RESEARCH ARTICLE

SABRAO Journal
of Breeding and Genetics
48 (4) 465-473, 2016

MARKERS FOR CYTOPLASMIC MALE STERILITY (CMS) TRAITS IN CHILI PEPPERS (*Capsicum annuum* L.). I: MULTIPLEX PCR AND VALIDATION

T. YEH[1], S. LIN[2], H. SHIEH[2], Y. TEOH[3] and S. KUMAR[2,*]

[1]Taiwan Agricultural Research Institute-Fengshan Tropical Horticultural Experiment Branch, Fengshan, Kaohsiung 830, Taiwan
[2]World Vegetable Center, Shanhua, Tainan 741, Taiwan
[3]Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sabah, Malaysia
*Corresponding author's email: sanjeet.kumar@worldveg.org
Email addresses of co-authors: tzuying139@fthes-tari.gov.tw; susan.lin@worldveg.org; yanjiteoh@yahoo.com

SUMMARY

Chili peppers (*Capsicum annuum* L.) are an important commercial crop valued for their pungent fruits, which are indispensable ingredients in many cuisines around the world. Cytoplasmic male sterility (CMS) is the most commonly used mechanism to produce commercial hybrid seeds of chili pepper. Using a set of 20 pepper (chili and sweet) CMS lines, their 20 maintainer lines and 10 restorer lines, we examined the validity of five male sterile cytoplasm (S-cytoplasm) specific (atp6-SCAR$_{607}$, Ψatp6-2$_{875}$ coxII-SCAR$_{708}$, orf$_{456}$, SCAR$_{130/140}$) and one *restoration-of-fertility* (*Rf*) locus specific (CRF-S$_{870}$) markers. Out of five markers evaluated, co-dominant sequence characterized amplified region (SCAR) marker (SCAR$_{130/140}$) was found to be most reliable and reproducible for detection of cytoplasm type (S-cytoplasm vs. normal, N-cytoplasm) in peppers. Hence, this CMS marker (SCAR$_{130/140}$) and *Rf* locus associated marker (CRF-S$_{870}$) were used in a multiplex polymerase chain reaction (PCR) protocol to facilitate efficient screening of cytoplasm types in peppers. This multiplex PCR can be used for very efficient and cost effective screening of a large number of pepper lines at the seedling stage in only 15-20 days to determine distribution of cytoplasm types (S vs. N), by passing tedious and time consuming conventional process involving three seasons in developing testcross F_1s (in the first season), growing and obtaining F_2 seeds (in the second season) and finally examining segregation in the F_2 progenies (in the third season).

Key words: Chili pepper**,** germplasm screening**,** fertility restoration, hybrid seeds, sweet pepper

Key findings: The SCAR$_{130/140}$ marker system was found to be most reliable for detection of CMS (S) and normal (N) cytoplasm in peppers (*Capsicum* spp.). Hence breeders can use this marker for rapid (~ 15-20 days) and highly cost effective determination of cytoplasm types (N vs. S) in pepper germplasm, by passing time consuming (~300 days) and tedious conventional approach.

Manuscript received: August 9, 2016; Decision on manuscript: August 30, 2016; Manuscript accepted: October 24, 2016.
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Communicating Editor: Naqib Ullah Khan

INTRODUCTION

Peppers (*Capsicum annuum* L.) are an important commercial crop valued for their pungent (chili pepper) and non-pungent (sweet pepper) fruits. Chili pepper fruits and products are an indispensable food ingredient in many cuisines worldwide.

One important aspect of pepper production is the development and implementation of hybrid cultivars, which can take advantage of hybrid vigor. Cytoplasmic

male sterility (CMS) is the most commonly used mechanism to produce commercial hybrid seeds of a number of crop plants, including chili pepper (Kaul, 1988; Lin *et al.,* 2013; Reddy *et al.,* 2015). The first pepper CMS plant (S-cytoplasm) was discovered in the USA in a landrace chili pepper population collected from India (Peterson 1958). S-cytoplasm is the most widely used for the development of hybrid cultivars in India, China, South Korea and other countries (Kumar *et al.,* 2009; Reddy *et al.,* 2015). This male sterile cytoplasm has been found to be genetically similar to the other independently isolated and commercially used male sterile cytoplasm in India (Kumar *et al.,* 2009). Therefore, diversification of pepper CMS cytoplasm is necessary to reduce the risks associated with predominant use of single male sterile cytoplasm. The susceptibility of Texas (T-) cytoplasm of corn to *Helminthosporium* blight in USA during 1970's, leading to devastation of T-cytoplasm based hybrid crops (Levings, 1990), is a well-known example of such risk.

Mitochondrial genes (*Ψatp6-2, CoxII*) and open reading frames (*orf456/orf507*) related to pepper CMS phenotype (S-cytoplasm) have been identified and studied. These genes and *orf* are located on the edges of highly rearranged CMS specific DNA regions and near to repeat sequences (Jo *et al.,* 2014). The *Ψatp6-2* gene is believed to be regulated through *restoration-of-fertility* (*Rf*) locus at the transcriptional level and the *orf456/orf507* is regulated at post transcriptional level or translational level (Kim *et al.,* 2006; Kim *et al.,* 2007). A number of S-cytoplasm specific (Kim *et al.,* 2005; Kim *et al.,* 2007; Gulyas *et al.,* 2010) and *Rf* locus specific (Gulyas *et al.,* 2006; Lee *et al.,* 2008a,b) molecular markers have been developed in peppers. One of these *Rf* locus markers (Gulyas *et al.,* 2006) was previously used to transfer the *Rf* allele from chili pepper into sweet pepper through marker-assisted backcrossing (Lin *et al.,* 2015). After validation, these markers can be used to increase efficiency of CMS hybrid pepper breeding in various ways (Kumar *et al.,* 2009). We examined the validity of CMS and *Rf* locus specific markers and developed a multiplex polymerase chain reaction (PCR) protocol using the most reliable markers. Results are decribed in light of their use in pepper genetic resources and breeding programs.

MATERIALS AND METHODS

Plant materials

A total of 50 pepper lines were used in this study (Table 1). We evaluated 10 pairs of near-isoplasmic chili pepper CMS (S-cytoplasm; A-lines) and their maintainer lines (B-lines; N-cytoplasm, designated as 1A/1B to 10A/10B) and 10 pairs of sweet pepper CMS and their maintainer lines (designated as 11A/11B to 20A/20B) developed by the World Vegetable Center (WorldVeg), as well as five each of chili pepper (21R to 25R) and sweet pepper (26R to 30R) known restorer lines (C-/R-lines, with unknown cytoplasm) (Table 1).

DNA extraction and markers for validation

Young, actively growing leaves (0.1 g) of two plants of each genotype were ground in liquid nitrogen using a mortar and a pestle; a modified CTAB extraction method was used for DNA extraction. To evaluate their validity, primers for six previously reported molecular markers associated with CMS (five markers) and *Rf* (one marker) (Table 2) were synthesized (Genscript Corporation, Taiwan) and used for PCR.

Polymerase chain reaction (PCR) and electrophoresis PCR for individual markers: The PCR reaction mixture (10 µl) consisted of 1µl DNA template (2 ng/ml), 2 µl 10x reaction buffer, 0.8 µl of 25 nM dNTPs, 2 µl of 10 µM primer set, and 0.1 µl HS DNA polymerase (Bioline, London, UK). The amplification profile consisted of 35 cycles of 30 sec at 95℃ for denaturation, 45 sec at annealing temperature, and 45 sec at 72℃ for extension and DNA synthesis. At the initial cycling profile, the reaction was heated for 5 min at 95℃ and the final cycle was extended to 10 min at 72℃. All the amplified products were initially separated by electrophoresis on 1.5% agarose gel, and visualized by staining with nucleic acid staining solution (EtB“Out”, Yeastern Biotech

Table 1. Identifier, pedigree, phenotype, and genotype of the pepper lines used.

Line code (P or NP)[a]	Line name	Pedigree	Phenotype	Genotype
1A(P)	AVPP0709-S	CCA-4916	CMS; A line	S-*rfrf*
1B(P)	VI060627;C05606	PBC362,C05606	Maintainer; B line	N-*rfrf*
2A(P)	AVPP0516-S	CCA7242;CCA4757	CMS; A line	S-*rfrf*
2B(P)	VI037614;TC06308	TC06308,PBC380	Maintainer; B line	N-*rfrf*
3A(P)	AVPP0517-S	CCA7243;CCA4758	CMS; A line	S-*rfrf*
3B(P)	VI060632;C05661	C05661,PBC483	Maintainer; B line	N-*rfrf*
4A(P)	AVPP9907-S	CCA7244;CCA4759	CMS; A line	S-*rfrf*
4B(P)	AVPP9907	9907-9611	Maintainer; B line	N-*rfrf*
5A(P)	AVPP9910-S	CCA7232	CMS; A line	S-*rfrf*
5B(P)	AVPP9910	9950-5633	Maintainer; B line	N-*rfrf*
6A(P)	AVPP0710-S	CCA4917	CMS; A line	S-*rfrf*
6B(P)	VI046838;TC06677	PBC292;TC06677	Maintainer; B line	N-*rfrf*
7A(P)	AVPP0309-S	CCA6475	CMS; A line	S-*rfrf*
7B(P)	AVPP0309	9849-5765	Maintainer; B line	N-*rfrf*
8A(P)	AVPP0310-S	CCA6476	CMS; A line	S-*rfrf*
8B(P)	VI060629;C05601	PBC378-2;C05601	Maintainer; B line	N-*rfrf*
9A(P)	AVPP0711-S	CCA7241;CCA4261	CMS; A line	S-*rfrf*
9B(P)	VI060630;C05671	PBC 534;C05671	Maintainer; B line	N-*rfrf*
10A(P)	AVPP9606-S	CCA7233	CMS; A line	S-*rfrf*
10B(P)	VI046844;TC06683	PBC308;TC06683	Maintainer; B line	N-*rfrf*
11A(NP)	AVPP9820-S	CCA7234	CMS; A line	S-*rfrf*
11B(NP)	AVPP9820	9847-4754	Maintainer; B line	N-*rfrf*
12A(NP)	AVPP9908-S	CCA7235	CMS; A line	S-*rfrf*
12B(NP)	AVPP9908	9946-2162	Maintainer; B line	N-*rfrf*
13A(NP)	AVPP9912-S	CCA7229	CMS; A line	S-*rfrf*
13B(NP)	AVPP9912	9946-2194	Maintainer; B line	N-*rfrf*
14A(NP)	AVPP9913-S	CCA7231	CMS; A line	S-*rfrf*
14B(NP)	AVPP9913	9946-2138	Maintainer; B line	N-*rfrf*
15A(NP)	AVPP9607-S	CCA7236	CMS; A line	S-*rfrf*
15B(NP)	VI037597	PBC84;TC06052	Maintainer; B line	N-*rfrf*
16A(NP)	AVPP9821-S	CCA7237	CMS; A line	S-*rfrf*
16B(NP)	AVPP9821	9852-1743	Maintainer; B line	N-*rfrf*
17A(NP)	AVPP1601-S	CCA13679	CMS; A line	S-*rfrf*
17B(NP)	AVPP1601	9950-5700	Maintainer; B line	N-*rfrf*
18A(NP)	AVPP1602-S	CCA13681	CMS; A line	S-*rfrf*
18B(NP)	AVPP1602	0407-7069	Maintainer; B line	N-*rfrf*
19A(NP)	AVPP1603-S	CCA13683	CMS; A line	S-*rfrf*
19B(NP)	AVPP1603, VI031628	C05464-B	Maintainer; B line	N-*rfrf*
20A(NP)	AVPP1604-S	CCA13684	CMS; A line	S-*rfrf*
20B(NP)	AVPP1604	0537-7007	Maintainer; B line	N-*rfrf*
21R(P)	VI037563	PBC473,C05625	Restorer; R line	S/N?-*RfRf*
22R(P)	VI059328	PBC142,C05573	Restorer; R line	S/N?-*RfRf*
23R(P)	AVPP9905	PP9955-15	Restorer; R line	S/N?-*RfRf*
24R(P)	AVPP0512	PP0537-7541	Restorer; R line	S/N?-*RfRf*
25R(P)	AVPP0605	PP0637-7505	Restorer; R line	S/N?-*RfRf*
26R(NP)	AVPP9807	PP9852-131	Restorer; R line	S/N?-*RfRf*
27R(NP)	AVPP9822	PP9852-190	Restorer; R line	S/N?-*RfRf*
28R(NP)	AVPP0515	PP0537-7044	Restorer; R line	S/N?-*RfRf*
29R(NP)	AVPP9904	PP9950-5558	Restorer; R line	S/N?-*RfRf*
30R(NP)	AVPP9808	PP9852-133	Restorer; R line	S/N?-*RfRf*

Table 2. Primer sequences of markers used for validation and multiplex studies.

Marker name	5′ to 3′ sequence	Product size, bp	Reference
Marker for S- or N-cytoplasm			
atp6-SCAR	AGTCCACTTGAACAATTTGAAATAATC GTTCCGTACTTTACTTACGAGC	607 (S)	Kim and Kim, 2005
coxII-SCAR	GTCGGGAGAACTACCTAACTA GGCTACCTAGTGATTTACAAGCA	708 (S)	Kim and Kim, 2005
orf456-SCAR	ATGCCCAAAAGTCCCATGTA TTACTCGGTTGCTCCATTGTTT	456 (S)	Kim *et al.*, 2007
Ψatp6-2	GTAGTTCATTCGGACCTAGTAG TGGATCTCGCTATTAACCAC	875 (S)	Ji *et al.*, 2013
SCAR	TTACGGCTCGTTACCGCAGC AATTGACCGACCCGCCAT	130 (S) 140 (N)	Ji *et al.*, 2014
Marker for *Rf* locus			
CRF-SCAR	GTACACACCACTCG-TCGCTCCT TTCTTGGGTCCCTTT-CTTCCAA	870 (*Rf*-)	Gulyas *et al.*, 2006

CO., Ltd., Taiwan). Multiplex PCR using most reliable markers: For PCR, each 15µl reaction mixture consisted of 1 µl DNA template (10 ng/ml), 1.5 µl of 10x reaction buffer, 1 µl of 25 mM dNTPs, 0.12 µl of (5 units) Gold Tag DNA polymerase (JMR Holdings Co., United Kingdom), 0.2 µl of 10 µM CRF-S primers and 0.05 µl of 10 µM $SCAR_{130/140}$ primers. The PCR cycle was performed following Lin *et al*. (2015) with minor modifications. The amplification profile consists of 35 cycles of 45 sec at 95℃ for denaturation, 1 min at annealing temperature, and 1 min 30 sec at 72℃ for extension and DNA synthesis. At the initial cycling profile, the reaction was heated for 10 min at 95°C and the final cycle was extended to 7 min at 72°C (Bio-RAD, Mexico).

All amplified products were initially separated by polyacrylamide gel electrophoresis (PAGE) on 6% polyacrylamide gels in 0.5 x TBE buffer, stained with ethidium bromide, and visualized using UVITEC Gel Documentation Systems & Software (Cambridge, United Kingdom).

RESULTS AND DISCUSSION

Substoichiometric shift in pepper CMS

Five CMS and one *Rf* locus specific markers were successfully applied in a panel of 50 pepper genotypes belonging to 20 pairs (40) of CMS and maintainer and 10 restorer (unknown cytoplasm, N/S-cytoplasm) lines. Data were analyzed for mismatch between phenotype and markers (Tables 3-5). In the initial screening with one pair of CMS and its maintainer lines with different concentrations of DNA, four CMS markers produced expected fragments in CMS lines (S-cytoplasm), but light intensity fragments were also amplified in maintainer lines (N-cytoplasm) with 50 ng and 10 ng template DNA (Figure 1).

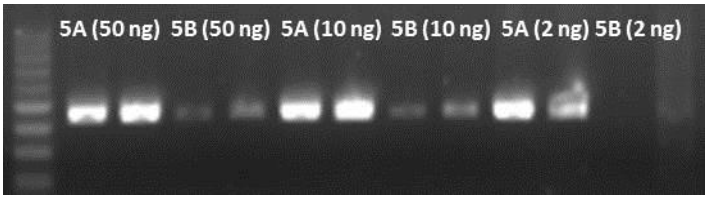

Figure 1. Intensity of fragment generated by CMS specific orf456 marker in CMS (S-cytoplasm, 5A) and maintainer (N-cytoplasm, 5B) plants with three concentrations of template DNA.

Light intensity of CMS-specific fragment amplification at higher concentration could be explained by the presence of a very low copy number of CMS specific mtDNA (substoichiometric shift, Jo *et al.,* 2014) in maintainer lines as mentioned in the case of pepper (Jo *et al.,* 2009), common bean (Janska *et al.,* 1998), and radish (Kim *et al.,* 2007). Our amplification results support this; when we used a low concentration (2 ng/ml) of template DNA, amplicons in maintainer lines were not detectable (Figure 1). Hence selected 2 ng/ml concentration.

Markers for CMS and *Rf* locus

All five S-cytoplasm specific markers produced fragments of expected size in 10 chili pepper CMS lines, and as expected, these fragments were absent in their 10 maintainer lines (Table 3). Hence, there was no mismatch between CMS phenotype and presence of markers and male fertile phenotype and absence of markers in all 20 CMS and their maintainer chili pepper lines (Table 3). These five markers were also amplified in 10 sweet pepper CMS lines and their four maintainer lines (11B, 12B, 18B, 20B). However, in the remaining six sweet pepper maintainer lines, four markers (atp6-$SCAR_{607}$, Ψatp6-2_{875}, coxII-$SCAR_{708}$ and orf_{456}) produced mismatched CMS specific fragments, while $SCAR_{130/140}$ did not (Table 4). The mismatch percentage for both atp6-$SCAR_{607}$ and Ψatp6-2_{875} was 5%, and 25% for both coxII-$SCAR_{708}$ and orf_{456} (Table 4). The levels of mismatch between the phenotypes and the markers found here are high enough to impede progress in breeding programs through limited accuracy of selection. Among the 10 restorer lines with unknown cytoplasm, $SCAR_{130}$ and other CMS specific markers were present in one chili pepper restorer (25R, AVPP0605), indicating the presence of S-cytoplasm (Table 5).

As expected CRF-SCAR did not produce *Rf* locus associated 870 bp fragment (CRF-S_{870}) in chili and sweet pepper CMS and maintainer lines (Tables 3 and 4). However, this marker (CRF-S_{870}) was found to be absent in one known chili pepper restorer line (21R) and three known sweet pepper restorer lines (27R, 28R and 30R) with 40% mismatches (Table 5). These results indicate the narrow distribution and applicability of CRF-S_{870} in improved restorer lines developed by the World Vegetable Center and examined in this study. Therefore, the use of CRF-S_{870} will be very limited in a wide range of pepper germplasm targeted for marker assisted selection of restorer and maintainer genes.

Multiplex PCR for selected CMS and *Rf* markers

The results of individual marker analyses revealed that among the five CMS specific markers, $SCAR_{130/140}$ was the most reliable. Therefore, $SCAR_{130/140}$ and *Rf* locus associated CRF-S_{870} markers were used in a multiplex PCR protocol to facilitate even more efficient screening of cytoplasm types in peppers. The results of $SCAR_{130/140}$ and CRF-S_{870} analysis of all 20 CMS lines (S-cytoplasm), their 20 maintainer (N-cytoplasm) lines and 10 restorer lines (unknown cytoplasm) (Figure 2) were found to be consistent with the results of individual marker analysis of these lines (Tables 3, 4 and 5).

Male sterility including CMS based hybrid seed is becoming necessary to produce cost effective pepper hybrid seeds in competitive seed markets. Unlike sweet pepper, chili pepper CMS lines are used commercially for hybrid seed production (Lin *et al.,* 2015). This also includes use of the World Vegetable Center's developed chili pepper CMS lines in India (Lin *et al.,* 2013; Reddy *et al.,* 2015; Schreinemachers *et al.,* 2016). Sweet pepper CMS lines are known to have unstable expression of male sterility and fertility restoration of known sweet pepper restorer lines is inconsistent. With the exception of a case-specific use of *Rf* gene associated marker in peppers (Lin *et al.,* 2015), markers developed for chili pepper *Rf* currently have limited applicability due to the lack of agreement between the marker and the phenotype (Kumar *et al.,* 2007; Min *et al.,* 2008; Jiang, 2015; this study). This lack of agreement could be because fertility restoration is influenced by temperature, quantitative trait loci (QTLs)/modifiers (Wang *et al.,* 2004) and the presence of either an additional partial restoration (*pr*) locus tightly

Table 3. Distribution of CMS and *Rf* specific markers in chili pepper CMS (S-*rfrf*) and maintainer (N-*rfrf*) lines.

Lines	Markers observed (expected)					
	atp6-SCAR$_{607}$	Ψatp6-2$_{875}$	coxII-SCAR$_{708}$	orf$_{456}$	SCAR$_{130/140}$	CRF-S$_{870}$
1A, 2A, 3A, 4A, 5A, 6A,7A, 8A, 9A, 10A	+ (+)	+ (+)	+ (+)	+ (+)	+/- (+/-)	-(-)
1B, 2B, 3B, 4B, 5B, 6B, 7B, 8B, 9B, 10B	- (-)	- (-)	- (-)	- (-)	-/+ (-/+)	- (-)
Mismatch (%)	0	0	0	0	0	0

+ = amplification; - = non-amplification

Table 4. Distribution of CMS and *Rf* specific markers in sweet pepper CMS (S-*rfrf*) and maintainer (N-*rfrf*) lines**.**

Lines	Markers observed (expected)					
	atp6-SCAR$_{607}$	Ψatp6-2$_{875}$	coxII-SCAR$_{708}$	orf$_{456}$	SCAR$_{130/140}$ (S/N)	CRF-S$_{870}$
11A, 12A, 13A, 14A, 15A, 16A, 17A, 18A, 19A, 20A	+ (+)	+ (+)	+ (+)	+ (+)	+/- (+/-)	- (-)
11B, 12B, 18B, 20B	- (-)	- (-)	- (-)	- (-)	-/+ (-/+)	- (-)
13B	+ (-)	+ (-)	+ (-)	+ (-)	-/+ (-/+)	- (-)
14B, 15B, 16B, 17B, 19B	- (-)	- (-)	+ (-)	+ (-)	-/+ (-/+)	- (-)
Mismatch (%)	5	5	25	25	0	0

+ = amplification; - = non-amplification

Table 5. Distribution of CMS and *Rf* markers in pepper restorer (S/N-*RfRf*) lines.

Lines/markers	atp6-SCAR$_{607}$	Ψatp6-2$_{875}$	coxII-SCAR$_{708}$	orf$_{456}$	SCAR$_{130/140}$	CRF-S$_{870}$
21R	+	-	-	+	-/+	-
22R	+	-	+	+	-/+	+
23R, 24R, 29R	-	-	-	-	-/+	+
25R	+	+	+	+	+/-	+
26R	-	-	+	-	-/+	+
27R, 28R, 30R	-	-	-	-	-/+	-
Mismatch (%)	Unknown cytoplasm (S/N)					40

+ = amplification; - = non-amplification

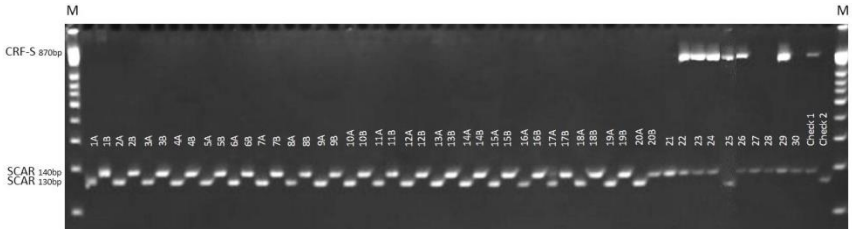

Figure 2. Amplification results of S-cytoplasm (130 bp) and N-cytoplasm (140 bp) and *Rf* gene associated markers in multiplex PCR in 50 pepepr genotypes (number corresponds to genotype in Table 1).

linked to *Rf* or a third allele of *Rf* locus (Lee *et al.,* 2008a,b). A very recent genome-wide analysis of chili pepper has revealed that 13 chili pepper domains have similarity to *Rf* genes of other species (Barchenger *et al.,* 2016). These *Rf* gene copies are mostly clustered on chromosome 6 (Jo *et al.,* 2010). This confirms the possible presence of many *Rf* loci and the reason for lack of a widely applicable *Rf* gene associated marker in peppers (Barchenger *et al.,* 2016).

Conventional germplasm characterization for cytoplasm type requires three crop growing seasons/generations. Cytoplasm (S or N) in a given accession can be tested by developing F_1 crosses using tester inbred accessions as female parents to known maintainer plants (in the first season), growing these F_1 crossed plants to produce F_2 seeds (in the second season) and examine F_3 progenies ability for their fertility restoration ability (in the third season). In contrast, most reliable CMS-associated marker ($SCAR_{130/140}$) developed by Ji *et al.* (2014) and validated during this study could be useful for efficient germplasm screening at the seedling stage (only 15-20 days) for cytoplasmic differentiation in peppers. Obtaining this strategic information is critical, as it will reveal the extent of cytoplasmic variability in widely grown cultivars, and anticipate any possible risk of vulnerability associated with monopolistic use and/or existence of genetically similar male sterile cytoplasm in pepper cultivars (Kumar *et al.,* 2009). Out of 10 known restorer lines screened in this study, one (AVPP0605) possessed S-cytoplasm. An Indonesian bacterial wilt resistant line, KR-B, is the donor of cytoplasm to AVPP0605 and other sister lines developed by WorldVeg (data not shown). Likewise, CM334, a famous Mexican landrace widely used as source of virus and *Phytophthora* blight resistant in pepper breeding program, also possess S-cytoplasm (Jiang, 2015). We used developed multiplex PCR protocol to rapidly screen (at seedling stage) more than 1000 *Capsicum* gemrplasm (open pollinated varieties, hybrids, improved breeding lines, landraces) and the results revealed about 8.3% of peppers have S-cytoplasm (data not shown). CMS causing cytoplasm has been found to be frequently distributed in open pollinated populations of cultivated onion (Havey, 1997), radish (Yamagishi and Terachi, 1996) and in wild species of radish (Yamagishi and Terachi, 1997). Evolutionary aspects of CMS (gynodioecious) in plant also suggest that CMS is predicted to be under balancing selection, under which the male sterilizing mitochondrial genome and *Rf* loci are favored, enabling their co-existence for a longer period of time (Lahiani *et al.,* 2013).

ACKNOWLEDGEMENTS

This work was supported by the Council of Agriculture (COA), Taiwan, under the "Strengthening the cooperation between World Vegetable Center and Taiwan research institutes on vegetable research and development" project (102AS-1.1.2-ST-a3).

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