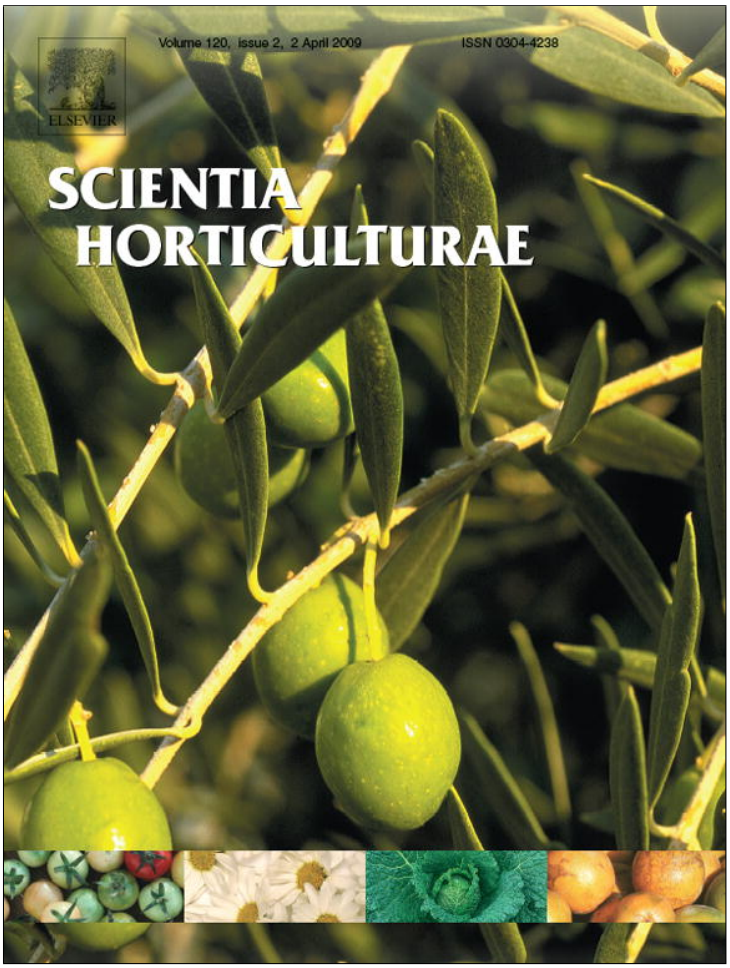

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Validation of SCAR markers, diversity analysis of male sterile (S-) cytoplasms and isolation of an alloplasmic S-cytoplasm in *Capsicum*

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ABSTRACTS

Cytoplasmic male sterility (CMS) is increasingly being utilized for hybrid seed production of hot pepper (*Capsicum annuum*). Two CMS specific sequenced characterized amplified regions (SCARs), viz., $atp6_{607}$ and $coxII_{708}$ developed elsewhere were validated in an array of genotypes possessing male sterile (S-) and normal/male fertile (N-) cytoplasms and their feasible uses in CMS based pepper hybrid breeding have been elaborated. A set of eight maintainer and restorer inbreds were crossed on four CMS lines possessing two independently isolated and commercially utilized S-cytoplasms (Peterson's and Reddy's). Based on fertility restoration/maintenance reaction of 32 resulted F_1s and on the presence of two SCARs ($atp6_{607}$ and $coxII_{708}$) in both the S-cytoplasms, it has been concluded that although two S-cytoplasms were isolated and commercially utilized independently, they are genetically same or similar. Through inter-specific hybridization between *C. chacoense* and *C. annuum*, a new alloplasmic S-cytoplasm in the genus *Capsicum* has been isolated and the CMS F_1 has been advanced to CMS BC_1F_1.

1. Introduction

Cytoplasmic male sterility (CMS) has been widely exploited for hybrid seed production of a number of agronomic and horticultural crops (Havey, 2007) including hot pepper (Kumar et al., 2007a). First CMS hot pepper (*Capsicum* spp.) plant was isolated in United States from an Indian introduction (Peterson, 1958) and this male sterile (S-) cytoplasm has so far been most commonly utilized for commercial hybrid development in South Korea, China and India (Zhang et al., 2000; Kim and Kim, 2005; Kumar et al., 2007a). A major fertility restoration locus (*Rf*) is known to restore fertility of this S-cytoplasm, but fertility restoration is also influenced by temperature, quantitative trait loci (QTLs)/modifiers (Shifriss, 1997; Wang et al., 2004) and an additional partial restoration (*pr*) locus (Lee et al., 2008a). This *pr* locus is suspected to be either tightly linked to *Rf* locus or is third allele of *Rf* locus (Lee et al., 2008b). More recently, another commercially utilized hot pepper S-cytoplasm of independent origin has been reported from India (Reddy et al., 2002). However, the genetic relationship between these two independently isolated S-cytoplasms (designated herein as Peterson's and Reddy's) is unclear. Nevertheless, like CMS specific open reading frames (ORFs) identified in several crops (Chase, 2006), the hot pepper mitochondrial gene causing CMS phenotype (Peterson's S-cytoplasm) has been characterized as *orf456* (Kim et al., 2007). Kim and Kim (2005) also developed two CMS (S-cytoplasm) associated sequenced characterized amplified region (SCAR) markers for Peterson's S-cytoplasm. The effective deployment of these SCAR markers for marker aided selection (MAS), however, needs its validation using an array of diverse genotypes possessing S-cytoplasm and normal male fertile (N-) cytoplasm. For the sustainability of CMS-based hybrid cultivars, it is imperative to avoid monopolistic use of single source of S-cytoplasm, hence diversification of S-cytoplasm has always been a major strategy. Alloplasmic situation resulting from association between nucleus and cytoplasm of two different species often lead to CMS phenotype and to achieve this phenotype in several crops, inter-specific hybridization has been successfully used (Kaul, 1988). We conducted a series of field and laboratory experiments to: (i) test validity of already reported two CMS specific SCAR markers, (ii) study the genetic relationship between two independently isolated S-cytoplasms and (iii) isolate a new S-cytoplasm derived from a wild *Capsicum* species. The results pertaining to all these objectives are presented and discussed in this communication.

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Table 1
Validation of SCARs in male sterile cytoplasm (S-) and normal cytoplasm (N-) sources.

SN	Accession name	Symbol assigned	PCR amplification[a]		
			SCAR $atp6_{607}$	SCAR $coxII_{708}$	Control $coxII_{1541}$
Peterson's S-cytoplasm in CMS[b]					
1	CCA-4261	A1	+	+	+
2	CCA-4757	A2	+	+	+
3	CCA-4758	A3	+	+	+
4	CCA-4759	A4	+	+	+
5	CCA-4916	A5	+	+	+
6	CCA-4917	A6	+	+	+
7	CCA-6475	A8	+	+	+
8	CCA-6476	A9	+	+	+
Peterson's S-cytoplasm in 40 F_1 plants[b,c]					
9	A2 x Pusa Jwala	CCH-4	+	+	+
Reddy's S-cytoplasm in CMS[d]					
10	IC-395318	A7	+	+	+
Alloplasmic S-cytoplasm in three BC_1F_1 plants[c]					
11	BC_1F_1s	A10	–	–	+
N-cytoplasm in maintainer counterparts of CMS					
12	PBC-534	B1	–	–	+
13	PBC-380	B2	–	–	+
14	9907-9611	B3	–	–	+
15	PBC-483	B4	–	–	+
16	PBC-362	B5	–	–	+
17	PBC-292	B6	–	–	+
18	IC-395319	B7	–	–	+
19	9849-5765	B8	–	–	+
20	PBC-378-2	B9	–	–	+
21	Kashi Anmol	B10	–	–	+

[a] Presence (+) and absence (–) of marker.
[b] Isolated in United States from an Indian introduction (Peterson, 1958).
[c] Developed during this study.
[d] Independently isolated and reported from India (Reddy et al., 2002).

2. Materials and methods

2.1. Sources of male sterile (S-) and normal (N-) cytoplasms

A total of nine pairs of CMS (S-cytoplasm) and their maintainer (N-cytoplasm) lines (designated as A1/B1 to A9/B9; Table 1) were initially utilized for SCAR markers validation study. The A7 had an independently isolated S-cytoplasm (designated as Reddy's) reported from India (Reddy et al., 2002) and the remaining eight CMS lines had first (Peterson, 1958) and commonly used S-cytoplasm (designated as Peterson's). These eight CMS and their maintainer counterparts were obtained from AVRDC-The World Vegetable Center (Liu and Gniffke, 2004) and maintained as active collections (Kumar et al., 2007a). In addition, three BC_1F_1 plants (A10) with a putative new S-cytoplasm of *C. chacoense* and 40 plants (S-cytoplasm) of a CMS (A2) based potential hybrid (CCH-4) developed during this study were also used for SCARs validation.

2.2. Diversity study of two S-cytoplasms

The genetic relationship between two independently isolated S-cytoplasms (i.e. Peterson's and Reddy's; Table 1) was studied based on fertility restoration/maintenance reaction of a given set of restorer/maintainer against both the S-cytoplasms. For this purpose, a set of three known maintainers (B1, B2, and B3) and four known restorers of Peterson's S-cytoplasm (Kumar et al., 2007a; Table 2) were crossed on A1, A2, A3 (Peterson's S-cytoplasm) and A7 (Reddy's S-cytoplasm). All these four CMS lines were also crossed with B7 line, the maintainer of Reddy's S-cytoplasm. Thus a total of 32 CMS based test-crosses involving eight male parents (four for each of maintainer and restorer) and four seed parents (CMS) were developed (2005–2006), evaluated under open field conditions (2006–2007) and data were tabulated for male fertility reaction (Table 2). Male fertility of 40 plants of each cross and 10 plants of parents were although examined using three methods (Kumar et al., 2007a), to obtain more reliable data under field conditions, results were finally presented based on the third method, i.e. ability (male fertile) or non-ability (male sterile) of a plant to produce selfed fruit with seeds.

2.3. Inter-specific F_1 and advancement

An inter-specific cross between a wild species, namely *C. chacoense* (as seed parent) and a cultivated species, namely *C. annuum* (cv. Kashi Anmol) was developed through conventional hybridization (2005–2006), putative F_1 plants were raised (2006–2007) under open field conditions and examined for male fertility expression (Kumar et al., 2007b). Five randomly selected flowers of all the 13 putative inter-specific F_1 plants were observed and number of stamens per flower scored. For hybridity confirmation, 100 random primers of OPO, OPP, OPS, OPY, OPZ series (Integrated DNA Technology, United States) were screened to identify primer pair/s producing male parent specific fragment. One of the F_1 plants was crossed with recurrent parent Kashi Anmol (*C. annuum*) to develop (2006–2007) and raise (2007–2008) BC_1F_1 plants for subsequent backcrossing and generation advancement.

2.4. DNA isolation

Total DNA from parents and inter-specific F_1 plants, three inter-specific BC_1F_1 plants, single plant of nine CMS and maintainer pairs and 40 morphologically confirmed hybrid plants of CCH-4 were isolated through polyvinylpyrrolidone (PVP) method (Kim et al., 1997). The extraction buffer consisted of 100 mM NaCl, 50 mM EDTA, 100 mM Tris–HCl (pH 8) and β-mercaptoethanol. Young leaf samples (1 g) were ground in liquid nitrogen and 3 ml of extraction buffer and 6% of PVP were added in ground samples followed by its incubation on ice for 5 min. Thereafter, 2% sodium dodecyl sulphate was added and the mixtures were again incubated for 15 min at 65 °C. The mixture was then centrifuged for 15 min at 14,000 rpm and supernatants were taken and mixed with 1/10 potassium acetate. Phenol:chlorophorm:isoamyl alcohol (25:24:1) treatment was given to the final supernatants and finally DNAs were precipitated with isopropanol. The DNAs were washed with 70% ethanol and stored in distilled water.

2.5. Synthesis of SCARs primers

Kim and Kim (2005) developed two CMS specific SCAR primer pairs based on the differences between the mitochondrial nucleotide sequences at the 3′ region of *atp6* and *coxII* of the N- and S-cytoplasms (Peterson's). The reported sequence data were used to commercially synthesize (IDT Inc., India) three sets of following primer pairs:

atp6 SCAR
5′-AGTCCACTTGAACAATTTGAAATAATC-3′ (F)
5′-GTTCCGTACTTTACTTACGAGC-3′ (R)
coxII SCAR
5′-GTCGGGAGAACTACCTAACTA-3′ (F)
5′-GGCTACCTAGTGATTTACAAGCA-3′ (R)
coxII Control
5′-GATGCAGCGGAACCATGGCAATTA-3′ (F)
5′-ACTGCACTGACCATAGTAAACTCC-3′ (R)

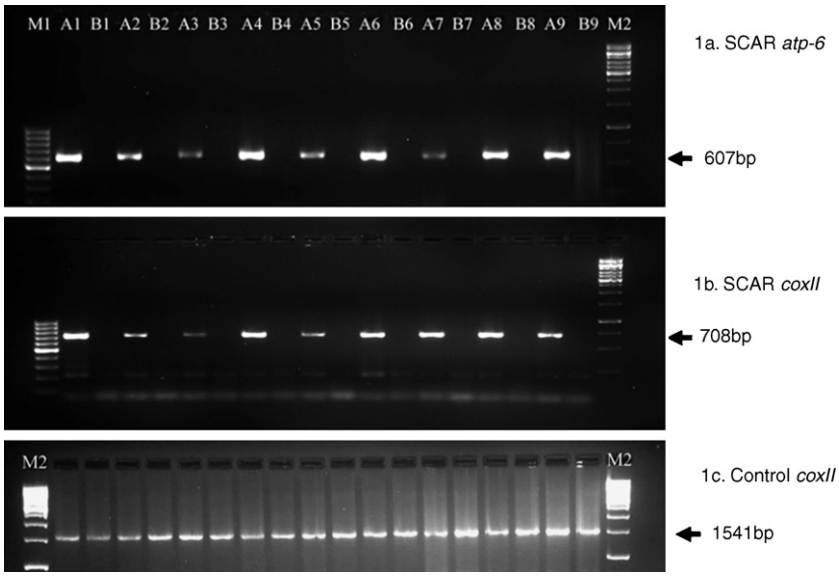

Fig. 1. Results of PCR amplification of nine CMS (A) lines (S-cytoplasm) and maintainer (B) lines (N-cytoplasm) using *atp6* SCAR primer (a), *coxII* SCAR primer (b) and *coxII* control primer (c). The size of PCR product is indicated on the right and details of A and B lines are given in Table 1; M1 and M2 are 100 bp and 1 kb markers, respectively.

2.6. PCR for SCARs

For polymerase chain reaction (PCR), master mix consisted of 10 mM of dNTPs mix, 50 mM $MgCl_2$, 0.4 U Taq polymerase (5 U/ml) with the supplied polymerase buffer (Bangalore Genei Pvt. Ltd., India), 10 μM of each primer and 50 ng genomic DNA. Predenaturation was performed at 94 °C for 4 min. The amplification profile consisted of 40 cycles of denaturation at 94 °C for 60 s, primer annealing at 55 °C for 60 s, primer extension at 72 °C for 60 s and DNA synthesis with final extension at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gel, stained with ethidium bromide, visualized and analyzed using Alpha ImagerTM 3400 Gel Documentation System (Alpha Infotech, United States).

3. Results

3.1. Validation of CMS specific SCAR markers

In this analysis, first we applied two CMS specific SCAR markers (Kim and Kim, 2005) in two independently isolated S-cytoplasms using nine pairs of CMS (S-cytoplasm) and their maintainer (N-cytoplasm) lines (Table 1). The *atp6* derived SCAR primer pair produced a 607 bp fragment in all the nine CMS lines possessing independently isolated two S-cytoplasms, i.e. Peterson's and Reddy's S-cytoplasms (Table 1). This 607 bp CMS (S-cytoplasm) specific fragment was absent in all of their maintainer counterparts, i.e. in N-cytoplasm (Table 1, Fig. 1a). Likewise, with *coxII* derived SCAR primer, S-cytoplasm specific 708 bp fragment was amplified in all the nine CMS (S-) lines and there was no amplification in maintainers (N-) lines (Table 1, Fig. 1b). In addition, both these SCAR markers were present in CMS (A2) based 40 hybrid (CCH-4) plants possessing S-cytoplasm (Peterson's). Both these SCARs, however, were absent in three CMS BC_1F_1 plants possessing putative S-cytoplasm derived from inter-specific cross (*C. chacoense* × *C. annuum*) and in backcross maintainer parent, i.e. Kashi Anmol (Table 1, Fig. 2). As expected, *coxII* control primer developed from coding region (highly conserved) of *coxII* (Kim and Kim, 2005) were able to amplify 1541 bp fragment in all the lines including in newly developed three BC_1F_1 CMS plants irrespective of S- or N-cytoplasms (Table 1, Figs. 1 and 2).

3.2. Diversity between two male sterile (S-) cytoplasms

In order to study the genetic relationship between two S-cytoplasms, a total of 32 CMS based F_1s using a set of known restorer and maintainer lines (as pollen parents) were successfully developed, raised and evaluated for male fertility expression (restoration/maintainer ability of involved male parents). It is evident from the fertility restoration data of F_1s (Table 2) that three known maintainers (B1, B2, and B3) of Peterson's S-cytoplasm could be able to maintain male sterility of Reddy's S-cytoplasm (A7). Likewise, all four restorer inbred lines (Pusa Jwala, Japani Longi, Kashi Anmol, Pant C-1) of Peterson's S-cytoplasm also restored fertility of Reddy's S-cytoplasm (Table 2). This indicated the same fertility restoration locus for both the commercially utilized S-cytoplasms. However, B1 for A3, B2 for A3 and B3 for A1 and A2 (maintainers and CMS of Peterson's S-cytoplasm) were unable to maintain sterility of Peterson's S-cytopalsm in all the plants, as among 40 F_1 plants derived from them, only respectively 52.2%, 47.4%, 21.1% and 20.0% were male sterile and the remaining proportions of plants were male fertile (Table 2).

Table 2
Fertility restoration reactions of 32 F_1s derived from a set of known restorer and maintainer lines (Kumar et al., 2007a) of two independently isolated male sterile (S-) cytoplasms.

	S-cytoplasm type			
The CMS lines →	Peterson's (P)			Reddy's (R)
Maintainer for (cytoplasm type)↓	A1	A2	A3	A7
B1 (P)	—	—	– (52.2)	—
B2 (P)	—	—	– (47.4)	—
B3 (P)	– (21.1)	—(20.0)	—	—
B7 (R)	—	—	—	—
Known restorer for (P)				
Pusa Jawla	++++	++++	++++	++++
Japani Longi	++++	++++	++++	++++
Kashi Anmol	++++	++++	++++	++++
Pant C1	++++	++++	++++	++++

++++ = all 40 F_1 plants were male fertile (stable fertility restoration); — = all 40 F_1 plants were male sterile (stable sterility maintenance); –(%) = certain proportion of plants were male sterile (unstable sterility maintenance).

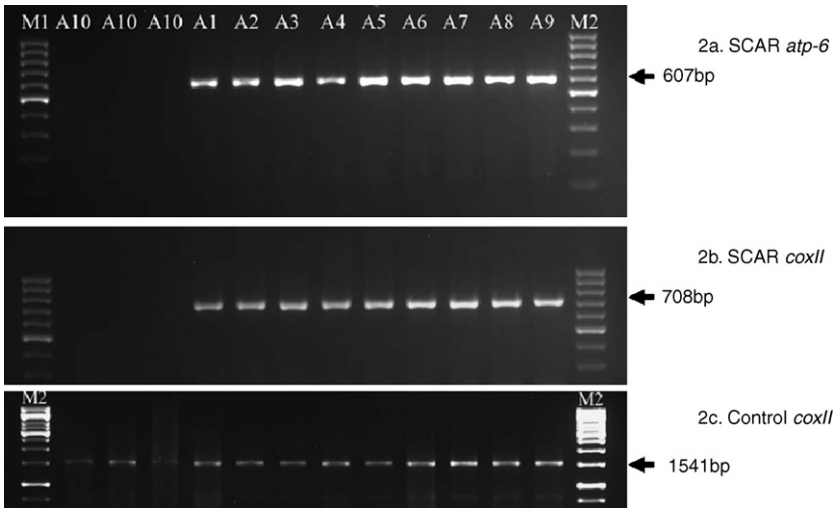

Fig. 2. Results of PCR amplification of three BC_1F_1s (A10) with alloplasmic S-cytoplasm and nine CMS (A1–A9) lines (S-cytoplasm) using *atp6* SCAR primer (a), *coxII* SCAR primer (b) and *coxII* control primer (c). The size of PCR product is indicated on the right and details of A lines are given in Table 1; M1 and M2 lanes are 100 bp and 1 kb markers, respectively.

3.3. Promising hybrid

Among the 32 CMS based crosses, one cross, viz., A2 × Pusa Jwala (CCH-4) was identified to be promising for commercialization based on general performances on profuse and early fruiting. First picking of green fruits starts only on 40–45 days after transplanting; fruits are light green and suitable for both fresh and dry market types. Hence after the seed increase, CCH-4 was included in All India Coordinated Research Project (AICRP) during 2007 and its suitability for commercial release is currently being evaluated at multiple locations.

3.4. Alloplasmic CMS F_1 and CMS BC_1F_1

Thirteen putative inter-specific F_1 (*C. chacoense* × *C. annuum*) plants were successfully raised and evaluated, however, crossed fruit could not be obtained from reciprocal attempts. Except for flower morphology, F_1 plants showed more resemblances to wild parent, i.e. *C. chacoense*. However, presence of male parent (*C. annuum*) specific 2.5 kb fragment ($OPS\text{-}1_{2500}$) in F_1 plants (Fig. 3) confirmed its hybridity and precludes their possible origin from the selfed seeds. Five plants had stamen-less flowers (Fig. 4) and the remaining eight plants had variable number of stamen per flower (0–5). However, stamens were rudimentary and no pollen grain could be seen under the microscope. Hence these plants had sporogenous male sterility with normal female fertility, as all these plants produced natural out cross fruits with seeds. The stamen-less five F_1 plants, however, did not produce natural out crossed fruits, which may be because pollinating insects might not have visited on stamen-less flowers. In view of this observation, for developing BC_1F_1, we used one F_1 plant with rudimentary stamens and eventually three CMS BC_1F_1 plants were able to attain reproductive maturity.

4. Discussion

4.1. SCARs validation and diversity between male sterile (S-) cytoplasms

Since both the previously developed CMS specific SCARs ($atp6_{607}$ and $coxII_{708}$) were reproducible and validated in two commercially utilized S-cytoplasms of *Capsicum*, i.e. Peterson's and Reddy's S-cytoplasms (Table 1, Figs. 1 and 2), it is suggested that CMS specific mitochondrial sequences of these two S-cytoplasms are perhaps similar or identical at the primer binding sites. We also studied the genetic relationship between these two commercially exploited S-cytoplasms and based on similar sterility maintenance and restoration ability of a given set of maintainer and restorer inbred lines of Petrerson's and Reddy's S-cytoplasms (Table 2), it is suggested that the two independently isolated and commercially utilized S-cytoplasms of the genus *Capsicum* are genetically similar. Similar results have been demonstrated in onion, wherein it was found that the same, or very similar, male-sterile cytoplasms were independently isolated and commercially exploited (Havey, 2000). Kashi Anmol is a known restorer for Peterson's S-cytoplasm (Kumar et al., 2007a) and Reddy's S-cytoplasm, but maintainer for *C. chacoense* derived cytoplasm (this study). This genetic evidence that S-cytoplasm in BC_1F_1 is different from the two already existing and utilized S-cytoplasms (Peterson's and Reddy's) also coincided with evidence that both the SCAR markers were absent in S-cytoplasm of BC_1F_1 plants (Fig. 2). Hence it is confirmed that *C. chacoense* derived S-cytoplasm isolated during this study is diverse than the two previously known S-cytoplasms in the genus *Capsicum*.

Three Peterson's S-cytoplasm maintainer lines (B1, B2, and B3) were not able to maintain complete sterility in one or other A line with Peterson's S-cytoplasm (Table 2). This could be due to the presence of fertility modifiers/QTLs in these maintainer inbreds because fertility restoration of Peterson's S-cytoplasm is known to be influenced by modifiers (Shifriss, 1997) and QTLs (Wang et al., 2004). This partial fertility restoration by maintainer inbreds in

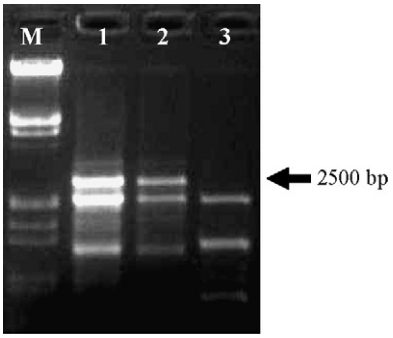

Fig. 3. Confirmation of inter-specific hybridity: presence of 2.5 kb fragments in male parent *C. annuum* (lane 1), hybrid (lane 2) and female parent *C. chacoense* (lane 3).

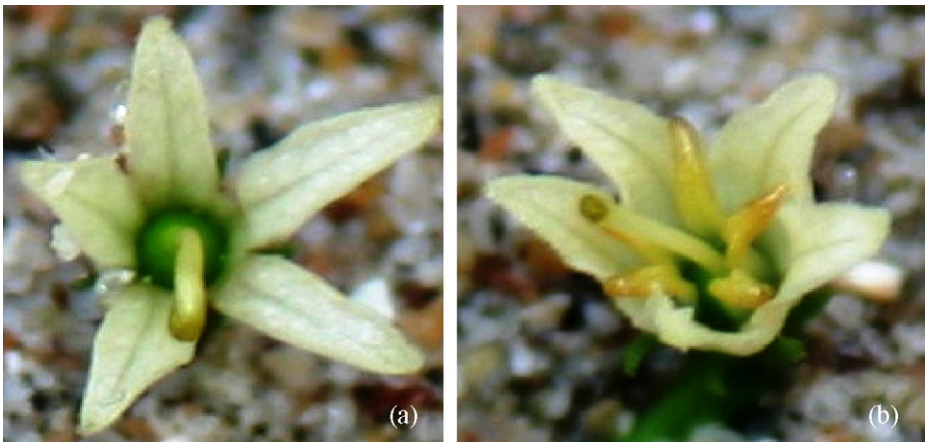

Fig. 4. Flowers of *C. chacoense* × *C. annuum* hybrid without stamen (a) and with rudimentary (pollen-less) stamens (b).

CMS (A) lines may also be due to the presence of partial fertility (*pr*) locus, which is known to express (restore partial fertility) under homozygous recessive condition in the presence of S-cytoplasm (Lee et al., 2008a,b). Nevertheless, Reddy's maintainer line (B7) completely maintained male sterility of all the four A (CMS) lines (Table 2), indicating better seed parent (CMS) breeding efficiency with A7 and B7 lines than the others.

4.2. Proposed utilization of validated SCAR markers

The genotypes with N- and S-cytoplasms are usually distinguished by conventional approaches including those proposed specially for hot pepper (Shifriss, 1997). These methods, however, are slow, laborious and expensive because they involve progeny testing that requires two growing seasons. The first season devoted for developing a large number of test- and reciprocal crosses involving genotypes with unknown cytoplasmic constitution, followed by the second season to evaluate progenies for male fertility reaction. The CMS based hot and sweet pepper hybrid breeding has several limitations and concerns and both these validated SCAR markers ($atp6_{607}$ and $coxII_{708}$) can be used to tackle some of these specific concerns effectively (Table 3) besides common use like in purity testing of parental and hybrid seed lots without performing grow-out-test (Kim and Kim, 2005). In the light of increased commercialization of CMS-based hot pepper hybrids in several countries (Zhang et al., 2000; Kim and Kim, 2005; Kumar et al., 2007a), it is anticipated that besides hybrid cultivars, many open pollinated cultivars may have S-cytoplasm with fertility restorer (*Rf*) gene. A similar situation exists in onion (Havey, 1993; Satoh et al., 1993; Engelke et al., 2003), which has long history of CMS exploitation to produce hybrid seeds (Havey, 2007). Likewise, occurrence of male fertile plants with N-cytoplasm as pollen shedders in CMS (S-*rfrf*) rows is a common problem faced in the hybrid seed production field. Lack of maintainer allele in hot pepper is a major limitation in rapid transfer of S-cytoplasm to develop new pair of CMS and maintainer lines (seed parent breeding), eventually leading to restricted parental choice for the hybrid combinations (Zhang et al., 2000; Kumar et al., 2007a). Therefore, identification of maintainer hot pepper genotype having N-cytoplasm and recessive allele at fertility restoration locus (N-*rfrf*) is highly desirable for faster nuclear diversification of CMS line through simple backcrossing and avoiding tedious maintainer breeding (Kumar et al., 2007a). Since both the SCARs ($atp6_{607}$ and $coxII_{708}$) are reproducible and proved to be S-cytoplasm specific in diverse materials, these can be used to screen a large number of lines/plants at seedling stage for quick and reliable determination of cytoplasm type (S- or N-) of individual plant and thus would be strategically useful in many ways (Table 3). For instance, in order to search for hot pepper maintainer genotype, in the first step both the SCARs should be used to screen a large number of plants to locate individuals with N-cytoplasm at the early growth stage. In the second step, only these individuals with N-cytoplasm should be considered as putative maintainer candidates and analyzed for allelic constitution at fertility restoration locus through test-cross progeny testing.

4.3. New alloplasmic CMS

The male sterility expression in the inter-specific (*C. chacoense* × *C. annuum*) F_1 plants, BC_1F_1 plants and natural out crossed plants obtained from BC_1F_1 clearly reveled that CMS expression in F_1 plants was attributed to the alloplasmic situation arising between nuclear genome of cultivated (*C. annuum*) and cytoplasmic genome of wild (*C. chacoense*) species. At the genetic level, CMS phenotype was previously considered as the consequence of incompatibility between nucleus and organelle encoded components of mitochondria, but now molecular evidence suggests that CMS usually occurs due to latent ORFs expression in the donor mitochondrial genome under alloplasmic condition (Pelletier and Budar, 2007). This is the reason that, with few exceptions, most of the CMS studied to date are gain of function mutants and mitochondrial ORFs causing CMS in several crops have been isolated (Chase, 2006) including from hot pepper (Kim et al., 2007). Inter-specific plants between *C. chacoense* and *C. annuum* were also obtained earlier (Kumar et al., 1988), but these plants were partially male fertile unlike plants of his study with an extreme type of male sterility, i.e. stamen-less flowers (Fig. 4). This kind of male sterility was also developed from *C. baccatum* × *C. annuum* hybrid backcrossed to *C. annuum*, but efforts to isolate fertility-restorer gene so far have been unsuccessful (Shifriss, 1997). Thus alloplasmic F_1 advanced to BC_1F_1 CMS plants possess a new male sterility causing S-cytoplasm derived from a wild *Capsicum* species (*C. chacoense*), as also evidenced from absence of CMS specific both the SCAR markers in this S-cytoplasm (Fig. 2). But, commercial exploitation of this S-cytoplasm would depend on extent of natural cross-pollination on the backcrossed introgressed plants and also on the availability of restorer gene/s for this S-cytoplasm. Therefore, instead of stamen-less F_1 plants, plant with rudimentary stamens producing natural out cross fruits was deliberately selected to create BC_1F_1 and this strategy should be followed during subsequent backcrossing. It is intended to initiate

Table 3
Proposed uses of SCAR markers in analyzing a large number of genotypes to facilitate CMS-based hybrid pepper breeding.

Concern of markers use	Use of markers in	Benefit of markers use
Specific for hot pepper		
Lack of availability of maintainer genotype (N-*rfrf*), leading to restriction in rapid nuclear diversification of CMS and in choice for the parents (Zhang et al., 2000; Kumar et al., 2007a)	Search for male fertile plants with N-cytoplasm (N-*RfRf or Rfrf or rfrf*) and use only them (N-) to develop testcross on CMS for maintainer (N-*rfrf*) identification through progeny testing	Reduced number of crosses with desirable N-cytoplasm (not with S-cytoplasm) will need to be developed and evaluated for maintainer identification, resulting in saving of time and cost.
Specific for sweet pepper		
Lack of availability of restorer (N-*RfRf* or S-*RfRf*) genotype, leading to unsuccessfully commercial exploitation of CMS (Kumar et al., 2007a)	Search for male fertile plants with S-cytoplasm (S-*RfRf* or *Rfrf*) because such genotype itself will be restorer and can directly be used as male parent	Cost and time effective identification of restorer without progeny testing, opening path for developing CMS based commercial hybrids
General for both peppers[a]		
Cytoplasmic heterogeneity within population due to the presence of plants with both N- and S-cytoplasms, interacting with nuclear fertility restoration locus, leading to impurity in parental and F_1 seed lots	Purification of population with respect to uniform cytoplasmic type by eliminating plants with S- or N-cytoplasm based on strategic need	No progeny testing needed, hence rapid (even at seedling stage) detection of genotypes to facilitate cost effective purification
Natural occurrence of male sterile plants in a population	Examination of male sterile plants, if they originated due to open reading frame (ORF) associated with these markers	Obtaining initial and early information on nature of male sterile plants of unknown origin.
Presence of male sterile plants (S-*rfrf*) in maintainer lines and male fertile (pollen shedder) plants (N-*rfrf*) in CMS lines	Identification and elimination of plants with S-cytoplasm from maintainer and plants with N-cytoplasm from CMS populations	More efficient and effective maintenance of seed stocks of CMS and maintainer lines

[a] These concerns are based on expected population dynamics because of increasing use of CMS-based hybrid cultivars of hot pepper. This kind of population dynamics has actually been reported in cross-pollinated crops like onion (Havey, 2000).

screening *Capsicum* germpalsm for the identification of restorer gene/s, while backcrossing efforts are under progress.

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