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ORIGINAL PAPER

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Characterization and high-resolution mapping of a late blight resistance locus similar to *R2* in potato

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Abstract Identification of resistance (*R*) genes to *Phytophthora infestans* is an essential step in molecular breeding of potato. We identified three specific *R* genes segregating in a diploid mapping population. One of the *R* genes is located on chromosome 4 and proved phenotypically indistinguishable from the *Solanum demissum*-derived *R2*, although *S. demissum* is not directly involved in the pedigree of the population. By bulked segregant analysis combined with a resistance assay, a genetic linkage map of the *R2-like* locus was constructed with 30 coupling and 23 repulsion phase AFLP markers. Two markers flanking the *R2-like* locus were applied to screen an extended population of 1,586 offspring. About 103 recombinants were selected, and an accurate high-resolution map was constructed. The *R2-like* resistance was localized in a 0.4 cM interval and was found co-segregating with four AFLP markers, which can be used to isolate the *R2-like* gene by map-based gene cloning. By analyzing race-specificity and *R* gene-specific molecular markers, we also found that an *R1-like* gene and an additional unknown *R* gene are segregating in the population.

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Introduction

Phytophthora infestans is the causal agent of late blight and one of the most important pathogens in cultivated potato (*Solanum tuberosum* L.). The economic value of the loss of global annual production and the cost of crop protection are estimated to be US $5 billion annually (Duncan 1999). In early breeding programmes for late blight resistance, 11 resistance (*R*) genes originating from the hexaploid wild species *S. demissum* were introduced into *S. tuberosum* (Black et al. 1953; Malcolmson and Black 1966). These *R* genes confer race-specific resistance, and they are controlled by major single dominant factors (Mastenbroek 1953; Malcolmson and Black 1966). The potato–*P. infestans* interaction is following the gene-for-gene model as proposed by Flor (1971). Although the *R* genes do not give durable resistance, the isolation of these *R* genes is of importance to understand the mechanism of the specific plant defence. Several *R* genes have been localized, e.g. *R1* on chromosome 5 (Leonards-Schippers et al. 1992) and *R3*, *R6* and *R7* clustered on chromosome 11 (El-Kharbotly et al. 1994, 1996). *R1* and *R3* have been isolated (Ballvora et al. 2002; Huang 2005). The *R2* locus has been localized on chromosome 4 using a tetraploid mapping population EJ96-4061 comprising 86 offspring (Li et al. 1998). Eleven AFLP markers linked to the *R2* locus have been identified using bulked segregant analysis [(BSA) Michelmore et al. 1991)], and the *R2* linkage group has been assigned using the reference map of AM3778-16. Three AFLP markers that are tightly linked to *R2* are present in the diploid mapping population RHAM026, which is used in this study.

Recently many different haplotypes of *R3-like* genes were discovered at the *R3* locus in *S. demissum* and ample variation for functional *R* genes to late blight appears to exist in *Solanum* species (Huang et al. 2004, Huang 2005). In this research we identified resistances by assaying race specificities and focused on a locus that is indistinguishable from *R2* in the diploid mapping

population RHAM026. Because the resistance was introgressed from different wild species, we designate the gene as *R2-like*. From the *R2-like* locus, a high-resolution map was constructed.

Materials and methods

Plant materials

The diploid mapping population RHAM026 was obtained from a cross between AM3778-16 (AM) and RH89-039-16 (RH). The dihaploid female parent AM is derived from the tetraploid breeding clone AM78-3778, which has various wild *Solanum* species in its pedigree, including *S. tuberosum* spp. *andigena*, *S. vernei*, *S. vernei* spp. *ballsii*, *S. oplocense* and *S. edinense*. The detailed pedigree of the mapping population is shown in Fig. 1.

The mapping population was generated from seeds that were sown in vitro after they were sterilized. When the seeds germinated and the seedlings reached approximately 3–5 cm, they were duplicated. One was used for maintenance and another for DNA isolation.

Inoculum preparation and resistance assay

Four different isolates were used to identify race-specific resistance (Table 1). For experiments, a plug of mycelium was transferred to a fresh plate with rye sucrose agar medium (Caten and Jinks 1968). One to 2 weeks later, ice-cold water was added to the mycelium that covered the agar plate. The sporangiospore suspension was pipetted into a tube and incubated at 4°C for 3 h.

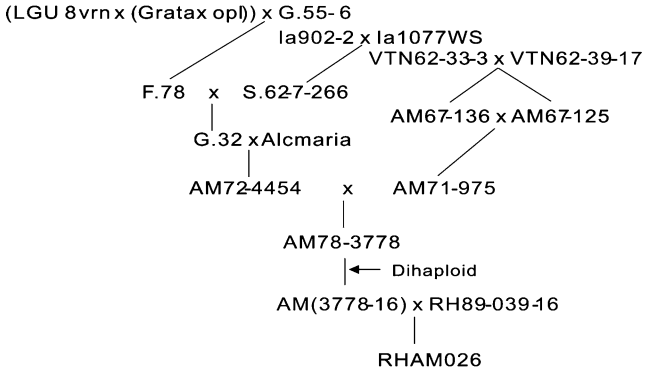

Fig. 1 Pedigree of the diploid mapping population RHAM026 (RH) used in this study

After the release of zoospores, the concentration of inoculum was adjusted to 5×10^4 spores/ml.

A resistance assay was performed by a detached leaf assay. Fully expanded and healthy leaves were collected from greenhouse plants and inoculated on wet paper in humid trays. Droplets of 10 µl inoculum were applied to the leaves, which were subsequently incubated at 15°C (Vleeshouwers et al. 1999). The symptoms on the leaves were evaluated 3 days and 5 days after inoculation.

DNA isolation

A high-throughput DNA isolation procedure was followed using a Retsch machine (Retsch, Haan, Germany) and 96-deep-well COSTER microtiter plates (Corning, Corning, N.Y., USA). Fresh leaf tissue was obtained by harvesting in vitro plants and grinding with two steel balls in the presence of nuclear lysis buffer (0.2 M Tris-HCl, 0.05 M EDTA, 2 M NaCl, 2% CTAB, with an end pH of 7.5), DNA extraction buffer (100 mM Tris-HCl, 5 mM EDTA, 0.35 M Sorbitol, 20 mM sodium bisulfite, with an end pH of 7.5) and 5% sarcosyl, followed by incubation at 65°C in a water bath for 1 h. Ice-cold chloroform isoamyl alcohol (24:1) was added, and the samples were mixed and centrifuged. The supernatant was transferred to new tubes and an equal volume of isopropanol was added. Another centrifugation step allowed the precipitation of DNA. The DNA pellet was dried and dissolved in $T_{0.1}$E-buffer (+0.5 µg RNAse).

AFLP analysis

The AFLP analysis was essentially carried out as described by Vos et al. (1995). Primary template DNA was prepared using *Eco*RI and *Mse*I and adaptors fitting to the restriction enzyme sites. Template DNA was diluted ten times prior to the selective pre-amplification with single-nucleotide extended primers, which decreases marker density. For the selective amplification, AFLP reactions with three nucleotides extended primers (*Eco* + 3/*Mse* + 3 primers) were performed to find markers linked to the resistance locus. The AFLP bands were separated on a 6% polyacrylamide gel in a Li-cor sequencer (Li-cor, Lincoln, Neb., USA).

The AFLP markers were named with the enzyme, the primer combination and the mobility of the fragment as described in reference autoradiograms created by Keygene NV, Wageningen, The Netherlands.

Table 1 Isolates of *Phytophthora infestans* and virulence factors

Isolate	Race specificity	Source
IPO-0	0	W. Flier, Plant Research International, The Netherlands
99018	1, 4	F. Govers, Wageningen University, The Netherlands
90128	1, 3, 4, 7, 8, 11	F. Govers, Wageningen University, The Netherlands
USA618	1, 2, 3, 6, 7, 11	W.E. Fry, Cornell University, USA

Genetic analysis of resistance

The AFLP markers linked to a resistance locus were identified by BSA (Michelmore et al. 1991). The data were analyzed using JoinMap software (Stam 1993). Marker order was determined by Record (van Os et al. 2000), and the map distance was calculated based on the frequency of the recombination between markers. A marker-saturated genetic linkage map was produced by MapChart (Voorrips 2002).

To identify race-specificity of the resistances, *R1*-specific marker 76_2s (forward primer: 5′-CAC-TCGTGACATATCCTCACTA-3′ and reverse primer: 5′-CAACCCTGGCATGCCACG-3′; Ballvora et al. 2002) and *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 (Li et al. 1998) were used.

Results

Characterization of resistance

A subset of the population of 28 genotypes and both parents AM and RH were evaluated for resistance to four different isolates, which differ in race specificity (Table 1). All 28 genotypes were susceptible to the isolate USA618, but segregated for resistance to the isolates IPO-0, 99018 and 90128 (Fig. 2a). The differential segregation for resistance to the three isolates suggests that three specific *R* genes are segregating in the population.

To examine whether known or unknown *R* genes segregate in RHAM026, specific molecular markers were designed. The presence of *R1* gene-specific sequences was tested by applying the 76_2s marker (Ballvora et al. 2002) on parents and progenies, and a clear segregation was found (Fig. 2b). The *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 (Li et al. 1998) were also tested. Both markers showed an identical segregation pattern in the progenies (Fig. 2b).

By comparing the genotypic results with the phenotypic results, we divided the 28 genotypes into five groups, which could have different candidate *R* genes (Fig. 2c). Presence of the 76_2s marker matched with the *R1*-specific resistance to *P. infestans* isolate IPO-0 (groups I and IV), and the *R2*-linked AFLP markers EATC/MCGA_186 and EACT/MCAC_189 perfectly matched with the *R2*-specific resistance to isolate 90128 (groups I and II). Some genotypes that do not contain the *R1* and *R2* markers were susceptible to isolate 90128, but resistant to isolates IPO-0 or 99018 (group III). This might be caused by another resistance gene that we designated R_{unknown}. In conclusion, the combination between race-specificity and molecular marker data suggests that R_{unknown}, *R1-like* and *R2-like* genes are segregating in the population.

Marker-saturated genetic linkage map

To determine the location of the *R2-like* locus, a genetic linkage study was initiated with the RHAM026 population comprising 78 genotypes. A detached leaf assay with *P. infestans* isolate 90128 revealed 24 resistant and 45 susceptible genotypes, which fits to a 1–2 segregation according to the χ^2 test. This segregation is skewed to susceptible, which might be caused by the relatively small population size or by postzygotic selection against unfavourable allelic combinations (Gebhardt et al. 1991; El-Kharbotly et al. 1994). Nine genotypes were not assigned due to unclear observation. A resistant and a susceptible bulk were composed of eight genotypes each. Two hundred fifty-six *Eco* + 3/*Mse* + 3 primer combinations were examined in the resistant parent, the susceptible parent, the resistant bulk and the susceptible bulk by BSA, and 33 coupling and 31 repulsion-phase candidate markers were revealed. After selecting the candidate markers, the 16 genotypes used to compose the resistant and susceptible bulks were separately checked for linkage to the resistance locus. Finally 30 coupling phase markers and 23 repulsion-phase markers were identified to be linked to the *R2-like* locus (Fig. 3). Seven AFLP markers co-segregate with the resistance locus. The linkage group was determined with two AFLP markers, EATC/MCGA_186 and EACT/MCAC_189, which were bridged to the genetic map of chromosome 4 where the *R2* locus is located (Li et al. 1998).

High-resolution map

To construct a high-resolution map, we selected two flanking AFLP markers (Fig. 3) to screen an extended population for selecting recombinants around the resis-

Fig. 2 Graphical genotyping of 28 offspring and parents AM and RH. **a** Phenotypic results with the *Phytophthora infestans* isolates IPO-0, 99018, 90128 and USA618. R, S and nd indicate resistant, susceptible and undetermined, respectively. **b** Genotypic results obtained with *R1*-specific marker 76_2s and the AFLP markers EATC/MCGA_186 and EACT/MCAC_189 linked to *R2*. aa and ab indicate absence and presence of the marker, respectively. **c** Classes distinguished based on the phenotypic and genotypic data

		AM	RH	28 progenies																											
A	IPO-0	R	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S
	99018	R	S	R	R	R	R	R	R	R	R	R	R	nd	nd	nd	S	S	S	S	nd	S	S	S	S	S	S	S	S	S	S
	90128	R	S	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	USA618	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
B	76_2s	ab	aa	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
	EATC/MCGA_186	ab	aa	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
	EACT/MCAC_189	ab	aa	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
C	Group			I		II					III						IV				V										

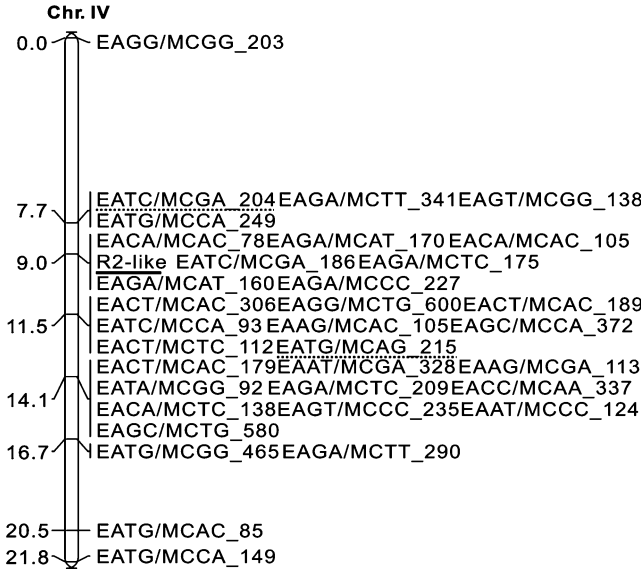

Fig. 3 Genetic linkage map of the *R2-like* locus. The resistance locus is *underlined*. Two flanking markers used for screening the extended population are *dot-underlined*. All 30 coupling phase markers, but only four repulsion phase markers and the resistance locus, are included in the map

tance locus. One is EATC/MCGA_204, which was mapped 1.3 cM telomeric from the resistance locus. Another is EATG/MCAG_215, which was mapped 2.5 cM centromeric from the resistance locus in the 78 offspring (Fig. 4). By screening the expanded population of 1,582 genotypes, 103 recombinants were selected resulting in a larger interval of a 6.4-cM genetic distance between these two AFLP markers. We screened the 103 recombinants with 14 AFLP markers located between EATC/MCGA_204 and EATG/MCAG_215 (Fig. 3) and tested them for their resistance to *P. infestans* isolate 90128. Subsequently a high-resolution genetic map of the 6.4-cM interval containing the resistance locus was constructed (Fig. 5), in which the AFLP markers are precisely separated and mapped. Closest markers on both sides of the resistance locus are EACA/MCAC_105 (three recombinants in 0.2 cM) and a group containing five markers (three recombinants in 0.2 cM). Four AFLP markers are still co-segregating with the resistance locus.

Comparative genetics

The *R2* and *R2-like* loci derived from two different mapping populations were compared. The bridged markers EATC/MCGA_186 and EACT/MCAC_189 are 0.2 cM apart in the *R2-like* diploid population (three recombinants in 1,582 genotypes) and 1.2 cM in the *R2* tetraploid population (one recombinant in 86 genotypes) (Li et al. 1998). One AFLP marker, EATC/MCGA_186, co-segregates with the *R2-like* locus, but is 1.2 cM separated from the *R2* locus. Another AFLP marker EACT/MCAC_189 is 0.2 cM separated from the *R2-like* locus, but co-segregates with the *R2* locus. The position of flanking markers that connect the two genetic linkage maps confirmed that the *R2* and *R2-like* loci are located at the same region of chromosome 4.

Discussion

In this research, we identified three resistance loci in the diploid segregating population RHAM026. One locus was localized on chromosome 4, where the *R2* locus from *S. demissum* was previously mapped using a tetraploid mapping population EJ96-4601 (Li et al. 1998). Accurate specificity studies with a panel of four different *P. infestans* isolates revealed that *R2-like* resistance of

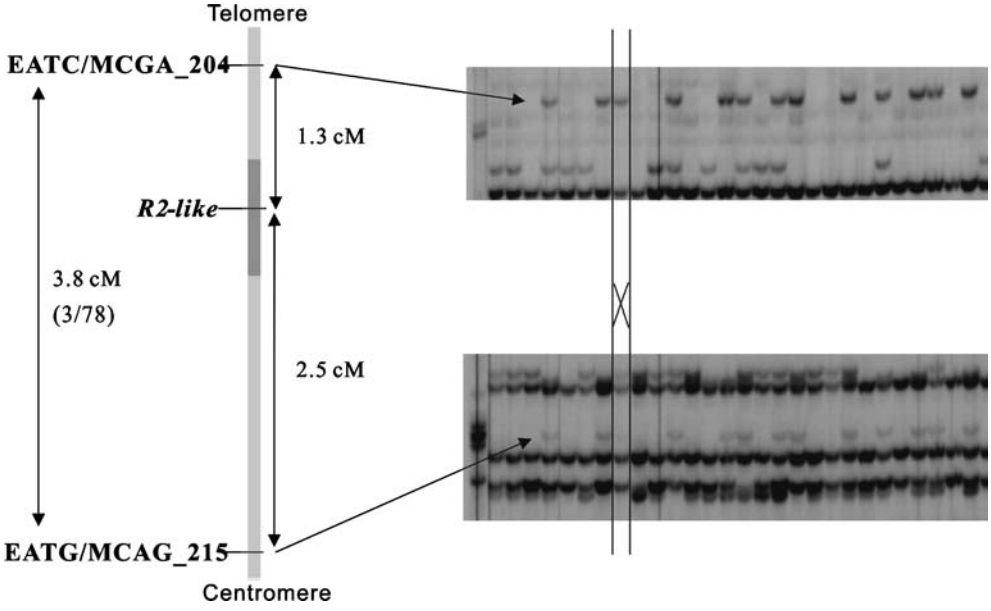

Fig. 4 Schematic representation for recombinant screening towards high-resolution mapping. The AFLP gel images of the two flanking AFLP markers EATC/MCGA_204 and EATG/MCAG_215, which were used to select recombinants in 1,582 genotypes. One example of a recombinant is indicated by *X*

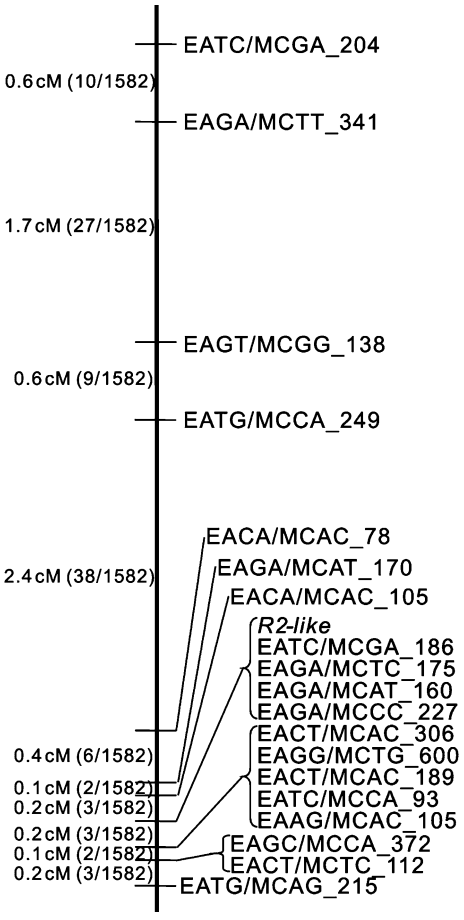

Fig. 5 High-resolution map of the *R2-like* locus. The 1,582 genotypes were screened with two AFLP markers, EATC/MCGA_204 and EATG/MCAG_215, which are *dot-underlined*, and 103 recombinants were selected. Four markers are co-segregating with the *R2-like* locus

this population is phenotypically indistinguishable from *R2*.

The origin of the AFLP markers EACT/MCAC_189 and EATC/MCGA_186 was previously thought to be *S. demissum*-specific, because the AFLP markers were identified in the fingerprints of three accessions of *S. demissum* (Li et al. 1998). However, we observed that EACT/MCAC_189 is also present in *S. acaule* accessions and EATC/MCGA_186 in *S. bulbocastanum* accessions (data not shown) and therefore conclude that these markers are present in a broader range of *Solanum* germplasm. Although *S. demissum* is not directly present in the ancestors of the *R2-like* diploid population, the introgression fragment could be of wild species origin, e.g. *S. edinense*, which is a natural hybrid between *S. tuberosum* and *S. demissum* (Hawkes 1990). It remains unknown what the origin of the *R2-like* locus is. Even if it is derived from *S. demissum*, definite identity with *R2* (Li et al. 1998) can only be determined after cloning both genes.

To construct a marker saturation and high-resolution map at the R2-like locus, we used a diploid population. Compared to the tetraploid level, mapping studies at the diploid level can avoid the complexities of tetrasomic inheritance and makes the study of potato genetics more feasible (Jacobs et al. 1995; Li et al 1998). The number of markers is at least fourfold higher in a diploid population than in a tetraploid population, because marker alleles with linkage in repulsion can be used at the diploid level (Wu et al. 1992). We identified 69 AFLP markers with linkage to *R2-like*, using 256 primer combinations. A similar high efficiency was achieved in another study in which 29 AFLP markers with linkage to *R1*, using 108 primer combinations were identified at the diploid level (Meksem et al. 1995). At the tetraploid level, much lower efficiency was achieved. Li et al. (1998) detected only 11 AFLP loci with linkage to *R2*, using 205 primer combinations.

Many R genes and resistance gene homologues are clustered in the genome (Meyers et al. 1998; Michelmore and Meyers 1998). In the potato genome, for instance, at least five R genes against diverse pathogens are located at the gene cluster on chromosome 5 (Leister et al. 1996; Marano et al. 2002), i.e. *Gpa* conferring resistance to the potato cyst nematode *Globodera pallida* (Kreike et al. 1994), *Grp1* conferring resistance to the potato cyst nematode *Globodera rostochiensis* (Rouppe van der Voort et al. 1998), *Nb* conferring resistance to potato virus X (de Jong et al. 1997), *Rx2* (Ritter et al. 1991) conferring resistance to potato virus X and *R1* conferring resistance to *P. infestans* (Leonards-Schippers et al. 1992). Also near the *R2-like* locus on chromosome 4, various known R genes are located. The *Hero* gene, which confers broad-spectrum resistance against *G. rostochiensis* in tomato, is located between CT229 and TG370 (Ganal et al. 1995), *Gpa4* conferring resistance to *G. pallida* (Bradshaw et al. 1998) and *R2* (Li et al. 1998), *Rpi-abpt* (Park et al. 2004) and *Rpi-blb3* (Park et al. 2004) conferring resistance to *P. infestans* are mapped at the same region on chromosome 4.

One of the most challenging methods for gene cloning in a plant species with a large genome is map-based cloning, which can be considered routine in model species such as *Arabidopsis* with a small genome (Lukowitz et al. 2000). Accurate mapping of the target gene and identification of recombinants in the population are essential steps for map-based cloning, and here we report a reliable high-resolution map of the *R2-like* locus within a 0.4-cM interval on chromosome 4 in the potato breeding line RHAM026. The initial research for tightly linked AFLP markers was facilitated using AFLP technology (Vos et al. 1995) in combination with BSA (Michelmore et al. 1991). The *R2-like* locus is flanked by one AFLP marker on the telomeric side, a group of five AFLP markers on the centromeric side and co-segregating with four AFLP markers (Fig. 5). The flanking and co-segregating markers will facilitate isolation of the *R2-like* gene by BAC walking. A 0.2-cM (4/2,109 recombinants) interval was covered by one BAC clone of 100 kb in the cloning of the potato late blight resistance gene *Rpi-blb1* (van der Vossen et al. 2003) and in the cloning of *R1*, a 0.2-cM (2/1049 recombinants) interval was estimated at 250–300 kb (Ballvora et al. 2002). We estimate that the 0.4-cM

interval spanning the *R2-like* gene could be covered by a single BAC clone or a few BAC-walking steps.

Currently a BAC library of diploid genotype AM is being constructed to establish a physical contig with the co-segregating markers and the flanking markers and isolation of *R2-like* gene in near future is quite feasible. After that, physical analysis of the gene cluster containing the *R2-like* gene might accelerate isolation of the homologous genes conferring resistance to *P. infestans* in different genetic backgrounds.

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