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Divergence and allelomorphic relationship of a soybean virus resistance gene based on tightly linked DNA microsatellite and RFLP markers

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Abstract The use of genetically diverse resistance sources is important in breeding for durable disease resistance. Detection and evaluation of resistance genes by conventional inheritance experiments, however, often require laborious screening and genetic testing. In the present study, a marker-assisted screening for resistance sources was initiated in soybean [*Glycine max* (L.) Merr] using one DNA microsatellite and two RFLP markers tightly linked to a soybean mosaic virus (SMV) resistance gene (*Rsv1*). The three marker loci were used to screen 67 diverse soybean cultivars, breeding lines, and plant introductions. Five variants were found at the microsatellite locus (HSP176L), and the two RFLP loci (pA186 and pK644a) near *Rsv1* show a remarkably higher level of restriction polymorphism than *Rsv1*-independent RFLP loci. Several specific variants at the three marker loci were found to be correlated with virus resistance, among which HSP176L-2 can be detected by PCR, thus may be useful for germplasm screening. The grouping of the 67 accessions according to their multilocus marker variants agrees with the available pedigree information. When all, or most, of the cultivars within a given group with the same *Rsv1*-linked marker variant are resistant, their SMV resistance is most likely conferred by *Rsv1*. These putatively *Rsv1*-carrying groups contain a total of 38 SMV-resistant lines including six differential cultivars that are known to carry *Rsv1*. The remaining seven resistant accessions (Columbia, Holladay, Peking, Virginia, FFR-471, PI 507403, and PI 556949) do not carry resistance marker variants, and at least some of them could be sources of resistance genes independent of *Rsv1*.

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Introduction

The use of genetically diverse resistance sources is a key to breeding for durable resistance to pests and diseases. Identification of different resistance genes in germplasm, however, usually requires allelism tests which involve extensive crossing and progeny testing. Therefore, a more efficient method of screening and evaluating host resistance genes would be desirable.

Closely linked RFLP (restriction fragment length polymorphism) marker loci have been identified for an increasing number of resistance genes in various crops. Unless they are separated by recombination, alleles at these marker loci are expected to be transmitted together with the targeted resistance genes as a chromosomal block. DNA markers provide the best alternatives or supplements to conventional disease screening, provided they can differentiate resistance alleles without the use of multiple pathogen strains and the influence from environmental factors. Hartl et al. (1993) found an RFLP marker for a wheat powdery mildew resistance gene (*Pm3*) which is capable of differentiating alleles at the *Pm3* locus. Graner and Bauer (1993) reported that RFLP markers closely linked to a barley mild-mosaic-virus resistance gene can distinguish resistant and susceptible German barley cultivars. Nonetheless, the effectiveness of "marker-assisted screening" in assessing genetic diversity and establishing allelic relationships of disease resistance genes has not been systematically studied.

Recently, DNA microsatellites or simple sequence repeats (SSRs) have been reported as a new class of markers with a wide range of applications in plant genetic studies (Akkaya et al. 1992; Wu and Tanksley 1993; Saghai Maroof et al. 1994). DNA sequences containing SSRs can be amplified

by the polymerase chain reaction (PCR), and SSR variants can be detected by gel electrophoresis of the amplified fragments (Yu et al. 1994). In the soybean genome, microsatellites with $(AT)_n$ repeats were found to be abundant, and more polymorphic than RFLP markers (Akkaya et al. 1992; Morgante and Olivieri 1993). Thus, PCR-based markers of this type may offer a greater opportunity for practical applications in breeding soybeans than is provided by other molecular markers.

Soybean mosaic virus (SMV) is one of the most prevalent soybean viral pathogens in the world, and has been found wherever soybeans [*Glycine max* (L.) Merr] are grown. At least eight SMV strain groups have been identified in the U.S. based on the reactions in differential soybean cultivars, and resistance appears to be the only practical method of SMV control (Buss et al. 1989). Kiihl and Hartwig (1979) found that SMV resistance in soybean line PI 96983 and 'Ogden' is controlled by a single dominant gene, now designated *Rsv1*. Roane et al. (1983) demonstrated that a single dominant gene in 'York' conditions resistance to SMV strain G1. Allelism tests of the genes in PI 96983, Ogden, York, 'Marshall', and 'Kwanggyo' (Chen et al. 1991; 1994) showed that resistance in each cultivar is controlled by a single dominant gene, and that these genes are alleles of the same locus (*Rsv1*). RFLP and microsatellite analysis of near-isogenic lines (NILs) indicated that SMV resistance in 'Buffalo' may also be due to an allele at *Rsv1* (Yu et al. 1994).

Buzzell and Tu (1984) reported that SMV resistance in 'Raiden' is controlled by a single gene at a locus independent of *Rsv1*, designated as *Rsv2*. They (1989) also proposed that a dominant gene at a third locus (*Rsv3*) derived from 'Columbia' confers a necrotic reaction to SMV. Bowers et al. (1992) showed that the single dominant genes in Buffalo and in the line HLS are located at two different loci. Lim (1985) reported that PI 486355 had a single gene for resistance, but Chen et al. (1993), using a different SMV strain, concluded that resistance in PI 486355 is controlled by two independent dominant genes, one of which is at the *Rsv1* locus.

Using microsatellite and RFLP markers, we recently mapped *Rsv1* in an F_2 population from PI 96983 × Lee 68 (Yu et al. 1994). Three marker loci, HSP176L (SSR), pA186 and pK644a (RFLPs), were located at a distance of 0.5, 1.5 and 2.1 cM from *Rsv1*, respectively. The availability of these SSR and RFLP markers, and the simple Mendelian inheritance of SMV resistance provide a model system to evaluate the use of closely-linked markers in assessing the divergence of disease resistance genes from various sources. The objectives of the present study were to (1) investigate the level of genetic heterogeneity near the *Rsv1* locus (2) examine the correlation between *Rsv1*-linked marker alleles and SMV resistance in 67 soybean accessions, and (3) explore a marker-assisted classification of the SMV resistance sources.

Materials and methods

Genetic materials

Sixty seven diverse soybean types, including plant introductions, cultivars, and breeding lines, were included in this study. Accessions of plant introductions were obtained from the USDA soybean germplasm collection maintained at the University of Illinois. Cultivars and breeding lines were from our collection or the Virginia cultivar evaluation tests. Among the accessions, PI 96983, 'Davis', Ogden, Marshall, Kwanggyo and York are known to contain *Rsv1* (Kiihl and Hartwig 1979; Chen et al. 1991). Approximately 30 seeds of each accession were sown in a single row 1-m long in a soybean nursery in Blacksburg, Virginia.

Virus screening

Field screening of reactions of soybean accessions to SMV was according to Roane et al. (1983). Three-week-old soybean seedlings were inoculated with the SMV-G1 strain, VA isolate (Hunst and Tolin 1982), and their reactions to the virus were classified as resistant (symptomless), stem-tip necrotic, or susceptible (systemic mosaic or wrinkling) about 1 month following inoculation.

Microsatellite procedure

DNA samples were prepared from soybean leaf tissues according to previously described protocols (Saghai Maroof et al. 1984). Leaf tissue was collected from 6-week-old plants grown in the field. Equal amounts of tissue from at least 12 plants per accession were pooled and frozen in dry ice. Frozen tissues were lyophilized in a Virtis Consol 25LL freeze dryer, ground to fine powder with a sample mill, and used for DNA extraction.

The procedure published by Yu et al. (1994) was used to detect SSR variants at the HSP176L (previously designated as SM176) locus. The primers 5'TTTTGTTAAGTTACTGTACTGTGG (forward primer) and 5'TATTTTAGCAGTTTTAGATGATTCG (reverse primer), also described by Akkaya et al. (1992), were synthesized based on the Gmhsp17.6L sequence (Nagao et al. 1985). The genomic fragments containing the SSR region were amplified by PCR for 30 cycles of 1 min at 95 °C (denaturing), 2 min at 55 °C (annealing) and 1.5 min at 72 °C (extension) in the presence of alpha-^{32}P-dCTP (ICN Biomedicals, Irvine, Calif.). The size variation of the PCR products were detected by a 6.0% denaturing polyacrylamide gel with 8 M urea, followed by autoradiography.

RFLP assays

RFLP analyses also followed the protocol described by Yu et al. (1994). The soybean genomic DNA clones used in this study were kindly provided by Dr. R. C. Shoemaker, USDA-ARS, Iowa State University. ^{32}P-labelled DNA probes were prepared from the insert DNA fragments by the random priming method and hybridized to Southern blots containing 8 µg of soybean genomic DNA digested with an appropriate restriction enzyme.

The clones pA186 and pK2 detect a single RFLP locus, while pK644 detects two loci, pK644a and pK644b, in two different linkage groups (Diers et al. 1992 a). At each of the four RFLP loci, variation in the length of restriction fragments was detected with three different restriction enzymes: *Hin*dIII (H3), *Eco*RI (R1) and *Eco*RV (R5) for pA186; H3, *Dra*I (D1) and R1 for pK644a and pK644b; and H3, D1 and R5 for pK2.

Results

Among the 67 soybean accessions screened, 45 were found to be resistant (R), one was stem-tip necrotic (N),

and 21 were susceptible (S) to SMV-G1. At the HSP176L locus, five variants (alleles) with different numbers of dinucleotide repeats $(AT)_n$ were detected in the 67 accessions by PCR reactions using specific primers. The variants were designated arbitrarily 1 through 5 with no regard to the number of AT repeats (Fig. 1). Variation at each of the four RFLP loci (pA186, pK644a, pK644b, and pK2) was detected with a combination of three different restriction enzymes in single digestions. At the pA186 locus, for instance, four variants (1, 2, 3, and 4) were found with H3, three (1, 2, and 3) with R1, and four (1, 2, 3, and 4) with R5. At pK644a, three variants (1, 2, and 3) were detected with H3, five (1, 2, 3, 4, and 5) with D1, and four (1, 2, 3, 4, and 5) with R1. The variants detected by the same probe but with different enzymes are correlated with each other. For instance, pA186 variant R1-1 was detected in the same lines with H3-1, R1-2 with H3-2, and R1-3 with either H3-3 or 4 (Table 1). Nonetheless, the use of multiple enzymes provides more information on the level of variation at the targeted chromosomal region.

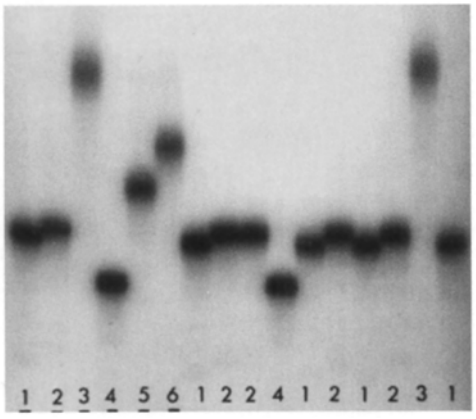

Fig. 1 SSR variants (alleles) at the HSP176L locus. *Lanes 1 through 5 (underlined)* show the five SSR variants observed in the 67 soybean accessions; *lane 6* is a variant found in one *G. soja* accession (PI 407162) which is not included in this study; *lanes 7 through 16* are random samples of the soybean accessions screened

The *Rsv1*-linked pA186 and pK644a loci exhibit a higher level of polymorphism than the pK2 and pK644b loci (Table 1). The pK2 locus is about 40 cM from *Rsv1*, and pK644b is on a different linkage group. Among 67 soybean accessions, six (A through F) and seven (A through G) multi-enzyme RFLP variant combinations (referred to as RFLP variants) were found at pA186 and pK644a, respectively. In contrast, only two RFLP variants (A and B) were detected at the pK2 locus, and four (A through D) at pK644b, two of which (B and C) were observed in only one line each. Five SSR variants (designated as 1, 2, 3, 4, and 5) were found at HSP176L, among which HSP176L-1 and -2 are most frequent, occurring in 31 and 26 accessions, respectively. The level of polymorphism detected by SSR analysis is generally higher than that detected by RFLP analysis (Akkaya et al. 1992). In the present study, we also observed a high level of SSR polymorphism at HSP176L. Using probes that correspond to marker loci closely linked to *Rsv1*, we found an atypically large number of RFLP variants, as opposed to the generally low level of RFLPs observed in soybeans, indicating an unusually high level of DNA rearrangements in the *Rsv1*-containing chromosomal region.

The association between an SSR or RFLP variant at *Rsv1*-linked marker loci and SMV resistance was examined by chi-square tests as shown in Table 2. SSR or RFLP variants (HSP176L-2, pA186-D, and pK644a-A) that were significantly associated with resistance were found at the three marker loci (Table 2). All 26 soybean accessions that carry HSP176L-2 are resistant to SMV. Soybean accessions with pA186-D are predominantly resistant to SMV, with an R:S ratio of 24:3. At the pK644a locus, 30 of the 32 lines that carry RFLP variant

Table 1 The number and distribution of marker variants at the *Rsv1* chromosomal region (HSP176, pA186, and pK644a) and elsewhere (pK2 and pK644b) in the soybean genome among 67 soybean accessions. There is no correspondence between different columns (loci) in this table

	HSP176		pA186			pK644a			pK2			pK644b		
	SSR[a]	No. obs.	$H_3R_1R_5$[b]	RFLP[c]	No. obs.	$H_3D_1R_1$	RFLP	No. obs.	$H_3D_1R_5$	RFLP	No. obs.	$H_3D_1R_1$	RFLP	No. obs.
	1	31	111	A	12	111	A	32	111	A	39	111	A	18
	2	26	221	B	3	222	B	5	222	B	28	311	B	1
	3	4	222	C	18	223	C	4				313	C	1
	4	4	332	D	27	224	D	1				222	D	42
	5	2	433	E	3	232	E	5						
			434	F	4	243	F	16						
						352	G	4						
N[d]	5		6			7			2			4		

[a] SSR variants that differ in the number of AT repeats
[b] Combinations of RFLP variants as detected using the following restriction enzymes: H_3 (*Hin*dIII), R_1 (*Eco*RI), R_5 (*Eco*RV), and D_1 (*Dra*I)
[c] Letter designations of multi-enzyme RFLP variants which will apear in Tables 2 and 3
[d] Frequency of SSR or RFLP variants observed in 67 soybean accessions. Data were collected for pK644b in only 62 soybean accessions

Table 2 Number of soybean lines that exhibit resistance (R) or susceptible (S) reaction to the SMV G1 strain as tabulated according to variants at the three *Rsv1*-linked SSR (HSP176L) and RFLP (pA186 and pK644a) marker loci

HSP176L					pA186					pK644a				
SSR[a]	R[b]	S	χ^{2c}	Class[d]	RFLP	R	S	χ^2	Class	RFLP	R	S	χ^2	Class
1	18[n]	13	1.7		A	10	2	1.1		A	30	2	9.1**	R
2	26	0	11.8**	R	B	3	0	1.3		B	4	1	0.2	
3	1	3	3.9*	S	C	7	11	7.6**	S	C	2	2	0.7	
4	1	3	3.9*	S	D	24[n]	3	5.1*	R	D	0	1	2.3	
5	0	2	4.6*	S	E	2	1	0.0		E	3	2	0.2	
					F	0	4	9.3**	S	F	7[n]	9	4.7*	S
										G	0	4	9.3**	S

[a] "SSR", and "RFLP" indicates SSR and multi-enzyme RFLP variants as shown in Table 1

[b] Number of *G. max* lines that exhibit resistance (R) or susceptible (S) reaction to SMV G1 strain. A superscript '*n*' following a number indicates that one (1) necrotic accession (PI 507389) is included as a resistant line for the purpose of χ^2 calculation

[c] In chi-square test for the independence of SMV resistance from marker variants, the expected ratio is based on the overall frequency of R (f = 0.69) and S (f = 0.31) accessions. A * or ** sign following a χ^2 value indicates significant ($P < 0.05$) or highly significant ($P < 0.01$) χ^2, respectively

[d] Marker variants associated with either R or S

A are resistant. These three marker variants (HSP176L-2, pA186-D, and pK644a-A) are hereafter referred to as resistance markers (R markers). On the other hand, pA186-C and pK644a-F are associated with SMV susceptibility. The sample size of HSP176L-3, -4, and -5, pA186-F, and pK644a-G is too small for a chi-square test, but they also appear to be associated with susceptibility.

According to their multilocus *Rsv1*-linked marker variants, the 67 soybean accessions screened were grouped into 20 classes, designated as C1–C20 (Table 3). The largest group, C12, includes 22 accessions, whereas the ten smallest groups have only one accession each. The grouping of cultivars based on the *Rsv1*-linked marker loci agrees with the available pedigree information with respect to the source of SMV resistance. The

Table 3 The grouping of 67 soybean accessions according to their multi-locus variants at *Rsv1*-linked HSP176L, pA186, and pK644a, and tentative allelism of the SMV resistance genes

Group	Variants[a]	Accessions[b]	Resistance genes[c]
C1	1AA	*PI 96983*	*Rsv1*
C2	1AB	*Davis, Brim, Young*, HT3550, *G83-198*	*Rsv1*
C3	1AF	*Marshall, Ogden, V73-178, N87-325*, PI 507376	*Rsv1*
C4	1CA	*Peking*, Williams, Md78L-0198, *FFR-471, PI556949*	*R?*
C5	1CE	*Kwanggyo, Suweon 97, PI 486355*	*Rsv1*
C6	1CF	Bay, Essex, *Holladay*, DP105, FFR-396, KS5292, Pioneer 9442, HT4290	*R?*
C7	1DF	PI 507389[n], 507453	*R?*
C8	1EC	*Columbia*	*Rsv3*
C9	1EG	Hartwig	–
C10	2AA	*Jizuka*	*Rsv1*
C11	2BA	*Buffalo, PI 507391, 507477*	*Rsv1*
C12	2DA	*York, Dorman, Hutcheson, Raiden, Toano, Youbian 30, Doujiao 44, TN6-90, V71-370, DP415, DP425, FFR-544, PI 80837, 399012, 468408A, 495020, 507474, 507690, 509096, 509098, 509106, 556950*	*Rsv1*
C13	3EC	*Virginia*	*R?*
C14	3FE	Underwood 607, TN85-157	–
C15	3FG	Pioneer 9691	–
C16	4DC	PI 508295	–
C17	4DD	PI 508298	–
C18	4DF	*PI 507403*	*R?*
C19	4FC	CNS	–
C20	5CG	Lee 68, Cumberland	–

[a] The multi-locus SSR and RFLP variants as shown in Table 1 in the order HSP176L, pA186, and pK644a

[b] All 45 *italicized* accessions are SMV-resistant. A superscript "*n*" following the accession PI 507389 indicates its necrotic reaction to SMV-G1. The remaining 21 accessions are susceptible

[c] Tentative allelism among SMV resistance genes. *Rsv3* is independent of *Rsv1* (Buzzell and Tu 1989), *R?* indicates that the SMV resistance genes in these groups need to be determined by inheritance studies, and "–" indicates no accession in the group is resistant to SMV-G1

group C2, for instance, includes 'Brim', 'Young', as well as their likely source of SMV resistance, Davis (Burton et al. 1987, 1994). Kwanggyo, Suweon 97, and PI 486355, three resistant lines from Korea, are clustered in the same class, C5. SMV resistance in 'Hutcheson' is derived from 'Dorman' via York (Buss et al. 1988). These three cultivars were found to belong to the same class, C12, with a multilocus variant of 2 D A (Table 3). Similarly, both 'Toano' and its SMV resistance donor (PI 80837, Buss et al. 1987) are in the C12 group.

Accessions belonging to a given group, with the same *Rsv1*-linked multilocus marker variant, appear to share the same linkage block around *Rsv1*. If all, or most, accessions in a group are resistant to SMV, then *Rsv1* which is contained in the linkage block shared by those accessions is probably responsible for the resistance. These putatively *Rsv1*-carrying groups, C1 (PI 96983), C2 ($n = 4$ accessions), C3 ($n = 4$), C5 ($n = 3$), C10 (Jizuka), C11 ($n = 3$) and C12 ($n = 22$), include a total of 38 SMV resistant lines (Table 3). The remaining seven resistant accessions and one necrotic (PI 507389) accession do not carry R markers, and at least some of them have resistance genes independent of *Rsv1*. Among them, Columbia is known to carry an independent gene (*Rsv3*, Buzzell and Tu 1989) as is PI 507389 (unpublished), but inheritance studies are needed for the identification of resistance genes in the other six lines: Holladay, 'Peking', 'Virginia', FFR-471, PI 507403, and PI 556949.

Discussion

Molecular diversity and resistance gene clusters

A number of RFLP surveys have indicated a low level of DNA polymorphism among soybean lines. Keim et al. (1992) reported that, among 91 genomic DNA probes that revealed polymorphism in 38 cultivars and ancestral lines, 88 probes detected two alleles, and the remaining three had three alleles. This predominantly two-allele phenomenon, or diallelism, of the soybean genome is consistent with the description by Apuya et al. (1988). Roth et al. (1989) found that a newly generated RFLP allele during the tissue-culture process was almost always identical to the existing alternative allele at a given locus.

In contrast to the norm of diallelism, multiple RFLP variants were found at the marker loci in the chromosomal region near *Rsv1*. Up to five RFLP variants were observed at the pA186 and pK644a loci among 67 soybean types when their genomic DNA was digested with each of three restriction enzymes. The use of multiple enzymes enabled us to detect a larger number of RFLP variants, six by the probe pA186 and seven by pK644a. The remarkably high level of polymorphism near, or around, the disease resistance gene cluster may suggest an association between the molecular mechanism of disease resistance and rapid sequence divergence in plants (Sudupak et al. 1993), similar to that found in human and mouse (Murphy 1993).

Several resistance genes, including resistance to peanut mottle virus (*Rpv*) (Roane et al. 1983), *Phytophthora* (Rps_3) (Diers et al. 1992b) and Javanese root-knot nematode (Tamulonis et al. 1994), have been located in the vicinity of the *Rsv1*-containing chromosomal region, possibly as a gene cluster. The clustering of host resistance genes conditioning resistance to pathogenic fungi has been documented in many plant species. The best examples are rust resistance genes (Rp) on chromosome 10 of maize (Hooker 1985), mildew resistance genes (*Ml-a*) on chromosome 5 of barley (Wise and Ellingboe 1985), and rust resistance genes clustered in the *L* group of flax (Shepard and Mayo 1972; Islam et al. 1993). A virus tolerance gene (*Bdv1*) was found to be either closely linked to or pleiotropic with *Lr34* and *Yr18* for adult plant resistance to rust in bread wheat (Singh 1993). In soybean, Lohnes et al. (1993) reported that two resistance genes (*Rmd* and *Rps2*) are closely linked to each other, and to a non-nodulation gene (*Rj2*).

Marker-assisted screening of SMV resistance sources

In this study, we explored the use of molecular markers as aids in plant genetics and plant-breeding programs based on the association between markers and the targeted disease resistance gene. Such uses of the resistance-correlated markers (R markers) include marker-assisted screening as alternatives, or supplements, to screening for the actual trait, and studies of the lineage of the target gene. R markers (HSP176L-2, pA186-D, and pK644a-A) were found at each of the three *Rsv1* marker loci. Of particular interest is HSP176L-2, which was observed exclusively in 26 resistant accessions. These R markers, especially HSP176L-2 which can be detected by PCR, may be suitable in the screening of SMV-resistant soybean germplasm for the presence or absence of *Rsv1*.

Associations between SMV resistance and R markers indicate that the transmission of the targeted gene (*Rsv1*) can be monitored as a linkage block. Therefore, analysis of SSR and RFLP variants at marker loci will provide clues about the ancestral source of SMV resistance alleles associated with the marker genotypes. The grouping of the soybean accessions based on marker genotypes was found in agreement with the available pedigree information with respect to the sources of *Rsv1*.

Based on the analysis of marker variants, *Rsv1* is the most likely source of SMV resistance in 38 resistant lines belonging to six groups. Differential cultivars, PI 96983, Ogden, Marshall, Kwanggyo and York, are known from previous inheritance studies to contain *Rsv1* alleles (Chen et al. 1991; 1994). Although no allelism test has been conducted for Buffalo, our marker analysis of a set of NILs indicated that the Buffalo gene is probably also at the *Rsv1* locus (Yu et al. 1994). Based on their marker

variants, all of these six cultivars were also found in the putatively *Rsv1*-carrying groups.

Buzzell and Tu (1984, 1989) reported that SMV resistance in Raiden and columbia was controlled by single genes at two different loci, *Rsv2* and *Rsv3*, respectively. Molecular-marker analysis supported the non-*Rsv1* control of resistance in Columbia, which belongs to a group by itself, separate from the *Rsv1*-carrying groups. Raiden, however, carries the same R markers as York and 20 other *Rsv1*-derived resistant lines. This observation, along with our previous analysis (Yu et al. 1994) in Williams and L88-8431 (an NIL with the SMV resistance gene from Raiden), suggests that Raiden may contain the *Rsv1* gene. Recent data (Buss et al., unpublished) from allelism tests of Raiden with cultivars carrying *Rsv1* alleles also suggest that the resistance gene in Raiden is at the *Rsv1* locus. Further studies are being conducted to clarify the true origin of the *Rsv2* gene.

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