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A new cytoplasmic nuclear male-sterility system derived from cultivated pigeonpea cytoplasm

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Summary

Cytoplasmic male-sterility (CMS) in pigeonpea has been reported when some wild relatives of pigeonpea were crossed as the female parent with cultivated types as the male parent. In this paper we report a new source of CMS developed by using the cultivated pigeonpea as the female parent and one of its wild relative *Cajanus acutifolius* as the pollen donor. This is the first report in pigeonpea where CMS has been developed using the cytoplasm of cultivated pigeonpea. Several pure line cultivars of pigeonpea restored pollen fertility whereas cv. HPL 24 partially maintained male-sterility. The wild species *C. acutifolius* used as one of the parents, maintained complete sterility. Cytological analysis revealed that both in male-sterile as well as the fertile floral buds, meiosis proceeded normally till the tetrad stage. However in the male-sterile genotypes during the formation of tetrads, the pollen mother cell (PMC) wall did not dissolve to release the tetrads unlike in the fertile genotypes and this major event was found to be responsible for male-sterility.

Introduction

In pigeonpea (*Cajanus cajan* (L.) Millsp.), cytoplasmic nuclear male-sterility (CMS) has been reported and it arises as a consequence of crossing cultivated types as male parent and their wild relatives as female parent. So far, four wild relatives of pigeonpea have been successfully used to breed CMS systems for developing commercial hybrid pigeonpea breeding technology. These are; *Cajanus sericeus* (Benth. ex Bak.) van der Maesen comb. nov. (Saxena et al., 2002), *C. scarabaeoides* (L.) Thou. (Tikka et al., 1997; Saxena & Kumar, 2003), *C. volublis* Blanco (Wanjari et al., 2001), and *C. cajanifolius* (Haines) van der Maesen comb. nov. (Saxena, 2004). In the present investigation, we report the development of a new CMS system which has been derived from crosses involving pigeonpea cultivars as female parent and a wild species, *C. acutifolius* (F.v. Muell.) van der Maesen comb. nov. as male parent. The unique feature of this system is that, unlike previous reports, the cytoplasm of cultivated pigeonpea and nuclear genes from its wild relative, *C. acutifolius* have been combined to produce a new CMS genotype.

Materials and methods

Cajanus acutifolius (F.v. Muell.) van der Maesen comb. nov. is a wild species and is endemic to Australia. Accession ICPW 15613 (ICC 12) was collected from northern territory, 16E Moline, Australia while ICPW 15605 (ICC 4) was collected from left bank of Walsh, Picnic Hole, Australia. Both the accessions have resistance to *Helicoverpa armigera*, the menacing pod borer of pigeonpea (ICRISAT, 2003).

Both the *C. acutifolius* accessions and six pigeonpea cultivars ICP 1140, ICPL 2, ICPL 85010, ICPL 85030, ICPL 88014, and ICPL 88034 were grown in a glasshouse in 10 in. × 10 in. plastic pots filled with sterilized Alfisol mixture (4 parts Alfisols: 2 parts

farm yard manure: 1 part sand). Hand emasculations of pigeonpea floral buds were followed by hand pollinations using *C. acutifolius* pollen grains each day before 10 am. To retain the pollinated buds on the mother plant, Gibberellic acid (50 mg/l) was applied to the base of the pistil for three consecutive days after pollination. Mature pods from these crosses were harvested and seeds collected. The F_1 progenies of all the crosses were also grown in a glasshouse and the individual plants were observed for male-sterility. Of these intra-cross variation for pollen-sterility was studied in only four F_1 crosses using the standard aceto-carmine test (Kaul & Singh, 1969). The F_1 male-sterile plants were crossed to five unrelated pigeonpea cultivars and the parental wild species accession ICPW 15613 to identify early generation male-sterility maintainers and restorers.

For cytological study of meiocytes, immature floral buds collected from male-sterile and male-fertile plants were fixed in Carnoy's II solution (acetic acid 1: chloroform 3: and ethanol 6) for 24 h at 4 °C and transferred to Carnoy's I solution (acetic acid 1 : ethanol 3). Meiocytes were squashed and stained with 2% aceto-carmine solution and well spread meiotic preparations were studied. For pollen fertility study, anthers were harvested on the day of flower opening and squashed in 2% aceto-carmine and the pollen fertility was estimated by counting the brightly stained pollen grains under the microscope.

Results and discussion

Evaluation of inter-specific hybrid progenies

The pod set in the crosses involving six pigeonpea cultivars and two accessions of *C. acutifolius* varied from 8 to 68%. There was no major difference for pod set between the groups of crosses involving the two accessions of *C. acutifolius*. The frequency of male-sterile and fertile plants in F_1 generation varied considerably from cross to cross (Table 1). The cross combinations with *C. acutifolius* accession ICPW 15613, on average, produced 18.3% male-sterile plants while it was 12.1% when crossed with accession ICPW 15605. The highest frequency (30.5%) of male-sterile plants was observed when pigeonpea cultivar ICPL 2 was crossed with ICPW 15613. On the contrary, the crosses made on ICPL 88034 produced only male-fertile F_1 progenies. The crosses involving pigeonpea line ICP 1140 produced contrasting results, with seven (17%) male-sterile plants recorded in its cross with ICPW 15613 while the progeny of cross ICP 1140 × ICPW 15605 yielded no male-sterile plant.

The detailed pollen fertility study was carried out within four F_1 progenies. In general there was a tendency to produce sterile plants as none of the F_1 plants was found to be fully male-fertile. The variation for pollen sterility was high (Table 2) and it ranged between 40–100%. Majority of the F_1 plants were in the

Table 1. Percent pod set in 12 inter-specific crosses and frequency of male-sterile plants observed in F_1 generation

Cross	Pollinations	Pod set (%)	Number of F_1 plants			Male-sterility (%)
			Total	Sterile	Fertile	
ICP 1140 × ICPW 15613	139	54	48	7	41	14.6
ICPL 2 × ICPW 15613	136	68	59	18	41	30.5
ICPL 85010 × ICPW 15613	133	32	45	6	39	13.3
ICPL 85030 × ICPW 15613	88	8	8	2	6	25.0
ICPL 88014 × ICPW 15613	52	38	34	9	25	26.5
ICPL 88034 × ICPW 15613	100	20	24	0	24	0.0
Total/mean	648	36.7	218	42	176	18.3
ICP 1140 × ICPW 15605	166	59	59	0	59	0.0
ICPL 2 × ICPW 15605	86	27	27	2	25	7.4
ICPL 85010 × ICPW 15605	107	27	23	4	19	17.4
ICPL 85030 × ICPW 15605	136	56	49	10	39	20.4
ICPL 88014 × ICPW 15605	80	22	22	6	16	27.3
ICPL 88034 × ICPW 15605	203	44	39	0	39	0.0
Total/mean	778	39.2	219	22	197	12.1

Table 2. Variation for pollen sterility in the F_1 hybrids from crosses between four pigeonpea cultivars and wild species *C. acutifolius* ICPW 15613

Pollen sterility (%)	Pigeonpea cultivars			
	ICPL 85010	ICPL 88014	ICPL 2	ICPL 88034
0–9	0	0	0	0
10–19	0	0	0	0
20–29	0	0	0	0
30–39	0	0	0	0
40–49	0	0	4	0
50–59	3	0	7	1
60–69	12	3	8	3
70–79	7	3	11	11
80–89	4	6	4	7
90–96	13	13	7	2
97–100	6	9	18	0

70–100% male-sterility range. However, for this study the plants with pollen-sterility of 97% or more were considered as male-sterile. This is in contrast to the CMS system developed using *C. scarabaeoides* where majority of the F_1 hybrids were highly male-fertile (Saxena & Kumar, 2003). The male-sterility observed in the present case when cultivated pigeonpea was used as a female parent, appears to be controlled by factors located in the pigeonpea cytoplasm. This is exemplified by the fact that when pigeonpea cultivar was used as the male parent, all the F_1 plants were male-fertile (Mallikarjuna & Saxena, 2002). On the contrary, in the present case a considerable proportion (0–31%) of F_1 plants was male-sterile (Table 1). These observations suggest that both the fertility restoration and male-sterility maintenance systems operating within the hybrid progenies were incomplete and it may be due to the presence of some major differential inter-genomic or cytoplasmic-genomic interactions. Such interactions usually arise due to complex phenomenon like complementation, inhibition, epistasis, or specific environmental factors (Kaul, 1988). Abdalla & Hermsen (1972) on the other hand, attributed the polymorphism, arising due to differential genes as the primary cause of inconsistent expression of male-sterility in such wide crosses.

Morphological and cytological studies

A close perusal of male-sterile and fertile segregants within any progeny showed close morphological similarities but they were distinct with respect to their anther characteristics. At anthesis, the anther filaments of the total male-sterile plants grew normally but in comparison to their male-fertile counterparts, the anther lobes in the male-sterile flowers were distinctly different with pale-white color, shrunken growth, and devoid of pollen grains. Cross section of the anther showed the collapse of the anther (Figure 1e).

The cytological studies of male-sterile floral buds showed normal meiosis with 11 chromosomes at each pole at anaphase and visible abnormalities were rare. The pollen mother cells at different stages of meiosis did not show abnormality that could render male-sterility in the plants. The process of meiosis proceeded normally till the tetrad stage (Figure 1a and b). However, during growth of the tetrads, unlike in fertile plants, the normal event of dissolution of PMC wall was inhibited (Figure 1c and d) and the tetrads remained enclosed within the persistent tetrad wall. Consequently, the further growth of tetrads ceased and they lost their cell contents leading to premature abortion of the pollen grains; but they remained together till the flowers opened. Hence, the degeneration of pollen grains at the late tetrad stage was identified as the prime cause for the manifestation of the male-sterility system.

Breakdown of microsporogenesis due to persistent tapetum, was also observed in the genetic male-sterile pigeonpea reported by Reddy et al. (1978). It was also observed that the genes causing male-sterility did not affect female-fertility. The fertility restoration factor(s) located in the nuclear genome are dominant in nature, since majority of the progenies obtained as a result of crossing different pigeonpea genotypes yielded predominantly male-fertile plants.

Maintenance of CMS system

For identifying effective maintainers of this male-sterility system, six male-sterile F_1 plants were used as female parents and crossed with diverse pigeonpea cultivars and *C. acutifolius* accession ICPW 15613. The resulting 24 F_1 crosses were evaluated under field conditions during 2003 rainy season. Of these, in 20 crosses no male-sterile plant was observed (Table 3) and, therefore pigeonpea lines MN 5, ICPL 88039, ICPL 129, and ICPL 86012 were classified as perfect male-fertility restorers. The progenies involving HPL 24 as pollen parent exhibited a differential response. In two crosses (MS sel. 6 × HPL 24 and MS sel. 7 × HPL 24), out of 32 plants grown three were complete male-sterile and

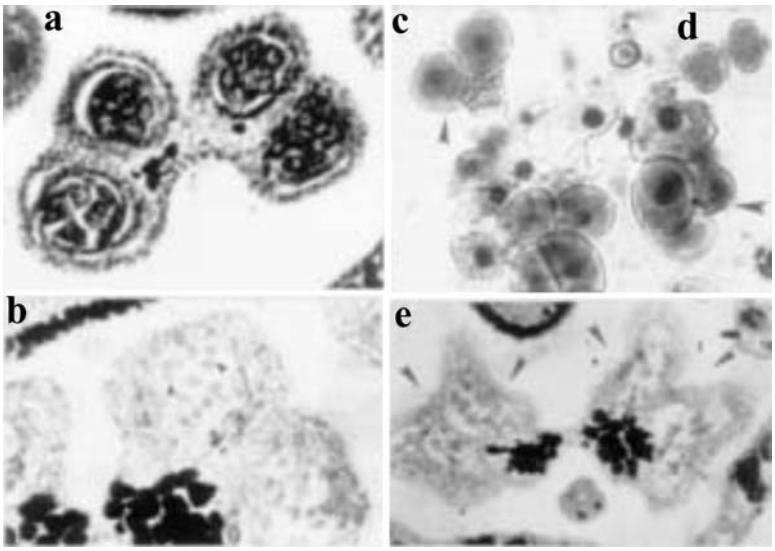

Figure 1. Cytoplasmic male sterility in the cross *C. cajan* × *C. acutifolius*. (a) Cross section of the anther showing four globular anther locules with pollen mother cells at the center. (b) An anther locule showing the formation of tetrads. (c) Anther squash showing diads and tetrads enclosed in thick callose wall. Inset: Persistant tetrads with thick callose walls and have not separated to form pollen grains (d). (d) Cross section of a pale white degenerating anther showing shrunken and empty anther locules.

the rest were male fertile, suggesting partial restoration of male fertility (Table 3).

In two back-crosses, where the male-sterile plants were crossed with *C. acutifolius* accession ICPW 15613 as pollen parent, all the 73 plants were male-sterile, exhibiting the ability of this wild relative of pigeonpea to effectively maintain male sterility system (Table 3). From this data it is concluded that the male-sterility maintaining factors were present in *C. acutifolius*, however when crossed with pigeonpea cultivar ICPL 129 or ICPL 86012 as male parents, it produced only male-fertile plants. Therefore it may be inferred that the fertility restoration nuclear genes are present in cultivated pigeonpea. The crosses involving pigeonpea cultivar HPL 24 as one of the parents yielded both male-fertile as well as male-sterile progenies. Since HPL 24 was also developed from an interspecific cross (Saxena et al., 2002) involving another wild species (*C. sericeus*), it appears that besides *C. acutifolius*, some other wild relatives of pigeonpea are also capable of maintaining this CMS system. However further studies are necessary to confirm this hypothesis.

General discussion

The CMS systems have been used for developing commercial hybrids in a number of cereal and horticultural crops with many fold increases in yield and stability. In legumes, this system could not be exploited for enhancing the productivity, primarily due to their cleistogamous nature of flowers that do not permit economical mass pollen transfer, necessary for large-scale hybrid seed production (Saxena et al., 1997). Pigeonpea, however, is an exception where insect-mediated natural out-crossing up to 70% has been reported (Saxena et al., 1990). The availability of CMS in this crop (Saxena, 2004; Saxena & Kumar, 2003; Wanjari et al., 2001; Tikka et al., 1997) has now opened up the possibilities of developing commercial hybrids in this legume crop. Experimental hybrids developed by using other CMS lines have demonstrated a yield advantage of over 25% Saxena (2004). It is a known fact that for a long-term commercially viable hybrid breeding programs both genetic as well as cytoplasmic diversity are essential. The present source of CMS, although not yet ready for direct use in the hybrid breeding programs, offers good opportunities for such diversification with some well planned breeding schemes.

For utilizing this new CMS source in a practical hybrid pigeonpea breeding programs, the male-sterility maintainers need to be identified among the cultivated types and this can be achieved by crossing a number of genetically diverse pigeonpea lines with the male-sterile genotypes which should be followed by back-crossing and selection. This cytoplasmic nuclear male

Table 3. Segregation for male-sterility in crosses involving CMS selections and diverse pigeonpea cultivars

Cross	Male sterile plant no.	Pollinator	Total	Fertile	Sterile	Remarks
ICPL 2 × ICPW 15613	2	MN5	13	13	0	R
		ICPL 88039	18	18	0	R
		HPL 24	7	7	0	R
		ICPL 129	8	8	0	R
ICPL 85010 × ICPW 15613	6	MN5	18	18	0	R
		ICPL 88039	8	8	0	R
		HPL 24	20	18	2	R+M
		ICPL 129	20	20	0	R
ICPL 85010 × ICPW 15613	7	MN5	9	9	0	R
		ICPL 88039	13	13	0	R
		HPL 24	12	11	1	R+M
		ICPL 129	6	6	0	R
ICPL 2 × ICPW 15613	11	MN5	6	6	0	R
		ICPL 88039	7	7	0	R
		HPL 24	13	13	0	R
		ICPL 129	11	11	0	R
		ICPL 86012	10	10	0	R
ICPL 2 × ICPW 15613	17	ICPL 86012	12	12	0	R
		ICPW 15613*	20	0	20	M
ICPL 2 × ICPW 15613	18	ICPL 88039	7	7	0	R
		HPL 24	13	13	0	R
		ICPL 86012	10	10	0	R
		ICPL 129	38	38	0	R
		ICPW 15613*	53	0	53	M

**C. acutifolius*.
R = Male-fertility restorer.
M = Male-sterility maintainer.

sterility is unique since it the first MS system which has been developed using the cytoplasm of cultivated pigeonpea.

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