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Near-Isogenic Lines for Genes Conferring Hypersensitive Resistance to Bacterial Spot in Chili Pepper

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In order to develop chili pepper bacterial spot resistant cultivars and near-isogenic lines (NILs) to prompt the molecular mapping of the resistance gene, we have run backcross breeding program since 1994. Two resistance genes against *Xanthomonas axonopodis* pv. *vesicatoria* *Bs2* from Fla. XVR 3-25 and *Bs3* from our breeding line 25-11-3-2, were introduced into a land race, Chilseong-cho (abbreviated to Chilseong hereafter) with good fruit guality. We report here the testing of BC_4F_3 to BC_4F_5. We found that BC_4F_5 lines of the crosses were homozygous with respect to the respective genes of introduction. The lines, in which *Bs2* gene was introduced, were hypersensitively resistant to both race 1 and race 3 of *X. axonopodis* pv. *vesicatoria*, whereas, those in which *Bs3* was introduced were resistant to race 1.

Keywords : breeding, *Chili pepper*, resistance, *Xanthomonas axonopodis* pv. *vesicatoria*

Bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria* is an important disease causing considerable damage on pepper (*Capsicum annuum*) in Korea. Hypersensitive resistance to the pathogenic bacterium is known as a good example of vertical resistance and gene-for-gene model. Hypersensitive resistance was found in PI163192, PI260435, PI271322, and PI235047 (Cook and Guevara, 1984; Cook and Stall, 1963; Sahin and Miller, 1998) and the genes conferring the hypersensitive resistance in PI163192, PI260435, and PI271322 were designated as *Bs1, Bs2*, and *Bs3*, respectively. In addition to the hypersensitive resistance, race-nonspecific general resistance has been found in PI241670, PI244670, 369994, and even in PI163192 and PI271322 where esistance genes were found (Kim, 1988; Sowell, 1960; Sowell and Dempsey, 1977).

A gene-for- gene model was observed between the hypersensitive genes in the host plants and virulence gene in the causal bacterium. Currently, isolates of *X. axonopodis* pv. *vesicatoria* may be classified into pathotype P0 to P8 on the basis of interaction between the HR genes and the bacterial strains (Kousik and Ritchie, 1998; Sahin and Miller, 1998). In Korea, however, race 2 of *X. axonopodis* pv. *vesicatoria* has not been but race 1 and race 3 were described. Some other pathotypes were not identified yet (Kim et al., 1990; Pae et al., 1994).

We have run a backcross breeding program to introduce *Bs2* from Fla. XVR 3-25 and *Bs3* from our breeding line, 25-11-3-2 (Kim et al., 1996), into a land race, Chilseong with good fruit quality since 1994 on dual purpose to develop bacterial spot resistant cultivars and near-isogenic lines (NILs) for molecular biologists studying markers linked with the resistance genes. We report here the results of selection from BC_4F_3 to BC_4F_5 generations of the crosses for homozygosity for the respective resistance genes in the breeding lines.

Materials and Methods

Initial crosses and backcrossing. The initial crosses were made between a susceptible local cultivar in Youngyang, Chilseong, and two breeding lines with different hypersensitive resistance genes, KC298, which is Fla. XVR 3-25 carrying *Bs1* and *Bs2* genes (Cook, 1984), and 25-11-3-2, which is a breeding line carrying *Bs3* gene (Kim et al., 1996). A backcross method of breeding procedure was applied to the crosses for transfer of the *Bs2* from KC298 and *Bs3* from 25-11-3-2 into Chilseong up to BC_4 during the period from 1997 to 2001. The BC_4 lines were self-pollinated, and the resultant BC_4F_2 lines were tested for hypersensitive resistance and hypersensitive resistant plants were selected to produce BC_4F_3 lines in 2002.

Bacterial strains used and culture of the bacterium. A strain of *X. axonopodis* pv. *vesicatoria* race 1, which was originally isolated from infected pepper plants in Milryang in 2002 and identified as race 1 on the basis of reaction on differential hosts, and a strain of race 3, which was originally isolated from Kyungpook National University experimental farm and identified as race 3, were used in testing for resistance in the study followed.

The bacterial isolates were routinely grown on Yeast extract Dextrose Calcium carbonate (YDC) agar (Dhingra

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Fig. 1. Water-soaked spots developed on the leaves of susceptible plants (Left) and necrotic spots on the leaves of plants with hypersensitive resistance (Right).

Fig. 2. Infiltration of bacterial suspension into leaf tissue through stomata with syringe without needle (Left), susceptible reaction 1 week after infiltration (Center), and hypersensitive reaction (HR) (Right).

and Sinclair, 1995) medium for 48 hrs at 28°C and bacterial cell suspensions were prepared by collecting the bacterial culture into distilled water. Density of the bacterial cells in the suspension was adjusted to approximately 10^8 cells per ml by measuring the optical density (OD) by spectrometer, comparing the OD with OD-viable count curve, and appropriate dilution.

Selection of true breeding BC_4F_3 lines for hypersensitive resistance in 2002-2003. Seeds of BC_4F_3 lines, parents and additional controls were sown in a commercial mix in 128 cell-trays on October 10 of 2002. The seedlings were transplanted to 32-cell trays filled with the commercial mix, GreenTech , about 1 month after sowing. The plants were inoculated with race 1 and race 3 of *X. axonopodis* pv. *vesicatoria* 45 days after sowing. The bacterial suspensions of race 1 and race 3 were infiltrated into separate leaves on a plant by pressing through stomata on abaxial side of leaves with syringes without needle. The inoculated plants were kept on the greenhouse bench after inoculation. Hypersensitive reaction of the plants was recorded by observation of the leaves from the third day to the seventh day after inoculation. After recording the data, segregating lines were discarded and single plants were selected in the lines breeding true in the hypersensitive reaction. The selected plants were transplanted to pots of 30 cm in diameter and grown for producing seeds of the next generation.

Confirmation of uniformity in HR genes in BC_4F_4. BC_4F_4 seeds were sown in a commercial mix, TKS-2, in a 50-cell tray on August 12, 2003. The plants were inoculated with race 1 of *X. axonopodis* pv. *vesicatoria* on 24th of September by the infiltration method as previously described. The disease was read 72 hrs after inoculation and the plants were classified into hypersensitive and non-hypersensitive or susceptible. Occasionally obscure reactions were found and the lines with obscure reactions were discarded.

Confirmation of uniformity in HR genes in BC_4F_5. Seeds

of the BC_4F_5 lines of the crosses between 'Chilseong' and KC297 (Fla. XVR 3-25) (Cook, 1984) and breeding line were sown in 'Wonjo mix' (Nongkyung Co., Ltd) in 72-cell trays in the greenhouse on Dec. 30, 2005. The seedlings at 4 to 5 true leaf stage were sprayed thoroughly with a cell suspension (10^8 cells per ml) of race 1 of *X. axonopodis* pv. *vesicatoria* on February 13, 2006, and the inoculated plants were incubated in the greenhouse bed by covering with plastic film and blanket for 48 hrs. The plants were grown as usual in the greenhouse beds thereafter, but covering the bed with plastic film and blanket in the evening and heating the wet mattress at the bottom of the beds induced the beds a humid and favorable condition for disease development. Disease was examined 10 days after inoculation. The plants were first classified into hypersensitive and non-hypersensitive plant based on the appearance of hypersensitive respone. Non-hypersensitive plants of them were graded into 1 to 5 degrees of disease severity on the basis of number and size of spots formed.

The plants were transplanted to 32-cell trays filled with 'Wonjo mix' after disease reading. Then, race 3 of *X. axonopodis* pv. *vesicatoria* was infiltrated into leaf tissues of the plants by syringe as described ahead. Reaction to the inoculation was classified into either hypersensitive or non-hypersensitive reaction 7 days after infiltration.

Results and Discussion

Reactions of BC_4F_3 lines to inoculation of race 1 and race 3 of *X. axonopodis* pv. *vesicatoria* are given in Table 1. In

Table 1. Segregation for hypersensitive resistance to race 1 and race 3 of *Xanthomonas axonopodis* pv. *vesicatoria* in BC_4F_3 lines of crosses between a susceptible cultivar, Chilseong and two resistant parents, KC297 and 25-11-3-2, carrying *Bs2* and *Bs3* genes, respectively, conferring hypersensitive resistance

Lines	Race 1				Race 3			
	Freq. of		Exp.[b]	X^2	Freq. of		Exp.	X^2
	HR[a]	NHR			HR	NHR		
BC_4F_3 (Chilseong × KC297)-1-2	13	6	3:1	0.44ns[c]	13	6	3:1	0.44ns
BC_4F_3 (Chilseong × KC297)-1-3	15	7	3:1	0.54ns	15	7	3:1	0.54ns
BC_4F_3 (Chilseong × KC297)-1-4	20	0			20	0		
BC_4F_3 (Chilseong × KC297)-1-5	17	7	3:1	0.22ns	17	7	3:1	0.22ns
BC_4F_3 (Chilseong × KC297)-1-7	15	5	3:1	0	15	5	3:1	
BC_4F_3 (Chilseong × KC297)-1-12	16	6	3:1	0.06ns	16	6	3:1	0.06ns
BC_4F_3 (Chilseong × KC297)-3-2	16	5	3:1	0.02ns	16	5	3:1	0.02ns
BC_4F_3 (Chilseong × KC297)-3-3	8	4	3:1	0.44ns	8	4	3:1	0.44ns
BC_4F_3 (Chilseong × KC297)-3-5	13	3	3:1	0.33ns	13	3	3:1	0.33ns
BC_4F_3 (Chilseong × KC297)-3-6	18	5	3:1	0.13ns	18	5	3:1	0.13ns
BC_4F_3 (Chilseong × KC297)-3-8	9	6	3:1	1.8ns	9	6	3:1	1.8ns
BC_4F_3 (Chilseong × KC297)-3-9	9	0			9	0		
BC_4F_3 (Chilseong × KC297)-3-12	16	7	3:1	0.36ns	16	7	3:1	0.36ns
BC_4F_3 (Chilseong × 25-11-3-2)-3-1	16	0			1	15		
BC_4F_3 (Chilseong × 25-11-3-2)-3-2	15	4	3:1	0.16ns	0	19		0.16ns
BC_4F_3 (Chilseong × 25-11-3-2)-3-3	16	9	3:1	1.61ns	0	25		1.16ns
BC_4F_3 (Chilseong × 25-11-3-2)-3-4	8	5	3:1	1.26ns	0	13		1.26ns
BC_4F_3 (Chilseong × 25-11-3-2)-3-5	17	0			0	17		
BC_4F_3 (Chilseong × 25-11-3-2)-3-6	21	0			0	21		
BC_4F_3 (Chilseong × 25-11-3-2)-3-8	9	4	3:1	0.23ns	0	13		
BC_4F_3 (Chilseong × 25-11-3-2)-3-9	12	2	3:1	0.86ns	0	14		
BC_4F_3 (Chilseong × 25-11-3-2)-3-11	7	0			0	7		
BC_4F_3 (Chilseong × 25-11-3-2)-3-12	19	0			0	19		
KC297-1-5	31	1			30	2		
25-11-3-2	43	0			0	43		
Chilseong	0	10			0	10		

[a]HR = hypersensitive; NHR = non-hypersensitive.
[b]Expected segregation ratio.
[c]Not significant at p=0.05.

general, about two thirds of the BC_4F_3 lines were segregating, and one third of them were breeding true in hypersensitive reaction as expected. Lines derived from cross Chilseong × KC297 responded the same to both race 1 and race 3 of *X. axonopodis* pv. *vesicatoria* as expected, since the hypersensitive plants are expected to carry *Bs2* gene, which is effective against both races. Lines derived from cross Chilseong × 25-11-3-2 were segregating or true breeding to race 1 but all susceptible to race 3 as expected since *Bs3* gene under transfer is effective to race 1 only. Segregating lines were well fitting to the expected 3:1 ratio of hypersensitive to susceptible reactions in lines of the both crosses. Single plants were selected among the plants in the lines breeding true in hypersensitive resistance to either or both races.

The results of testing BC_4F_3 are presented in Table 2. Only race 1 of the pathogen was used in inoculation because uniformity was only concern of the study. All the lines derived from either Chilseong × KC297 or Chilseong × 25-11-3-2 were uniform in their reaction to race 1 since *Bs2* and *Bs3* under transfer confer hypersensitivity to race 1 of the pathogen.

All the BC_4F_5 lines bred by introduction of *Bs2* from KC297 (Fla. XVR 3-25) (Cook, 1984) into Chilseong were uniformly hypersensitive to both race 1 and race 3 of *X. axonopodis* pv. *vesicatoria*, indicating that the lines were fixed with respect to *Bs2*. All the lines bred by introduction of *Bs3* from 25-11-3-2 (Kim et al., 1996) into Chilseong were also uniformly hypersensitive to race 1 of *X. axonopodis* pv. *vesicatoria* but non-hypersensitive to race 3. However, Chilseong and commercial cultivars included as control cultivars developed water-soaked spots at first and then prematurely defoliated. KC177, which was included in the experiment as a non-hypersensitive but quantitatively resistant control, remained almost disease-free. KC177 was originally received as PI163192 from Southern Regional Plant Introduction Station in Georgia, U.S.A, but was not hypersensitive as reported by Cook and Stall (1963) although it was highly resistant.

KC297 (Fla. XVR 3-25) is known to have *Bs1* and *Bs2* genes (Cook, 1984) but race 2 of *X. axonopodis* pv. *vesicatoria*, to which *Bs1* gene induces hypersensitive reaction, is not present in Korea. So, *Bs1* gene could not be identified for selection or for incorporation together into Chilseong.

Thus, the recurrent parent, Chilseong, and the breeding lines with either *Bs2* or *Bs3* would correspond to near-isogenic lines. The near-isogenic lines may be used as differential hosts in identification of pathotypes of *X. axonopodis* pv. *vesicatoria* and as materials for molecular research for host-parasite interaction in addition to as a new variety. The value of the new breeds for commercial growing is very limited due to susceptibility to some other diseases including viral diseases. The lines where *Bs3* gene was introduced will be resistant to race 1 only, so their value for genetic control of the disease is even lower because *Bs3* is effective against race 1 only but not against race 3. However, they may be used as building blocks in breeding for multiple disease resistance.

Table 2. Segregation for resistance to *Xanthomonas axonopodis* pv. *vesicatoria* race 1 of BC_4F_4 lines of cross Chilseong × 25-11-3-2 and cross Chilseong × KC297

Line	R gene contained	Reaction frequency of plants	
		HR[a]	NHR[a]
BC_4F_4(Chilseong × KC297)-1-4-1	*Bs2*	13	0
BC_4F_4(Chilseong × KC297)-1-4-2	*Bs2*	16	0
BC_4F_4(Chilseong × KC297)-3-9-1	*Bs2*	24	0
BC_4F_4(Chilseong × KC297)-3-9-2	*Bs2*	17	0
BC_4F_4(Chilseong × KC297)-3-9-3	*Bs2*	21	0
BC_4F_4(Chilseong × 25-11-3-2)-3-5-1	*Bs3*	24	0
BC_4F_4(Chilseong × 25-11-3-2)-3-5-2	*Bs3*	25	0
BC_4F_4(Chilseong × 25-11-3-2)-3-5-3	*Bs3*	25	0
BC_4F_4(Chilseong × 25-11-3-2)-3-6-1	*Bs3*	23	0
BC_4F_4(Chilseong × 25-11-3-2)-3-6-2	*Bs3*	23	0
BC_4F_4(Chilseong × 25-11-3-2)-3-6-3	*Bs3*	19	0
BC_4F_4(Chilseong × 25-11-3-2)-3-6-4	*Bs3*	22	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-1	*Bs3*	21	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-2	*Bs3*	15	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-3	*Bs3*	11	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-4	*Bs3*	21	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-5	*Bs3*	22	0
BC_4F_4(Chilseong × 25-11-3-2)-3-11-6	*Bs3*	23	0
BC_4F_4(Chilseong × 25-11-3-2)-3-12-1	*Bs3*	20	0
BC_4F_4(Chilseong × 25-11-3-2)-3-12-3	*Bs3*	23	0
KC297	–	22	0
25-11-3-2	–	15	0
Chilseong	–	0	75

[a]HR = hypersensitive; NHR = non-hypersensitive.

The hypersensitive resistance genes were introduced into bell pepper varieties in Florida but the resistance genes was readily overcome by new races. Thus, in view of genetic control of the disease, effectiveness of hypersensitive resistance alone was very limited. Therefore, integration of the hypersensitive genes with quantitative but race-non-specific resistance will be necessary for breeding varieties for more durable and dependable resistance.

For integration of hypersensitive and quantitative resistance, backcross method would not be an appropriate procedure, but recurrent selection, pedigree or bulk method, or combination of them would be more promising alter-

Table 3. Reaction to race 1 and race 3 of *Xanthomonas axonopodis* pv. *vesicatoria* of BC_4F_5 lines of the crosses between Chilseong and sources of hypersensitive resistance genes, *Bs1* and *Bs3*

Line or cultivar	R gene contained	Reaction frequency of plants to pathotype			
		Race 1		Race 3	
		HR[a]	NHR[a]	HR	NHR
BC_4F_5 (Chilseong × KC297)-1-4-1-2	*Bs2*	12	0	8	0
BC_4F_5 (Chilseong × KC297)-1-4-2-2	*Bs2*	12	0	8	0
BC_4F_5 (Chilseong × KC297)-3-9-1-1	*Bs2*	12	0	8	0
BC_4F_5 (Chilseong × KC297)-3-9-2-1	*Bs2*	12	0	8	0
BC_4F_5 (Chilseong × KC297)-3-9-3-2	*Bs2*	12	0	8	0
BC_4F_5 (Chilseong × 25-11-3-2)-3-5-2-2	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-5-3-2	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-5-5-1	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-5-6-2	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-6-1-2	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-6-3-2	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-1-1-1	*Bs3*	12	0	0	8
BC_4F_5 (Chilseong × 25-11-3-2)-3-12-4B	*Bs3*	12	0	0	8
KC297 (Fla. XVR 3-25)	*Bs2*	12	0	8	0
25-11-3-2-4-6	*Bs3*	12	0	0	8
KC177 (PI163192)	–	0	12 (1.8)[b]	0	8
Chilseong	–	0	12 (5.0)	0	8
Cheonhatongil	–	0	12 (4.9)	0	8
Daejangbu	–	0	12 (5.0)	0	8
Dokyacheongcheong	–	0	12 (5.0)	0	8
Yeokganghongjangun	–	0	12 (5.0)	0	8
PR Gangja	–	0	12 (5.0)	0	8
PR Data	–	0	12 (4.4)	0	8
Yeongyang Mat	–	0	12 (4.0)	0	8

[a]HR = hypersensitive; NHR = non-hypersensitive.
[b]Value in parenthesis, 1 = No lesion; 2 = pin-point or arrested spot; 3 = spots with halo or less than or equal to 3 mm in diameter; 4 = spots larger than 3 with water or oil-soaked edge; 5 = many type-4 spots with coalition.

natives (Bartual et al., 1991; Palloix et al., 1990). Pepper cultivars that most Korean farmers are cultivating are F_1 hybrids. The F_1 hybrid seeds are produced by manipulation of cytoplasmic male sterility. Therefore, hypersensitive resistance genes may be introduced into male sterile parent and general resistance may be introduced into male fertile paternal parent or vice versa to produce F_1 hybrid with more durable resistance.

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