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# Linkage analysis of *er-1*, a recessive *Pisum sativum* gene for resistance to powdery mildew fungus (*Erysiphe pisi* D.C.)

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**Abstract** Linkage analysis was used to determine the genetic map location of *er-1*, a recessive gene conditioning resistance to powdery mildew, on the *Pisum sativum* genome. Genetic linkage was demonstrated between *er-1* and linkage group 6 markers after analyzing the progeny of two crosses, an $F_2$ population and a set of recombinant inbred lines. The classes of genetic markers surrounding *er-1* include RFLP, RAPD and allozyme markers as well as the morphological marker *Gty*. A RAPD marker tightly linked to *er-1* was identified by bulked segregant analysis. After DNA sequence characterization, specific PCR primers were designed to convert this RAPD marker into a sequence characterized amplified region (SCAR).

**Key words** Pea · Powdery mildew · Genome mapping · Molecular markers · Bulked segregant analysis

## Introduction

Powdery mildew of peas (*Pisum sativum*) is caused by the fungus *Erysiphe pisi* D.C. and has worldwide distribution (Dixon 1978). This disease has been shown to affect a number of agronomically important plant traits, which in turn adversely affects yield and quality (Gritton and Ebert 1975). Powdery mildew poses the greatest threat to crops of dried peas (Dixon 1978), which are an important source of dietary protein in many countries. Naturally-occurring resistance to this pathogen exists in *P. sativum,* and a number of lines resistant to powdery mildew have been described (Ford and Baggett 1965; Heringa et al. 1969; Marx 1971; Kumar and Singh 1981; Reeser et al. 1983; Haglund and Anderson 1987). Using these and other sources, plant breeders are incorporating powdery mildew resistance into commercial cultivars.

Resistance to powdery mildew is inherited as a recessive trait, with a number of studies suggesting single gene inheritance (Harland 1948; Pierce 1948; Heringa et al. 1969; Marx 1971). Heringa et al. (1969) made crosses between various resistant lines and obtained results that suggest that there may be two distinct genes for resistance to powdery mildew. One report has indicated that two recessive genes may be necessary to achieve resistance to powdery mildew (Kumar and Singh 1981). Although there are no previously published reports of linkage mapping of the gene(s) conferring resistance to powdery mildew, Marx (1971) showed a clear genetic linkage between resistance to powdery mildew, designated as *er*, and the morphological marker "Gritty" (*Gty*) and as a result assigned *er* to linkage group 3. Later, Marx (1986) reconsidered his assignment of *er* and *Gty* to linkage group 3 but did not assign them to a different linkage group. Subsequently, Wolko and Weeden (1990) placed *Gty* on linkage group 6.

DNA markers provide powerful tools for mapping economically important genes. Since a large number can be identified, DNA markers provide the opportunity for developing tightly linked, phenotypically neutral genetic tags for trait introgression in plant breeding programs. DNA markers have been used to map and tag a number of disease resistance genes occurring in crop species (for example, Waugh and Powell 1991; Debener et al. 1991; Diers et al. 1992; Martin et al. 1992; Paran et al. 1992; Timmerman et al. 1993). In this paper we report the genetic mapping of *er-1* to linkage group 6 using DNA markers and other loci known to map to this linkage group (Timmerman et al. 1993). In addition, we report the application of the bulked segregant analysis approach (Michelmore et al.

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1991) to identifying a random amplified polymorphic DNA (RAPD) marker tightly linked to *er-1* and the conversion of this RAPD into a sequence characterized amplified region (SCAR; Paran and Michelmore 1993), a more reliable polymerase chain reaction (PCR) marker.

## Materials and methods

### Plant material

Two distinct *P. sativum* populations were used to map the gene for resistance to powdery mildew. An $F_2$ population of 111 individuals was produced from a genetic cross between the powdery mildew resistant cultivar 'Almota' (A Gufstafson, Crites Moscow Growers Inc, Moscow, Idaho, USA) and a powdery mildew susceptible breeding line, '88V1.11' (W Jermyn, Crop & Food Research, Lincoln, NZ). $F_3$ and $F_4$ descendants were derived by self-pollination. A set of single-seed-descent recombinant inbred lines (RILs) (Weeden et al. 1994a) resulted from a cross between *P. sativum* ssp. *humile* accession JI1794 (John Innes Institute, Norwich, UK) and *P. sativum* ssp. *sativum* accession Slow (N Weeden, Cornell University, Geneva, N.Y., USA). The parental line Slow is resistant to powdery mildew. The population used for linkage mapping comprised 51 RILs.

### Powdery mildew susceptibility testing

Plants were tested for susceptibility to powdery mildew fungus (*Erysiphe pisi* D.C.) in Lincoln, New Zealand and in Geneva, N.Y., USA. The inoculum sources were derived from the populations endemic to each region. Inoculations were carried out by blowing or shaking fungal spores from heavily infected pea plants onto young plants. In addition, heavily infected susceptible plants were grown adjacent to the plants being tested as a continuing source of inoculum. Plants were grown in a greenhouse. Susceptible plants eventually became covered with lesions. In general, no visible lesions developed on resistant plants; however, under some conditions a few small lesions which were slow to develop appeared on the lower leaves of resistant plants.

From the cross 'Almota'×'88V1.11', both the $F_3$ and $F_4$ descendants were tested for powdery mildew susceptibility. Three plants from each of 44 RILs were grown and tested for susceptibility to powdery mildew. The susceptibility or resistance of parental plants was confirmed at the same time.

### Analysis of molecular markers

DNA isolations, RFLP (restriction fragment length polymorphism) and RAPD analyses were conducted as described previously (Timmerman et al. 1993). To analyze the progeny of the 'Almota'×'88V1.11' cross, DNA for RAPD amplifications was isolated from individual $F_2$ plants, while DNA for RFLP analysis was isolated from young leaves pooled from at least 4 descendant $F_3$ plants. For RAPD and RFLP analyses of the RILs, DNA samples were isolated by pooling young leaf tissue from 3 plants representing each RIL. The sequences of the primers used for RAPD amplifications were: NW04, GTTAGGTCGT; PD10, GGTCTACACC.

Segregation patterns for the allozyme *PRX-3* (peroxidase) were obtained as previously described (Timmerman et al. 1993). For *ACP-4* (acid phosphatase) scoring, beta-acid phosphatase phenotypes were determined on horizontal starch gels using the citrate/*N*-(3-aminopropyl) morpholine pH 6.1 buffer system of Clayton and Tretiak (1972). Seeds were nicked and allowed to imbibe overnight before a 0.05 mg piece was removed from one of the cotelydons and crushed in 0.5 ml 0.1 *M* phosphate pH 7.0 containing 20% glycerol and 14 m*M* 2-mercaptoethanol. The extract was applied to the starch gel in a filter paper wick. Electrophoresis was performed at 5°C for 5 h. The beta-acid phosphatase assay consisted of 25 ml 0.1 *M* sodium acetate, pH 5.0, 20 mg sodium beta-naphthyl acid phosphate and 25 mg Fast Garnet GBC salt.

### Bulked segregant analysis

DNA samples from $F_2$ individuals derived from the cross '88V1.11'×'Almota' were pooled for bulked segregant analysis to identify a RAPD marker or markers linked to powdery mildew resistance. The DNA pools were constructed by mixing equal amounts of total leaf DNA isolated from $F_2$ plants. The final concentration of DNA in the pools was adjusted to 20 ng/μl.

The pools of DNA from progeny resistant or susceptible to powdery mildew were each from 10 $F_2$ individuals. The "susceptible" bulk was made up of DNA from individuals presumed to be homozygous susceptible on the basis of testing $F_3$ progeny. RAPD amplifications were carried out as described previously (Timmerman et al. 1993). The primers added to the PCR reactions were 10-mers obtained from Operon Technologies Inc, kits A – J (Alameda, Calif., USA).

### Cloning and sequencing of the $PD10_{650}$ RAPD

Unless otherwise specified, DNA manipulations were performed using standard methods (Sambrook et al. 1989). For cloning, the $PD10_{650}$ RAPD product was reamplified from a small agarose plug taken from the $PD10_{650}$ band using a P200 tip, placed in 100 μl of $H_20$ and vortexed. One microliter was used as template for reamplification. Amplifications conditions were identical to our standard RAPD parameters (Timmerman et al. 1993) except that the final elongation time was only 2 min. After phenol-chloroform extraction, primers and dNTPs were removed using a Millipore Ultrafree-MC 30,000 NMWL filter unit. The RAPD product was then 5′-end phosphorylated using T4 polynucleotide kinase and blunt-end cloned into *Sma*I-cut and dephosphorylated pUC19. Two clones were sequenced by the dideoxy chain termination method.

### Amplification of the $PD10_{650}$ product using a specific primer pair

On the basis of the DNA sequence data, two 24 nucleotide primers were synthesized: 5′-GGTCTACACCTCATATCTTGATGA; 5′-GGTCTACACCTAAACAGTGTCCGT. The conditions for amplification were: 20–40 ng of plant DNA, PCR buffer (50 m*M* KCl, 10 m*M* TRIS-HCl pH 8.3, 1.5 m*M* $MgCl_2$, 0.01% gelatin), 200 μ*M* of each dNTP, 1.0 μ*M* of each primer, 0.5 U of Taq DNA polymerase (Boehringer Mannheim) in a 25 μl reaction capped with 2 drops of paraffin oil. Products were amplified for 40 cycles (94°C 1 min, 65°C 1 min, 72°C 2 min) in a Perkin Elmer Cetus thermal cycler. Electrophoresis was carried out as previously described (Timmerman et al. 1993).

### Linkage analysis

Linkage of loci segregating in both the $F_2$ and RIL populations was analyzed using MAPMAKER, version 3.0 (Lander et al. 1987, Lincoln et al. 1992). For the $F_2$ population, the genotypes for *er-1* were inferred after examining the susceptibility of the $F_3$ and $F_4$ descendants. A few $F_2$ plants produced 3 or less $F_3$ progeny, and for these individuals the ambiguous code "D" (not homozygous 'Almota') was used. All the progeny requiring this treatment of the data were susceptible to powdery mildew.

## Results

### Segregation of resistance to powdery mildew

The $F_2$ population derived from the cross 'Almota' ×'88V1.11' consisted of 85 susceptible and 25 resistant plants. These results are in agreement with powdery mildew resistance being conferred by a single recessive gene ($\chi_2$=0.30; $P$=0.58). The RIL population included 21 resistant and 23 susceptible lines, approximating the 1:1 ratio expected for segregation of a single gene ($\chi_2$=0.09;

$P$=0.76). The results of susceptibility tests conducted on segregating progeny in New Zealand and New York were in agreement.

## Analysis of linkage with linkage group 6 markers

To locate *er-1* on the genetic map of pea, the segregation of resistance to powdery mildew in the 'Almota' ×'88V1.11' progeny was compared with the segregation patterns for RFLP markers previously mapped to various pea chromosomes (Weeden and Wolko 1990). Early in the study, a clear linkage to linkage group 6 marker pI49 was demonstrated. This progeny set was characterized for the following polymorphic linkage group 6 loci: RFLP loci *Fed-1*, *Gs-p*, pI49 and pID18; allozyme locus *Prx-3*; RAPD loci $NW04_{950}$ and $PD10_{650}$; and disease resistance locus *sbm-1*. A linkage map for linkage group 6, including all of these loci except $PD10_{650}$ was published recently (Timmerman et al. 1993). Joint segregation analysis of *er-1* and the nearest marker loci, pI49, pID18 and $PD10_{650}$, is presented in Table 1.

The RILs segregated for many markers on linkage group 6 (Wolko and Weeden 1990; Timmerman et al. 1993), including *Acp-4* and *Gty*, which were not segregating in the 'Almota'×'88V1.11' cross. Joint segregation analysis revealed linkage between *er-1* and a number of these markers (Table 2). In addition, joint segregation analysis was performed with *er-1* and the many markers assigned to other linkage groups that segregate in this RIL set. All of these markers assorted independently of powdery mildew resistance (data not presented).

Linkage maps are presented in Fig. 1 showing the position of *er-1* relative to neighboring linkage group 6 markers for each cross. The order and distances presented are the result of multipoint analysis of the data and therefore differ slightly from the pairwise comparisons presented in Tables 1 and 2. The locus orders presented are the most favored and are the same as the best order determined previously when mapping the *sbm-1* gene (Timmerman et al. 1993). The map in Fig. 1A shows the best order for linkage group 6 loci surrounding *er-1* based on data from the 'Almota'×'88V1.11' progeny. This map is characterized by the presence of three pairs of tightly linked loci. A precise order for the loci within these pairs is not given since the favored order for each pair of markers is less than 100 times more likely than the next best order (i.e. $\Delta$ LOD < 2.0). Similarly, the map derived from the RILs (Fig. 1B) shows tight linkage between *er-1* and $PD10_{650}$. The precise order of these loci is also not given because of the lack of strong statistical support for a particular order. Comparison of the estimated distances between loci for the linkage maps in Fig. 1 shows they are not the same but are roughly proportional. The map distances estimated using the $F_2$ population are larger than the estimates based on the RILs. To determine whether the differences in the lengths

**Table 1** Joint segregation analyses involving *er-1* and chromosome 6 loci in $F_2$ progeny of the cross 'Almota'× '88V1.11'

| Markers | Number of $F_2$ progeny in each phenotypic class[a] | | | | | | | | | *n* | Pairwise LOD | Pairwise distance in centiMorgans (Kosambi function) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 11,11 | 12,11 | 11,12 | 12,12 | 11,22 | 12,22 | 22,11 | 22,12 | 22,22 | | | |
| *er-1*[b], pID18 | | 23 | | 53 | | 4 | 1 | 4 | 16 | 101 | 17.48 | 8.7 |
| *er-1*[b], pI49 | | 25 | | 37 | | 6 | 1 | 4 | 18 | 91 | 9.53 | 18.0 |
| pID18, pI49 | 8 | 15 | 3 | 29 | 6 | 5 | 1 | 6 | 12 | 85 | 2.44 | 36.0 |
| *er-1*[b], $PD10_{650}$[c] | | 20 | | 62 | | | 0 | 22 | | 104 | 12.63 | 2.1 |

[a] Phenotypic designations: 1, 88V1.11; 2, Almota
[b] 11 and 12 cannot be distinguished because of dominance of *Er-1* (susceptible allele)
[c] 12 and 22 cannot be distinguished beacuse of dominance of RAPD marker $PD10_{650}$ (2 = band present)

**Table 2** Joint segregation analyses involving chromosomes 6 loci and *er-1* in RILs

| Markers | Number of single seed descent RILs in each phenotypic class[a] | | | | *n* | Pairwise LOD | Pairwise distance in centiMorgans (Kosambi function) |
|---|---|---|---|---|---|---|---|
| | 11,11 | 11,22 | 22,11 | 22,22 | | | |
| *er-1*, pI49 | 17 | 4 | 7 | 15 | 43 | 2.32 | 17.9 |
| *er-1*, *Gty* | 18 | 2 | 3 | 17 | 40 | 5.48 | 7.2 |
| *er-1*, $PD10_{650}$ | 16 | 2 | 1 | 19 | 38 | 6.87 | 4.3 |
| *er-1*, pID18 | 19 | 1 | 4 | 18 | 42 | 5.97 | 6.8 |
| *Gty*, *PRX-3* | 14 | 5 | 5 | 11 | 35 | 1.44 | 21.2 |
| *Gty*, pI49 | 20 | 3 | 3 | 17 | 43 | 5.38 | 8.2 |
| *Gty*, $PD10_{650}$ | 17 | 4 | 2 | 16 | 39 | 4.46 | 9.2 |
| *Gty*, pID18 | 18 | 4 | 6 | 15 | 43 | 2.81 | 15.6 |
| $PD10_{650}$, pI49 | 17 | 4 | 8 | 14 | 43 | 1.88 | 20.4 |
| $PD10_{650}$, pID18 | 20 | 0 | 5 | 17 | 42 | 5.97 | 6.8 |
| pID18, *ACP-4* | 24 | 2 | 2 | 19 | 47 | 8.19 | 4.7 |

[a] Phenotypic designation: 1, 'Slow'; 2, 'JI1794'

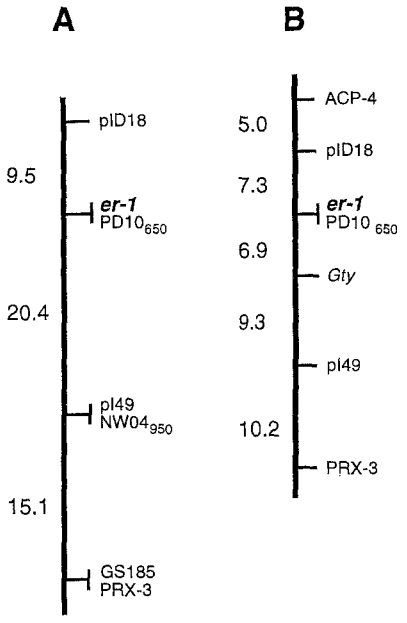


**Fig. 1A, B** Linkage maps of the region of linkage group 6 of pea surrounding the *er* gene. Map distances, written on the *left*, are in centiMorgans. **A** Map obtained from analysis of $F_2$ progeny of the cross 'Almota'×'88V1.11'; **B** map obtained from analysis of RILs produced from the cross 'Slow'×'JI1794'

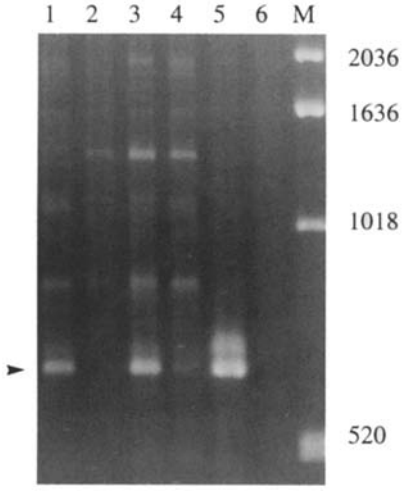


**Fig. 2** Bulked segregant analysis using RAPD primer PD10 and amplification of the $PD10_{650}$ SCAR. RAPD amplifications were carried out using the following template DNAs: 'Almota' (*lane 1*), '88V1.11' (*lane 2*), *er-1* resistant DNA pool (*lane 3*), *er-1* susceptible pool (*lane 4*). Amplifications of the $PD10_{650}$ SCAR were carried out using DNA from: 'Almota' (*lane 5*) and '88V1.11' (*lane 6*). *Lane 7* contains marker DNAs (BRL 1-kb DNA ladder). The *arrow* indicates the $PD10_{650}$ product

of the two maps are significant, recombination fractions (±SE) were calculated for corresponding intervals using the LINKAGE-1 program (Suiter et al. 1983). The differences in recombination fractions were within 1 SE and therefore may not be significant.

### Bulked segregant analysis to identify linked RAPDs

DNA pools were constructed from *er-1*-resistant and -susceptible progeny and these, as well as parental DNAs, were screened for RAPD product polymorphisms. From the 200 primers used, there were 112 RAPD products that showed polymorphism between the two parents, 'Almota' and '88V1.11'. Of these RAPD products, 6 showed differences in band intensity in one or the other pair of DNA pools, thereby suggesting a possible linkage of the RAPD product with *er-1*. Individual $F_2$ progeny were then tested for cosegregation of the RAPD phenotypes with *er-1* as well as flanking linkage group 6 markers. Only $PD10_{650}$ showed tight linkage to the genomic region containing *er-1*. The RAPD products amplified from 'Almota' and '88V1.11' and the *er-1* DNA pools using primer PD10 are shown in Fig. 2. The remaining 5 RAPD products segregated independently of linkage group 6 markers (data not presented), suggesting that the DNA pools making up the *er-1* bulks are enriched for at least one other region of the genome.

### Development of primer pairs specific for the $PD10_{650}$ RAPD product

To produce a pair of primers that would specifically amplify the $PD10_{650}$ fragment linked to *er-1*, the sequence of the $PD10_{650}$ RAPD product was determined and used to design a pair of 24-mer oligonucleotide primers. PCR amplification at a high annealing temperature (65°C) produced a single DNA fragment that comigrated with the $PD10_{650}$ RAPD product when 'Almota' DNA was used as the template (Fig. 2). In contrast, no product was amplified when the template was '88V1.11' DNA; therefore, the primers amplify a dominant marker that is tightly linked to the recessive *er-1* allele. Similarly, in the 'Slow'×'JI1794' cross, the primers generated a dominant marker linked to the recessive *er-1* allele in Slow. The primers amplified a dominant marker at lower annealing temperatures as well. Segregation of the $PD10_{650}$ product amplified using the specific primer pair was examined using both the $F_2$ progeny derived from the 'Almota'×'88V1.11' cross and the RILs derived from the 'Slow'×'JI1794' cross. In all cases the segregation of the specific amplification product was identical to the segregation of the $PD10_{650}$ RAPD product.

## Discussion

The gene conferring resistance to powdery mildew in pea has been previously suggested to be on linkage group 1 (Harland 1948), 3 (Marx 1971), 5 (Gupta et al. 1992) or 6 (Wolko and Weeden 1990). In an attempt to resolve the confusion about the location of the gene for powdery mildew resistance, as well as to identify a useful marker for this important trait, we performed a detailed genetic anal-

ysis of this trait in two populations also segregating for a large number of marker loci. Our results demonstrate that powdery mildew resistance is linked to markers on linkage group 6. The closest morphological marker appears to be *Gty*, a linkage previously identified by Marx (1971). However, in contrast to the results obtained by Marx, we could clearly associate *Gty* and *er-1* with other markers on linkage group 6. In addition, neither the segregation pattern of *Gty* nor that of *er-1* displayed strong correlations with markers on other linkage groups, most specifically linkage groups 1, 3 or 5, which have been suggested as alternative locations for the powdery mildew resistance gene.

Several sources of powdery mildew resistance have been reported in pea (Harland 1948; Yarnell 1962; Heringa et al. 1969), and it is possible that the resistance in these different lines represents distinct genes. Marx (1986) reported powdery mildew resistant $F_1$, $F_2$ and $F_3$ progeny from a cross between PI 185183 (believed to be material collected by Harland in Peru) and one of Marx's "*er*" lines (derived from 'Stratagem'), indicating that these two sources of resistance were conferred by the same locus. In contrast, Heringa et al. (1969) reported that their Peruvian material contained a different gene, which was designated *er-2*. The resistance phenotype of *er-2* lines differed from that of *er* lines and was described by Heringa et al. (1969) as leaf resistance, while Marx (1986) pointed out that there was heavy stem attack of *er-2* plants by powdery mildew.

Our results show that the genes for powdery mildew resistance found in 'Slow' and 'Almota' map to the same location. We suspect that the source of resistance for both 'Slow' and 'Almota' is either 'Stratagem' or PI 185183, however our knowledge of the genetic background of both lines is incomplete. 'Slow' came from material supplied by Dr. G. A. Marx who primarily used 'Stratagem'-derived material in his breeding program, whereas 'Almota' is a product of crosses made by A. Gufstafson, then at Western Valley Seed Co. However, this breeding program relied on breeding lines supplied by Dr. Marx. As we know the 'Stratagem'-derived resistance displays linkage with *Gty* (Marx 1971), we have used the designation *er-1* in the present study.

The existence of an *er-2* locus might explain some of the confusion in the literature regarding the genomic location of powdery mildew. On the basis of our data, we can neither confirm nor reject the existence of an independent *er-2* locus. Harland (1948) initially placed his Peruvian-derived resistance on linkage group 1, but this linkage was very weak (35 map units from *A*, the only marker with which it showed linkage). Marx (1986) reported his failure to confirm that *er* was on linkage group 3, concluding the location of *er* remained uncertain. Our results confirm the linkage with *Gty* that was observed by Marx (1971) and do not necessarily contradict those of Harland, who may have been investigating a different gene. The more recent placement of resistance to powdery mildew on linkage group 5 (Gupta et al. 1992) is also based on very weak linkage with linkage group 5 markers (30 map units from *Fs* and 35 map units from *cp*). We were also unable to confirm this linkage.

To identify a DNA marker more closely linked to *er-1* and therefore suitable for use in marker-assisted selection, we applied bulked segregant analysis (Michelmore et al. 1991). After an analysis of segregation in both the $F_2$ progeny and RILs, 1 RAPD product, $PD10_{650}$, was confirmed as being closely linked to *er-1*, at a distance estimated by multipoint mapping of about 2 cM (Fig. 1). This RAPD locus is in repulsion phase with *er-1*; that is, 'Almota' and 'Slow' are both homozygous dominant (band present) for $PD10_{650}$ but homozygous recessive (disease resistant) for *er-1*. This RAPD marker was successfully converted into a SCAR. The specific primers direct amplification of a product from the resistant parents of the crosses used in this study but not from the susceptible parents. For mapping using $F_2$ progenies, while being linked but in repulsion phase has strong disadvantages, it will be very valuable for marker-assisted selection of backcross populations. The use of either the RAPD or the SCAR marker will enable identification $BC_1$ plants with a high probability of containing the *er-1* (resistant) allele. Without such a linked marker, heterozygous $BC_1$ plants (*Er-1 er-1*) can only be identified after self-fertilization and progeny testing. A further advantage of the dominant $PD10_{650}$ SCAR is that it can be used with appropriate segregating populations to permit quick identification of individuals carrying the *er-1* locus without electrophoresis (Weeden et al. 1994b), thereby providing a valuable tool for plant breeding programs.

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