PEPPER (*CAPSICUM ANNUUM* L.) CYTOPLASMIC MALE STERILTY
PAPRIKA (*CAPSICUM ANNUUM* L.) CITOPLAZMÁS HÍMSTERILITÁSÁNAK VIZSGÁLATA

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ÖSSZEFOGLALÁS

A citoplazmás hímsterilitás (CMS) olyan anyai úton öröklõdõ tulajdonság, amely feltételezhetõen a mitokondriumban lejátszódó intra- és intermolekuláris rekombinációs események eredményeként jön létre, és az utódok csökkent vagy életképtelen pollentermelõ képességében nyilvánul meg. A legtöbb CMS rendszerben a fertilitást specifikus nukleáris resztorer (*Rf*) gének helyreállíthatják. Habár a CMS/Rf rendszert, korlátozottan ugyan, de alkalmazzák a paprikanemesítésben, eddig kevés ismeretünk van a rendszer sikeres mûködéséröl és a mûködés molekuláris mechanizmusáról.

Szántóföldi és üvegházi kísérleteket végeztünk öt hímsteril (201-205) és négy resztorer (206-210) paprika (*Capsicum annuum* L.) vonal szülői, F_1 és F_2 nemzedékével. A növények pollentermelését vizuálisan és mikroszkóposan vizsgáltuk. A környezeti tényezők – különösen a hőmérséklet - a paprika növények pollentermelő képességét befolyásolhatják, ezért a szülői vonalak pollentermelő képességét három tenyészidőszakon keresztül figyeltük meg. A hímsteril vonalak - a 201. hímsteril vonal kivételével - teljesen pollen sterilek voltak (1. táblázat). A 201. CMS vonal egyedeinél májusban részleges fertilitást figyeltünk meg. Hasonló, de ellentétes irányú instabilitást figyeltünk meg a pollentermelő képességben a 206. és 207. resztorer vonalaknál. Az F_1 nemzedék minden egyedében a pollen fertilitás helyreállt, jelezve a helyreállító vonalakban a resztorer gén jelenlétét (2. táblázat).

A CMS és resztorer vonalak közötti genetikai különbségek molekuláris szinten történő meghatározására specifikus nukleinsav kezdő szekvenciákat (primer) alkalmaztunk. A resztorer specifikus markerek segítségével először ún. „bulk szegregáns" majd később egyedi vizsgálatokat végeztünk. A vizsgálatok során egyértelmű megerősítést nyert, hogy az alkalmazott primerekkel a hímsteril és fertilis növények molekuláris szinten jól megkülönböztethetők. A resztorer specifikus primerek egy kb. 870 bp nagyságú DNS fragmentumot határoztak meg, és a 207. vonal kivételével kizárólag csak a resztorer növényekben amplifikálódtak (1. kép). A resztorer gén markerezését az F_2 nemzedékre is kiterjesztettük és közel száz egyedet vizsgáltunk meg. Az *Rf* gén specifikus fragmentumok az F_2 egyedeiben is tisztán megjelentek (2. kép). A vizsgált száz egyed 75 (marker jelenléte): 25 (marker hiánya) hasadási arányt mutatott (3:1, $\chi^2 = 0.01$, $0.9 < P < 0.95$). A pollen fertilitási adatok és gélanalízissel kapott eredmények rekombinációs értékének kiszámításával a resztorer gén és a marker közötti távolságot 20cM-ban határoztuk meg.

KULCSSZAVAK: citoplazmás hímsterilitás, paprika, pollen fertilitás, resztorer gén specifikus marker, F_2

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ABSTRACT

In the present study the molecular basis and mechanism of pepper cytoplasmic male sterility (CMS) and its restoration system (*Rf*) has been characterized in detail. Pollen fertility of five CMSs (No. 201 to 205), four restorer lines (No. 206, 207, 209 and 210) and their F_1, F_2 generations were investigated during different growing seasons to study the response of male sterility to various environmental conditions. Restorer gene specific primers were applied to reveal the molecular genetic differences between the CMS and restorer lines. „Bulk segregant" and individual analysis screened DNA markers linked to the fertility restorer (*Rf*) gene for cytoplasmic male sterility.

By the application for conventional breeding and molecular genetic methods co-segregation of the restorer specific markers and pollen viability data were observed on hundred individuals of the F_2 generation in order to construct a physical linkage map.

KEY WORDS: cytoplasmic male sterility, pepper, pollen fertility, restorer gene specific marker, F_2

DETAILED ABSTRACT

Cytoplasmic male sterility (CMS) is a maternally inherited inability of a plant to produce functional pollen. The CMS phenotype is suggested to originate from some mutations in the mitochondrial genome as a result of some mutations of intra- or intermolecular recombination events. In many CMS systems, fertility can be restored in the presence of specific nuclear genes (*Rf* genes). Although the CMS/Rf system is used in pepper genetics and breeding, very little is known on CMS characterization, and only a few applications for breeding have been reported.

In this study nine CMS (201-205), four restorer pepper (*Capsicum annuum* L.) lines and their F_1, F_2 generations were investigated. Plants were grown either under greenhouse or field condition. All plants were visually observed at flowering for the presence of anthers and for pollen production. Pollen grains were counted using light microscope. Since the influences of environmental conditions –especially temperature – over the degree of pollen sterility of pepper can be appreciable, the pollen fertility of the parental lines were checked during three growing seasons (Table 1.). The CMS lines were completely sterile, except the CMS line 201. Partial instability of pollen sterility of the CMS line 201 occurred in May. A reverse instability of the pollen fertility could be observed in the restorer line 206 and 207. In all F_1 hybrids fertility was restored when the CMS lines were crossed to the restorer lines, indicating the presence of the nuclear fertility restorer gene (Table 2.).

Specific primers were applied to reveal the molecular genetic differences between the CMS and restorer lines. DNA markers linked to the fertility restorer (*Rf*) gene for cytoplasmic male sterility were screened by "bulk segregant" and individual analysis. The PCR amplification result of the parental lines showed that by using the restorer gene specific primers, fertile and CMS plants could be distinguished (Fig. 1.). An 870bp fragment was amplified for all the fertile restorer plants, except the restorer line 207. The fragments specific to the *Rf* gene clearly appeared in the F_2 individual plants. A hundred of F_2 individuals were studied in total, segregated in the ratio of 75 presence of *Rf* marker: 25 absence of *Rf* marker (3:1, χ^2= 0.01, $0.9 < P < 0.95$). By the comparison of the pollen viability data obtained from the F_2 individuals and the results of the gel analysis, the physical distance between the marker and the restorer gene was determined in 20cM.

INTRODUCTION

Cytoplasmic male sterility (CMS) is a maternally inherited trait characterized by the inability of a plant to shed viable pollen [2, 15]. Although it can appear spontaneously in nature, either inter - or intraspecific crosses, commercial purposes made, often induce it. The CMS phenotype is suggested to originate from some mutations in the mitochondrial genome of the male fertile progenitors as a result of intra- or intermolecular recombination events [17, 6, 10, 14, 16]. The association of CMS with abnormal mitochondrial gene expression has been established in many plant species including maize [1] petunia [20, 13,], sunflower [3, 8, 9, 12] and common bean [11, 4]. In most of the CMS systems the pollen deficiency can be restored by the interaction of nuclear fertility restorer (*Rf*) genes [2], which is desirable requirement of the F_1 commercial hybrid seed production. These fertility restorer genes are thought to block or compensate for cytoplasmic dysfunctions that are phenotypically expressed during pollen development. This direct nuclear-cytoplasmic interaction provides an ideal opportunity to characterize the role of particular nuclear genes in the regulation of cytoplasmic function. Although pepper is evaluated intensively as a leading vegetable crop, the mechanism underlying cytoplasmic male sterility has not been sufficiently characterized, yet. The mechanism by which the nuclear restorer gene acts to restore fertility is also poorly understood: up to date no nuclear restorer gene has been characterized at molecular level.

Since the control of cytoplasmic male sterility and its fertility restoration system proved to behave differently in pepper, the genetic mechanisms underlying these interactions need to be investigated further using genotypes of different origin.

In the present study, the molecular basis and mechanism of pepper CMS and its restoration system has been characterized further. By the application for conventional breeding and molecular genetic methods co-segregation of the restorer specific markers and pollen viability data were observed on hundred of individuals of the F_2 generation in order to construct a physical linkage map.

MATERIALS AND METHODS

Five CMS No. 201-205 and four restorer No. 206, 207, 209 and 210 pepper (*Capsicum annuum* L.) lines were investigated. Plants were grown either under greenhouse or field condition at the Tokyo University of Agriculture and Technology. A total of 180 F_1 hybrids derived from the crosses between CMS and restorer lines were evaluated for pollen fertility, and morphological characters. F_2 generation was obtained from F_1 self-pollinated hybrids. The F_2 generation was deemed as one population exceeded the 203×210 population. For an easier maneuverability, this population was selected, in which both crossing partner were stably male sterile or male fertile under various environmental conditions. Pollen fertility was determined as the percentage of pollen stained with 1% of acetocarmin. Pollen was shaken from dehisced anthers into a drop of stain on a slide glass, and then counted under microscope. Pollen viability percentage was calculated using the "fertile/ fertile + sterile" quotient. Total DNA was extracted from young-fresh leaves using the crude nuclear pellet method [18]. Amplification was performed in ASTEC Program Temp Control System PC-700 machine, following an initial denaturation step at 94 ºC for 3 minutes. Amplification program was 35 cycles of 94 ºC for 30 seconds, 55 ºC for 1 min. 72 ºC for 1.5 minutes. For the PCR amplification restorer gene specific primers [19] and *atp9* primers were used. DNA fragments were separated in a mini-gel electrophoresis instrument on 1.5% agarose gel. Separated fragments were visualized by staining with ethidium-bromid and photographed under ultraviolet light.

RESULTS

The pollen fertility of the parental lines was checked during three growing seasons as given in Table 1. The CMS lines proved to be almost completely sterile. The sterile plants of the line No. 201 that did not produce completely viable pollens in summer were found to shed around 20 percent of pollen quantity in May. When those plants were transferred from the greenhouse to field conditions they stopped to produce fertile pollens. This indicates that the change from sterility to partial fertility is not a permanent alteration.

Table 1: Pollen fertility of the CMS and restorer parental lines
1. táblázat A hímsteril (CMS) és resztorer (Rf) szülõi vonalak pollen fertilitása

Parental lines	Pollen fertility (%)		
	May	June-July	August-September
201	21.07±7.03 (16)	0 (4)	0 (6)
202	0 (7)	0 (8)	0 (7)
203	0 (5)	0 (7)	0 (9)
204	0 (7)	0 (6)	0 (8)
205	0 (5)	0 (7)	0 (8)
206	48.73±4.81 (6)	53.85±14.16 (3)	only sterile
207	only sterile	43.99±14.33 (4)	22.19±10.13 (6)
209	35.47±7.07 (7)	66.52±7.26 (14)	77.00±4.87 (8)
210	52.08±7.16 (7)	56.25±13.55 (4)	69.30±7.42 (4)

(n)= number of investigated flowers (2 anthers x 2 replicates x 2 observation field)
(n)= a vizsgált virágok száma (2 antéra x 2 ismétlés x 2 megfigyelési terület)

The No. 206-restorer line showed relatively higher pollen fertility during two seasons (over 50 percent), but did not produce any viable pollen grains at the beginning of autumn. Probably the pollen mother cells reach a later stage of maturity due to delay or absence of the pollen abortion under higher temperature [5]. In the case of the No. 207 restorer line, we could not observe fertile pollen in May, and it showed a slight reduction in the production of pollens during all seasons. This restorer line resulted in the lowest pollen fertility percentage seasonally (43.99±14.33; 22.19±10.13, respectively).

From the eight possible combinations five hybrid populations were selected to form the base of the investigation for pollen viability. In all F1 hybrids fertility was restored. Within the hybrid populations, the combination of No. 204x209 showed the highest pollen fertility percentage evaluated monthly (data shown bimonthly in Table 2.), suggesting stronger restoration ability of the No. 209 restorer line as C (restorer) breeding line. The hybrids from the cross between lines No. 202 and 210 showed vestigial flower development in August and September. The first flowers appeared at the beginning of October, although the seeds were sown at the same time, together with other F1 populations. In October all the individuals were highly male fertile.

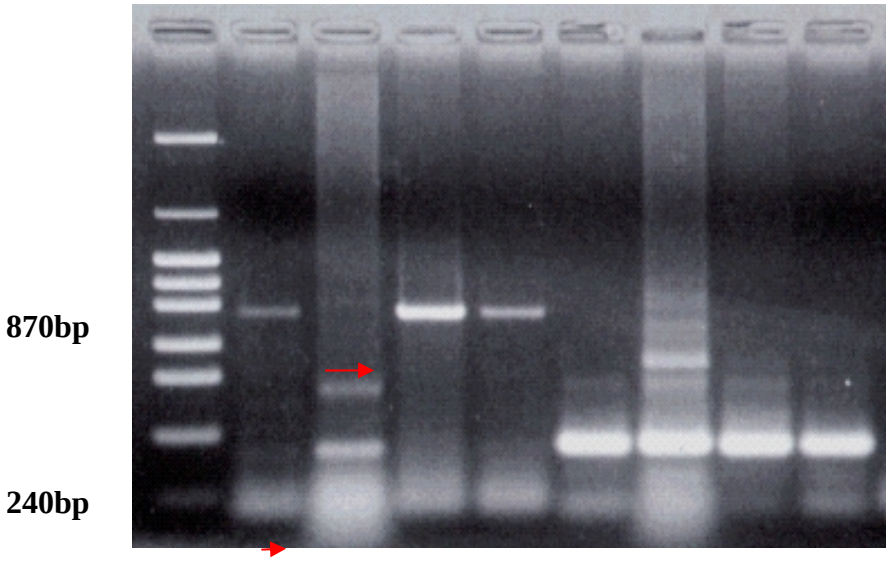
Table 2. Pollen fertility of the hybrid populations
2. táblázat A hibrid populációk pollen életképessége

Hybrid populations	Pollen fertility (%)	
	August-September	October
201 × 210	44.28±12.13 (11)	64.63±12.01 (8)
202 × 210	-	87.34±6.26 (12)
203 × 210	65.03±12.15 (8)	66.03±7.09 (12)
205 × 210	72.38±5.58 (8)	85.69±5.11 (12)
204 × 209	77.32±7.52 (12)	96.77±0.64 (4)

-: no flower, 202 × 210 F_1 did not developed flower in August and September
-: nem volt virág, a 202 × 210-hez tartozó egyedek csak augusztus szeptemberben hoztak virágot

DNA markers linked to the fertility restorer (Rf) gene for cytoplasmic male sterility in pepper were screened by bulked segregant analysis using the PCR method. To identify the DNA fragments linked to the Rf gene, six individuals from each line were arbitrarily selected for bulking. The PCR amplification results showed that the restorer gene specific markers [19] could be distinguished in fertile and CMS plants. Only the restorer specific DNA fragments of the restorer plants No. 206, 207, 209 and 210 were amplified. Further investigation of the fertile plants showed that the Rf gene specific primers produced different banding patterns. With the restorer specific primers an 870bp fragment was amplified for all the fertile restorer plants except one. The exceptional line No. 207 does possess a fragment of 240bp, which differs from the other restorer lines. This difference may suggest the recombinant nature of the line No. 207. The atp9 mitochondrial gene specific primer was used as inner control, whose amplification product does not affect the expression of fertility.

Figure 1: PCR amplification results of the restorer lines using the restorer gene specific (2-5 lanes) and mt specific *atp9* primers (6-9 lanes). Arrows point to the restorer specific 870bp fragment amplified in most of the restorer lines and the unique 240bp fragment, amplified only in the restorer line No. 207.



Approximately a hundred of progenies from the F2 population were arbitrarily selected for the same PCR analysis in order to characterize the restoration response. Identification of the marker linked to the restorer gene was conducted by analyzing these individuals. Using the restorer specific primers tested on the parental lines, the same PCR fragments specific to the Rf gene clearly appeared in the F2 individual plants. The 870bp fragment was amplified for the fertile restorer plants were identified in 75 individuals. On the other hand, in other individuals this specific fragment did not appeare. A hundred of F2 individuals were studied in total, segregated in the ratio of 75 presence of Rf marker: 25 absence of Rf marker (3:1, $\chi 2 = 0.01$, $0.9 < P < 0.95$). By the comparison of the pollen viability data obtained from the F2 individuals and the results of the gel analysis, the physical distance between the marker and the restorer gene was determined in 20cM.

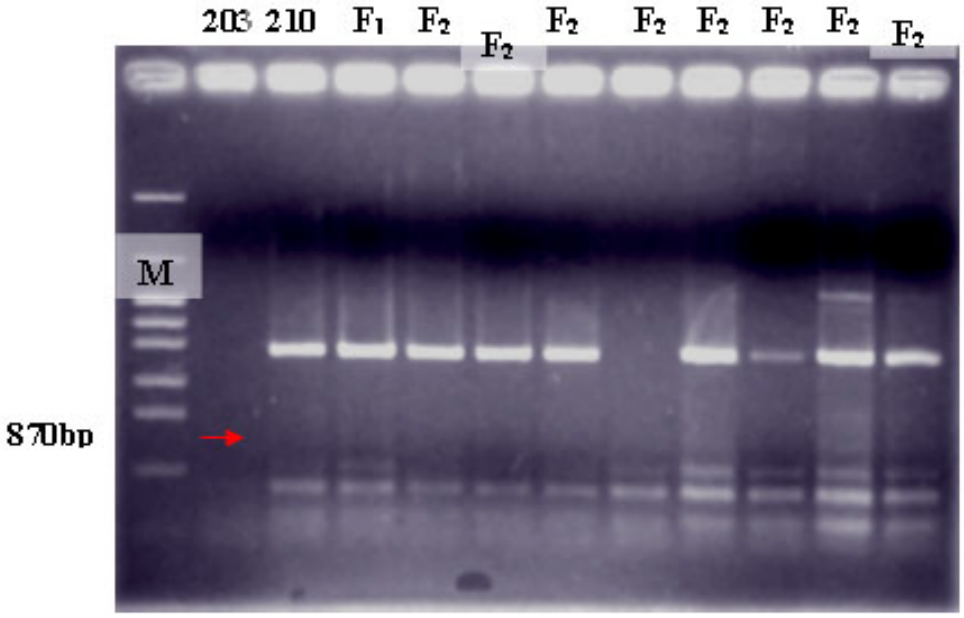
DISCUSSION

Pollen viability was investigated to study the response of male sterile plants or genes to different environmental conditions during three growing seasons. For the CMS lines No. 202, 203, 204 and 205 no viable pollen was formed, and this complete pollen sterility was hold stable in a wide range of environmental conditions. The fact, that these CMS plants maintained sterility under altered environmental conditions indicates non-sensitivity of the *ms* gene to different environments and the functional stability under changing environments. In

all of these lines a single recessive gene supposed to control male sterility.

Partial instability of pollen sterility of the CMS line No. 201 occurred in May under greenhouse conditions. The increased pollen production of this CMS line under cool conditions may be caused by (Figure 1) temporary alteration in the abortive properties of the sterile cytoplasm or (Figure 2) in the functioning of the gene products of the non-restorer *ms* gene. Being abolished the possible stress factors by transferring those plants from the greenhouse to the field, plants stopped to shed fertile pollens. This confirmed that the change from sterility to partial fertility is not a permanent alteration in the cytoplasm.

Figure 2. The fragments specific to the Rf gene clearly appeared in the F_2 individual plants. A hundred of F_2 individuals were studied in total, segregated in the ratio of 75 presence of Rf marker : 25 absence of Rf marker (3:1, χ^2= 0.01, 0.9 < P < 0.95).



The restorer line No. 206 shed pollen profoundly, but only sterile deformed pollens could be observed in August and September. The pollens of the restorer line No. 207 were phenotypically normal and stained well, but were non-functional. This might be the reason why successful crosses under current circumstances using these two restorer lines as male parents could not have been obtained.

For characterization of the restoration response, CMS lines were crossed to the restorer lines and F_1 hybrid populations were obtained. The fact, that all the F_1 hybrids were male fertile indicated the unequivocal presence of the nuclear fertility restorer gene in the male parents. Forming hybrid populations the restorer line No. 209 could only be used in one combination as male parent and resulted in the highest pollen fertility percentage, suggesting its stronger restoration ability as C-pollinator line. The restorer lines No. 209 and 210 are applicable for hybrid breeding.

Recently Yanagawa et al. [19] detected a molecular marker linked to the restorer gene in Capsicum sp.. This marker is applicable in the discrimination of CMS and restorer plants. By the application of these restorer specific primers, an approximate 870bp fragment was amplified from the bulks of restorer lines No. 206, 209, and 210. Screening of additional twenty individuals from the line No. 207 ascertained the recombination nature of this unique restorer line and moreover segregated. For these its usage as a restorer line in commercial hybrid pepper breeding is limited.

However the applied restorer specific primers were effective to distinguish the CMS from male fertile plants, the distance of the marker from the restorer gene indicates that a number of another *Rf* marker would be required to get closer to the restorer gene. An adequate linkage namely would offer the possibility to isolate the gene itself. On the other hand, searching CMS associated markers also would be desired, because up to now CMS-associated sequences in Capsicum genus have not been identified, yet. Specific sterility linked mitochondrial loci have been characterized in several species, but those genes share no significant sequence homology. Furthermore based on the recent results [7] CMS seems to show an unusual behavior in Capsicum sp. by a 14kb deletion was found in the mitochondria. By the exploration of these molecular genetic possesses, which control the CMS characters in pepper, would open new perspectives in the large-scale hybrid breeding.

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