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Mapping the *R10* and *R11* genes for resistance to late blight (*Phytophthora infestans*) present in the potato (*Solanum tuberosum*) R-gene differentials of Black

Abstract The R10 and R11 late blight differentials of Black (tetraploid clones 3681ad1 and 5008ab6) were crossed with the susceptible potato (*Solanum tuberosum*) cultivar Maris Piper and the progeny were assessed for blight resistance in a whole plant glasshouse test using race 1,2,3,4,6,7 of *Phytophthora infestans*. The disease scores for the R10 population displayed a continuous distribution whereas the progeny in the R11 population could be categorised as resistant or susceptible. A bulk segregant analysis using amplified fragment length polymorphism assays was done on the ten most resistant and ten most susceptible progeny in each population and two closely linked markers were found to be associated with resistance. *R11* mapped to 8.5 cM from marker PAG/MAAG_172.3 and *R10* mapped as a quantitative trait locus in which marker PAC/MATC_264.1 explained 56.9% of the variation in disease scores. The results were consistent with *R10* and *R11* being allelic versions of genes at the *R3* locus on chromosome 11. The implications are discussed for mapping R-genes which fail to give complete immunity to a pathogen.

Introduction

Late blight disease of potato (*Solanum tuberosum*), caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, first made its impact outside of Mexico in the mid-1840s when severe epidemics swept through North America and Europe and resulted in the

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Irish potato famine (Large 1940). The need for resistant cultivars was clear and an apparent breakthrough came in 1909 when Salaman recognised the Mexican wild species *S. demissum* as a source of extreme resistance that could be backcrossed into *S. tuberosum* (Muller and Black 1951). Breeding for late blight resistance therefore concentrated on using *S. demissum's* major dominant R-genes, of which 11 were identified (Muller and Black 1951; Malcolmson and Black 1966; Malcolmson 1969). For example, at the Scottish Plant Breeding Station, cultivar Pentland Ace (with resistance gene *R3*) resulted from a cross made in 1937 between *S. phureja* and *S. demissum*, and was released in 1951 after just three backcrosses to *S. tuberosum*. The commercially more successful cultivar Pentland Dell (having resistances *R1*, *R2* and *R3*) required two further backcrosses, and entered ware production in 1963 at a time when race 4 was the prevalent race of *P. infestans*, but succumbed to blight in 1967. In 1967 and 1968 races overcoming the more recently discovered R-genes (*R5–R11*) were widely distributed in Great Britain although these genes were not present in common commercial varieties, and it was clear that they would not provide durable resistance either singly or in combination due to the evolution of new races of *P. infestans* (Malcolmson 1969). As a consequence, many breeders switched to selecting for quantitative field resistance either by using races of *P. infestans* compatible with the R-genes present in their material or by creating R-gene free germplasm so that screening could be done with any race (Toxopeus 1964; Black 1970; Wastie 1991; Ortiz 2001).

Today there is renewed interest in the use of R-genes for resistance to late blight. Since 1984, new populations of *P. infestans* have been spreading from Mexico to the rest of the world (Goodwin and Drenth 1997). These new populations are seen as an emerging threat to food security because they comprise both the A1 and A2 mating types, some strains have increased aggressiveness and there is insensitivity to the widely used fungicide metalaxyl (GILB 1999). Breeding for resistance has therefore intensified and new sources of

major R-gene resistance have been found in *Solanum* species other than *S. demissum*. These include: *S. berthaultii* (Ewing et al. 2000), *S. bulbocastanum* (Naess et al. 2000; Park et al. 2003; van der Vossen et al. 2004), *S. mochiquense* (Smilde et al. 2005) and *S. pinnatisectum* (Kuhl et al. 2001). Some authors advocate the independent cloning and transfer of these new R-genes into a single cultivar by genetic transformation to produce a multi-line cultivar as a way of achieving durable resistance (Niederhauser et al. 1996; Huang 2005; Smilde et al. 2005). There is also some evidence that defeated R-genes may contribute to late blight resistance and that combining R-genes with high levels of field resistance is therefore a desirable goal (Stewart et al. 2003). Hence there is now great interest in mapping R-genes and quantitative trait loci (QTLs) for field resistance, including those from *S. demissum*, to see if new R-genes are alleles at a known genetic locus or a new locus and to see if QTLs are also alleles at known R-gene loci or closely linked to them. Of the 11 *S. demissum* R-genes, *R1* has been mapped to chromosome 5 of the 12 potato chromosomes (Leonards-Schippers et al. 1992), *R2* to chromosome 4 (Li et al. 1998), *R3*, *R6* and *R7* to a complex locus on chromosome 11 (El-Kharbotly et al. 1994; Huang et al. 2004) and *R5*, *R8*, *R9, R10* and *R11* have been shown to be allelic versions of the *R3* complex locus by marker analysis and mapping results (Huang 2005). However, *R10* and *R11* have not been mapped in the original R-gene differentials of Black, namely tetraploid clones 3681ad1 and 5008ab6, which are maintained at SCRI. This paper therefore presents the results of mapping them in these clones, both as quantitative and qualitative traits through their linkage to molecular markers, and demonstrates that *R10*, unlike *R11*, behaves more like a QTL than a major dominant R-gene.

Materials and methods

Crosses and plant material

In the year 2000, the R10 and R11 differentials (tetraploid clones 3681ad1 and 5008ab6, respectively) were crossed as female parents with the cultivar Maris Piper which does not carry any R-genes. In 2001, 200 seeds of each cross were sown in a glasshouse and 150 seedlings raised and their tubers harvested to provide up to 150 clones of each cross. These clones were maintained by planting three tubers of each in the glasshouse in 2002 and 2003, and the blight resistance tests were done in 2003 on the 146 clones from each cross with enough tubers. Leaf samples for molecular marker analysis were taken from the maintenance plants during the week beginning 12 May 2003. Between 5 and 10 g of young, fully expanded leaves were harvested from each clone, "flash-frozen" in liquid nitrogen, and stored at −80°C.

Blight isolates

Two isolates were used in the blight tests. Isolate 99/23, race 1,2,3,4,6,7 (isolated in 1999), originated in Scotland, and isolate CP, race 1,3,4,7,10,11 (isolated in 1994) came from Wales. Both isolates were passaged through potato tubers to select for increased aggressiveness before use in the resistance tests. The virulence characteristics of each isolate were verified in a whole plant test using Black's differential series of R-gene-bearing clones (Black et al. 1953; Malcolmson and Black 1966; Malcolmson 1969).

Blight resistance tests

A whole-plant glasshouse test, similar to that described by Stewart et al. (1983), was used to determine blight resistance. Blight inoculum was prepared as described by Malcolmson (1976), using the zoospore suspension resulting from a sporangial suspension at an initial concentration of 14×10^3 sporangia/ml. A hand sprayer was used to apply the inoculum and the spray was allowed to fall evenly on the plants from above. For each progeny, two plants of each of the 146 clones, and six of each parent, in flower bud, were inoculated with race 1,2,3,4,6,7 (avirulent against the genes *R10* and *R11*). The plants were dampened, inoculated and incubated for 24 h in either a misted controlled environment cabinet or a misted cooled glasshouse maintained at 15°C and 95–100% humidity, then kept at 15°C in a cooled glasshouse. Seven days after inoculation each plant was scored using Malcolmson's 1–9 disease scoring scale where 9 is resistant (Cruickshank et al. 1982). Disease on lower leaves was ignored. The apparent presence or absence of the R-gene was noted: plants that were symptomless or had only limited necrotic lesions, or spreading lesions in the oldest leaves only, were assumed to carry the R-gene, whilst plants with spreading lesions on the other leaves were not. In 29 cases where the R-gene status was unclear, and two where it was clear, (31 clones of the R10 progeny), two further plants per clone (and six per parent) were inoculated with the avirulent race, and two plants per clone (six per parent) were inoculated with the virulent race 1,3,4,7,10,11 for comparison. Clones that showed R-gene phenotype (described above) with the avirulent race but spreading lesions with the virulent race were considered to carry the R-gene, whilst clones that developed spreading lesions similarly with both isolates were not. The differences in disease scores between the two races were also taken into account (see Results).

DNA isolation from plants

DNA was extracted from frozen plant tissue using the DNeasy Plant DNA Extraction kit (Qiagen cat.no 69106) according to the manufacturer's instructions.

Amplified fragment length polymorphism (AFLP) assays

Amplified fragment length polymorphism assays were performed using a modification of the protocol of Vos et al. (1995) as described in Bryan et al. (2002). AFLP fragment nomenclature has been described elsewhere (Bryan et al. 2002). The products of selective AFLP amplification were run on acrylamide gels and the products detected by autoradiography.

Bulked segregant analysis (BSA)

Two bulks (resistant and susceptible) were constructed from the offspring of each of the populations using the blight score data. The resistant bulks consisted of the ten clones with the least disease symptoms (highest scores on 1–9 scale) and the susceptible bulks consisted of the ten clones with the most disease (lowest scores on 1–9). DNA samples, in the form of AFLP secondary template taken from each of the selected progeny clones, were pooled in equimolar amounts to form the bulk samples.

Each of the bulk and parental DNA samples were subjected to AFLP analysis using 65 *Pst*I/*Mse*I AFLP primer combinations. Primer combinations showing at least one AFLP band present in the resistant parent and the resistant pool, and absent from the susceptible parent and the susceptible pool, were selected for application to all of the individuals in the bulk. Primer combinations, which produced a band that was present in all individuals in the resistant bulks but absent from all individuals in the susceptible bulks, were then applied to all individuals within both populations. Parents of a Ultra High Density mapping population, SH and RH, were included in the AFLP analyses to enable putative identification of markers which cosegregated with previously mapped markers (Isidore et al. 2003).

Analyses of the data

Analyses of variance of the disease scores and regressions of clone means on the presence and absence of the molecular markers were done using GENSTAT 5 Release 3 (GENSTAT 5 Committee 1993).

Segregation ratios of the markers and *R11* (*R10* could not be analysed as a qualitative trait) and the association of the markers with *R11* were tested using Pearson's chi-square statistic. As the markers and *R11* segregated in 1:1 ratios, simplex inheritance was inferred and the recombination frequency (*r*) for a marker and *R11* was calculated as: $r = (b + c)/n$ with standard error the square root of $r(1-r)/n$ where $b + c$ is the number of recombinant clones and n is the total number of clones in the R11 population.

Results

Late blight resistance

The analyses of variance for the two populations (R10 and R11) are shown in Table. 1 and 2. For both populations there were statistically significant ($P < 0.001$) differences between clones and most of the variation between clone means (mean of two replicates) was genetical rather than environmental (79.3% for R10 and 89.0% for R11). The histograms of the clone means for the two populations are shown in Figs. 1 and 2. The distribution for R10 is continuous and skewed with its mean (5.90) closer to the maximum value (8.50) than to the minimum value (1.5). In contrast, the distribution for R11 is clearly bimodal with the two peaks coinciding with the blight scores of Maris Piper (the susceptible parent) and the R11 differential (the resistant parent).

Bulked segregant analysis using AFLP markers

Bulks containing the most late blight resistant (R), and susceptible (S), clones from each of the crosses were screened using BSA. The ten resistant clones from the R10 population all had scores of 8.0 or 8.5 and the ten susceptible clones all had scores of 1.5, 2.0 or 2.5. Likewise for the R11 population, the disease scores were 7.5 or 8.0 for the resistant bulk and 1.0 or 1.5 for the susceptible bulk.

Each of the bulks and parents were subjected to AFLP analysis using 65 *Pst*I/*Mse*I AFLP primer combinations. Nineteen primer combinations showing at least one AFLP band present in the R10 resistant parent and the R10 resistant pool, and absent from the corresponding susceptible parent and the susceptible pool, were selected for application to all of the individuals in the bulks. Likewise, six primer combinations were found and applied to all individuals within the bulks of the R11

Table 1 Analysis of variance of blight scores for R10 population

| Source of variation	df	Mean squares
Replicates	1	0.507[NS]
Clones	145	6.575***
Residual	143	1.360

[NS]$P > 0.05$; ***$P < 0.001$

Table 2 Analysis of variance of blight scores for R11 population

Source of variation	df	Mean squares
Replicates	1	30.679***
Clones	145	10.677***
Residual	144	1.177

***$P < 0.001$

Fig. 1 Blight scores (mean of two replicates) for 146 clones in R10 population on 1 (susceptible) to 9 (resistant) scale
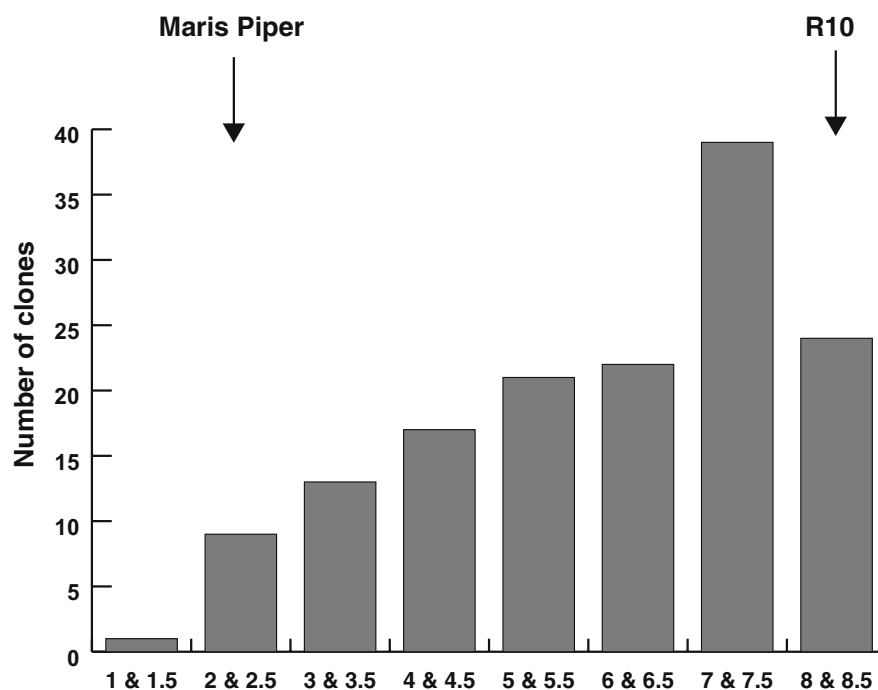

population. Two primer combinations, PAC/MATC and PAG/MAAG, produced a fragment (PAC/MATC_264.1 and PAG/MAAG_172.3) which was present in all individuals within the resistant bulks but absent from all individuals within the susceptible bulks of both populations. The results for PAC/MATC_264.1 in the R11 population are shown in Fig. 3 (those for R10 are identical). These bands were also present in SH and absent in RH, the parents of the UHD mapping population, and had been mapped in adjacent bins, SH11B066 (Bin 66) and SH11B065 (Bin 65)

(http://www.dpw.wageningen-ur.nl/uhd). These bins are located on the distal end of chromosome 11 and according to Huang et al. (2004) the *R3* locus also maps to this location.

Association of bands PAC/MATC_264.1 and PAG/MAAG_172.3 with blight scores

The results of the regressions of blight scores on the presence and absence of the markers are shown in

Fig. 2 Blight scores (mean of two replicates) for 146 clones in R11 population on 1 (susceptible) to 9 (resistant) scale
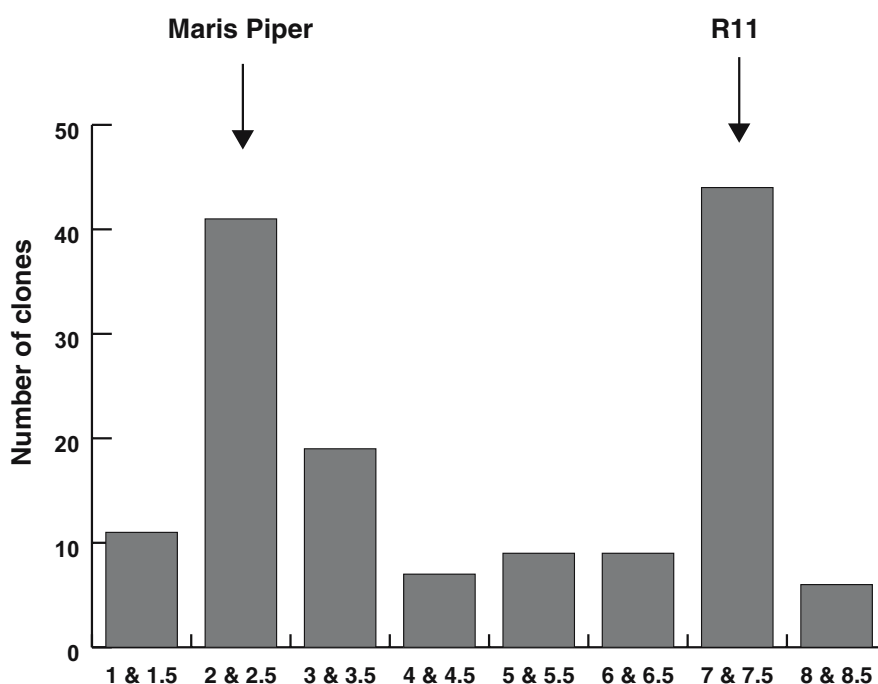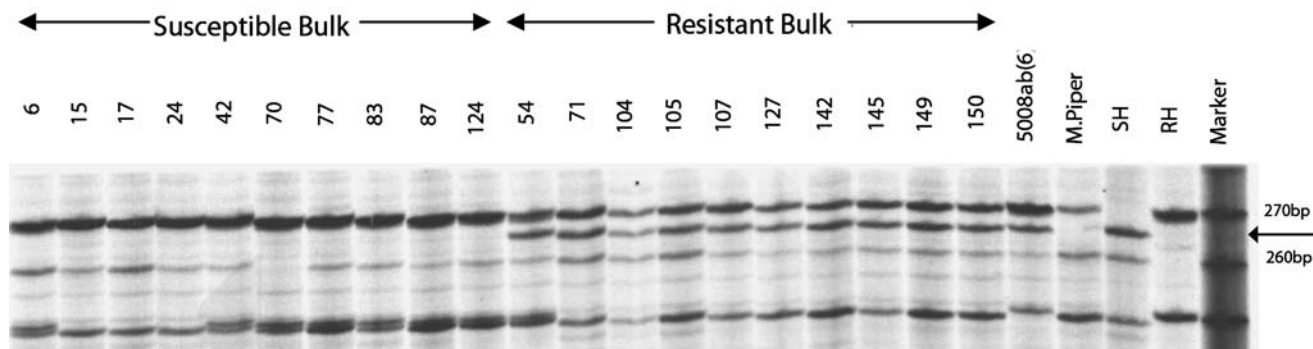
Fig. 3 Bulked segregant analysis of R11 population showing the ten susceptible clones, the ten resistant clones, the R11 differential (clone 5008ab6), cultivar Maris Piper and SH and RH, the parents of a UHD mapping population, and marker PAC/MATC_264.1

Tables 3 and 4. The regressions were statistically significant ($P < 0.001$) and explained between 48 and 64% of the variation in blight scores. With the R10 population, the average differences in blight scores between the presence and absence of the markers were 2.75 and 2.49 for PAC/MATC_264.1 and PAG/MAAG_172.3, respectively, compared with 5.66 for the difference between the R10 differential and Maris Piper. With the R11 population, the corresponding differences were 3.41 and 3.73 compared with 4.67 for the difference between the R11 differential and Maris Piper. Hence the markers explained more of the variation in the R11 population than in the R10 population.

Classification of clones with respect to *R10*

The blight scores for the R10 population displayed a continuous distribution, and hence could not be simply classified into resistant and susceptible. Although there was a big difference between the scores of the R10 parent (7.83) and Maris Piper (2.17), many clones had intermediate values with both replicates in agreement. The parents and 31 clones including 29 with uncertain R-gene status were tested with an avirulent and a virulent race to see if their R-gene status could be determined. As expected, Maris Piper was overcome by both the avirulent and virulent races of *P. infestans* resulting in mean disease scores of 3.67 and 3.50, respectively, with a maximum difference in disease scores of 1.5. The disease scores recorded on the R10 parent were 7.17 when the clone was challenged with the avirulent isolate and 4.14 when challenged with the virulent isolate. As a result of

these further tests, 11 clones were categorised as having *R10* based on a reduction in blight score of 3.0 or more when tested with the virulent as opposed to the avirulent isolate and seven of these clones had the expected type of lesion. Eleven clones were thought to lack *R10* based on a reduction in blight score of 1.5 or less and nine of these clones had the expected lesion type. The presence of *R10* in nine clones remained undetermined based on a reduction in blight score of 2.0 or 2.5. Two of these clones had a resistant lesion type, five had a susceptible type and two were intermediate. The mean disease scores of the three groups (*R10* present, *R10* absent and undetermined) with the avirulent race in the original test were almost identical, namely 6.95, 6.77 and 6.83. It was concluded that the R-gene status of the clones with intermediate disease scores could not be determined with sufficient certainty to test the association of the presence and absence of the markers with the presence and absence of *R10*. We have therefore treated *R10* only as a QTL.

Association of bands PAC/MATC_264.1 and PAG/MAAG_172.3 with *R11*

In contrast, in the R11 population, the clones scoring 6 and above were clearly resistant and contained *R11*, and those scoring 3.5 and below were clearly susceptible and lacked *R11* (Fig. 2). When the raw data were examined for the 16 clones scoring 4.0, 4.5, 5.0 and 5.5, it was found that for 13 of them one replicate was resistant and the other one was susceptible so that it was not possible to tell whether the clone contained the R-gene or not.

Table 3 Regression of blight scores on presence and absence of molecular markers in population segregating for *R10*

Marker	Percentage variation explained	Marker present	Marker absent	Difference
PAC/MATC_264.1	56.9	7.19	4.44	2.75 ± 0.199
PAG/MAAG_172.3	47.6	7.12	4.63	2.49 ± 0.217

Table 4 Regression of blight scores on presence and absence of molecular markers in population segregating for *R11*

Marker	Percentage variation explained	Marker present	Marker absent	Difference
PAC/MATC_264.1	51.6	5.98	2.57	3.41 ± 0.280
PAG/MAAG_172.3	63.6	6.36	2.64	3.73 ± 0.238

Hence these clones were omitted from the analysis of the association of the presence and absence of the markers with the presence and absence of *R11*. The two clones scoring 5.5 were classed as resistant and the one scoring 4.0 as susceptible. The results of the classification are presented in Table 5. A few clones (7 and 4, respectively) are missing because of lack of marker data. Both the markers and *R11* displayed a 1:1 segregation ratio (chi-square test statistic, $P > 0.05$) from which simplex inheritance can be inferred. The tests for linkage were statistically significant ($P < 0.001$) and the estimates of the recombination frequency were $r = 0.143 \pm 0.031$ and $r = 0.085 \pm 0.025$ for *R11* with PAC/MATC_264.1 and PAG/MAAG_172.3, respectively. As the markers were linked with a recombination frequency of $r = 0.081 \pm 0.024$, the gene order on the linkage map is PAC/MATC_264.1–PAG/MAAG_172.3–*R11*.

Discussion

Mapping confirmed that the *R10* and *R11* genes in Black's R10 and R11 differentials map to the distal end of chromosome 11 (as defined by the UHD map), as found by Huang (2005) using the Dutch differential set of Mastenbroek which is equivalent to Black's for R5–R11. Huang (2005) was able to conclude from his marker analysis and mapping results that the R5–R11 differentials all contain allelic versions of genes at the *R3* locus. The mapping in this paper was not quite so clear. Two molecular markers on the distal end of chromosome 11 were 8.1 cM apart compared with being in adjacent bins, separated by a single recombination event, in the UHD mapping population (http://www.dpw.wageningen-ur.nl/uhd). *R11* mapped to 8.5 cM from PAG/MAAG_172.3 and *R10* mapped as a QTL in which PAC/MATC_264.1 explained 56.9% of the variation in disease scores, and 2.75 of the 5.66 difference in blight scores between the R10 differential and cultivar Maris Piper. The apparent lack of precision in chromosomal locations could be due to increased recombination in our tetraploid mapping populations or errors in the blight scores or marker data. Considerable additional resources would be required to eliminate all possible errors in this kind of experiment, and precision in mapping can only be increased by increasing the population size (not the number of replicates) as it depends upon the number of recombinants produced during meiosis. Nevertheless, there was good agreement

from mapping *R11* as both a qualitative and quantitative trait. The estimated effect of *R11* when treated as a QTL (the difference in marker means divided by $1 - 2r$, where r is the recombination frequency between marker and QTL) was 4.49 for marker PAG/MAAG_172.3 compared with 4.67 for the difference in blight scores between the R11 differential and cultivar Maris Piper.

In this work, *R10* could only be mapped as a quantitative trait because of the continuous distribution of blight scores in the progeny resulting from the cross between the *R10* containing parent and the susceptible cultivar Maris Piper. There was also a lack of clear differences in disease score and lesion appearance when clones with intermediate values were inoculated with compatible (virulent) and incompatible (avirulent) isolates. This result was unexpected but can be related to previous results from SCRI with the R10 and R11 differentials where neither gene gave complete immunity to *P. infestans*. Stewart (1990) found that the resistances of R10 and R11 to avirulent isolates were not always fully expressed in detached potato leaflets and depended on plant age and inoculum concentration. In her results, resistance was best expressed in R5 in two experiments and R8 in a third experiment. In a further experiment, Stewart and Gourlay (1995) compared the results from glasshouse detached leaflet tests with glasshouse whole plant tests and field tests and found that R5, R8 and R10 were all highly resistant in all of the tests whereas R11 was highly resistant only in the glasshouse whole plant test. Compared with R5 and R8, R10 had a slightly lower field score (7 compared with 8 on a 1 to 9 scale of increasing resistance) and a slightly larger lesion size in the detached leaflet test. Again, Stewart and Bradshaw (2001) found that both R10 and R11 were highly resistant but not immune in whole plant glasshouse tests with avirulent isolates. The results of field experiments using virulent isolates (Stewart and Gourlay 1995; Stewart and Bradshaw 2001) have shown that Black's R10 differential possesses more background field resistance to *P. infestans* than R11 and the other differentials tested (R1, R3, R4 and R7). However, in the whole plant glasshouse experiments of Stewart and Bradshaw (2001), the field resistance of R10 was underestimated and that of R11 was overestimated, although the former was still higher than the latter. Interestingly, in whole seedling tests, Malcolmson and Killick (1980) found that out of nine clones and cultivars, the offspring of the R10 differential had the highest average score (general combining ability) for overall blight resistance and small lesion size in the

Table 5 Association of presence and absence of molecular markers with presence and absence of *R11*: number of clones in each category

R11	PAC/MATC_264.1			PAG/MAAG_172.3		
	Present	Absent	Total	Present	Absent	Total
Present	57	2	59	59	2	61
Absent	16	51	67	9	59	68
Total	73	53	126	68	61	129

leaf, petiole and stem. The higher field resistance of R10 compared with R11 was also apparent in the experiments of Stewart et al. (2003) which provided evidence of a small contribution to field resistance from defeated R-genes, including similar sized effects from *R10* and *R11*. Where comparisons could be made, the differences in blight scores between virulent and avirulent isolates were smallest for the R10 differential (Stewart and Gourlay 1995; Stewart and Bradshaw 2001). In conclusion, it seems reasonable to attribute the continuous distribution of blight scores in the cross between the R10 differential and cultivar Maris Piper to the background field resistance of the R10 differential.

The contrasting results for *R10* and *R11* suggest that the differences between major dominant R-genes and QTLs of large effect are not always clear cut. Both *R10* and *R11* are accepted as major dominant R-genes for blight resistance although their expression is variable. When mapped as QTLs, associated markers explained similar amounts of the phenotypic variation, namely 56.9 and 63.6%, although the distributions of blight scores were continuous and bimodal, respectively. Interestingly, a major QTL for field resistance to late blight has been found on chromosome 4 in cultivar Stirling which explains 37.2% of the variation in blight scores in the progeny of a cross to a susceptible clone (Bradshaw et al. 2004). Stirling also possesses a major dominant R-gene and the molecular mechanisms of R-gene mediated and field resistance are being compared in appropriate crosses, but the genes which are specifically upregulated in the two types of resistance are still to be identified (Avrova et al. 2004).

Isolates of *P. infestans* capable of overcoming the resistance of *R10* and *R11* are common throughout the world and hence these resistances are of little value in breeding for durable resistance (Swiezynski et al. 2000). However, the results in this paper, and the previous work done at SCRI and discussed above highlight some of the difficulties in working with these resistance genes which should be avoided in seeking new R-genes for use in breeding. Ideally, R-genes should be sought which give immunity or consistently high levels of resistance in detached leaflets (for speed of screening), whole plants and the field. It is easier to study the genetics of R-gene mediated resistance in a background of low levels of field resistance. However, in breeding for resistance, one wants to combine R-genes with high levels of field resistance in anticipation of breakdown of the R-gene resistance. Molecular markers that are always associated with the R-genes would be extremely useful in achieving this aim. Finally, one needs to remember that the durability of the R-gene resistance will be determined by the relative fitnesses of strains of *P. infestans* with and without the corresponding virulence genes, so that trying to determine these fitnesses would be worthwhile.

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