wash buffer (1 M Tris base and 2 M NaCl for 20X stock) for 5 min at room temperature. Detection reagent (30 - 40 μl/ cm²) was pipetted on to the blot and left for 2 - 5 min. Blot was developed after exposing the blot on x-ray film for 1 h.

RESULTS AND DISCUSSION

In the present study, male sterile lines of *Arabidopsis* (*Eco. Columbia*) were developed through stable transformation. The work is based on silencing of *Bcp1* gene which is 587bp in size and responsible for fertile pollen development and active in both diploid tapetum and haploid microspores. Perturbation of this gene in either tapetum or microspores prevents production of fertile pollen. Thus, mature anthers contain dead shriveled pollens (Xu et al., 1995). The group targeted 0.77 kb regulatory region of *Bcp1* gene through antisense but the efficiency of gene silencing through antisense technology was found to be 25 - 35%, which is not satisfactory. *Bcp1* gene can be divided into two parts based on sequence homology. The first one is non-conserved region, which is 163 bp in size while the other is conserved region (372 bp) which has 87% sequence homology with the female fertility gene *Bgp1*. So to avoid silencing of female part, only non-conserved region was selected to be targeted. Primers were designed with respect to dsRNA binary vector pFGC5941 which has two multiple cloning sites, *bar* gene for resistance to the herbicide Glufosinate Ammonium and 35S constitutive promoter within left and right borders (Kerschen et al., 2004). The two multiple cloning sites are flanked by an intron of 1.364 kb to increase the efficacy of PTGS (Chuang and Meyerowitz, 2000; Smith et al., 2000). 163 bp region of *Bcp1* gene was cloned in both sense and antisense orientations in pFGC5941 with the help of restriction sites present in the amplified PCR products. The PCR products used for cloning were sequenced to confirm intact gene. Cloning of gene in sense and antisense orientations in the same vector (Figure 1) is considered to produce dsRNA inverted repeat molecules which induce PTGS in plant cells. To obtain *Bcp1* transgenic *Arabidopsis* plants, the constructs were transformed into the *A. tumefaciens* strain LBA4404 by electroporation (Bio-Rad). The *Agrobacterium* culture was confirmed with PCR amplification. The construct was transformed in *Arabidopsis* (*Eco. Columbia*) using leaf disc method. Different transformation methods have been used for *Arabidopsis* transformation like floral dip method (Steven and Andrew, 1998), root transformation (Cardoza and Stewart, 1994) but the leaf disc method (Graaff and Hooykaas, 1998) was found to be more efficient for *Agrobacterium*-mediated plant transformation in *Arabidopsis*.

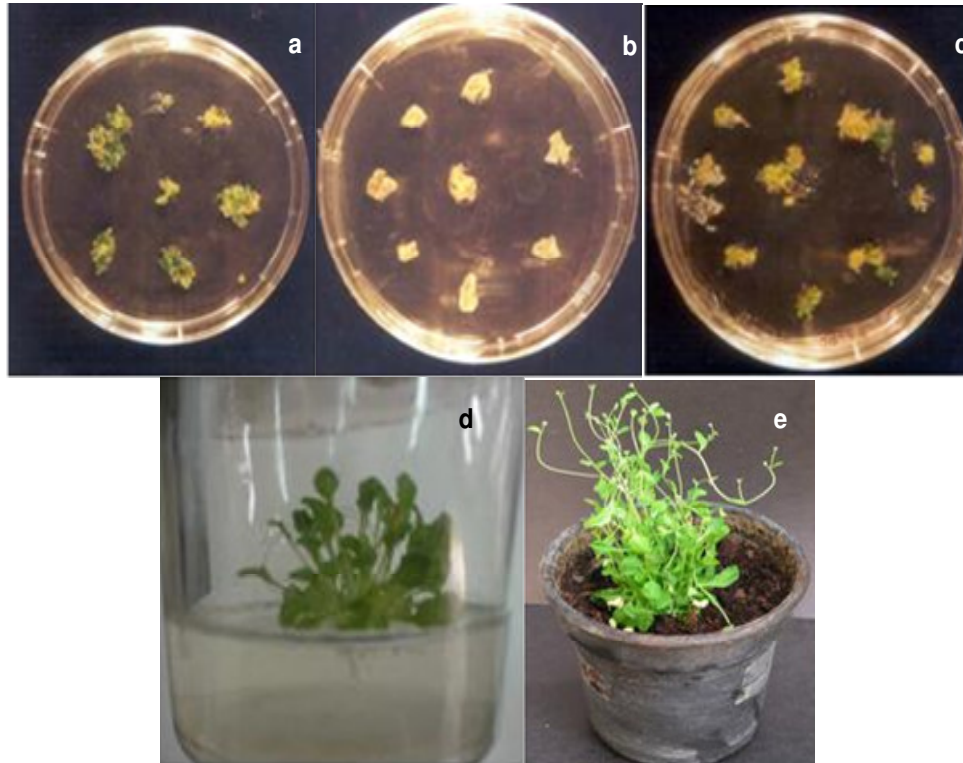


Figure 2. Transformation of *Bcp1/pFGC* in *Arabidopsis thaliana*. (a) Positive control (leaf discs of *Arabidopsis* (*Eco. Columbia*) on C_2 medium without antibiotic); (b) Negative control (leaf discs of *Arabidopsis* *Eco. Columbia* on C_2 medium with antibiotic); (c) Leaf discs after inoculation with *Agrobacterium* bearing *Bcp1/pFGC* construct on regeneration (C_2 medium); (d) Regenerated shoots were shifted on rooting media; (e) Transgenic *Arabidopsis* (*Eco. Columbia*) plant transplanted in soil showing development of flowers but no seed setting.

Three batches of explants were selected on herbicide glufosinate ammonium (Figure 2). The putative transgenic plants were confirmed through PCR using *bar* gene specific primers and Southern hybridization (Figure 3), which gives amplification along with positive and negative controls. Southern hybridization was performed using *bar* gene specific probe along with positive control and negative control and formation of sharp bands confirm the presence of transgene. It was deduced that transcribed mRNA of RNAi construct will result into a dsRNA with a hairpin loop and the resultant dsRNA triggered on the RNAi machinery. We found that the dsRNA interfere the *Bcp1* gene function in the transgenic *Arabidopsis* plants and consequently male sterile plants were obtained. About 49 out of 58 *Arabidopsis* lines transformed with RNAi construct containing *Bcp1* sequences were male sterile (Table 1). Transgenic plants were phenotypically indistinguishable from non-transgenic plants except for aborted or malfunctioning pollen grains. These transgenic plants were used as female plants for crossing with wild-type non-transgenic plants to produce hybrid seeds. In these plants there were non-viable pollens to fertilize their own female partners on self-pollination. By using the same strategy, it will be easy to produce hybrid seeds on

large scale, for higher yield and quality of the crop.

The use of RNAi for hybrid seed development through male sterile pollens is very important. Due to sequence homology in different crop species, RNAi of *Bcp1* gene can be used for hybrid seed development. For this purpose, another study was made to observe conservation of *Bcp1* gene in high value crops like cotton, squash, chillies and tomato. For this purpose, using *Bcp1* specific primers, about 372 bp region of in chillies and tomato was also amplified which shows the diversity of *Bcp1* gene in other crops.

From this study, it is concluded that silencing of *Bcp1* through RNAi is responsible for male sterility in *Arabidopsis*. A sequence homology in other crop species of this gene also shows that this method can be employed to obtain hybrid seeds of commercially high valued crops.

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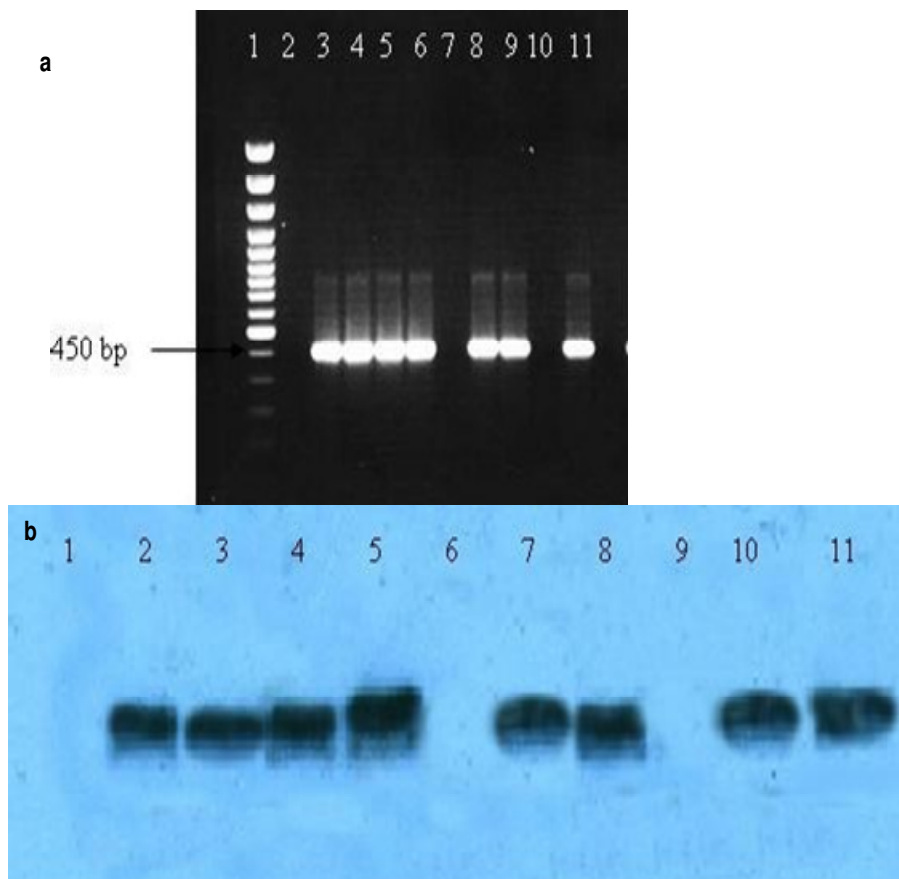


Figure 3. a) PCR analysis of selected transgenic plants with *bar* specific primers (Lane 1, Marker; Lane 2, Negative control; Lane 3, Positive control (pFGC5941); Lanes 4,5,6,8,9, and 11, PCR products amplified from DNA extracted from transgenic *Arabidopsis* plants. b) Southern hybridization of selected transgenic plants using *bar* specific probe (Lane 1, Negative control; Lane 2, Positive control (pFGC5941); Lanes 3,4,5,7,8,10, and 11, Strong signals showing the presence of transgene in DNA extracted from transgenic *Arabidopsis* plants.

Table 1. Transformation of *Arabidopsis thaliana* (*Eco. Columbia*) with *Agrobacterium tumefaciens* (LBA4404) bearing plasmid containing 163 bp region of male fertility (*Bcp1*) gene under 35S promoter.

Batch no.	Leaf discs treated (no.)	Explants regenerated on selected media*	Plants which developed roots (no.)	Positive plants for <i>bar</i> genes**	Number of sterile plants	Seed production upon cross-pollination
1	71	49	28	20	17/ 20	Yes
2	84	51	22	17	12/ 17	Yes
3	60	43	27	21	19/ 21	Yes

* Containing 10mg/l BASTA®.

** Analysed by PCR using *bar* gene specific pair of primers.

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