


Development of molecular markers linked to three spinach downy mildew resistance loci

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Abstract Downy mildew, caused by the obligate oomycete pathogen *Peronospora effusa*, is the most important disease of spinach. Growing resistant cultivars is the most economical way to manage the disease, particularly for organic spinach production. Therefore, the tools that assist and expedite the development of resistant cultivars are urgently needed. Resistance to *P. effusa* is typically controlled by major qualitative dominant genes, and at least six resistance loci are known to exist in spinach. Three of these resistant loci, *RPF1*, *RPF2* and *RPF3* have been genetically characterized and each locus controls resistance to more than nine of the 17 described races of *P. effusa*. However, as downy mildew is an obligate pathogen, the bioassays used to evaluate resistance are especially challenging. Thus, having robust molecular markers closely linked to resistance loci would greatly expedite genetic selections. In this study, multiple approaches, including bulked segregant analysis (BSA) using genotyping-by-sequencing, were deployed to develop thirteen, two, and seven markers linked to the *RPF1*, *RPF2*, and *RPF3* resistance loci, respectively. One marker, RPF3-3, was linked to *RPF2* and *RPF3*, and three markers, RPF3-1, RPF3-2, and RPF3-6, were linked to all three loci, indicating that the three resistance loci are closely linked. In blind

tests, the markers effectively discriminated the presence of *RPF1*, *RPF2*, and *RPF3* in diverse spinach germplasm. Thus, the markers developed in this study accurately predict the presence of *RPF1*, *RPF2*, and *RPF3* loci, and can accelerate the development of downy mildew resistance in spinach breeding programs.

Keywords Spinach · Downy mildew · Resistance · Molecular marker

Introduction

Spinach, a highly nutritious leafy green vegetable, has steadily increased in popularity in recent years (Correll et al. 2011). As a result, fresh baby-leaf spinach production has increased dramatically. Due to the overall increase in acreage, and substantially higher plant densities for fresh market production, downy mildew continues to limit production in California and Arizona, where approximately 90% of fresh market spinach is grown in the U.S. (<https://www.nass.usda.gov>). High density plantings, combined with year-round production, provides highly conducive conditions for disease development and pathogen dissemination.

Downy mildew, caused by the obligate oomycete pathogen *Peronospora effusa* (= *Peronospora*

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farinosa f. sp. *spinaciae*), is the most economically important disease of spinach (Correll et al. 1994, 2011). The disease affects spinach production worldwide and can greatly reduce the yield and quality of fresh market spinach. A very low threshold (< 5%) of infected leaves can cause fields to be unharvestable due to strict quality standards. Similarly, low levels of disease can incur substantial additional costs, particularly when labor crews are required to manually cull symptomatic leaves in fields prior to harvest (Correll and Feng unpublished).

The most cost-effective and environmentally sound strategy to manage spinach downy mildew is deploying effective genetic resistance in commercial cultivars. However, resistant cultivars have historically been rapidly compromised by the emergence of novel races of *P. effusa* (Feng et al. 2014, 2018). New races of *P. effusa* have been emerging rapidly, with 14 of the 17 named races having emerged in last two decades with few cultivars resistant to all contemporary races (Feng et al. 2018). Although some fungicides effectively control downy mildew in conventional spinach production systems, no effective products are available for organic production (Correll et al. 2016; Matheron et al. 2017). Considering that organic spinach production is approaching 50% of total U.S. production, and still increasing, the continual development of effective resistance is a primary objective of all spinach breeding programs (Morelock and Correll 2008).

Screening for downy mildew disease resistance in spinach is labor intensive and time consuming. Sporangia of the pathogen are required, as well as spinach plants of a suitable age, temperature-controlled growth chambers and dew chambers, and a 3-weeks period from planting to disease evaluation. Additionally, as an obligate pathogen, *P. effusa* must be propagated on living spinach plants, and thus cannot be preserved easily for long periods of time. In light of these challenges, marker assisted selection would greatly expedite the process of selecting for disease resistance and would also reduce associated costs and resources.

Due to the limited information regarding the genetic basis of resistance to spinach downy mildew, and the number of loci involved in resistance, a series of near isogenic lines containing various resistance genes were developed (Correll et al. 2007; Feng et al. 2018). These efforts revealed that *RPF1* segregated as

a single genetic locus and that resistance was dominant (Irish et al. 2008). Subsequently, based on disease reactions and genetic background, at least six loci conveying resistance to downy mildew were hypothesized to exist (Correll et al. 2011; Feng et al. 2014). Each resistance locus provides resistance to multiple races of *P. effusa*. For example, *RPF1* provides resistance to 12 of the 17 described races, *RPF2* provides resistances to 11 races, and *RPF3* provides resistance to nine races. Theoretically, a cultivar containing all these three loci would be resistant to races 1–16 of *P. effusa* (Feng et al. 2018) and would have durable resistance.

Molecular markers for plant resistance genes can be developed by several approaches. Disease resistance genes in plants commonly encode nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins (McHale et al. 2006). Markers for resistance genes can be developed from resistance gene analogs (RGAs), and the marker 5B14r for the *RPF1* locus was developed from an RGA identified from BAC-end sequencing (Feng et al. 2015). Bulk segregant analysis (BSA) also has been widely used to develop markers for disease resistance genes (Michelmore et al. 1991), and the first molecular marker (Dm1) for spinach downy mildew resistance was developed using this approach (Irish et al. 2008). The 5B14r and DM1 markers cosegregated and were estimated to be approximately 1.7 cM from the *RPF1* locus (Feng et al. 2015; Irish et al. 2008). More recently, next generation DNA sequencing technologies have enabled rapid and inexpensive whole-genome sequencing. Relatedly, genotyping by sequencing (GBS) (Elshire et al. 2011) has emerged as a powerful tool to develop markers for major resistance genes and quantitative trait loci (QTLs) in plants. For example, a locus conveying resistance to stem rust has been mapped in wheat via GBS (Edae et al. 2016). The objective of the current research was to identify robust molecular markers closely linked to the spinach downy mildew resistance loci *RPF1*, *RPF2*, and *RPF3*.

Materials and methods

Spinach populations for marker development

Spinach cultivars Lion, Lazio, and Califlay were the sources of the resistance loci *RPF1*, *RPF2*, and *RPF3*,

respectively. Each of these three cultivars was crossed with the cultivar Viroflay, which is susceptible to all known races of *P. effusa*. Viroflay was the recurrent parent in backcrosses with each of the three F₁ generations to create BC₃ populations. The (Viroflay × Lion) BC₃ population contained 120 progenies, the (Viroflay × Lazio) BC₃ population consisted of 160 progenies, and the (Viroflay × Califlay) BC₃ consisted of 144 progenies. Each backcross generation from the three segregating populations was scored for resistance to *P. effusa* races 6, 10, or 12 provided by *RPF1*, *RPF2* and *RPF3*, respectively (Feng et al. 2014). Assays for disease resistance followed previously published protocols (Feng et al. 2014, 2018). Briefly, each segregating population was grown in 25 cm × 50 cm plastic trays filled with the Sunshine LC1 potting soil (Sun Gro Horticulture, Canada), seed were planted in 10 rows per tray and 10–15 seed per row. Five female resistant plants in the BC₁ and BC₂ generations were backcrossed with the recurrent susceptible Viroflay male plants. In the BC₃ population, individual seedlings were labeled with plastic tags, and leaf tissue was collected from each plant of the segregating populations as well as the four parental cultivars for DNA extraction (Feng et al. 2015). Then, the populations were inoculated with *P. effusa* races 6 (isolate SP1), 10 (isolate INT1) and 12 (UA2209) on the *RPF1*, *RPF2* and *RPF3* segregating populations following previously described protocols (Feng et al. 2014, 2018). The disease reaction of each plant was recorded. Each plant identified as resistant was re-inoculated to confirm that it was not a false negative (escape) from the original inoculation.

Bulked segregant analysis and genotyping by sequencing (GBS)

In each segregating population, Bulked DNA from 10 resistant and 10 susceptible plants, with four replications, were digested with the restriction enzyme ApeK I, and sequenced with an Illumina HiSeq-2000 platform at the Genomic Diversity Facility, Institute of Biotechnology, Cornell University, Ithaca, NY. The raw reads were processed with TASSEL5.0. Polymorphic SNPs for each resistance locus were identified using this software. Primers were designed based on the reference genome of the susceptible parent Viroflay (Dohm et al. 2014) using the polymorphic

SNPs from the resistant bulks at the 3' end of the primers.

Developing markers based on the insertions/deletions of the resistant and susceptible parents

A spinach BAC library was probed with the Dm1 and 5B14r markers linked to the *RPF1* locus (Feng et al. 2015; Irish et al. 2008) using the Amersham ECLTM Direct Nucleic Acid Labeling and Detection System (Cat. # RPN3001, GE Healthcare Life Sciences, PA) following the manufacture's instruction. A total of 43 and 36 BAC clones were identified, respectively, and 20 of the clones hybridized with both probes. The 59 BAC clones harboring the DM1 and/or 5B14r fragments were reactivated from glycerol stocks stored at – 80 °C, streaked to isolation on Luria–Bertani agar plates, and individual colonies were cultured in Luria–Bertani broth medium with chloramphenicol at 37 °C overnight. DNA of each BAC clone was extracted from bacterial cultures via alkaline lysis (Sambrook et al. 1989), quantified via Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, MA), and an equal amount of DNA from each BAC clone was pooled for sequencing. The pooled DNA of the 59 BAC clones and DNA of the spinach cultivar Califlay were sequenced in one flow cell lane of Illumina HiSeq-2000 at BGI, Shenzhen, China. Sequence assembly was performed with CLC Genomics 7.0 (<https://www.qiagenbioinformatics.com/>) using the default settings. The contigs containing the Dm1 and/or 5B14r fragments, and the SNPs specific to the *RPF3* genotypes were identified from the assembled sequences of the cultivars NIL1 and Califlay using megaBLAST in the software Geneious 7.1.9 (Biomatters Inc., NJ). Then, the identified contigs were aligned to the reference genome of the susceptible parent Viroflay (Dohm et al. 2014) using BLAST (<https://blast.ncbi.nlm.nih.gov/>). Primers were designed using the online tool Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) at the insertion/deletion regions according to the sequence alignments of the resistant and susceptible genotypes.

SCAR marker development

A reliable sequence characterized amplified region (SCAR) marker was developed based on the sequence information of a fragment specific to the *RPF2* locus.

After screening 800 10-mer random primers in the three backcross populations using bulk segregant analysis, only one fragment specific to RPF2 amplified by primer UA529 was found to be polymorphic between the resistant and susceptible bulks. After the polymorphism between the resistant and susceptible genotypes was confirmed by PCR with genomic DNA from each individual in the segregating population, this polymorphic band was excised from the agarose gel, purified with a Qiagen Gel Extraction kit, ligated to the pGEM-T vector, and transformed into *E. coli* competent cells (Promega, WI). Plasmid DNA was extracted from ten white colonies with inserts and sequenced at the DNA core laboratory at the University of Arkansas, Fayetteville, AR. Primers were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) from the sequence of this RPF2 resistance-specific fragment, and evaluated on the individuals of the segregating population.

Marker testing and construction of linkage maps for resistant loci

Primers were designed and initially tested on the various resistant and susceptible parents and the bulked samples. The PCR conditions were: 94C 2 min, followed by 35 cycles of 94C 15 s, 56C 30 s, and 72C 30 s, and a final extension 72C 5 min, except the extension time 1 min was used for marker RPF2-1 in the cycles. Primer sets that discriminated the resistant and susceptible parents and bulks were further evaluated on individuals from the segregating populations. The genotypic data were recorded based on the presence or absence of the amplified bands of each primer on each individual of the segregating populations. Linkage maps for each resistance locus were constructed using the software JoinMap 4.1 (Van Ooijen 2011). To validate the primers, blind tests were performed by testing the identified markers on phenotype-unrevealed DNA samples from various seed companies. The resistance phenotypes to race 6, 10 and 12 of *P. effusa* of these samples were predicted by the presence/absence of chosen markers in each DNA sample when amplified with the same conditions for testing the markers on the segregating populations. Seed companies then revealed the phenotypes of their samples and confirmed the match/mismatch of the samples to evaluate the robustness of the markers.

Results

Markers associated with the *RPF1* locus

Multiple potential markers were designed from the insertion/deletion regions and SNPs identified by comparing the sequences of the resistant genotype NIL1 and susceptible parent Viroflay. Thirteen primer sets produced amplicons that were specific to resistant genotypes (Table 1). Of these, seven primer sets (RPF1-3, RPF1-4, RPF1-5, RPF1-6, RPF1-7, RPF1-12, and RPF1-13) were designed from insertion/deletion regions, and six other sets (RPF1-8, RPF1-9, RPF1-10, RPF1-11, RPF1-14, and RPF1-15) were designed from SNPs. RPF1-8 was a co-dominant marker, which amplified a monomorphic amplicon in all genotypes and another amplicon specific to the resistant genotypes. The amplicon associated with resistance was detected in one susceptible plant. However, in a blind test of 192 samples, the presence and absence of the *RPF1* locus in all samples were correctly predicted using this co-dominant marker RPF1-8. Similarly, the two previously developed co-dominant markers Dm1 and 5B14r amplified one monomorphic band in all genotypes, and an additional band that was present exclusively in resistant progenies, which indicated these two markers co-segregated with *RPF1*. Other dominant markers each amplified one band that was specific to the resistant genotypes, only one to three recombinants were found between the markers and the *RPF1* locus.

Markers associated with the *RPF2* locus

Among the 800 random 10-mer primers tested, primer UA529 (5'-CACTCCTACA-3') produced an amplicon specific to genotypes resistant to race 10, including the resistant parent, resistant bulk, and resistant progenies. Based on the 1121 bp sequence of this amplicon, a pair of sequence characterized amplified region (SCAR) primers designated RPF2-1 (Table 1) was designed to amplify the full length of this fragment, and only one amplicon was detected from resistant genotypes. When tested in the segregating population, the amplicon was detected in all resistant progenies, but none of the susceptible progenies, which indicated co-segregation of this marker with *RPF2*. In a blind test of 131 samples from various seed companies, resistance to *P. effusa* race 10 was

Table 1 Primers of markers closely linked to the three resistance loci *RPF1*, *RPF2*, and *RPF3* to spinach downy mildew disease

Marker	Forward	Reverse	Target locus	Source
RPF1-3	ACGGAGGGACTAATGGACTA	GTGAACCAACACATATCACAA	<i>RPF1</i>	Indel
RPF1-4	GAGGTGATTCAGAGTTCAGA	TCCGTGTTGAAAATGTCTTG	<i>RPF1</i>	Indel
RPF1-5	TGTGTTTGGTTTCACTAAGTG	GGGAATCCAACAGAAACAATC	<i>RPF1</i>	Indel
RPF1-6	GAGGACCAGAATTTTTAGGA	CAGTGCATGGGTAGGTAGG	<i>RPF1</i>	Indel
RPF1-7	TTTGGGCTAGGCTCGGGCAA	TTGTTAGTTAACGTACACCC	<i>RPF1</i>	Indel
RPF1-8	ATTCCCCCTCTCCTCA	CTGCATAGTCTCGTGAGCTC	<i>RPF1</i>	SNP
RPF1-9	ATAGGGCCACAAATGTGACA	GGAACGGGTACCTGTACGC	<i>RPF1</i>	SNP
RPF1-10	AACCCGACATGGTGGCCCCC	TCACCGGGTTTATATGACCC	<i>RPF1</i>	SNP
RPF1-11	GTCGAAAATGACTTTGTCTG	AGAAATCAGCTCAAAACAG	<i>RPF1</i>	SNP
RPF1-12	CGACACGCACTCGTAGAAAA	TCCATATACGTTCCGGGAAGC	<i>RPF1</i>	Indel
RPF1-13	TGAGGGGTGGGGTGTAATA	CGGGTTTTAAAAGTCCACGA	<i>RPF1</i>	Indel
RPF1-14	CTACAATATCCATTCCCCCA	GATTCATCTCATAATTCCCC	<i>RPF1</i>	SNP
RPF1-15	TGCGGCGAATAGTCAATTTG	CGAAGATGCTGCAGAGCTTC	<i>RPF1</i>	SNP
RPF2-1	CACTCCTACACATTTATATC	CACTCCTACAATAAGAA	<i>RPF2</i>	SCAR
RPF2-2	TCTGCGAAATACAACGCCGT	CTCTTTGTTTAGCTCCATATTT	<i>RPF2</i>	SNP
RPF3-1	GATGCACGCATAACACCAAG	TCAGTAATTTTCCATCGC	<i>RPF1</i> , 2, 3	Indel
RPF3-2	GAATTTGAGCTCTTCTTTGAG	TAGGGCACACGATGACACGA	<i>RPF1</i> , 2, 3	Indel
RPF3-3	TGGTCATCAAGGACCCAAA	AAACGGGTAACATGACGGACCC	<i>RPF2</i> , 3	Indel
RPF3-4	GTCTGTGGTTGATCACCATA	GAAGCATAACATCTCTGTAAT	<i>RPF3</i>	SNP
RPF3-5	CCAAAAACATCATCATACTC	GCGACTAATAACACATCTTC	<i>RPF3</i>	Indel
RPF3-6	GTATGCAGCCTACAGATATG	AAGGCCTGTATTGTGATAGG	<i>RPF1</i> , 2, 3	SNP
RPF3-7	CTAATGCCCATATTATAGGAAA	GTAATTCCTTATTCGCCAAGT	<i>RPF3</i>	Indel

correctly predicted in 125 samples with the RPF2-1 marker. An amplicon was not produced with the RPF2-1 marker for six samples and thus, these six samples were predicted to be susceptible to race 10. However, these six lines were reported to be resistant to race 10. The source of resistance was due to a different resistant gene to *P. effusa* race 10 other than the RPF2 locus (Jan Dijkstra, personnel communication). Primer set RPF2-2 (Table 1), designed based on the SNPs between the resistant and susceptible bulks, produced an amplicon from all but three resistant individuals and was absent in all but one susceptible individual, indicating a close linkage between this marker RPF2-2 to the *RPF2* locus.

Markers associated with the *RPF3* locus

Based on SNPs between the resistant and susceptible bulks and insertions/deletions between the resistant parent Califlay and the susceptible parent Viroflay,

seven markers were developed that were associated with *RPF3* (Table 1). Of these, six markers were dominant, which amplified a fragment specific to the resistant genotypes, and one marker (RPF3-5) was co-dominant, which amplified a monomorphic fragment in all genotypes, and a polymorphic fragment that was specific to the resistant genotypes. The polymorphic bands produced by primers RPF3-3 and RPF3-5 were present in all resistant progenies, and absent in all susceptible progenies, indicating that these two markers were co-segregating with *RPF3*. In a blind test of 192 samples, the presence and absence of the *RPF3* locus were correctly predicted using the co-dominant marker RPF3-5. For other five markers, two to three recombinants were found between the *RPF3* locus and the marker depending on the marker.

Markers associated with multiple *RPF* loci

After testing the markers developed for *RPF1*, *RPF2*, and *RPF3* in the corresponding segregating populations, the markers were also tested on other spinach genotypes. Markers for *RPF1* or *RPF2* were only detected in genotypes containing *RPF1* or *RPF2*, respectively (Fig. 1a, b), which indicated a high level of specificity. Of the seven markers developed for *RPF3*, markers RPF3-4, RPF3-5, and RPF3-7, were exclusively present in genotypes containing *RPF3*

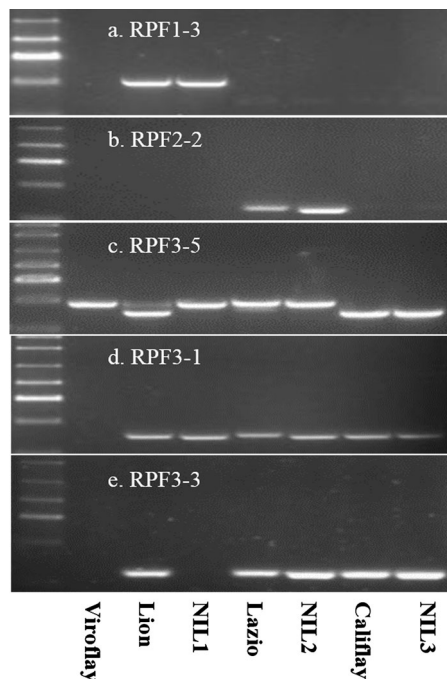


Fig. 1 Validation of the five markers designed for spinach resistance loci *RPF1*, *RPF2* and *RPF3* on seven genotypes: Lane 2, Viroflay, no resistance locus; Lane 3, Lion, containing the *RPF1* and *RPF3* loci; Lane 4, NIL1, containing the *RPF1* locus; Lanes 5–6, Lazio and NIL2, containing the *RPF2* locus; Lanes 7–8, Califlay and NIL3, containing the *RPF3* locus. Lane 1 was the 100-bp ladder. **a** RPF1-3 only amplified from Lion and NIL1; **b** RPF2-2 only amplified from Lazio and NIL2; **c** co-dominant marker RPF3-5 amplified one band from Viroflay, NIL1, Lazio, NIL2, and two bands for heterozygous Lion, and one band from homozygous Califlay and NIL3; **d** RPF3-1 amplified from genotypes containing either of the resistance loci; **e** RPF3-3 amplified one band from *RPF2* and *RPF3* containing genotypes

(Fig. 1c), indicating specificity. However, markers RPF3-1, RPF3-2, and RPF3-6 were also present in genotypes containing the *RPF1* and *RPF2* loci (Fig. 1d), and marker RPF3-3 was present in genotypes containing *RPF2* and *RPF3* (Fig. 1e). In populations segregating for *RPF1* or *RPF2*, markers RPF3-1, RPF3-2, and RPF3-6 were also closely linked to the *RPF1* and *RPF2* loci, suggesting that the *RPF1*, *RPF2* and *RPF3* loci were also linked. Marker RPF3-3 was found to be associated with the *RPF2* and *RPF3* loci.

Linkage maps for *RPF1*, *RPF2*, and *RPF3* were constructed with markers specific to the corresponding resistant locus and common markers for two or three loci (Fig. 2). The linkage map of *RPF1* covered 15.4 cM, and the markers originally developed for *RPF1* spanned 4.5 cM. Markers DM1 and 5B14r co-segregated with *RPF1*, with RPF1-3 and RPF1-6 0.7 cM on one side, and RPF1-5 and RPF1-8 0.7 cM on the other side of the *RPF1* locus. The closest common marker RPF3-2 was 5.3 cM from the *RPF1* locus (Fig. 2a). The linkage map for *RPF2* covered 8.9 cM. Marker RPF2-2 was 2.1 cM on one side, and RPF3-6 0.8 cM on the other side of the *RPF2* locus (Fig. 2b). The linkage map for *RPF3* covered 6.4 cM, with RPF3-3 and RPF3-5 co-segregating with the locus, and RPF3-6 1.5 cM on one side, and RPF3-2 0.6 cM on the other side of the *RPF3* locus (Fig. 2c).

Discussion

Growing resistant cultivars is the most economical and effective practice for managing the downy mildew disease in spinach (Correll et al. 1994; Morelock and Correll 2008). The major gene resistance used in spinach has been readily overcome by the appearance of new races of the downy mildew pathogen (Feng et al. 2014, 2018; Irish et al. 2007). Moreover, screening for disease resistance is labor intensive and time consuming process (Morelock and Correll 2008). The aim of the current research was to expedite this selection and breeding process by identifying robust molecular markers linked to the loci that provide resistance to the downy mildew pathogen. In this research, multiple (2–13) robust molecular markers were identified that were closely linked to the *RPF1*, *RPF2* or *RPF3* loci. Furthermore, these co-dominant markers that distinguish homozygous

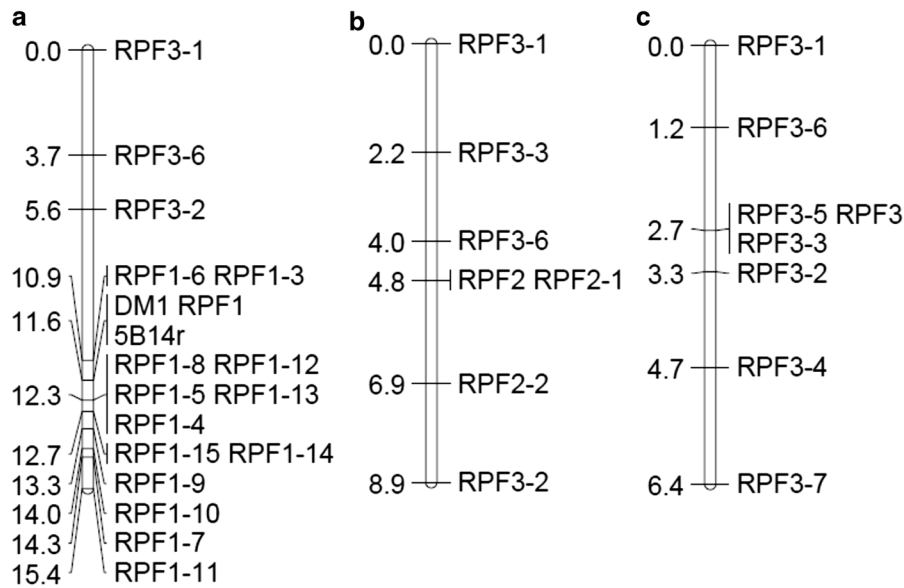


Fig. 2 Linkage maps for the spinach downy mildew resistance loci *RPF1*, *RPF2* and *RPF3*. **a** Linkage map for the spinach downy mildew resistance locus *RPF1* based on 120 progenies of (Viroflay × Lion) BC3 population; **b** linkage map for the

spinach downy mildew resistance locus *RPF2* based on 160 progenies of (Viroflay × Lzaio) BC3 population; **c** linkage map for the spinach downy mildew resistance locus *RPF3* based on 144 progenies of (Viroflay × Califlay) BC3 population

resistant versus heterozygous resistant individuals would significantly reduce the time needed to develop inbred parental lines used in hybrid cultivar development.

Debate is ongoing regarding the genetic basis of resistance to spinach downy mildew. Data from earlier studies suggested that resistance to races 1 and 3 were controlled by two closely linked genes (Eenink 1976). However, subsequently, many new races of the pathogen have been identified, and some of the original resistance genes have been effective against these new races (Feng et al. 2018). This has led to speculation that resistance may be controlled by a single locus with multiple alleles versus multiple closely linked resistance loci, whereby linkage has not been broken. Theoretically, a spinach line containing the resistance loci *RPF1*, *RPF2* and *RPF3* would be resistant to races 1–16 of *P. effusa* (Feng et al. 2018). So far, however, no commercial cultivars or hybrids containing these three resistance loci have been developed, since no cultivars resistant to races 1–16 of *P. effusa* have been developed by combining the three resistant loci.

Among the markers identified in this study, three markers (RPF3-1, RPF3-2, and RPF3-6), were closely linked to *RPF1*, *RPF2* and *RPF3* and one marker

(RPF3-3) was closely linked to *RPF2* and *RPF3*. These data suggest that the resistance loci are either very closely linked or are alleles of a single locus. Either scenario could explain why combining all three resistance loci into one line or hybrid has proven to be elusive. These results are consistent with the assumption in the patent WO2018060474A1 (Kock et al. 2018). In the interval between the two markers RPF3-6 and RPF3-2 associated with all three loci, the *RPF2* locus was close to the marker RPF3-6, and the *RPF3* locus was close to the marker RPF3-2 (Fig. 2). The *RPF1* locus was not placed in the interval but was closer to the RPF3-2 marker. Thus, these data would indicate that the *RPF3* locus could be between the *RPF1* and *RPF2* loci, which may be why some of the markers originally developed for the *RPF3* locus were also linked to the *RPF1* and *RPF2* loci. Efforts are underway to determine the genetic basis of resistance by crossing near-isogenic lines containing *RPF1*, *RPF2*, or *RPF3*, previously developed (Feng et al. 2018), in various combinations and then generating F2:3 lines for phenotyping and genotyping to help resolve the question of linkage.

The size of the segregating population is critical for accurate estimation of genetic distance. The markers Dm1 and 5B14r were estimated to be approximately

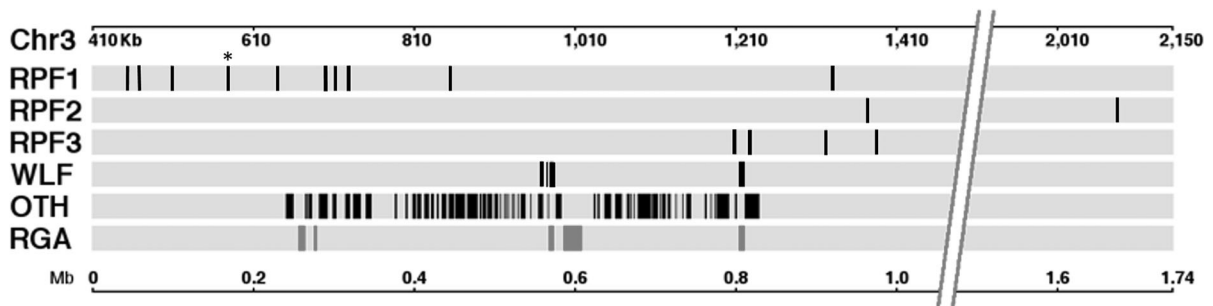


Fig. 3 Putative downy mildew resistance genes, *RPF1*, *RPF2*, and *RPF3*, have been mapped to a 1.5 Mb region on spinach chromosome 3. The *RPF1*, *RPF2*, and *RPF3* markers identified in this study and the two markers for the *RPF1* locus (Irish et al. 2008; Feng et al. 2014) have been mapped to a 1.5 Mb region on the spinach chromosome 3. Five NBS-LRR resistance gene

analogs (*RGA*) and 68 other genes (*OTH*) have been predicted in this region (Xu et al. 2017). While the ‘Wolf’ genes (*WLF*) listed in the patent WO2018060474A1 have been mapped to two distinct locations in this region. *indicates the position of the first *RPF1* marker Dm-1

1.7 cM from the resistance locus *RPF1* (Feng et al. 2015; Irish et al. 2008), based on a segregating BC3 population (Viroflay × Lion) with 120 individuals, but these two markers were co-segregating with *RPF1* in this study. The mapping populations used in this research were relatively small (120, 160, and 144 individuals for the *RPF1*, *RPF2* and *RPF3* loci, respectively). Increasing the population size would aid in accurate estimation of the genetic distances between the molecular markers and the *RPF* loci.

Each of the three resistance loci are effective to between 9 and 12 of the 17 named races of *P. effusa* (Feng et al. 2018). The durability of resistance to the downy mildew pathogen remains a challenge due to the rapid appearance of new races of the pathogen. The use of the robust molecular markers identified in the current study will allow for a more efficient approach to breeding for disease resistance to this economically important disease. Furthermore, as sequence information on spinach becomes available, the molecular markers will allow for the identification of functional and non-functional resistance genes/alleles. Based on the spinach genome sequences available, the *RPF* loci have been localized to a 1.5 Mb region on chromosome 3 (Fig. 3), where five NBS-LRR resistance gene analogs (Xu et al. 2017) and some putative resistance genes (Kock et al. 2018) have been mapped. This information could be valuable for improving traditional breeding approaches to improve the durability of resistance to the downy mildew pathogen, as well as possibly improving the resistance through targeted gene editing.

Bulked segregant analysis (BSA) has been proven to be an excellent strategy for marker development (Michelmore et al. 1991), and genotyping-by-sequencing (GBS) is a powerful tool for identification of a large number of single nucleotide variants (Elshire et al. 2011). It is much more efficient to develop markers when combining the BSA with GBS approaches, as used in the effort for development of markers for all three resistance loci in this research. Such an approach will continue to be valuable for marker development to improve the durability of resistance to this important disease. Efforts are underway using the discovery from this research to demonstrate the functionality of specific candidate resistance genes.

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