

# The development of a PCR-based marker linked to resistance to the blackcurrant gall mite (*Cecidophyopsis ribis* Acari: Eriophyidae)

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**Abstract** Gall mite (*Cecidophyopsis ribis*) is the most serious pest of blackcurrant (*Ribes nigrum* L.), causing the damaging condition known as ‘big bud’ and also transmitting blackcurrant reversion virus (BRV) within and between plantations. The identification of resistant germplasm is at present a time-consuming and expensive process, dependent on field infestation plots. Resistance based on gene *Ce* introgressed from gooseberry has been used in UK breeding programmes for blackcurrant. Using a bulked segregant analysis, 90 AFLP primer combinations were screened and a linkage map constructed around the resistance locus controlled by *Ce*. Sixteen of the primer combinations produced a fragment in the resistant bulked progeny and the gall mite-resistant parent, but not in the susceptible bulked progeny and parent; subsequent testing on individual progeny identified an AFLP fragment closely linked to gall mite resistance. This fragment, designated E41M88-280, was converted to a PCR-based marker based on sequence-specific primers, amplifying only in resistant individuals. Validation of this marker across a range of susceptible and resistant blackcurrant germplasm with different genetic backgrounds confirmed its reliability in the identification of mite-resistant germplasm containing gene *Ce*. The conversion of an AFLP fragment to a sequence-based PCR marker simplifies its application and therefore

increases its utility for selection of mite-resistant germplasm in high-throughput breeding programmes for blackcurrant.

## Introduction

Gall mite (*Cecidophyopsis ribis* Westw.) is the most serious pest of blackcurrant (*Ribes nigrum* L.), causing the damaging condition known as ‘big bud’. Additionally, *C. ribis* is the vector of blackcurrant reversion virus (BRV), which renders the affected plants sterile within 2 years (Jones 2002). Gall mite is ubiquitous throughout Europe, and also occurs in parts of New Zealand and Asia (Sabitov et al. 2002); there are at present no reports of this pest from North America.

Measures to control *C. ribis* in Europe are restricted to sulphur sprays, since previously available chemical agents are now withdrawn on environmental grounds. The effective application of sulphur requires careful timing, and needs to be supported by the identification and removal of infested plants at the earliest stage that symptoms are visible. Additionally, there is a growing movement towards organic or integrated crop management of blackcurrants throughout Europe (Gordon 2008), and as a result, breeding for resistance to *C. ribis* is a major objective in most blackcurrant breeding programmes (Brennan 1996).

Most commercially-grown blackcurrant cultivars in Western Europe are susceptible to *C. ribis* (Brennan, 1996). Resistance is available from other *Ribes* species, notably *R. grossularia* (Knight et al., 1974) where the resistance is controlled by a single gene, *Ce*. In order to utilise this gene in breeding mite-resistant blackcurrant cultivars, its introgression into blackcurrant was achieved by the development of resistant F<sub>1</sub> allotetraploids (Knight et al. 1974)

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using colchicine. Following an extensive backcrossing programme to BC<sub>7</sub> and beyond at the Scottish Crop Research Institute (SCRI), there are now resistant diploid hybrids with fruit of commercial processing quality in advanced trials. However, within the breeding programme, the identification of resistant segregants has proved to be a major limiting factor in the long generation time for resistant hybrids. At the present time, the only available means for phenotyping is through the use of infestation plots with infector rows of galled bushes and test germplasm planted between them. However, this method has the inherent potential for possible escapes due to uneven mite pressure across the plot, and is also time-consuming, taking approximately 4 years to produce reliable results. A rapid high-throughput screening method is therefore required.

Initial studies in the development of markers for mite resistance involved investigations of the biochemical composition of blackcurrant buds using a metabolomics approach (Austin et al. 1983; Brennan et al. 1992), in which some correlations between mono- and sesquiterpene composition and resistance status were found. However, more recently, work has focused on the development of molecular markers linked to the *Ce* resistance gene.

Various marker types have been developed in *Ribes*, including AFLPs (Lanham and Brennan 1999), SSRs [genomic (Brennan et al. 2002) and EST-derived (Brennan et al. 2008a)] and SNPs (Brennan et al. 2008a). The first linkage map for blackcurrant was published by Brennan et al. (2008a), using a population created from two breeding lines at SCRI segregating for a number of important agronomic and fruit quality traits. Several of these traits were mapped (Brennan et al. 2008a), including resistance to gall mite based on the *Ce* gene which mapped to linkage group 2. In the development of the linkage map, the segregation of the gall mite-resistant phenotype confirmed that a single gene is involved (Knight et al. 1974).

The use of markers for selection of key traits in fruit germplasm has become widespread in some species, particularly in members of the Rosaceae (Dirlewanger et al. 2004); the most obvious targets are traits that either require complex phenotyping or those that cannot be evaluated until the plant is mature, such as fruit quality. Examples are markers for root knot nematode in *Prunus* rootstocks (Claverie et al. 2004), for resistance to *Venturia* scab (Xu and Korban 2002; Hemmat et al. 2002) and woolly aphid (Bus et al. 2000) in *Malus*, for resistance to *Colletotrichum acutatum* (Lerceteanu-Köhler et al., 2002) in strawberry and for resistance to *Phytophthora* root rot in raspberry (Weber et al. 2008). However, although marker systems have been reported in various berry fruit crop species, to date there remain relatively few reports of the use of marker-assisted breeding, and none in blackcurrant.

This paper describes the development of an AFLP marker linked to resistance to blackcurrant gall mite, its conversion to a readily applied PCR-based marker and the subsequent deployment of this marker in breeding germplasm at SCRI, to identify resistant seedlings for progression as new resistant cultivars for blackcurrant growers.

## Materials and methods

### Plant material

#### Mapping population

The population used was as described by Brennan et al. (2008a), designated 9328, comprising of a diploid F<sub>1</sub> full-sib progeny from a pseudo-testcross (Grattapaglia and Sederoff 1994) between two diverse breeding lines from the Scottish Crop Research Institute. The population, segregating for resistance to *C. ribis* based on the *Ce* gene, was raised in the glasshouse from seed and subsequently planted in the field at SCRI in an infestation plot for blackcurrant gall mite. The population was grown using standard agronomic practices, although no pest or pathogen control measures were used. A total of 96 segregants were phenotyped for resistance.

The parental types for the 9328 population were:

- Seed parent—SCRI S36/1/100 (cv. Ben Alder × cv. Ben Loyal)—gall mite-susceptible.
- Pollen parent—EMRS B1834 (EMRS B1426 × cv. Ben Lomond)—gall mite-resistant BC<sub>6</sub> from *Ribes grossularia*.

#### Other germplasm

Fifty-nine genetically diverse accessions from the SCRI blackcurrant breeding programme and the SCRI *Ribes* germplasm collection were used to assess the applicability of the marker. Breeding lines were phenotyped for mite resistance in the infestation plot (details below), while the named cultivars' resistance status was already established through agronomic performance data. A list of germplasm used in the experimentation is given in Table 1.

#### Infestation plot/phenotyping

Resistance to gall mite was assessed in an infestation plot at SCRI, with four replicates of two plants of each member of the mapping population planted between highly infested guard rows in a randomised block design. Plants were assessed for symptoms of mite infestation for 4 years, after which time the individual segregants were classified as

**Table 1** *Ribes* germplasm used in the study along with their gall mite resistance status

Cultivar/Breeding no.	Ce in parentage ( $\checkmark$ / $X$ )	Phenotype (R/S)	Marker Present/Absent
<i>Cultivars</i>			
Baldwin	X	S	X
Ben Alder	X	S	X
Ben Avon	X	S	X
Ben Gairn	X	S	X
Ben Hope	$\checkmark$	S	X
Ben Klibreck	X	S	X
Ben Lomond	X	S	X
Ben More	X	S	X
Ben Tirran	X	S	X
Big Ben	X	S	X
<i>SCRI breeding lines</i>			
88111-4	$\checkmark$	R	Present
894-3	X	S	X
896-4	X	S	X
8912-1	X	S	X
8930-2	X	S	X
8942-5	X	S	X
8944-4	$\checkmark$	R	Present
8944-13	$\checkmark$	R	Present
8949-15	$\checkmark$	R	Present
8955-2	X	S	X
8962-1	X	S	X
8966-9	X	S	X
8982-6	$\checkmark$	R	Present
8986-13	$\checkmark$	R	Present
8992-11	$\checkmark$	R	Present
8999-9	X	S	X
9110-1	$\checkmark$	R	Present
9114-1	$\checkmark$	R	Present
9134-7	X	S	X
9137-2	X	S	X
9141-6	X	S	X
9148-9	X	S	X
9154-1	$\checkmark$	R	Present
9198-1	X	S	X
9199-4	$\checkmark$	R	Present
91129-1	$\checkmark$	R	Present
91130-1	$\checkmark$	R	Present
91192-1	X	S	X
9213-2	$\checkmark$	R	Present
9216-4	$\checkmark$	R	Present
9253-1	X	S	X
9260-20	X	S	X
9265-6	X	S	X
92100-33	X	S	X

**Table 1** continued

Cultivar/Breeding no.	Ce in parentage ( $\checkmark$ / $X$ )	Phenotype (R/S)	Marker Present/Absent
92101-1	$\checkmark$	R	Present
92105-13	$\checkmark$	R	Present
92127-1	X	S	X
934-10	$\checkmark$	R	Present
934-58	$\checkmark$	R	Present
934-60	$\checkmark$	R	Present
934-74	$\checkmark$	R	Present
938-56	$\checkmark$	R	Present
9311-25	$\checkmark$	R	Present
9311-66	$\checkmark$	R	Present
9311-82	$\checkmark$	R	Present
9382-45	$\checkmark$	R	Present
S36-1-100	X	S	X
<i>EMR breeding line</i>			
B1834-120	$\checkmark$	R	Present
<i>Mapping population</i>			
9328—total of 55 segregants	$\checkmark$	S	X
9328—total of 41 segregants	$\checkmark$	R	Present

resistant or susceptible. Symptoms of mite infestation in even a single replicate were taken to indicate susceptibility for that segregant.

#### Sample material

Leaf material from a total of 155 cultivars, breeding lines and seedlings from the mapping population was used for the validation of putative markers. Resistance status of each phenotype was assigned on the basis of infestation plot information.

#### Marker development

In order to develop a marker closely linked to gall mite resistance a bulked segregant analysis (BSA) approach was taken. Using the 9328 mapping population, bulk DNA pools of, respectively, 41 resistant and 50 susceptible progeny were constructed. Bulks and parents were subjected to AFLP analysis using 90 *Eco*R1 and *Mse*I primer combinations as described by Brennan et al. (2008a). Potentially useful primer combinations were verified in separate amplification reactions with individuals from the resistant bulk population.

Primer combinations which co-segregated with gall mite resistance were then subjected to sequence analysis for use

as potential markers. Briefly, autoradiographs were matched to the corresponding area of the gel, and the target fragments in the resistant progeny were excised into 10 × 50 µL sterile distilled water and incubated at 4°C overnight. Fragments were pooled (5 × 100 µL), purified using MinElute PCR purification columns (Qiagen) and eluted into a final volume of 10 µL. Eluted DNA was subjected to PCR (94°C for 5 min followed by 40 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 30 s and a final extension of 72°C for 10 min) with the corresponding E and M primers, and separated by electrophoresis on 1.5% agarose gels. Positive PCR fragments were excised and purified using MinElute gel extraction columns (Qiagen). DNA was eluted from each column in 20 µL EB buffer (Qiagen) and 5 µL was directly sequenced using the corresponding E and M primer using Big Dye Terminator version 2.0 chemistry (Applied Biosystems), and analysed on an ABI Prism 3730. Specific primers were designed using the primer 3 programme (Rozen and Skaletsky 1996, 1997) and tested on the segregating progeny and the other *Ribes* germplasm listed in Table 1.

#### Map development

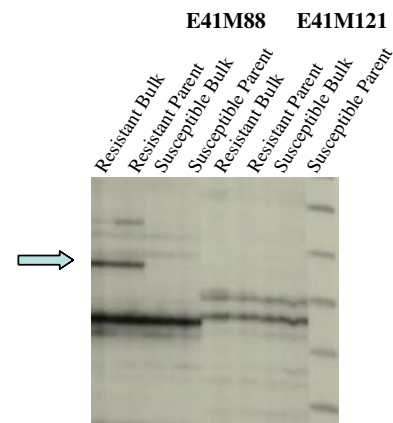
The markers linked to the gall mite resistant locus were ordered using a simulated annealing approach (Hackett et al. 2003). This uses the same ordering criterion as JoinMap 3.0 (Van Ooijen and Voorrips 2001) but has a more exhaustive search algorithm based on simulated annealing (Goffe et al. 1994). The linkage map was drawn with the programme MapChart (Voorrips 2002).

#### Results

Sixteen of the 90 primer combinations revealed a fragment in the resistant bulked progeny and the gall mite resistant parent, but no fragment in the susceptible bulked progeny and parent (Fig. 1). In order to confirm the linkage to gall mite resistance, these 16 primer combinations were then tested on 91 progeny (41 resistant and 50 susceptible progeny) and both mapping parents. As a result, the AFLP fragment E41M88-280 bp was identified as being closely linked to gall mite resistance in the mapping population (Figs. 1, 3).

Products from resistant individuals were converted into a PCR-based marker by sequencing and alignment, producing a final set of primers designed to amplify only in the resistant individuals. These primers (GMresaF 5' TTACC GCAGATACAAGGTGAAG 3' and GMresaR 5' GGACT AGGCTTCTTATGAC 3') amplified a 130 bp fragment only in the resistant parent and progeny (Fig. 1).

To confirm whether this dominant marker was linked to gall mite resistance in unrelated germplasm, a comprehen-



**Fig. 1** Representative AFLP fragments (E41M88-280 bp) derived from resistant parent and bulks and susceptible parent and bulks. The arrow indicates the polymorphic band present in resistant parent and bulks. An unlinked AFLP (E41M121), showing no amplification due to lack of homology, is included for comparison

sive range of available *Ribes* germplasm (Table 1), from a wide variety of genetic backgrounds, was tested. The diversity of test material was as extensive as possible within *R. nigrum*, with ancestral lines derive from a wide range of northern European, Scandinavian and Russian germplasm. All of the material known to be gall mite-resistant through phenotypic tests in the field amplified a 130 bp fragment using the primers described above, while all the susceptible germplasm showed no amplification (Fig. 2). The marker was accurate in predicting the phenotype in all of the test germplasm (Table 1).

In order to verify that the absence of a fragment was due to a lack of sequence homology and not simple amplification failure, another set of primers (mapped SSR) was used as controls, which amplified across all germplasm (Fig. 1).



**Fig. 2** PCR products obtained using the GMRes primers in phenotypically scored *Ribes nigrum* germplasm. A band is observed in resistant (R) accessions, with no amplification in susceptible (S) accessions. Germplasm used: top row from left 8944-4, 8986-13, 8982-6, Ben Finlay, 9311-82, 9328-45, 938-56, 934-74, 9311-66, 9199-4, B1834-120, 8942-5, 894-3, 8992-11, 8930-2, 8999-9, 8966-9, 896-4. Bottom row from left Ben Lomond, Ben Tirran, 9137-2, 9148-9. X empty lanes

## Discussion

The conversion of AFLP fragments to simple PCR-based markers has been previously reported in a range of crops, e.g. maize (Dussie et al. 2002), soybean (Meksem et al. 2001) and wheat (Shan et al. 1999). The advantage of this approach is to produce a readily applicable and accurate marker for easier deployment in high-throughput breeding situations. AFLPs are generally too expensive and labour-intensive for the analysis of many large breeding progenies, so the conversion to PCR-based markers is an important development. The success in this study of converting an AFLP fragment into a PCR-based marker whilst retaining the very high degree of linkage between the marker and the *Ce* resistance gene will ensure its use in appropriate breeding programmes for blackcurrant. As such, it is the first reported marker to be developed and ultimately deployed in this crop species, and one of the first in berry fruits generally.

The results presented show that there is a very close linkage between the AFLP E41M88 and gall mite resistance based on gene *Ce*, and that the marker can accurately identify resistant germplasm containing *Ce*. Whilst the initial development used one mapping population, the successful validation of the marker encompassed a wide range of genetic backgrounds in which the *Ce*-based resistance was located. In the resistant germplasm used in the validation (Table 1), there was no indication from parentage or phenotyping results that any other resistance genes apart from *Ce* were present.

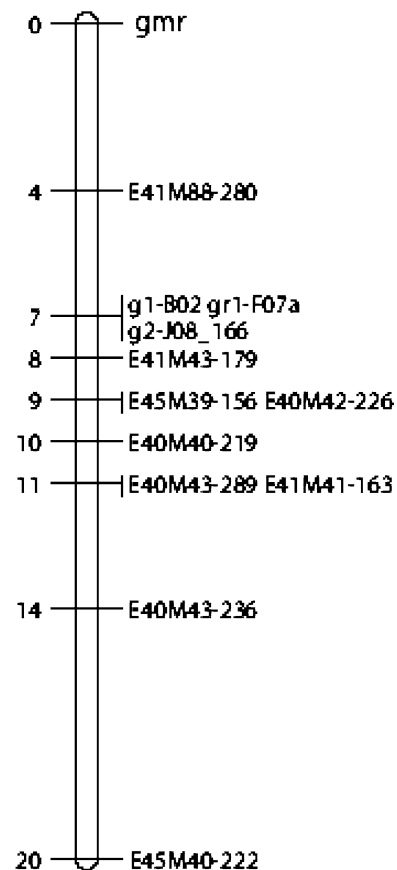
The breeding of mite-resistant blackcurrants depends on the introgression of the *Ce* gene from *R. grossularia* (cv. Green Ocean); as this species is located in a different subgenus to *R. nigrum*, interspecific hybrids between the two do not occur naturally and induced hybrids are mostly infertile (Knight et al. 1974). The large genetic differences between these two species would suggest that recombination within the introgressed region, including the *Ce* gene, is uncommon. The majority of the *R. grossularia* genome is lost during the earliest stages of backcrossing (Knight et al. 1974), with most of the BC<sub>2</sub> progenies showing close morphological similarity to *R. nigrum*, although the same authors recognised that *Ce* may represent a small section of chromosome showing single gene-type segregation rather than a single gene itself.

The identification of mite-resistant plants has proven to be one of the most time-consuming parts of many blackcurrant breeding programmes in Europe, dependent on phenotypic data from field observations or infestation plots. The accuracy of the marker derived from the AFLP E41M88 is sufficiently high to give confidence that it offers a robust means of early screening for the presence or absence of the *Ce* resistance gene in blackcurrant.

The use of the marker to investigate gall mite resistance in cv. Ben Hope confirmed that this cultivar does not pos-

sess *Ce*-based resistance. It had been thought by breeders and growers that cv. Ben Hope was a *Ce*-containing genotype, due to its significantly reduced level of mite susceptibility over that found in other commercial types such as cvs. Ben Tirran and Ben Alder and the presence of the *Ce* gene in one of its parents. However, symptoms of mite infestation have been found on cv. Ben Hope, potentially leading to the erroneous conclusion that resistance conferred by the *Ce* gene was breaking down. The findings of this study confirm that whilst Ben Hope may have some other resistance genes that can delay mite infestation, it does not contain *Ce* and this resistance therefore remains unbroken at the present time.

The development of the E41M88 marker was initially based on the same 9328 population as used for the creation of the linkage map for blackcurrant (Brennan et al. 2008a). In the latter study, the population of 125 individuals was phenotyped for a range of characters, including gall mite resistance, and the resistant phenotype was mapped to linkage group 2. The E41M88 marker maps at a distance of 4 cM to the phenotypic resistance based on *Ce* (Fig. 3), but



**Fig. 3** Linkage map of region around gall mite-resistant phenotype (*gmr*) in blackcurrant. Distances (cM) between successive marker pairs are shown to the left of the schematic chromosome segment. E41M88-280 is shown 4 cM from *gmr* location



its conversion to a PCR-based marker has massively enhanced its effectiveness as a selection tool, to a level beyond what one might expect from the relatively large map distance between the original AFLP marker and phenotype.

Mite resistance remains a high priority for most blackcurrant breeding programmes located in regions where *Cecidophyopsis ribis* is found (Brennan et al. 2008a, b), particularly as blackcurrant growers are now moving towards Integrated Pest Management (IPM) systems (Gordon 2008) with associated reduction in pesticide applications. Gene *Ce* from *R. grossularia* remains the most effective source of resistance, and has proved durable for over 40 years in trials in both the UK and abroad, e.g. Finland (Jones et al. 1998). However, the increasing commercial use of mite-resistant blackcurrant cultivars containing *Ce* may lead to a rise in pressure on the mite to overcome the resistance for survival, and it is therefore crucial that other sources of resistance are identified for the longer term. It is likely that this will involve some element of interspecific hybridisation, which can be difficult if the parental species are in different subgenera, as in the case of the introgression of *Ce* from *R. grossularia*. Of course, alternative sources of resistance will necessarily require further development of linked markers.

It is also important that future breeding work aims to combine resistances to gall mite and also to Blackcurrant Reversion Virus, which it transmits. The mode of resistance of *Ce* prevents multiplication of the mite, but it can sometimes still feed for long enough to transmit BRV (Herr 1986), although the incidence of BRV in *Ce*-containing plants is relatively low (Jones et al. 1998).

The development and validation of a PCR-based marker for gall mite resistance in blackcurrant is the first step towards a marker-assisted breeding strategy for blackcurrant. Further work is currently in progress to develop markers for other important traits, including those linked to fruit quality and plant development.

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