

Development of Sequence Characterized Amplified Region Markers Linked to Downy Mildew Resistance in Broccoli

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ABSTRACT. Downy mildew, caused by the fungal parasite *Peronospora parasitica* (Pers.: Fr.) Fr., is a destructive disease of *Brassica oleracea* L. crops, including broccoli (*B. oleracea*, Italica Group). The development and deployment of downy mildew resistant broccoli cultivars is a priority for breeders and producers. Identification of genetic markers linked to downy mildew resistance genes should facilitate selection for resistance and pyramiding of resistance genes into cultivars. The objectives of this study were to 1) identify RAPD markers linked to a single dominant gene for resistance in broccoli, 2) clone and sequence the linked RAPD markers, and 3) develop and evaluate SCAR markers as screening tools for resistance. Bulk segregant analysis led to the identification of eight linked RAPD markers following a screen of 848 decamers. Two of the linked RAPD fragments, UBC359₆₂₀ and OPM16₇₅₀, were converted to dominant SCAR markers linked in coupling to the resistance locus at 6.7 and 3.3 cM, respectively. The SCAR marker based on UBC359₆₂₀ sequence exhibited less accuracy (94%) than the original RAPD (96%) in differentiating resistant and susceptible plants, but the accuracy (97%) of the OPM16₇₅₀-SCAR was not different than the original RAPD. These SCAR markers are among the first genetic markers found linked to a gene conferring cotyledon-stage downy mildew resistance in *B. oleracea*. Results of this work provide breeders with useful information and tools for the systematic development of resistant cultivars.

Downy mildew, caused by the obligate fungal parasite *Peronospora parasitica* (Pers.: Fr.) Fr., is among the most destructive diseases of *Brassica oleracea* L. crops, including broccoli (*B. oleracea*, Italica Group). This disease is distributed worldwide and can be found on many economically important species in the Brassicaceae (Channon, 1981). Broccoli can be susceptible to infection during all growth stages, but cotyledon stage infection is particularly damaging. When cotyledons and young tissues are infected with downy mildew, seedlings are often stunted or killed, and crop stand and yield are reduced (Sherf and MacNab, 1986). Fungicides can provide some control of downy mildew in broccoli (Brophy and Laing, 1992), but the development and deployment of downy mildew resistant cultivars offers a more practical, long-term solution to effective disease control in broccoli.

The genetic basis of downy mildew resistance has been described at both the cotyledon stage and the true leaf stage in *B. oleracea* crops against several isolates of *P. parasitica*. Natti et al. (1967) studied two sources of downy mildew resistance expressed at the cotyledon stage and found each to be controlled by single dominant genes. In a study of resistance at the four to five true leaf stage, Hoser-Krauze et al. (1987) concluded that a single recessive gene was responsible for downy mildew resistance. None of these resistance genes have been incorporated into cultivars.

The broccoli cultivar 'Everest' exhibits a relatively high level of downy mildew resistance (Dickson and Petzoldt, 1993; Wang et al., 2000), that had not been characterized genetically until recently. Work from our lab (Wang et al., 2001) showed that one double haploid (DH) line derived from 'Everest' exhibits downy mildew resistance at the three to four true leaf stage that is controlled by two complementary, dominant genes. In addition, we (Farnham et al., 2001) identified another DH line that expresses downy mildew resistance at the cotyledon stage controlled by a single dominant gene. Not only does this single dominant gene for resistance protect plants at the cotyledon stage, it also protects against infection at later stages (Wang et al., 2000). In total, these recent studies indicate that at least three genes contribute to the resistance exhibited by 'Everest.' These resistance genes have been shown to be effective against *P. parasitica* isolates sampled from different geographic regions (Wang et al., 2000).

Conventional methods for evaluating downy mildew resistance are effective (Williams, 1986), but they are also time-consuming and expensive. A general problem is inoculum preparation, which requires maintenance of *P. parasitica* isolates on live hosts with weekly transfers or alternatively, several rounds of sporulation on a host following isolate preparation from infected leaves or frozen stocks of spores. A less complicated and faster method for identifying resistant plants, such as marker-assisted selection, is likely to accelerate breeding progress in broccoli.

Bulk segregant analysis, used in conjunction with random amplified polymorphic DNA (RAPD) (Williams et al., 1990), provides one possible method for identification of markers linked to disease resistance genes (Michelson et al., 1991). Once linked RAPD markers are identified, they can be readily isolated, cloned, and sequenced, and the sequences can then be used to develop sequence characterized amplified regions (SCARs) (Paran

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and Michelmore, 1993). Examples where this approach has been used successfully to mark genes include a fertility restorer gene in canola (*B. napus* L.) (Delourme et al., 1994), a fusarium wilt resistance gene in chickpea (*Cicer arietinum* L.) (Mayer et al., 1997), and a rust resistance gene in bean (Miklas et al., 1993). To our knowledge, no formal results identifying markers linked to downy mildew resistance in *B. oleracea*, and broccoli in particular, have been published at this time.

In this study, our objectives were to 1) identify RAPD markers linked to a single dominant gene for cotyledon stage resistance in broccoli previously described by Farnham et al. (2001), 2) clone and sequence the linked RAPD markers, and 3) develop SCAR markers based on RAPD sequences and test these for use in identifying individuals resistant to downy mildew.

Materials and Methods

PLANT MATERIALS AND DNA EXTRACTIONS. An F_2 population segregating for cotyledon stage downy mildew resistance was developed previously (Farnham et al., 2001). The DH lines that served as parents of this segregating population were characterized by Wang et al. (2000). One of the lines, USVL089, exhibited cotyledon stage downy mildew resistance. In addition, USVL089 also exhibited resistance at true leaf stages (Wang et al., 2000). This line is derived from the commercial hybrid cultivar 'Everest' (Syngenta Seed, Gilroy, Calif.) and served as the resistant (R) parent. A second line, USVL047, is highly susceptible to downy mildew at the cotyledon stage and is also susceptible at true leaf stages (Wang et al., 2000). USVL047 was derived from the commercial hybrid cultivar 'Marathon' (Sakata Seed Inc., Salinas, Calif.) and served as the susceptible (S) parent. USVL089 and USVL047 were crossed to generate an F_1 , which was self-pollinated to generate an F_2 population. This F_2 population of 100 individuals segregated 76 resistant to 24 susceptible, a ratio consistent with inheritance of a single dominant gene (Farnham et al., 2001). This previously described F_2 , designated $F_{2(RXS)}$, served as test population for marker studies described herein.

After the parents and the F_2 study population were evaluated for downy mildew resistance as described by Farnham et al. (2001), genomic DNA was extracted using DNeasy plant mini-kits (Qiagen Inc., Valencia, Calif.). DNA concentrations of all extracts were measured using a Hoechst dye-based protocol for a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, Calif.). To facilitate their use in RAPD reactions, extracts were diluted to 10 ng DNA/ μ L in water.

BULKED SEGREGANT ANALYSIS AND RAPD SCREENING. Bulk segregant analysis was performed following protocols outlined by Michelmore et al. (1991). Two different bulked DNA solutions were prepared: a resistant bulk (R-bulk) and a susceptible bulk (S-bulk). These bulks were composed of equal amounts of DNA from the eight most resistant and eight most susceptible F_2 plants, respectively, as determined by reaction phenotype screening of the $F_{2(RXS)}$.

DNA from the R and S parents and the R- and S-bulks were screened for RAPD marker polymorphisms using a series of 10-base pair (bp) oligonucleotides. A total of 848 primers were used in the analysis; 199 were Operon primers (Operon Technologies Inc., Alameda, Calif.) and 649 were UBC primers (Biotechnology Laboratory of the University of British Columbia, Vancouver, B.C.). Each RAPD reaction was carried out as described previously by Farnham (1996), with the addition of 25 mg bovine serum albumin. Amplified products were electrophoresed on

1.5% agarose gels, and a 100-bp ladder (GIBCO/BRL; Life Technologies, Rockville, Md.) served as a molecular weight standard for estimation of band size. Gels were stained with ethidium bromide and photographed using a Gel Doc 2000 gel documentation system (Bio-Rad Laboratories, Hercules, Calif.).

Any primer detecting a polymorphism between both R and S and the R- and S-bulks was retested on the individual samples comprising the bulks. Markers putatively linked to resistance were then further examined by conducting RAPD analysis (in duplicate) of all 100 F_2 individuals. Percent recombination was calculated for selected markers, and any marker exhibiting <5% recombination with resistance was isolated, cloned, and sequenced for SCAR development. Genetic linkages between RAPD markers and the resistance locus were determined with MAPMAKER software (Lander et al., 1987), using a minimum logarithm of odds ratio (LOD score) of 3.0.

CLONING AND SEQUENCING RAPD PRODUCTS. QIAquick gel extract kits (Qiagen Inc.) were used to excise and purify RAPD fragments linked to cotyledon stage resistance from agarose gels. The amplified products were cloned and transformed using TOPO TA cloning kits for sequencing (Invitrogen Corp., Carlsbad, Calif.). The cloned RAPD fragments were sequenced by means of a standard fluorescence-based protocol for an A.B.I. 377 DNA sequencer. For each cloned RAPD fragment, two pairs of oligonucleotide SCAR primers were synthesized, a pair of 20-mers and a pair of 30-mers, with the first 10 bases of each matching the original 10 bases of the RAPD primer. Both pairs of 20-mers and 30-mers were evaluated to determine if one or both would reveal easily scored SCAR markers. The SCAR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa).

SCAR ANALYSIS. The SCAR primers were used to test all 100 F_2 individuals of the study population using relatively standard polymerase chain reaction (PCR) conditions. Specifically, each PCR was carried out in a 25-mL volume and included 15 ng of genomic DNA, 1 \times buffer, 2.5 mM $MgCl_2$, 0.2 mM primer, 10 mM of dNTPs, and 0.5 unit of *Taq* polymerase (Promega Corp., Madison, Wis.).

Reaction conditions first used to test SCARs included an initial denaturation at 94 °C for 1 min, 35 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 1 min, and a final extension at 72 °C for 7 min. PCR conditions were further optimized for each pair of SCAR primers by systematically testing different annealing temperatures. Amplification products were examined by gel electrophoresis, stained with ethidium bromide, and visualized as described for RAPD markers. Percent recombination was also calculated from the SCAR analysis of the 100 F_2 plants, and genetic linkage between the SCAR markers and the resistance locus were calculated as before.

Table 1. Sequences of 10-mer oligonucleotide primers that generated markers linked to cotyledon stage downy mildew resistance.

Primer	Sequence (5' to 3')
UBC302	CGG CCC ACG T
UBC324	ACA GGG AAC G
UBC359	AGG CAG ACC T
UBC459	GCG TCG AGG G
UBC497	GCA TAG TGC G
UBC598	ACG GGC GCT C
OPD07	TTG GCA CGG G
OPM16	GTA ACC AGC C

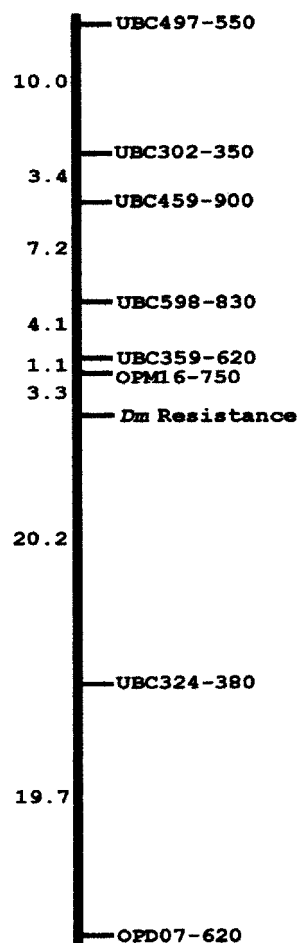


Fig. 1. Relative positions of random amplified polymorphic DNA (RAPD) markers to the cotyledon stage downy mildew resistance locus (*Dm*) in a segregating broccoli population derived from the cross of USVL089 (R) x USVL047 (S). Map distances (in centimorgans) between markers are indicated to the left of the vertical line.

Results

Successful amplification of parent and bulk DNA was obtained with 772 (91.0%) of the 848 primers tested in this study. On average, 1.37 polymorphisms per primer (or a total of 1160 polymorphisms) were observed between R and S DNA samples. From bulked segregant analysis, two types of polymorphism were noted: 1) the appearance of a coupling parental fragment in R-bulk but not in S-bulk or 2) the appearance of a repulsion parental fragment in S-bulk but not in R-bulk. Twenty-seven primers amplified a fragment in R- but not S-bulk, 28 primers amplified a fragment in S- but not R-bulk, and six primers produced both polymorphisms. Primers amplifying a fragment in R-bulk but not S-bulk were scrutinized further. Ultimately, eight primers (Table 1) generated markers that appeared to be linked to the downy mildew resistance locus. Based on the primer used and the size, in bp, of the marker generated, these markers were designated UBC302₃₅₀, UBC324₃₈₀, UBC359₆₂₀, UBC459₉₀₀, UBC497₅₅₀, UBC598₈₃₀, OPD07₆₂₀, and OPM16₇₅₀.

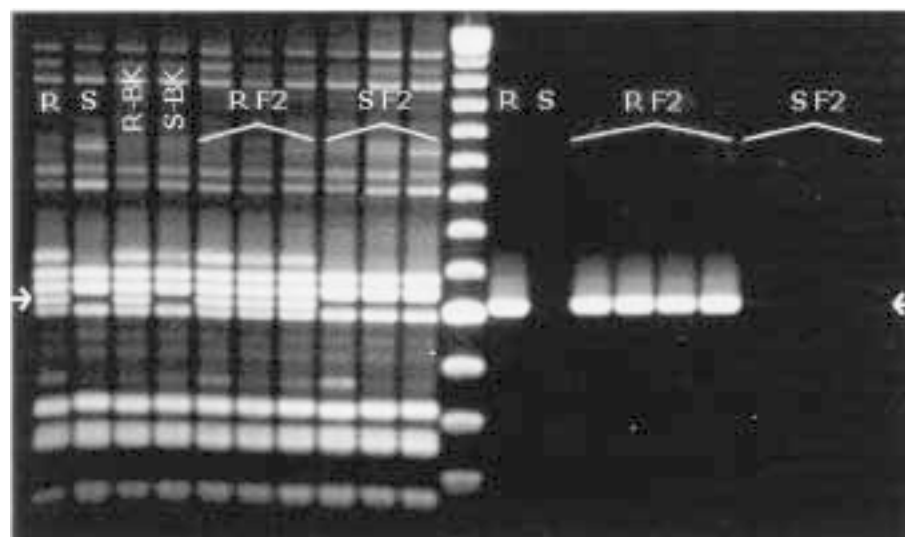


Fig. 2. Electrophoretic results of a RAPD amplification (left 10 lanes) using the 10-mer UBC359 and corresponding results of a SCAR amplification (right 10 lanes) using the 30-mer primers constructed based on the sequence of UBC359₆₂₀. Resistant (R) and susceptible (S) parents, resistant bulk (R-BK), susceptible bulk (S-BK), and resistant (R F₂) and susceptible (S F₂) F₂ samples are indicated. The middle lane contains the 100-bp DNA ladder for size estimation. Arrows indicate the position of the UBC359₆₂₀ fragments.

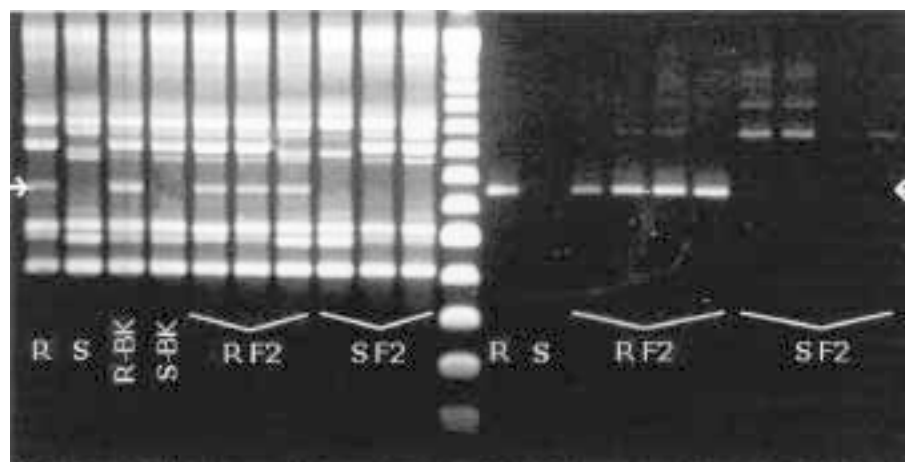


Fig. 3. Electrophoretic results of a RAPD amplification (left 10 lanes) using the 10-mer OPM16 and corresponding results of a SCAR amplification (right 10 lanes) using the 20-mer primers constructed based on the sequence of OPM16₇₅₀. Resistant (R) and susceptible (S) parents, resistant bulk (R-BK), susceptible bulk (S-BK), and resistant (R F₂) and susceptible (S F₂) F₂ samples are indicated. The middle lane contains the 100-bp DNA ladder for size estimation. Arrows indicate the position of the OPM16₇₅₀ fragments.

UBC359₆₂₀ exhibited 4% recombination with and mapped 4.4 cM from the resistance locus (Fig. 1). This marker was observed in all cases when resistant plant DNA was amplified, but absent in most susceptible plants (Fig. 2). UBC359₆₂₀ was observed when susceptible plant DNA was amplified in only four cases. OPM16₇₅₀ was observed in all cases where resistant plant DNA served as the template but not in most cases where susceptible plant DNA was the template (Fig. 3). Only three susceptible (of 24 total) plant DNA samples amplified OPM16₇₅₀. This marker exhibited 3% recombination with the downy mildew (*Dm*) resistance locus and mapped only 3.3 cM from it. Only 1% recombination was observed between UBC359₆₂₀ and OPM16₇₅₀.

Table 2. Sequences of the 30-mer and 20-mer oligonucleotide Sequence Characterized Amplified Region (SCAR) primers derived by sequencing RAPD markers linked to cotyledon stage downy mildew resistance in broccoli. The original 10-mer RAPD primers are underlined.

Primer	Sequence
UBC359	
Forward	<u>AGGCAGACCT</u> AAGGTAGACA AGTATTGTAG
Reverse	<u>AGGCAGACCT</u> ATGAGCACTC TAGAGTTATA
OPM16	
Forward	<u>GTAACCAGCC</u> CCTTGTGAAC
Reverse	<u>GTAACCAGCC</u> TTGAAGCCCA

Genetic sequences for the UBC359₆₂₀ and OPM16₇₅₀ clones revealed fragment lengths similar to the sizes estimated from agarose gels. The UBC359 marker was determined to be 610 compared to 620 bp and the OPM16 marker was 776 compared to 750 bp. All sequences contained the original RAPD primer sites. These observations verified successful band isolation and cloning.

When 20-mer SCAR primers based on the sequence of UBC359₆₂₀ (Table 2) were tested, several bands were amplified in bulk individuals, including a band of the same size (620 bp) as the original RAPD fragment in most susceptible samples. Several different annealing temperatures were assessed, and the number of observed bands did not change with different temperatures. As the marker was amplified in susceptible individuals, the 20-mers were not used to evaluate the F₂ study population.

Initial PCR using UBC359₆₂₀ 30-mers at an optimized annealing temperature of 68 °C revealed a single 620-bp band in resistant bulk individuals only (Fig. 2). Therefore, the 30-mers were tested on the F₂ study population, in which they either amplified a single 620-bp fragment or no fragment at all. Evaluation of the UBC359 30-mers with the F₂ revealed slightly different results than the original RAPD screening. As with RAPDs, the 620-bp SCAR marker was observed in the same four cases where susceptible plant DNA amplified UBC359₆₂₀. In addition, the marker was lacking in two cases in which resistant DNA served as template. This raised recombination between the resistance locus and the marker from 4% for the RAPD analysis to 6% for the SCAR, and increased the linkage distance from 4.4 to 6.7 cM, respectively.

Results for the SCAR primer pairs based on OPM16₇₅₀ were different than those based on UBC359₆₂₀. Essentially, the OPM16₇₅₀ 30-mers were not deemed useful for distinguishing resistant and susceptible F₂ plants. On the contrary, preliminary screening of the OPM16₇₅₀ 20-mers using an optimized annealing temperature of 70 °C produced a band of the same size (750 bp) as the original RAPD fragment in R-bulk individuals, but not in S-bulk individuals. Although the pair of 20-mers generated some other faint bands in all bulk individuals, these bands were distant from the 750-bp band and did not interfere in scoring the marker (Fig. 3). Therefore, the 20-mers were used to examine the F₂ study population. In this screen, the 750-bp band was observed in all resistant samples, and comparison of the original RAPD reaction results to the SCAR results revealed no differences. In both instances, the marker was observed in the same three cases where susceptible plant DNA served as the template, while it was observed in all resistant samples. Recombination with resistance based on OPM16 20-mer analysis was 3%, and the calculated distance from the resistance locus was 3.3 cM.

Discussion

In this study, genetic markers linked to cotyledon-stage downy mildew resistance were identified using bulked segregant analysis in combination with RAPD screening. On average, each RAPD primer that resulted in successful amplification yielded 1.5 polymorphisms between R and S, indicating that the parents exhibit a relatively high level of genetic divergence and that RAPDs were useful markers for meeting the objectives of this study. Two of eight identified markers, UBC359₆₂₀ and OPM16₇₅₀, were deemed suitable candidates for SCAR development providing 96% and 97 % accuracy, respectively, in identifying resistant and susceptible F₂ individuals. Mapping revealed that these two dominant markers are located relatively close to each other and on the same side of the resistance locus (Fig. 1). To date, we are unaware of any other published reports describing a DNA marker linked to a gene conferring cotyledon-stage resistance in *B. oleracea*.

By themselves, the UBC359₆₂₀ and OPM16₇₅₀ RAPD markers could prove useful in plant breeding programs due to the relatively high accuracy they display in identifying resistant individuals without the need for downy mildew inoculation. In addition, these RAPD markers could be placed onto existing maps (Cheung et al., 1997; Hu et al., 1998), providing information on chromosomal placement of the resistant locus in the genome. A limitation of these RAPD markers could result due to problems in reproducibility of RAPD results among different laboratories (Weeden et al., 1992). To overcome this potential problem, we developed SCARs from the RAPD markers.

UBC359₆₂₀ and OPM16₇₅₀ were successfully cloned in this study. The 30-mer primers for UBC359₆₂₀ were most effective SCAR primers, producing a 620-bp marker in four susceptible samples that also exhibited UBC359₆₂₀, and failing to amplify the SCAR marker in only two resistant samples that exhibited UBC359₆₂₀.

Although the UBC359₆₂₀ SCAR marker exhibited less accuracy (94%) than the original RAPD (96%), it still may prove useful from a plant breeding standpoint. Despite the challenges associated with determining the accuracy of any disease evaluation, breeders often assume that 90% to 100% accuracy in resistance screening is acceptable, although at least 95% accuracy is desired (C.E. Thomas, personal communication). It is difficult to discern why two resistant individuals of the population that exhibited the UBC359₆₂₀ RAPD failed to exhibit the corresponding SCAR marker. However, other researchers (Brahm et al., 2000) have observed similar phenomena.

The OPM16₇₅₀-derived primers gave different results than those based on UBC359₆₂₀. The OPM16₇₅₀-derived 20-mers were more accurate primers, producing the SCAR marker in only three susceptible samples in addition to all of the resistant individuals. This SCAR displayed the same high accuracy (97%) in identifying resistant and susceptible individuals as the original OPM16₇₅₀. This dominant marker should be a valuable tool in facilitating marker-assisted selection.

We have observed that the dominant resistance genes described herein are functional when our resistant lines (e.g., USVL089) are crossed to various susceptible lines to form different hybrid combinations or genetic backgrounds (unpublished data). Thus, we presume that the SCAR markers from USVL089 will also function in different genetic backgrounds. However, this must be confirmed in additional studies.

Because SCARs can be useful in identifying resistant and

susceptible plants for use in breeding programs, they can aid breeders in incorporating a number of desirable alleles into a single cultivar. This process, called pyramiding, combines several identified sources of disease resistance into one line. The result is a cultivar having several resistance genes that protect the crop against the prevalent strain of the pathogen and possibly against new races of the pest pathogen (Fehr, 1987). The SCARs described herein may be useful in such a gene pyramiding strategy for combating downy mildew in *B. oleracea*. Recent informal reports (Agnola et al., 2000; Farinho et al., 2000) indicate that several genes for cotyledon stage downy mildew resistance are under study. If these genes prove different from one another, it is possible they might be incorporated into a single resistant cultivar in the future.

This paper formally describes the production of useful SCAR markers linked to downy mildew resistance in *B. oleracea*. This work emulates the successful application of bulked segregant analysis and SCAR development demonstrated in other species (Correa et al., 2000; Deng et al., 1997; Murayama et al., 1999) and indicates these techniques can be readily applied to *B. oleracea* crops.

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