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CMS-*Rf* Genotype of Resistance Sources to Gray Leaf Spot in Pepper (*Capsicum annuum* L.)

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A total of 19 selections derived from 4 sources of peppers with resistance to gray leaf spot (KC43, KC47, KC220, and KC319) were tested for their nuclear genotype of the gene conferring the ability to restore the cytoplasmic male sterility. All the selections derived from KC220 and KC319 were maintainers with a genotype of *Nrfrf*, while all the selections from KC43 and KC47 were restorers with a genotype of *N(S)RfRf*.

Keywords **:** *Capsicum annuum*, cytoplasmic male sterility, *Stemphylium lycopersici*, *S. solani*

Gray leaf spot caused by *Stemphylium solani* and *S. lycopersici* (Blazquez, 1969; Hannon and Weber, 1955; Sinclair et al., 1958; Weber, 1930) is found commonly in pepper fields in mountainous areas in Korea such as northern part of Gyeongbuk and Gangwon provinces (Kim et al., 2004; Yu, 2001). Symptoms include tiny, numerous spots, usually less than 2 mm in diameter, forming mainly on the leaves and occasionally on the calyx and pedicels of the pepper fruits. The disease may be controlled by spraying chemicals, but resistance to the disease is conferred from many different sources (Cho et al., 2001). Therefore, it was thought to be necessary to develop resistant cultivars for an environmentally friendly and labor-saving system of pepper production. Almost of the pepper cultivars grown in Korea are F_1 hybrids, and the majority of the hybrid seeds are produced by utilization of cytoplasmic male sterility (CMS) (Yu, 1990). The CMS-system consists of three lines. The male sterile line is often called the A-line and holds a male sterile cytoplasm and recessive nuclear genotype for restore-gene (*Rf*), and expresses *Srfrf*, *S* designating male sterile cytoplasm. Maintainers of the male sterile lines are called B-lines and carry normal cytoplasm and the recessive nuclear genotype for *Rf*-gene forming *Nrfrf* genotype. The restorers for the CMS lines are called C-lines and carry the dominant *Rf* gene in the homozygote with normal or sterile cytoplasm being expressed as either *NRfRf* or the *SRfRf* genotype, respectively, both of which are expressed together as *N(S)RfRf*. Male sterile lines (*Srfrf*) are maintained and reproduced by crossing them with the maintainers (*Nrfrf*).

In hybrid-seed production, male sterile lines (*Srfrf*) are crossed with the restorers (*N(S)RfRf*) to produce male fertile F_1 hybrids (*SRfrf*). Therefore, genetically pure genetic resources and breeding lines are classified as either maintainers (*Nrfrf*) or restorers (*N(S)RfRf*) depending on their nuclear genotype for the CMS-restoring gene, *Rf* (Hwang and Kim, 1997, 2002; Kim and Hwang, 1998; Kim et al., 2004; Shifriss, 1997; Yu, 1990). Nuclear genotyping of the sources of resistance to gray leaf spot is crucial for planning a program to breed cultivars resistant to the disease. The current study identified the nuclear genotype of the sources of resistance to gray leaf spot with respect to fertility-restoring gene (*Rf*) for male sterile cytoplasm.

Materials and Methods

Selection from the sources of resistance and making crosses. Selections from the sources of resistance to *Stemphylium* spp. (Cho et al., 2001) were evaluated by inoculation and for uniformity again in 2003 for resistance to the disease. The uniform selections were transplanted to a greenhouse and were crossed with a cytoplasmically male sterile line, Chilseong-A (*Srfrf*) in order to identify the nuclear genotype of the *Rf* gene, which confers the ability to restore cytoplasmic male sterility.

Confirmation of resistance to the gray leaf spot disease for parental lines and F_1 hybrids. The seeds of the parental lines and the F_1 hybrids between 'Chilseong-A' and the resistant selections were sown in a growing mix in 128-cell trays on December 8, 2003. The number of the F_1 hybrid seeds sown was the double of that of the parental lines. One month after sowing, the seedlings were transplanted to the same growing medium of 32-cell trays (16 plants each line). The seedlings of the F_1 hybrids were divided into two sets of 16 plants, one for evaluation of resistance to the gray leaf spot disease and the other for testing fertility. The seedlings of the parental line and the

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F_1's for testing resistance were inoculated with a spore suspension of *Stemphylium lycopersici* 45 days after seeding. Sporulation of *S. lycopersici* was induced as previously described (Kim et al., 2004). The V-8 juice agar plates were initially seeded with mycelial pieces of a pure isolate of *S. lycopersici*. The inoculated plates were cultured on a shelf under a 12 hr light/dark cycle with fluorescent light, in a temperature range between 16 and 25°C. As the fungal colonies sporulated, mycelial plugs with spores were removed from the actively sporulating area of the mycelial mats by a cork borer. The mycelial plugs were put in sterile distilled water in test tubes. The tubes were agitated vigorously so as to shed spores from the mycelial plugs. For the mass production of spores, the V-8 juice agar plates were seeded by flooding the plates with the spore suspension, which was decanted from the tubes. The extra spore suspension was removed from the plates. Then, the plates were cultured under the same conditions as described for the initial culturing. As the culture sporulated abundantly for 4 days after seeding, the spores were washed from the culture plates to make spore suspension by use of a camel-hair brush with sterile distilled water. The spore suspension was filtered through 4 layers of cheese cloth to separate sporangiophores and agar pieces. Pepper seedlings were inoculated by spraying the spore suspension on the seedling foliage and incubating the inoculated plants in a hot bed, heated by an electric heat cable and covered with a sheet of plastic film and a blanket for 48 hrs. The temperature range in the hot bed during the incubation was similar to the temperature range for sporulation. After incubation, the plants were handled as usual in the hot bed: they were covered with a plastic film and a blanket in the evening. The disease was scored 10 days after inoculation, using a 1-5 scale based on the number of spots and the level of discoloration on the most diseased leaf of a plant; 1=no spots found; 2=one to three spots; 3=4 to 6 spots; 4=7 or more spot but no discoloration; 5=7 or more spots with discoloration.

Testing fertility and quantification of pollen. As the plants begin to bloom, the F_1 hybrid plants were examined for fertility by observing the anthers and by quantifying pollens per anther to identify the *Rf* genotype of the pollen parents. Fertility of the F_1 plants was determined by visual observation with an aid of magnifier on the basis of production of pollen or not. Quantification of the amount of pollen per anther was done according to the method described by Yu (1990). Five plants per line were randomly chosen for quantification of pollen. An anther that was about to dehisce was picked from a mature flower bud on the plants and put in a test tube. Tubes with an individual anther were then incubated at 25°C for 24 hours so that the anthers could split open. The tubes were then lightly shaken on a vortex mixer to shed pollen from the anther. Then 1 ml of acetocarmine solution was dropped into the test tube and mixed with the pollen by shaking. Five microliters of the pollen suspension in acetocarmine solution was taken with a micropipet, dropped onto a slide glass, and covered with an 18×18 mm cover slip. The cover slip exactly covered the surface area of the 5 μl solution. The mean of five cover glass area counts from the five plants was taken, and the number of pollen per anther was calculated by multiplying the mean by 200.

Results and Discussion

Selections crossed to a cytoplasmic male sterile line, Chisleong-A, were tested for nuclear genotype identification and again for resistance to gray leaf spot for confirmation and reference. The results of testing parental lines for resistance are given in Table 1. All of the selections from KC220, one selection each from KC319, and KC43 (PI241670) remained disease-free. Although pin-point specks were occasionally found, they remained arrested up to the end of the experiment. The rest of the selections from

Table 1. Gray leaf spot index of selections of resistance sources 10 days after inoculation with *S. lycopersici*

Resistant parent	Origin	No. plants tested	Disease index[a]
KC220-1-2-2-1	Beopjeon, Bonghwa	16	1.0 a[b]
KC220-1-2-2-3		16	1.0 a
KC220-1-2-2-4		16	1.0 a
KC220-1-5-3-1		16	1.0 a
KC220-1-5-3-2		16	1.0 a
KC220-1-5-3-3		16	1.0 a
KC220-1-6-3-2		16	1.0 a
KC220-1-6-4-3		16	1.0 a
KC220-1-6-5-4		16	1.0 a
KC319-1-2-3	Unknown	14	1.0 a
KC43-3	PI241670	16	1.0 a
KC319-1-3-1	Unknown	16	1.1 ab
KC319-1-3-3	"	16	1.1 ab
KC319-1-1-1	"	16	1.2 abc
KC319-1-3-4	"	16	1.2 abc
KC319-1-2-1	"	16	1.3 bcd
KC47-1	PI244670	16	1.3 cd
KC319-1-4-2	Unknown	16	1.4 d
Chilseong	Illwol, Youngyang	16	5.0 e

[a]Disease index of the most diseased leaf on a plant: 1, no spots; 2, 1 to 3 spots; 3, 4-6 spots; 4, 7 or more spots formed but no yellowing; 5, 7 or more spots on a leaf with discoloration and defoliation.
[b]Mean separation within column by Duncan's multiple range test, $P \leq 0.05$.

Table 2. Gray leaf spot index of F_1's between susceptible 'Chilseong-A' and selections of resistance sources 10 days after inoculation with *S. lycopersici*

Common maternal parent	Pollen parent	No. plants tested	Disease index[a]
Chilseong-A	KC220-1-5-3-1	16	2.8 a[b]
	KC220-1-2-2-3	16	3.3 b
	KC220-1-6-2-3	16	3.3 b
	KC220-1-2-2-4	16	3.5 c
	KC220-1-6-2-4	16	3.5 c
	KC220-1-5-3-2	16	3.6 c
	KC319-1-1-3	16	3.6 c
	KC319-1-1-1	16	3.7 c
	KC319-1-3-1	16	4.0 d
	KC319-1-3-3	16	4.0 d
	KC43-3-4	16	4.0 d
	KC47-1-1	16	4.0 d
	KC47-1-2	16	4.0 d
	KC47-1-3	16	4.0 d
	KC47-1-4	16	4.0 d
	KC43-3-1	16	5.0 e
	KC43-3-2	16	5.0 e
	KC43-3-3	16	5.0 e
Chilseong-B		16	5.0 e

[a]Disease index of the most diseased leaf on a plant: 1, no spots; 2, 1 to 3 spots; 3, 4-6 spots; 4, 7 or more spots formed but no yellowing; 5, 7 or more spots on a leaf with discoloration and defoliation.
[b]Mean separation within column by Duncan's multiple range test, $P \leq 0.05$.

Table 3. Fertility of F_1 plants between Chilseong-A (*Srfrf*) and selections of sources of resistance to gray leaf spot in *Capsicum annuum*

Maternal parent	Pollen parent	Fertility of F_1 plants		No. pollen/ anther ($\times 10^3$)	Pollen parent genotype
		MS	MF		
Chilseong-A[a]	KC220-1-2-2-3	16			*Nrfrf*
	KC220-1-2-2-4	16			*Nrfrf*
	KC220-1-5-3-1	16			*Nrfrf*
	KC220-1-5-3-2	16			*Nrfrf*
	KC220-1-5-3-3	16			*Nrfrf*
	KC220-1-6-2-3	16			*Nrfrf*
	KC220-1-6-2-4	16			*Nrfrf*
	KC319-1-1-1	16			*Nrfrf*
	KC319-1-1-3	16			*Nrfrf*
	KC319-1-3-1	16			*Nrfrf*
	KC319-1-3-3	16			*Nrfrf*
	KC43-3-1		16	12.2	*N(S)RfRf*
	KC43-3-2		16	7.1	*N(S)RfRf*
	KC43-3-3		16	9.0	*N(S)RfRf*
	KC43-3-4		16	11.5	*N(S)RfRf*
	KC47-1-1		16	10.0	*N(S)RfRf*
	KC47-1-2		16	9.4	*N(S)RfRf*
	KC47-1-3		16	10.3	*N(S)RfRf*
	KC47-1-4		16	11.1	*N(S)RfRf*
Chilseong-B		16			*Nrfrf*

[a]Chilseong-A and Chilseong-B are isogenic lines carrying *Srfrf* and *Nrfrf* genotype, respectively.

KC319 and KC47-1(PI244670) developed only a few arrested spots on the leaves. 'Chilseong', a local cultivar in Yeongyang in Gyeongbuk province that we used as a susceptible check, was severely infected with numerous spots and defoliation at the end of the study period.

The F_1's between a cytoplasmic male sterile 'Chilseong-A' line (*Srfrf*) and the above lines selected from the resistance sources are given in Table 2. The F_1 plants had formed many spots and the leaves were defoliating. They were close to the susceptible maternal parent in susceptibility, suggesting that resistance to gray leaf spot tends to be recessive. The F_1 plants whose paternal parents were selections from KC220 appeared to be somewhat less affected by the disease, suggesting that KC220 is more resistant to gray leaf spot than KC43, KC47, and KC319. The fact that resistance tends toward the recessive side suggests that a highly resistant F_1 hybrid can be obtained only when both parents are resistant to the disease. Further study is necessary to elucidate the mode of inheritance of resistance to this disease. Although *S. lycopersici*, one of the two species of the fungal pathogens causing the same disease symptoms, was used in this study, resistance is expected to be true to other species, *S. solani*, because it had been already proven before (Cho et al., 2001).

The fertility rates of the F_1 plants between a cytoplasmic male sterile, 'Chilseong-A', and the selections from the resistance sources are shown in Table 3. Selections derived from KC220 and KC319 resulted in all male sterile F_1 plants. Therefore, they were classified as maintainers with *Nrfrf* genotype. The F_1 plants between a common male sterile parent, 'Chilseong-A', and selections derived from KC43 and KC47 were all fertile males. Therefore, selections from KC43 and KC47 were identified as restorers with *N(S)RfRf* genotype. As expected, plants of the cross between Chilseong-A (*Srfrf*) and Chilseong-B (*Nrfrf*), which was included as a check and reference, were all sterile males.

The amount of pollen on an anther of the fertile crosses ranged from 7,120 to 12,200. It is not a new discovery that KC43 and KC47 are restorers. KC43 and KC47 are similar with each other and are also resistant to bacterial spots caused by *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. Their nuclear genotypes have been reported as sources of resistance to bacterial spot (Kim and Hwang,

1998). In conclusion, KC220 and KC319 may be considered first as sources of resistance in breeding maintainers for resistance to gray leaf spot, and KC43 and KC47 first in breeding restorers. This is in contrast to sources of resistance to *Phytophthora capsici* and to bacterial spot. The nuclear genotype, all of which are restorers (*N(S)RfRf*) (Hwang and Kim, 1997; Kim and Hwang, 1998). In such a case, additional effort for back-crossing to maintainer and testing for the nuclear genotype of the breeding lines is necessary to obtain maintainers that are resistant to the respective diseases as reported by Hwang et al. (2004).

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